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Genetic mapping of leaf-related traits in autotetraploid alfalfa (*Medicago sativa* L.)

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Abstract Understanding the genetic architecture of leaf-related traits is important for improving alfalfa yield. Leaf size has a great influence on the protein content and yield for alfalfa. In this study, a low-yielding precocious alfalfa individual (paternal parent) and a high-yielding late-maturing alfalfa individual (maternal parent) were used to build a hybrid F_1 population of 149 individuals. The linkage map was constructed using simple sequence repeat and single nucleotide polymorphism markers, and quantitative trait loci (QTL) for leaf length, leaf width, and leaf area were mapped using 3 years phenotypic data. We identified a total of 60 QTLs associated with leaf size. These QTLs were located on chromosomes 1 to 8, and the percent of phenotypic variation explained by QTL ranged from 2.97% to 18.78%. There were 13 QTLs explain more than 10% of phenotypic variation, most of which represent novel loci controlling leaf traits that have not been found in previous studies. The nearest markers of QTL may be used in marker-assisted selection and breeding alfalfa new varieties with high yield.

Keywords Alfalfa · Leaf length · Leaf width · Leaf area · Quantitative trait loci

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Introduction

Alfalfa (*Medicago sativa* L.) is one of the most important legumes used for forage worldwide because of its high yield and good nutritional value (Adhikari et al. 2018). Leaves are one of the most important targets for improvement by breeders because they are essential organs with high protein content and good palatability, providing the plant with a large amount of organic matter and energy (Zhang et al. 2016), and thus playing

a vital role in increasing yield. Leaves are the primary sites of photosynthesis in plants, and leaf size is related to photosynthetic capacity (Bhagsari 1990). For example, photosynthetically active radiation use efficiency is reduced in smaller leaves. Because leaf area (LA) and leaf distribution affect the amount of solar radiation captured, these traits affect crop photosynthesis (Stewart et al. 2003) and growth rate and transpiration (Lieth et al. 1986). Thus, to improve the yield and quality of alfalfa, it is necessary to increase photosynthetic efficiency by enhancing leaf-related traits, such as leaf length (LL), leaf width (LW), and leaf area (LA).

The genetic control of leaf traits in alfalfa is not well known, and investigation into the endogenous factors influencing leaf traits will be valuable for developing cultivars with high yield. In QTL mapping, phenotypic information and genotype information are combined to determine the chromosomal locations of genetic variants that are associated with a trait (Tanksley 1993). It is a powerful tool for analyzing the genetic basis of complex agronomic traits, and it is also a useful method for elucidating the genetic architecture of agronomic traits such as leaf size. Therefore, identifying QTLs underlying phenotypic variation in alfalfa leaf size will facilitate the breeding of alfalfa cultivars with high protein content and high yield.

There has been extensive QTL mapping of leaf-related traits in many crops such as rice, wheat, and maize. In maize, different QTLs for leaf morphology, which is usually only studied in terms of size, length, and width, have been mapped in different chromosomes and locations. In addition, genetic regions associated with leaf-related traits under different environmental conditions have also been identified by performing genome-wide association analysis of maize nested association mapping populations (Feng et al. 2015) and QTL mapping in F_2 and recombinant inbred line (RIL) populations (Li et al. 2015a). Indicating that the genetic control of leaf morphology is complex (Cai et al. 2012; Ku et al. 2012; Wassom 2013) and QTL mapping results differ between environments (Hou et al. 2015). QTLs for leaf size-related traits have also been mapped in other crops. For example, QTLs for leaf area (Cui et al. 2003; Ishimaru et al. 2001) and LL and LW have been identified in rice (Kobayashi et al. 2004; Tong et al. 2007) and the QTL loci related to leaf traits were also identified in wheat and white clover (Cogan et al. 2006; Jia et al. 2013; Wu et al. 2015). Relatively few studies of leaf-related traits have been identified in alfalfa, and these QTLs provide a theoretical method for alfalfa localization.

Genetic analysis of leaf-related traits in plants through QTL mapping requires adequate genome coverage with molecular markers. A large number of single nucleotide polymorphisms (SNPs) can be obtained cost effectively through next-generation sequencing methods such as RAD-seq (restriction-site associated DNA), even in species with no prior genome assemblies (Mansur et al. 1993). The extensive natural variation in alfalfa and the availability of a high-density genetic linkage map provide the basis for accurate localization of alfalfa leaf QTLs and the causal genes. The published QTL studies in alfalfa have mainly focused on traits such as flowering time (Pierre et al. 2008), seed mineral concentration and content (Sankaran et al. 2009), seed germination and growth before seedling emergence (Dias et al. 2011), and seed vigor (Vandecasteele et al. 2011). Previous conducted QTL mapping for the fall dormancy and winter hardiness traits of alfalfa, they used the GBS (genotyping-by-sequencing) technique and two varieties as materials, and they mapped 45 QTLs that were significantly associated with fall dormancy and 35 QTLs related to winter hardiness (Adhikari et al. 2018). Previous identified 71 QTLs related to plant height and winter injury in an F1 population of alfalfa (Li et al. 2015b). However, these QTLs were concentrated on sites containing QTLs for yield-related alfalfa traits, and there have been few QTL studies on alfalfa leaf traits. Avia (Avia et al. 2013) identified a few QTLs related to LA on 1, 3, and 4 chromosome of *Medicago truncatula*. Previous studies have also found QTLs associated with LA in *M. truncatula* (Arraouadi et al. 2011). These studies provide useful information for our current study.

Leaf-related traits are essential and vary widely in nature. Studies to quantify overall leaf shape variation are necessary to identify complete complements of genes that determine differences in leaves between populations (Chitwood et al. 2012a, b, c). Considering the difference in gene composition, we can analyze the genetic traits of specific in a species by comparing the similar traits in different populations. Identifying QTLs underlying leaf traits in alfalfa will enable the identification of the genetic factors controlling these traits and aid the discovery of markers associated with yield-related trait. Nearest markers of these QTL can be used for marker-assisted selection and breeding alfalfa cultivars with high yield after validation.

Table 1 Traits associated with leaf size in the F1 population and parental lines in 2016, 2017 and 2018

Year	Trait	Number of plants	Female parent mean	Maternal parent mean	Population mean	CV	Z test	Skewness	Kurtosis	H^2
2016	LL	149	2.6	5.4	2.69 ± 0.32	0.14	$P < 0.01$	− 0.38	0.06	0.45
	LW	149	1.2	2.4	1.67 ± 0.18	0.17	$P < 0.01$	− 0.4	0.23	0.51
	LA	149	2.33	6.52	2.93 ± 0.26	0.32	$P < 0.01$	0.55	− 0.27	0.63
2017	LL	149	2.1	4.9	1.77 ± 0.27	0.26	$P < 0.01$	1.13	0.66	0.41
	LW	149	0.9	2	1.89 ± 0.15	0.23	$P < 0.01$	0.65	− 1.21	0.49
	LA	149	2.14	4.64	1.30 ± 0.23	0.36	$P < 0.01$	0.84	0.5	0.62
2018	LL	149	2.8	5.5	3.55 ± 0.26	0.31	$P < 0.01$	0.71	1.12	0.41
	LW	149	1.2	1.8	1.81 ± 0.14	0.19	$P < 0.01$	0.68	0.48	0.52
	LA	149	2.8	5.73	4.4 ± 0.28	0.31	$P < 0.01$	0.95	0.57	0.66

LL leaf length, *LW* leaf width, *LA* average leaf area, *CV* coefficient of variation, H^2 broad-sense heritability

Materials and methods

Plant material and growth conditions

Two parental genotypes, separated from local variety "Cangzhou" (CF000735) (maternal parent) and variety Zhongmu NO.1 (CF032020) (paternal parent), the two individuals were crossed to generate an F_1 population consisting of 149 progeny lines. The phenotypic data of LL, LW, and LA were collected in 2016, 2017, and 2018 at the field of Chinese Academy of Agricultural Sciences in Langfang, Hebei province, China. The annual average temperature is 11.9 °C, the average temperature in the coldest month (January) is minus 4.7 °C, and the average temperature in the hottest month (July) is 26.2 °C. The annual precipitation is 554.9 mm, and precipitation is unevenly distributed throughout the year and is mostly concentrated in summer. The soil is medium loam soil, containing 1.69% organic matter, with a pH value of 7.37. The detail of field experiment design was described in our previous study (Zhang et al. 2019). No fertilizer or irrigation was applied, and weeding was done manually. The remaining 5 cm mowing was performed on each individual plant before winter, thus ensuring consistency between individuals.

Table 2 Correlation coefficients for leaf size-related traits in 2016, 2017 and 2018

Year	Trait	LA	LL	LW
2016	LA	1.00	0.818**	0.866*
	LL		1.00	0.434**
	LW			1.00
2017	LA	1.00	0.506**	0.609**
	LL		1.00	0.307**
	LW			1.00
2018	LA	1.00	0.617**	0.789**
	LL		1.00	0.370**
	LW			1.00

LA leaf area, *LL* leaf length, *LW* leaf width

**A significant correlation at the 0.01 level (two-sided)

Phenotype measurement

LL, LW, and LA were measured using a handheld leaf area meter. The handheld leaf area meter uses the principle of photoelectric induction, and the blade can be measured by simply spreading the blade on the photosensitive plate. Beginning on May 1, each individual plant randomly selected three leaves for measurement. After entering the normal measurement state, the photosensitive plate of the instrument was opened, the petiole was removed, and measurements were made from three blades randomly selected from each individual plant for leaf traits. When the instrument displayed the measurement, the blade was pulled slowly and smoothly, that is, the LL, LW, and LA are completed. Finally, the average result of multiple measurements was obtained by pressing the "Average" key.

Genetic linkage map

The linkage map information is reported in a separate study (Zhang et al. 2019). In brief, *Medicago sativa* L. were sequenced using the RAD-seq method. SNP data

Table 3 Variance components of leaf length, leaf width, and leaf area in an alfalfa population

		df	Type III SS	Mean square	F-value	Significance
Leaf area	Genotype (G)	149	350.63	2.29	2.51	***
	Year (Y)	2	1747.11	873.55	957.48	***
	G × Y	301	471.68	1.57	1.72	***
Leaf length	Genotype (G)	149	141.69	0.93	1.4	***
	Year (Y)	2	603.94	301.97	456.62	***
	G × Y	297	256.37	0.86	1.31	***
Leaf width	Genotype (G)	149	90.09	0.59	0.72	***
	Year (Y)	2	5.61	2.81	3.42	*
	G × Y	297	197.07	0.66	0.81	***

*Significant at the 0.05 probability level; **significant at the 0.01 probability level; ***significant at the 0.001 probability level

were called using the Universal Network Enable Analysis Kit (UNEAK) pipeline (Lu et al. 2013). SNP markers with more than 50% missing values were removed, and single-dose alleles (SDA, AAAB X AAAA) with a segregation ratio of less than 2:1 among F1 progenies were used to construct a genetic linkage map using Joinmap. SNP (single nucleotide polymorphism) data were added during linkage map construction. There were 2317 SDA SNP markers in P1 (paternal parent) and 4553 SDA SNP markers in P2 (maternal parent). For SNP markers, there were 56 and 84 SDA markers in P1 and P2, respectively. The final P1 linkage map spanned a total of 3455 cM with 1153 mapped markers and an average marker density of 3.00 cM. The P2 linkage map spanned a total of 4381 cM with 3312 mapped markers and an average marker density of 1.32 cM.

QTL mapping

QTL analysis was performed to identify QTLs related to leaf traits and to calculate the contribution rate and additive effect of each QTL. QTL mapping was analyzed using the additive composite interval mapping method (ADD-ICIM) of the QTL IciMapping software (Institute of Crop Science, Chinese Academy of Agricultural Sciences CAAS, Beijing, China) (Lei et al. 2015). The phenotype (LL, LW, and LA) and genotype data for each plant in the F1 population were combined, and QTLs were mapped using the BIP function (Lei et al. 2015) in QTL IciMapping with a LOD threshold of 3. QTL information for the F1 population was integrated using Mapchart software (Voorrips 2002) to determine the total number of leaf trait-related QTLs.

Results

Phenotypic data analysis

Extensive phenotypic variations were observed for all the measured quantitative traits in this alfalfa leaf traits, as shown by the descriptive statistics in Table 1. The range of values for F1 plants was wider than that of the parents, reflecting the presence of transgressive segregation. The kurtosis and skewness of leaf traits were close to zero. The coefficient of genetic variation ranged from 0.14 for LL in 2016 to 0.36 for LA in 2017 (Table 1). Broad-sense heritability (H^2) was calculated as described in a previous study (Tornqvist et al. 2018). Our finding broad-sense heritability ranged from 0.41 to 0.66. Correlation analysis showed a significant correlation according to Pearson's test ($P < 0.01$) among four yield-related traits (Table 2). In 2016, 2017, and 2018, the correlation coefficients for LL and LA were 0.8, 0.57, and 0.62, respectively; those for LA and LW were 0.87, 0.61, and 0.79, respectively; and those for LL and LW were 0.43, 0.31, and 0.37, respectively. Genotypic variation, variation between years and genotype × year interactions were significant for all leaf-related traits ($P < 0.001$) (Table 3). As can be seen from the probability density distribution in Fig. 1, the difference in leaf traits between the two parents was significant ($P < 0.01$). The leaf sizes of female parent were obviously smaller than that of the maternal parent.

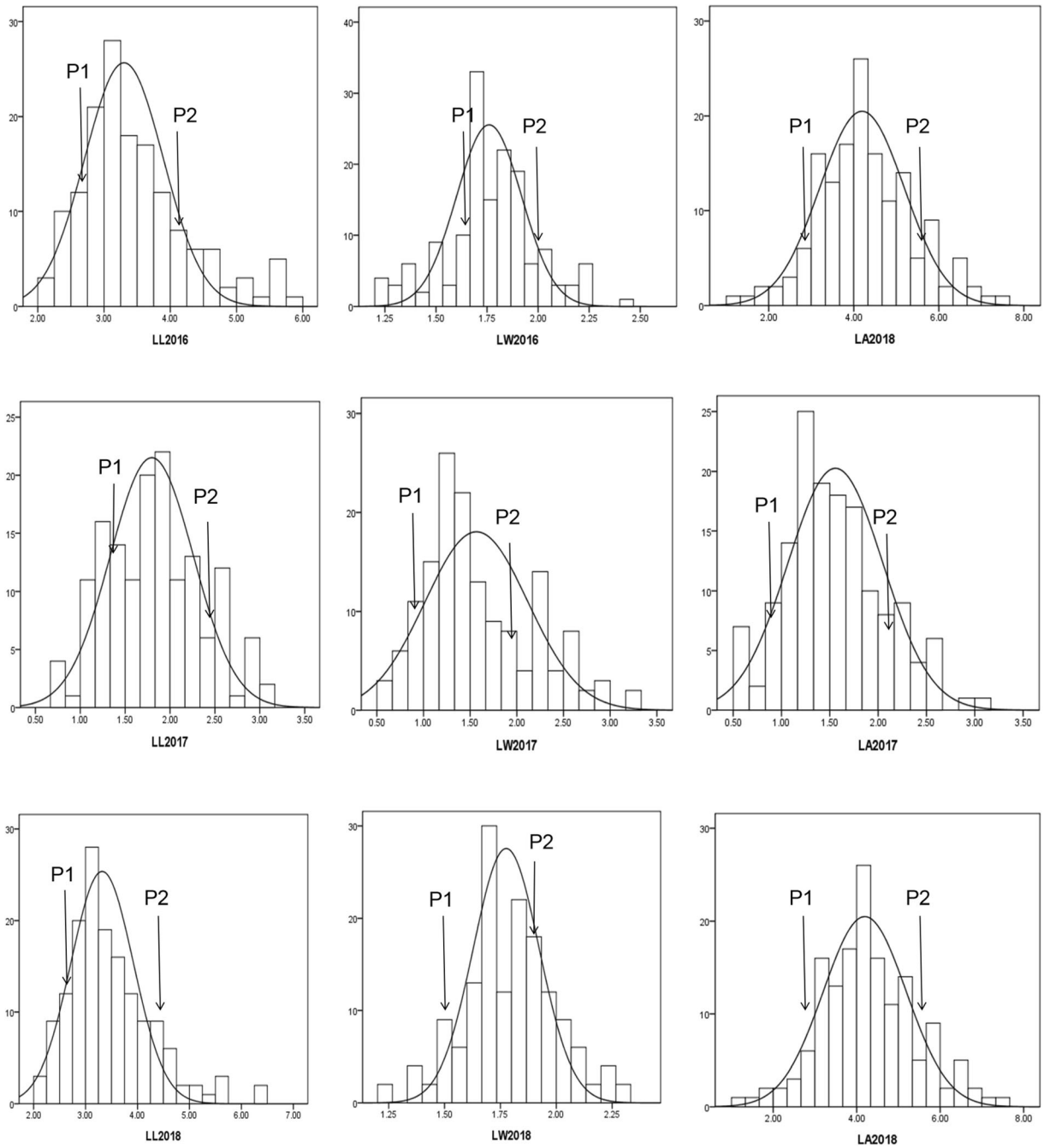

Fig. 1 Distribution of quantitative traits in the *Medicago sativa* L. F1 population. Arrows indicate the mean values of the two parents. P1 stands for the female parent and P2 stands for the maternal parent

Identification of QTLs for leaf-related traits

We performed interval mapping for LA, LL, and LW using the phenotypic data of 2016, 2017, and 2018 (Tables 4, 5, and 6). A total of 60 leaf size-related QTLs were identified over the 3-year experiment, and these QTLs were distributed for chromosomes 1 to 8. The percent phenotypic variance explained by individual QTLs ranged from 2.99 to 18.78%, with 13 QTLs each accounting for more than 10% of the phenotypic

Table 4 Quantitative trait loci (QTL) parameters in the *Medicago sativa* L. leaf length traits

Parent	Trait name[a]	QTL	LG[b]	Position[c] (cM)	LOD interval (cM)	Left marker	Right marker	LOD[d]	PVE (%)[e]	Add[f]
Paternal	2016-leaf length	qLL-1	3C	90.5	89.65–91.77	TP16142	TP28355	2.8213	5.0925	0.2906
	2018-leaf length	qLL-2	2C	81.0	80.4–81.1	TP11421	TP102553	2.7998	6.2817	− 0.2555
	2018-leaf length	qLL-3	2D	32.0	30.41–34.97	TP5760	TP66523	3.6489	8.9753	− 0.3031
Maternal	2016-leaf length	qLL-4	1B	120.0	119.34–120.73	TP119651	TP96230	3.4626	18.775	− 0.2342
	2016-leaf length	qLL-5	2D	7.5	6.32–12.51	TP116788	TP49959	5.049	7.5305	− 0.1482
	2016-leaf length	qLL-6	4B	73.5	73.5–73.8	TP92963	TP121286	4.2618	5.4382	0.1253
	2016-leaf length	qLL-7	6B	98.0	97.01–98.78	TP59911	TP112047	2.5053	3.1423	− 0.0973
	2017-leaf length	qLL-8	6A	122.0	121.85–123.21	TP54361	TP12469	2.6659	9.2553	0.2711
	2018-leaf length	qLL-9	1D	35.5	33.54–37.11	TP5157	TP100870	2.586	6.4935	− 0.2392
	2018-leaf length	qLL-10	4C	114.0	113.61–115.77	TP13043	TP40661	3.1843	11.110	0.3083

[a] The leaf traits described in the "Materials and methods"

[b] Chromosomal position

[c] The genetic distance of the QTL on the relevant chromosome in the genetic linkage map

[d] The threshold LOD values determined with 1000 permutations of the data

[e] The proportion of phenotypic variation explained by each QTL

[f] Estimated additive effect of the QTL

variation explained (PVE). There were 27 QTLs related to LA, 10 QTLs related to LL, and 23 QTLs related to LW.

Identification of leaf length QTLs in the F_1 population

Table 4 lists QTL for LL leaf traits identified by CIM in 3 years and combined over years. These QTLs were located on chromosomes 1B, 1D, 2C, 2D, 3C, 4B, 4C, 6A, and 6B, and the phenotypic variance explained by the individual QTLs ranged from 3.14% to 18.77% (Table 4). QTLs on chromosomes 4C and 1B contributed 11.11% and 18.78% in 2016 and 2018, respectively, to the phenotypic variation. A QTL accounting for 18.78% of the total variation was detected on chromosome 1B within the marker interval TP119651-TP96230. Another QTL mapping to 4C within TP13043-TP40661 accounted for 11.11% of the total phenotypic variation. In addition, contrary to QTL in 1B, 1D, 2C, 2D, and 6B had negative effects (add < 0) on LA (Table 4).

Identification of leaf width QTLs in the F_1 population

A total of 23 QTLs related to LW (38.3% of all leaf size-related QTLs) were identified in the 3 years of the experiment, and these QTLs were mapped to chromosomes 1B, 1D, 2A, 2C, 2D, 3A, 3B, 3C, 4B, 4C, 4D, 5A, 6B, 6D, 7A, and 8A (Table 5). The phenotypic variance explained by individual QTLs ranged from 2.99 to 14.07%. Seven QTLs on chromosomes 3B, 3C, 4B, 4D, 6B, and 8A explained more than 10% of the phenotypic variation, with genetic contribution rates ranging from 10.76 to 14.07% (PVE). The QTLs on chromosomes 4C, 6B, 6D, and 8A were identified in multiple years, suggesting they have a greater impact on LW (6.47–12.93%). QTL impact analysis showed that 30.43% (7/23) of the QTLs were associated with increased LW, indicating different allelic effects.

Table 5 Quantitative trait loci (QTL) parameters in the *Medicago sativa* L. leaf width traits

Parent	Trait Name[a]	QTL	LG[b]	Position[c] (cM)	LOD interval (cM)	Left marker	Right marker	LOD[d]	PVE (%)[e]	Add[f]
Paternal	2016-leaf width	qLW-1	2C	107.0	106.18–108.11	TP118790	TP43156	3.7836	8.5875	− 0.1335
	2016-leaf width	qLW-2	6B	70.0	67.66–75.32	SSR44	TP51277	4.3961	12.801	− 0.1629
	2017-leaf width	qLW-3	8A	18.0	17.56–19.16	TP111076	TP2146	4.2782	5.5656	− 0.2268
	2017-leaf width	qLW-4	8A	90.0	88.36–90.15	TP26454	TP82500	3.839	10.806	0.3123
	2018-leaf width	qLW-5	3A	52.0	51.67–53.01	TP38054	TP73336	2.5706	8.7284	− 0.0841
	2018-leaf width	qLW-6	3B	89.0	87.30–89.91	TP68289	TP70313	4.4533	11.475	− 0.0967
Maternal	2016-leaf width	qLW-7	1B	56.75	56.73–56.79	TP20480	TP13207	5.1418	4.5248	− 0.0748
	2016-leaf width	qLW-8	1D	39.0	38.72–39.53	TP124060	TP95981	3.521	2.9864	0.061
	2016-leaf width	qLW-9	2D	53.0	50.10–55.74	TP106191	TP15553	4.906	5.4625	− 0.0836
	2016-leaf width	qLW-10	4B	116.0	115.18–116.58	TP65847	TP44955	11.174	10.757	0.116
	2016-leaf width	qLW-11	4C	50.0	50.21–50.93	TP17099	TP62659	4.7755	4.2022	− 0.0725
	2016-leaf width	qLW-12	6B	113.0	111.45–115.42	TP92765	TP77093	7.1692	6.4695	− 0.0908
	2016-leaf width	qLW-13	7A	25.0	24.07–25.21	TP11839	TP78641	5.4747	5.2899	− 0.0821
	2016-leaf width	qLW-14	8A	35.0	34.89–35.93	TP60448	TP35978	8.3551	7.7281	0.0984
	2016-leaf width	qLW-15	8A	43.5	43.22–43.74	TP99687	TP27214	13.021	12.927	− 0.1264
	2017-leaf width	qLW-16	4C	109.5	109.47–109.86	TP32203	TP25090	3.5918	6.2304	0.1851
	2017-leaf width	qLW-17	4D	22.0	21.59–22.91	TP125366	TP68765	6.2212	12.305	− 0.2579
	2017-leaf width	qLW-18	4D	56.5	56.25–56.63	TP40032	TP11560	3.181	6.5066	0.1887
	2017-leaf width	qLW-19	5A	94.0	94.69–94.71	TP19012	TP7685	3.3289	7.778	0.2064
	2017-leaf width	qLW-20	6D	115.5	113.20–119.16	TP32265	TP34930	2.7874	4.7817	− 0.1603
	2018-leaf width	qLW-21	2A	64.0	63.90–65.82	TP22321	TP89578	3.302	6.7458	0.0733
	2018-leaf width	qLW-22	3C	26.0	25.64–27.47	TP125264	TP105519	5.6692	14.070	− 0.104
	2018-leaf width	qLW-23	6D	60.0	58.42–63.33	TP58492	TP89224	3.1298	6.3514	− 0.0699

Identification of QTLs for leaf area in the F1 population

Twenty-seven QTLs were detected for LA chromosomes 1–8(Table 4), and together, they contributed 45% of the phenotypic variation (individual variance ranged from 3.41 to 18.56%). The LOD values ranged between 2.67 and 9.26, and the additive effect values ranged between − 0.52 and 0.96. The QTLs on chromosomes 1A, 1C, 2D, 3B, 4C, and 5B were identified in multiple years, and these QTLs had a large effect on LA (4.03 to 13.97%). Among these QTLs, the QTL on chromosome 4B explained the largest amount of

Table 6 Quantitative trait loci (QTL) parameters in the *Medicago sativa* L. leaf area traits

Parent	Trait Name[a]	QTL	LG[b]	Position[c] (cM)	LOD interval (cM)	Left marker	Right marker	LOD[d]	PVE (%)[e]	Add[f]
Paternal	2016-leaf area	qLA-1	5B	24.0	23.70–24.29	TP90528	TP111237	2.7998	8.1377	− 0.2519
	2017-leaf area	qLA-2	1A	97.0	96.59–97.47	TP6437	TP24150	3.9881	1.1253	0.9401
	2017-leaf area	qLA-3	1A	98.0	97.47–98.72	TP24150	TP60413	4.1613	1.1267	0.9486
	2017-leaf area	qLA-4	2B	99.0	97.41–99.25	TP31503	TP69543	4.0438	1.1247	0.9612
	2017-leaf area	qLA-5	8D	75.0	72.76–77.09	TP110657	TP118899	3.3673	1.1033	0.9335
	2018-leaf area	qLA-6	2C	82.0	81.11–84.58	TP11421	TP102553	2.9911	5.0939	− 0.3161
	2018-leaf area	qLA-7	2D	32.0	30.41–34.97	TP5760	TP66523	5.046	8.7522	− 0.4093
	2018-leaf area	qLA-8	3A	52.0	51.67–53.01	TP38054	TP73336	2.9029	7.1781	− 0.3771
	2018-leaf area	qLA-9	3B	91.5	89.91–93.02	TP70313	TP49598	6.283	13.966	− 0.5222
Maternal	2016-leaf area	qLA-10	1C	47.5	47.08–48.02	TP91677	TP105407	5.1591	5.1207	− 0.209
	2016-leaf area	qLA-11	2D	53.0	50.10–55.74	TP106191	TP15553	5.3046	5.5166	− 0.219
	2016-leaf area	qLA-12	3D	69.0.0	68.61–69.55	TP57248	TP8555	3.5987	3.4814	− 0.1723
	2016-leaf area	qLA-13	4B	116.0	115.18–116.58	TP65847	TP44955	9.2568	18.564	0.3939
	2016-leaf area	qLA-14	4C	50.5	50.21–50.93	TP17099	TP62659	3.816	4.0314	− 0.1849
	2016-leaf area	qLA-15	4D	88.5	88.22–88.85	TP103833	TP77009	4.4514	6.3862	− 0.2316
	2016-leaf area	qLA-16	5A	23.0	22.14–23.32	TP103349	TP20782	4.8877	5.0186	− 0.2053
	2016-leaf area	qLA-17	6A	130.0	128.32–133.14	TP20995	TP37415	3.5105	3.4125	0.1732
	2016-leaf area	qLA-18	6C	50.0	48.49–50.23	TP80549	TP108406	3.9436	5.7398	0.2223
	2016-leaf area	qLA-19	8A	49.5	49.04–50.06	TP59420	TP53438	5.1641	6.0708	− 0.2267
	2017-leaf area	qLA-20	5B	98.0	97.40–98.48	TP49465	SSR50	2.9843	10.029	− 0.2879
	2018-leaf area	qLA-21	1C	135.0	133.90–136.04	TP60798	TP8894	6.8395	8.5581	0.4222
	2018-leaf area	qLA-22	2B	126.5	125.59–127.18	TP61589	TP123947	4.9804	6.0729	− 0.3538
	2018-leaf area	qLA-23	3B	65.0	64.61–65.47	TP19780	TP27949	3.8772	6.3491	− 0.3609
	2018-leaf area	qLA-24	3C	5.5	4.55–7.23	TP70173	TP66342	2.6724	3.4219	− 0.2667
	2018-leaf area	qLA-25	4C	39.5	39.25–39.97	TP106798	TP68992	4.3787	5.2694	− 0.3278
	2018-leaf area	qLA-26	6D	53.0	51.70–54.13	TP62599	TP69225	8.1678	13.172	− 0.5249
	2018-leaf area	qLA-27	7D	105.0	104.3–105.91	TP96019	TP12903	6.685	8.4479	0.4162

phenotypic variance (18.56%). QTLs on chromosomes 3B, 5B, and 6D between marker intervals TP70313-TP49598, TP65847-TP44955, and TP699-TP69225, respectively, also explained a large amount of phenotypic variance (13.97%, 10.03%, and 13.17%, respectively).

Analysis of QTL mapping results

A total of six QTLs were identified in the same location in all 3 years (Figs. 2 and 3). It can be seen from the QTL map of the female parent plant (Fig. 2) that two QTLs associated with LA and two associated with LL were mapped to the same location on 2C and 2D in 2018 (8 9cM and 52 cM). These QTLs had LOD values ranging from 2.79 to 2.99. The percent phenotypic variance explained by the individual QTLs ranged from 5.09 to 6.28%, and the additive effect ranged between − 0.31 and − 0.25. Two QTLs related to LW and LA were detected on chromosome 3A in 2018 (64 cM). The LOD values ranged from 2.57 to 2.90, and the percent phenotypic variation explained ranged from 7.18% to 8.73%. QTLs for LW and LA were mapped to the same position on chromosome 2D in 2016 (119 cM)(Fig. 2). QTLs for LA and LW were also mapped to the same location on chromosomes 4B and 4C (70 cM and 101 cM, respectively).

Discussion

The two parental lines used for creating the mapping population varied in leaf-related trait phenotypes

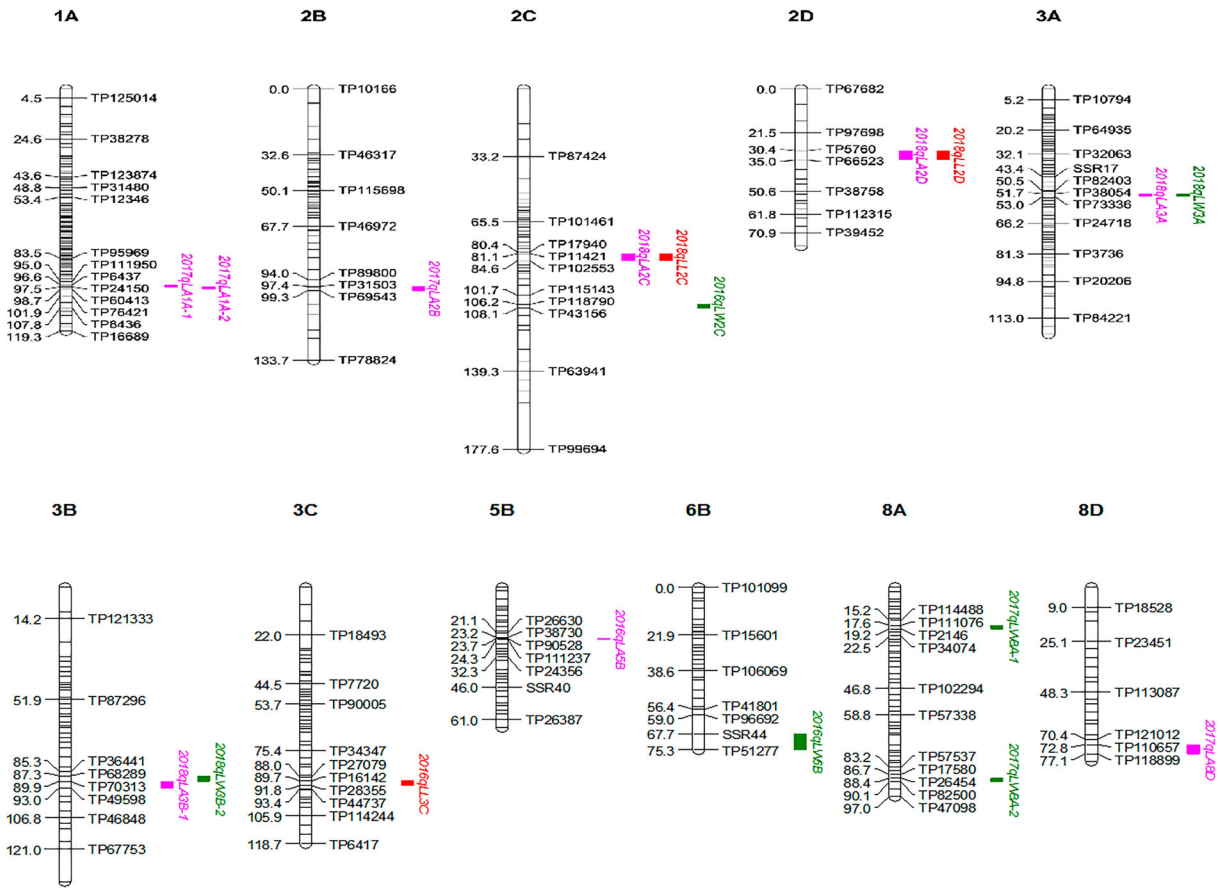

Fig. 2 Locations of leaf trait QTLs on the constructed paternal linkage map

(Table 1), and there was variation in these traits in our population. Leaf size has been reported to be highly heritable (Caradus and Chapman 1996). Previous study found that the broad-sense heritability of LA in *M. truncatula L.* is 0.45, which is significantly less than the heritability of 0.66 that we observed (Avia et al.

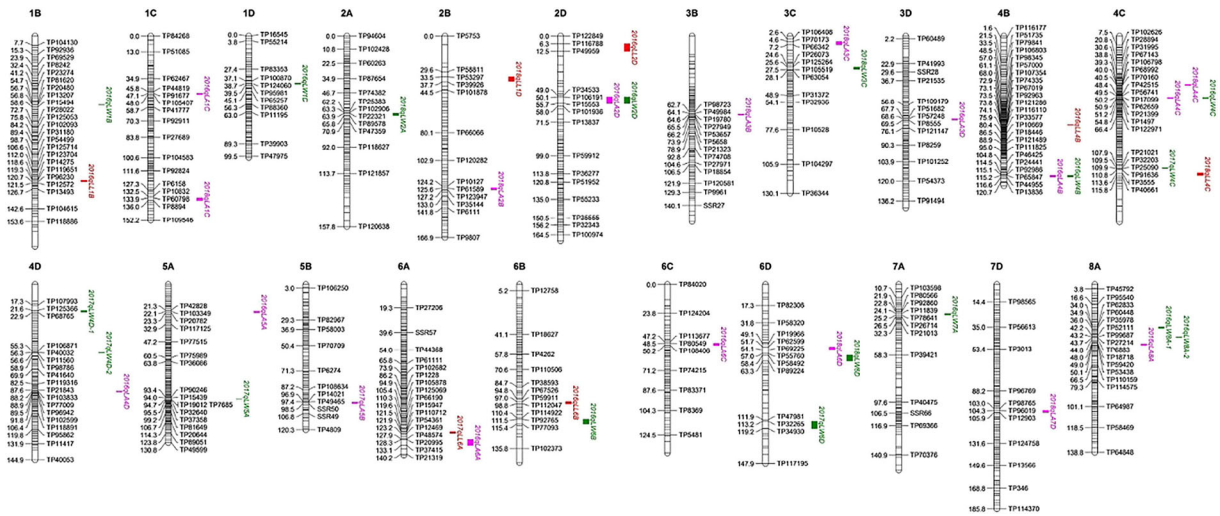

Fig. 3 Locations of leaf trait QTLs on the constructed maternal linkage map

2013). We also found that there was a high correlation between LL, LW, and LA, which is the same as in previous studies (Avia et al. 2013; Badri et al. 2011; Cogan et al. 2006). There was also a positive correlation between leaf traits measured over 3 years, and they are dependent on environmental changes. This change may be due to differences in heredity caused by differences between climated or the environment in which they are located. We observed a genotype × year interaction. It can be seen from the results that leaf traits are clearly influenced by environmental factors; therefore, selecting markers from QTLs that have similar effect sizes across different years will be necessary to efficiently breed alfalfa.

Comparison of QTL associated with leaf-related traits in alfalfa

We have identified 60 major QTLs affecting LL, LW, and LA in this population, and the QTLs discovered include new ones that have not been previously identified. In this study, QTLs for LA, LL, and LW were identified by first looking for significant linkages between SNP markers, and then CIM analysis was performed to predict more precise genomic locations for each QTL. Some alfalfa QTLs have been reported for winter injury and fall dormancy, but the QTL intervals were large (> 10 cM)(Li et al. 2015b), and thus there is need for further research to narrow down the QTL positions, using SDA markers and the composite interval mapping method, and QTL intervals were greatly reduced (< 3 cM) in the present analysis. In our study, we found 27 QTLs controlling LA, and four QTLs with a heritability greater than 10% were mapped in qLA-9, qLA-13, qLA-20, and qLA-26; the locations of these QTLs are consistent with those identified in previous studies (Foroozanfar et al. 2014; Moreau et al. 2012).

To reduce the effect of the interaction between genetics and the environment (G × E), we used BLUP to estimate phenotypic variation across three traits and identified QTL for each trait analyzed in this study. Most QTLs identified in the present study were co-located with previously reported yield-related QTL (Zhang et al. 2019) and fall dormancy QTL (Li et al. 2015b). Their mapping results are consistent with those of this study and provide further evidence that the corresponding QTLs in alfalfa may be important for the control of LA in alfalfa. In addition, we identified 23 QTLs controlling LW in the alfalfa population, and seven major QTLs were distributed on chromosomes 3B, 3C, 4B, 4D, 6B, and 8A. We compared the physical locations of these QTLs with those of published QTLs locations. We found major QTL loci on qLW-15 and qLW-22, located at 43.5 cM and 26 cM on chromosomes 4 and 8, which is consistent with the sites previously found in winter hardiness (40.8–43.6 cM) (Adhikari et al. 2018). LW QTLs on alfalfa chromosomes 4 and 6 have not been previously found and may represent new QTLs. We found several QTLs (qLA-11, qLW-9, qLA-13, qLW-10, qLA-14, qlw-11, qLA-6, qLL-2, qLA-7, qLL-3, qLW-5, and qLA-8) identified at the same location, which have been identified for many years and have a large contribution rate; these QTLs may play a major role in controlling leaf traits. We also identified ten novel QTLs for LL, and none of the previously reported QTLs were found in chromosome 1, 2, 3, 4, and 6. Therefore, we not only identified stable QTLs identified by other studies but also identified some novel QTLs, further illustrating the complexity of the genetic architecture that controls leaf trait.

Given that the average heritability (52%) of leaf-related traits was reasonable for genetic analysis, the QTL identified in the present analysis need further validation. According to the results obtained over 3 years, it can be speculated that there are large-effect, stable, and reliable leaf size-related QTLs that play an important role in controlling leaf-related traits; their PVE is higher than 10% (qLL-1, qLL-4, qLW-3, qLW-4, qLW-6, qLW-8, qLA-3, qLA-4, qLA-5, qLA-6). These stable QTLs may be useful for breeding alfalfa with altered leaf size. We also identified novel QTLs, which provide new target genomic regions for further identification of alfalfa genes regulating leaf traits. Furthermore, in the present analysis, we were able to narrow down the QTL interval with high PVE, which will facilitate further investigations such as fine mapping and gene cloning.

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Author contribution Tiejun Zhang and Qingchuan Yang designed the experiments and developed the mapping population. Fei He and Junmei Kang performed data analysis and wrote the manuscript. Fan Zhang, Ruicai Long, and Long-Xi Yu performed the genotyping and sequencing and constructed genetic maps. Zhen Wang and Zhongxiang Zhao managed field work and investigated phenotypic data. All the authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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