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REVIEW

Genetic diversity and relationship of global faba bean (*Vicia faba* L.) germplasm revealed by ISSR markers

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Abstract Genetic diversity and relationships of 802 faba bean (*Vicia faba* L.) landraces and varieties from different geographical locations of China and abroad were examined using ISSR markers. A total of 212 repeatable amplified bands were generated with 11 ISSR primers, of which 209 were polymorphic. Accessions from North China showed highest genetic diversity, while accessions from central China showed low level of diversity. Chinese spring faba bean germplasm was clearly separated from Chinese winter faba bean, based on principal component analysis and UPGMA clustering analysis. Winter accessions from Zhejiang (East China), Jiangxi (East China), Sichuan (Southwest China) and Guizhou (Southwest China) were quite distinct to that from other provinces in China. Great differentiation between Chinese accessions and those from rest of the world was shown with a UPGMA dendrogram. AMOVA analyses demonstrated large variation and differentiation within and among groups of accessions from China. As a continental geographic group, accessions from Europe were genetically closer to those from North Africa. Based on ISSR data, grouping results of accessions from Asia, Europe and Africa were obviously associated with their geographical origin. The overall results indicated that the genetic relationship of faba bean germplasm was closely associated with their geographical origin and their ecological habit.

Keywords Faba bean · ISSR · Genetic diversity · Gene pool · Landraces · Varieties

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Introduction

Faba bean (*Vicia faba* L.) is a major food and feed legume for its high seed yield, high seed protein content and high biomass. Its critical role in crop rotation, effective nitrogen fixation, soil improvement abilities has long been recognized (Ye et al. 2003). Faba bean is a partially allogamous plant with fewer chromosome number ($2n = 2x = 12$) than other species in genus *Vicia* L. (Raina and Ogihara 1995). However, the nuclear genome of faba bean is remarkably large (~13,000 Mb, Bennett and Smith 1982; Johnston et al. 1999). This large genome size poses a serious challenge to marker-assisted genetic studies on faba bean.

There are approximately 38,360 faba bean germplasm conserved by 37 collections in the world, the largest world collection is at the International Center for Agricultural Research in the Dry Areas (ICARDA) Syria. ICARDA has collected more than 9,000 accessions, followed by CAAS in China, with more than 5,200 accessions (Duc et al. 2010). China is the largest faba bean producer in both sowing area and production globally. There are two main production regions in China, for spring and for winter faba bean. The winter ecotype faba bean is sown in October and

November and harvested in March–May of the following year, in the area between 21°N and 35°N latitude in China; while the spring ecotype faba bean is sown in March–April and harvested between June and September (Ye et al. 2003) in the area between 31°N and 53°N latitude.

Despite numerous studies, the geographic origin of faba bean and its evolution is still debated. The morphologically most similar *Vicia* species (*V. narbonensis*, *V. galilea*, *V. johannis*) do not cross with faba bean (Jose and Suso 1981), and the wild progenitor of faba bean remains unknown. Cubero (1974) supposed that the center of origin of faba bean was the Near East, with four different routes radiating from the Near East: (1) via the Mediterranean regions to the rest of Europe; (2) along the Mediterranean coast to the Magreb and the Iberian Peninsula; (3) following the Nile Delta through to Abyssinia; (4) from Mesopotamia eastwards to India. Although Ladizinsky (1975) reported that the geographic origin of faba bean was in the central Asia, recent archeological excavation in Israel and northwest Syria has indicated that southwest Asia is the major center of faba bean diversity (Duc et al. 2010). The timing of the introduction of faba bean to China is uncertain. However, the faba bean archaeological site in the Guanghe county of Gansu province in northern China and grain fossils found in Wuxi county of Zhejiang province demonstrated that faba bean was introduced to China about 4,000–5,000 years ago (Zheng et al. 1997; Zong et al. 2009a, 2010).

The taxonomic classification of faba bean is various and contentious. Muratova (1931) defined two subspecies according to the seeds characters: *paucijuga* and *eu-faba*, the *eu-faba* having three variants: *minor* Beck, *equina* pers and *faba* (*Major* Harz). Hanelt (1972) has proposed a new taxonomy for faba bean according to different shapes, weights and sizes of seeds and pods. He recognized two subspecies: *minor* (the oldest one) and *faba*, the latter having two variants, *faba* and *equina.* In China, faba bean is divided into three types according to seed size: large seeded varieties with 100-seeds weights greater than 120 g, medium seeded varieties (100-seeds weights of 70–120 g), and small seeded varieties with 100 seeded weights less than 70 g. Traditionally, large seeded varieties are grown in spring sowing region for human consumption (Ye et al. 2003), while varieties of different seed size produced in autumn sowing region are mainly used for food, feed and vegetable.

It is problematical if taxonomic classification is only based on morphological and agronomic traits since these traits are either affected by stage of plant development and environment factors or they reveal only limited variation (Terzopoulos and Bebeli 2008). Recently, various molecular markers have been successfully used to characterize genetic diversity in faba bean accessions. Link et al. (1995) examined three groups of faba bean inbred lines from European and Mediterranean by RAPDs assays. Winter ecotype (Zong et al. 2009a) and spring ecotype (Zong et al. 2010) faba bean germplasm from China were separately compared with accessions from the rest of the world using AFLP. Kwon et al. (2010) used TRAP markers to assess the genetic diversity and relationship among faba bean germplasm entries. Recently, Terzopoulos and Bebeli (2008) analyzed large diversity for morpho-agronomic traits in Mediterranean landraces using ISSR primers.

The previous studies did not combine different ecotypes of faba bean germplasm grown in China with those from the rest of the world. The main goal of this study was to compare genetic relationship of winter ecotype faba bean accessions with spring ecotype faba bean germplasm from China, and analyze the genetic diversity of global faba bean germplasm as a whole, to evaluate their genetic relationship and population structure, in order to provide essential information for effective evaluation and utilization of faba bean genetic resources.

Materials and methods

A total of 802 faba bean accessions consisted of 538 Chinese accessions and 264 accessions from outside China. Chinese accessions comprised 195 spring ecotype faba bean accessions from 5 provinces of northwestern China and 3 provinces of northern China, 343 winter ecotype accessions from 2 provinces of central China, 4 provinces of eastern China and 3 provinces of southwestern China, respectively. Other accessions selected from the rest of the world included 91 Asian accessions (except China) from 10 countries, 103 European accessions from 14 countries, and 70 African accessions from 6 countries. The seeds were obtained from the National Genebank of China, Institute of Crop Science, Chinese Academy of Agricultural Sciences, Beijing, China (Table 1).

Genomic DNA was extracted from pooled eight randomly young seedlings of each accession which directly issued from collected seeds, using the CTAB method (Dellaporta et al. 1983; Doyle and Doyle 1990) with minor modification (Liu et al. 2007). ISSR-PCR amplifications were performed in 20 µl reaction volumes of 20–50 ng of genomic template DNA, 2 µl of 10× Taq buffer (Zhexing, Beijing, China), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM primer, and 0.5 U of *Taq* DNA polymerase. PCR reactions were performed on a Heijingang Thermal Cycler (Eastwin, Beijing, China), under the following conditions: initial denaturation at 95°C for 5 min, followed by 35 cycle of 95°C for 30 s, annealing at optimal temperature for 1 min, and 72°C for 1 min, with a final 10 min elongation step at 72°C. Fragments were separated on 6% denaturing polyacrylamide gels in 10× TBE buffer and visualized by silver nitrate staining.

Table 1 Geographic origin of 802 faba bean accessions from worldwide geographical origins

Origin	Sample	Origin (number of accessions)	Latitude of province of China	Longitude of province of China
Spring ecotype (Northwest China)	122	Xinjiang (17)	34°25′–48°10′	73°40′–96°18′
		Gansu (38)	32°31′–42°57′	92°13′–108°46′
		Qinghai (24)	31°39′–36°12′	89°45′–102°23′
		Ningxia (14)	35°14′–39°23′	104°17′–107°39′
		Shaanxi (29)	31°42′ –39°35′	105°29′ –111°15′
Spring ecotype	73	Hebei (13)	36°01′–42°37′	113°04′–119°53′
(North China)		Shanxi (31)	34°34′–40°43′	110°14′–114°33′
		Inner Mongolia (29)	37°20′–53°20′	97°10′–126°29′
Winter ecotype (Central China)	77	Hubei (42)	29°05′–33°20′	108°21′–116°07′
		Hunan (35)	24°37′–29°55′	108°56′–114°09′
Winter ecotype (East China)	138	Anhui (51)	29°41′–34°38′	114°54′–119°37′
		Jiangsu (35)	30°45′–35°20′	116°18′–121°57′
		Jiangxi (17)	24°31′–29°58′	114°02′–118°28′
		Zhejiang (35)	27°12′–31°30′	118°04′–122.25°
Winter ecotype (Southwest China)	128	Guizhou (35)	24°37′–29°13′	103°36′–109°35′
		Sichuan (46)	26°03′–34°19′	97°21′–108°31′
		Yunnan (47)	21°09′–29°15′	97°31′–106°12′
Asia	91	Japan (7), Afghanistan (14), India (2), Iran (9), Iraq (13), Yemen (1), Jordan (1), Syria (17), Lebanon (25), Cyprus (2)		
Europe	103	Sweden (3), England (14), France (19), Holland (10), Germany (7), Spain (25), Portugal(4), Greece (1), Turkey (14), Poland (1), Hungary (2), Bulgaria (1), Yugoslavia (1), Russia (1)		
Africa	70	Morocco (5), Algeria (11), Tunis (12), Egypt (14), Sultan (15), Ethiopia (13)		

The genetic diversity parameters, genetic distance and cluster analysis of faba bean accessions from different geographical groups were carried out by POPGEN1.32 (Yeh and Boyle 1997). Polymorphism information content (PIC) of each primer pair were calculated by using the following formulas: $PIC = \Sigma(1 - pi^2)/n$, where pi is the frequency of the i th allele, n is the total number of genotypes (Weir 1990). The cluster analysis of different geographical groups was carried out using unweighted pair-group method with arithmetic average (UPGMA), and the dendrogram was drawn by MEGA3.1 (Tamura et al. 2007). Analysis of molecular variance (AMOVA) was used to assess the variance among and within populations from different geographical origin with GenAlEx 6.4 software (Peakall and Smouse 2006). Principle components analysis (PCA) was applied to show the distribution of individual accessions in scatter diagram and 2-dimension PCA graph was drawn using the NTSYS-PC 2.2 statistical package (Rohlf 2006).

Results

ISSR polymorphism

A total of 100 ISSR primers were obtained from the public biotechnology website of University of British Columbia. These primers were screened in a preliminary experiment with 8 accessions from different countries, of which 11 primers amplified the unambiguous, polymorphic and reproducible bands (Table 2). The ISSR analysis using the 11 primers produced a total number of 212 unambiguous and repeatable bands, of which 209 were polymorphic, with an average of 19 polymorphic fragments per primer. The scored fragment size ranged from 300 to 2,000 bp. Percentage of polymorphic bands ranged from 100% to a minimum of 91% with an average of 93%. Polymorphism information content of each primer ranged from 0.0017 to 0.0161 with an average of 0.0103. In general, dinucleotide repeats

Table 2 Amplification result and polymorphism of the 11 ISSR primers used in this study

Primer	Sequence (5′→3′)	Annealing temperature (°C)	No. of bands	No. of polymorphic bands	Percentage of polymorphic bands (%)	Polymorphism information content
807	$(AG)_8T$	50	17	17	100	0.0123
808	$(AG)_8C$	52	20	20	100	0.0122
809	$(GA)_8G$	52	28	28	100	0.0073
810	$(GA)_8T$	50	17	17	100	0.0161
811	$(GA)_8C$	52	17	17	100	0.0148
812	$(GA)_8A$	50	13	13	100	0.0071
842	$(GA)_8YG$	54	29	29	100	0.0151
857	$(AC)_8YG$	54	24	24	100	0.0148
885	$BHB(GA)_7$	56	5	5	100	0.0068
886	$VDV(TC)_7$	52	20	19	95	0.0057
890	$VHV(GT)_7$	52	22	20	91	0.0017
Average			19	19	98	0.0103

B = (C,G,T) (i.e. not A), D = (A,G,T) (i.e. not C), H = (A,C,T) (i.e. not G); V = (A,C,G)

Table 3 Analysis of genetic diversity for 8 groups of faba bean germplasm resources from worldwide geographical origins

Geography origin	Sample size	Polymorphic loci	Effective number of alleles	Shannon's information index	Nei's gene diversity
Northwestern China	122	168	1.4067	0.3719	0.2437
Northern China	73	181	1.4313	0.3991	0.2610
Central China	77	129	1.2994	0.2768	0.1806
Eastern China	138	166	1.3888	0.3621	0.2362
Southwestern China	128	162	1.4029	0.3701	0.2439
Asia (except China)	91	159	1.4111	0.3703	0.2447
Europe	103	164	1.4303	0.3874	0.2568
Africa	70	134	1.3401	0.3171	0.2083

(AG)8 and (GA)8 anchored with A, T, C or G, showed clearer patterns and best polymorphism.

Genetic diversity and classification analysis among populations within and outside China

The genetic diversity parameters of different populations were summarized in Table 3. Gene diversity index and Shannon's information index of different geographic groups of germplasm ranged from 0.1806 to 0.2610 and from 0.2768 to 0.3991, respectively. The Northern China group showed the highest genetic diversity while the germplasm from central China showed the lowest level of diversity. The Europe, Asia, Northwestern China and Southwestern China groups had similar genetic diversity parameters which indicated that those groups had higher diversity. Accessions from Africa showed a low level of diversity.

A cluster analysis was carried out based on the UPGMA method using POPGEN1.32 (Yeh and Boyle 1997), and a dendrogram was generated (Fig. 1). In the dendrogram, all the faba bean accessions were distinctly separated into three major groups. Group I includes accessions from northwestern and northern China, where the spring ecotype faba bean is sown at the beginning of spring season; group II consists of accessions from central, eastern, and southwestern China, where the winter ecotype faba bean is sown in autumn; and group III comprise accessions from outside China. The spring ecotype faba bean germplasm was clearly separated from winter ecotype faba bean germplasm of China in UPGMA clustering analysis based on ISSR molecular marker data. Germplasm from China is quite distinct to that from exotic accessions. Accessions from Europe had a closer genetic relationship with that from North Africa.

Variance among and within populations from different geographical origin

The results of AMOVA analyses indicated that the majority of the genetic variation in faba bean was due to within population variation (Table 4). There was a highly significant difference between Chinese populations and other populations from the rest of the world. Chinese winter ecotype populations showed the highest genetic differentiation among populations ($\Phi_{PT} = 0.34$). The largest variation

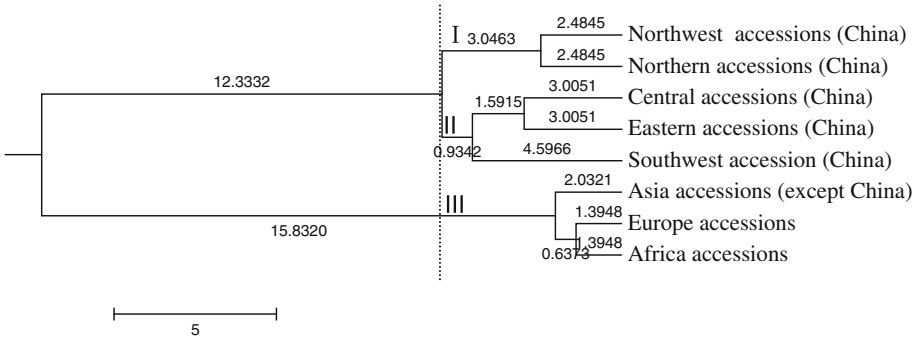


Fig. 1 UPGMA dendrogram of 8 groups of faba bean germplasm from worldwide geographical origins

Table 4 Analysis of molecular variance (AMOVA) of faba bean germplasm on the basis of ISSR data for different groups

Groups	Subgroups	Samples size	No. subgroups	V_{AP}	V_{WP}	Φ_{PT}	*p* value*
All faba bean accessions	Worldwide	802	8	10.09	21.79	0.32	0.0001
Chinese accessions	Spring vs. winter ecotypes	538	2	5.68	25.29	0.18	0.0001
Spring ecotype, China	Province	195	8	6.14	19.96	0.24	0.0001
Winter ecotype, China	Province	343	9	9.08	17.27	0.34	0.0001
Asia	Country	91	9	2.57	20.05	0.11	0.0001
Europe	Country	103	10	2.94	20.2	0.13	0.0001
Africa	Country	70	6	2.23	17.91	0.11	0.0001

V_{AP} variance among populations, V_{WP} variance within populations, Φ_{PT} is calculated as the proportion of the variance among populations, relative to the total variance, $\Phi_{PT} = V_{AP}/(V_{AP} + V_{WP})$

* Probability value *p* calculated by 1,000 random permutations of individual across populations. *P* is calculated as the number of values ≥ observed value (including observed value) ÷ (number of permutations + 1)

within populations ($V_{WP} = 25.29$) were detected in spring and winter ecotype accessions from China.

Genetic differentiation among different ecotypes of faba bean germplasm in China

The clustering analysis of groups from different provinces of China were carried out by POPGEN1.32 (Yeh and Boyle 1997) based on the UPGMA method, and the dendrogram was drawn by MEGA3.1 (Tamura et al. 2007). The results of clustering analysis divided all the faba bean accessions into four major groups (Fig. 2). One group included all the spring ecotype faba bean accessions which are mainly distributed in northwestern and northern China. The second group comprised mostly winter ecotype faba bean accessions. The third group consisted of winter ecotype faba bean germplasm from Zhejiang and Jiangxi provinces in eastern China. The fourth group contained winter ecotype faba bean germplasm from Guizhou and Sichuan provinces in southwestern China. The accessions from different ecotypes of faba bean grown in different regions of China were closely related to their geographical distribution and growth habit. The spring ecotype faba bean germplasm was clearly separated from winter ecotype faba bean germplasm, while winter ecotype germplasm from Zhejiang, Jiangxi, Sichuan and Guizhou is quite distinct to that from other provinces. Principle components analysis and 2-dimension PCA graph was drawn using the NTSYS-PC 2.2 statistical package (Rohlf 2006). The PCA graph (Fig. 3) showed the greatest differentiation between spring ecotype faba bean germplasm and winter ecotype faba bean germplasm in China. Accessions from Zhejiang and Sichuan are clearly separated from other germplasm in China. The results of PCA graph were consistent with the dendrogram. The results indicated that the genetic relationship of faba bean in China is closely associated with their growth habit, geographical origin, and ecological distribution.

Classification and PCA analysis of accessions from outside of China

The genetic distances and cluster analysis of different groups from worldwide countries (except China) were carried out by POPGEN1.32 (Yeh and Boyle 1997). The dendrogram (Fig. 4) separated 19 groups into 4 major clusters. The results showed that accessions from Asia, Europe, and Africa clustered together, respectively. The dendrogram indicated that the accessions from Japan had the furthest relationship with other Asian germplasm while the closest relationship was between Egyptian and Sultanese

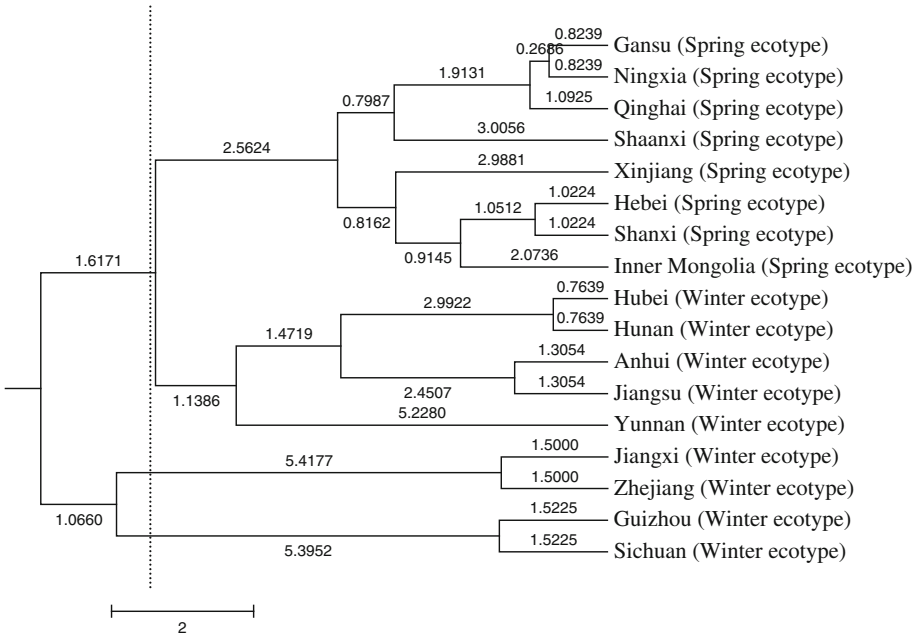


Fig. 2 UPGMA dendrogram of 18 groups of faba bean germplasm from different provinces in China

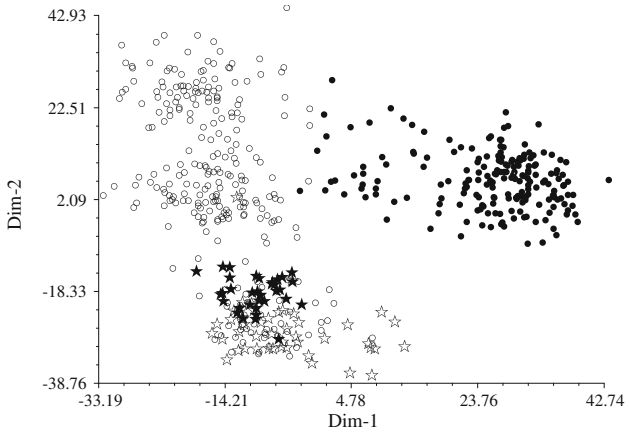


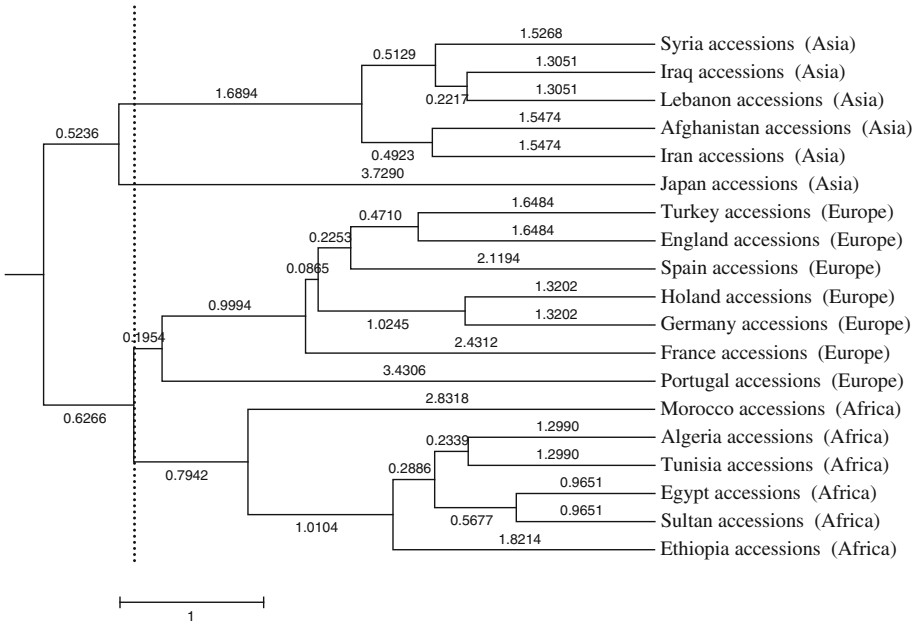
Fig. 3 Two-dimension principal components analysis of faba bean accessions from China. *Closed circle* spring-sown accessions, China; *open circles* winter-sown accessions, China (except Sichuan and Zhejiang); *closed star* Zhejiang accessions, *open star* Sichuan accessions

accessions which both from North Africa. The Portuguese accessions had the largest genetic distance from other European accessions, as well as Dutch accessions showed closer genetic relationship with German accessions. In general, accessions from Europe had a closer genetic relationship with that from Africa. The results indicated the germplasm resources from Asia, Europe and Africa are closely related to their geographical origin. The genetic relationship of individual accessions was analyzed using principle components analysis (PCA); all the accessions were labeled according to their geographical origin. The PCA graph (Fig. 5) showed that accessions from Asia, Europe, and Africa were largely associated with their geographical origins. It is clear that accessions from Asia, Europe and Africa were quite distinct from each other.

Discussion

The genetic diversity and relationship of a large number accessions (802) from different faba bean ecotype regions grown in China and from the rest of the world were analyzed in this study. The Northern China and the European groups had the greatest genetic diversity and largest number of effective alleles, despite a small sample size for the Northern group. Asian, Northwestern China and Southwestern China groups showed intermediate genetic diversity, while the Central China and African groups had low level of diversity. The Northern China accessions were sourced from three provinces (Inner Mongolia, Hebei and Shanxi), which have a wide geographic range with altitudes from 800 to 1,600 m in the faba bean growing area. These regions have a wide fluctuation in weather conditions which may contribute to selection for genetic distinctness and differentiation of faba bean gene pool.

Chinese faba bean landraces have been compared in diversity with accessions from the rest of the world using AFLPs and the results showed that the spring faba bean accessions were generally associated ecologically their genetic diversity, and Chinese accessions showed a similar genetic diversity as Asian and European sources both in Nei's gene diversity and number of polymorphic bands, but genetic diversity was less for accessions from Africa, America and the ICARDA breeding program (Zong et al. 2010). Results from ISSR marker data presented in this



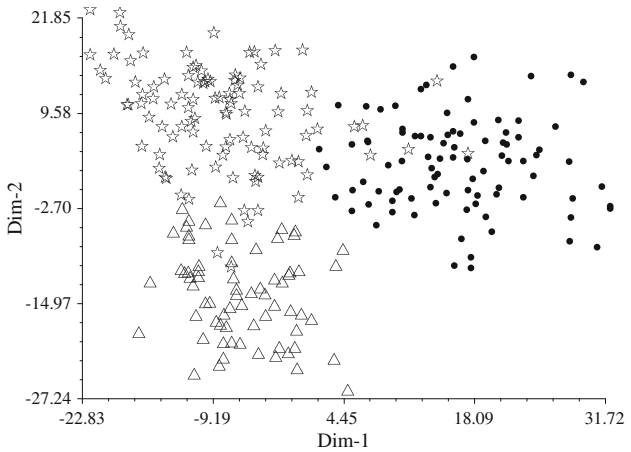


Fig. 4 UPGMA dendrogram of 19 groups of faba bean germplasm from outside China

Fig. 5 Two-dimension principal components analysis of faba bean accessions from outside China. *Closed circle* Asia accessions (except China), *open star* Europe accessions, *open triangle* Africa accessions

study revealed significant differentiation among germplasm from different provinces in China and the rest of the world. AMOVA analyses demonstrated large variation and differentiation among populations from China (Table 4). We may reasonably concluded that China is another secondary center of diversity for faba bean genetic resources, as well as having been reproductively isolated from the African, European and Asian gene pools.

The spring ecotype faba bean germplasm was clearly separated from winter ecotype faba bean germplasm in China, based on principal component analysis and UPGMA clustering analysis in this study. The number of spring landraces from China also was larger in this study (195) than the 39 in a previous AFLP study of Chinese landraces (Zong et al. 2009a). In the current study, the spring germplasm was found to be more widely separated from the rest of the world than previously when spring landraces from some provinces were clustered with breeding lines from ICARDA, or with landraces from Asia or from Europe. In particular, the landraces from Qinghai were heterogeneous in their cluster associations in Zong et al. (2009a, 2010), and two appeared likely to be introductions from ICARDA. This study with a larger sample of Chinese spring landraces broadly indicates separation from the rest of the world and confirms their separation from winter landraces in China.

The winter germplasm groups from Zhejiang–Jiangxi provinces in eastern China, Sichuan–Guizhou and Yunnan provinces in southwestern China are quite distinct from those of both the other winter faba bean and spring faba bean provinces of China. In this paper, there is broad agreement with previous AFLP studies of winter and of spring faba bean from China (Zong et al. 2009a, 2010), although the sample of winter landraces from China was larger in this study and with 47 from Yunnan (vs. 14 in Zong et al. 2009a) and the Yunnan accessions were found to differ from other provinces but not to be unique as suggested in the previous study. The Chinese winter ecotype germplasm was clearly separated from the rest of the world in principal components analysis and clustering analysis (Zong et al. 2009a). Both Chinese winter ecotype and spring ecotype faba bean accessions are clearly distinguished from the rest of the world.

The genetic distinctness and differentiation of gene pools from different regions within cultivated species may be associated with reproductive isolation, due to wide geographic separation, as well as to non-overlapping ecological adaptation (Zong et al. 2009a, b; Rajaram 1999). Polignano

et al. (1999) described the extent and patterns of phenotypic diversity of the Bari faba bean germplasm and found that phenotypic variation was closely associated with geographical origin. Genetic diversity of faba bean accessions from Ethiopia and Afghanistan was assessed base on eight quantitative characters; differences among origins were highly significant for plant height and yield characters, with an obvious association between phenotypic differences and geographic origin (Polignano et al. 1993). Robertson and El-Sherbeeny (1991) described significant variation of pure line faba bean germplasm collection from eight regional groups, and many important regional variations were detected. Diverse morphological trait combinations were associated with diverse regional origins, and it was also the case for some abiotic or biotic stress tolerance traits (Duc et al. 2010). These morphological data demonstrated great differentiation of faba bean germplasm from different regions. Results from the molecular data in this study confirmed that the distinct differentiation of faba bean genotypes was obviously associated with their geographic origin. Therefore, reproductive isolation and divergent natural selection arising from wide geographic separation may be one of major reason for the differentiation of faba bean gene pools from different regions.

The dendrogram (Fig. 5) demonstrated that Asian groups were convincingly separated from all the other groups, and that accessions from Europe had a closer genetic relationship with that from North Africa. Wide crosses between materials from the Asian germplasm and that from European may have a great potential for further cultivar improvement and hybrid-breeding programs. Zeid et al. (2003) assessed the genetic diversity of 79 elite faba bean cultivars in Asia, Europe (Northern and Southern) and North African origin, using AFLP primers. Based on clustering with Jaccard's similarity coefficient and principal coordinate analysis, Asian lines were distinct as a group, South European and North African groups clustered together. It is believed that the Near or Middle East was the center of origin of faba bean, the spread of faba bean took place towards Central and Northwestern Europe through North Africa, and the spread of faba bean to China may have been on repeated occasions with winter faba bean possibly preceding the introduction of spring faba beans (Zong et al. 2009a, 2010). Some similarity is also detected between European and North African germplasm in molecular data which confirmed the spread routes proposed by Cubero (1974).

These results indicated that the genetic relationship and diversity of worldwide faba bean germplasm are closely associated with their growth habit, geographical origin, and ecological distribution. However, a wider investigation of morphological and agronomical characters is needed to confirm the distinct differentiation of gene pools between China and the rest of the world.

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