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Transferability of SSR markers from distantly related legumes to *Glycyrrhiza* species

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Abstract: Licorice (*Glycyrrhiza* spp.) is an important medicinal plant and its distribution of natural habitats has been shrinking day by day due to extensive collection. Genetic diversity identification will likely assist in conserving the different *Glycyrrhiza* species. A total of 127 simple sequence repeat (SSR) markers from *Medicago truncatula* Gaertn., *Phaseolus vulgaris* L., and *Cicer arietinum* L. were used and 26 of them were amplified on *Glycyrrhiza* genomes. The highest transferability rate (33%) was obtained from *M. truncatula* markers, while the highest genetic diversity values were obtained in *P. vulgaris* markers. The markers BM153 and PV-ag004 from *P. vulgaris* had the largest polymorphism information content. The amplified primers were also used to identify genetic diversity among three *Glycyrrhiza* species. The gene diversity values among *Glycyrrhiza* species appeared to be similar; however, donor species mostly had lower diversity values than those of *Glycyrrhiza* species. Additionally, the genetic analysis showed that *G. flavescens* Boiss. subsp. *flavescens* is more distantly related to species *G. glabra* L. var. *glandulifera* and *G. echinata* L. The number of alleles was mostly higher than in the donor species, possibly proposing a multiallelic and/or polyploid structure in *Glycyrrhiza* species.

Key words: *Glycyrrhiza* species, legumes, licorice, markers transferability, SSRs

1. Introduction

Glycyrrhiza species are members of the family Fabaceae. *Glycyrrhiza* is an important medicinal plant and contains glycyrrhizin, a glycoside making it sweeter than sugar. The roots of licorice are traditionally used for prolonging lifespan, improving health, detoxification, and cures for injury and swelling in China (Nomura and Fukai, 1998). In addition to its pharmaceutical uses, licorice is also a drought-tolerant and deep-rooted plant and very important for wind breaking, sand fixing, and soil formation in semiarid ecosystems (Zhang and Ye, 2009). It has also been used in the tobacco, wine, cosmetics, beer, cola, and sweetener industries (Yao et al., 2008). There are 30 *Glycyrrhiza* species in the world, of which 8 taxa are found in Turkey (Akan and Balos, 2008; Asl and Hosseinzadeh, 2008). Sumbul et al. (2003) reported that *G. asymmetrica* Hub.-Mor.; *G. iconica* Hub.-Mor.; *G. flavescens* Boiss. subsp. *flavescens*; *G. flavescens* Boiss. subsp. *antalyensis* Sümbül, Tufan, O.D.Düşen & Göktürk; *G. aspera* Pall.; *G. echinata* L.; *G. glabra* L. var. *glabra*; and *G. glabra* L. var. *glandulifera* (Waldst. & Kit.) Galushko are found in Turkey and the first 3 of these species are endemic to Turkey. In general, *G. glabra* L. is cross-pollinated and has a diploid (2n = 16) genome (Darlington and Wylie, 1955; Mehrotra et al., 2012).

Most medicinal plant species are harvested from the wild and extensive usage has led to endangerment or even extinction of some species (Schippmann et al., 2002; Larsen and Olsen, 2007). Conservation of medicinal plants is an important part of biodiversity conservation (Kate and Laird, 1999). Because of great market demand, the wild sources of this species are much reduced and the conservation of wild populations becomes urgent (Zhang et al., 2006; Zhang and Ru, 2010). Conservation of medicinal plants is a main concern of environmental policies in many countries and regions.

Divergence of wild populations has been successfully identified using molecular tools such as DNA markers. Molecular genetic diversity studies for *Glycyrrhiza* species were performed with random amplified polymorphic DNA (RAPD) (Khan et al., 2009), inter-simple sequence repeat (ISSR) (Yao et al., 2008), and amplified fragment length polymorphism (AFLP) (Zhang et al., 2010) markers. Yamazaki et al. (1994) used RAPD and restriction fragment length polymorphism (RFLP) analyses to determine genetic relationships among *Glycyrrhiza* species

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and found that *G. glabra* L. and *G. uralensis* Fisch. ex DC., which are richer in glycyrrhizin, are more closely related than *G. echinata* L. and *G. pallidiflora* Maxim. Although there are several studies dissecting the *Glycyrrhiza* genome with DNA markers systems such as RFLP, RAPD, and ISSR (Heubl, 2010), there are limited numbers, if any, of studies using simple sequence repeat (SSR) markers. SSR markers are known to be more polymorphic and render more consistent results than the other marker types indicated above. However, developing SSR markers is costly. SSR markers are known to be transferred across species and are widely used to detect genetic diversity in various crop plants (Lichtenzveig et al., 2005). For example, 39 highly polymorphic SSR markers were transferred to blackgram (*Vigna mungo* L. Hepper) from other food legumes (Gupta et al., 2013). Additionally, *Medicago* SSRs were transferred to the *Trifolium repense* L. genome and a genetic map was constructed accordingly (Demdoum et al., 2012).

The objective of this study was to explore the transferability of SSR markers from *Medicago truncatula* Gaertn., *Phaseolus vulgaris* L., and *Cicer arietinum* L. genomes to distantly related *Glycyrrhiza* species found in the East Mediterranean region. Therefore, more consistent relationships among *Glycyrrhiza* species will be established.

2. Materials and methods

2.1. Plant material

The three species of *Glycyrrhiza* used in this study were collected from Hatay Province (East Mediterranean region) in Turkey. These were *G. glabra* L. var. *glandulifera* (Waldst. & Kit.) Galushko, *G. echinata* L., and *G. flavescens* Boiss. subsp. *flavescens*. Including the endemic *G. flavescens*, these 3 species are widespread in the East Mediterranean region. For genomic comparisons, legume species, including *M. truncatula* Gaertn. obtained from the US National Plant Germplasm System (NPGS; PI 190083), *P. vulgaris* L. (cv. Terzibaba), and *C. arietinum* L. (cv. Gökçe), were germinated and tissue samples were collected when the plants were 3 weeks old. The donor legume seeds were kindly provided by Dr Cahit Erdoğan of the Mustafa Kemal University Agricultural Faculty, Hatay, Turkey. All tissue samples were preserved at –80 °C until analyses were performed.

2.2. Amplification of SSRs

From the published reports, a total of 127 SSR primers previously developed and assayed for bean, chickpea, and barrel clover were utilized to determine whether SSR primers of such distantly related species are transferable to *G. glabra*, *G. echinata*, and *G. flavescens* genomes. Of those, we used 27 primers from common bean BM, BMD, PV, and GATS primer sources (Yu et al., 2000; Blair et al., 2003); 36 primers from chickpea NCPGR, TA, TAA, TR, and TS primer sources (Winter et al., 1999); and 64 primers from barrel clover MTIC, AW, AJ, AW, CaSTMS, and CB primer sources (Julier et al., 2003; Chandra, 2011). In addition to the primers depicted in Table 1, *P. vulgaris* markers such as PV-tttc001, PV-at001, PVBR14, BMd-1, BMd-15, BMd-18, BMd-42, BM154, BM160, BM161, BM175, BM183, BM187, BM188, BM199, BM200, BM209, BM210, BM211, GATS91, BM053, BM114, BM137, and BM143; *C. arietinum* markers such as CaSTMS15, CaSTMS2, CaSTMS21, NCPGR12, NCPGR19, NCPGR4, NCPGR6, TA113, TA117, TA118, TA130, TA135, TA14, TA142, TA200, TA206, TA21, TA22, TA27, TA28, TA46, TA64, TA72, TA76S, TAA58, TR2, TR29, TR31, TR43, TR7, and TS84; and *M. truncatula* markers such as MTIC12, MTIC14, MTIC19, MTIC21, MTIC210, MTIC237, MTIC238, MTIC248, MTIC251, MTIC258, MTIC27, MTIC273, MTIC289, MTIC299, MTIC304, MTIC314, MTIC318, MTIC331, MTIC338, MTIC339, MTIC345, MTIC347, MTIC35, MTIC354, MTIC365, MTIC432, MTIC441, MTIC470, MTIC471, MTIC475, MTIC48, MTIC51, MTIC58, MTIC64, MTIC7, MTIC72, MTIC79, MTIC82, MTIC84, MTIC90, MTIC93, MTIC94, AJ248338, AJ410087, AW698723, AW698894, and CB858137 were used for polymorphism studies.

Genomic DNA obtained from leaf samples was extracted using a CTAB method (Murray and Thompson, 1980) with minor modifications. Genomic DNA was quantified on nanodrop (ACTGene UVS-99, USA), at $A_{260/280}$ nm. The quality was confirmed on 0.8% agarose gel. Polymerase chain reaction (PCR) analysis was performed in 20 µL of reaction volume containing 30 ng of genomic DNA, 2.5 mM $MgCl_2$, 2 µM of each primer pair, 0.6 U of Taq polymerase, and 2 mM of dNTPs in 10X reaction buffer. PCR amplifications were carried out using the MultiGene Thermal Cycler (Labnet International, USA) with an initial denaturation for 5 min at 95 °C, then 35 cycles of 30 s denaturation at 94 °C and 30 s annealing at 50–60 °C, 1 min extension at 72 °C, and final extension for 10 min at 72 °C. Amplified PCR products were electrophoresed on 3% Nu Micropor agarose (Prona, Spain) agarose gels containing 1X Tris-borate-EDTA buffer. The genomic DNA was stained with 1 µg/mL EtBr. The gels were run at 140 V for 80 min. Gel photos were taken under UV light using the DNR MiniLumi (DNR Bio-Imaging Systems, Israel) gel documentation system.

2.3. Statistical analysis

Data were scored as presence of band as "1" and absence of band as "0" from SSR amplifications. To characterize genetic variation, the observed number of alleles (Na), effective number of alleles (Ne), Nei's gene diversity (He), and Shannon's information index (I) were calculated. All of the above calculations were performed using the POPGENE program, ver. 1.31 (Yeh et al., 1999). We used

chi-square analysis to compare whether the transferability rates of donor species were the same. Therefore, expected and observed number of transfers for donor species were calculated to find a chi-square value. Coefficient of similarity among species was calculated according to Nei (1972). The UPGMA tree was constructed using NTSYS v. 2.02 (Rohlf, 1998).

3. Results

Of the 127 SSR primers tested, 26 primers produced clear amplifications and polymorphic bands were observed on *G. glabra*, *G. echinata*, and *G. flavescens*. Three *Glycyrrhiza* species significantly differed in terms of marker transferability (chi-square value = 8.626; P = 0.013). The highest rate of transferability was from *M. truncatula* (33%), followed by *P. vulgaris* and *C. arietinum* (11% and 6%, respectively). Twenty-one *M. truncatula* primers produced a total of 121 bands, of which 85 bands were polymorphic (Table 1). Three *P. vulgaris* L. primers produced 32 bands, of which 22 were polymorphic (Table 1). Two *C. arietinum* primers produced 29 bands, of which 21 were polymorphic (Table 1). The average of allele numbers and polymorphic bands for *M. truncatula* was 5.76 and 4.05; for *P. vulgaris* it was 10.67 and 7.33; and for

Table 1. Genetic analysis of markers and diversity values.

Donor species	Marker	Number of total bands	Number of polymorphic bands	Gene diversity value	Polymorphism information content
P. vulgaris	BM140	16	12	0.375	0.305
	BM153	7	4	0.500	0.375
	PV-ag004	9	6	0.500	0.375
C. arietinum	TA71	17	12	0.427	0.334
	TaaSH	12	9	0.417	0.328
M. truncatula	MTIC230	7	3	0.375	0.305
	MTIC232	4	3	0.417	0.328
	MTIC233	4	3	0.417	0.328
	MTIC247	4	4	0.375	0.305
	MTIC249	7	5	0.425	0.333
	MTIC250	7	5	0.425	0.333
	MTIC272	4	3	0.417	0.328
	MTIC278	11	4	0.344	0.264
	MTIC289	3	3	0.375	0.305
	MTIC326	6	4	0.438	0.340
	MTIC332	4	4	0.417	0.328
	MTIC343	6	5	0.400	0.319
	MTIC354	2	2	0.375	0.305
	MTIC356	10	5	0.450	0.347
	MTIC446	2	2	0.375	0.305
	MTIC447	5	5	0.375	0.305
	MTIC451	4	4	0.406	0.322
	MTIC452	11	6	0.396	0.316
	MTIC475	2	2	0.375	0.305
	AW698672	6	6	0.375	0.305
	AW698994	12	7	0.411	0.325
	Total	182.00	128.00		

C. arietinum it was 14.5 and 10.5, respectively. Although the number of amplified markers were the highest in *M. truncatula*, gene diversity (h*) and polymorphism information content values were the highest in *P. vulgaris* markers (Table 1). The highest number of bands was obtained from *C. arietinum* primer TA71 while the lowest was obtained from *M. truncatula* primers MTIC354, MTIC446, and MTIC475 (Table 1). However, the most informative loci were from *P. vulgaris* markers BM153 and PV-ag004 (Table 1). Although MTIC278 produced one of the highest band numbers among *M. truncatula* Gaertn. markers, it was found to be the least informative (Table 1).

Using *M. truncatula*, *P. vulgaris*, and *C. arietinum* primers, no significant differences were observed among the *Glycyrrhiza* species in terms of number of effective alleles, polymorphic loci, Shannon diversity index, or total number of bands ($P > 0.05$; Table 2). The number of bands in *Glycyrrhiza* species was mostly higher than those of donor legume species. The *P. vulgaris* primers seemed to distinguish *Glycyrrhiza* species better since the band production rate (ranging 25%–33% more than the *P. vulgaris* genome produced for itself) was higher than those of other 2 donor legumes (Table 2).

We also used the successfully transferred 26 SSR markers derived from 3 donor legume species to detect genetic diversity among *G. glabra*, *G. echinata*, and *G. flavescens* species. The transferred markers form each donor genome were used to construct dendrograms separately to identify how each marker system formed the genetic similarity among the 3 *Glycyrrhiza* species. According to genetic similarity tests, *G. glabra* and *G. echinata* were more similar to each other than to *G. flavescens* (Table 3). The dendrogram using *M. truncatula* primers appeared to be more consistent with the taxonomic separation of the *Glycyrrhiza* species since they were grouped separately from the *M. truncatula* (Figure). Within that group, *G. glabra* and *G. echinata* were within the same subgroup and were genetically similar to each other. The dendrogram using *P. vulgaris* markers formed 2 groups and although they are genetically distant from each other, *P. vulgaris* and G. *flavescens* were within the same group while *G. glabra* and *G. echinata* were in the other group (Figure). Similarly, in the dendrogram formed using *C. arietinum* primers, the donor species was within the same subgroup as *G. flavescens*, while *G. glabra* and *G. echinata* were in the other subgroup (Figure). Overall, using the 26 primers from 3 related donor legume genomes, *G. glabra* and *G. echinata* were found to be more similar to each other than to *G. flavescens*.

4. Discussion

There have been many examples of SSR markers transferred within legume genera: from soybean to peanut (He et al., 2006); from *M. truncatula* to pea, fava bean, and *C. arietinum* (Gutierrez et al., 2005); and from *M. truncatula*, *Pisum sativum*, and *Trifolium pratense* to *Lens culinaris* (Reddy et al., 2010). However, there have been limited studies to transfer SSR markers to *Glycyrrhiza* species. In the present study, all the transferred markers amplified within *Glycyrrhiza* species were polymorphic. Additionally, the amplified primers were used to detect genetic diversity within the genus *Glycyrrhiza*. To our

Table 3. Genetic similarity matrix constructed using *M. truncatula*, *P. vulgaris*, and *C. arietinum* primers.

	G. glabra	*G. echinata*	*G. flavescens*
G. glabra	1.000		
G. echinata	0.810	1.000	
G. flavescens	0.464	0.417	1.000
M. truncatula	0.381	0.333	0.393
G. glabra	1.000		
G. echinata	0.870	1.000	
G. flavescens	0.261	0.217	1.000
P. vulgaris	0.391	0.348	0.522
G. glabra	1.000		
G. echinata	0.952	1.000	
G. flavescens	0.381	0.333	1.000
C. arietinum	0.286	0.238	0.429

Table 2. Observed number of alleles (na*), effective number of alleles (ne*), Nei's (1973) gene diversity (h*), and Shannon's information index (I*) values. Each donor species was analyzed separately.

Species	na*	ne*	h*	I*	Total bands
G. glabra	2	1.849	0.459	0.652	30
G. echinata	2	1.893	0.472	0.665	32
G. flavescens	2	1.872	0.466	0.658	33
M. truncatula	**2**	**1.747**	**0.427**	**0.619**	**26**
Total					121
G. glabra	2	1.830	0.454	0.646	8
G. echinata	2	1.910	0.476	0.669	9
G. flavescens	2	1.910	0.476	0.669	9
P. vulgaris	**2**	**1.628**	**0.386**	**0.574**	**6**
Total					35
G. glabra	2	1.893	0.472	0.665	8
G. echinata	2	1.960	0.490	0.683	9
G. flavescens	2	1.570	0.363	0.549	5
C. arietinum	**2**	**1.800**	**0.444**	**0.637**	**7**
Total					29

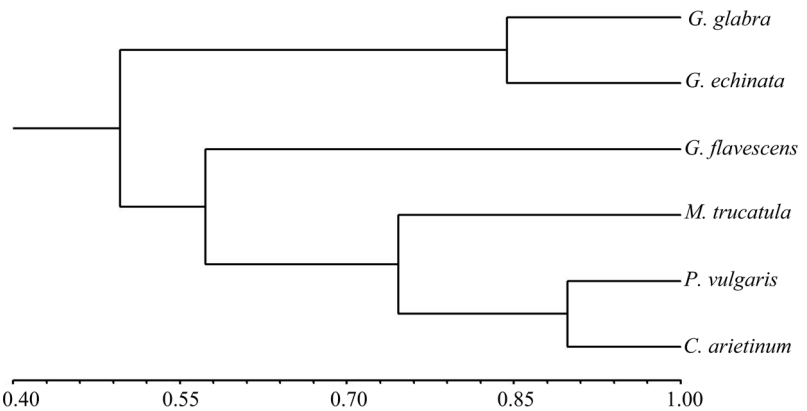

Figure. Dendrogram constructed using *M. truncatula* Gaertn., *P. vulgaris* L., and *C. arietinum* L. markers.

best knowledge, the present study is the first attempt to transfer SSR markers from distantly related legume species to *Glycyrrhiza* species. The degree of polymorphism was also suitable to construct dendrograms that depicted the genetic relatedness of the 3 different *Glycyrrhiza* species. The results showed that *G. flavescens* is distantly related to *G. glabra* and *G. echinata*. Interestingly, *G. flavescens* was as genetically distant to the other *Glycyrrhiza* species as the other donor legumes. This result may suggest that *G. glabra* and *G. echinata* diverged from *G. flavescens*. However, further studies such as plastid genome comparisons are needed to confirm such a relationship (Jansen and Ruhlman, 2012).

Amplified markers were usually weaker than the amplifications in donor species. Weaker bands amplified in related genomes were typical in transferability studies (Kuleung et al., 2004). Amplified markers from donor genomes generally resulted in similar banding patterns in

the *Glycyrrhiza* genome, suggesting that they were derived from the same loci and that these allelic regions of the primer binding sites are conserved. However, many of the primers could not be amplified in *Glycyrrhiza* genomes. This degree of site loss due to insertion or deletion is likely reflective of the genomic divergence in legume genomes.

Out of 127 SSR primers tested, only 26 primers were amplified and polymorphic in *Glycyrrhiza* species. A transferability rate of about 20% from such distantly related species could be considered a relatively good success, since within pulse crops or even closely related legume species SSR marker transferability rates could be as low as 16% (Choudhary et al., 2008; Reddy et al., 2010; Gupta et al., 2013). The success rate is dependent upon the suitability of donor species, as well. In our study, *M. truncatula* resulted in the highest transferability rate (33%). With a small genome of about 500 Mbp, *M. truncatula* has been used as a model plant for various features of legume genetics and genomics (Cook, 1999). However, such information has not yet been used to understand the syntenic relationship with the genus *Glycyrrhiza*. In the present study, all of the amplified primers were polymorphic and, interestingly, the number of allelic fragments was mostly higher than in the donor species. This implies that once SSR primers are transferred to the *Glycyrrhiza* genome, especially from *P. vulgaris* or *C. arietinum*, markers could be even more informative than they were within the donor species. Although the number of *P. vulgaris* primers was lower, they appeared to be more informative than the either of the 2 other donor species. In addition, an increased number of alleles may imply polyploidy or multiallelic loci in *Glycyrrhiza* species. In the present study, MTIC237, MTIC289, and MTIC332 markers observed only 1 locus in *G. flavescens* and *M. truncatula*, while they observed more than 3 loci in *G. glabra* and *G. echinata*. There are also examples that many plant species are actually ancient polyploids (Soltis et al., 2004). So far, there has not been any information about the ploidy or multiallelic structures of *Glycyrrhiza* genomes. However, there are examples of multiallelic loci and polyploidy structures in several plant genomes such those of as vines (Frank et al., 2002; Hocquigny et al., 2004), Jerusalem artichoke (Buteler et al., 1999), and olive (Bandelj et al., 2004). It is also possible that the higher number of allelic order may suggest higher polyploidy level (Essenlink et al., 2004). The present data are not strong evidence about the ploidy structure; however, more research is needed to reveal information about the chromosomal structure and ploidy level of *Glycyrrhiza* species.

The cost of developing SSR markers may be high; however, the wealth of sequence information in legume databases and the published literature may be a better source for dissecting *Glycyrrhiza* genomes. In the present study, we used SSR primers from distantly related legume species and were able to amplify those on *Glycyrrhiza* genomes. The results of this initial study are expected to help generate tools to identify genetic distributions of such an important medicinal plant and possibly understand the genomic synteny with other legumes in a relatively feasible way. Therefore, more SSR markers should be searched to dissect the genomic diversity among *Glycyrrhiza* species and to make conservation plans for the future.

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