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ARTICLE

# Genetic diversity and association analysis of leafminer (*Liriomyza langei*) resistance in spinach (*Spinacia oleracea*)

Ainong Shi and Beiquan Mou

**Abstract:** Leafminer (*Liriomyza langei*) is a major insect pest of many important agricultural crops, including spinach (*Spinacia oleracea*). Use of genetic resistance is an efficient, economic, and environment-friendly method to control this pest. The objective of this research was to conduct association analysis and identify single nucleotide polymorphism (SNP) markers associated with leafminer resistance in spinach germplasm. A total of 300 USDA spinach germplasm accessions were used for the association analysis of leafminer resistance. Genotyping by sequencing (GBS) was used for genotyping and 783 SNPs from GBS were used for association analysis. The leafminer resistance showed a near normal distribution with a wide range from 1.1 to 11.7 stings per square centimeter leaf area, suggesting that the leafminer resistance in spinach is a complex trait controlled by multiple genes with minor effect in this spinach panel. Association analysis indicated that five SNP markers, AYZV02040968_7171, AYZV02076752_412, AYZV02098618_4615, AYZV02147304_383, and AYZV02271373_398, were associated with the leafminer resistance with LOD 2.5 or higher. The SNP markers may be useful for breeders to select plants and lines for leafminer resistance in spinach breeding programs through marker-assisted selection.

*Key words:* association mapping, genotyping by sequencing, leafminer, single nucleotide polymorphism, spinach, *Spinacia oleracea*.

**Résumé :** La mouche mineuse (*Liriomyza langei*) est un insecte ravageur important chez plusieurs cultures dont l'épinard (*Spinacia oleracea*). Le recours à la résistance génétique constitue une méthode de lutte efficiente, économique et écologique contre ce ravageur. L'objectif de ce travail était de réaliser une analyse d'association et d'identifier des marqueurs SNP (polymorphismes mononucléotidiques) associés à la résistance à la mineuse au sein des ressources génétiques de l'épinard. Au total, 300 accessions de la collection du USDA ont été employées pour l'analyse d'association avec la résistance à la mineuse. Le génotypage a été réalisé au moyen du génotypage par séquençage (GBS) et 783 marqueurs SNP ont été utilisés pour l'analyse d'association. La résistance à la mineuse ressemblait à une distribution normale, le nombre de piqures par centimètre carré de surface foliaire variant entre 1,1 et 11,7; ceci suggère que la résistance à la mineuse est un caractère complexe contrôlé par de nombreux gènes à effet mineur au sein de cette collection d'accessions d'épinard. L'analyse d'association a révélé que cinq marqueurs (AYZV02040968_7171, AYZV02076752_412, AYZV02098618_4615, AYZV02147304_383 et AYZV02271373_398) étaient associés à la résistance à la mineuse, avec un score LOD de 2,5 ou plus. Ces marqueurs aideront possiblement les sélectionneurs à développer des plantes et des lignées dotées d'une résistance à la mineuse dans le cadre de programmes d'amélioration génétique assistée de marqueurs. [Traduit par la Rédaction]

*Mots-clés :* cartographie par association, génotypage par séquençage, mouche mineuse, polymorphisme mononucléotidique, épinard, *Spinacia oleracea*.

## Introduction

Leafminers are important insect pests of many agricultural crops throughout the world (Parrella 1987), and major leafminer species affecting vegetables include *Liriomyza brassicae* (Riley), *L. sativae* Blanchard, *L. trifolii* (Burgess), *L. huidobrensis* (Blanchard), and *L. langei* Frick. Scheffer et al. (2001) identified the leafminers in the principal spinach production area of central California to be the morphologically cryptic species *L. langei* by using polymerase chain reaction (PCR) amplification of mitochondrial DNA.

Leafminer adults are small black flies with a bright yellow triangular spot on the upper thorax between the wings. Adult flies puncture leaves to feed on plant sap

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and females lay white, oval eggs within the leaf tissue. Feeding and oviposition result in leaf damage as "stings" that appear as holes or bumps on the leaves, and adult feeding on cotyledons may stunt seedling growth. Larvae hatch from eggs and feed in leaves, generating the winding, whitish tunnels or mines that are initially narrow, but increase in width as the larvae grow. Larvae drop out of the mines after completing three instars and pupate in the soil or on the leaf surface, and adult flies emerge from pupae in about 8–11 days. The entire life cycle can be completed in less than 3 weeks in warm weather in California and many generations are produced each year. Damages caused by adult sting and larval mining of leaves reduce photosynthetic capacity, render spinach leaves unmarketable, and provide an entrance for disease organisms (LeStrange et al. 1999). About 75% of the spinach produced in the Salinas Valley is used for fresh market consumption (Monterey County Agricultural Commissioner's Office 2001). The percentage of spinach acreage grown for fresh markets versus processing markets has increased significantly in the United States (Morelock and Correll 2006, 2008). Quality standards for fresh market spinach are extremely high, so the leafminer pest poses a serious threat for growers in California and other states who need to produce defect-free products.

Use of host genetic resistance is an alternative strategy to chemicals for leafminer management, and resistant varieties were recognized as the most economical method to control leafminer in vegetables (Basij et al. 2011). Evaluations of germplasm for leafminer resistance have been conducted in vegetables. Mou and Liu (2003, 2004) screened more than 200 lettuce accessions, found a large range of variation in reactions to leafminer attack, and identified sources of resistance to leafminers. Trumble and Quiros (1988) did not observe any cultivated celery with resistance to leafminers (*L. trifolii*), but they found that an accession of a wild species, *Apium prostratum*, was immune to the pest. Basij et al. (2011) evaluated leafminer (*L. sativae*) resistance in 17 cucumber cultivars in greenhouse conditions and found that the 17 cultivars can be divided into four groups: susceptible, semi-susceptible, semi-resistant, and resistant based on indices such as the number of leafminer stings, the number of larval mines, the proportion of larval mines to leafminer stings, and the rate of injury. In spinach, Mou (2008) screened 345 USDA accessions and commercial cultivars for resistance to leafminer, and he found that no genotype was immune to leafminers, but significant genotypic differences existed for leafminer stings per unit leaf area, mines per plant, and mines per 100 g plant weight among the genotypes tested. Mou (2008) also observed some spinach accessions that had much lower levels of leafminer stings and mines than commercial cultivars and reported two accessions, PI274065 and PI174385, with the lowest sting density and with the fewest mines per unit plant weight, respectively, among genotypes in the field. It has been indicated that the leafminer-resistant accessions can be used for genetic improvement of spinach for leafminer resistance. So far, two spinach germplasms with resistance to leafminer mines have been released (Mou 2007*a*, 2007*b*).

No information is available for the genetics of leafminer resistance in spinach. Leafminer resistance in spinach seems a complex trait because a large range of responses exists in spinach genotypes (Mou 2008). It would be time-consuming to transfer these complex traits through a classic plant breeding approach. However, molecular plant breeding can be an efficient way to select quantitative traits through marker-assisted selection (MAS). Single nucleotide polymorphism (SNP), with its abundance, cost efficiency, and high-throughput scoring, has become a powerful tool in genome mapping, association studies, diversity analysis, germplasm identification, and tagging of important genes in plant genomics (Collard et al. 2005; Collard and Mackill 2008; Xu and Crouch 2008; Fang et al. 2014; Wang et al. 2015). Therefore, identification of SNP markers associated with leafminer resistance will provide breeders with a useful tool to assist in selecting for insect resistance in spinach breeding programs. Genotyping by sequencing (GBS) is one of the next-generation sequencing platforms to discover SNPs without prior knowledge of the genome in spinach (Elshire et al. 2011; Sonah et al. 2013; He et al. 2014). The spinach genome sequences AYZV01 and AYZV02 are available to the public (http://www.ncbi.nlm.nih.gov/Traces/wgs/?val=AYZV01 and http://www.ncbi.nlm.nih.gov/Traces/wgs/?val=AYZV02) and represent approximately half of the spinach genome (Dohm et al. 2014; Minoche et al. 2015). In addition, a more comprehensive version of the spinach genome assembly will be made publicly available in 2016 (van Deynze 2014; van Deynze et al. 2015; Allen van Deynze, personal communication). Recently, Xu et al. (2015) reported the comparative transcriptomes of cultivated and wild spinach postulated from RNAseq, assembled 72 151 unigenes, and identified a total of ~320 000 high-quality SNPs, and this information can be viewed and downloaded at http://spinachbase.org/cgi-bin/spinach/index.cgi. Yang et al. (2016) built a spinach genome database, SpinachDB (http://222.73.98.124/spinachdb), where 21 702 spinach genes were annotated; a total of 131 592 SSRs and 1 125 743 potential SNPs located in 548 801 loci of spinach genome were identified in 11 cultivated and wild spinach varieties. These resources provide a reference for SNP discovery and association analysis in spinach.

To date, knowledge of the spinach genome is limited and few reports have been published on the use of molecular markers in spinach. Khattak et al. (2006) published a genetic linkage map with six linkage groups, constructing the map with 101 amplified fragment length polymorphisms (AFLPs) and nine simple sequence repeats (SSRs). This genetic map has a total length of 585 cM, and an average distance of 5.18 cM between

markers, but it does not offer a great amount of detail about the linkage groups. AFLPs and SSRs, while useful, are less specific than SNP markers. Recently, Chan-Navarrete et al. (2016) reported a SNP genetic map with six linkage groups (P01–P06), consisting of 283 SNP markers, ranging in size from 46 to 116 cM, and they identified 39 quantitative trait loci (QTLs) related to nitrogen use efficiency (NUE) in spinach. The identification of SNP markers for spinach traits of interest, including insect resistance, will provide breeders with powerful tools to develop improved spinach cultivars more efficiently. Therefore, the development of robust SNP markers and SNP genetic maps would be a valuable resource for spinach breeding efforts. The objective of this research was to conduct association analysis and identify SNP markers associated with leafminer resistance in USDA spinach germplasm.

## Materials and methods

### Plant materials

A total of 300 spinach genotypes were used for the association analysis of leafminer resistance in this study (supplementary data, Table S1[1]). The 300 spinach genotypes were USDA spinach germplasm accessions, originally collected from 31 countries, mainly from eight countries including Turkey, the United States (US), Afghanistan, Serbia, England, Iran, China, and Belgium having 236 accessions, which consisted of 78.7% of all tested accessions, and the other 23 countries only had 64 accessions with 21.3% of the tested accessions (Table S1[1]). All seeds were kindly provided by the North Central Regional Plant Introduction Station, USDA-ARS, Iowa State University, Ames, Iowa.

### Leafminer phenotyping

Experiment for leafminer pest evaluation was conducted at the Agricultural Research Station of the USDA, Salinas, California (Mou 2008). Sixteen seeds from each accession were planted in a plastic pot (10 cm × 10 cm × 10 cm) with a mixture of 2 sand: 1 soil (by volume) in a greenhouse, and seedlings were thinned to 10 plants per pot. Plants were moved into an outdoor insect cage (2 m high × 4 m wide × 8 m deep) made of polypropylene shadecloth for resistance screening 5 weeks after planting. Lettuce leaves with leafminer mines were collected from newly harvested fields around Salinas and hung in the shade to allow leafminer larvae to emerge from the leaves and pupate. Pupae were collected and put in plastic containers to allow adult flies to emerge. Approximately 3500 flies were then released in the outdoor cage to feed on the spinach plants. After 10 days, the number of stings per unit area was counted on the leaf with most leafminer stings on each plant using an optical glass binocular magnifier (OptiVisor; Donegan Optical Co., Lenexa, Kans.).

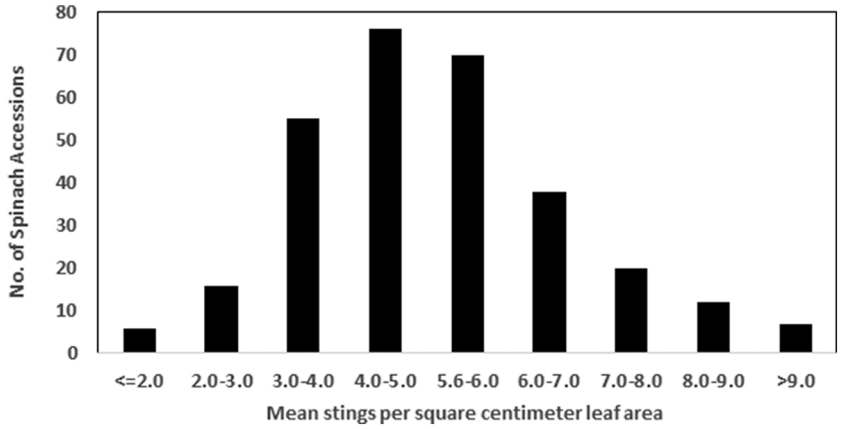
For each spinach genotype, the stings per square centimeter was accounted based on a single plant and a total of 10 plants in one pot were evaluated. The mean stings per square centimeter for each spinach genotype was used for further data analysis. The Tabulate procedure in JMP Genomics 7 (SAS Institute, Cary, NC) and Microsoft (MS) Excel 2013 were used to estimate the average, range, standard deviation (SD), and standard error of leafminer stings per square centimeter leaf area. The distribution of mean leafminer stings per square centimeter in the 300 accessions was drawn using MS Excel.

### DNA extraction, GBS, and SNP discovery

Genomic DNA was extracted from leaves of spinach plants using the CTAB (hexadecyltrimethyl ammonium bromide) method (Kisha et al. 1997). A DNA library was prepared using the restriction enzyme *ApeK*I following the GBS protocol described by Elshire et al. (2011). The 90-bp double-end sequencing was performed on each spinach accession using GBS protocol by an Illumina HiSeq 2000 at the Genomics Research Institute (BGI), Hong Kong. GBS data assembly, mapping, and SNP discovery were done using SOAP family software (http://soap.genomics.org.cn/) by the bioinformatics team at BGI. The GBS data provided by BGI averaged 3.26 M 90-bp short-read nucleotides for each spinach sample. The short reads of the GBS data were first aligned to spinach genome reference Viroflay-1.0.1 with AYZV01 project (http://www.ncbi.nlm.nih.gov/Traces/wgs/?val=AYZV01) using SOAPaligner/soap2 (http://soap.genomics.org.cn/). Following the Spinach-1.0.3 spinach genome reference released on 22 July 2015, the AYZV01 series of contig accessions were changed to AYZV02 accessions (http://www.ncbi.nlm.nih.gov/Traces/wgs/?val=AYZV02), so all SNP information was updated to AYZV02 version. The two versions of spinach genome references were also published at http://bvseq.molgen.mpg.de/Genome/Download/Spinach/. The SOAPsnp v 1.05 was used for SNP calling (Li et al. 2009; Li 2011). Approximately one half-million SNPs were discovered from the GBS data among the 300 spinach germplasm accessions and provided by BGI. The spinach accessions and SNPs were filtered before conducting genetic diversity and association analyses. If the spinach accession had greater than 20% missing SNP data and the heterozygous SNP genotype >30%, the spinach genotype was removed from the panel. The SNP data were filtered by minor allele frequency (MLF) >2%, missing data <7%, and heterozygous genotype <20%. After filtering, 783 SNPs for 300 spinach accessions were used for genetic diversity and association analysis.

[1]Supplementary data are available with the article through the journal Web site at http://nrcresearchpress.com/doi/suppl/10.1139/gen-2016-0075.

**Fig. 1.** The distribution of mean leafminer stings per square centimeter leaf area in 300 spinach germplasm accessions.




### Population structure and genetic diversity

The model-based program STUCTURE 2.3.4 (Pritchard et al. 2000) was used to assess the population structure of the 300 spinach accessions/cultivars based on 783 loci. To identify the number of populations (*K*) making up the structure of the data, the burn-in period was set at 10 000 with the Markov Chain Monte Carlo iterations and the run length set at 20 000 in an admixture model. The analysis then correlated allele frequencies that were independent for each run (Lv et al. 2012). Ten runs were performed for each simulated value of *K*, which ranged from 1 to 11. For each simulated *K*, the statistical value delta *K* was calculated using the formula described by Evanno et al. (2005). The optimal *K* was determined using STRUCTURE HARVESTER (Earl and von Holdt 2012; http://taylor0.biology.ucla.edu/structureHarvester/). After the optimal *K* was determined, a Q-matrix was obtained and was used in TASSEL 5 for association analysis. Each spinach accession was then assigned to a cluster (Q) based on the probability determined by the software that the genotype belonged in that cluster. The cut-off probability for assignment to a cluster was 0.5225 for only two clusters (structure populations). Based on the optimum *K*, a Bar plot with 'Sort by Q' was obtained to show the visual population structure among the 300 spinach accessions.

Genetic diversity was also assessed and the phylogeny trees were drawn using MEGA 6 (Tamura et al. 2013) based on the Maximum Likelihood tree method with the following parameters. Test of Phylogeny: Bootstrap Method; No. of Bootstrap Replications: 500; Model/Method: General Time Reversible model; Rates among Sites: Gamma distributed with Invariant sites (G + I); Number of Discrete Gamma Categories: 4; Gaps/Missing Data Treatment: Use all sites; ML Heuristic Method: Subtree-Pruning-Regrafting-Extensive (SPR level 5); Initial Tree for ML: Make initial tree automatically (Neighbor Joining); and Branch Swap Filter: Moderate. To compare the results from the two software programs, during the drawing of the phylogeny trees by MEGA, the colored shape and branch of each spinach genotype was drawn using the same color that was located at the cluster (Q) from STRUCTUR. For sub-tree of each Q (cluster), the shape of 'Node/Subtree Marker' and the 'Branch Line' was drawn with the same color as in the figure of the Bar plot of the population clusters from the STRUCTURE analysis.

### Association analysis

Association analysis was performed using the single marker regression (SMR) without structure and without kinship, the general linear model (GLM), and the mixed linear model (MLM) methods as described in TASSEL 5 (Bradbury et al. 2007; http://www.maizegenetics.net/tassel). Population structure (Q) was estimated using STUCTURE 2.3.4 (Pritchard et al. 2000), and Kinship (K) was estimated by the tool Kinship with Scald_IBS method built in TASSEL 5.

## Results

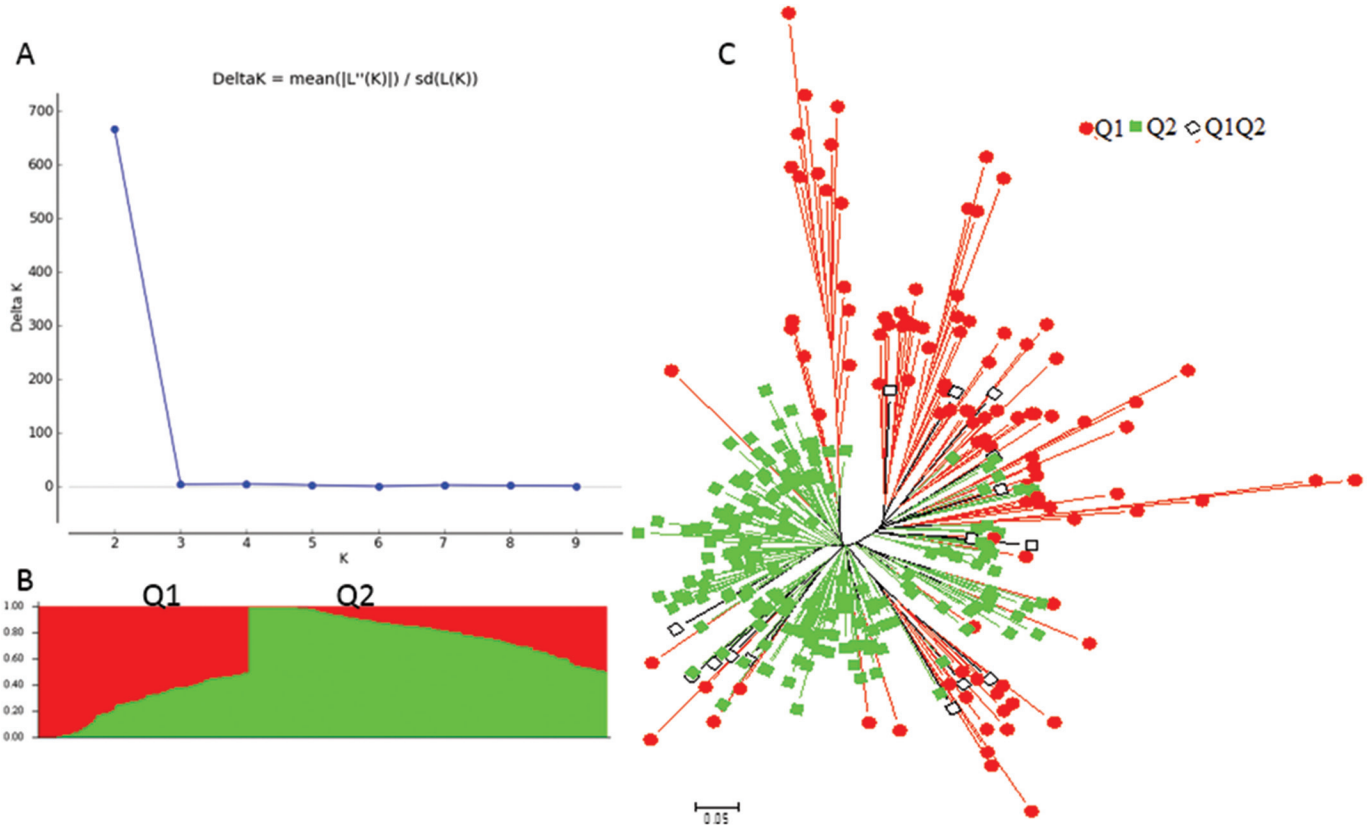
### Phenotyping of leafminer resistance

None of the genotypes tested were immune to leafminers, because all genotypes had at least a few stings. Significant genotypic differences were found for leafminer stings per unit leaf area (Table S1[1]). Leafminer stings per square centimeter leaf area ranged from 1.1 to 11.7 and averaged 5.2 with a near normal distribution (Table S1[1]; Fig. 1), suggesting that the leafminer resistance in spinach is a complex trait controlled by multiple genes with minor effect in the spinach panel. The standard deviation was 1.73 with the standard error 0.0058, indicating that there were significant genetic differences of leafminer resistance among the 300 spinach accessions (Table S1[1]).

### Genetic diversity and population structure

The population structure of the 300 spinach accessions was initially inferred using STRUCTURE 2.3.4 (Pritchard et al. 2000) and the peak of delta *K* was observed at $K = 2$, indicating the presence of two main populations (clusters, Q1 and Q2) in the spinach panel (Figs. 2A and 2B). The classification of accessions into populations based on the model-based structure from STRUCTURE 2.3.4 is shown in Fig. 2B and Table S1[1]. We used Q-value = 0.525 as the value to divide the clusters, i.e., if a spinach had its Q1

**Fig. 2.** Model-based populations in the association panel: (A) Delta *K* values for different numbers of populations assumed (*K*) in the STRUCTURE analysis. (B) Classification of 300 USDA spinach accessions into two populations using STRUCTURE 2.3.4. The distribution of the accessions to different populations is indicated by the color code (Q1: red and Q2: green). (C) Maximum likelihood (ML) tree of the 300 accessions drawn by MEGA 6. The color codes for each population are consistent in parts B and C, and the empty black square are the admixture Q1Q2. [Colour online.]




value ≥ 0.525, it was assigned to Cluster Q1; else if its Q2 value ≥ 0.525, it was assigned to Cluster Q2; and the leftover (0.475 < Q1 < 0.525 or 0.475 < Q2 < 0.525) was assigned to the admixture Q1Q2. In total, 286 accessions (95.3%) were assigned to one of the two populations (Q1 or Q2). Population 1 and 2 (Q1 and Q2) consisted of 103 (34.3%) and 183 (61.0%) accessions, respectively. The remaining 14 accessions (4.7%) were categorized as having admixed ancestry between Q1 and Q2 and was called Q1Q2 (Table S1[1]).

The genetic diversity among spinach accessions was also assessed using the Maximum Likelihood (ML) method by MEGA 6 (Tamura et al. 2013). We defined Q1 and Q2 as the two main clusters and used the same colors as the population structure Q1 (red) and Q2 (green) from STRUCTURE 2.3.4 (Fig. 2B) to draw the subtrees of the phylogenetic tree (Fig. 2C) with Q1 (red and round shape), Q2 (green and square shape), and the admixture Q1Q2 (black empty square). Two phylogenetic trees were included: (*i*) Fig. 2C, without taxon names to compare it to the structure populations from STRUCTURE and to view them easily and clearly; (*ii*) Fig. S1[1], the format of the traditional rectangular phylogenetic tree with taxon name. The phylogenetic trees from MEGA 6 (Fig. 2C; Fig. S1[1]) were good but not fully consistent with the structure populations (Q1-Q2) from STRUCTURE 2.3.4 (Figs. 2A and 2B), indicating that there were two differentiated genetic populations and admixtures in the spinach panel, which was not completely divided into two clusters.

### Association analysis

Based on the genetic diversity analysis from STRUCTURE and MEGA and by viewing the phylogenetic tress from Fig. 2 and Fig. S1[1], the 300 spinach accessions can be organized into two structured populations. Therefore, we used the Q matrix with two structures in the association mapping in TASSEL. In total, three models in TASSEL were used to do association analysis of leafminer resistance, including SMR, GLM (Q), and MLM (Q+K). We also used a LOD value (or likely LOD = ~(–LOG(*P*)), where *P* is the P value) equal to or greater than 2.5 as the threshold value to identify the SNP marker associated with the leafminer resistance in the study.

With LOD value of 2.5 or higher in all three models (SMR, GLM, and MLM) from TASSEL, there were five SNPs shown to be associated with leafminer resistance (Table 1). Among the five SNP markers, AYZV02040968_7171, AYZV02076752_412, and AYZV02271373_398 had 2.6 or higher LOD values in all three models, and AYZV02098618_4615 and AYZV02147304_383 had a 2.5 or higher LOD in both SMR and GLM models

**Table 1.** Five SNP markers associated with leafminer resistance identified from three modes using TASSEL in 300 spinach accessions.

| Spinach genome Spinach-1.0.3 information | | | | Viroflay-1.0.1 | | LOD (–LOG(*P*)) | | | $R^2$ (%) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| SNP name[a] | SNP type | Contig at AYZV02 project | SNP position | Contig at AYZV01 project | SNP position | SMR | GLM | MLM | SMR | GLM | MLM |
| AYZV02040968_7171 | C/T | AYZV02040968 | 7171 | AYZV01031587 | 7171 | 3.4 | 3.5 | 2.7 | 5.3 | 5.3 | 4.2 |
| AYZV02076752_412 | T/G | AYZV02076752 | 412 | AYZV01058628 | 412 | 2.9 | 2.9 | 3.0 | 4.3 | 4.3 | 4.7 |
| AYZV02098618_4615 | T/C | AYZV02098618 | 4615 | AYZV01074880 | 4615 | 3.5 | 3.5 | 2.2 | 5.5 | 5.4 | 3.5 |
| AYZV02147304_383 | T/C | AYZV02147304 | 383 | AYZV01109497 | 383 | 2.5 | 2.5 | 2.2 | 3.7 | 3.7 | 3.5 |
| AYZV02271373_398 | A/G | AYZV02271373 | 398 | AYZV01198119 | 398 | 2.6 | 2.7 | 3.1 | 4.2 | 4.3 | 5.1 |

**Note:** SMR, single marker regression; GLM, general linear model; MLM, mixed linear model using TASSEL 5 (Bradbury et al. 2007; http://www.maizegenetics.net/tassel).

[a]SNP name is defined as the contig name plus the SNP position on the contig.

and a 2.2 LOD value in MLM model, indicating that the five SNP markers were associated with leafminer resistance. However, the $R^2$ values were very low from 3.5% to 5.5% for all five SNP markers in three models (Table 1), indicating that the markers had only a minor effect for leafminer resistance. The five SNPs were located at five different contigs, which may be located at different chromosomes or different regions of chromosomes, further suggesting leafminer resistance was a quantitative trait controlled by multiple genes with minor effect.

## Discussion

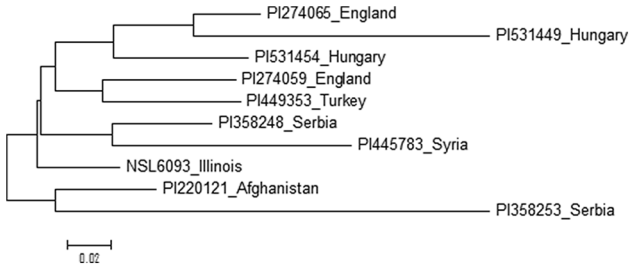
From this research, the leafminer resistance in the tested 300 spinach genotypes showed a near normal distribution with a wide range from 1.1 to 11.7 leafminer stings per square centimeter leaf area, suggesting that the leafminer resistance in spinach was a complex trait governed by multiple genes with minor effect. Mou (2008) reported that no genotype was immune to leafminers, but significant genotypic differences were found for leafminer stings per unit leaf area, mines per plant, and mines per 100 g plant weight among the spinach genotypes tested. So far, it is not clear whether leafminer resistance in spinach is a quantitative or qualitative trait controlled by major genes or minor genes. We did not find major QTLs for leafminer resistance in this study. All identified SNP markers had very low $R^2$ values, further indicating that multiple genes with minor effect existed in these spinach genotypes for controlling leafminer resistance. But there is no evidence either to deny that major genes exist or do not exist for leafminer resistance in spinach. For resistance to *Liriomyza* spp. in other crops, major genes have been identified. Dogimont et al. (1999) reported a dominant gene, *Lt*, for leafminer (*L. trifolii*) resistance in melon based on $F_2$ and backcross progenies from a cross between the resistant line Nantais Oblong and Vedrantais, a Charentais line susceptible to the leafminer. However, Kennedy et al. (1978) reported that two melon accessions, PI282448 and PI313970, had recessive or incompletely dominant resistance to another leafminer species (*L. sativae*). In other reports, QTL and multiple genes were also identified for leafminer resistance, such as Moreira et al. (1999) who identified one major QTL for leafminer resistance located on chromosome 2 using random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) markers, and Cardoso et al. (2014) who identified four candidate genes for coffee leafminer resistance. Further QTL mapping using bi-parent populations derived from highly susceptible and highly resistant spinach lines will confirm the genetics of resistance to leafminer resistance in spinach.

In this study, there was no replicates for the phenotyping experiment with the 300 spinach genotype; however, three experiments have been conducted for evaluation of leafminer resistance in spinach germplasm: one in a cage and two in the field during the years 2002 and 2004 (Mou 2008). Leafminer stings per square centimeter leaf area in spinach was found to be a very stable trait with strong correlations among the three experiments (Mou 2008). Mou (2008) identified the correlation coefficients ($r$) to be 0.770, 0.746, and 0.802 between cage and 2002 field, between cage and 2004 field, and between 2002 field and 2004 field, respectively.

Three models, SMR, GLM, and MLM, were used to conduct association analysis of leafminer resistance in this study. We observed that a lot of SNP showed different results in different models (data not shown). We supposed that if it gave significant association in different models, the SNP marker should be a reliable one. Based on LOD (–Log($P$)) values of 2.5 or higher in three models, five SNP markers were identified to be strongly associated with leafminer resistance from this study (Table 1), indicating that the three SNP markers may be used as reliable molecular markers in breeding programs through MAS.

Among the 300 spinach genotypes, six accessions, PI220121, PI274059, PI358248, PI445783, PI449353, and PI531454, had 2.0 or fewer mean stings per square centimeter leaf area. In addition, NSL6093, PI274065, PI358253, and PI531449 also had fewer mean stings per square centimeter leaf area across three experiments (Mou 2008). Theses 10 accessions showed high resistance to leafminer and may be used as parents in spinach breeding programs to develop leafminer-resistant culti-

**Fig. 3.** A phylogenetic tree drawn by MEGA 6 among 10 spinach germplasm accessions with low mean stings per square centimeter leaf area.




vars. Using different genetic sources will allow breeders to create a wider range of variation in a given trait among progeny derived from two parents with a broader genetic background or larger genetic distance. A phylogenetic tree among the 10 spinach accessions was built using 783 SNP alleles by MEGA 6 (Fig. 3). From the phylogenetic tree, accession PI274065 (from England) is merged to PI531449 (Hungary), closer to PI531454 (Hungary), and then clustered together with PI274059 (England) and PI449353 (Turkey); the two accessions, PI358248 (Serbia) and PI445783 (Syria) are merged together and closer to the above five accessions; another two accessions, PI220121 (Afghanistan) and PI358253 (Serbia), merged together as a separate group; and the Illinois accession NSL6093 does not merge to anyone as a outlier but is closest to PI220121 and PI358248 (Fig. 3). The phylogenetic analysis provides breeders with knowledge about how to select the 10 leafminer-resistant accessions in a breeding program. Thus, these accessions may provide good sources of leafminer resistance to be used as parents in spinach breeding.

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## References

Basij, M., Askarianzaeh, A., Asgari, S., Moharramipou, S., and Rafezi, R. 2011. Evaluation of resistance of cucumber cultivars to the vegetable leafminer (*Liriomyza sativae* Blanchard) (Diptera: Agromyzidae) in greenhouse. Chilean J Agric. Res. **71**: 395–400. doi:10.4067/S0718-58392011000300008.

Bradbury, P.J., Zhang, Z., Kroon, D.E., Casstevens, T.M., Ramdoss, Y., and Buckler, E.S. 2007. TASSEL: software for association mapping of complex traits in diverse samples. Bioinformatics, **23**: 2633–2635. doi:10.1093/bioinformatics/btm308. PMID:17586829.

Cardoso, D.C., Martinati, J.C., Giachetto, P.F., Vidal, R.O., Carazzolle, M.F., Padilha, L., et al. 2014. Large-scale analysis of differential gene expression in coffee genotypes resistant and susceptible to leafminer—toward the identification of candidate genes for marker assisted-selection. BMC Genomics, **15**: 66. doi:10.1186/1471-2164-15-66. PMID:24460833.

Chan-Navarrete, R., Dolstra, O., van Kaauwen, M., van Bueren, E.T.L., and van der Linden, C.G. 2016. Genetic map construction and QTL analysis of nitrogen use efficiency in spinach (*Spinacia oleracea* L.). Euphytica, **208**: 621–636. doi:10.1007/s10681-015-1618-6.

Collard, B.C.Y., and Mackill, D.J. 2008. Marker-assisted selection: an approach for precision plant breeding in the twenty-first century. Philos. Trans. R. Soc. B Biol. Sci. **363**: 557–572. doi:10.1098/rstb.2007.2170. PMID:17715053.

Collard, B.C.Y., Jahufer, M.Z.Z., Brouwer, J.B., and Pang, E.C.K. 2005. An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: the basic concepts. Euphytica, **142**: 169–196. doi:10.1007/s10681-005-1681-5.

Dogimont, C., Bordat, D., Pages, C., Boissot, N., and Pitrat, M. 1999. One dominant gene conferring the resistance to the leafminer, *Liriomyza trifolii* (Burgess) Diptera: Agromyzidae in melon (*Cucumis melo* L.). Euphytica, **105**: 63–67. doi:10.1023/A:1003436428847.

Dohm, J.C., Minoche, A.E., Holtgräwe, D., Capella-Gutiérrez, S., Zakrzewski, F., Tafer, H., et al. 2014. The genome of the recently domesticated crop plant sugar beet (*Beta vulgaris*). Nature, **505**: 546–549. doi:10.1038/nature12817. PMID:24352233.

Earl, D.A., and von Holdt, B.M. 2012. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conserv. Genet. Res. **4**: 359–361. doi:10.1007/s12686-011-9548-7.

Elshire, R.J., Glaubitz, J.C., and Sun, Q. 2011. A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. PLoS ONE, **6**(5): e19379. doi:10.1371/journal.pone.0019379. PMID:21573248.

Evanno, G., Regnaut, S., and Goudet, J. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol. Ecol. **14**: 2611–2620. doi:10.1111/j.1365-294X.2005.02553.x. PMID:15969739.

Fang, W.P., Meinhardt, L.W., Tan, H., Zhou, L., Mischke, S., and Zhang, D. 2014. Varietal identification of tea (*Camellia sinensis*) using nanofluidic array of single nucleotide polymorphism (SNP) markers. Hortic. Res. **1**: 14035. doi:10.1038/hortres.2014.35. PMID:26504544.

He, J., Zhao, X., Laroche, A., Lu, Z., Liu, H., and Li, Z. 2014. Genotyping-by-sequencing (GBS), an ultimate marker-assisted selection (MAS) tool to accelerate plant breeding. Front. Plant Sci. **5**: 484. doi:10.3389/fpls.2014.00484. PMID:25324846.

Kennedy, G.G., Bohn, G.W., Stoner, A.K., and Webb, R.E. 1978. Leafminer resistance in muskmelon. J. Am. Soc. Hort. Sci. **103**: 571–574.

Khattak, J., Torp, A.M., and Andersen, S.B. 2006. A genetic linkage map of *Spinacia oleracea* and localization of a sex determination locus. Euphytica, **148**: 311–318. doi:10.1007/s10681-005-9031-1.

Kisha, T., Sneller, C.H., and Diers, B.W. 1997. Relationship between genetic distance among parents and genetic variance in populations of soybean. Crop Sci. **37**: 1317–1325. doi:10.2135/cropsci1997.0011183X003700040048x.

LeStrange, M., Koike, S., Valencia, J., and Chaney, W. 1999. Spinach production in California. University of California, Division of Agriculture and Natural Resources. Publication 7212. pp. 3–4.

Li, H. 2011. A statistical framework for SNP calling, mutation discovery, association mapping and population genetical parameter estimation from sequencing data. Bioinformatics, **27**: 2987–2993. doi:10.1093/bioinformatics/btr509. PMID:21903627.

Li, R., Yu, C., Li, Y., Lam, T., Yiu, S., Kristiansen, K., and Wang, J. 2009. SOAP2: an improved ultrafast tool for short read alignment. Bioinformatics, **25**: 1966–1967. doi:10.1093/bioinformatics/btp336. PMID:19497933.

Lv, J., Qi, J., Shi, Q., Shen, D., Zhang, S., Zhang, A., et al. 2012. Genetic diversity and population STRUCTURE of cucumber (*Cucumis sativus* L). PLoS ONE, **7**: 46919. doi:10.1371/journal.pone.0046919.

Minoche, A.E., Dohm, J.C., Schneider, J., Holtgrawe, D., Viehover, P., Montfort, M., et al. 2015. Exploiting single-molecule transcript sequencing for eukaryotic gene prediction. Genome Biol. **16**: 184. doi:10.1186/s13059-015-0729-7. PMID:26328666.

Monterey County Agricultural Commissioner's Office. 2001. Vegetable crops. Monterey County Crop Report 2000, pp. 6.

Moreira, L.A., Mollema, C., and van Heusden, S. 1999. Search for molecular markers linked to *Liriomyza trifolii* resistance in tomato. Euphytica, **109**: 149–156. doi:10.1023/A:1003728302493.

Morelock, T.E., and Correll, J.C. 2006. History of spinach production in the United States. *In* Proceedings, 2006 International Spinach Conference, 13–14 July 2006, La Conner, Wash.

Morelock, T.E., and Correll, J.C. 2008. Spinach. *In* Vegetables I: Asteraceae, Brassicaceae, Chenopodiaceae, and Cucurbitaceae. *Edited by* J. Prohens and F. Nuez. Springer, New York. pp. 189–218.

Mou, B. 2007*a*. Leafminer-resistant spinach germplasm 03-04-9. HortScience, **42**: 699–700.

Mou, B. 2007*b*. Leafminer-resistant spinach germplasm 03-04-63. HortScience, **42**: 1717–1718.

Mou, B. 2008. Leafminer resistance in spinach. HortScience, **43**: 1716–1719.

Mou, B., and Liu, Y.B. 2003. Leafminer resistance in lettuce. HortScience, **38**: 570–572.

Mou, B., and Liu, Y.B. 2004. Host plant resistance to leafminers in lettuce. J. Am. Soc. Hort. Sci. **129**: 383–388.

Parrella, M.P. 1987. Biology of Liriomyza. Annu. Rev. Entomol. **32**: 201–224. doi:10.1146/annurev.en.32.010187.001221.

Pritchard, J.K., Stephens, M., and Donnelly, P. 2000. Inference of population structure using multilocus genotype data. Genetics, **155**: 945–959. PMID:10835412.

Scheffer, S.J., Wijesekara, A., Visser, D., and Hallett, R.H. 2001. Polymerase chain reaction restriction fragment-length polymorphism method to distinguish *Liriomyza huidobrensis* from *L. langei* (Diptera: Agromyzidae) applied to three recent leafminer invasions. J. Econ. Entomol. **94**: 1177–1182. doi:10.1603/0022-0493-94.5.1177. PMID:11681681.

Sonah, H., Bastien, M., Iquira, E., Tardivel, A., and Legare, G. 2013. An improved genotyping by sequencing (GBS) approach offering increased versatility and efficiency of SNP discovery and genotyping. PLoS ONE, **8**: 54603. doi:10.1371/journal.pone.0054603.

Tamura, K., Stecher, G., Peterson, D., Ailipski, A., and Kumar, S. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. **30**: 2725–2729. doi:10.1093/molbev/mst197.

Trumble, J.T., and Quiros, C.F. 1988. Antixenotic and antibiotic resistance in *Apium* species to *Liriomyza trifolii* (Diptera: Agromyzidae). J. Econ. Entomol. **81**: 602–607. doi:10.1093/jee/81.2.602.

van Deynze, A. 2014. A de novo draft assembly of spinach using Pacific Biosciences technology. Plant & Animal Genomics XXII Conference, 10–15 January 2014. San Diego, CA. Available at http://aa314.gondor.co/webinar/a-de-novo-draft-assembly-of-spinach-using-pacific-biosciences-technology/ [accessed 3 March 2016].

van Deynze, A., Ashrafi, H., Hickey, L., Peluso, P., Rank, D., Chin, J., et al. 2015. Using spinach to compare technologies for whole genome assemblies. Plant & Animal Genomics XXIII Conference, 10–14 January 2015, San Diego, Calif.

Wang, B., Tan, H., Fang, W., Meinhardt, L.W., Mischke, S., Matsumoto, T., and Zhang, D. 2015. Developing single nucleotide polymorphism (SNP) markers from transcriptome sequences for identification of longan (*Dimocarpus longan*) germplasm. Hortic. Res. **2**: 14065. doi:10.1038/hortres.2014.65. PMID:26504559.

Xu, C., Jiao, C., Zheng, Y., Sun, H., Liu, W., Cai, X., et al. 2015. De novo and comparative transcriptome analysis of cultivated and wild spinach. Sci. Rep. **5**: 17706. doi:10.1038/srep17706. PMID:26635144.

Xu, Y., and Crouch, J.H. 2008. Marker-assisted selection in plant breeding: from publications to practice. Crop Sci. **48**: 391–407. doi:10.2135/cropsci2007.04.0191.

Yang, X., Tan, H., and Zhu, W. 2016. SpinachDB: A well-characterized genomic database for gene family classification and SNP information of spinach. PLoS ONE, **11**(5): e0152706. doi:10.1371/journal.pone.0152706. PMID:27148975.