ANALYSIS OF LEAF MORPHOLOGY AND POPULATION STRUCTURE IN A MULTIFOLIATE ALFALFA BC_3 PROGENY USING SSR MARKERS

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ABSTRACT

The multifoliate leaf trait in alfalfa is a key agronomic trait associated with enhanced forage yield and quality. In this study, a backcross (BC_3) progeny was developed from crosses between alfalfa (*Medicago sativa* L. cv. Huaiyin) and PL34HQ multifoliate leaves to analyze the leaf morphology and population structure using SSR markers. Leaf morphological traits were observed and recorded at different growth stages to assess structural and seasonal variations. Genomic DNA was extracted from each progeny, and Simple Sequence Repeat (SSR) markers were employed to evaluate the genetic diversity and population structure of the 147 BC_3 progeny and their two parents. The observations of leaf morphology revealed diverse compound leaf types (with 4, 5, 6, or 7 leaflets) on the main stem of multifoliate plants. The frequency of multifoliate leaves was notably higher in spring and autumn than in winter and summer, with an overall multifoliate rate of 90.48% in the BC_3 progeny. The highest and lowest rates of multifoliate plants were 88.75% and 5.35%, respectively. Using 15 SSR primers, a total of 68 bands were amplified across the 147 hybrids, with an average of 4.53 alleles per locus. Primer MES 3 generated the highest number of alleles (7), while MES 37 and MES 64 produced the fewest (2). The polymorphism rate ranged from 66.67% (W6007) to 100% (MES 37 and MES 64). The average PIC and gene diversity values were 0.672 and 0.224, respectively. Population structure analysis using STRUCTURE, principal coordinate analysis PCoA, and neighbor-joining methods identified three distinct genetic groups. Group III exhibited the greatest genetic diversity (0.2543) and highest Shannon index (0.3831), whereas Group I had the lowest values (0.2413 and 0.3560, respectively). These findings provide valuable insights for developing mapping populations, selecting breeding parents, and evaluating SSR marker polymorphism in alfalfa improvement programs.

Keywords: Alfalfa; Microsatellite; Population structure; Genetic diversity; BC_3 progeny

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INTRODUCTION

Alfalfa (*Medicago sativa* L.) is a premier legume forage crop globally, valued for its high protein, vitamin, and mineral content, which provides essential nutrition for livestock (Feng *et al*., 2022). Varieties of alfalfa that exhibit multifoliate (ML) leaf traits are considered superior in terms of forage quality. Enhancing the leaf-to-stem ratio is one strategy to improve the nutritional value of alfalfa, and multifoliolate genotypes offer potential in achieving this goal (Petkova, 2010; Olom *et al*., 2023).

Alfalfa varieties exhibiting the multifoliolate (ML) leaf trait producing more than three leaflets per leaf are considered superior. Breeding for this trait has the potential to improve forage quality by increasing leaf number without compromising dry matter yield (Juan *et al*., 1993). The genetic inheritance of the multifoliolate trait in cultivated, tetraploid alfalfa remains poorly understood despite its agronomic value; however, early studies in diploid alfalfa suggested control by a recessive gene (mf) and two other genes that impacted its expression.(Bingham and Murphy, 1965), the genetic architecture in the more complex tetraploid, allogamous (cross-pollinating) system is unknown. This complexity, characterized by polysomic inheritance, has historically made genetic analysis in alfalfa challenging (Osborn *et al*., 1999; Wang *et al*., 2011). In cross-pollinated crops, inbreeding depression occurs in several economically significant biological traits, including productivity, tolerance and resistance to biotic and abiotic stresses, nutritional quality, etc. It is well known that inbreeding aims to create a homogenous crop population (Acquaah, 2015).

The development of compound leaves, which are the norm in alfalfa, is a key morphological process. Unlike simple leaves with a single blade, compound leaves have multiple leaflets arranged in specific patterns, offering significant morphological diversity (Mo *et al*., 2022). Understanding the development and genetic control of this trait in multifoliolate alfalfa is therefore a fundamental botanical and breeding objective (Mo *et al*., 2022).

To investigate and utilize this trait, effective breeding strategies are required. The backcross method is a powerful technique for transferring specific traits, such as multifoliolate leaves, from a donor parent into the genetic background of a high-yielding, well-adapted recurrent parent. This approach allows breeders to correct a specific defect (e.g., low leaf-to-stem ratio) in an otherwise elite cultivar while recovering most of its desirable genes over successive generations. Evaluation at the BC_3 generation is strategic, as this stage achieves 94% genetic recovery of the Huaiyin genome, minimizing confounding background variation and making it the optimal point to select for superior recombinants at the target traits.

Furthermore, unlocking the genetic potential of this trait requires assessing the diversity within germplasm collections. Modern molecular tools such as (RFLP) (Brummer *et al*., 1991; Kidwell *et al*., 1994; Pupilli *et al.*, 2000), (SRAPs) (Ariss and Vandemark, 2007), (RAPD) (Mengoni *et al*., 2000), (SSR) (He *et al*., 2019), and (AFLP) (Keivani *et al*., 2010) have been successfully employed to estimate genetic diversity and population structure in alfalfa, overcoming some of the challenges posed by its tetraploid nature. Such genetic diversity is the foundation for crop improvement, enabling the development of resilient varieties with superior traits.

Given the lack of information on the inheritance and development of the multifoliate trait in tetraploid alfalfa, a comprehensive study is necessary. Therefore, the objectives of this study were to: (1) Study the leaf morphological traits of BC_3 progeny to assess the structural variation in leaflet number within the population; (2) determine the stability and expression of the multifoliate trait by tracking the multifoliate rate within the BC_3 progeny across different growing seasons; and (3) evaluate the genetic diversity and population structure to understand the genetic relatedness and sub-grouping within the hybrid population.

MATERIALS AND METHODS

Plant Material: The backcross population was developed to produce the multifoliate leaf trait from the donor parent PL34HQ multifoliate leaves (Australian germplasm) to the genetic background of the recipient parent Huaiyin alfalfa (local Yangzhou germplasm). A field trial was established in February 2021 at the grassland field to assess the multifoliate leaf populations at Yangzhou University, China. Initially, F_1 hybrids were obtained by crossing PL34HQ as the recurrent female parent with Huaiyin as the non-recurrent male parent. The F_1 plants with high multifoliate traits and favourable comprehensive traits were selected and then backcrossed to the recurrent parent Huaiyin to generate the first-generation backcross (BC_1) progeny. Plants showing high multifoliate traits were selected as backcross parents for subsequent generations. The second-generation backcross progeny (BC_2) was produced by crossing selected BC_1 plants with Huaiyin. Selection continued based on high multifoliate traits and agronomic performance. Finally, third-generation backcross hybrids (BC_3) were developed by crossing BC_2 plants exhibiting stable multifoliate expression with the recurrent parent Huaiyin.

Seeds of the BC_3 hybrids were sown in the field in PVC pipes (16 cm in diameter, 50 cm in height) were placed in rows on the soil surface (Figure 1). The experimental unit was 15 meters long and 10 meters wide; all genotypes were planted in 10 rows; the distance between each row was 1 m, and the distance between the plants within the row was 1 meter (15 plants per row). In this study, 147 BC_3 populations (named BC_3-1 to BC_3-147) and their parents were used.

Figure 1. BC_3 progeny of alfalfa multifoliate leaves

Determination of alfalfa multifoliate traits: To evaluate the multifoliate trait, data were collected from the each plant of 147 BC_3 progeny and their two parents. For each plant, the overall multifoliate rate was defined as the percentage of its branches that exhibited at least one multifoliate leaf. To get a more accurate measurement, three main branches were chosen at random from each plant. The multifoliate rate for each of these branches was calculated as the percentage of its leaves that were multifoliate. The values from the three branches were then averaged to produce a mean branch multifoliate rate.

Compound leaf type: Alfalfa compound leaves can be divided into 3, 4, 5, 6, and 7 leaf types according to the number of leaflets on a single compound leaf. According to the combination of compound leaf types on individual alfalfa plants, individual alfalfa plants can be divided into 3, 4, 4+5, 4+5+6, and 4+5+6+7 leaf types.

During the study period, multifoliate rate per single plant and multifoliate rate on the main branches were recorded in 147 progeny and their two parents. The multifoliate rate per single plant and multifoliate on the main branches were calculated as follow:

$$\text{Multifoliate rate per plant} = \frac{\text{number of branches with multifoliate}}{\text{Total number of branches per plant}} * 100 \quad (1)$$

$$\text{Multifoliate rate on the main branches} = \frac{\text{multifoliate leaves on the branches}}{\text{Total number of leaves in sigle branch}} * 100 \quad (2)$$

Isolation of genomic plant DNA: Genomic DNA was extracted from fresh young leaves of all 147 BC_3 progeny and their two parents using the cetyltrimethyl ammonium bromide (CTAB) technique described by (Doyle, 1991). DNA from healthy leaves of each plant was isolated and stored at -80 °C for further use.

DNA quantification: The concentration and purity of the extracted DNA samples were determined using a NanoDrop spectrophotometer and by resolving on 1% agarose gel (1 g of agarose powder in 100 ml of sodium borate buffer). This was to detect degradation and the presence of contaminants. Samples with low-quality DNA were re-extracted. DNA samples were diluted to a working concentration of 50ng/µl. The DNA was diluted to 50 ng/µl and stored at -20 °C.

PCR amplification: The polymerase chain reaction (PCR) was amplified in a 10 µl reaction system containing 5×2 reaction mix, 0.2 µl golden DNA polymerase, 1 µl (10µmol/L) of each forward and reverse primer, 1 µl of DNA template, and 2 µl of ddH2O. The following is the protocol that was used during the PCR amplification: (3 min at 94 °C) for the initial denaturation step, then (35 cycles of 30 sec at 94 °C for the denaturation step, 30 sec at 47-52 °C for the annealing step, and 1 min at 72°C for the extension step), and the final extension (5 min at 72°C). Electrophoresis was used to separate and measure the PCR amplification products in a 6% denatured polyacrylamide stock solution in 1× TAE buffer at 200 V for 90 minutes. Then, the gel was put in a silver staining solution (add 0.8 g of AgNO3 to 1 L of distilled water and mix well) and shaken in the silver stain for 10 min. To make the solution, mix 15 g of NaOH with 1 L of distilled water and 4 mL of formaldehyde, then shake until the bands can be seen.

Data analysis: The data of morphological structure and change of multifoliate rate in different season were recorded and then analyzed by using EXCLE 2010 and SPSS 25.0. Genetic diversity parameters for each microsatellite marker including the number of alleles, gene diversity, and polymorphic information content (PIC) were calculated. PIC values for each SSR primer pair were determined according to (Botstein *et al*., 1980). The Shannon diversity index and overall genetic diversity were computed using the POPGENE software (Yeh *et al*., 1997). To assess genetic relationships, a phylogenetic dendrogram of the BC_3 alfalfa germplasm was generated using the DICE similarity matrix and the UPGMA clustering method within the DARwin 6.0.021 software (Perrier and Jacquemoud-Collet, 2006) with 30,000

bootstraps. Population structure was analyzed using STRUCTURE software (Pritchard *et al*., 2000). A Bayesian clustering approach based on fifteen SSR markers was applied to 147 BC_3 hybrids and their parents. The analysis included a burn-in period of 10,000 and 10,000 iterations Markov chain Monte Carlo (MCMC) replications, with 10 independent runs for each K value (ranging from K = 1 to K = 10). The STRUCTURE HARVESTER was used to compile and interpret the results across 20 independent runs for each K.

RESULTS

Morphology and structure of different types of alfalfa multifoliate leaves: In our observations, plant development followed the standard pattern: after sowing, the two cotyledons emerged first, followed by the first true leaf from the first node. The common leaf type of alfalfa is three leaflets (Figure 2 A). A key finding of this study is the characterization of a multifoliate alfalfa line, where the compound leaves on the main stem exhibited a variety of leaflet numbers, including 4, 5, 6, and 7 leaflets. The five-leaflet type was the most frequent, followed by the seven-leaflet type. We documented several distinct morphologies for these multifoliate leaves; the most common 4 leaf type is the leaflet on both sides of the tri-lobed type, or a leaflet on the left or right side (Figures 2 B); the size is similar to that of the leaflets on both sides, and it grows in the same position as the side leaves, without a petiole; secondly, a leaflet grows on the left or right side of the terminal leaflet and grows on a small petiole (Figures 2 C).

The most common leaf type is one leaflet on each side, which is uniform in size and symmetrical (Figure 2 D); the second most common phenotype is two leaflets on both sides of the terminal leaflet, which are similar in size and symmetrical. However, other types can be observed in the field, as indicated in (Figure 2 E). The 6-leaf type is usually based on the traditional 5-leaf type (Figure 2 F), and a leaflet grows on the left or right side of the terminal leaflet, which is smaller than the other leaflets. The two terminal leaflets have small petioles (Figure 2 G). There are two most common types of the 7-leaf type. One is that there is a leaflet on each side of the proximal leaflet and the terminal leaflet, which is similar in size and symmetrical in position (Figure 2 H); the other is symmetrical near the leaflets on both sides. A pair of leaflets are attached, similar to typical pinnate compound leaves, and the terminal leaflets are symmetrically grown on both sides and have the same small petiole (Figure 2).

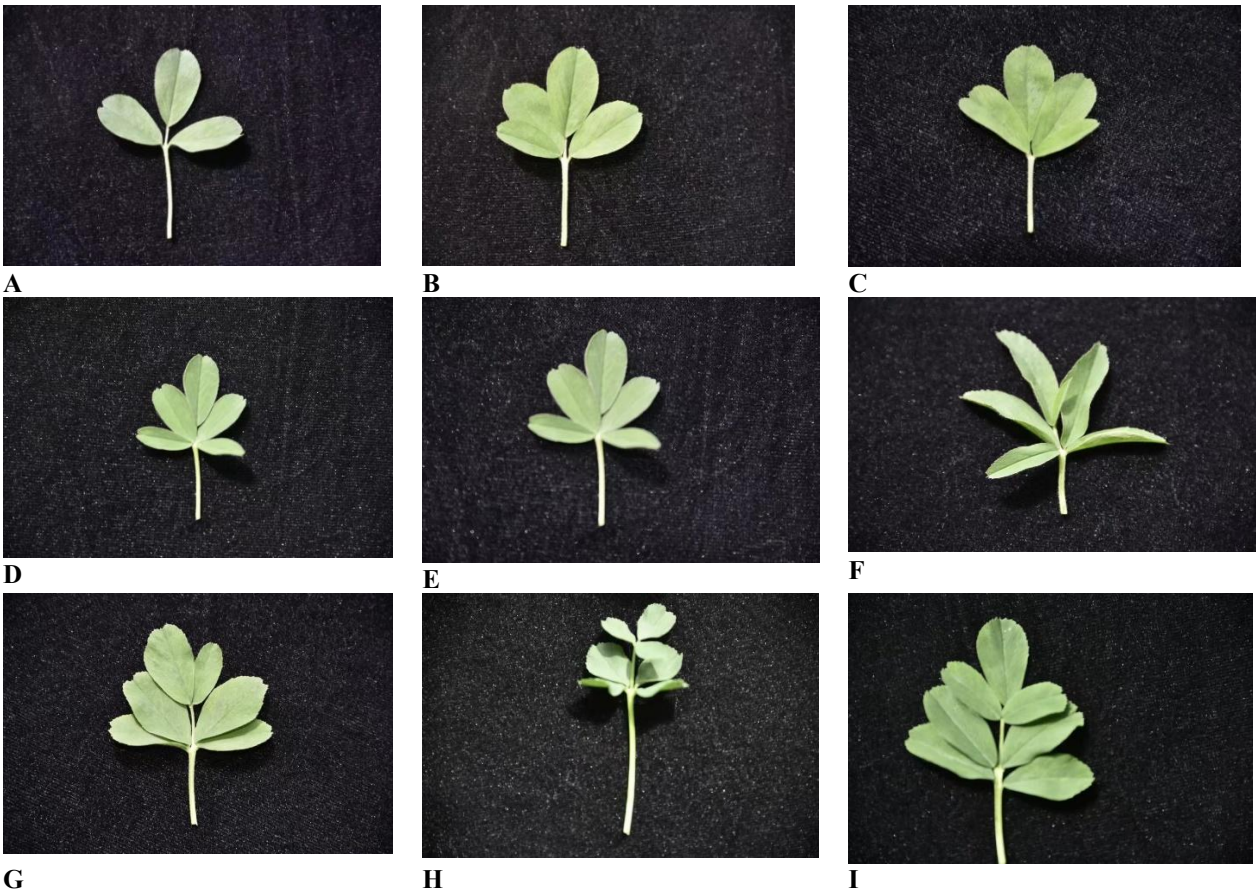

Figure 2. Different leaf type's structure of alfalfa BC_3 germplasm.

Comparative analysis of multifoliate rate between BC_3 progeny and parents: In this study, 147 BC_3 progeny, and their two parents were obtained. Among the 147 individual plants, 14 plants had trifoliate leaves, the remaining 133 plants had multifoliate leaves, and the population multifoliate rate reached 90.48% (Figure 3). Among the plants with multifoliate leaves, the plant with the highest multifoliate rate was plant No. 121, with a multifoliate rate of 88.75%, and the plant with the lowest multifoliate rate was plant No. 126, with a multifoliate rate of 5.35%. The average parent value was 44.65%, and the average multifoliate rate per plant was 48.68%. Among the multifoliate rate, 91 plants were higher than the average of two parental values, accounting for 68.42%, while 42 plants was lower than the average of two parental values, accounting for 31.58%.

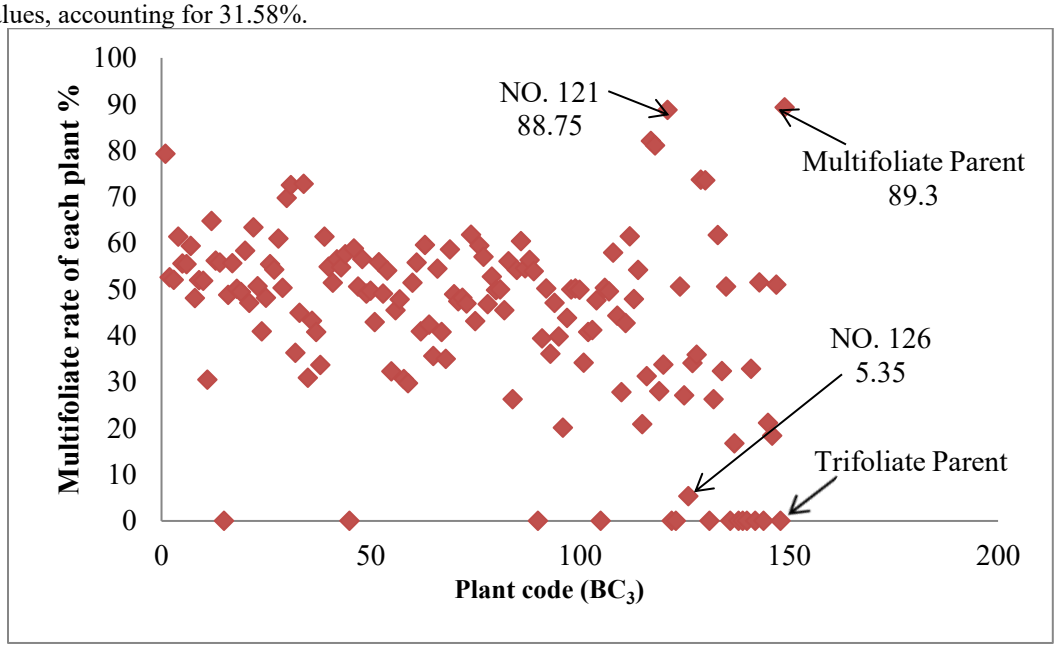

Figure 3. Multifoliate rate of BC_3 progeny and parents.

Study the change in the multifoliate rate of BC_3 progeny in a different season: The study showed that the multifoliate expression of alfalfa was affected by the length of sunshine and temperature, and it was different in four seasons. The expression of multifoliate traits in the BC_3 progeny in four seasons is shown in (Figure 4). The multifoliate rate of the BC_3 population reached 90.48% in spring, 84.93% in autumn, 54.43% in winter, and 50.83% in summer. The multifoliate rate in spring and autumn is higher than in summer and winter.

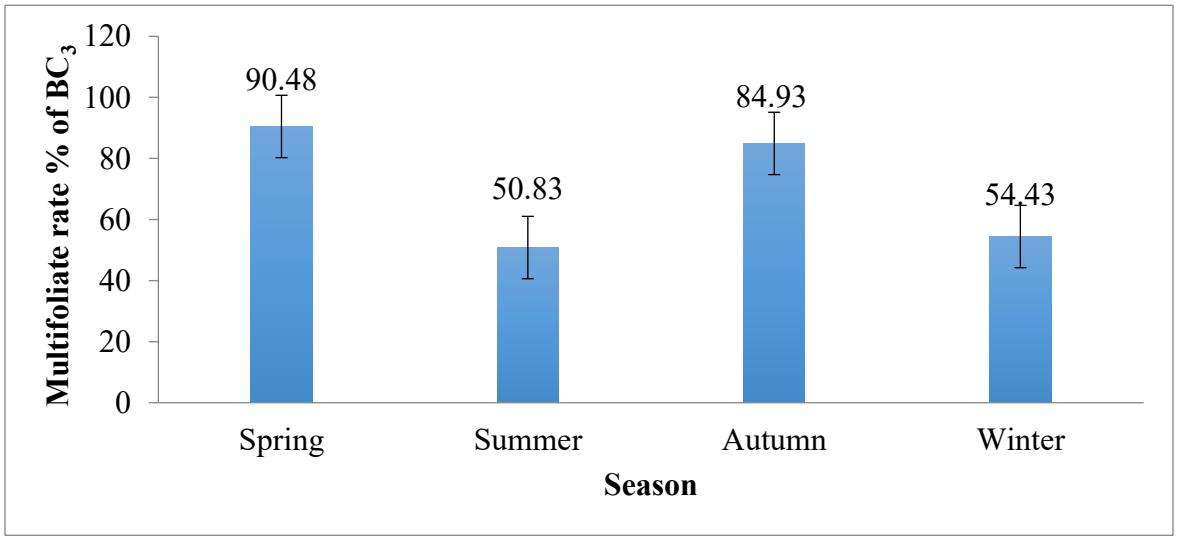

Figure 4. The Change of the multifoliate rate of BC_3 progeny in different season.

Table 1. Information of fifteen Pairs of SSR Markers

Primers	Forward primer	Reverse primer	Ann. Temp. ℃
MES 3	GACAGAGTTGGGTTGTATTC	AGAAGTAGCTGCATAAGATCA	52
MES 22	GTTAAAGCAAGTGATGAACC	CAAAGTTCATACAAGGACACT	52
MES 29	CCAACTCAGCTAACCTATTC	GACATCACCGATCTACTTTC	52
MES 30	ACTCTCCGTTGGAATTGCTG	CGAGGAGGAAGAGGATGTTG	52
MES 37	AGTCATTGGTACTTCCATGT	GACCTTGAAAGCAACATAGT	47
MES 59	CTGAAATCACCGTTCTACTT	CATCATCATCATCGTCATC	50
MES 60	GAACATAAGAAAGCGCAC	ACCAACTGGTTGTTACTGTT	50
MES 64	GGTGAACATAGAAACCTGTC	ATTCTGGATCCTCAACAGTA	47
MT 34	GAAGAAGAAAAAGAGATAGATCTGTGG	GGCAGGAACAGATCCTTGAA	50
MT 66	CTGCAATGCAATGTATTTTCG	TGAACGCAGTAACGTGGAGA	52
W6002	CATATTGTTAGATTTGTGG	GTGAGCGTTAAGTTGGTAGAG	52
W6007	GATTTGGGCCTCATTCCTTCTTGT	CCTGAAGGGGGAAAATTGCCCAC	50
W6018	AGCAGGATTTGGGACAGTTGT	ACCGTAGCTCCCTTTTCCA	50
W6019	TGGAATTTGGGATATAGGAAG	GCCATAAGAACTTCCACTT	50
MTB73	TGGAAGAGACCGGAGAAGAA	TCGAGAGCTCGGTATTCGAT	52

Polymorphism of SSR markers: The electrophoresis gels generated from the fifteen microsatellite loci were analyzed by scoring the allele bands based on their base pair sizes. Across the 15 primer combinations, the number of amplified bands observed in the 147 BC_3 progeny and their two parents ranged from 2 to 7, with an average of 4.53 bands per primer pair. Details of the amplification results for all fifteen primer pairs are presented in Table 1. Among these, the primer MES 3 produced the highest number of amplified bands, whereas primers MES 37 and MES 64 yielded the fewest (Table 2). The overall mean for polymorphism bands was 3.66. The primers MES 37 and MES 64 had the highest percentage of polymorphism loci (100%), followed by MES 3 (85.71%), with a mean of 81.89% polymorphic loci per primer. The polymorphism information content (PIC) ranged from 0.373 (MES 30) to 0.927 (MES 3), with an average PIC value of 0.608. Based on the value of gene diversity, the primers MES 29 and MES 30 had the highest gene diversity.

Population structure of BC_3 progeny: To investigate the population structure of the BC_3 progeny, a Bayesian model-based clustering analysis was performed using STRUCTURE software. The results revealed the presence of three distinct genetic clusters among the 147 BC_3 progeny and their two parents. The optimal number of clusters was determined to be K = 3, based on the analysis of genotypic data (Figure 5). These clusters were visually represented in different colors: Cluster I in red, Cluster II in green, and Cluster III in blue (Figure 6). Through the analysis of Q values for different groups of alfalfa, it was found that 144 genotypes (96.65%) had Q values equal to or greater than 0.5 in a specific group. This suggests that these genotypes have relatively homogeneous genetic components and can be classified into one of the three groups. The Q values of the other 5 individuals (3.35%) in the three groups were all less than 0.5, and there was no clear characteristic of group belonging, forming a mixed group (Figure 7). The three groups each have a different number of individual plants: group I consisted of 51 plants, accounting for 34.23% of the total population; group II had 53 plants, accounting for 35.57% of the total population; and group III had the lowest plants, with 40 plants, accounting for 26.85% of the total population.

Genetic relationships and diversity of BC_3 progeny: The neighbor-joining (NJ) tree was constructed using the DARwin 6.0.021 program to illustrate the genetic relationship among the 147 BC_3 progeny and their two parents (Figure 7). The results were consistent with those from principal coordinate analysis and STRUCTURE.

The neighbor-joining (NJ) tree constructed based on the genetic distances showed 147 BC_3 and their parents in three major groups by STRUCTURE, PCoA, and cluster analysis (Figures 6, 7 and 8). However, some genotypes were divided between the three populations.

The first cluster grouped 92 hybrids, including male and female parents, accounting for 61.75% of the total population. The second cluster contains 55 hybrids, accounting for 36.91% of the total progeny. Cluster three includes two hybrids, accounting for 1.34% of the total variation.

Table 2. Summary of genetic statistics for each locus in the 147 BC_3 progeny and their two parents.

Primer	Amplified bands	Polymorphism bands	Polymorphic loci rate	Gene diversity	PIC Value
MES 3	7	6	85.71%	0.181	0.927
MES 22	6	5	83.33%	0.121	0.715
MES 29	5	4	80.00%	0.369	0.383
MES 30	4	3	75.00%	0.409	0.373
MES 37	2	2	100.00%	0.04	0.725
MES 59	5	4	80.00%	0.201	0.787
MES 60	4	3	75.00%	0.174	0.436
MES 64	2	2	100.00%	0.128	0.519
MT 34	6	5	83.33%	0.262	0.823
MT 66	5	4	80.00%	0.255	0.760
W6002	4	3	75.00%	0.145	0.723
W6007	3	2	66.67%	0.238	0.703
W6018	4	3	75.00%	0.158	0.792
W6019	5	4	80.00%	0.320	0.674
MT B73	6	5	83.33%	0.363	0.741
Mean	4.53	3.66	81.49	0.224	0.672

Principal coordinate analysis and neighbor-joining based clustering: Principal Coordinate Analysis (PCoA) was conducted to further validate the population structure revealed by the STRUCTURE analysis. The first and second principal coordinates accounted for 13.4% and 8.7% of the total genetic variation, respectively (Figure 7). The PCoA results were largely in agreement with the STRUCTURE findings, with the first principal coordinate (PCo1) effectively distinguishing the 147 BC_3 progeny and their two parents into the three groups (I, II, and III) identified by STRUCTURE.

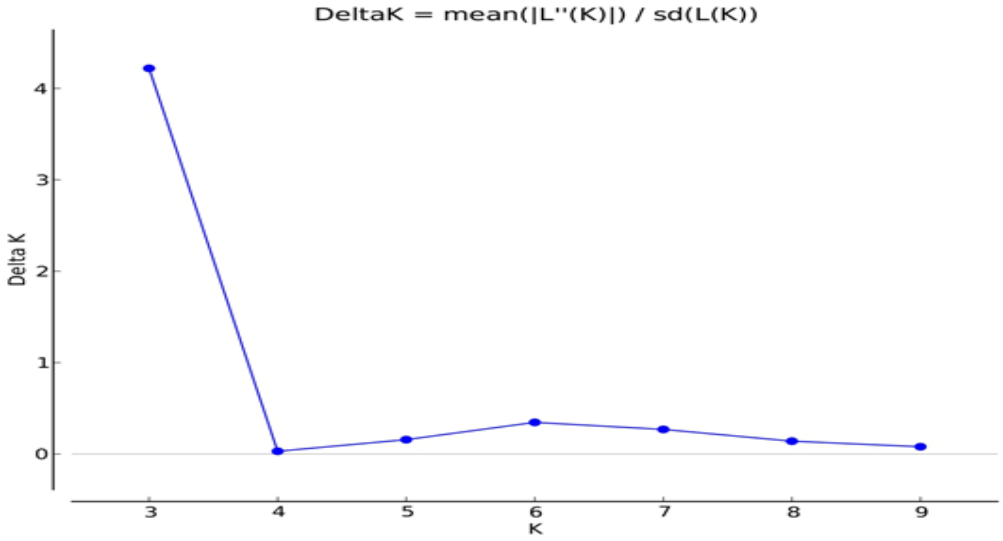

Figure 5. Estimation of populations in 147 BC_3 progeny and their two parents of trifoliate and multifoliate alfalfa using LnP(D) derived delK for K from 1 to 10. The maximum value of delK was considered to be the value of K (subpopulations /groups). Results indicate the optimal partition is K=3.

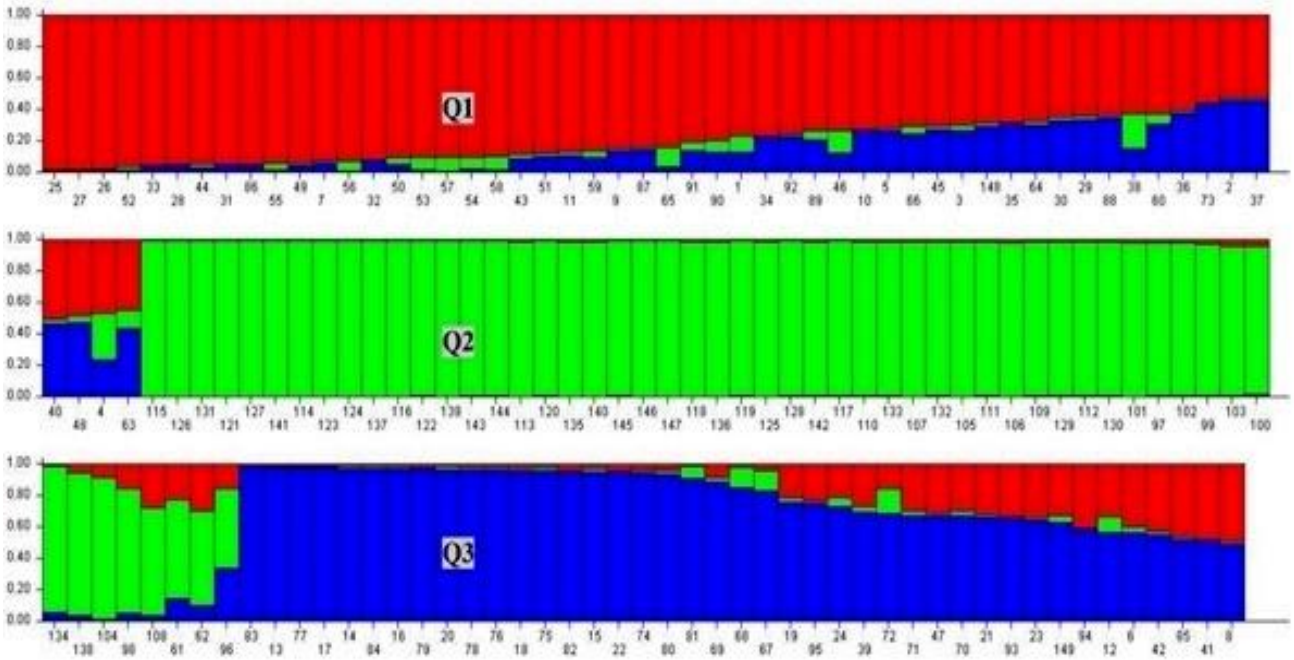

Figure 6. Alfalfa population structure among 147 progeny of BC_3 and their two parents analyzed with 15 SSR markers assuming Three clusters, K = 3: Population Q1 (Red bars, n=51), Population Q2 (Green bars, n=53) and Population Q3 (Blue bars, n=40). X-axis indicates the name of the genotypes and the numbers on the y-axis denote genetic proportion in different groups.

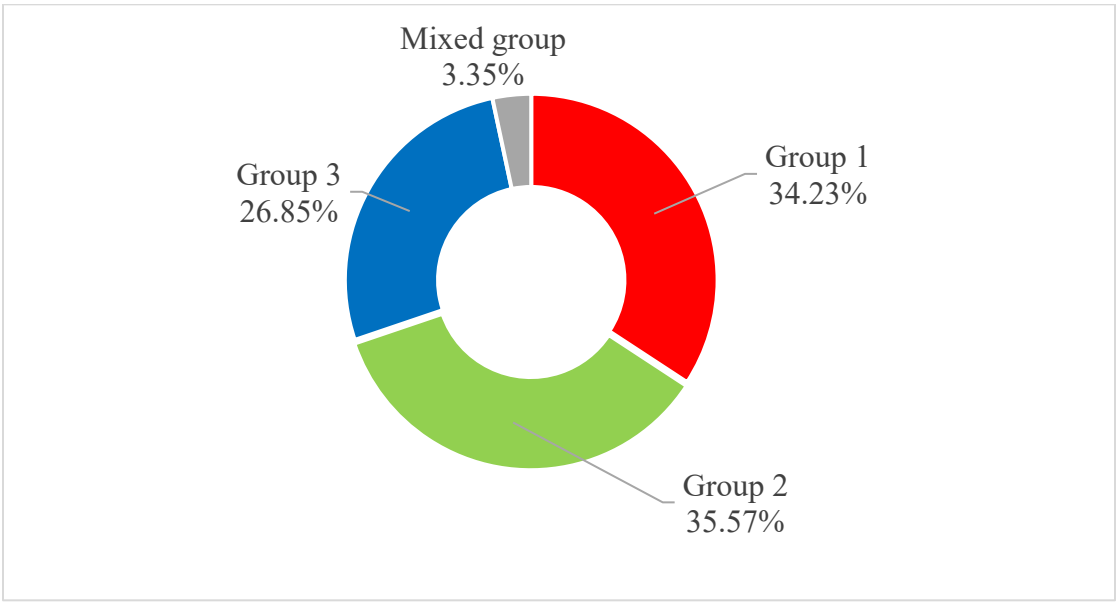

Figure 7. The proportion of each subgroup from the population structure analysis.

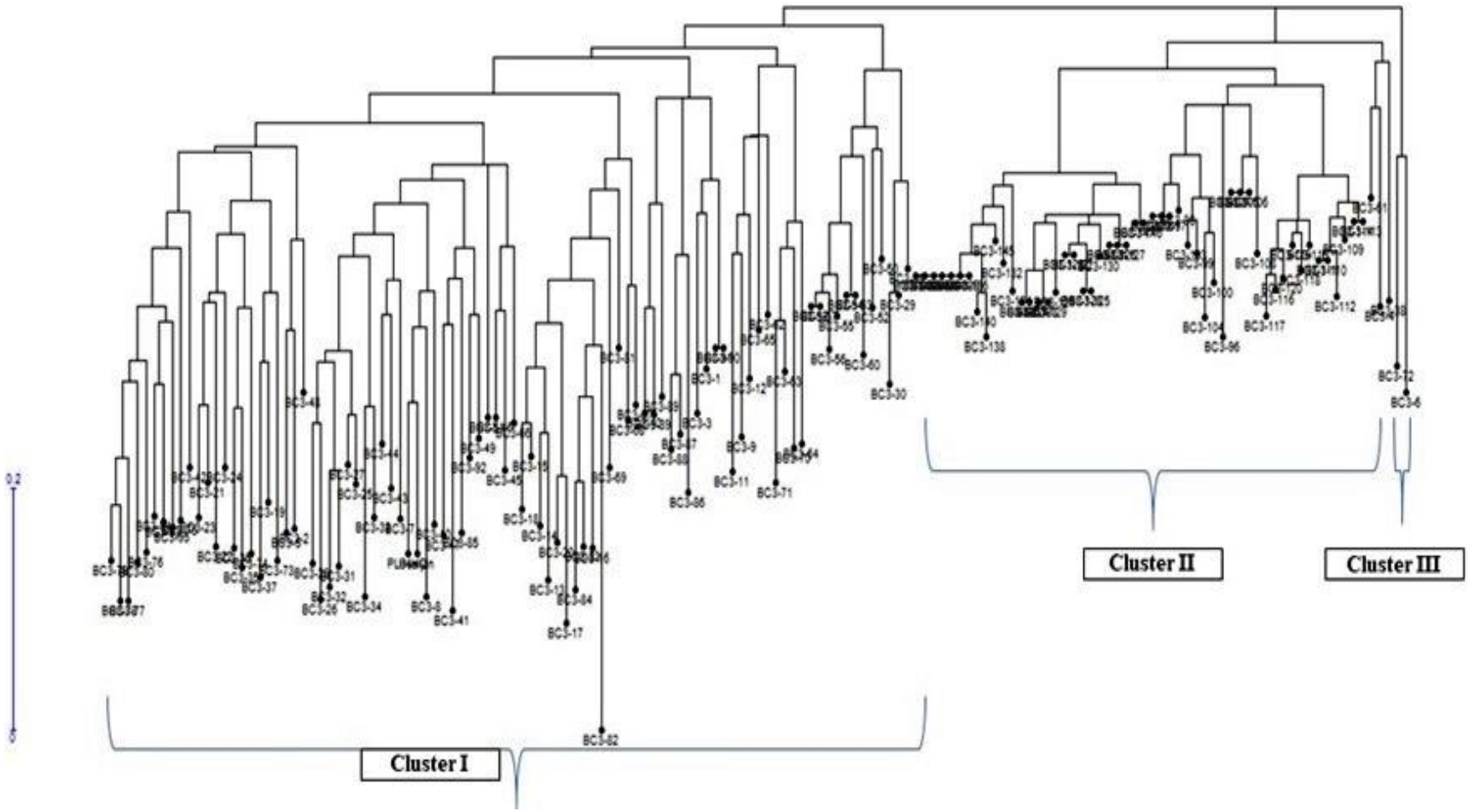

Figure 8. Unweighted Neighbor-joining dendrogram of 147 BC_3 progeny and their two parents of trifoliate and multifoliate alfalfa based on the UPGMA dendrogram generated from eight SSR markers.

Genetic diversity and population differentiation: Genetic diversity and Shannon index analyses were conducted for each of the three identified subpopulations (Table 3). Among them, Group III exhibited the highest genetic diversity (0.2543), whereas Groups I and II showed lower values of 0.2413 and 0.2419, respectively. Similarly, Group III also recorded the highest Shannon index (0.3831), compared to Group I (0.3560) and Group II (0.3644). These results indicate that Group III possesses significantly greater genetic variability than the other two groups. Consequently, Group III may serve as a valuable genetic resource for developing inbred lines with enhanced diversity.

Table 3. Summary statistics of Genetic diversity and Shannon index for model-based groups

Group	Sample size	Gene diversity	Shannon index
I	51	0.2413[b]	0.3560[b]
II	53	0.2419[b]	0.3644[b]
III	40	0.2543[a]	0.3831[a]

DISCUSSION

This study successfully introgressed the multifoliolate trait from the donor parent (PL34HQ) into the genetic background of an elite cultivar (Huaiyin) through a backcrossing program. The high mean multifoliolate rate of 90.48% in the BC_3 progeny is a primary indicator of this successful trait transfer. This rate is comparable to the 91.11% reported for F_1 progeny by (Zhu *et al*., 2015) and significantly higher than the 86% observed in self-pollinated (S_1) populations (Gan *et al*., 2011). This contrast strongly justifies the use of cross-pollination and backcrossing over selfing, as the latter can lead to inbreeding depression and a reduction in the expression of this valuable trait. Our results align with modern breeding strategies that emphasize the use of marker-assisted backcrossing (MABC) to efficiently transfer qualitative traits while minimizing linkage drag, a significant advantage in a complex polyploid like alfalfa (Yunus *et al*., 2025).

The backcrossing strategy was specifically chosen to recover the high-yielding genes of the recurrent parent (Huaiyin) while incorporating the specific multifoliate trait from the donor. The resulting BC_3 progeny exhibited abundant genetic variation in leaf morphology, featuring compound leaves with four, five, six, and seven leaflets. This diversity aligns with the concept that the multifoliate trait arises from variations in the patterning and differentiation of primary leaf primordia, as previously suggested by (Wu and Wei, 2013). Recent studies on simple and compound leaves may develop through fundamentally different mechanisms. KN1 or the KNAT1 gene, either through misexpression in leaf primordia or constitutive expression, is reported to have a role in compound leaf formation (Malaviya *et al*., 2021).The absence of a unified classification standard for these complex leaf forms underscores the importance of our morphological characterization, which provides a crucial foundation for subsequent genetic studies and practical breeding (Zaman *et al*., 2025).

A key finding of our study is the stability of the introgressed multifoliate trait across generations and its dynamic expression in response to seasonal environments. The expression was significantly higher in the optimal growing conditions of spring and autumn compared to summer and winter, a pattern consistent with previous reports (Zhu *et al*., 2015). This demonstrates that, while backcrossing has successfully fixed the trait's genetic potential, its phenotypic expression is still influenced by environmental conditions, particularly temperature (Pathak *et al*., 2015). This genotype-by-environment (G×E) interaction is a critical consideration in the era of climate-resilient breeding (Cooper and Messina, 2023). Recent research highlights that traits like multifoliolate expression can be modulated by temperature-sensitive pathways involving phytohormones, which may offer targets for breeding more stable phenotypes under fluctuating climates (Li *et al*., 2021).

Alfalfa is a cross-pollinated autotetraploid legume with a complex genetic background. Molecular analysis using SSR markers in our BC_3 progeny revealed a total of 68 alleles, with an average gene diversity of 0.224 and a Polymorphism Information Content (PIC) of 0.672. While these values are lower than those reported in studies assessing vast, diverse germplasm collections (Flajoulot *et al*., 2005; Qiang *et al*., 2015), this is expected. High genetic diversity in alfalfa provides the resources needed to identify, preserve, and breed improved varieties (Halbauer *et al*., 2017). Conversely, low diversity reduces resistance to new pests and adaptability to environmental changes (Fu *et al*., 2003). The extent of this diversity is measured by Heterozygosity and PIC values.

Population structure analysis allows for an understanding of genetic diversity in a certain collection as well as the determination of the most relevant population for association mapping (Zhang *et al*., 2010). In this study, population structure analysis clearly divided the BC_3 progeny into three distinct genetic groups, with 96.65% of individuals cleanly assigned. This stratification confirms successful genetic recombination between the parents and demonstrates that the multifoliate traits has been incorporated alongside different genomic backgrounds. The presence of a small admixed

portion (3.35%) is characteristic of alfalfa's outcrossing nature and polysomic inheritance, which can lead to complex segregation (Flajoulot *et al*., 2005). For example, BC_3-4, BC_3-8, BC_3-48, BC_3-63, and BC_3-121 were divided between groups I, II, and III. It may be due to a combination of several factors: reproductive aspects of alfalfa (outcrossing) and the effects of artificial breeding.

The parental lines were clearly separated, with the recurrent parent, Huaiyin (Parent 148), grouping with Q1, and the donor parent, PL34HQ (Parent 149), grouping with Q3. This clear grouping confirms the significant genetic distance between the two founder lines and validates the successful creation of a diverse mapping population. The distribution of the BC_3 hybrids across all three groups indicates a mixture of parental genomes, with individuals in Q1 being genetically closer to Huaiyin, those in Q3 being closer to PL34HQ, and those in Q2 representing admixed individuals with substantial contributions from both parents.

Furthermore, the genetic diversity varied among the groups. Group III exhibited the highest gene diversity (0.2543) and Shannon index (0.3831). These values are notably higher than those reported in studies of natural and cultivated alfalfa populations (Sabokbari *et al*., 2013; Ertus and Sensoy, 2021). This identifies Group III as a particularly valuable genetic reservoir. For breeders, this group represents a pool of genotypes where the high-yielding background of the recurrent parent is successfully combined with the multifoliate traits and a rich source of genetic variation. Selecting from this group will maximize the probability of identifying elite, stable multifoliolate lines for future cultivar development. The low genetic diversity (PIC=0.224) observed in the BC_3 progeny is an expected outcome of the backcrossing process, not a shortcoming. This strategy intentionally reduces genetic variation to recover the recurrent parent's (Huaiyin) genome while incorporating the multifoliolate trait. Therefore, the low PIC value confirms the breeding scheme successfully created a population that is mostly uniform with the high-yielding Huaiyin cultivar. The remaining diversity is mainly around the multifoliolate traits, making the significant variation we found even more useful for selecting superior plants.

Finally, the clustering of genotypes with a high degree of genetic similarity demonstrates the relationship between genetic background and traits expression. Maintaining biodiversity is essential. As our findings show, the local environment shapes genetic resources; thus, conserving such specifically developed breeding populations is as important as preserving wild germplasm for ensuring the genetic resources needed to meet future agricultural challenges.

Conclusion: The multifoliolate trait was successfully stabilized in a cultivated alfalfa background, as shown by its high expression rate (90.48%) in BC_3 progeny. Stability was confirmed across seasons, with peak expression in optimal conditions. Molecular analysis revealed successful genetic recombination and identified a specific subgroup with the highest genetic diversity as the optimal source for future selection of multifoliolate lines. Future research should use this population to validate the trait's agronomic value through field tests and to identify its genetic basis via fine-mapping and cloning.

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