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Full Length Research Paper

Identification of SSR and RAPD markers associated with QTLs of winter survival and related traits in *Brassica napus* L.

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Because of importance of winter survival in winter type of *Brassica napus*, this study was performed to identify the QTLs controlling winter survival and related traits using SSR and RAPD markers. For this, an $F_{2:3}$ population of 200 families derived from crossing between cv. 'SLMO46' (winter type and cold resistant) and cv. 'Quantum' (spring type and susceptible to low temperature) were used. Winter survival (WS), leaves per plant in rosette stage (L/P), crown wet weight (CWW), crown dry weight (CDW) and crown water content (CWC) were measured in F_3 families. 350 SSR primer pairs and 250 RAPD primers were used to assess the parental polymorphism. The 32 SSR primer pairs and 47 RAPD polymorphic markers between parental lines were used to screen F_2 individuals. Linkage map was constructed using polymorphic markers. The markers were assigned into 14 linkage groups with total length of 1199.1 cM and an average distance of 17.13 cM between adjacent markers. The relationship between measured traits and genotypic data was analyzed using CIM method and totally 12 putative QTLs were detected for studied traits. The explained phenotypic variance by identified QTLs ranged between 0.5 and 11%. The identified QTLs had positive and negative additive effects and transferred from both parents to F_2 plants and F_3 families. Some of these QTLs located in the same genomic regions.

Key word: *Brassica napus*, molecular markers, QTL, rapeseed and winter survival.

INTRODUCTION

One important strategy for increasing crop productivity is to minimize losses due to biotic and abiotic stresses by developing more stress-tolerant varieties. In temperate and high elevation areas, low temperature stress remains one of the most important limitations on production. In these areas, winter survival is an important characteristic of plants and depends on the expression of many interacting traits such as leaf characteristics (Kole et al., 2002; Andaya and Tai, 2006). A number of investigators have used leaf characteristics as a measure of winter survival of plants (Andrews and Morrison, 1992; Kole et al., 2002). Increased leaf numbers was accompanied by increased expression of low temperature tolerance confirming that the length of the vegetative phase determines a plants ability to maintain a high level of low temperature tolerance gene expression (Mahfoozi et al., 2006). It has been shown that full expression of low temperature resistance genes only occurs in the vegetative stage and plants in the reproductive phase have a limited ability to cold acclimate (Kole et al., 2002). The cold resistance at one development stage is therefore not necessarily correlated with the resistance at other stages (Fujino et al., 2004). Thus, the assessment of resistance and the identification of useful resistance components must be carried out separately for each of the development stages (Toth et al., 2003).

Kole et al. (2002) evaluated a double haploid lines po-

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pulation of *Brassica napus* in multiple winters, and six significant QTLs for winter survival were detected in more than one winter. Segregating populations of this species derived from crosses of annual and biennial types was analyzed for flowering time (Teutonico and Osborn, 1994; Ferreira et al., 1995; Osborn et al., 1997) and freezing tolerance (Teutonico et al., 1995) in order to map and compare QTLs controlling these cold related traits. In *B. rapa*, loci controlling non-acclimated and acclimated freezing tolerance were mapped to different regions of the genome that were also different from those of flowering time genes (Kole et al., 2002). In *B. napus*, none of the genome regions covered by markers were significantly associated with freezing tolerance (Teutonico et al., 1995).

In recent years, various molecular markers were developed and applied for mapping QTLs and marker assisted selection (MAS) and several QTLs conferring cold tolerance related traits in several stages of plants have been identified (Teutonico et al., 1995; Kole et al., 2002; Andaya and Mackill, 2003; Toth et al., 2003; Fujino et al., 2004; Andaya and Tai, 2006). These studies support the idea that cold tolerance is a complex trait involving multiple genes. For this approach, this study was performed to constructing the linkage map of SSR and RAPD markers in order to identify association of these markers with winter survival and related traits QTLs in *B. napus*.

MATERIALS AND METHODS

Plant materials and phenotyping

A set of 200 $F_{2:3}$ families of rape seed (*Brassica napus* L.) obtained by crossing between cv. SLMO46 (winter type and cold resistant) and cv. Quantum (spring type and susceptible to low temperature) were used as genetic materials. The parental lines differed in winter survival and related traits. F_2 Plants were grown in greenhouse with photoperiod of 14/10 (day/night) and in rosette stage, leaf samples were harvested from individual plants for DNA extraction. F_3 families were sown in randomized complete block design with 2 replication in September 11, 2006 in Ardabil (a cold region in northwest of Iran with -2.7℃ mean temperature in winter season in 10 years ago). In each replication, F_3 families sown in rows with 40 cm space between rows and 10 cm space between plants in each row, 4 m long. In November 11, 2006, the number of plants for F_3 families was counted in each replication. The number of plants was counted again in April 20, 2007 and the winter survival was measured as percentage of plants surviving the winter. The average percentage over replication was used for QTL analysis. Also, in November 11, 2006, 10 plants from each replication of F_3 families randomly selected and transferred to laboratory and average number of leaves per plant, wet weight of crown (leaves and apical meristem), crown dry weight (samples incubated at 80℃ for 72 h and then weighted) and crown water contents ([(CWW-CDW)/CWW]*100) measured as cold resistance related-traits in F_3 families. Obtained data were subjected to analysis of variance and QTL analysis.

Genotyping

The DNA of parental lines and F_2 plants was extracted using the CTAB procedure according to Saghai-Maroof et al. (1984). The quality and quantity of DNA samples were assessed using Biophotometer (Eppendorf, Germany) and 0.8% agarose gel electrophoresis. All of the DNA samples were diluted to 25 ng/µl and used in PCR reactions. A total of 350 microsatellite primer pairs (The BBSRC primers, for which the sequences were obtained from http/ukcrop.Net/perl/ace/tree/brassicaDB) and 250 RAPD primers (NAPS unit standard primers: set # 5, set # 6 and set # 7) were used to analyze polymorphism in the parents and the polymorphic primers were used to genotype 200 F_2 individuals.

Microsatellite analysis was carried out using PCR reaction of 10 µl volume containing 50 ng of DNA template, 3 mM MgCl2, 0.25 mM each dNTP, 0.25 µM each primer, 1 U Taq DNA polymerase and 1x reaction buffer. The amplification profile consisted of an initial 3 min denaturation step at 94°C followed by 35 cycles of 1 min at 94°C, 1 min for annealing at 55 - 65°C (depending on each primer pair), 2 min extension at 72°C and a final 7 min extension step at 72°C. PCR products were detected using 6% denaturing polyacrylamide gels and silver staining method based on CIMMYT protocols (CIMMYT, 1984). For RAPD primers, PCR reaction, the amplification profile and PCR product detection published by Asghari et al. (2007).

Constructing of linkage groups and QTL analysis

Map Manager/QTL v. 2 (Manly and Olson, 1999) and QTL Cartographer v. 2.5 (Basten et al., 2001) were used for constructing linkage groups and QTL analysis. After the testing of segregation distortion in each primer locus, the linkage map of polymorphic primers was constructed using map manager/QTL, considering of a LOD score 3.0 and a maximum distance of 50 cM between adjacent markers. The Kosambi mapping function was used for calculating of genetic distances in cM (Kosambi, 1944). QTL analysis was performed using composite interval mapping (CIM) method of QTL Cartographer software. Putative QTL were chosen based on LOD score of 3.0 or above.

RESULTS

The winter survival and related traits of the susceptible parent, Quantum, the resistant parent, SLMO46, and the minimum and maximum values of these traits in F_3 families were shown in Table 1 and Figure 1. Parents and F_3 families had significant differences in studied traits ($p \leq 0.01$).

In this study, SSR and RAPD markers were used together for constructing linkage groups and rescanning the genome of rapeseed to identify QTLs controlling winter survival and related traits. For this, the parental polymorphism was evaluated using 350 SSR primer pairs and 250 RAPD primers and 32 polymorph SSR primer pairs and 47 polymorphic RAPD primers between parents were used to screen 200 individuals of F_2 population. The linkage map based on polymorphic markers was constructed considering of maximum 50 cM distance between two adjacent primers and the minimum LOD score three. To ensure Mendelian segregation at individual marker locus, segregation distortion was tested in each locus. The 70 markers were assigned into 14 linkage groups with total length of 1199.1 cM and an average distance of 17.13 cM between the adjacent mar-

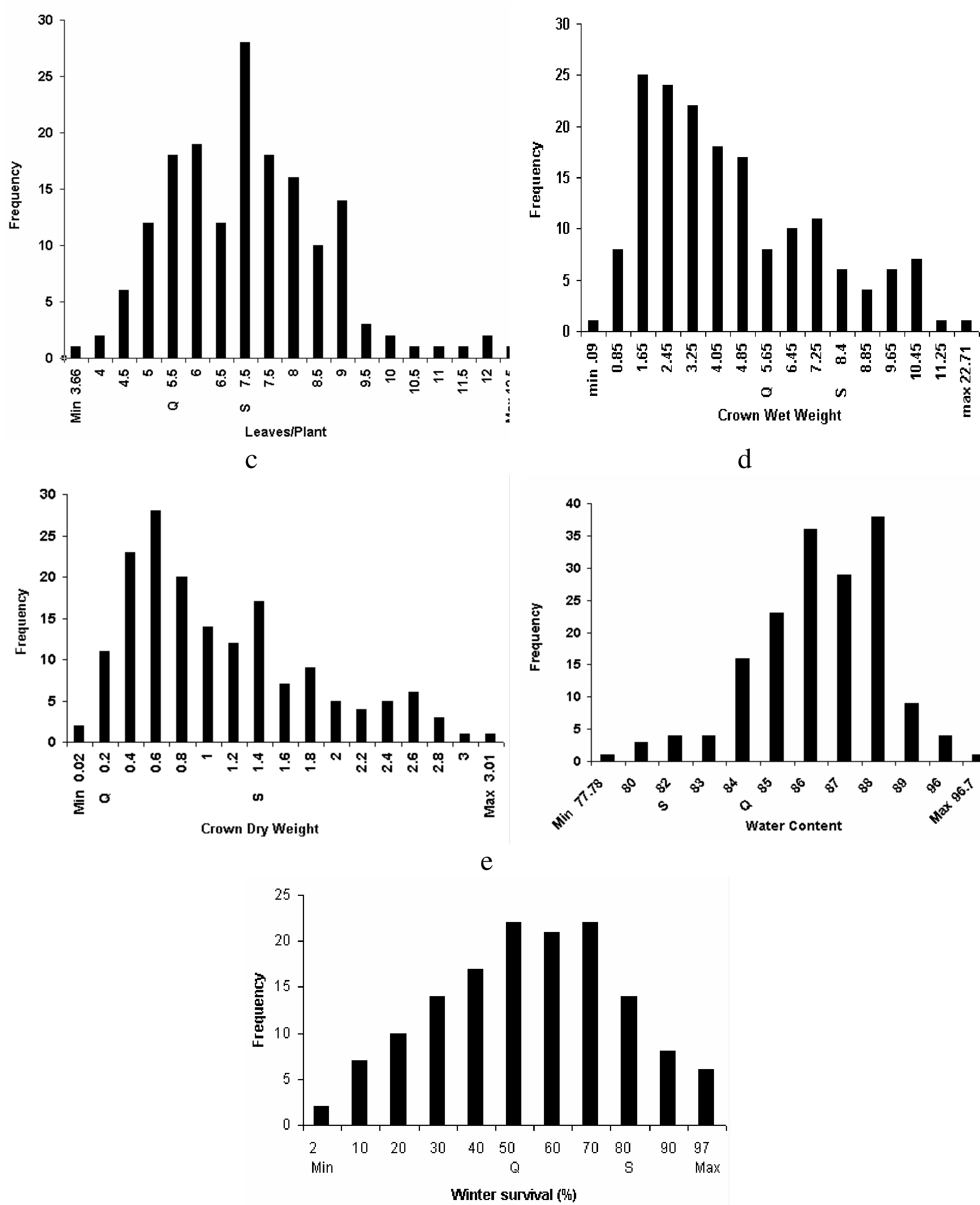

Figure 1. Distribution of parental lines Quantum (Q), SLMO46 (S) and F_3 families in: a) L/P, b) CWW, c) CDW, d) CWC and e) WS.

kers (Figure 2). The other nine markers were not assigned at any linkage groups.

The relationship between phenotypic and genotypic data was analyzed using CIM method and 12 putative

Table 1. Measurements of studied traits in parents and the minimum and maximum values of these traits in F_3 families.

Genotypes	Traits				
	L/P	CWW (g)	CDW (g)	CWC (%)	WS (%)
Quantum	5.5	5.65	0.2	85	50
SLMO46	7.5	8.4	1.4	82	80
F_3 families: min	3.66	0.9	0.02	77.78	2
F_3 families: max	12.5	22.71	3.01	96.7	97

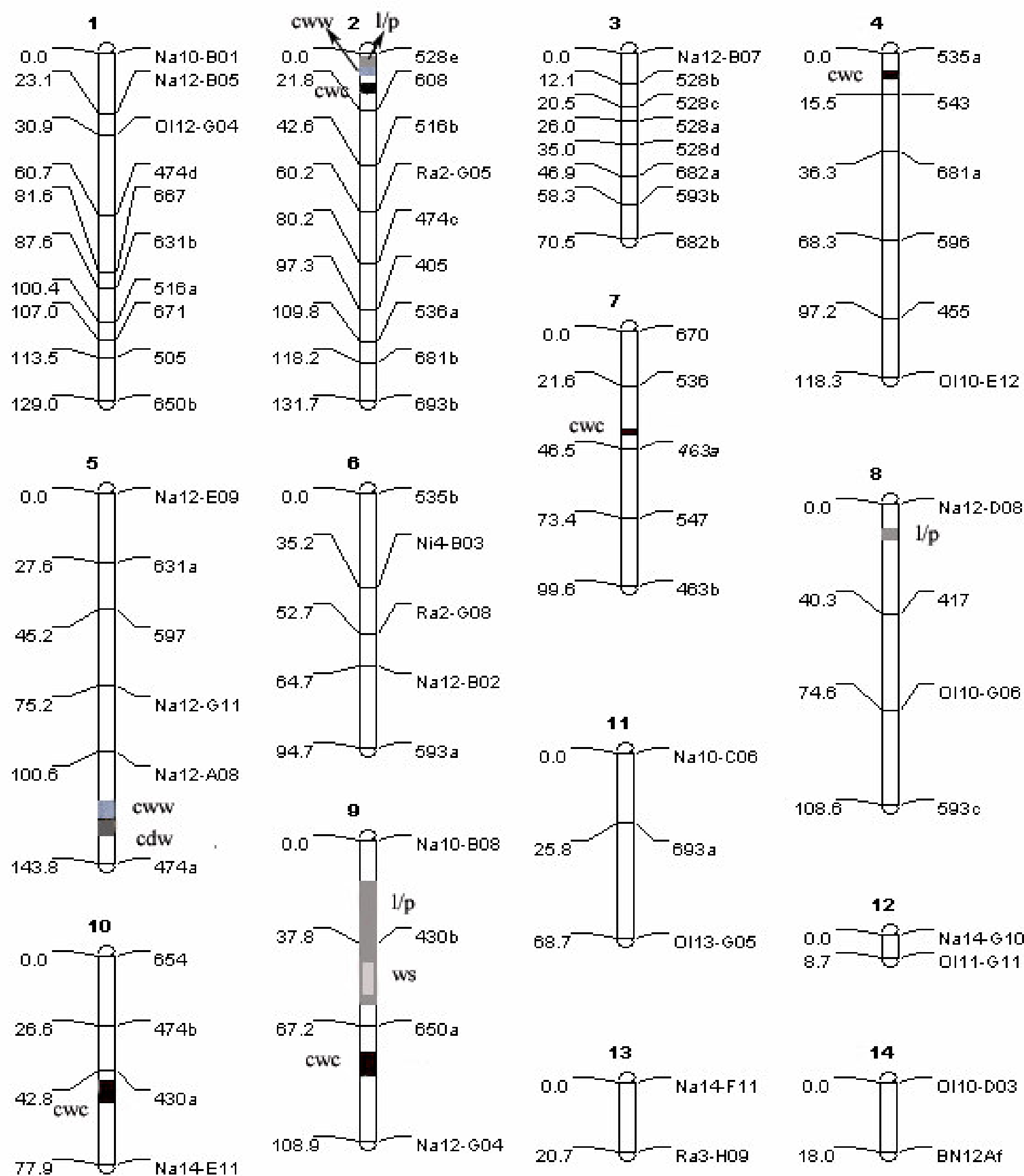

Figure 2. Linkage groups of SSR and RAPD markers and the position of QTLs controlling winter survival and related traits in the $F_{2:3}$ populations of *Brassica napus* cross 'SLMO46'×'quantum'. RAPD markers are shown with numbers (The same number with different subscript are the different polymorphic bands of one RAPD primer) and the name of SSR markers is the same as the name of these markers in *Brassica* data base. The sequences of flanking markers with identified QTLs are shown in Table 3. Map distances in cM were indicated on the left side of the linkage groups.

Table 2. Linkage group, additive effect, peak position and LOD score of the identified QTL and traits phenotypic variance.

Trait	QTL	Linkage group	Peak position (cM)	LOD	Additive effect	Explained phenotypic variance (%)
L/P	1	2	4.2	16.4	0.48	8
	2	8	11	10.2	0.27	7
	3	9	38	3.4	-0.38	5
CWW	1	2	8	5.9	0.87	2
	2	5	126.8	4.6	-1.82	6
CDW	1	5	127	3.7	-0.21	11
CWC	1	2	12.2	181	3	1
	2	4	8.2	5.6	-9.5	3
	3	7	40.1	155.1	0.22	0.5
	4	9	82	473.4	10.13	5
	5	10	55.8	3.1	-1.07	0.5
WS	1	9	48	7.1	-9.2	8

QTLs were detected for studied traits (Table 2). In this analysis 3, 2, 1, 5 and 1 QTLs were detected for L/P, CWW, CDW, CWC and WS, respectively. The three detected QTLs for L/P, situated on linkage groups 2, 8 and 9 (Figure 2) and explained 20% of the phenotypic variance of this trait. These QTLs had positive and negative additive effects (Table 2), indicating that alleles at these loci increasing L/P, come from both parents, SLMO46 and Quantum, to the F_2 plants and F_3 families. The located QTL on linkage group 9 for L/P covered a large portion of this linkage group and in this genomic region; one QTL was detected for WS with negative additive effect that explained 8% of WS phenotypic variance. This result showed that the allele transmitted from susceptible parent, Quantum, to F_2 and F_3 families, controlled both L/P and WS traits. The two identified QTLs for CWW located on linkage groups 2 and 5 that explained 2 and 6% of CWW phenotypic variance respectively (Figure 2 and Table 2). The first QTL had positive and the next one had negative additive effects, indicating that alleles increasing of CWW in F_3 families come from both parents. In the same genomic region on linkage group 5, one QTL also was detected for CDW with negative additive effect and this showed that alleles of QTL that located on linkage group 5 transmitted from susceptible parent, Quantum, and increased CDW and CWW in F_3 families. This QTL explained 11% of CDW phenotypic variance (Table 3).

In this study five QTLs for CWC also detected on linkage groups 2, 4, 7, 9 and 10 with positive and negative additive effects (Figure 2 and Table 2). From this, we can conclude that the alleles increasing CWC come from both parents to offspring. The explained phenotypic variance of CWC with these QTL ranged between 0.5 and 5% and totally 9% of CWC phenotypic variance explained with them.

The position of SSR primers Ol12-G04, Na12-A08, Na10-B08 and Na14-E11 were identified on *B. napus* chromosomes (8, 6, 15 and 14, respectively) based on BBSRC linkage maps (http://brassica.bbsrc.uk/ace). In this study, these primers were assigned to linkage groups 1, 5, 9 and 10, respectively, and we can assign QTL on these linkage groups and other linked markers to chromosomes 8, 6, 15 and 14 of *Brassica napus*.

DISCUSSION

The number of identified QTLs and the amount of explained phenotypic variance of studied traits in F_3 families (0.5 to 11%) revealed the minor effects of QTLs on winter survival and related traits in rapeseed and emphasized on quantitative inheritance and polygenic control of these traits. The complex genetic regulation of freezing tolerance and winter survival was reported in most or all crop species (Teutonico et al., 1995; Kole et al., 2002). Also, transgressive segregation was seen in all of the studied traits in this investigation (Figure 1). In *B. napus* and *B. rapa* populations transegressive segregation were reported for freezing tolerance, acclimation ability and ion leakage (Teutonico et al., 1995).

In many studies, QTLs with the major and minor effects for winter survival and related traits have been detected. In an acclimated F_2 population of *B. rapa*, four QTLs for freezing tolerance ability were detected and their effects ranged from 3.0 to 20.5% (Teutonico et al., 1995). Kole et al. (2002) evaluated the *B. napus* and *B. rapa* populations in multiple winters and 16 significant QTLs for winter survival and related traits were identified. The explained variance by each QTL ranged between 5.3 and 39.3%. In wheat, one QTL on chromosome 5B was detected that explained 31.5% of the cold resistance phenotypic variance (Toth et al., 2003). In the $F_{2:4}$ population of maize, forty QTLs were identified under cold stress that associated with the shoot, root and seed traits. The QTLs effects ranged from 0.4 to 30.1% (Hund et al., 2004).

Table 3. Name, sequence, type and linkage group of adjacent markers with identified QTLs in $F_{2:3}$ population of rapeseed cross 'SLMO46'×'Quantum'.

Markers name	Sequence		Marker type	Linkage group
Na10-B08	Forward Reverse	5′-AGA GAA AAA CAC TTC CCG CC-3′ 5′-GTG AGC TTT GCG AAA CAC G-3′	Microsatellite	9
Na12-D08	Forward Reverse	5′-ACGACGATTCAACTCATCTTC -3′ 5′-TTAACCAACTTCGCTTTTTG -3′	Microsatellite	8
Na12-G04	Forward Reverse	5′-CGAATTGAAGGATGAGTTTGG-3′ 5′-CACATGTTTTATCATTCACAAGTCC-3′	Microsatellite	9
Na14-E11	Forward Reverse	5′-TCATCCTTCTCACACCAAAATC-3′ 5′-CCTCGAAATAGCTCCAACCC-3′	Microsatellite	10
Na12-A08	Forward Reverse	5′-AACACTTGCAACTTCATTTTCC-3′ 5′-CATTGGTTGGTGAATTGACAG-3′	Microsatellite	5
474		5′-AGG CGG GAA C-3′	RAPD	5
528		5′-GGA TCT ATG C-3′	RAPD	2
430		5′-ATG CGG CAC C-3′	RAPD	9
608		5′-GAG CCC GAA A-3′	RAPD	2
535		5′-CCA CCA ACA G-3′	RAPD	4
463		5′-AGG CGG AAG C-3′	RAPD	7
543		5′-CGC TTC GGG T-3′	RAPD	4
536		5′-GCC CCT CGT C-3′	RAPD	7
417		5′-GAC AGG CCA A-3′	RAPD	8
650		5′-AGT ATG CAG C-3′	RAPD	9

The alleles of identified QTLs of studied traits transmitted to F_2 plants and F_3 families are from both parents based on their negative and positive additive effects. All of identified QTLs had small additive effects and other studies reported that the negative and positive additive effects were quite small and dominance effects were more significant in freezing tolerance of rapseed (Teutonico et al., 1995; Kole et al., 2002).

The identified QTLs in linkage groups 2, 5 and 9 are located on the nearest genomic regions that contained QTLs for some traits and this showed the possibilities for correspondence between these traits. The QTLs located on the same genomic region at linkage group 5, come from Quantum parent to F_2 plants and F_3 families and caused increasing CWW and CDW. The two QTLs located on same genomic region at linkage group 9 had negative additive effects and come from Quantum parent to offspring and caused increasing L/P and WS. Also, the same genomic region on linkage group 2 caused increasing L/P, CWW and CWC. In a previous study using RAPD markers, near the 528b, 474a, 474b and 430 markers, QTLs for some of the studied traits were identified (Asghari et al., 2007). These QTLs were confirmed in new constructed linkage groups using SSR and RAPD markers. If these QTLs are confirmed in the next generations after several meiotic cycles, they could be used more efficiently in marker-assisted selection for winter survival and related traits. Therefore, additional Q-TLs are probably involved which we did not detect in this study.

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