RFLP Mapping in Cultivated Sugarcane (*Saccharum* spp.): Genome Organization in a Highly Polyploid and Aneuploid Interspecific Hybrid

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Manuscript received June 6, 1995
Accepted for publication November 22, 1995

ABSTRACT

Sugarcane cultivars are polyploid, aneuploid, interspecific hybrids between the domesticated species *Saccharum officinarum* and the wild relative *S. spontaneum*. Cultivar chromosome numbers range from 100 to 130 with ~10% contributed by *S. spontaneum*. We have undertaken a mapping study on the progeny of a selfed cultivar, R570, to analyze this complex genome structure. A set of 128 restriction fragment length polymorphism probes and one isozyme was used. Four hundred and eight markers were placed onto 96 cosegregation groups, based on linkages in coupling only. These groups could tentatively be assembled into 10 basic linkage groups on the basis of common probes. Origin of markers was investigated for 61 probes and the isozyme, leading to the identification of 80 *S. officinarum* and 66 *S. spontaneum* derived markers, respectively. Their distribution in cosegregation groups showed better map coverage for the *S. spontaneum* than for the *S. officinarum* genome fraction and occasional recombination between the two genomes. The study of repulsions between markers suggested the prevalence of random pairing between chromosomes, typical of autopolyploids. However, cases of preferential pairing between *S. spontaneum* chromosomes were also detected. A tentative Saccharum map was constructed by pooling linkage information for each linkage group.

SUGARCANE is probably the most genetically complex crop for which genome mapping has been attempted. Sugarcane and all wild species related to it are highly polyploid. The first domesticated clones originated in Papua New Guinea and Indonesia. These clones constitute the species *Saccharum officinarum*. Their chromosome number is generally $2n = 80$. Various types of meiotic irregularities have been described (BREMER 1923), but *S. officinarum* clones usually form 40 bivalents (PRICE 1963). Octoploidy with $x = 10$ is suspected but has not yet been clearly demonstrated (BREMER 1961). *S. spontaneum* is the second major species that has contributed to modern cultivars. It is a wild species with very low sugar content but with many other useful characteristics for breeding, including vegetative vigor and resistance to abiotic stresses (*e.g.*, drought and cold) and various parasites (*e.g.*, the fungus *Ustilago scitaminea* and the sugarcane mosaic virus). Clones of this species have different chromosome numbers, ranging from 40 to 128, but multiples of eight are most frequent, suggesting a basic number of $x = 8$ (SREENIVASAN *et al.* 1987). Early in this century, breeders in Java produced interspecific hybrids between the two species and backcrossed twice with *S. officinarum* as the recurrent parent. In India at the same time, breeders conducted three-way interspecific hybridizations involving *S. officinarum*, *S. spontaneum* and *S. barberi*, another sugar-producing species that may be a natural hybrid between *S. officinarum* and *S. spontaneum* (DANIELS and ROACH 1987; GLASZMANN *et al.* 1990; D'HONT *et al.* 1993). In both Java and India, the resulting clones were highly productive.

Introgression considerably increased the genome complexity. The *S. officinarum* clones, used as females, transmitted their somatic chromosome number during the first interspecific cross and the first backcross. This phenomenon can be due to either endoduplication or fusion of two megaspore nuclei after the second meiotic division (BHAT and GILL 1985). Cytological observations indicate that the first interspecific cultivars had chromosome numbers in the 100–130 range. Statistical considerations predict a probable 10% coming from *S. spontaneum* (SIMMONDS 1976). Modern sugarcane breeding essentially entails intercrossing hybrid clones and their derivatives. Current cultivar pedigrees reveal that no more than five to seven meioses, *i.e.*, opportunities for recombination between the two genomes, have occurred from the first interspecific hybrids to modern breeding materials (TEW 1987). Meiosis appears to be fairly regular in modern cultivars, with prevalent bivalents, rare univalents and multivalents. PRICE (1963) found, at the most, six univalents and one multivalent in a study on four Hawaiian cultivars. The genomes of present cultivars are thus very complex since they are highly polyploid, aneuploid, and of multispecific origin.

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Biochemical (GLASZMANN *et al.* 1989) and molecular (D'HONT *et al.* 1994) markers provide the only means for monitoring unifactorial inheritance in such complex genomes.

WU *et al.* (1992) developed the theoretical aspects of genetic mapping in high polyploids with bivalent pairing. Molecular techniques often simultaneously reveal several markers, or alleles, for a given locus in polyploids, *i.e.,* several fragments of distinct sizes revealed by one restriction fragment length polymorphism (RFLP) probe, or several electromorphs for isozymes. Due to limited polymorphism, it is often not possible to identify as many distinct alleles as the ploidy level could allow for a given locus: some alleles are *simplex* (only one copy is present at the locus), others *duplex* (two copies) or *multiplex* (multiple copies). The pairing behavior is often unclear or involves a combination of *di-* and *polysomy* (*i.e.,* systematic predetermined diploid-like and random chromosome assortment within the bivalents, respectively, generally distinguishing between allo- *vs.* autopolyploidy), sometimes further disturbed by aneuploidy. Complications, such as possible duplication in the genome and confusion between alleles at *paralogous* loci (*i.e.,* loci derived from this duplication), are particularly difficult to unravel. The only way to handle the data is to separately analyze each allele segregation and subsequently look for linkages in coupling and in repulsion. For mapping purposes, simplex markers are by far more informative than multiplex, especially in F_2 type progenies.

A linkage map for a *S. spontaneum* clone ($2n = 64$ chromosomes) has been constructed using 279 random amplified polymorphic DNA (RAPD) markers (AL JANABI *et al.* 1993) and 216 RFLP markers detected by 116 probes (DA SILVA *et al.* 1993). The data were recently pooled into a single unified map (DA SILVA *et al.* 1995). The results suggest that this *S. spontaneum* representative is autopolyploid. The domesticated species *S. officinarum* is also being mapped on the basis of a cross between a representative of *S. officinarum* and a clone of its putative wild ancestor *S. robustum.* The first results suggest incomplete polysomy (AL JANABI *et al.* 1994a).

Linkage analysis of modern cultivars has been carried out in exploratory studies using RFLPs and a small number of progeny of a selfed cultivar (D'HONT *et al.* 1994; GRIVET *et al.* 1994). An overall rationale was thus developed, focusing particularly on the coexistence of genomic components derived from *S. officinarum* and *S. spontaneum.* Several linkage groups were identified, but the number of progeny was not sufficient to arrange the order of most markers. These studies made use of maize probes, thus revealing marked genomic similarities in sugarcane, maize and sorghum, three members of the Andropogoneae tribe.

We present a new genetic map constructed for a sugarcane cultivar with 408 markers generated by 118 anonymous RFLP probes, two cloned genes and one isozyme. The high number of markers per probe or isozyme permitted us to merge homologous cosegregation groups and draw up a tentative composite map for each linkage group.

MATERIALS AND METHODS

Plant material: Sugarcane is a clonally propagated crop, in which most mature plants can produce seeds. The mapping population in this study has been described previously (GRIVET *et al.* 1994). It involved 77 progeny derived from the self-fertilization of the elite cultivar R570 (pseudo F2). Centre d'Essai, de Recherche et de Formation (CERF), Réunion developed this cultivar. It was selected from progeny of the cross H32 8560 [Hawaiian Sugar Planters Association (HSPA)] × R445 (CERF). It is commercially very successful in Réunion and Mauritius and is now used as a parent stock in many breeding stations worldwide. The total number of chromosomes in R570 was estimated to be ~107–115 on the basis of karyotypic observations (unpublished data).

The species origins of some of the markers (whether specific to *S. spontaneum* or *S. officinarum*) were established by comparing RFLP patterns between R570 and *S. officinarum, S. spontaneum* and *S. barberi* clones supposedly involved in the genealogy of R570. Other clones were added to widen the coverage of the parental species. These were as follows: for *S. officinarum,* Badila, Bandjarmasin Hitam, Black Cheribon*, Cristalina, EK28*, Fiji 24, Kaludai Boothan, Loethers, Mauritius Guinghan, POJ100*, Vellai; for *S. spontaneum,* Coimbatore local*, Glagah*, Mandalay, SES14, US56158; for *S. barberi,* Chunnee*, Saretha and Uba (clones with an * are probably involved in the genealogy of R570). Clones surveyed were obtained from the Bureau of Sugar Experiment Stations (Australia); Copersucar (Brazil); the Sugarcane Breeding Institute, Coimbatore (India); the West Indies Central Sugar Cane Breeding Station (Barbados); and CERF and CIRAD (Réunion and Guadeloupe).

Detection of RFLPs and isozyme markers: The procedures for extraction of genomic DNA, isolation and labeling of cloned inserts and Southern hybridizations have been presented elsewhere (HOISINGTON 1992; D'HONT *et al.* 1994; GRIVET *et al.* 1994). DNA was digested with either *Dra*I, *Hin*dIII or *Sst*I. Digested DNA samples (10 μg/lane) were separated by electrophoresis in 0.8% TAE agarose gels at 1.7 V/cm for 24 hr.

The probes used were of several origins. Among them, 37 were obtained by developing a *S. spontaneum* genomic library (*SSCIR* probes). Total plant DNA (clone SES 278) was digested with the methylation-sensitive enzyme *Pst*I and was size separated on a 10–40% sucrose gradient. The 500- to 2500-bp size fraction was ligated into a pUC18 plasmid, and DH5-α bacterial cells were then transformed with ligated plasmid. Of the 230 clones screened, 72% yielded low copy sequences.

A set of 20 other sugarcane probes, corresponding to genomic DNA (*SG* probes) and cDNA (*CDS* probes), and one oat cDNA clone were kindly provided by Drs. J. DA SILVA and W. BURNQUIST (Copersucar), Dr. P. MOORE (U.S. Drug Administration-HSPA) and Dr. M. SORRELS (Cornell University). The probes were chosen to represent several large linkage groups detected by DA SILVA *et al.* (1993).

A set of 71 maize probes (*BNL* and *UMC* probes) were kindly supplied by Dr. B. BURR of Brookhaven National Laboratory and Dr E. COE of the University of Missouri, Colombia (BURR and BURR 1991; GARDINER *et al.* 1993). They were all genomic probes, except *BNL1.297,* which is a cDNA; 54 of them were already used by DA SILVA *et al.* (1993) or D'HONT *et al.* (1994), and the other 17 were chosen in the gaps of the maize map not yet targeted in our previous study and were retained for mapping on the basis of RFLP pattern quality.

Two cloned genes were also used: the rDNA wheat gene [probe *pTA71* (GERLACH and BEDBROOK 1979)] and the maize alcohol dehydrogenase gene [*Adh1* (GERLACH *et al.* 1982)].

Isozyme analysis of sugarcane was conducted for peroxidase (POX) as described by GLASZMANN *et al.* (1989).

Single locus segregation data analysis: RFLP hybridization patterns on the progeny revealed both monomorphic and polymorphic DNA fragment size classes. The former corresponded to multiplex markers (or possibly homozygous markers in case of disomic inheritance) and the latter to simplex, duplex or, less frequently, triplex markers. Each distinguishable DNA fragment size class was scored independently as a dominant marker in a presence *vs.* absence fashion (D'HONT *et al.* 1994). When two fragment size classes generated by the same probe (with the same or with different restriction enzymes) had the same segregation pattern, they were considered as a single marker.

The analysis was conducted using simplex markers only. If there is no segregation distortion, the expected segregation ratio for simplex markers is 3:1, irrespective of the type of chromosome pairing. For duplex markers, the expected segregation ratio is 15:1 in the case of disomic inheritance and larger for polysomic inheritance. For triplex markers, the ratio is 63:1 for disomic inheritance and larger for polysomic inheritance, increasing rapidly as the ploidy level decreases. Since simplex markers are by far the most informative type, our first aim was to distinguish them from all the others. We thus retained all markers with a segregation ratio lower than 6.7:1 ($\sqrt{3} \times 15{:}1$). This ratio gives equal χ^2 for both 3:1 and 15:1 hypotheses (MATHER 1957), the latter being the smallest theoretical ratio for all nonsimplex markers. Markers showing segregation distortion toward lower values on the basis of a χ^2 test at $\alpha = 0.05$ with the 3:1 hypothesis were considered as skewed and were marked on the map.

Map construction: Linkage analysis of simplex markers was performed with the program MAPMAKER 3.0 (LANDER *et al.* 1987). The markers were coded as dominant markers with the same phase (D'HONT *et al.* 1994), thus only linkages in coupling could be detected. Indeed, estimation of distances and ordering of dominant simplex markers in repulsion on F_2-type segregation data is very inefficient when inheritance is disomic and is impossible when inheritance is polysomic, with the number of progeny surveyed here (ALLARD 1956; WU *et al.* 1992). Estimation of genetic distances between a simplex and a duplex marker in coupling is also very poor. The amounts of information, I_p, for these three linkage detection situations can be calculated according to MATHER (1957). Their value relative to the I_p value of the simplex-simplex coupling configuration are given in Figure 1.

Grouping of markers was performed by two point analysis. The groups of linked markers identified at this stage are referred to as cosegregation groups. A basic set of cosegregation groups was built up at a LOD score of 5. This score leaves very little chance for artifacts to cause false linkages. However, for a given locus pair, it may lead to selection of the closest apparent linkages from all parental bilocus allelic combinations. To avoid such artificial map compression, we then decreased the LOD score by one unit, then by two units, retaining a new linkage only when it involved two probes (or isozymes) that were already associated with one another in a cosegregation group at a LOD score of 5.

Markers were ordered by multipoint analysis using standard MAPMAKER procedures. When the most likely hypothesis was <10 times more likely than the second one (relative log-likelihood lower than one), the order was considered ambiguous and this was highlighted on the map.

Since a cosegregation group is defined on the basis of linkage in coupling only, it will correspond to a single chromosome among all the homo- or homoeologous chromosomes of the same linkage group. As a first attempt to assemble cosegregation groups into linkage groups, we tentatively considered that two cosegregation groups having at least two probes in common belonged to the same basic linkage group.

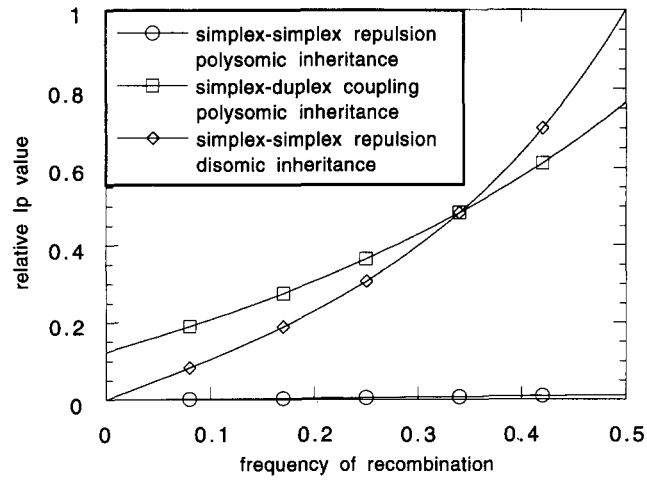

FIGURE 1.—Individual offspring information, I_p, of three different linkage detection situations relative to the information of the simplex-simplex coupling configuration and their dependence on the recombination frequency. Simplex-simplex repulsion and simplex-duplex coupling configurations concern decaploids.

Cosegregation groups associated within the same tentative linkage group were then compared, focusing particularly on the possibility that the genomes contributed by *S. officinarum* and *S. spontaneum* could be significantly different. Pairing between these cosegregation groups was investigated by testing linkage in repulsion between markers. The segregation data matrix was thus doubled, and markers of the second data set were encoded as dominant markers provided by the other parent. We looked at linkage in repulsion between tentative allelic markers generated by the same probe.

A composite map of each linkage group was then constructed by pooling segregation data of all constitutive cosegregation groups, as already described (D'HONT *et al.* 1994).

RESULTS

Sorting of probes and polymorphism: A total of 131 probes were surveyed. For each probe, a choice was made among the restriction enzymes *Dra*I, *Hin*dIII and *Sst*I, retaining the enzyme(s) that yielded the highest numbers of bands with a low intensity signal in R570. This maximized the number of potential simplex markers that would segregate in the progeny. This revealed scorable segregating bands for all 131 probes but three (*BNL7.49, UMC157, CDO1056*). Segregation was then investigated with 154 probe-enzyme combinations. For isozymes, peroxidase exhibited a remarkable segregation for two markers.

A total of 630 segregating markers were generated by the 128 probes and one isozyme system. The segregation ratios (presence *vs.* absence) were always higher than 1:1 and their distribution exhibited a peak around

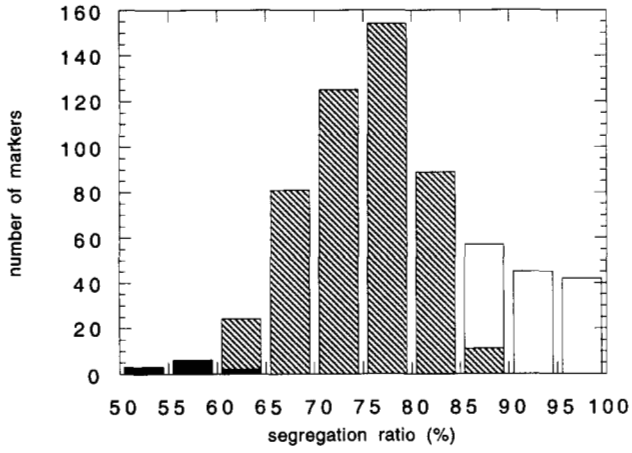

FIGURE 2.—Distribution of segregation ratios (percentage of progenies showing the marker) of the 630 segregating markers revealed by 128 RFLP probes and one isozyme in the self progeny of cultivar 570. The distribution of the 505 markers retained as simplex markers is shown by the hatched area. The distribution of the 11 simplex markers skewed toward lower values is double hatched.

the expected 3:1 simplex marker pattern (Figure 2). The overall distribution was skewed toward higher values, in accordance with the existence of multiplex markers. Application of Mather's criterion excluded 125 possible multiplex markers and retained 505 tentative simplex markers. Among those, only 11 (2%) were skewed toward lower values at a $P = 0.05$ significance level. The mean number of simplex and total segregating markers per probe was 3.9 and 4.8, respectively.

Map construction: The genetic map is presented in Figure 3. The grouping of the markers was first performed with a LOD score of 5. This high threshold was chosen to exclude most false linkages anticipated from the high number of pairwise comparisons (127,260). The results showed that many markers were tightly linked, which reduced the total number of truly independent comparisons to <100,000. A LOD score of 5 gives a false positive rate of $<10^{-5}$ thus bringing the expected number of false linkages down to less than one. Grouping with simplex markers at a LOD score of 5 assigned 381 markers into 98 cosegregation groups. When the LOD score was decreased to 4 and 3, new linkages that confirmed existing probe associations were obtained. At a LOD score of 4, 11 previously unlinked markers were added to the map, three group clusterings occurred and three new groups appeared, adding another six markers to the map. At a LOD score of 3, six previously unlinked markers were added, four clusterings occurred and two new groups appeared, adding another four markers to the map. This gave a total of 408 linked markers assembled into 96 cosegregation groups. Ten of the 11 skewed markers were scattered on seven cosegregation groups.

Marker orders preferred by a LOD of 1 over alternate orders were considered significant. The order was often ambiguous, especially when linkage was tight. In this case, the markers were clustered along a bold line on the map. When the position of a single marker was not clearly determined according to the same threshold, often due to missing data, this was highlighted on the map by a "T" bar on the right of the cosegregation group (KESSELI *et al.* 1994).

The resulting genetic map of R570 comprises 408 markers generated by 120 probes and one isozyme linked in 96 cosegregation groups. Ninety-six simplex markers remained unlinked. Eight probes had no markers involved in any cosegregation group. The size of the cosegregation groups ranged from 0 (complete cosegregation) to 128 cM. The cumulative length for all groups was 2008 cM. The average distance between loci was 6.1 cM, but distribution along chromosomes was irregular, with both sparsely populated map regions and clusters of loci.

Establishment of linkage groups: Gathering cosegregation groups on the basis of common probes led to 10 basic linkage groups with four or more cosegregation groups each (Figure 3). Thus 85 of the 96 cosegregation groups were assigned on the basis of two probes or more, as defined in the MATERIALS AND METHODS. The remaining 11 cosegregation groups were small with four markers at the most (groups u1–u11, Figure 3). Two of them formed one pair (u1 and u2) and nine remained single.

The composition of putative linkage groups led to several critical observations:

1. There was a consistent gene repertoire inside each linkage group. If we exclude probes or isozymes that yielded only one segregating marker (*BNL1.297, UMC167, UMC54, CDSR63, UMC6),* there were only seven probes represented in groups by a single linked marker (*BNL7.28, SSCIR230, SG54, SSCIR103, BNL3.04, UMC29, UMC108*). All the others had two or more representatives, reaching up to nine (*BNL16.06,* group X) and 10 (*BNL12.06,* group I). On the other hand, only eight probes had markers in two different linkage groups (*BNL8.39, BNL12.06, SSCIR103, SG426, BNL3.04, SG54, SSCIR230, UMC10*). They seemed to be randomly distributed, possibly corresponding to individually duplicated small genome regions. Other such cases could appear, considering the probes involved both in linkage groups and in unassigned cosegregation groups, but no further conclusions can be drawn at this time.

2. There was a consistent marker order between cosegregation groups of the same linkage group. Taking into account locally ambiguous orders mentioned previously, only three cases of inconsistency were observed. The first involved probes *SSCIR69* and *SSCIR73,* whose order seemed to be inverted between groups I5 and I10. The second involved probes *UMC113* and *SSCIR76,* inverted between groups IX1 and IX2. The third involved probe *SSCIR51* in group X, whose position was unclear. The three cases involved terminal markers, which are more

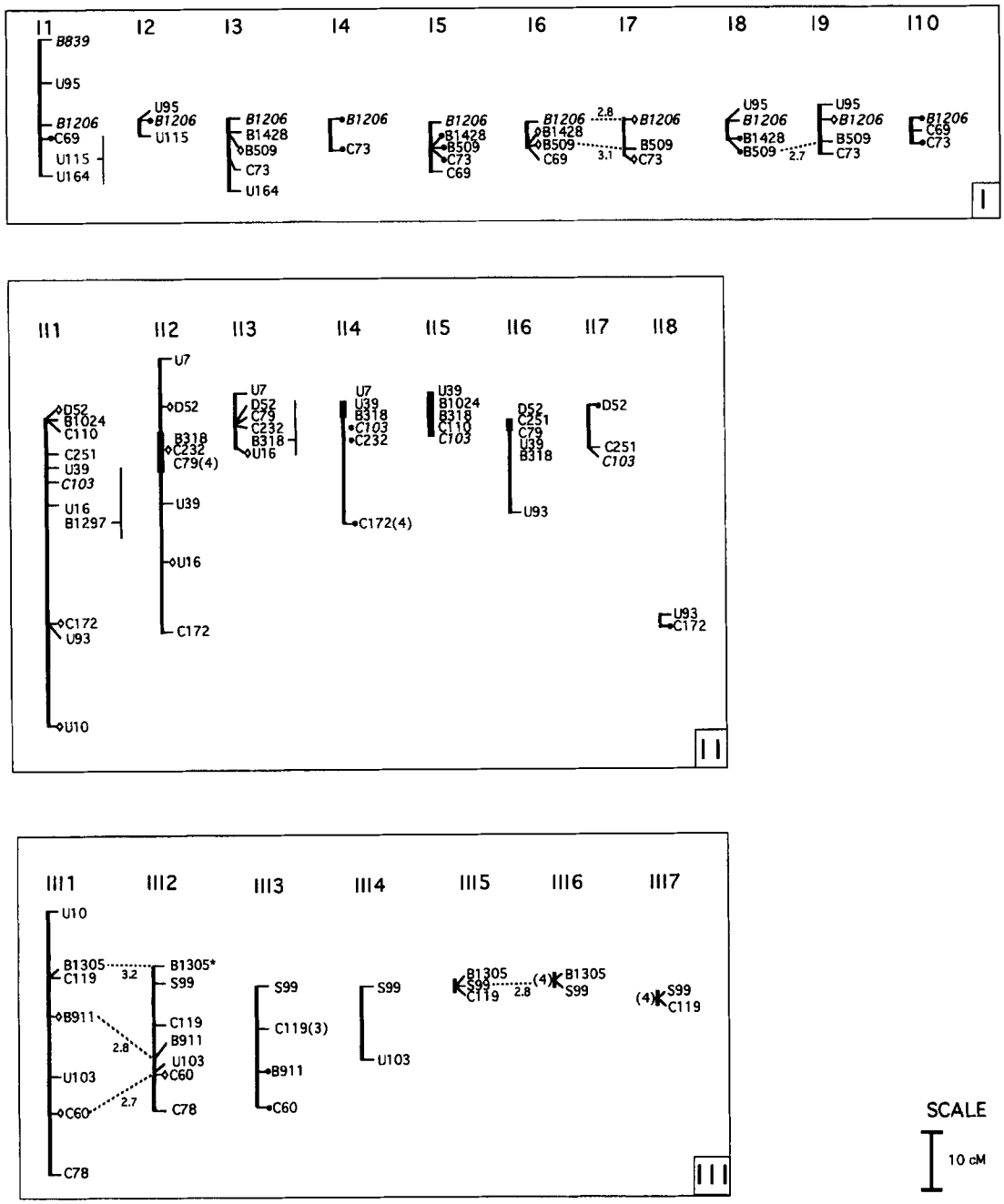

FIGURE 3.—Genetic map of the sugarcane cultivar R570. The segregation data permitted assembly of 408 markers generated by 120 probes and one isozyme gene into 96 cosegregation groups. Those could be assembled into 10 basic linkage groups. The RFLP probe prefix and isozyme designations were abbreviated as follow: B, *BNL;* C, *SSCIR;* D, *CDSC;* E, *CDSB;* R, *CDSR;* S, *SG;* U, *UMC;* POX, peroxidase). Cosegregation groups, corresponding to linkages between markers in coupling, are represented by vertical bars. Grouping was first performed with an LOD score of 5. New linkages appearing at LOD scores 4 or 3 are identified on the map by symbols (4) or (3), respectively. Uncertain orders (LOD score < 1) are represented by a bold line for clustered markers or by a T bar on the right of the cosegregation group for a single marker. Linkages in repulsion between two markers generated by the same probe are indicated by dashed lines, together with the LOD threshold (see text). Cosegregation groups coresponding to the same linkage group are assembled in a rectangle. Linkage groups are designated with roman numerals, additional unassigned cosegregation groups with a "u" letter. Duplicated probes giving markers in two different linkage groups are italicized. Skewed markers are identified with *. When the species origin of a marker is known, it is indicated either with ● for *S. officinarum* or ◇ for *S. spontaneum.*

difficult to order due to less coinformative data, as already reported in several species such as maize (GARDINER *et al.* 1993) and rice (MCCOUCH *et al.* 1988).

3. Markers were unevenly distributed along chromosomes. Some chromosome segments are densely marked and appeared as poorly ordered clusters (linkage groups I and VI, top part of group II, top part of group VIII, top and bottom parts of groups X) and

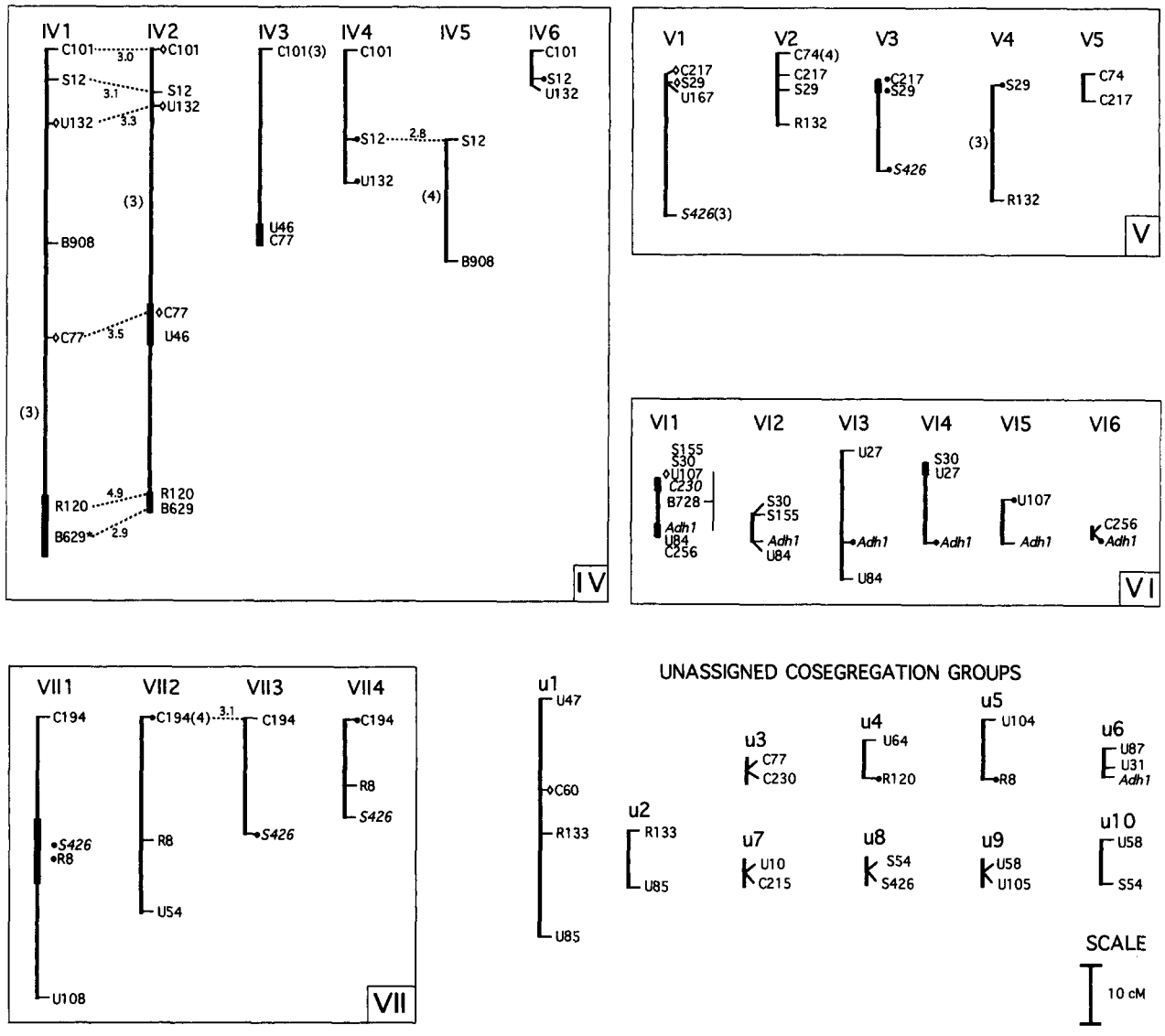

FIGURE 3.—*Continued*

others were sparsely marked (groups IV and IX, middle part of group X).

4. There was complete homogeneity of genetic distances between cosegregation groups of the same linkage group. The only pairs of probes that showed a genetic distance discrepancy at a significance level lower than $P = 0.05$ among instances where they appeared linked were probes *SG298* and *BNL15.40* in cosegregation groups X2 (38 cM) and X16 (10 cM) at $P = 0.016$ and probes *BNL9.11* and *SSCIR60* in cosegregation groups III1 (17 cM) and III2 (3 cM) at $P = 0.037$. Given the high number of such comparisons that could be performed at the whole map scale, a few single tests are expected significant at $P = 0.05$; the global multiple test, however, is not significant.

In an attempt to cluster apparently unrelated cosegregation groups that might correspond to a single chromosome, we decreased the linkage LOD threshold within each linkage group. For this purpose, we analyzed segregations for each linkage group separately, taking into account all markers of the group. In group IX, two cosegregation groups (IX1 and IX3) could be assembled at LOD 2.5, giving a larger group that was colinear to the other cosegregation groups. In group X, group u11 joined X1 at LOD score 2.5 and groups X4 and X9 could be lumped when the LOD score was reduced to 2 (data not shown). Surprisingly, no lumping occurred in group VIII until a LOD score of 1, despite the abundance of small nonoverlapping cosegregation groups.

Species markers distribution on the map: The species origins of the markers were identified by comparing RFLP patterns of R570 and representative clones of ancestral species. A band was declared of *S. officinarum* origin when it was absent in all *S. spontaneum* representative clones and present in at least one *S. officinarum*, and of *S. spontaneum* origin when it was absent in all *S. officinarum* clones and present in at least one *S. sponta-*

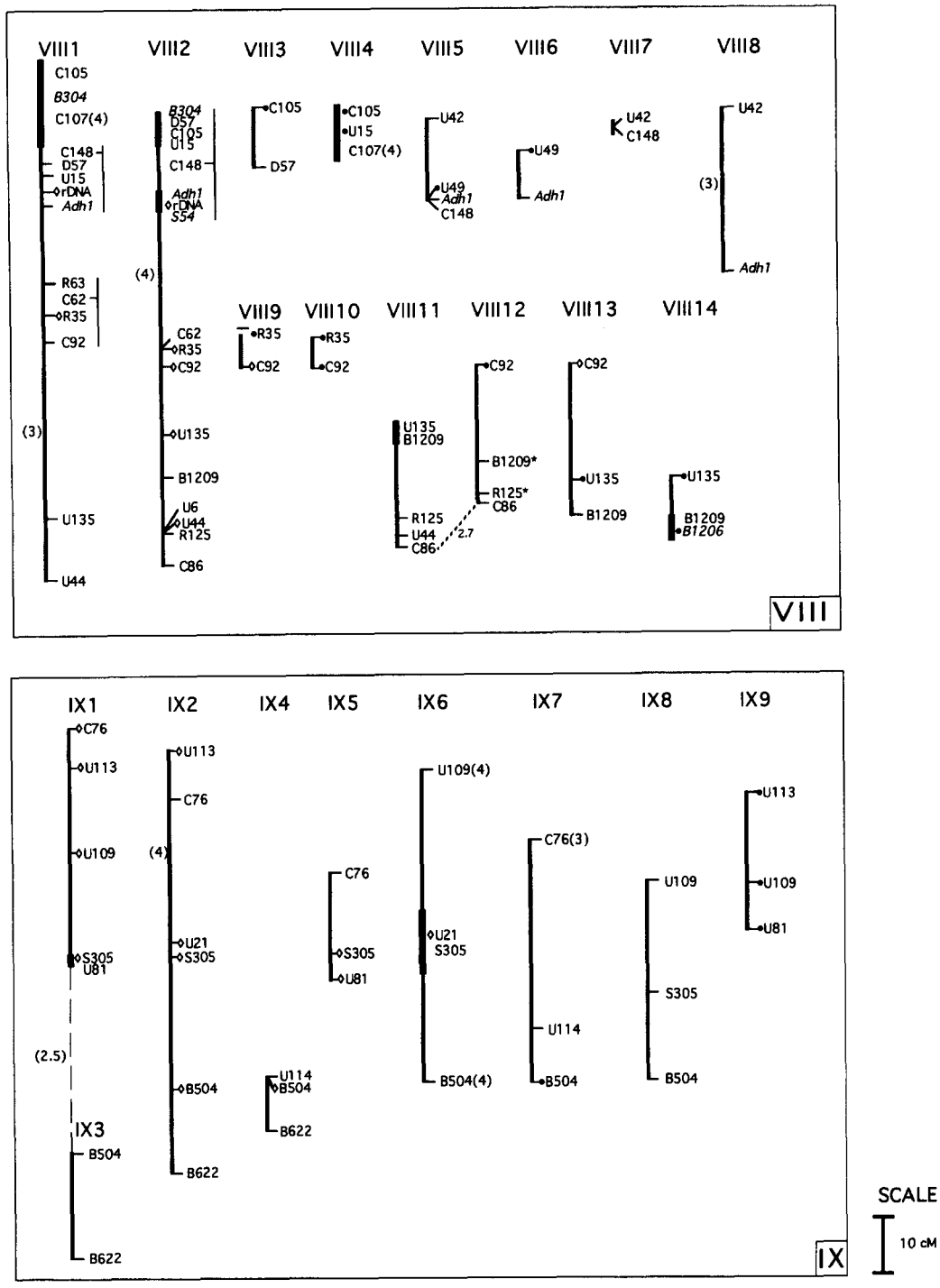

FIGURE 3.—*Continued*

neum clone. This was investigated for 62 probes, chosen from among those with the largest numbers of simplex markers having easy-to-read banding patterns, and for the peroxidase isozyme system. This permitted study of the origin of 284 simplex markers. For 138 of them, the origin was ambiguous, with 112 cases of bands present in both *S. officinarum* and *S. spontaneum* clones and 26 cases of bands absent in all reference clones. Tentative origins could be given to the other 146 markers. The band was of putative *S. officinarum* origin for 80 of them and of *S. spontaneum* origin for the other 66, among those, seven and three, respectively, were unlinked markers. No band appeared to be *S. barberi* specific. Referring back to the map, the species-specific markers brought additional insight into the genome organization in the following ways:

1. The intervals delimited by markers of known species origin covered a total of 745 cM within the map; 156 cM corresponded to 26 intervals of 0–23 cM each between *S. officinarum* specific markers, 461 cM to 34

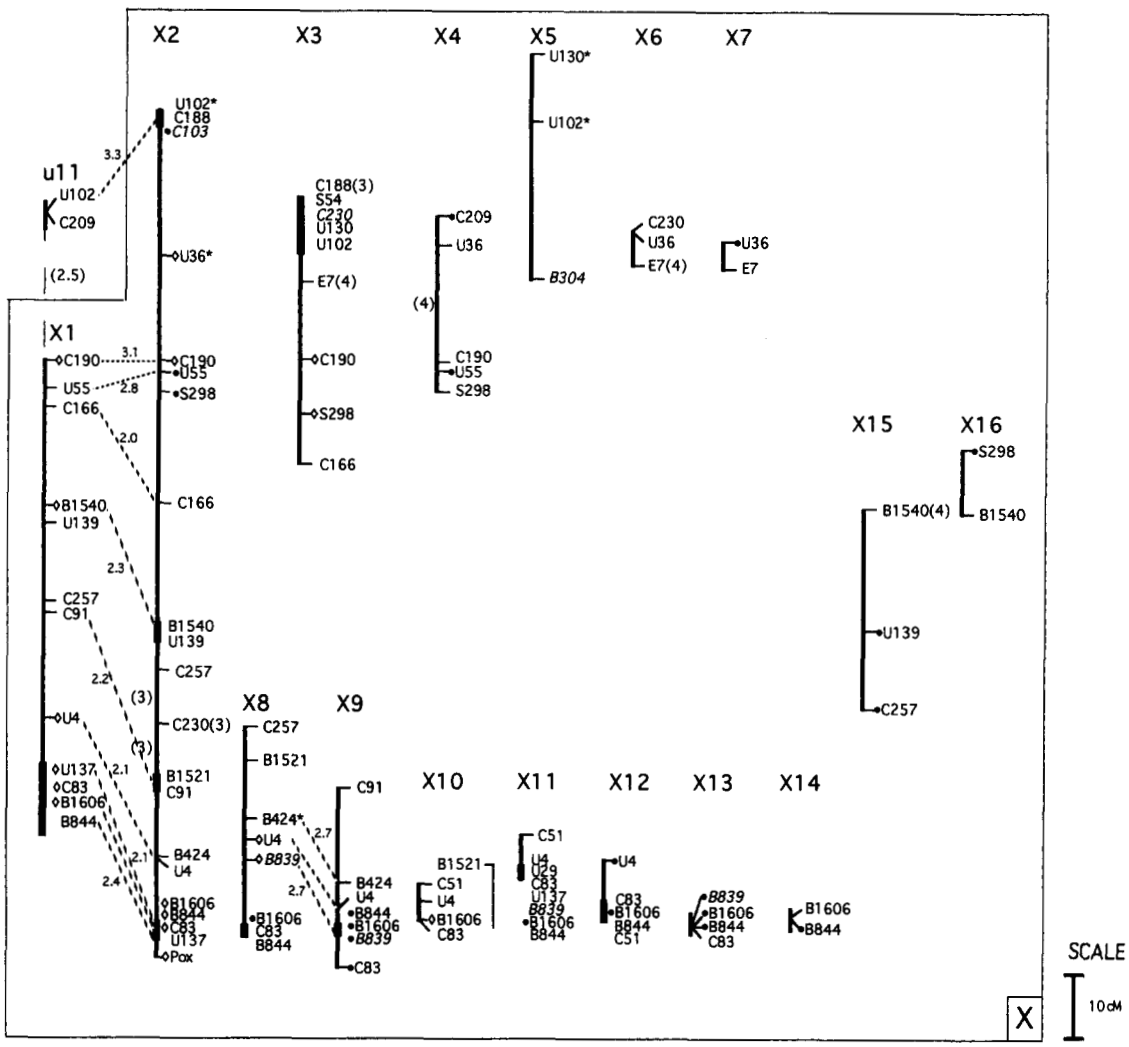

FIGURE 3.—*Continued*

intervals of 0–32 cM each between *S. spontaneum* markers, and 128 cM to six intervals of 2–80 cM with edges of different specificities. This suggests that at least six recombination events between chromosomes contributed by the two ancestral species occurred in the ancestry of R570 within the 745 cM genome portion where this test was possible. This contradicts the assumption that interspecific intrachromosomal recombination is absent (PRICE 1967; BERDING and ROACH 1987).

2. Of the 96 cosegregation groups, 27 had no species-specific markers, 32 had one (24 *S. officinarum* and eight *S. spontaneum*) and 38 had two or more. Among the latter, 34 showed consistent intragroup specificity (19 *S. officinarum* and 15 *S. spontaneum*). For the other four groups (VIII9, VIII13, X2, X8), the cosegregation group appeared to have a double origin, indicating that they were derived from recombinations between homoeologous chromosomes. The most significant example is that of X2 where three recombination events may have taken place.

3. Cosegregation groups that involved *S. spontaneum* markers were usually longer and composed of more markers. This is consistent with our previous mapping study (D'HONT *et al.* 1994). It is also in line with the results of diversity studies, which showed a much larger allelic diversity in *S. spontaneum* as compared to *S. officinarum* (GLASZMANN *et al.* 1990; EKSOMTRAMAGE *et al.* 1992; LU *et al.* 1994a,b). The existence of a larger number of alleles that can be discriminated among as RFLPs in *S. spontaneum* probably limits the occurrence of multiplex fragments in segregation analysis and enhances the chances of detecting linkages between loci on *S. spontaneum*-derived chromosomes.

4. The data did not pinpoint any unambiguous structural heterogeneity related to the bispecific origin of the genome, since the loci order was conserved between all cosegregation groups of the same linkage group. Nevertheless, in the large group VIII, the small cosegregation groups that involved *S. officinarum* markers did not seem to be randomly distributed. They were composed of probes roughly mapping the ends of the two larger *S. spontaneum*-derived groups; they did not

overlap, and no group clustering was possible until analyzed with a LOD score as low as 1. This could have been due to a sparse distribution of probes in the median part of the linkage group or their low polymorphism, which would be expected if the 2n gametes involved during introgression were derived from the fusion of two nuclei after the second meiotic division (BHAT and GILL 1985). This could also be indicative of a *S. spontaneum* chromosome being homologous to two different *S. officinarum* chromosomes.

Hence, the species origins of the markers along the map suggest that the two ancestral genomes have remained largely conserved but that heterologous recombination does sometimes occur. The portion of the map homogeneously bearing *S. spontaneum* markers was about thrice as large as that bearing *S. officinarum* markers, whereas it seems that only about one-tenth of the genome could be derived from *S. spontaneum*. Even though this genome ratio may be strongly underestimated, it can be concluded that the resolution of the map is considerably higher in this genome portion derived from *S. spontaneum*.

Chromosome pairing behavior: The study of repulsion between markers generated by the same probe permitted investigation of chromosome pairing behavior within linkage groups. At each locus, we took into account markers involved in cosegregation groups as well as unlinked simple markers that are putatively borne by undetected homologous chromosomes. The total number of pairwise comparisons was 974. A LOD threshold as low as 2.5 was accepted to account for the low I_p of dominant markers in repulsion, but it was kept in mind that artifacts could also be involved. When this threshold was matched for at least one pair of homologous markers, linkages with LOD scores as low as 2 were also rated for other pairs of homologous markers of the same two cosegregation groups.

Out of the 96 cosegregation groups, 21 had markers involved in pairwise repulsion and one had two markers linked in repulsion with homoeologous unlinked counterparts (group VIII5 with probes *UMC42* and *Adh1*) (data not shown). We distinguished three types of situations. (1) In four cases, the linkage in repulsion was strong (one or several repulsions with LOD > 3 between the two cosegregation groups) and well distributed between the groups (I6 and I7, III1 and III2, IV1 and IV2, X1-u11 and X2). For the pairs of chromosomes I6-I7, IV1-IV2, and X1-X2, no double recessive genotype was observed for any pair of homologous markers, thus suggesting systematic pairing. For pair III1-III2, one double recessive was observed for probe *SSCIR78*. (2) In four cases, repulsion was limited to one pair of markers or a few pairs generated by tightly linked probes (I8-I9, VI4-VI5, VIII11-VIII12, X8-X9). (3) In three cases, repulsion involved small cosegregation groups of two to four markers (III5-III6, VII2-VII3, VIII5 with unlinked markers) and it was difficult to investigate the distribution of repulsion between groups.

A strong repulsion, indicative of strong preferential pairing, seemed to be more frequent between chromosomes with *S. spontaneum* markers: of the four strong repulsions detected, three involved chromosomes of putative *S. spontaneum* origin (I6-I7, III1-III2, IV1-IV2), and the last one involved a putative *S. spontaneum* chromosome (X1-u11) and a putatively recombined chromosome (X2).

In groups II and IX, no pairwise repulsions were observed, although *3* and *5* chromosomes bore at least one *S. spontaneum*-specific marker, respectively. We tried to test the hypothesis of preferential pairing within the *S. spontaneum* subset *vs.* general random pairing. For this purpose, we used a χ^2 test (D'HONT *et al.* 1994) on probes generating alleles for all putative *S. spontaneum* chromosomes of the group, with no missing data (probes *CDSC52* and *SG305* for groups II and IX, respectively). The test was significant at $\alpha = 0.01$ in both cases, indicating probable preferential pairing between chromosomes bearing *S. spontaneum* markers.

Polysomic inheritance in cultivar R570 could not be ruled out for 74 cosegregation groups for which no markers were involved in any significant pairwise repulsion. When pairwise repulsion was detected, it was often weak, with a low linkage LOD threshold and an uneven distribution of repulsion in the cosegregation groups concerned. In a limited number of cases, however, preferential pairing may exist, especially between *S. spontaneum* chromosomes. Overall preferential pairing inside the *S. spontaneum* chromosome subset may also occur. This general pairing behavior was confirmed for markers produced by probe *BNL16.06* in group X on the basis of a larger progeny sample of 157 individuals (data not shown).

Incidentally, the assignment of cosegregation group u11 to linkage group X was confirmed by the repulsion with X2.

Composite map: Considering that recombination between chromosomes of both origins may be possible and that no definite structural difference was detected between chromosomes of *S. spontaneum* and *S. officinarum*, we built a tentative Saccharum composite map (Figure 4). The map integrates segregation information from all cosegregation groups of each linkage group without considering their specific origins. Marker ordering was not improved by pooling the data and remained poor in some parts of the map, but this enabled us to synthesize information scattered between different cosegregation groups and propose a first core map for sugarcane. This map may overlook possible differences between the genomes of *S. officinarum* and *S. spontaneum*. However, since there was strong internal consistency in the linkage groups defined in our map concerning the gene repertoire and the accessible locus order, it would be reasonable to state that any possible differences

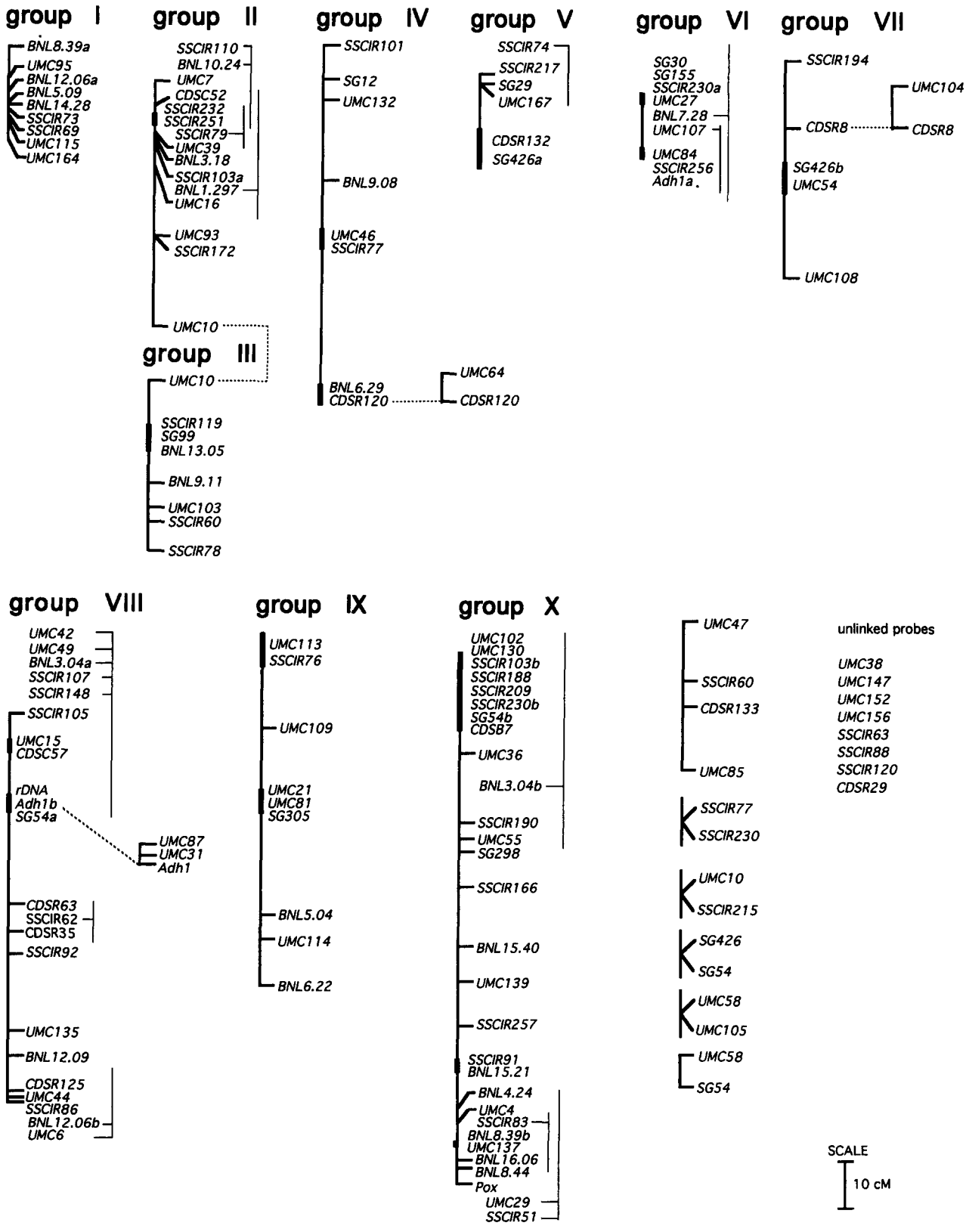

FIGURE 4.—Composite map derived from sugarcane cultivar R570. This map integrates information of all simplex markers involved in cosegregation groups of each linkage group (see text). Uncertain orders of clustered loci (LOD < 1) are denoted by a bold line. Uncertain positions of isolated loci are indicated by a T bar on the right of the linkage group. When probes are duplicated, each locus is identified by a letter, a or b. For three unassigned cosegregation groups, assignment to a particular linkage group was very likely, on the bases of one probe in common and similarity with other sugarcane, maize or sorghum maps; this is indicated by a dashed line. Possible fusion of groups II and III is indicated the same way (see text).

TABLE 1

Correspondence between linkage groups defined according to the present study, those defined in previous mapping studies in Saccharum genus, and maize chromosomes

Linkage groups	Correspondence with other sugarcane maps		Correspondence with maize chromosomes or chromosome arms[c]
	Cultivar SP701006[a]	Saccharum spontaneum[b]	
I	A		(1S, 9L)
II	F	II	(3, 8)
III		II	(3, 8)
IV			(6, 8)
V			
VI	C	V	(1L, 5S)
VII	G	VII	(5L)
VIII	B	VI–VIII	(2S, 10L) + 4
IX	H		(6, 9S)
X	D	IV	(2L, 7) + 10S

Putative chromosome homoeology was based on conserved linkage of several markers. Brackets assemble those chromosome regions that share many duplicate loci in the maize genome (see HELENTJARIS *et al.* 1988).

[a] D'HONT *et al.* (1994).

[b] DA SILVA *et al.* (1993).

[c] BURR and BURR (1991); GARDINER *et al.* (1993).

would involve only simple intrachromosomal rearrangements rather than interchromosomal translocations. The composite map could thus be interpreted as a representation of larger linkage groups between the *S. officinarum* or *S. spontaneum* genomes. A finer interpretation may be possible when more data are available. The cumulative genetic length of the 10 basic linkage groups was 487 cM. The definite "haploid" map length will certainly be larger when more markers have been located. Note, however, that the existence of polymorphic probes left unlinked and of small cosegregation groups left unassigned in our map does not automatically imply that the map length was underestimated, as would be the case for a diploid plant.

Comparison with other genetic maps: This map and that of D'HONT *et al.* (1994), derived from the self progeny of another sugarcane cultivar (SP701006), had 49 probes or isozymes in common. The composition of the linkage groups were highly congruent. The correspondance is given in Table 1. The only difference occurred with probe *BNL6.22;* this could either indicate a duplication for this probe or an artifact in D'HONT *et al.* (1994), since the assignment to linkage group F was only due to one linkage, whereas the assignment to linkage group IX relied on three linkages in this study. The study of D'HONT *et al.* (1994) was performed on a small number of progeny that permitted detection of linkage between two markers only when the recombination rate was lower than 10%. Indeed, the comparison between the two maps revealed that the linkage groups of D'HONT *et al.* (1994) corresponded to low recombination segments on the present map.

Another comparison is possible with the map of DA SILVA *et al.* (1993) on *S. spontaneum* material on the basis of 26 common probes. This established a correspondence between some of the groups defined in both studies (Table 1). It also permitted tentative clustering of some of our linkage groups. The most likely grouping concerned groups II and III. They had one common probe (*UMC10*), located in both cases at one end of the group; the surrounding probes (*UMC93, SSCIR72, SSCIR119, SG99, BNL13.05*) were distant and had few unlinked simplex markers, which could explain why linkage was not detected between the two groups. Probes *CDSC52* (II) and *SG99* (III) were linked on the map of DA SILVA *et al.* (1993).

The locations of the probes on the maize genome and availability of information on their locations in partial sorghum genome maps permit comparisons with these two related crops. Some of our linkage groups are syntenic with certain chromosomes or chromosomal regions of the maize genome (Table 1), usually by pairs due to the possible allotetraploid origin of maize (MOORE *et al.* 1995). Unification of groups II and III closely matches the results from the maize map and the sorghum map where maize probes of groups II and III are scattered, respectively, on maize chromosomes *3* and *8,* two highly related regions (HELENTJARIS *et al.* 1988) and on sorghum linkage group F (WHITKUS *et al.* 1992). Possible clusterings of groups IV and VI, and groups VIII and V were not supported by as much congruent evidence. More information is needed to confirm their possible relationships, especially because of possible duplication of isolated probes.

Among the 11 unassigned cosegregation groups, three could tentatively be associated to linkage groups on the basis of one common probe and map comparisons with *S. spontaneum,* maize and sorghum (Figure 4). These are u4, assigned to linkage group IV (probe *CDSR120* in common, compatible with the sorghum map) (WHITKUS *et al.* 1992); u5, assigned to group VII [probe *CDSR8* in common, compatible with the SP701006 map (D'HONT *et al.* 1994) and the maize map (GARDINER *et al.* 1993)]; and u6 assigned to group VIII [*Adh1* in common, compatible with the *S. spontaneum* map (DA SILVA *et al.* 1993) and the maize map (GARDINER *et al.* 1993)].

The status of cosegregation groups u1 and u2 remains ambiguous. They could be lumped into group III on the basis of probe *SSCIR60,* but this is unlikely considering the high number of unlinked simplex markers. They could also be lumped into linkage group VIII according to the maps of D'HONT *et al.* (1994) (group E, *S. spontaneum* chromosomes) and of DA SILVA *et al.* (1993) (cosegregation group 28).

Comparison of genetic distances between our composite map, representative of a varietal hybrid genetic structure, and the map of DA SILVA *et al.* (1993) on *S. spontaneum* material was possible for 11 pairs of probes.

The genetic distances were larger in *S. spontaneum* by an average of 10–30% (the variation being due to uncertain marker orders), but the difference was not significant (*t*-test at $\alpha = 0.05$). This suggests that recombination rates are similar in these very different genetic structures.

DISCUSSION

Constructing the map of an atypical polyploid genome: The use of locus-specific markers such as RFLPs and isozymes was essential in undertaking genetic mapping within the complex structure of a sugarcane cultivar. It allowed investigation of synteny and colinearity between homo(eo)logous cosegregation groups and assessment of the ancestral origin of some of the alleles. The process that we followed involved a progressive search for the simplest explanations, often neglecting alternative possible interpretations. All sorts of unusual behaviors cannot be excluded, such as occasional whole chromosome losses during somatic multiplication upstream from the germinal lines that might affect part of the gametes or a gametic counter selection affecting certain chromosomal combinations. Such phenomena would result in segregation distortions. The primary consequences for linkage estimations between two loci would be an artificial increase of one parental combination (with both markers absent) causing an artificial underestimation of recombination rates and overall map compression. In the case of our progeny, the pattern of segregation ratios (Figure 2) suggests that such artifacts would be exceptional and weak. A secondary consequence could be to mistake a duplex marker as a simplex marker. The segregation of a duplex marker is determined by the segregation of two homo(eo)logous chromosomes. Such a marker might therefore appear linked with simplex markers of the two corresponding cosegregation groups and cause an artificial bridge between them. We found no such cases in our results. Another source of uncertainty concerns identification of species origins of the markers. We tried to collect most clones in the ancestry of R570, but some are unknown, some are questionable, and some possibly mislabeled. For instance, we could unambiguously confirm that R445 and H32 8560 are the genuine parents of R570, but some of the grandparents (at least the clones we had) were definitely questionable (data not shown). These uncertainties hindered the monitoring of allele inheritance through the pedigree. The origin of alleles thus had to be assessed on the basis of presence *vs.* absence of a band among a few representatives of the ancestral species, which is certainly less conclusive than examining the most ancestral founder clone that contributed the markers.

Despite these constraints, the overall segregation data confirmed the information obtained in our previous study (D'HONT *et al.* 1994) and broadened the general understanding of genome perspectives in sugarcane. No significant structural differences were uncovered between genome portions inherited from *S. officinarum* and *S. spontaneum*. Nevertheless, a simple rearrangement between the two genomes was suspected in linkage group VIII. Adding more markers to the map will provide greater insight. However, this may take time since independence between two loci cannot be proven as long as the map is not saturated, due to the possible lack of suitable polymorphism. In this respect, precise comparisons with RFLP maps constructed for intraspecific crosses in *S. spontaneum* (DA SILVA *et al.* 1993) and *S. officinarum* will be very informative. Our data provide strong evidence that recombination is possible between *S. spontaneum* and *S. officinarum* chromosomes. This justifies the construction of a composite map for Saccharum. Such recombination events must, however, be rare since only a few were detected on the map. Preferential pairing between *S. spontaneum* chromosomes clearly contributes to limiting their occurrence.

Global length of the genetic map in cultivated sugarcane: Our previous results (D'HONT *et al.* 1994) revealed a very low amount of recombination (<10%) between loci that were scattered over a great chromosome length in maize (as high as 70 cM), suggesting a possible *in toto* transmission of chromosomes in sugarcane varieties. This hypothesis can be excluded on the basis of the present data. Nevertheless, the loci mapped did not seem to be distributed randomly between chromosomes. In several regions, there appeared to be clusters of almost nonrecombining loci. These regions closely corresponded to areas of low recombination identified in our previous study. Recombination rate heterogeneity along chromosomes is not a novel observation and has been reported from studies of several species, such as hexaploid bread wheat (DVORAK and CHEN 1984; CHAO *et al.* 1989; GILL and GILL 1994) and common bean (VALLEJOS *et al.* 1992). Although the map was larger than we initially expected, the length of the composite map remained close to 500 cM. We found no significative difference of recombination rate between R570 and *S. spontaneum* "SES208" on the basis of 11 specific pairs of probes. DA SILVA *et al.* (1995) estimated the cummulated length of cosegregation groups to be 6600 cM in SES208. Assuming autooctoploidy, a rough estimation of the composite map length of this clone is 825 cM (6600/8). This could indicate either a better coverage of the SES208 genome or a slightly higher mean recombination rate in the genome of this wild euploid relative. Our map will certainly be extended by increasing the number of mapped loci, but it already comprises a sample of maize probes sufficiently scattered on maize chromosomes to make it possible to roughly compare genetic map lengths. These are 2300 cM in maize (BEAVIS and GRANT 1991) and at least 1445 cM in sorghum (CHITTENDEN *et al.* 1994). Our study thus confirms that the recombination rate per basic ge-

nome is much lower in cultivated sugarcane (D'HONT *et al.* 1994; GRIVET *et al.* 1994).

Directing future mapping experiments for breeding applications: Mapping of cultivated hybrid material naturally led to preferential targeting of *S. spontaneum* chromosomes owing higher number of segregating loci. We showed that although *S. spontaneum*-derived chromosomes constitute a small part of the total genome, the largest part of the map spans chromosome regions probably inherited from this wild species. Multiplying the number of restriction enzymes used on each RFLP probe would improve the coverage and thus enhance that of the *S. officinarum* genome. Nevertheless, isolating all alleles at each locus would probably be a very hard task given the low level of polymorphism in *S. officinarum*. In fact, it cannot be excluded that simplex markers might be completely absent from large *S. officinarum* chromosome segments due to 2n gamete transmission during nobilization and high inbreeding in the ancestry of R570, *e.g.*, the clone POJ2878 occurs twice as its grandparents. This genome sector would be almost inaccessible for mapping.

Due to this unequal map coverage, a complete repertoire of alleles influencing useful quantitative traits could be constituted for the *S. spontaneum* part of the genome only. For the *S. officinarum* part, large gaps in the map may considerably disturb the identification of chromosome segments having a significant influence on agronomic traits. Concentrating efforts on the easily accessible *S. spontaneum* part of the genome would not reduce the importance of the approach since *S. spontaneum* is supposed to have brought many interesting characters to modern cultivars. Conservation of orthologous loci colinearity with maize and sorghum will provide additional guidance for locating specific important genes in the sugarcane genome. Sorghum seems particularly relevant as a diploid model owing to taxonomical closeness (AL JANABI *et al.* 1994b) and apparent conservation of chromosome organization with sugarcane (GRIVET *et al.* 1994). The emergence of DNA genomic *in situ* hybridization techniques, for distinguishing various genomic components (D'HONT *et al.* 1995 and A. D'HONT, L. GRIVET, P. FELDMANN, P. S. RAO, N. BERDING and J. C. GLASZMANN, unpublished results), will provide a very useful complement to purely analytical approaches. Altogether, molecular tools should enhance interspecific introgression, which is the key to further genetic improvement in sugarcane.

We thank Dr. M. LORIEUX for helpful advices in computer analysis, Drs. W. BURNQUIST, J. DA SILVA, P. MOORE and M. SORRELLS for kindly providing probes, Drs. A. H. D. BROWN, C. L. BRUBAKER and anonymous reviewers for helpful suggestions and comments. French Ministère de l'Enseignement Supérieur et de la Recherche supported this research.

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Communicating editor: A. H. D. BROWN