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A COMBINED AFLP AND RAPD GENETIC LINKAGE MAP OF OIL RADISH (*Raphanus sativus* L.)

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ABSTRACT

A combined genetic linkage map of an intra-specific F_2 population of radish was constructed by the integration of 290 AFLP markers to the existed RAPD map. It consisted of 424 markers distributed over 9 linkage groups. Integration of the AFLP markers increased the RAPD map length by 362 cM. The combined map covered 1044 cM of the radish genome. Our combined map was greater than the maps previously constructed by others in length and markers number. The differences in maps length can be attributed to the differences in map construction algorithms. This map is considered an important step towards the construction of a saturated map for radish.

Key words: ***AFLP, Raphanus sativus, Genetic mapping, JOINMAP, Brassicasea, Chromosome rearrangement, Beet Cyst Nematode resistance.***

INTRODUCTION

Historically, genetic maps have provided very few advantages for the plant breeding programs and crop improvement, unless for few species such as maize and tomato. This is because of: 1) the type of markers predominantly used to construct these maps (morphological, cytological and macro-mutation markers); 2) the existence of very poor integrated maps that were constructed based on crosses of single population, and 3) the polyploidy nature of many crop genomes which increased the genotype environment interactions and, therefore, modifying the expression of the quantitatively inherited traits (Bradeen *et al* 2001, Burr and Burr 1991 and Lee 1995). The discovering of molecular marker technology (e.g., hybridization-based markers and/or PCR-based markers) and the related computer technologies (molecular analysis softwares e.g., MAPMAKER, LINKAGE1 and JOINMAP) have allowed the geneticists and the breeders to construct high-density genetic maps that cover a large part of the genome. These technologies have enhanced the contribution of genetic linkage maps in plant breeding programs and crop improvement (Lee 1995).

High-resolution genetic maps serve to: 1) locate or tag genes responsible for important traits; 2) study the inheritance of gene(s) of interest; 3) positional (map-based) cloning of gene(s), and 4) the target

markers located at these maps can be usefully utilize for marker-assisted selection of important traits, and therefore, increase breeding efficiency (Quedraogo *et al* 2002; Vuylsteke *et al* 1999). Moreover, high-density genetic maps are principle tools for physical mapping of plant chromosomes. The knowledge provided from the localized markers is also useful for accurate estimation of the genetic relationship between plant cultivars that is helpful in cultivars registration and protection (Lombard and Delourme 2001).

Recently, there are few number of molecular attempts have been conducted to construct a genetic map of radish (*Raphanus sativus*). The first study was based on restriction fragment length polymorphism (RFLP) markers and was carried out by Bett (2001). The author used two different mapping populations (F_2 and BC). The F_2 map consisted of 204 markers, spanned 844 cM, while the BC map contained 221 markers, and spanned 915 cM. The combined map (constructed from the F_2 and BC maps) consisted of 236 RFLPs and spanned 900 cM. Using the advantages of the PCR-based marker techniques, Kandeel *et al* (2004a and b) constructed two different genetic maps of an intra-specific F_2 radish population using RAPD and AFLP markers. The RAPD genetic map comprised 202 markers, spanned 682 cM, while the AFLP map contained 321 markers, and spanned 1064 cM. To date, a saturated genetic map of radish does not exist. Enhanced map saturation may be possible by characterization and adding new markers generated by powerfully molecular techniques (e.g., AFLP and SSR markers) to the existence maps. Additionally, this can be achieved by the integration of existing maps that were constructed from different populations.

Our goals in the present study were: 1) the construction of radish-combined map; 2) the comparison of the efficiency of RAPD and AFLP markers to saturate the radish map, and 3) the comparison of this map with the previously published maps.

MATERIALS AND METHODS

PLANT MATERIAL AND DNA EXTRACTION

A population of 245 F_2 plants derived from self-pollinating of single F_1 plant of the intra-specific oil radish (*Raphanus sativus* L.) cross 'Pegletta' x 'Siletta Nova' (Kandel *et al* 2004a), was used. Plant DNA was extracted by a method slightly modified from that reported by Dellaporta *et al* (1983), as described by Kandeel *et al* (2004a).

MARKER ANALYSES

The AFLP markers were performed using the modified protocol of Vos *et al* (1995) as described by Kandeel *et al* (2004b). Briefly, the genomic DNA (125 ng) was digested using two restriction enzymes *Eco*RI and *Mse*I. Two appropriate linkers were ligated to the restricted DNA sites to generate suitable template for PCR. The restricted/ligated genomic DNA was used for two PCR rounds with increasing selectivity in order to generate the AFLP polymorphisms. Forty pre-screened AFLP primer combinations were used for selective amplification of DNA from the 245 F_2 individuals.

RAPD assay was done essentially as described by Williams *et al* (1990). PCR reaction (8µl) contained 16 ng genomic DNA, 1 x PCR buffer (10mM Tris-HCl pH 8.3, 50 mM KCl), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP (dATP, dCTP, dGTP and dTTP), 0.1 U Taq DNA polymerase (InVitek) and 0.4 mM primer. Forty-five cycles were used for amplification with PE 9600 Gene Amp system (PE Applied Bio systems). PCR conditions were, primary denaturation (94°C for 5 min); 45 cycles of denaturation (at 94°C for 30s), annealing (at 36°C for 30s) and extension (at 72°C for 1 min). In the last cycle, the extension period was increased to 10 min. A total of 60 pre-screened decamers primers (Operon technologies) were used to generate the RAPD polymorphisms for mapping. Forty-three primers were applied with a subset of 64 F_2 individuals and 17 primers were applied with the whole mapping population along with DNA samples of the parents and the F_1 hybrid.

LINKAGE ANALYSES

Chi-square goodness of fit was used to test the marker segregation against the expected 3:1 ratio. Only markers that did not significantly ($P \leq 0.005$) differ from the expected ratio (dominant markers) were utilized for map construction.

The computer software JOINMAP 3.0 (Van Ooijen and Voorips 2001) was used for map construction. Maps of RAPD and AFLP techniques were constructed separately and then combined. Initially, JOINMAP organizes the markers that fitted the expected segregation ratio in groups (Gs) based on the modified algorithm of odds (LOD) values. The modified LOD scores are based on chi-square test for independence of segregation. The test of independence segregation avoids spurious linkages of markers with skewed segregation to the chromosomes (Van Ooijen and Voorips 2001). Range values of LOD grouping threshold were set to construct either the individual RAPD and AFLP maps or the combined map. In the second step, the

recombination frequencies were computed for all marker pairs within each marker group. Third, the markers were assigned in linkage groups (LGs) based on the recombination frequency (RF). Data points with LOD score below 0.1 and recombination fraction above 0.4 were selected in the final computation. The more stringent linkage threshold setting caused error in marker order (Stam and van Ooijen 1995). Recombination frequencies were converted to centiMorgans (cM) with Kosambi's mapping function (Kosambi 1944). A ripple was applied after adding each new marker and the JUMP value was set to 7.

Assigning of the markers to their chromosomes is obtained during three rounds. In the first round, the markers were ordered one by one to the map starting from the most informative pair of loci (Low RFs). JOINMAP is searched for the best position and measured goodness-of fit (revealed by the JUMP values) for each added locus. JOINMAP removed all loci that revealed too low goodness-of fit (high JUMP value) and/or gave negative genetic distances from the map at this round. All loci previously removed were used again in an attempt to assign to the map during the second round. This was to increase the map distance and number of mapped markers. However, when there were high JUMP values or negative distance, JOINMAP removed these markers once more. Finally, JOINMAP added the removed markers close to their location in their LGs at the end of the third round. This may be attributed to the ignoring of the requirements for maximum allowed goodness- of fit reduction (JUMP values), which made by the JOINMAP program (Van Ooijen and Voorips 2001). It has been suggested to use the map constructed after the second round for linkage and map distance calculations (Powell *et al* 1997 and Van Ooijen and Voorips 2001).

MARKERS DISTRIBUTION

Poisson distribution function was used to test the distribution of RAPD and AFLP markers over linkage groups. This was done to study the ability of RAPD and AFLP markers to distribute uniformly on the linkage groups. The chromosomes lengths were divided into intervals each of 10 cM. The number of markers per interval was counted. In addition, Poisson law $P_{(x)}= e^{-\mu} \mu/X!$ (μ = the average number of markers/10 cM interval) was used to calculate the expected markers distribution and chi-square test ($P < 0.05$) was applied to test the significances between the expected and the observed markers distribution (Young *et al* 1999 and Saal and Wricke 2002).

RESULTS AND DISCUSSION

The 60 informative RAPD primers generated a total of 372 polymorphic loci with distinct segregation (Table 1). Of these loci 222 were produced by 43 primers and 64 F_2 individuals and 150 were produced by 17 primers and the whole mapping population. Each RAPD primer revealed a mean of 6.2 polymorphic bands. For AFLP markers, 40 primer combinations (PCs) produced a total of 408 readable and reproducible bands segregating in the whole F_2 individuals. The mean number of polymorphic bands per PC was 10.2. The majority of RAPD and AFLP markers (83.1% and 89.5%, respectively) followed the Mendelian segregation 3:1 ratio (hypothesis of dominant monogenic inheritance). Sixty-three and 43 RAPD and AFLP loci, respectively, were extremely distorted ($P < 0.005$) from the hypothesized 3:1 ratio (Table 1). Overall, the AFLP technique was more efficient in detecting genetic differences than RAPD system. This can clearly be observed from the high polymorphisms rate detected and the high number of segregating markers which can be utilized for map construction (Table 1). The low rate of polymorphisms revealed by the RAPD markers is due to the sensitivity of this technique to the reagents and the PCR conditions (He *et al* 1994, Perez *et al* 1998 and Williams *et al* 1993).

Table 1. An overview of the RAPD and AFLP marker efficiencies to detect polymorphisms in *Raphanus sativus* cross Pegletta x Siletta nova.

Marker types	No. of primers or PCs[a]	Amplified fragments	Segregation 3:1	Skewed	Total	Mean[b]
RAPD	60	372	309(83.1%)	63(16.9%)	372	6.2
AFLP	40	408	365(89.5%)	43(10.5%)	408	10.4

[a] number of pre-screened primers and PCs [b] mean number of amplified fragments per primer or PC.

Using a range of LOD scores (7 to 10), 434 markers (136 RAPDs and 298 AFLPs) organized in 9 groups (Table 2). A set of 240 markers (173 RAPDs and 67 AFLPs) remained ungrouped. This may be attributed to the risk of using dominant markers (RAPDs and AFLPs) in the F_2 populations (Burr and Burr 1991 a and Kennard *et al* 1994). Park *et al* (2000) reported that the dominant markers are useful tools for mapping and assigning genetic relationships in the recombinant inbred populations. Otherwise, the high number of ungrouped markers may be attributed to either RAPD or AFLP loci which were produced by a low number of the F_2 individuals (Horejsi and Staub 1998 and Kandeel *et al* 2004a). The results showed 80.9 per cent ungrouped RAPD markers (140 markers from total of 173 unlinked markers) were produced by 64 F_2 individuals.

Table 2. Combined grouping of RAPD and AFLP markers that generated using an intra-specific F_2 population of the radish cross 'Pegeletta' x Siletta nova, based on the algorithm of odd (LOD score) values.

Group	No. of markers			LOD score
	RAPD	AFLP	Total	
1	22	46	68	10.0
2	17	34	51	9.0
3	16	33	49	8.0
4	7	29	36	9.0
5	9	13	22	9.0
6	19	38	57	9.0
7	23	37	60	7.0
8	20	39	59	7.0
9	3	29	32	9.0
Grouped	136	298	434	
Ungrouped	173	67	240	
Total	309	365	674	

With mapping stringency of LOD scores of 7 to 10, the combined map consisted of 424 markers distributed over 9 major linkage groups (Fig. 1). Ten markers (8 AFLPs and 2 RAPDs) could not be assigned to any of these LGs and remained unmapped. These markers were E41M60-186, E41M60-293, E41M59-530, E39M60-292, E39M62-207, E33M59-530, E32M48-292, OPH-03-374, OPI-17-516 and OPH-15-1240. As presented in Table (3), the number of markers assigned to Map1, Map2 and Map3 was 235, 241 and 424, respectively. Map2 was used to perform the combined map framework. A set of 189 markers caused rising of the JUMP values during the first and the second rounds and, therefore, did not assign to Map1 and/or Map2. The program ordered these markers after the third round close to their location on Map3 LGs (Fig. 1). We listed these markers on the core Map2, however, they omitted from further linkage analysis and map calculation. The constructed combined map covered a total length of 1044 cM with a mean distance of 4.3 cM between markers (1044 cM/241 markers assigned to Map2). LG5 contained the least number of markers (18 loci) and covered the shortest part of the genome (62 cM). In contrast, the most dense LG was LG7 (35 markers) and the longest LG was LG2 (173 cM) (Table 3). RAPD and AFLP markers

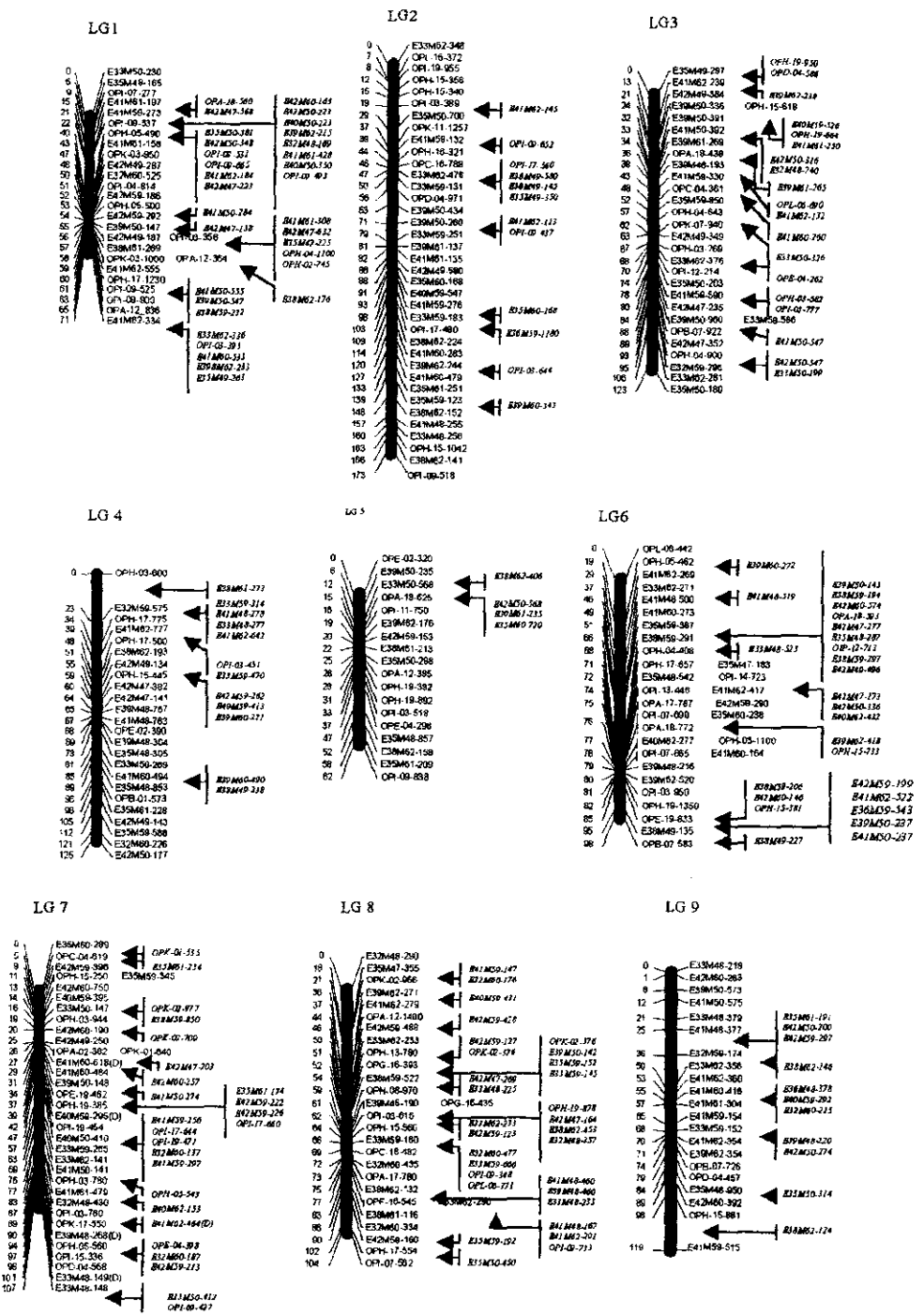

Fig. 1. Radish combined map based on RAPD and AFLP markers using an F_2 population of the intra specific cross Pegletta x Siletta nova. Map2 is used to make the map framework. The markers are assigned on the right side and the map distance on the left side of each linkage group. The markers listed on the right side (markers with arrows) could not be assigned to Map2 without ambiguity. These markers have been forced to sign to Map3 at the third round and excluded from further analysis.

Table 3. Constructing of the combined map linkage groups of the radish cross Pegletta x Siletta nova. Map1, Map2 and Map3 were constructed after the first, second and the third round, respectively. Recombination frequenceies (RFs) were converted to centiMorgens (cM) using Kosambi map function.

LGs	LOD	LG length (cM)	Number of Mapped markers								
			Map1			Map2			Map3		
			RAPD	AFLP	Total	RAPD	AFLP	Total	RAPD	AFLP	Total
1	10.0	71	12	14	26	13	14	27	20	39	59
2	9.0	173	12	15	27	12	15	27	17	33	50
3	8.0	123	9	20	29	9	21	30	16	33	49
4	9.0	125	6	18	24	6	18	24	7	29	36
5	9.0	62	8	8	16	9	9	18	9	13	22
6	9.0	98	14	15	29	15	16	31	19	38	57
7	7.0	107	14	21	35	14	21	35	23	37	60
8	7.0	104	13	15	28	13	15	28	20	39	59
9	9.0	119	3	18	21	3	18	21	3	29	22
Mapped	-	-	91	144	235	94	147	241	134	290	424
Unmapped	-	-	45	154	199	42	151	193	2	8	10
Total	-	1044	135	298	434	136	298	434	136	298	434

were distributed uniformly among all LGs. However, some clusters were observed at LG1 and LG6 (comprising 11 and 16 markers, respectively). These clusters spanned 20 cM of the total map length and the mean distance between markers was 0.74 cM.

As presented in Fig. (1), integration of the AFLP markers to the RAPD map presented by Kandeel *et al* (2004a) expanded the map length by 362 cM. Despite the great number of mapped markers (241) to the combined map in comparison to the RAPD map (176), the mean distance between adjacent markers was increased (4.3 cM for combined map and 3.9 cM for the RAPD map). Table (4) presents the currently exists genetic maps of radish. The RAPD and AFLP genetic maps presented by Kandeel *et al* (2004a and b) spanned 682 cM and 1064 cM, respectively. The BC, F_2 and BC+F_2 genetic maps presented by Bett (2001) spanned 844, 915 and 900 cM, respectively. The combined map presented herein is longer than these reported maps (except the AFLP map of Kandeel *et al* 2004b) and it comprised a greater number of markers (424 markers) than others.

Table 4. Previously published genetic linkage maps in radish (*Raphanus sativus* L.)

Population	Number of individuals	Markers		LGs	Map length (cM)	Reference
		Type	Number			
F2	245	RAPD	202	10	682	Kandeel *et al* (2004a)
F2	245	AFLP	321	10	1064	Kandeel *et al* (2004b)
F2	85	RFLP	204	9	844	Bett (2001)
BC	54	RFLP	221	9	915	Bett (2001)
F2+BC[a]	85 and 54	RFLP	236	9	900	Bett (2001)

[a] combined map

The differences in map length between the combined map presented in this study and the other reported maps may be attributed to the addition of new markers to the combined map (Lombard and Delourme 2001) since the chromosome length increased as the number of linked markers increased. The differences in maps length were observed in several studies on other crop species (cucumber, Bradeen *et al* 2001, barley, Castiglioni *et al* 1998 and maize, Vuylsteke *et al* 1999). The authors attributed the differences in the maps length to the distinct map functions and/or mapping algorithm used to construct those maps. For instance, Vuylsteke *et al* (1999) constructed two high-density genetic maps of two maize recombinant inbred lines (RILs) using the AFLP markers on the base of JOINMAP 2.0 software (Stam and van Ooijen (1995). They found that both maps were shorter (for both total genome length and the individual linkage groups) than the other published maps (which performed using MAPMAKER software), despite the larger number of markers mapped to

both maps. While MAPMAKER estimates the distance between each marker pair using the information of that pair of markers, JOINMAP uses all of the pair-wise recombination estimates in a dataset simultaneously. Otherwise, MAPMAKER ignoring the interference at the initial estimation procedure and, therefore, only the adjacent recombination frequencies are converted to map distance with a given mapping function (e.g. Kosambi or Haldane map functions). JOINMAP estimations are accounted for the interference levels given by applying mapping function to recombination frequencies. Thus, maps constructed using JOINMAP are always shorter than those of MAPMAKER (Bradeen *et al* 2001and Vuylsteke *et al* 1999). Despite the fact that the RAPDs map of Kandeel *et al* (2004a) and the combined map presented herein are produced with JOINMAP, the total genome coverage and the length of most linkage groups in the RAPD map were shorter than those in the combined map. We attributed this to the differences in data reliability which can affect the recombination frequency rates and, therefore, the map distance estimations.

As presented in Table (5) the combined map in this study has a total of 76 RAPD and 140 AFLP markers in common with the framework of the *Raphanus* maps proposed by Kandeel *et al* (2004a and b). The order of these markers is consistent between both maps (Fig. 2). The largest differences between the marker distance were discovered at: 1) LG2: the distance between the RAPD loci OPI-17-480 and OPD-04-971 is 57 cM in the combined map, while it was 95 in that of Kandeel *et al* (2004a), and 2) LG8: the distance between OPI-07-562 and OPA-17-780 and OPK-02-966 is 78 and 7 cM in the combined map but it was 27 and 82 cM, respectively, in that of Kandeel *et al* (2004a), between OPH-17-554 and OPH-17-780 and OPK-02-966 is 75 and 4 cM in the combined map in comparison to 25 and 80 cM, respectively, in Kandeel *et al* (2004a). With respect to the AFLP map presented by Kandeel *et al* (2004b), the largest differences between the marker distances were observed at LG8 between E38M61-116 and E32M60-435, E33M62-290 and E32M62-334 (129, 127 and 122 cM in combined map in comparison to 13, 6, 3 cM for the map of Kandeel *et al* (2004b), respectively). In addition, there are great differences in genetic distances observed between E42M59-160 and E32M60-435, E33M62-290 and E32M62-334 in the present map (18, 22 and 4 cM, respectively) and the AFLP map of Kandeel *et al* (2004b) (127, 125 and 120 cM, respectively).

Table 5. The combined map linkage groups versus the corresponding recently published map linkage groups. Number in parentheses represents the number of shared markers between the combined map and that was recently constructed.

Reference	Combined map LGs								
	1	2	3	4	5	6	7	8	9
Kandeel *et al* (2003a)[1]	2[6](10)	1(10)	5(3)	6(5)	3(8)	7(12)	9(12)	4(13)	8(3)
Kandeel *et al* (2003b)[2]	1(9)	2(24)	3(19)	4(17)	5(9)	8(15)	10(20)	7(10)	9(17)
Bett (2001)[3]	-	-	-	-	-	-	-	-	-
Bett (2001)[4]	-	-	-	-	-	-	-	-	-
Bett (2001)[5]	-	-	-	-	-	-	-	-	-

[1] **RAPDs and F2 population**
[2] **AFLPs and F2 population**
[3] **RFLPs and F2 population**
[4] **RFLPs and BC population**
[5] **Combined map**
[6] **comparable linkage groups to the combined map linkage groups**

In conclusion, the total length of the presented combined map was as greater as the maps reported by others (Kandeel *et al* 2004a and b and Bett 2001). In comparison to the genetic maps of Kandeel *et al* (2004a and b) which consisted of 10 LGs for each, the number of the combined map linkage groups was comparable to the nine radish chromosomes. Efforts, however, are needed to enhance the number of common markers in different radish maps and to facilitate map merging and integration. A new powerful marker techniques as SSRs should be developed to map markers to the poorly covered regions of *Raphanus* genome. The map presented herein is currently used for mapping the Beet Cysts Nematode (BCN) resistance gene(s) in *Raphanus sativus* as a first step to transfer this trait to the related *Brassica* species.

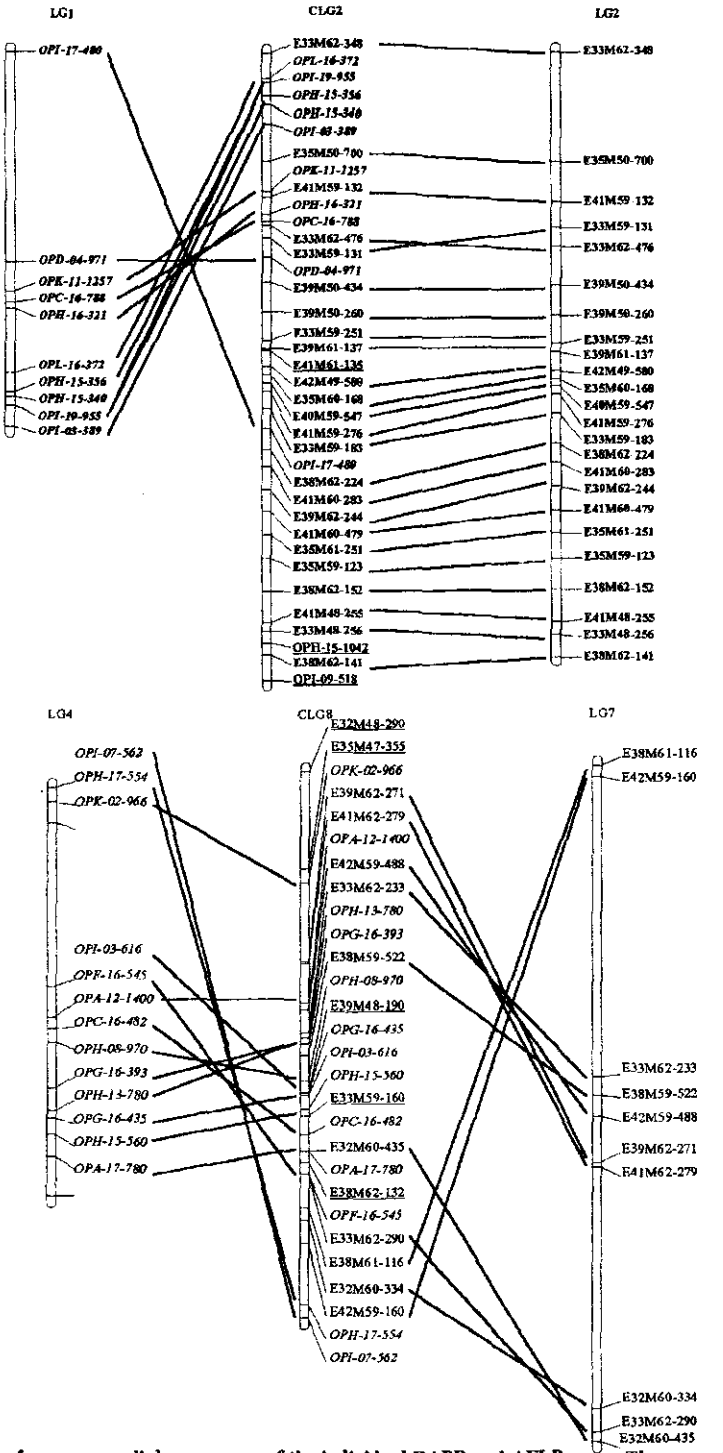

Fig. 2. Construction of a consensus linkage groups of the individual RAPD and AFLP maps. The presented linkage groups from left to right are for: RAPD Map2, combined Map2 and AFLP Map2, respectively. The underlined markers were mapped to the combined map2, whereas were not mapped to RAPD and/or AFLP Map3.

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انشاء خريطة ارتباط وراثي مجمعة لنبات الفجل باستخدام تكنيكات التذايد العددي العشوائي (RAPD) و التذايد في الطول (AFLP) لجزيئات ال "دي ان اية"

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فـــي هـــذه الدراســـة تـــم انشاء خريطة ارتباط وراثي مجمعة باستخدام عشيرة الجيل الثاني الانعزالـــي(F2) لنبات الفجل وذلك بادخال عدد ٢٩٠ من واسمات التزايد في طول جزيئات ال " دى ان ايه " (AFLP) إلى خريطة التزايد العددى العشوائى لجزيئات ال " دى ان ايه " (RAPD) الموجودة بـــالفعل . وقد احتوت الخريطة المجمعة على ٤٢٤ من الواسمات الوراثية التى انتشرت في ٩ مجاميع ارتباطـــية . ادى ادخـــال الواســـمات الوراثية (AFLP) إلى زيادة قدرها ٣٦٢ سنتيمورجن في طول خريطة ال(RAPD) وقد غطت خريطة الارتباط الوراثى المجمعة ١٠٤٤ سنتيمورجن من جينوم نبات الفجـــل. تعد خريطة الارتباط الوراثى المجمعة من الخطوات الهامة لانشاء خريطة ارتباط وراثى كاملة لجينوم نبات الفجل .

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