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RFLP mapping of *Brassica napus* using doubled haploid lines

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Abstract The combined use of doubled haploid lines and molecular markers can provide new genetic information for use in breeding programs. An F_1-derived doubled haploid (DH) population of *Brassica napus* obtained from a cross between an annual canola cultivar ('Stellar') and a biennial rapeseed ('Major') was used to construct a linkage map of 132 restriction fragment length polymorphism loci. The marker loci were arranged into 22 linkage groups and six pairs of linked loci covering 1016 cM. The DH map was compared to a partial map constructed with a common set of markers for an F_2 population derived from the same F_1 plant, and the overall maps were not significantly different. Comparisons of maps in *Brassica* species suggest that less recombination occurs in *B. napus* (n = 19) than expected from the combined map distances of the two hypothesized diploid progenitors, *B. oleracea* (n = 9) and *B. rapa* (n = 10). A high percentage (32%) of segregating marker loci were duplicated in the DH map, and conserved linkage arrangements of some duplicated loci indicated possible intergenome homoeology in the amphidiploid or intragenome duplications from the diploid progenitors. Deviation from Mendelian segregation ratios ($P < 0.05$) was observed for 30% of the marker loci in the DH population and for 24% in the F_2 population. Deviation towards each parent occurred at equal frequencies in both populations and marker loci that showed deviation clustered in specific linkage groups. The DH lines and molecular marker map generated for this study can be used to map loci for agronomic traits segregating in this population.

Key words *Brassica napus* · Doubled haploid · Linkage map · Restriction fragment length polymorphism

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Introduction

Rapeseed oil obtained from *Brassica napus* L. is a major commodity in agriculture. The importance of the crop and its derivatives for human and animal consumption has been reviewed recently (Downey and Rakow 1987; Sahidi 1990). An understanding of the genetic control of important agronomic traits in *B. napus* is crucial for the improvement of the crop. Techniques such as microspore culture and molecular marker analysis can potentially accelerate the accumulation of useful genetic information for breeding programs.

Techniques based on the anther or microspore culture of *B. napus* have been used to generate completely homozygous lines (Keller and Armstrong 1978; Coventry et al. 1988) and have potential for the development of improved cultivars (Chen and Beversdorf 1990). Doubled haploid (DH) lines are desirable for genetic studies because fixed genotypes can be propagated indefinitely by sexual means. The use of fixed genotypes in replicated experiments facilitates the estimation of genetic and environmental components of phenotypic variation. Furthermore, doubled haploids usually are obtained in a single generation, whereas recombinant inbred lines require many generations to develop (Burr et al. 1987).

Restriction fragment length polymorphisms (RFLP) have been used to construct genetic linkage maps in many species. These maps have provided insight on genome organization and evolution (Helentjaris et al. 1988; Slocum et al. 1990; Song et al. 1991), and they have been used to locate loci controlling important agronomic traits (Edwards et al. 1992; Paterson et al.

1988). Information from these types of studies can be used to develop better breeding strategies for a crop. Also, a linkage map is a first step towards detailed genetic analysis that can culminate with the cloning of specific genes (Orkin 1986).

Genetic linkage maps based on DH lines offer the possibility of integrating the advantages of segregating homozygous lines and molecular marker techniques. The objectives of the study presented here were to develop a linkage map of RFLP loci using an F_1-derived DH population of *B. napus* and to compare this map to a partial map constructed with an F_2 population derived from the same F_1 individual. The linkage map based on DH lines was also studied in relation to recombination frequencies in other *Brassica* species, genome duplication and deviation from Mendelian segregation ratios.

Materials and methods

Population development

A single plant from the biennial cultivar 'Major' was crossed to a DH line derived from the annual cultivar 'Stellar'. Approximately 155 plants were obtained from the microspore culture of a single F_1 hybrid plant (Coventry et al. 1988), from which 21% (33/155) had spontaneous chromosome doubling. The haploid plants were treated with colchicine to induce chromosome doubling by immersing the roots of 3- to 4- week-old rooted cuttings for 4 h in a 0.05% colchicine solution. Alternatively, small pieces of cotton soaked with colchicine solution (0.1%) were applied to axillary buds for 16 h. Each colchicine-induced and spontaneously DH plant was self-pollinated. Lines with good seed set were selected randomly from the original population. Many of the plants that produced few or no progenies upon extensive self-pollination were probably haploids or aneuploids, and were not used. Random samples of S_1 seeds from 105 plants were grown and self-pollinated. The S_2 plants from these 105 lines were used as a source of the DNA for RFLP analysis.

The same F_1 hybrid plant that was used for microspore culture was self-pollinated to generate an F_2 population. Ninety-eight F_3 families obtained by self-pollination of individual F_2 plants were used as a source of DNA for RFLP analysis.

Plants were grown in Com-Pack® 812 pots filled with a 1:1:1 mix of autoclaved soil:sand:Jiffy® mix for 2–4 weeks, during which time young leaves were harvested for DNA extraction. The plants were fertilized daily with 0.5 × Hoagland's solution and kept in a growth chamber under a constant cool white fluorescent irradiance of 240 μE/m^2 per second and 24 °C. For seed production, the plants were vernalized for 6 weeks in a cold room (4 °–6 °C) with a 16-h photoperiod and light intensity of 150 μE/m^2 per second from fluorescent bulbs. Plants were then transplanted to 1500-ml pots and grown in a greenhouse for seed production (22 °C, minimum light irradiance of 300 μE/m^2 per second). Under these conditions, all of the DH and F_2 plants flowered. Delnet® bags were used to cover and isolate inflorescences during pollination.

DNA isolation

Leaves were harvested and bulked for 24 individuals of each progeny of the self-pollinated parents 'Major' and 'Stellar' and for 12 individuals from each of the 105 DH lines and 98 F_3 families. The leaves were kept on ice throughout harvesting, lyophilized immediately and stored at −20 °C. DNA was extracted from the DH lines and F_3 families following the procedures described by Kidwell and Osborn (1992). DNAs from the parents were further purified by ultracentrifugation in cesium chloride-ethidium bromide (Murray and Thompson 1993).

RFLP analysis

RFLPs were detected as described by Song et al. (1988). Essentially, 5 μg of *Hin*dIII- or *Eco*RI-digested DNAs was loaded onto 1% agarose gels, and the restriction fragments were separated by electrophoresis on the basis of size. DNAs from the gels were transferred to Magna NT nylon membranes (MSI, Westboro, Mass.), and treated for 2 h in a 90 °C vacuum oven and then for 30 s under UV. The DNA clones were radiolabeled with [^{32}P] by random priming (Feinberg and Vogelstein 1983) and hybridized to the Southern blots. Final washes were with 0.5 × SSC at 60 °C, and the blots were exposed to X-ray film with intensifying screening for 8 h to 10 days at −80 °C. Blots were reprobed up to 15 times by first being treated with 0.2 *M* NaOH for 20 min and then with 0.5 *M* Tris/HCl pH 7.5, 0.1 × SSC, 0.1% SDS for 20 min.

DNA clones used as probes were from three libraries: 1) a *Pst*I genomic DNA library (WG clones) and 2) a cDNA library (EC clones) from *B. napus* cv 'Westar' (Thormann et al. 1994), and 3) an *Eco*RI genomic DNA library from *B. rapa* cv. Tobin (TG clones) that was constructed and screened essentially as described for the Westar genomic DNA library. Probes from these libraries (280 WG clones, 82 TG clones and 8 EC clones) were prescreened on Southern blots containing *Eco*RI- and *Hin*dIII-digested DNAs from the parents and F_1. A subset of the probes that detected polymorphism between the parents was selected for mapping on the basis of simplicity and clarity of the banding patterns, as well as on the detection of polymorphism between the parents of other *Brassica* mapping populations. One *B. napus* and 10 *Arabidopsis thaliana* cloned genes were also prescreened for RFLPs: pCR-1 (Simon et al. 1985), PR1, PR2 and PR3 (Uknes et al. 1992), cor15 (Lin and Thomashow 1992), UBC 1015 and p112 (Burke et al. 1988), CHS 3.8 and Gpa3 (B. Hauge, personal communication), and PAHAZ-11 and ATPase P (Harper et al. 1990). Clones detecting RFLPs between the parents were used for mapping.

Segregation analysis and map construction

Selected DNA clones were used as probes on Southern blots containing DNAs from parents, F_1 and segregating lines (DH population) or individuals (F_2 population) digested with *Eco*RI or *Hin*dIII. Segregating RFLP loci were scored by recording the genotype of each line or individual in the populations: homozygous for alleles from 'Major' (M/M) or for alleles from 'Stellar' (S/S) in the DH population and M/M, S/S or heterozygous (M/S) in the F_2 population. RFLP loci were given the same name as the clone used for hybridization, which for *Brassica* clones included the library designation followed by a number, a letter and a number. Multiple segregating loci identified by the same probe were coded with the letters a, b, etc., at the end of the locus name (e.g. *tg2b4a* and *tg2b4b*). Some probes were hybridized to both *Eco*RI- and *Hin*dIII-digested DNAs, and only data for the most informative of the enzymes were used for linkage analysis.

For mapping purposes, a DH population can be treated as a backcross population with an expected Mendelian segregation of 1:1 for the two alleles present in the F_1 used for microspore culture. Deviations from the expected segregation ratios for DH (1:1) and F_2 (1:2:1) populations were calculated based on chi-square contingency tables. Linkage analysis and map construction were performed separately for the DH and F_2 data using the computer program Mapmaker v2.0 (Lander et al. 1987). Only RFLP loci with 70 or more scored DH lines or F_2 individuals were included in the analysis. A minimum LOD score of 3.0 and maximum recombination frequency of 0.35 were used to group the RFLP loci into potential linkage groups. Three-point and multipoint analyses were performed to find the most probable locus order within each linkage group. The final locus order of each linkage group was tested by removing 1 locus at a time and checking for inconsistencies. Double crossover events were examined, and the original scores re-checked before a final linkage group order was assigned. Recombination frequencies were corrected based on Kosambi's map distance function as executed by Mapmaker.

Adjacent locus recombination frequencies of linkage groups from the DH map and the F_2 map were compared by two-way chi-square contingency tests. The overall homogeneity of the two maps was also

tested by the two-way chi-square contingency test based on the total number of recombinant and parental marker classes.

Results

Polymorphic markers

Approximately 38% and 44% of the 280 WG clones detected RFLPs between the parental DNAs cut with *Eco*RI and *Hin*dIII, respectively, and 61% detected RFLPs in DNAs digested with either enzyme. Of the 82 TG clones, 29% and 38% detected RFLPs between the parent DNAs digested with *Eco*RI and *Hin*dIII, respectively, and 46% detected RFLPs in DNAs digested with either enzyme. Eighty-two WG clones, 14 TG clones and 3 EC clones were selected for mapping. Of the 11 cloned genes 2 (PR2 and cor15) detected RFLPs between the two parents and were used for mapping.

Segregation and linkage analysis

The 101 DNA clones detected 138 segregating RFLP loci in the DH population. Two RFLP alleles were scored for most marker loci. However, a single segregating fragment was scored for 4 loci since the allelic fragments were clearly missing, suggesting that these loci had null alleles. Some other loci were scored based on a single segregating fragment because the allelic fragment was either faint or obscured by other fragments. Thirty-two clones detected multiple segregating RFLP loci (average of 2.25 loci per clone), with 8 clones (WG1G5, WG1G8, WG1G10, WG1H4, WG3F7, WG4D5, WG4D7, WG4F4) detecting 3 or more segregating loci. Additional monomorphic fragments were detected for some clones, which may represent separate RFLP loci. These loci might segregate in the DH population when other restriction enzymes are used. All 105 DH lines were homozygous at all RFLP marker loci, and none of the lines had identical RFLP genotypes at all loci.

Linkage analysis of the 138 marker loci using a LOD threshold of 3.0 and maximum $r = 0.35$ resulted in 19

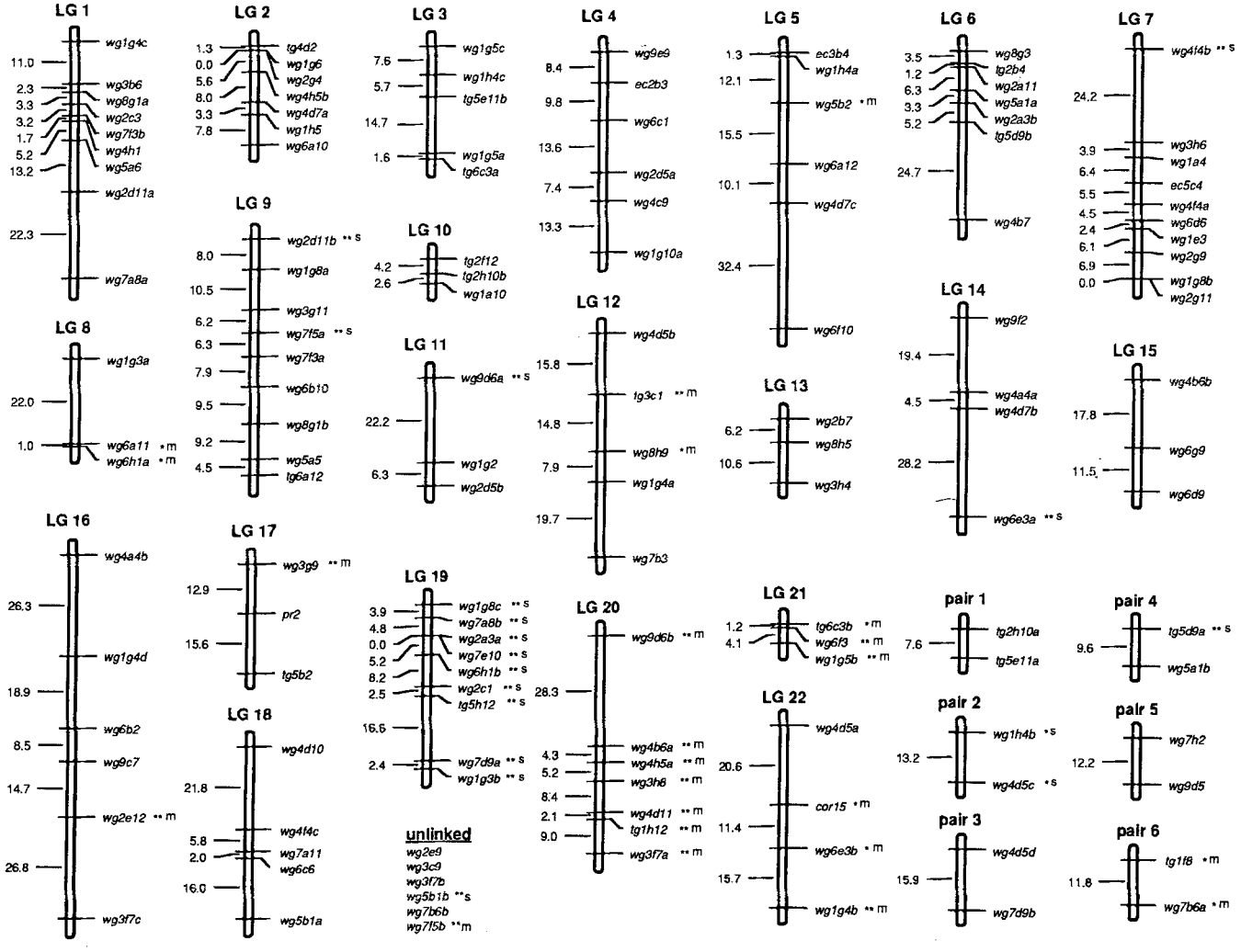

Fig. 1 RFLP linkage map of *B. napus*. Map distances in cM are indicated on the *left side* of linkage groups and locus names are on the *right*. Loci marked * and ** deviated significantly from a 1:1 ratio at $P < 0.05$ and $P < 0.01$, respectively, and *s* and *m* indicate that S/S or M/M genotypes predominated, respectively

potential linkage groups. Of these groups, 4 were very large (12–34 loci), and these were subsequently re-analyzed using progressively higher LOD thresholds (up to 6.0). As the LOD threshold was increased, the 4 large groups were split into smaller linkage groups. The final map included 120 marker loci in 22 linkage groups (LGs) of 3 or more loci and six pairs of linked loci covering 1016 cM with an average spacing of 7.7 cM between loci (Fig. 1). Six marker loci were unlinked. The 4 large linkage groups that formed at LOD 3.0 included: LG 17, LG 6 and pair 4 in 1 group; LG 1, LG 7, LG 16, LG 18 and LG 22 in a second group LG 11, LG 12, LG 20 and wg7f5b in a third group; and LG 9 and LG 19 in a fourth group.

Of the 132 loci, 33 were used to develop a partial map for the F_2 population and 5 additional loci were mapped in the F_2 population but not in the DH population (Table 1). Linkage was detected for 60.5% (23/38) of the marker loci, and these loci were organized into linkage groups corresponding to those in the DH map (Fig. 2).

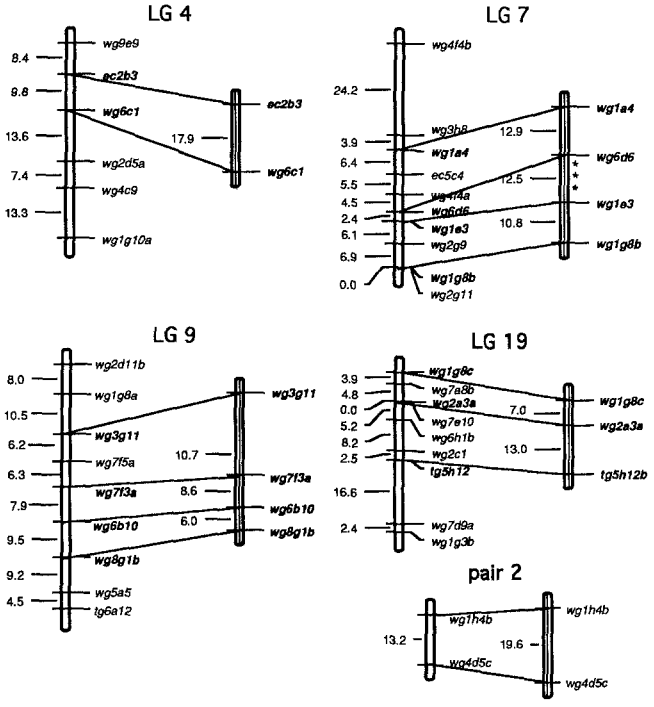

Fig. 2 Comparison of locus order and map distances between two RFLP linkage maps of *B. napus* based on DH (*left group* of each pair) and F_2 (*right*) populations derived from the same F_1 plant. Map distances in cM are indicated on the *left side* of linkage groups and locus names are on the *right*. One interval that had a significantly different map distance ($P < 0.05$) in the two maps is indicated by *

Duplicated marker loci

Thirty-two of the clones used for mapping detected multiple segregating loci distributed throughout the genome. For example, RFLP loci homologous to clone WG4D7 were found in LGs 2, 5 and 14, and to clone WG1G8 in LGs 7, 9 and 19 (Fig. 1). The order of some duplicated loci in linkage groups was conserved in other

Table 1 Segregation ratios of RFLP loci in DH and F_2 populations of *B. napus* obtained from the same F_1 plant

Linkage group	Locus[a]	DH ratio[b]	F_2 ratio[b]	Linkage group	Locus[a]	DH ratio[b]	F_2 ratio[b]
1	*wg8g1a*	41:49	24:49:16	9	*wg3g11*	38:57	19:48:17
	wg7f3b	41:53	27:44:23		*wg7f3a*	38:57	25:36:19
					wg6b10	42:59	26:43:18
2	*wg2g4*	45:41	26:43:24		*wg8g1b*	36:52	29:37:17
3	*tg5e11b*	47:34	11:50:18*	12	*wg8h9*	50:32*	26:35:24
	wg1g5a	52:39	20:24:18				
				14	*wg4a4a*	42:52	20:31:24
4	*ec2b3*	43:53	26:36:10*				
	wg6c1	51:51	27:25:22**	17	*tg5b2*	54:48	21:42:25
	wg4c9	39:52	11:41:8*				
	wg1g10a	36:51	12:43:19	19	*wg1g8c*	30:75**	9:29:18
					tg5h12b	26:71**	17:45:18
5	*wg1h4a*	48:43	23:43:20				
	wg6f10	45:55	19:43:13	20	*wg3h8*	81:17**	23:49:12
6	*tg2b4*	60:42	18:38:24	22	*cor15*	34:55*	14:40:18
	wg2a3b	41:36	17:52:22				
				pair 2	*wg1h4b*	26:45*	14:27:20
7	*wg1a4*	46:50	17:29:25		*wg4d5c*	31:52*	11:46:28*
	wg6d6	51:47	21:39:24				
	wg1e3	48:40	30:15:29**	pair 3	*wg4d5d*	41:42	23:40:27
	wg1g8b	51:43	31:31:23*				
				pair 5	*wg9d5*	44:43	26:36:26
8	*wg6a11*	62:40*	25:34:21				

*,** Indicate significant deviation from expected segregation ratios 1:1 for DH and 1:2:1 for F_2 at the 5% and 1% levels, respectively

[a] Loci are listed in linkage order

[b] Ratios are numbers of M/M:S/S genotypes (DH lines) and M/M:M/S:S/S genotypes (F_2 plants)

linkage groups. For example, LGs 1 and 9 each had loci detected by the same probes (WG8G1, WG7F3, WG2D11) and arranged in the same linkage order (Fig. 3). There were pairs of segregating duplicated loci at similar distances on LG 12 and LG 22 and on LG 6 and pair 4 (Fig. 3). Another duplication involving two closely linked loci (*wg1g5* and *tg6c3*) was observed in LGs 3 and 21. LG 3 had two RFLP loci homologous to the WG1G5 probe (Fig. 3).

Deviation from Mendelian segregation ratios

Thirty-four percent of the markers showed significant deviation ($P < 0.05$) from the expected Mendelian ratio in the DH population (Fig. 1). Of these marker loci, 20 had an excess of M/M genotypes and 21 had an excess of S/S genotypes. The overall proportion of M/M genotypes (0.495) was not significantly different from 0.5. Most of the linkage groups had no loci with significant deviation from expected Mendelian ratios, whereas LGs 19–21 were composed entirely of marker loci showing significant deviation (Fig. 1). Within these three linkage groups, all loci had an excess of alleles from either one or the other parent.

Twenty-one percent of the marker loci segregating in the F_2 population had distorted allelic segregation ratios ($P < 0.05$). Except for 1 locus in pair 2 (*wg4d5c*), different loci showed distorted segregations in the DH and F_2 populations (Table 1). In four cases, the allelic segregation ratios at loci in the two populations were clearly different: loci *tg5h12b* and *wg1g8c* (LG 19) and *wg3h8* (LG 20) had highly distorted allelic ratios in the DH population ($P < 0.01$) but not in the F_2 population ($P > 0.05$), and locus *wg1e3* (LG 7) was not distorted in the DH population but highly distorted in the F_2 population ($P < 0.01$).

Comparison of DH-and F_2-derived RFLP maps

Recombination frequencies between ten pairs of adjacent marker loci in both population maps were compared based on two-way chi-square contingency tests. Only one pair of marker loci had significantly different recombination frequencies ($P < 0.05$) between the two maps (Fig. 2). In LG 7, fewer recombinants were obtained between loci *wg6D6* and *wg1E3* in the DH population than in the F_2 population ($\chi^2_{1df} = 4.65$, $P = 0.036$). Intervals for the remaining pairs of loci and three interval comparisons involving the common distal loci on LGs 7 (*wg1a4-wg1g8b*), 9 (*wg3g11-wg8g1b*) and 19 (*wg1g8c-tg5h12b*) were not significantly different ($P > 0.05$) between the two maps ($\chi^2_{1df} = 0.004$, $\chi^2_{1df} = 0.14$, $\chi^2_{1df} = 0.66$, respectively). The heterogeneity chi-square test based on all adjacent locus pairs in the two maps was not significant ($\chi^2_{9df} = 7.29$, $P > 0.60$).

Discussion

On the basis of linkage analysis in a DH population, 132 loci were assembled into 22 linkage groups and six pairs of linked loci. This number of linkage groups is greater than the number of chromosomes in *B. napus* ($n = 19$), probably due to incomplete coverage of the entire genome. Also, some real linkage association may have been separated by use of a higher LOD threshold for a few large groups. However, this approach could have resolved some spurious associations, and it is a more conservative representation of the linkage groups. As more markers are added to the map, complete linkage groups will be resolved.

Linkage relationships of RFLP loci in the DH map were compared to those in an F_2 population using a

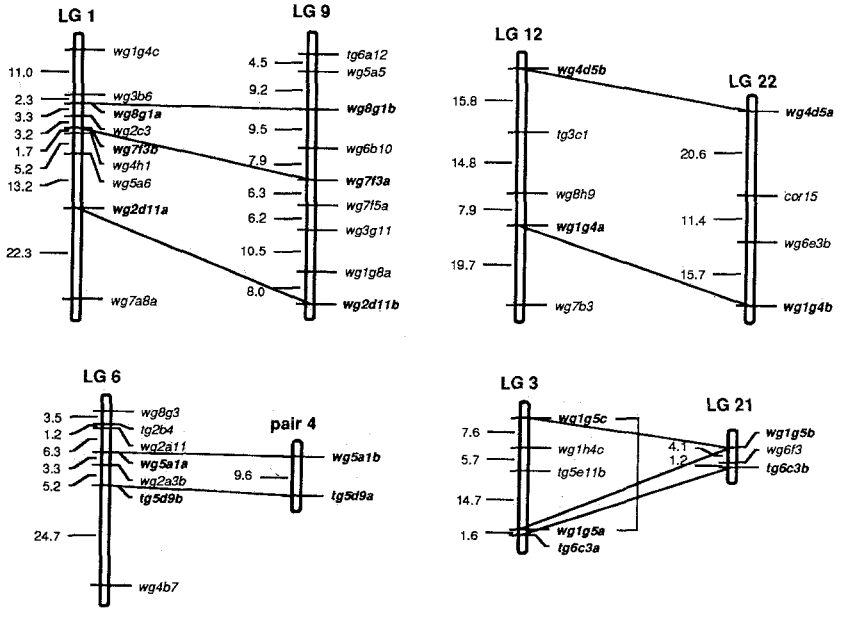

Fig. 3 Linkage groups of *B. napus* containing duplicated RFLP loci with conserved linkage arrangements detected in the DH population

common subset of probes. Identification of allelic bands and RFLP scoring in the F_2 population were facilitated by comparison to the simple segregation patterns of the corresponding loci in the DH population and by the use of only loci with co-dominant alleles. The maps were constructed simultaneously; therefore, no a priori knowledge of linkage relationships was available. Comparison of linkage relationships in the two populations suggested that the F_2 map does not differ significantly from the DH map.

B. napus has a genome size based on a nuclear DNA content (2.45 ± 0.5 pg DNA/2C) that is about equal to the sum of its hypothetical diploid progenitor species, *B. oleracea* (1.02 ± 0.5 pg DNA/2C) and *B. rapa* (1.33 ± 0.5 pg DNA/2C) (Arumuganathan and Earle 1991). However, the map distances for *B. napus* reported in this study (1016 cM, with 132 markers) and by Landry et al. (1991) (1413 map units with 103 markers) are much less than the sum of the map distances reported for the diploid progenitors: 1876 map units with 360 marker loci (Chyi et al. 1992) and 1850 cM with 280 marker loci (Song et al. 1991) for *B. rapa*, and 820 map units with 258 marker loci (Slocum et al. 1990) and 1112 cM with 201 marker loci (Landry et al. 1992) for *B. oleracea*. Also, cytological observations have indicated that the chromosomes of *B. napus* are much more condensed than those of diploid *B. rapa* and *B. oleracea* during late prophase of mitosis (Olin-Fatih and Heneen 1992), and that the nuclear size of natural *B. napus* is much smaller (approximately 60%) than that of amphidiploids newly synthesized from the diploid species (Verma and Rees 1974). The reported map distances are not directly comparable, since they are dependent on the degree of genome coverage by marker loci and on recombination frequency, which are influenced by the genetic diversity of the parents and/or environmental effects on meiosis. However, it is possible that alterations in the chromosome structure of natural *B. napus* have lead to reduced recombination per unit of DNA compared to the diploid progenitor species. Additional studies are needed to more thoroughly compare recombination frequencies in these species.

Almost all of the clones used for mapping detected multi-copy sequences, as would be expected for an amphidploid speices, although most clones detected only 1 segregating locus. Multiple segregating loci were detected by 32% of the clones, and some sets of duplicated loci had conserved linkage arrangements on pairs of linkage groups. These linkage groups may represent homoeologous chromosomes from the two diploid progenitor species. However, evidence for extensive intragenome duplication of chromosomal regions has been reported for *B. oleracea* and *B. rapa* (Slocum et al. 1990; Landry et al. 1992; Song et al. 1991; Kianian and Quiros 1992; Chyi et al. 1992). Hence, it is difficult to determine if sequence duplications detected in *B. napus* reflect homoeologous chromosomes of diploid progenitors or duplications within diploid progenitor genomes. Analysis of additional markers and comparisons to *B. rapa* and *B. oleracea* linkage maps using a common set of clones should provide additional information and insight on the nature of genome duplication. However, the inability to map exact progenitor genomes and the possibility of rearrangement since the synthesis of *B. napus* are limitations to conclusive studies.

High percentages of loci showed deviation from expected Mendelian ratios in the DH (30%) and F_2 (24%) populations, and also in another study of *B. napus* (21%) utilizing an F_2 population (Landry et al. 1991). In other studies reporting molecular marker segregation data, distorted allelic ratios ranged from 3% (8/273) (Song et al. 1988) to 70% (69/96) (Bentolilas et al. 1992) of the loci scored. Deviations from Mendelian ratios could be caused by differential viability or selection of certain genotypes at various stages in the development of gametophytes and/or sporophytes. However, the two parents did not differ significantly for numbers of haploid plants obtained by microspore culture (data not shown). Also, the high frequency of markers with distorted allelic ratios observed in the F_2 population derived from the same F_1 plant suggests that factors other than microspore culture influenced the selection of specific genotypes. Since different loci showed allelic deviation from expected ratios in the two populations derived from the same F_1 plant, different environmental and/or random effects may have caused distorted segregation in the two populations.

The RFLP linkage map for the DH population of *B. napus* will be useful for mapping loci controlling traits of agronomic importance and for studying the genetics of these traits. Since the DH lines are genetically fixed, the assessment of the genetic and environmental components of the phenotypic variation of quantitative traits can be greatly enhanced by replicated trials (Powell et al. 1990). The integration of the information generated by this and other maps may be of value in breeding programs for rapeseed improvements.

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