

RESEARCH ARTICLE

A High-Density Genetic Map of Tetraploid *Salix matsudana* Using Specific Length Amplified Fragment Sequencing (SLAF-seq)

Jian Zhang^{1,3*}, Huwei Yuan^{2*}, Min Li³, Yujuan Li³, Ying Wang³, Xiangjian Ma³, Yuan Zhang³, Feng Tan³, Rongling Wu^{1,4*}

1 Center for Computational Biology, College of Biological Sciences and Technology, Beijing Forestry University, Beijing, China, **2** National Engineering Laboratory for Forest Tree Breeding, Key Laboratory for Genetics and Breeding of Forest Trees and Ornamental Plants of Ministry of Education, College of Biological Sciences and Technology, Beijing Forestry University, Beijing, China, **3** Jiangsu Riverine Institute of Agricultural Sciences, Rugao, Jiangsu, China, **4** Center for Statistical Genetics, Pennsylvania State University, Hershey, United States of America

* These authors contributed equally to this work.

* rwu@bjfu.edu.cn; rwu@phs.psu.edu



CrossMark
click for updates

OPEN ACCESS

Citation: Zhang J, Yuan H, Li M, Li Y, Wang Y, Ma X, et al. (2016) A High-Density Genetic Map of Tetraploid *Salix matsudana* Using Specific Length Amplified Fragment Sequencing (SLAF-seq). PLoS ONE 11(6): e0157777. doi:10.1371/journal.pone.0157777

Editor: Song Wu, The State University of New York at Stony Brook, UNITED STATES

Received: March 7, 2016

Accepted: June 3, 2016

Published: June 21, 2016

Copyright: This is an open access article, free of all copyright, and may be freely reproduced, distributed, transmitted, modified, built upon, or otherwise used by anyone for any lawful purpose. The work is made available under the [Creative Commons CC0](https://creativecommons.org/licenses/by/4.0/) public domain dedication.

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work was supported by “the Fund for Independent Innovation of Agricultural Sciences and Technology in Jiangsu Province (No. CX (14)5094)”, “the Fund for 333 Engineering Project in Jiangsu Province (No. BRA2015125)”, “the Fund for the 12th Six Peak Talent Project in Jiangsu Province (No. 2015-NY-042)”, and “the Fund for 226 Engineering Project in Nantong City (No. 2014008)”.

Abstract

As a salt-tolerant arbor tree species, *Salix matsudana* plays an important role in afforestation and greening in the coastal areas of China. To select superior *Salix* varieties that adapt to wide saline areas, it is of paramount importance to understand and identify the mechanisms of salt-tolerance at the level of the whole genome. Here, we describe a high-density genetic linkage map of *S. matsudana* that represents a good coverage of the *Salix* genome. An intraspecific F₁ hybrid population was established by crossing the salt-sensitive “Yanjiang” variety as the female parent with the salt-tolerant “9901” variety as the male parent. This population, along with its parents, was genotyped by specific length amplified fragment sequencing (SLAF-seq), leading to 277,333 high-quality SLAF markers. By marker analysis, we found that both the parents and offspring were tetraploid. The mean sequencing depth was 53.20-fold for “Yanjiang”, 47.41-fold for “9901”, and 11.02-fold for the offspring. Of the SLAF markers detected, 42,321 are polymorphic with sufficient quality for map construction. The final genetic map was constructed using 6,737 SLAF markers, covering 38 linkage groups (LGs). The genetic map spanned 5,497.45 cM in length, with an average distance of 0.82 cM. As a first high-density genetic map of *S. matsudana* constructed from salt tolerance-varying varieties, this study will provide a foundation for mapping quantitative trait loci that modulate salt tolerance and resistance in *Salix* and provide important references for molecular breeding of this important forest tree.

Introduction

Willows, the general name of species in *Salix* (*Salicaceae*), are deciduous trees or shrubs distributed mainly in the temperate and frigid zones of the Northern Hemisphere. There are > 500

Competing Interests: The authors have declared that no competing interests exist.

willow species worldwide, about half of which can be found in China. Willows are important tree species for energy production, afforestation, and greening [1–3]. Some willow varieties, including *S. matsudana* and *Salix psammophila*, have been receiving increasing attention because of their salt tolerance [4–8]. Some varieties of *S. matsudana* have become potential strategic resources in coastal forestry exploitations of China [9, 10]. To date, the responses of *S. matsudana* to salt stress have been studied at the levels of physiology [10], gene expression [9], and miRNA expression [11]. However, the mechanisms of salt tolerance at the whole genome level of *S. matsudana* has not been extensively explored thus far.

Genetic maps that are constructed according to the linkage relationships among genetic markers at the whole genome level are the basis for quantitative trait locus (QTL) mapping, map-based gene cloning, comparative genomics, and marker-assisted breeding. Willows have relatively high recombination rates and low levels of linkage disequilibrium (LD) [12], which makes them suitable for genetic mapping. The first genetic linkage map of *Salix* was constructed using a population of 87 hybrids derived from a cross between "Björn" (the male hybrid clone of *Salix viminalis* × *Salix schwerinii*) and "78183" (the female clone of *Salix viminalis*). The map consisted of 325 amplified fragment length polymorphisms (AFLP) and 38 restriction fragment length polymorphisms (RFLP) markers with an average density of markers of 14 cM [13]. Later, two linkage maps of *Salix* containing 495 single nucleotide polymorphisms (SNP) and 221 AFLP markers were constructed, with the average distances of 5.0 and 8.1 cM, respectively [14]. All these maps were constructed using diploid ($2n = 38$) willows and were of low density, with the average marker densities of 5.0–14 cM. Barcaccia et al. [15] have successfully constructed a genetic map for tetraploid ($2n = 4x = 76$) *Salix*, but its marker density needs to be increased for a better understanding of the genome structure and organization of tetraploid *Salix*.

Single nucleotide polymorphisms (SNP) represent DNA sequence variation among individuals caused by single base mutations. SNP markers have become a powerful tool in genetics due to their abundant and even distribution. Recent advances in next-generation sequencing (NGS) technologies have provided enormous impetus for the rapid development and extensive application of SNP markers [16]. Technologies that can develop SNP markers in a short time include complexity reduction of polymorphic sequences (CroPS) [17], restriction-site-associated DNA sequencing (RAD-seq) [18], and genotyping-by-sequencing [19]. Specific length amplified fragment sequencing (SLAF-seq) is a newly developed NGS technology that can be used to rapidly develop SNP markers by constructing a SLAF-seq library [20]. Development of SNP markers and construction of high-density genetic maps based on SLAF-seq have been applied to a number of species [20–24].

In this study, "Yanjiang" (a salt-sensitive variety of *S. matsudana* native to the Jiangsu riverine areas of China) and "9901" (a salt-tolerant variety of *S. matsudana* in Shandong coastal areas of China) were used as female and male parents, respectively, and the intraspecific F_1 hybrid population containing 3,520 individuals were obtained through controlled pollination. A total of 200 individuals, along with its parents, were selected randomly from the F_1 population and used as the mapping population. SLAF-seq technology [20] was used to develop SLAF markers (SLAFs) and construct a high-density intraspecific genetic map of tetraploid *S. matsudana* constructed from salt tolerance-varying varieties. Results from this study will provide a foundation for genetic map-based QTL fine mapping, gene cloning, comparative genomics, and marker-assisted breeding of salt-tolerant related traits in this important tree species.

Materials and Methods

Plant Material, DNA Extraction, and Identification of Ploidy Levels

Main branches of "Yanjiang" (female parent) and "9901" (male parent) with ≥ 5 cm diameters were collected on December 15, 2013, and then cultured hydroponically in an intelligent

greenhouse at 20–25°C (This work was conducted in Jiangsu Riverine Institute of Agricultural Sciences. We are members in Jiangsu Riverine Institute of Agricultural Sciences and this Institute granted us full permission of the work. This study did not involve endangered or protected species.). Mature pollens were collected from “9901” and then crossed with receptive female flowers on “Yanjiang”. A total of 3,520 F₁ offspring were obtained and sown. Next, 200 individuals were selected randomly from the F₁ population on Jul. 3, 2014, and used as a mapping population. Genomic DNA of the mapping population was extracted using the cationic detergent cetyltrimethylammonium bromide (CTAB) method [25]. The extracted DNA was detected by agarose gel electrophoresis (1%) and then analyzed on the ND-1000 spectrophotometer platform (NanoDrop, Wilmington, DE, USA) for concentration and purity. Ploidy levels of the parents and offspring were examined based on flow cytometry [26] according to the method of Serapiglia et al. [27].

Construction and Sequencing of the SLAF Library and Development of Polymorphic SLAF Markers

The genome of *Populus trichocarpa* (http://www.ncbi.nlm.nih.gov/assembly/GCF_000002775.3) was chosen as the reference genome for pre-restriction enzyme digestion according to the genome size and GC content information. *Hae*III and *Hpy*166II restriction enzymes were finally chosen to digest genomic DNA of the mapping population. After digestion by *Hae*III and *Hpy*166II, the obtained SLAFs (314–364 bp in length) of the mapping population had an A-tail added to the 3' ends, was ligated with Dual-index sequencing adaptors, amplified by PCR, screened, and then used to construct the SLAF library of *S. matsudana* (for detailed processes of SLAF library construction refer to the methods of Sun et al. [20]).

SLAFs in the quality-tested library were sequenced using the Illumina HiSeq 2500 platform (Illumina, San Diego, CA, USA). The genome of *Oryza sativa*, used as a control, underwent the same treatments of library construction and sequencing as the *S. matsudana* mapping population to check the reliability of testing processes.

Reads of the samples were obtained by identifying Dual-index sequences. The adaptor-filtered reads were evaluated for quality and data size, and then clustered to develop SLAFs in the parents and offspring. Polymorphic SLAFs were selected according to the single nucleotide mutations (SNP markers) and insertion and deletion mutations (InDel markers).

Construction of High-Density Genetic Maps

The high-quality polymorphic SLAFs were allocated into different linkage groups (LGs) according to their method limit of detection (MLOD) values. Genetic maps were constructed and corrected using HighMap software according to the methods of [22]. The genetic map was evaluated according to the haplotype maps, percentage of missing SLAFs, and heat maps of each LG.

Results

Ploidy Levels of the Parents and Offspring

Salix suchowensis, the already known diploid ($2n = 2x = 38$), was used as a control to identify ploidy levels of the parents and offspring. Our results showed that the peak values of the parents were twice that of *S. suchowensis*, indicating that both the female parent and male parent were tetraploid ($2n = 4x = 76$) (Fig 1). Similarly, the randomly selected 15 offspring in the mapping population were all tetraploid ($2n = 4x = 76$) (Fig 2).

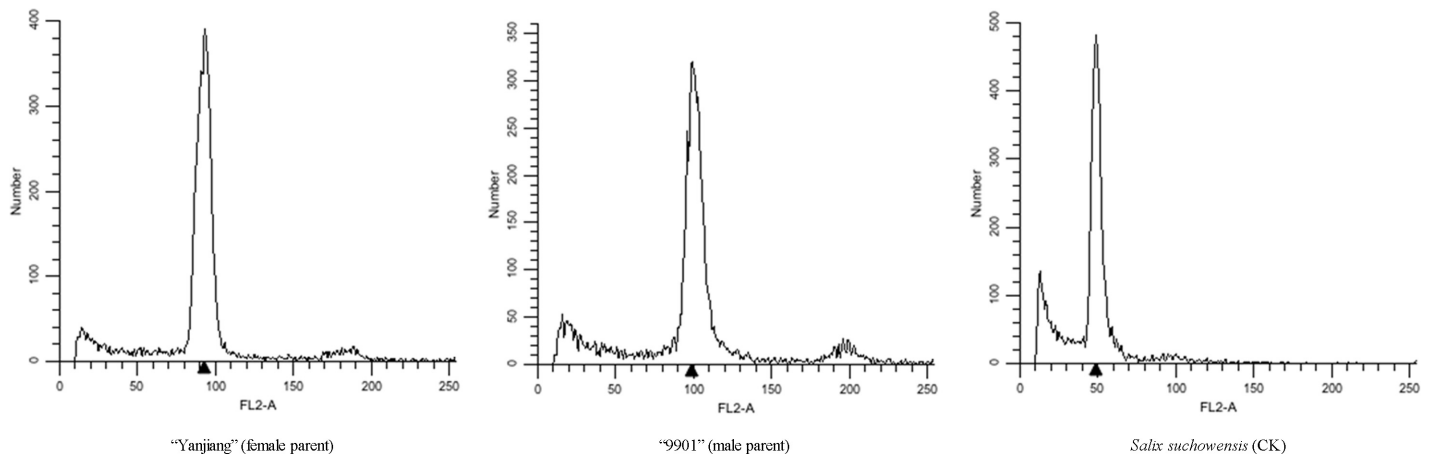


Fig 1. Ploidy levels of *S. matsudana* parents.

doi:10.1371/journal.pone.0157777.g001

Quality of SLAF-seq Data

Evaluations of the *Oryza sativa* SLAF library showed that cleavage efficiency of the *Hae*III and *Hpy*166II restriction enzymes was 92.06%, and the paired-end reads accounted for 96.67% of all reads obtained. These results indirectly reflected that the SLAF library of *S. matsudana* was of high quality. DNA sequencing generated a total of 472.53 M reads. Mean Q30 percentage (the percentage of bases with sequencing values ≥ 30 in the total bases) and mean GC percentage (the percentage of G and C bases in the total bases) of the *S. matsudana* parents and offspring were 90.15% and $\approx 38\%$, respectively. Basic statistics of the SLAF-seq data are listed in [Table 1](#).

Development of SLAF Markers

After analyzing the SLAF-seq data, five offspring with the percentage of abnormal SLAFs in total SLAFs of $\geq 0.3\%$ were removed from the mapping population and the remaining 195 offspring and the parents were retained. In total, 277,333 SLAFs were detected, of which the average depth of the male parent, female parent, and offspring were 53.20-fold, 47.41-fold, and 11.02-fold, respectively ([Table 2](#)).

The 277,333 SLAFs were classified into the three categories of polymorphic, non-polymorphic, and repetitive according to differences in allele number and sequences. Of all the SLAFs obtained, 99,526 (accounting for 35.89% of the total SLAFs) were polymorphic ([Table 3](#)).

After filtering out the 99,526 polymorphic SLAFs lacking parent information, 58,763 were retained and further classified into eight segregation patterns ([Fig 3](#)). The number of SLAFs ranged from 806 to 16,442 in different patterns. The detailed distribution of the markers is shown in [Fig 3](#).

Since F_1 offspring were used as the mapping population, SLAFs with an aaxbb pattern were filtered. The remaining seven segregation patterns of SLAFs (42,321) were candidate markers to construct the genetic map.

Construction of the High-Density Genetic Map

To confirm the quality of the genetic map, the 42,321 polymorphic SLAFs with the following characteristics were further filtered: 1) number of SNPs ≥ 3 ; 2) sequencing depths of the

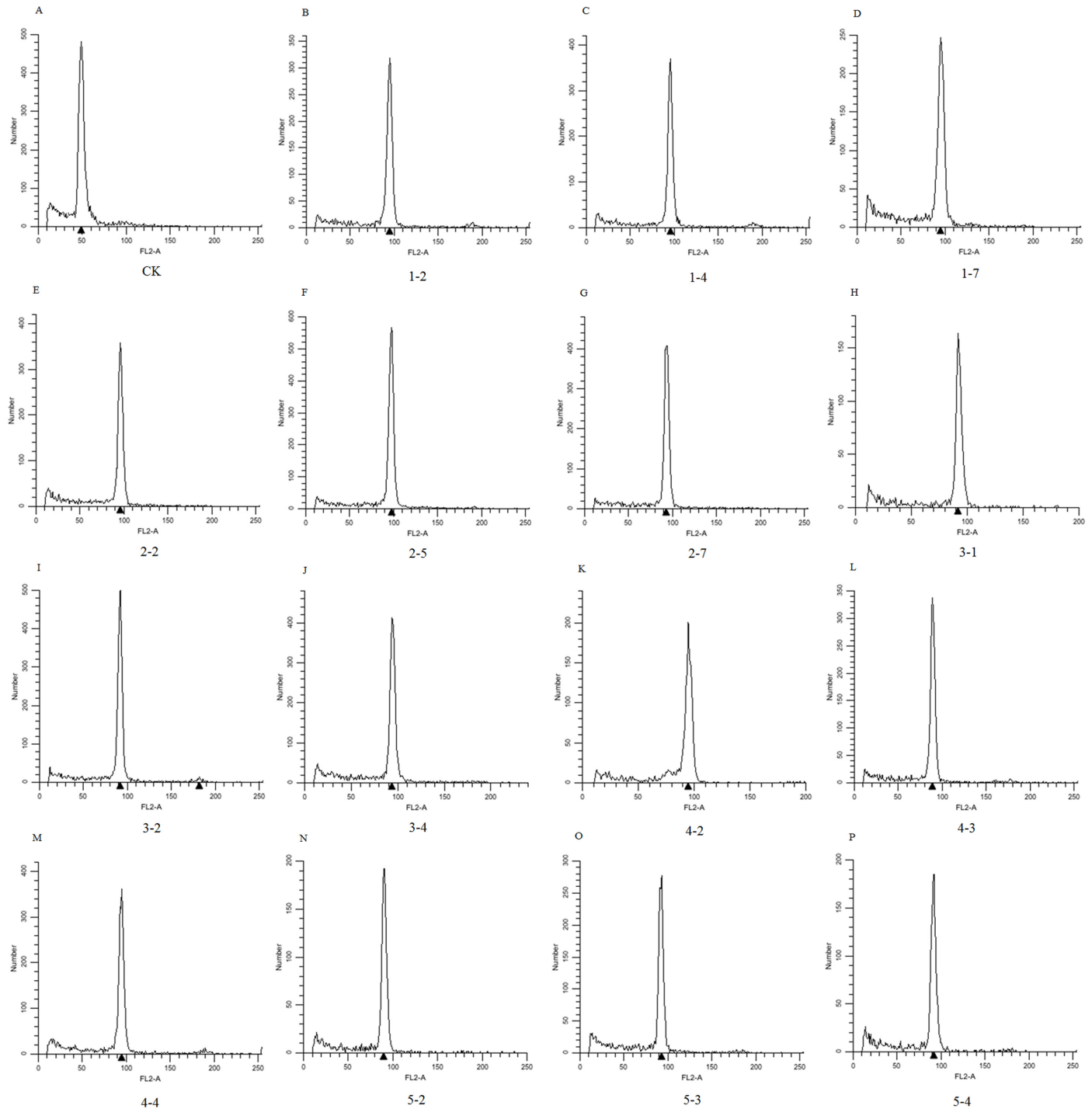


Fig 2. Ploidy levels of *S. matsudana* offspring.

doi:10.1371/journal.pone.0157777.g002

parents ≤ 10 -fold; 3) integrity $\leq 85\%$; 4) distorted segregation; 5) homozygous parents. Finally, 6,744 high quality SLAFs belonged to five segregation patterns (Fig 4) were suitable to construct the genetic map.

Table 1. Basic statistics of the SLAF-seq data in *S. matsudana*.

Sample ID	Total reads	Q30 Percentage (%)	GC Percentage (%)
9901 (male parent)	14,160,572	93.98	37.59
Yanjiang (female parent)	13,640,990	93.84	38.03
Average of 195 offspring	2,223,636	90.11	38.14
Rice (Control)	397,584	93.26	42.23

Note: Total reads represents the number of total reads; Q30 percentage represents the percentage of bases with sequencing values ≥ 30 in the total bases; GC percentage represents the percentage of G and C bases in the total bases.

doi:10.1371/journal.pone.0157777.t001

The MLOD values between the 6,744 SLAFs were calculated. Finally, 6,737 SLAFs were allocated into 38 LGs (S1 Fig). The linear arrangements of all SLAFs and genetic distances of adjacent SLAFs within each LG were analyzed using HighMap software. An integrated genetic map, 5,497.45 cM in total length and 0.82 cM in average length, was finally constructed (S1 Fig, Table 4). A total of 9,488 SNP markers were included on the map. The number of SNP markers in different LGs ranged from 125 to 511 on the map (Fig 5). The mean sequencing depth of the SLAFs on the map were 190.38-fold for the parents and 28.68-fold for the offspring. The basic characteristics of the LGs on the male map and the female map are listed in Tables 5 and 6, respectively.

Evaluation of the Genetic Map

The genetic map of *S. matsudana* was evaluated using haplotype maps and heat maps. Haplotype maps were generated for each of the 195 F₁ individuals using 6,737 SLAFs (S1 File). The recombination events of each individual on LGs of the integrated genetic map were displayed intuitively on the haplotype maps. As can be seen in S2 File, the majority of recombination blocks were clearly defined. The missing percentage of markers in each LG of the integrated genetic map ranged from 0.14% to 0.52% (Fig 6), which did not significantly affect the quality of the genetic map. It can also be seen that all LGs distribute uniformly. Heat maps were also generated to evaluate the quality of genetic map using pair-wise recombination values for the 6,737 mapped SLAFs (S2 File). Heat maps could reflect the recombination relationships between markers in each of the LGs and were used to find the potential ordering errors. It can be seen in S2 File that most of the LGs performed well in visualization, indicating that the markers were well-ordered in each LG. Consequently, the genetic map of *S. matsudana* was of high quality.

Discussion

Willows show high-level variations in chromosome numbers, including diploid, tetraploid, hexaploid, and even dodecaploid [28, 29]. Variations in chromosome numbers of willows occur not only among species, but among varieties of the same species, which has led to difficulties in improving willows. *S. matsudana* is an important arbor willow species, certain

Table 2. Summary of sequencing depth of SLAF markers.

Sample ID	The number of SLAFs	Total depth	Average depth
9901 (male parent)	207,443	11,036,037	53.20
Yanjiang (female parent)	206,718	9,801,466	47.41
Average of 195 offspring	150,292	1,666,374	11.02

doi:10.1371/journal.pone.0157777.t002

Table 3. Classification of SLAF markers.

Type	Polymorphic SLAFs	Non-Polymorphic SLAFs	Repetitive SLAFs	Total SLAFs
Number	99, 526	175,800	2,007	277, 333
Percentage	35.89%	63.39%	0.72%	100%

doi:10.1371/journal.pone.0157777.t003

varieties of which have become potential strategic resources in coastal developments of China because of their salt tolerances [9, 10]. The chromosome number of *S. matsudana* remains unknown, which has hindered studies on this species. To ensure accuracy in the subsequent experiments, ploidy levels of chromosome in the parents and the offspring were determined by comparing the peak values of their chromosomes with diploid *S. suchowensis* ($2n = 2x = 38$) [27, 30] using flow cytometry [26]. Our results showed that both the parents and the 15 randomly selected offspring were tetraploid ($2n = 4x = 76$) (Figs 1 and 2), which has provided a reference for genetic map construction of *S. matsudana*.

Genetic maps are the basis for QTL fine mapping of interesting traits, map-based gene cloning, and marker-assisted breeding. A suitable mapping population is the basis for successful construction of the genetic map. In this report, salt-sensitive “Yanjiang” and salt-tolerant “9901”, the intraspecific varieties of *S. matsudana*, were chosen as female and male parents, and their F₁ hybrid population was used as the mapping population for genetic map construction. The segregation population may present with a large number of variations due to

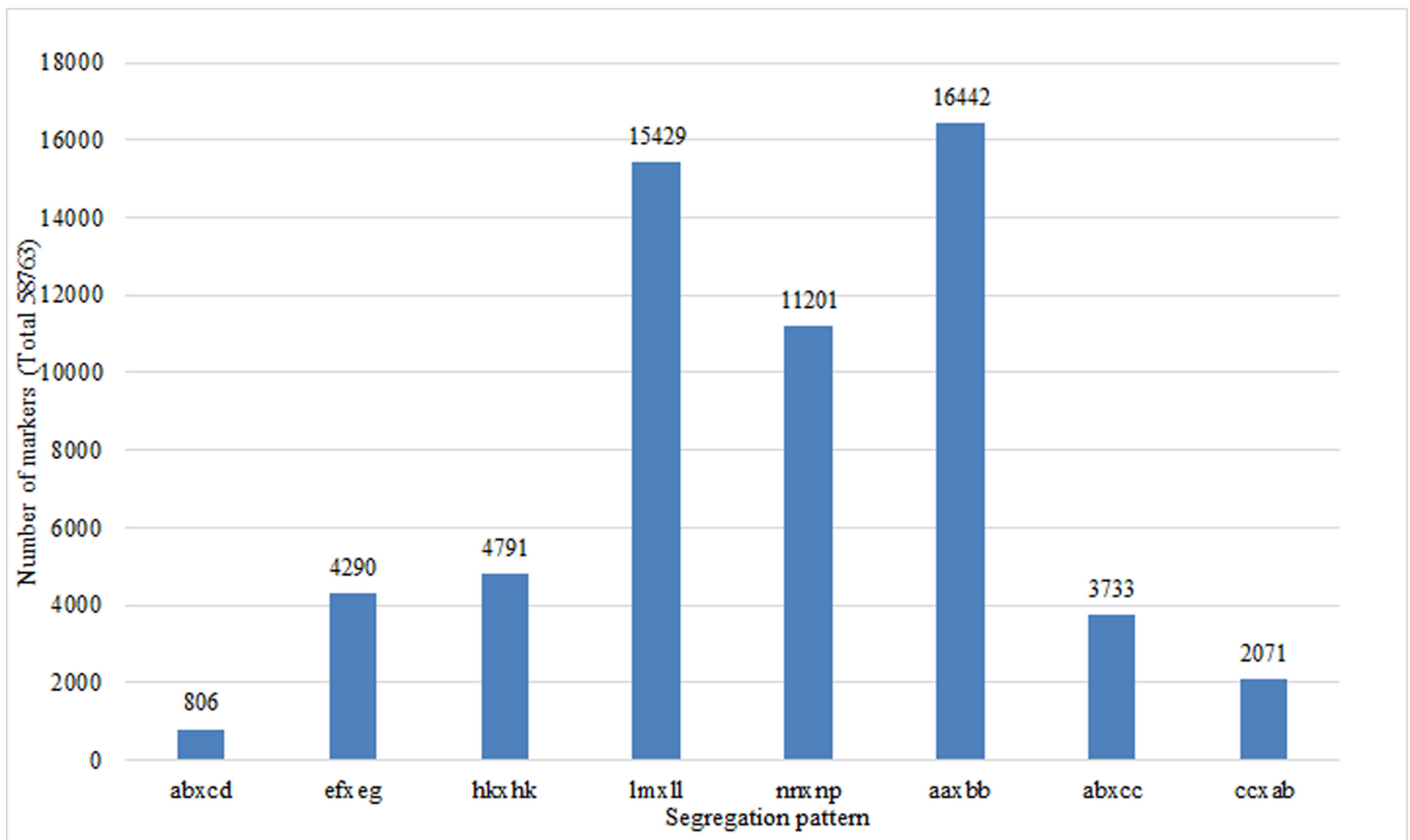


Fig 3. Classification of the filtered polymorphic specific length amplified fragment sequencing (SLAF) markers.

doi:10.1371/journal.pone.0157777.g003

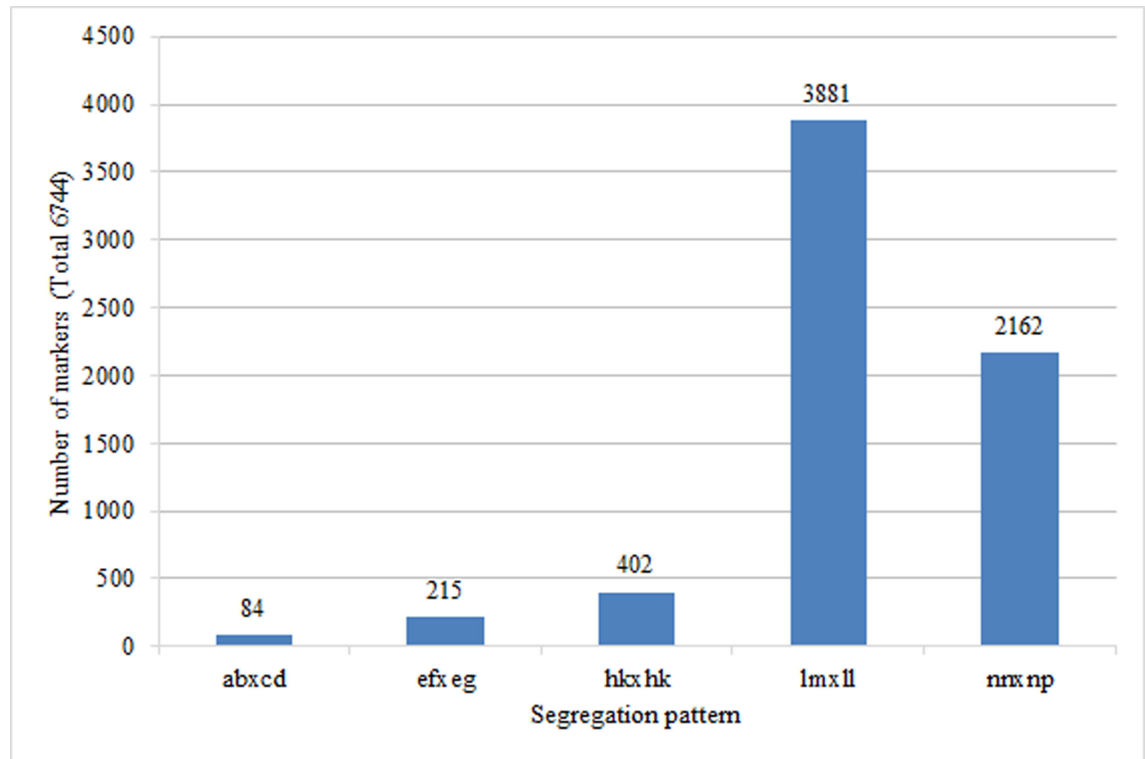


Fig 4. Classification of SLAF markers suitable to construct the genetic map.

doi:10.1371/journal.pone.0157777.g004

significant variations in the salt tolerance of the parents, which could facilitate QTL mapping of salt-tolerant related traits.

Genetic markers are powerful tools for genetic map construction. The selection of molecular markers is key for the success of genetic map construction. The molecular markers used to construct the genetic maps of willows included amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR), and SNPs [13–15]. Among all the markers developed, SNPs are ideal markers for constructing the genetic map due to the advantages of abundance and even distribution across the genome. SNP molecular markers were used in this report, and are suitable for constructing the genetic map of *S. matsudana*.

The number of molecular markers is one of the indices for evaluating the quality of the genetic map. A small number of markers will lead to a map with low-density and large distance, thus decreasing the effective utilization of the map. Although previous genetic maps of willows have been utilized to a certain degree [13–15], the number of markers on the maps was only several hundred, which has hindered effective utilization. SNP markers are used widely in genetic map construction not only because of the advantages of the marker itself, but also because advances in high throughput sequencing technologies allow us to detect a large number of SNPs in a short time [17–20]. RAD-seq and SLAF-seq are among the sequencing technologies that can detect large-scale SNPs. RAD-seq is a reduced-genome sequencing technology that randomly digests genomic DNA with restriction enzymes followed by sequencing. SLAF-seq is a reduced-genome sequencing technology that digests genomic DNA with double restriction enzymes followed by sequencing of the paired-end reads with a specific length. Compared with RAD-seq, the largest advantage of SLAF-seq is its repeatability. SLAF-seq has been used to construct the genetic maps of a number of species in the last few years

Table 4. Basic characteristics of the linkage groups in the integrated genetic map of *S. matsudana*.

Linkage group ID	Marker number	Total distance (cM)	Average distance (cM)	Max gap
LG1	90	110.12	1.24	11.64
LG2	94	104.83	1.13	9.09
LG3	115	96.02	0.84	5.94
LG4	305	240.00	0.79	8.52
LG5	206	175.73	0.86	6.81
LG6	120	123.86	1.04	12.62
LG7	236	166.48	0.71	5.23
LG8	158	129.30	0.82	7.39
LG9	301	175.40	0.58	10.47
LG10	123	156.42	1.28	12.10
LG11	187	137.38	0.74	5.29
LG12	130	111.51	0.86	6.82
LG13	232	151.98	0.66	7.76
LG14	117	121.46	1.05	13.03
LG15	186	155.46	0.84	6.73
LG16	192	126.16	0.66	8.59
LG17	153	137.23	0.90	16.77
LG18	243	182.24	0.75	10.83
LG19	198	138.26	0.70	7.12
LG20	144	121.48	0.85	6.13
LG21	207	165.44	0.80	8.02
LG22	169	123.62	0.74	7.00
LG23	189	142.91	0.76	5.29
LG24	204	150.69	0.74	8.33
LG25	106	106.93	1.02	10.54
LG26	117	113.58	0.98	11.38
LG27	115	103.07	0.90	7.62
LG28	361	252.34	0.70	7.31
LG29	229	174.32	0.76	13.78
LG30	127	129.50	1.03	13.51
LG31	152	144.46	0.96	9.72
LG32	155	153.93	1.00	7.50
LG33	273	174.28	0.64	6.8
LG34	190	144.56	0.76	7.55
LG35	136	106.10	0.79	8.23
LG36	160	148.81	0.94	11.62
LG37	185	172.34	0.94	19.89
LG38	132	129.25	0.99	7.67
Total	6,737	5,497.45	0.82	19.89

doi:10.1371/journal.pone.0157777.t004

[21–24]. This report has also adopted the more advanced SLAF-seq technology, which was suitable for constructing the genetic map.

Using intraspecific varieties of tetraploid *S. matsudana*—“Yanjiang” and “9901” as parents, the randomly selected 200 F₁ hybrid offspring as a mapping population, and the high-throughput SLAF-seq to construct the genetic map of *S. matsudana*, it can be seen from the map that a total of 277,333 SLAFs were obtained, of which 99,526 were polymorphic (Table 3). After

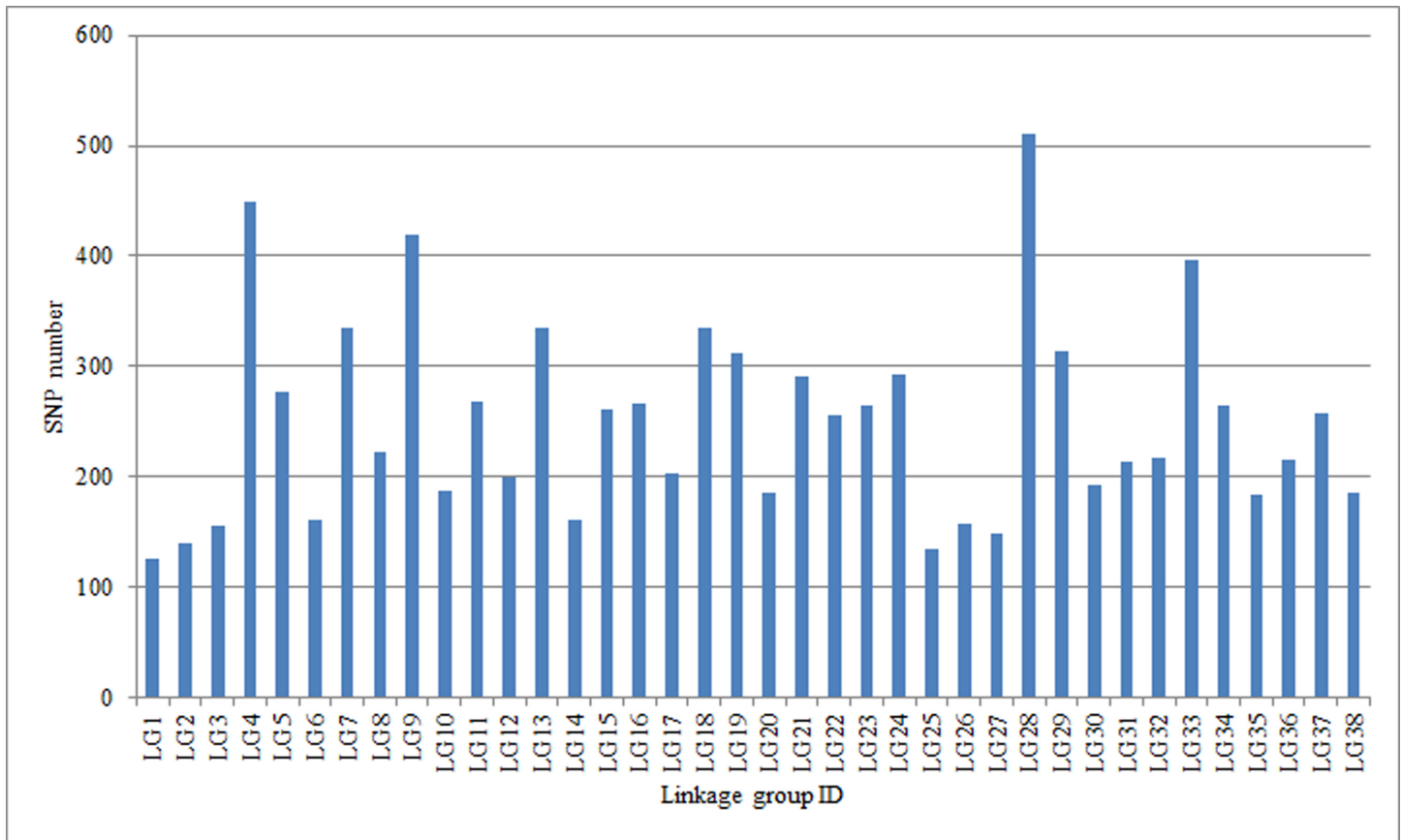


Fig 5. The number of single nucleotide polymorphism (SNP) markers on the linkage groups.

doi:10.1371/journal.pone.0157777.g005

filtering, the final number of SLAFs were 6,737 for the integrated genetic map (Table 4), 4,575 for the male genetic map (Table 5), and 2,857 for the female genetic map (Table 6), respectively. The integrated genetic map consisted of 38 linkage groups with total and average genetic distances of 5,497.45 cM and 0.82 cM, respectively (S1 Fig, Table 4). The number of SLAFs in each LG of the integrated genetic map ranged from 90 to 361. A total of 9,488 SNP markers were included on the integrated map. The map was of high quality based on evaluation of typlotype sources and linkage relationships (S1 and S2 Files). These results showed that SLAF-seq is a reliable technology to detect large-scale SNPs and construct genetic maps.

The intraspecific F₁ offspring used for map construction were derived from two tetraploid parents. Previous cytological and marker-segregation analyses show that tetraploid willows are allotetraploid, a group of tetraploids with two sets of chromosomes, each from a different ancestral species [15]. Because allotetraploids usually undergo disomic inheritance, approaches for their linkage analysis can be directly borrowed from those for diploids. However, these approaches do not characterize the difference of meiotic pairing occurring between homologous chromosomes from that between homeologous chromosomes, and therefore, the genome structure, organization and evolution of allotetraploids. By implementing the preferential pairing factor, Wu and colleagues have developed a series of statistical models that can identify the difference between homologous pairing and homeologous pairing [31, 32]. These models can be used to analyze our tetraploid marker data and identify the degree of the preferential pair of

Table 5. Basic characteristics of the linkage groups in the male genetic map of *S. matsudana*.

Linkage group ID	Marker number	Total distance (cM)	Average distance (cM)	Max gap
LG1	56	110.86	2.02	38.11
LG2	60	105.83	1.79	28.17
LG3	90	102.32	1.15	6.68
LG4	219	236.64	1.09	8.00
LG5	147	167.01	1.14	16.21
LG6	70	118.73	1.72	14.13
LG7	172	172.33	1.01	18.39
LG8	115	131.19	1.15	7.75
LG9	208	180.94	0.87	9.77
LG10	79	151.02	1.94	18.84
LG11	119	139.98	1.19	10.21
LG12	86	114.69	1.35	18.85
LG13	150	139.82	0.94	15.48
LG14	88	126.55	1.45	10.00
LG15	134	162.26	1.22	7.79
LG16	128	115.44	0.91	8.59
LG17	82	135.49	1.67	22.24
LG18	157	175.56	1.13	8.75
LG19	125	147.88	1.19	11.48
LG20	90	119.89	1.35	15.37
LG21	149	154.52	1.04	13.39
LG22	106	115.41	1.10	12.76
LG23	135	147.34	1.10	6.57
LG24	121	128.54	1.07	14.81
LG25	80	97.05	1.23	13.95
LG26	75	118.86	1.61	19.58
LG27	75	100.27	1.36	7.75
LG28	274	257.32	0.94	12.79
LG29	152	187.12	1.24	26.40
LG30	101	116.52	1.17	12.15
LG31	102	148.57	1.47	7.54
LG32	120	137.75	1.16	8.96
LG33	173	185.30	1.08	11.11
LG34	131	153.16	1.18	11.48
LG35	100	95.35	0.96	10.84
LG36	122	147.13	1.22	17.23
LG37	88	107.11	1.23	24.70
LG38	96	127.28	1.34	8.36
Total	4575	5,379.03	1.19	38.11

doi:10.1371/journal.pone.0157777.t005

homologous chromosomes over homeologous chromosomes. This information helps to gain new insight into the evolution of *Salix* genomes.

In nature, the meiotic recombination may differ between the two sexes, a phenomenon called heterochiasmy [33]. Wu et al. [34] modified a linkage analysis model to estimate and test heterochiasmy using marker data generated from a controlled cross. The application of this model provides additional insight into the genome-wide occurrence of heterochiasmy in *Salix*.

Table 6. Basic characteristics of the linkage groups in the female genetic map of *S. matsudana*.

Linkage group ID	Marker number	Total distance (cM)	Average distance (cM)	Max gap
LG1	40	88.33	2.26	17.65
LG2	51	101.24	2.02	30.95
LG3	34	79.09	2.40	13.41
LG4	116	235.45	2.05	27.65
LG5	79	176.43	2.26	19.64
LG6	56	113.76	2.07	35.06
LG7	85	159.07	1.89	15.55
LG8	56	126.89	2.31	15.48
LG9	126	164.63	1.32	17.63
LG10	52	160.00	3.14	30.11
LG11	87	130.64	1.52	12.79
LG12	58	97.02	1.70	9.92
LG13	117	149.81	1.29	7.76
LG14	41	110.40	2.76	18.17
LG15	74	148.66	2.04	14.03
LG16	85	115.78	1.38	16.93
LG17	85	135.91	1.62	20.66
LG18	109	187.89	1.74	23.04
LG19	92	123.69	1.36	16.21
LG20	67	121.00	1.83	14.82
LG21	73	174.22	2.42	24.69
LG22	79	116.95	1.50	12.13
LG23	76	129.90	1.73	18.39
LG24	114	157.13	1.39	40.36
LG25	35	110.54	3.25	23.00
LG26	54	108.29	2.04	16.76
LG27	53	105.87	2.04	20.62
LG28	134	240.13	1.81	24.53
LG29	102	158.89	1.57	21.35
LG30	34	128.89	3.91	29.00
LG31	71	140.36	2.01	38.66
LG32	49	168.08	3.50	24.51
LG33	146	161.68	1.11	10.21
LG34	70	132.87	1.93	14.81
LG35	46	116.34	2.59	17.65
LG36	52	123.91	2.43	17.63
LG37	111	171.97	1.56	28.12
LG38	48	119.30	2.54	17.27
Total	2857	5,291.01	1.88	40.36

doi:10.1371/journal.pone.0157777.t006

The high-density genetic map constructed in this study provides a basis for QTL mapping, map-based gene cloning, and molecular breeding of willows, and gives important information for selection, breeding, protection, and utilization of salt-tolerant willow varieties; furthermore, the map will advance the genetic improvement of willows. Since no whole genomes of willows are available, the 38 linkage groups could not be further divided into two sets. This genetic map will be improved once the whole genome of willows is available.

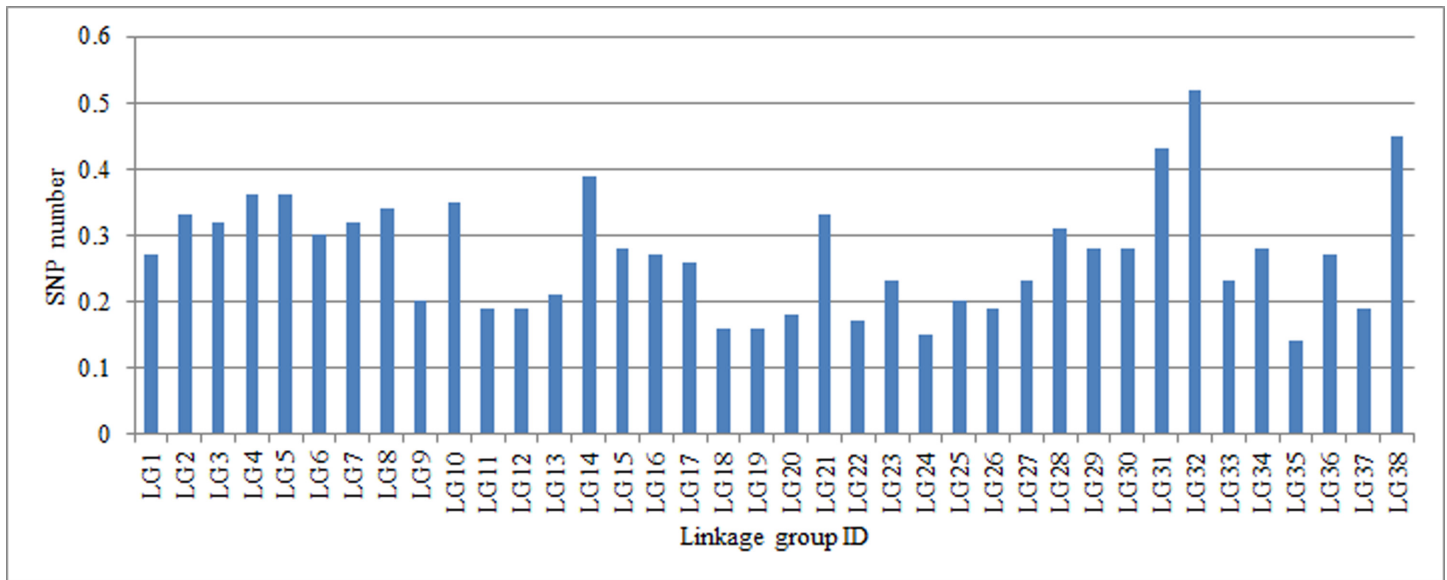


Fig 6. Missing percentage of markers in each of the linkage groups.

doi:10.1371/journal.pone.0157777.g006

Conclusion

Using the intraspecific F_1 hybrid population of salt-tolerant and salt-sensitive varieties of tetraploid *S. matsudana* as the mapping population, a total of 277,333 SLAFs were obtained, among which 99,526 were polymorphic, and 42,321 of the polymorphic markers could be used as potential markers for constructing the genetic map. After filtering the low quality markers and unsuitable markers for the F_1 population, 6,737 high quality SLAFs were used to construct a high quality genetic map. The genetic map consisted of 38 LGs, with total and mean genetic distances of 5,497.45 cM and 0.82 cM, respectively. The mean sequencing depth of SLAFs for the parents and offspring were 190.38-fold and 28.68-fold, respectively. The results from this study will provide a basis for fine mapping of salt-tolerant related QTLs and molecular breeding of *S. matsudana*.

Supporting Information

S1 Fig. High-density genetic map of *S. matsudana*.

(PDF)

S1 File. Haplotype maps of the genetic map. Green represents “9901”, blue represents “Yanjiang”, white means the parent could not be estimated, grey represents deletions.

(RAR)

S2 File. Heat maps of the genetic map. Each cell represents the recombination rate of two markers. Yellow indicates a lower recombination rate and purple a higher one.

(RAR)

Author Contributions

Conceived and designed the experiments: RW JZ. Performed the experiments: JZ HY ML YZ. Analyzed the data: HY YW. Contributed reagents/materials/analysis tools: YL XM FT. Wrote the paper: RW JZ HY.

References

1. Gullberg U. Towards making willows pilot species for coppicing production. *For Chron.* 1993; 69: 721–726.
2. Ahman I, Larsson S. Genetic improvement of willow (*Salix*) as a source of bioenergy. *Norwegian J Agric Sci.* 1994; 18: 47–66.
3. Lindegaard KN, Barker JHA. Breeding willows for biomass. *Aspects Appl Biol.* 1997; 49: 155–162.
4. Dimitriou I, Aronsson P, Weih M. Stress tolerance of five willow clones after irrigation with different amounts of landfill leachate. *Bioresource Technol.* 2006; 97(1): 150–157.
5. Stolarska A, Klimek D. Free proline synthesis in leaves of three clones of basket willow (*Salix viminalis*) as a response to substrate salinity. *Environ Prot Eng.* 2008; 34(4): 97–101.
6. Aronsson P, Dahlin T, Dimitriou I. Treatment of landfill leachate by irrigation of willow coppice plant response and treatment efficiency. *Environ Pollut.* 2010; 158(3): 795–804. doi: [10.1016/j.envpol.2009.10.003](https://doi.org/10.1016/j.envpol.2009.10.003) PMID: [19883963](https://pubmed.ncbi.nlm.nih.gov/19883963/)
7. Hangs RD, Schoenau JJ, Van Rees KCJ, Steppuhn H. Examining the salt tolerance of willow (*Salix* spp.) bioenergy species for use on salt-affected agricultural lands. *Can J Plant Sci.* 2011; 91(3):509–517.
8. Mirck J, Volk TA. Mass balances and allocation of salt ions from Solvay storm water for shrub willow (*Salix* spp.). *Biomass Bioenerg.* 2012; 39: 427–438.
9. Li M, Yu C, Wang Y, Li W, Wang Y, Yao Y, et al. Cloning and characterisation of two H⁺ translocating organic pyrophosphatase genes in *Salix* and their expression differences in two willow varieties with different salt tolerances. *Curr Genomics.* 2014; 15(5): 341–348. doi: [10.2174/138920291505141106102544](https://doi.org/10.2174/138920291505141106102544) PMID: [25435797](https://pubmed.ncbi.nlm.nih.gov/25435797/)
10. Wang Y, Yuan H, Li M, Li Y, Ma X, Tan F, et al. Phenotypic and physiological responses of two willow varieties to salt stress. *Israel J Plant Sci.* 2014; 61(1–4): 73–82.
11. Zhou J, Liu M, Jiang J, Qiao J, Lin S, Li H, et al. Expression profile of miRNAs in *Populus cathayana* L. and *Salix matsudana* Koidz under salt stress. *Mol Biol Rep.* 2012; 39(9): 8645–8654. doi: [10.1007/s11033-012-1719-4](https://doi.org/10.1007/s11033-012-1719-4) PMID: [22718503](https://pubmed.ncbi.nlm.nih.gov/22718503/)
12. Berlin S, Fogelqvist J, Lascoux M, Lagercrantz U, Rönnerberg-Wästljung AC. Polymorphism and divergence in two willow species, *Salix viminalis* L. and *Salix schwerinii* E. Wolf. *G3—Genes Genom Genet.* 2011; 1: 387–400.
13. Tsarouhas V, Gullberg U, Lagercrantz U. An AFLP and RFLP linkage map and quantitative trait locus (QTL) analysis of growth traits in *Salix*. *Theor Appl Genet.* 2002; 105: 277–288. PMID: [12582530](https://pubmed.ncbi.nlm.nih.gov/12582530/)
14. Berlin S, Lagercrantz U, Arnold S, Öst T, Rönnerberg-Wästljung AC. High-density linkage mapping and evolution of paralogs and orthologs in *Salix* and *Populus*. *BMC Genomics.* 2010; (11): 129–143. doi: [10.1186/1471-2164-11-129](https://doi.org/10.1186/1471-2164-11-129) PMID: [20178595](https://pubmed.ncbi.nlm.nih.gov/20178595/)
15. Barcaccia G, Meneghetti S, Albertini E, Triest L, Lucchin M. Linkage mapping in tetraploid willows: segregation of molecular markers and estimation of linkage phases support an allotetraploid structure for *Salix alba* × *Salix fragilis* interspecific hybrids. *Heredity.* 2003; 90: 169–180. PMID: [12634824](https://pubmed.ncbi.nlm.nih.gov/12634824/)
16. Kumar S, Banks TW, Cloutier S. SNP discovery through next-generation sequencing and its applications. *Int J Plant Genom.* 2012; 2012: 1–15.
17. Van Orsouw NJ, Hogers RC, Janssen A, Yalcin F, Snoeijers S, Verstege E, et al. Complexity reduction of polymorphic sequences (CRoPS): a novel approach for large-scale polymorphism discovery in complex genomes. *PLoS One.* 2007; 2(11): e1172. PMID: [18000544](https://pubmed.ncbi.nlm.nih.gov/18000544/)
18. Baird NA, Etter PD, Atwood TS, Currey MC, Shiver AL, Lewis ZA, et al. Rapid SNP discovery and genetic mapping using sequenced RAD markers. *PLoS One.* 2008; 3(10): e3376. doi: [10.1371/journal.pone.0003376](https://doi.org/10.1371/journal.pone.0003376) PMID: [18852878](https://pubmed.ncbi.nlm.nih.gov/18852878/)
19. Elshire RJ, Glaubitz JC, Sun Q, Poland JA, Kawamoto K, Buckler ES, et al. A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. *PLoS One.* 2011; 6(5):e19379. doi: [10.1371/journal.pone.0019379](https://doi.org/10.1371/journal.pone.0019379) PMID: [21573248](https://pubmed.ncbi.nlm.nih.gov/21573248/)
20. Sun X, Liu D, Zhang X, Li W, Liu H, Hong W, et al. SLAF-seq: an efficient method of large-scale de novo SNP discovery and genotyping using high-throughput sequencing. *PLoS One.* 2013; 8: e58700. doi: [10.1371/journal.pone.0058700](https://doi.org/10.1371/journal.pone.0058700) PMID: [23527008](https://pubmed.ncbi.nlm.nih.gov/23527008/)
21. Li B, Tian L, Zhang J, Huang L, Han F, Yan S, et al. Construction of a high-density genetic map based on large-scale markers developed by specific length amplified fragment sequencing (SLAF-seq) and its application to QTL analysis for isoflavone content in *Glycine max*. *BMC Genomics.* 2014; 15: 1086. doi: [10.1186/1471-2164-15-1086](https://doi.org/10.1186/1471-2164-15-1086) PMID: [25494922](https://pubmed.ncbi.nlm.nih.gov/25494922/)

22. Qi Z, Huang L, Zhu R, Xin D, Liu C, Han X, et al. A high-density genetic map for soybean based on specific length amplified fragment sequencing. *PLoS One*. 2014; 9(8): e104871. doi: [10.1371/journal.pone.0104871](https://doi.org/10.1371/journal.pone.0104871) PMID: [25118194](https://pubmed.ncbi.nlm.nih.gov/25118194/)
23. Ma J, Huang L, Ma C, Jin J, Li C, Wang R, et al. Large-scale SNP discovery and genotyping for constructing a high-density genetic map of Tea plant using specific-locus amplified fragment sequencing (SLAF-seq). *PLoS One*. 2015; 10(6): e128798.
24. Xu X, Xu R, Zhu B, Yu T, Qu W, Lu L, et al. A high-density genetic map of cucumber derived from specific length amplified fragment sequencing (SLAF-seq). *Front Plant Sci*. 2015; 5: 768. doi: [10.3389/fpls.2014.00768](https://doi.org/10.3389/fpls.2014.00768) PMID: [25610449](https://pubmed.ncbi.nlm.nih.gov/25610449/)
25. Porebski S, Bailey L, Baum B. Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. *Plant Mol Biol Rep*. 1997; 15: 8–15.
26. Hedley DW, Friedlander ML, Taylor IW, Rugg CA, Musgrove EA. Method for analysis of cellular DNA content of paraffin-embedded pathological material using flow cytometry. *J Histochem Cytochem*. 1983; 31(11): 1333–1335. PMID: [6619538](https://pubmed.ncbi.nlm.nih.gov/6619538/)
27. Serapiglia MJ, Gouker FE, Smart LB. Early selection of novel triploid hybrids of shrub willow with improved biomass yield relative to diploids. *BMC Plant Biol*. 2014; 14: 74. doi: [10.1186/1471-2229-14-74](https://doi.org/10.1186/1471-2229-14-74) PMID: [24661804](https://pubmed.ncbi.nlm.nih.gov/24661804/)
28. Suda Y, Argus GW. Chromosome numbers of some North American *Salix*. *Brittonia*. 1968; 20: 191–197.
29. Macalpine WJ, Shield IF, Trybush SO, Hayes CM, Karp A. Overcoming barriers to crossing in willow (*Salix* spp.) breeding. *Asp Appl Biol*. 2008; 90: 173–180.
30. Dai X, Hu Q, Cai Q, Feng K, Ye N, Tuskan GA, et al. The willow genome and divergent evolution from poplar after the common genome duplication. *Cell Res*. 2014; 24(10): 1274–1277. doi: [10.1038/cr.2014.83](https://doi.org/10.1038/cr.2014.83) PMID: [24980958](https://pubmed.ncbi.nlm.nih.gov/24980958/)
31. Wu RL, Gallo-Meagher M, Littell RC, Zeng Z-B. A general polyploid model for analyzing gene segregation in outcrossing autotetraploid species. *Genetics*. 2001; 159: 869–882. PMID: [11606559](https://pubmed.ncbi.nlm.nih.gov/11606559/)
32. Yang XX, Lv YF, Pang XM, Tong CF, Wang Z, Li X, et al. A unifying framework for bivalent multilocus linkage analysis of allotetraploids. *Brief Bioinform*. 2013; 14: 96–108. doi: [10.1093/bib/bbs011](https://doi.org/10.1093/bib/bbs011) PMID: [22508791](https://pubmed.ncbi.nlm.nih.gov/22508791/)
33. Phillips D, Jenkins G, Macaulay M, Nibau C, Wnetrzak J, Fallding D, et al. The effect of temperature on the male and female recombination landscape of barley. *New Phytol*. 2015; 208: 421–429. doi: [10.1111/nph.13548](https://doi.org/10.1111/nph.13548) PMID: [26255865](https://pubmed.ncbi.nlm.nih.gov/26255865/)
34. Wu RL, Ma CX, Wu SS, Zeng Z-B. Linkage mapping of sex-specific differences. *Genet Res*. 2002; 79: 85–96. PMID: [11974606](https://pubmed.ncbi.nlm.nih.gov/11974606/)