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ORIGINAL PAPER

Genetic analysis of the sugarcane (*Saccharum* spp.) cultivar 'LCP 85-384'. I. Linkage mapping using AFLP, SSR, and TRAP markers

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Abstract Sugarcane hybrids are complex aneu-polyploids ($2n = 100$–130) derived from inter-specific hybridization between ancestral polyploid species, namely *S. officinarum* L. and *S. spontaneum* L. Efforts to understand the sugarcane genome have recently been enhanced through the use of new molecular marker technologies. A framework genetic linkage map of Louisiana's popular cultivar LCP 85-384 was constructed using the selfed progeny and based on polymorphism derived from 64 AFLP, 19 SSR and 12 TRAP primer pairs. Of 1,111 polymorphic markers detected, 773 simplex (segregated in 3:1 ratio) and 182 duplex (segregate in 77:4 ratio) markers were used to construct the map using a LOD value of ≥ 4.0 and recombination threshold of 0.44. The genetic distances between pairs of markers linked in the coupling phase was computed using the Kosambi mapping function. Of the 955 markers, 718 simplex and 66 duplex markers were assigned to 108 co-segregation groups (CGs) with a cumulative map length of 5,617 cM and a density of 7.16 cM per marker. Fifty-five simplex and 116 duplex markers remained unlinked. With an estimated genome size of 12,313 cM for LCP 85-384, the map covered approximately 45.6% of the genome. Forty-four of the 108 CGs were assigned into 9 homo(eo)logous groups (HGs) based on information from locus-specific SSR and duplex markers, and repulsion phase linkages detected between CGs. Meiotic behavior of chromosomes in cytogenetic studies and repulsion phase linkage analysis between CGs in this study inferred the existence of strong preferential chromosome pairing behavior in LCP 85-384. This framework map marks an important beginning for future mapping of QTLs associated with important agronomic traits in the Louisiana sugarcane breeding programs.

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Introduction

The sugarcane plant is a tall perennial grass, which is typically grown in tropical and sub-tropical climates for its stalks that accumulate sucrose. Sugarcane is a member of the *Poaceae* family like rice and *Andropogoneae* tribe like maize and sorghum. By the end of the nineteenth century, sugarcane cultivars were mostly clones of *Saccharum*

officinarum, the species with high sugar content, domesticated from the species *S. robustum* in New Guinea (Brandes 1958; Berding and Roach 1987). Early in the twentieth century, hybridization attempts between *S. officinarum* ($2n = 80$) and its wild relative *S. spontaneum* ($2n = 40$–128) and then backcrossing of the hybrids to *S. officinarum* resulted in high sugar yields and disease resistance (Roach 1972). An unequal transmission of chromosome number ('$2n$' from *S. officinarum* and 'n' from *S. spontaneum*) had taken place during the initial hybridization and subsequent backcrossing events (Bremer 1961; Bhat and Gill 1985). Consequently, modern sugarcane cultivars have chromosome numbers ranging between $2n = 100$–130 with a strong prevalence of aneuploidy and with the ancestral species contributing about 85% (*S. officinarum*) and 15% (*S. spontaneum*), respectively, of the genome (d'Hont et al. 1996). Subsequent analysis of these ancestral species using the FISH (fluorescence in situ hybridization) technique has demonstrated that the basic chromosome number is $x = 10$ in *S. officinarum* and $x = 8$ in *S. spontaneum* (d'Hont et al. 1998).

Sugarcane is the source of 70% of the world's sugar and is a major source of export earning in some countries. In recent times, sugarcane has also received attention as a potential bio-energy crop. Despite its economic importance worldwide, the complexity of the sugarcane genome has limited classical genetic studies while other genetically simpler crops have made remarkable gains (Barnes and Bester 2000). The genetic complexity is due to the coexistence of simplex and multiplex alleles and irregular chromosome numbers in various homo(eo)logy groups due to its aneuploidy (Hoarau et al. 2001). The elevated ploidy levels, cytogenetic complexity of interspecific hybrids, and the difficulty of controlled hybridization have further complicated genetic dissection studies (Hogarth 1987). However, with the advent of a diverse array of molecular marker systems in recent times, the efficiency in developing genetic linkage maps in sugarcane has increased and these markers have eventually been used in gene tagging, QTL mapping, and map-based cloning (Cunff et al. 2008). The initial difficulty in mapping polyploids using molecular markers was due to the inability to identify the genotypes of marker phenotypes where a large number of genotypes for each marker phenotype are possible in a segregating population (Wu et al. 1992). Efforts in unraveling the sugarcane genome now seem to be promising with the development of theoretical aspects of genetic mapping in polyploids by Wu et al. (1992) using single dose fragments (SDF).

Genetic linkage maps have been developed for sugarcane cultivars as well as for their ancestral species using the full-sib (F_1) individuals (pseudo-test cross strategy) and random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), simple sequence repeats (SSR), sequence related amplified polymorphism (SRAP), target region amplification polymorphism (TRAP), and expressed sequence tag-SSR (EST-SSR) markers (da Silva et al. 1993; Al-Janabi et al. 1993; Guimaráes et al. 1999; Atienza et al. 2002; Ming et al. 2002; Aitken et al. 2005, 2007; Raboin et al. 2006; Edmé et al. 2006; Oliveira et al. 2007; Alwala et al. 2008). However, few maps have been constructed using populations derived from selfing. In France, Hoarau et al. (2001) developed a sugarcane map using the selfed progeny of the commercial cultivar R570 ($2n = 112$; d'Hont et al. 1996). The map based on 939 simplex AFLP markers was distributed onto 120 CGs arranged into 10 putative HGs. The cumulative length of 5,849 cM was postulated to cover one-third of the R570 genome. The same population had been used previously to construct a map using RFLP markers by the same group (Grivet et al. 1996). This RFLP map consists of 408 loci on 96 CGs arranged in 10 putative HGs. The mapping effort by this group led to the identification of a major rust resistance gene '*Bru1*' (Daugrois et al. 1996; Asnaghi et al. 2000, 2004) and efforts are underway to isolate it by map-based cloning (Cunff et al. 2008).

LCP 85-384 is considered as one of the most successful sugarcane cultivars of the Louisiana sugar industry and has achieved significant monetary gains after its release in 1993. The sugar yields of LCP 85-384 were superior over those of previously grown cultivars by about 25% (Gravois and Bischoff 2008). It was commercially successful and occupied 91% of the Louisiana sugarcane acreage in 2004 because of its superior agronomic characters (good cane yield, ratooning ability and planting ratio), and resistance to various biotic (leaf scald by the *Xanthomonas albilineans* and mosaic viral disease by the Carla virus group) and abiotic (post-freeze recovery) stresses. For this reason, LCP 85-384 has been frequently used as a parent in Louisiana breeding programs, although, the cultivar has recently become susceptible to the common rust (*Puccinia melanocephala*) (Gravois and Bischoff 2008). A molecular genetic linkage map of LCP 85-384 was, therefore, considered useful to understand the coexistence of genomic components derived from its parents and the genetic basis of the heterosis observed in the F_1 generation.

The objective of this study was to construct a molecular genetic linkage map of LCP 85-384 using AFLP, SSR, and TRAP markers. The mapping population segregates in relation to various diseases and a number of agronomic traits. This study presents results concerning the development of the framework linkage map as a first step towards subsequent identification of QTLs for important agronomic traits.

Materials and methods

Plant materials

Because sugarcane clones are highly heterozygous, the S_1 progeny from selfing a sugarcane clone is considered as pseudo F_2 population. The progeny derived from self-fertilization of LCP 85-384 was used to develop a molecular linkage map. The cultivar, LCP 85-384, was selected from the progeny of a cross between CP 77-310 and CP 77-407 (Milligan et al. 1994). LCP 85-384 was developed through the joint efforts of the Louisiana State University Agricultural Center, Sugar Research Station, St. Gabriel, the USDA-ARS Sugarcane Research Unit, Houma and the American Sugarcane League of the USA, Inc, Thibodaux. More than 1,000 seedling progeny of the mapping population was germinated in flats in the greenhouse. These seedlings were transplanted to speedling trays after about 3 weeks and eventually to the field. The population has been maintained both as clones in field plots and as one-eye sets in the greenhouse. A random sample of 300 individual plants was taken from the population and used in the linkage mapping study. The parents of LCP 85-384 (CP 77-310 and CP 77-407) were also included to track the origin of markers segregating in the population.

DNA extraction

Young and actively growing leaves were collected from the pot-grown plants in the green house, placed on ice and eventually stored in a refrigerator until DNA extraction. Genomic DNA was extracted using both the Plant DNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's protocol and the CTAB procedure. Concentrations of extracted DNA were estimated by the *Nanodrop* 1000 spectrophotometer (*Nanodrop*, Bethesda, MD) at 260 nm of UV wavelength and the DNA was stored at −20°C. The quality of the DNA was checked by taking the ratio of UV wavelengths at 260 nm/280 nm.

AFLP protocol

AFLP marker analysis was performed according to the protocol of Vos et al. (1995) with some modifications. The DNA (~200 ng/µl) of each genotype was double-digested with *Eco*RI and *Mse*I restriction enzymes. The restricted DNA fragments were ligated to adapters specific for the *Eco*RI and *Mse*I restriction sites. A pre-selective amplification was carried out with *Eco*RI+ A and *Mse*I+ C primers. The resultant PCR product was then tenfold diluted and used as template for the selective amplifications. The selective amplifications were performed with three selective nucleotides in a final volume of 10 µl containing 1.5 µl of the diluted pre-amplification product, 1 µl of 1 µM IR-labeled *Eco*RI primer, 1 µl of 1 µM *Mse*I primer, 0.25 µl of 5 U/µl Taq DNA polymerase (Promega, Madison, WI), 1.5 µl of 2.5 mM dNTPs, 2.0 µl of 5× PCR buffer (supplied with Taq), 1.2 µl of 25 mM $MgCl_2$, and 1.55 µl of nano pure water. The selective amplification products were mixed with 5 µl of 5× Bromophenol Blue loading dye. The mixture was denatured at 95°C for 5 min and 0.4 µl was loaded on a 6.5% polyacrylamide denaturing gel in a LiCor 4300 DNA Analyzer (LiCor Inc., Lincoln, NE). A total of 64 AFLP primer pairs were used to fingerprint all the individuals.

TRAP protocol

The design and sequence information of the primers used in this study was previously described in Alwala et al. (2006). The forward primers were designed using the gene/EST sequences of sucrose synthase (susy), soluble acid invertase (sai), calcium dependent protein kinase (cdpk), sucrose phosphate synthase (sups), pyruvate orthophosphate dikinase (podk), and starch synthase (stsy). The genes susy, sai, sups, podk, and stsy are associated with sucrose metabolism whereas cdpk is believed to be associated with cold tolerance. The two reverse primers employed were IR-labeled with IR dye-700 and -800. The PCR protocol used was as described by Alwala et al. (2006). A total of 12 TRAP primer pairs were used in this study.

SSR protocol

Nineteen SSR primers from the Sugarcane Microsatellite Consortium (Cordeiro et al. 2000; Pan 2006) were used in this study. The Hamilton's Microlab Star Liquid Handling Station (Hamilton Company, Reno, NV) was used to prepare 384-well PCR amplification reaction plates with each well containing a 5-µl PCR reaction mixture. The mixture consisted of 0.25 µl of DNA sample, 0.5 µl of 10× Buffer, 0.3 µl of 25 mM $MgCl_2$, 0.1 µl of 10 mM dNTPs, 0.41 µl each of 3 pM/µl forward and reverse primers, 0.5 µl of 10 mg/ml BSA-V, 0.5 µl of 100 mg/ml PVP-40, 0.025 µl of 5 U/µl *Taq*, and 2.0 µl of PCR water. PCR amplification reactions were conducted on a DNA Engine Tetra equipped with four 384-well Alpha blocks with heated lids (Bio-Rad Laboratories, Hercules, CA) under the program of 95°C for 15 min, 40 cycles of (94°C for 15 s, annealing for 15 s, and 72°C for 1 min), with a final extension at 72°C for 10 min, and holding at 4°C. The annealing temperature varied with each SSR primer pair. The robot was used again to prepare 384-well CE sample plates by first diluting the amplified SSR DNA fragments and then dispensing in each well 1 µl of the diluted products and 9 µl Hi-Dye formamide solution premixed with the Rox™ 500 size standards following

the manufacturer's instructions (Applied Biosystems, Inc., Foster City, CA) (Pan 2006).

Marker scoring

The PCR fragments amplified by the AFLP and TRAP techniques were run on a LiCor 4300 DNA analyzer (LiCor Inc., Lincoln, NE) while the SSR amplified fragments were run on an ABI3730 Genetic Analyzer (Applied Biosystems, Inc., Foster City, CA). The digital images of marker profiles were automatically saved onto a computer hard drive after electrophoresis. The images from the AFLP and TRAP amplified fragments were manually scored as '1' for presence and '0' for absence of clear and unambiguous fragments. Individual GeneScan files from the ABI3730 Genetic Analyzer were analyzed with the GeneMapper™ software (Applied Biosystems, Inc., Foster City, CA). Presence of an SSR allele was scored as '1' and its absence as '0'. AFLP markers were denoted by three respective selective nucleotides of *Eco*RI–*Mse*I primer pairs along with their band size as suffix. TRAP markers were denoted by the codes for the forward and reverse primers along with the marker size as suffix (Alwala et al. 2006). SSR markers were coded by name and identity number from the Sugarcane Microsatellite Consortium (Cordeiro et al. 2000) along with the allele size as suffix.

Segregation analyses

Both monomorphic and polymorphic fragments were produced by all three marker systems. Several segregation ratios are possible in the S_1 population and dosage of the markers was estimated based on their expected segregation ratios in the progeny (Table 1). Under the assumptions of polysomic inheritance and absence of segregation distortion and double reduction, simplex and duplex markers are expected to segregate in 3:1 and 77:4 ratios in the S_1 with gametic (*A—:aaaa*) segregation ratios of 1:1 and 7:2, respectively (Ripol et al. 1999). The markers selected from this analysis were tested for the expected 3:1 and 77:4 for simplex and duplex theoretical Mendelian ratio using the χ^2 test (1 *df*) at 5% error level (Type I). A Bonferroni correction was applied to limit the experiment-wide error rate associated with multiple testing (Sokal and Rohlf 1995). The critical χ^2 values were calculated by dividing the alpha (0.05) by the number of markers. Markers that deviated from the theoretical expected ratios even after the Bonferroni correction were considered as distorted and marked with an asterisk.

Linkage map construction

Mapping of simplex and duplex markers onto co-segregation groups (CGs) (Grivet et al. 1996) was implemented using the software JoinMap 3.0 (van Ooijen and Voorrips 2001). Linkages in coupling phase were detected using an upper recombination fraction (r) threshold of 0.44 [max $r = \{0.5 - z\ (\alpha)\ \sqrt{0.5\ (1 - 0.5)/n}\}$, where z $(0.01) = 2.3264$, and n (population size) $= 300$] (Wu et al. 1992). Only coupling phase linkages between simplex/simplex, duplex/duplex and simplex/duplex markers were included on the linkage map because all the coded dominant markers designate the same phase and have high Fisher's information content (d'Hont et al. 1994; Qu and Hancock 2001). The final map was constructed in two steps. Initially, only simplex markers were used to build the framework map with fixed orders. In order to avoid false linkages, multiple two-point linkage analyses were

Table 1 Gametic and offspring (S_1) probabilities for marker pair configurations between simplex/simplex, duplex/duplex and simplex/duplex markers used in the study

		Single dose (coupling)	Double dose (coupling)	Single versus double (asymmetric coupling 1–2)
Autodecaploid (2× ~10)		Aaaaaaaaaa	AAaaaaaaaa	Aaaaaaaaaa × AAaaaaaaaa
Gametic ratio		1:1	7:2	
S_1-offspring ratio		3:1	77:4	
Gametic phenotype probabilities[a]	AB	$(1 - r)/2$	$(7 - 4r+2r^2)/9$	$(1/2 - 2r/9)$
	Ab	$r/2$	$(4r - 2r^2)/9$	$2r/9$
	aB	$r/2$	$(4r - 2r^2)/9$	$(5/18 + 2r/9)$
	ab	$(1 - r)/2$	$(2 - 4r+2r^2)/9$	$(2 - 2r)/9$
Offspring phenotypic probabilities (S_1)	A_B_	$(3 - 2r+r^2)/4$	$(4r^4 - 16r^3 + 24r^2 - 16r + 77)/81$	$(16r^2 - 32r + 243)/324$
	A_b_	$r(1 - r)/4$	$(-4r^4 + 16r^3 - 24r^2 + 16r)/81$	$(-4r^2 + 8r)/81$
	a_B_	$r(1 - r)/4$	$(-4r^4 + 16r^3 - 24r^2 + 16r)/81$	$(-16r^2 + 32r + 65)/324$
	a_b_	$(1 - r)^2/4$	$(4r^4 - 16r^3 + 24r^2 - 16r + 4)/81$	$(4r^2 - 8r + 4)/81$

[a] Gametic phenotype probabilities were taken from Ripol et al. (1999)

performed at LOD score ≥4.0. Co-segregation groups (CGs), which correspond to a single chromosome among all the homo(eo)logous chromosomes, were identified by grouping the linked markers. The genetic distances (in cM) between markers on the map were computed from recombination fractions using the Kosambi mapping function. Under the assumption of autodecaploidy and polysomic inheritance, recombination fraction (r) and LOD values for the rest of the marker configurations between duplex/duplex and simplex/duplex were computed by numerical maximization of the log-likelihood as in Kriegner et al. (2003). The S_1 phenotypic probabilities for the marker configurations were computed by taking the gametic phenotypic probabilities provided in Ripol et al. (1999) (Table 1). LOD scores for each marker pair were computed as: $\log_{10}$ (likelihood for $r = \hat{r} - \log_{10}$ (likelihood for $r = 0.5$). All pair-wise estimates between duplex/duplex and simplex/duplex marker pairs were inputted into JOINMAP 3.0 (Van Ooijen and Voorips 2001). Finally, the positions of the duplex markers were found on the CGs based on the simplex marker positions from the fixed order map (Kriegner et al. 2003).

Meiotic behavior of LCP 85-384 and ploidy type

The meiotic behavior of LCP 85-384 was studied using cytogenetic analysis. An inflorescence of LCP 85-384 was collected at early to mid-boot stage for meiotic analysis using standard cytogenetic procedures (Smith 1947; Burner 1991). Anthers were squashed and microsporocytes were examined at metaphase I (MI) to determine chromosome number and chromosome pairing.

Repulsion phase linkages between markers were also tested to investigate pairing behavior between CGs as described by Grivet et al. (1996) and Hoarau et al. (2001). For this purpose, only simplex markers were used. To test for repulsion phase linkages, marker scores were inverted (0 → 1 and 1 → 0). For JoinMap 3.0, the initial coding of d (+)/b (−) was re-coded as a/c ($d \rightarrow a$ and $b \rightarrow c$: or vice versa if the original coding was a/c). The original data was doubled by appending the inverted scores to the original data set. Linkages between the original/original and original/inverted markers were assessed. This step yielded markers in all possible pair-wise combination. Linkages are said to be in coupling if the pairing occurs between original/original (or inverted/inverted) and in repulsion if they occur between original/inverted markers. In autopolyploids, however, the observed repulsion phase recombination frequency (R) is composed of the recombination fractions due to crossing-over (R_c) and independent assortment (R_i). The R_i value is generally high (0.44 for an autodecaploid) and thus, the value of default linkage frequency to detect repulsion phase linkages should be set at >0.44 (Qu and Hancock 2001; Kriegner et al. 2003). However, we examined repulsion phase linkages at the recombination frequency value of above and below 0.44 and LOD score ≥4.0. The observed ratio of coupling to repulsion phase linkages was tested against the theoretical ratio of 1:1 (for disomic pairing) and 1:0 (for random paring) using χ^2 value at (1 df) (Wu et al. 1992; Kriegner et al. 2003).

CGs were assigned into homologous groups (HG) based on markers produced by the same SSR and duplex markers that were located on two or more groups. Repulsion phase linkages between long segments of chromosomes of any two CGs were also considered for HG grouping of CGs.

Results

Simplex and duplex markers

A total of 1,111 unambiguous polymorphic markers were detected after genotyping 300 S_1 progeny of the cultivar LCP 85-384 with 64 AFLP, 12 TRAP, and 19 SSR primer pairs. Simplex markers occurred at a rate of 1–22 with a mean of 12 per primer pair (Table 2). A total of 773 provisional simplex markers were retained for the linkage analysis. Most of the simplex marker polymorphism was generated by the AFLP (84%) with the TRAP and SSR markers generating 12.1 and 3.8%, respectively. Thirty-two simplex markers deviated from theoretical expectations after the Bonferroni procedure. The distribution of simplex markers exhibited a peak in the class of Mendelian expected 3:1 ratio which implies that the majority (∼70%) of the markers could be considered to be simplex markers (Fig. 1).

Duplex markers were produced at a rate of 0–11 with a mean of 1.9 per primer pair (Table 2). A total of 182 duplex markers were produced with all of them fitting the expected Mendelian ratio of 77:4 and, therefore, retained for the construction of the linkage map. As in simplex markers, the polymorphism in duplex markers was produced by the three markers systems in the order of AFLP (91.2%) > SSR (7.1%) > TRAP (1.7%). Bonferroni procedure was not further applied to find any distorted markers. The distribution of duplex markers also showed a peak in the class of expected segregation ratio of 77:4 (∼19) among the S_1 progeny (data not shown).

Linkage map construction

A simplex frame work map was built using 718 simplex markers which were assigned onto 108 CGs with a genome length of 5,387 cM. The grouping of the simplex markers was consistent with LOD scores ranging from 4 to 12. Fifty-five markers remained unlinked. Of 182 duplex markers, 66 markers were placed onto 40 CGs based on the

Table 2 A list of the AFLP, TRAP, and SSR polymorphic markers (simplex and duplex) used for constructing the S_1 map of LCP 85-384

Primer pair	Simplex markers	Duplex markers	Co-segregation groups covered[a]
64 AFLP primer pairs			
aaccaa	10	7	8,19,37,43,44,46,48,49,61,75,88 (11)
aaccac	14	1	1,2,3,15,20,34,36,37,46,54,55,63,83,93 (14)
aaccag	16	6	5,16,18,27,28,34,42,47,48,49,50,53,77,80,82,84 (16)
aaccat	13	4	2,17,18,51,67,72,73,75,78,90,96 (11)
aaccta	10	3	6,13,20,27,31,36,43,44,73,92,100 (11)
aacctc	11	5	1,6,10,17,22,27,49,62,65,76,85,96 (11)
aacctg	18	6	4,5,12,13,23,27,33,36,45,48,54,62,70,71,76, 91(16)
aacctt	3	4	16,47,73,75,88 (5)
aagcaa	6	3	21,40,41,49,50 (5)
aagcac	8	0	1,5,19,44,49,66,81,90 (8)
aagcag	11	1	12,16,17,31,39,46,49,75,89,103 (10)
aagcat	10	2	6,14,17,21,25,56,57,94,96,101,104 (11)
aagcta	15	3	1,7,16,20,22,24,35,44,45,47,76,78,83,89 (14)
aagctc	15	2	1,17,39,45,54,56,65,74,75,82,92,99,101 (13)
aagctg	15	3	10,13,14,50,52,53,61,74,78,84,85,87,88,89 (14)
aagctt	14	5	5,10,16,31,39,43,44,46,51,69,70,79,83,88 (14)
acccaa	11	7	4,7,9,12,18,21,55,75,82,85,100,104 (11)
acccac	11	1	27,29,44,48,54,74,75,85 (8)
acccag	20	6	1,2,9,17,20,21,23,39,51,59,60,67,75,78,89,93,97,99,108 (19)
acccat	7	0	43,44,46,75,90,91,105 (7)
acccta	18	6	1,4,8,9,10,12,17,27,31,45,47,50,53,79,80,107 (16)
accctc	13	11	1,12,15,21,24,31,34,35,37,39,46,50,59,75,85,90,91 (17)
accctg	22	4	1,4,7,20,22,23,34,42,43,50,56,71,73,76,78,79,83,85,88,96,103 (21)
accctt	11	3	7,20,34,43,56,63,75,83,85,87,105 (10)
acgcaa	11	3	11,20,34,35,43,46,49,55,57,94 (10)
acgcac	6	1	33,47,57,86,106,107 (6)
acgcag	14	1	5,24,25,31,42,44,47,70,76,78 (10)
acgcat	10	1	5,33,43,47,50,51,74,75,89,98,101 (11)
acgcta	10	2	2,20,31,37,39,52,78,98 (8)
acgctc	7	2	27,34,39,43,48,70,78 (7)
acgctg	12	1	1,16,18,26,27,28,35,46,54,74,85,89 (12)
acgctt	6	2	2,22,40,71,78,77,90 (7)
actcaa	5	1	4,12,24,35,53,93 (6)
actcac	14	2	17,23,28,30,31,37,39,43,55,80,82,86 (12)
actcag	10	4	8,11,23,37,49,68,70,76,83 (9)
actcat	10	0	2,10,11,62,69,75,78,80,86, 97 (10)
actcta	14	1	3,4,12,20,36,46,54,56,83,84,97,102 (12)
actctc	10	0	20,21,25,39,52,78,83,94 (8)
actctg	11	0	1,24,34,36,40,44,55,73,89,93 (10)
actctt	8	0	11,17,28,47,73,75 (6)
agacaa	6	3	1,39,47,61,81,88,94,102 (8)
agacac	14	5	10,11, 20,21, 24,25,26,28,30,39,49,58,80,81 (14)
agacag	13	6	7,16,18,23,34,43,50,51,52,53,80,82,85 (13)
agacat	8	2	4,35,37,70,92,94 (6)
agacta	6	0	12,30,45,59,91,93 (6)
agactc	13	5	1,4,6,15,22,23,32,37,47,50,70,76,84,98 (14)
agactg	13	7	4,10,13,20,21,34,46,57,58,63,81,83,95,102 (14)

Table 2 continued

Primer pair	Simplex markers	Duplex markers	Co-segregation groups covered[a]
agactt	4	1	15,37,52,77 (4)
agccaa	5	1	20,24,51,76,79,80 (6)
agccac	7	1	2,43,46,68,77,92 (6)
agccag	10	1	2,4,6,16,27,31,44,60,91,108 (10)
agccat	5	2	3,12,40,81,85,87 (6)
agccta	11	2	1,2,13,39,60,64,73,77,84 (9)
agcctc	8	2	4,9,18,24,39,41,42,65,80,90 (10)
agcctg	7	2	24,44,45,58,80,81,91 (7)
agcctt	9	2	1,12,16,17,18,23,50,86,103 (9)
aggcaa	3	1	22,78,80 (3)
aggcac	8	1	1,20,34,37,51,57,74,84 (8)
aggcag	4	1	6,46,52 (3)
aggcat	6	2	42,43,59,76,85,106 (6)
aggcta	1	0	14 (1)
aggctc	9	2	4,7,14,31,32,42,64,78 (8)
aggctg	16	2	2,11,12,13,15,16,17,23,42,47,80,81,83,84 (14)
aggctt	4	1	1,8,15,38 (4)
Sub-total	650	166	
Mean	10.15	2.6	
Range	1–22	0–11	
12 TRAP primer pairs			
susy_r1	9	0	2,42,43,45,58,71,72,85,98 (9)
susy_r3	5	0	21,24,53,71 (4)
sups_r1	15	0	4,37,38,45,48,81,92 (7)
sups_r3	6	0	2,27,31,44,90 (5)
podk_r1	7	1	4,10,15,37,42,45,81 (7)
podk_r3	7	1	16,20,45,52,82,87 (6)
sai_r1	6	0	31,45,59,75,86,100 (6)
sai_r3	4	0	6,8,47 (3)
cdpk_r1	8	0	1,16,20,46,56,66,84 (7)
cdpk_r3	12	0	1,8,21,24,37,39,48,81,84,99 (10)
stsy_r1	5	0	12,20,33,73,83 (5)
stsy_r3	10	0	24,37,39,55,74,75,83,85,95 (9)
Sub-total	94	2	
Mean	7.84	0.17	
Range	4–15	0–1	
19 SSR primer pairs			
SMC119CG	1	0	70 (1)
SMC1604SA	2	1	4,5 (2)
SMC1751CL	2	0	44,49 (2)
SMC18SA	3	0	12,23,40 (3)
SMC278CS	2	0	10,11 (2)
SMC31CUQ	2	0	10,11 (2)
SMC334BS	1	0	13 (1)
SMC336BS	2	0	10,11 (2)
SMC36BUQ	1	1	20 (1)
SMC486CG	1	1	0
SMC569CS	3	0	49 (1)

Table 2 continued

Primer pair	Simplex markers	Duplex markers	Co-segregation groups covered[a]
SMC597CS	1	1	64 (1)
SMC7CUQ	1	0	73 (1)
mSSCIR19	2	0	70,97 (2)
mSSCIR29	3	2	27,29,75 (3)
mSSCIR66	1	2	27 (1)
mSSCIR74	1	0	93 (1)
SMC703BS	0	5	0
mSSCIR3	0	0	0
Sub-total	29	13	
Mean	1.52	0.68	
Range	0–3	0–5	

[a] The total number of co-segregation groups covered by each primer pair is shown in the brackets

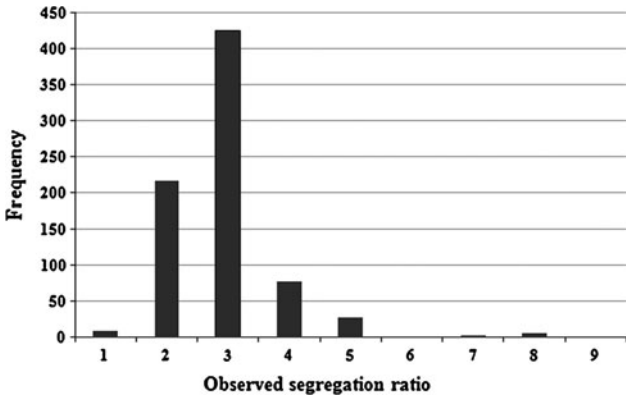

Fig. 1 Frequency distribution showing the segregation ratios of presence: absence of all 773 simplex markers detected among 300 selfed progeny of the cultivar LCP 85-384

fixed order frame work linkage map and the remaining 116 markers were not mapped onto the final map.

The final map had a cumulative genome length of 5, 617 cM (Fig. 2). The length of the CGs varied from 4 cM (CG-68) to 147 cM (CG-39) with an average of 7.16 cM between any two adjacent markers. The total number of markers per CG varied from 2 to 22. The number of CGs containing markers from each primer pair ranged from 0 to 21. Four primer pairs, namely, accctg (AFLP), cdpk_r3 (TRAP), SMC18SA (SSR), and mSSCIR29 (SSR), covered the most number of CGs (Table 2). Although some CGs (CG-1, CG-4, CG-20, CG-39, and CG-75) were dense, the marker loci were not well distributed across CGs and gaps still remained with clustering of markers. For the recombination threshold (0.44) and the mapping function (Kosambi) used in this map, the theoretical maximum distance between any two adjacent markers can be 73.6 cM. However, no interval between two adjacent markers was observed to be greater than 43 cM (CG-97). Of the 32 distorted simplex markers, 19 were scattered on 11 CGs and 13 remained unlinked. Extensive clustering of distorted simplex markers was not observed on the CGs.

Chromosomal paring behavior and ploidy type

Meiotic analysis of LCP 85-384 showed a mean of 3.4 univalents, 6 rod bivalents, and 45.3 ring bivalents per cell based on ten MI microsporocytes. No multivalent configurations were observed. Five cells gave unambiguous counts of $2n = 106$ chromosomes. In addition to the cytogenetic study, repulsion phase linkages were investigated. Recombination frequency estimates were computed for 597,529 (773 × 773) pair-wise marker combinations. Both coupling and repulsion phase linkages were found among the markers in CGs of LCP 85-384. Each pair of repulsion phase markers was examined individually. Only eight pairs of markers on six CGs were found to have significant repulsion phase recombination fractions above the threshold value ($R_i = 0.44$) at a LOD value of $\geq$4.0. Repulsion phase linkages between two pairs (CGs-31 and -39 and, CGs-5 and -89) of the six CGs involved more than two markers, whereas the remaining two CGs had one significant repulsion phase marker pair (Fig. 2). No repulsion phase linkages were found below the observed recombination frequency value of 0.44.

Ploidy type is usually studied by taking the ratio of simplex markers linked in coupling and repulsion phases. The ratio is expected to be equal to 1:1 in allopolyploids and deviate in autopolyploids. A ratio of 4,265:8 coupling to repulsion phase linkages were detected which does not conform to a 1:1 ratio (χ^2 at 0.05). The markers in coupling and repulsion phase linkages within each CG of the LCP 85-384 also did not conform to a 1:1 (χ^2 at 0.05) ratio.

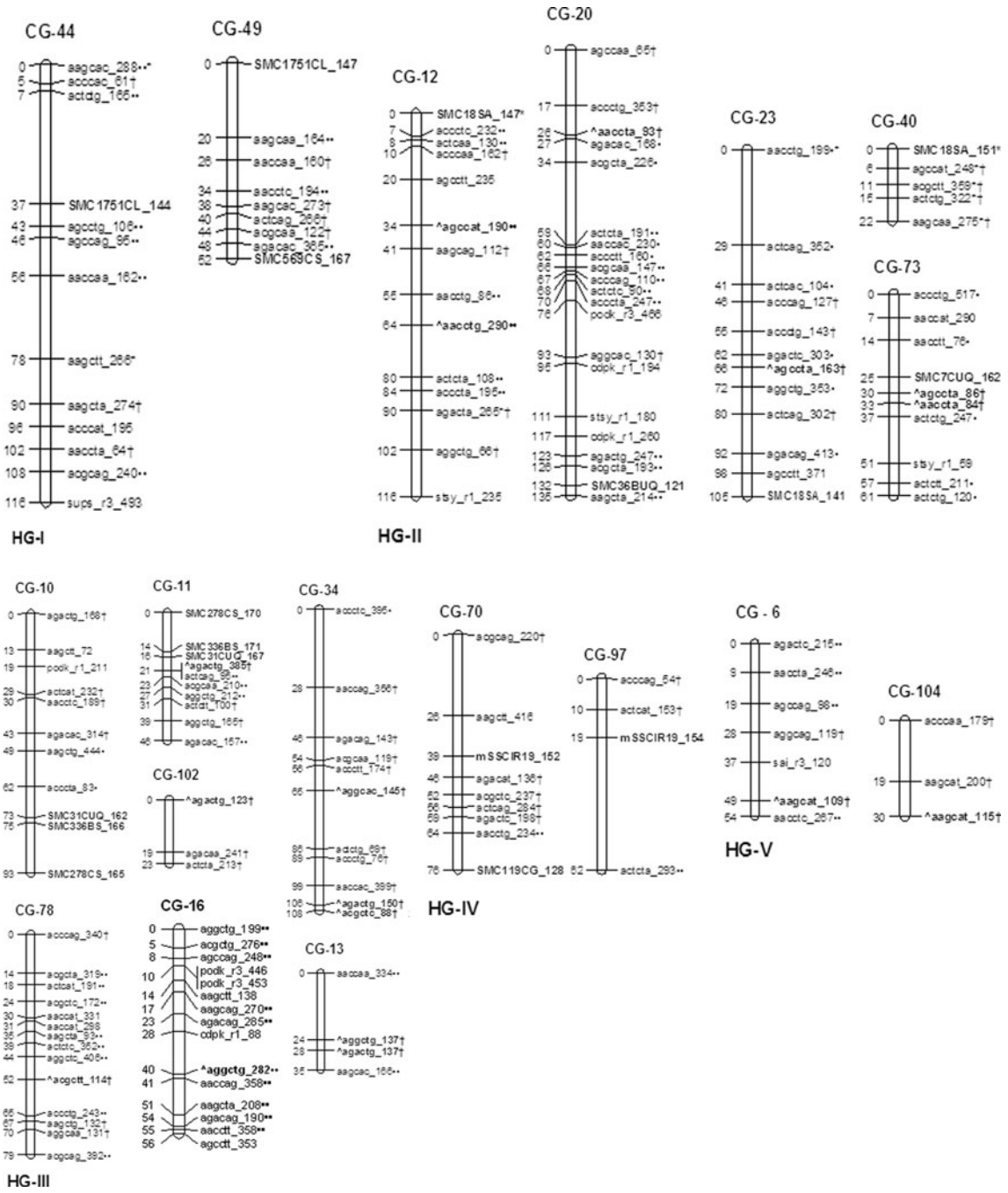

Fig. 2 A genetic linkage map of sugarcane cultivar LCP 85-384 based upon 300 selfed progeny and AFLP, SSR and TRAP markers. The map was constructed using a LOD score >4.0 and a recombination fraction of 0.44. A total of 713 simplex (3:1) and 66 duplex (77:4) markers were assigned onto 108 co-segregation groups (*CGs*). The vertical bars indicate CGs with markers in coupling phase linkages. The Kosambi map distances (cM) and marker names are indicated on the *left* and *right* sides, respectively, of each CG. AFLP markers denoted by three selective nucleotides of *Eco*RI–Mse*I* primer pairs and TRAP markers are denoted as per Table 2. The remaining are SSR markers. Co-segregation groups were grouped into homo(eo)logous groups (*HGs*) using SSR loci, duplex markers, and repulsion phase linkages. Linkages in repulsion between CGs is shown using lines. Co-segregation groups belonging to the same HG are Roman numbered. The SSR alleles and duplex markers responsible for each HG are represented in *capital and bold letters*. The grandparents (parents of LCP 85-384) specific markers are represented by *one dot* or *two dots* for CP 77-407 and CP 77-310, respectively. The *hat symbol* as a prefix to the marker name represents duplex markers. Markers present in both parents and segregating in the S_1 population are denoted by the *tagger* symbol. The marker names with an *asterisk* represent distorted markers

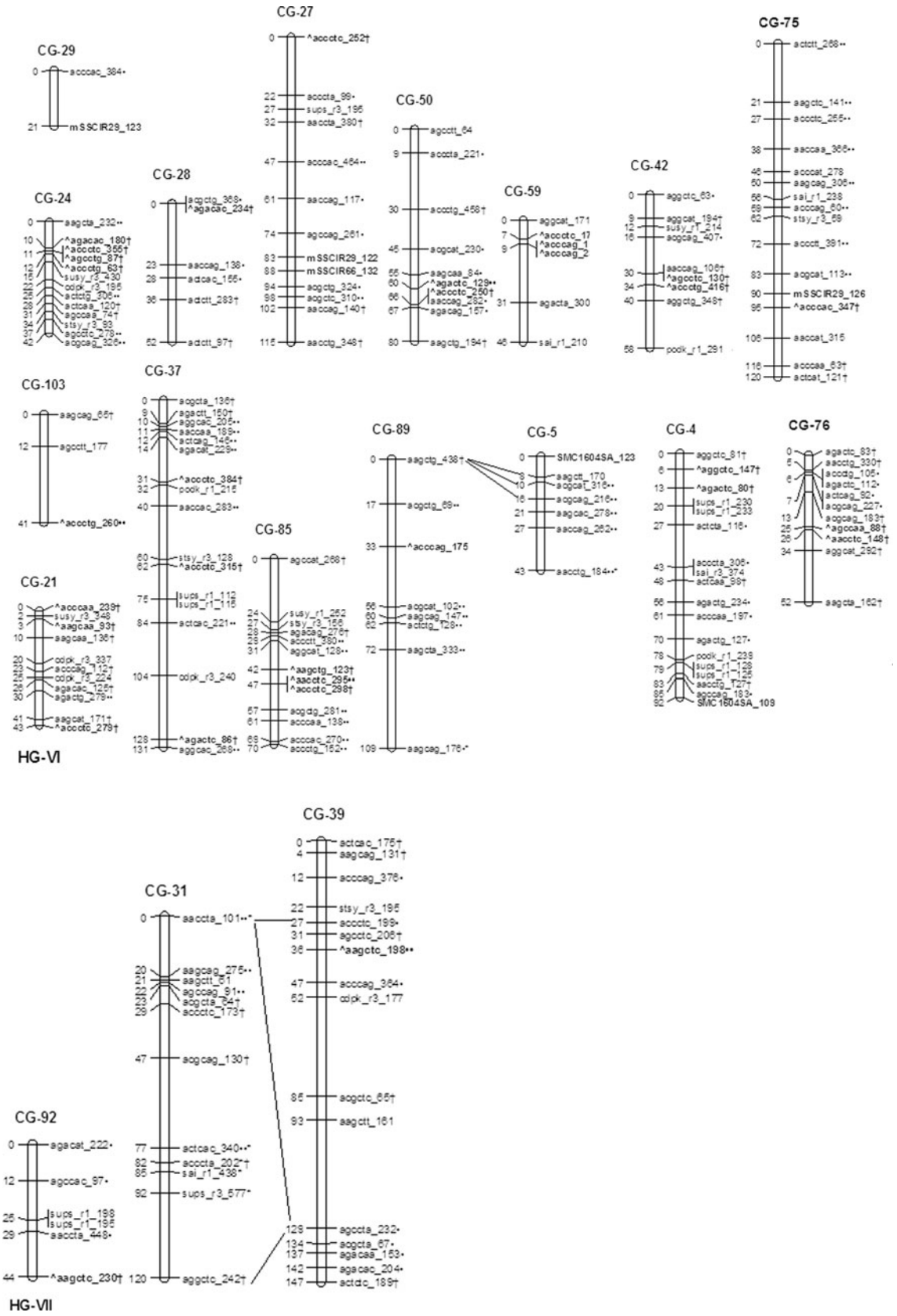

Fig. 2 continued

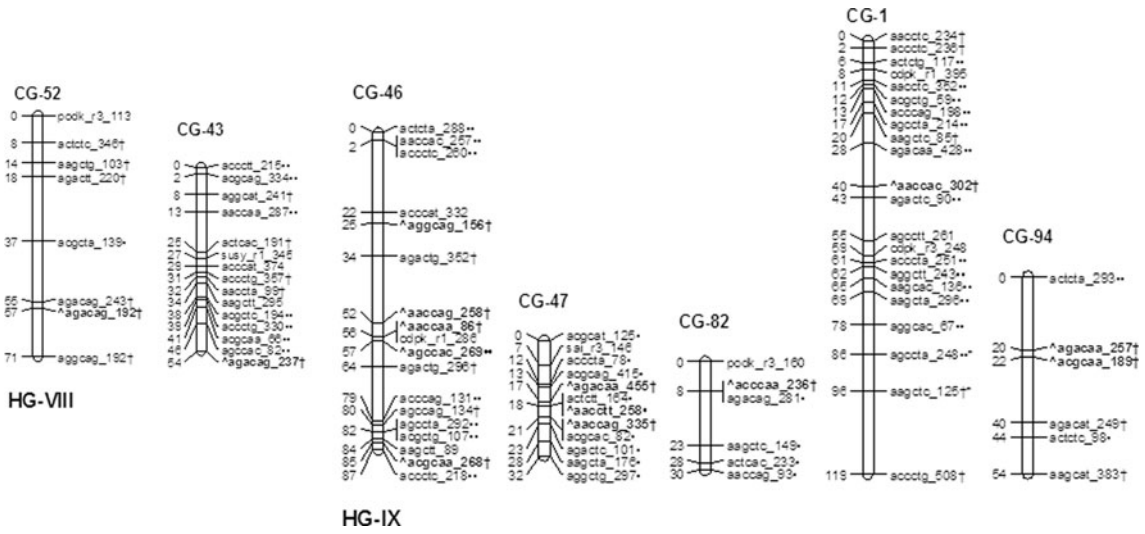

Fig. 2 continued

Genome size and genome coverage

Approximate genome size of LCP 85-384 was estimated using the method followed by Aitken et al. (2005) and Hoarau et al. (2001). The estimated genome size of LCP 85-384, 106 × 112 = 12,313 cM, was obtained by multiplying the estimated number of chromosomes ($2n = 106$; Burner1994) with the average size (112 cM) of the remaining long CGs after excluding the longest CGs (CG-20, -37, and -39). The ratio between the cumulative genome length and estimated genome size indicated that approximately 45.6% (5,617/12,313) of the LCP 85-384 genome has been covered in this study.

Homo(eo)logous groups (HGs)

In this study, co-dominant SSR loci, duplex (chromosomal bridges), and repulsion phase linkages were helpful to provide associations between homologous chromosomes (Fig. 2). By complementation of these three methods, 44 CGs were gathered into 9 putative HGs with a range of 2 (HG-I,-IV,-V, and -IX) to 16 (HG-VI) CGs per HG. The remaining 64 CGs that remained unassigned either had no SSR markers in common or chromosomal bridges established by duplex markers. CGs containing a single pair of repulsion linkages were not considered in HG assembly. Some CGs containing duplex markers (CGs -56, -79, -80 and -93) remained unassigned into any one of the HGs.

Discussion

Sugarcane is a highly heterozygous and genetically complex aneu-polyploid species, which suffers from severe inbreeding depression upon selfing (Stevenson 1965). For this reason, many published sugarcane linkage maps have been developed by applying the pseudo-testcross strategy on the full-sib populations using simplex markers (Garcia et al. 2006; Aitken et al. 2007; Edmé et al. 2006; Alwala et al. 2006; Mudge et al. 1996; Ming et al. 2002). However, genetic linkage maps of sugarcane founded on the S_1 population of the cultivar R570 have been developed using RFLP and AFLP markers to unravel the genomic contributions of the respective ancestral species (Grivet et al. 1996; Hoarau et al. 2001). In this study, we report on a genetic linkage map of Louisiana's popular cultivar LCP 85-384 that was developed using 300 S_1 progeny and polymorphism derived from AFLP, SSR and TRAP markers.

In comparison with diploids, sugarcane has a large genome and generally requires a large number of progeny and markers to construct genetic linkage maps. The population size of 300 S_1 progeny used in this study is comparable to that used in another mapping effort (295 S_1 progeny) in sugarcane that produced one of the most comprehensive maps (Hoarau et al. 2001). High reliability in estimating useful genetic distances was assured in this study by using mostly simplex and duplex markers in coupling phase linkage because they are the most informative and have high Fisher's information content (Wu et al. 1992; Grivet et al. 1996; Hoarau et al. 2001). Simplex markers are abundant in polyploids and make up about 70% of the polymorphic loci detected in this and other studies (Da Silva et al. 1993; Hoarau et al. 2001; Aitken et al. 2005; Garcia et al. 2006; Alwala et al. 2008). However, the total number of simplex markers scored in the present study is low compared to the 939 simplex markers reported by Hoarau et al. (2001) despite the similar sized

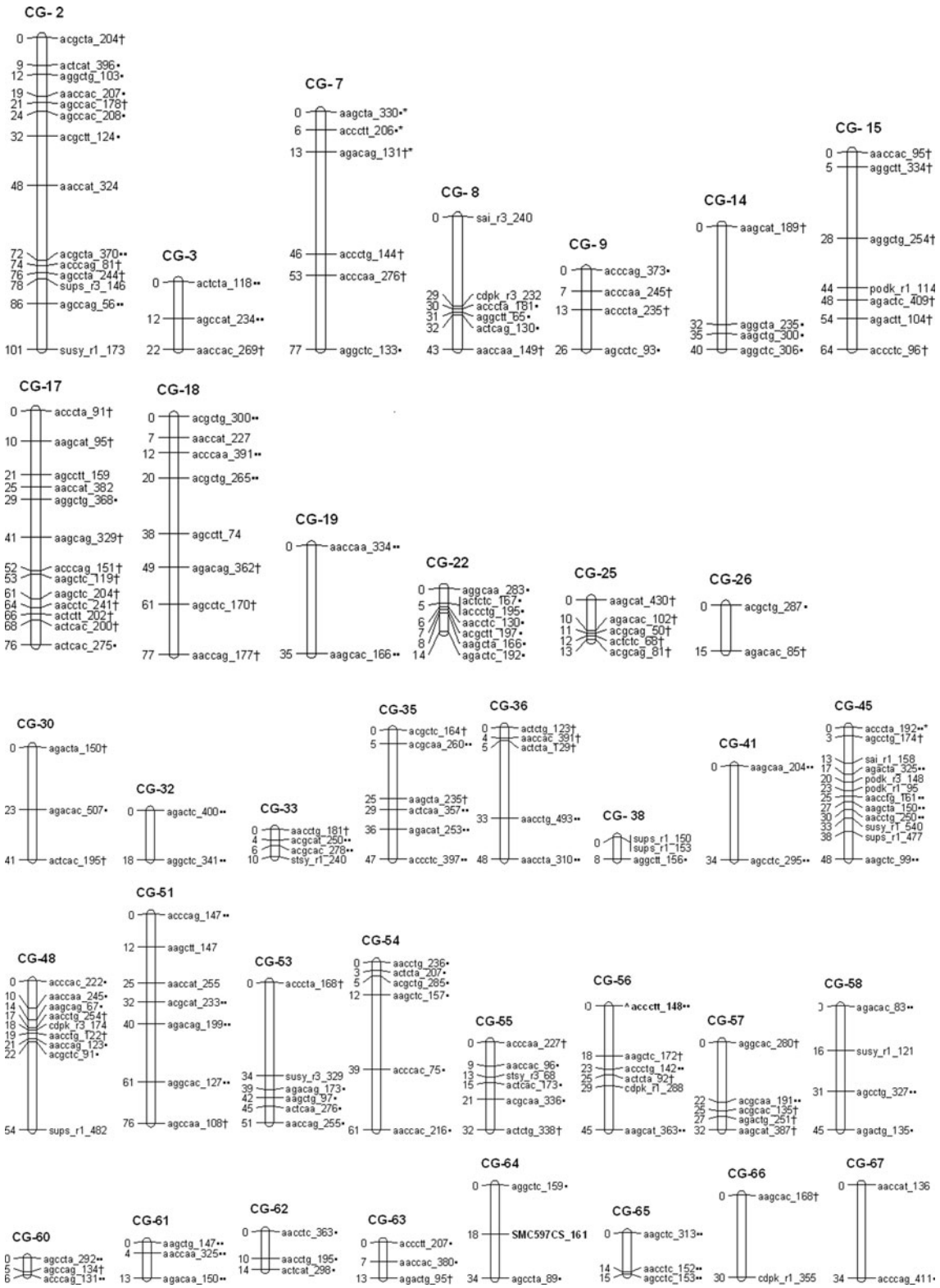

Fig. 2 continued

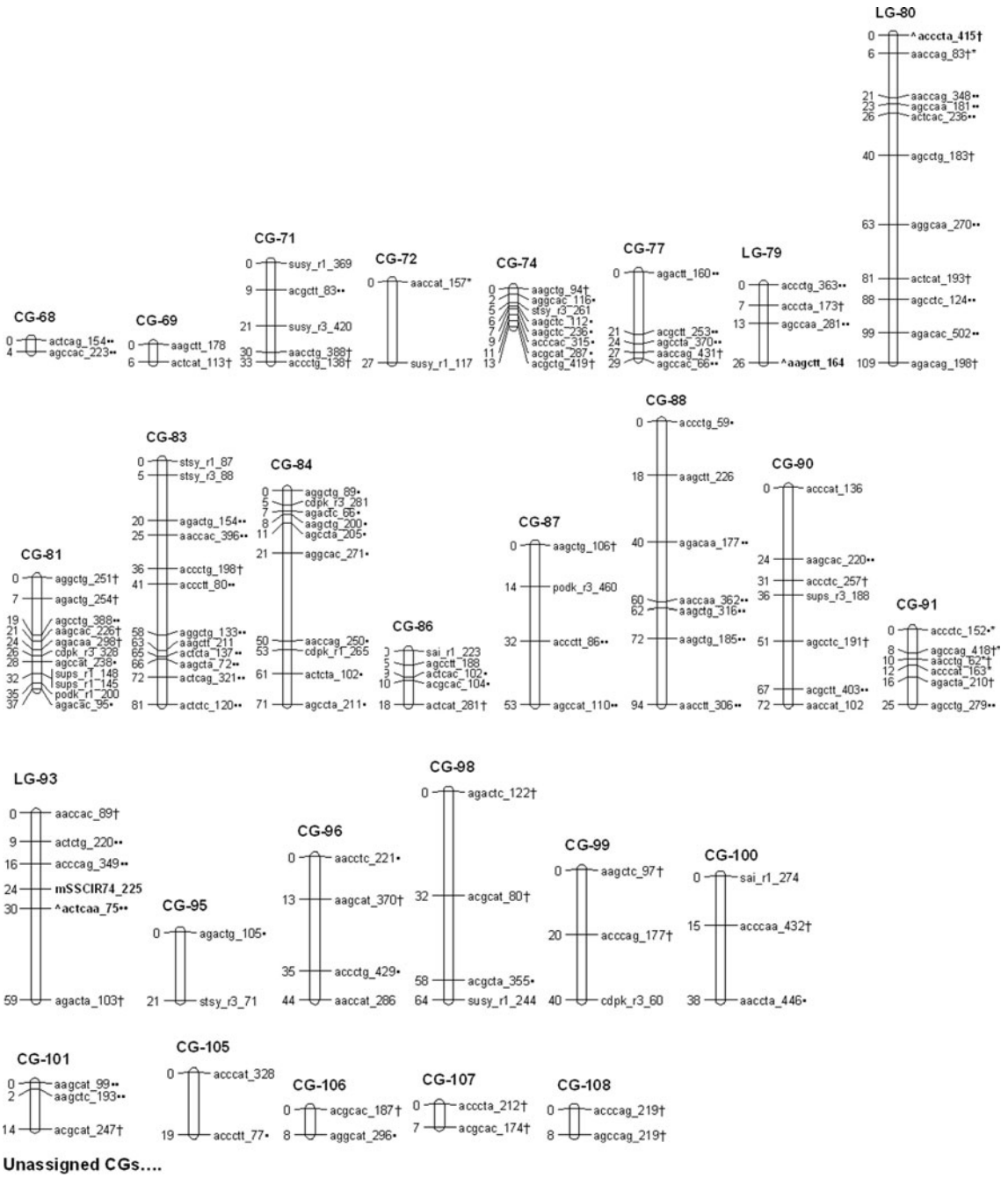

Fig. 2 continued

populations used. Several reasons, including differences in the level of heterozygosity and chromosome numbers in the mapped hybrids could account for this difference. Also Hoarau et al. (2001) used high resolution X-ray film to visualize gel images of 37 AFLP primer pairs while in this study the gel images from 64 AFLP primer pairs were captured using the LiCor analyzer and the digital files saved onto a computer and scored manually. A great number of visually polymorphic fragments beyond 550 base pairs obtained from the LiCor Analyzer were irresolvable and therefore were not scored.

Linkage map construction, genome size, and genome coverage

LOD scores and upper recombination threshold generally determine the number of CGs present in a linkage map. Genetic linkage mapping in sugarcane has used LOD

scores of ≥3.0 and recombination fraction values ranging between 0.25 and 0.45 (Grivet et al. 1996; Al-Janabi et al. 1993; Da Silva et al. 1993; Alwala et al. 2008). A maximum detectable recombination threshold of 0.44 and LOD score values of ≥4.0 (one allowed error in 10,000 linkages) were used in this study to avoid spurious linkages. However, the maximum detectable recombination generally depends on the size of the mapping population. The S_1 map of the commercial hybrid R570 reported by Grivet et al. (1996) and Hoarau et al. (2001) contained 96 CGs spanning 2,008 cM and 120 CGs spanning 5,849 cM, respectively. The S_1 map from this study contained 108 CGs covering a cumulative map length of 5,617 cM. The number of CGs observed in the LCP 85-384 S_1 map was close to the expected number of chromosomes in LCP 85-384 ($2n = 106$). Garcia et al. (2006) and Hoarau et al. (2001) obtained similar results.

The S_1 map of LCP 85-384 was not saturated even after including duplex markers and, covered about 45.6% of the genome because most of the duplex markers were removed while developing the final map due to ambiguity in marker location and order on the map. The S_1 map of R570 covered about 33% of the genome (Hoarau et al. 2001). The S_1 map of LCP 85-384 has achieved more genome coverage probably due to the smaller estimated genome size of LCP 85-384 compared to that of R570 (Hoarau et al. 2001). The linkage map in the current study has uneven marker distribution along the CGs. Other studies have shown that the *S. spontaneum* portion of the genome is better mapped when compared to the *S. officinarum* portion (Lu et al. 1994; Grivet et al. 1996) and this could be the cause of the unevenness. Unsaturated linkage maps from the current study were also evident by the high number of unlinked markers coupled with short CGs (those with fewer than three markers per CG). A comparable number of unlinked markers were also reported by Hoarau et al. (2001). In contrast, a significantly high number of unlinked markers were reported by Garcia et al. (2006) and Alwala et al. (2008) while mapping an F_1 population containing 100 individuals. The reasons for this disparity could be associated with the number of progeny and the different types of populations used in these studies. Several short CGs, which may actually be part of larger CGs, could be a consequence of using the higher LOD values (≥4.0) while developing the linkage map (Alwala et al. 2008). However, spurious linkages were avoided by adopting the higher LOD values. Besides, gaps in sugarcane maps are expected because LCP 85-384 and its parents are complex aneuploids and have a large genome requiring a large number of markers to saturate the linkage maps (Al-Janabi et al. 1993; Garcia et al. 2006). Therefore, more markers will be needed on this S_1 map for it to be saturated and to make it amenable to QTL discovery.

Segregation distortion

Segregation analysis of 773 simplex markers generated based on the theoretical Mendelian ratio (3:1) revealed segregation distortions (~4.5% after Bonferroni correction), which could be a reflection of the diverse genomes present within LCP 85-384. Using a similar type of population, Grivet et al. (1996) and Hoarau et al. (2001) reported 2 and 8% distorted markers, respectively. Interspecific hybrids in sugarcane are fit in F_1 and have a general tendency of exhibiting a high proportion of distorted markers upon selfing. Segregation distortion may also be more elevated in hybrid genomes within regions experiencing selection (Woram et al. 2004). The female parent of LCP 85-384 (CP 77-310) originates from the cross between CP 52-068 and L 65-69. The male parent CP 77-407 (CP 71-021 × CP 66-035) is a BC_3 progeny of a *S. spontaneum* genotype US 56-15-8. Thus, the parents of LCP 85-384 came from very distant parents, which could have an impact on the segregation distortion observed in the S_1 population. Deviations in expected ratios could also be related to the significantly different sizes and chromosome number of the parental genomes (Edmé et al. 2006; Alwala et al. 2008).

Segregation distortion is also an indication of the linkage between molecular markers and distorting factors (deleterious recessive alleles) (Zamir and Tadmor 1986). If the linkage is tight, they usually have similar segregation patterns and thus, skewed markers would appear to be clustered (Jenczewski et al. 1997). However, the distorted markers in this study did not show extensive clustering, which could signify a lower level of inbreeding depression due to recessive alleles. Similarly, in a grapevine S_1 map (Hvarleva et al. 2009), distorted markers did not cluster together. The clustering of distorted markers may not necessarily imply linkage, but linkage disequilibrium could be suspected (Jenczewski et al. 1997). Furthermore, the amplification of two fragments of the same length from non-allelic regions (homoplasy) and co-migration of two different fragments amplified at paralogus loci could also be responsible for some of the markers showing segregation distortion. However, more markers and larger population sizes would be required to identify distorted loci and determine their likely biological significance in sugarcane (Alwala et al. 2008).

Chromosome pairing behavior and ploidy type

Predominantly bivalent pairing at meiosis have been reported in cytological studies of the two ancestral species (*S. spontaneum* and *S. officinarum*) (Bremer 1961; Sreenivasan 1975) as well as in cultivated sugarcane (Price 1963; Burner 1994; Burner and Legendre 1994). We studied the

meiotic behavior of LCP 85-384 chromosomes and confirmed the high incidence of bivalent pairing among homologous chromosomes in this cultivar. Although suggestive of disomic inheritance, bivalent pairing alone is not a good indication of allopolyploidy as homologues of autopolyploids often associate randomly into bivalents rather than multivalents (Qu and Hancock 2001; Crawford and Smith 1984). Additional, albeit indirect, insights into chromosome pairing behavior during meiosis are now possible in sugarcane mapping studies thanks to analysis of marker segregation in the repulsion phase. Working with ancestral species of cultivated sugarcane, the *S. officinarum* clones LA Purple (Mudge et al. 1996), Green German (Edmé et al. 2006) and IJ 76-514 (Aitken et al. 2007) were implied to be allopolyploid on the basis of the presence of linkage in repulsion phase in mapping studies. Conversely, a general lack of repulsion phase linkages in mapping studies of progeny derived from the *S. spontaneum* clones SES 208 (Al-Janabi et al. 1993; da Silva et al. 1993), IND 81-146 (Edmé et al. 2006) and SES 147B (Alwala et al. 2008) led to the suggestion that these clones are autopolyploids undergoing random chromosome pairing. In this study, repulsion phase linkages (at a LOD value of >4.0) found among three or more markers in some CGs is suggestive of disomic inheritance or preferential pairing of chromosomes in LCP 85-384. Evidence of preferential pairing of chromosomes has been reported in other sugarcane mapping studies involving cultivars (Hoarau et al. 2001; Aitken et al. 2005). However, further statistical analysis not performed by the previous authors show a statistical deviation from the 1:1 ratio (χ^2 at 0.05) of coupling: repulsion phase markers, which is indicative of polysomic inheritance. Most of the polymorphism found in cultivars is supposedly derived from *S. spontaneum* (Jannoo et al. 1999; Hoarau et al. 2001) and the *S. spontaneum* portion of the genome is better mapped when compared to the *S. officinarum* portion (Lu et al. 1994; Grivet et al. 1996). Thus, it is quite conceivable that these results reported here reflect a bias in the disproportionate amount of *S. spontaneum* relative to *S. officinarum* alleles involved in the analysis. On the other hand, the results may well represent a reflection of the unsystematic meiotic behavior of a complex polyploid such as cultivated sugarcane, which comprises of two ancestral species differing in pairing affinity and capable of displaying a continuous range of paring affinity (Jannoo et al. 2004).

Homo(eo)logous groups (HGs)

Given the basic chromosome number of *Saccharum* species hybrid ($\sim x = 10$), it is predicted that 10–11 homo(eo)logus sets of chromosomes should exist within the genome if homo(eo)logus chromosomes are preserved during the diplodization process in meiosis. Previous studies identified ten HGs from an S_1 population in the R570 map based on the joint segregation of RFLP (Grivet et al. 1996) and AFLP (Hoarau et al. 2001) markers and eight HGs in Q165 (Aitken et al. 2005) using AFLP and SSR markers. In the current study, we found nine HGs, which is lower than the predicted number of HGs. It is apparent from the HGs in Fig. 2 that CGs were under represented and this could be attributed to the prevalence of aneuploidy in the sugarcane genome. In addition, many CGs were not identified as belonging to a HG due to the lack of sufficient SSR and duplex markers and, very few markers linked in the repulsion phase. Most of the HGs were also formed with small and less dense CGs. Low levels of polymorphism in the regions of the *S. officinarum* part of the genome could be contributing to the lack of dense CGs (Ming et al. 1998; Hoarau et al. 2001; Aitken et al. 2005). Garcia et al. (2006) and Aitken et al. (2005) found probable duplicated loci derived from a common SSR locus on a CG. However, we did not find any evidence of genome duplication but this assertion is based on limited information of SSR markers. The number of CGs per HG as well as marker density in the CGs will continue to be enhanced by including more locus specific SSR markers to achieve a HG number that is closer to the basic chromosome number of *Saccharum* species.

Future studies

The linkage map of LCP 85-384 from the present study is not saturated and is based on 773 simplex and 182 duplex markers generated by 64 AFLP, 19 SSR and 12 TRAP primer pairs. The map covered about 45.6% of the genome and indeed, none of the published genetic maps of sugarcane are saturated. The main reason attributed to this is the genetic complexity of sugarcane. The density of the LCP 85-384 map in the current study is 7.16 cM per marker. However, gaps still exist. Filling such large gaps in the map, which could be from the less polymorphic *S. officinarum* fraction of the genome, will be an enormous task despite the availability of new marker technologies. However, more markers need to be added to the current map to make the map more resourceful in finding QTLs for several agronomic traits.

Most of the genetic diversity found among modern cultivars was reportedly from the *S. spontaneum* genome, probably because *S. officinarum* was used as the recurrent parent during nobilization and transmitted 2*n* gametes to its progeny. In Louisiana, a few *S. spontaneum* genotypes have been used extensively to develop new cultivars. One such *S. spontaneum* genotype is US 56-15-8 (Thailand origin), which is found in the pedigree of most of the popular cultivars (LCP 85-384, HoCP 85-845, L 97-128,

and HoCP 96-540) in Louisiana (Arro et al. 2006). For this reason, it would be vital to estimate the genomic contribution of the *S. spontaneum* clone US 56-15-8 and other ancestral clones (*S. officinarum* and legendary cultivars) to LCP 85-384. Tracking the source of each allele (from the ancestry) and eventually identifying and tagging the markers in successful cultivars could be one way of mapping useful alleles in sugarcane.

Summary

In the current study, a linkage map of LCP 85-384 was developed using the selfed progeny and AFLP, SSR and TRAP markers. The current map allowed us to study the segregation pattern in the mapping population and chromosome pairing behavior during meiosis. LCP 85-384 possesses desirable agronomic traits and resistance to biotic and abiotic stresses and the S_1 progeny developed by selfing LCP 85-384 segregate in relation to these traits. The framework map in this study will provide an important background for mapping QTLs associated with sugar-related traits and this information will be useful for crossing and selecting of clones in the breeding program.

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