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ORIGINAL ARTICLE

QTL Mapping for Frond Length and Width in *Laminaria japonica* Aresch (Laminarales, Phaeophyta) Using AFLP and SSR Markers

Fuli Liu · Zhanru Shao · Haining Zhang · Jidong Liu · Xiuliang Wang · Delin Duan

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Abstract In *Laminaria japonica* Aresch breeding practice, two quantitative traits, frond length (FL) and frond width (FW), are the most important phenotypic selection index. In order to increase the breeding efficiency by integrating phenotypic selection and marker-assisted selection, the first set of QTL controlling the two traits were determined in F_2 family using amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) markers. Two prominent *L. japonicas* inbred lines, one with "broad and thin blade" characteristics and another with "long and narrow blade" characteristics, were applied in the hybridization to yield the F_2 mapping population with 92 individuals. A total of 287 AFLP markers and 11 SSR markers were used to construct a *L. japonica* genetic map. The yielded map was consisted of 28 linkage groups (LG) named LG1 to LG28, spanning 1,811.1 cM with an average interval of 6.7 cM and covering the 82.8% of the estimated genome 2,186.7 cM. While three genome-wide significant QTL were detected on LG1 (two QTL) and LG4 for "FL," explaining in total 42.36% of the phenotypic variance, two QTL were identified on LG3 and LG5 for the trait "FW," accounting for the total of 36.39% of the phenotypic variance. The gene action of these QTL was additive and partially dominant. The yielded linkage map and the detected QTL can provide a tool for further genetic analysis of two traits and be potential for maker-assisted selection in *L. japonica* breeding.

Keywords *Laminaria japonica* Aresch · Genetic linkage map · QTL mapping · Marker-assisted selection · AFLP · SSR

F. Liu · Z. Shao · H. Zhang · J. Liu · X. Wang (✉) · D. Duan (✉)
Institute of Oceanology, Chinese Academy of Sciences,
Qingdao 266071, China
e-mail: xliangw@yahoo.com
e-mail: dlduan@ms.qdio.ac.cn

F. Liu · Z. Shao · H. Zhang
Graduate School of the Chinese Academy of Sciences,
Beijing 100059, China

Introduction

Useful as food and as raw materials for algin, mannitol, and iodine extraction, *Laminaria japonica* Aresch is one of the most economically important seaweeds in China (Tseng 1983). In this kelp commercial production, prominent variety breeding is very crucial. Through years of studying genetic and breeding, many good varieties were obtained and cultivated widely (Zhang et al. 2001; Li et al. 2007a), and to some degree, this indeed enhanced the *Laminaria* cultivation and promoted the industry development of this economic seaweeds (Zemke-White and Ohno 1999).

However, many problems still existed in this kelp breeding. Firstly, so far, many cultivated *Laminaria* in China exhibited comparatively high degrees of heterozygosities (Wu and Lin 1987; Wang et al. 2004). Obtaining homozygous strains by multigeneration inbreeding and directional selection is time-consuming and labor-intensive (Feng et al. 2005). Secondly, many traits related to commercial production of *L. japonica,* such as stipe length, frond length, width, thickness, and iodine content, are quantitative characteristics (Wang 1984). It may be believed that such complex traits could be controlled by many of quantitative trait loci (QTL) and will be susceptible to environmental changes. Usually, phenotypic variances of these traits were depended on the interaction between the genes and the environment, leading that the phenotype has

no specific corresponding genotype. This will decrease the accuracy and efficiency of conventional breeding depending on directly phenotype selection. Therefore, an integrated breeding strategy of phenotypic selection and maker-assisted selection (MAS) would be more effective.

Molecular markers have several advantages over the traditional phenotypic markers that were previously available to breeders. They offer great scope for improving for the efficiency of conventional breeding by carrying out selection not directly on phenotype but on genotype. Of course, selection on genotype would require a molecular marker linked to the trait of interest tightly (Mohan et al. 1997). Genetic mapping and QTL analysis is not only one of the important methods to identify the markers linked to the objective trait but also one of the crucial tools to dissect the genetic mechanism of complex traits. Genetic mapping and QTL studies have been successfully applied to most farm animal species and crops (Andersson and Georges 2004) and more recently to aquaculture species such as Atlantic salmon, rainbow trout, tilapia, and so on (Korol et al. 2007). The first genetic linkage map has only recently become available (Li et al. 2007b), but QTL mapping has still not been undertaken for *L. japonica* to today. Base on our previous work on the genetic mapping analysis, we wish to add more stable marker loci to construct higher density genetic map and then map the QTL controlling the traits related to commercial production of *L. japonica*.

Two quantitative traits, frond length (FL) and frond width (FW), are crucial traits in breeding prominent high-yielding variety in *L. japonica*. Previous studies showed that the phenotypic variances of two traits were continuous and controlled by polygene (Fang et al. 1965). In order to dissect the inheritance model of the two complex traits and map the genetic loci linked to the traits, QTL analysis was conducted on a high-density genetic map for the first time. This work will benefit to MAS in *L. japonica* breeding.

Materials and Methods

Mapping Population Construction

Two *L. japonica* parental strains, one with "broad and thin blade" characteristics and another with "long and narrow blade" characteristics, were selected as paternal and maternal, respectively, in the hybridization. Both of them were prominent cultivar obtained by continuous multi-generation inbreed. Suitable genetic difference between the parental strains (Wang et al. 2005) can result in a higher proportion of polymorphic loci in the hybrid progeny which was beneficial to the construction of high-density genetic linkage map. Additionally, the opposite trait phenotype between the patent stains should be favorable for detecting the QTL controlling the traits. The parental gametophytes were isolated and preserved in the Institute of Oceanology, Chinese Academy of Sciences and were used for the hybridization to yield F_1 generation with the method described by Li et al. (2007a). The F_2 mapping population of 92 individuals was obtained by F_1 self-crossing.

Frond Length and Width Examination

Juvenile sporophytes of hybridized *Laminaria* (about 1 cm) were transplanted on the cultivating rope and transferred to the cultivation rafts in the sea on November 11, 2006. After the management of the cultivation, the *Laminaria* were harvested on March 1, 2007, and the sporophyts fronds' length and width were measured. The Shapiro–Wilks' test of normality for the two traits phenotypic variances and the Pearsonian correlation analysis between the two traits were applied with the Statistics Package for Social Science (SPSS) software.

AFLP and SSR Analysis

Genomic DNA of F_2 progeny and the parents were extracted from sporophytes using a Plant genomic DNA kit (Tiangen Biotech Co., Ltd, Beijing, China) according to manufacturer instructions. Amplified fragment length polymorphism (AFLP) analysis was based on the method described by Vos et al. (1995). Twenty-four *EcoR*I primers (coded by numbers) and 12 *Mse*I primers (coded by letters) were synthesized (Sangon, Co. Shanghai) and used to form 288 primer combinations. These primer combinations were screened against two patents to identify the informative primers. Primer combinations that generated unambiguous, highly polymorphic, and reproducible DNA bands between two parents were selected and used in subsequent experiments. The products of selective amplified were separated on 6% polyacrylamide gels and detected with silver staining method. Clear and unambiguous AFLP markers were scored as dominant markers and markers that were present in one parent, absent in the other parent, and segregated in a 3:1 Mendelian segregation ratio ($P \leq 0.05$) in the F_2 mapping population were used to construct genetic linkage map. AFLP markers were named by primer combinations code following a letter f (fragment) and four digits representing the size measured in base pairs.

Simple sequence repeat (SSR) analysis was performed according to the procedure described by Shi et al (2007) with minor modification. Together with 18 primers inferred from Shi et al. (2007) and our developed SSR primers from the EST database (Liu et al. 2009), a total of 35 primers were synthesized (Sangon Co., Shanghai, China) and screened for polymorphism between the parents. The polymorphic primers were used to genotyping the mapping

Table 1 Basic statistic characteristics of the two traits frond length and frond width phenotypic variance in parent lines and F_2 family

Trait	Parents		F_1	F_2 family						
	Paternal	Maternal		Maximum	Minimum	Mean	SD	VC %	Skewness	Kurtosis
FL (cm)	360.00	180.00	260.00	214.00	20.20	91.27	45.24	0.50	0.56	-0.20
FW (cm)	15.00	30.00	20.00	14.90	1.80	6.52	2.85	0.46	0.84	0.40

population, and the PCR products were separated and detected using the same method with AFLP analysis. SSR marker loci were scored as codominant markers, and the segregation at each marker locus was checked for deviation from Mendelian segregation ratios (1:2:1) by a Chi-square goodness-of-fit test. The primer code was taken as the marker name if the primer yielded only one locus. However, if the primer yielded two or more loci, the marker loci were named by the primer code following a lowercase letter.

Linkage Analysis and QTL Detection

MAPMAKER/exp software (Lander et al. 1987) was used to calculate recombination fractions between marker pairs and map distances. A LOD score of 5.0 and maximum recombination fraction (θ=0.30) were set as the linkage threshold for grouping markers. Groups were analyzed using multipoint mapping functions to define the most likely map orders with significance value (P=0.00001). After framework linkage groups were established, relatively less stringent criteria (LOD=3.0 and θ=0.40) were applied to test whether or not additional markers could be added to the framework map. To compensate for interference, map distance was calculated using the Kosambi function (Kosambi 1944). Genome size was estimated using methods described by Postlethwait et al. (1994). Maps were drawn using MapChart software (Voorips 2002).

QTL detection was performed on the software package Windows QTL Cartographer 2.0 (Wang et al. 2003) according the user manual. Mapping of QTL and estimation of effects were performed using composite interval mapping (CIM) method (Zeng 1994). The genome-wide significant threshold for each trait was determined by carrying out 1,000 permutations at P<0.01. A QTL position was determined at the local maximum of the LOD plot curve in the region under consideration. The proportion of phenotypic variance explained by a single QTL was calculated as the square of the partial correlation coefficient. Gene action type of QTL was determined according to criterion suggested by Stuber et al (1987). The names of the mapped QTL were made up of four parts: q + L/W (frond length or width) + a number (the serial number of the QTL controlling a trait) + a number (the serial number of the linkage groups).

Results

Phenotypic Data Analysis

The two parent strains are inbreeding lines with prominent agronomic traits. They show opposite phenotypes in frond

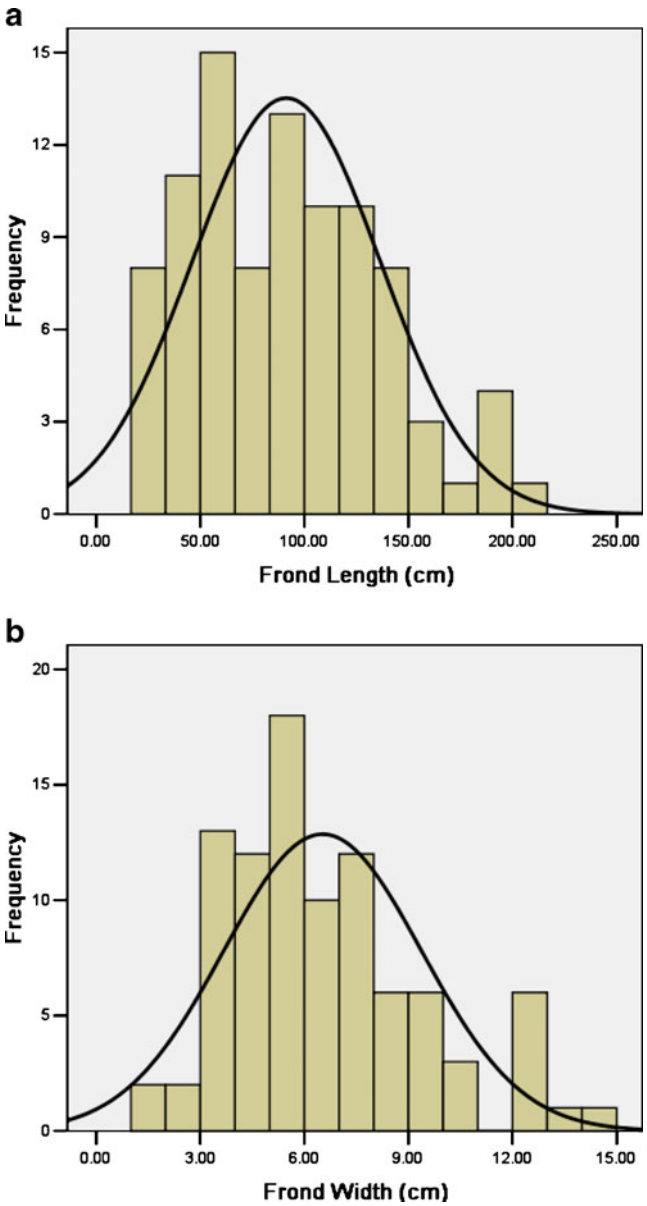

Fig. 1 Frequency distribution of the phenotypic value of the two traits "FL" (**a**) and "FW" (**b**) in F_2 family

Table 2 AFLP primer combinations information

	M-caa (B)	M-cat (C)	M-cag (D)	M-cac (E)	M-cta (F)	M-ctt (G)	M-ctc (I)	M-cga (J)	M-cgt (K)	M-cgg (L)	M-cgc (M)
E-aa (1)	11				27		10				14
E-ac (2)	11			15		13		12		1	10
E-ag (3)			24	17							
E-at (4)								3			
E-aac (17)	3					14		9		11	
E-aag (18)				9							
E-aca (19)	9		6					8		9	
E-acc (20)	2		8					9	9	9	9
E-acg (21)		9						11	9		
E-act (22)	9							6		7	8
E-agc (23)										8	9
E-agg (24)			18						8		

*Eco*RI selective primers were coded by numbers and *Mse*I selective primers were coded by letters. The numbers of marker loci following a 3:1 Mendelian segregation ratio ($P \leq 0.05$) yielded by the primer combinations were shown

length and width (Table 1). The average frond length and width of the F_1 family were close to the average value of the parents. The statistical parameters for the frond length and width were examined in F_2 mapping population (Table 1). The frond lengths were ranged from 20.2 to 214.0 cm, with an average of 89.8 cm, while the blade was ranged from 1.8 to 14.9 cm, with an average of 6.4 cm. The Shapiro–Wilks' test indicated the two traits phenotypic variances were coordinate with normal distribution (Fig. 1). Pearsonian correlation analysis showed that the correlation between "FL" and "FW" ($r=0.761$) was significant ($P<0.01$).

Genotyping Analysis

To the AFLP analysis, 39 screened primer combinations were selected for genotyping the F_2 mapping population finally (Table 2), and each of these primer combinations could yield clear, unambiguous polymorphic loci. Totally 899 loci were obtained from the 92 progeny of the mapping population, with an average of 23.1 loci detected per primer combination. Of the 899 loci detected, 440 (48.9%) loci followed the 3:1 Mendelian segregation ratio ($P \leq 0.05$), and 206 (22.9%) exhibited significant segregation distortion ($P \leq 0.05$).

Table 3 Information of primers yielding the marker loci mapped on linkage map

Code	Accession no.	SSR motif	Primer sequence (5′–3′)	Ta (°C)
SSR2	CX943173	$(ta)_5$	TGAACTGACGAGGTAGAGC TTGTATTAGAGGCAAGAGC	51
SSR4	CX943083	$(agac)_5$	GCGTGGGAGGCTTTCTTC CGTGGGATTGCTGATACTG	56
SSR6	CX943061	(gcct)18	CGGCTTCATCTCCCACAG TGACGGAATAGACCCAAA	56
SSR8	CX942986	$(cag)_5$	AACCGCTCTTTCCACCCA TCAAGGCAAGGACCGTAG	56
SSR9	CX942983	$(ac)_5$	GCAGGCTCGTGTCGTGTA CGCAGCATCAAGAAGGTA	54
SSR10	CX942859	$(tgc)_7$	CAATAATCGCAGAAAGGG CCGTCCGAACAACCAACT	53
SSR22	DQ978350	(AC)8	TCTGCTGATGCTAATATGGTG GAAATATCTGCAAAACTAAGGGGC	50
SSR25	DQ985173	(AC)8(TA)5	AGCAAAGAAGGGCGAAGATAA GGTTGGAATCATATTTGGCGTT	60
SSR27	DQ978340	(CAA)9	TATCCCGTTCGTTCCACTC CGACCCTAATAAGCTCTACCT	57

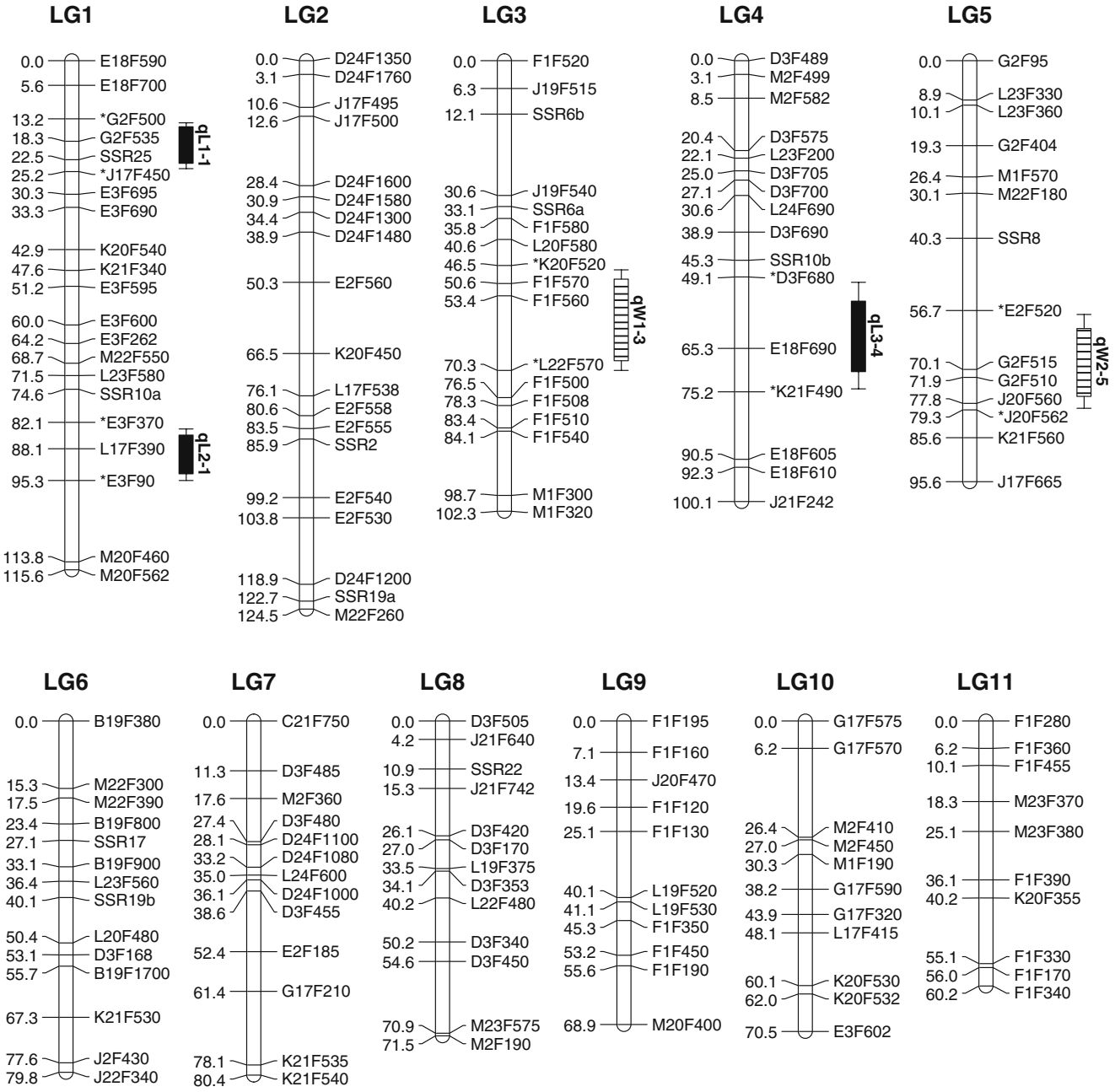

Fig. 2 Genetic linkage map and QTL location for the two traits "FL" and "FW" for *L. japonica*

While to the SSR study, totally 35 primers were screened through the detection on parental samples, finally 20 primers with high polymorphic yielding were applied for the genotyping the mapping population. Nearly each SSR primers could yield single locus, only a few primers yielded two or more loci. Of the total of 24 loci marker, 16 (66.6%) fitted to the Mendelian segregation ratios (1:2:1) by the Chi-square goodness-of-fit tests and eight (33.3%) exhibited significant segregation distortion ($P \leq 0.05$). The information of primers yielding the marker loci mapped on linkage map was present in Table 3.

Genetic Linkage Mapping

A total of 440 AFLP loci and 16 SSR loci fitted the Mendelian segregation ratios were used for genetic mapping construction. Of those markers, 287 AFLP marker loci and 11 SSR marker loci were anchored on 28 linkage groups (LG) named from LG1 to LG28 (Fig. 2); the remaining 158 markers were not mapped on any linkage group. The linkage group lengths were between 24.3 to 124.5 cM, with an average of 42.2 cM and a total length of 1,811.1 cM. The number of mapped loci on each linkage

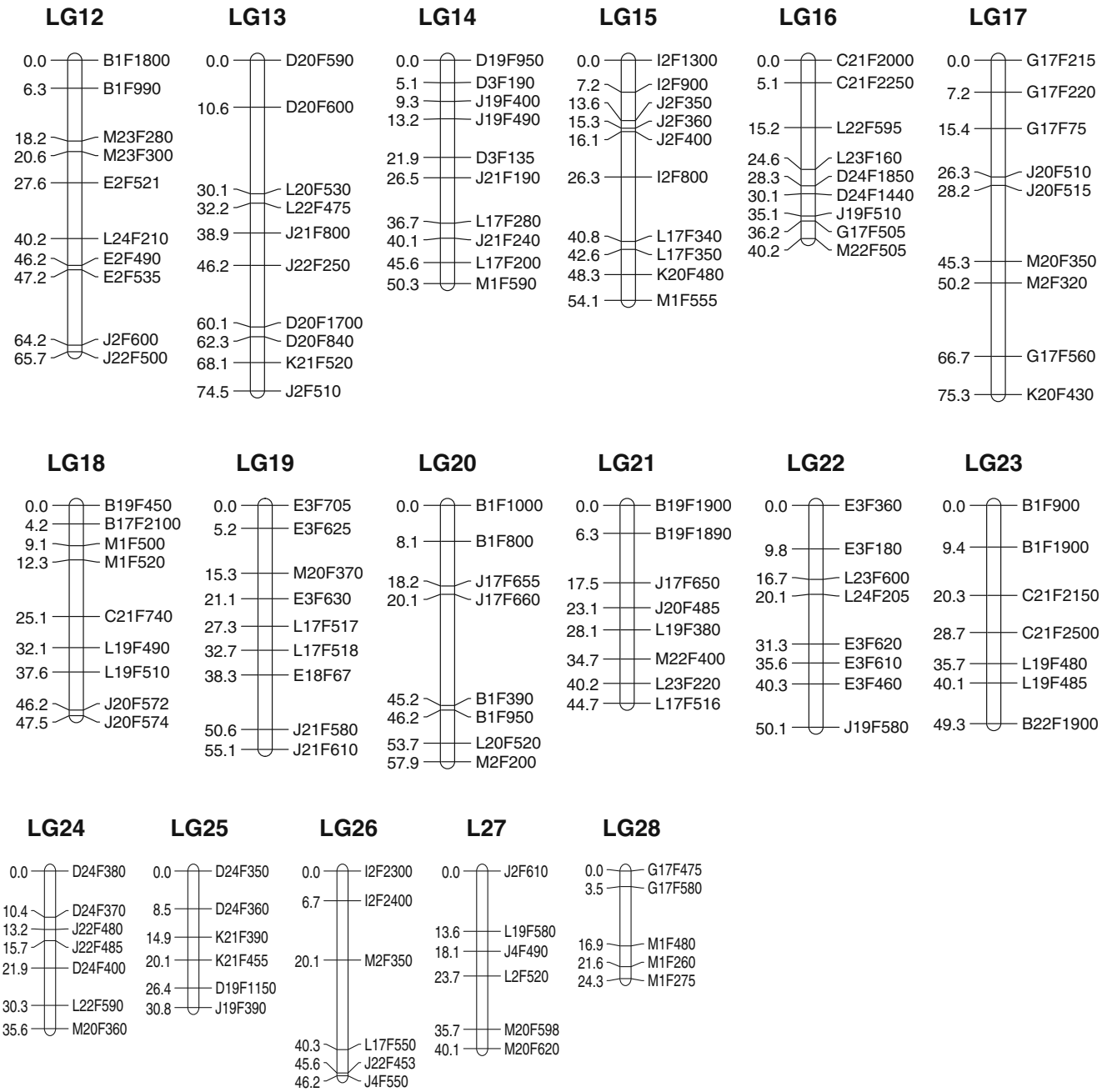

Fig. 2 (continued)

group was ranged from five to 19, with an average 10.6 loci per group. The yielded map density of the marker loci was 1 per 6.7 cM. The total length of the map covered 82.8% of the estimated *L. japonica* genome (2,186.7 cM) by the method of Postlethwait et al. (1994). Statistical data of the linkage map was summarized in Table 4.

QTL Analysis

Based on the linkage map, QTL correlated with the two objective economic traits, "FL" and "FW," were detected in F_2 mapping population with CIM method. With selected five loci as cofactors and a window size of 10 cM in the CIM method, the permutation rest (1,000 times) of the two traits were conducted, and the derived genome-wide threshold value for "FL" and "FW" (at 5% significance level) were LOD=2.8 and LOD=3.0, respectively. Three QTL, qL1-1, qL2-1, and qL3-4, correlated with the trait "FL" were anchored on LG1 (two QTL) and LG4, respectively, accounting for 18.37%, 10.87%, and 13.12% of the phenotypic variance, respectively. Alleles from the QTL were originated from the maternal strain, and the gene

Table 4 Summary of yielded genetic linkage map of *L. japonica*

Parameters the linkage map	
Number of markers mapped	298
Number of linkage groups	28
Average number of markers per group	10.6
Minimum number of markers per group	5
Maximum number of marker per group	19
Average marker spacing (cM)	6.7
Minimum length of linkage group (cM)	24.3
Maximun length of linkage group (cM)	124.5
Total map length (cM)	1,811.1
Estimated genome length (cM)	2,186.7
Estimated genome coverage (%)	82.8

action was additive and partial dominant. Two QTL, qW3-1 and qW5-2, correlated with the trait of "FW," were anchored on LG3 and LG5, accounting for 16.1% and 20.3% of the phenotypic variance, respectively. Alleles from the QTL were originated from the maternal strain, and the gene action was partially dominant. The detailed information of the QTL was presented in Table 5.

Discussion

Efficiency of AFLP and SSR Analysis

The AFLP method has already been used successfully in genetic mapping and is proven to be reliable, stable, rapid, and reproducible without prior knowledge of genomic sequence (Bai et al. 1999). In our study, the polymorphic ratio (48.9%) of AFLP systems exhibited highly effectiveness, and the applied AFLP primers yielded averaged 23.1 loci per primer. Due to the widespread distribution, high level of polymorphism, high reliability, and codominant mode of inheritance, SSR markers have been used in construction of genetic linkage map widely. However, a few SSR marker loci were available in *L. japonica* so far because developing SSR involves time-consuming and labor-intensive (Edwards et al. 1996). In our study, 20 pairs of polymorphic SSR primers were used to genotype the F_2 family and only 11 marker loci were anchor on the linkage group. Given that the SSR markers will serve as anchor points in comparative mapping and integration different linkage maps in future, it is very necessary to develop more SSR marker loci for *L. japonica*.

Segregation distortion usually ascribed to the close linkage of loci to genes or chromosomal region (Abe and Tsuda 1988), genetic drift and natural selection (Jung et al. 1996), chromosomal rearrangements (Gebhardt et al. 1991; Kianian and Quiros 1992), and loci sampling error or small population size (Lu et al. 1998). Here in our analysis, 206 (22.9%) AFLP marker loci and eight (33.3%) SSR marker loci showed segregation distortion ($P \leq 0.05$). Murigneux et al. (1993) suggested that more stringent requirements (higher LOD value) be adopted in genetic linkage analysis if there existed highly distorted loci; however, we exclude the distorted loci in genetic mapping because it might decrease the accuracy of the map distances and QTL detection.

Linkage Mapping and Genome Coverage

The chromosome number of *L. japonica* haploid has not yet been determined confidently, ranging from 16 to 32 reported by different researchers (Abe 1939; Yabu 1973; Yabu and Yasui 1991; Lewis et al. 1993; Zhou et al. 2004). Although the yielding map consisted of 28 linkage groups, and a one-to-one correspondence between the linkage groups and the chromosomes was not determined, the number of the linkage groups can reflect the number of chromosomes from another standpoint. The map was totally 1,811.1 cM with an average marker distance of 6.7 cM, covering 82.8% of *L. japonica* genome. The suitable density and the high coverage of the map offered favorable condition for QTL analysis.

QTL Mapping for Objective Traits

Cultivating high-yield variety is the most important breeding objective in *L. japonica*. The coefficient of the traits "FL," "FW" and the fresh weight, dry weight is high, showing that the frond length and width is crucial

Table 5 QTL analysis for the two traits "blade length" and "blade width" in F_2 family with CIM

Trait	QTL	LG	Markers interval	Posotion (cM)	LOD	Additive	Dominant	D/A[a]	R^2 (%)
FL	qL1-1	1	G2F500-J17F450	18.9	5.01	2.42	0.86	0.35	18.37
	qL2-1	1	E3F370-E3F90	88.6	3.75	2.53	1.21	0.47	10.87
	qL3-4	4	D3F680-K21F490	63.5	4.01	3.25	0.56	0.17	13.12
FW	qW1-3	3	K20F520-L22F570	54.6	3.96	−1.32	0.83	−0.63	16.06
	qW2-5	5	E2F520-J20F562	67.3	3.29	−1.33	0.76	−0.57	20.33

[a] Gene action: dominant/additive

to the kelp production (Wang 1984). Additionally, previous studies showed that the heritability of the traits "FL" and "FW" was high (Fang et al. 1965) which indicate the two traits have high selection potential and can be apt to achieve the breeding objective by inbred and selection. However, the two traits were typical quantitative traits controlling by polygene and susceptible to environment. The traditional research on the traits using statistic method cannot satisfy the need of the breeding development. Here, we used genetic mapping and QTL analysis to study the heredity and variation mechanism of the traits on molecular level in order to serve breeding on these traits.

In the present study, three and two QTL controlling the traits "FL" and "FW" were mapped and explain totally 42.4% and 36.4% of the phenotype variance, respectively. The identified AFLP markers (such as E3F370 and E3F90) tightly linked to the QTL can be used as selection indicator in *L. japonica* MAS and heighten the selection efficiency. Pearsonian correlation analysis between the two traits phenotype data indicated that the correlation between "FL" and "FW" (r=0.761) was significant (P<0.01). Generally, it can be presumed that the significant phenotype correlation between the traits was originated from genetic correlation. In another words, the QTL controlling the traits may correlate or link with each other in some chromosome regions. In cotton, maize, and other crops, QTL analysis, some correlated or linked QTL, controlling the correlated traits, were detected in certain chromosome regions (Ulloa et al. 2002; Lima et al. 2006). However, in our study, all QTL controlling the two correlated traits distributed on different linkage group. This might result from that the linked QTL might not be detected because of their low effect. Additionally, the genetic correlation was lower than the phenotype correlation for some quantitative traits like "FL" and "FW" in *L. japonica* (Wang 1984), implying that QTL controlling the correlated traits unlink on chromosomes. Question about that QTL controlling the correlated traits link or unlink on chromosomes needs in-depth study in future.

For the two traits, "FL" and "FW", three and two QTL were mapped and explained 42.4% and 36.4% of the phenotypic variance, respectively. The unexplained part of the genetic variation for two traits indicated that the two traits "FL" and "FW" were controlled by at least three and two QTL separately, and other potential QTL were not mapped in the present study. The incomplete coverage of the linkage map and the somewhat limited size of our sample pool may lead to undetected minor effect QTL. Temporal and spatial expression changes of the genes controlling traits influence the QTL analysis. Since the phenotype data were surveyed when kelp saprophytes were not fully mature, some genes controlling the trait might not express or these genes might not act on the phenotypic variance completely. This factor might lead that QTL containing these genes were not detected in QTL analysis.

In conclusion, the first set QTL controlling the two traits "FL" and "FW" were mapped, and the molecular inherence model of the two traits was dissected. This study opens new avenues for the two traits selection breeding. In future, a complete linkage map with higher density will be constructed by adding more markers, and the stability of the detected QTL and the other minor effect QTL will be tested in other genetic background and environment.

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