

Physical Mapping of the *liguleless* Linkage Group in *Sorghum bicolor* Using Rice RFLP-Selected Sorghum BACs

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ABSTRACT

Physical mapping of BACs by fluorescent *in situ* hybridization (FISH) was used to analyze the *liguleless* (*lg-1*) linkage group in sorghum and compare it to the conserved region in rice and maize. Six *liguleless*-associated rice restriction fragment length polymorphism (RFLP) markers were used to select 16 homeologous sorghum BACs, which were in turn used to physically map the *liguleless* linkage group in sorghum. Results show a basic conservation of the *liguleless* region in sorghum relative to the linkage map of rice. One marker which is distal in rice is more medial in sorghum, and another marker which is found within the linkage group in rice is on a different chromosome in sorghum. BACs associated with linkage group I hybridize to chromosome I_1 , which was identified by using FISH in a sorghum cytogenetic stock trisomic for chromosome I (denoted I_1), and a BAC associated with linkage group E hybridized to an unidentified chromosome. Selected BACs, representing RFLP loci, were end-cloned for RFLP mapping, and the relative linkage order of these clones was in full agreement with the physical data. Similarities in locus order and the association of RFLP-selected BAC markers with two different chromosomes were found to exist between the linkage map of the *liguleless* region in maize and the physical map of the *liguleless* region in sorghum.

THE family Gramineae contains 10,000–11,000 species, including many important crop plants, such as wheat, rice, maize, oat, rye, sorghum, barley, millet, and sugarcane. Results from comparative genomic mapping within this family (Helentjaris *et al.* 1988; Hulbert *et al.* 1990; Binelli *et al.* 1992; Berhan *et al.* 1993; Dunford *et al.* 1995), examination of phylogenetic relationships using conserved DNA sequences (Hsiao *et al.* 1993), and crosses between distantly related species producing intergeneric and interspecies hybrids (Laurie *et al.* 1990) all suggest that grasses evolved from one common ancestor (Tzelev 1989; Moore *et al.* 1995a,b). The evolutionary lines leading to rice (*Oryza sativa*) and maize (*Zea mays*) are believed to have diverged ~50 mya (Bennetzen and Freeling 1993), while those leading to Sorghum and Zea are believed to have diverged about 20 mya (Doebly *et al.* 1990) from a common ancestor that had a base number of $n = 5$ (Anderson 1945; Garber 1950; Rhodes 1951; Celarier 1956). Maize and sorghum appear to have a polyploid ancestry but it is not clear whether polyploidy occurred in the ancestor of maize and sorghum or independently after divergence. The divergence between sorghum and maize has apparently been accompanied by various duplications of chromosome segments, inversions,

and intrachromosomal translocations (Whitkus *et al.* 1992).

Comparative mapping within this family is generally straightforward because probes generated in one species can frequently be used for mapping in another species (Hulbert *et al.* 1990; Binelli *et al.* 1992; D'Hont *et al.* 1994; Dufour *et al.* 1996; Sherman *et al.* 1995). The genetic blocks containing the *liguleless* gene apparently vary in copy number and are arranged in different orders among the various members of the Gramineae, but the locus order is similar across species (Moore *et al.* 1995a,b). The conservation of genes and gene order is irrespective of the base chromosome number in grasses, which varies from 5 in maize and sorghum, 7 in wheat, barley, and rye, 9 in millet, 10 in sugarcane, to 12 in rice (Moore *et al.* 1995a). It is also independent of the haploid DNA content (C value), which varies by a factor of ~40, with rice being the smallest (386–579 Mb; Bennett and Smith 1991), and wheat the largest (16,695–17,423 Mb; Bennett and Smith 1976; Michaelson *et al.* 1991).

Comparative mapping between rice and maize indicates that the *liguleless* (*lg-1*) linkage group is a highly conserved region of the genome (Ahn and Tanksley 1993). Because the *liguleless* linkage group is highly conserved between rice and maize, it is expected to be conserved in other cereals as well. Based on this premise, restriction fragment length polymorphism (RFLP) markers from a linkage group of one member of the Gramineae can be used to screen a library of a related

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cereal. Clones selected based on these markers should then correspond to that linkage group within the test species and may be used for fluorescent *in situ* hybridization (FISH) to physically map a linkage group. This approach to physical mapping has great potential in that it is efficient, *i.e.*, a linkage map of the targeted species is not required, and wild relatives and progenitor species can be rapidly mapped. It can also provide insight into the homeologies of regions between species, as well as provide evidence of the ploidy of a targeted species.

The presented results describe the physical mapping of the *liguleless* linkage group in *Sorghum bicolor* (L.) Moench using FISH. Sorghum bacterial artificial chromosome (BAC) clones were isolated from a sorghum BAC library (Woo *et al.* 1994) using RFLP markers from the *liguleless* linkage groups of rice and maize. Using physical mapping and trisomic stocks, we anchored *liguleless* to chromosome *I*₁ in *S. bicolor* (Chromosome *I* in sorghum is denoted as *I*₁ to prevent confusion with linkage group I of sorghum). Transgenomic BAC FISH revealed a basic conservation of the sorghum linkage group relative to the genetic maps of rice and maize. Deviations in sorghum from that of rice and maize include a single transposition of one of the RFLP-selected BAC markers to a more internal position within the linkage group, and the occurrence of a separate RFLP-selected BAC marker on a different chromosome.

MATERIALS AND METHODS

***liguleless* RFLP probes:** Vectors containing *liguleless*-associated RFLP clones CDO36, RZ596, BCD135, RZ590, CDO93, CDO539, RZ53, RZ467, RZ86, and CDO1417 were kindly provided by S. McCouch (Cornell University). Clones are designated in capital letters, *e.g.*, CDO1417, as opposed to markers, which are in lowercase italic letters and numbers, *e.g.*, *cdo1417*. Samples were rehydrated in 20 μ l TE (10 mM Tris-HCl, 10 mM EDTA), and 1 μ l was electroporated into *Escherichia coli* ElectroMAX DH10B cells (Bethesda Research Labs, Gaithersburg, MD) using the BRL Cell Porator system as recommended by the manufacturer. Transformants were selected on LB plates containing 50 μ g/ml kanamycin. Culture tubes containing 5 ml LB broth plus 50 μ g/ml kanamycin were inoculated and grown overnight at 37° with shaking. Cultures were pelleted in a Beckman (Fullerton, CA) (CS-6R) benchtop centrifuge, and plasmids were isolated by alkaline lysis minipreps as described by Silhavy *et al.* (1984). Samples were restriction-digested using (1) *EcoRI* (BRL) for RZ53 and RZ590, (2) *HindIII* for CDO93, (3) *EcoRI* and *XhoI* for CDO36, CDO539, and CDO1417, and (4) *BamHI* and *EcoRI* for BCD135, RZ86, RZ467, and RZ569. Samples were loaded onto a 1% TAE gel, which was stained with ethidium bromide. Visible DNA bands were cut from the gel, incubated at 65° for 10 min, treated with 1 unit of GELase (Epicenter, Madison, WI) per 100 mg gel, and allowed to digest for 2 hr at 40°. Samples were stored at -20° until needed.

Screening of the sorghum BAC library: Three filter copies of the sorghum BAC library were made as described by Woo *et al.* (1994). Six extra filters were also made for use as test filters. RFLP probes were random primer-labeled using α -³²CTP (Amersham, Arlington Heights, IL) and hybridized

to the test filters, as well as filters containing genome-equivalent amounts of *EcoRI*-digested rice, maize, and sorghum DNAs to determine whether the probes shared enough homology to sorghum to effectively screen the BAC library. RFLP probes that passed prescreening tests were hybridized, as above, to a filter set (one copy of the library), using three probes simultaneously. Filters were washed three times, 30 min each with 0.5 \times SSC, 0.1% SDS, and autoradiography was conducted using Kodak X-OMAT AR film (Rochester, NY) and a single intensifying screen.

Clone verification and BAC purification: BAC clones giving potentially positive signals were removed from the sorghum BAC library, and grown overnight at 37° on LB plates containing chloramphenicol (CM, 12.5 μ g/ml). Colonies were replated on LB/CM plates in three sets (one set for each probe) and again grown overnight. The colonies were transferred to a single sheet of Hybond N⁺ (Amersham) for Southern hybridization and processed as described by Woo *et al.* (1994). After processing, the filter was cut into three sets, and each set was hybridized individually with one of the probes from the previous hybridization. Clones positive for specific RFLP markers: (69D11, 86B10, 88C12, 109H3; RZ86), (97B4, 110C3; RZ467), (112F2, 118B1; RZ590), (66C12, 102H11, 116B10; RZ569), (112H4, 125C8, 129E8; CDO539), (67B3, 144A5; CDO1417) were selected and used to inoculate 100 ml (LB/CM) cultures for plasmid isolation. Cultures were maxiprep using a 20-fold scale up of the procedure described by Silhavy *et al.* (1984). BAC DNA was resuspended in 200 μ l TE and RNase treated (25 μ g) for 2 hr at 37°. Samples were then phenol, phenol/chloroform, and chloroform extracted and BAC plasmids were ethanol precipitated overnight at -20° (Sambrook *et al.* 1989). Plasmids were resuspended in 100 μ l TE, and the DNA was quantitated on a fluorometer (Hoeffer, San Francisco). BAC samples were digested with *NotI* for 4 hr at 37°, run on a CHEF (contour-clamped homogeneous electric field) gel, and blotted. Clones derived from the same RFLP marker were run side-by-side to compare restriction patterns and to determine if the clones were multiple copies of the same region or if another locus possibly exists within the genome. BAC DNA was stored at -20° until needed.

BAC end-cloning and RFLP mapping: BAC ends were isolated by plasmid rescue. All procedures were conducted as described by Woo *et al.* (1994). Clones 69D11, 86B10, 88C12, 109H3, 97B4, 110C3, 112F2, and 118B1 were digested separately with 0.5 units of *BamHI*, *ScaI*, *SphI*, and *NotI* (BRL) for 4 hr at 37°. Samples were run on a 1% agarose gel, which was blotted and hybridized with ³²P-labeled pBeloBAC11. Clones digested with *SphI* that were slightly larger than the *NotI*-digested pBeloBAC11 vector were selected for self-ligation. Clones 66C12, 102H11, 116B10, 112H4, 125C8, 129E8, 67B3, and 144A5 were only digested with *SphI*, and self-ligated following gel analysis. One microliter of each ligated end clone product was transformed as previously described, and colonies were selected on LB/CM (12.5 mg/ml) plates. Five colonies from each ligation reaction were picked, replated on LB/CM plates, and used to inoculate a 5 ml miniprep culture. Samples were miniprep and digested with *SphI* and *HindIII* to release the insert, and verify that all clones contained the same fragment. The gel was blotted and hybridized with ³²P-labeled total genomic sorghum DNA to detect repetitive end-fragments. Clones that tested negative were used for RFLP mapping in sorghum. Five micrograms of each end clone were digested with *SphI* and *HindIII* for 4 hr at 37°. Samples were fractionated on a 1% TAE gel, and DNA fragments were removed. DNA was purified from the gel pieces by electroelution. DNA fragments were ethanol precipitated as described, and DNA was quantified on an agarose gel. To detect polymorphisms, ~100 ng

BAC-end DNA was ^{32}P -labeled, and hybridized to test filters of the parental cross BTx623 X IS3620C (Xu *et al.* 1994) that had been digested with *Bam*HI, *Eco*RI, *Eco*RV, *Hind*III, and *Xba*I. Probes that showed polymorphisms were hybridized to the F2 mapping population filters, polymorphisms were scored, and the data were processed using MAPMAKER (Dupont, Wilmington, DE).

Incorporation of haptins into BAC FISH probes: Whole BAC plasmid DNA was either biotin-14-dATP-labeled (BRL) using the GIBCO (Grand Island, NY) BRL BioNick Labeling System or labeled with digoxigenin-11-UTP using the Boehringer Mannheim (Indianapolis) Nick Translation Kit according to the manufacturer's recommendations.

C₀t-1 DNA isolation: Total genomic DNA was isolated from greenhouse-grown sorghum (BTx623), maize (VA35), and rice (IR36) leaf tissues using the technique described by Ayres *et al.* (1997) with slight modifications. All leaf tissues were ground with liquid nitrogen to a fine powder and then mixed with PEX potassium ethyl xanthogenate [(Fluka Chemical, Buchs, Switzerland)] buffer. Purified, RNase-treated, phenol/chloroform-extracted DNA was then used to make species-specific C₀t-1 DNA as described by Zwick *et al.* (1997).

Plant materials and metaphase slide preparation: Roots from 4- to 6-day-old seedlings of sorghum (*S. bicolor* BTx623) were treated with 0.4% 8-hydroxyquinoline aq. (8HQ) in the dark at room temperature (RT) for 5 hr. Roots were fixed in ethanol-acetic acid (4:1) overnight at RT. Roots harvested from 5- to 7-day-old maize (*Z. mays* VA35) seedlings were treated with 8HQ for 5 hr. Rice (*O. sativa* IR36) roots were harvested from 7- to 10-day-old seedlings, and were treated for 3 hr with 8HQ. Both maize and rice roots were fixed as described above. Microscope slides with metaphase chromosomes were prepared using techniques modified from Jewell and Islam-Faridi (1994).

Fluorescent *in situ* hybridization (FISH): The FISH procedures were those of Hanson *et al.* (1995) as modified by Zwick (1997). Slides were analyzed with an Olympus Vanox epifluorescence microscope (Olympus, New York) using standard filters. Chromosomes were photographed with an attached camera on Fuji HG ASA 400 professional film. Photographic prints were scanned using a standard desktop RGB scanner, and digital images were processed, cropped, and assembled digitally. Images were printed by a Kodak dye-sublimation printer.

RESULTS

BAC library screening: The sorghum BAC library was screened with probes of 10 RFLP markers for the *liguleless* (*lg-1*) linkage groups of rice and maize (Figure 1). RFLP clone CDO93 was eliminated from further screening because it hybridized intensely to all BAC clones on a BAC library testing filter. This marker may have been contaminated with vector sequences because there were problems excising the insert from the vector. Clones RZ53 and BCD135 were excluded from library screening due to lack of signal following hybridization to sorghum DNA present on test filters containing genome equivalent amounts of DNA of rice, maize, and sorghum, although these markers did show hybridization to both rice and maize. The remaining seven clones were used to screen the sorghum BAC library (Table 1). Of these, CDO36 showed no hybridization to any of the sorghum BAC clones, suggesting either that a posi-

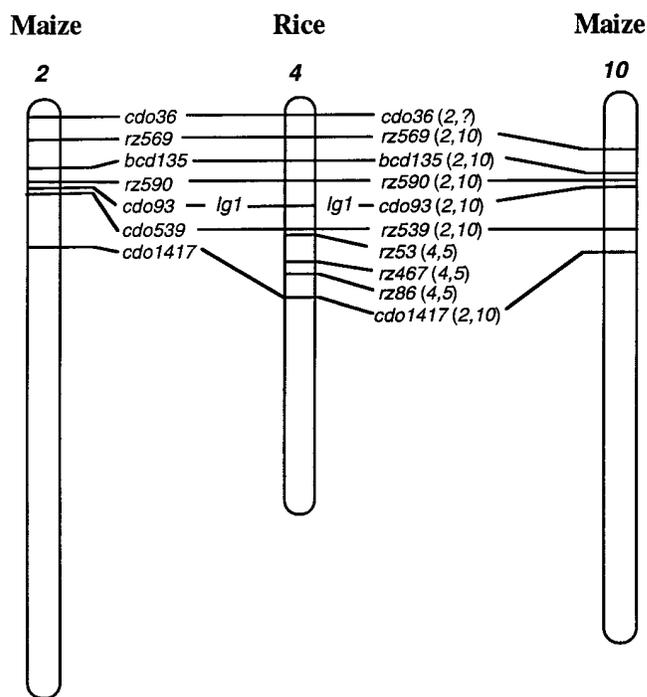


Figure 1.—Comparative linkage maps of maize chromosomes 2 and 10 and rice chromosome 4 showing the conserved *liguleless* linkage group (Figure taken in part from Ahn and Tanksley 1993). Bracketed numbers following the RFLP markers on chromosome 4 of rice indicate the chromosome location of these markers in maize. All of these loci are single copy in rice and are duplicated in maize. RFLP markers are designated as follows, based on their origin: cdo, oat leaf cDNA; bcd, barley leaf cDNA; rz rice leaf DNA.

tive clone was interpreted as background when evaluating the Southern blots or that this locus segment was not represented among the clones of the BAC library. We deem the latter more likely because the library contains only 2.8 genomic equivalents, providing only a 91% chance of finding a particular genomic region within the sorghum BAC library (Woo *et al.* 1994). Nonetheless, 16 BAC clones were identified as having homology to a total of six *liguleless*-associated RFLP clones.

RFLP mapping of *liguleless* BACs: The selected BACs were subjected to end-cloning procedures (Woo *et al.* 1994). Hybridization of ^{32}P -labeled total genomic sorghum DNA revealed that the end clones derived from BACs 66C12 (RZ569), 144A5 (CDO1417), 88C12 (RZ86), and 97B4 (RZ467) contained repetitive DNA sequences, rendering them ineffective as probes for linkage mapping. The remaining end clones were hybridized to the sorghum survey filters. End clones 109H3 (RZ86), 102H11 (RZ569), 129E8 (CDO539), and 116B10 (RZ569) revealed no polymorphisms, whereas the remaining end clones detected polymorphisms and were used for mapping. Linkage analysis using these clones revealed that the corresponding RFLP loci are in two linkage groups, namely I and E. The end clones from

TABLE 1
FISH signal intensities of *liguleless*-selected sorghum BACs to sorghum, rice, and maize metaphase chromosomes

Rice RFLP	Sorghum BAC	BAC Size (kb)	FISH signal intensity		
			Sorghum	Rice	Maize
<i>rz569</i>	66C12	140	strong	very weak	very weak
	102H11	315	strong	NI	NI
	116B10	315	strong	NI	NI
<i>rz590</i>	112F2	275	strong	moderate	strong
	118B1	190	NI	NI	NI
<i>cdo539</i>	112H4	240	strong	NI	NI
	125C8	225	strong	NI	NI
	129E8	200	strong	moderate	strong
<i>rz467</i>	97B4	265	strong	NI	NI
	110C3	275	strong	very weak	weak
<i>rz86</i>	69D11	235	strong	NI	NI
	86B10	170	moderate	NI	NI
	88C12	190	strong	moderate	strong
	109H3	240	NI	NI	NI
<i>cdo1417</i>	67B3	245	strong	moderate	strong
	144A5	195	strong	NI	NI
<i>cdo36</i>	none detected	NI	NI	NI	NI

Signal intensities are defined as strong (very intense), moderate (easily seen), weak (visible), and very weak (difficult to distinguish). NI indicates that the BAC was not hybridized and no information exists. BACs are designated by their location in the sorghum BAC library and were sized by *NotI* restriction digest and CHEF gel electrophoresis.

BACs 112F2 (RZ590) and 110C3 (RZ467) were associated with linkage group I at the positions indicated in Figure 2 (LOD > 3), and an end clone derived from BAC 112H4 (CDO539) mapped to the interval indicated on linkage group I (Figure 2) with a LOD score of 2.57. The end clone from BAC 69D11 (RZ86) mapped to a position on linkage group E (Figure 2, LOD > 3). The end clone from another BAC (125C8) screened with CDO539 also mapped to linkage group E in the interval between *Xbcd147* and *Xumc51* (LOD > 3), but the data for this marker expanded the map by about 11 cM. This indicates that some of the data points are incorrect and therefore its map position is uncertain. The map order of these BAC end clones is in agreement with that of the RFLP markers in the rice *liguleless* linkage group.

Restriction analysis of RFLP-selected BACs: The *NotI* restriction digest patterns of the BACs (Figure 3) indicate that all of the clones isolated using the same RFLP originated from the same region of the genome. All of the BACs isolated using the same RFLP shared at least one common fragment, representing shared regions of overlap between the clones. Uncommon fragments represent the unshared regions, which can be attributed to the partial restriction digest of the sorghum DNA when constructing the BAC library.

FISH of BACs: All RFLP-selected BACs, except 109H3 (RZ86) and 118B1 (RZ590), were individually analyzed by FISH to root-tip metaphase chromosome spreads of *S. bicolor*. An example of a sorghum FISH karyotype is

shown in Figure 4. A pair of signals were visible from all BAC hybridizations, but some BACs produced more intense signals than others and had lower amounts of overall background (Figure 5, A-N).

Because the *lg-1* linkage group is conserved between rice and maize, a BAC homologous to each of the *lg-1* RFLP markers was selected, and these BACs were hybridized simultaneously to sorghum metaphase chromosomes, according to the order of the RFLP markers found in rice (Figure 6). BAC probes were labeled in an alternating order with biotin and digoxigenin so they would be detected as sites of red and green fluorescence. The BAC signals occurred in the following order: (red) RZ569, (green) RZ590, (green) CDO1417, (red) CDO539, and (green) RZ467. The unexpected rearrangement of CDO1417 in sorghum to the middle of the linkage group placed two consecutive biotin-labeled probes adjacent to each other, producing one large yellow signal and, thus, only four distinct hybridization sites.

To determine the true order of the *lg-1* region in sorghum, BACs 66C12, 112F2, 129E8, 110C3, 88C12 and 67B3, each having sequences homologous to a different RFLP marker, were used pairwise for physical mapping of the *liguleless* linkage group by dual FISH. Each BAC probe was labeled with either biotin or digoxigenin so individual BACs could be subsequently detected as red or green signals and properly identified (Figure 7, A-G). In several cases, signals appear yellow or white, instead

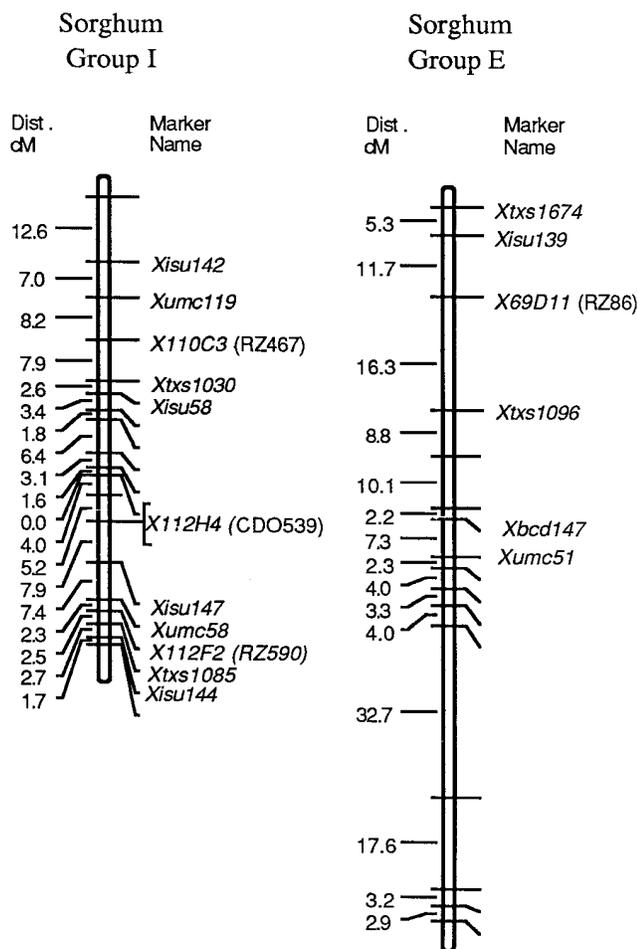


Figure 2.—RFLP mapping of BAC end clones 110C3, 112F2, 112H4, and 69D11 in *S. bicolor*, using the parental cross BTx623 XIS3620C of Xu *et al.* (1994). End clones from BACs 110C3, 112H4, and 112F2 (in that order) all mapped to linkage group I. This order is consistent with the order of the homologous RFLP markers in rice and the physical order of the homologous BAC probes in sorghum as determined by FISH. Marker 112H4 mapped between markers 112F2 and 110C3, although its exact position could not be precisely mapped due to conflicting data points (area defined by a vertical line). An end clone from BAC 69D11 mapped to linkage group E as indicated, and an end clone from BAC 125C8 mapped to linkage group E in the interval between *Xbcd147* and *Xumc51* (see results). The linkage maps of sorghum Groups I and E are from G. E. Hart (unpublished results).

of green. This is due to the overlapping of the red and green signals, which blend and form a white signal. The order of probes hybridized to sorghum was based on the arrangement of the homeologous linkage group in rice. When *liguleless* BACs were FISHed two at a time in an overlapping order, *i.e.*, 66C12/112F2, 112F2/129E8, 67B3/129E8, 129E8/110C3, 110C3/67B3, and 110C3/88C12, collective results indicated the order of markers in the *liguleless* region of sorghum to be *rz569* (66C12), *rz590* (112F2), *cdo1417* (144A5), *cdo539* (129E8), and *rz467* (110C3) (Figure 8). FISH of BAC 88C12 was associ-

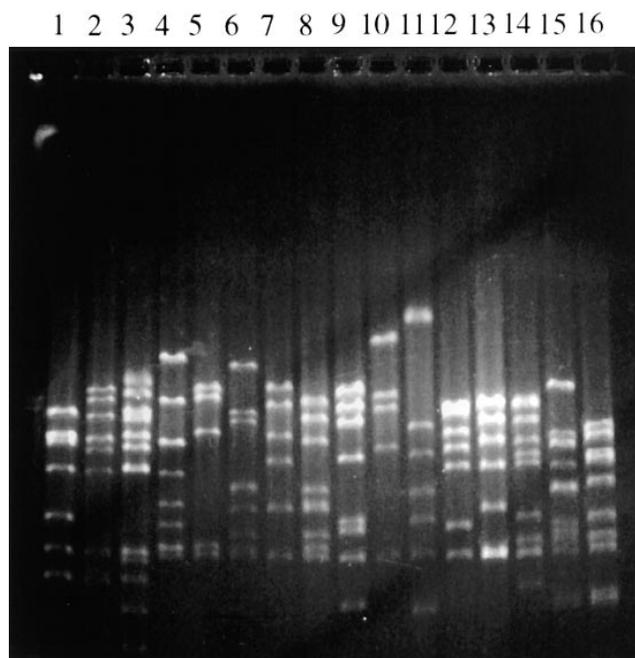


Figure 3.—Ethidium bromide-stained CHEF gel of *liguleless*-associated RFLP-selected sorghum BACs. BACs were prepared by using a standard miniprep and restriction-digested with *NotI*. All BACs selected from the same RFLP marker share at least one common band. BAC clones are shown as follows: (lane 1) 66C12, (lane 2) 102H11, (lane 3) 116B10, (lane 4) 112F2, (lane 5) 118B1, (lane 6) 112H4, (lane 7) 125C8, (lane 8) 129E8, (lane 9) 97B4, (lane 10) 110C3, (lane 11) 69D11, (lane 12) 86B10, (lane 13) 88C12, (lane 14) 109H3, (lane 15) 67B3, and (lane 16) 144A5.

ated with a different chromosome pair. Figure 9 shows FISH of BAC clones 112F2 and 69D11 to sorghum as trisomic for chromosome *I_r* (Schertz 1972). Three pairs of signals are clearly seen from BAC 112F2, while BAC 69D11 produced two pairs of signals on separate chromosomes. These data not only identified the location of *liguleless* in sorghum but also anchored linkage group I to chromosome *I_r*. The identity of the chromosome with which RZ86-selected BAC 88C12 associated has not been resolved.

The sorghum BACs used for physical mapping in sorghum were also hybridized *in situ* to both rice and maize chromosomes (Figures 10 and 11, A–D). In maize, probes 67B3 (CDO1417, Figure 10A), 129E8 (CDO539, Figure 10B), and 112F2 (RZ590, Figure 10C) generated intense signal pairs on two pairs of chromosomes, while probe 88C12 (RZ86, Figure 10D) produced a strong pair of signals on one pair of chromosomes, and possibly a second less-intense pair of signals on a different pair of chromosomes. In maize, where the major *lg-1* group of markers has been mapped, the RFLP loci RZ86 are in chromosomes 4 and 5, as opposed to chromosomes 2 and 10. Probe 110C3 (RZ467) gave four moderately weak signals on two pairs of chromosomes, and 66C12 (RZ569) produced extremely weak signals on four chro-

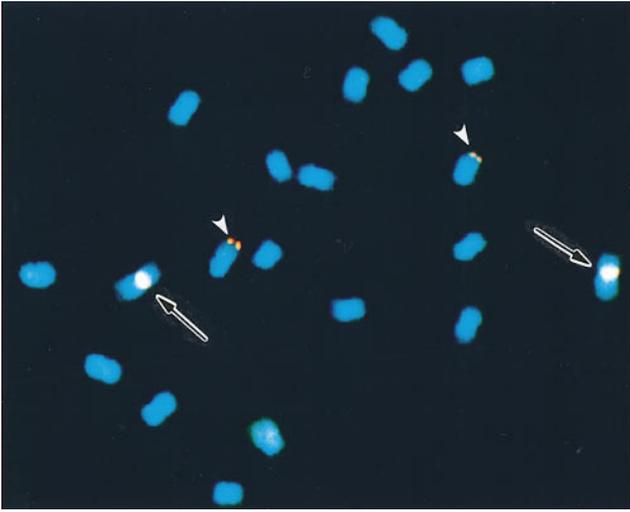


Figure 4.—FISH of BAC 66C12 (arrowheads) and 18S rDNA (arrows) to metaphase chromosomes of *S. bicolor*. Probes were labeled with biotin and both sites were detected with Cy3-conjugated anti-biotin. The chromosomes were counterstained with DAPI.

mosomes. For these last two probes, signal intensities quenched rapidly, making photography difficult, so analysis of the hybridization sites could only be made by observing numerous karyotypes (data not shown).

Signals from sorghum BAC probes were more difficult to detect in rice than in maize. Probes 67B3 (CDO1417, Figure 11A), 129E8 (CDO539, Figure 11B), 112F2 (RZ590, Figure 11C), and 88C12 (RZ86, Figure 11D) generated a moderately intense pair of signals on two chromosomes. The remaining probes 110C3 (RZ467) and 66C12 (RZ569) produced an extremely faint pair of signals which were difficult to detect even with a Texas red UV filter (data not shown). Again, analysis of the hybridization of these sites was dependent on

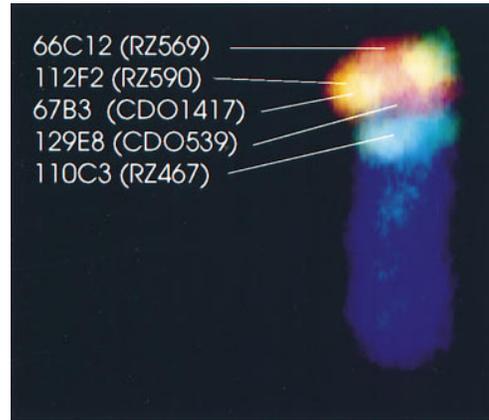


Figure 6.—Simultaneous hybridization of *liguleless* BACs 66C12, 112F2, 129E8, 110C3, and 67B3 to chromosome I of sorghum. Four intense signals are seen rather than the expected five due to the rearrangement of BAC 67B3 from its expected terminal position in rice to a more medial position in sorghum. Since probes were labeled with digoxigenin and biotin in an alternating manner according to the *liguleless* linkage group in rice, the rearrangement of this BAC placed probes 67B3 and 122F2 next to each other, thus creating one large FITC (yellow) signal.

visualizing numerous karyotypes. Results from FISH of these probes to sorghum, rice, and maize are summarized in Table 1.

DISCUSSION

Comparative mapping between species often involves the cross-hybridization of molecular markers such as RFLPs, *e.g.*, Gebhardt *et al.* (1991) and Dunford *et al.* (1995). In the Gramineae, probes that reveal RFLPs in one species will often cross-hybridize with genomic DNA from other genera within the family (Hulbert *et al.* 1990; Whitkus *et al.* 1992; Ahn *et al.* 1993; Ahn and

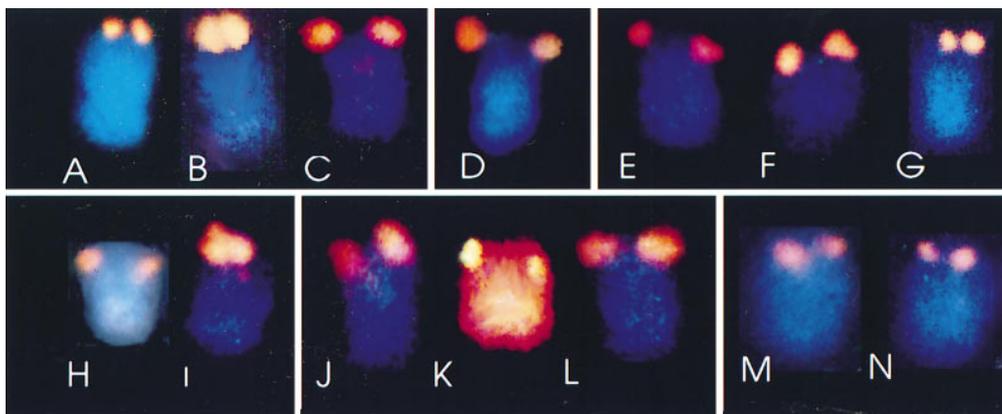


Figure 5.—(A–N). FISH of *liguleless*-associated sorghum BACs to sorghum metaphase chromosome. Chromosomes displaying FISH signals from corresponding RFLP markers are grouped together; variable signal intensities are seen within these groups: (A) 66C12; (B) 102H11; (C) 116B10, all from RZ569; (D) 112F2, from RZ590; (E) 112H4; (F) 125C8; (G) 129E8, all from CDO539; (H) 97B4; (I) 110C3, both from RZ467; (J) 69D11; (K) 86B10 (FITC detection and propidium iodide counterstaining); (L) 88C12, all from RZ86; (M) 67B3; (N) 144A5, both from CDO1417.

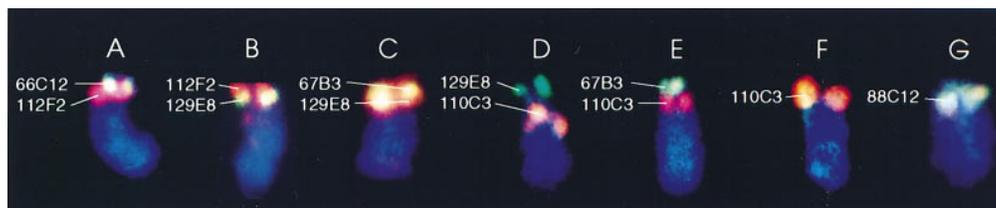


Figure 7.—(A–G). Physical mapping of the *liguleless* linkage group in *S. bicolor*, using dual FISH of *liguleless*-associated sorghum BACs. Probes were either labeled with biotin or digoxigenin, and detected with Cy3-conjugated anti-digoxigenin (red) or FITC-conjugated anti-biotin (green). BACs were simultaneously hybridized in an alternating manner (*i.e.*, 1 and 2, 2 and 3), and order was determined by either a red and green signal or a green and red signal, respectively: (A) BACs 66C12 (RZ569) and 112F2 (RZ590), (B) BACs 112F2 (RZ590) and 129E8 (CDO539), (C) BACs 67B3 (CDO1417) and 129E8 (CDO539), (D) BACs 129E8 (CDO539) and 110C3 (RZ467), (E) BACs 67B3 (CDO1417) and 110C3 (RZ467), (F and G) The simultaneous hybridization of BACs 110C3 (RZ467) and 88C12 (RZ86) showing that BACs associated with RZ86 are not syntenic to the *liguleless* linkage group located on sorghum chromosome *I_r*.

Tanksley 1993; Dunford *et al.* 1995). The intrafamily conservation of sequences among low-copy sites has been widely exploited for homeology-based cloning and marker development, and it should be possible to use these markers in other genomic libraries to select clones in a homeology-specific, if not site-specific, manner. Another potential use of such clones will be extremely rapid comparative physical mapping of related genomes.

In this study, we chose probes for 10 RFLP loci that collectively span 30.4 cM in chromosome 4 of rice

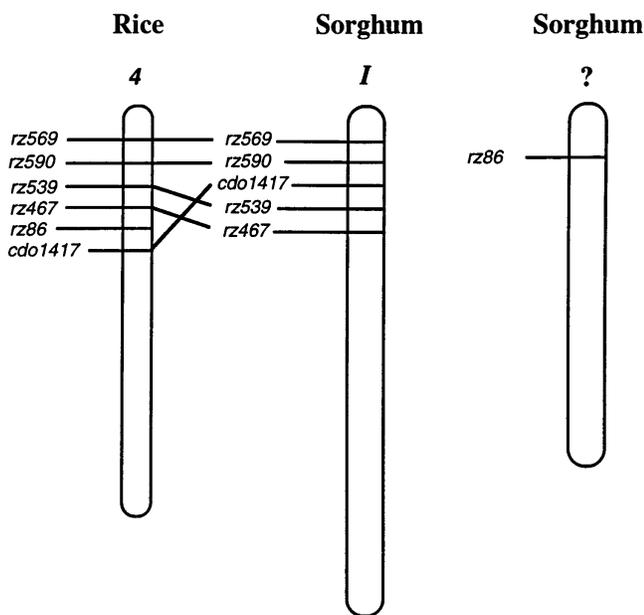


Figure 8.—Comparison of the rice *liguleless* linkage map and the physical map of *liguleless* determined by BAC FISH in *S. bicolor*. The linkage group is conserved in sorghum as it is in rice, with two exceptions. First, the region homologous to *cdo1417* is rearranged to the middle of the linkage group between markers *rz590* and *rz539*. Second, the sequence homologous to RZ86 is found on a chromosome other than chromosome *I_r*. The location of this marker in sorghum is similar to its position in maize, as this marker is found on chromosomes 4 and 5, and not within the major *liguleless* cluster found on chromosomes 2 and 10.

(Causse *et al.* 1994) to screen the sorghum BAC library. Six markers were effective at selecting at least two homeologous clones per marker. When restriction-digested with *NotI* and run side-by-side on a CHEF gel (Figure 3), shared DNA fragments were easily seen, suggesting that all BACs pertaining to a particular marker were cloned from the same region of the genome. To further test this hypothesis and to determine the genetic distances in that region, the BACs were end-cloned (the right ends via plasmid-rescue) for RFLP mapping. Of the 16 selected BAC clones, five were RFLP mapped to two linkage groups. Markers 112F2 (RZ590), 110C3 (RZ467), and 112H4 (CDO539) mapped to linkage group I, and 69D11 (RZ86) mapped to linkage group E. The mapping of 125C8 to linkage group E was surprising because this probe, like 112H4, is from a BAC with a sequence complementary to RFLP probe CDO539. In maize, a close relative of sorghum, *cdo539* is located in the middle of the *liguleless* linkage group on chromosomes 2 and 10 (Ahn and Tanksley 1993). For this reason, we expected both of these markers to map to

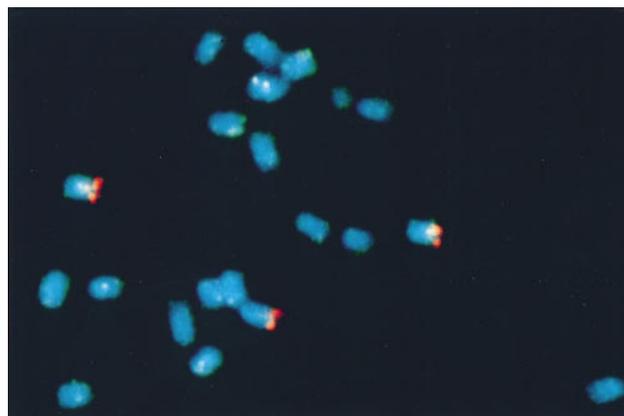


Figure 9.—FISH of sorghum BACs 112F2 and 69D11 to sorghum trisomic for chromosome *I* (denoted *I_r*). BAC clone 112F2 produced three pair of signals on chromosome *I_r*, whereas BAC clone 69D11 produced only two pair of signals on an unidentified chromosome.

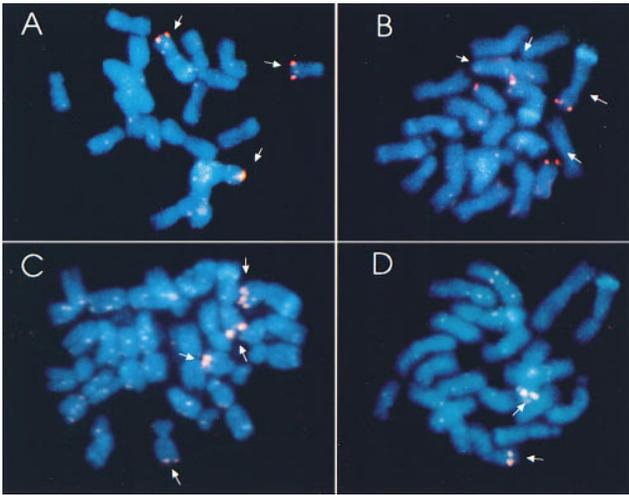


Figure 10.—(A–D). The cross-hybridization of sorghum BACs to maize metaphase chromosomes. Only BACs (A) 67B3, (B) 129E8, (C) 112F2, and (D) 88C12 hybridized intensely to the chromosomes of the maize genome (arrows).

the same region of linkage group I. It is possible that 125C8 mapped to an alternative position due to the tetraploid nature of *S. bicolor* (Hoang-Tang *et al.* 1991; Gomez *et al.* 1988), *i.e.*, duplications on homeologous chromosomes or to other chromosomal rearrangements such as translocations or transpositions. The association of 69D11 (RZ86) with sorghum linkage group E coincides with the linkage data of maize, where RZ86 detected polymorphisms in chromosomes 4 and 5 rather than the major *liguleless* clusters in chromosomes 2 and 10.

Physical mapping placed the sorghum BACs in the same order as determined by linkage mapping of the BAC end clones in sorghum. Both physical and segregation data indicated that RZ86 is nonsyntenic with the other *liguleless* BACs in sorghum. Physical mapping to trisomic lines placed the *liguleless* region in chromosome I_r . The chromosome associated with BAC 69D11 and linkage group E has not been determined.

The *liguleless* linkage group was physically mapped in *S. bicolor* by hybridizing BACs in pairs, according to the arrangement of the linkage group in rice (Causse *et al.* 1994). Probes 112F2 (RZ590), 66C12 (RZ569), 129E8 (CDO539), and 110C3 (RZ467) hybridized in the same sequence as the map order in rice, suggesting conservation of the gene order across genera. BAC 67B3 (CDO1417), however, hybridized to a site in the middle of the chromosome between *rz590* and *cdo539*, indicating a transposition or a double inversion of the region (Figure 8). The position of *cdo1417* is conserved among the linkage groups of rice and maize, thus the rearrangement in sorghum suggests that the rearrangement occurred some time after the divergence of sorghum and maize. All *liguleless* markers are found on chromosome 4 of rice (Figure 1), while in maize these markers are

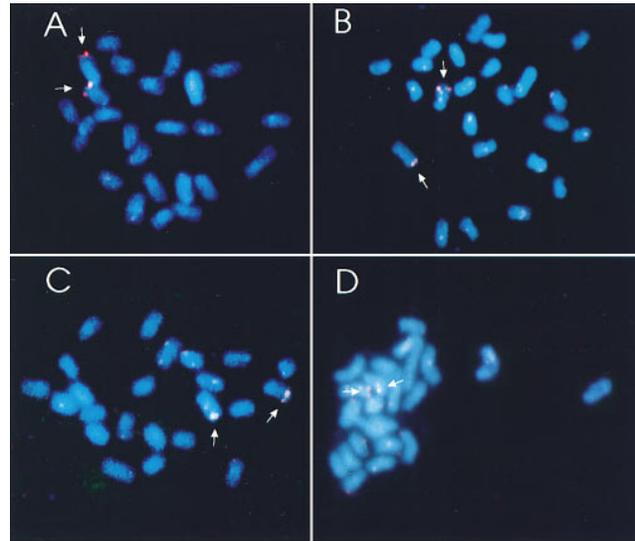


Figure 11.—A–D. Hybridization of sorghum BACs (A) 67B3, (B) 129E8, (C) 112F2, and (D) 88C12 to rice metaphase chromosomes. Arrows indicate hybridization sites.

found mainly on chromosomes 2 and 10, with several other markers dispersed on chromosomes 4 and 5 (*rz53*, *rz467*, and *rz86*). Similarities of marker dispersal in sorghum were seen with probes 88C12 and 69D11 (both from RZ86), which hybridized to a different chromosome and were not found to cohybridize with any BACs that make up the *liguleless* linkage group on chromosome I_r . The finding that *rz86* is syntenic with *liguleless* in rice, but not maize or sorghum, may indicate that the genomic event leading to this rearrangement occurred some time after the shared ancestor of maize, sorghum, and rice. It is noteworthy, however, that 110C3, an RZ467 screened clone, did not cohybridize with 88C12 as might be predicted from the maize map. Instead, it hybridized to the major *liguleless* group in sorghum as seen in rice chromosome 4. This suggests that the evolutionary line leading to maize underwent rearrangement following divergence from the line leading to sorghum some 20 mya (Doebley *et al.* 1990).

The *in situ* hybridization of *liguleless*-associated sorghum BACs to both rice and maize chromosomes shows the potential application of using these probes across species to physically map conserved regions of the genome. The cross-hybridization of these sorghum BACs to maize chromosomes generally produced strong signals, although some probes (*e.g.*, 66C12 and 110C3) were difficult to detect (Table 1). The same probes which produced strong signals in maize also hybridized to rice but with less overall intensity; the two probes that were weak in maize were virtually undetectable in rice. This suggests that maize and sorghum are more closely related than sorghum and rice, which is compatible with their taxonomic positions.

Comparative mapping within families provides clues to the degree of sequence conservation, ploidy of a

species, ancestral relationships, and the rates at which individual genomes are evolving. Virtually all comparative mapping has been done by cross-hybridizing molecular markers across species within a given family. In this article, we have demonstrated the feasibility and facility of comparative mapping in plants by means of physical mapping using BAC FISH. As in genetic mapping using RFLPs, molecular markers are needed, but instead of direct hybridization to mapping filters, the markers are used to select large insert clones from a total genomic DNA library of a related species. The selected clones, each a representative of a single RFLP marker, can then be used to physically map the region in the target species. The advantage of this method for comparative mapping is that no mapping population or linkage map of the target species is needed and, as demonstrated, the clones may also be used in other closely-related species. The cross-hybridization to related species may provide clues to the rates of evolution and the evolutionary paths that species may have taken. Thus, this approach can be applied quickly and to diverse germplasm. RFLP-selected BAC FISH can also rapidly correlate physical and genetic maps, provide starting points for chromosome-walking experiments, and anchor RFLP loci in contig maps. This type of mapping can play a critical role in mapping wild relatives, progenitor species, and species used for germplasm surveys for which mapping populations or linkage maps typically do not exist.

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