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BRIEF COMMUNICATION

ISSR primer screening and preliminary evaluation of genetic diversity in wild populations of *Glycyrrhiza uralensis*

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Abstract

Fourteen efficient inter-simple sequence repeat (ISSR) primers were screened and optimized for detecting the genetic diversity in wild populations of *Glycyrrhiza uralensis* Fisch. By using these primers, 249 polymorphic bands out of a total of 270 (92.2 %) were generated from 70 individuals of 4 wild *G. uralensis* populations sampled from Inner Mongolia Province of China. Nei's gene diversity (*h*) and Shannon index (*I*) calculated from the data matrix of the ISSR phenotypes revealed a high level of genetic diversity with $h = 0.268$ and $I = 0.415$ within this plant. Analysis of molecular variation (*AMOVA*) showed that most of the genetic variation (81 %) occurred within the populations, whereas the variance among populations was only 19 %. The UPGMA tree based on Nei's unbiased genetic diversity illustrated that populations from Bulage and Bayanwusu were genetically close related, while the population from Shanghaimiao was found to be the most diverse from the other three. The high genetic diversity implies that the wild resources of this species could be restored soon if an appropriate and efficient protection strategy was employed. Our results also provided an optimized method for evaluating genetic diversity of *G. uralensis* using ISSR markers which was useful for further investigation.

Additional key words: *Fabaceae*, licorice, wild resources.

Glycyrrhiza uralensis Fisch. (*Fabaceae*) is a perennial herb widely distributed from Central Asia to northeast China. Its root, named licorice, has been used as a traditional drug for at least 2000 years in China. In addition, it contains glycyrrhizin, a non-sugar sweetener, and is widely used in the manufacture of food, tobacco, cosmetic, *etc*. (Hayashi *et al.* 2005). Overcollection has resulted in a rapid decline of the wild resources of this species and, as a consequence, a severe ecological disaster in North China. Investigation on population genetic diversity using various molecular markers is of great importance for the genetic resource characterization, protection and sustainable utilization of important medicinal plants (Narasimhan *et al.* 2006, Padmesh *et al.* 2006). However, few reports on this topic for *G. uralensis* are available in the literature up to now (Wu *et al*. 2003). In this communication, we report the optimization of primer screening and preliminary evaluation of genetic diversity in wild populations of *G. uralensis* through ISSR markers.

Seventy *G. uralensis* individuals of 4 wild populations (Table 1) were collected from Inner Mongolia Province of China in August 2005. Fresh leaves of each individual were placed in a plastic bag containing silica gel until DNA isolation. Genomic DNA from the dried leaves (0.1 g) was extracted using the CTAB method modified by Murray and Thompson (1980). Extraction buffer consisted of 100 mM Tris-HCl, pH 8.0; 20 mM EDTA, pH 8.0; 1.4 M NaCl, 2 % cetyltriethylammonium bromide (CTAB), 0.3 % β-mercaptoethanol and 0.06 cm^3

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Abbreviations: CTAB - cetyltrimethylammonium bromide; ISSR - inter-simple sequence repeat; PCR - polymerase chain reaction.
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20 % PVP. Precipitation buffer consisted of 50 mM Tris-HCl, pH 8.0; 10 mM EDTA, pH 8.0; 1 % CTAB, and 0.15 % β-mercaptoethanol. After washed twice with 70 % ethanol, the total DNA was resuspended in 0.05 cm^3 distilled water and quantified by UV-spectrophotometer (*Eppendorf Biophotometer*, Hamburg, Germany).

A total number of 100 ISSR primers, purchased from the University of British Columbia (UBC set No. 9), were screened using a few DNA samples. PCR amplification was performed in a *PTC100* thermocycler (*MJ Research*, Watertown, USA). A denaturation period of 5 min at 94 °C was followed by 45 cycles of 1 min at 94 °C, 45 s at 52 °C, 2 min at 72 °C, and then 7 min at 72 °C for final extension. Reaction was carried out in a 0.025 cm^3 reaction mix containing 1× PCR buffer (10 mM Tris-HCl, pH 8.3 and 50 mM KCl), 2 mM $MgCl_2$, 1 U Taq DNA polymerase (*TaKaRa Inc*., Dalian, China); 200 μM each of dATP, dTTP, dCTP, dGTP (*Shenerg Biocolor*, Shanghai, China); 0.5 μM of primer and approximately 20 ng of template DNA. The amplification products was analyzed by electrophoresis in 2 % agarose gel in 0.5× TBE buffer and detected by ethidium bromide staining. *Gene Ruler* 100 bp DNA ladder (*Shenerg Biocolor*) was used to determine the size of the ISSR fragments.

From the preliminary screening, 20 primers that could amplify visible bands were selected for further examination. Different anneal temperature were examined to optimize the amplification condition for the 20 selected primers (Table 2). Eventually, 14 ISSR primers that produced clear and reproducible bands were selected for the amplification of all samples. The selected primers generated an average of 19.3 of total and 17.8 of polymorphic bands per primer (Table 2). The size of the DNA fragments ranged from 100 to 2500 bp. The maximum number of fragments (23 bands) was found from primers 845 and 856, respectively, whereas the least number (13 bands) was produced by primer 828 (Table 2). Samples of population SH generated the maximum number of polymorphic bands (206, 76.3 %), while individuals from population EJ showed the least polymorphism (158, 58.5 %). Examples of the ISSR patterns generated by primers 844 and 807 were shown in Fig. 1.

The amplified DNA polymorphic fragments were scored as binary presence (1) or absence (0), and the data matrix of the ISSR phenotypes was assembled for further analysis. A total of 270 bands were generated from 14 ISSR primers of which 249 were found to be

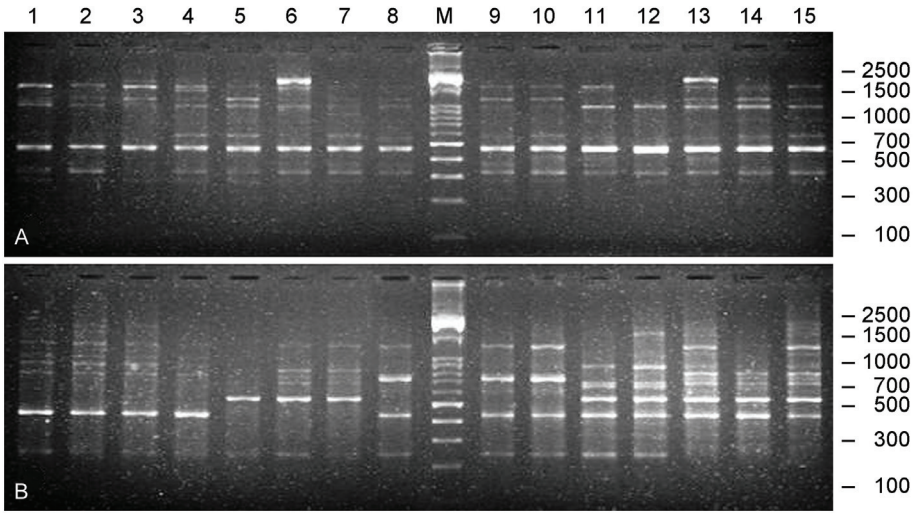

Fig. 1. ISSR patterns of *Glycyrrhiza uralensis* generated by primers 844 and 807. *A* - Products of primer 844; *Lanes 1 - 15* are samples of BL population; *B* - Products of primer 807; *Lanes 1 - 15* are samples of EJ population.

Table 1. Genetic diversity statistics and differentiation parameters for four wild populations of *Glycyrrhiza uralensis*. *Ss* - *s*ample size, *Np* - *n*umber of polymorphic loci, *Pp* - *p*ercentage of polymorphic loci, *h* = Nei's gene diversity, *I* = Shannon index, *Gst* = diversity among populations, *Nm* = gene flow 0.25 (1 - *Gst*)/*Gst*.

Population	Latitude	Longitude	*Ss*	*Np*	*Pp*	*h*	*I*	*Gst*	*Nm*
Shanghaimiao (SH)	38°14.682′	106°50.573′	20	206	76.3	0.236 ± 0.185	0.361	-	-
Bulage (BL)	38°29.268′	107°13.137′	20	174	64.4	0.204 ± 0.191	0.271	-	-
Eqianqijiaoqu (EJ)	38°10.848′	107°29.258′	15	158	58.5	0.212 ± 0.200	0.316	-	-
Bayanwusu (BY)	40°13.940′	108°17.007′	17	182	67.4	0.218 ± 0.191	0.332		
Total			70	249	92.2	0.268 ± 0.162	0.415	0.059	7.978

Table 2. ISSR Primers used for PCR amplification of *Glycyrrhiza uralensis* and total number of amplified fragments generated from 70 individuals.

Primer	Sequence(5'-3') R = (A,G); Y = (C,T)	Annealing temperature [°C]	Total number of bands	Number of polymorphic bands
807	AGAGAGAGAGAGAGAGT	52	22	20
810	GAGAGAGAGAGAGAGAT	52	16	13
823	TCTCTCTCTCTCTCTCC	54	15	13
826	ACACACACACACACACC	52.5	21	20
828	TGTGTGTGT GTG TGT GA	52	13	11
834	AGAGAGAGAGAGAGAGYT	52.5	22	21
835	AGAGAGAGAGAGAGAGYC	52	16	14
841	GAGAGAGAGAGAGAGAYC	52	21	19
843	CTCTCTCTCTCTCTCTRA	53	18	17
844	CTCTCTCTCTCTCTCTRC	54	22	21
845	CTCTCTCTCTCTCTCTRG	53	23	22
848	CACACACACACACACARG	52.5	17	16
855	ACACACACACACACACYT	53	21	20
856	ACACACACACACACACYA	53	23	22
Total			270	249

polymorphic (92.2 %) (Table 1). Percentage of polymorphic loci (*Pp*), Nei's gene diversity (*h*), Shannon index (*I*) were calculated to estimate genetic variation level. Gene flow (*Nm*) was estimated from the $Nm = 0.25 \times (1 - Gst)/st$. All the statistical analyses were performed using *POPGENE* program (version *1.31*) (Yeh *et al*. 1999). The populations included in this study showed a relatively high level of genetic diversity with $h = 0.268$ and $I = 0.414$, respectively. Population SH showed the highest level of genetic diversity ($h = 0.236$, $I = 0.361$), while population BL exhibited the lowest ($h = 0.204$, $I = 0.271$). The gene flow (*Nm*) among all populations was 7.978 (Table 1).

Analysis of molecular variation (*AMOVA*) (version *1.55*) was performed to examine differences among and within populations. Analysis was carried out at three hierarchical levels. The significance of this analog was evaluated by 1000 random permutations of sequences among populations (Miller 1998). The results of *AMOVA* with a high significant ($P < 0.001$) showed that most of the genetic variation (81 %) occurred within the populations, whereas the variance among populations was only 19 %.

Relationships of populations were estimated from the ISSR dada using the *UPGMA* clustering method on the basis of Nei's (1978) unbiased genetic diversity. The UPGMA tree (Fig. 2) revealed that population BL and BY were genetically close related, while population SH showed the most diverse from the other three.

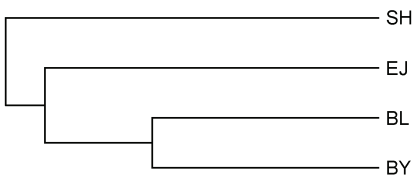

Fig. 2. UPGMA dendrogram showing the relationship among 4 populations of *Glycyrrhiza uralensis* sampled from Inner Mongolia Province of China. SH - Shanghaimiao, EJ - Eqianqi-jiaoqu, BL - Bulage, BY - Bayanwusu.

The level of genetic diversity in wild populations of *G. uralensis* obtained in our study is significantly higher than the average values for long-lived perennial herbs (*Pp* = 0.413, *He* = 0.116) and widespread distributed species (*Pp* = 0.589, *He* = 0.202) (Hamrick and Godt 1989). The high genetic diversity implies that, if an appropriate and efficient protection strategy was employed, the wild resources of this species could be restored soon. Our results also provided an optimized method for evaluating the genetic diversity of *G. uralensis* using ISSR markers which was useful for further investigation. To our knowledge this is the first report on the assessment of genetic variation in this medicinal plant.

References

Hamrick, J.L., Godt, M.J.W.: Allozyme diversity in plant species. - In: Brown, A.H.D., Clegg, M.T., Kahler, A.L., Weir, B.S. (ed.): Plant Population Genetics, Breeding and Genetic resources. Pp. 43-46. Sinauer Associates, Massachusetts 1989.

Hayashi, H., Inoue, K., Ozaki, K., Watanabe, H.: Comparative analysis of ten strains of *Glycyrrhiza uralensis* cultivated in Japan. - Biol. pharm. Bull. **28**: 1113-1116, 2005.

Miller, M.P.: *AMOVA-PREP*, a Program for the Preparatition of *AMOVA* Input Files from Dominant Marker Raw Data

(Release 1.01). - Dep. Biol. Sci., Northern Arizona Univ., Flagstaff 1998.

Murray, M.G., Thompson, W.F.: Rapid isolation of high molecular weight plant DNA. - Nucl. Acid. Res. **8**: 4321-4325, 1980.

Narasimhan, S., Padmesh, P., Nair, G.M.: Assessment of genetic diversity in *Coscinium fenestratum*. - Biol. Plant. **50**: 111-113, 2006.

Nei, M.: Estimation of average heterozygosity and genetic distance from a small number of individuals. - Genetics **89**: 583-590, 1978.

Padmesh, P., Reji, J.V., Dhar, M.J., Seeni, S.: Estimation of genetic diversity in varieties of *Mucuna pruriens* using RAPD. - Biol. Plant. **50**: 367-372, 2006.

Wu, X., Liu, Q.H., Ma, Y.H. Dang, R.L.: [RAPD analysis of genetic relationship among different populations of *Glycyrrhiza uralensis* from six growing area in Sinkiang.] - Chin. J. Biochem. Pharm. **24** (4): 191-193, 2003. [In Chin.]

Yeh, F.C., Young, R.C., Boyle, T.: Microsoft Window-based freeware for population genetic analysis (*POPGENE*, ver. 1.31). ftp://ftp.microsoft.com/softlib/mslfiles/hpgl.exe, 1999.

Pugnaire, F.I., Valladares, F. (ed.): **Functional Plant Ecology.** 2nd Edition. - CRC Press, Taylor and Francis Group, Boca Raton - London - New York 2007. 724 pp. ISBN 978-0-8493-7488-3.

Modern ecological research is hardly possible without detail knowledge of structural and functional traits of individual plant components of the pertinent ecosystem. Rapid development of new portable instruments has facilitated collection of data on physiological activity of plants, even in the field and at multiple scales. Subsequently, it is possible to use them for causal analysis of recent or expected changes in nature at higher, ecologically more relevant levels (whole plant, population, community). This is why the interest in integrative studies based on functional approach has been recently much increasing.

This volume on "Functional Plant Ecology" is an extensively revised and updated second edition of a very successful compendium, which was published several years ago under the title "Handbook of Functional Plant Ecology". The book provides in 23 chapters extensive up-to-date reviews of selected important topics, presented in a very readable style, easily understandable for a broad audience. The aims, historical development and perspectives of functional approach to plant ecology are shortly summarized in the first chapter. The following chapters can be divided into several groups, although such division is not explicitly done in the book.

To the first group belong contributions reviewing structural and functional traits of plant components of some less known biomes, including terrestrial Antarctic and Arctic vegetation, Mediterranean-type ecosystems, and tropical forests. Synthetic view on adaptation of different physiological processes is nicely presented also in a chapter devoted to ecological success of desiccation tolerant plants and lichens. To the second group belongs reviews on more specific aspects of plant functioning as, *e.g.*, responses of plants to heterogeneous irradiance, water relations in plants with different hydraulic architecture, or mineral nutrients acquisition and use. In addition to this, a separate chapter is devoted to structure and function of root system. More structural aspects are discussed in contributions devoted to the architecture of plant crowns, but consequences of shoot structure for optimisation of light capture are also described.

Unusually large space in the book is devoted to the biotic interactions of plants. This is undoubtedly beneficial for potential readers, because good review articles on these important ecological processes are not much abundant in scientific literature. Recent findings in the fields of competition in plant communities, plant-herbivore interactions, and facilitation, are reviewed in separate chapters. Quite interesting and useful is the chapter describing very complex interactions in the rhizosphere, and their effect on ecosystem functioning. Interactions of plants with pollinators, reviewed in the next contribution, are relatively less complicated, nevertheless indispensable for plant sexual reproduction of many plant species.

I would like to point out three remaining, very interesting synthetic articles on topics of more general character. One of them is devoted to evaluation of ecological significance of inherent variation in relative growth rate and its components. Growth rate as an integrative plant function is more relevant for prediction of plant performance in nature, but it is still less frequently taken into account than traditionally measured individual metabolic processes. The other synthetic article discusses various approaches to the application of remote sensing in ecological research at multiple scales. And, finally, the last chapter of the book is focused on such a delicate topics, like selection of representative species for experimental work in functional ecology, selection of the most suitable criteria on which to compare species, and inference rules for generalizing from vegetation samples.

This concise and nicely printed compendium will be undoubtedly of continuous interest to students and teachers of biological disciplines as an advanced textbook, as well as to scientists and practising plant ecologists as an invaluable source of basic information.

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