

# Molecular Maps and Mapping of Genes and QTLs of *Salvia miltiorrhiza*

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## Abstract

*Salvia miltiorrhiza* is an important medicinal crop in traditional Chinese medicine (TCM). Knowledge of its genetic foundation is limited. The first genetic linkage map of *S. miltiorrhiza* was constructed in 94 F<sub>1</sub> individuals from an intraspecific cross, consisting of 53 SSR, 38 SRAP, and 2 ISSR loci in eight linkage groups. Another genetic map was constructed based on 111 genomic SSRs markers. Moreover, specific length amplified fragment sequencing (SLAF-seq) is a recently developed high-throughput strategy for large-scale SNP, which was used to construct a high-density genetic map for *S. miltiorrhiza*. The results not only provide a platform for mapping quantitative trait loci but also offer a critical new tool for *S. miltiorrhiza* genome and comparative genomics as well as a valuable reference for TCM studies. A total of six QTL have been identified through interval mapping. It includes three salvianolic acid B (SAB) content-related QTL on linkage

group LG7, two lithospermic acid (LA) content markers on LG2 and LG4, and one rosmarinic acid (RA) content-linked QTL on LG7. These QTL could be used in marker-assisted breeding for phenolic acid contents of *S. miltiorrhiza*. The results provide important basis for QTL mapping, map-based cloning, and association studies for commercially important traits in *S. miltiorrhiza*.

## 3.1 Introduction

*Salvia miltiorrhiza* Bunge, also known as Danshen in Chinese, is a typical herb plant used for traditional Chinese medicine (TCM). This plant has been extensively used for thousands of years to treat various diseases, particularly coronary heart disease and cerebrovascular diseases, in China and Japan and recently in the USA and many European countries (Zhou et al. 2005). Currently, more than 200 compounds have been isolated, of which diterpenoid quinones and hydrophilic phenolic acids are the major constituents (Ma et al. 2015). Moreover, recent studies have found several new bioactivities of Danshen constituents, such as antioxidant, antitumor, and protective effects on the kidney and liver. It suggests the potential for new applications. More than 320 Danshen preparations are produced by different pharmaceutical manufacturers.

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There is constantly increasing demand for *S. miltiorrhiza* because of its varied and diverse pharmacologic properties. Currently, the annual demand for Danshen in China is approximately 10 million kg. Interest in biotechnology research on Danshen is increasing in many research groups. A large number of genes involved in the biosynthesis of phenolics and terpenoids have been identified through either molecular cloning or transcriptome-wide analysis (Gao et al. 2014; Luo et al. 2014; Xu et al. 2015). However, the genetic background and regulators of these two biosynthetic pathways, especially in the later steps, remain unknown. Danshen is of considerable research interest, and its superior genetic characteristics, such as its modest genome size, low number of chromosomes, vitality, short generation cycle, and mature tissue culture technology, have resulted in Danshen becoming an ideal model plant among TCM (Song et al. 2013).

At present, almost all of the Danshen preparations are primarily obtained by extraction from plants. An important and urgent task is to focus on improvements in Danshen for optimizing desirable traits, e.g., effective components, resistance, and yield. According to the literature on *S. miltiorrhiza*, several molecular markers, including amplified fragment length polymorphisms (AFLPs) (Yang et al. 2012), sequence-related amplified polymorphisms (SRAPs) (Song et al. 2010), EST-simple sequence repeats (SSRs), and inter-SSRs (ISSRs), have been used to analyze the genetic diversity of Danshen. Previous studies have shown that *S. miltiorrhiza* is a cross-pollinated plant with high differentiation of its germplasm (Song et al. 2010). These results provide an important basis for further construction of genetic maps with the aim of selecting parents and markers. Genetic linkage maps, particularly high-density genetic maps, are valuable tools in meeting the requirement of high-throughput superior trait selection among various germplasms, including plants and animals.

Secondary metabolites are usually synthesized in specific organs or tissues of plants with a relatively low level of contents, which were

generally considered as complex agronomic traits, and controlled by multiple genes known as quantitative trait loci (QTL). QTL analysis based on linkage maps is useful for screening closely linked molecular markers of complex traits. It can effectively elucidate the genetic basis of complex agronomic traits, estimate gene actions and genetic parameters, and further accelerate the breeding progress through marker-assistant selection (MAS) in different species (Shehzad and Okuno 2016).

Since the construction of genetic map of *Artemisia annua* L. and the identification of QTL affecting yields of artemisinin were reported (Graham et al. 2010), progresses have been obtained for various plant species, such as *Bupleurum chinense* DC., *Siraitia grosvenorii*, *Eucommia ulmoides*, and *Dendrobium officinale* (Zhan et al. 2010; Liu et al. 2011; Li et al. 2015; Lu et al. 2018). Although this opens a new door for genetic breeding researches of medicinal plant, genetic researches about active compound contents of medicinal or aromatic plants only conducted in a few species. The studies on medicinal or aromatic plants are behind far from other crops.

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## 3.2 Molecular Linkage Map Construction

### 3.2.1 Map Strategy and Mapping Populations

An appropriate mapping population, which generally includes RIL, DH, F<sub>2</sub> or backcrossed progeny, is very important in the construction of genetic maps (Grattapaglia and Sederoff 1994). However, similar to most medicinal species, it is very difficult to obtain a typical family-based population in *S. miltiorrhiza* due to its high heterozygosity resulting from a long history of natural cross-pollination and inbreeding depression. The double pseudo-testcross strategy was first proposed by Grattapaglia and Sederoff (1994) and was successfully applied to construct a genetic map of forest trees. In the pseudo-testcross, an F<sub>1</sub> progeny is developed as a

mapping population by hybridizing two unrelated and highly heterozygous individuals, where gene segregation patterns can be interpreted as a backcross. This strategy has been widely used in plant species that lack appropriate pedigrees (Myburg et al. 2003; Feng et al. 2013; Sudarshini et al. 2014).

Because of its cross-pollinated habit and lack of basic genetic and breeding study, it is difficult to construct the  $F_2$  and RIL populations in *S. miltiorrhiza*. In many fruit and forest trees with cross-pollination and asexual reproduction, the  $F_1$  mapping populations were used for genetic study of interested agronomic traits based on the principle of ‘pseudocrossing’, such as *Mangifera indica* L. (Luo et al. 2016) and *Sesamum indicum* L. (Zhang et al. 2013a, b). Because both parents were heterozygous, the genotype among different  $F_1$  progeny was different, which provides a feasible method for most medicinal plants. Many medicinal plants have a cross-pollinating habit. There is no doubt that this tendency provides favorable conditions for the selection of mapping parents. If there is a poor genetic basis and the lack of a typical family-based population, the pseudo-testcross is the most promising method for creating genetic maps of medicinal plants at the present time.

Study route for genetic mapping and QTL location of *S. miltiorrhiza* showed on Fig. 3.1. The first step is to select female and male parents with significant different characteristics, which is the guarantee for the analysis of QTL for important trait. A considerable difference between the parents was required to generate large polymorphic markers. The next step is to create  $F_1$  interspecific hybrid population, which usually contains at least 100  $F_1$  lines. The third step is the main part of work, which includes two parallel elements. One is DNA extraction, genotyping by various molecular markers and further genetic map construction. Another is to investigate agronomic traits. The fourth step is association of agronomic traits with genotyping by software to identify the location, number of loci, and genetic effect of QTL. The final aim is

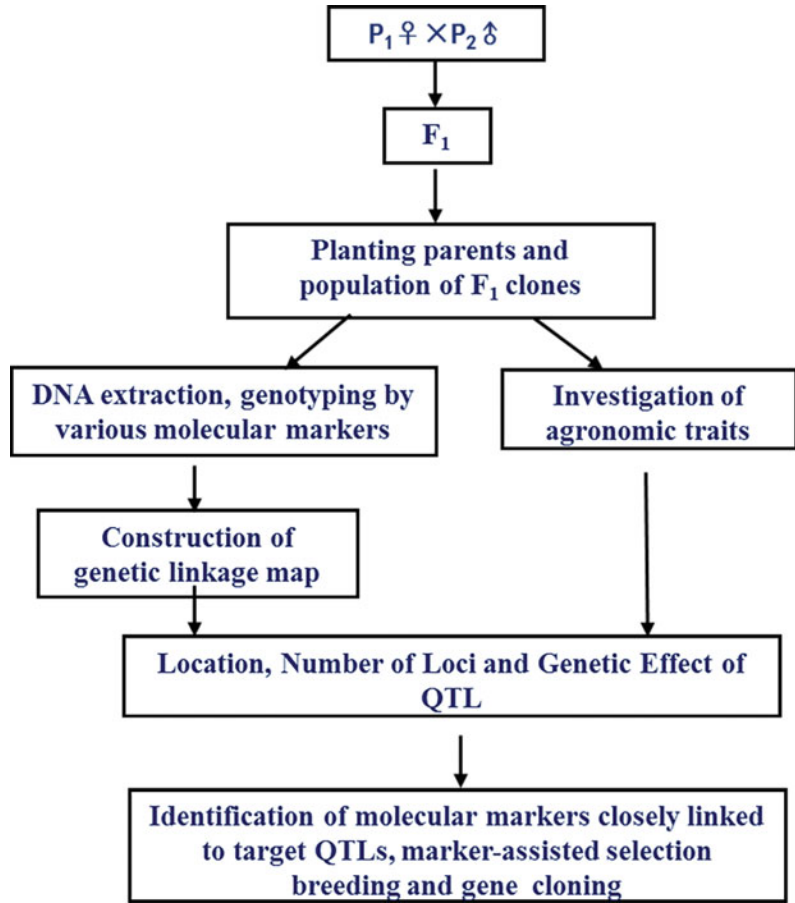
to identify molecular markers closely linked to important trait, providing important basis for marker-assisted selection (MAS) and map-based gene cloning.

### 3.2.2 $F_1$ Genotyping, Data Analysis and Map Construction Method

The marker analysis was carried out according to the isolation types of parents and  $F_1$  population. Marker segregation types can be divided into three categories: parental heterozygous markers, maternal-specific markers, and paternal-specific markers, including  $ab \times cd$ ,  $ef \times eg$ ,  $hk \times hk$ ,  $lm \times ll$ , and  $nn \times np$  (Fig. 3.2). There are four different amplification bands in the  $ab \times cd$  segregation type with  $ab$  derived from maternal parent and  $cd$  from paternal parent, which produce four types including  $ac$ ,  $ad$ ,  $bc$ , and  $bd$  with same ratio of 1:1:1:1 in their  $F_1$  population. For the  $ef \times eg$  type, the “e” amplification band was a common one from two parents, but “f” and “g” were the unique markers from maternal parent and paternal parent, respectively. There will appear four types including  $ee$ ,  $eg$ ,  $fe$ , and  $fg$  in  $F_1$  population with the ratio of 1:1:1:1. The two parents have the same bands type in the  $hk \times hk$  type, and the  $F_1$  population show  $hh:hk:kk$  with the ratio of 1:2:1. There are two different amplification bands in  $lm \times ll$  type or  $nn \times np$  type, with “l” or “n” as a common band of two parents, “m” and “p” as unique band in maternal parent and paternal parent, respectively. Therefore, their  $F_1$  population displays two types with same ratio of 1:1.

Markers with blurred or missing data are labeled as “-”. According to different types of markers, the data were analyzed by statistical analysis, and the obtained data were tested for segregation ratios using the Chi-squared ( $\chi^2$ ) test. The distorted markers ( $p < 0.05$ ) were assigned a “\*” suffix. JoinMap 4.0 software was employed to establish the genetic linkage map at likelihood of odds (LOD) score at least 3.0 with a

**Fig. 3.1** Study route for genetic mapping and QTL location of *S. miltiorrhiza*



cross-pollinator (CP) population type. Map distances were calculated using Kosambi's mapping function. MapChart 2.2 was used to draw the genetic map (Cui et al. 2015).

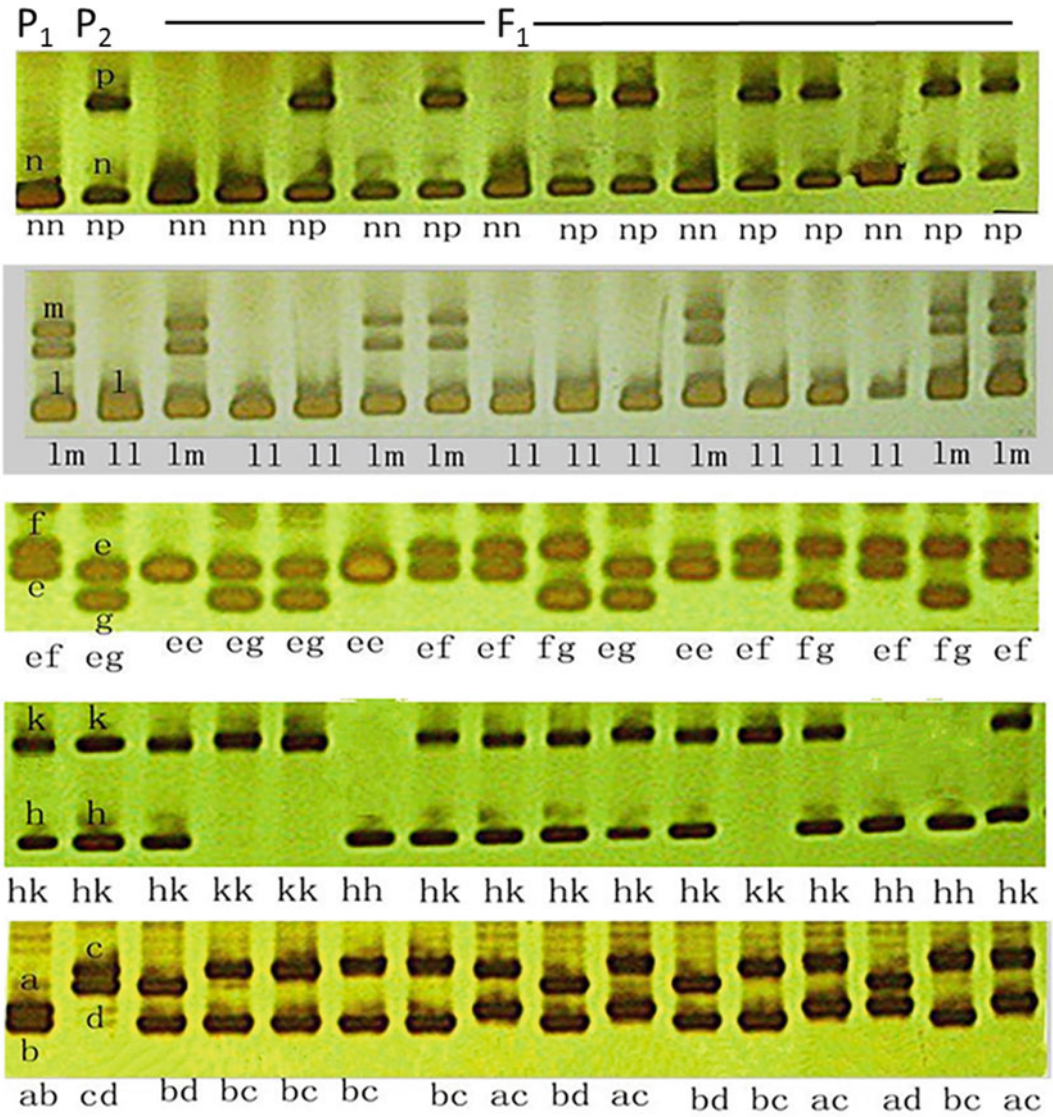
### 3.2.3 Genetic Linkage Map Construction of *S. miltiorrhiza*

In our group, we selected the lines ZH74, ZH23, and ZH105 as female parents and BH18 as male parent (Fig. 3.3) to create three F<sub>1</sub> interspecific hybrid population of *S. miltiorrhiza* for marker genotyping and the construction of genetic linkage maps using the double pseudo-testcross mapping strategy. A total of four genetic linkage maps were constructed using the above three F<sub>1</sub> populations.

#### 3.2.3.1 The First Linkage Genetic Map and Another Map Based on Integration of Multiple Markers

The first genetic linkage map of *S. miltiorrhiza* was constructed in 94 F<sub>1</sub> individuals from crossing ZH74 and BH18 by using simple sequence repeat (SSR), sequence-related amplified polymorphism (SRAP) and inter-simple sequence repeat (ISSR) markers. A total of 93 marker loci in the linkage map, consisting of 53 SSR, 38 SRAP, and 2 ISSR loci, were made up of eight linkage groups, covering a total length of 400.1 cm with an average distance of 4.3 cm per marker. The length of linkage groups varied from 3.3 to 132 cm and each of them included 2–23 markers, separately (Zong et al. 2015).

Another map was constructed in 138 F<sub>1</sub> individuals from crossing ZH105 and BH18



**Fig. 3.2** Five segregation types in F<sub>1</sub> population of *S. multiorrhiza*

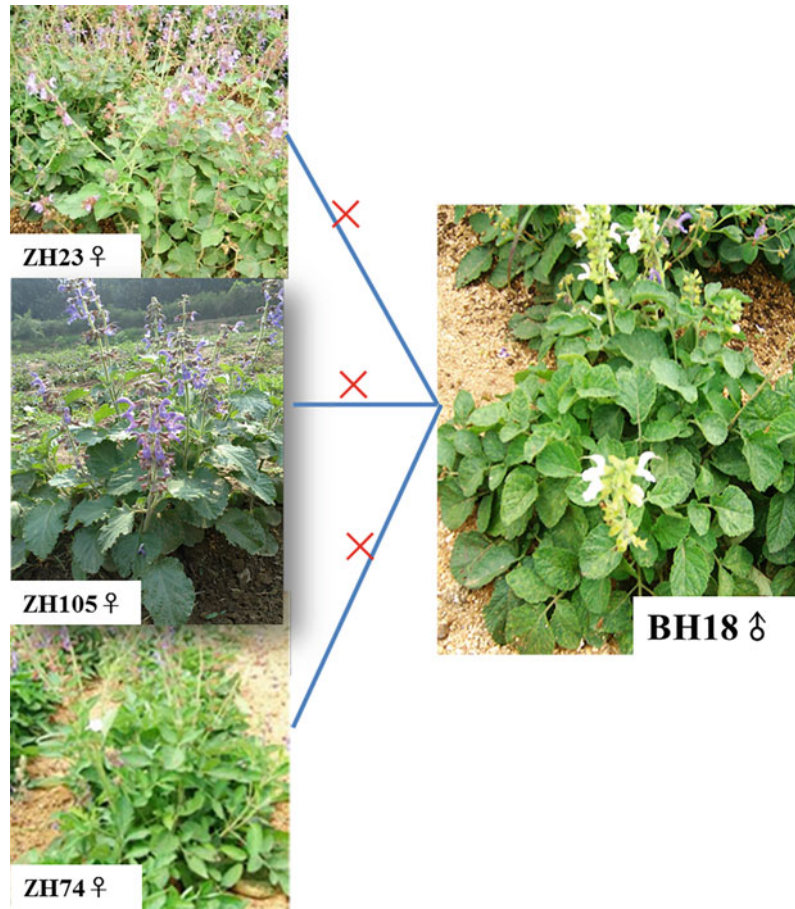
based on integration of multiple markers. A set of 503 SSR primers, 100 SRAP primers, and 8 ISSR primers developed from *S. multiorrhiza* were screened in the two parents. A total of 340 primers yielded amplification effectively, with 206 primers possess polymorphism. It contained 158 SSR primers, 46 SRAP primers, and 2 ISSR primers with the percentage to be 31.4, 46.0, and 25.0%, respectively. It included 9 linkage groups, contained 111 loci, and covered genome 857.4 cM with a mean marker interval of

7.7 cM. The length of linkage groups varied from 41.6 to 149.7 cM and each of them included 2–27 markers, respectively. Seventeen point one percent partially segregated markers distributed in the map were mainly located on LG1, LG2, and LG3 linkage groups.

### 3.2.3.2 The Genomic SSR Linkage Genetic Map

Genome SSR was developed by simplified genome sequencing of female parent ZH23.

**Fig. 3.3** Lines ZH74, ZH23, and ZH105 as female parents and BH18 as male parent for creating three F<sub>1</sub> interspecific hybrid population of *S. miltiorrhiza*



A total of 665 pairs of SSR primers were synthesized and used to analyze the polymorphism between ZH23 and BH18, of which 568 pairs of primers generated clear bands. A total of 138 pairs of genomic SSR primers that amplified 151 loci were polymorphic and stable among the parents and 206 F<sub>1</sub> lines. Among 138 pairs of polymorphic SSR primers, a total of five separation types were produced. The separation types of  $ab \times cd$ ,  $ef \times eg$ ,  $hk \times hk$ ,  $lm \times ll$ , and  $nn$   $np$  involved 1, 13, 0, 48, and 49 primer pairs, respectively. One hundred fifty-one polymorphic loci were subjected to the  $\chi^2$  test to determine if they met the expected segregation ratio given by the JoinMap4.0 software program. The LOD value was selected to 3.0, and finally, 111 SSR markers were used to construct the genetic linkage map.

The second genetic map of *S. miltiorrhiza* was constructed based on genomic SSRs. Simple sequence repeat (SSR) is one of the most popular and versatile marker type and is considered as an ideal co-dominant marker for plant genetic mapping (Morgantae and Olivieri 2010). It has several prior characteristics, such as neutrality, abundance and polymorphism, high information content, and mostly single-gene inheritance. Totally, 111 SSR markers were finally assigned into eight linkage groups, which corresponded to the haploid chromosome number of *S. miltiorrhiza* ( $2n = 16$ ). The genetic distance spanned 397.5 cM in this maps accounting for about 92.7% of the entire genome of *S. miltiorrhiza*. The length of individual linkage group ranged from 24.8 cM (LG4) to 102.0 cM (LG1) with the number of markers on each group ranged from 6

to 24. Average distance between markers was 3.6 cM, with intervals between loci ranged from 1.8 to 9.7 cM (Feng et al. 2019).

### 3.2.3.3 The High-Density Linkage Genetic Map

To construct a high-density genetic map, specific length amplified fragment sequencing (SLAF-seq) was used for the rapid discovery of SNPs in the  $F_1$  population. Analysis of the  $F_1$  mapping population indicated that 83,154 SLAFs were generated, with an average depth of 6.17-fold for each offspring. Among the 151,035 high-quality SLAFs, 62,834 were polymorphic, resulting in a polymorphism rate of 41.60%. Of the 62,834 polymorphic SLAFs, 47,701 were classified into eight segregation patterns. For the  $F_1$  population, five segregation patterns, including  $ab \times cd$ ,  $ef \times eg$ ,  $hk \times hk$ ,  $lm \times ll$ , and  $nn \times np$ , were used for genetic map construction, and a total of 5198 SLAFs were used for map construction (Fig. 3.4) (Liu et al. 2016).

Then, the high-density genetic map was constructed. After completing the data preparation, 5164 of the 5198 SLAFs were mapped onto the genetic map (i.e., a ratio of 99.34%). There were a total of 2966 BH18 (male) markers, 3038 ZH74 (female) markers, and 5164 SLAFs (7554 SNPs), which fell into 8 LGs, for the integrated map. The

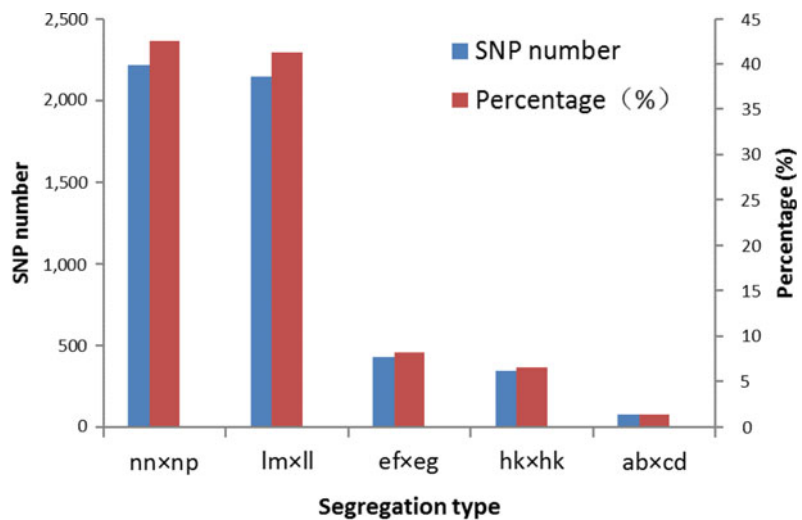
coverage of the markers was 75.67-fold in the female parent, 91.20-fold in the male parent, and 10.36-fold in each  $F_1$  individual (on average). The final map was 1516.43 cM in length, with an average inter-marker distance of 0.29 cM (Table 3.1). According to the formula for genetic linkage maps, the coverage ratio of the total length to the expected length was 99.83%, which is considerably stronger compared to the value of 84.4% obtained for the first map. The current linkage map covers nearly the entire genome with a resolution of 0.29 cM (Liu et al. 2016).

## 3.3 Quantitative Trait Loci Analysis of Important Agronomic Traits in *S. miltiorrhiza*

### 3.3.1 Quantitative Trait Loci (QTL) Mapping Method

Quantitative trait loci (QTL) mapping was performed using the MapQTL 6.0 software based on the interval mapping (IM) algorithm with a significance level of 0.05, 1000 times of permutation and walking speed of 1 cM. Quantitative trait loci (QTL) identified in this way were marked by the marker with the highest LOD score in the corresponding QTL region.

**Fig. 3.4** SNP number and percentage of five segregation types in  $F_1$  population of *S. miltiorrhiza*



**Table 3.1** Genetic map for eight linkage groups (LGs)

	Total marker			Total distance (cM)			Max gap (cM)		
	Female map	Male map	Integrated map	Female map	Male map	Integrated map	Female map	Male map	Integrated map
LG1	347	316	559	141.153	107.028	132.85	15.79	6.68	7.88
LG2	341	371	613	186.544	180.181	190.39	17.24	13.01	7.88
LG3	426	430	747	195.187	216.418	213.66	21.86	9.12	9.6
LG4	378	390	652	183.249	175.127	181.17	10.38	18.74	9.11
LG5	279	266	478	153.085	108.194	130.64	13.01	26.95	10.45
LG6	404	372	675	196.998	202.857	207.41	114.00	18.44	11.53
LG7	352	353	610	115.325	170.161	163.048	6.68	20.27	19.22
LG8	511	468	830	293.022	297.244	297.25	26.95	17.24	20.67
Total	3038	2966	5164	1464.563	1457.21	1516.43	26.95	26.95	20.67

### 3.3.2 QTL Analysis for Phenolic Acid Content

The main peaks in the chromatograms of rosmarinic acid (RA), salvianolic acid (SAB), and lithospermic acid (LA) were identified by HPLC, based on their retention time and UV absorptions. Measurement of three phenolic acid contents showed significant differences between two parents. The variation coefficients of these traits varied from 33.64% (SAB content) to 53.03% (RA content), which showed a significant variation among  $F_1$  individuals. Skewness and peakedness test indicated that these traits were segregated continuously and controlled by multiple genes, suggesting their suitability for QTL analysis.

Six QTL were identified for three phenolic acids content traits with phenotypic variance explained from 10.8 to 15.9%. Among them, 4 QTL was anchored on LG7, 1 QTL on LG2, and 1 QTL on LG4, respectively. Three QTL for SAB content were detected in a continuous interval between DSSR-540 and DSSR-98 markers on LG7 with a length of 27.2 cM (Fig. 3.5), in which, three continuous QTL explained 13.8, 12.9, and 13.6% of the phenotypic variation, respectively. Two QTL for LA content were located on LG2 and LG4, explained 10.8 and 15.9% of the phenotypic variation, respectively. These two QTL were localized with

a single marker DSSR-140 and DSSR-77, respectively. Only one QTL for RA content was detected on LG7, which accounted for 12.4% of the phenotypic variation, and this locus was in the same region of QTL for SAB content.

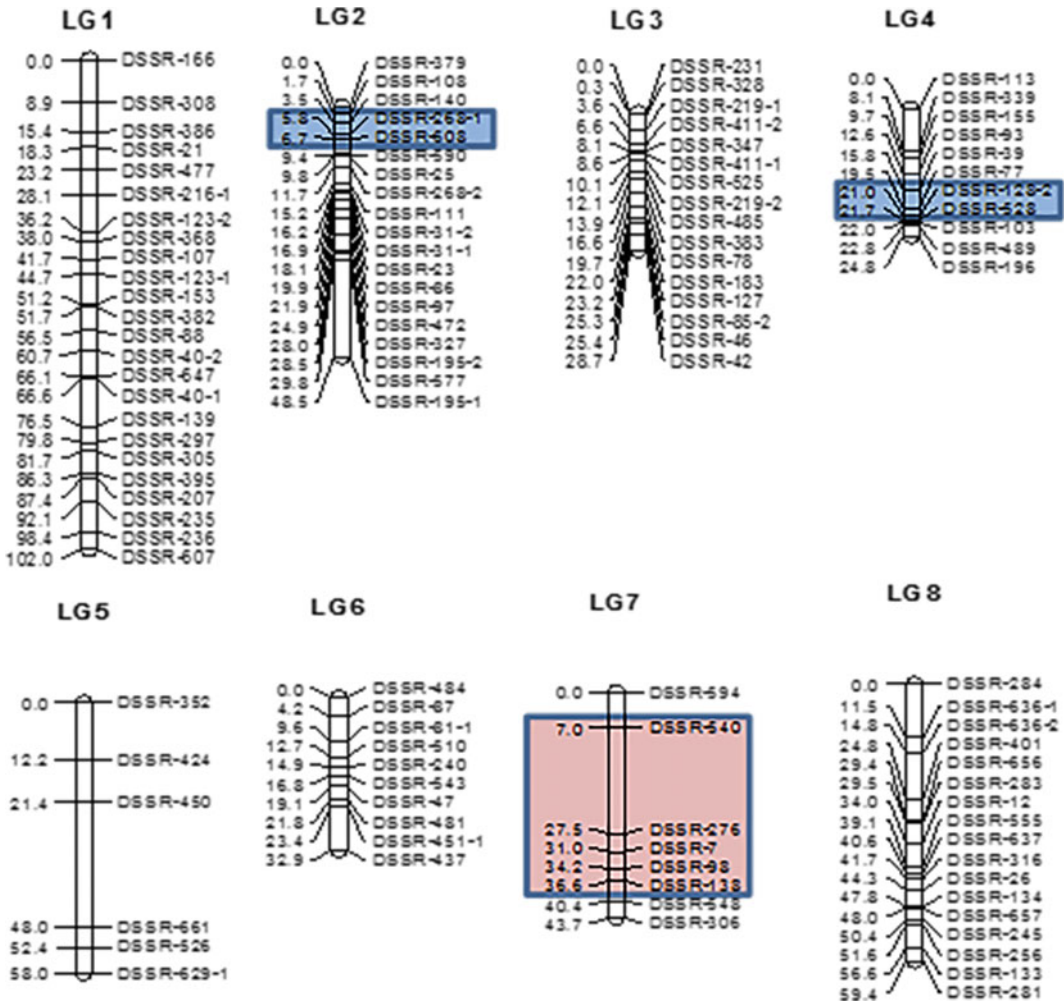
### 3.3.3 QTL Analysis for Root Yield

Various characters in ZH105/BH18  $F_1$  generation groups showed continuous distribution of the root traits of *S. miltiorrhiza*. These characters conformed the quantitative trait loci for genetic and normal distribution. Twenty-six loci were detected in the ten phenotypic traits of *S. miltiorrhiza* by the interval mapping method. Root diameter, root range, root fresh weight, and root number were detected QTL loci, explaining the phenotypic variation rate ranged from 8.0 to 13.7%.

## 3.4 Discussion

Most of the genetic maps constructed in medicinal plants mainly use the universal markers (ISSR, SRAP, and AFLP), which indicates that the related genetic research of medicinal plants is still in its infancy, far behind that of food crops and other crops. The genetic map of Danshen, even though it is an emerging model medicinal





**Fig. 3.5** Genetic map of the F<sub>1</sub> population (SNDS1 × BH18) based on the simple sequence repeat (SSR) markers and distribution of quantitative trait loci (QTL) effecting phenolic acid concentration. Genetic linkage map of *S. miltiorrhiza* presented as eight pseudo-chromosomes. The names of loci are shown on the right, and the positions of

the loci are shown, in Kosambi centiMorgan (cM), on the left. QTL are shown on the light blue boxes on LG2 and LG4 for two loci of lithospermic acid content. The light red box showed an important cluster consisting of four QTL, one for rosmarinic acid and three for salvianolic acid B content

plant, only recently begun to be constructed. The first map, which was constructed using SRAPs, ISSRs, and SSRs in the F<sub>1</sub> population, includes 94 loci with an average interval distance of 4.3 cM. However, this unsaturated genetic map has limited future application. SNPs are the most abundant and stable form of genetic variation in most genomes and have become the marker type of choice in many genetic studies. Subsequently, a high-density genetic map of *S. miltiorrhiza* was

constructed, which contained 5164 high-quality SLAFs and spanned 1516.43 cM, with an average marker interval of 0.29 cM. Due to different parents and different population, the genetic maps usually have poor versatility. In integration, the genomic SSR was essential because of the characteristics of co-dominance, easy to score, simple banding, and convenient identification (Wu et al. 2016; McCallum et al. 2016). Species-specific SSR markers need to be further

developed and widely used in TCM. The methods used in this study for genetic mapping and for the development of markers provide a valuable reference for other medicinal plants.

Quantitative trait loci analysis based on linkage map can effectively elucidate the genetic basis of complex agronomic traits (Ma et al. 2014). A large number of QTL have been mapped in many crops related to various important agronomic traits, such as quantity (Tharanya et al. 2018), quality (Vallejo et al. 2015), plant adaptation and tolerance to stresses (Pilet-nayel et al. 2017), and some of which had been successfully utilized in breeding programs (Singh and Miklas 2015).

Phenolic acids are one of the main active components in *S. miltiorrhiza* root. Thus, improvement of phenolic acid contents has been an important objective of its genetic and breeding research. In our group, QTL analyses of phenolic acid contents in *S. miltiorrhiza* were firstly reported. It opened a new window for future MAS breeding of phenolic acids and identification of genes associated with phenolic acids biosynthesis.

Quantitative trait loci for correlated traits often position on the same chromosomal regions. Consistently, QTL for rosmarinic acid (RA) and three QTL for salvianolic acid (SAB) content were located within the same region. This region can be considered as further target region for fine mapping and marker-assisted selection in *S. miltiorrhiza* breeding programs. Besides, SAB content also had a significantly high correlation with the other two secondary compounds contents. Selection of SAB content will have positive effects on other traits. The same phenomenon of overlaps in QTL regions was also reported in many plant species, such as *Pyrus L.* (Zhang et al. 2013a, b), *Glycine max L.* Merr. (Akond et al. 2014), and *Cynodon dactylon* var. *dactylon* (Guo et al. 2017a, b). The above findings may be due to two genes controlling different traits. These QTL are likely to be either within the same region or in adjacent locations on a chromosome or a single gene tended to have pleiotropic effect (Zhang et al. 2013a, b). A limited number of QTL for phenolic acid contents were detected due to the genetic maps with low

density. Improvement in the marker density would increase the identification of QTL number. It is suggested that the density of the genetic map is a key to perform QTL fine mapping of interested agronomic traits.

In future breeding of *S. miltiorrhiza*, attention should be paid to the continuous recording and dynamic analysis of agronomic traits for many years. It will greatly improve the predictability and accuracy of the selection of good genotypes for quantitative traits and will effectively improve the MAS efficiency. In addition, expanding mapping population, increasing the number of markers in genetic map, constructing ultra-high density map, and integrating and locating physical map can effectively improve the establishment of closely linked QTL and lay an important foundation for molecular breeding of *S. miltiorrhiza*.

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