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A high-density linkage map in *Brassica juncea* (Indian mustard) using AFLP and RFLP markers

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Abstract A high-density genetic linkage map of *Brassica juncea* (2n = 36) was constructed with 996 AFLP (amplified fragment length polymorphism) and 33 RFLP (restriction fragment length polymorphism) markers using a F1-derived doubled-haploid (DH) population of 123 individuals. This mapping population was developed by crossing a well-adapted, extensively grown Indian variety Varuna and a canola quality line Heera. The two lines are highly divergent and contain a number of contrasting qualitative and quantitative traits of high agronomic value. AFLPs were generated by the use of restriction enzymes *Eco*RI or *Pst*I in combination with either *Mse*I or *Taq*I. Using 91 primer pairs, a total of 1,576 parental polymorphic bands were detected of which 996 were used for mapping. In addition, 33 RFLP markers, developed from genomic clones of *B. napus*, were added to the map. The segregation of each marker and linkage analysis was performed using the program JoinMap version 2.0. The 1,029 mapped-markers were aligned in 18 linkage groups, which is the haploid chromosome number of the species, at LOD values ranging from 5 to 8. The total map length was 1,629 cM with an average marker interval of 3.5 cM. AFLP markers generated by *Eco*RI were more clustered, whereas *Pst*I markers showed more extensive distribution. A set of 26 primer pairs (9 *Eco*RI/*Mse*I, 6 *Eco*RI/*Taq*I, 6 *Pst*I/*Mse*I and 5 *Pst*I/*Taq*I) generating 385 markers were identified for AFLP-based whole-genome selection as these markers covered 96% of the genome mapped with the 91 primer pairs. The map developed in the present study could be used for dissection and the transfer of agronomically important traits and favourable QTLs from ill-adapted exotic germplasm to cultivated Indian varieties.

Keywords AFLP · RFLP · *Brassica juncea* · Linkage map

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Introduction

Brassica juncea (Indian mustard) is a major oilseed crop of India and is grown in around six million hectares, primarily in the north-western part of the country during the winter season. It is also a potential crop in western prairies of Canada and in Australia, China and Russia. In the past, *B. juncea* breeding programmes, particularly in India, mainly exploited the variability that exists among the adapted pool of elite germplasm for the development of new varieties. Hybridizations within such germplasm have resulted only in marginal improvements in the productivity of this crop. To enhance productivity in a significant manner, it would be necessary to utilize new sources of variations. The exotic *B. juncea* lines from Canada, eastern Europe and Australia are ill-adapted to Indian agro-climatic conditions but constitute a rich source of agronomically important traits such as quality, seed coat colour, disease resistance (Srivastava et al. 2001) and many yield components like the number of primary and secondary branches, pod density and number, and oil content (Pradhan et al. 1993). Exploitation of these genetic resources through conventional plant breeding has largely been unsuccessful due to non-availability of desirable segregants in the F2 and subsequent pedigrees of the crosses between Indian and exotic germplasm (unpublished results).

DNA-based markers have been extensively used for developing linkage maps, and this information is being effectively used in the crop-improvement programmes for rapid transfer of recessive loci, the transfer of favourable QTLs from unadapted germplasm to the elite cultivars (Tanksley et al. 1996) and the cloning of oligogenes (Young 1995) and QTLs (Frary et al. 2000). The

first DNA-based marker technique used for the construction of linkage maps was based on the use of restriction fragment length polymorphism (RFLP). RFLP-based linkage maps have been developed in some of the important *Brassica* species (Quiros 1999), including *B. juncea* (Cheung et al. 1997; Axelsson et al. 2000). A major advantage of RFLP markers is that these are predominantly co-dominant. However, genotyping by RFLP is time and labour consuming and requires large amounts of DNA. The PCR-based techniques such as RAPD (random amplified polymorphic DNA) and SSR (simple sequence repeats) have also been used to construct linkage maps. However, the lack of reproducibility of RAPD and the cost and time required for generating SSR primers have limited their application in many plant species.

The PCR-based amplified fragment length polymorphism (AFLP) technique does not require *a priori* sequence information and has high reproducibility and locus specificity (Vos et al. 1995). Due to the high multiplex ratio, AFLP provides a rapid way to construct a linkage map. Hence, a number of integrated maps using AFLPs have been constructed in many crop species such as potato (Van Eck et al. 1995), barley (Qi et al. 1998), sugar beet (Schondelmaier et al. 1996), soybean (Keim et al. 1997), tomato (Haanstra et al. 1999), rice (Maheswaran et al. 1997) and maize (Vuylsteke et al. 1999). In the case of *Brassica* species, AFLP markers have been used to construct a linkage map in *Brassica oleracea* (Sebastian et al. 2000) and *Brassica napus* (Lombard and Delourme 2001). However, no such map has been constructed in *B. juncea*. We report here the construction of a high-density linkage map in *B. juncea* containing 996 AFLP and 33 RFLP markers using a F1-derived doubled-haploid (DH) population of 123 individuals from a cross between a widely adapted Indian cultivar, Varuna, and a canola quality line, Heera. The two lines are highly divergent and belong to two distinct gene pools, Varuna to Indian and Heera to a eastern European pool (Srivastava et al. 2001).

Materials and methods

Plant material and DNA isolation

The two parental lines, Varuna, the most adapted Indian variety, and Heera, the canola quality mustard line used for the development of doubled-haploids (DH), were maintained by selfing for more than six generations. The DH population was derived from the F1 of a cross between Varuna and Heera through microspore culture, following Mollers et al. (1994). Out of 752 DH lines obtained, a total of 123 DH lines were selected randomly as the mapping population. DNAs from the parents, F1 and DH plants, were isolated from the mature leaves following Rogers and Bendich (1994).

AFLP analysis

AFLP markers were generated following Vos et al. (1995). DNAs of parents, F1 and the DH mapping population were digested with four different enzyme combinations, i.e. *Eco*RI/*Mse*I (E/M), *Eco*RI/*Taq*I (E/T), *Pst*I/*Mse*I (P/M) and *Pst*I/*Taq*I (P/T). For pre-amplification, E/M and E/T digests were amplified with AFLP primers having a single selective nucleotide, whereas P/M and P/T digests were pre-amplified with primers without any selective nucleotide at the 3′ end. In the second step, selective amplification was carried out using primers having three selective nucleotides at the 3′ end for all the enzyme digests. *Eco*RI and *Pst*I primers were end labeled with γ^{33}P ATP in their respective digests. All the PCR amplifications were carried out using a Perkin Elmer 9600 thermocycler.

AFLP marker scoring and nomenclature

AFLPs were scored as dominant markers on the basis of the presence or absence of the band at a corresponding position among the segregating DH population. Only clear and unambiguous bands were scored for genotyping. Segregating AFLP markers in the mapping population were named according to the primer combinations employed, followed by the parental line from which they were derived (v, Varuna; h, Heera) and the estimated molecular weight of the fragment. For example, e32m62v261 is a varuna-specific AFLP of 261-bp length amplified from primer pair E-AAC/M-CTT.

RFLP analysis

DNA clones used as probes were from a *Pst*I genomic library (WG clones) of *B. napus* cv Westar (Thormann et al. 1994). The inserts were isolated as *Pst*I fragments and used as probes for the detection of polymorphism between the parents, and subsequent genotyping of the mapping population.

For detection of polymorphism, approximately 10 µg of DNA from each of the two parents was digested with either of the four restriction enzymes, namely, *Bam*HI, *Eco*RI, *Eco*RV and *Hin*dIII, electrophoresed in 0.8% agarose gels and transferred to a Hybond N^+ membrane (Amersham) by alkaline transfer. The DNA probes were radiolabelled with α^{32}P-dCTP by random priming (Amersham) and used for Southern hybridization as described earlier by Pradhan et al. (1992). Clones that detected polymorphism between the parents were selected for mapping. Segregation analysis was done by hybridizing each of the selected probes to parents, F1 and 123 DH DNAs digested with a selected restriction enzyme that gave clear polymorphism. RFLP clones were named using the nomenclature suggested by Thormann et al. (1994). Multiple loci detected by a single probe were designated by the same name followed by a different lower-case letter (a, b and c etc.).

Segregation analysis and map construction

The segregation of each marker and linkage analysis was done using the program JoinMap version 2.0 (Stam 1993; Stam and Van Ooijen 1996). The segregating markers were grouped at a wide range of LOD scores (4.0–8.0) to identify the linkage groups. Markers grouped at a LOD score of 6.0 were mapped using the JMREC32 and JMMAP32 module (LOD value 1.0 and recombination fraction 0.45). The Kosambi mapping function was used to convert recombination frequencies into map distance (Kosambi 1944). In order to verify the final map order, a subset of informative markers, defined as those with <5% missing data separated at an average interval of 9 cM, were grouped at LOD score of 4.0 and were mapped following the above-mentioned mapping criteria to generate a framework map. The markers of the framework map belonging to different linkage groups were used as fixed-order files to verify the order of the final map and map distances.

Results

Screening and scoring of polymorphic markers

Prior to the construction of a linkage map, the two parents, Varuna and Heera, were screened for polymorphism

Table 1 Comparison of four types of AFLP primers (E/M, P/M, E/T and P/T) to detect AFLP markers for mapping the *B. juncea* genome

Type of AFLP primer	No. of primers used	No. of polymorphic bands	No. of mapped markers[a]	No. of TDLM	No. of distorted markers	No. of mapped points[b]	Total coverage[c] (cM)	Mean coverage index[d]
*Eco*RI/*Mse*I	24	667	423(63%)	29	84	252(59.6%)	1,432.8(5.7)	0.62
*Eco*RI/*Taq*I	22	363	235(65%)	9	34	150(63.8%)	1,295.0(8.6)	0.72
*Pst*I/*Mse*I	22	313	183(58%)	12	22	136(74.3%)	1,137.6(8.4)	0.78
*Pst*I/*Taq*I	23	233	155(66%)	21	19	118(76.1%)	1,076.7(9.1)	0.83
Total	91	1,576	996	69	159			

[a] Numbers in parentheses are the percentage over the total no. of polymorphic bands
[b] Numbers in parentheses are the percentage over the total no. of mapped marker
[c] Numbers in parentheses indicates the average interval size
[d] Coverage index was determined as the number of linkage groups covered per marker from a primer pair

with 2,186 AFLP primers consisting of 619 E/M, 432 E/T, 560 P/M and 575 P/T primer pairs. This screening was done to select a subset of primer pairs from all four enzyme combinations on the basis of the following criteria: primers should reveal high polymorphism between the two parental lines, and amplification from highly repetitive restriction fragments, as revealed by very intense bands, should be absent or minimal. On the basis of these criteria, a total of 91 primer pairs consisting of 24 E/M, 22 E/T, 22 P/M and 23 P/T combinations were selected for genotyping the mapping population (Table 1). A total of 1,576 polymorphic bands were detected between the parental lines with the 91 selected primer pairs at an average of 17.3 bands per primer pair. Maximum polymorphism was observed with E/M primer combinations (average of 28 bands per primer) and a minimum with P/T primers (average of 10 bands per primer).

When all the 91 primer pairs were used on the 123 DH mapping population, it was observed that all the parental polymorphic bands could not be scored unambiguously due to poor separation of amplification products of nearly identical sizes. As a result, the scoring of bands was confined to only clearly visible polymorphic fragments. Consequently, out of the 1,576 polymorphic bands generated by 91 primer pairs, 996 (63%) bands consisting of 423 E/M, 235 E/T, 183 P/M and 155 P/T markers were finally identified as usable mapping markers (Table 1), with an average of 17.6, 10.7, 8.3 and 6.7 markers per primer pair for E/M, E/T, P/M and P/T primer combinations, respectively.

A total of 110 genomic clones (WG clones, Thormann et al. 1994) were used as probes to screen for RFLPs between the two parental lines Varuna and Heera using four different restriction endonucleases, *Eco*RI, *Bam*HI, *Eco*RV and *Hin*dIII. It was observed that 45 genomic clones (41% of the total number tested) showed unambiguous polymorphism between the parents with either one or more restriction enzymes. Twenty two of these genomic clones were used for mapping. These 22 clones detected 33 RFLP markers ranging from 1 to 3 markers per clone. Of the 33 segregating RFLP loci, 7 (21%) were dominant and remaining 26 (79%) were co-dominant in nature.

Segregation of markers

Among 996 AFLP markers detected with the selected 91 primer combinations, 507 were Varuna-specific and 489 were Heera-specific. The majority (84%) of the AFLP markers showed 1:1 segregation for the two parental alleles ($P < 0.05$) and the remaining 16% (159 markers) deviated from the expected 1:1 ratio showing distortion in the segregation values (Table 1). Among the 159 distorted segregation markers, 87 skewed towards the Varuna-specific allele while 72 skewed towards the Heera-specific allele. Sixty nine marker pairs were found to be Trans Dominant Linked Markers (TDLM) due to their complementary segregation in the repulsion phase when amplified from the same primer combination (Table 1). Sixty seven of these TDLM pairs differed by less than 20 bp in size from each other. Since these bi-allelic markers mapped at the same position, only one marker from each pair was considered for mapping. Of the 33 RFLP markers used, 11 (33%) showed distortion in segregation. Eight of these loci exhibited a segregation bias for the Varuna allele and remaining three showed bias for the Heera allele.

Construction of a linkage map

A total of 1,029 markers consisting of 996 AFLPs and 33 RFLPs were used on the mapping population of 123 DH lines for the construction of a linkage map. The total dataset consisted of 51% Varuna and 49% Heera specific alleles with an average missing data of 2.3%. The markers were grouped at LOD values of 4, 5, 6, 7 and 8. It was observed that at a LOD value of 5–8 all the markers were arranged into 18 linkage groups (LGs) that putatively corresponded to the haploid chromosome number of *B. juncea*. However, at a LOD value of 4.0, two of these 18 linkage groups merged to give rise to 17 linkage groups. The map showing 18 LGs in Fig. 1 was generated at a LOD value of 6.0. The linkage groups were numbered LG 1–18 in descending order of their length. The total length of the map was 1,628.7 cM. The individual linkage groups ranged from LG1 of 132.5 cM to LG18 of 52.1 cM in length (Fig. 1, Table 2). The number of mark-

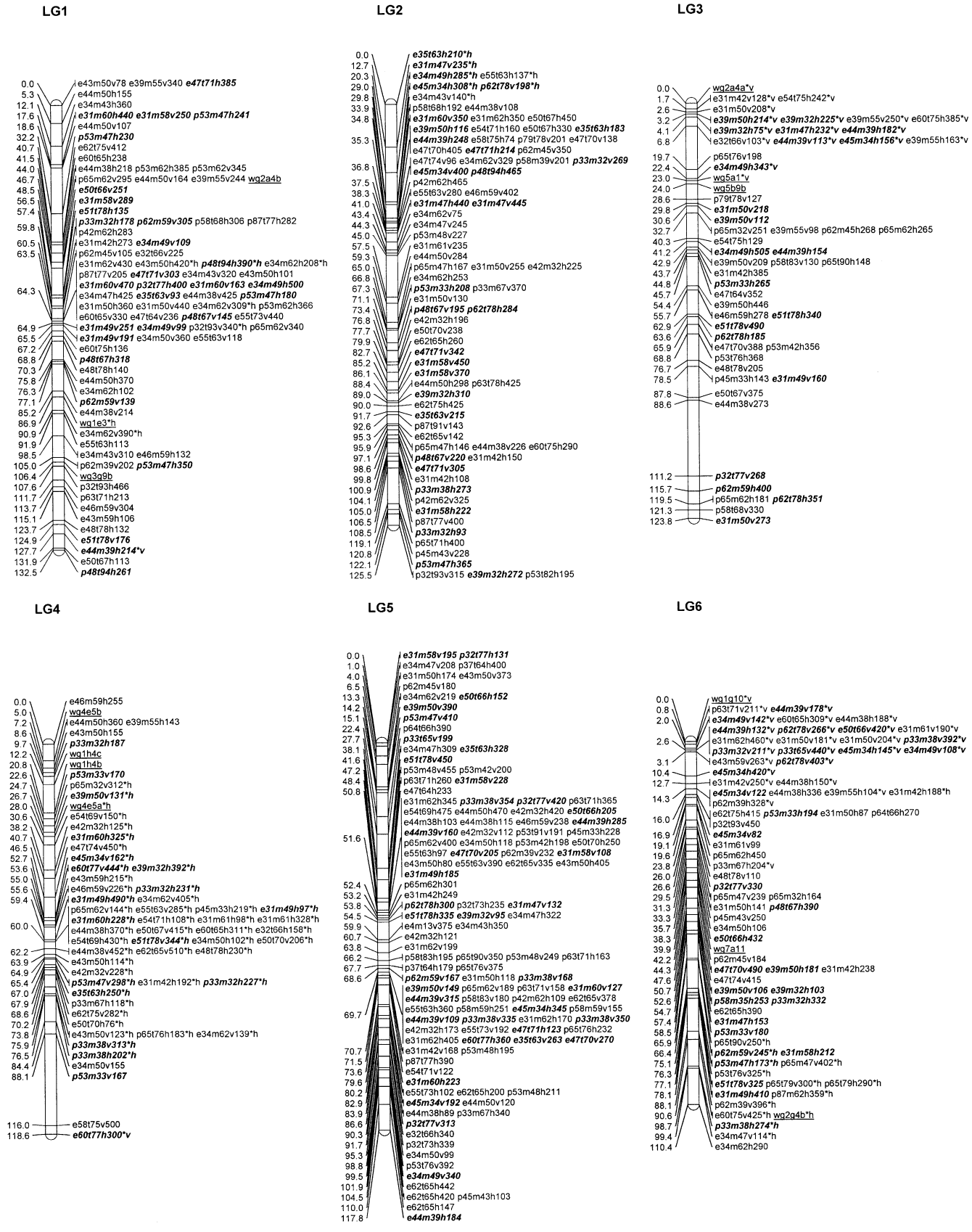

Fig. 1 A *B. juncea* linkage map with AFLP and RFLP markers. The map shows the distribution of 1,029 markers among 18 linkage groups (LG1–LG18). LGs are arranged in descending order of length. Cumulative recombination distances are shown on the left and marker loci on the right of the LGs bar diagram. RFLP markers have been *underlined*. Maker loci showing segregation distortion are indicated with asterix (*) and the *letter* following the asterix indicates skewness toward a particular parent (*v*, Varuna; *h*, Heera). The distribution of markers generated by 26 selected AFLP primers are printed *boldface in italics*

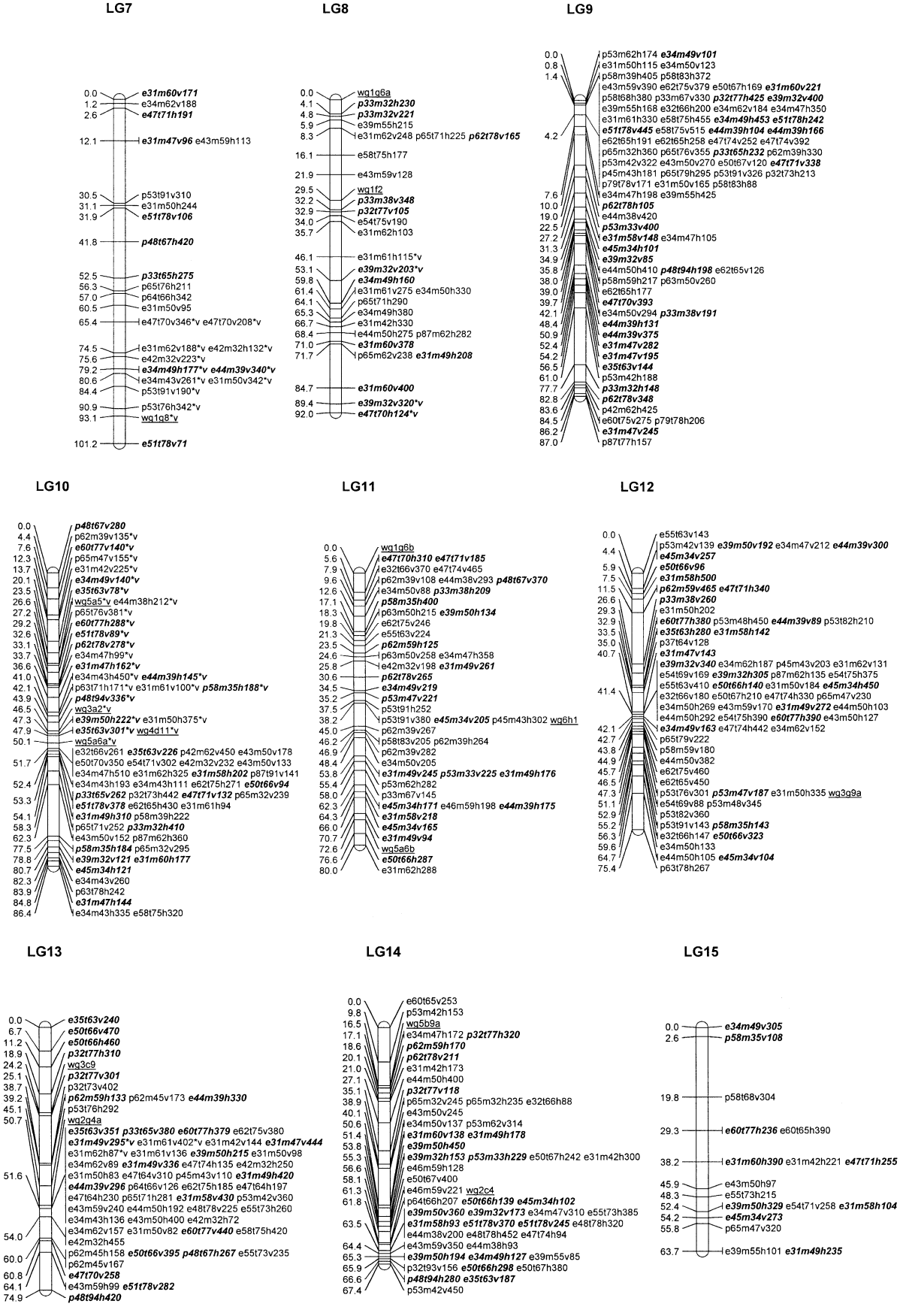

Fig. 1 Legend see page 610

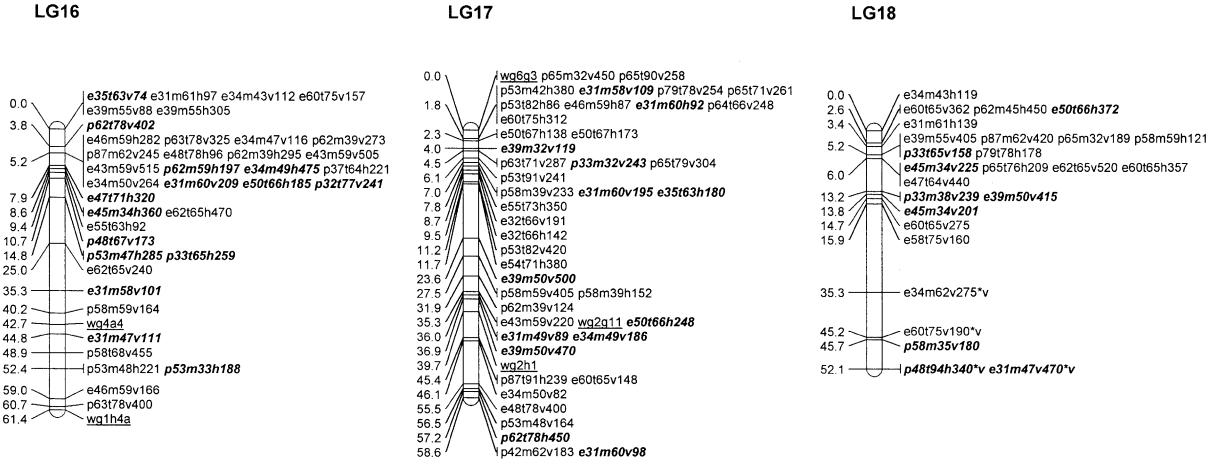

Fig. 1 Legend see page 610

Table 2 Characteristics of 18 LGs of the *B. juncea* map constructed with 996 AFLP and 33 RFLP markers

LG	No. of markers	Density (marker/cM)	No. of intervals	No. of gaps[a]	Av. interval size(cM)	No of clusters	Length (cM)
1	86	0.65	40	0	3.2	1	132.5
2	79	0.63	46	0	2.6	1	125.5
3	54	0.44	34	1	3.6	0	123.8
4	61	0.51	35	1	3.4	1	118.6
5	118	1.00	42	0	2.9	2	117.8
6	76	0.69	39	0	2.8	1	110.4
7	26	0.26	20	0	5.1	0	101.2
8	30	0.33	24	0	3.8	0	92.0
9	76	0.87	28	0	3.1	1	87.0
10	66	0.76	33	0	2.6	1	86.4
11	47	0.59	30	0	2.7	0	80.0
12	67	0.89	25	0	3.0	1	75.4
13	61	0.81	16	0	4.5	1	74.9
14	52	0.77	24	0	2.8	1	67.4
15	17	0.27	10	0	6.4	0	63.7
16	41	0.67	17	0	3.6	1	61.4
17	46	0.78	24	0	2.4	1	58.6
18	26	0.50	12	0	4.3	1	52.1
∑/mean	1,029	0.63	499	2	3.5	14	1,628.7

[a]A distance of more than 20 cM between two adjacent markers is termed 'a gap'

ers in each LG varied from 118 (LG5) to 17 (LG15). The number of intervals as determined on the basis of mapped points in the LGs varied from 46 (LG2) to 12 (LG18), with an average interval size of 3.5 cM ranging from an interval size of 6.4 cM in LG15 to an interval size of 2.4 cM in LG17. Two major gaps of more than 20 cM length were observed in LG3 and LG4 (Fig. 1, Table 2).

To verify the map order of the final map (Fig. 1), a framework map was constructed consisting of 199 informative markers selected at the average interval of 9 cM. The size of the framework map was 1,626.7 cM and the length of each of the LGs was almost similar to the length of the various LGs in the final map (data not shown). When the markers of the framework map were used in a fixed order and mapping was undertaken with all the 1,029 markers, the order and length of LGs remained unchanged as compared to the final map.

The 170 markers (159 AFLP and 11 RFLP markers) showing distortion in segregation were distributed in ten linkage groups (LGs 1, 2, 3, 4, 6, 7, 8, 10, 13 and 18). These markers are highlighted by an asterix in Fig. 1. The majority of the markers showing an excess of Varuna alleles were found to cluster in LGs 3, 6, 7 and 10, whereas those with an excess of Heera alleles were clustered in LGs 2, 4 and 6. In other linkage-groups markers showing distorted segregation were either found in minor clusters or were dispersed.

Distribution of AFLP markers

It was observed that a considerable number of AFLP markers were clustered in specific regions of different linkage groups. A survey of the clustering of the AFLP markers indicated the presence of 14 clusters in 13 linkage groups with the exception of LGs 3, 7, 8, 11 and 15 where no apparent clustering of markers was observed. Among the 13 linkage groups showing a clustering of markers, all the LGs were found to have at least one

cluster each. In LG5 two clusters were observed (Table 2). Detailed analysis of AFLP markers based on the four different restriction enzyme combinations, E/M, E/T, P/M and P/T, showed that markers amplified with *Pst*I primers were more randomly distributed with a high percentage of markers (76.1% and 74.3% for P/T and P/M respectively) showing a distribution all over the genome, thus providing a higher coverage index (0.83 and 0.78 for P/T and P/M respectively, Table 1). However, *Pst*I-based AFLPs showed a low level of polymorphism and were represented poorly on some of the LGs. As an example, no P/M primer combination marker was represented on LG7; LG4 and LG15 had only one marker each from the P/T primers. A map solely based on P/M primers would give a coverage of 1,137.6 cM and P/T primers a coverage of 1,076.7 cM (Table 1).

Markers generated with *Eco*RI primers were found to be less randomly distributed. Only 59.6% and 63.8% of the markers were uniquely distributed with a coverage index of 0.62 and 0.72 for E/M and E/T, respectively (Table 1). However, the E/M primers being capable of generating more markers per primer pair could cover more area in the genome (1,432.8 cM) with an average interval of 5.7 cM as against an average of 8.4 cM and 9.1 cM for P/M and P/T primers, respectively, indicating that E/M primers are more suitable for saturation mapping of the *B. juncea* genome as compared to the other primers.

Markers for whole-genome selection

Based on the constructed map (Fig. 1), we identified a subset of primer combinations that would allow high or very extensive coverage of the mapped part of the genome with the minimum number of primer combinations. These combinations were selected on the following two criteria, primers should allow extensive coverage of the genome and should give adequate representation of the minor LGs. A set of 26 primer pairs (9 E/M, 6 E/T, 6 P/M and 5 P/T) was identified. These primer pairs were e31m47, e31m49, e31m58, e31m60, e34m49, e39m32, e39m50, e44m39, e45m34, e35t63, e47t70, e47t71, e50t66, e51t78, e60t77, p33m32, p53m33, p33m38, p53m47, p58m35, p62m59, p32t77, p33t65, p48t67, p48t94 and p62t78 (the detailed primer combinations could be obtained from the authors on request). A total of 385 markers generated by these primers could cover 95.8% (1,560.5 cM) of the mapped genome. The remaining 4.2% region that was not covered corresponded to the terminal regions of some of the linkage groups. The markers generated by the selected primers were distributed with an average marker interval of 6.8 cM. This set of selected AFLP primers could be used for whole-genome selection in backcross breeding programmes in *B. juncea*.

Discussion

The present investigation was undertaken to construct a high-density linkage map in *B. juncea* primarily using AFLP markers. The high multiplex ratio and extensive level of polymorphism observed in *B. juncea* for AFLP markers enabled us to generate 996 mappable markers from only 91 AFLP primer pairs belonging to four enzyme combinations, *Eco*RI/*Mse*I, *Eco*RI/*Taq*I, *Pst*I/*Mse*I and *Pst*I/*Taq*I. The developed map also includes 33 RFLP markers, 26 of which were found to be codominant. The total of 1,029 AFLP and RFLP markers grouped into 18 LGs over LOD values that ranged from 5 to 8. This consistency of grouping over a wide range of LOD scores, coupled with the fact that no marker was left stranded out of the 18 LGs, indicates that the *B. juncea* map generated in the present study is a saturated one and the 18 LGs in all probability represent the putative haploid chromosome number of *B. juncea*.

Results showed that the *Eco*RI-based AFLP markers were more prone to clustering compared to *Pst*I-based AFLP markers. However, due to the high marker generation capability of *Eco*RI-based AFLP markers, these were more suitable for saturation mapping. On the other hand, *Pst*I-based AFLP markers were less clustered and showed a better genome distribution due to their higher coverage index. Hence, a combination of both *Eco*RI- and *Pst*I-based AFLP markers resulted in the construction of a high-density linkage map in *B. juncea*. However, some gaps remain, including two major gaps of more than 20 cM on LG3 and LG4. Earlier studies also showed higher clustering of *Eco*RI-based AFLP markers compared to *Pst*I-based AFLP markers in the molecular maps of maize (Castiglioni et al. 1999) and tomato (Haanstra et al. 1999).

Linkage maps in *B. juncea* were published earlier by Cheung et al. (1997) and Axelsson et al. (2000). In both these studies, RFLP markers have been used to construct the linkage maps. The map of Cheung et al. (1997) consisted of 343 markers arranged in 18 major and five minor groups covering a total map distance of 2,073 cM, whereas the map of Axelsson et al. (2000) consisted of 183 markers arranged in 18 major groups and four unlinked loci with a total coverage of 1,269 cM. In our study, we obtained 18 LGs with 1,029 markers without any minor group or any marker left out, providing a coverage of 1,629 cM. Although, we mapped more numbers of markers compared to the study of Cheung et al. (1997), the total genetic length of our *B. juncea* map was shorter than the one reported by the other group and we did not observe any evidence of map inflation due to genotypic errors. Vuylsteke et al. (1999) reported similar observations while mapping the maize genome. They observed that despite the larger number of markers, the total genome coverage and the lengths of the individual linkage groups were shorter than the ones already published. They also did not observe the inflation of the total genetic map length consequent to more genotypic errors when a larger number of markers was put on to the maps. They explained that the observed discrepancy could be due to the difference in the mapping algorithms used by the two extensively used mapping packages, MapMaker and JoinMap. Due to this difference, the JoinMap would produce shorter maps than MapMaker.

Castiglioni et al. (1998) also noticed a drastic contraction in map length using JoinMap compared to MapMaker. In *B. juncea*, while Cheung et al. (1997) used MapMaker, we used JoinMap for construction of the map. Another explanation could be the difference in the recombination rates in the two mapping populations due to environmental influences (Allard 1963) or the extent of diversity between the two parents used for generating the mapping population (Axelsson et al. 2000).

The *B. juncea* map developed in the present study will be utilized for dissection of quantitative traits and the identification of superior QTLs from the exotic eastern European lines, and these will be subsequently introgressed into Indian varieties by recurrent backcrossing through marker-assisted breeding. The set of 26 AFLP primers identified for whole-genome selection will be used for rapid recovery of the recipient genome in the backcross breeding programme. Earlier studies on heterosis breeding in *B. juncea* (Pradhan et al. 1993) indicated that Indian and exotic lines belong to two different gene pools and exhibit heterosis for yield in many cross combinations. Hence the transfer of component QTLs through backcross breeding would also help to preserve the diversity pattern or the 'heterotic blocks' for any future-breeding programme for producing high yielding hybrids in *B. juncea*.

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