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RESEARCH ARTICLE

RAPD, ISSR and SSR Based Integrated Linkage Map From an F_2 Hybrid Population of Resynthesized and Natural *Brassica carinata*

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Abstract A genetic linkage map of *Brassica carinata* (BBCC; $2n = 34$) has been constructed with 23 RAPD, 29 ISSR and 17 SSR markers using an F_2 hybrid population of 150 individuals. The mapping population was developed by selfing single F_1 plant following hybridization between a resynthesized *B. carinata* genotype, Ar29 and natural *B. carinata* cv. PC5. The segregation of each marker and linkage analysis was performed using the program MapMaker v3.0. The 69 mapped markers were aligned into 17 major linkage groups and a subgroup 5_A at LOD value of 3.0. The total length of the map was 2,166 cM, with an average marker interval of 31.39 cM. To the best of our knowledge, this is the first report on genetic linkage mapping of *B. carinata* and the map developed in the present study will be highly useful for anchoring of more loci in future investigations.

Keywords RAPD · ISSR · SSR · Linkage map · *Brassica carinata*

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Introduction

Ethiopian mustard (*Brassica carinata* A. Braun) is an amphidiploid species (BBCC; $2n = 34$), believed to have originated in Ethiopian plateau from interspecific hybridization between two diploid progenitor species *Brassica nigra* (BB; $2n = 16$) and *Brassica oleracea* (CC; $2n = 18$) [1]. Current work on the crop suggests that it has a great potential as an oilseed crop for many regions, especially those with semi-arid or mediterranean climates, and can withstand wide range of biotic stresses, more effectively than conventional *Brassica* oilseed crops [2–4]. Wide scale adoption of the crop has, however, been hampered by its late maturity, poor harvest index, low oil content and unattractive grain colour, besides poor oil and meal quality [5]. In this sense, it is imperative to exploit the primary gene pool available in the country of its origin or introgress desirable alleles from related *Brassica* digenomics for the improvement of this crop species. However, exploitation of these genetic resources, through conventional plant breeding so far has not been very encouraging and only limited selection advances for yield and component traits have yet been achieved [6]. Under these circumstances, DNA markers-based genetic linkage maps would provide great potential for increasing the speed and precision of cultivar improvement programmes of *B. carinata.*

The members of the Triangle of U species [7] have been subjected to extensive genetic mapping using molecular markers to identify loci associated with various qualitative and quantitative traits of agronomic interest [8–15]. However, most studies have focused on more economically important species and due importance has not been given to *B. carinata*. To our knowledge, there is no previous report in the literature on the genetic linkage mapping or development of molecular markers specific to *B. carinata*.

Keeping this in mind, the present study was aimed at developing a genetic linkage map of *B. carinata* using random amplification of polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) markers. Moreover, microsatellite or simple sequence repeat (SSR) markers already available in different *Brassica* species were also evaluated for their cross-transferability to *B. carinata* and subsequently integrated to the genetic linkage map. It is expected, the linkage map developed in the study will allow better genetic analysis of target agronomic traits through localization of major genes and QTLs and their utilization in crop improvement programmes through marker assisted selection (MAS). Besides, it will serve as a skeleton to be used for anchoring of more loci in future investigations.

Materials and Methods

Plant Material and DNA Isolation

A set of 150 randomly selected F_2 individuals derived from a single F_1 plant of a cross between a resynthesized *B. carinata* genotype, Ar29 and natural *B. carinata* cv. PC5, was used as a mapping population for developing the linkage map. DNAs from the parents, F_1 and F_2 plants, were isolated from fresh young expanded leaves following Doyle and Doyle [16].

DNA Marker Analysis

Forty-eight decamer RAPD primers obtained from Operon Technologies (Alameda, CA, USA), 100 ISSR primers chosen from the #9 ISSR primer kit (801-900) of the Biotechnology Laboratory, University of British Columbia (Vancouver, Canada) and 359 SSR primers derived from *Brassica rapa*, *B. oleracea*, *B. nigra* and *B. napus*, whose nucleotide sequences information are publicly available in the *Brassica* microsatellite information exchange database (www.brassica.info/resourse/markers/ssr-exchange.php), were used for detection of polymorphism between resynthesized *B. carinata* genotype, Ar29 and natural *B. carinata* cv. PC5. PCR amplifications of the genomic DNA with different primers were carried out using an MJ Research Thermocycler PTC 200.

RAPD and ISSR analyses were performed on the basis of protocols devised by Williams et al. [17] and Zietkiewiez et al. [18], respectively. PCR amplifications of ISSR loci were performed in a 20 µl reaction volume containing approximately 40 ng template DNA, 0.5 mM of a single primer, 200 µM each of dNTPs and 1 U of Taq DNA polymerase (MBI Fermentas, Vilnius, Lithuania) in 1× PCR buffer, and 2 mM $MgCl_2$. For RAPD, the conditions were the same as those of ISSR except that 25 ng of template DNA was utilized. The PCR cycling conditions for ISSR analysis was 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 2 min, and a final extension of 10 min at 72 °C, while it was 94 °C for 5 min followed by 40 cycles of 94 °C for 1 min, 37 °C for 1 min, 72 °C for 2 min, and a final extension of 10 min at 72 °C for RAPD.

SSR amplifications were performed in 20 µl reactions consisting of 2.25 mM $MgCl_2$, 250 µM of each dNTP, 10 pmol of each primer, 1× PCR buffer, 1 U Taq DNA Polymerase, and approximately 50 ng DNA. The PCR cycling conditions for SSR analysis was 94 °C for 5 min for the initial denaturation of the template DNA, followed by 35 cycles of 94 °C for 45 s, annealing temperatures of 52.5–66 °C for 30 s, and 72 °C for 2 min, and a final extension of 10 min at 72 °C.

The RAPD and ISSR amplification products were size separated by standard horizontal electrophoresis on 1.5 % agarose gels whereas, SSR-amplified products were resolved on 3.0 % Super Fine Resolution (SFR) agarose gels, at 70 V for 3 h in 1× Tris–borate-EDTA (TBE) buffer and stained with ethidium bromide (0.5 µg/ml) for 30 min. The gels were viewed and photographed in gel documentation and image analysis system (Syngene, Synoptics Group, Cambridge, UK).

Segregation Analysis and Map Construction

RAPD and ISSR banding patterns were scored from gel photographs for presence (1) and absence (0) of bands assuming that each band represents a unique genetic locus. However, the genotype of loci with SSR markers was scored as A (homozygous for allele present in resynthesized *B. carinata* genotype, Ar29), B (homozygous for allele present in *B. carinata* cv. PC5), and H (heterozygous for both the alleles). Deviation from the expected Mendelian segregation ratio of 3:1 for RAPD and ISSR, and 1:2:1 for SSR markers were tested using χ^2 analysis at 5 % level of significance with degree of freedom, 1 and 2, respectively.

Table 1 Primer specificity and segregation distortion shown by various marker systems in *B. carinata*

Type of marker	No. of primers used	Primer specificity		No. of distorted markers	Segregation biasness	
		Ar29	PC5		Ar29	PC5
RAPD	35	18	17	7 (20.0 %)	2	5
ISSR	57	27	30	11 (19.3 %)	6	5
SSR	48	14	0	11 (22.9 %)	0	11
Total	140	59	48	29 (20.4 %)	8	21

Table 2 List of markers used for genetic mapping of *B. carinata*

Primers	Total no. of bands	Parent 1 (Ar29)	Parent 2 (PC5)	No. of polymorphic bands
RAPD				
OPAK18	10	7	9	2
OPL11	12	12	10	2
OPH12	8	7	8	1
OPN13	7	6	7	1
OPG04	12	10	12	2
OPQ12	8	6	7	1
OPY15	13	11	13	2
OPT04	11	8	9	1
OPK06	10	7	10	3
OPR10	12	11	12	1
OPAE12	8	8	7	1
OPN01	7	6	7	1
OPG05	13	13	12	1
OPF06	10	9	10	1
OPF20	11	11	10	1
OPA07	10	8	9	1
ISSR				
UBC 819	6	4	5	1
UBC 821	8	6	5	1
UBC 827	7	4	5	1
UBC 828	7	6	5	1
UBC 830	5	5	4	1
UBC 834	10	7	8	1
UBC 835	7	5	6	1
UBC 836	9	9	8	1
UBC 843	7	6	7	1
UBC 845	7	5	3	1
UBC 849	5	5	4	1
UBC 850	10	10	8	2
UBC 851	6	6	5	1
UBC 853	8	7	8	1
UBC 856	6	6	4	2
UBC 857	8	9	8	1
UBC 864	8	6	2	4
UBC 866	7	6	7	1
UBC 873	6	6	5	1
UBC 878	6	6	5	1
UBC 889	10	9	10	1
UBC 890	10	9	10	1
UBC 891	11	11	9	2
UBC 900	8	4	5	1
SSR				
Ni2 B07	10	10	9	1
Ni2 C08	2	1	1	1
Ni4 B10	3	3	2	1
Ni4 D10	3	2	2	1
Ni4 D12	2	1	1	1
Ni4 F10	3	3	3	1
Ni4 F11	2	2	1	1
O110A09	3	2	2	1
O110B12	2	1	1	1
O110D01	2	1	1	1
O110E05	2	2	1	1
O110E12	3	3	2	1
O112G04	3	2	2	1
Na10 H06	3	2	1	1
Na12 B09	3	2	1	1
Bn 25C2	3	2	1	1
Ni4D09	2	1	1	1

Linkage analysis of polymorphic marker loci was performed using the program MapMaker v3.0 [19]. A minimum LOD threshold of 3.0 was used to group all the markers into potential linkage groups (LGs). Two-point, three-point and multi-point analyses were used in order to determine the best order of marker loci within the LGs. Three-point and multipoint analyses were used to find the most likely locus orders within each LG. The final linkage orders were determined with 'ripple' command [20]. In case of more than one possible arrangement of LGs; the one with smallest genetic linkage distance between adjacent marker loci of the LGs was chosen to construct the genetic map. The Kosambi mapping function was used to convert recombination frequencies into map distance [21]. Linkage map was drawn using the computer software 'MAPCHART' version 2.1 [22].

Results and Discussion

Primer Selection

Parental polymorphism survey was performed using 507 different markers comprising of 48 RAPD, 100 ISSR and 359 SSRs. Poor level of polymorphism (13.3 %) was apparent for SSR and 45.0 % for ISSR markers. However, RAPD showed relatively higher polymorphism (62.5 %) as reported earlier [23]. Finally, only 140 markers comprising of 35 RAPD, 57 ISSR and 48 SSR markers were found to be polymorphic and utilized for genotyping the mapping population. Apparent lack of polymorphism for SSR markers have been reported in the past for related *Brassica* species [24]. However, there is no previous reference indicating the level of polymorphism with ISSR markers.

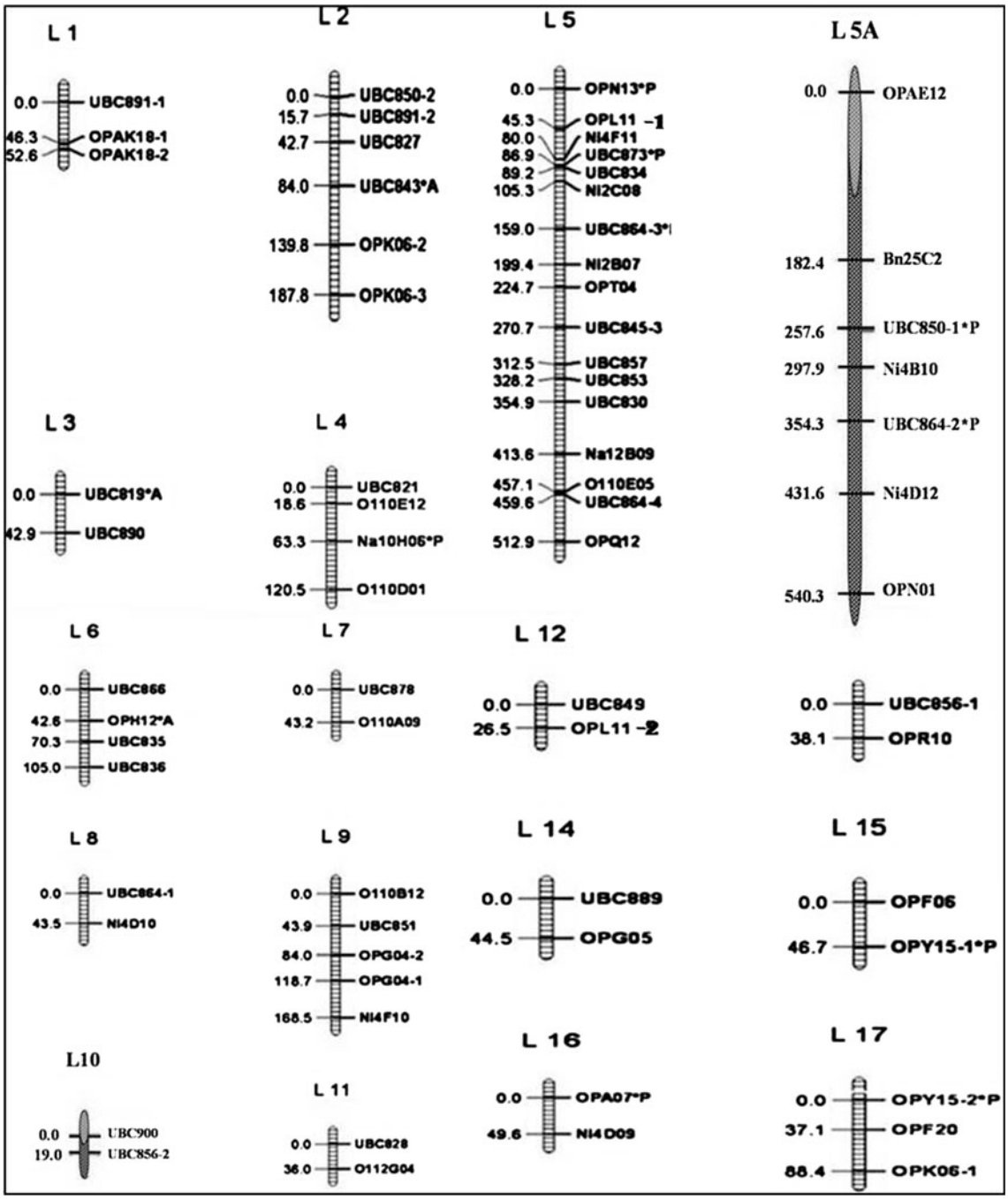

Fig. 1 Linkage map of *B. carinata*. Map distances in cM are indicated in *left side* and locus names are on the *right side* of LGs. Loci marked in *asterisk* deviated significantly from Mendelian ratio (1:3 and 1:2:1) at 5 % level of significance. A and P indicate that Ar29 or PC5 genotypes predominated, respectively

Extensive distortion of allele transmission ratios was observed for all marker types. A total of 7 (20 %) RAPD and 11 (19.3) ISSR markers showed significant deviations from expected Mendelian segregation ratio of 3:1, and out of them 10 markers showed segregation biasness towards *B. carinata* cv. PC5. Furthermore, 11 (22.9 %) SSR markers also showed segregation distortion from expected Mendelian segregation ratio of 1:2:1 and all the 11 loci showed segregation biasness towards *B. carinata* cv. PC5 (Table 1). Such a high frequency of segregation distortion could be attributed to the fact that one of the parents, *B. carinata* Ar29, was resynthesized and may still be suffering from a degree of genomic instability. Distortions in segregation ratios are known to increase with the level of divergence of the parents

Table 3 Characteristics of 17 linkage groups of *B. carinata*

Linkage groups	No. of markers	Density (marker/cM)	Size (cM)
1	3	0.057	52.6
2	6	0.031	187.8
3	2	0.046	42.9
4	4	0.033	120.5
5	17	0.033	512.9
5_A	7	0.012	540.3
6	4	0.04	105.0
7	2	0.05	43.2
8	2	0.05	43.5
9	5	0.03	168.5
10	2	0.11	19.0
11	2	0.06	36.0
12	2	0.08	26.5
13	2	0.05	38.1
14	2	0.04	44.5
15	2	0.04	46.7
16	2	0.04	49.6
17	3	0.03	88.4

Total length of linkage map = 2,166 cM

and have been attributed to differentiation of parental chromosomes [25]. Moreover, high level of segregation distortion could also be indicative of chromosome rearrangements such as translocations, as observed earlier [26]. Relatively higher polymorphism observed for RAPD markers and lower segregation distortion associated with them underlined the usefulness of RAPD markers for *B. carinata* map construction. In the present study, RAPDs constitute an abundant source of markers loci dispersed randomly across BC genomes of *B. carinata*.

Construction of Linkage Map

A total of 140 RAPD, ISSR and SSR markers, which were found to be polymorphic among the resynthesized *B. carinata* genotype, Ar29 and natural *B. carinata* cv. PC5, were used for the construction of the linkage map. However, MapMaker allowed the assembly of only 69 marker loci amplified using 16 RAPD, 24 ISSR and 17 SSR primers (Table 2) into 17 major LGs and one subgroup of LG5 (Fig. 1). A very large number of markers (71) were left stranded out of the 17 LGs constructed. The number of major LGs obtained in the study is in agreement with the haploid chromosome number ($n = 17$) of *B. carinata*. The total map distance was 2,166 cM with an average distance of 31.39 cM between marker loci. Although, it compared poorly with the map lengths recorded in *B. napus* (2,429 cM) and *B. juncea* (1,629 cM) by more elaborate studies of Lombart and Delourme [26] and Pradhan et al. [27] with average marker interval of 3–4 cM, the map present in the present study can serve as a framework map and will be highly useful for anchoring of more loci in future investigations.

The present study reveals a non-random distribution of the assigned markers for some of the LGs. It is an indication of non-random sampling of the genome, by an uneven recombination rate along the LGs [28] or by a clustering tendency of some markers due to their preferential targeting of specific genome regions. The size and number of markers, density of markers of each LGs are summarized in Table 3.

The size of LGs varied substantially between 19.0 cM (LG10) to 512.9 cM (LG5). It was possible to align 21 RAPD, 27 ISSR 14 SSR markers to 17 different LGs. Besides, some markers clustered to develop another subgroup and appeared separately as $LG5_A$ within LG5 due to unavailability of linked markers that could fill the large gap. The length of the subgroup $LG5_A$ was 540.3 cM having 7 markers comprised of 2 RAPD, 2 ISSR and 3 SSR markers. The map reported in the study represents the first skeleton map of *B. carinata* for anchoring of more loci in further investigations. The F_2 mapping population generated in the study and the genetic linkage map obtained will be highly useful to locate genes controlling several traits of agronomic interest and enrich the genetic map of *B. carinata* with more markers.

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