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Full Length Research Paper

Using microsatellite (SSR) and morphological markers to assess the genetic diversity of 12 falcata (*Medicago sativa* spp. *falcata*) populations from Eurasia

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Falcata (*Medicago sativa* spp. *falcata* L.), with its high resistance to cold weather, drought and disease, plays an important role in alfalfa breeding. The aim of this study was to assess the genetic diversity of the 12 falcata populations in Eurasia using SSR markers and morphological traits. Regressions for genetic distance, phenotypic distance and geographic distance were also computed to study whether the origin of these populations influenced their genetic or morphological behavior. A total of 22 SSR alleles were detected in 12 populations, and the average genetic diversity of each population ranged from 0.2517 to 0.4965, indicating substantial variation within populations. Among the 12 populations, three had flowers of colors other than yellow, indicating introgression before seed collection. These three populations grew in a more erect manner and clustered into a sub-group for phenotypic distance. Regression analysis showed no relationship between genetic distance and phenotypic distance. Latitude, longitude and altitude of origin were not correlated with genetic distance among populations, while altitude had a correlation with phenotypic distance among these populations ($p < 0.0001$).

Key words: Genetic diversity, *Medicago sativa* spp. *falcata* L., microsatellite, SSR.

INTRODUCTION

Falcata (*Medicago sativa* spp. *falcata* L.), is a close relative of alfalfa (*M. sativa* spp. *sativa* L.). It grows naturally under more diverse conditions than most perennial *Medicago* species (Hanson, 1972a), especially in hostile environments, with a range of adaptation extending far north into Siberia and the Eurasian region of comparable climate (Hanson, 1972b; Lesins and Lesins, 1979). Exhibiting substantial winter hardiness, drought resistance, and disease resistance, falcata has been an important breeding material that has been used all over the world (Small and Bauchan, 1984). According to Bolton et al. (1972), local endemic ecotypes of falcata played a very important role in enriching the gene pool of common alfalfa. For optimal utilization of the species, it is necessary to research and understand the genetic and morphological variation of falcata and to estimate the relationship of these wild falcata germs to alfalfa.

Morphological characteristics are the strongest determinants of the agronomic value and taxonomic classification of plants. For legumes, growth habit, flower color, leaf shape, pod and seed shape are usually used as indexes to estimate diversity between species. Riday and Brummer (2004) assessed the morphological performance of 125 *Medicago* genotypes, including 105 falcata genotypes derived from 37 Eurasian populations. They found that falcata genotypes from Europe had greater plant width and vegetative density, more erect growth and greater height at harvest than those from Asia.

Compared with other means, morphological evaluations are direct, inexpensive and easy. However, errors can arise; furthermore, morphological estimations are more dependent on environment and are more subjective than other measurements.

Neutral, DNA-based molecular markers allow a more precise and environment-independent way to evaluate the genetic diversity of a particular species. Several studies have been done on alfalfa using DNA-based

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Table 1. Information on the falcata germplasms investigated in this study.

Inventory	Locality	Latitude and longitude (deg)†	Elevation (m)	Improvement Status‡
A0000000	Inner Mongolia	N(42.267)E(118.87)	1000	w
PI325384	Ukraine	N (46.450)E (33.867)	175	w
PI464725	Sivas, Turkey	N(41.01)E(28.58)	1290	w
PI464729	Kars, Turkey	unknown	1830	w
PI491407	Inner Mongolia	N(43.63)E(122.28)	1000	u
PI494660	Cluj, Romania	N(46.767)E(23.600)	unknown	w
PI499548	Jilin, China	N(43.967)E(116.033)	1000	w
PI502445	Kazakhstan	N (43.15)E (76.57)	300-500	w
PI561456	Mongolia	unknown	unknown	c
PI631635	Mongolia	N(47.200)E(108.678)	1448	w
PI631650	Bulgaria	N(41.991)E(24.853)	736	w
W614159	Nepal	N (29)E (84)	2774	w

†N: north; E: east.
‡w: wild material; c: cultivated material; u: improvement status is unknown.

markers like AFLP (Zaccardelli et al., 2003), RAPD (Kidwell et al.,1994a, b; Brouwer et al., 1995; Crochemore et al., 1996, 1998; Mengoni et al., 2000), RFLP (Brouwer et al., 1991,1993; Pupilli et al., 1996; Kidwell et al., 1999) and SSR (Diwan et al., 1997, 2000; Brouwer et al., 1999; Mengoni et al., 2000). Among these markers, SSR is accepted as an effective means of assessing the level of variation among alfalfa populations and describing the relationships among various alfalfa genotypes.

Simple sequence repeats (SSR), also known as microsatellite DNA markers, is polymerase chain reaction (PCR)-based genetic markers. Compared with other markers, SSR has many advantages: highly polymorphic, co-dominant inherited, reproducible and reliable. SSR markers are therefore widely used in studying genetic diversity and the population structure of plants and animals (Haiyan Wang et al., 2007; Cheng Yang et al., 2008; Solomon et al., 2008).

Previous work on falcata genetics primarily served to create a species against which to compare alfalfa. Kidwell et al. (1994a) evaluated the genetic diversity of nine *Medicago* accessions representing the original germplasm sources for North American alfalfa cultivars. They included one falcata accession in their study and separated falcata from alfalfa accessions successfully using RFLP markers. However, they studied only one falcata cultivar and did not declare any relationship among falcata populations.

In the present study, we examined 12 falcata populations from Eurasia with seven SSR markers and seven morphological traits. The objectives of the current study were to estimate the genetic diversity within and among falcata populations coming from Eurasia, and to compare morphological diversity and genetic diversity among these populations.

MATERIALS AND METHODS

Plant material and field conditions

We investigated 12 diploid falcata populations from eight countries in Eurasia; the information collected is shown in Table 1. All germplasms except A0000000 were obtained from the U. S. department of agriculture; A0000000 was collected from the Keshiketeng county of China. Each population was represented by 40 plants. Seeds were grown in a greenhouse and seedlings were transplanted three months later to the experiment station in Hebei province (Guyuan State Key Monitoring and Research Station of Grassland Ecosystem), on May 9, 2005.

The experimental design was a randomized complete block design with four blocks. Plant materials in each block were planted at intervals of 1 m to allow for creeping branches. Blocks were weeded by hand and irrigation was available. The lowest and average temperatures in the Guyuan were about -30 and -12°C, respectively, during winter, and the average temperature over the course of a year was about 3°C. The highest and lowest temperatures appeared in July and January, respectively. Rainfall was about 400 mm annually and was mainly concentrated in July (>100 mm).

Field evaluation and temporal analysis

24 plants randomly selected from each of the 12 populations were evaluated for the following six morphological characteristics:

1.) Plant height, including the natural height (without stretching) and absolute height (after stretching).
2.) Plant width (the mean of the largest width and the vertical width).
3.) Decumbent coefficient, showing the degree of creeping for each plant (denoted by the ratio of natural height to width)
4.) Leaf shape, represented by the ratio of leaf length to leaf width.
5.) Leaf area, the product of leaf length and leaf width (leaf shape and area were recorded from three leaflets of the trifoliate leaves positioned on the median inter-node of the longest stem)
6.) Flower color (yellow, varied or purple).

Field data were collected twice in 2006 and twice in 2007, when 1/3 of plants were flowering. Statistical analysis was performed by ANOVA using the GLM procedure of the SAS program; a Duncan

test was also performed.
Euclidean phenotypic distance (PD) values were calculated among populations using the formula

$$PD = \sum_{k=1}^{7} (M_{ik} - M_{jk})^2,$$

where M_{ik} and M_{jk} are means for the k character of populations i and j (k ranged from 1 to 7, representing the seven morphological characters detected in the experiment). Population means were standardized to μ = 0 and σ = 1 before calculation of PD (Casler, 2004). The un-weighted pair group method utilized arithmetic average (UPGMA) dendrograms, drawn using the SAHN clustering of NTSYSpc 2.10, based on PDs.

We also performed regression and correlation analysis on PD and geographical differences using the SAS8.0 program. Geographical factors including latitude, longitude and altitude of the populations' origin were examined. Geographic distance was calculated by the formula

$$GED = \sum_{k=1}^{3} (M_{ik} - M_{jk}),$$

where M_{ik} and M_{jk} are data for the k factor of populations i and j (k ranged from 1 to 3, representing the latitude, longitude and altitude of the place of origin). Data were standardized and subtraction was used to calculate the difference between populations for each factor.

SSR analysis

Young leaves were collected from 25 plants of each population; total genomic DNA was extracted from individual plants according to the cetyltri-methylammonium bromide (CTAB) technique of Mengoni et al. (2000). Plant material was ground in liquid nitrogen, resuspended in 750 µl of CTAB 2× buffer and incubated at 65°C for 15 min. After two chloroform-isoamylic (24:1) extractions, the aqueous phase was collected and the nucleic acid was precipitated with isopropanol and 3 M sodium acetate, then washed with 75% ethanol and resuspended in 1×TE buffer after being air-dried.

Seven SSR primer pairs were selected from the study of *M. truncatula* (Table 2) (Julier et al., 2003; Flajoulot et al., 2005). PCR was performed for a 20 µl total volume containing 1×buffer, 0.2 mM dNTP, 0.4 µM SSR primers, 1.5 mM $MgCl_2$, 2 µl genomic DNA and 0.6 units of *Taq* polymerase. Reactions were performed in a Biometra gradient thermocycler programmed for an initial melting at 94°C for 4 min followed by 35 cycles at 94°C for 30 s, at melting temperature (Tm) for 1 min, a 72°C for 1 min, and then a final extension step at 72°C for 10 min. PCR products (5 µl/lane) were denatured at 105°C for 8 min, separated in 6% polyacrylamide denaturing gels at 300 v constant power for 4 - 5 h, and then visualized with silver staining protocol (Sandrine et al., 2005).

Data analysis

The bands visualized on the gels were identified to determine whether they were a single allele or just replicates of alleles to be discarded (Sandrine et al., 2005). Within-population genetic diversity was estimated by the number of alleles (A), the Shannon Index and the expected heterozygosities (H_E). Pair-wise population differentiation among populations was estimated and tested according to Weir and Cockerham (1984). Genetic distance (GD) between populations was calculated as done by Nei (1972).

DNA data were analyzed using the POPGENE software package (Yeh et al., 1997). Un-weighted pair group method using arithmetic average (UPGMA) dendrograms were drawn using the SAHN clustering of NTSYSpc 2.10, based on GDs. Regression and correlation analysis were carried out to describe the relationship between genetic distance and geographical differences or morphological distance using SAS8.0.

RESULTS

Morphological diversity

Plants from PI561456 and PI631635 produced flowers were with varied colors, those from PI464725 produced purple flowers, indicating that these 3 populations were misidentified (Table 3).

Plant height and creeping habit divided the populations into several groups. Those plants from PI561456, PI631635 and PI464725 with purple flowers had much larger decumbent coefficients (0.71, 0.69, and 0.67, respectively) than those with yellow flowers (the largest decumbent coefficient was 0.59 [W614159]), meaning that the first group grew in a more erectly.

Leaf shape differentiated PI464729, PI502445, PI494660 and PI325384 from the others. Plants from these first three populations had longer and narrower leaves than those from other populations, while leaves from PI325384 were wider and shorter than those of other plants.

Absolute height was measured by the length of the longest branch. While the longest value was recorded for W614159 at 50.55 cm and the shortest value was recorded for PI502445 at 31.24 cm, most between-population differences were not significant for plant width or decumbent coefficient (Table 3). Correlation analysis between these three parameters showed that there was a close correlation between absolute height and plant width ($p < 0.05$).

The phenotypic distance among populations ranged from 1.146 to 37.566, averaging 14.000. The largest phenotypic distance among all populations was between PI464725 and PI464729, while both of them came from Turkey. Therefore, there was no relationship between morphological characteristics and the origin of these falcata populations.

Regression and correlation analysis between phenotypic distance and geographic difference showed similar results. Neither latitude nor longitude had a linear relationship with phenotypic distance ($p > 0.05$). However, plant height had a relationship with longitude and altitude ($p < 0.05$) and phenotypic distance had a close relationship with altitude ($p < 0.0001$). Data were clustered according to phenotypic distance (Figure 1).

Polymorphisms of SSR loci

In this study, genetic diversity was examined at seven SSR loci, and the percentage of polymorphic loci was

Table 2. The list of loci used in this study, the expected heterozygosity (H_E) for each locus in each population; and the mean gene diversity (MGD) calculated by H_E for each population.

Locus	A0000000	PI325384	PI491407	PI561456	PI502445	PI631635	PI464725	PI464729	PI494660	PI499548	W614159	PI631650
MTIC250	0.3911	0.4978	0.4911	0.4800	0.4978	0.4800	0.1244	0.4978	0.4974	0.4444	0.4911	0.2449
MTIC251	0.5044	0.3541	0.4178	0.4378	0.2911	0.1800	0.4911	0.1244	0.2551	0.5244	0.0644	0.4911
MTIC332	0.6200	0.5867	0.4978	0.6822	0.6289	0.6956	0.4244	0.5844	0.5408	0.7000	0.4978	0.5578
B14B03	0.4200	0.4911	0.4800	0.2311	0.2311	0.4644	0.3750	0.4233	0.4770	0.4200	0.2778	0.3457
AFCT45	0.3378	0.2778	0.5400	0.4378	0.2911	0.5578	0.0644	0.0644	0.5944	0.2778	0.2778	0.1244
MTIC183	0.5267	0.5933	0.5511	0.5244	0.4733	0.5956	0.3200	0.4911	0.4628	0.3911	0.4444	0.4911
MTIC103	0.4200	0.3200	0.4978	0.4800	0.2778	0.4978	0.2164	0.1782	0.3750	0.3911	0.2778	0.2311
MGD	0.4600	0.3952	0.4965	0.4676	0.3844	0.4959	0.2570	0.2517	0.4575	0.4498	0.3330	0.3552

Table 3. Morphological characteristics of the 12 falcata populations studied*†.

Inventory	Plant height		Plant width (cm)‡	Decumbent coefficient§	Leaf shape¶	Leaf area (mm^2) #	Flower color††
	Natural height (cm)	Absolute height (cm)					
A0000000	22.73±0.921cd	47.49±1.877abc	67.82±4.339a	0.31±0.017c	2.14±0.043fg	86.75±2.569c	-1c
PI325384	17.75±0.804d	32.39±2.202cd	49.64±2.835abcd	0.33±0.025c	1.86±0.044h	90.52±3.102c	-1c
PI464725	35.23±1.881ab	46.23±1.835abcd	54.95±4.751abcd	0.67±0.108ab	2.03±0.031gh	121.53±2.752a	1a
PI464729	22.01±1.772cd	38.50±2.111abcd	66.33±3.837ab	0.33±0.036c	3.23±0.072a	58.96±1.296d	-1c
PI491407	23.14±1.992cd	40.24±6.267abcd	51.97±6.868abcd	0.42±0.035bc	2.38±0.047def	84.40±3.406c	-1c
PI494660	18.63±1.008d	39.29±2.342abcd	59.22±3.786abcd	0.30±0.026c	2.61±0.061c	63.40±2.599d	-1c
PI499548	33.67±1.529ab	49.30±2.414ab	61.38±4.331abc	0.52±0.040bc	2.45±0.082cd	121.09±4.628a	-1c
PI502445	20.46±3.872cd	31.24±3.912d	39.25±6.198d	0.54±0.130abc	2.81±0.100b	87.01±4.968c	-1c
PI561456	30.79±2.972b	34.13±3.502cd	38.75±3.275d	0.76±0.076a	2.32±0.058def	87.97±3.164c	0b
PI631635	28.54±2.979bc	34.93±2.427bcd	45.97±3.600cd	0.62±0.070ab	2.18±0.049efg	104.06±6.530b	0b
PI631650	18.87±1.633d	32.40±2.650cd	41.47±2.877cd	0.43±0.023bc	2.41±0.055cde	32.35±1.471e	-1c
W614159	39.21±1.702a	50.55±2.599a	61.28±2.743abc	0.59±0.031ab	2.33±0.049def	108.28±3.582b	-1c

*Date represent mean values ± SE. Means followed by the same letter are not significantly different at p=0.05 within every measured character.
†Data were measured twice in 2006 and 2007 when 1/3 plants were in flower. Means with the same letter are not significantly different.
‡Plant width is the mean of the largest width and the vertical width.
§Decumbent coefficient was calculated by the ratio of vegetable height and largest width.
¶Leaf shape was calculated by the leaf length and leaf width.
#Leaf area was the product of the leaf length and leaf width.
††Flower color, -1: yellow, 0: cream, 1: purple.

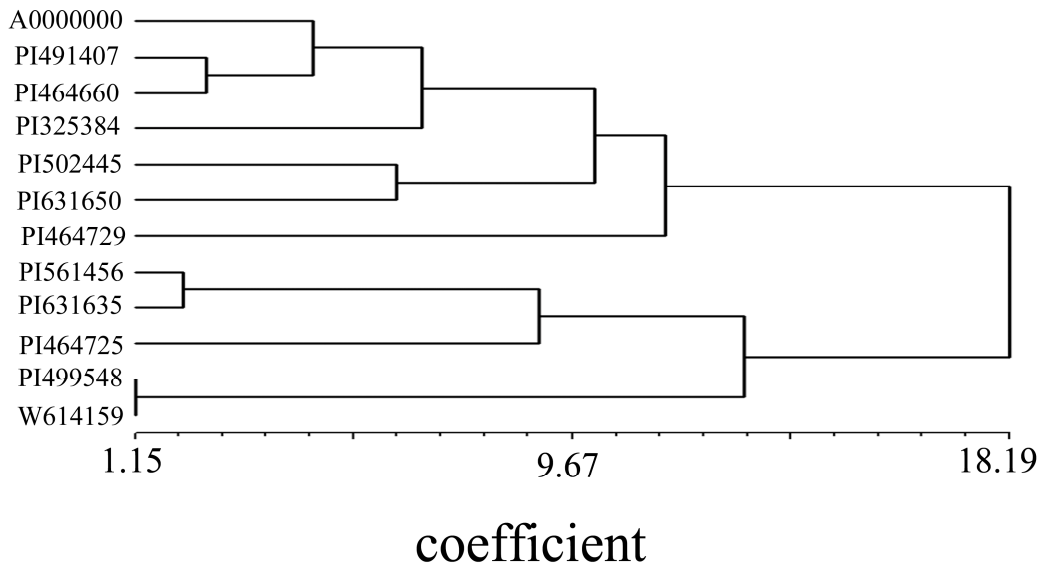

Figure 1. Cluster tree showing the relationships among 12 populations, based on morphological distance.

found to be 100%. Three SSR loci had polymorphisms in population 12, three in population 11, and one in population 10, indicating that these markers were suitable for diversity research on falcata.

Genetic diversity of falcata populations

The extent of polymorphism present in 12 populations of falcata was analyzed in 25 individuals from each population. Allele doses from seven microsatellite loci were counted; a total of 22 alleles were detected in the 12 falcata populations, with sizes ranging from 110 to 220 bp. The number of alleles per locus ranged from two for MTIC 250, B14B03, and MTIC103, to five for MTIC183. Table 2 shows the expected heterozygosity (H_E) and the mean gene diversity (MGD) among each population for each marker; the MGD ranged from 0.4965 to 0.2517 for PI491407 and PI464729, respectively. The Shannon Index (SI) is also a measure of genetic diversity. Although the SI values were higher than H_E values, these two indexes showed a high correlation ($r^2 = 0.78$, $p < 0.0001$). The F-statistic showed that population variance contributed 16.30% of total variation; within-population variation represented most of the total variation.

Genetic relationships among populations

The average genetic distance (GD) of the populations ranged from 0.1004 for PI561456 to 0.1772 for PI499548, with highest genetic distance detected between PI464725 and PI631635. To elucidate the genetic relationships among falcata populations, a dendrogram was produced using Nei's genetic distances over seven SSR loci (Figure 2). The populations clustered into two main groups, with population PI499548 comprising a singular group.

Regression and correlation analysis between genetic distance and phenotypic distance showed no significant correlation ($p > 0.05$). But PI499548 was differentiated from the others by both genetic and phenotypic data (Figures 1 and 2).

DISCUSSION

In this study, we investigated the morphological diversity and genetic diversity of falcata populations. To our knowledge, this is the first report on the morphological and genetic diversity of falcata populations. The main aim of our study was to evaluate the genetic diversity of falcata populations and to compare morphological distance with genetic distance.

Among seven morphological traits studied, absolute height was the least effective in distinguishing the falcata populations. We examined absolute height because it was one of the components that determines forage yield. However, in our study, the difference among populations was not significant, possibly because we measured the longest branch of the plant rather than all branches. The yield parameter is defined by the collective contribution of all branches and may distinguish falcata populations.

In previous studies, natural height was often used as a distinguishing trait of alfalfa or other plants (Riday and Brummer, 2004; Dias et al., 2007), especially in describing heterosis. In our study, natural plant height ranged from 17.75 to 39.21, with broad variability that would

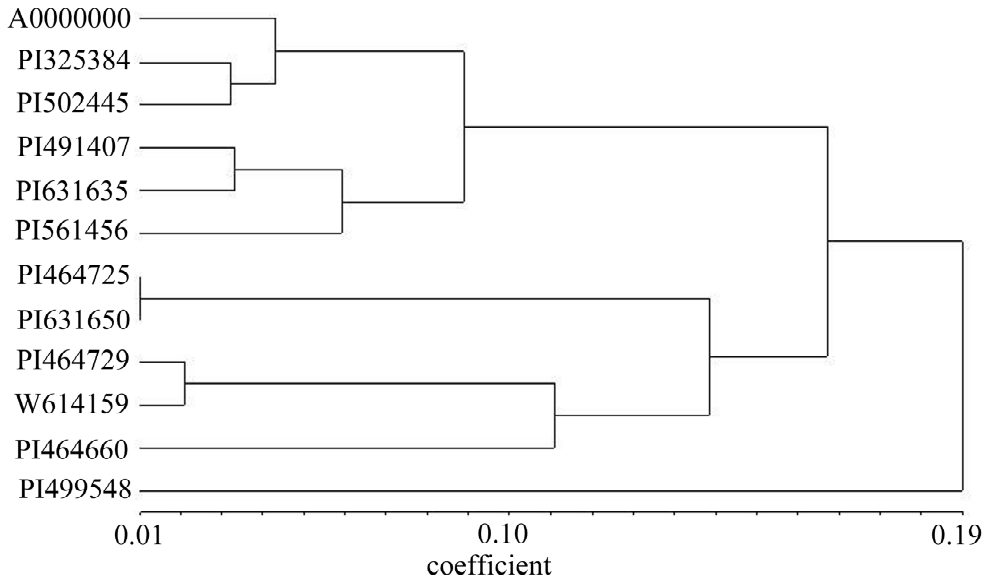

Figure 2. UPGMA tree showing the relationship among 12 populations, based on Nei's unbiased genetic distance calculated on 7 SSRs.

facilitate improving plant height by selecting taller plants for breeding.

In our study, three populations which had been identified as falcata were proved to be other species. PI561456 and PI631635 were variegated and PI464725 was alfalfa. These three populations exhibited more erect growth habit than falcata populations. This result confirms previous observations regarding plant morphology among *M. sativa* groups. According to phenotypic distance, the two variegated populations were clustered into the subgroup closest to the subgroup with alfalfa. The two variegated populations were closer molecularly, either.

The mean genetic diversity for each population, represented by $H_{E,}$ ranged from 0.2517 to 0.4965 (Table 2). These values were much lower than those in alfalfa cultivars (Flajoulot et al., 2005) because the falcata populations used in our study were diploid (we detected this before). Unexpectedly, the cultivated population PI561456 had higher genetic diversity than most wild populations (Table 2); however, this phenomenon has been documented previously (Dias et al., 2007).

We performed regression analysis between the morphological diversity and genetic diversity of 12 falcata populations. In agreement with Crochemore's (1998) work, phenotypic distance and genetic distance did not define the same pattern of population clustering. This may have resulted from the random selection of DNA markers and the environmental dependence of morphological traits, as well as uncertainty regarding whether the alleles correlated with the phenotypic characteristics studied. Therefore we could not determine whether or not the SSR markers were related to phenotype. To estimate the relationship or genetic diversity of populations more accurately, more DNA loci should be examined.

This study is the first in the published literature to document a close relationship between phenotypic distance and altitude of origin ($p < 0.0001$). This phenomenon could be explained by the influence of altitude on the environment in terms of temperature, rainfall and ultraviolet radiation, all of which affect plant growth.

In contrast to work by Cazacarro et al. (2000), we did not find a correlation between phenotypic or genetic distance and origin latitude. This may be due to the fact that the populations in our study came from a narrow latitude range (from northern latitude 29° to northern latitude 47.2°, mostly north of 41°).

In conclusion, the study confirmed that genetic and morphological diversity work in different ways to determine the relationships among populations. To effectively exploit germplasms, we should utilize both methods in breeding work.

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