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High-Density Linkage Mapping Revealed Suppression of Recombination at the Sex Determination Locus in Papaya

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ABSTRACT

A high-density genetic map of papaya (*Carica papaya* L.) was constructed using 54 F_2 plants derived from cultivars Kapoho and SunUp with 1501 markers, including 1498 amplified fragment length polymorphism (AFLP) markers, the papaya ringspot virus coat protein marker, morphological sex type, and fruit flesh color. These markers were mapped into 12 linkage groups at a LOD score of 5.0 and recombination frequency of 0.25. The 12 major linkage groups covered a total length of 3294.2 cM, with an average distance of 2.2 cM between adjacent markers. This map revealed severe suppression of recombination around the sex determination locus with a total of 225 markers cosegregating with sex types. The cytosine bases were highly methylated in this region on the basis of the distribution of methylation-sensitive and -insensitive markers. This high-density genetic map is essential for cloning of specific genes of interest such as the sex determination gene and for the integration of genetic and physical maps of papaya.

PAPAYA (*Carica papaya*) is a member of the family Caricaceae that is the flowering plant family closest to the family Brassicaceae (which includes Arabidopsis, whose genome is completely sequenced) (Bremer *et al.* 1998; Arabidopsis Genome Initiative 2000). Papaya is a widely cultivated fruit crop in tropical and subtropical regions worldwide. It is believed to be native to tropical America where it has undergone a long period of selection (Storey 1976). Papaya is polygamous with three basic sex types: female, male, and hermaphrodite. Hermaphrodite trees produce a pyriform-shaped fruit that is preferred in the market. However, seeds from hermaphrodite trees always segregate into hermaphrodites and females at the ratio of 2:1 and the sex types of the plants can be determined only by inspection of the flowers. Therefore, it is a general practice for farmers to plant three to five seedlings in one hill, allowing them to grow for 4 to 6 months until the sex types are identified, and then to remove the undesired plants to develop the orchards with only hermaphrodite plants.

On the basis of segregation ratios from crosses among three sex types, Storey (1938) and Hofmeyr (1938) proposed that sex determination in papaya is controlled by a single gene with three alleles: M, M^h, and m. Male individuals (Mm) and hermaphrodite individuals (M^h m) are heterozygous, whereas female individuals (mm) are homozygous recessive. The dominant combinations of MM, M^hM^h, and MM^h are lethal, resulting in a 2:1 segregation of dominant markers on the linkage group (LG) where the sex determination gene is located. Later, Storey (1953) revised the hypothesis to state that sex is determined not by one gene, but rather by a complex of genes that are confined to a small region on the sex chromosome within which crossing over is precluded. The different segments in this region are so closely linked together that they behave as unit factors. Hofmeyr (1967) proposed that the symbols M1 (M) and M2 (M^h) represent inactivated regions of slightly different lengths from which vital genes are missing. On the basis of interspecific hybridization in Caricaceae, Horovitz and Jiménez (1967) proposed that the sex determination is of XX-XY type. The genotype of the male is XY, female XX, and hermaphrodite XY_2, where Y_2 is the modified Y chromosome. The Y chromosome has a lethal region and the Y_2 chromosome preserves this lethal region. A more recent modification of the model proposed to explain the papaya sex expression is that the three alleles encode different *trans*-acting factors to direct the expression of the different flower forms (Sondur *et al.* 1996).

Papaya is diploid with nine pairs of chromosomes and a small genome size of 372 Mbp (Storey 1941; Arumuganathan and Earle 1991). Over the past 10 years, three major advancements have enhanced papaya genetic and genomic research:

i. Transgenic improvement. Papaya transformed with

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TABLE 1

List of AFLP primers used in linkage mapping in papaya

Primer	Code	Primer	Code	Primer	Code	Primer	Code	Primer	Code
M-CAA	01	M-ATGA	19	E-AAC	01	E-GT	19	E-GTT	37
M-CAC	02	M-ATGG	20	E-AAG	02	E-AA	20	P-AGG	51
M-CAG	03	M-CAGG	21	E-ACA	03	E-AG	21	P-AGT	52
M-CAT	04	M-CAAT	22	E-ACC	04	E-GAA	22	P-ACA	53
M-CTA	05	M-CATT	23	E-ACG	05	E-GAC	23	P-ACC	54
M-CTC	06	M-CTTA	24	E-ACT	06	E-GAG	24	P-ACG	55
M-CTG	07	M-AA	25	E-AGC	07	E-GAT	25	P-ACT	56
M-CTT	08	M-AC	26	E-AGG	08	E-GCA	26	P-AAT	57
M-CA	09	M-AG	27	E-AAT	09	E-GCC	27	P-AGC	58
M-CC	10	M-AT	28	E-AGT	10	E-GCG	28	P-AGA	59
M-CG	11	M-GA	29	E-ATC	11	E-GCT	29	P-AAG	60
M-CT	12	M-GC	30	E-ATT	12	E-GGA	30	P-ATC	61
M-ACAA	13	M-GG	31	E-AGA	13	E-GGC	31	P-ATA	62
M-ACAT	14	M-GT	32	E-ATA	14	E-GGG	32	P-AAC	63
M-ACTG	15	M-TA	33	E-ATG	15	E-GGT	33	P-AAA	64
M-ACTT	16	M-TC	34	E-GA	16	E-GTA	34	P-ATT	65
M-AGCT	17	M-TG	35	E-GC	17	E-GTC	35	P-ATG	66
M-AGGC	18	M-TT	36	E-GG	18	E-GTG	36		

the papaya ringspot virus coat protein (PRSVCO) gene was successfully developed and released to save Hawaii's papaya industry from collapse because of susceptibility to papaya ringspot virus disease (Fitch *et al.* 1992; Gonsalves 1998).

ii. Sex-linked DNA markers. Four sequence-characterized amplified region (SCAR) markers tightly linked to sex forms were developed (Parasnis *et al.* 2000; Deputy *et al.* 2002; Urasaki *et al.* 2002) as ways to sex the plant prior to flowering since known sex-linked vegetative characters are too far from the sex-determining locus to be of practical use. These DNA sex markers are now used in selection of desired sex types at the seedling stage for more efficient papaya production (Deputy *et al.* 2002).

iii. The genetic linkage map. The first genetic map was reported more than 60 years ago and consisted of only three morphological markers: sex form, flower color, and stem color (Hofmeyr 1939). In 1996, Sondur *et al.* (1996) developed a second map based on 62 randomly amplified polymorphic DNA (RAPD) markers and mapped the sex determination gene on linkage group 1.

Considerable efforts have gone into the genetic mapping of many plant species. High-density genetic mapping not only is the first step toward isolating and cloning the genes of interest via chromosome walking or chromosome landing but also is an important tool for genomic dissection, marker-assisted selection, comparative analysis of the plant genomes, and integration of genetic, physical, and cytomolecular maps (Martin *et al.* 1993; Tanksley *et al.* 1995; Klein *et al.* 2000; Paterson *et al.* 2000; Draye *et al.* 2001). Genetic linkage maps have been reported for many plant species over the last 15 years. However, high-resolution genetic maps, which are essential for genomic studies, have been limited to the model plant Arabidopsis (Peters *et al.* 2001) and to major crop species, such as maize (Davis *et al.* 1999), rice (Harushima *et al.* 1998; Wu *et al.* 2002), tomato, potato (Tanksley *et al.* 1992; Haanstra *et al.* 1999), wheat (Boyko *et al.* 2002), soybean (Keim *et al.* 1997), and rapeseed (Lombard and Delourme 2001).

Among the crop species, the genetic mapping of papaya lagged behind that of many other plant species, due partly to the low level of polymorphism among existing germplasm (Sharon *et al.* 1992; Stiles *et al.* 1993; Kim *et al.* 2002). The objective of this study was to develop a high-density genetic map of papaya and to characterize its sex locus. This map will serve as the first and essential step for conducting extensive genomic research on this crop.

MATERIALS AND METHODS

Plant materials: The F_2 mapping population was grown at the Kunia substation on Oahu, Hawaii, along with the parents Kapoho, SunUp, and a set of F_1 plants. Plant tissues from young leaves of 34 hermaphrodite and 20 female plants were collected for DNA isolation.

AFLP analysis: DNA extraction protocol used to extract the papaya DNA was described previously (Steiger *et al.* 2002). Amplified fragment length polymorphism (AFLP) analyses were performed on a Li-Cor IR^2 automated DNA sequencer (Li-Cor, Lincoln, NE) with selective amplification prepared from fluorescent-labeled *Eco*RI or *Pst*I primers and *Mse*I primers as described by Kim *et al.* (2002). Selective amplifications were carried out using various combinations of two- to four-nucleotide extensions to *Eco*RI (E-) or *Pst*I (P-) primers with

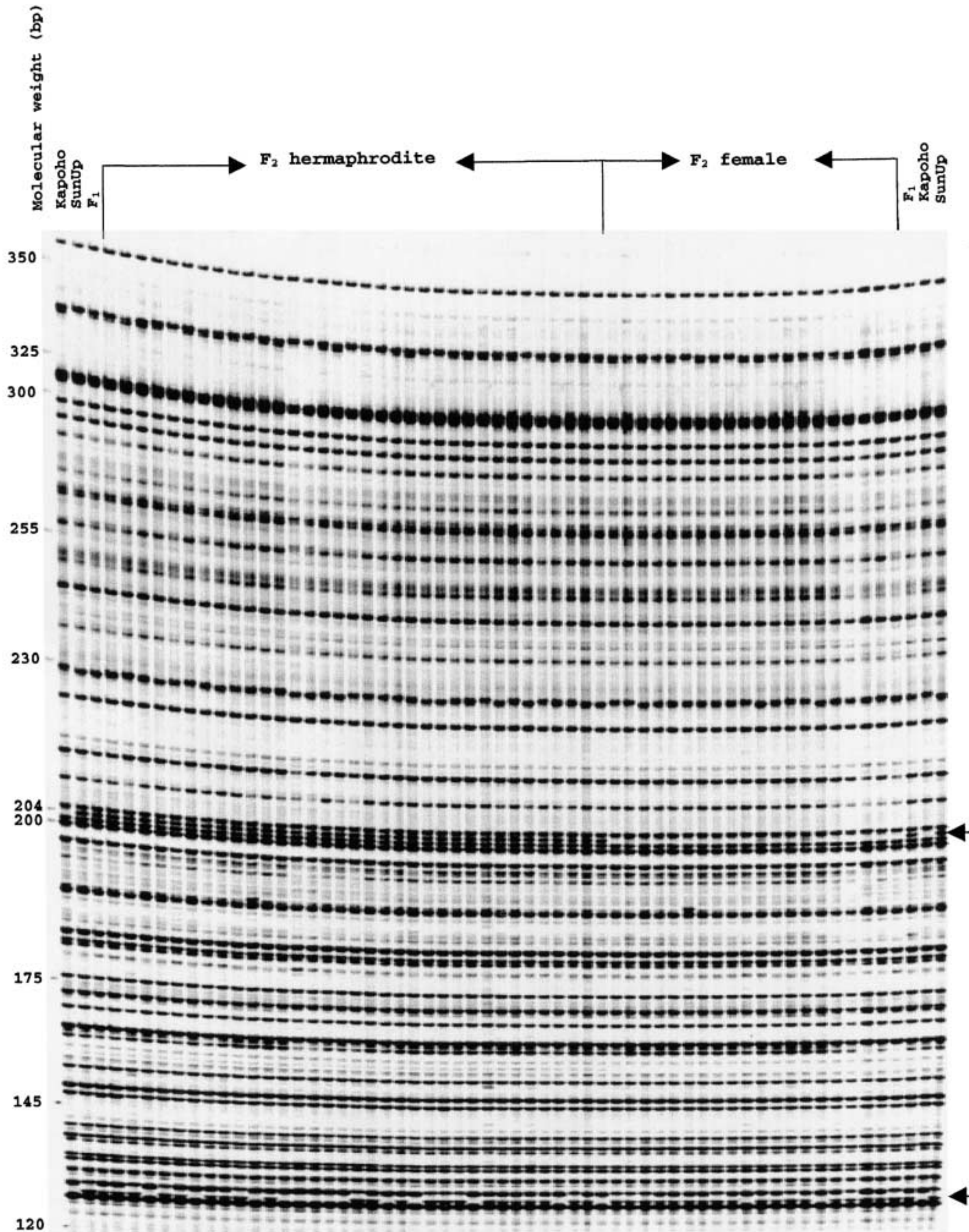


FIGURE 1.—A portion of a TIF image showing AFLP products amplified by the primer pair E-GCT/M-AG. The wells are denoted at the top of the figure. Polymorphic markers are identified as fragments that are present in one of the original parents and some of the F_2 samples but absent from others (arrows). The SunUp dominant marker between 200 and 204 bp is cosegregating with sex.

*Mse*I (M-) primers (E-2/M-2, E-2/M-3, E-2/M-4, E-3/M-3, E-3/M-2, P-3/M-2, P-3/M-3, and P-3/M-4; Table1).

Map construction: Goodness of fit was tested by chi-square analysis for the expected segregation of dominant (3:1), sex-linked (2:1), and codominant (1:2:1) markers in the F_2 population. The linkage map was constructed using the program MAPMAKER 3.0 (LANDER *et al.* 1987) employing a minimum LOD score of 5.0 and a maximum recombination rate (θ) of 0.25. First, the codominant markers were used for defining linkage groups. After this, the codominant markers in a total of nine linkage groups were combined with the middle-sized (80–350 bp) markers, fitting either 3:1 or 2:1 ratios, and assigned to linkage groups. After ordering the markers in each group, the anchor markers were selected at regular intervals (one marker/15–20 cM). The anchor markers were mixed with all the remaining markers for a final grouping and ordering by using "first order," "try," and "ripple" functions of the program. Linkage maps were generated using the Kosambi mapping function.

Poisson distribution test: Marker distribution on our map was determined by counting the number of AFLP markers in a sliding 10-cM interval over the total length of each linkage group. The AFLP marker distribution was analyzed using a Poisson distribution function $P(x) = e^{-\mu}\mu^x/x!$, where μ is the average number of markers in a 10-cM interval over the entire map and x is the actual marker count in each interval.

Marker distribution and DNA methylation analysis: At any site where the number of markers in the interval was greater than, or equal to, the minimum upper limit number of markers of the chi-square-tested expectation, that site was designated as a cluster. The distribution of *Pst*I/*Mse*I markers was compared to that of the *Eco*RI/*Mse*I markers. If methylation occurred randomly throughout the genome, the frequency of *Pst*I/*Mse*I markers in each linkage group or on the sex locus

TABLE 2

AFLP fragments generated with different primer combinations

Primers	*Mse*I-**		*Mse*I-***		*Mse*I-****	
	No. of bands	No. of markers	No. of bands	No. of markers	No. of bands	No. of markers
*Eco*RI-A*	78	1.1				
*Eco*RI-G*	67	1.4	50	1.4	43	1.3
*Eco*RI-A**	73	2.5	53	1.9	31	1
*Eco*RI-G**	59	2				
*Pst*I-A**	50	1.2	39	1.4	38	0.6

Asterisks represent any one, two, three, or four of the four nucleotides (A, T, G, or C).

would be expected to be equal to the frequency of *Eco*RI/*Mse*I markers observed in the same group or region. Significant deviation from an equal distribution supports the hypothesis of an alternate methylation status.

RESULTS

DNA polymorphism: The genomic DNA of parental cultivars Kapoho and SunUp, with F_1 and 54 F_2 plants, was amplified with 987 AFLP primer combinations (Table 1). Of the 987 primers, 781 generated 58,173 bands. A portion of a TIF image for AFLP products amplified by the primer pair E-GCT/M-AG is shown in Figure 1. The number of nucleotides in *Eco*RI/*Mse*I and *Pst*I/*Mse*I primer extensions were significantly correlated to the number of bands ($r = -0.8703$***) but not to the number of markers ($r = -0.3468$). A two-nucleotide extension of *Mse*I-** primer with a two-nucleotide extension of *Eco*RI-A* generated the most bands, with an average of 78 bands/primer pair (Table 2). A two-nucleotide extension of *Mse*I-** primer with a three-nucleotide extension of *Eco*RI-A** generated the most polymorphic markers, with an average of 2.5 markers/primer pair.

Of the 987 primer pairs used in this study, 106 did not generate any polymorphic markers; the remaining 881 primer pairs generated 1812 AFLP markers. Theoretically, the AFLP markers generated by the shorter nucleotide extension primer pairs should include the markers generated by longer nucleotide extension primer pairs as well as additional new markers. Forty-five of the *Eco*RI/*Pst*I and *Mse*I primer pairs having two to four extra nucleotide extensions were found to generate redundant markers. After pairing the data set to remove the redundant markers, a total of 1767 polymorphic markers, ranging from 25 to 700 bp, remained for linkage analysis

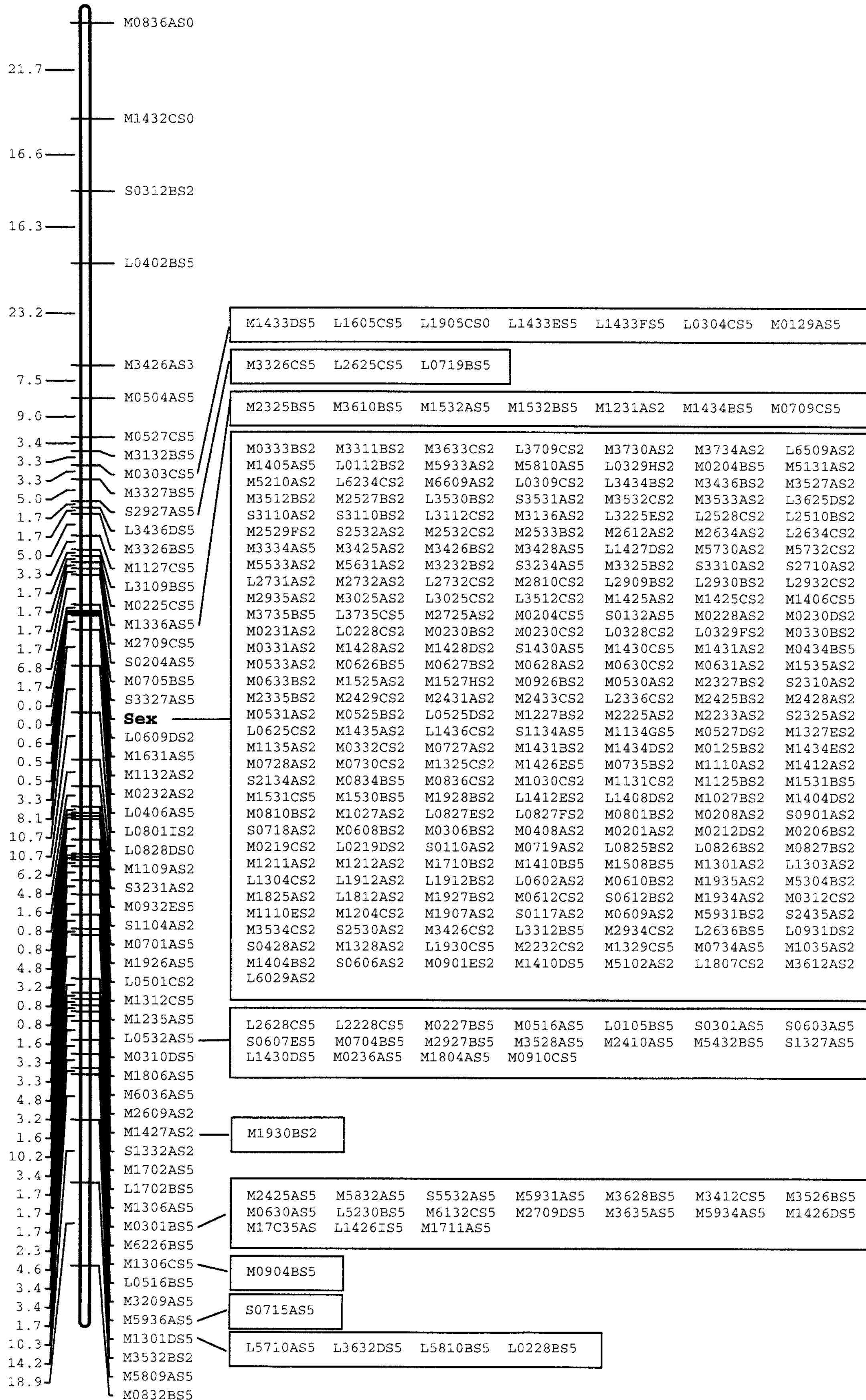
Among all 1767 markers, 644 were derived from the female parent, Kapoho, 983 were from the hermaphrodite (pollen donor) parent, SunUp, and 140 were inherited in a codominant fashion. Each primer pair generated an average of 1.8 polymorphic markers. The *Eco*RI/*Mse*I primer pairs generated more polymorphic markers than the *Pst*I/*Mse*I primer pairs did, with an average of 2.0 and 1.2 markers, respectively.

Segregation analysis: The χ^2 test showed that 71.4% of the AFLP markers fit the expected 3:1 and 1:2:1 Mendelian segregation ratios. When the papaya sex-linked segregation (2:1) markers were added, those fitting the expected ratios increased to 89.4%. The remaining 187 markers were skewed significantly from the expected ratios.

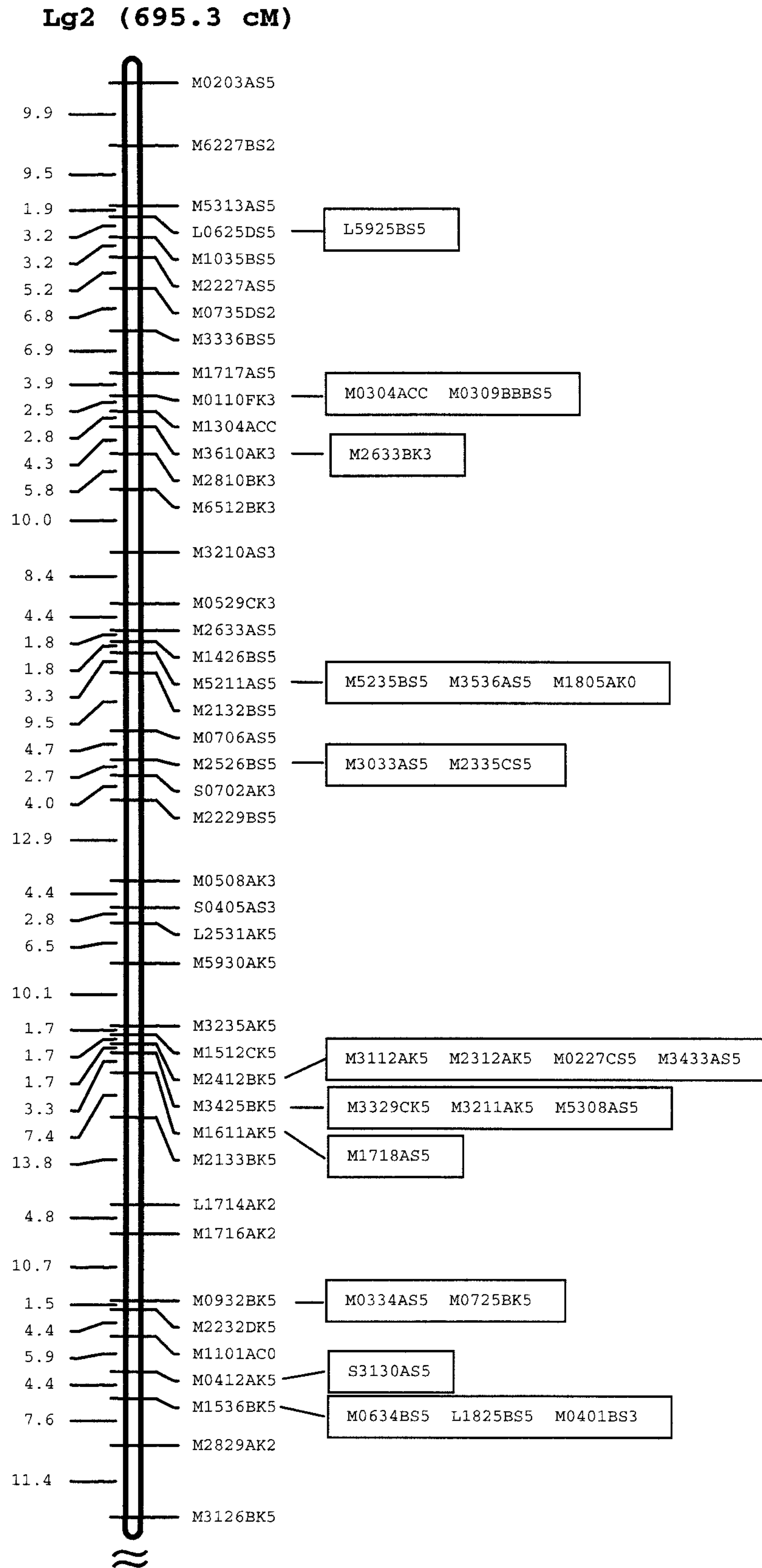
Genetic linkage map: A total of 1501 markers, including 1498 AFLP markers, the papaya ringspot virus coat protein marker, morphological sex type, and fruit flesh color, were mapped into 12 linkage groups at a LOD score of 5.0 and a recombination frequency of 0.25. The 12 major linkage groups covered a total length of 3294.2 cM, with an average distance of 2.2 cM between adjacent markers. The remaining 269 AFLP markers were assigned into smaller groups or remained unlinked. Our linkage group 1 corresponded to the LG1 of the RAPD map where the sex locus was located (Figure 2; Sondur *et al.* 1996). Because we have no common markers with the previous RAPD map, we designated LGs 2–12 in descending order according to the length of each group generated from our AFLP data (Figure 2).

Striking features of LG1 include 225 markers that

Figure 2.—AFLP genetic map of papaya linkage groups 1–12. The sex determination locus was mapped on LG1 with 225 cosegregating markers. Transgenic PRSVCO and Fcolor were mapped on LG7. The alphanumeric code describes the AFLP marker as scored. The first letter of the marker represents the size category of the fragments ("S," <80 bp; "M," 80 ~ 350 bp; and "L," >350 bp); the four numerals following represent *Eco*RI-/*Pst*I- and *Mse*I-primer combination code (see Table 1), and the first two numerals are the code for the *Eco*RI- or *Pst*I-primer, while the last two numerals are the code for the *Mse*I-primer; the sixth element of the code is an alphabetical order of the polymorphic loci generated by each primer pair; then the seventh element indicates from which parent the marker was derived (K, Kapoho; S, SunUp; C, codominant); the last element of the code is information about the χ^2 test (0, not fit expected segregation; 2, fit 2:1; 3, fit 3:1; 5, fit 2:1 and 3:1; C, fit 1:2:1). Cosegregating markers are in boxes.



Lg1 (289.7 cM)
M0836AS0
21.7
M1432CS0
16.6
S0312BS2
16.3
L0402BS5
23.2
M3426AS3
7.5
M0504AS5
9.0
3.4 3.3 3.3 5.0 1.7 1.7 5.0 3.3 1.7 1.7 1.7 1.7 6.8 1.7 0.0 0.0 0.6 0.5 0.5 3.3 8.1 10.7 10.7 6.2 4.8 1.6 0.8 0.8 4.8 3.2 0.8 0.8 1.6 3.3 3.3 4.8 3.2 1.6 10.2 3.4 1.7 1.7 1.7 2.3 4.6 3.4 3.4 1.7 10.3 14.2 18.9
M0527CS5 M3132BS5 M0303CS5 M3327BS5 S2927AS5 L3436DS5 M3326BS5 M1127CS5 L3109BS5 M0225CS5 M1336AS5 M2709CS5 S0204AS5 M0705BS5 S3327AS5 Sex L0609DS2 M1631AS5 M1132AS2 M0232AS2 L0406AS5 L0801IS2 L0828DS0 M1109AS2 S3231AS2 M0932ES5 S1104AS2 M0701AS5 M1926AS5 L0501CS2 M1312CS5 M1235AS5 L0532AS5 M0310DS5 M1806AS5 M6036AS5 M2609AS2 M1427AS2 S1332AS2 M1702AS5 L1702BS5 M1306AS5 M0301BS5 M6226BS5 M1306CS5 L0516BS5 M3209AS5 M5936AS5 M1301DS5 M3532BS2 M5809AS5 M0832BS5
M1433DS5 L1605CS5 L1905CS0 L1433ES5 L1433FS5 L0304CS5 M0129AS5
M3326CS5 L2625CS5 L0719BS5
M2325BS5 M3610BS5 M1532AS5 M1532BS5 M1231AS2 M1434BS5 M0709CS5
M0333BS2 M3311BS2 M3633CS2 L3709CS2 M3730AS2 M3734AS2 L6509AS2
M1405AS5 L0112BS2 M5933AS2 M5810AS5 L0329HS2 M0204BS5 M5131AS2
M5210AS2 L6234CS2 M6609AS2 L0309CS2 L3434BS2 M3436BS2 M3527AS2
M3512BS2 M2527BS2 L3530BS2 S3531AS2 M3532CS2 M3533AS2 L3625DS2
S3110AS2 S3110BS2 L3112CS2 M3136AS2 L3225ES2 L2528CS2 L2510BS2
M2529FS2 S2532AS2 M2532CS2 M2533BS2 M2612AS2 M2634AS2 L2634CS2
M3334AS5 M3425AS2 M3426BS2 M3428AS5 L1427DS2 M5730AS2 M5732CS2
M5533AS2 M5631AS2 M3232BS2 S3234AS5 M3325BS2 S3310AS2 S2710AS2
L2731AS2 M2732AS2 L2732CS2 M2810CS2 L2909BS2 L2930BS2 L2932CS2
M2935AS2 M3025AS2 L3025CS2 L3512CS2 M1425AS2 M1425CS2 M1406CS5
M3735BS5 L3735CS5 M2725AS2 M0204CS5 S0132AS5 M0228AS2 M0230DS2
M0231AS2 L0228CS2 M0230BS2 M0230CS2 L0328CS2 L0329FS2 M0330BS2
M0331AS2 M1428AS2 M1428DS2 S1430AS5 M1430CS5 M1431AS2 M0434BS5
M0533AS2 M0626BS5 M0627BS2 M0628AS2 M0630CS2 M0631AS2 M1535AS2
M0633BS2 M1525AS2 M1527HS2 M0926BS2 M0530AS2 M2327BS2 S2310AS2
M2335BS2 M2429CS2 M2431AS2 M2433CS2 L2336CS2 M2425BS2 M2428AS2
M0531AS2 M0525BS2 L0525DS2 M1227BS2 M2225AS2 M2233AS2 S2325AS2
L0625CS2 M1435AS2 L1436CS2 S1134AS5 M1134GS5 M0527DS2 M1327ES2
M1135AS2 M0332CS2 M0727AS2 M1431BS2 M1434DS2 M0125BS2 M1434ES2
M0728AS2 M0730CS2 M1325CS2 M1426ES5 M0735BS2 M1110AS2 M1412AS2
S2134AS2 M0834BS5 M0836CS2 M1030CS2 M1131CS2 M1125BS2 M1531BS5
M1531CS5 M1530BS5 M1928BS2 L1412ES2 L1408DS2 M1027BS2 M1404DS2
M0810BS2 M1027AS2 L0827ES2 L0827FS2 M0801BS2 M0208AS2 S0901AS2
S0718AS2 M0608BS2 M0306BS2 M0408AS2 M0201AS2 M0212DS2 M0206BS2
M0219CS2 L0219DS2 S0110AS2 M0719AS2 L0825BS2 L0826BS2 M0827BS2
M1211AS2 M1212AS2 M1710BS2 M1410BS5 M1508BS5 M1301AS2 L1303AS2
L1304CS2 L1912AS2 L1912BS2 L0602AS2 M0610BS2 M1935AS2 M5304BS2
M1825AS2 L1812AS2 M1927BS2 M0612CS2 S0612BS2 M1934AS2 M0312CS2
M1110ES2 M1204CS2 M1907AS2 S0117AS2 M0609AS2 M5931BS2 S2435AS2
M3534CS2 S2530AS2 M3426CS2 L3312BS5 M2934CS2 L2636BS5 L0931DS2
S0428AS2 M1328AS2 L1930CS5 M2232CS2 M1329CS5 M0734AS5 M1035AS2
M1404BS2 S0606AS2 M0901ES2 M1410DS5 M5102AS2 L1807CS2 M3612AS2
L6029AS2
L2628CS5 L2228CS5 M0227BS5 M0516AS5 L0105BS5 S0301AS5 S0603AS5
S0607ES5 M0704BS5 M2927BS5 M3528AS5 M2410AS5 M5432BS5 S1327AS5
L1430DS5 M0236AS5 M1804AS5 M0910CS5
M1930BS2
M2425AS5 M5832AS5 S5532AS5 M5931AS5 M3628BS5 M3412CS5 M3526BS5
M0630AS5 L5230BS5 M6132CS5 M2709DS5 M3635AS5 M5934AS5 M1426DS5
M17C35AS L1426IS5 M1711AS5
M0904BS5
S0715AS5
L5710AS5 L3632DS5 L5810BS5 L0228BS5



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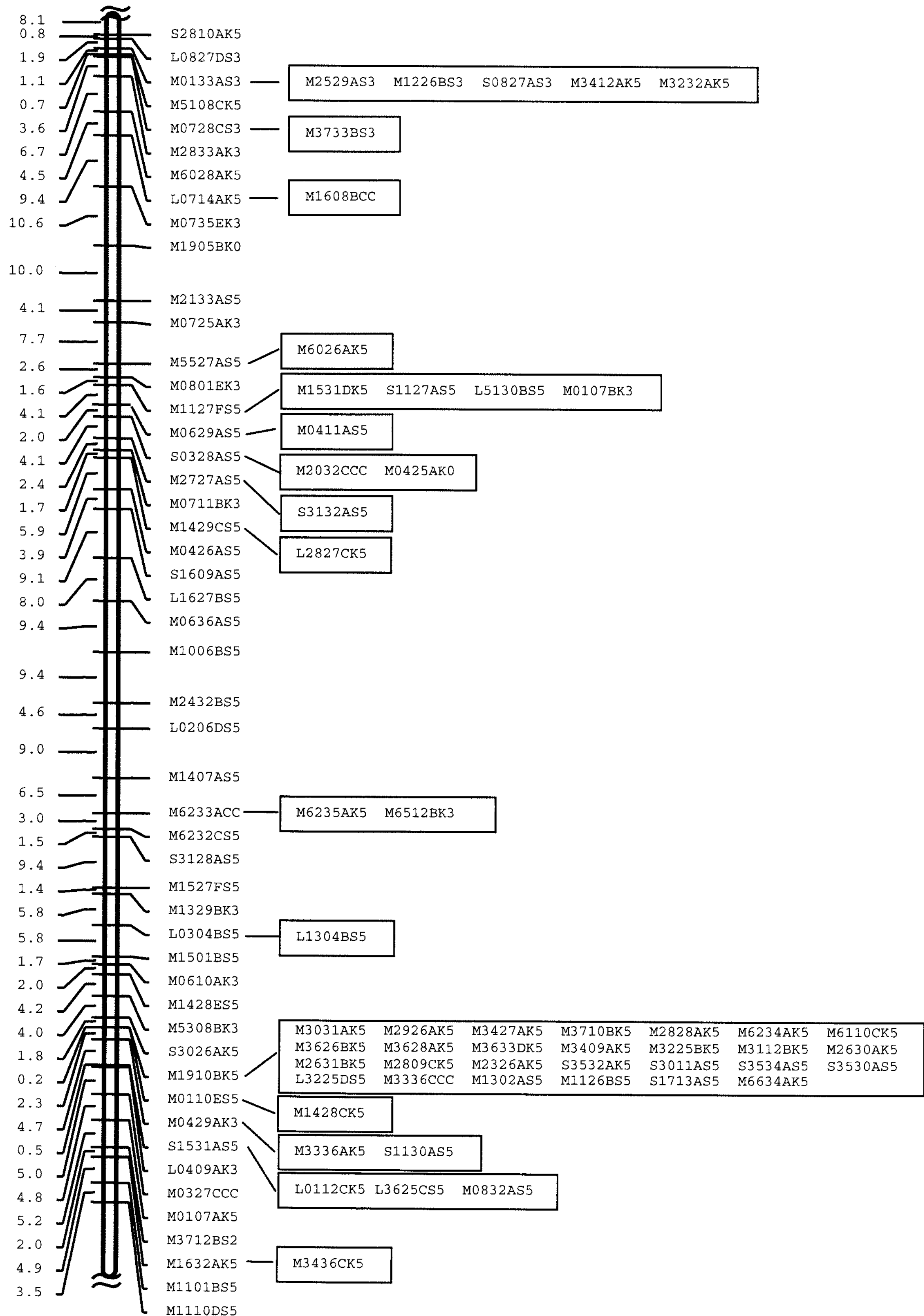

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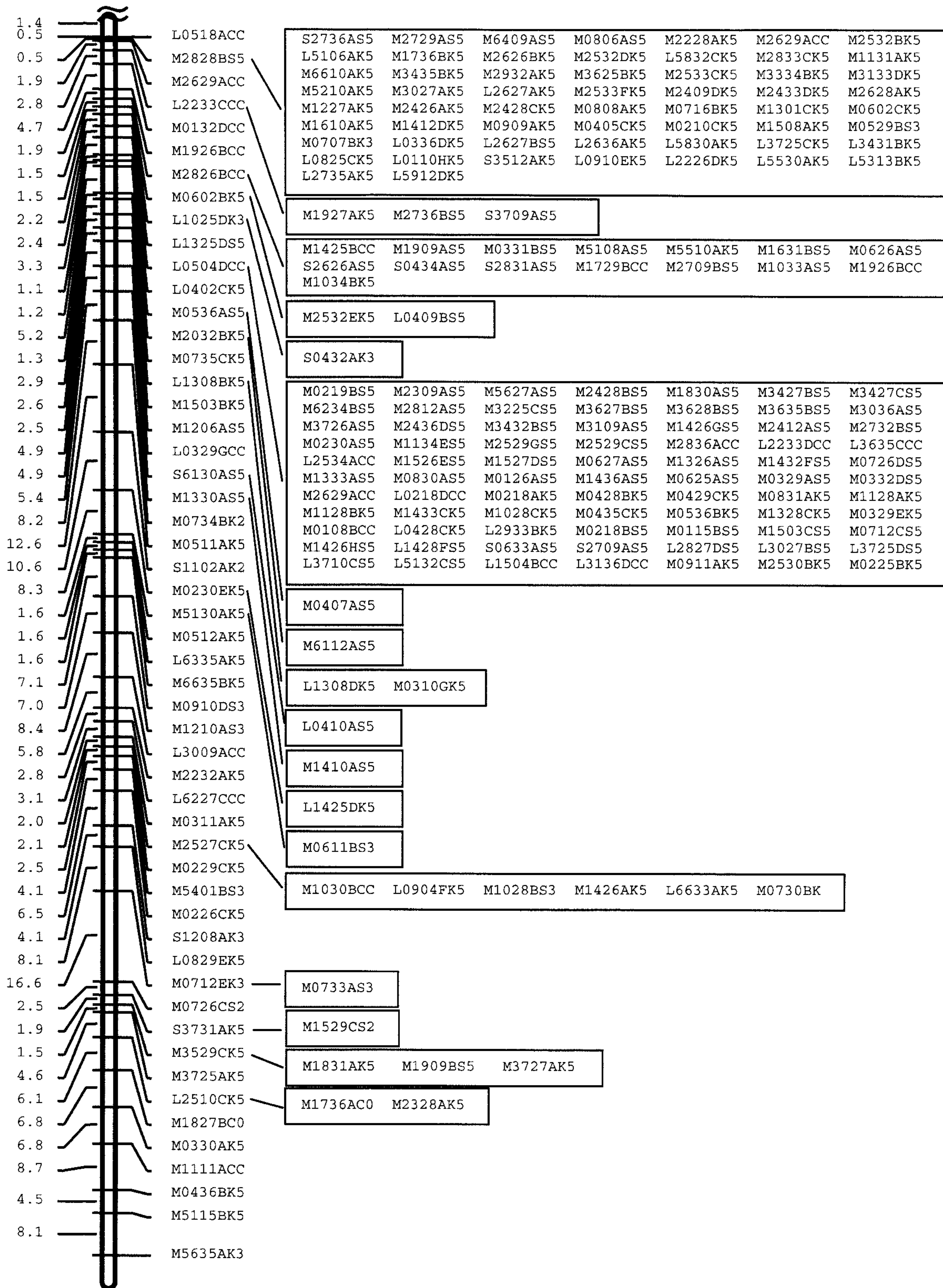

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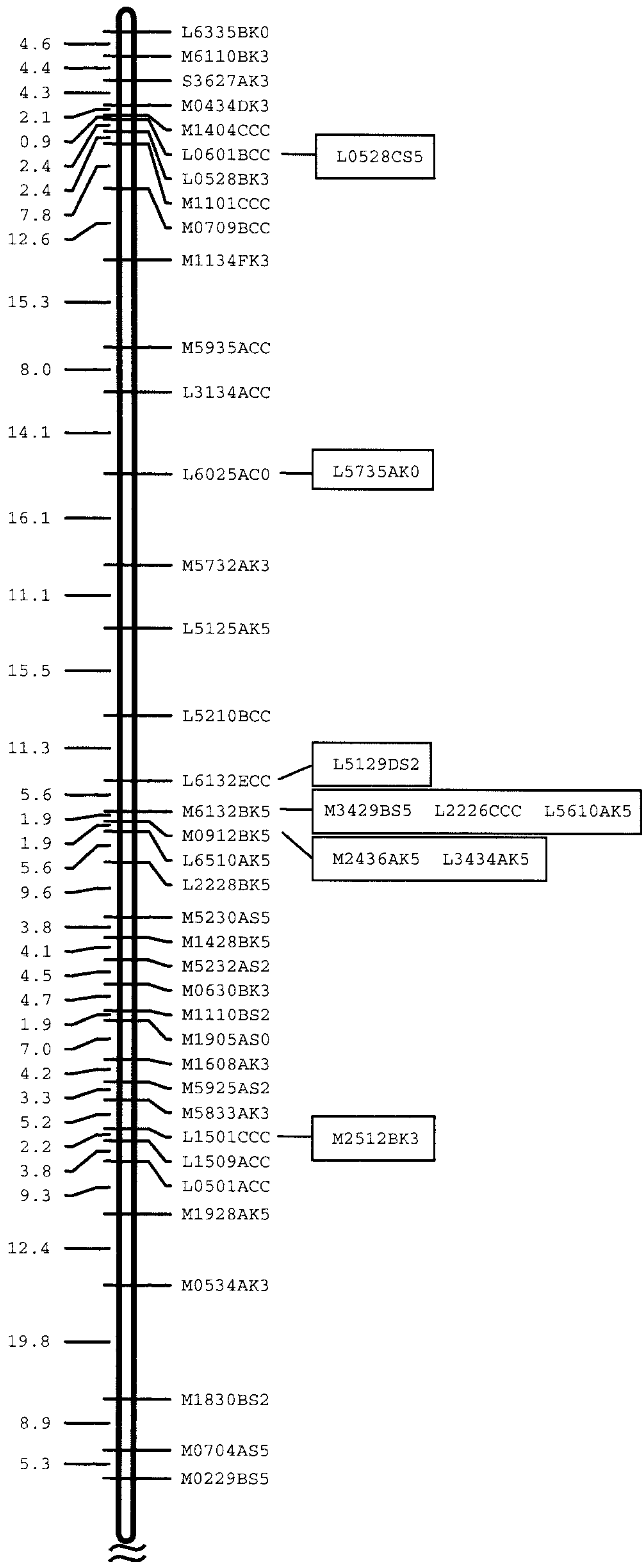

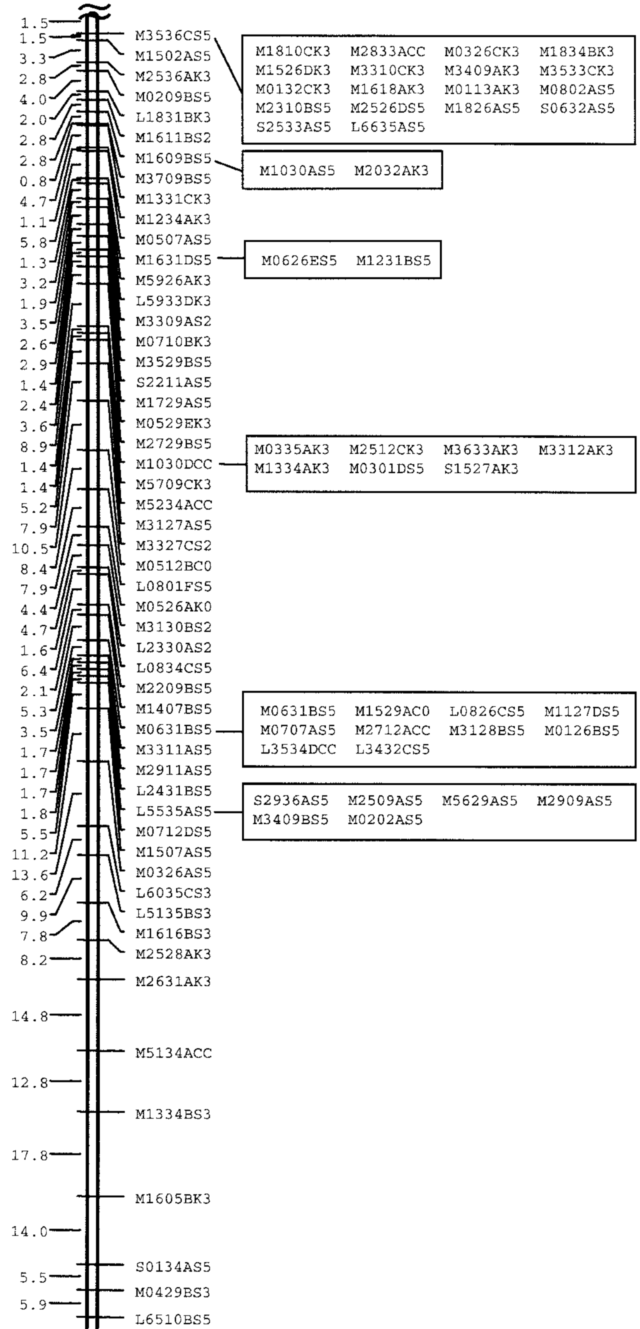

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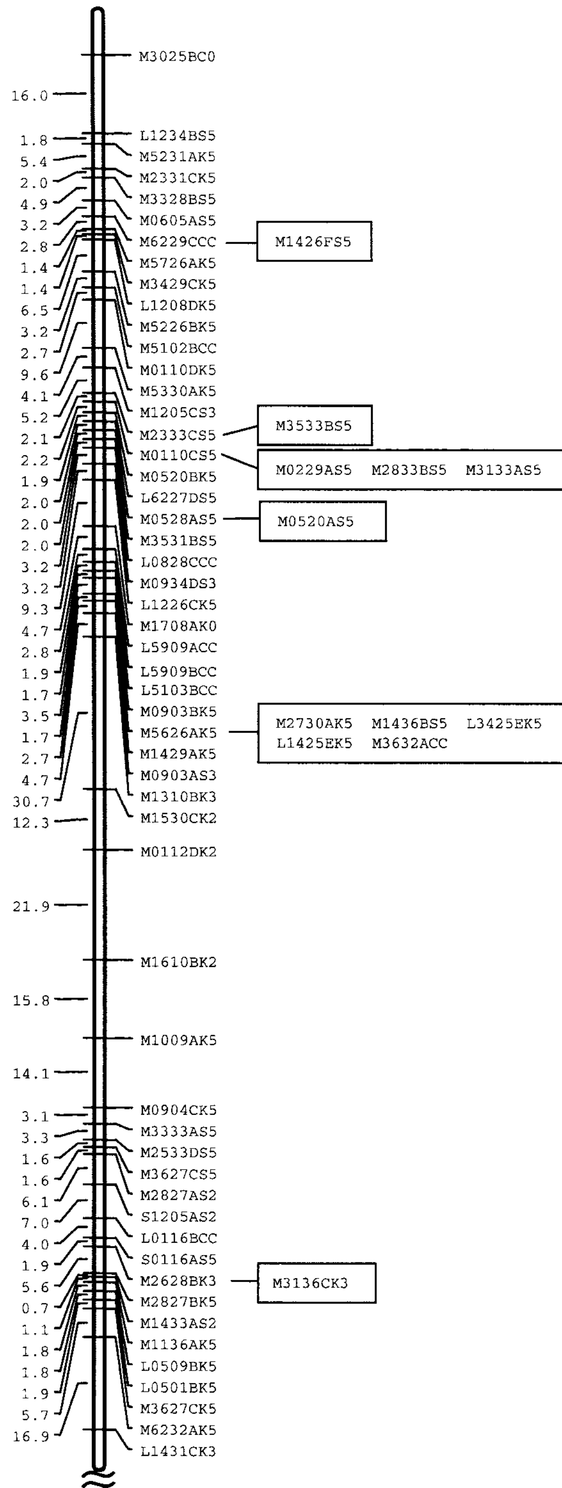


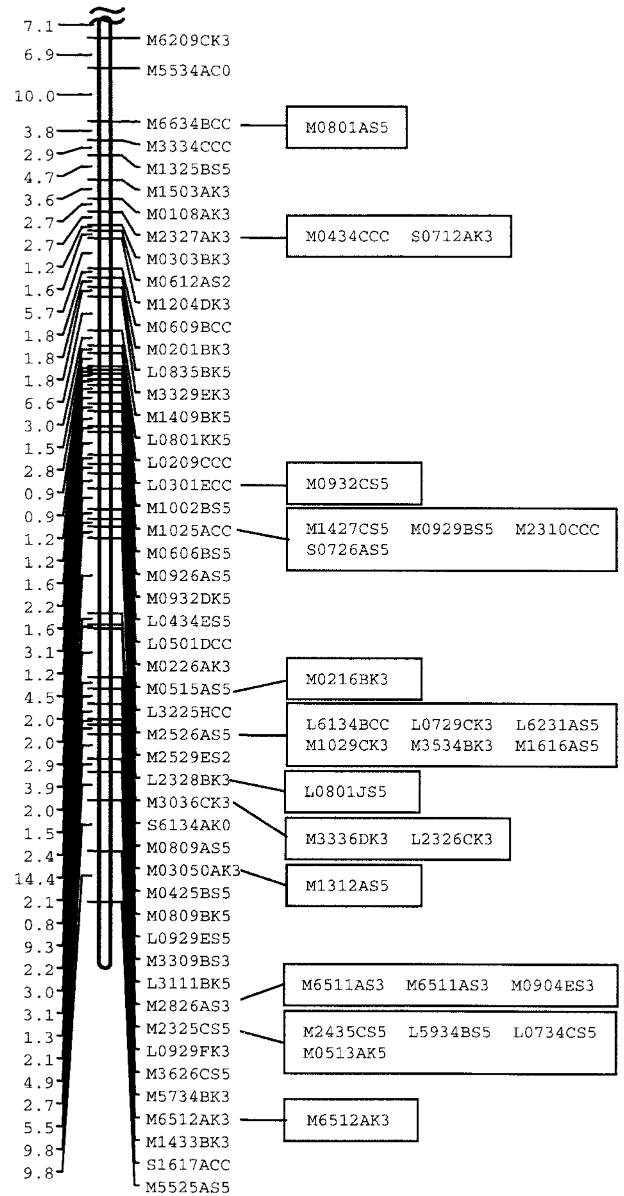
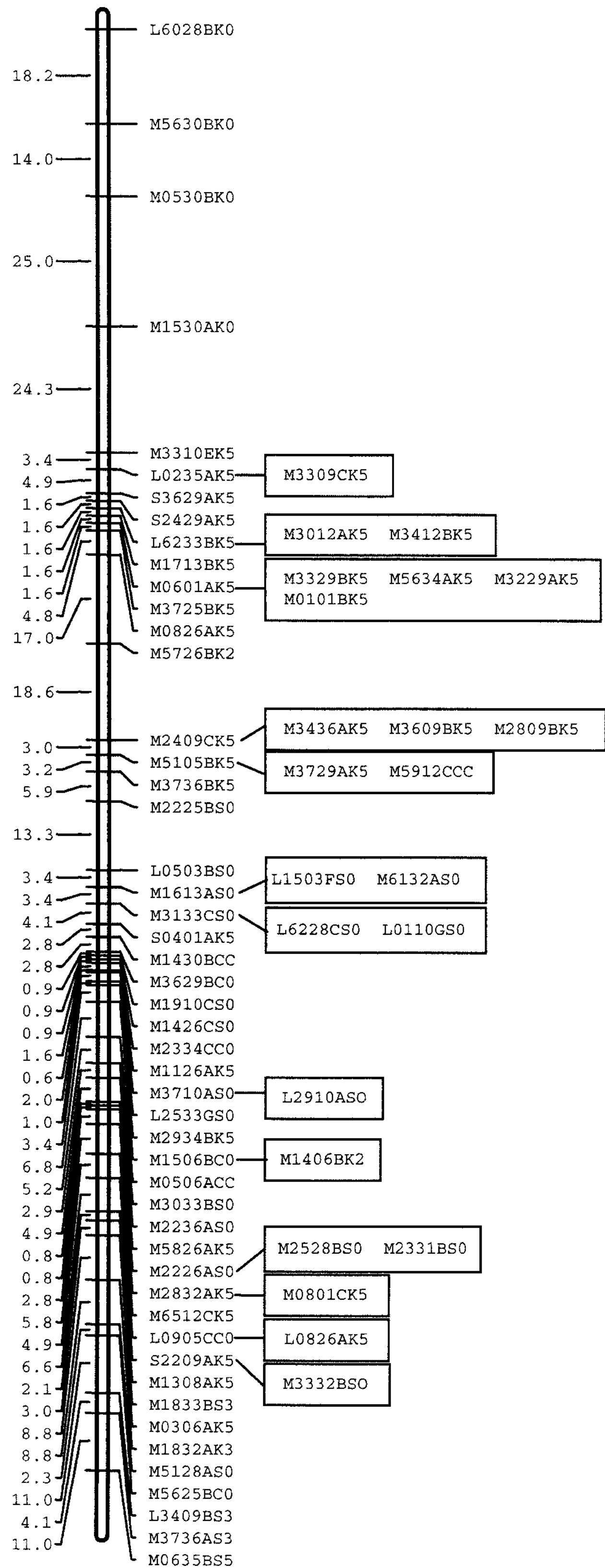


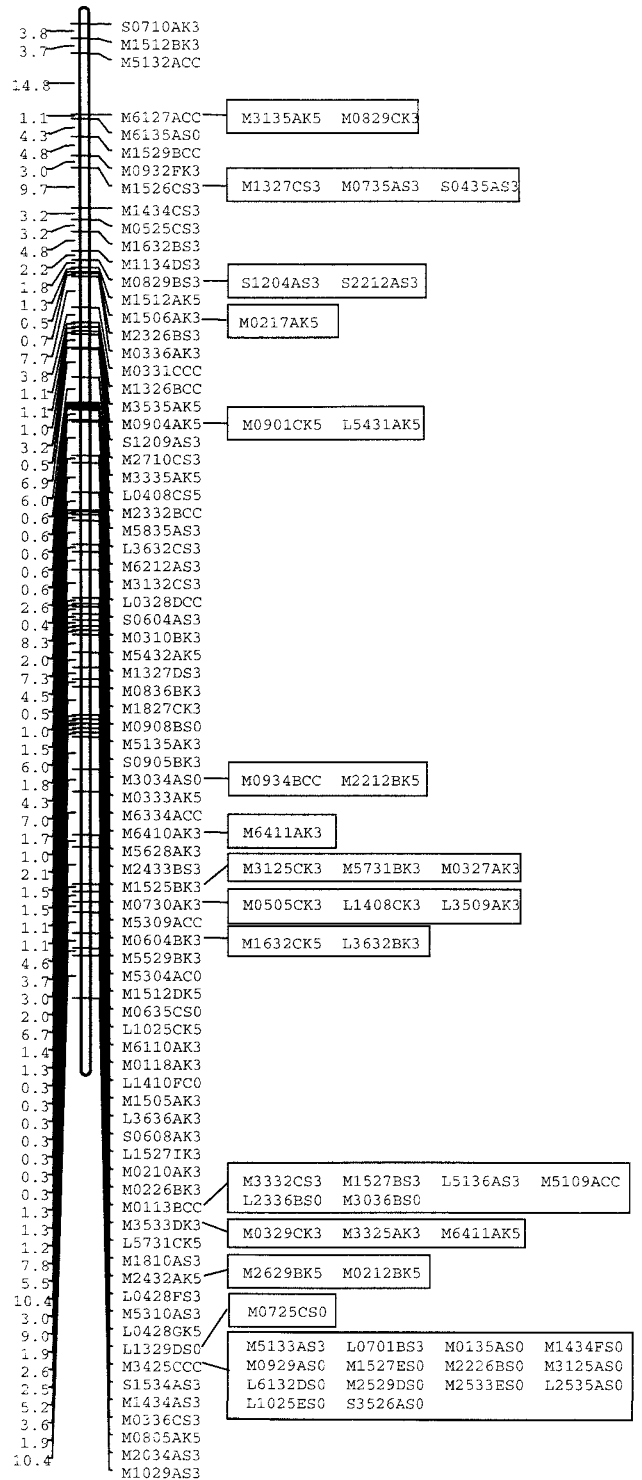
Figure 2.—*Continued.*

cosegregated with the sex locus and all 344 dominant markers on LG1 that were derived from the pollen donor parent SunUp.

The PRSVCO and fruit flesh color (Fcolor) were mapped to LG7 where each was flanked by two markers that were 3.7/4.7 cM and 3.4/3.7 cM distant from them, respectively (Figure 2).

Distribution of AFLP markers over the papaya genome: The distribution of the AFLP markers over the 12 linkage groups varied greatly. LG1 had the highest





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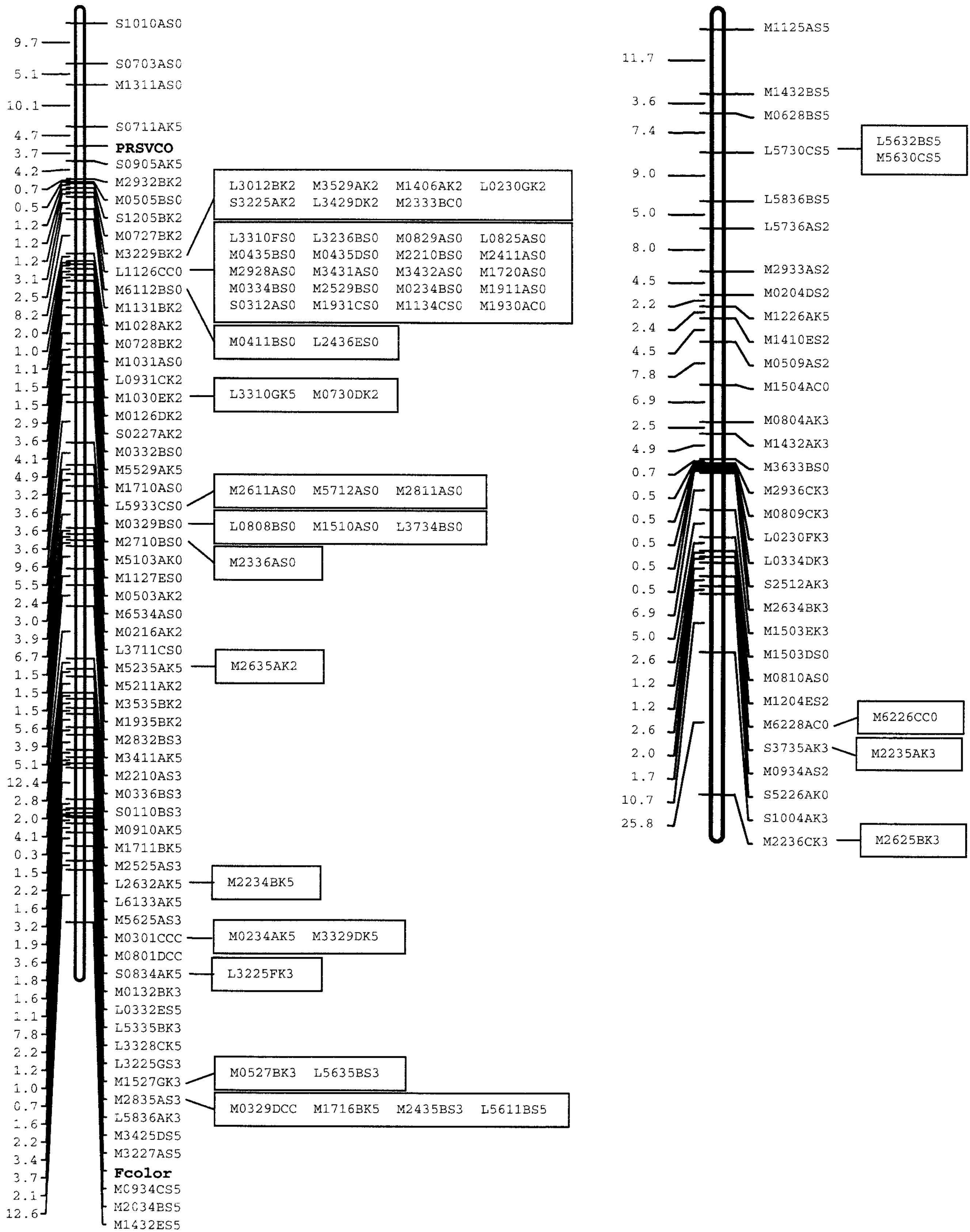

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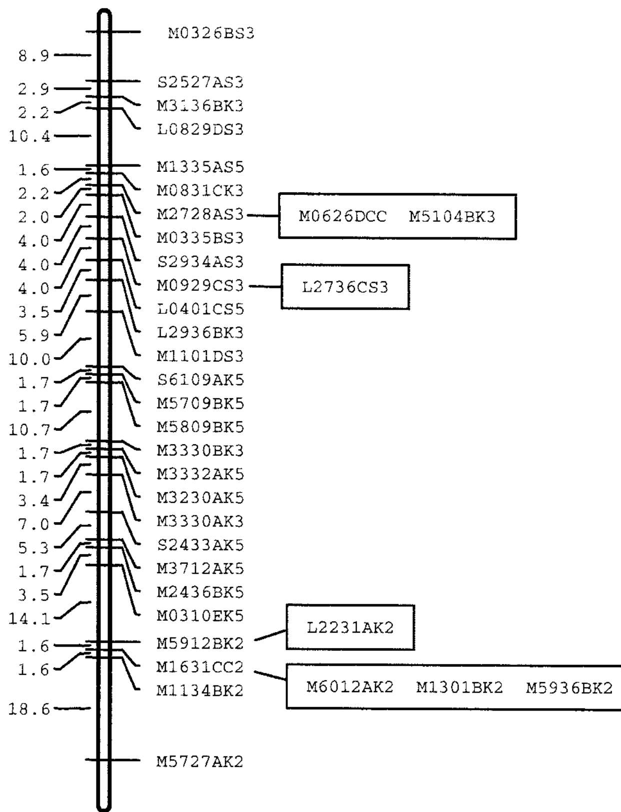

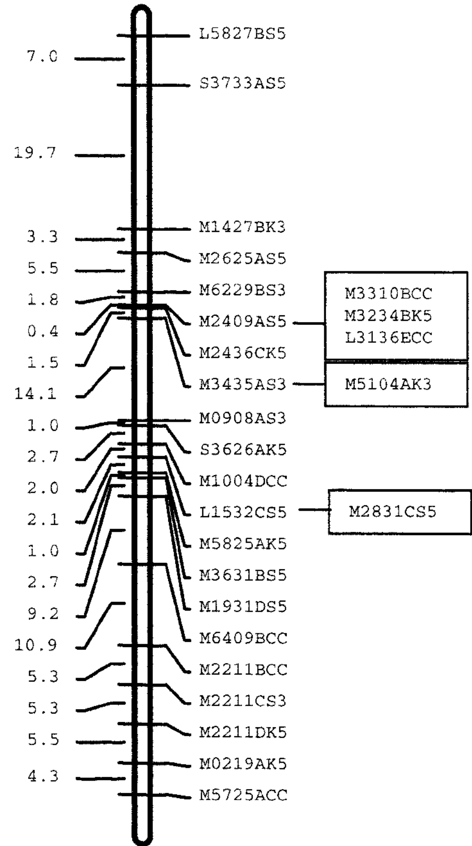

Figure 2.—*Continued.*

marker density (0.85 cM/interval), while LG10 had the lowest (3.9 cM/interval). The number of markers per linkage group ranged from 27 to 400; the length of the linkage groups ranged from 86.2 to 695.3 cM. Under the assumption of random marker distribution, the number of 10-cM intervals containing a given number of markers would follow a Poisson distribution. Our observed and expected number of frame markers/10-cM interval for all the linkage groups deviated significantly from Poisson expectations ($\chi^2 = 717.42$, d.f. = 8, $P < 0.0001$; Figure 3). The deviation was greatest in those intervals containing 1, 7, or >7 markers.

A χ^2 test confirmed that the mapped AFLP markers were not evenly distributed throughout the linkage groups. Clustering was prevalent for all markers, frame markers (markers on each linkage group excluding cosegregating markers), and *Eco*RI-only frame markers in each group analyzed (Table 3). Although *Pst*I frame markers distributed evenly when the χ^2 test included all the linkage groups, in LG1 (obs = 5; exp = 13) and LG6 (obs = 21; exp = 11), they still deviated significantly from the expected number.

On the basis of the total number of frame markers and the length of the linkage groups, 6 markers/interval would be the minimum upper limit for the number of markers that is statistically different from the average of 2.2 markers/10 cM ($P = 0.05$). When the frame markers were counted, 14 clusters were identified on 8 linkage groups. When the cosegregating markers were included in the analysis, 59 clusters were identified on the 12 linkage groups. The cluster covering the sex locus and containing 232 markers is the largest among the 59 clusters.

DNA methylation analysis: If the *Eco*RI and *Pst*I restriction sites were distributed randomly throughout the genome and the genome were not methylated, then the *Pst*I markers should occur in each linkage group at the same frequency as the *Eco*RI markers. This was not the case. We calculated a significant deviation from equal distribution of the *Pst*I markers in all the linkage groups (Table 3). This deviation was caused mainly by LG1 (obs = 28, exp = 50) and LG2 (obs = 45, exp = 58). These results suggest a higher level of DNA methylation for LG1 and LG2 compared to the level of methylation in the other 10 linkage groups.

We assessed the potential level of DNA methylation of the sex locus by determining the frequency of sex cosegregating markers derived from *Eco*RI/*Mse*I *vs.* those from the *Pst*I/*Mse*I primer pairs. A total of 16.3% of the 1285 mapped markers or 14.2% of all the 1479

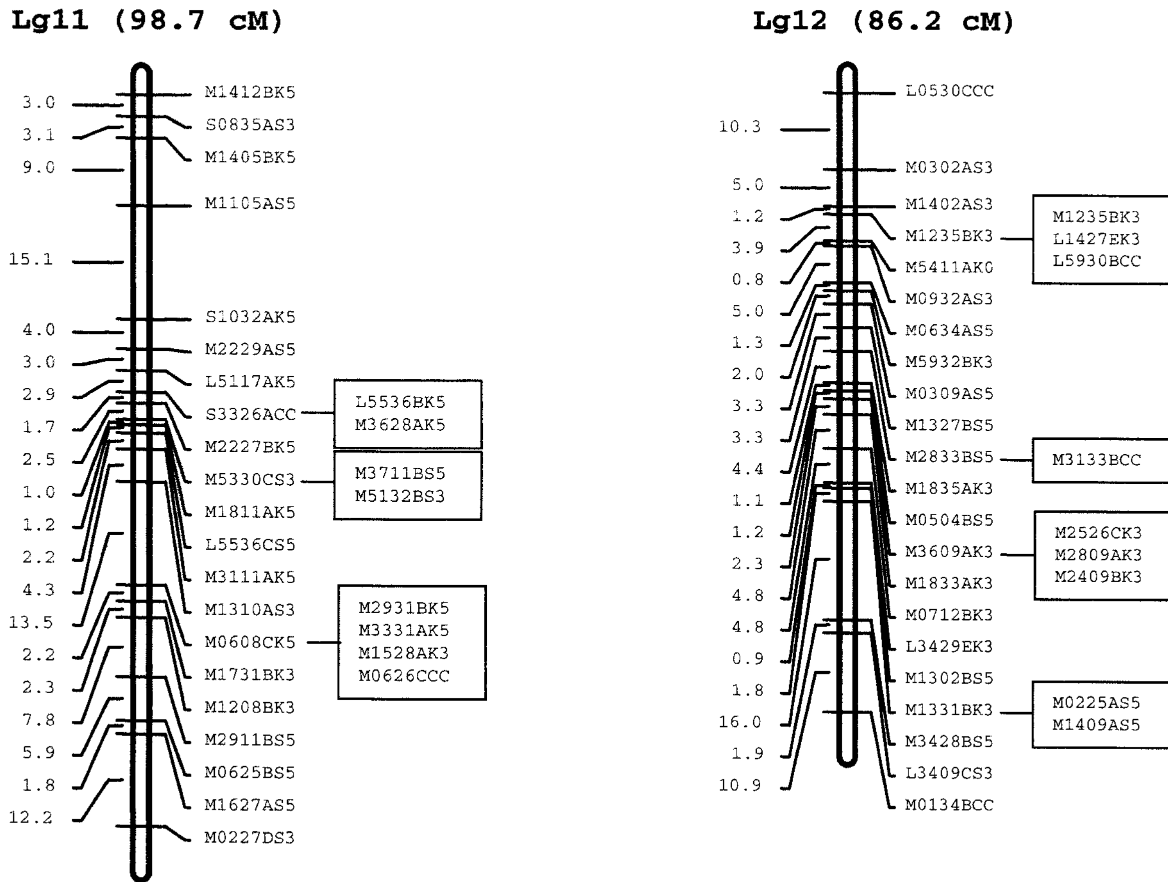

FIGURE 2.—*Continued.*

polymorphic markers produced by *Eco*RI/*Mse*I primers cosegregated with sex; only 7.3% of the 204 mapped markers or 5.2% of all 288 *Pst*I/*Mse*I markers cosegregated with sex. Thus, there were significantly fewer ($P < 0.01$) *Pst*I/*Mse*I markers than *Eco*RI/*Mse*I markers associated with the sex locus.

DISCUSSION

Characteristics of the sex determination locus: A total of 225 markers, or a total of 66% of all 342 markers on LG1, cosegregated with the sex determination locus. Our results showed that recombination was severely suppressed in the region surrounding the sex determination locus and validated the hypothesis that crossing over is repressed within the region containing the sex determination gene of papaya so that it behaves as a unit factor (STOREY 1953). DNA sequence divergence often causes regional suppression of recombination (LUKACSOVICH and WALDMAN 1999). The large number of cosegregating markers at the sex determination locus suggested extensive sequence divergence resulting in a high polymorphism rate. This might be the consequence of evolution to preserve the function of the sex determination gene that led to dioecy. Suppression of recombination was reported around another gene with evolutionary significance that controls apospory in buffelgrass (OZIAS-AKINS *et al.* 1998; JESSUP *et al.* 2002).

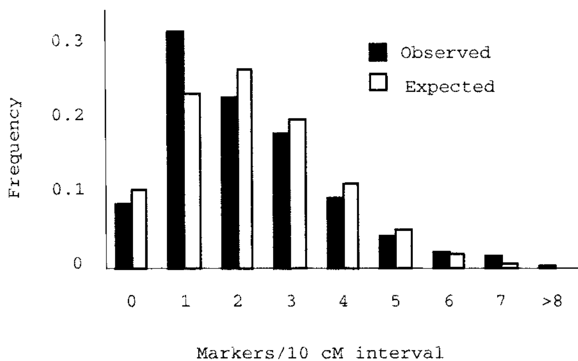

FIGURE 3.—The distribution of AFLP markers compared to that expected by Poisson distribution.

All 342 dominant markers on LG1 were derived from the pollen donor parent SunUp, and all but 2 of these markers fit a 2:1 segregating ratio. This situation arises from the lethal effect of the homozygous dominant genotype at the sex determination locus. For a dominant marker linked to the recessive sex allele m from the female parent Kapaho to be mapped, its counterpart recessive marker (absent band) is linked to the dominant sex allele M^h from the hermaphrodite parent

TABLE 3

χ^2 tests for the distribution of AFLP markers among the linkage groups

Marker types	χ^2 value	Linkage groups
All the markers	480.35***	LG1,*** LG3,*** LG4,*** LG5,*** LG8,*** LG9,** LG11*
Frame markers	27.51**	LG3,* LG6,*** LG7*
*Eco*RI frame markers	25.71**	LG3,** LG6,** LG7*
*Pst*I frame markers	16.29	LG1,* LG6**
All the *Pst*I markers	33.43***	LG1,*** LG2*

*Significance level at 0.5, **significance level at 0.1, ***significance level at 0.01.

SunUp since only one hermaphrodite F_1 plant was used to generate the mapping population. When the seeds of homozygous dominant genotypes (M^hM^h) were aborted, the class of homozygous recessive markers as the counterparts to the Kapaho-derived dominant markers were aborted as well. The segregating ratio of sex-linked dominant markers from Kapoho become 3:0 and thus cannot be mapped.

Excluding the SunUp dominant markers on LG1, the dominant markers derived from each parent are almost equal: 644 from Kapoho and 639 from SunUp, suggesting a similar level of genetic variation on each parental cultivar as reported previously (Kim *et al.* 2002).

It is known that DNA markers are clustered in the centromeric region due to the suppression of recombination in the heterochromatic regions surrounding centromeres (Alonso-Blanco *et al.* 1998; Copenhaver *et al.* 1999; Haanstra *et al.* 1999). However, the scale of suppression of recombination that we found at the sex determination locus of papaya has not been previously reported in any centromeric clusters found in high-density maps of any other plant species (Davis *et al.* 1994; Round *et al.* 1997; Boyko *et al.* 2002; Menz *et al.* 2002). Suppression of recombination in the papaya sex region is less likely the effect of the centromere and more likely the consequence of sex evolution in this plant species. This hypothesis is supported by the fact that another four clusters with at least seven cosegregating markers are mapped on this linkage group. Because several marker clusters, not just one, were mapped on LGs 2–8, it is difficult to predict the position of centromeres.

DNA methylation: In LG1, the sex locus had an extremely low frequency of *Pst*I markers. The lower level of sex cosegregating markers generated by the methylation-sensitive enzyme than by the nonsensitive enzyme indicates that the cytosine bases are highly methylated around the sex locus. A positive correlation has been reported for other plant species among marker clusters, centromeric regions, high levels of cytosine methylation, and heterochromatic regions (Vuylsteke *et al.* 1999; Young *et al.* 1999). Our analysis of DNA methylation is in accord with the marker cluster results. Since most expressed genes are associated with hypomethylated regions (Young *et al.* 1999), our results suggest that relatively few active genes are located around the sex locus of papaya. These results strongly support the hypothesis that Hofmeyr proposed more than 30 years ago (Hofmeyr 1967).

High-density genetic map: Although our high-density linkage map was constructed from 1767 AFLP markers, 2 morphological markers, and 1 transgene marker, gaps still remain. A total of 1501 markers were mapped into 12 linkage groups corresponding to the nine pairs of chromosomes. Theoretically, the 3 "extra" linkage groups should eventually cojoin the other 9 groups to eliminate those gaps that are so large they currently necessitate the reported 12 linkage groups. The differences in length among the linkage groups might be due to recombination rate and nucleotide composition difference of each chromosome, or they may be due to chromosomal translocations during meiosis and/or methods used in our map construction.

If chromosomal translocation had occurred, at least half of the seeds derived from F_1 might not be viable (Klug and Cummings 1994). A seed germination test that we conducted (data not shown) failed to support the hypothesis that a translocation event was the basis for the large linkage groups. The largest linkage group, LG2, likely represents more than one chromosome since cytological observations indicate its chromosomes are physically similar in size (Storey 1941). However, we were not able to break this linkage group using our current mapping population and the type of DNA markers used. This may be resolved in the future by integration of the genetic and physical maps of papaya and by chromosome *in situ* hybridization of selected bacterial artificial chromosome clones on the physical map.

Crop species such as maize, rice, and sorghum have a bigger genome size than papaya (Arumuganathan and Earle 1991), but the lengths of their high-density genetic maps are <2000 cM (Harushima *et al.* 1998; Vuylsteke *et al.* 1999; Menz *et al.* 2002). Although papaya has a relatively smaller genomic size, the length of its genetic map is 3294.2 cM. This disparity could be due partially to a high genome homology between the two papaya parental cultivars that were derived from the same gene pool (Kim *et al.* 2002). A higher genome

homology results in a higher recombination rate, which expands the genetic map. For example, in potatoes, the genetic map generated from an intraspecific cross is 165% the size of the map from an interspecific population (Bonierbale *et al.* 1988; Gebhardt *et al.* 1991). Another reason for the large size of the papaya map is the nature of dominant markers that were used for its construction. Since dominant markers represent both homozygous and heterozygous dominant genotypes, the resolution of dominant markers is 50% lower than that of codominant markers for mapping in an F_2 population of a diploid species. The direct effect of mapping dominant markers expands the map size by 50%.

The inserted PRSVCO was mapped on LG7 on this high-density linkage map, verifying that there was a single insertion when this gene was transformed (Fitch *et al.* 1992). Currently, a high-density map of the inserted gene is required for registering genetically modified food products. This map can be used for registration of transgenic papaya in foreign countries to open new markets.

Papaya breeders have long been interested in developing DNA markers linked to fruit flesh color for selection. Dominant yellow color is associated with firmness, while recessive red color is associated with good flavor but shorter shelf life. Experiments are underway to develop SCAR markers linked to flesh color from the flanking AFLP markers on LG7.

Efficiency of the length of nucleotide extension: The number of amplified products generated by the AFLP technique is theoretically related to the size of the genome and the length of the nucleotide primer extensions (Vos *et al.* 1995). Papaya has a relatively small genome with 372 Mb (Arumuganathan and Earle 1991). The combination of *Eco*RI-A** primer with *Mse*I primer that contained two-, three-, and four-nucleotide extensions gave 73, 53, and 31 bands, respectively (Table 2). As reported for barley, flax, and onion (Van Treuren 2001), shortening the nucleotide extension increased the number of bands in steps by 1.7- and 2.4-fold. This was considerably less than the 4-fold increase expected on a theoretical basis. However, the varied length of extended nucleotides of primers from four to seven in our study resulted in a statistically significant negative correlation with the number of amplified products.

Likewise, the number of polymorphic markers was not strictly related to the length of nucleotide extensions of the primer pairs. The short length of selective nucleotides, such as two-nucleotide extensions of *Eco*RI-A* and *Eco*RI-G* with *Mse*I-**, produced less informative profiles than did a three-nucleotide extension of the same *Eco*RI primer with *Mse*I-**. The reasons for the less informative profile appear to be saturation and homoplasy, which were produced during selective amplification. Saturation and homoplasy have been reported in a range of crop species (Van Treuren 2001). Other plant species may have optimal nucleotide extensions for an effective AFLP amplification on the basis of fragment saturation and homoplasy (Han *et al.* 1999). In papaya the five primer extensions of *Eco*RI-A**/*Mse*I-** and *Eco*RI-G**/*Mse*I-** were most efficient for AFLP marker generation (Table 2). The *Pst*I primers generated fewer markers than the *Eco*RI primers did, possibly because the *Pst*I restriction nuclease is a methylation-sensitive enzyme and large portions of plant genomes are methylated (Inamdar *et al.* 1991; Warner 1996; Young *et al.* 1999), thus dramatically reducing the number of restriction sites.

Polymorphisms and segregation: Although the primer pairs of *Eco*RI-A**, *Eco*RI-G**, and *Pst*I-A** with *Mse*I-** resulted in an average of 73, 59, and 50 bands, respectively (Table 2), the number of bands is fewer than that produced in other plant species such as *Alstroemeria* spp. (Han *et al.* 1999), lettuce (Van Treuren 2001), sugarcane (Hoarau *et al.* 2001), and tomato (Haanstra *et al.* 1999). Also each primer pair generated an average of only 1.8 AFLP markers in papaya, which is very low compared to polymorphism observed in other plant species such as *Populus deltoids* (Wu *et al.* 2000), tomato (Haanstra *et al.* 1999), rice (Mackill *et al.* 1996), and Arabidopsis (Peters *et al.* 2001). The low rate of polymorphic AFLP markers in papaya is the result of the low level of polymorphism between the two original parents, since Kapoho and SunUp are both Hawaiian Solo types derived from the same gene pool. This is at first surprising because the two parents are morphologically quite different. This result is consistent with other reports that papaya has a narrow genetic base (Sharon *et al.* 1992; Stiles *et al.* 1993; Sondur *et al.* 1996; Kim *et al.* 2002).

The effect of AT/GC content in selective nucleotide sequence: The effect of AT/GC content in selective nucleotide sequences on the number of AFLP bands has been reported in soybean (Keim *et al.* 1997), Alstroemeria (Han *et al.* 1999), and Pinyon pine (Travis *et al.* 1998). Those studies used *Eco*RI/*Mse*I primer pairs and showed that a high level of GC content will produce a low number of bands for soybean and Alstroemeria, but a high number of bands for pinyon pine. Our data were generated from both *Eco*RI/*Mse*I and *Pst*I/*Mse*I primer pairs and indicated that the number of AFLP bands and markers was not statistically associated with either the AT or the GC content of the nucleotide extensions in the primers. However, AT-rich *Eco*RI and *Pst*I selective nucleotide primers produced significantly more sex cosegregating markers than did the GC-rich primers. In *Alstroemeria aurea*, De Jeu *et al.* (1997) found that heterochromatic regions were related to AT-rich sequences. A more detailed description of the genomic structure of the papaya sex locus will require studies involving *in situ* hybridization and sequence analyses.

Marker clusters: Because greatly suppressed recombination would produce an apparent tight clustering of

markers while high levels of recombination would produce widely dispersed markers, the clusters found in our linkage map can be interpreted as reflecting possible chromosome structures. A similar pattern has been reported for other plant species, including tomato (Tanksley *et al.* 1992), Arabidopsis (Alonso-Blanco *et al.* 1998), soybean (Young *et al.* 1999), and maize (Vuylsteke *et al.* 1999). In those cases, marker clusters were frequently associated closely with the chromosome centromere. If we limit our present analysis to only the frame markers, papaya has only a few marker clusters that are present on only eight linkage groups. A possible reason for this paucity of clusters in papaya compared to other plant species, with the notable exception of Arabidopsis, could be the small size of the Arabidopsis and papaya genomes with a greatly reduced "junk" DNA component in the heterochromatin regions. Notably, if the cosegregating markers were added to the analysis, the sex locus is the biggest cluster. This indicates that the sex locus is particularly differentiated from the other cluster regions in our map.

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