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An integrated AFLP and RFLP *Brassica oleracea* linkage map from two morphologically distinct doubled-haploid mapping populations

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Abstract Genetical maps of molecular markers in two very different F_1-derived doubled-haploid populations of *Brassica oleracea* are compared and the first integrated map described. The F_1 crosses were: Chinese kale×calabrese (var. *alboglabra*×var. *italica*) and cauliflower×Brussels sprout (var. *botrytis*×var. *gemmifera*). Integration of the two component maps using Joinmap v.2.0 was based on 105 common loci including RFLPs, AFLPs and microsatellites. This provided an effective method of producing a high-density consensus linkage map of the *B. oleracea* genome. Based on 547 markers mapping to nine linkage groups, the integrated map covers a total map length of 893 cM, with an average locus interval of 2.6 cM. Comparisons back to the component linkage maps revealed similar sequences of common markers, although significant differences in recombination frequency were observed between some pairs of homologous markers. Map integration resulted in an increased locus density and effective population size, providing a stronger framework for subsequent physical mapping and for precision mapping of QTLs using substitution lines.

Key words *Brassica oleracea* · Integrated map · Molecular markers · Doubled-haploid · Comparative mapping

Introduction

Brassica oleracea is a vegetable *Brassica*, giving rise to a morphologically diverse range of crops including cabbage, cauliflower, broccoli, kale, kohlrabi and Brussels sprouts. The advent of molecular markers has enabled the construction of genetic linkage maps, and in some species, e.g. *Brassica napus* (Hansen et al. 1997), the subsequent marker-assisted selection of agronomically important traits. Detailed genetic maps have been constructed for several *Brassica* species using predominantly RFLPs, but also AFLPs, RAPDs, isozymes and some morphological markers (see Cheung et al. 1997 for examples). For a genus such as *Brassica*, where the cultivated species have a close genomic relationship based on interspecific hybridisation and polyploidy, examining *Brassica* genome structure and organisation is of great interest, not only in terms of locating quantitative trait loci (QTLs) or major genes controlling traits of agricultural interest, but also in clarifying *Brassica* evolution, taxonomy and synteny with related species and genera, and not the least with *Arabidopsis*.

Comparative mapping is effective in identifying homoeologous or homologous loci, and therefore collinear chromosomal segments, provided that common markers are used which segregate in at least two of the crosses under consideration. A few intraspecific comparative mapping studies involving *B. napus* (Foisset et al. 1996) and *B. oleracea* (Hu et al. 1998) exist, which examine genome organisation and the resulting level of chromosomal rearrangement within species of *Brassica*. In both examples some variation in the relative positions of homologous loci was found although, in general, a good conservation of marker orders was observed.

The integration of genetic maps is facilitated by the development of the computer software JOINMAP 2.0 (Stam 1993; Stam et al. 1995) which relies on segregating markers common to these maps. Homologous loci can easily be identified for RFLP and microsatellite markers which locate single- or low-copy number DNA. Recently, comparative mapping in barley (Waugh et al. 1997), potato (Van der Voort et al. 1997) and eucalyptus (Marquees et al. 1998) has revealed that co-migrating AFLP loci reliably represent homologous markers between mapping populations. Map integration

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can resolve variation between common markers on different maps and enable comparisons between maps to be undertaken more easily. In addition it can give insight into the reliability of marker order and map intervals. Integrated maps have been produced for *Arabidopsis thaliana* (Hauge et al. 1993) and barley (Qi et al. 1996) where 14% and 22% of the markers, respectively, were common to at least two independent data sets. In both examples no significant re-ordering of markers was found although localised rearrangements were observed.

The present paper reports the construction of the first integrated map of *B. oleracea* based on genetic maps generated from the segregation data of two very different F_1-derived doubled-haploid (DH) mapping populations. The Chinese kale×calabrese linkage map incorporates RFLP markers (Bohuon et al. 1996) with as yet unpublished AFLP and microsatellite (SSR) marker data. It comprises 433 markers mapping to nine linkage groups spanning a total map length of 889 cM (using the Kosambi (1944) function), with an average locus interval of 3.3 cM. The cauliflower×Brussels sprout map represents a novel *B. oleracea* genetic map. This is based on 223 markers mapping to nine linkage groups, with a total map length of 831 cM (Kosambi) and an average locus interval of 4.7 cM. The common polymorphic loci include RFLP, AFLP and microsatellite molecular-marker types. Map integration resulted in increased locus density and effective mapping population size. The greater density and precision will be invaluable for supporting current programmes to correlate genetical and physical maps, both within the *Brassicas*, and between *Brassica* and *Arabidopsis*. It will also provide a wider choice of markers for positional cloning.

Materials and methods

Plant material and DNA extraction

The first of two mapping populations (A12×GD), consisting of 210 DH lines, was produced from a cross between two DH parents, a rapid-cycling Chinese kale line, *B. oleracea* var. *alboglabra* (A12DHd), and a calabrese, *B. oleracea* var. *italica* (GDDH33), through microspore culture of the F_1 (Bohuon et al. 1996). A genetic map with 303 RFLP-defined loci was previously produced using a subset of 169 lines from this population (Bohuon et al. 1996). For the present analysis, 206 lines were used to generate molecular-marker data.

A second mapping population (N×G) of 97 DH lines was supplied by Horticulture Research International (HRI), Wellesbourne, UK. The two parents, DJ7032 and DJ3753, were both F_1-derived (DH) lines of *B. oleracea*. DJ7032, derived from a cauliflower line, Nedcha; *B. oleracea* var. *botrytis*, was crossed with DJ3753, derived from a Brussels sprout hybrid, Gower, *B. oleracea* var. *gemmifera*, in order to produce a set of genetically uniform F_1 progeny. The cross was carried out by bud pollination with emasculation of DJ7032 flowers. The DH lines were produced through anther culture of the F_1 (Ockendon 1984). DNA from both populations was extracted from freeze-dried leaf material as described by Sharpe et al. (1995).

Marker data

RFLP markers

Seventy eight highly informative *Brassica* RFLP probes were selected from those known to be polymorphic in the A12×GD population (Bohuon et al. 1996) and were hybridised to the N×G population. Restriction-enzyme digestion, gel electrophoresis, alkaline transfer and Southern hybridisation all were carried out as described in Sharpe et al. (1995). Genomic DNA was digested with the restriction endonuclease *Eco*R1. The 'pO', 'pR' and 'pN' probes were selected from three libraries of small *Pst*1 fragments of genomic DNA from *B. oleracea*, *Brassica rapa* and *B. napus*, respectively (Sharpe et al. 1995). A second *Pst*1 genomic DNA library from *B. napus* and an *Eco*R1 genomic library from *B. rapa* (Teutonico and Osborn 1994) provided the 'pW' probes.

AFLP markers

AFLP data were produced for both populations using identical primer combinations and methodology. Both sets of parental DNA samples were run on all gels for a strict comparison of the resulting banding patterns. The AFLP protocol developed and described by Vos et al. (1995) was followed, with minor modifications. Adapter and primer sequences were as described by Vos et al. (1995) and were synthesised by Operon Technologies Inc. The PCR pre-amplification of adapter-ligated restriction fragments was performed using the primer combination E+A/M+C (The code following E or M corresponds to the selective nucleotides at the 3´ end of the *Eco*R1 or *Mse*1 primers respectively). Selective amplification followed, as described by Vos et al. (1995), for two and three selective nucleotides, except that the annealing temperature was reduced from 65°C to 56°C over ten cycles in steps of 1°C. The primer combinations used were, E+AC with M+CAA, M+CAC, M+CAG, M+CAT, M+CTA or M+CTC and E+AA/M+CAT. The PCR reaction mixture was the same as for pre-amplification except that in each case 5 ng of *Eco*R1 primer, end-labelled with [γ^{33}P] ATP using T4 polynucleotide kinase, was used. After electrophoresis (Vos et al. 1995), the gel was removed using a 3MM Whatman filter paper, dried under vacuum for about 1 h at 80°C, and autoradiography was carried out with Kodak Biomax film at room temperature for 2–7 days depending on signal intensity.

Microsatellite markers

Five primer pairs were used to generate microsatellite molecular markers in both the N×G and A12×GD populations. BN12A, BN72A, BN83B1 and BN35D (Altabioscience) from *B. napus* (Szewc-McFadden et al. 1996), and primer nga111 (Research Genetics) from *A. thaliana* (Bell and Ecker 1994) were used with specific PCR annealing temperatures of 54°C, 63°C, 52°C, 51°C and 53°C, respectively. PCR was performed in a 25-µl reaction mixture composed of 50 ng of template DNA, 1.0-µM of each primer (the forward primer of each pair was end-labelled with [γ^{33}P]ATP using T4 polynucleotide kinase), 200-µM of each dNTP, 0.6 U of *Taq* polymerase, 1×KCl reaction buffer (including 1.5 mM $MgCl_2$) and 0.012% formamide. PCR reactions had one denaturing step of 1 min 10 s at 94°C, followed by 30 cycles of 20 s at 94°C, 2 min of specific annealing temperature, 1 min 30 s at 72°C with a final cycle of 3 min 30 s at 72°C and a holding temperature of 25°C. Electrophoresis was carried out as for AFLPs, but at a constant power of 80 W.

Linkage analysis and map integration

Data were generated for all three marker types for all 97 lines of the N×G population. In the A12×GD population, 84 of the 206 lines had data for all three marker types, 112 lines possessed

RFLP data only and ten lines had AFLP and microsatellite data only. Polymorphic loci were identified by comparing DNA banding patterns within the two pairs of parental lines. Data quality was assured by only recording clear, unambiguous bands for each marker type and by scoring all gels independently on two separate occasions.

Separate linkage analyses were carried out for both mapping populations using JoinMap 2.0 (Stam 1993; Stam and van Ooijen 1995) with the parameters set for DH-derived progeny. For the A12×GD segregation data, markers were assigned to linkage groups with LOD scores ≥4.5. For the N×G population, markers were assigned to linkage groups with LOD scores ≥6.3. Some of the groups were merged when markers were linked to more than three other markers in another group by a LOD score ≥4.0. The LOD score ≥7.5 was used to separate two groups associated through a skewed segregation of markers. Map distances were calculated using Kosambi's (1944) mapping function. Any data points involved in improbable double-recombination events were re-scored against the original autoradiographs.

Integration of the N×G and A12×GD linkage maps was also achieved using JOINMAP 2.0 after merging the two data files containing the pairwise recombination frequencies and corresponding LOD scores. The linkage groups were constructed using DRAWMAP (Van Ooijen 1994).

Results

Integrated map

An integrated genetic linkage map of *B. oleracea* was generated based on 547 markers mapping to nine linkage groups covering a total map length of 893 cM using the Kosambi function (or 1042 cM using the Haldane function) (see Fig. 1[1]). Six loci were removed by JoinMap analysis due to internal conflicts within the linkage groups. Comparison of the RFLP loci with the original A12×GD map allowed the order and orientation of the corresponding linkage groups to be established and numbered LG1 through to LG9 (Bohuon et al. 1996). This map is referred to as the integrated map, and summary data together with that of the component N×G and A12×GD linkage maps are given in Table 1.

Integration of the N×G and A12×GD linkage maps has resulted in the production of a high-density composite map covering most (99%) of the *B. oleracea* genome, based on a chiasma frequency of 18 chiasmata per meiosis, as scored in the F_1 of the A12×GD cross (G.H. Jones, personal communication), giving an expected map length of 900 cM. The number of markers in the integrated map has increased by 114 from the A12×GD map, which results an the increased locus density of the integrated map. The number of loci increased by 75, from 275 loci in the A12×GD map to 350 in the integrated map, and therefore the average locus interval decreased from 4.6 cM and 3.2 cM in the N×G and A12×GD maps, respectively, to 2.6 cM in the integrated map. Clustering, involving more than five markers mapping to the same position on the linkage group, was observed on LG1 (51 cM), LG2 (70–73 cM), LG5 (51 cM), LG7 (13 cM), LG8 (9 cM) and LG9 (56 cM). Except for the cluster at the top of LG8, which involved only RFLP markers, all other cases incorporated both AFLP and RFLP loci. In contrast, some areas of the linkage groups remain low in marker density, such as the top of LG1 and LG2, and the bottom of LG6. The largest marker interval of 31 cM is observed at the top of LG2.

Comparison of integrated map with NxG and A12xGD maps

The 78 RFLP probes used to generate marker data for the N×G mapping population were a subset of those already segregating in the A12×GD map. These common RFLP probes resulted in 91 and 134 segregating loci in the N×G and A12×GD maps, respectively. Out of a total of 163 different polymorphic loci identified between the N×G and A12×GD maps, 62 were common to both populations, 29 were only polymorphic in the N×G cross, while the remaining 72 loci only showed segregation in the A12×GD cross. Ten (12.8%) of the probes were monomorphic in the NxG cross. Because of the replicated nature of the *B. oleracea* genome, banding patterns of the parental lines for the multiple-copy clones were checked to ensure that only homologous loci were being compared. Similarly, in producing AFLP and microsatellite segregation data, identical primer combinations were utilised. Bands migrating to identical positions on the gel and segregating in both pairs of parents were considered to be homologous markers. From the seven AFLP primer combinations used, a total of 126 and 127 segregating loci were scored in the N×G and A12×GD populations, respectively, averaging 18 polymorphic loci per primer combination for both populations. With regard to the microsatellite data, the four common primer pairs each generated a single segregating locus in each cross, resulting in four homologous markers.

A simple correlation analysis involving the position of the homologous markers in the N×G and A12×GD linkage maps revealed close conservation of marker order even though the populations were derived from very different genetic backgrounds. Correlation coefficients (r) for all nine linkage groups ranged from 0.91 to 0.99 with a mean of r=0.97. Scattergrams for linkage group 2 (r=0.91) and linkage group 3 (r=0.99) are shown in Fig 2. Direct observation of collinearity, involving the alignment of maps of the joint markers for the integrated map and both component maps (Fig. 3 shows the results for LG2 and LG3), emphasises the lack of major structural alterations between the two *B. oleracea* populations. Marker order was identical in all three maps for linkage groups 1 and 5. Linkage groups 4, 6, 7, 8 and 9 each had a single-sequence simple inversion spanning less than 3 cM. Linkage group 2 exhibited several sequence inversions over very short cM distances which is reflected in the more scattered nature of its correlation analysis (Fig. 2). A rearrangement was also observed at the top of joined linkage group 2 involving the loci pO85J2 and

[1] See WWW.biology.bham.ac.uk/brassica_map/ for more detailed diagrams of the integrated map, including marker names, and for the alignment of the integrated map with the two component linkage maps for all nine linkage groups

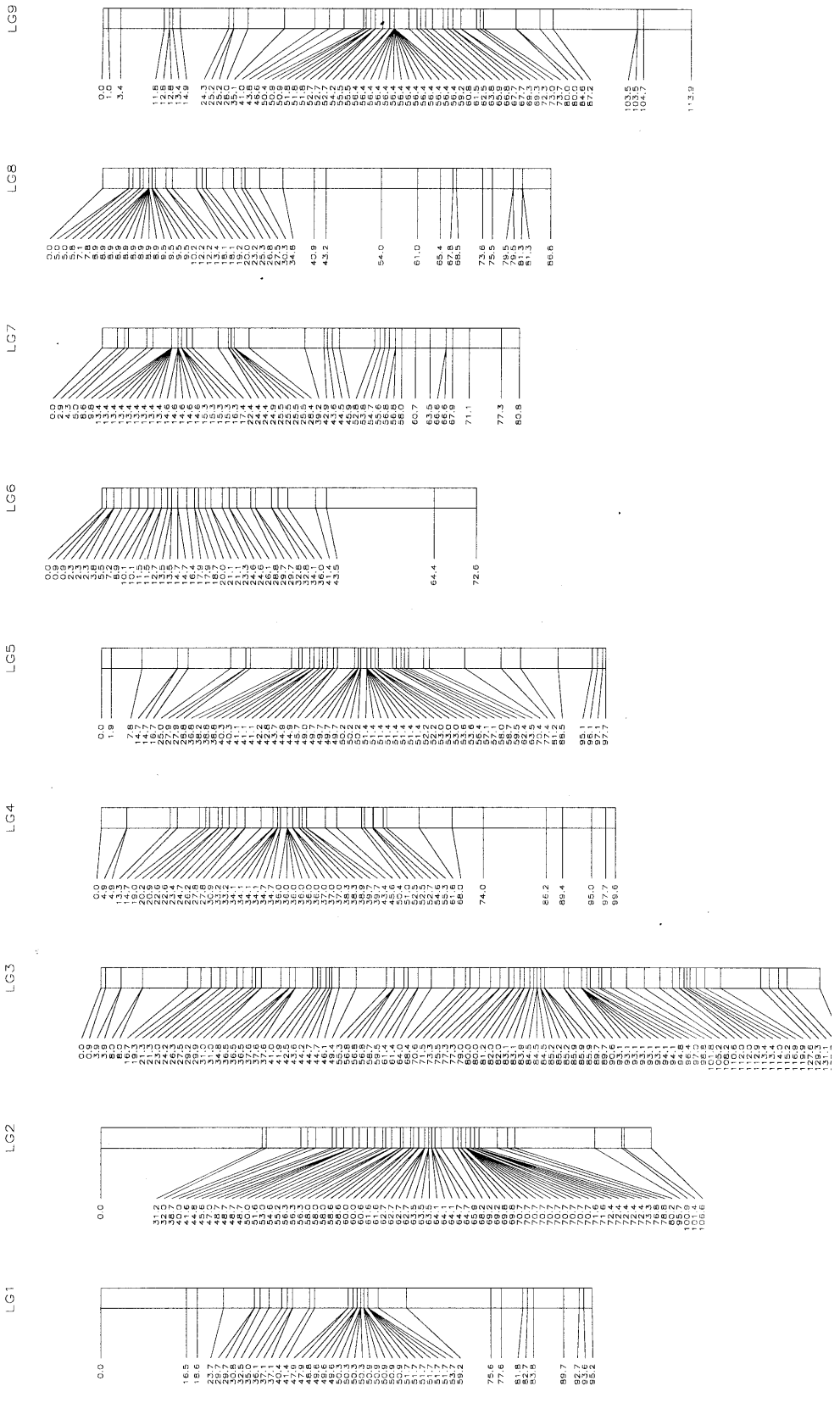

Fig. 1 Integrated linkage map of *B. oleracea*. Linkage groups are numbered *LG1 through to LG9*, with their order and orientation as defined by Bohuon et al. (1996). Recombination distances are in Kosambi's cM. Only the marker positions are shown here, illustrating the high marker-density and good coverage obtained through map integration

Table 1 Comparison of linkage group size, number of markers, average marker interval and distribution of RFLP, AFLP and SSR marker types across the linkage groups of the integrated, A12×GD and N×G *B. oleracea* linkage maps. * includes one morphological marker for flower colour

LG	No. loci	Length cM	Average marker interval cM	No. RFLPs	No. AFLPs	No. SSRs	No. joint markers
Integrated map							
1	46	95.2	3.28	28	18	0	7
2	74	106.6	2.67	38	36	0	18
3	101*	139.0	1.99	61	38	1	24
4	55	99.0	2.68	34	21	0	9
5	64	97.7	2.44	35	29	0	11
6	41	72.6	2.42	18	20	3	7
7	55	80.8	2.38	37	16	2	7
8	47	86.8	2.80	30	16	1	10
9	64	113.9	2.92	45	18	1	12
$\bar{x}/\Sigma$	547	892.6	2.62	326	212	8	105
A12×GD Map							
1	32	108.6	4.72	24	8	0	
2	58	116.2	3.63	37	21	0	
3	86*	140.1	2.50	56	28	1	
4	45	100.1	3.34	33	15	0	
6	33	72.5	2.90	17	13	3	
7	42	72.0	2.88	33	8	1	
8	39	79.3	3.60	29	10	0	
9	53	106.8	3.14	40	12	1	
$\bar{x}/\Sigma$	433	888.5	3.34	299	127	6	
N×G Map							
1	21	70.3	5.40	9	12	0	
2	34	59.7	3.14	9	25	0	
3	39	142.3	3.85	19	19	1	
4	21	87.4	5.83	6	15	0	
5	30	107.7	4.49	12	18	0	
6	15	56.1	4.68	3	11	1	
7	20	97.0	4.85	9	9	2	
8	18	106.8	5.93	9	8	1	
9	25	104.0	4.52	15	9	1	
$\bar{x}/\Sigma$	223	831.3	4.74	91	126	6	

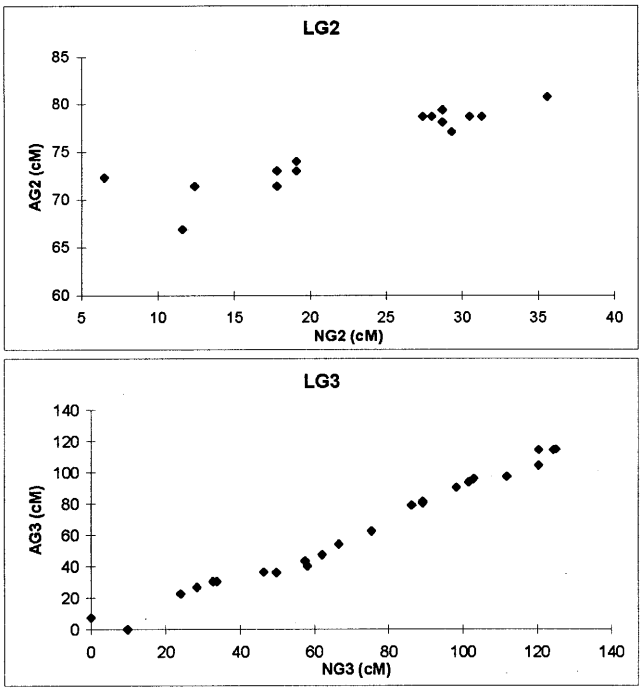

Fig. 2 Scattergrams showing the correlation between homologous loci of A12×GD and N×G for linkage group 2 (r^2=0.83) and linkage group 3 (r^2=0.98)

pR86J1 which are 5.1 cM apart. Two apparent simple inversions were found on linkage group 3 spanning less than 1 cM in both cases. However, an apparent inversion spanning 10 cM was observed at the top of linkage group 3 involving the markers pW153J1 and pW116J1. The majority of the observed rearrangements involved very short map distances and hence are likely to be artefacts of the mapping process. Joinmap gives only the most likely sequences as output, without revealing other likely orders.

The homology of linkage groups revealed by the common RFLP, AFLP and microsatellite markers showed that the N×G, A12×GD and integrated map had regions covered by homologous loci spanning in total 683 cM (82%), 567 cM (64%) and 558 cM (63%) of their genomes respectively. Linkage group 2 showed the shortest region of homology spanning distances of only 27 cM (N×G), 14 cM (A12×GD) and 17 cM (Integrated map) of each linkage group. Linkage groups 3, 5, 7 and 8 revealed conserved regions that spanned more than 80% of the linkage group in all three maps. A contingency chi-square test, based on the recombination frequency between all pairs of homologous loci, revealed that in 91% of cases the distance between pairs of collinear loci did not differ significantly (P>0.01) between the N×G and A12×GD linkage maps. In 66% of the significantly different collinear loci the N×G recombination frequen-

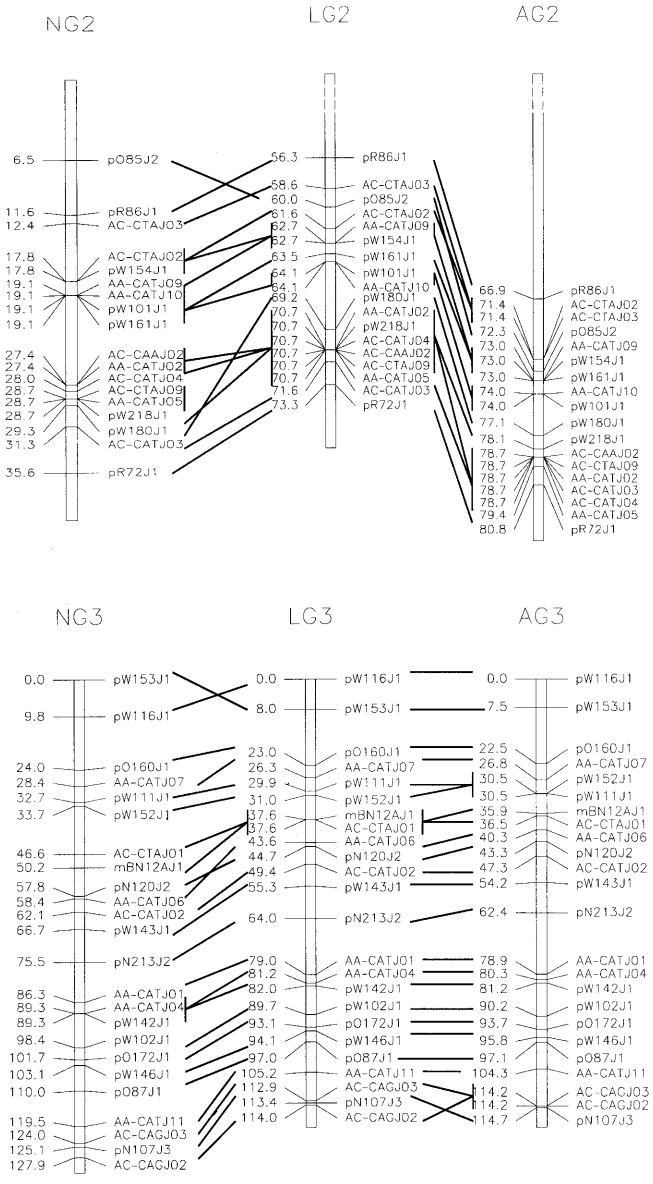

Fig. 3 Comparison of marker order of the component A12×GD and N×G linkage maps with the integrated map for linkage groups 2 and 3.The linkage groups of the A12×GD and N×G maps are numbered AG2 and AG3, and NG2 and NG3 respectively. Only common markers are shown. The RFLP markers are denoted by the prefix '*p*' and are named after the probe used for hybridisation. Multiple loci resulting from hybridisation with a single probe are given identical names with the addition of a number *suffix*. The microsatellite markers have the prefix '*m*' and are similarly named after their primer pair. Nomenclature of the AFLP markers is based on their primer extensions with band number, denoted by a *suffix*, corresponding to the polymorphic bands numbered sequentially in order of increasing mobility. The upper case '*J*' refers to markers common to the component linkage maps

cies were significantly greater than those of A12×GD. Also it did not necessarily follow that the regions encompassing the apparent inversions accounted for the discrepancies observed in the map distances. Linkage group 1, which had identical marker orders in all three maps, had significant differences in recombination frequency for a number of pairs of markers. In contrast, linkage group 7, which has an apparent inversion at the bottom of the linkage group, had no significant differences in recombination frequency.

Discussion

This is the first reported integrated genetic map of *B. oleracea* derived from two distinct DH mapping populations. Based on 105 homologous loci common to the component A12×GD and N×G linkage maps, integrated mapping has served to increase the total number of markers in the integrated map by 114 from the A12×GD linkage map, thus providing an effective method of producing a good-quality, high-density consensus linkage map of the *B. oleracea* genome.

AFLP marker data showed very similar levels of polymorphism in both the A12×GD and N×G mapping populations, as approximately equal numbers of segregating markers resulted from the seven randomly chosen primer combinations emloyed. All the RFLP probes hybridised on to the N×G population had already been shown to segregate in the A12×GD population, thereby aiding the identification of homologous loci between the two component linkage maps. As expected, a proportion of these probes were monomorphic in the N×G population, giving the impression of greater variation in the A12×GD population compared with the NxG material. In identifying homologous loci, for both RFLP and AFLP markers, the ability to refer back to the original autoradiographs was invaluable.

The present study has shown that AFLP markers can be successfully used across populations for comparative mapping and therefore map integration, based on the assumption that fragments migrating to the same position on a gel constitute homologous loci (Van der Voort et al. 1997; Waugh et al. 1997). Sequence information from homologous AFLP bands across species and genomes confirms this result (P.S. Virk, personal communication). AFLPs proved useful in filling gaps in the integrated map remaining from mapping solely RFLP markers, although, some large intervals still exist. For example, a large (31.2 cM) gap remains at the top end of LG2 and between 43.5 cM and 64.4 cM of LG6. These intervals may indicate regions of high recombination which may prove difficult to populate no matter how many new markers are added to the map. Previous mapping studies based on AFLPs have reported much clustering, particularly around the centromeres (Marques et al. 1998; Qi et al. 1998) where recombination may be suppressed (Tanksley et al. 1992). The clustering of AFLPs may be linked to regions of repetitive DNA where several markers are located at the same site regardless of the primer sequence employed. In the current study 43% of AFLP markers mapped to unique positions on the integrated map. The remainder mapped to the same locations as other AFLP or RFLP markers. No more than three AFLP markers were clustered at a single locus without other marker types also being present, and the clustered AFLPs did not always arise from the same primer combinations.

The existence of two AFLPs at a single locus which are in repulsion can be very useful because jointly they behave as a co-dominant marker. On 22 occasions clustering of AFLPs effectively produced co-dominant markers for either the N×G or A12×GD populations. The addition of substantial numbers of AFLP loci to the component maps, and therefore the integrated map, will be useful for future work involving *Brassica* BAC libraries. As an easy to use PCR-based marker, AFLPs could be employed to screen clones for specific fragments from the genomic DNA. Although denser maps do not help QTL location in segregating populations (Hyne et al. 1995), they will be particularly valuable for identifying end points of donor regions in backcross-derived substitution lines. This, in turn, will facilitate precision QTL mapping.

Conservation of marker order between the N×G, A12×GD and integrated maps is good and gives greater confidence in the reliability of marker order. Some localised differences in map order were observed as with the *Arabidopsis* integrated map (Hauge et al. 1993). These differences are unlikely to represent real variation between the N×G and A12×GD linkage maps as Joinmap gives a 'best fit' map as output, when in reality several sequences may be almost equally likely (Hauge et al. 1993). However, significant variation in the recombination frequency between pairs of homologous markers occurred regardless of any rearrangements in marker order. Identical orders of homologous markers were observed in all three maps for linkage group 1 despite significant differences in recombination frequency for a number of pairs of markers between the maps of linkage group 1. This could be due to shifts in the chiasma frequency of the parental subspecies. It has been reported that A12 has an extra nucleolar-organising region (NOR) (Armstrong et al. 1998) compared with other varieties of *B. oleracea*, but so far the physical and genetical maps have not been fully aligned. This may result in one region of suppressed recombination in the A12×GD map, accounting for one of the differences in observed recombination frequency between this and the N×G linkage map.

The similarity of the A12×GD, N×G and the integrated linkage maps, means that the integrated map will have many uses as a *B. oleracea* consensus genetic map. It will help solve the problem of markers being polymorphic in one population and not another by identifying other markers as substitutes in the same region. Additionally, the close similarity of the A12×GD and N×G linkage maps will allow inferences of gene or QTL positions to be made across populations.

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