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Construction of an oilseed rape (*Brassica napus* L.) genetic map with SSR markers

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Abstract We constructed a *Brassica napus* genetic map with 240 simple sequence repeats (SSR) primer pairs from private and public origins. SSR, or microsatellites, are highly polymorphic and efficient markers for the analysis of plant genomes. Our selection of primer pairs corresponded to 305 genetic loci that we were able to map. In addition, we also used 52 sequence-characterized amplified region primer pairs corresponding to 58 loci that were developed in our lab. Genotyping was performed on six F2 populations, corresponding to a total of 574 F2 individual plants, obtained according to an unbalanced diallel cross design involving six parental lines. The resulting consensus map presented 19 linkage groups ranging from 46.2 to 276.5 cM, which we were able to name after the *B. napus* map available at http://ukcrop.net/perl/ace/search/BrassicaDB, thus enabling the identification of the A genome linkage groups originating from the *B. rapa* ancestor and the C genome linkage groups originating from the *B. oleracea* ancestor in the amphidiploid genome of *B. napus*. Some homoeologous regions were identified between the A and the C genomes. This map could be used to identify more markers, which would eventually be linked to genes controlling important agronomic characters in rapeseed. Furthermore, considering the good genome coverage we obtained, together with an observed homogenous distribution of the loci across the genome, this map is a powerful tool to be used in marker-assisted breeding.

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Introduction

Genetic linkage maps offer the possibility of developing genetic studies on various agronomic traits through the localization of major genes and quantitative trait loci (QTLs), as well as helping breeding programs with marker-assisted selection (MAS). In addition, positioning molecular markers on a genetic map is of great help when evaluating genetic diversity of resources and establishing genetic relationships between cultivars in order to carry out the optimal breeding strategy. Homogenous distribution of markers and good genome coverage are two central desirable features of markers in this case.

A map using multiple segregating populations has many advantages over a map based on a single population. The former can map a larger number of loci, thus providing a higher number of potential useful markers in various genetic backgrounds. Consequently, greater genome coverage is obtained. In *Brassica napus*, many genetic maps based on single populations were reported (Landry et al. 1991; Ferreira et al. 1994; Parkin et al. 1995; Sharpe et al. 1995; Uzunova et al. 1995; Foisset et al. 1996; Cheung et al. 1997). Furthermore, some studies reported maps based on multiple populations. Parkin and Lydiate (1997) generated an integrated map (using only RFLP markers) using two populations with three different parents. Lombard and Delourme (2001) used three populations to construct a map of 540 RFLP, AFLP or RAPD loci spread over 19 linkage groups, covering a total distance of 2,429 cM. In addition, Udall et al. (2005) aligned RFLP maps from four independent mapping populations.

As far as PCR markers are concerned, AFLP and RAPD have complex electrophoretic patterns. Furthermore, their heritability is dominant; they are biallelic

and hence poorly polymorphic. For these reasons, they are not easy to use in MAS, and PCR-specific markers need to be developed for targeted applications. Microsatellites, defined as simple sequence repeats (SSR), are randomly interspersed within eukaryotic genomes. They are highly variable with regard to repeat number, mostly co-dominant and highly efficient in the pedigree analysis of most crops (Plieske and Struss 2001). Microsatellite loci have already been mapped by different teams (Uzunova and Ecke 1999; Lowe et al. 2004) relative to a framework of RFLP markers. In this article, we describe the construction of an SSR-based *B. napus* map. Our ambition was to map the robust and polymorphic markers that were available to us on reliable linkage groups with precise distances. To elaborate this map, we used six F2 populations, corresponding to a total of 574 plants, obtained according to an unbalanced diallel cross design involving six parental lines chosen with regards to their genetic distance, to optimize the polymorphism rate. All the SSR markers used in this work are publicly available (sequences provided as Electronic Supplementary Material), the originally private SSR primer pair sequences having been released to the public in June 2005. Furthermore, we consolidated our genotyping data with those obtained with sequence-characterized amplified region (SCAR) markers developed in our laboratory. The resulting consensus map presented 19 linkage groups which we were able to name after the *B. napus* map available at http://ukcrop.net/perl/ace/search/BrassicaDB. We positioned a total of 363 genetic loci which were distributed in a relatively homogenous manner across the genome. The occurrence of homoeologous loci allowed us to indicate some regions of homoeology.

Materials and methods

Mapping populations

Six F2 populations were obtained as shown in Table 1. Parental lines A, B and C are spring-type rapeseed lines (Euralis Semences proprietary germplasm, available on request under Material Transfer Agreement for research purposes only), whereas D, E and F, respectively, Comet (INRA), Vivol (CARGILL) and Jaguar (Ets. Lecureur), are winter-type rapeseed lines. These lines were chosen after a study on their genetic distances, based on the results obtained with a subset of SSR markers (data not shown). The number of genotyped plants per population is as reported in Table 1. These six populations were primarily chosen because of their high rate of polymorphism. Furthermore, the choice of these populations implied that each parent was involved in two different crosses and thus, when only one line displayed a different allele for a locus, as compared with the allele from the five other lines, two populations were polymorphic for this locus. A table provided as Electronic Supplementary Material summarizes which populations were genotyped for each primer pair.

Table 1 Unbalanced diallel cross design representing the lines used in the nine crosses (A, B, C, D, E, F) and the total number of genotyped F2 plants

	B	C	D	E	F
A	92	–	93	–	–
B		–	–	–	101
C			96	100	–
D				–	–
E					92

Genetic markers

Public SSR primer pair sequences were obtained at http://ukcrop.net/perl/ace/search/BrassicaDB. Most of these SSR are originally from the BBSRC microsatellite programme (a collaboration between IACR Long Ashton Research Station and the John Innes Centre). They have different genomic sources (*B. rapa*, *B. oleracea*, *B. napus* and *B. nigra*, respectively, prefixed by Ra, Ol, Na and Ni). A48350 and S77096 are two accessions on which primer pairs were designed, leading to the corresponding markers. Primer pairs denominated "BRAS" followed by three numbers and "CB" followed by five numbers were developed by Celera AgGen, the following rapeseed breeding companies providing the funding: Advanta, Koipesol, Calgene, Caussade, Danisco, DLF, Euralis, Limagrain, KWS, Syngenta, Pioneer, PGS, Monsanto, Seminis, Serasem and SW seeds. Primer pairs prefixed "MR" and "MD" were developed by the Institute of Agronomy and Plant Breeding of the University of Goettingen. Funding was provided by the German Federal Ministry for Education and Research and the companies of the Rapool-Ring-GmbH. All these primer pair sequences, as well as the allele sizes, are provided as Electronic Supplementary Material.

In addition, we included in our work a set of SCAR markers, prefixed "RCS", developed in our lab. These were very useful, in some situations, to bridge large intervals. When a primer pair corresponded to more than one mapped genetic locus, the chosen nomenclature for the different loci was the one for the primer pair, eventually followed by a letter to distinguish the different loci, for instance primer pair Ol12-F11 corresponded to two genetic loci Ol12-F11A and Ol12-F11B.

DNA was extracted according to Dellaporta et al. (1983). PCR reactions were performed in 384-well plates with a volume of 10 µl. The composition of the mix was the following: Taq DNA polymerase (Eurobio) 0.0125 U/µl, Eurobio Taq buffer ten-times diluted, $MgCl_2$ 3 mM, dNTP 0.2 mM, forward primer coupled to a 19-base tail in 5′ 0.02 µM, reverse primer 0.4 µM and the 19-base tail primer with the 700 nm or the 800 nm chromophore (IR_700 or IR_800) 0.4 µM.

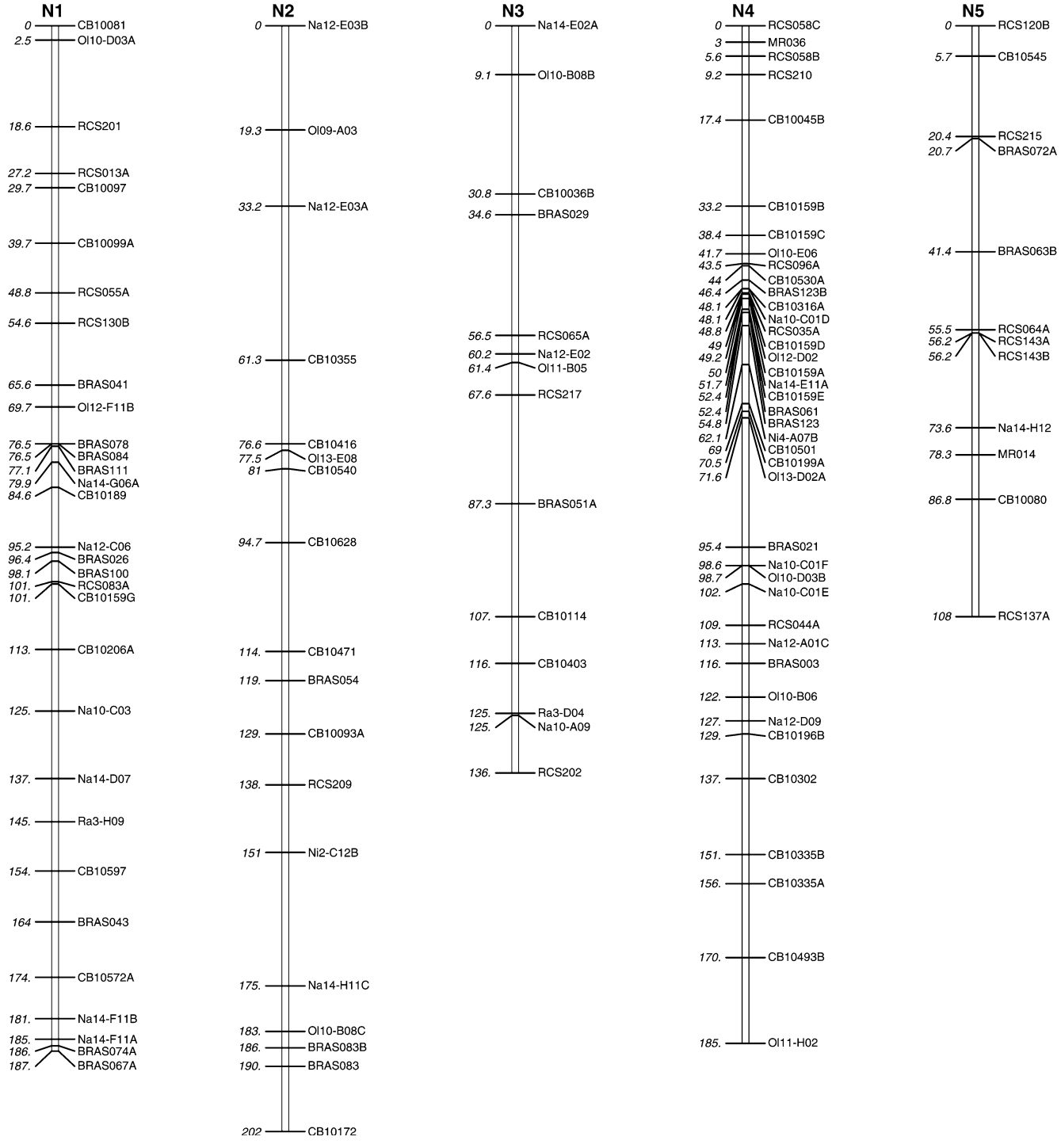

Fig. 1 Representation of the *B. napus* map. The cM distances reported on the *left part* of each linkage group have been calculated from recombination frequencies according to Kosambi

DNA was present in the PCR reaction to a concentration of 1 ng/µl. The PCR was performed on an MJ research PTC-200 thermal cycler with the following program: 94°C for 4 min; 15 cycles with 94°C denaturation, 60°C annealing, 72°C elongation, 1 min each step, with a 0.7°C decrease in annealing temperature at each cycle; then 23 cycles with 94°C for 0.5 min, 53°C for 0.5 min and 72°C for 1 min; then a final elongation step of 5 min. PCR products were loaded on a LICOR DNA Analyzer (gene readir 4200), using 18 cm polyacrylamide electrophoresis gels according to the recommendations of the manufacturer.

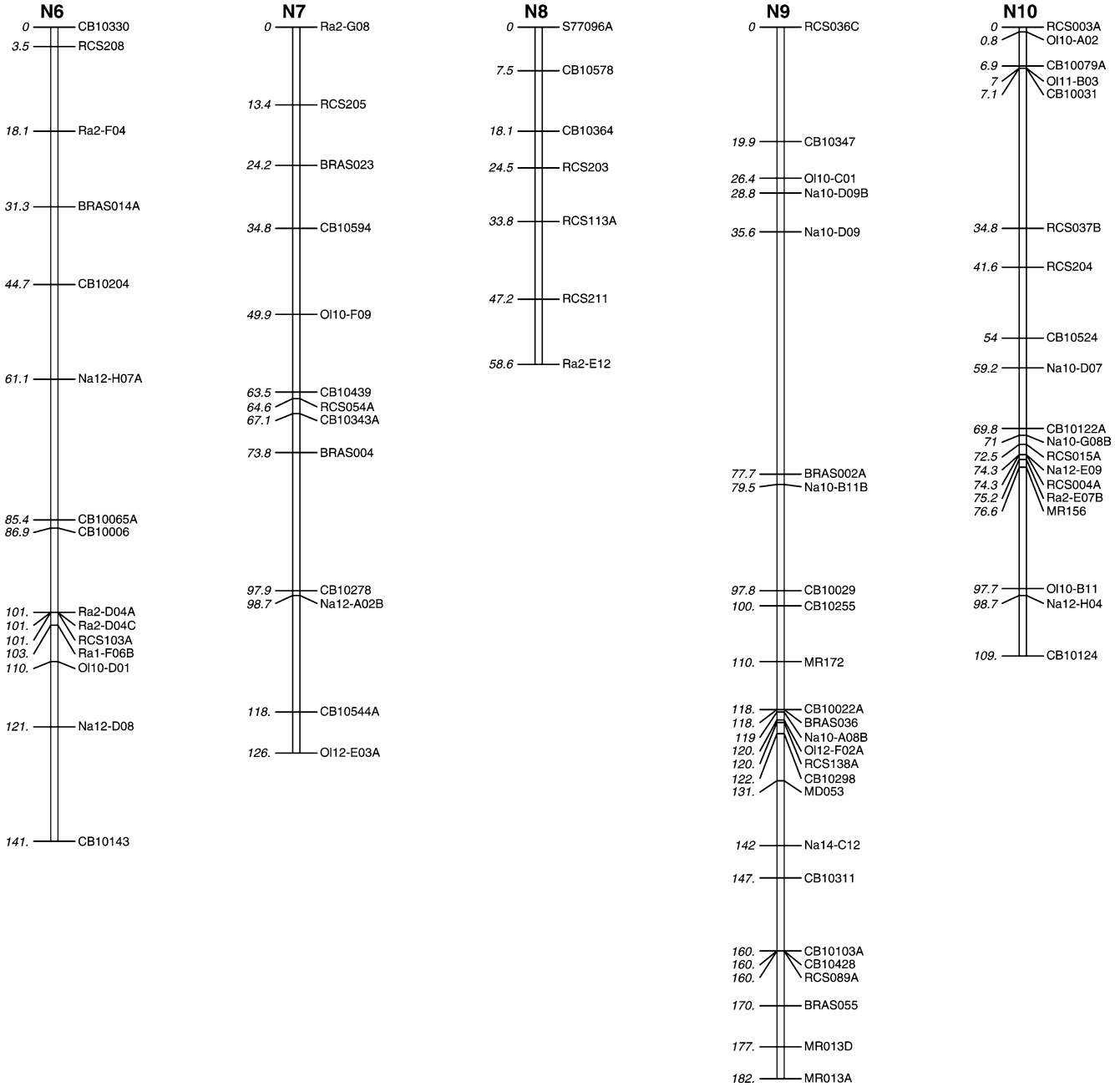

Fig. 1 (Contd.)

Linkage analysis

Genotyping data were assembled on a unique file to elaborate the consensus map. At first, MAPMAKER/EXP 3.0 was used to build the consensus map. A minimum LOD score of 4.0 with a maximum genetic distance of 30 cM was first used to associate SSR loci into initial linkage groups, then the LOD score was reduced to bridge some intervals. A full multipoint linkage analysis was performed to determine the most probable locus order for each linkage group. The marker order in linkage groups was slightly modified in some cases after running the software CARTHAGENE (Schiex and Gaspin 1997), using the flips and polish commands. "Flips" applies all possible permutations in a sliding window on the current best map and reports likelihood variations. This command was performed with the arguments 5/1/1 for, respectively, the size of the sliding window, the maximum difference of log likelihood with the best map to report the permutation and a Boolean indicating that the flips command should be iterated as long as a new improved map has been found. "Polish"

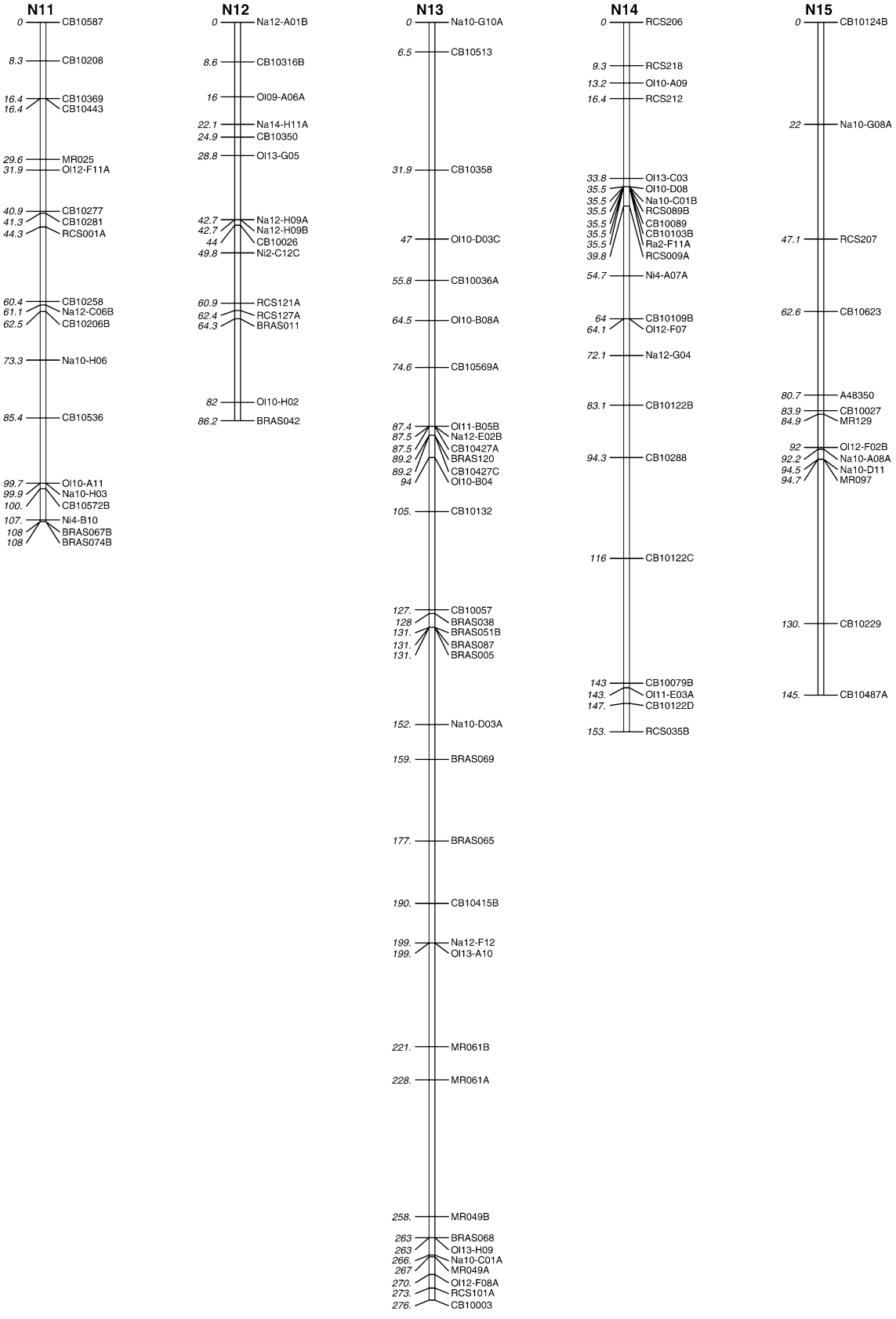
N11
0 CB10587
8.3 CB10208
16.4 CB10369
16.4 CB10443
29.6 MR025
31.9 OI12-F11A
40.9 CB10277
41.3 CB10281
44.3 RCS001A
60.4 CB10258
61.1 Na12-C06B
62.5 CB10206B
73.3 Na10-H06
85.4 CB10536
99.7 OI10-A11
99.9 Na10-H03
100. CB10572B
107. Ni4-B10
108 BRAS067B
108 BRAS074B
N12
0 Na12-A01B
8.6 CB10316B
16 OI09-A06A
22.1 Na14-H11A
24.9 CB10350
28.8 OI13-G05
42.7 Na12-H09A
42.7 Na12-H09B
44 CB10026
49.8 Ni2-C12C
60.9 RCS121A
62.4 RCS127A
64.3 BRAS011
82 OI10-H02
86.2 BRAS042
N13
0 Na10-G10A
6.5 CB10513
31.9 CB10358
47 OI10-D03C
55.8 CB10036A
64.5 OI10-B08A
74.6 CB10569A
87.4 OI11-B05B
87.5 Na12-E02B
87.5 CB10427A
89.2 BRAS120
89.2 CB10427C
94 OI10-B04
105. CB10132
127. CB10057
128 BRAS038
131. BRAS051B
131. BRAS087
131. BRAS005
152. Na10-D03A
159. BRAS069
177. BRAS065
190. CB10415B
199. Na12-F12
199. OI13-A10
221. MR061B
228. MR061A
258. MR049B
263 BRAS068
263 OI13-H09
266. Na10-C01A
267 MR049A
270. OI12-F08A
273. RCS101A
276. CB10003
N14
0 RCS206
9.3 RCS218
13.2 OI10-A09
16.4 RCS212
33.8 OI13-C03
35.5 OI10-D08
35.5 Na10-C01B
35.5 RCS089B
35.5 CB10089
35.5 CB10103B
35.5 Ra2-F11A
39.8 RCS009A
54.7 Ni4-A07A
64 CB10109B
64.1 OI12-F07
72.1 Na12-G04
83.1 CB10122B
94.3 CB10288
116 CB10122C
143 CB10079B
143. OI11-E03A
147. CB10122D
153. RCS035B
N15
0 CB10124B
22 Na10-G08A
47.1 RCS207
62.6 CB10623
80.7 A48350
83.9 CB10027
84.9 MR129
92 OI12-F02B
92.2 Na10-A08A
94.5 Na10-D11
94.7 MR097
130. CB10229
145. CB10487A

Fig. 1 (Contd.)

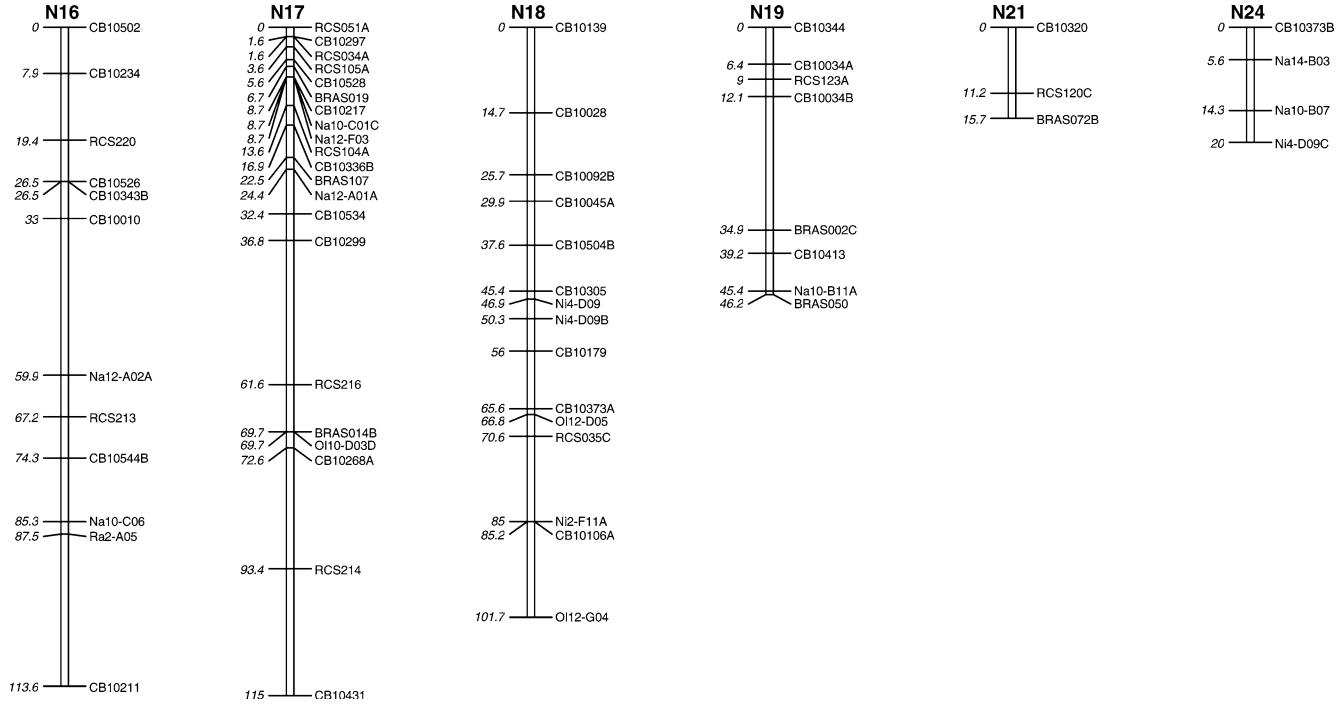

Fig. 1 (Contd.)

displays each marker in all possible intervals. Genetic distances were calculated according to the Kosambi formula.

Results

Polymorphism of SSR and SCAR markers

A total of 911 SSR primer pairs were tested on the six progenitors of the different F2 populations to determine their polymorphisms. For 267 primer pairs, no substantial amplification products were observed with our PCR program. These primer pairs were not used in further work. Of the remaining SSR, 48 were unspecific as shown by smears or very complex electrophoretic patterns. The remaining 596 primer pairs were analysed for polymorphism and carefully selected with a priority towards codominant markers. Using these criteria, 240 primer pairs were selected. It is noteworthy that among them were six primer pairs originating from *B. nigra* (B-genome according to U 1935), corresponding to nine genetic loci, suggesting that markers originating from *Brassica* species other than *B. rapa*, *B. oleracea* or *B. napus* can be mapped on the rapeseed genome. Out of the 240 SSR, 190 displayed one polymorphic genetic locus, eventually accompanied by a monomorphic locus, and 50 SSR corresponded to two or more loci. A total of 305 SSR markers were finally found polymorphic on our six F2 populations. Out of the 125 SCAR markers, we were able to distinguish a total of 52 polymorphic primer pairs, corresponding to 58 polymorphic loci that we were able to map. Three populations were polymorphic, on average, for each SCAR marker whereas the average of polymorphic population was higher (3.5) for the SSR markers. Altogether, a total of 363 genetic loci were used to elaborate the map.

Construction of the consensus map

High proportion of distorted loci is a frequent feature in genotyping analyses when using doubled haploid lines (Foisset and Delourme 1996), and this can trigger problems in mapping analyses, since genetic distances are skewed. Here we performed our work on F2 populations, and 14 loci out of 363 displayed segregation distortions on at least one of the six populations. However, these distortions only concerned one of the populations in each case and considering the small number of such loci, we chose to keep them in the datasets, since they would not have a significant skewing effect on the map. The distribution of these loci did not seem to be restricted to some clusters but rather were randomly interspersed over the genome (data not shown).

Nineteen major linkage groups (N1–N19) were obtained (Fig. 1). A total of 356 genetic loci were positioned on these major linkage groups. In addition, we obtained two small (less than 20 cM) linkage groups N21 and N24 carrying three and four loci, respectively. By adding the length of all linkage groups, we obtained a total distance of 2,619 cM. The numbering of the 19 major groups was based on the map of Parkin et al.

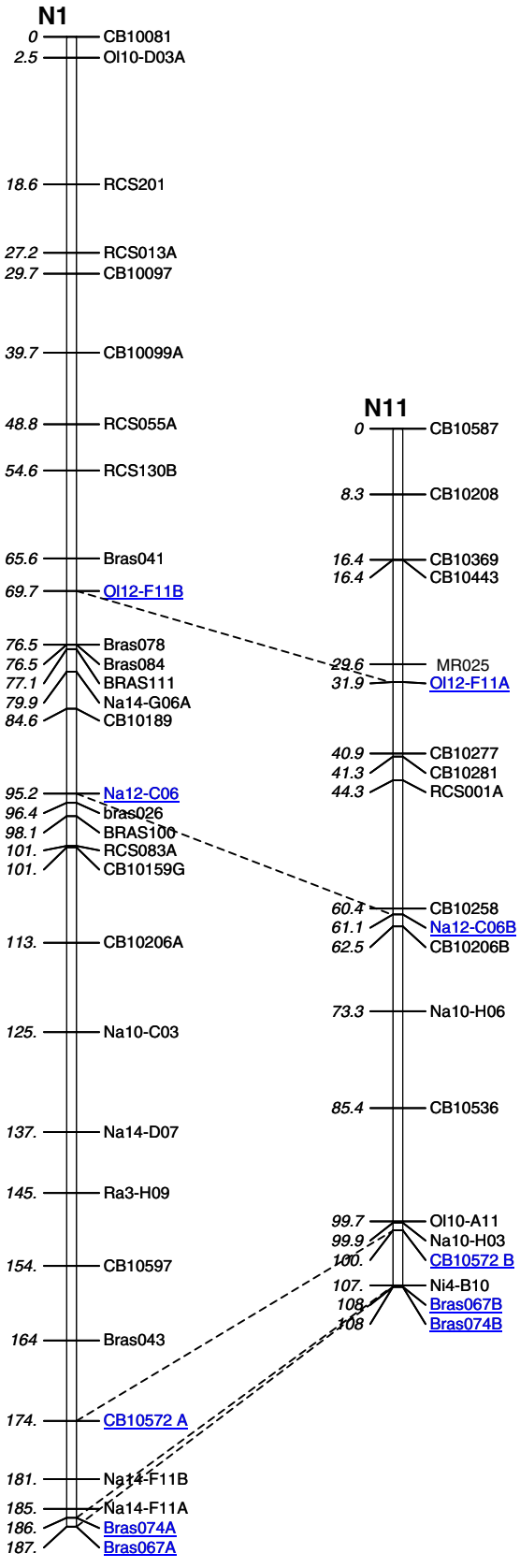

Fig. 2 Alignment of homoeologous markers in linkage groups N1 and N11. Homoeologous markers are *underlined* and joined by *dashed lines*. Genetic distance (cM) is reported in Kosambi

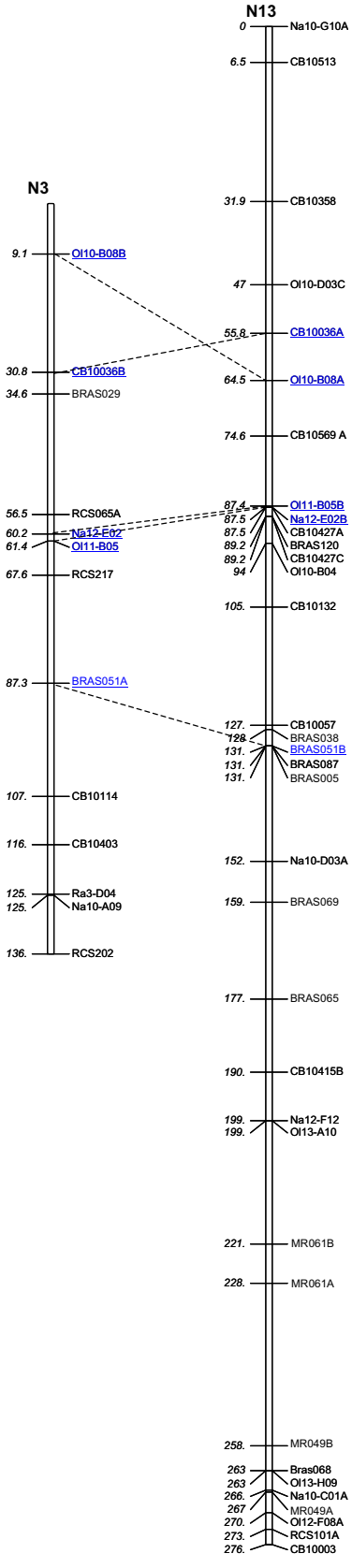

Fig. 3 Alignment of homoeologous markers in linkage groups N3 and N13. Homoeologous markers are *underlined* and joined by *dashed lines*. Genetic distance (cM) is reported in Kosambi

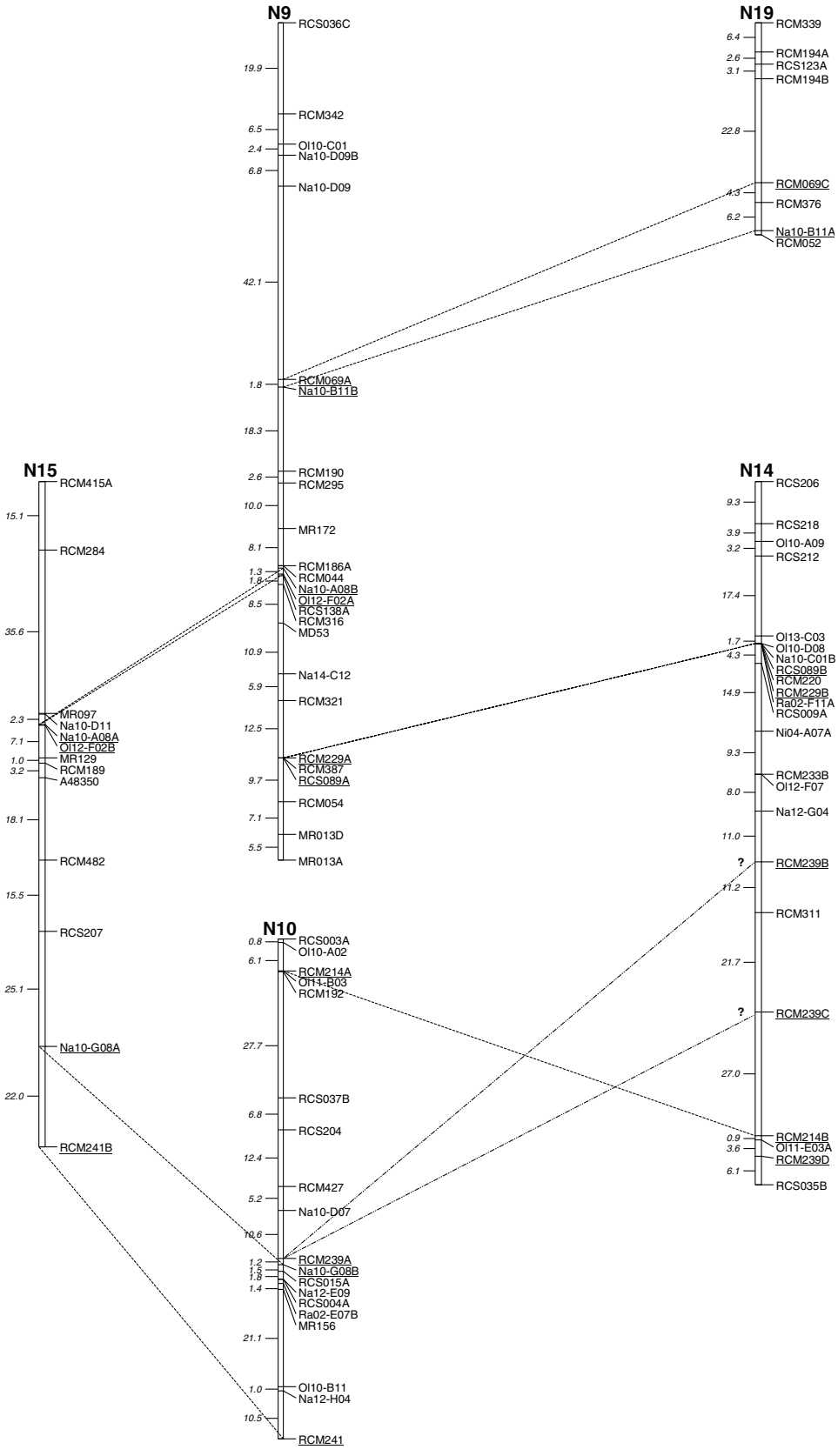

Fig. 4 Alignment of homoeologous markers in linkage groups N9, N10, N14, N15, N19. Homoeologous markers are *underlined* and joined by *dashed lines*. Genetic distance (cM) is reported in Kosambi

(1995) for which the chromosome maps are available at http://ukcrop.net/perl/ace/search/BrassicaDB. Indeed, the public microsatellites we used helped us to establish the links. There were a few linkage groups where no link was possible between the map of Parkin et al. (1995) and our map via the public microsatellites. We circumvented

this problem by genotyping a limited number of public RFLP markers on our populations (data not shown).

Detection of regions of homoeology between the A and C genomes

The presence of the highly homoeologous A (origin *B. rapa*) and C (origin *B. oleracea*) genomes in the amphidiploid genome of *B. napus* explains the duplicate nature of *B. napus* loci. Taking into account the thorough selection of markers we performed in our work, i.e. codominant markers with a simple pattern, most of the markers we used corresponded to one genetic locus on the map. Therefore, our results are not optimal for homoeology analyses. It would have been preferable for this objective to favour multicopy primer pairs. However, we still had a substantial amount of such primer pairs. A total of 47 primer pairs out of 240 corresponded to more than one mapped locus, with seven primer pairs having from three to six corresponding loci and a majority (38) corresponding to two mapped loci. The majority of these primer pairs (28) amplified one locus on each genome (A and C), seven amplified two loci on the A genome, and seven amplified two loci on the C genome. The 28 primer pairs with loci on the A and C genome enabled us to detect regions where homoeology is obvious. One example of a clear homoeology involving six primer pairs appears on N1 as compared with N11 (Fig. 2). The order and distribution of the corresponding loci on each linkage group appears to be similar, indicating there would be a large syntenous region covering 100 and 80 cM on N1 and N11, respectively. Another example of homoeology was given by N3 and N13 (Fig. 3), where a homoeologous region covering 70 cM seems to be conserved in these two linkage groups (Fig. 3). More syntenous regions were highlighted such as N7 and N16, involving three common primer pairs, and a complex relation between N9, N10, N14, N15 and N19 (Fig. 4).

Two primer pairs displayed six corresponding loci each (CB10159 and Na10-C01), CB10159 having five different loci on N4 and Na10-C01 having three loci, also on N4. Considering the important size of N4 (186 cM), this could be an indication of the high level of duplication on this linkage group.

Discussion

Good genome coverage and high rate of polymorphism between parental lines are the main advantages of our map. However, there are still important gaps in our map, for instance three gaps of more than 20 cM on N13. Our lab is currently densifying these genomic zones with additional markers by screening RAPD oligonucleotides in a bulked segregant analysis (BSA) approach. Also, we are in the process of positioning on our map a set of about 100 more SSR markers, the primer pair sequences having been kindly provided by Agriculture and Agri-food Canada.

This map was first elaborated with the Mapmaker software, which made it possible to establish linkage groups with a fairly correct order of markers. However, considering the relatively high number of markers on some linkage groups, we sometimes generated small inversions. This was corrected in some cases with CARTHAGENE, which performed an exhaustive analysis on each linkage group. Despite these minor corrections, the two software gave similar results.

Following this work, we were able to make an unambiguous connection with linkage groups from the map of Lombard and Delourme (2001), by genotyping 60 doubled-haploid lines, from the population Darmor *Bzh* X Yudal, with 96 SSR markers (data not shown). Comparing the sizes of the corresponding linkage groups, it is noteworthy that the largest linkage group LG717 (246 cM) shown by Lombard and Delourme (2001) also corresponds to the largest linkage group (N13, 276 cM) on our map. The equivalent N13 linkage group of the BBSRC map, with a size of 170 cM, is also among the largest linkage groups of the map.

Despite the fact that our results are not optimal for homoeology analyses, we were able to show that in most cases where two loci are detected by a single primer pair, this is not the result of a duplication event within the A or the C genome (even if this occurs in some situations) but is rather due to amplification of a single loci from each genome. This is in accordance with the results of Saal et al. (2001), who analysed the assignation of the two loci amplified with each of 34 primer pairs flanking microsatellites. In our work, 38 primer pairs corresponded to two genetic loci on the map. The majority of these primer pairs could help us point out zones of homoeology. The most striking example of homoeology is shown between linkage groups N1 and N11. This result is in line with those of Parkin et al. (2003), who showed that linkage groups N1 and N11 are completely syntenous.

This work is a prerequisite for genetic analyses aimed first at positioning QTL involved in important rapeseed agronomic traits, such as yield, disease resistance and oil quality. At the same time, the knowledge of gene position on the genetic map will be of great help to focus on the ones which colocalize with QTL. For instance, single nucleotide polymorphism (SNP) markers or PCR-specific markers designed on genes of interest could be positioned on the map and later the use of desirable alleles for the creation of optimal genotypes could be carried out. Research programs in partnership with different labs, aiming at positioning genes and QTL on the map, are currently in process. An oilseed rape BAC library with insert sizes ranging between 200 and 300 kb has been made available within the French Genoplante project (Budin et al. 2004). This library is currently being contigued and ordered with PCR markers developed from *Arabidopsis thaliana* and oilseed rape EST coding sequences. Genetic mapping of such markers as

well as SSR and SNP markers on our map could, as a consequence, lead on to a physical/genetic database. This will facilitate rapid gene and QTL cloning in oilseed rape using both forward and reverse genetic approaches.

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