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Full Length Research Paper

Genetic diversity in three morphological types of *Astragalus membranaceus* (Fisch.) Bge. var. *mongholicus* (Bge.) Hsiao as revealed by inter simple sequence repeat markers

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Inter simple sequence repeat (ISSR) markers were used to detect the genetic diversity in three morphological types of cultivated *Astragalus membranaceus* Bge. var. *mongholicus* (Bge.) Hsiao discovered in Gansu province of China. Eight primers used for analysis generated 165 scorable bands of which 162 (98.18%) were polymorphic. The percentage polymorphic bands (PPB), Shannon's information index (I) and Nei's gene diversity (h) for 3 types ranged from 59.39%, 0.2912, 0.1901 to 78.18%, 0.3411, 0.2238 respectively, indicating each type had a high genetic diversity. Analysis of molecular variance (AMOVA) showed 90.41% of the total genetic diversity existed within the 3 morphological types, whereas only 9.59% occurred among them. The UPGMA tree based on SM similarity coefficients implied that the 3 types had genetically differentiated though not obviously. The high genetic diversity suggested that each type had a potential to develop new strains or cultivars and ISSR was a potentially useful tool in the process of selective breeding of *A. membranaceus* var. *mongholicus*.

Key words: *Astragalus membranaceus* var. *mongholicus*, morphological type, genetic diversity, ISSR, Radix astragali.

INTRODUCTION

Astragalus membranaceus Bge. var. *mongholicus* (Bge.) Hsiao, a perennial herbaceous plant, is defined as one of the genuine botanical sources of Radix astragali ("Huangqi" in Chinese) in the Chinese Pharmacopoeia. As a commonly used traditional Chinese medicine, Radix astragali has the functions of reinforcing "qi" (vital energy), of strengthening the superficial resistance, of inducing diuresis and of promoting the discharge of pus and the growth of new tissue (Pharmacopoeia Commission of People's Republic of China, 2005). In China, the wild resources of Radix astragali are gradually decreasing due to its exhaustive exploitation and hence the commercial supply of it is mainly taken from farming sources, especially from cultured *A. membranaceus* var. *mongholicus* (Wang and Xie, 2004; Ma et al., 2000; Feng and Xiao, 2002).

According to our investigation into *A. membranaceus* var. *mongholicus* in Longxi county, Gansu province, there exist 3 morphological types (R type, RG type and G type) in the cultivated population (Xie et al., unpublished data). R type possesses red dapples on both stems and pods more or less, while G type has completely green stems and pods without any flammulation. For RG type, the stems are same as the G type ones while the pods are

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Abbreviations: AMOVA, analysis of molecular variance; **CTAB,** cetyltrimethylammonium bromide; **ISSR,** inter simple sequence repeat; **PCR,** polymerase chain reaction; **UPGMA,** unweighed pair-group method using arithmetic average.

Table 1. ISSR primers used for PCR amplification of *A. membranaceus* var. *mongholicus,* together with optimum Mg^{2+} concentration and annealing temperature for each primer.

Primer	Sequence of the primer(5' - 3')*	Mg^{2+} (mM)	Annealing temperature (℃)
UBC809	AGA GAG AGA GAG AGA GG	2.10	56.2
UBC836	AGA GAG AGA GAG AGA GYA	1.95	50.0
UBC841	GAG AGA GAG AGA GAG AYC	2.10	51.0
UBC842	GAG AGA GAG AGA GAG AYG	1.95	53.4
UBC856	ACA CAC ACA CAC ACA CYA	1.80	48.3
UBC861	ACC ACC ACC ACC ACC ACC	1.80	59.4
UBC868	GAA GAA GAA GAA GAA GAA	1.95	47.4
UBC891	HVH TGT GTG TGT GTG TG	2.10	54.2

*Y = (C,T); H = (A,C,T), that is, not G; V = (A,C,G), that is, not T.

same as the R type ones. The morphological diversity as well as the results of our previous studies (Xie et al., 2004, 2007) showed the cultivated population is very heterogeneous, which has resulted in a rapid decline of the yield and quality of Radix astragali in this region. On the other hand, the different morphological types may be useful genetic resources for the breeding program of *A. membranaceus* var. *mongholicus*. The objective of the present study was to provide genetic references for Radix astragali selection and breeding by analyzing the genetic diversity in the 3 morphological types using ISSR markers.

MATERIALS AND METHODS

Plant material and DNA extraction

A total of 32 individuals comprised of 16 plants of R type, 6 plants of RG type and 10 plants of G type were sampled from Longxi county for this study. Fresh leaves of per plant were desiccated with silica gel in a plastic zip-lock bag and stored at -20℃ until DNA isolation.

Total DNA was extracted by a cetyltrimethylammonium bromide (CTAB) protocol of Tian et al. (2004) with minor modifications. Dried leaf materials were rapidly ground with about 0.025 g PVP powder and a little quartz sand and mixed with 900 ul preheated 2 × CTAB extraction buffer [100 mM Tris-HCl (pH 8.0), 1.4 M NaCl, 20 mM EDTA, 2%(w/v) CTAB] containing 0.1% (v/v) β-mercaptoethanol in a 2.0 ml centrifuge tube. The mixture was extracted twice with an equal volume of chloroform:isoamyl alcohol (24:1,v/v) followed by centrifugation at 10,000 g for 10 min. The resulting supernatant was mixed with 2/3 volumes of ice-cold isopropanol and kept at -20℃ for 2 h. The total DNA, recovered by centrifugation at 10,000 g for 6 min, was washed twice with 70% ethanol, air dried and finally re-suspended in 150 ul sterilized double distilled water. The extracted DNA was quantified with a λDNA/*Hind*III marker in 0.8% agarose gel.

ISSR-PCR amplification

38 ISSR primers of the UBC set #9 (Sangon, Shanghai, China) were screened with three DNA samples. Reaction volumes were 20 ul and consisted of 2.8 ul 10 × PCR buffer (100 mM Tris-HCl, pH 8.3 and 500 mM KCl), 1.8 - 2.1 mM $MgCl_2$ (Table 1), 325 uM dNTPs (Sangon, Shanghai, China), 1U *Taq* DNA polymerase (TaKaRa Inc., Dalian, China), 0.3 uM primer and 0.84 ng DNA template. All the amplifications were carried out in a Dyad Disciple™ Peltier Thermal Cycler (Bio-RAD, USA) with the following program: an initial denaturation step at 94℃ for 5 min; followed by 40 cycles of 45 s at 94℃, 60 s at a specific annealing temperature depending on a specific primer (Table 1), 90 s extension at 72℃ and a final 7 min at 72℃. After resolved by electrophoresis in 1.5% agarose gel in 1 × TAE buffer, amplification products were stained with ethidium bromide (0.5 ug/ml) and photographed under UV light. Molecular weights were estimated with a 200 bp DNA ladder marker (TaKaRa Inc., Dalian, China).

Data analysis

The ISSR amplification products were scored as "1" for presence and "0" for absence across the analyzed samples. The binary data matrix, thus obtained was used for further analyses. Genetic diversity parameters including the percentage of polymorphic bands (PPB), Shannon's information index (I), Nei's (1973) gene diversity (h) and effective number of alleles (ne) were calculated with POPGENE version 1.31 (Yeh et al., 1999). Analysis of molecular variance (AMOVA) was made as described by Excoffier et al. (1992) to examine variability among and within types and the significance of variance components was tested by 1000 random permutations. Cluster analysis of the individuals was performed with the unweighed pair-group method using arithmetic average (UPG MA) method based on SM similarity coefficients using NTSYSpc version 2.0 (Rohlf, 1998).

RESULTS AND DISCUSSION

Eight primes that produced clear, reproducible and polymorphic bands were selected for PCR analysis (Table 1). A total of 165 bands ranging from 270 to 1650 bp in size, were generated with an average of 20.63 bands per primer, of which 162 (98.18%) were polymorphic among 32 plants with a mean of 20.25 bands. The percentage of polymorphic bands of each primer varied from 94.74% (primers UBC841 and UBC861) to100% (UBC809, UBC836, UBC842 and UBC856) (Table 2).

Of the 3 morphological types studied, R type produced

Table 2. Number of amplification products generated with 8 ISSR primers from 32 individuals of three morphological types of *A. membranaceus* var. *mongholicus*.

Primer	Total number of bands	Number of polymorphic bands	Percentage of polymorphic bands (%)	Bands size (bp)
UBC809	24	24	100	380-1600
UBC836	25	25	100	350-1250
UBC841	19	18	94.74	270-1600
UBC842	20	20	100	300-1500
UBC856	16	16	100	520-1550
UBC861	19	18	94.74	420-1600
UBC868	22	22	100	370-1650
UBC891	20	19	95	380-1400
Total	165	162		
Average	20.63	20.25		

Table 3. Genetic diversity parameters for three morphological types (R type, RG type and G type) of *A. membranaceus* var. *mongholicus*.

Type	ne*(±SD)	h**(±SD)	I***(±SD)	Number of polymorphic bands	Percentage of polymorphic bands (%)
R	1.3514 ± 0.3428	0.2155 ± 0.1793	0.3358 ± 0.2485	129	78.18
RG	1.3138 ± 0.3503	0.1901 ± 0.1872	0.2912 ± 0.2690	98	59.39
G	1.3742 ± 0.3605	0.2238 ± 0.1890	0.3411 ± 0.2657	116	70.30
Total	1.3875 ± 0.3174	0.2432 ± 0.1608	0.3847 ± 0.2109	162	98.18

*ne = Effective number of alleles, **h = Nei's gene diversity, ***I = Shannon's information index.

the highest number of polymorphic bands (129, 78.18%) while RG type gave the least polymorphism (98, 59.39%). Although the minimums of Nei's (1973) gene diversity (0.1901), Shannon's information index (0.2912) and effective number of alleles (1.3138) still existed in RG type, the maximums (h = 0.2238, I = 0.3411, ne = 1.3742) occurred in G type instead of R type (Table 3). However, these parameters showed that each morphological type of *A. membranaceus* var. *mongholicus* had a high level of genetic diversity comparing with the long-lived perennial herbs and widespread distributed species (Yao et al., 2008), indicating each type had a potential to develop strains or cultivars. The high genetic diversity detected may be mostly attributed to 2 reasons:

1) *A. membranaceus* var. *mongholicus* is a cross-pollinating species, thus it can maintain higher heterozygosity as compared to self-pollinating plant species (Joshi and Dhawan, 2007; Xie et al., 2004).
2) Being informative and polymorphic at intraspecies level, ISSR markers can reveal more variability (Joshi and Dhawan, 2007; Qian et al., 2001; Goulão et al., 2001).

The results of AMOVA with a high significance ($P < 0.001$) showed that most of the whole genetic diversity (90.41%) resided within the 3 morphological types, whereas only 9.59% existed among types.

An UPGMA dendrogram based on SM coefficients between all studied individuals is shown in Figure 1. In this dendrogram, except few individuals including RG1, R1, R2, R13, R14, G1 and G2, most from the same morphological type formed a separate group without any other types' individuals involved. This indicated:

1) 3 morphological types have genetically differentiated to some degree, which could be corroborated by the results of AMOVA and hence it is feasible to classify plants by the forenamed morphological characteristics.
2) If RG1, R1, R2, R13, R14, G1 and G2 are excluded, each type can be further purified.
3) The excluded individuals are genetic materials as important as those remained in each type.

Generally, in the process of breeding by selection, traditional approach of genotype identification depends on morphological differences. However, morphological identification has several disadvantages such as large scale and long term field evaluation trials, environmental and developmental plasticity of the traits and limited number

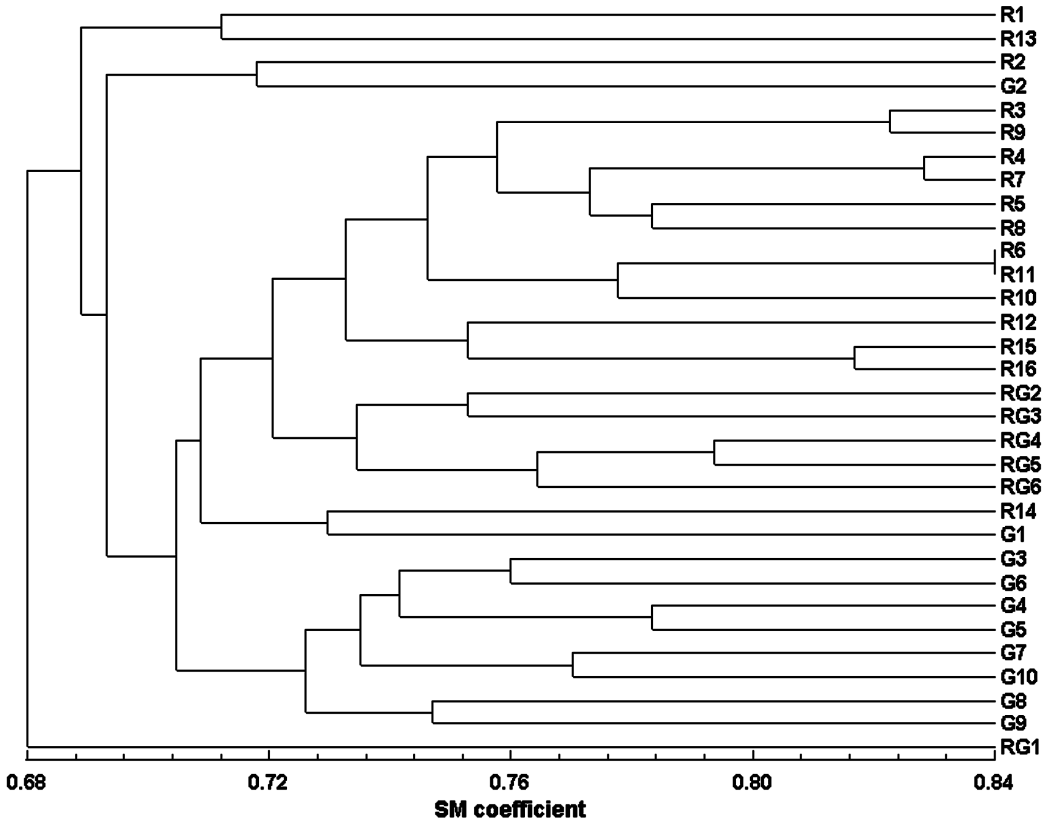

Figure 1. Dendrogram illustrating genetic relationships among 32 individuals of three morphological types of *A. membranaceus* var. *mongholicus* generated by UPGMA method based on SM similarity coefficients calculated on ISSR analysis (Individuals with R, RG and G belong to R, RG and G type, respectively).

of phenotypic makers available (Goulão et al., 2001; Manimekalai and Nagarajan, 2006). Furthermore, *A. membranaceus* var. *mongholicus* being a perennial herb, some morphological markers such as pod color are available for use until the sowed seeds grow at least for 2 years. On the contrary, molecular markers are available in unlimited numbers, irrespective of environment and the development stage of the plant and can be accomplished in a relatively shorter period (Goulão et al., 2001; Manimekalai and Nagarajan, 2006). Therefore, if conventional approach of selective breeding is assisted with appropriate molecular markers, reasonable and efficient strategies will be employed and consequently the period of artificial selection will be shortened. In this study, ISSR effectively revealed the levels of genetic diversity and differentiation in the 3 morphological types, which breeders would benefit from, suggesting ISSR is a potentially powerful and helpful tool for breeding program of *A. membranaceus* var. *mongholicus*.

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