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ORIGINAL PAPER

SSR-based genetic maps of *Miscanthus sinensis* and *M. sacchariflorus*, and their comparison to sorghum

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Abstract We present SSR-based genetic maps from a cross between *Miscanthus sacchariflorus* Robustus and *M. sinensis*, the progenitors of the promising cellulosic biofuel feedstock *Miscanthus* × *giganteus*. cDNA-derived SSR markers were mapped by the two-way pseudo-test-cross model due to the high heterozygosity of each parental species. A total of 261 loci were mapped in *M. sacchariflorus*, spanning 40 linkage groups and 1,998.8 cM, covering an estimated 72.7% of the genome. For *M. sinensis*, a total of 303 loci were mapped, forming 23 linkage groups and 2,238.3 cM, covering 84.9% of the genome. The use of cDNA-derived SSR loci permitted alignment of the *Miscanthus* linkage groups to the sorghum chromosomes, revealing a whole genome duplication affecting the *Miscanthus* lineage after the divergence of subtribes Sorghinae and Saccharinae, as well as traces of the pan-cereal whole genome duplication. While the present maps provide for many early research needs in this emerging crop, additional markers are also needed to improve map density and to further characterize the structural changes of the *Miscanthus* genome since its divergence from sorghum and *Saccharum*.

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Introduction

Global warming associated with combusting a diminishing supply of fossil fuels has motivated the search for renewable energy sources that have minimal effects on environments. Unlike hydro-, wind, solar, and geothermal power, biomass can directly replace fossil energy by burning, or by producing liquid fuels. Feedstocks for "second generation" cellulosic bioethanol such as *Miscanthus* have gained attention because they are highly efficient biomass accumulators, non-food crops, and adaptable to marginal lands not well suited to food crop production.

The genus *Miscanthus* is a member of the grass subtribe Saccharinae, including *Saccharum* (sugarcane), *Erianthus, Narenga,* and *Sclerostachya*. These grasses form an interbreeding group called the *Saccharum* complex, and are members of the tribe Andropogoneae (Daniels et al. 1975). Currently, 14 *Miscanthus* species are recognized with most of them native to southeastern Asia, but some are endemic to Polynesia, the Himalayas and southern Africa (Hodkinson et al. 2002a). *Miscanthus* × *giganteus* is a species used as an ornamental that has been considered as a biomass crop for at least two decades in Europe and the United States (Clifton-Brown et al. 2001). One clone of *Miscanthus* × *giganteus*, a sterile and vegetatively propagated triploid ($2n = 3x = 57$), is believed to originate from a cross

between allotetraploid *M. sacchariflorus* × *M. sinensis* ($2n = 4x = 76$) and *M. sinensis* ($2n = 2x = 38$), and generally yields 2 ~ 3 times more biomass in the US Midwest than another prospective biomass crop, switchgrass (Heaton et al. 2008; Hodkinson et al. 2002c). *M. sacchariflorus* accessions from China, the primary center of diversity for the genus, are nearly always diploid ($2n = 2x = 38$) (J. Jiang, Hunan Agricultural University, personal communication). In contrast, Japanese accessions that have often been referred to in the literature as *M. sacchariflorus* are typically tetraploid though sometime triploid or rarely pentaploid ($2n = 5x = 95$), with no diploids reported from Japan (Adati and Shiotani 1962; Hirayoshi et al. 1957). Multiple lines of evidence indicate that the Japanese polyploids that have often been called *M. sacchariflorus* are in fact allopolyploid hybrids of *M. sacchariflorus* and *M. sinensis* (Adati and Shiotani 1962; Hodkinson et al. 2002c; Lafferty and Lelley 1994; Linde-Laursen 1993). Hodkinson and Renvoize (2001) have defined the nothospecies, *Miscanthus* × *giganteus*, as a hybrid of *M. sinensis* and *M. sacchariflorus*.

Genetic linkage maps, particularly those based on DNA markers, have provided an efficient tool for many applications in crop breeding. Genetic mapping is particularly valuable to identify genetic factors that are quantitatively inherited and/or only expressed under particular conditions (such as pest or pathogen attack, or drought). Genetic markers linked to agriculturally important traits can be used for marker-assisted selection at early stages to accelerate breeding of desirable cultivars. Genetic maps using sequence-based markers, such as restriction fragment length polymorphism (RFLP) (Botstein et al. 1980), simple sequence repeat (SSR) (Akkaya et al. 1992), and single nucleotide polymorphism (SNP) (Jordan and Humphries 1994), provide transferable genetic information, enabling various comparative genomics approaches.

Since the first molecular study in *Miscanthus*, using 13 isozymes for the estimation of genetic variation (Von Wuhlisch et al. 1994), AFLP (Greef et al. 1997; Hodkinson et al. 2002b) and inter-simple sequence repeat (ISSR) (Hodkinson et al. 2002b) have been used to evaluate the genetic diversity of *Miscanthus* species. The intergenic spacer (IGS) of the nuclear genes for ribosomal RNA (rDNA) (Chou et al. 1999), RAPD (Chou et al. 2000), the IGS between *trnL* and *trnF* genes of chloroplastic DNA (cpDNA) (Chiang et al. 2003), RFLP (Iwata et al. 2005), and microsatellite markers (Hung et al. 2009) have also been implemented to study structures of natural populations of *Miscanthus*. The first *Miscanthus* genetic map was constructed using RAPD markers (Atienza et al. 2002). Most *Miscanthus* species are self-incompatible (outcrossing), resulting in a high level of heterozygosity which makes this species similar to many forest or fruit trees using the two-way pseudo-testcross model (Grattapaglia and Sederoff 1994) in genetic mapping studies. This strategy has also been applied to the construction of genetic maps in several grasses such as sugarcane (*Saccharum* spp.) (Al-Janabi et al. 1993; Grivet et al. 1996; Ming et al. 1998), Kentucky bluegrass (*Poa pratensis* L.) (Porceddu et al. 2002), Italian ryegrass (*Lolium multiflorum* Lam.) (Inoue et al. 2004), bermudagrass (*Cynodon* spp.) (Bethel et al. 2006), bahiagrass (*Paspalum nonatum* Flugg) (Stein et al. 2007), and switchgrass (*Panicum virgatum*) (Missaoui et al. 2005; Okada et al. 2010).

We present genetic maps of *M. sacchariflorus* Robustus ($2n = 2x = 38$) and *M. sinensis* ($2n = 2x = 38$) as well as their structural relationships to the sorghum genome. Although the first *Miscanthus* map was generated almost a decade ago (Atienza et al. 2002), its comparison to sorghum or other relatives was not possible due to the use of a semi-arbitrary marker system (RAPD). *Miscanthus* has a basal set of 19 chromosomes while 10 is typical of subtribes Saccharinae (*Saccharum*) and Sorghinae (sorghum). The transition of chromosome numbers from 10 to 19 may be hypothesized to be due to a polyploidization after the divergence of Sorghinae and Saccharinae about 8 million years ago (Kim et al. 2009), however, the alternative possibility of chromosomal fission(s) has not yet been falsified. Cai et al. (2005) successfully applied sugarcane SSRs to the *Saccharum* complex including *Miscanthus*. In a similar manner, we designed SSRs from sugarcane expressed sequence tags (ESTs) and applied them to a *Miscanthus* mapping population, in order to generate EST-SSR-based genetic maps of *Miscanthus*.

Materials and methods

Description of the mapping population

The mapping population comprised 98 F1 individuals from a controlled cross between heterozygous single plants of *M. sacchariflorus* Robustus (female, $2n = 2x = 38$, accession 98m0002) and *M. sinensis* (male, $2n = 2x = 38$, accession 99m0036). *M. sacchariflorus* Robustus is diploid like *M. sinensis*, as is typical of *M. sacchariflorus* from China, the primary center of diversity for the genus.

DNA extraction

Leaf samples were harvested and shipped overnight to the laboratory, then frozen at −80°C and lyophilized for 48 h. About 30 mg dried leaf material was pulverized with the Retsch MM400 bead mill. Genomic DNA was extracted using the NucleoSpin® 96 Plant II kit (Macherey–Nagel) and stored in TE buffer.

SSR design

A set of 12,067 SSRs identified in sugarcane ESTs were assembled from various public databases. This collection contained short ESTs ranging in length from 150 to 400 base pairs (bp), with an average of 290 bp. To assure adequate surrounding sequences for primer design, we attempted to obtain full length ESTs for each of the SSR-containing sequences, by blasting against the full set of sugarcane ESTs collected from the NCBI's dbEST (www.ncbi.nlm.nih.gov/dbEST/) and the TGI database (compbio.dfci.harvard.edu/tgi/). As a result, the following cases were accepted for downstream analysis: (1) if a sugarcane SSR-containing EST sequence perfectly matched one larger EST, the larger one was used, (2) if a SSR did not show a perfect match to any of the ESTs, the original SSR sequence was used, and (3) if a SSR sequence perfectly matched more than one larger EST, each of the ESTs were aligned with MUSCLE (Edgar 2004) and assembled to create a consensus sequence. Then, the extended set of SSRs was aligned using a spliced alignment program, SPLIN (Kapustin et al. 2008), to the sorghum genome ver. 1.0 (Paterson et al. 2009). The split alignments to a single SSR sequence were considered exons of a single gene if two split alignments were less than 5 kbp apart; otherwise, they were treated as alignments to multiple genes. A SSR sequence was chosen for primer design when (1) at least 75% of the length of the SSR sequence was present in the alignment, (2) SSR repeats were located in the center of alignment, and (3) an SSR sequence did not have multiple regions that aligned to greater than 75% of the SSR sequence in length. Approximately 75% of the 12,067 SSR sequences met the criteria. In order to increase the likelihood of cross-taxon application to *Miscanthus*, candidate primer locations were required to have 100% conservation between sugarcane and sorghum in the SSR alignment. Finally, PCR primers were designed with primer3 (Rozen and Skaletsky 2000) using default parameters. Each PCR amplicon was required to span SSR repeats and be greater than 50 bp. Approximately 27% of the 12,067 sugarcane SSRs yielded candidate primers for further analysis.

SSR analysis

The PCR reactions were carried out under standard conditions for all primer pairs using 1 U Taq polymerase with 10× PCR buffer (100 mM Tris–HCl at pH 9, 500 mM KCl and 15 mM $MgCl_2$), 2 mM dNTP, 3 mM $MgCl_2$, 0.2 mM of each primer, and 20 ng of DNA template with a final reaction volume of 10 μL. The thermo-cycling was performed with the following program: (1) preheat at 95°C for 3 min, (2) denaturation at 95°C for 30 s, (3) annealing at 65°C for 1 min (−1°C/cycle), (4) extension at 72°C for 1 min, (5) 10 cycles of steps (2)∼(4), (6) denaturation at 95°C for 30 s, (7) annealing at 55°C for 1 min, (8) extension at 72°C for 1 min, (9) 32 cycles of steps (6)∼(7), and (10) final extension at 72°C for 5 min. The amplified products were visualized in 10% polyacrylamide gels with silver staining. Polymorphic bands were surveyed for presence or absence of an SSR allele based on the two-way pseudo-testcross model (Grattapaglia and Sederoff 1994).

Selection of SSRs for parental screening

A total of 3,271 sugarcane ESTs used for designing SSR primers were blasted against the sorghum genome ver.1.0 (Paterson et al. 2009) at $e < 10^{-10}$ to find putative locations in sorghum chromosomes. Owing to the high level of similarity between sorghum and sugarcane, 2,924 ESTs (6,943 loci) could be located in the sorghum chromosomes. We screened a total of 2,592 SSRs derived from the matched ESTs with the mapping parents. A total of 901 SSRs showing polymorphisms between the parents were further tested with six F1 progenies to confirm their segregation patterns and to select SSRs for genotyping.

Linkage analysis

A total of 98 individuals were genotyped based on the two-way pseudo-testcross model, generating separate genetic maps of the respective parental genomes. The markers fell into three categories: (1) maternal testcross markers which are heterozygous in *M. sacchariflorus* Robustus (MSA), (2) paternal testcross markers which are heterozygous (band present) in *M. sinensis* (MSI), and (3) bi-parental markers that are heterozygous in both parents. The Chi-square test ($P < 0.05$) verified marker loci which showed a Mendelian segregation ratio of 1:1 (marker types 1 and 2) or 3:1 (marker type 3). Two separate datasets were assembled, one for the genetic map of MSA (marker types 1 and 3) and the other for the genetic map of MSI (marker types 2 and 3). Since the linkage phase of each marker locus is unknown in the pseudo-testcross model, the original dataset was duplicated for each parent and allelic designations were reversed. MAPMAKER/EXP ver. 3.0 (Lander et al. 1987) was used for map construction with the data type 'F2 backcross' which is suitable for the pseudo-testcross configuration. Map distances, in centiMorgans (cM), were calculated using the Kosambi function. Marker loci were grouped by two-point linkage analysis with a LOD threshold of 4.0 and a maximum distance of 30 cM. Local maximum likelihood orders of marker loci were confirmed using the 'ripple' command. The genetic maps were drawn using MapChart ver. 2.2 (Voorrips 2002).

Marker distribution, genome length and map coverage

To examine marker distribution over the entire map, the Pearson correlation coefficient was used to test the relationship between linkage group (LG) size (in cM) and number of markers. For the estimation of genome length, two different approaches were implemented. First, the average marker spacing/interval (s) was calculated by dividing the total map length by the number of intervals (number of markers minus number of LGs). The estimated genome length (G_{e1}) was determined by adding $2s$ to the length of each LG to account for chromosome ends (Fishman et al. 2001). Second, an estimated genome length (G_{e2}) was calculated by multiplying the length of each LG by $(m + 1)/(m-1)$, where m is the number of markers in each group (Chakravarti et al. 1991). The observed map coverage was estimated by G_o/G_e, where G_o and G_e are the length of genetic map and the average of the estimated genome length from the two approaches, respectively.

Comparative mapping

The putative locations of EST-SSRs in sorghum chromosomes were compared to the locations of the EST-SSRs used for *Miscanthus* mapping. As proposed by Ming et al. (1998) for *Saccharum*, *Miscanthus* homology groups (HGs) were defined by counting the numbers of corresponding markers based on the blast results between the EST-SSRs mapped in *Miscanthus* and sorghum genome sequences ($e < 10^{-10}$). Linkage groups were generally considered homologous when three or more orthologous loci were in common between sorghum and *Miscanthus*. However, for small LGs ($\leq$6 loci), HGs were accepted when sorghum and *Miscanthus* shared two orthologous loci. The alignments between *Miscanthus* LGs and sorghum chromosomes were visualized by Circos (Krzywinski et al. 2009).

Results

Marker segregation and map construction

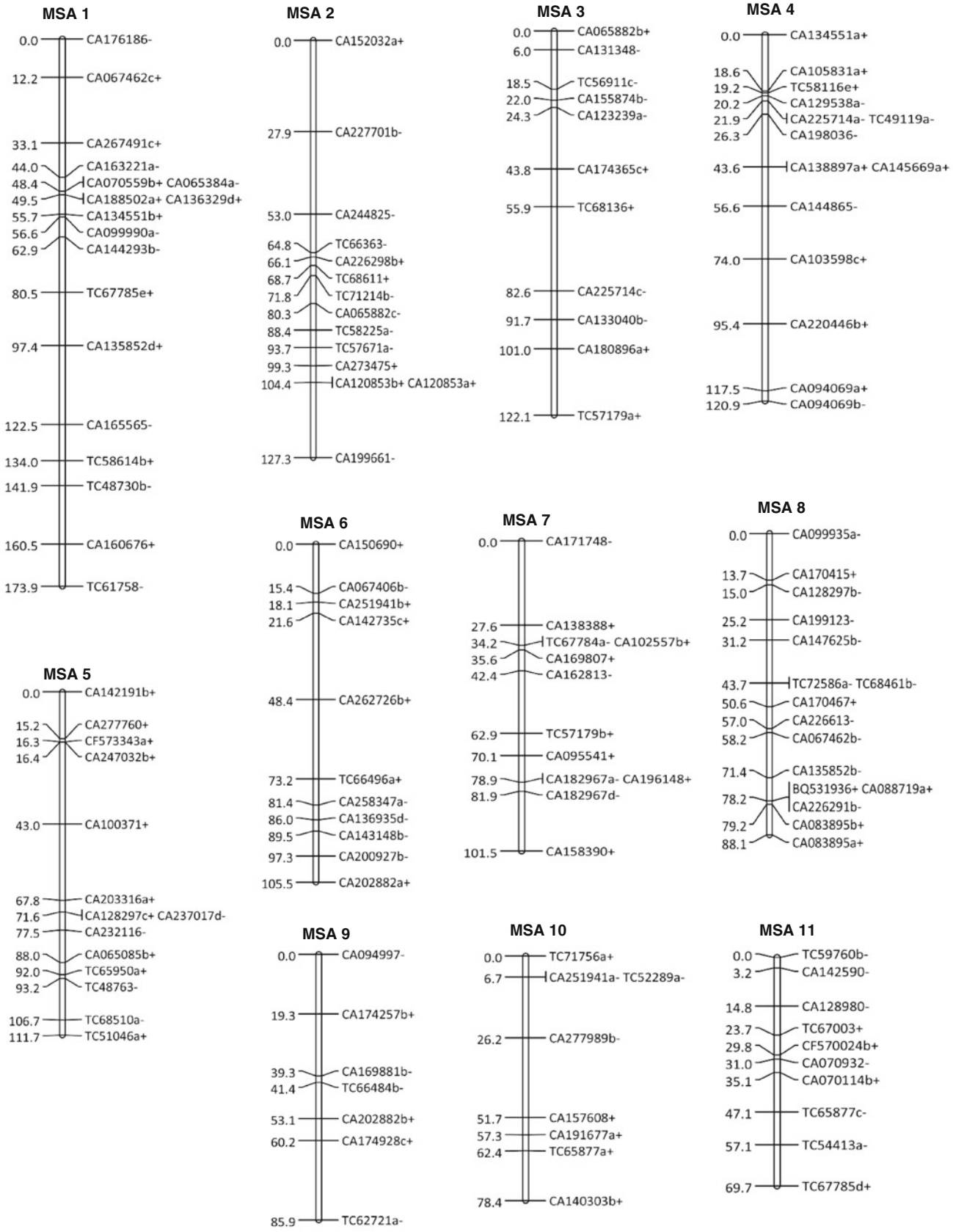
We initially selected 669 SSRs for genotyping based on similarities to sorghum sequences and parental screening (see "Materials and methods"). Nonetheless, only 409 SSRs (segregating for 673 marker loci) were actually genotyped, with the remainder rejected due to weak signals or false positives. Out of the genotyped SSRs, 317 and 349 loci were pseudo-testcross markers (1:1 segregation) in MSA and MSI, respectively, whereas only seven bi-parental markers (3:1 segregation) were genotyped (Table 1). Of the genotyped loci, 279 in MSA and 326 in MSI did not deviate from expected Mendelian segregation ratios at $P < 0.05$. We excluded distorted loci to avoid potential ambiguity in constructing the maps at this early stage. We used a two-stage mapping strategy to take maximum advantage of the sorghum genome sequence: First, we constructed frameworks of markers that were more or less evenly distributed in sorghum based on the blast search, and then tried to join fragmented LGs by selecting additional markers from 'gaps' in the sorghum sequence not covered by the fragmented LGs. Both maps were constructed using a LOD score of 4.0 with maximum distance of 30 cM. For the MSA map, LOD scores of 3.0 and 4.0 resulted in 40 LGs while 41 LGs and 42 LGs were found under LOD 5.0 and 6.0, respectively. For the MSI map, LOD scores from 3.0 to 7.0 all provided 23 LGs. Although fewer LGs were generated in both maps when maximum distance was increased to 50 cM, the maximum distance was held at 30 cM to avoid large gaps in LGs.

The MSA map contained 261 marker loci in 40 LGs (Fig. 1) with 18 unlinked markers. The length of the MSA

Table 1 Parental origins of the marker loci used for each step of mapping

	M. sacchariflorus	*M. sinensis*
Numbers of genotyped loci		
Pseudo-testcross	317	349
Bi-parental	7	7
Sum	324	356
Numbers of Mendelian-segregating loci at $P < 0.05$		
Pseudo-testcross	274	321
Bi-parental	5	5
Sum	279	326
Numbers of mapped loci		
Pseudo-testcross	259	301
Bi-parental	2	2
Sum	261	303
Numbers of unlinked loci	18	23

Fig. 1 Genetic linkage map of *M. sacchariflorus* Robustus (MSA) ▶ constructed from an outbred population between *M. sacchariflorus* Robustus ($2n = 2x = 38$) and *M. sinensis* ($2n = 2x = 38$). The map consists of 40 linkage groups with 261 markers and 1,999.8 cM in total length. EST-SSR markers are labeled with GenBank accession numbers of ESTs from which SSRs are derived, except the names beginning with TC. The TC-type markers indicate the TGI database marker names that are derived from assembled ESTs (compbio.dfci.harvard.edu/tgi/). Markers are indicated on the *right* while distances are on the *left* in Kosambi cM (cumulative). The *alphabetical suffix* at the end of the accession numbers indicates SSRs that were genotyped multiple times. The suffix '±' designates alternative linkage phases. Markers covered by vertical lines have no detectable distances among them. The 40 linkage groups were arranged according to map length


MSA 1
0.0 CA176186-
12.2 CA067462c+
33.1 CA267491c+
44.0 CA163221a-
48.4 CA070559b+ CA065384a-
49.5 CA188502a+ CA136329d+
55.7 CA134551b+
56.6 CA099990a-
62.9 CA144293b-
80.5 TC67785e+
97.4 CA135852d+
122.5 CA165565-
134.0 TC58614b+
141.9 TC48730b-
160.5 CA160676+
173.9 TC61758-
MSA 2
0.0 CA152032a+
27.9 CA227701b-
53.0 CA244825-
64.8 TC66363-
66.1 CA226298b+
68.7 TC68611+
71.8 TC71214b-
80.3 CA065882c-
88.4 TC58225a-
93.7 TC57671a-
99.3 CA273475+
104.4 CA120853b+ CA120853a+
127.3 CA199661-
MSA 3
0.0 CA065882b+
6.0 CA131348-
18.5 TC56911c-
22.0 CA155874b-
24.3 CA123239a-
43.8 CA174365c+
55.9 TC68136+
82.6 CA225714c-
91.7 CA133040b-
101.0 CA180896a+
122.1 TC57179a+
MSA 4
0.0 CA134551a+
18.6 CA105831a+
19.2 TC58116e+
20.2 CA129538a-
21.9 CA225714a- TC49119a-
26.3 CA198036-
43.6 CA138897a+ CA145669a+
56.6 CA144865-
74.0 CA103598c+
95.4 CA220446b+
117.5 CA094069a+
120.9 CA094069b-
MSA 5
0.0 CA142191b+
15.2 CA277760+
16.3 CF573343a+
16.4 CA247032b+
43.0 CA100371+
67.8 CA203316a+
71.6 CA128297c+ CA237017d-
77.5 CA232116-
88.0 CA065085b+
92.0 TC65950a+
93.2 TC48763-
106.7 TC68510a-
111.7 TC51046a+
MSA 6
0.0 CA150690+
15.4 CA067406b-
18.1 CA251941b+
21.6 CA142735c+
48.4 CA262726b+
73.2 TC66496a+
81.4 CA258347a-
86.0 CA136935d-
89.5 CA143148b-
97.3 CA200927b-
105.5 CA202882a+
MSA 7
0.0 CA171748-
27.6 CA138388+
34.2 TC67784a- CA102557b+
35.6 CA169807+
42.4 CA162813-
62.9 TC57179b+
70.1 CA095541+
78.9 CA182967a- CA196148+
81.9 CA182967d-
101.5 CA158390+
MSA 8
0.0 CA099935a-
13.7 CA170415+
15.0 CA128297b-
25.2 CA199123-
31.2 CA147625b-
43.7 TC72586a- TC68461b-
50.6 CA170467+
57.0 CA226613-
58.2 CA067462b-
71.4 CA135852b-
78.2 BQ531936+ CA088719a+
CA226291b-
79.2 CA083895b+
88.1 CA083895a+
MSA 9
0.0 CA094997-
19.3 CA174257b+
39.3 CA169881b-
41.4 TC66484b-
53.1 CA202882b+
60.2 CA174928c+
85.9 TC62721a-
MSA 10
0.0 TC71756a+
6.7 CA251941a- TC52289a-
26.2 CA277989b-
51.7 CA157608+
57.3 CA191677a+
62.4 TC65877a+
78.4 CA140303b+
MSA 11
0.0 TC59760b-
3.2 CA142590-
14.8 CA128980-
23.7 TC67003+
29.8 CF570024b+
31.0 CA070932-
35.1 CA070114b+
47.1 TC65877c-
57.1 TC54413a-
69.7 TC67785d+

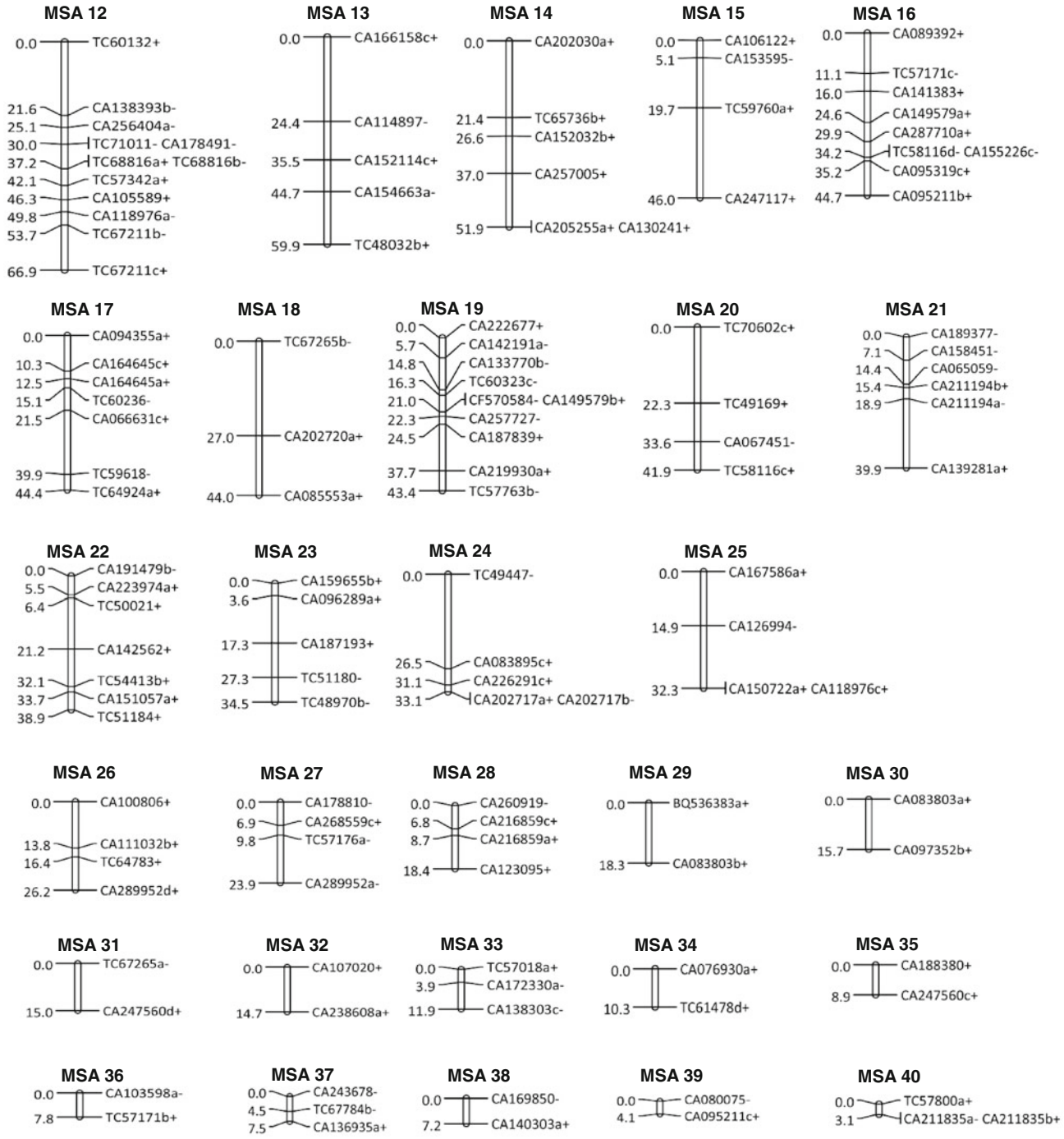


Fig. 1 continued

map was 1,999.8 cM and the average interval between loci was 9.0 cM (Table 2). The MSI map consisted of 303 marker loci in 23 LGs (Fig. 2) with 23 unlinked markers. The length of the MSI map was 2,238.3 cM, and the average interval between loci was 8.0 cM, with the larger number of markers more than compensating for its greater length than the MSA map.

Excluding two LGs with only three markers (MSIs 20, 21) and two with only two markers (MSIs 22, 23), the remaining number of LGs in the MSI map is consistent with the basal chromosome number of the parental species ($2n = 2x = 38$, $x = 19$). On the other hand, the MSA map is fragmented when compared to the MSI map, indicating that more markers are needed to join small LGs to other

Table 2 Length, number of markers, average spacing, and largest intervals of *M. sacchariflorus* Robustus (MSA) and *M. sinensis* (MSI) genetic maps

LGs	Length (cM)	Number of loci	Average interval (cM)	Largest interval (cM)
MSA				
1	173.9	18	10.2	25.1
2	127.3	14	9.8	27.9
3	122.1	11	12.2	26.7
4	120.9	14	9.3	22.1
5	111.7	14	8.6	26.6
6	105.5	11	10.6	26.8
7	101.5	12	9.2	27.6
8	88.1	16	5.9	13.7
9	85.9	7	14.3	25.7
10	78.4	8	11.2	25.5
11	69.7	10	7.7	12.6
12	66.9	12	6.1	21.6
13	59.9	5	15.0	24.4
14	51.9	6	10.4	21.4
15	46	4	15.3	26.3
16	44.7	9	5.6	11.1
17	44.4	7	7.4	18.4
18	44	3	22.0	27.0
19	43.4	10	4.8	13.2
20	41.9	4	14.0	22.3
21	39.9	6	8.0	21.0
22	38.9	7	6.5	14.8
23	34.5	5	8.6	13.7
24	33.1	5	8.3	26.5
25	32.3	4	10.8	17.4
26	26.2	4	8.7	13.8
27	23.9	4	8.0	14.1
28	18.4	4	6.1	9.7
29	18.3	2	18.3	18.3
30	15.7	2	15.7	15.7
31	15	2	15.0	15.0
32	14.7	2	14.7	14.7
33	11.9	3	6.0	8.0
34	10.3	2	10.3	10.3
35	8.9	2	8.9	8.9
36	7.8	2	7.8	7.8
37	7.5	3	3.8	4.5
38	7.2	2	7.2	7.2
39	4.1	2	4.1	4.1
40	3.1	3	1.6	3.1
Total	1,999.8	261	9.0[a]	
MSI				
1	211.8	16	14.1	27.2
2	187.5	24	8.2	22.6
3	162	20	8.5	25.9
4	144.4	15	10.3	21.3
5	140.9	19	7.8	26.6
6	137.9	16	9.2	24.4
7	129	24	5.6	19.2
8	118.1	15	8.4	27.9
9	114.4	20	6.0	23.6
10	110.8	14	8.5	18.1
11	110.7	12	10.1	27.7
12	101	15	7.2	17.2
13	92.6	11	9.3	21.9
14	84.5	10	9.4	21.9
15	83.2	18	4.9	21.8
16	73.1	14	5.6	14.5
17	52.4	13	4.4	10.2
18	49.6	6	9.9	19.2
19	46.3	11	4.6	18.9
20	31.5	3	15.8	20.2
21	22.2	3	11.1	16.0
22	17.5	2	17.5	17.5
23	16.9	2	16.9	16.9
Total	2,238.3	303	8.0[a]	

[a] Average of all linked markers

LGs. Bi-parental markers are commonly used in pseudo-testcross mapping to connect two parental maps into a consensus map but we could map only two bi-parental markers. Thus, homologous chromosomes between MSA and MSI could not be assigned directly because of the lack of bi-parental marker information (but see below for indirect assignments based on alignments to sorghum). Linkage groups were numbered according to their length.

Marker distribution and genome coverage

Although there are several methods to estimate genome length, two methods (see "Materials and methods") were implemented to estimate *Miscanthus* genome length from the genetic maps. Since the two methods assume that markers are randomly distributed in a map, we first calculated the Pearson correlation coefficient to test the relationship between LG size (in cM) and number of markers. The coefficient values of MSA and MSI were 0.91 and 0.82, respectively, indicating that the marker distributions in both maps are approximately random. Genome length, which was estimated by two methods, showed a slight difference, so we used the average of the two estimates as expected genome length (Table 3). On the basis of the expected genome length, genome coverage for the MSA

and MSI maps is 72.7 and 84.9%, respectively. Considering estimated 1C genome sizes of *M. sacchariflorus* Robustus (2.6×10^9 bp) and *M. sinensis* (2.7×10^9 bp) (Rayburn et al. 2009), the present average interval between marker loci is equivalent to 8.6 Mbp in MSA and 8.2 Mbp in MSI. In the same way, 1 cM represents 0.9 Mbp in MSA and 1.0 Mbp in MSI on average. We were not able to establish a putative monoploid map (defined herein as a map having LGs consistent with the basal chromosome number) of MSA due to fragmentation; however, the MSI map is near monoploidy, excluding the short LGs 20 ~ 23. When the monoploid map is only taken into consideration, genome coverage increases to 87.6%, with marker intervals and cM estimated at 8.6 and 1.1 Mbp, respectively.

Alignment of *Miscanthus* maps to the sorghum genome

By virtue of high similarity among sorghum, sugarcane, and *Miscanthus*, SSRs derived from sugarcane ESTs were located on sorghum chromosomes, which were compared to the parental maps of *Miscanthus*. Although redundancy of some SSR loci in the sorghum genome complicated the alignment between sorghum and *Miscanthus*, many LGs had clear matches to sorghum chromosomes (Table 4). Lacking bi-parental markers, this information also identifies putative HGs between MSA and MSI. The comparison was mainly dependent upon the alignment between sorghum and MSI since the MSA map showed many more LGs than its monoploid number.

Most sorghum chromosomal regions had at least two corresponding LGs in both MSA and MSI, supporting the hypothesis that the common ancestor of MSA and MSI experienced a whole genome duplication after the divergence of subtribes Sorghinae and Saccharinae. Different members of *Miscanthus* HGs show no evidence of either coupling or repulsion phase associations, indicating that they are not regularly pairing or recombining. While present data are not sufficient to evaluate the possibility of localized associations such as 'gene conversion', it appears that the pairs of chromosomes formed by whole-genome duplication in the *Miscanthus* lineage have largely established independent transmission genetics, i.e. that *M. sinensis* and *M. sacchariflorus* Robustus are each largely allopolyploids. For sorghum chromosome (SBI)-MSI, for example, only SBIs 2, 8, and 9 have four corresponding MSI LGs (Table 4), which could indicate additional duplication or partial residual autopolyploidy, and perhaps also some remaining fragmentation of linkage groups due to insufficient DNA marker density.

In addition to evidence of a new genome duplication in the *Miscanthus* lineage, we also observed traces of the pan-cereal genome duplication (Paterson et al. 2004; Wang et al. 2005) (*rho*) (Fig. 3). Theoretically, a sorghum chromosome may correspond to two homeologous chromosomes resulting from recent genome duplication in *Miscanthus*, and two more resulting from the pan-cereal genome duplication. Multiple matches between MSI and duplication blocks in sorghum chromosomes formed by the pan-cereal duplication, such as MSI 1-SBI 3/9, MSI 2-SBI 2/7, MSI 2-SBI 4/6, MSI 5-SBI5/8-SBI4/10, MSI 7-SBI 1/2-SBI4/10, and MSI 9-SBI 4/10 largely involved sorghum 'paralogs', tracing to the pan-cereal genome duplication (Paterson et al. 2004; Wang et al. 2005).

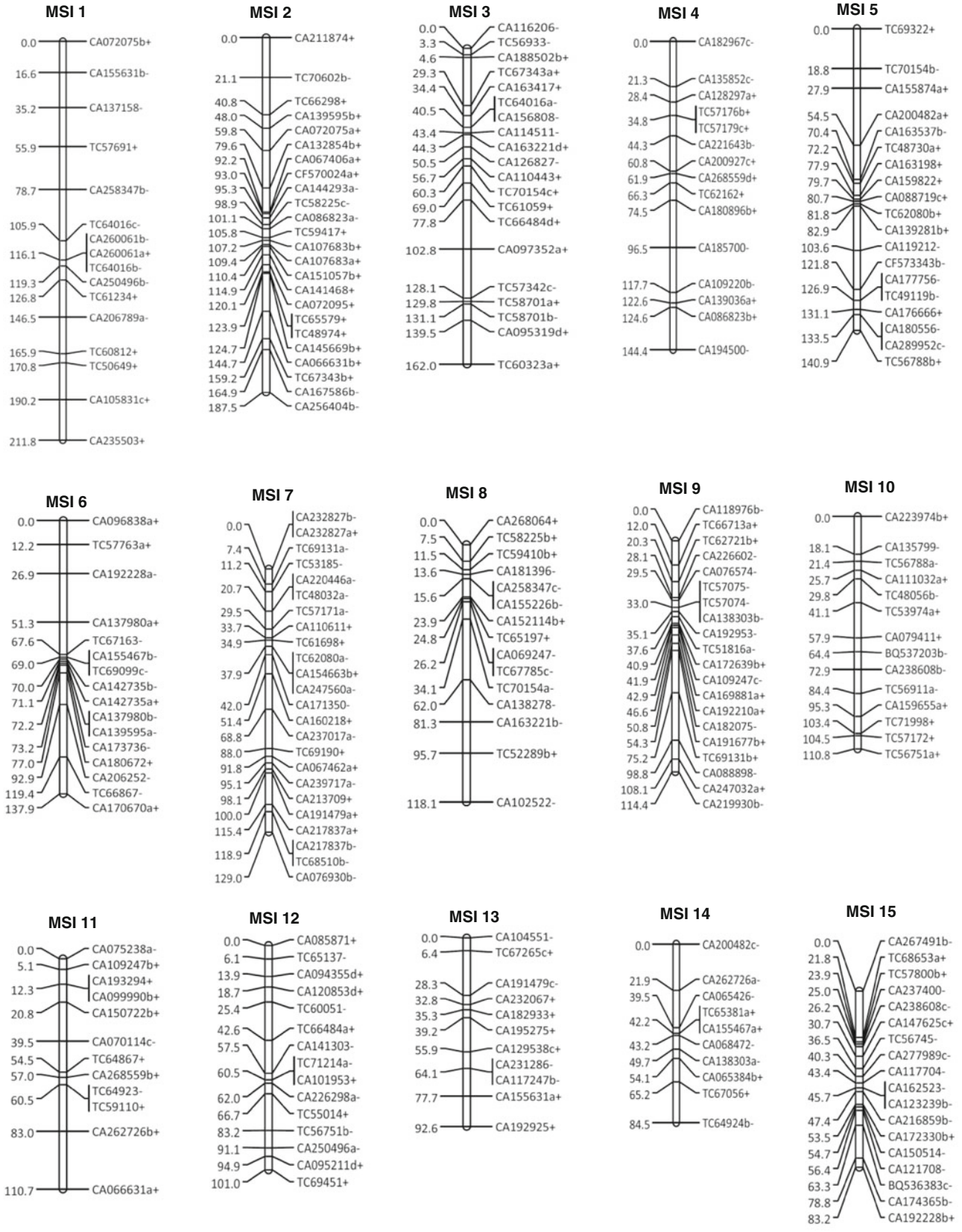
Fig. 2 Genetic linkage map of *M. sinensis* (MSI) constructed from an outbred population between *M. sacchariflorus* Robustus ($2n = 2x = 38$) and *M. sinensis* ($2n = 2x = 38$). The map consists of 23 linkage groups with 303 markers and 2238.3 cM in total length. EST-SSR markers are labeled with GenBank accession numbers of ESTs from which SSRs are derived, except the names beginning with TC. The TC-type markers indicate the TGI database marker names that are derived from assembled ESTs (compbio.dfci.harvard.edu/tgi/). Markers are indicated on the *right* while distances are on the *left* in Kosambi cM (cumulative). The *alphabetical suffix* at the end of the accession numbers indicates SSRs that were genotyped multiple times. The suffix '±' designates alternative linkage phases. Markers covered by *vertical lines* have no detectable distances among them. MSI1 ~ MSI19 are considered as a monoploid map. The 23 linkage groups were arranged according to map length ▶

Discussion

We report SSR-based genetic maps of *M. sacchariflorus* Robustus and *M. sinensis*, progenitors of the promising cellulosic bioethanol feedstock, *Miscanthus* × *giganteus*. A *Miscanthus* genetic map was previously published using a population derived from an intraspecific cross between two *M. sinensis* genotypes (Atienza et al. 2002). However, comparative genomics research was constrained in this prior study because the map was constructed using a semi-arbitrary marker system. Owing to the cross-taxon applicability of SSRs and the full genome sequence of sorghum, we could gain insight into the genomic relationship between *Miscanthus* and sorghum.

Parental genetic maps of *Miscanthus*

Since *Miscanthus* is highly outcrossing and heterozygous, the two-way pseudo-testcross model with an F1 population was used for mapping. In the current study, the population was generated by a cross between *M. sacchariflorus* Robustus and *M. sinensis*. In general, an interspecific cross provides many markers following pseudo-testcross (i.e. 1:1) segregation. In contrast, such a cross yields an extremely low number of bi-parental markers (i.e. 3:1 segregation) which are useful to integrate the two parental maps. For example, the genetic mapping of interspecific crosses


MSI 1
0.0 CA072075b+
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55.9 TC57691+
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105.9 TC64016c-
CA260061b-
116.1 CA260061a+
TC64016b-
119.3 CA250496b-
126.8 TC61234+
146.5 CA206789a-
165.9 TC60812+
170.8 TC50649+
190.2 CA105831c+
211.8 CA235503+
MSI 2
0.0 CA211874+
21.1 TC70602b-
40.8 TC66298+
48.0 CA139595b+
59.8 CA072075a+
79.6 CA132854b+
92.2 CA067406a+
93.0 CF570024a+
95.3 CA144293a-
98.9 TC58225c-
101.1 CA086823a-
105.8 TC59417+
107.2 CA107683b+
109.4 CA107683a+
110.4 CA151057b+
114.9 CA141468+
120.1 CA072095+
123.9 TC65579+
TC48974+
124.7 CA145669b+
144.7 CA066631b+
159.2 TC67343b+
164.9 CA167586b-
187.5 CA256404b-
MSI 3
0.0 CA116206-
3.3 TC56933-
4.6 CA188502b+
29.3 TC67343a+
34.4 CA163417+
40.5 TC64016a-
CA156808-
43.4 CA114511-
44.3 CA163221d+
50.5 CA126827-
56.7 CA110443+
60.3 TC70154c+
69.0 TC61059+
77.8 TC66484d+
102.8 CA097352a+
128.1 TC57342c-
129.8 TC58701a+
131.1 TC58701b-
139.5 CA095319d+
162.0 TC60323a+
MSI 4
0.0 CA182967c-
21.3 CA135852c-
28.4 CA128297a+
34.8 TC57176b+
TC57179c+
44.3 CA221643b-
60.8 CA200927c+
61.9 CA268559d+
66.3 TC62162+
74.5 CA180896b+
96.5 CA185700-
117.7 CA109220b-
122.6 CA139036a+
124.6 CA086823b+
144.4 CA194500-
MSI 5
0.0 TC69322+
18.8 TC70154b-
27.9 CA155874a+
54.5 CA200482a+
70.4 CA163537b-
72.2 TC48730a+
77.9 CA163198+
79.7 CA159822+
80.7 CA088719c+
81.8 TC62080b+
82.9 CA139281b+
103.6 CA119212-
121.8 CF573343b-
CA177756-
126.9 TC49119b-
131.1 CA176666+
CA180556-
133.5 CA289952c-
140.9 TC56788b+
MSI 6
0.0 CA096838a+
12.2 TC57763a+
26.9 CA192228a-
51.3 CA137980a+
67.6 TC67163-
CA155467b-
69.0 TC69099c-
70.0 CA142735b-
71.1 CA142735a+
CA137980b-
72.2 CA139595a-
73.2 CA173736-
77.0 CA180672+
92.9 CA206252-
119.4 TC66867-
137.9 CA170670a+
MSI 7
0.0 CA232827b-
CA232827a+
7.4 TC69131a-
11.2 TC53185-
CA220446a-
20.7 TC48032a-
29.5 TC57171a-
33.7 CA110611+
34.9 TC61698+
TC62080a-
37.9 CA154663b+
CA247560a-
42.0 CA171350-
51.4 CA160218+
68.8 CA237017a-
88.0 TC69190+
91.8 CA067462a+
95.1 CA239717a-
98.1 CA213709+
100.0 CA191479a+
115.4 CA217837a+
CA217837b-
118.9 TC68510b-
129.0 CA076930b-
MSI 8
0.0 CA268064+
7.5 TC58225b+
11.5 TC59410b+
13.6 CA181396-
CA258347c-
15.6 CA155226b-
23.9 CA152114b+
24.8 TC65197+
CA069247-
26.2 TC67785c-
34.1 TC70154a-
62.0 CA138278-
81.3 CA163221b-
95.7 TC52289b+
118.1 CA102522-
MSI 9
0.0 CA118976b-
12.0 TC66713a+
20.3 TC62721b+
28.1 CA226602-
29.5 CA076574-
TC57075-
33.0 TC57074-
CA138303b-
35.1 CA192953-
37.6 TC51816a-
40.9 CA172639b+
41.9 CA109247c-
42.9 CA169881a+
46.6 CA192210a+
50.8 CA182075-
54.3 CA191677b+
75.2 TC69131b+
98.8 CA088898-
108.1 CA247032a+
114.4 CA219930b-
MSI 10
0.0 CA223974b+
18.1 CA135799-
21.4 TC56788a-
25.7 CA111032a+
29.8 TC48056b-
41.1 TC53974a+
57.9 CA079411+
64.4 BQ537203b-
72.9 CA238608b-
84.4 TC56911a-
95.3 CA159655a+
103.4 TC71998+
104.5 TC57172+
110.8 TC56751a+
MSI 11
0.0 CA075238a-
5.1 CA109247b+
CA193294+
12.3 CA099990b+
20.8 CA150722b+
39.5 CA070114c-
54.5 TC64867+
57.0 CA268559b+
60.5 TC64923-
TC59110+
83.0 CA262726b+
110.7 CA066631a+
MSI 12
0.0 CA085871+
6.1 TC65137-
13.9 CA094355d+
18.7 CA120853d+
25.4 TC60051-
42.6 TC66484a+
57.5 CA141303-
TC71214a-
60.5 CA101953+
62.0 CA226298a-
66.7 TC55014+
83.2 TC56751b-
91.1 CA250496a-
94.9 CA095211d+
101.0 TC69451+
MSI 13
0.0 CA104551-
6.4 TC67265c+
28.3 CA191479c-
32.8 CA232067+
35.3 CA182933+
39.2 CA195275+
55.9 CA129538c+
CA231286-
64.1 CA117247b-
77.7 CA155631a+
92.6 CA192925+
MSI 14
0.0 CA200482c-
21.9 CA262726a-
39.5 CA065426-
TC65381a+
42.2 CA155467a+
43.2 CA068472-
49.7 CA138303a-
54.1 CA065384b+
65.2 TC67056+
84.5 TC64924b-
MSI 15
0.0 CA267491b-
21.8 TC68653a+
23.9 TC57800b+
25.0 CA237400-
26.2 CA238608c-
30.7 CA147625c+
36.5 TC56745-
40.3 CA277989c-
43.4 CA117704-
CA162523-
45.7 CA123239b-
47.4 CA216859b-
53.5 CA172330b+
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56.4 CA121708-
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78.8 CA174365b-
83.2 CA192228b+

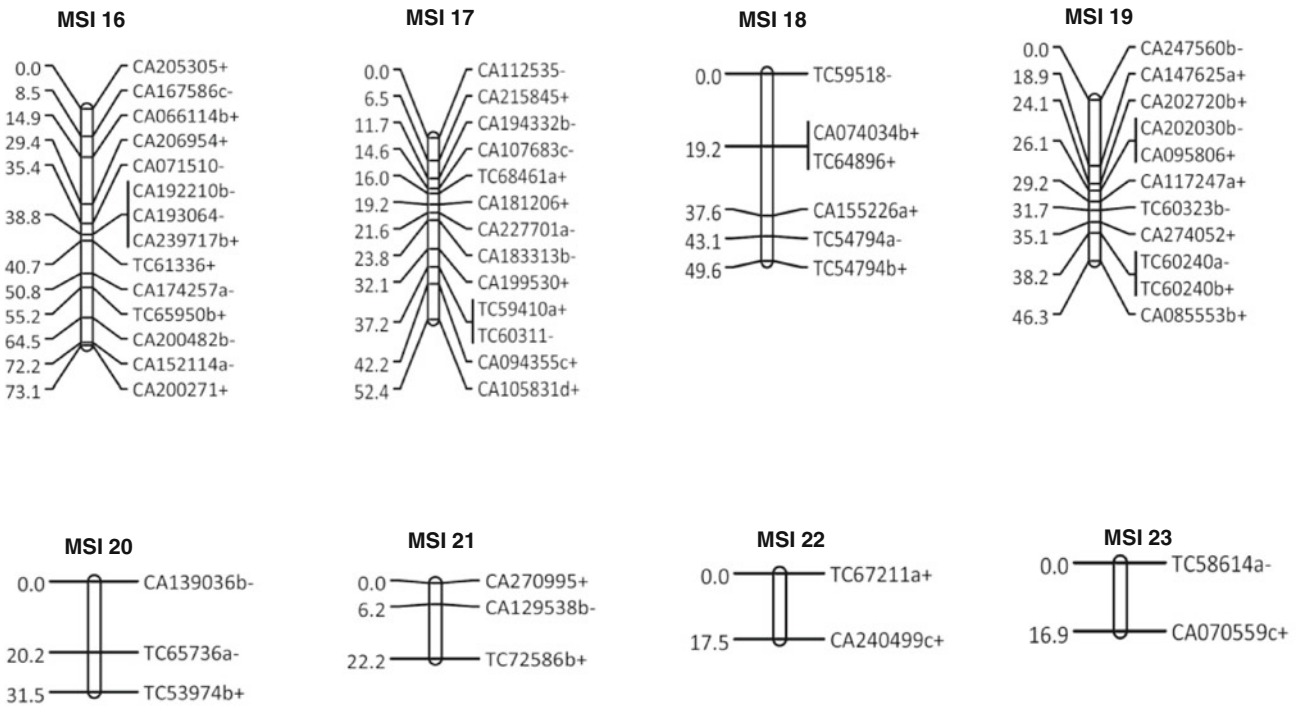

Fig. 2 continued

Table 3 Estimated and observed map length and genome coverage of *M. sacchariflorus Robustus* (MSA) and *M. sinensis* (MSI) genetic maps

	MSA	MSI	MSI-monoploid map
G_{e1} (cM)	2,723.7	2,607.3	2,455.1
G_{e2} (cM)	2,775.4	2,665.0	2,454.4
G_e (cM)	2,749.6	2,636.2	2,454.7
G_o (cM)	1,999.8	2,238.3	2,150.2
Genome coverage (%)	72.7	84.9	87.6
1C genome size (Mbp)	2,600	2,700	2,700
Genome coverage (Mbp)	1,891	2,293	2,365
Mbp per marker interval	8.6	8.2	8.6
Mbp per cM	0.9	1.0	1.1

The monoploid map of MSA is not established because of fragmentation. MSI-monoploid map; from MSI 1 to MSI19. 1C genome size was estimated based on Rayburn et al. (2009)

Mbp mega base pair, G_{e1} estimated length by Fishman et al. (2001), G_{e2} estimated length by Chakravarti et al. (1991), G_e average of G_{e1} and G_{e2}, G_o observed length

between *Eucalyptus grandis* × *E. urophylla* (Grattapaglia and Sederoff 1994), *Populus adenopoda* × *P. alba* (Yin et al. 2001), and *Dendrobium officinale* × *D. hercoglossum* (Xue et al. 2010) reported 1.97, 2.24, and 3.58% bi-parental markers, respectively. We only found seven (2.1%) bi-parental markers. The number of bi-parental markers can be significantly improved when intraspecific hybrids are used. For example, the previous genetic map of *M. sinensis* (Atienza et al. 2002) took advantage of intraspecific hybrids by crossing two F1 individuals to produce a pseudo-testcross mapping population, which provided 51.5% bi-parental markers. An intraspecific cross of *Morus* spp. (mulberry) generated 61.3% bi-parental markers, facilitating the assembly of a consensus map (Venkateswarlu et al. 2006). While we could not integrate the two parental maps using bi-parental markers due to their rarity, homologous chromosomes between MSA and MSI could be indirectly determined by aligning linkage groups to the sorghum genome as a reference.

The Chi-squared test at $P < 0.05$ identified 45 markers for MSA and 30 markers for MSI showing segregation distortion, representing 13.9 and 8.4% of genotyped marker loci in MSA and MSI, respectively. Many of the distorted loci may be associated with alleles affecting viability (Carr and Dudash 2003). Marker genotypes linked to loci that reduce viability tend to be underrepresented in sets of progeny. Therefore, analysis of distorted loci may be a valuable tool to seek deleterious alleles in crop breeding. To find segregation-distorted loci in the *Miscanthus* maps, we added these markers after constructing the maps with Mendelian-segregated markers. However, the distorted markers were not considered in the current mapping because most of them were unlinked or scattered in the LGs (data not shown). Those markers will be added as we enhance map resolution with additional markers.

Table 4 Sorghum chromosomes corresponding to the linkage groups from *M. sacchariflorus Robustus* (MSA) and *M. sinensis* (MSI) genetic maps based on the number of matched SSRs

Sorghum chromosomes (SBI)	MSA		MSI	
	LGs	No. matches	LGs	No. matches
1	4	9	4	5
	5	5	7	13
	8	5	9	5
	12	4	16	5
	13	3	18	3
	16	3	20	2
	20	2		
	21	4		
	27	2		
	34	2		
	38	2		
	40	2		
2	2	6	5	5
	12	5	7	5
	20	2	12	5
	21	3	15	7
	26	2		
	27	2		
	40	2		
3	3	5	1	7
	5	4	3	6
	11	5	7	5
	12	4	9	6
	23	3	14	4
	25	2	15	5
	28	2		
4	4	4	2	8
	9	2	3	5
	19	3	5	6
	22	2	7	5
	30	2	9	7
			21	2
5	7	4	5	5
	14	2	7	3
	15	2	8	4
6	4	5	2	5
	6	6	8	7
	10	3	10	5
			11	5
			19	3
7	16	3	2	7
	22	3	17	6
8	1	6	5	3
	14	3	10	3
			14	3
			16	3
9	7	4	1	6
	8	9	2	7
	24	4	5	5
	37	2	6	7
10	18	3	5	5
	26	3	7	5
	31	2	9	5
			13	5
			19	4
Orphans	17	0	22	0
	29	0	23	0
	32	0		
	33	0		
	35	0		
	36	0		
	39	0		

Orphans indicate *Miscanthus* linkage groups that did not have any matches against sorghum chromosomes under given criteria. LGs that have matches to the same sorghum chromosomes are defined as putative homology groups

This study also provides the first estimated genetic map length of *Miscanthus*: 2,749.6 cM for MSA and 2,636.2 cM for MSI (Table 3). The genome coverage was 72.7% for the MSA map and 84.9% for the MSI map. The genome coverage of sorghum (2,512 loci; Bowers et al. 2003) and sugarcane (492 ~ 739 loci; Ming et al. 1998) was also calculated using the same method according to the data provided, indicating 99.2% for the sorghum map and 75.4 ~ 82.2% for the sugarcane maps. While the sorghum map has been shown to reflect most of the individual recombination events that are available in the mapping population (Bowers et al. 2003), *Miscanthus* more closely resembles sugarcane in that the parental maps have not yet been resolved into the expected basal chromosome numbers. Small LGs, particularly in the MSA map, might represent unconnected parts of other LGs. For instance, the genome coverage of the MSI monoploid (without small LGs) is 87.6%, slightly higher than that of the MSI map. To cover the entire genome and facilitate merging fragmented pieces, additional markers will be required. More bi-parental markers would be especially desirable to confirm HGs between the parental maps.

Comparative genomics

SSRs from sugarcane were readily located on sorghum chromosomes and genotyped in *Miscanthus* due to high

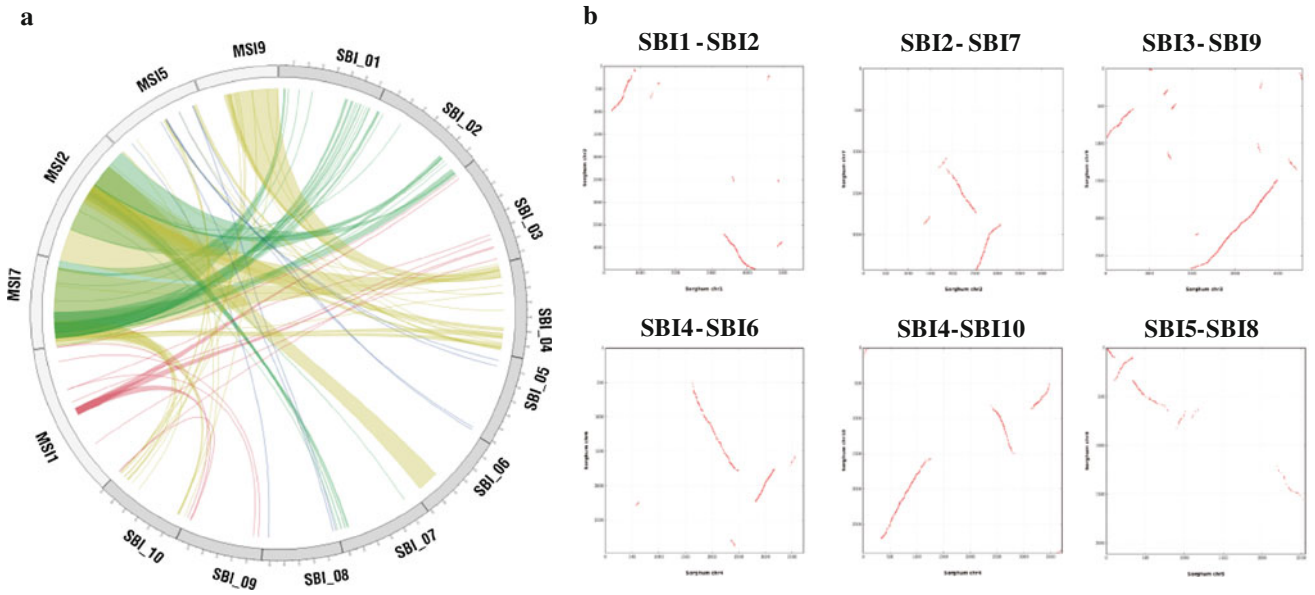

Fig. 3 Linkage groups of *M. sinensis* (MSI) that may contain duplication blocks formed by the pan-cereal whole genome duplication, and their comparison to sorghum chromosomes (SBI). **a** A circle alignment of MSIs and SBIs. **b** *Dot plots* illustrating the whole genome duplication blocks among sorghum chromosomes. The title above each *dot plot* indicates *X* axis–*Y* axis based on the order of genes on two chromosomes. The plots were downloaded from the Plant Genome Duplication Database (PGDD, chibba.agtec.uga.edu/duplication)

similarities among sugarcane, sorghum, and *Miscanthus*. This cross-taxon application also made it possible to compare *Miscanthus* maps to the sorghum genome. The comparative study was mostly dependent upon the MSI map in that the MSA map has 40 LGs, more than twice the basal chromosome number of *Miscanthus*.

Since a consensus map of MSA and MSI with shared markers (e.g. bi-parental markers) could not be constructed, putative HGs were indirectly predicted when *Miscanthus* LGs corresponded to the same sorghum chromosome (Table 4). HGs may include false matches resulting from homeologous LGs. Indeed, some LGs from MSI have matches to the *rho* duplication blocks in sorghum that trace to a pan-cereal genome duplication thought to have occurred about 70 million years ago (Fig. 3). For example, MSI 1 in Fig. 3a has matches to SBI3-SBI9, which is consistent with locations of the *rho* duplication blocks in sorghum (Fig. 3b). This suggests that the MSI 1 potentially has traces of the *rho* duplication and MSI 1 is homeologous to some MSI LGs in the same HG. However, we could not find the homeologous LGs because the number of markers was not yet sufficient for the comparison between LGs. Thus, more common markers between two parental species and within species must be added to identify homologous and homeologous LGs. In addition, each sorghum chromosome has at least two matches to LGs both in MSA and MSI, providing evidence that the entire *Miscanthus* genome probably duplicated after the divergence of the common ancestor of sorghum and *Miscanthus*.

Our inference that an additional genome duplication occurred after the divergence of *Miscanthus* from sorghum would explain an increase in the basal chromosome number of *Miscanthus* to 20, but we are not yet able to definitively explain why the basal chromosome number of *Miscanthus* is now 19. An attractive hypothesis is the fusion of either entire chromosomes or parts thereof, resulting in a net chromosome reduction of 1. The current genetic maps suggest some possible scenarios, however, more densely-mapped markers are needed to definitively answer this question.

Miscanthus has recently gained attention as a promising cellulosic bioethanol feedstock, however the cost of cellulosic bioethanol production is still high. Sustainable biomass production by breeding elite cultivars is one way to reduce costs. A well-saturated molecular linkage map is a valuable tool to accelerate breeding of high yielding, locally adapted *Miscanthus* varieties for biomass production. In this study, EST-derived SSRs have been implemented to construct genetic maps of *Miscanthus*, also facilitating the comparison between *Miscanthus* and the fully-sequenced sorghum genome by virtue of marker sequence information. Although the current *Miscanthus* maps must be enhanced with additional markers, they provide a valuable starting point for marker-assisted studies in *Miscanthus* germplasm. Further, the comparative study provides an initial framework for transferring information from model species (sorghum), and will also be an important tool for efficiently increasing marker density

near genetic loci of specific interest. The two-stage mapping strategy used for the current mapping may enable us to complete maps with fewer markers than a truly random set would require. In particular, the comparative study permits us to infer the locations in *Miscanthus* corresponding to previously mapped domestication or biomass-determining genes/QTLs from sorghum and other cereals that may also prove important in the early stages of *Miscanthus* improvement. An enhanced map will provide further details of *Miscanthus* genome structure and organization such as the discrepancy of the basal chromosome numbers between *Miscanthus* and many of the Saccharinae, the levels and patterns of homoeologous gene duplication, and the types and frequencies of genetic polymorphism in both diploids and polyploids that are important to *Miscanthus* improvement.

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