Theor Appl Genet (1995) 90: 1079–1086

L. Gentzbittel · F. Vear · Y.-X. Zhang · A. Bervillé
P. Nicolas

Development of a consensus linkage RFLP map of cultivated sunflower (*Helianthus annuus* L.)

Received: 18 August 1994 / Accepted: 22 November 1994

Abstract This paper provides the first description of a consensus map of the cultivated sunflower genome (*Helianthus annuus* L., n=17 chromosomes), based on RFLP. A total of 180 probe-enzyme combinations were mapped on at least one of five segregating progenies (three F_2 and two BC_1 populations), revealing 237 loci that did not show any distortion of segregation. The consensus linkage map obtained with these loci covers 1150 cM and consists of 16 linkage groups of more than 20 cM, 7 groups of less than 20 cM and 18 unlinked loci. The mean distance between loci is 7 cM, but in some regions intervals of 20 cM remain. Genotypic and gametic segregation distortions affect about 7% of loci. It was found that 25% of the probes mapped using several different restriction enzymes or that on different progenies they revealed 2 or more loci.

Key words RFLP · *Helianthus annuus* · Linkage map · Consensus map · CDNA

Communicated by J.W. Snape

L. Gentzbittel (✉)[1]
G.I.E. Cartisol, 12 avenue George V, 75008 Paris, France

F. Vear
INRA, Station de Génétique et d'Amélioration des Plantes, Domaine de Crouelle, 63039 Clermont-Ferrand Cedex 02, France

A. Bervillé
ENSA-INRA SGAP, 2 place P. Viala, 34060 Montpellier Cedex 1, France

Y.-X. Zhang[2] · P. Nicolas
Unité associée Université Blaise Pascal-Clermont II-INRA, Organisation et Variabilité des Génomes Végétaux, 4 rue Ledru, 63038 Clermont-Ferrand, Cedex, France

Present adresses:
[1] G.I.E Cartisol, 4 rue Ledru, 63038 Clermont-Ferrand Cedex, France
[2] GIP GEVES, Unité expérimentale de la Minière, 78285 Guyancourt Cedex, France

Introduction

Sunflower (*Helianthus annuus* L.) is currently one of the most important oil-producing crops in large parts of the world. The European Union is the largest producer of sunflower seed, and the majority of all breeding programmes are carried out in Europe. However, breeding programmes to produce hybrid varieties are relatively recent. For both Mendelian traits (Leclercq 1966; Kahler and Lay 1985) and agrophysiological characters (Quillet et al. 1992; Tersac et al. 1994) relatively, little data is available as compared to other crop species.

A recent study (Gentzbittel et al. 1994) showed that the restriction fragment length polymorphism (RFLP) technique could be used to identify sunflower genotypes and that there was sufficient variability to develop an RFLP linkage map of sunflower. This technique has enabled the rapid development of chromosome maps for many crop species, such as maize (Helentjaris 1987; Burr et al. 1988; Gardiner et al. 1993), rice (McCouch et al. 1988) and potato (Gebhardt et al. 1989). The distribution of RFLP markers over the whole genome has helped in the analysis of monogenic traits such as resistance to *Pseudomonas* in tomato (Martin et al. 1993), *Phytophthora infestans* in potato (Elkharbotly et al. 1994) or *Erysiphe graminis* (powdery mildew) in wheat (Hartl et al. 1993), and of complex characters such as fruit pH or fruit mass in tomato (Paterson et al. 1988) or seed protein and oil content in soybean (Diers et al. 1992).

These results led us to produce an RFLP chomosome map of cultivated sunflower based on the progeny of intraspecific crosses. Although interspecific variability in the *Helianthus* genus (i.e. between cultivated sunflower and the other species of its section) is known to be greater than that within *H. annuus* (Choumane and Heizmann 1988; Rieseberg et al. 1990; Gentzbittel et al. 1992), it was thought that such a map would be of more immediate use if one used intraspecific crosses. In order to construct a map that was not restricted to the cross from which it had been deduced, a strategy of consensus mapping was cho-

sen involving different crosses and genetic backgrounds. In addition, the probes used to construct this map were chosen not only on the basis of the polymorphism between the parents of the crosses, but also for their polymorphism in a set of representative inbred lines of cultivated sunflower. This map could thus be of nearly universal use for all the inbreds of agronomic importance. It was also thought that this map would be more accurate by mixing information obtained from different progenies, in particular by comparing linkage data of F_2 and BC_1 progenies. This map would then serve in breeding programmes, for example for genealogical analyses, identification of cultivated inbreds or mapping of agronomic traits.

Materials and methods

Probe screening

A total of 168 genomic probes, isolated from *Pst*I (53 probes) and *Hind*III (115 probes) libraries, as well as 967 cDNAs (from five different libraries of etiolated seedlings, green leaves, ovaries and florets) were screened against two inbred lines (HA89 and RHA266) with four restriction endonucleases (*Eco*RI, *Eco*RV, *Hin*dIII and *Bgl*II). Using this procedure, we selected 463 low-copy-probes, were then screened against a set of 17 inbred lines of cultivated sunflower (Gentzbittel et al. 1994). To be considered for mapping, these probes had to be polymorphic both between the parents of the progenies studied and also on at least 20% of the 17 inbreds. A total of 209 low-copy DNA (11 genomic probes and 198 cDNAs) probes were thus selected after digestion with four restriction enzymes (*Bgl*II, *Eco*RI, *Eco*RV and *Hin*dIII). Of these probes, 157 were mapped on the five progenies described below.

Sunflower genotypes and data collection

The lines HA89, CX, RHA266 and PAC2, whose origins are given in Table 1, were used to construct three F_2 populations and two BC_1 progenies (Table 2). In order to increase the information obtained from the crosses, the progenies always had one common parent with respect to the other crosses. Progenies C1 and C2 segregated for male fertility restoration, C5 for recessive branching and C2 for stigma colour. The plants were grown under netting or polythene-covered cages in the field. Leaves were collected from each individual, just before flowering, and kept in a freezer until DNA extraction.

DNA was isolated from each leaf sample, and digestion, Southern blotting and hybridizations were carried out as described previously (Gentzbittel et al. 1994).

Linkage analysis

The hybridization bands were identified on the five segregating progenies, and the linkage phase of each band was determined by comparison with the hybridization pattern of the parents of the cross. In some cases it was not possible to define an allelic band for a given hybridization signal, probably because such a band was present in a part of the gel difficult to read (>10 kb or < 500 bp) or because there was a nonsegregating band superimposed on the allelic band. When this occurred the segregating band was noted by presence/absence, like a dominant marker (giving 3:1 segregations).

The genotypic and gametic segregations were tested against the expected F_2 or BC ratios by the G test (Holloway and Knapp 1993) at a 1% significance level. The maps for each cross were then constructed from the loci that did not show any segregation distortion using the programs GMendel 3.0 (Holloway and Knapp 1993) and MapMaker 3.0 (Lander et al. 1987). For each programme, calculation conditions were identical (maximum recombinant fraction=0.3; minimum LOD score=3.0). The Haldane function was used to obtain the centiMorgan (cM) equivalence. In all cases, the loci orders obtained with GMendel 3.0 and with MapMaker 3.0 were compared.

To construct the consensus map and to define allele and loci names in the different crosses, we compared the hybridization patterns of all the parents. Loci nomenclature is as follows: SxxxYY (-Z), with Sxxx, the SUN probe number and YY the restriction enzyme used for mapping (BG: *Bgl*II; E1: *Eco*RI; E5: *Eco*RV; H3: *Hin*dIII). Probes that hybridized with more than one site in the genome were arbitrarily identified on the maps with the suffix -Z (-1, -2, -3 etc.) to indicate each duplicate locus. If a locus was mapped with two different enzymes and the loci detected mapped to the same place, the locus is numbered SxxxAABB, with AA and BB being the abbreviations of the 2 enzymes, respectively. The consensus map was constructed using GMendel 3.0, under the same conditions as for the individual crosses. The order of markers in each group was then determined by using sequentially three different ordering functions based on minimizing the sum of adjacent recombination frequencies (SAR). At each stage, the number of recalculations of each order for each ordering function was increased. For linkage groups in each progeny where the most likely gene order was not consistent with the consensus map, MapMaker 3.0 was used to compare the log-likelihood of the most likely gene order in each progeny with the log-likelihood of the most likely order on the consensus map. If a difference of loglikelihood greater than 3.0 between the two orders was obtained, it could be concluded that there was a significant difference in marker order (Beavis and Grant 1991). The validity of each order on the consensus map was estimated by Monte-Carlo simulations in the GMendel 3.0 package.

Table 1 Origins of the sunflower inbred lines used as parents of the segregating progenies on which the RFLP map was built

Inbred line	Origin	Breeder[a]
HA89	VNIIMK 8931 - CM303	USDA
CX	Peredovik - HA300	INRA
RHA266	Wild *H. annuus* × Peredovik	USDA
PAC2	*H. petiolaris* restorer × USDA line (HA61)	INRA

[a] USDA, United States Department of Agriculture (USA); INRA, Institut National de la Recherche Agronomique (France)

Table 2 Crosses used for RFLP mapping of sunflower

	Type	Cross	Number of individuals	Segregating phenotypic character
C1	F2	HA89 × RHA266	80	
C2	F2	CX × RHA266	130	Restoration, stigma colour
C3	BC1	(HA89 × CX) × HA89	100	
C4	BC1	(HA89 × CX) × CX	100	
C5	F2	PAC2 × RHA266	150	Branching

Results

Probe screening

Out of the 1135 probes screened, 209 were identified as suitable for mapping (19%) in that they exhibited polymor-

phism both between the lines HA89, CX, RHA266 and PAC2 and also among the set of 17 inbred lines studied (Gentzbittel et al. 1994). There was a considerable difference in the frequency of mappable markers between the probe sources, especially when comparing *Hin*dIII genomic libraries and *Pst*I or cDNA libraries. For the former, 9 mappable markers were identified from 115 probes (7.8%), whereas the latter two allowed the identification of 200 mappable markers from 1020 probes (20%). This result is mainly due to the fact that *Hin*dIII genomic libraries tend to detect more repetitive sequences than cDNA or *Pst*I genomic libraries.

Of the 209 mapping markers identified, 157 probes were mapped on at least one progeny (SUN probes). This slight reduction in the number of available probes is partly due to the fact that line CX was found to present some heterogeneity for RFLP (data not shown) and that the different plants used for parental characterization and the three crosses involved were not identical.

Maps for individual crosses

Each of the five progenies was mapped separately. The basic characteristics of these maps are given in Table 3. The RFLP maps of crosses C2 and C5 are examplified in Fig. 1A and B. On average, 6% of the loci showed a highly significant ($P< 0.01$) distortion of segregation (genotypic or gametic) and were not included in the computations of the unit maps nor of the consensus map. Progenies C2 and C5 allowed the identification of more than 100 loci; a smaller number of loci were mapped on crosses C3 and C4. This was due partly to the problem with CX described above, but also to the fact that these were backcross progenies and, since about 30% of the probes gave hybridization signals that were coded presence/absence, there was no segregation when the backcross was made with the parent having the band. The C3 and C4 segregating populations were also designed to allow the study of a potential difference in the recombination rates between inbreds, as the F_1 hybrid was backcrossed by each of the two parental lines. No effect was detected, probably because the number of loci mapped in these two crosses was too small (60 and 26 loci for C3 and C4, respectively).

Some probes revealed several loci, even when they were mapped on the same progeny but with a different enzyme. For example, this was the case for SUN040, mapped by *Eco*RI on progeny C2: the locus SUN040E1-1 is in group 2, the locus SUN040E1-2 in group 4. Another example is the probe SUN006 with progeny C5: the locus SUN006E1 was mapped in group 11, the locus SUN006H3-1 in group 8 and SUN006H3-3 appeared to be unlinked. Without exception, for all the probes that showed duplicated loci, in particular when they were mapped with different enzymes, a non-segregating band appeared in 1 of the probe-enzyme combinations. It is reasonable to suppose that a band seen as monomorphic with one restriction enzyme will often be rendered polymorphic by the use of a different enzyme.

Table 3 Summary of linkage analyses in each of the five crosses

	Number of probes used	Number of combinations	Number of loci detected	Disturbed loci[a] (%)	Number linkage groups	Overall size (in cM)[b]
C1	46	46	64	6	14	176
C2	83	94	123	8	18	582
C3	52	54	60	5	15	186
C4	22	23	26	8	5	71
C5	106	117	146	7	21	763

[a] The distorsion was tested with an risk of 1%; results are given in percentage of the number of loci detected

[b] The recombination fraction was converted into cM using the Haldane function

Consensus map

Genome size and locus ordering

The results presented come from analysing 157 RFLP probes mapped on at least one progeny with 180 probe-enzyme combinations. A total of 237 loci showing no distortion of segregation were included in the consensus linkage map (Fig. 2). This map covers at least 1150 cM in 23 linkage groups. Three groups exceeded 100 cM in length, 10 groups measured between 50 and 100 cM and 18 loci (7.5%) are at present considered to be unlinked. Analysis of the lengths of groups in relation to the number of loci per group showed that there is a very close correlation (r^2=0.81, data not shown) between the two, indicating that loci are randomly distributed over the genome. The mean distance between loci is 7 cM, but there are a few regions of more than 30 cM with no markers, for example in groups 1 and 3. Such gaps may reflect an under-representation of probes from these regions or, alternatively, an uneven distribution of recombination events along the chromosome. The large number of individuals analysed for some loci (up to 480 individuals for loci segregating in four progenies) allow us to be reasonably confident of the results. Only 1 locus (SUN064H3, at the end of group 3) is removed from a linkage group when the LOD score is increased to 4.0.

In a few cases, the order of loci on the consensus map was different from the optimal order defined for unit maps. To test whether there were significant differences in the order of the consensus map compared with that of the unit maps, the method defined by Beavis and Grant (1991) was used. The order of loci defined in the consensus map was compared with the best order of these loci defined by the unit maps. If a difference of loglikelihood greater than 3.0 between the two orders was obtained, it would be concluded that there was a significant difference in marker order. This comparison was made for the three F_2 progenies, C1, C2 and C5, and in no case was there any significant difference with LOD=3.0 between the consensus map and the unit maps defined by GMendel 3.0 or MapMaker 3.0.

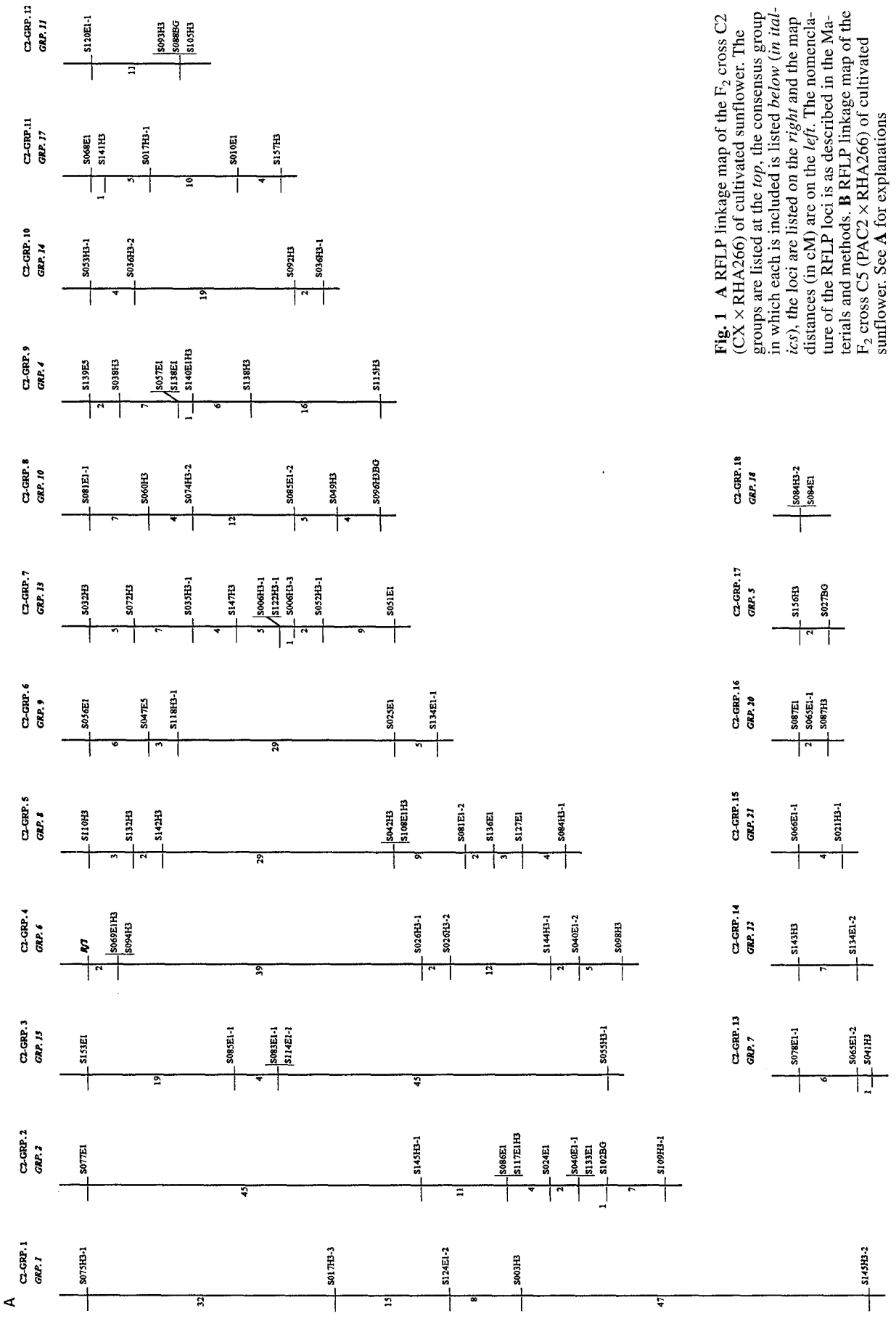
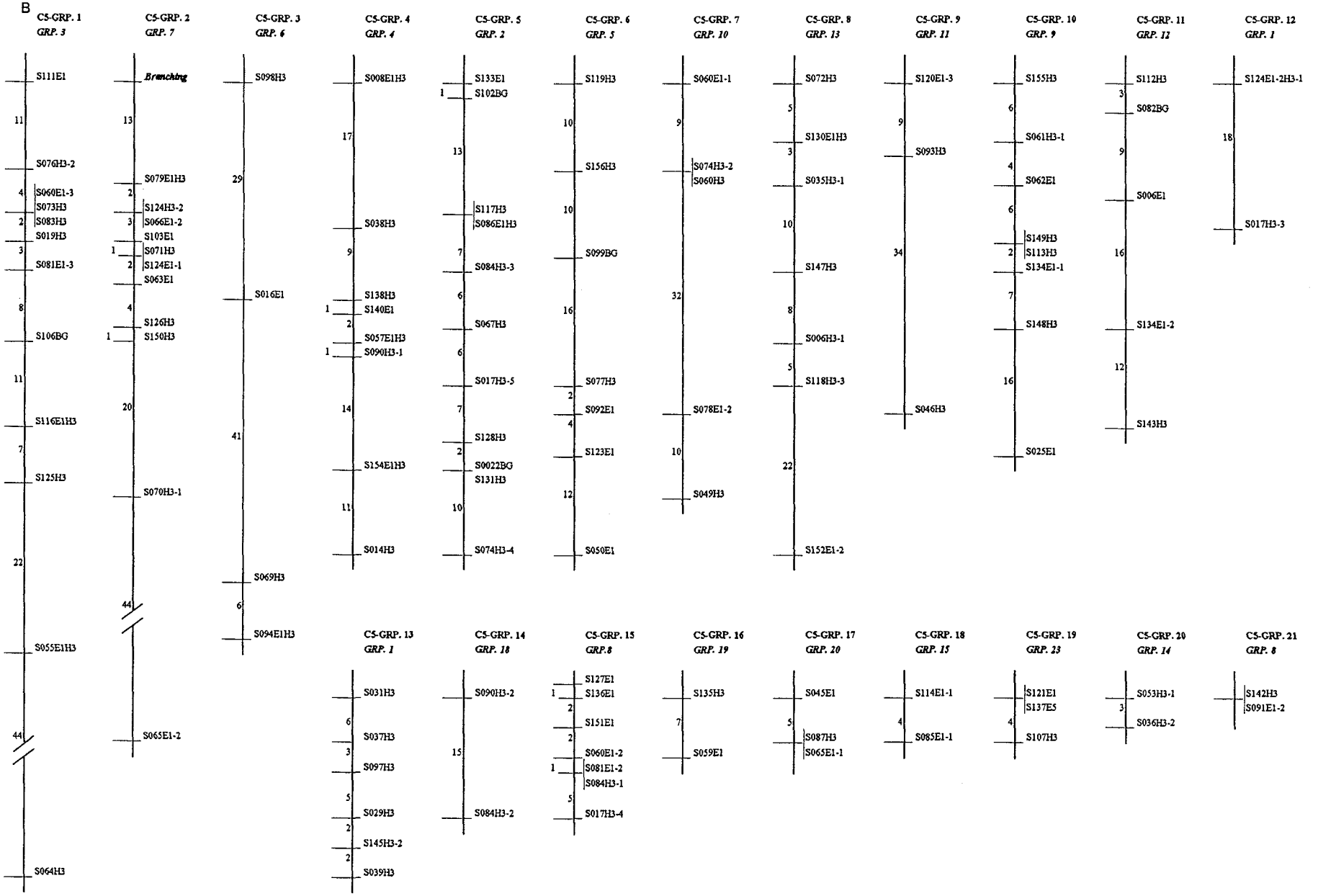
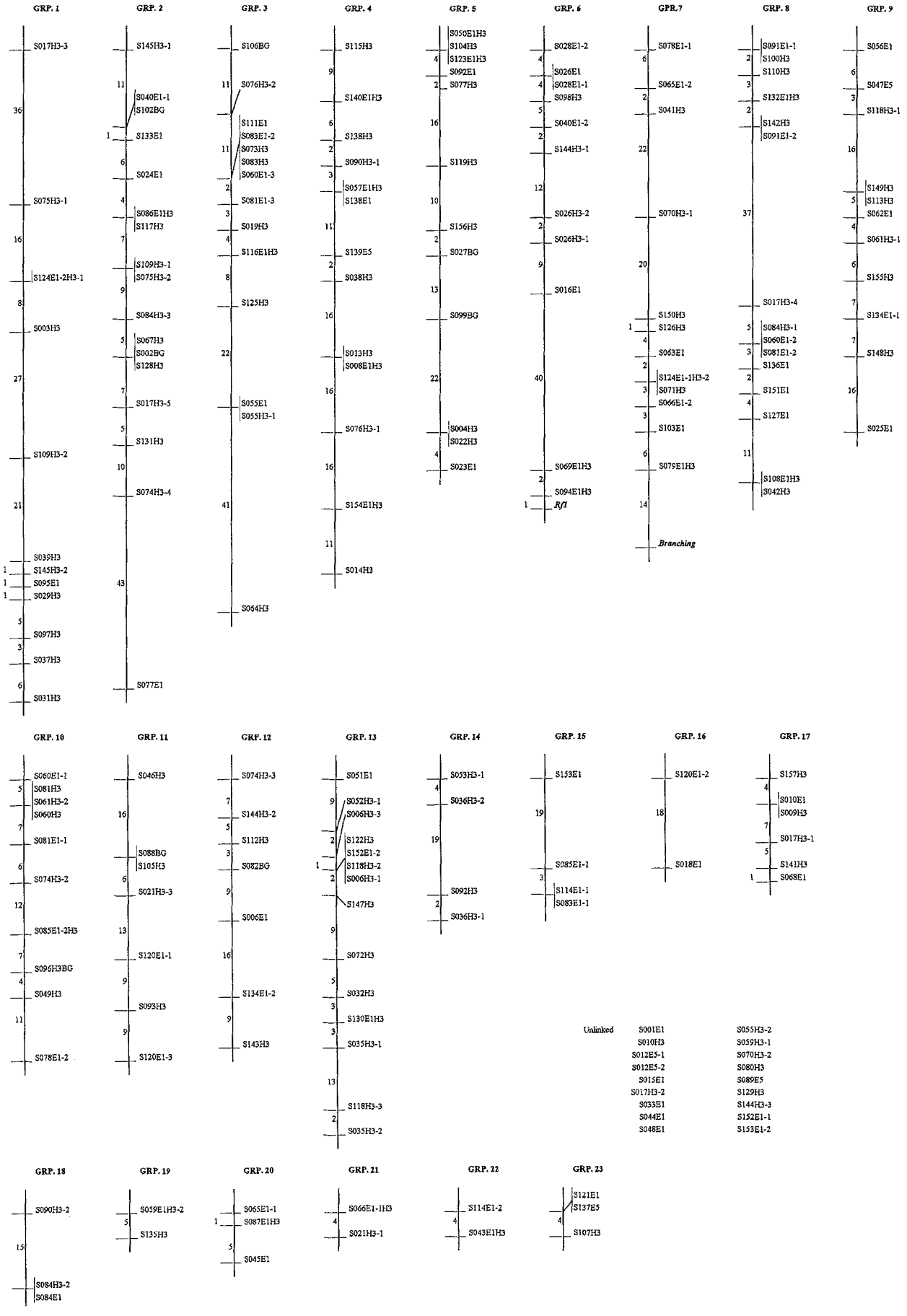


Fig. 1 **A** RFLP linkage map of the F_2 cross C2 (CX × RHA266) of cultivated sunflower. The groups are listed at the *top*, the consensus group in which each is included is listed *below* (*in italics*), the loci are listed on the *right* and the map distances (in cM) are on the *left*. The nomenclature of the RFLP loci is as described in the Materials and methods. **B** RFLP linkage map of the F_2 cross C5 (PAC2 × RHA266) of cultivated sunflower. See **A** for explanations


B
C5-GRP. 1
GRP. 3
S111E1
11
S076H3-2
4
S060E1-3
S073H3
2
S083H3
S019H3
3
S081E1-3
8
S106BG
11
S116E1H3
7
S125H3
22
S055E1H3
44
S064H3
C5-GRP. 2
GRP. 7
Branching
13
S079E1H3
2
S124H3-2
3
S066E1-2
S103E1
1
S071H3
2
S124E1-1
S063E1
4
S126H3
1
S150H3
20
S070H3-1
44
S065E1-2
C5-GRP. 3
GRP. 6
S098H3
29
S016E1
41
S069H3
6
S094E1H3
C5-GRP. 4
GRP. 4
S008E1H3
17
S038H3
9
S138H3
1
S140E1
2
S057E1H3
1
S090H3-1
14
S154E1H3
11
S014H3
C5-GRP. 5
GRP. 2
S133E1
1
S102BG
13
S117H3
S086E1H3
7
S084H3-3
6
S067H3
6
S017H3-5
7
S128H3
2
S0022BG
S131H3
10
S074H3-4
C5-GRP. 6
GRP. 5
S119H3
10
S156H3
10
S099BG
16
S077H3
2
S092E1
4
S123E1
12
S050E1
C5-GRP. 7
GRP. 10
S060E1-1
9
S074H3-2
S060H3
32
S078E1-2
10
S049H3
C5-GRP. 8
GRP. 13
S072H3
5
S130E1H3
3
S035H3-1
10
S147H3
8
S006H3-1
5
S118H3-3
22
S152E1-2
C5-GRP. 9
GRP. 11
S120E1-3
9
S093H3
34
S046H3
C5-GRP. 10
GRP. 9
S155H3
6
S061H3-1
4
S062E1
6
S149H3
2
S113H3
S134E1-1
7
S148H3
16
S025E1
C5-GRP. 11
GRP. 12
S112H3
3
S082BG
9
S006E1
16
S134E1-2
12
S143H3
C5-GRP. 12
GRP. 1
S124E1-2H3-1
18
S017H3-3
C5-GRP. 13
GRP. 1
S031H3
6
S037H3
3
S097H3
5
S029H3
2
S145H3-2
2
S039H3
C5-GRP. 14
GRP. 18
S090H3-2
15
S084H3-2
C5-GRP. 15
GRP. 8
S127E1
1
S136E1
2
S151E1
2
S060E1-2
1
S081E1-2
S084H3-1
5
S017H3-4
C5-GRP. 16
GRP. 19
S135H3
7
S059E1
C5-GRP. 17
GRP. 20
S045E1
5
S087H3
S065E1-1
C5-GRP. 18
GRP. 15
S114E1-1
4
S085E1-1
C5-GRP. 19
GRP. 23
S121E1
S137E5
4
S107H3
C5-GRP. 20
GRP. 14
S053H3-1
3
S036H3-2
C5-GRP. 21
GRP. 8
S142H3
S091E1-2


GRP. 1
S017H3-3
36
S075H3-1
16
S124E1-2H3-1
8
S003H3
27
S109H3-2
21
S039H3
1 S145H3-2
1 S095E1
1 S029H3
5
S097H3
3
S037H3
6
S031H3
GRP. 2
S145H3-1
11
S040E1-1
S102BG
1 S133E1
6
S024E1
4
S086E1H3
S117H3
7
S109H3-1
S075H3-2
9
S084H3-3
5 S067H3
S002BG
S128H3
7
S017H3-5
5
S131H3
10
S074H3-4
43
S077E1
GRP. 3
S106BG
11
S076H3-2
S111E1
S083E1-2
11 S073H3
S083H3
S060E1-3
2
S081E1-3
3
S019H3
4
S116E1H3
8
S125H3
22
S055E1
S055H3-1
41
S064H3
GRP. 4
S115H3
9
S140E1H3
6
S138H3
2
S090H3-1
3
S057E1H3
S138E1
11
S139E5
2
S038H3
16
S013H3
S008E1H3
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S076H3-1
16
S154E1H3
11
S014H3
GRP. 5
S050E1H3
S104H3
4 S123E1H3
S092E1
2 S077H3
16
S119H3
10
S156H3
2
S027BG
13
S099BG
22
S004H3
S022H3
4
S023E1
GRP. 6
S028E1-2
4
S026E1
4 S028E1-1
S098H3
5
S040E1-2
2
S144H3-1
12
S026H3-2
2
S026H3-1
9
S016E1
40
S069E1H3
2
S094E1H3
1 Rf1
GPR.7
S078E1-1
6
S065E1-2
2
S041H3
22
S070H3-1
20
S150H3
1 S126H3
4
S063E1
2
S124E1-1H3-2
3 S071H3
S066E1-2
3
S103E1
6
S079E1H3
14
Branching
GRP. 8
S091E1-1
2 S100H3
S110H3
3
S132E1H3
2
S142H3
S091E1-2
16
37
S017H3-4
5 S084H3-1
S060E1-2
3 S081E1-2
S136E1
2
S151E1
4
S127E1
11
S108E1H3
S042H3
GRP. 9
S056E1
6
S047E5
3
S118H3-1
16
S149H3
5 S113H3
S062E1
4
S061H3-1
6
S155H3
7
S134E1-1
7
S148H3
16
S025E1
GRP. 10
S060E1-1
5 S081H3
S061H3-2
S060H3
7
S081E1-1
6
S074H3-2
12
S085E1-2H3
7
S096H3BG
4
S049H3
11
S078E1-2
GRP. 11
S046H3
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S088BG
S105H3
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S021H3-3
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S120E1-1
9
S093H3
9
S120E1-3
GRP. 12
S074H3-3
7
S144H3-2
5
S112H3
3
S082BG
9
S006E1
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S134E1-2
9
S143H3
GRP. 13
S051E1
9
S052H3-1
S006H3-3
2
S122H3
S152E1-2
1 S118H3-2
2
S006H3-1
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9
S072H3
5
S032H3
3
S130E1H3
3
S035H3-1
13
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GRP. 14
S053H3-1
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S036H3-2
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S092H3
2
S036H3-1
GRP. 15
S153E1
19
S085E1-1
3
S114E1-1
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GRP. 16
S120E1-2
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GRP. 17
S157H3
4
S010E1
S009H3
7
S017H3-1
5
S141H3
1 S068E1
Unlinked
S001E1
S010H3
S012E5-1
S012E5-2
S015E1
S017H3-2
S033E1
S044E1
S048E1
S055H3-2
S059H3-1
S070H3-2
S080H3
S089E5
S129H3
S144H3-3
S152E1-1
S153E1-2
GRP. 18
S090H3-2
15
S084H3-2
S084E1
GRP. 19
S059E1H3-2
5
S135H3
GRP. 20
S065E1-1
1 S087E1H3
5
S045E1
GRP. 21
S066E1-1H3
4
S021H3-1
GRP. 22
S114E1-2
4
S043E1H3
GRP. 23
S121E1
S137E5
4
S107H3

This suggested that there were, at the present state of analysis, no significant differences in the order of markers between the different segregating progenies studied.

Cross-mapping and duplicated loci

Considerable differences were present between the numbers of probes and loci analysed on more than one cross. This may be explained in part by the fact that 1 probe may reveal several loci in one progeny, or different loci in different progenies. The mean number of loci revealed by 1 RFLP probe was 1.2 for individual progenies and 1.3 for the consensus map. Some probes detect up to 5 loci, as for example SUN017E1. Overall, 25% of the probes studied revealed at least 2 loci over the five progenies. A search was made for blocks of markers common to different linkage groups but, in most instances, the duplicate RFLP loci occur in different linkage groups. As described in *Brassica rapa* (Song et al. 1991; Chyi et al. 1992) and in soybean (Keim et al. 1990), no homeologous groups could be formed for sunflower.

Discussion and conclusions

In this paper we report the first description of a consensus RFLP linkage map for cultivated sunflower that is based on 237 non-distorted segregating loci detected by 180 probe-enzyme combinations. It covers 1150 cM with 23 linkage groups, 6 more than the haploid number of chromosomes. If we refer to the estimated size of the sunflower genome (3.10^9 bp; Arumuganathan and Earle 1991) and include the 18 unlinked loci, this map probably represents about 70% of the sunflower genome. The distorted loci were not included in the analyses because they might affect the test of independent segregation and the estimation of recombination frequencies between two loci (Bailey 1961). However, this could lead to unidentified regions of the genome being subject to segregation distortions. Additional RFLP markers are therefore necessary to obtain 17 linkage groups and to cover the regions lacking markers.

This map was obtained with combined analysis of three F_2 and two BC_1 progenies with one common parent in each comparison pair. This system was used to increase the information obtained from different progenies, in particular by allowing the identification of common loci between segregating populations having one parent in common. The consensus mapping was found to be much more efficient than unit maps, which are always much shorter and less well-structured, for example, in terms of locus order. For example, progenies C2 and C5 gave the greatest amounts of information (with 100 and 124 non-distorted loci mapped, respectively), but these two maps gave more than 17 linkage groups and covered about 50% of the genome covered by the consensus map. This indicates that, in some cases, there is a coverage of the same regions in the different crosses studied, and in other cases, some regions of the genome are only covered by one cross. Thus, if the unit maps are not saturated, the consensus map could be of greater length, both in number of linkage groups and in total recombination fraction. Another example is the different ordering found in certain cases on the consensus map in comparison with that obtained with the unit maps. In all the cases, the difference was not significant, indicating that mixing of loci from different progenies, given the presence of common loci between these segregating populations, increases the efficiency of the ordering steps.

The finding that 25% of RFLP markers detect 2 or more loci was unexpected. In maize, for example, Henlentjaris et al. (1988) stated that similar observations could be attributed to the existence of ancient homeologous chromosomes. From our results, it was not possible to demonstrate such homeologous linkage groups. It may be that either there are a large number of independent duplications in the sunflower genome, mainly due to pseudogene families, or that the sunflower may have evolved by allopolyploidy (amphidiploid). This hypothesis was suggested from cytogenetic data by Jackson and Murray (1983), but the present results are not sufficient enough to draw a conclusion. Analysis of further duplicate markers or in situ hybridization will be necessary to progress further in this aspect of sunflower genome evolution.

The RFLP map of sunflower is of direct interest to breeders, particularly in view of the previous lack of information on genetic linkages. The cross C2 allowed mapping of the locus (*Rf1*) for male fertility restoration in RHA266 (Leclercq 1969; Laver et al. 1991) by 2 closely positioned loci (SUN069E1 and SUN094H3, linkage group 4 of C2). Similarly, in cross C5 it was possible to map the recessive branching gene (*b1*, Putt 1964) at 13 cM from the locus SUN079E1H3 (linkage group 2 of C5). The locus controlling stigma colour showed a gametic distortion in C2 and so could not be mapped. Thus, male fertility restoration and recessive branching were both placed on the consensus map (on linkage group 6 and 7, respectively), and their presence in different linkage groups agrees with classical genetic data that they are independent characters.

The consensus RFLP map of sunflower will provide an immediate new tool in breeding programmes and should help in the analysis of the genetics of agronomically important characters such as resistance to major diseases as well as in the development of marker-assisted selection in sunflower.

Fig. 2 Consensus linkage map of cultivated sunflower, based on 237 loci identified by 180 probe-enzyme combinations. The groups are listed at the *top*, the loci listed at the *right* and the map distance (in cM) on the *left*. The nomenclature of the RFLP loci is as described in the Materials and methods

Acknowledgements We would like to thank Véronique de Conto (laboratory), Annie Perrault (laboratory) and Jacqueline Philippon (field) for their expert technical assistance. Mathilde Causse, Yvette Dattée and Marianne Lefort-Buson are also acknowledged for critical reading of the manuscript. This programme was funded by the G.I.E. CARTISOL (group of 16 seed companies including Agrosem,

Semences Cargill, Caussade Semences, Coop de Pau, Eurograin Semences, Hilleshoeg NK, Limagrain, Maïsadour, Pioneer France, RAGT, Rhône Poulenc Agrochimie, Rustica Semences, SDME, SES France, UCST, Van der Have), the French Ministry of Agriculture and a State/Region contract "Semences et plants".

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