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Original Research Article

Genetic Diversity and Population Structure of Korean Mint *Agastache rugosa* (Fisch & Meyer) Kuntze (Lamiaceae) Using ISSR Markers

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Abstract - *Agastache rugosa*, a member of the mint family (Labiatae), is a perennial herb widely distributed in East Asian countries. It is used in traditional medicine for the treatment of cholera, vomiting, and miasma. This study assessed the genetic diversity and population structures on 65 accessions of Korean mint A. *rugosa* germplasm based on inter simple sequence repeat (ISSR) markers. The selected nine ISSR primers produced reproducible polymorphic banding patterns. In total, 126 bands were scored; 119 (94.4%) were polymorphic. The number of bands generated per primer varied from 7 to 18. A minimum of seven bands was generated by primer 874, while a maximum of 18 bands was generated by the primer 844. Six primers (815, 826, 835, 844, 868, and 874) generated 100% polymorphic bands. This was supported by other parameters such as total gene diversity (H_T) values, which ranged from 0.112 to 0.330 with a mean of 0.218. The effective number of alleles (N_E) ranged from 1.174 to 1.486 with a mean value of 1.351. Nei's genetic diversity (H) mean value was 0.218, and Shannon's information index (I) mean value was 0.343. The high values for total gene diversity, effective number of alleles, Nei's genetic diversity, and Shannon's information index indicated substantial variations within the population. Cluster analysis showed characteristic grouping, which is not in accordance with their geographical affiliation. The implications of the results of this study in developing a strategy for the conservation and breeding of A. rugosa and other medicinal plant germplasm are discussed.

Key words - *Agastache rugosa*, Genetic diversity, ISSR, Polymorphism, Population structure

Introduction

The Labiatae comprises a large family of over 6000 species (Thorne, 1992). The family is known for its fine ornamental and culinary herbs like basil, lavender, mint, oregano, rosemary, sage, and thyme, as well as representing a rich source of essential oils for the flavoring and perfume industry. They have traditionally been considered closely related to the Verbenaceae (Harley *et al*., 2004), but in the 1990s, phylogenetic studies suggested that many genera classified in Verbenaceae belong instead in Lamiaceae (Cantino *et al*., 1992). The genus *Agastache* (Lamiaceae) comprises 12 species with stiff, angular stems clothed in tooth-edged lance-shaped leaves and broad that vary by species. They have an upright spike of tubular, two-lipped flowers. The flowers are usually white, pink, mauve, or purple with bracts that back the flowers, being of the same or a slightly contrasting color. Leaf tips can be eaten and made into teas.

The species *Agastache rugosa* Kuntze (syn. *Lophanthu rugosus*) is a perennial herb belonging to the Labiatae family. The plant is widely distributed in Korea, China, India, Japan, and other East Asian countries and used in Chinese folk medicine. Antitumor and cytotoxic activities of the plant have been reported (Weverstahl *et al*., 1992) and the whole plant has been used as an agent for the treatment of cholera, vomiting, and miasma. Leaves can also be used as a spice. The species is traditionally used as a medicinal plant in Korea (Jung and Shin, 1990).

In recent years, the breeding and registration of medicinal plant cultivars have been reported from several different countries (Bernath, 1996). The most important goals of any

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medicinal plant-breeding program are to improve the morphological characteristics and increase the accumulation of biologically active agents. Studying the genetic diversity of available plant germplasm is a prerequisite for each breeding program (Bernath, 2002).

Molecular markers have been used in determining genetic diversity and reconstructing evolutionary processes (Ikbal *et al*., 2010). In contrast to traditional selection based on phenotypic screening, molecular markers refractory to environmental variation are fully inheritable and available in large numbers. To develop an efficient identification method, molecular techniques have been used since they are reliable, unaffected by environmental conditions, and can aid in varietal identification. In recent years, DNA-based on molecular markers have been used to assess the genetic diversity among the germplasm of many plant species. DNA-based molecular markers have the advantage of being free from environmental modulation. Inter simple sequence repeats (ISSR) based on PCR amplification have been widely used for the study of population genetics in several medicinal plants (Bornet *et al*., 2002; Chen *et al*., 2006; Fracaro and Echeverrigaray, 2006; Li and Jin, 2008). ISSR primers of repeat motifs (microsatellites) that are abundant and dispersed throughout genomes anchor at either the 5' or 3' end with one or a few specific nucleotides. Sequences are amplified between two microsatellite loci (Wang *et al*., 1994; Wang *et al*., 2008; Zietkiewicz *et al*., 1994; Ratnaparkhe *et al*., 1998). Because of the higher annealing temperature and longer sequence of ISSR primers, they can yield more reliable and reproducible bands than random amplification of polymorphic DNA (RAPD; Nagaoka and Ogihara, 1997; Wolfe *et al*., 1998; Goulao *et al*., 2001; Qian *et al*., 2001), and the cost of these analyses is lower than that of some other markers such as restriction fragment length polymorphisms (RFLPs), simple sequence repeats (SSRs), and amplified fragment length polymorphisms (AFLPs; Yang *et al*., 1996; Wang *et al*., 2008). In addition to freedom from the necessity of obtaining genomic sequence information, ISSR markers are technically simpler than many other marker systems in the genetic diversity studies of plants (Ratnaparkhe *et al*., 1998; Bornet and Branchard, 2001; Ye *et al*., 2005). To date, no report is available on applications of molecular markers in studies on the genetic diversity of *A. rugosa*. In this investigation, a first attempt was made to study the genetic diversity and population structure of *A. rugosa* using ISSR markers.

Materials and Methods

Plant material and DNA isolation

Sixty-six accessions of *A. rugosa* germplasm (Table 1) representing different countries of origins (61 accessions from Korea, two accessions from Russia, one accession from North Korea, one accession from China, and one accession from Canada) were selected for the present study. Seeds were obtained from the National Agrobiodiversity Center at the Rural Development Administration, Republic of Korea. ISSR analyses were conducted using a single plant from each accession. DNA was extracted from the freeze-dried leaves of 15-day-old seedlings of each accession, according to the modified cetyltrimethyl ammonium bromide (CTAB) method (Dellaporta *et al*., 1983). The relative purity and concentration of extracted DNA were estimated using an ND-1000 spectrophotometer (Dupont Agricultural Genomics Laboratory, NanoDrop Technologies, Wilmington, DE, USA). The final DNA concentration was adjusted to 10ng/μl.

ISSR primer screening

In total, 139 primers (UBC, University of British Columbia, Canada) were used in the ISSR-PCR reaction. After a preliminary test, we selected nine primers that had clear, repeatable, amplified polymorphic bands to analyze the polymorphism of *A. rugosa* germplasm accessions. Each experiment was performed at least twice.

ISSR-PCR amplification

The ISSR-PCR amplification of 20 ng of DNA was performed in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM spermidine, 2% formamide, 0.1 mM dNTPs, 0.3 μM primer, and 1 U of Taq DNA polymerase (NeoTherm) in a 20 μl reaction using a PTC-220 thermal cycler (MJ Research, Watertown, MA, USA). The following reaction conditions were used: initial denaturation for 5 min at 94 °C, followed by 40 cycles of 1 min at 94 °C (denaturation), 45 s at 50 °C (annealing), and 2 min at 72 °C (extension), with a final

Table 1. Effects of the Red Ginseng on the thymus and spleen index in BALB/c mice

Sl. No.	Accession No.	Species name	Country code	Country	Sl. No.	Accession No.	Species name	Country code	Country
1	175074	*Agastache rugosa*	KOR	Korea	38	K134675	*Agastache rugosa*	KOR	Korea
2	183182	*Agastache rugosa*	KOR	Korea	39	K134676	*Agastache rugosa*	KOR	Korea
3	195160	*Agastache rugosa*	KOR	Korea	40	K134677	*Agastache rugosa*	KOR	Korea
4	196719	*Agastache rugosa*	KOR	Korea	41	K136422	*Agastache rugosa*	Unknown	Unknown
5	196720	*Agastache rugosa*	KOR	Korea	42	K144963	*Agastache rugosa*	KOR	Korea
6	209893	*Agastache rugosa*	KOR	Korea	43	K144964	*Agastache rugosa*	KOR	Korea
7	209894	*Agastache rugosa*	KOR	Korea	44	K144965	*Agastache rugosa*	KOR	Korea
8	807260	*Agastache rugosa*	KOR	Korea	45	K144966	*Agastache rugosa*	KOR	Korea
9	K001011	*Agastache rugosa*	CAN	Canada	46	K144967	*Agastache rugosa*	KOR	Korea
10	K001475	*Agastache rugosa*	KOR	Korea	47	K144968	*Agastache rugosa*	KOR	Korea
11	K001476	*Agastache rugosa*	KOR	Korea	48	K146120	*Agastache rugosa*	RUS	Russia
12	K001477	*Agastache rugosa*	KOR	Korea	49	K146200	*Agastache rugosa*	RUS	Russia
13	K001478	*Agastache rugosa*	KOR	Korea	50	K153751	*Agastache rugosa*	PRK	North Korea
14	K001479	*Agastache rugosa*	KOR	Korea					
15	K001480	*Agastache rugosa*	KOR	Korea	51	K160463	*Agastache rugosa*	KOR	Korea
16	K001481	*Agastache rugosa*	KOR	Korea	52	195180	*Agastache rugosa*	KOR	Korea
17	K001482	*Agastache rugosa*	KOR	Korea	53	195188	*Agastache rugosa*	KOR	Korea
18	K001483	*Agastache rugosa*	KOR	Korea	54	911019	*Agastache rugosa*	KOR	Korea
19	K001484	*Agastache rugosa*	KOR	Korea	55	K124860	*Agastache rugosa*	KOR	Korea
20	K001485	*Agastache rugosa*	KOR	Korea	56	K124861	*Agastache rugosa*	KOR	Korea
21	K001486	*Agastache rugosa*	KOR	Korea	57	K124862	*Agastache rugosa*	KOR	Korea
22	K001487	*Agastache rugosa*	KOR	Korea	58	K124864	*Agastache rugosa*	KOR	Korea
23	K001488	*Agastache rugosa*	KOR	Korea	59	K124865	*Agastache rugosa*	KOR	Korea
24	K001489	*Agastache rugosa*	KOR	Korea	60	K124866	*Agastache rugosa*	KOR	Korea
25	K001490	*Agastache rugosa*	KOR	Korea	61	K124867	*Agastache rugosa*	KOR	Korea
26	K001491	*Agastache rugosa*	KOR	Korea	62	K124868	*Agastache rugosa*	KOR	Korea
27	K001492	*Agastache rugosa*	KOR	Korea	63	K124869	*Agastache rugosa*	KOR	Korea
28	K001493	*Agastache rugosa*	KOR	Korea	64	K124870	*Agastache rugosa*	KOR	Korea
29	K001494	*Agastache rugosa*	KOR	Korea	65	K124871	*Agastache rugosa*	KOR	Korea
30	K001495	*Agastache rugosa*	KOR	Korea					
31	K024673	*Agastache rugosa*	CHN	China					
32	K026440	*Agastache rugosa*	KOR	Korea					
33	K040600	*Agastache rugosa*	KOR	Korea					
34	K109330	*Agastache rugosa*	KOR	Korea					
35	K109639	*Agastache rugosa*	KOR	Korea					
36	K124859	*Agastache rugosa*	KOR	Korea					
37	K124863	*Agastache rugosa*	KOR	Korea					

extension at 72 °C for 5 min. Amplified products were electrophoresed on 2 % agarose gel using 0.5 × TAE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) and visualized using ethidium bromide staining. The patterns were photographed and stored as digital pictures in a gel documentation system.

Data analysis

Unequivocally reproducible bands were scored and entered into a binary character matrix (1 for presence and 0 for absence). Nei's similarity matrix was subjected to cluster analysis by the unweighted pair group method for arithmetic mean averages (UPGMA) and a dendrogram was generated using the software MEGA 4. POPGENE 1.3 software was used to calculate Nei's unbiased genetic distance among the different genotypes for all markers. Data for total gene diversity (H_T), effective number of alleles (N_E), Nei's genetic diversity (H) and Shannon's information index (I) were also

analyzed across all 165 accessions (Yeh *et al*., 1999).

Results

ISSR polymorphisms

The *A. rugosa* specimens were analyzed using 139 primers, of which nine produced reproducible polymorphic banding patterns. In total, 126 bands were scored, 119 (94.4 %) of which were polymorphic. The number of bands generated per primer varied from 7 to 18. The minimum of seven bands was generated by primer 874, while the maximum of 18 bands was generated by primer 844. Six primers (815, 826, 835, 844, 868, and 874) generated 100% polymorphic bands. A high level of polymorphism was detected by the ISSR markers in this study.

Genetic variability details from inter simple sequence repeat (ISSR) markers

The data for total gene diversity (H_T) values ranged from 0.112 to 0.330 with a mean value of 0.218, while effective number of alleles (N_E) ranged from 1.174 to 1.486 with a mean value of 1.351 (Table 2). Nei's genetic diversity (H) values ranged from 0.112 to 0.330 with a mean value of 0.218 and Shannon's information index (I) ranged from 0.198 to 0.494 with a mean value of 0.343. The values of H and I were found to be highest for primer 880 and lowest for primer 874 in all 65 accessions, which were analyzed using nine ISSR markers. The high values for total gene diversity, effective number of alleles, Nei's genetic diversity, and Shannon's information index displayed substantial variations within the population.

Genetic structure

Genetic relationships among the accessions were established using UPGMA cluster analysis (Fig. 1). Nei's unbiased measure of genetic distance was employed to further elucidate the genetic differentiation in all the accessions. Nei's genetic distance ranged from 0.482 to 1.000 and genetic identity ranged from 0.000 to 0.730. A dendrogram was developed for 65 accessions of *A. rugosa* using ISSR markers. The genetic relationships revealed among the accessions using nine ISSR markers were grouped into two major clusters denoted as cluster A and cluster B. Cluster A was subdivided in to two subclusters, A1 and A2. The subcluster A1 contained 49 accessions, including 43 accessions from Korea, two accessions from Russia, one accession from Canada, one accession from North Korea, one accession from China, and one unknown accession. The subcluster A2 contained 13 accessions from Korea only and cluster B contained only three accessions also from Korea. Despite the identification

Table 2. Genetic diversity of A. *rugosa* detected by ISSR marker analysis

Primer	Nucleotide sequence (5'-3')	Total no. of scorable bands	No. of polymorphic bands	Polymorphic (%)	H_T	N_E	H	I
811	(GA)8C	16	12	75	0.205	1.334	0.205	0.330
815	(CT)8G	17	17	100	0.175	1.263	0.175	0.284
826	(AC)8C	10	10	100	0.292	1.486	0.292	0.447
834	(AG)8YG	17	15	88.23	0.231	1.380	0.231	0.360
835	(AF)8YC	17	17	100	0.163	1.251	0.163	0.266
844	(CR)8RC	18	18	100	0.184	1.276	0.184	0.298
868	(GAA)6	11	11	100	0.236	1.387	0.236	0.360
874	(CCCT)4	7	7	100	0.112	1.174	0.112	0.198
880	(GGAGA)3	13	12	92.31	0.330	1.563	0.330	0.494
Total		126	119	-	-	-	-	-
Mean		14.0	13.22	-	0.2178	1.3514	0.2178	0.3427
S.D.		-	-	-	0.2178	0.3346	0.1744	0.2386

Y (C, T), R (A, G); NE, effective number of alleles; I, Shannon's information index; HT, total gene diversity; H, Nei's gene diversity

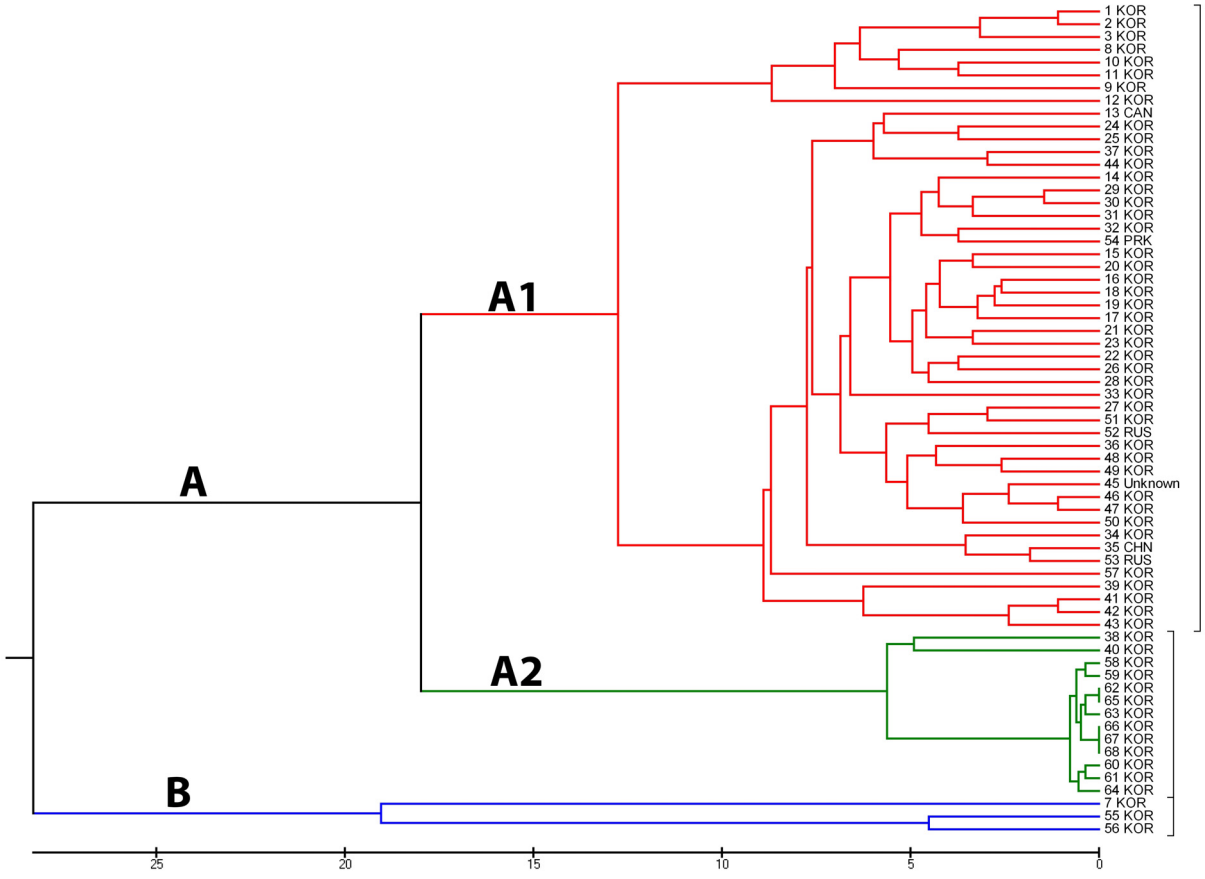

Fig. 1. Dendrogram of 65 A. rugosa accessions based on Jaccard's similarity coefficient.

of several groups, the dendrogram showed no strict relationship with respect to geographical distribution.

Discussion

In the rapid development of molecular biology, molecular markers have increasingly been shown to be useful for diversity assessments in many plant species (Waugh and Powell, 1992). Compared with other molecular markers, ISSR markers are more reproducible, stable, simple, and have been demonstrated to be very powerful in analyzing genetic structure and diversity in many medicinal plant species including *Carthamus tinctorius* (Peng *et al*., 2008), *Pogostemon cablin* (Wu *et al*., 2010), and *Rheum tanguticum* (Hu *et al*., 2010). In our study using 139 primers, nine produced reproducible polymorphic banding patterns. In total, 126 bands were scored, and 119 (94.4 %) were polymorphic. The number of bands generated per primer varied from 7 to 18, with the minimum of seven bands generated by primer 874, and the maximum of 18 bands generated by primer 844. Six primers (815, 826, 835, 844, 868, and 874) generated 100 % polymorphic bands. Therefore, the observed high levels of polymorphism detected in otherwise self-pollinated accessions of *A. rugosa* may be attributable to the broad genetic base of the species that in the process of speciation may have acquired novel gene combinations for better adaptability to changing environmental conditions. A similar observation of high diversity in a self-pollinated species has been reported in *Mucuna pruriens* (Capo-chichi *et al*., 2003; Padmesh *et al*., 2006). The high genetic diversity observed in *A. rugosa* may be due to its wide geographical and climatic distribution in both tropical and temperate regions that must have accelerated the evolvement of novel and diverse genotypes.

In the present study, the gene diversity (H_T) values ranged from 0.112 to 0.330 with a mean value of 0.218, while

effective number of alleles (N_E) ranged from 1.174 to 1.486 with a mean value of 1.351. The values of H and I were found to be highest for primer 880 and lowest for primer 874 for all 65 accessions and were analyzed using nine ISSR markers. The high values for total gene diversity, effective number of alleles, Nei's genetic diversity, and Shannon's information index indicated substantial variations within the population.

Yang *et al*. (2010) similarly reported total genetic diversity (H_T) was 0.2954 and Shannon's information index (I) was 0.4371, suggesting a relatively high rate of genetic variation at the species level in *Meconopsis quintuplinervia* Regel. The average within-population diversity also appeared to be high, with He and I values of 0.2408 and 0.3347, respectively. Nei's coefficient of differentiation (G_{ST}) was also found to be high (0.2320), further confirming the relatively high level of genetic differentiation within populations. Genetic variability within populations is a very important measure of species adaptation to environmental changes and of species survival (Sofia *et al*., 2006). When the gene pool of a population narrows and loses genetic plasticity, it becomes increasingly susceptible to changes in the environmental conditions and hence more prone to extinction (Louis, 1980). In the present study, Nei's unbiased measure of genetic distance was employed further to elucidate the genetic differentiation in all the accessions. Nei's genetic distance ranged from 0.482 to 1.000 and genetic identity ranged from 0.000 to 0.730. A dendrogram was developed for 65 accessions of *A. rugosa* using ISSR markers. The genetic relationships among accessions using nine ISSR markers were grouped into two major clusters denoted as cluster A and cluster B. Cluster A was subdivided into two subclusters, A1 and A2; subcluster A1 contained 49 accessions. Subcluster A2 contained 13 accessions and cluster B contained only three accessions. Despite the identification of several groups, these dendrograms showed no strict relationship with respect to geographical distribution.

Padmesh *et al*. (2012) reported in their cluster analysis of *Rauvolfia serpentina* L. that the grouping of samples did not strictly follow the geographical affiliation of the accessions, since all samples from southern and central Kerala clustered along with the lone member from a distant location (Andhra Pradesh, India) to form three clusters with few outliers. Gajera *et al*. (2010) reported that dendrograms showed no clear pattern of clustering according to the locations from where genotypes were collected, indicating little or no location specificity among castor genotypes. Similar results were also reported in Azuki bean (Yee *et al*., 1999) and in groundnut (Dwivedi *et al*., 2001). The high genetic diversity observed implies that an appropriate and efficient protection strategy if employed would help restore the species stability to a significant extent. As *A. rugosa* is widely used in traditional medicine in China and Korea, the results of our study on the genetic diversity and population structure of *A. rugosa* using ISSR markers, and the integration of information obtained by genetic analysis, allow for the development of effective conservation strategies. In this context, the persistence of high genetic diversity within and among the population, and the presence of high polymorphism, total gene diversity, effective number of alleles, Nei's gene diversity, Shannon's index, and UPGMA analysis, ultimately resulted in a nonsignificant correlation between geographical distance and genetic diversity. The aforesaid investigations indicate that large germplasm collections are necessary to represent the overall genetic diversity of *A. rugosa.* Moreover, the genetic diversity information obtained from this study may provide detailed inputs to devise a useful and appropriate tool for the conservation and management of breeding programs for medicinal plant species.

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