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Assembly of the 373K gene space of the polyploid sugarcane genome reveals reservoirs of functional diversity in the world's leading biomass crop

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Abstract:

Background
Sugarcane cultivars are polyploid interspecific hybrids of giant genomes, typically with 10-13 sets of chromosomes from two Saccharum species. The ploidy, hybridity and size of the genome, estimated to have in excess of 10 Gb, pose a great challenge for sequencing.
Results
Here we present a gene-space assembly of SP80-3280, including 373,869 putative genes and their potential regulatory regions. Their alignment to single copy genes of diploid grasses indicates that we could resolve 2-6 (up to 15) gene copies

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ABSTRACT

Background

Sugarcane cultivars are polyploid interspecific hybrids of giant genomes, typically with 10-13 sets of chromosomes from two Saccharum species. The ploidy, hybridity and size of the genome, estimated to have in excess of 10 Gb, pose a great challenge for sequencing.

Results

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Conclusions

This gene-copy resolved assembly represents a large step towards a whole genome assembly of a commercial sugarcane cultivar providing a large diversity of genes and homo(eo)logs useful for improving biomass and food production.

Keywords: Allele; Bioenergy; Biomass; Genome; Polyploid

BACKGROUND

Sugarcane is the world's most cultivated crop in tonnage (more than rice, maize and wheat) [1], and is considered the most sustainable of energy crops [2] with high potential to mitigate climate change without affecting food security [3]. Already produced in over 100 countries, high productivity of sugar, bioethanol and bioelectricity [4] make it a highly expandable green alternative to petroleum [5–7]. The International Energy Agency projects a 150 EJ (17% of energy demand) contribution of bioenergy by 2060, delivering 18% of the emission reductions needed to achieve the 2DS (2ºC Scenario). Sugarcane bioenergy production by 2045 could displace up to 13.7% of crude oil consumption and 5.6% of the world's CO_2 emissions relative to 2014. This can be achieved without using forest preservation areas or land necessary for food production systems. Additionally, the myriad of products that can derive from sugarcane biomass [8] further enhance opportunities for sugarcane in a portfolio of technologies needed to transition to a low carbon 'bioeconomy'.

Opportunities to accelerate breeding progress and enrich knowledge of the fundamental biology of this important plant motivate efforts to produce a high-quality reference genome, a challenge that is unusually complex. Unlike wheat cultivated species known to be either tetraploid (AABB) or hexaploid (AABBDD), the *Saccharum* (sugarcane) genus is considered to be a species complex. A recent study [9] proposed independent polyploidization events within *Saccharum* after divergence from the last ancestor shared with *Sorghum*, superimposed upon an additional whole genome duplication since the diversification of grasses. As a consequence, the sugarcane genome is redundant and harbors genes in multiple functional copies. Adding further complexity, sugarcane cultivars are polyploid interspecific hybrids, typically with 10-13 sets of their 10 basic chromosomes, 80-85% from *Saccharum officinarum* (2n=80), which is known for its sweetness, 10-15% from *S. spontaneum* (2n=40-128) known for its robustness, and ~5% with recombined chromosomes between those two progenitors [10,11]. The ploidy, hybridity and sheer size of the genome, estimated to have in excess of 10 Gb, pose a great challenge for sequencing [12]. Recently released sequences of the modern cultivar R570 yielded a mosaic monoploid reference (382 Mb single tiling path) [13] and a *S. spontaneum* AP85-441 haploid assembly (3.13 Gb) [14].

Worldwide sugarcane yield (~84 ton/ha) is currently only ~20% of the theoretical potential yield (~381 ton/ha), spurring great interest in conventional or molecular breeding approaches to improve yield. However, progress by conventional breeding towards closing the gap between current and potential yield has been slow with yield gains in the order of 1.0–1.5% a year [15]. Sugarcane commercial cultivars distribute roughly one

third of their carbon into sucrose and two thirds into tops and stems which, due to high lignin content, are burned to fuel boilers, contributing to the favorable energy balance of industrial processes [16]. As sugarcane can accumulate large amounts of sucrose in its stems, up to ~650 mM [17], it is important to study sucrose metabolism and the key players in its regulation. Also, of interest is the revealing of regulators of cell wall biosynthesis. Altering these pathways may help shift carbon partitioning from sucrose storage to biomass accumulation, rich in fiber content, mostly composed of secondary cell walls formed by cellulose, hemicellulose and lignin [18]. The latter compound is a hydrophobic polymer that provides strength and rigidity to the plant, but also is responsible for cell wall recalcitrance, which is the natural plant resistance to hydrolytic attacks that hampers cellulosic ethanol production [19].

RESULTS

The SP80-3280 assembly reveals a gene space of 373,869 genes

Here, we report a representative gene space assembly of the genome sequence of SP80-3280 (GenBank accession number QPEU01000000), the cultivar used in Brazilian breeding programs with the largest collection of transcriptomic data available [20]. On average, 6 sugarcane haplotypes, putatively homo(eo)logs, could be resolved from 4.26 Gb of assembled data and 373,869 putative genes and promoter regions. This is the first release of a resolved gene assembly of such a giant hybrid polyploid genome and their potential regulatory regions.

The assembly was constructed using 26 libraries sequenced using Illumina Synthetic Long-Read technology, obtaining 19 Gb, ~19x haploid genome coverage (~1.9X genome coverage) with >99% of bases having >99% accuracy (**Additional file 1: Fig. S1**). The final assembly includes 450,609 contigs (unitigs + singletons), with average length of 9,452 bp and NG50 of 41,394 bp (**Table 1)**, adding over 3Gb of sequence not previously reported (**Additional file 2: Table S1**) [21]. The gene space described here is organized in the SUCEST-FUN public database (http://sucest-fun.org/wsapp/).

Several indicators support the comprehensiveness of the SP80-3280 gene space: (*i*) among 39,441 sorghum transcripts, 39,207 (99.4%) matched the assembly, at least partially; of these, 71.1% matched at least one sugarcane contig with 90% or higher coverage (**Additional file 1: Fig. S2**); (*ii*) the assembly completely

covers 217 (87.5%) of the 248 ultra-conserved Core Eukaryotic Genes Mapping Approach (CEGMA) [22] proteins, and partly covers 18 (7.3%), with only 13 (5.2%) not detected (**Additional file 2: Table S2)**; (*iii*) among 1,440 genes in the Benchmarking Universal Single-Copy Orthologs (BUSCO) [23] Plantae lineage, the assembly completely covers 1,309 (90.9%) and partially covers 53 (3.7%) (**Additional file 2:Table S3)**. By including tBLASTn of the 78 (5.4%) missing Plantae lineage BUSCO genes, only 8 (0.5%) are absent; (*iv*) assembled chloroplast (MG969494) and mitochondrial (MG969495 and MG969496) genomes were over 99% similar (at gene level) to published *Saccharum* genomes [24–26]; and (*v*) 94.9% of 134,840 SP80-3280 expressed sequence tags (ESTs) match the assembled gene-space sequence.

The assembly revealed 373,869 putative genes with 374,774 transcripts (**Table 1**), far more than the 72,269 unigenes inferred from six sugarcane genotypes [27]; 85,151 transcripts of sugarcane genotypes with contrasting lignin contents [28]; and 195,765 transcripts inferred from *de novo* assembly of ORFeomes from *S. officinarum*, *S. spontaneum* and SP80-3280 [29]. The number of genes, high quality of alignments, and the following analysis indicates that the assembly provides a high-quality resolution of homo(eo)logs genes.

Among the predicted transcripts, 302,627 (80.7%) aligned to a Uniref50 protein [30], and 195,651 were annotated with 10,362 GO terms [31] (**Additional file 1: Fig. S3**). Our previously published SP80-3280 ORFeome was reassembled using the genome as a reference, revealing 269,050 genes and 275,807 transcripts from leaves, immature and intermediate internodes (**Additional file 2: Table S4)**. Further, a set of 134,840 SP80-3280 ESTs from a Sugarcane EST Project – SUCEST [20] – were mapped to assembled contigs and compared to predicted genes, in order to further estimate the completeness of the predicted gene space. A total of 127,940 ESTs (94.9%) have at least one match in the assembly, which is in accordance with similar analysis of other plant genomes [32], and only 7.6% of aligned ESTs (9,772) do not correspond with predicted genes. This result resembles the BUSCO results, for which only 5.4% of conserved genes could not be identified in the assembly. Performing the same approach using a set of 43,141 sugarcane assembled sequences (SAS) – derived from an EST project [20] – produces similar results regarding the overall alignment rate, with 93% of the sequences (40,147) matching at least one location in contigs. It is important to note that the SUCEST database included ESTs from 10 sugarcane cultivars including SP80-3280 which may explain the reduced correspondence with genic regions, with 16% of aligned SAS having no correspondence to predicted genes of SP80-3280.

To verify how the assembled genes reflected the expected content of homo(eo)logs genes, the gene content was compared to those of other grasses. Single-copy genes from diploid grasses (sorghum, rice and *Brachypodium*) are present in up to 15 copies in sugarcane, mostly with 2-6 copies (total of 1,592 coding sequences (CDS) in sugarcane) (**Fig. 1A**). Dissimilarities among gene copies increase from the coding region to the promoter region, with median divergence of 0.90% between CDS, 1.03% for the 100 nucleotides (nt) upstream, 4.47% for 500 nt and 7.50% for 1,000 nt (**Fig. 1B**). Frame-preserving INDELs are more abundant than frameshifts (**Fig. 1C**) and short frameshift INDELS were relatively less frequent in the sugarcane exons than in sorghum [33].

Differential homo(eo)logs expression is observed

The SP80-3280 gene series that correspond to diploid grass single-copy genes showed expression of sense copies for multiple homo(eo)logs (**Fig. 2A**), with very few copies transcribed in antisense orientation (**Fig. 2B**) based on alignment with the SP80-3280 cDNA reads [29] from leaves, immature and intermediate internodes. For some genes, not all copies are expressed in SP80-3280 (**Fig. 2A, Additional file 1: Fig. S4**). The number of expressed homo(eo)logs is different across the three tissues (**Additional file 1: Fig. S4A**). This difference among copies is consistent with the divergence of upstream regions (putatively gene promoter regions). The increase in the number of expressed copies is not accompanied by an increase in the level of expression (**Additional file 1: Fig. S4B**).

As an example of the complexities in data mining of such a complex gene-space for future reference, we offer an example using some well-known genes involved in biomass production.

Gene family analysis of SuSy and PAL shows differences in their regulatory regions in SP80-3280 and *S. spontaneum*

Sucrose Synthases (SuSy) catalyze the reversible breakdown of sucrose into UDP-glucose and fructose in carbon partitioning [34]. In agreement with previous work on sugarcane progenitors [35] (*S. officinarum*, *S. robustum* and *S. spontaneum*), phylogenetic analysis of 44 ScSuSy members identified in the SP80-3280 assembly supports that this hybrid has 5 SuSy genes (hereafter ScSuSy1-5) in three clades: I (ScSuSy1 and 2), II (ScSuSy3 and 5) and III (ScSuSy4) (**Fig. 3A)**. Sorghum shares these 5 SuSy genes, indicating that they

evolved before the sugarcane/sorghum divergence. RNA-Seq data from leaves and internodes of SP80-3280 [29] shows expression of 36 of the 44 ScSuSy members, suggesting ScSuSy1-2 (clade I) and ScSuSy5 might control carbon flux from source to biomass conversion in stems, as they show higher expression in internodes than in leaves (**Fig. 3C**).

SuSy produces the substrate for cellulose biosynthesis (UDP-glucose) and is commonly associated with cell wall and cellulose synthesis [36,37]. In view of the myriad of possibilities to convert lignocellulosic compounds into chemicals and fuels, defining phenylpropanoid biosynthesis pathway members in sugarcane is of great interest. Phenylalanine ammonia-lyase (PAL), the first enzyme in phenylpropanoid biosynthesis [38–40], is correlated with lignin content [38–41], a major component of plant cell walls [18], and is responsive to the ethylene-releasing ripener (ethephon) in both leaf and internode [42].

Mapping of predicted proteins from SP80-3280 against the SUCEST-FUN Cell Wall Catalogue [41] (731 transcripts of 20 protein categories) identified 3,054 similar proteins (**Additional file 2:Table S5)**, including 47 PAL copies. Phylogenetic analysis together with sorghum, *S. spontaneum* and mosaic monoploid R570 PAL sequences reveals 5 clusters (**Fig. 3B**), each containing at least one representative with a sorghum ortholog. *S. spontaneum* has 33 putative PAL genes, somewhat more than expected considering that the sequenced genotype is a tetraploid. The higher number may be due to expansion of PAL members in clade I that occurred also for sorghum and the sugarcane hybrid genomes of R570 and SP80-3280. Clade V has a higher number of SP80-3280 PAL members and all except one (ID 37780.4) showed expression evidence (**Fig. 3D**). In addition, the CCR (Cinnamoyl-CoA reductase), COMT (Caffeic acid 3-O-methyltransferase) and 4CL (4-coumarate-CoA ligase) gene families, also related to phenylpropanoid biosynthesis, have much higher numbers of genes (620, 453 and 375, respectively) in sugarcane than sorghum [43] (44, 41 and 15, respectively). This is another challenge and opportunity for future functional characterization (**Additional file 2: Table S6)**.

The sheer number of sugarcane genes found so far, the large size of multi-gene families and the finding that homo(eo)logs are differentially expressed point to a very complex role of regulation in the determination of phenotypic differences. Consistent with the gene copy-richness of sugarcane, we inferred 15,737 transcription factors (TFs) from 57 families (**Additional file 2: Table S7)**, versus ~2,000 previously estimated [44]. The classification of core promoters and identification of Transcription Factor Binding Sites (TFBSs) in

proximal promoters was performed *in silico* and the percentage of core promoter regions with a TATA-box element was 47.72% and 12.76% for SuSy and PAL genes, respectively. The TFBS identification pointed to a wealth of regulatory elements differentially distributed among members of the same gene family, i.e. SuSy and PAL (**Fig. 3C and D and Additional file 2: Table S8)**. In addition, using gene expression data of SP80-3280 plants grown in field conditions for 13 months, we have found evidence of a co-expression module, enriched for phenylpropanoid and lignin biosynthesis gene ontology terms (**Additional file 1: Fig. S5A**). This module comprises 116 transcripts, including one PAL (**Additional file 1: Fig. S5B**), whose expression is higher in internodes 5 and 9, than in leaves and immature internode (**Additional file 1: Fig. S5C**). It was possible to identify the TFBSs, predicted as putative regulators of the PAL gene family (**Fig. 3D**) within the upstream region of these co-expressed genes, suggesting that ABF, ERF, ZF-HD/C2H2, and ARF3 (**Additional file 1: Fig. S5D**) may also regulate other genes involved in lignin biosynthesis and metabolism.

The most significant motifs found for each gene family (SuSy and PAL) were mapped to the promoter region of the remaining sequences from both SP80-3280 and R570 hybrids and *S. spontaneum* (**Additional file 2: Table S8c and Table S9**). Interestingly, only ScSuSy2 and ScSuSy3 motifs mapped in all species, suggesting that SP80-3280 hold particular regulatory elements involved in sucrose synthesis. Conversely, SP80-3280 and *S. spontaneum* share all predicted motifs for PAL genes (**Additional file 2: Table S9)**, suggesting that this gene family may be derived from the *S. spontaneum* ancestor.

Transposable element insertions may affect SuSy and PAL expression

Fewer transposable elements (TE) were identified in SP80-3280 gene space than in the AP85-441 *S. spontaneum* and mosaic monoploid R570 assembly, probably due to repetitive regions collapsing in the assembly even with the use of long synthetic-read sequencing (**Additional file 1: Fig. S6**, **Additional file 2: Table S10)**. All previously described TE families are represented in the three genome assemblies, disclosing few cultivar specific amplifications. The two modern cultivars have fewer TE counts than the *S. spontaneum* progenitor in normalized monoploid genomes. LTR retrotransposons are large contributors to genome composition at the chromosome assembly level. However, scMaximus (Copia) and scDel (Gypsy) LTR-retrotransposon families are similarly represented in both gene-space and chromosome assemblies supporting their presence in transcriptionally active regions [45]. We also note that scCACTA transposons are more represented at the gene-space assembly than schAT while the scMutator family is similarly represented in both.

Functionally important TE insertions were identified in the ScSuSy gene family (**Fig. 3**). ScSuSy2 copies have a contrasting pattern, most *S. spontaneum* having TE insertions while most SP80-3280 homo(eo)logs do not – although SP80-3280 and *S. spontaneum* share one ancient insertion of schAT159 at similar distances from the ATG. ScSuSy3 genes are polymorphic between species and within SP80-3280, with 6 copies having no TE and 5 in which different TEs may impact expression. In particular, scga7_uti_cns_0020964:7575-17575 (-) harbors a full LTR at 280 bases from the ATG. Most ScSuSy4 copies have no TE insertion but interestingly, as described for ScSuSy2, SP80-3280 (scga7_uti_cns_0226458:7638-16073 (-)) and *S. spontaneum* (Chr1B:33406669-33416669 (-)) share one ancient schAT159 insertion. Finally, ScSuSy1 has similar patterns of TE presence and absence in both genomes, and ScSuSy5 genes have no insertions in the promoter regions of either *S. spontaneum* or SP80-3280. Furthermore, PAL genes from clade I exhibit most of the copy variation and harbor TEs inserted near the promoter region. Only two copies from SP80-3280 and *S. spontaneum* lack TE insertion in PALs from group I.

Sugarcane and sorghum polymorphisms support recent allotetraploidy

Despite a common foundation for evolving high sugar content with similar Susy genes (ScSuSy1-5), sugarcane and closely related sorghum have taken different paths since sharing ancestry. We identified 4,750 natural SNP variations (SNVs) between sorghum and sugarcane gene regions, mostly bi-allelic (3,840 (80.8%)), but 6.2% tri-allelic (295) and 0.97% tetra-allelic (46) (**Fig. 4**). Further, 1,334 SNVs that differentiate sugarcane from sorghum in 585 single copy genes include frameshifts, premature splicing, loss of stop codons and translation initiation (**Additional file 1: Fig. S7**, **Additional file 2: Table S11)** in genes significantly enriched in transcription, DNA-dependent cell organization and biogenesis in the nucleus and endoplasmic reticulum (**Additional file 2: Table S12)** comprise a rich slate of candidates for causes of morphological and physiological differences between these taxa.

The gene-space contribution towards a chromosome level assembly of a sugarcane commercial hybrid

Notwithstanding the fragmented nature of our assembly, we explored how it could contribute beyond the gene space toward a whole genome assembly of the hybrid sugarcane genome. Previous analysis of grass genomes revealed extensive conservation of gene order overlaid with a background of small-scale chromosomal rearrangements and numerous localized gene deletions, insertions and duplications [46].

Recently published estimates of the levels of gene synteny between *Sorghum bicolor* and the sugarcane cultivar R570 found that 83% of the genes are arranged co-linearly in the two genomes [13]. In our assembly of SP80-3280, 79,094 contigs had at least two predicted genes and could therefore be used to compare the order of genes in SP80-3280 to those of sorghum. To avoid the need to resolve multiple comparisons to duplicated regions in the sorghum genome, we generated a sequence similarity-based clustering of all coding sequences from both genomes and used the genes in clusters with only one sorghum gene as anchors to evaluate synteny (**Additional file 1: Fig. S8**). We found that 9,319 SP80-3280 contigs had at least two synteny anchors and 85% (7,906) of these contigs were fully syntenic (**Additional file 1: Fig. S9A, B**), *i.e.* had all genes in the same order and orientation in SP80-3280 contigs and the sorghum chromosomes (**Additional file 2: Table S13)**. To evaluate the effect of SP80-3280 assembly fragmentation on the distribution of contigs per syntenic block length, we sampled 10,000 contigs from SP80-3280 and 10,000 chromosome fragments from both R570 and *S. spontaneum*, while preserving the overall distribution of contig lengths observed in SP80-3280. The distributions converged to similar shapes, with most contigs and chromosome fragments harboring a single syntenic block in all genomes (**Additional file 1: Fig. S9C)**. While the number of syntenic blocks per contig were identical for the two cultivars, a larger frequency of fully syntenic contigs was observed for SP80-3280, suggesting that our assembly is enriched in genomic neighborhoods that are co-linear to sorghum, presumably comprising euchromatin. While an increase in sequencing coverage would lead to improved estimates of co-linearity, our results agree with widespread findings on the conservation of gene order among grass species and support the conclusion that, albeit fragmented, our assembly does not contain an excess of chromosomal rearrangements, as would be expected if there was a significant amount of chimeric contigs.

Finally, to allocate the gene space into potential physical groupings we aligned the SP80-3280 transposable element (TE) masked BWA-SW to chromosome level assemblies of the *S. spontaneum* tetraploid AP85-441 genome [14] and the R570 [13] monoploid genome data. Multiple correspondence analysis (MCA) with hierarchical clustering of the sequences enabled us to allocate the gene space contigs into 6 clusters, an important contribution to future scaffolding efforts. From the total of 450,609 contig sequences, 418,471 (92,86%) produced a BWA-SW alignment against the *S. spontaneum* [14] and R570 [13] assemblies (**Fig. 5A**) and protein alignment among these three species are consistent with MCA results (**Fig. 5B and C**). Contigs were also mapped against a collection of 778 targeted sequenced BACs of which 347 are from SP80-3280 and 431 from R570. All BACs had a corresponding contig match against the assembly. This collection shows

centromeric regions and non-TE multigene families are the most covered (64x). An R gene locus (I2C-2) found in cluster 3 of SP80-3280 and in chromosome 9 of R570, was verified for co-location with a Ca^{+}-dependent kinase, a *dog1* (delay of germination 1) and an aminotransferase. The co-location was confirmed in R570 and SP80-3280 BACs showing up to eight copies of each gene (**Additional file 1: Fig. S10**).

DISCUSSION

This assembly presents 373,869 genes. The gene space described here represents a significant step in understanding the haplotype origin of the hybrid genome. Approximately 12.25% of the SP80-3280 sequences are of *S. spontaneum* origin [14], supporting previous studies [10,11]. The comparison against different sets of genes (sorghum, CEGMA, BUSCO, mitochondrial and chloroplast) supported the comprehensiveness of the gene space. The total of predicted genes (373,869) is around 10x, 14x and 13x higher than those for monoploid genome assemblies of *S. spontaneum* [14], sugarcane R570 [13] and sorghum [48], respectively. This is in agreement with the predicted 8 to 14 copies for *S. spontaneum*, depending on the cytotypes, and for modern sugarcane varieties [49]. Genes that are single-copy in diploid grasses are present in up to 15 copies in the SP80-3280 assembly and the sequence differences are present mainly in the upstream regulatory region. This highlights the importance and complexity of studying homo(eo)logs expression in sugarcane and adds great value to the development of molecular markers for breeding in gene promoter regions. The differences in gene upstream sequences causes the differential expression among the copies and across the studied tissues. This was also reported for the polyploids cotton [50] and wheat [51]. Expression differences among homo(eo)logs in polyploid species may play a crucial role in increasing adaptability to environmental stresses (such as salinity [52], heat and drought [53]) and in improving performance of new cultivars. These differences highlight the importance of our assembly which discriminates homo(eo)logs, for example providing information important for the selection of target sequences (genes or promoters) to produce transgenic sugarcane plants. With the homo(eo)logs identified, one could discard a sequence that is not expressed or use genome editing tools to modify a target sequence to increase its expression. It is also possible to identify the progenitor contributing a homo(eo)log (e.g., *S. spontaneum*, *S. officinarum* or a parent in a cross) and select the homo(eo)log from the progenitor that has the phenotype of interest.

We also show how the data can be useful for gene promoter analysis. Expansion of SuSy genes might by selected for fiber development in cotton [54]. Different members of the SuSy gene family may have different functional roles and in sugarcane this was observed as different expression levels related to different TFBs identified. We identified five different top-ranked TFBs (with the highest score) in the ScSuSy1-5 members. Three of them are related to auxin and abscisic-acid hormone signaling (ScSuSy1, 3, 5). For ScSuSy1 genes, the TFBS analysis predicted the motif wATATATATw (MA1184.1) that is associated with RVE1, a morning-phased transcription factor integrating the circadian clock and auxin pathway genes that bind to the evening element (EE) of promoters [55]. For ScSuSy2 genes, we found the motif GACrAATryA (MA1374.1) that is associated with IDD which regulates photoperiodic flowering by modulating sugar transport and metabolism [56]. For ScSuSy3 genes, we found the AyACTAGTrT (MA0930.1) motif in 64% of its SP80-3280 copies and in all copies in the *S. spontaneum* and R570 monoploid genomes. It is associated with ABA-responsive elements (ABRE) that regulate stress response via ABA signaling. For ScSuSy4 genes, we found the TAGyAynTTT (MA1012.1) motif that is probably involved in regulation of the photoperiod and vernalization pathways. Finally, for ScSuSy5 genes, we found a CTGCTAGCAG (MA0564.1) conserved motif exclusively for ScSuSy5 genes in SP80-3280. This motif allows binding with an element associated with ABI3, which participates in abscisic acid (ABA)-regulated gene expression. Previous studies from our group had already pointed out ABA- and sucrose-induced genes associated with higher sucrose content in sugarcane [57].

On the other hand, for ScPAL I genes, the TFBS analysis predicted an ArCAyATnTG (MA0930.1) element, which is associated with ABF3, a transcription factor involved in ABA and stress responses and acting as a positive component of glucose signal transduction. For ScPAL III genes, we found the element GGTCsGGCkC (MA0992.1), an element associated with AP2/ERF, a transcription factor involved in the regulation of gene expression by stress factors and by components of stress signal transduction pathways. For ScPAL Va genes, we found the element TCTAAAGTTT (MA0064.1), which is associated with PBF, a transcription factor involved in ABA, stress response and components of stress signal transduction pathways. Finally, for ScPAL Vb genes, we found the motif GCCGGAACGG (MA1009.1). This element is associated with ARF3, a transcription factor involved in auxin and ABA-regulated gene expression. In summary, our results corroborates reported findings [57] which reveal that PAL genes were induced by ABA.

Fewer TEs were identified in SP80-3280 in comparison to the two other published genome [13,14] and few cultivar specific amplifications were observed as all previously described TE families were identified.

Around 81% of the SNVs identified were bi-allelic with only 0,97% being tetra-allelic. The predominance of biallelic variations supports the theory that sugarcane experienced allotetraploidy shortly after divergence with sorghum ca. 3.8~4.6 MYA [58], creating two 'subgenomes'. Further, autotetraploidization after Saccharum speciation ca. 3.1~3.8 MYA may have contributed to allelic richness within each sugarcane 'subgenome', with occasional exchanges between subgenomes likely. Recent published results from Vieira et al. [59], demonstrate that sugarcane meiotic chromosomes behave as bivalents, supporting these inferences.

In an attempt to organize the contigs, we allocate them in 6 clusters using MCA with hierarchical clustering of the sequences. MCA results suggest that 4 out of 6 clusters correspond to single chromosomes in *S. spontaneum* and R570. On the other hand, clusters 3 and 4, which contain multiple chromosomes, include those in which chromosomal rearrangement events were demonstrated in comparison to sorghum: SsChr5, SsChr6 and SsChr7 from *S. spontaneum* [14] and six R570 hom(oe)ology groups HG5-HG10 [13].

CONCLUSION

The singular challenge associated with sugarcane breeding makes genomic tools and genome assembly of a polyploid interspecific hybrid of especially high value. Its large autopolyploid genome, predominantly clonal propagation, and need for extensive phenotyping to determine breeding values, have contributed to the relatively slow (~1% per year at most) rate of progress in improvement of sugarcane [60] and perhaps other autopolyploids. The demonstration that most of its many homo(eo)logs are expressed, often with tissue-specificity, and that transcription factor binding sites and TE insertions differ among homo(eo)logs, suggests complex constraints that may necessitate unusual richness of information to make effective decisions about selecting some homo(eo)logs alleles at the expense of others in autopolyploid breeding populations. These principles may apply widely to many plants with large polyploid genomes that include many of those most efficient at converting solar radiation to biomass.

The present work discloses a large collection of gene-space homo(eo)logs diversity, taking advantage of novel sequencing technologies, adding over 3Gb of sequence not previously reported, in addition to genome

annotation, data mined homo(eo)logs, and explored regulatory regions. The resolved gene-space of the sugarcane genome is a fundamental step towards a high-quality chromosome resolved assembly from a current commercial hybrid. The genome sequence released for this interspecific polyploid supports its recent allotetraploid nature, reveals differences in promoter regions associated to differentially expressed genes and transposable elements contributing to fine tuning of the rich diversity in a genome that is otherwise highly syntenic with its close relative, sorghum.

METHODS

Plant material

Leaves from SP80-3280 were collected and frozen in liquid nitrogen. Genomic DNA was extracted using DNeasy Plant Mini Kit (Qiagen) following the standard protocol. DNA integrity was analyzed using the Agilent High Sensitivity DNA Analysis Kit (Agilent Technologies) and Agilent 2100 Bioanalyzer Instrument. Quantification was done using Quant-it^{TM} PicoGreen® dsDNA Assay Kit (ThermoFisher Scientific) and SpectraMax M2 microplate reader (Molecular Devices).

Sequencing Illumina Long-reads and Assembly

We used Illumina Synthetic Long-read sequencing technology, which provides very accurate long reads with a mean read length of roughly 5 kb, thus being able to represent polymorphisms across all copies of chromosomes. Genomic DNA was sheared into 5-10 kb fragments and diluted in 384-well plates. DNA fragments were ligated with PCR primers and specific sequences, which identify the 5' and 3' ends. The fragments were amplified, fragmented and barcoded to create 26 TruSeq Synthetic Long-Read DNA libraries. The short fragments created in the second step of fragmentation were pooled and sequenced on the HiSeq instrument at the Illumina Service Genome Network. The reads from each of the 384 wells were pre-processed to correct sequencing and PCR errors. Contigs were produced from the paired-end information and further scaffolded together to resolve repeats and fill in gaps. More details on the informatics pipeline for short read scaffolding into long reads are available in the Fast Track Services Long Reads Pipeline User Guide [61].

To assemble sequences we used a two step approach: *i)* the Celera Assembler [62] (CA) was used for overlap computation and layout building; *ii)*the *tig-sense* module of the HBAR-DTK (Hierarchical-Based AssembleR Development ToolKit) from Pacific Biosciences [63] was used to construct consensus sequences. This was motivated by the fact that the CA, which uses the overlap-layout-consensus method, is more robust than *de Bruijn* graph approaches. However, some adjustments needed to be made. CA, designed for Sanger reads, only accepts quality scores between 0 and 40. Since synthetic long reads are very accurate and some of the base qualities exceeded this upper bound, we modified the quality scores of our long-read data to allow them to be appropriately parsed. The consensus module was also adapted for the analysis of big complex genomes. The substantial number of contigs generated initially (roughly 450.000, half of them singletons) incurred in several files in a folder that hindered I/O operations. So, we *i)* modified *tig-sense* to automatically create subdirectories that contained not more than a thousand contig FastA files, reducing delays for file lookup; *ii)* divided contig processing into non-singletons and singletons, prioritizing non-singleton contigs; and *iii)* created a work history so that the program could be resumed after a halt. Overall, these modifications allowed us to reduce the running time of the consensus pipeline by one or two orders of magnitude.

Sequencing BAC clones and assembly

A total of 780 independent BACs were sequenced using Roche454 and PACBio sequencing technologies. Each BAC clone was tagged with a unique barcode and sets of 12 BACs were pooled in one gasket. We assembled BACs individually as described [64] and obtained a total of 49.6 Mbp of assembled sequence, with a mean length of 107 kbp. The BAC data includes 317 R570 BACs[18], 116 additional R570 BACs and 347 from SP80-3280.

Assembly Validation

Comparison with Sugarcane BACs

Assembled contigs were aligned against a set of 780 BACs with BWA mem. Alignment data was processed for coverage with the aid of Samtools (v1.1) and Bedtools (v2.25) and selected matches were at least 10 kbp long and covered 90% or more of the contig. Additionally, the unassembled synthetic long reads were aligned

to the same set of BACs, to check for discrepancies among contigs and long reads, which could be indicative of regions that were not assembled.

Comparison with Sorghum CDS

The set of 39,207 annotated sorghum coding sequences (CDS), release version v2.1, were downloaded from Phytozome [65]. These were aligned against the assembled contigs with BLASTn (v2.2.30+) using default parameters. For each sorghum CDS, we identified the longest fraction of the coding sequence contained within a single unitig. Only hits with at least 80% identity at the nucleotide level were considered for computing coverage.

Comparison with CEGMA

A total of 248 Ultra-conservative core eukaryotic genes classified by Korf Lab [22] were assessed in our sugarcane assembly with '-g' and other default options of CEGMA v2.5.

Comparison with BUSCO

Assembly completeness was assessed by searching for the 1,440 core genes from the Plantae lineage of Benchmarking Universal Single-Copy Orthologs (BUSCO) [23]. BUSCO performs gene prediction and orthogonality assessment using Augustus [66] and HMMER3 [67]. Since these steps demand huge resources, we parted sugarcane contigs (4.3Gbp) into six groups with similar volume and processed BUSCO in parallel. After we merged results, we applied orthogonality assessment algorithm once again as thresholds that BUSCO exploits to discern actual single copy orthologs from paralogs.

Comparison of the mitochondrial and chloroplast genomes

To reconstruct the SP80-3280 mitochondrial and chloroplast genomes, we have used as reference the complete genomes of *Saccharum* hybrid chloroplast (NC_005878.2) [68] and the *Saccharum officinarum* mitochondrial chromosome 1 (LC107874.1) and chromosome 2 (LC107875.1) [25], downloaded from NCBI. The SP80-3280 genome contigs were aligned using BLASTN against their respective references and the best hits were selected based on cutoff E-value ≤ 1.0E-15, with contig coverage ≥ 90% and identity ≥ 70%. The BLASTN

alignment results identified 2,482 and 909 contigs for the two mitochondrial chromosomes, respectively; and 51,768 contigs for the chloroplast genome. To reconstruct the consensus sequences and do the genome annotation we have used the CLC Genomics Workbench tools [69]. Using the CLC Tools and the Genome Finishing Module, the selected contigs were aligned to their respective references and consensus sequences extracted, filling the gaps with N's. The reconstructed consensus sequence aligned against the chloroplast genome presented 100% of coverage and identity. Alignment against mitochondrial chromosomes 1 and 2 presented over 99% of coverage and identity. The consensus sequences were annotated using their respective NCBI references with the CLC tool "Annotate from Reference", where all genes, tRNAs rRNAs and miscellaneous features were totally transferred.

Genome Annotation

Gene prediction

Contigs were annotated using a pipeline developed in house, previously used for BAC annotation. Transposable element (TE) discovery and masking was done using LTR harvest, LTR digest, CrossMatch against *Utricularia gibba* TE DB and RepeatMasking [70] of Viridiplantae [71] and previously known sugarcane TEs [45].

Genes were discovered and annotated using masked contig sequences. *De novo* predictions were done with Augustus [66], Glimmer HMM [72], GeneMark HMM [73], SNAP and PASA [74] with rice models and sugarcane EST and RNAseq data. Alignments were also generated against reference protein DBs (sorghum, known sugarcane and Phytozome) using Exonerate [75] and BLAST [76] (v2.2.30+). Both *de novo* and alignment evidence were used for consensus annotation with EVidenceModeler [77] with greater weight given to experimental and alignment information. Functional assignment was derived from protein DB best hits and InterProScan 5 [78] results.

GeneOntology annotation

For functional annotation of predicted proteins from SP80-3280, all sequences were aligned to UniRef50 clusters, a dataset of representative sequences clustering high similarity proteins from UniProtKB [30], using BLASTP (v2.2.30+, *-evalue 1e-5*). Sequences that fail to align in this first approach were also searched against

the RefSeq non-redundant protein database. Gene Ontology mapping and annotation of sequences with positive BLAST results was performed using Blast2Go framework [79].

Reference-guided RNAseq Assembly

We used Trinity version 2.0.6 for reassembly of the Sugarcane ORFeome [29] using the genome as a reference, with a minimum contig length of 250 bp (genome_guided_max_intron 3000, genome_guided_min_coverage 5, genome_guided_min_reads_per_partition 10) to identify transcript models. SP80-3280 RNA-seq reads from 3 tissues (leaves and immature and intermediate internodes) were used for alignment against the reference genome and partitioned into read clusters, which were then individually assembled using Trinity genome-guided methods. Trinity and genome-guided methods used a fixed k-mer size of 25nt. In this new assembly, 269,050 genes and 275,807 transcripts were recovered. The quantity of transcripts recovered by the reference guided-assembly was higher, and thus closer to the number of predicted genes (374,774), than the *de novo* assembly.

Identification of Gene Copies and Count Estimation

We downloaded the *Sorghum bicolor* genome assembly v2.1 from Phytozome and took 2,051 single copy genes according to Han *et al*. [80], which were also present as single copies in the genomes of *Oryza sativa* and *Brachypodium distachyon*. We aligned the coding sequences of these sorghum genes to the coding sequences of predicted sugarcane genes from the SP80-3280 assembly, using the BLASTn (v2.2.30+, *-evalue 1e-6*). We filtered alignments with at least 80% nucleotide identity, covering at least 70% of both the sugarcane and sorghum sequences. For sorghum coding sequences with multiple hits to sugarcane genes, we further required that each hit had at least 80% identity, based on Wang *et al*. [46]. Sugarcane gene models aligned to the same single copy sorghum gene were denoted as putative homo(eo)logues. Finally, we counted the number of copies for each gene.

We clustered all gene copies based on each single copy sorghum gene to get estimates of sequence differentiation. We aligned the coding sequences for each pairwise combination in each gene cluster, using BLAT v35 [81] (*–minIdentity=0 –minScore=60*), disregarding clusters with more than 16 putative homo(eo)logs. Next, we parsed the alignments to obtain estimates of copy differentiation considering both

SNPs and INDELs. We gathered distance estimates from all pairs, from all clusters, to obtain dissimilarity distributions.

Gene copy characterization

Upstream region analysis

We also assessed the dissimilarity levels of regions upstream (potential promoter regions) of the predicted sugarcane gene copies. We initially collected three different sequence ranges (100 bp, 500 bp and 1000 bp) upstream of the predicted gene start site. Next, we aligned these upstream sequences for each pairwise combination in each cluster, again using BLAT v35 [81] (*–minIdentity=0 –minScore=30*). Finally, for each distance range, we parsed the alignments and computed the dissimilarity level considering both mismatches and gaps. To avoid partial alignments of the upstream sequences, only alignments up to 20% shorter or longer than the expected sequence length were considered.

Insertions and Deletions between gene copy Coding Sequences

To investigate the occurrence of frameshift mutations between gene copies, we built multiple alignments of the coding sequences of putative copies, for each cluster, with MUSCLE v3.8.31 [83], using default parameters. We then computed the length distribution of insertions and deletions in the coding sequences, to differentiate between frame-preserving and frameshift indels. We parsed the CDS alignment for each pairwise combination of gene copies and counted the number of occurrences of gaps of a given length. We then pooled counts from all copy combinations to get a joint estimated distribution.

Tissue-Specific Homo(eo)logs Expression Analysis

We used RNA-Seq data [29] from leaves (*L*), immature (*I1*) and intermediate (*I5*) internodes of SP80-3280 to find the expression of putative tissue-specific gene copies. These reads were initially aligned to the Sugarcane genome assembly using TopHat2 [84] version 2.0.9 (*library-type fr-firststrand*). We allowed reads to be aligned to up to 20 contigs of the genome assembly to identify alignments to different homo(eo)logs (*--max-multihits 20*) and supplied TopHat2 with the putative homo(eo)logs' annotation as a GTF file (*--GTF CDSMapping-homo(eo)logs.gtf*), in order to direct TopHat2 to align the reads to this transcriptome first.

Besides the *TopHat2* alignment, we used the RSEM tool rsem-calculate-expression (version 1.2.31) to quantify the expression of predicted genes (bowtie2, fragment-length-mean, fragment-length-sd and calc-ci parameters). An in house perl script was used to estimate the mean length and standard deviation for each RNA-seq library. The main output of *Tophat2 BAM* formatted file [85] *accepted_hits.bam* was used with *RSEM* to estimate the transcriptome expression profile. We developed in-house perl and R language (version 3.3.2) scripts to find the number of putative expressed homo(eo)logs for each single copy Sorghum gene, using the information from *genome annotation* file (GFF format), showing the gene structure, the transcriptome annotation and respective TPM (Transcript Per Million) abundance. The previous information allowed the creation of the homo(eo)logs GFF file. We also applied TopHat2 to find the number of putative homo(eo)logs expressed only in *antisense* orientation, using the same protocol described above, and the *antisense* reads of RNA-Seq previously identified by Nishiyama *et al*. [29].

ScSuSy and ScPAL gene family analysis

We used the sugarcane and sorghum SuSy protein sequences reported by Zhang et al. [35] as query for a tBLASTn (v2.2.30+) search in the predicted proteins from SP80-3280, *S. spontaneum*[46] and R570 genome assemblies [13]. Putative SuSy genes were then filtered by query coverage >=80% of at least one of the five ScSuSy from Zhang et al. [35] and by PFAM [86] domain search, considering only those containing both the conserved sucrose synthase and glucosyl-transferase 1 domains.

Based in BLAST and keyword search in two databases (Plant GDB, http://www.plantgdb.org/ and Phytozome [65]) we found 8 different PAL genes in the sorghum genome, the same number previously reported [87]. For sugarcane, PAL genes were retrieved from an EST Cell Wall catalogue [41], which was used as query together with sorghum PAL genes for a BLASTn (v.2.2.30+) search to identify PAL genes in the predicted proteins from *S. spontaneum* [47] and R570 genome assemblies [13]. Putative PAL genes were then filtered by query coverage ≥ 80% of the sorghum PAL genes and by PFAM [86] domain search, considering only those containing the Aromatic amino acid lyase domain. Also, sequences not containing the PAL conserved amino acid motif Ala-Ser-Gly [88,89] and an essential Tyr110 [90] were excluded.

For both SuSy and PAL, nucleotide sequences (CDS) were aligned with clustalw [91] software in MEGA 7.0 [92] and maximum likelihood trees were constructed with default parameters except for 1,000 bootstraps and

Gaps/missing data treatment "*use all sites*". Expression heatmap was constructed using log2 transcript per million (TPM) from previous RNA-seq data [29].

Cell wall-related genes

For the identification of cell wall-related genes in the sugarcane genome we used the Sugarcane SAS Cell Wall catalogue [41] as a reference. The search was carried out using tBLASTn (v2.2.30+, *-evalue 1e-6*). These were manually re-annotated to produce a sugarcane cell wall catalogue with 3,054 sequences, classified in 10 cell wall categories.

Transcription Factor analysis

For the identification and classification of sugarcane predicted proteins into transcription factor (TF) families, we used the classification rules and tools described in GRASSIUS [44]. The search was carried out using HMMER v3.1b1 [93] and all significant HMM hits with *e*-value smaller than $1e^{-3}$ were kept.

Promoter region analysis

Transcription Start Site (TSS) and promoter region classification

We evaluated promoter regions of genes associated with cell wall and sugar metabolism,ScPAL and ScSuSy, respectively, as described above. A total of 47 ScPAL and 44 ScSuSy was used. To extract the candidate promoter region, we selected, when available, up to 1500 nt upstream from the annotated start position of the gene, consisting of a core promoter (500 nt upstream of the start position) and proximal promoter (1000 nt upstream of the core promoter). Next, we used TSSPlant [94] to predict the TSS of the genes and the type of promoter (TATA-box, TATA-less). The software was set to report high score, sense only TSSs.

Transcription Factor Binding Site (TFBS) in silico characterization

The annotation of TFBSs in the proximal promoter regions was performed in two steps: *de novo* prediction of TFBS motifs in smaller subsets of sequences and mapping the predicted TFBSs in the remaining promoter sequences. Sequences were partitioned in 10 subsets: five ScPAL groups and five ScSuSy groups. We then applied MEME [95] and MotifSampler [96], with default parameters, to each of these datasets to determine putative TFBS motifs. Both were restricted to search for at most 6 motifs with 10nt or less. MEME candidates

were a subset of MotifSampler's. MotifSampler ran for 100 cycles; following the manual we selected, from the 10 top-ranked motifs, the first 5 that occurred at least 10 times in the different cycles. Each of the resulting 35 candidate motifs was searched in the JASPAR public database [97], with partial positive matches for all of them.

To evaluate the significance of the motifs we measured their frequency in promoter regions of each of the original gene families and compared them with the frequency of each of these motifs in the promoter regions of the other SP80-3280 predicted genes. We also mapped the motifs of each ScSuSy and ScPAL gene family respectively in the promoter region of the ScSuSy and ScPAL genes from *S. spontaneum* and R570. Candidate motifs were mapped with MotifLocator [96]. For characterizing background sequences, we trained a first order Markov chain [96] trained on SP80-3280 coding regions that were previously shuffled using the fasta-shuffle-letters tool [95]. The parameters were set to full match of the motif in the target sequence and score 95% above of the background.

Co-expression analysis

A field experiment was conducted at the Agricultural Sciences Center of the Federal University of São Carlos in Araras (22°21’25’’S and 47°23’3’’W) in the state of Sao Paulo, Brazil. Trial plots of SP-3280 consisted of four rows of 10 m long and spaced 1.35m apart. The field experiment was initiated in October 2012 and extended up until November 2013, representing the conditions under which “one-year” sugarcane crops are cultivated. Aiming to carry out observations throughout growth and development, tissue samples of the +1 leaves (L1) and upper (I1), immature (I5) and mature (I9) internodes were collected from two plots (two technical replicates) after 4, 8, 11 and 13 months of planting.

RNA was extracted for four biological replicates, two from each plot, using the TriZol method, treated with DNase I and purified. A pool of samples from leaves and a pool of internodes was used as a 'reference sample' for hybridization experiments on a customized 4×44 K oligoarray (Agilent Technologies) for sugarcane (CaneRegNet), conducted following the recommendations proposed by Lembke et al. [97]. The oligoarrays were read using the GenePix 4000B scanner device (Molecular Devices) and the fluorescence data was processed by Feature Extraction software 9.5.3 (Agilent Technologies).

Log2 transformed expression data was used for discovery and the analysis of co-expression modules, on CEMiTool R package [98]. The adjacency matrix was calculated by estimating the Spearman's correlation coefficient between all pair of genes and raised to a soft thresholding power (β) of 14. TopGO R package [99] was used for gene ontology enrichment analysis for each module and node and edge files were generated for use with the Cytoscape network visualization program [100].

SNP variants (SNVs) analysis compared to genic regions in *Sorghum bicolor*

The 450,609 sugarcane contigs (183,322 singletons and 267,287 unitigs) were aligned to the sorghum genome sequence [48] using the BWA MEM v0.7.10 [101] and reads with alignment score larger than 20 were used for variant calling. SNVs were called using samtools v1.1 and bcftools v1.1 [85]. Using in-house python scripts, extracted SNVs were screened when sugarcane contigs were located on the genic regions of the sorghum genome and two or more sugarcane contigs were aligned to the same sorghum gene. Then, the number of SNVs in each gene was counted according to four-base changes.

SNVs that are homozygous in sugarcane were extracted for further analysis. Large-effect SNVs were identified as those mapped to coding regions, splicing sites, stop codons and transcription initiation sites.

Functional Enrichment Test

Arabidopsis GO-slim gene annotation was used for functional enrichment analysis. GO-slim terms were assigned to sugarcane genes based on sequence similarity inferred from best BLASTp (v2.2.30+) hit. We used a binomial distribution based on the proportion of a GO-slim term among all annotated genes in the sorghum genome as the null distribution. The binomial test was used to assess functional enrichment, with a significance threshold of $p > 0.05$.

Conserved Synteny Blocks

DNA sequences for all CDSs from *S. spontaneum* [47], R570 [13], *Sorghum bicolor* [102] and SP80-3280 were aligned using the BLASTn program. Results from BLAST searches, with e-value <= 10^{-5}, were parsed using an in-house Python script to filter alignments covering at least 70% of the length of both the query and hit sequences. A second filter, requiring at least 80% identity was also applied and the resulting pairs of

queries and hit sequences were classified into putative orthologous groups using the union-find algorithm. We selected putative orthologous groups present in all three organisms but with only one *Sorghum* gene to be used as markers to detect blocks of conserved gene order (syntenic bocks) in comparisons of SP80-3280 and *S. spontaneum* against the genome of *S. bicolor*, thus avoiding the complications of a direct comparison of the two polyploid genomes (**Additional file 1: Fig. S8**). Another Python script was used to detect the syntenic blocks in both *Saccharum* genomes and to count the number of syntenic blocks in each contig.

Chromosome Synteny Multiple Correspondence Analysis with Clustering

We performed a multiple correspondence analysis (MCA) with clustering of the best local alignment hit of masked contigs. Input data were the 450,609 contigs of the sugarcane synthetic long read assembly and the masked genomic sequences of *S. spontaneum* [47] and R570 [13]. We used the masked sugarcane contig sequence produced by the annotation pipeline, excluding 69,879 sequences that were fully masked.

The contigs were aligned to the grass genomes using BWA-SW v0.7.12-r1044 [101]. We used an in-house Perl 5 script to retrieve the highest scoring hit for each contig and generate a table for input into R v3.2.1 [82]. This table contained the chromosome hit, if any, for each contig against each reference genome.

We then used the FactoMineR R package v1.31.3 [103], along with the missMDA missing data handling auxiliary package v1.8.2 [104]. We performed MCA with these data, *i.e.*, chromosome hit number information for each contig was treated as a set of categorical variables and represented in the two principal component dimensions. This was followed by hierarchical clustering in these two dimensions, as well as figure rendering, using the Hierarchical Clustering on Principal Components (HCPC) function of FactoMineR.

In order to identify the correspondence between *S. spontaneum* and R570 chromosomes and SP80-3280 clusters, protein sequence alignment between the cultivar variety and the ancestor and R570 was performed with BlastP considering an e-value threshold of 1e-5. The best hit with a minimum query coverage of 90% was selected for visual representation of the alignment results with Circos plot.

ADDITIONAL FILES

Additional file 1.doc contains Supplemental Figures S1 to S10

Additional file 2.xls contains Supplemental Tables S1 to S13

DECLARATIONS

List of abbreviations

CEGMA: Core Eukaryotic Genes Mapping Approach

BUSCO: Benchmarking Universal Single-Copy Orthologs

ESTs: expressed sequence tags

CDS: coding sequences

SuSy: Sucrose Synthases

PAL: Phenylalanine ammonia-lyase

CCR: Cinnamoyl-CoA reductase

COMT: Caffeic acid 3-O-methyltransferase

4CL: 4-coumarate-CoA ligase

TFBSs: Transcription Factor Binding Sites

TE: transposable elements

MCA: Multiple correspondence analysis

I2C-2: R gene locus

dog1: (delay of germination 1

ABRE: ABA-responsive elements

ABA: abscisic acid

Consent for publication: Not applicable

Availability of data and material

Genomic data is publicly available at NCBI under GenBank Bioproject PRJNA431722. Contig sequence and annotation are also available in a genome browser framework at http://sucest-fun.org/. The microarray data

have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE124990.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Project leaders: GMS, MAVS and DH;

Sample collection and DNA extraction: CGL;

Genome sequencing and assembly: HL, MCS, GRAM, RP and BD;

Genome assembly supervision: DH;

Genome annotation: MAVS, GJW, MYNJ and FTC;

Saccharum spontaneum genome assembly: JZ, XZ, QZ and RM;

BWA-SW analysis: GJW;

BAC sequencing and assembly: MAVS, GJW, GTR, HB and SV;

Synteny analysis: AMD, RFS and GGS;

Reference-guided RNAseq Assembly: MYNJ;

Tissue-Specific Allelic Expression Analysis: MYNJ, CGL and PMA;

Phylogeny analysis: SSF and ALD;

SP80-3280 growth and maturation experiment: MSC, GMS, CGL and ALD

Co-expression analysis: ALD

Regulatory region analysis (TE and TFBS): MAVS, MMO, AMD, GMS, CTH and ALD;

SNP variants (SNVs) analysis: CK, HG and AP;

Organization and management of the author's contributions: CGL, ALD, GMS and MAVS;

Data availability (NCBI and Sucest-fun): FTC;

All authors have read and approved the final version of the manuscript.

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Table 1 – Genome sequencing: Technology and assembly details and gene prediction features.

	Description	Genomic DNA	BAC clones
Sequencing and assembly data	Sequencing Data	26 Illumina synthetic long-read libraries	Single end Roche 454 of BAC library clones
	Total Sequence	19 Gb	6,6 Gb
	Genome	1.9 x	0.66 x
	Read length Min/Max	1,500 bp / 22,904 bp	8 bp / 2611 bp
	Mean read length	4,930 bp	368.5 bp
	Assembler Software	Celera Assembler (Overlap Graph)	PHRAP/CONSED
	Total reads used in assembly	3,857,849	17,894,306
	Total assembly size	4.26 Gb	49.6 Mb
	Number of unitigs/contigs + singletons	450.609	463
	Contigs Length Min/Max/Mean	1,500 bp / 468,011 bp / 9,452 bp	11,723 bp / 235,533 bp / 107,129 bp
	NG50	41,394 bp	109,618 bp
	N50	13,157 bp	N/A
Gene prediction features	# genes	373,869	3,550
	# transcripts	374,774	-
	# exons	1,035,764	13,132
	Average GC content	43.20%	44.99%
	Average # exons per gene	2.8	3.7
	Average exon size [bp]	291	271.8
	Median exon size [bp]	171	154
	Average intron size [bp]	352.6	539.2
	Median intron size [bp]	132	139
	Average gene size [bp] with UTR	1,437.80	2,429.20
	Median gene size [bp] with UTR	806	1,260.50
	Average gene size [bp] without UTR	1,318.80	2,351.30
	Median gene size [bp] without UTR	771	1,199.50
	Average gene density (kb per gene)	11.4	14

Figure captions

Fig. 1 – Gene copy number estimation. (**A**) Distribution of copy counts for putative single copy genes. A total of 1,592 single copy genes from sorghum, rice and *Brachypodium* matched sugarcane predicted genes. More than 99.9% of the aligned single copy genes are present between one and 15 times in the sugarcane gene models. (**B**) Copy differentiation between sugarcane coding sequences (CDS) and upstream regions, based on pairwise sequence alignment of gene clusters. Genetic dissimilarity increases with increasing distance from the translation start site. (**C**) Indel length distribution in sugarcane gene copies. Frame preserving indels are more common than frameshifts for this set of genes.

Fig. 2 – Homo (eo)log expression: Frequency of sugarcane genes plotted against the total number of homo(eo)logs per gene and the number of expressed homo(eo)logs per gene. Genes with cDNAs aligned with FPKM > 1 were considered expressed. Plots show sense (**A**) and antisense (**B**) transcripts. Reads from Ion PGM Sequencing were used, as strand orientation is maintained [29].

Fig. 3 – Phylogenetic, expression and TFBS categorization of SuSy and PAL gene family. Phylogenetic analysis of (**A**) sucrose synthase (SuSy) and (**B**) phenylalanine-ammonia lyase (PAL) genes from SP80-3280, R570, *S. spontaneum*, and sorghum. SuSy sequences from *Saccharum* ssp [35] were also included. Core promoter analysis suggests ScSuSy2 (**C**) and most ScPAL (**D**) as TATA-less and TFBS specific for each clade. The three ScPAL genes marked (*) are present in the same contig. Transposable elements (TEs) were identified within 10 kb upstream from the gene. Heatmap analysis of RNA-Seq data [29] shows more pronounced expression in SP80-3280 internodes of ScSuSy1, ScSuSy2, ScSuSy5 and ScPAL from Clade V. RNA-Seq of leaf tissues indicates more pronounced expression of ScPAL from Clades II and III. ScSuSy4 presents high numbers of TFBS and TE and low expression in all samples.

Fig. 4 – SNP variants. Alignment of sugarcane contigs to the genic regions of sorghum chromosomes (chromosome 1 is on top and 10 is at the bottom). X and Y axes indicate physical distance on each chromosome (mega base pairs, Mb) and the number of single nucleotide variants compared to the sorghum reference genome, respectively. Each dot indicates sorghum genes matching two or more sugarcane contigs.

Fig. 5 – Pseudoassembly of contigs. Multiple correspondence analysis (MCA) with hierarchical clustering of the SP80-3280 assembly against the *S. spontaneum* tetraploid AP85-441 homo(eo)log-resolved assembly [14] and the R570 [13] monoploid genome. **A**: SP80-3280 contigs best hits against AP85-441 and R579 chromosomes and corresponding size of the preliminary scaffolds; Cluster = hierarchical cluster from the MCA. **B** and **C**: Circos plot of the proportion of proteins from SP80-3280 (classified into one of the 6 clusters or as ‘non-clustered’) that align to the AP85-441 and R570 putative chromosomes, respectively.

Figure 1

Click here to access/download;Figure;Fig 1.pdf

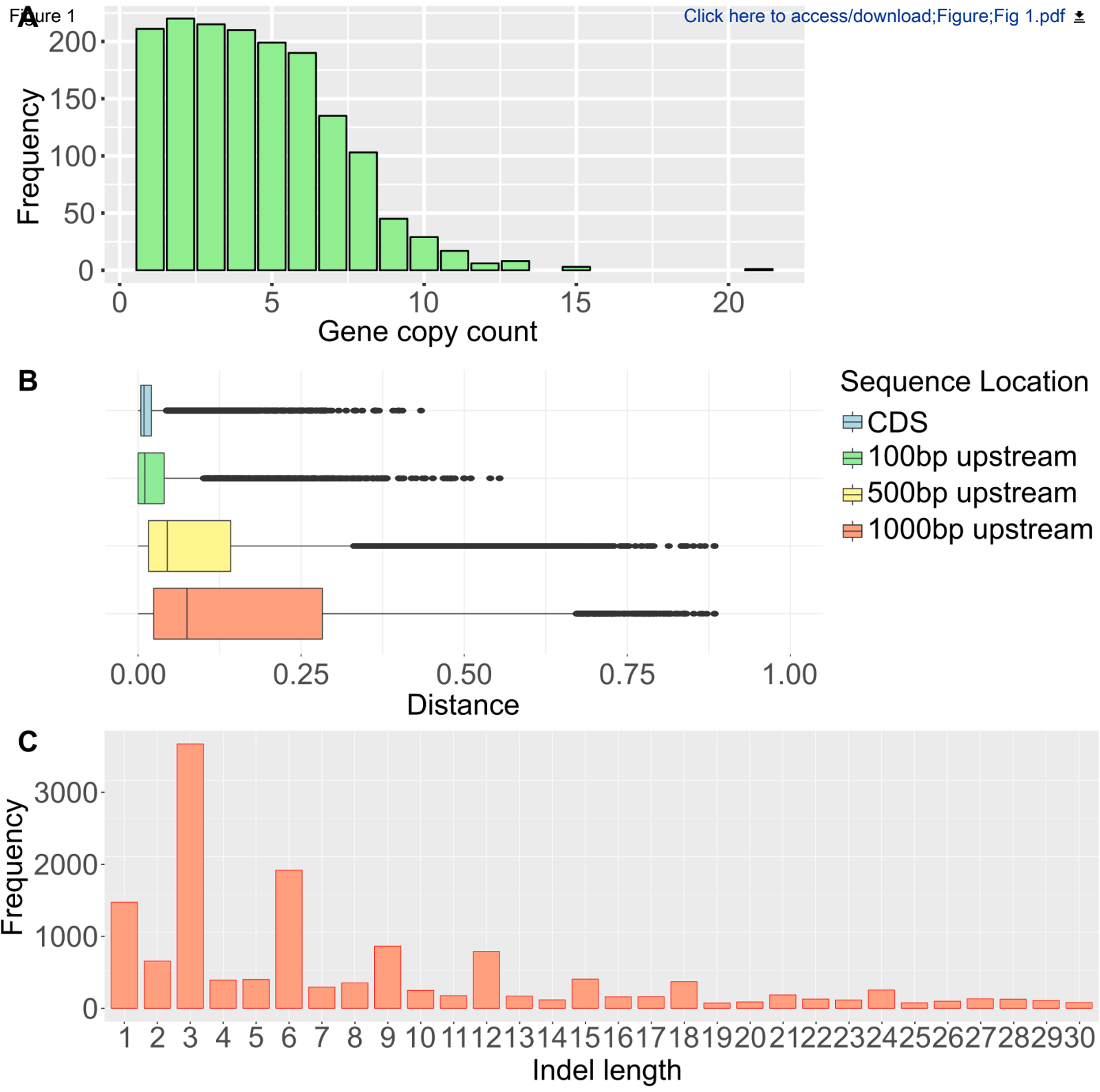

Figure 2

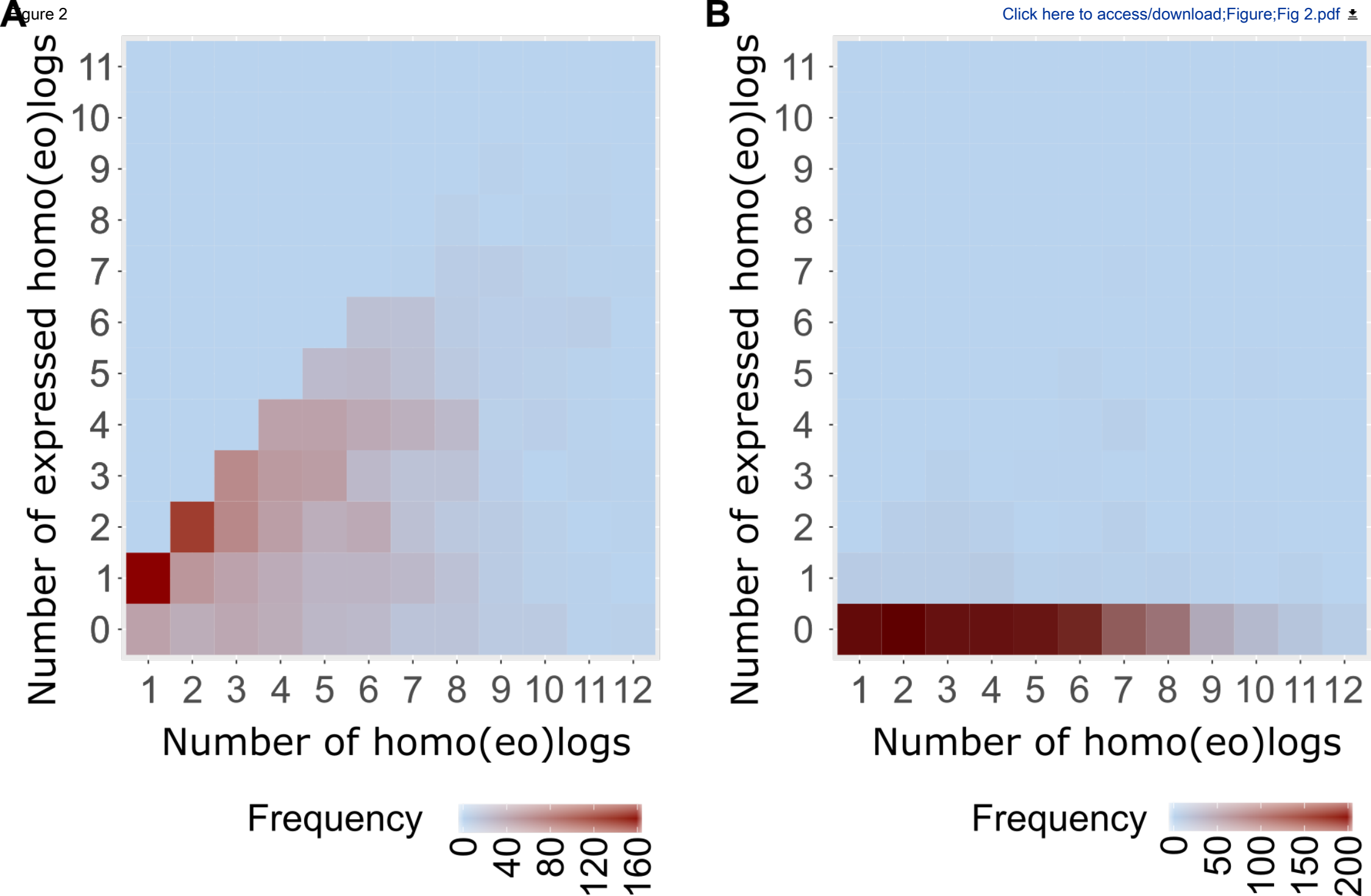

Figure 3

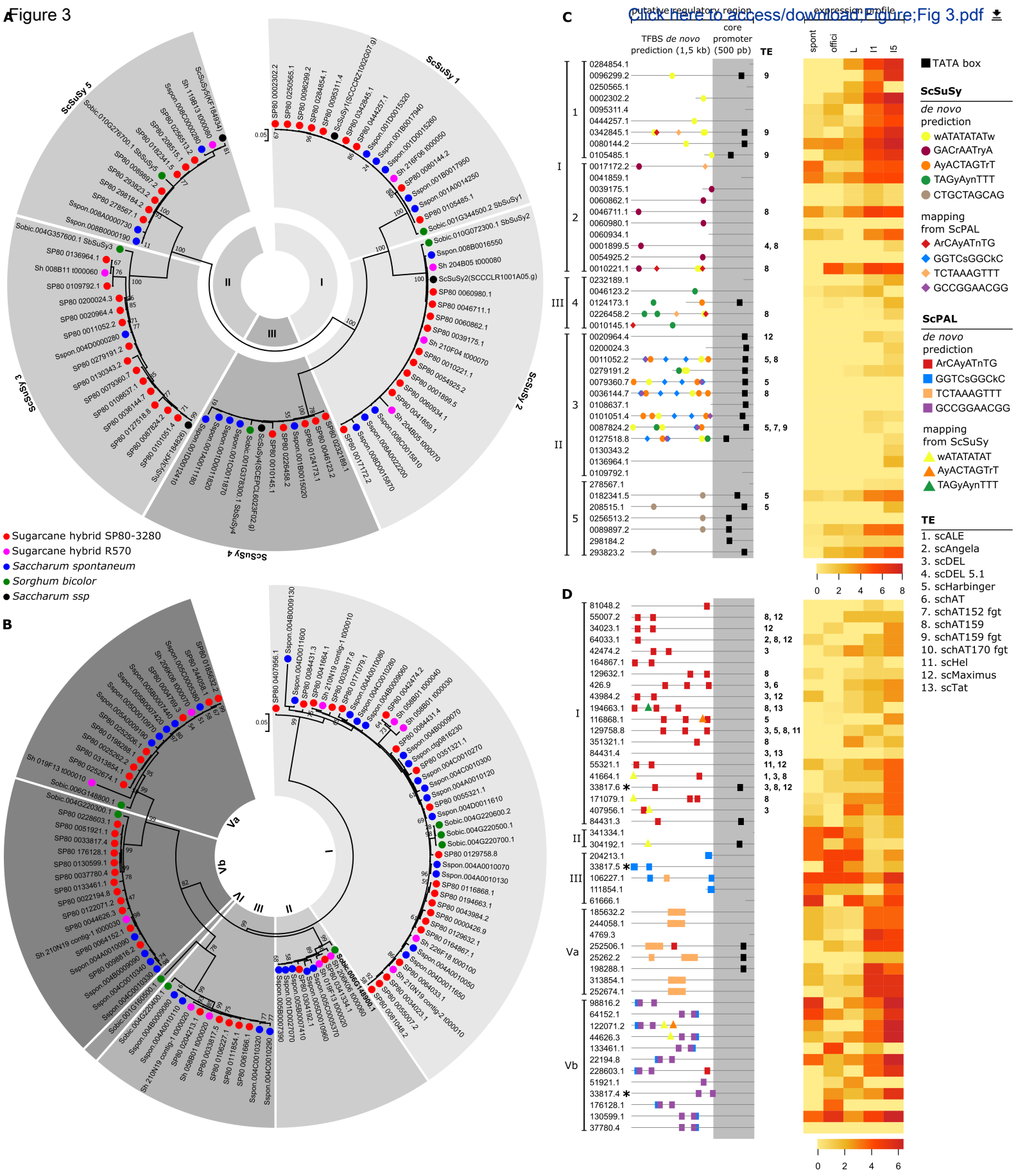

Figure 4

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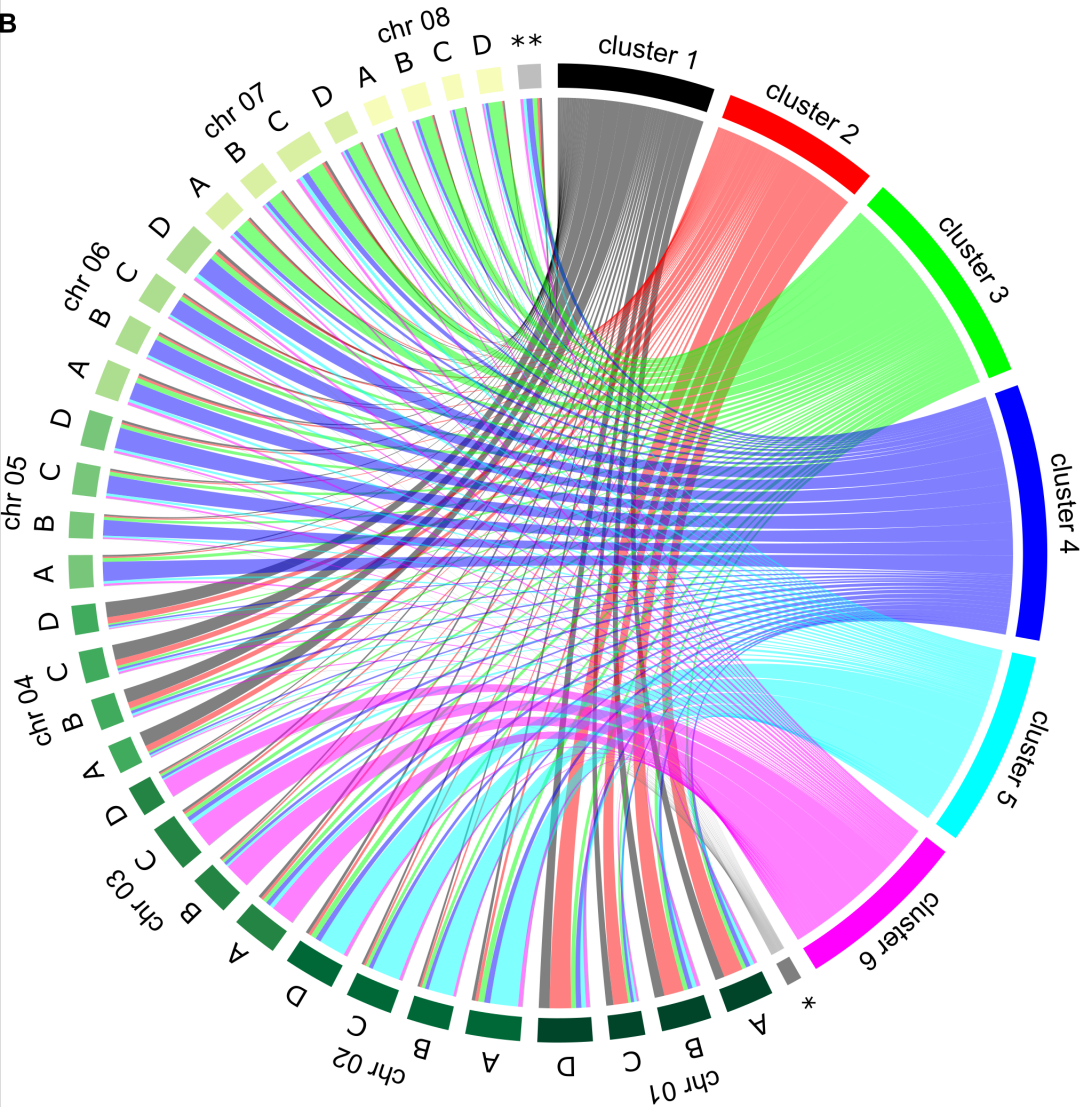
Figure 5

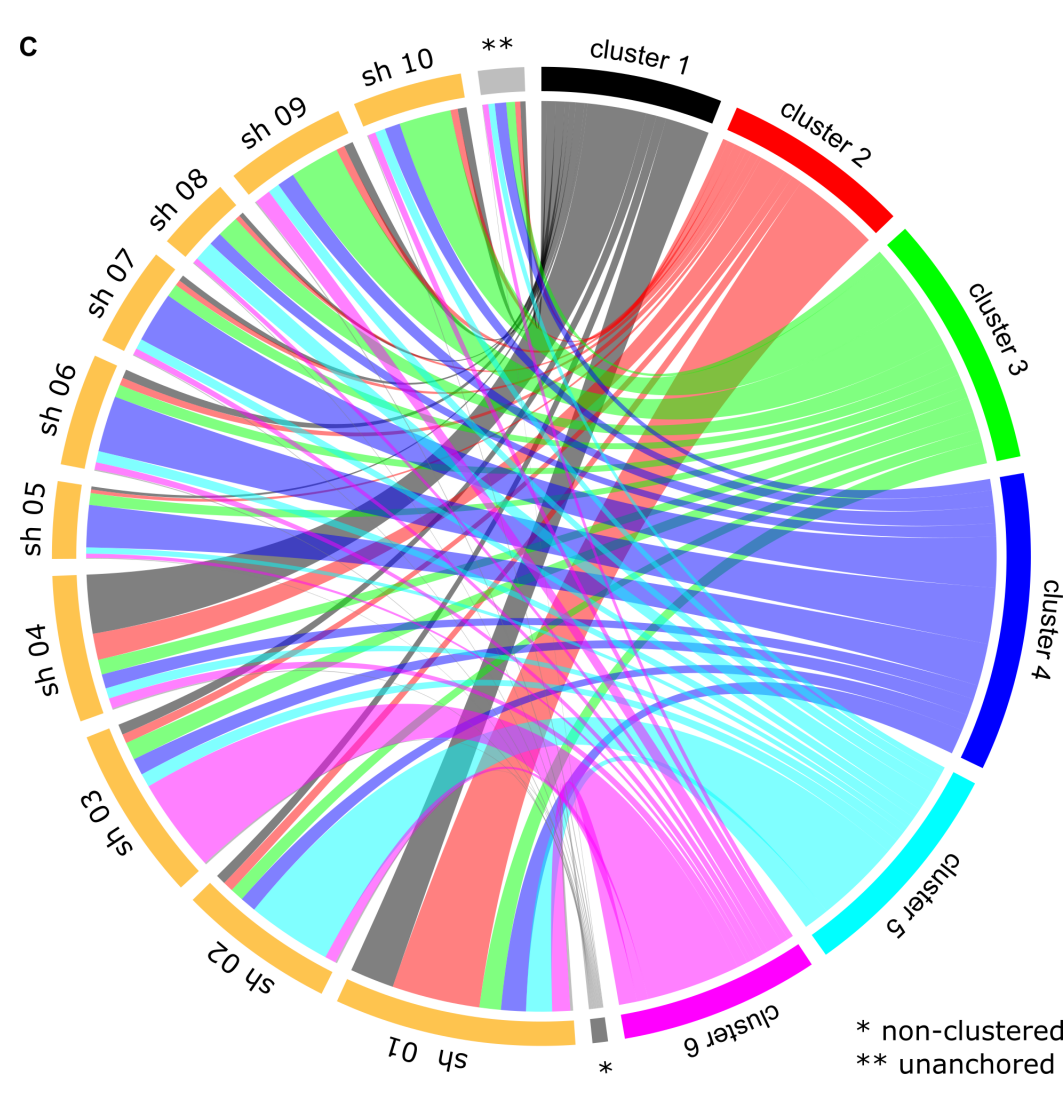
A

Cluster	Number of Contigs	bp	Chromosomal Correspondence	
			S. spontaneum	R570
1	60,150	567,792,642	4	4
2	61,705	574,401,531	1	1
3	87,155	823,254,612	7, 8	8, 9 10
4	90,152	896,362,990	5, 6	5, 6, 7
5	63,996	679,392,733	2	2
6	55,313	565,012,329	3	3
Total	**418,471**	**4,106,216,837**	-	-
Original	**450,609**	**4,259,506,050**	-	-

B



C



Additional file 1

Additional file 2

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Supplementary Material

Souza and Van Sluys et al Additional file 2.xls

Universidade de São Paulo
Instituto de Química

Departamento de Bioquímica

Dear Laurie Goodman,

Editor-in-Chief

GigaScience

On behalf of the co-authors, we would like to submit for your consideration the manuscript by Souza, Van Sluys and colleagues entitled “Assembly of the 373K gene space of the polyploid sugarcane genome reveals reservoirs of functional diversity in the world’s leading biomass crop”.

We sequenced the genome of a sugarcane commercial hybrid and were able to produce a high-quality assembly that resolved the homo(eo)logs of the intricate *Saccharum* complex.

Our assembly contains 373,869 genes from a modern commercial cultivar (SP80-3280) used in breeding programs to generate new varieties and that for many years has been the reference of genomic studies. It is important to note that the assembly offers for the first-time access to the gene-space copy-resolved content of interspecific polyploid commercial sugarcane including gene promoters and a gene network analysis. We produced transcriptome data of field grown sugarcane harvested from planting to maturation including four tissues and found regulatory elements of co-expressed genes within a gene network associated with fiber metabolism. They show promoter regions with different transcription factor binding sites associated to differentially expressed genes. In addition, annotation of transposable elements contributed to fine-tuning the observed gene diversity in an otherwise highly syntenic genome with Sorghum.

Our gene space of 373 thousand genes represents a much closer representation of the gene content diversity of Saccharum adding valuable new data to previous studies by Gasmeur et al., 2018 (doi: 10.1038/s41467-018-05051-5), Zhang et al., 2018 (https://www.nature.com/articles/s41588-018-0237-2), Riaño-Pachón and Mattiello, 2017 (doi: https://doi.org/10.12688/f1000research.11859.2). In addition, our genome sequence supports its recent allotetraploid nature.

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The data set is a fundamental and large step toward a high-quality chromosome resolved assembly from a current commercial hybrid. We note that as the wheat sequencing genome effort, a multi-initiative is necessary to approach complex genomes, but different from wheat, sugarcane genetics is hindered by it being mainly propagated through cuttings and crosses being performed only for breeding purposes. The availability of a polyploid gene-space will be a valuable step towards breeding in a plant extraordinarily difficult to yield genetic maps.

This Whole Genome Shotgun project is publicly available at NCBI under GenBank Bioproject PRJNA431722. Finally, we assure that all gene/protein names and symbols used in this manuscript are in accordance to approved nomenclature guidelines for Saccharum species.

We hope you find this achievement to be of interest to GigaScience and look forward to hearing from you.

Sincerely,

Glaucia Mendes Souza
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