AN INTEGRATED AND COMPOSITE LINKAGE MAPS BASED ON INFORMATION FROM THREE *B. RAPA* DOUBLE HAPLOID SUBSETS

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Abstract: three segregating double haploid populations were derived from 3 FI plants from a single cross between vegetable Pack Choi and oilseed Yellow Sarson Brassica rapa. However, the Pack Choi accession is heterogeneous and heterozygous due to its self-incompatibility. This means that the different FI seeds may ha\'e obtained different alleles. YS is almost homozygous. In addition, we do not have DNA from the original FI plants, so we cannot predict which alleles will segregate. We describe the efficiency of two chosen approaches for genetic linkage map construction using segregating data derived from three DH lines subpopulations and JoinMap 4 software. The first "Composite " linkage map is calculated based on merged marker data of all three DH subpopulations in one matrix, second one "Integrated" - the result of combining of two independently developed genetic linkage map for most informative DH subpopulations. The Integrated DH68 genetic map has a total length of 1,045 cM, with an average distance of 2.1 cM between 2 loci, 91.1% of the genome within 5 cM of a marker; a total 502 loci were assigned to 10 linkage groups with LOD values of 5.0-9.0. The Composite DH68 linkage map has a total length of1,232 chi, with an a\'erage distance of 2.8 chi between 2 loci, 83.5% of the genome within 5 chi of a marker; a total 441 loci were assigned to 10 linkage groups with LOD values of 8.5-15.5.

Keywords: genetic linkage map, Integrated, Composite, DH subpopulations.

Introduction

The construction of detailed genetic maps with high levels of genome coverage is a first step for some of the applications of molecular markers in plant breeding (Tanksley et al., 1989). Assigning molecular markers to the linkage groups and constructing genetic maps is an important step towards analyzing the genomes of crop species. Genetic linkage maps provide a better insight into genome organization, evolution of the crop species, and synteny with related species. In addition, the maps are useful for tagging and cloning genes of economically important traits, marker assisted breeding and gene pyramiding.

Genetic linkage mapping dates back to the early 20th century when scientists began to understand the recombinational nature and cellular behavior of chromosomes. The first genetic map was published in 1913 by Alfred Sturtevant the student of T.H. Morgan (Sturtevant, 1913). The principles of genetic mapping and linkage analyses are still used in much the same way but with far more advanced methodologies. First, markers have to be partitioned into linkage groups. Second, it's necessary to determine the correct order of a given set of markers in the same linkage group. Third, the genetic distances between adjacent markers have to be estimated.

The introduction of DNA-based markers such as restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), simple sequence repeats (SSR) and amplified fragment length polymorphism (AFLP) caused genetic maps to become much more densely populated, generally into the range of several hundred to more than a thousand markers per genome.

A genetic map usually is built using input data composed of the states of loci in a set of meiotically derived individuals obtained from controlled crosses. When an order of the markers is computed from the data, the recombinational distance is also estimated. Most of the computational packages proposed for genetic linkage mapping, for example, JoinMap (Stam, 1993; Van Ooijen, 2006), Tmap (Cartwright et al., 2007), Carthagene (Schiex and Gaspin, 1997; Givry et al., 2004) and AntMap (Iwata and Ninomiya, 2006) produce accuracies of the maps on conditions of high quality of data (Wu et al., 2008). However, a molecular marker data for linkage mapping is normally complicated by missing values, genotyping errors and the unexpected ratio of markers segregation. Moreover, the effects of mentioned factors depend on the population size: the smaller the population, the more severe the effects are likely to be. Simulation study has shown that when markers are separated by distances of about 10 cM, a map based on 150 individuals is quite robust to the presence of typing errors, missing values and segregation distortion. However, finer scale genetic maps are of increasing interest, and in this case the map order and length are increasingly affected (Hackett and Broadfoot, 2003).

The effect of genotyping errors, especially errors arising in data entry, was quickly recognized in the human genetics mapping literature (Shields et al., 1991). Simulation studies have shown that undetected typing errors at a rate of 1% can lead to incorrect map orders and inflation of map lengths, particularly as marker density increases (Buetow, 1991).

Segregation distortion, which is defined as a deviation of the observed genotypic frequencies from their expected values, violates the law of segregation and renders conventional genetic theory and analysis to be invalid (Lu et al., 2002). Segregation distortion is a problem often encountered in mapping studies (Nozaki et al., 1997; Chyi et al., 1992; Suwabe et al., 2006; Soengas et al., 2007). It has been shown that the analysis of linkage may be influenced by deviations of single-locus segregation ratios from expected frequencies, and several authors have discussed methods to test for linkage or to estimate recombination frequencies between genes showing segregation distortion (Garcia-Dorado and Gallego, 1992; Lorieux et al., 1995). The effects of inclusion of loci with significant segregation distortion in the final linkage map seem contradictory. According to Hackett and Broadfoot (2003), segregation distortion had very little effect both on marker order and map length but others have reported reduction in map length.

The presence of missing values in the marker data means that information about the number of true recombinations that have taken place along the chromosome is lost. Missing values has less effect than typing errors, but they reduce the number of correctly ordered maps, especially for a marker separation of 2 cM. The variability among the order in bootstrap replicates also increases when missing values are present. There was also a tendency for missing values to lead to slightly shorter map lengths for more widely separated markers, especially in the presence of distorted segregation ratios and/or when using weighted least squares (Hackett and Broadfoot 2003).

A number of genetic linkage maps have been produced for *B. rapa* (Song et al., 1991; Chyi et al., 1992; Teutonico and Osborn, 1994; Kole et al., 1997; Nozaki et al., 1997; Kim et al., 2006; Suwabe et al., 2006; Soengas et al., 2007; Choi et al., 2007).

In our study we used molecular data from three double haploid (DH) lines subpopulations constructed from one cross of vegetable and oilseed type *B. rapa.* We describe the efficiency of pooling segregating data derived from all three DH subsets for genetic linkage map construction and integration of two independent genetic linkage map developed for two most informative DH subpopulations. For any design, we want some way of evaluating the quality of the resulting maps, i.e., how much better one map is than other.

Materials and Methods

Population development. Three *B. rapa* doubled haploid subpopulations were obtained from three F1 plants developed from a cross between vegetable type Pak Choi PC-175 *(B. rapa* ssp. *chinensis)* as the male parent to the oil type Yellow Sarson YS-143 **(***B. rapa* ssp. *trilocularis).* The Pack Choi accession is heterogeneous and heterozygous due to its self- incompatibility. YS is assumed to be almost homozygous. The different F1 plants may have obtained different alleles.

The cross was carried out by bud pollination with emasculation of Pak Choi PC-175 flowers. The DH lines were produced through microspore culture using a protocol described in Lou et al. (2008), based on Coventry et al. (1988) and Custers et al. (1994, 2001). Atotal of 249 DH lines were produced and named as the DH68 population. For the present analysis a maximally informative subsets of 89 DH68F1-1 lines, 19 DH68F1-2 lines and 123 DH68F1-3 of these (total 231) lines were used for map construction.

Molecular marker assays. *DNA isolation.* Genomic DNA was extracted from fresh leaves DH plants with the DNAeasy kit (Qiagen, USA) using KingFisher 96 instrument (Thermo Electron, Finland) according to the protocol supplied by manufacturer. Fresh leaf tissue was ground by shaking tubes containing leaf material and iron beads in a Retsch shaker at maximum speed (Retsch BV, Ochten, The Netherlands).

Amplified Fragment Length Polymorphism (AFLP) markers. The AFLP procedure was performed as described by Vos et al. (1995). Total genomic DNA (100 ng) was digested with two different enzyme combinations, *EcoRI/MseI* and *PstI/MseI* and ligated to adaptors in a total volume of 25 µ1 at 37°C overnight.

Pre-amplifications were performed in 20 µl volume of lx PCRbuffer, 0.2mM dNTPs, 30ng of adaptor primers, 0.4 units Taq polymerase and 5 µ1 of a restriction ligation mix, using 24 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 60 s.

Seventeen selective primer combinations, 11 of EcoRI/Msel (E32M16, E32M19, E32M47, E32M52, E34M15, E34M16, E36M15, E37M47, E39M20, E39M22, E44M16) and 6 of Pstl/Msel (P13M48, P14M51, P21M47, P23M47 (P21M47 Jan), P23M48, P23M50) were used to generate AFLP fragments from l0x diluted pre-amplified fragment templates. Selective amplifications were performed in 10 µl volume of lx PCR buffer, 0.2mM dNTPs, 50ng of unlabeled Mse-primcr. 0.05 pMol/µl IRD-labelled *Eco(Pst)*-primer 0.2 unit Taq polymerase and 5 µ1 of a lOx diluted pre-amplification templates, using touch-down PCR profile 12 cycles of 94°C for 30 s, 65-56°C for 30 s decrease 0.7°C each cycle and 72°C for 60 s, continued with 24 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 60 s.

To generate AFLP marker data, *EcoRl* and *Pstl* primers were labeled with IRD 700 and IRD 800 infrared dyes at the 5' end for the selective amplification. All amplifications were performed on Veriti® 96-Well Thermal Cycler (Applied Biosystems, USA).

Following the selective amplification, the reaction products were mixed with an equal volume of formamide-loading buffer (98% Formamide, 10 mM EDTA pH 8.0 and 0.1% Bromophenol Blue), denatured for 5 min at 94°C, cooled on ice and run on a 5.5% denaturing polyacrylamide gel with a 4200 LI-COR DNA Analyzer (LI-COR® Biosciences, Lincoln, NE). The marker data from AFLP digital gel images were scored using semi-automated scoring software Quantar PRO (Keygene, The Netherlands) as dominant markers on the basis of the presence or absence of the band at a corresponding position among the segregating population. Only clear and unambiguous bands in the range of 50 bp to 500 bp were scored for genotyping. Segregating AFLP markers in the mapping populations were named according to the primer combinations employed, followed by the molecular weight of marker band.

Simple Sequence Repeat (SSR) markers. Public SSR primer pair sequences information of *Brassica* was obtained from the Brassica information website (http://www.brassica.info), and previous publications (Suwabe et al., 2002; Kim et al., 2006, Lou et al., 2008, Choi et al., 2007). PCR reactions were performed in 96-well plates in a volume of 10 ц1. The composition of the mix included 1 unit of Taq DNA polymerase, 5 mM of dNTP, 2.5 µ1 10x supertaq buffer and 50 ng of each primer (forward and reverse primers). DNA was present in the PCR reaction to a concentration of 1 ng/µ1. The PCR was performed on GeneAmp PCR system 9700 (Applied Biosystems, USA) with the following program: 94°C for 2 min; 10 cycles with 94°C denaturation for 1 min, 65°C annealing for 1 min, 72°C elongation for 1.5 min, with a 1°C decrease in annealing temperature at each cycle; then 30 cycles with 94°C denaturation, 55°C annealing, 72°C elongation, 1 min each step; then a final elongation step of 5 min. PCR products were loaded on 2% agarose electrophoresis gels with loading buffer in 0.5x TBE buffer or 5.5% denaturing polyacrylamide gel with a 4200 LI-COR DNA Analyzer (LI-COR® Biosciences, Lincoln, NE). Alleles were scored as dominant markers visually and bands of the same size were assumed to be identical. Multiple segregating loci detected with one SSR primer pair were indicated by addition of a suffix (a, b) to the locus names.

Gene-targeted (GT) markers. GT markers were designed on the basis of Flowering Time (FT) gene sequence information available from *B. rapa*, *Arabidopsis* and *B.napus* genetic databases. Primer pairs were designed from the sequences using Primer 3 software (Rozen and Skaletsky, 2000). The criteria used for designing the primers were that the size of amplified DNA fragments were in the range of 100-400 bp, the difference of 7m between the two primers within a pairs was less than 3°C, the primer 7m ranged from 55 to 63°C, and the GC contents were greater than 35%.

PCR amplifications were performed in black frame white 96-well plates in 10 µ1 volume of lx PCR Phire reaction buffer, 0.2 mM dNTPs, 30 ng of each (forward and reverse) primers, 0.1 µ1 Phire Hot Start II DNA polymerase and 1 µ1 of LC-green (xlO) fluorescent dye covered with 20 µ1 of mineral oil. DNA was present in the PCR reaction in a concentration of 1-2 ng/µl. The PCR was performed on Veriti® 96-Well Thermal Cycler (Applied Biosystems, USA) with the following program 40 cycles of 98°C for 10 s, 60°C for 10 s and 72°C for 30 s continued with 1 cycle of 72°C for 30 s, 94°C for 30 s and 25°C for 30 s.

PCR products were analyzed by Light Scanner Hi-Res Melting system (Idaho Technology Inc., USA) and markers were scored using Light Scanner Software package.

Construction of a genetic linkage maps. Markers which were reproducibly polymorphic between the parent lines were scored in the DH68 subpopulations. Total number of markers 706 including Amplified Fragment Length Polymorphism. Simple Sequence Repeat and Gene-targeted markers were used for genetic map construction.

Three extensively studied *B. rapa* DH 68 mapping subpopulations, DH68F1-1 (89 lines), DH68F1-2 (191ines)andDH68F 1-3 (123 lines), share all loci derived from common genetic marker assays. Two genetic linkage maps were constructed and characterized: the "Composite DH68" genetic map was developed on the base of composite genotype matrix generated from three DH68F1-1, DH68F1-2 and DH68F1-3 subpopulations; and the “Integrated DH68" - result of integration of two separately developed maps for DH68F1-1 and DH68F1-3 subpopulations.

Linkage analysis and maps construction were performed using JoinMap 4 (Stam 1993; Van Ooijen, 2006). Linkage groups were identified in the LOD grouping threshold range of 5.0-7.5 and 5.5-9.0 for DH68F1-1 and DH68F1-3 subpopulation maps, respectively, used for construction of the “Integrated DH68" map and LOD grouping threshold range of 8.5-15.5 for “Composite DH68" map. Linkage groups were named using the nomenclature

convention internationally adopted for *Brassica* maps primarily via the set of SSR markers as A01-A10.

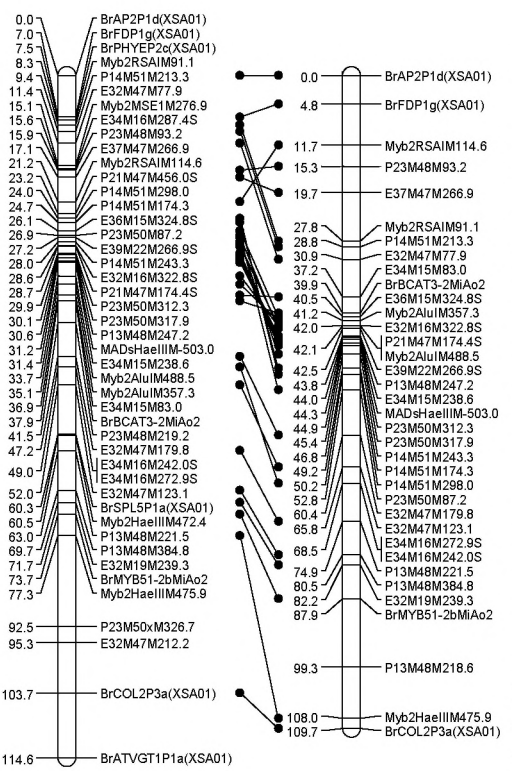
Maps were generated for each linkage group using a recombination frequency below 0.40 and LOD score above 1.0 for all the marker pairs within each linkage group. A "ripple" procedure was performed after the addition of each marker and the "jump" thresholds were set to 5. Recombination frequencies were converted to centiMorgans (cM) with Haldane's method for map-distance calculation (Haldane, 1919).

Results and discussions

Linkage analysis and map construction. The Integrated DH68 genetic map has a total length of 1,045 cM, with an average distance of 2.1 cM between two loci; a total of 502 loci were assigned to 10 linkage groups with LOD values of 5.0-9.0. The Composite DH68 linkage map has a total length of 1,232 cM, with an average distance of 2.8 cM between two loci; a total of 441 loci were assigned to 10 linkage groups with LOD values of 8.5-15.5. Using SSR markers common to the reference A genome of *B. rapa* (Lou et al. 2008, Kim et al. 2006, Choi et al. 2007; Suwabe et al. 2002) linkage groups A01-A10 were assembled (fig. 1).

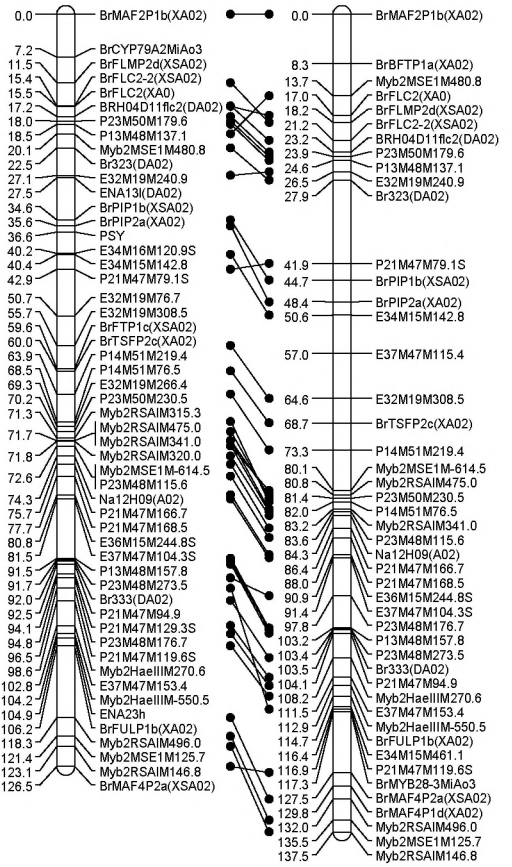
A01

IntegratedDH68 CompositeDH68



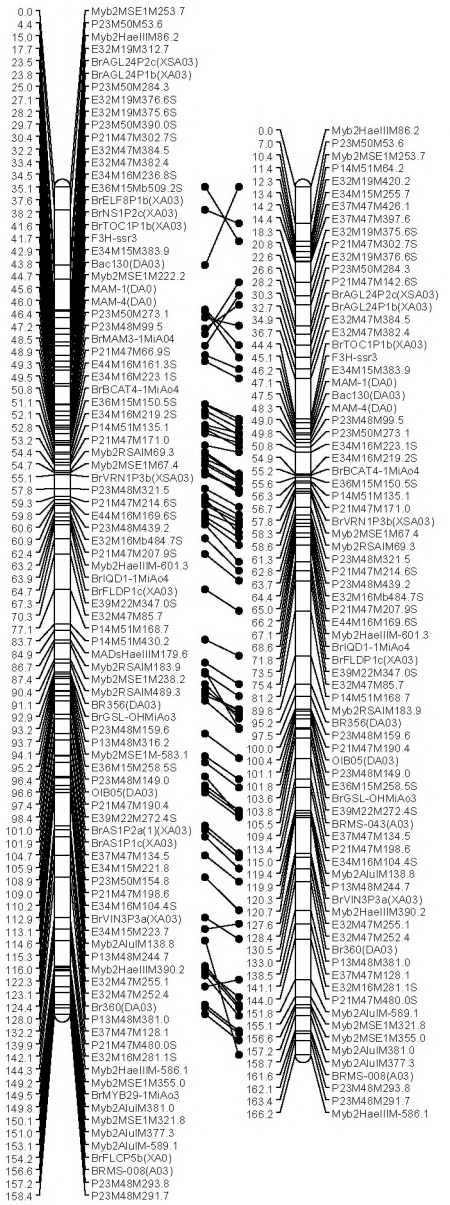
A02

IntegratedDH68 CompositeDH68



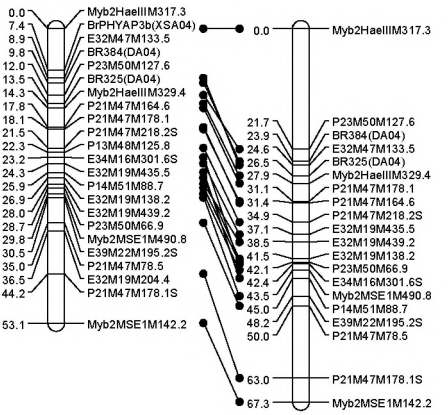
Continued

A03
IntegratedDH68 CompositeDH68

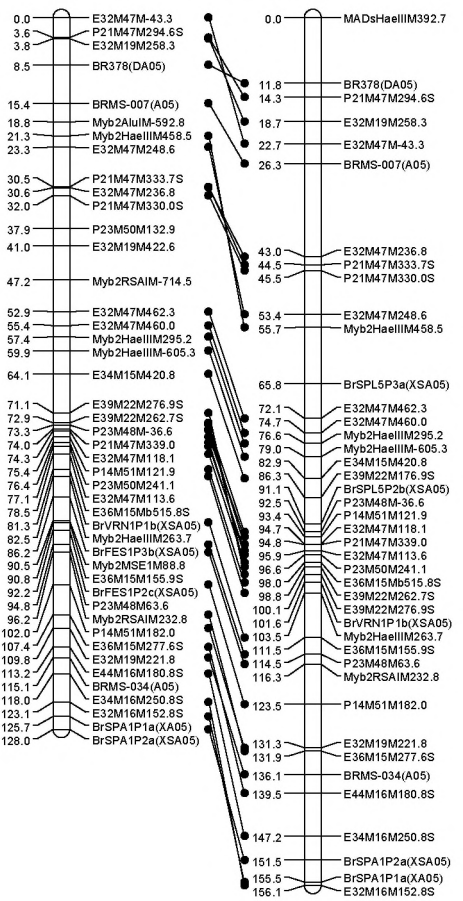


Continued

A04
IntegratedDH68 CompositeDH68



A05
IntegratedDH68 CompositeDH68



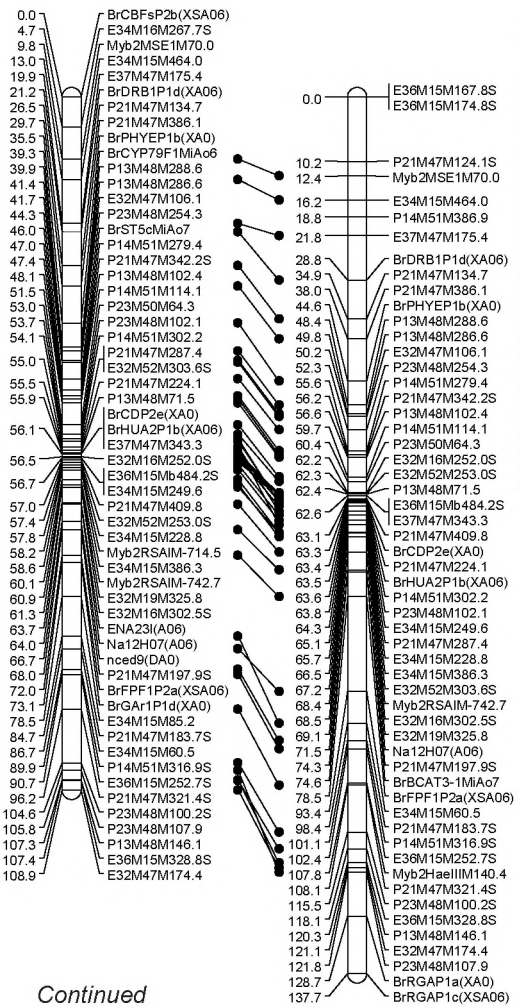
The length of linkage groups ranged from 52 to 158 cM for A07 and A03, respectively for Integrated DH68 map and the length of linkage groups ranged from 67 to 166 cM for A04 and A03, respectively for Composite DH68 map (table 1).

The number of markers in the 10 linkage groups ranged for Integrated DH68 map from 23 (A04) to 95 (A03), for Composite DH68 - from 20 (A04) to 79 (A03). The markers density in the linkage groups varied for Integrated DH68 map from 1,67 to 2,85 cM for A03 and A05, respectively with the average marker density 2.16 cM. The markers density in the linkage groups varied for Composite DH68 map from 2.10 to 3.72 cM for A03 and A05, respectively with the average marker density 2.97 cM.

In the Integrated DH68 linkage map 91.1% of the genome was within 5 cM of a marker; the largest gaps observed on LG's were 15.2, 12.2, 10.6, 8.8, 7.2, 8.4, 8.3, 8.9,

A06

IntegratedDH68 CompositeDH68



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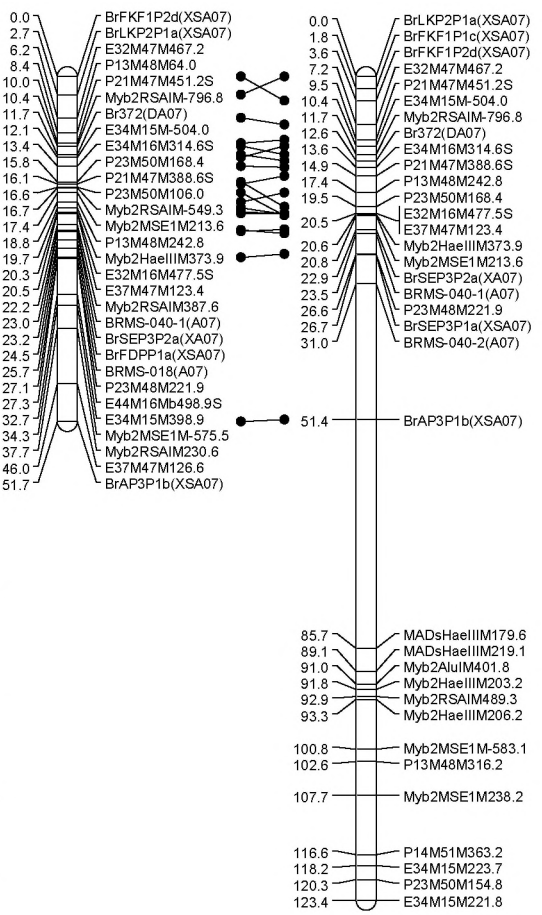
A07

IntegratedDH68 CompositeDH68



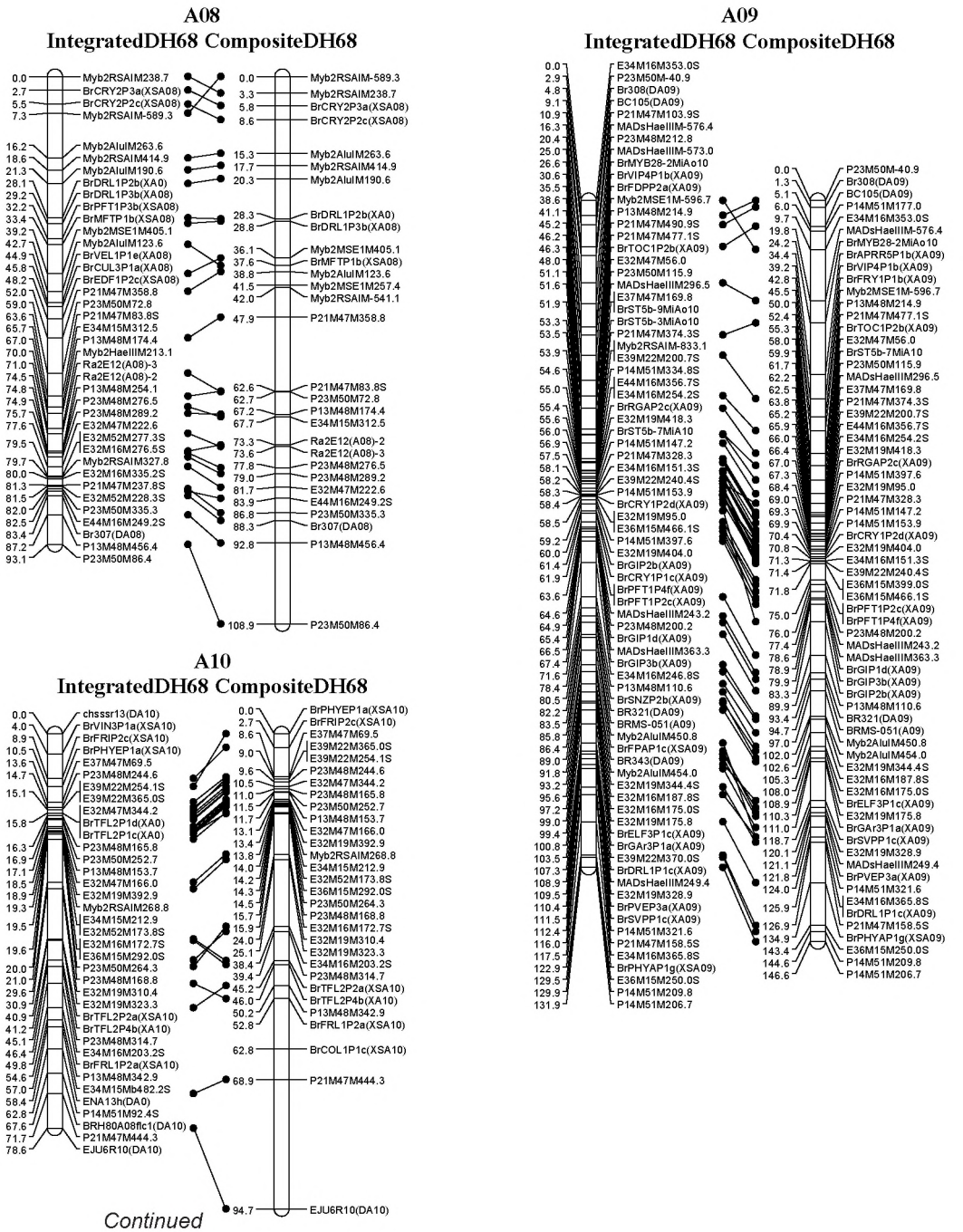


Fig. 1. Integrated DH68 and Composite DH68 *B. rapa* L. linkage maps. Cumulative recombination distances are shown on the left side and marker loci on the right of the linkage groups. The map shows the distribution of 502 and 441 loci along 10 linkage groups (A01-A10) of Integrated DH68 (total length 1,045 cM) and Composite DH68 (total length 1,232 cM) maps respectively. Black lines show homologues markers

Table 1

Salient features of Integrated DH68 and Composite DH68 genetic linkage maps

Linkage group	Integrated DH68					Composite DH68				
	No. of markers	Length (cM)	Density (markers/cM)	Number of gaps (> 5 cM)	Lagest gap (cM)	No. of markers	Length (cM)	Density (markers/cM)	Number of gaps (> 5 cM)	Lagest gap (cM)
A01	45	114,63	2,55	7	15,2	36	109,66	3,05	10	11,4
A02	53	126,51	2,39	5	12,1	47	137,53	2,93	9	14,0
A03	95	158,39	1,67	6	10,6	79	166,17	2,10	7	8,6
A04	23	53,06	2,31	3	8,8	20	67,29	3,36	2	21,7
A05	45	128,04	2,85	9	7,0	42	156,09	3,72	9	16,7
A06	57	108,87	1,91	7	8,4	56	137,72	2,46	9	14,9
A07	30	51,67	1,72	3	8,3	35	123,41	3,53	4	34,3
A08	39	93,10	2,39	5	9,0	29	92,98	3,21	7	16,1
A09	78	131,95	1,69	3	6,8	67	146,59	2,19	7	10,2
A10	37	78,64	2,13	3	10,0	30	94,68	3,16	7	25,8
Total / average	502	1044,86	2,16	51	96,2	441	1232,12	2,97	71	173,7

6.8 and 10.0 cM on A01, A02, A03, A04, A05, A06, A07, A08, A09 and A10, respectively. In the Composite DH68 linkage map 83.5% of the genome was within 5 cM of a marker; the largest gaps observed on LG's were 11.4, 14.0, 8.6, 21.7, 16.7, 14.9, 34.3, 16.1, 10.2 and 25.8 cM on A01, A02, A03, A04, A05, A06, A07, A08, A09 and A10, respectively.

Potential problems with pooling information from three segregating subpopulations to construct Composite DH68 map include the fact that not all genetic markers are polymorphic in all subpopulations and due to scoring mistakes it is not possible to dissect monomorphic and highly distorted markers accurately, but exclusion of a part of distorted markers leads to loses of informative markers and map density; estimates of linkage differ among populations; and population sizes are different.

The disadvantage of the Integrated DH68 map is the reduced number of plants in mapping populations - 89, 19 and 123 for DH68F1-1, DH68F1-2, DH68F1-3 respectively compare to whole set of genotyped plants 231. At the same time increasing of number of markers with high percentage of missing values after division of DH68 population on subpopulations was not confirmed.

Skewed segregation of markers. Skewed segregation of markers is a common feature in most of the Brassica linkage maps, and particularly ones based on DH lines due to preferential selection of genotypes responsive to microspore or anther culture (Chuong et al. 1988; Ferreira et al. 1994; Cloutier et al 1995; Uzunova et al. 1995; Foisset et al. 1996; Takahate and Keller 1991). Of the 441 markers assigned to the linkage groups of Composite DH68 map, 219 (49.7%) showed deviation from the expected segregation ratio of 1:1 (P • 0.05). This was a general property of the markers and not restricted to a particular marker type. In general, the loci showing distorted segregation were more skewed

towards the male Pack Choi PC-175 (63% of markers) alleles irrespective of the type of marker used.

The markers showing biased segregation were distributed along all the linkage groups.
In five linkage groups, A01, A02, A03, A04 and A06, the cluster of markers were distorted towards Pack Choi PC-175 (male). However, for A05, A07, A08, A09 and A10 the most of the clusters of segregating markers were skewed towards Yellow SarsonYS-143 (female) alleles (table 2).

The clusters of distorted markers were generally randomly distributed across the linkage groups A03, A06, A08 and A09. All markers in A01 and almost all in A04 and A10 linkage groups were skewed (table 2).
However, in A02 cluster of markers showing skewed segregation was located in the central part of the chromosome, in A05 and A07 on the distal end of the chromosomes.

Table 2

Skewness of distorted segregating markers clusters towards Yellow Sarson YS-143 and Pack Choi PC-175 alleles

Linkage group	Accession number	Skewed markers, % (Poe)
A01	Pack Choi PC-175	100
A02	Pack Choi PC-175	51
A03	Pack Choi PC-175	46
A04	Pack Choi PC-175	70
A0 5	Yellow Sarson YS-143	43
A06	Pack Choi PC-175	46
A0 7	Yellow Sarson YS-143	51
A0 8	Yellow Sarson YS-143	48
A0 9	Yellow Sarson YS-143	15
A10	Yellow Sarson YS-143	77

Missing values. The number of plants in mapping populations Fl-1 DH68, Fl-3 DH68, Composite DH68, number of mapped markers and number of markers with missing values is summarized in table 3.

Table 3

Comparison between genetics maps developed from F1-1 DH68, F1-3 DH68 populations and Composite DH68 map pooling genotypic data of three F1-1, F1-2 and F1-3 DH68 subpopulations

Genetical map	Number of				Number of mapped markers with missing values, %				Number of ungrouped markers
	plants	markers used for mapping	mono-morphic markers[b]	potential mono-morphic markers[c]	<25	25-50	50-75	>75	
F1-1 DH68	89	576	16	—	328	18	228	2	191
F1-3 DH68	123	682	24	5	228	137	253	64	195
Composite DH68[a]	249	706	—	16	170	242	287	7	159

[a] Pool of F1-1, F1-2 and F1-3 DH68 genotypic data;[b] segregation 1 : >10;[c] segregation from 1:10 to 1:7.

Number of markers with missing values <25% is decreasing with increasing size of mapping population, but number of markers with missing values < 50% is proportional to a number of markers (57%, 54%, 58% for Fl-1 DH68, Fl-3 DH68, Composite DH68, respectively). It means that division of DH68 population on mapping Fl-1 DH68 and Fl-3 DH68 subpopulations had no effect on the number of mapped markers with missing values.

Conclusion

Integration of two independent genetic linkage maps developed for two most informative DH subpopulations DH68F1-1, DH68F1-2 was more efficient compare to developing genetic map based on pooled segregating data derived from three DH subsets. Map integration resulted in an increased number of locus and locus density, less number and shorten gaps, and general accuracy of Integrated DH68 genetic maps, providing a stronger framework for subsequent precision mapping of QTLs. The greater density and precision will be invaluable for supporting current programmes to correlate genetical and physical maps.

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ИНТЕГРИРОВАННАЯ И КОМБИНИРОВАННАЯ ГЕНЕТИЧЕСКАЯ КАРТЫ НА ОСНОВЕ ТРЕХ СУБПОПУЛЯЦИЙ ЛИНИЙ УДВОЕННЫХ ГАПЛОИДОВ *B. RAM*

Аннотация: в работе представлены результаты сравнительного анализа основных характеристик интегрированной и комбинированной генетических карт B. rapa и эффективности выбранных подходов к их построению с использованием программного пакета JoinMap 4. Исходным материалом для создания генетических карт послужили данные молекулярного анализа (706 генетических маркеров) трех субпопуляций дигаплоидных линий DH68 F1 (89 линий), DH68F2 (19 линий) и DH68 F3 (123 линии), полученных на основе трех гибридных растений от скрещивания овощного ssp. chinensis и масличного ssp. trilocularis представителей вида B.rapa. Комбинированная генетическая карта построена на основе объеденных на одной матрице молекулярных данных сегрегационного анализа 231 линии; интегрированная - интеграцией генетических карт разработанных двух наиболее информативных популяций DH68 F1 и DH68 F3 с общим числом генотипируемых линий 212. Показано, что подход интеграции двух независимых генетических карт является более эффективным и позволяет получить генетическую карту с большим числом картированных маркеров, большей их плотностью, меньшим количеством участков, свободных от маркеров. Аккуратно выполненные генетические карты являются основой для последующего точного картирования локусов количественных признаков (OTL) и эффективной маркеропосредованной селекции.

Ключевые слова: генетическая карта, интегрированная, комбинированная, линии удвоенных гаплоидов.

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