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Construction of an RFLP linkage map for cultivated sunflower

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Abstract An RFLP linkage map was constructed for cultivated sunflower *Helianthus annuus* L., based on 271 loci detected by 232 cDNA probes. Ninety-three F_2 plants of a cross between inbred lines RHA 271 and HA 234 were used as the mapping population. These genetic markers plus a fertility restoration gene, Rf_1, defined 20 linkage groups, covering 1164 cM of the sunflower genome. Of the 271 loci 202 had codominant genotypic segregation, with the rest showing dominant segregation. Thirty-two of the 232 probes gave multiple locus segregation. There were 39 clusters of tightly linked markers with 0 cM distance among loci. This map has an average marker-to-marker distance of 4.6 cM, with 11 markerless regions exceeding 20 cM.

Key words RFLP (restriction fragment length polymorphism) · Linkage map · *Helianthus annuus* L · cDNA

Introduction

Cultivated sunflower (*Helianthus annuus* L., 2n=2x=34) is one of the few major crops indigenous to the USA. It was first domesticated by the Native Americans from wild *Helianthus annuus* L., which is one of the 49 wild *Helianthus* species of North American origin. In the sixteenth century, Spanish explorers first brought sunflowers to Europe, where they were initially admired primarily as an ornamental flower. By the late nineteenth century the sunflower was planted over a large area of Russia, and in the early twentieth century Russian scientists began serious breeding efforts on cultivated sunflower. The narrow genetic base of modern cultivated sunflower has been a concern among sunflower researchers.

Sunflower is the second largest oilseed crop in the world, after soybean. Its future improvement is dependent upon: (1) the continued introduction of useful genetic diversity from wild species into the breeding programs, and (2) the utilization of genetic information contained in the genomes of the cultivated and wild sunflower species. Inheritance and linkage relationships have been reported for only 11 isozyme loci in sunflower (Torres 1974; Torres and Diedenhofen 1976; Kahler and Lay 1985; Gerdes et al. 1998). Also, fewer than 50 morphological markers and only a limited number of genes controlling important traits such as disease resistance, fertility restoration, branching, fatty acid composition, plant height, and maturity have been described (Miller and Fick 1997). No linkage map of classical genes in *Helianthus* exists; thus, the assignment of individual sunflower genes to specific chromosomes is not possible. Molecular restriction fragment length polymorphism (RFLP) markers offer an opportunity for the sunflower geneticist to circumvent this difficulty.

RFLP genetic markers have unique characteristics useful for crop improvement (Beckman and Soller 1986; Tanksley 1983). RFLPs have been used to identify crop varieties or hybrids (Smith and Smith 1991; Soller and Beckman 1983; Smith et al. 1990) and to evaluate genetic diversity and phylogenetic relationships among both cultivars and wild species (Livini et al. 1992; Menancio et al. 1990; Song et al. 1990; Debener et al. 1990; Kesseli et al. 1991). In a number of crops, DNA markers have been linked with both qualitative and quantitative traits. The association between specific DNA markers and such agronomic traits will

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facilitate marker-assisted selection in crop breeding programs and the eventual cloning and manipulation of desirable genes (Jung et al. 1992; Diers et al. 1992; Nienhuis et al. 1987; Knapp et al. 1990; Ahn et al. 1992; Michelmore et al. 1992).

Several studies have demonstrated the utility of molecular markers in sunflower genetic analysis. Gentzbittel et al. (1992) described the phylogeny of 44 *Helianthus* species with the use of 10 DNA probes. Relationships among inbred lines of cultivated sunflower were studied by Gentzbittel et al. (1994) and by Berry et al. (1994) using low-copy genomic and cDNA probes. Rieseberg et al. (1995), using 197 RAPD markers, concluded that gene introgression in *Helianthus* involved both chromosomal and genic barriers. Berry et al. (1995) reported the first RFLP map of cultivated sunflower using an F_2 population of ZENB8 × HA 89.

The objective of this project was to construct a low-density RFLP map for cultivated sunflower utilizing the high polymorphism between USDA inbred lines RHA 271 and HA 234.

Materials and methods

Plant material

Twenty-three USDA inbred lines including 12 restorer (RHA) lines, 10 maintainer (HA) lines, and a cytoplasmic male-sterile (cms) line were used in this study (Table 1). A total of 409 cDNA probes were screened with these lines using three restriction enzymes, *Eco*RI, *Eco*RV, and *Hin*dIII. An additional 206 probes were screened using a subset of 3 RHA and 3 HA lines that represented the highest polymorphism among lines. These 6 lines were grown in the field in 1992 at Fargo, N.D. A half-diallel cross combination was completed using single plants as male and female parents. F_1 plants were grown and selfed in the greenhouse in 1993. F_2 seeds were planted in the field and were sampled for the RFLP mapping project in the summer of 1993. Ninety-three random F_2 plants of RHA 271 × HA 234 were used for the RFLP linkage mapping. The fertility restoration gene, Rf_1, in RHA 271 was also mapped.

Library construction

A sunflower cDNA library was developed using seedling leaves of HA 89 (Ullrich et al. 1977; Gilsin et al. 1974; Gubler and Hoffman 1983). Total RNA was extracted by homogenization of fresh tissue in a guanidinium thiocyanate solution and purified by sedimentation through CsCl. The poly(A)$^+$ RNA fraction was obtained by oligo(dT)-cellulose chromatography and employed as the template in a reverse transcription reaction primed with a dT-tailed *Not*I primer/adaptor oligonucleotide. The cDNA was constructed with 5′-*Eco*RI and 3′-*Not*I sequences and was ligated with the pGEM-11Zf vector. The sunflower cDNAs were inserted in an "anti-sense" orientation relative to the *lac* promoter in the vector to avoid bias against sequences that could generate toxic fusion products in the *E. coli* host, DH5-alpha. Following transformation and selection, the presence and lengths of sunflower cDNA inserts were determined by agarose gel electrophoresis of polymerase chain reaction (PCR) products that were produced either with T7 and SP6 promoter primers or the pUC/M13 "forward" and "reverse" primers.

DNA extraction, digestion, Southern blotting, hybridization, and RFLP probes

Leaf material from plants was rapidly frozen in liquid nitrogen, lyophilized, and ground to a fine powder using a sample mill equipped with a 0.4-mm screen. DNA extraction and purification were performed as described by Murray and Thomspon (1980) with some minor modifications. Restriction enzyme digestion was carried out according to the manufacturer's protocol (Life Technologies). Restriction fragments were separated by agarose gel electrophoresis

Table 1 Inbred lines screened for polymorphism and their pedigrees or sources

Line	Pedigree or source
RHA 265	2* Peredovik/953-102-1-1-41 = T66006-2-1-3-1
RHA 271	CMS PI343765/HA119//HA62-4-5/2/T66006-2-1-31-1 = T70020
RHA 273	CMS PI343765/HA119//HA62-4-5/2/T66006-2
RHA 274	CMS PI343765/HA119//HA62-4-5/2/T66006-2
RHA 294	CMS PI343863/BONITA GIANT-MANCHURIAN/HA61/MENNONITE RR
RHA 358	RHA 274*3/DDR Short Height
RHA 365	SELECT
RHA 373	RHA 274/82ROM-R31
RHA 374	ARG-R43
RHA 377	RHA 299//SOREM HT 58/RHA 801
RHA 801	Derived from a Restorer Composite
RHA 858	P1161/RHA 298
HA 89	VNIIMK 8931 Sel
HA 124	VNIIMK 8883
HA 234	2*Smena/HA6/HA8
HA 370	RK-74-198
HA 371	H52 Sel
HA 372	H55 Sel
HA 369	ARG 8018
HA 821	HA 300 Selection
HA 822	HA 400 Selection – Canada
HA 852	Chernyanka 77/HA 89
CMS HA 822	

and transferred to charge-modified nylon membrane by capillary action under alkaline conditions. Southern hybridization was based on procedures described by Budowle and Boechtel (1990). The final post hybridization wash was performed in 0.2× SSPE buffer at 65°C for 10 min.

Among the 615 cDNA probes screened, 267 cDNA probes were identified to be single- or low-copy polymorphic probes for the two parents, RHA 271 and HA 234. Only the best probe-enzyme combination for the 267 probes was used to screen the F_2 segregating population of this parent pair.

Linkage analysis

The linkage map was constructed using MAPMAKER 3.0 (Lander et al. 1987). Linkage groups were obtained using the "group" command with a recombination value of less than 0.30 and an LOD score of less than 3.5. For each group, three-point analysis was first performed, followed by the "order" command, and the resulting marker order was examined using the "ripple" command to obtain the unique marker sequences. Loci not in the unique sequence were placed using the "try" command. Unlinked loci, and loci that were excluded by the "try" command because of their three-point analysis, were not mapped. The "error detection" command was used to check for unexpected mistakes in scoring, data entering, or double cross-over. Multiple loci detected by a single probe had suffix a, b, c, . . . added following the regular probe names. Kosambi's (1944) mapping function was used to generate map distances.

Multiple probes that mapped to the same locus were compared for their RFLP banding patterns against the 23 inbred lines or against the subset of 6 lines containing 3 RHA and 3 HA lines. Probes with the same banding pattern were considered to be the same cDNA clone. Thus, only 1 probe was selected for the construction of the map.

Results

Our preliminary evaluation eliminated 34 duplicated probes, 1 unlinked probe (9C2), and 1 marker (7A2a) which failed to be placed properly onto its linkage group. The 232 unique cDNA probes used for the RFLP linkage analysis represented 38% of the 615 screened probes and revealed 271 polymorphic loci (Fig. 1). The probe-enzyme combinations selected indicated equal effectiveness of DNA digestion with *Eco*RI, *Eco*RV, and *Hin*dIII, with respective probe numbers of 84, 75, and 73. Of the 271 loci, 202 (75%) had codominant segregation and 69 had dominant segregation of allelic forms.

We mapped 232 probes (271 loci) and the Rf_1 gene onto 20 linkage groups, covering 1164 cM of the sunflower genome, with an average marker-to-marker distance of 4.6 cM (Fig. 2 and Table 2). The number of markers per group varied from 2 to 27, and the average between-marker distance varied from 2 to 16 cM. Throughout the genome there were 39 clusters that contained from 2 to 9 tightly linked markers showing no recombinations, including 20 dominant and 78 codominant markers. The clustering of so many markers reduced the resolving power of this map as well as the practical utilization of some of the markers for sunflower improvement. The Rf_1 fertility restoration gene was mapped to one end of Group 4 at a distance of 25 cM from an adjacent marker.

Among the 232 probes, 200 identified single loci while 26, 5, and 1 probes identified 2, 3, and 4 loci, respectively. These duplicated loci or small multigene families represented 14% of the total probes and 26% (71) of the markers on the map. The majority of the loci of the multiple-locus probes segregated independently. The distribution of multiple loci of the 32 probes is shown in Table 3. In general, the multiple loci of each probe were unlinked or distantly linked. The only

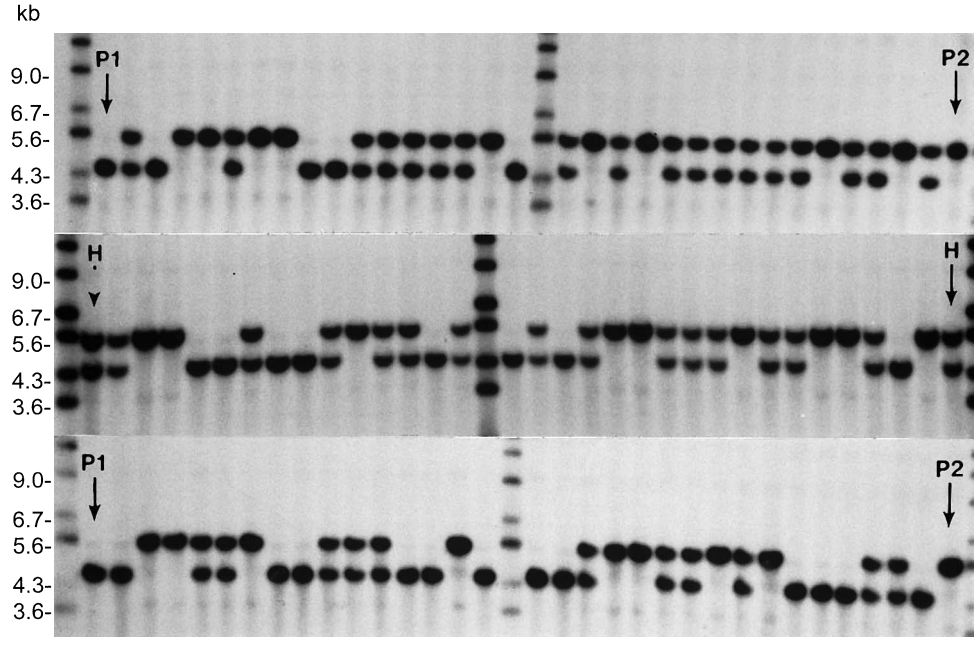

Fig. 1 Segregation pattern of 93 F_2 plants of RHA 271 × HA 234 for a single-copy cDNA probe 1D2 with restriction enzyme *Eco* RV. *P1* RHA 271, *P2* HA 234, *H* RHA 271 × HA 234, F_1

exceptions were the loci of probes 1A6, 8B3, and 13B3, which were tightly linked at less than 2 cM.

The distribution of markers, including the Rf_1 gene, can be seen in Table 4. There were 11 gaps of over 20 cM, covering 292 cM map distance, which represents 25% of the total mapped genome.

Discussion

Twenty-three USDA sunflower inbred lines were screened with 409 cDNA probes and three restriction enzymes in order to determine the degree of

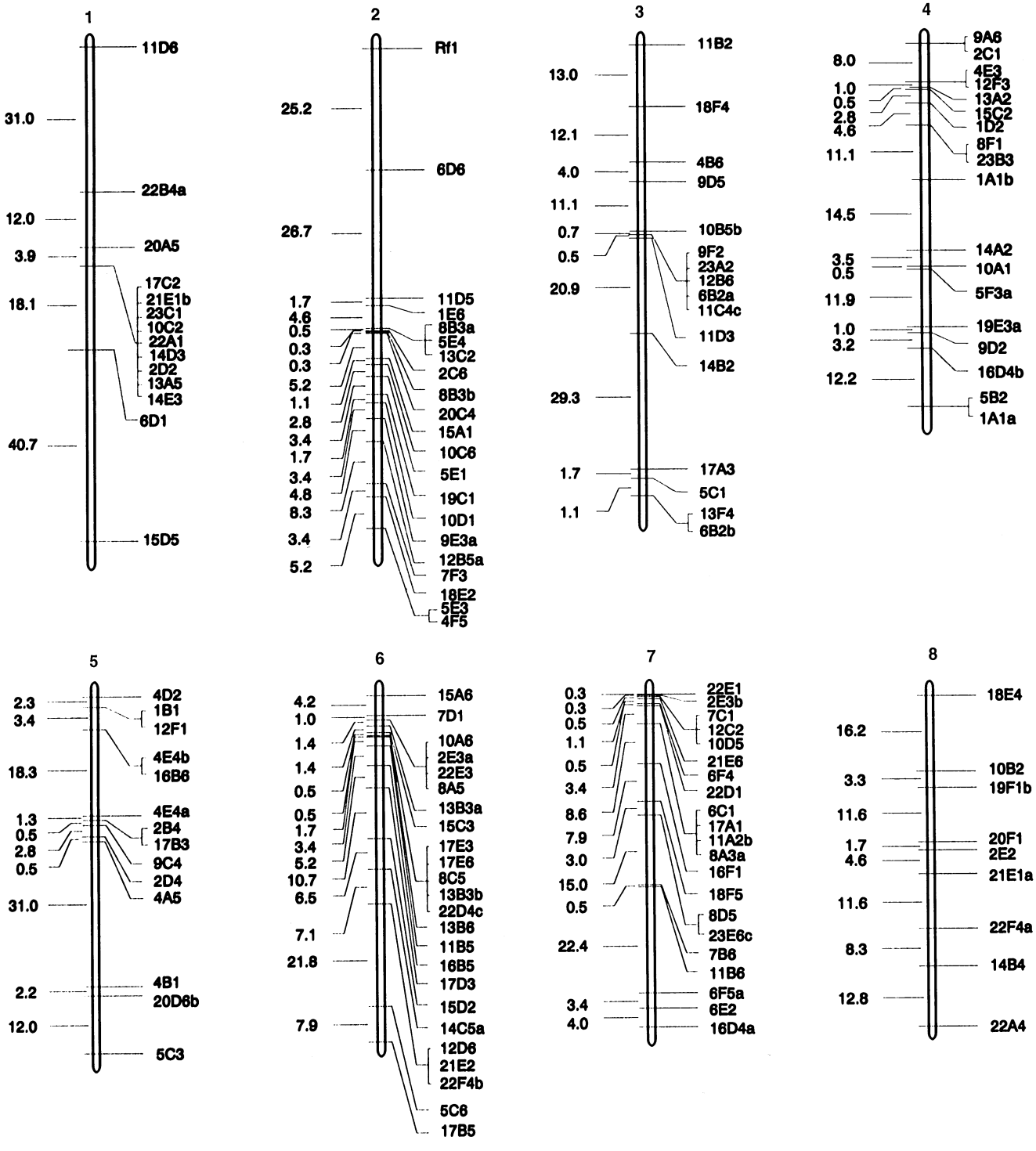

Fig. 2 Sunflower RFLP linkage map of 271 loci identified by 232 cDNA probes. Linkage group numbers are indicated on *top*. The loci are listed on the *right* and the map distances given in centi-Morgans (cM) are on the *left*. Duplicated loci detected with the same probe are labelled with a different *lowercase letter* following the name

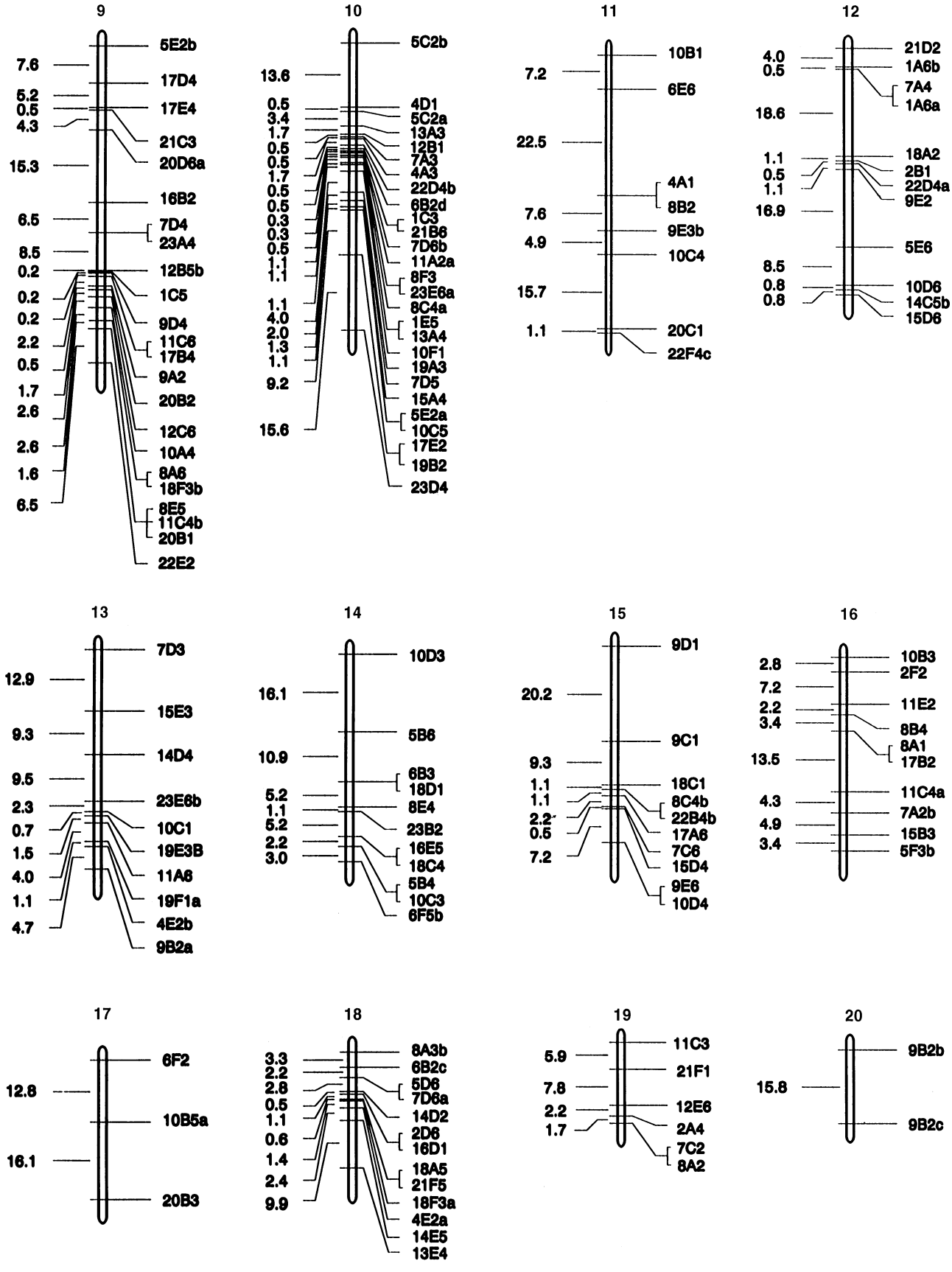

Fig. 2 Continuation (see page 4 for legend)

Table 2 Number of markers, group size, and the average between-marker distance of each of the 20 linkage groups, and the distribution of marker clusters with 0 cM between-marker number distance

Linkage group	Number of markers	Group size (cM)	Average marker-marker distance (cM)	Number of clusters with markers of				
				2	3	4	5	9
1	14	106.1	8.16					1
2	21	98.8	4.94	1	1			
3	16	94.7	6.31	1			1	
4	18	75.2	4.42	4				
5	14	74.6	5.74	3				
6	24	73.9	3.21	1		1	1	
7	21	71.2	3.56	1	1	1		
8	9	70.1	8.76					
9	23	66.6	2.90	3	1			
10	27	60.9	2.34	5				
11	8	59.1	8.44	1				
12	12	53.1	4.83	1				
13	10	45.9	5.10					
14	11	43.8	4.38	3				
15	10	41.8	4.64	2				
16	10	41.7	4.63	1				
17	3	28.9	14.45					
18	13	24.4	2.03	3				
19	6	17.7	3.54	1				
20	2	15.8	15.8					
Total	272	1164.3		31	3	2	2	1

Table 3 Linkage group of the loci for the 32 multiple-locus probes

Probe	Number of loci	Linkage group
1A1	2	4 (46.8)[a]
1A6	2	12 (0.5)
8A3	2	7, 18
11A2	2	10, 7
6B2	4	18, 10, 3, (53.5)
9B2	3	13, 20 (15.8)
8B3	2	2 (0.8)
10B5	2	17, 3
13B3	2	6 (1.9)
12B5	2	2, 9
22B4	2	1, 15
5C2	2	10 (14, 1)
8C4	2	10, 15
11C4	3	16, 9, 3
14C5	2	6, 12
7D6	2	10, 18
16D4	2	7, 4
20D6	2	9, 5
22D4	3	6, 12, 10
2E3	2	6, 7
4E2	2	18, 13
4E4	2	5 (18.3)
5E2	2	10, 9
9E3	2	2, 9
19E3	2	4, 13
21E1	2	8, 1
23E6	3	10, 13, 7
5F3	2	4, 16
6F5	2	7, 14
18F3	2	18, 9
19F1	2	13, 8
22F4	3	6, 8, 11

[a] Numbers in parenthesis indicate distance of the 2 loci in the same linkage group

Table 4 The frequency distribution of between-marker space of the RFLP map

Distance between markers (cM)	Number of intervals for a given size	Percentage of total
0.0–4.9	181	71.8
5.0–9.9	30	11.9
10.0–14.9	18	7.1
15.0–19.9	12	4.8
20.0–24.9	5	2.0
25.0–29.9	3	1.2
30.0–34.9	2	0.8
35.0–39.9	–	–
40.0–44.9	1	0.4
Total	252	100.0

polymorphism of the inbred lines. Three RHA and 3 HA lines that displayed the highest polymorphism were further screened with an additional 206 probes. The inbreds RHA 271 and HA 234 were the most divergent, and were crossed to produce the F_2 progeny used to construct an RFLP linkage map based on the multilocus segregation of cDNA probes in the F_2 population.

Of the 615 screened cDNA probes, we found 232 distinctive probes that segregated for 271 polymorphic loci. The 232 probes and the Rf_1 gene mapped onto 20 linkage groups covering 1164 cM of the sunflower genome. There were 11 regions on the map which had between-marker distances of more than 20 cM. These results were similar to those of Berry et al. (1995), who

also mapped 11 regions with between-marker distances greater than 20 cM. We expect that most of the large gaps will disappear with the addition of more markers to the map. The use of different mapping populations or the use of genomic microsatellite simple-sequence repeat (SSR) probes will reduce the size of the gaps present in the current RFLP map.

The duplication of loci for many probes supports the polyploid origin of *H. annuus* (Heiser and Smith 1995; Jackson and Murray 1983). The fact that most multiple-loci probes are represented twice suggested the merging of two related ancient genomes and its subsequent polyploidization. The frequent chromosome rearrangement during the course of sunflower evolution explained the widespread distribution of duplicated loci into different linkage groups (Chandler 1991; Chandler et al. 1986). Natural and intentional introgression of other *Helianthus* species into cultivated *H. annuus* may have contributed more than 1 additional loci. Future studies on the duplication of loci using other diploid, tetraploid, and hexaploid *Helianthus* species will likely provide new information regarding sunflower evolution.

It is apparent that our 20 linkage groups are more than the 17 haploid chromosome number of the cultivated sunflower. The RFLP map of Berry et al. (1995) had a coverage of 1380 cM, which they believed to represent 60–80% of the sunflower genome. Our map of 1129 cM is even more likely to have one or two large gaps that split a large group into smaller groups. We anticipate that these small groups will be brought together as we identify new markers. In addition, RFLP markers will eventually be linked on physical maps of the sunflower genome. The work of Rooney et al. (1994) and Wang et al. (1995) suggested the use of representative probes from each group on a set of trisomics to link the groups to specific chromosomes. Accordingly, we are presently producing a set of sunflower trisomics (Jan et al. 1988). This work will expedite the construction of 17 chromosome linkage maps and also simplify our concurrent cytological studies.

Many studies have shown the usefulness of molecular maps for the manipulation of crop genomes. Critical genes for disease resistance or quantitative traits have been mapped and are now being cloned. Recently in sunflower Mouzeyar et al. (1995) linked a downy mildew resistance gene to RFLP and RAPD markers. Additional work in mapping simply inherited genes such as resistance to different races of downy mildew and rust, fertility restoration, and nuclear male sterility would enhance breeding efforts. RFLP probes will also be valuable for studying interspecific gene introgression and interspecific and intraspecific relationships (Rieseberg et al. 1995; Gentzbittel et. al. 1992; Berry et al. 1994) of sunflower.

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