Genetic Relationships between *Gardenia jasminoides* var. *radicans* and G. *jasminoides* for. *grandiflora* Using ISSR Markers

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Inter simple sequence repeat (ISSR) markers were performed in order to analyse the genetic relationships of both taxa of *Gardenia jasminoides* var. *radicans* and *G. jasminoides* for. *grandiflora*. Over the 88 fragments, only one locus (ISSR-11-05) was specific to *G. jasminoides* var. *radicans* and only one (ISSR-09-05) *G. jasminoides* for. *grandiflora*. Although *G. jasminoides* var. *radicans* showed low levels of alleles and Shannon's information index than *G. jasminoides* for. *Grandiflora*, however, there was not significant differences ($p > 0.05$). For both taxa the mean genetic diversity of natural populations was higher than that of cultivation populations. It was suggested that domestication processes via artificial selection do not have eroded the high levels of genetic diversity. ISSR markers were more effective in classifying natural populations of wild *G. jasminoides* in East Asia as well as cultivated *G. jasminoides*. The information about the phylogenetic relationship of *G. jasminoides* var. *radicans* and its closely related species is very valuable of the systematics of genus *Gardenia*, the origin of cultivated *G. jasminoides*, and future *G. jasminoides* breeding.

Key words – *Gardenia jasminoides* var. *radicans*, *G. jasminoides* for. *Grandiflora*, ISSR markers

Introduction

Gardenia jasminoides var. *radicans* Makino and *G. jasminoides* for. *grandiflora* Makino (Rubiaceae) are shrub species that are mostly distributed throughout East Asia.

G. jasminoides is diploid ($2n = 22$) with white flowers. Although this family grows low mountains with very fertile soil, it is also partly cultivated near agricultural house as medicines or ornamentals. For example, the flower of *G. jasminoides* var. *radicans* (thereafter var. *radicans*) is also as a very good aroma and as ornamentals with many beautiful petals. *G. jasminoides* for. *grandiflora* (thereafter for. *grandiflora*) is also used as a vegetable crop with flavor and its seeds are mostly used natural yellow dyes with many fruits. Because of these two distinct usages, *G. jasminoides* is conventionally classified into two different varieties, var. *radicans* for a flower type, and for. *grandiflora* for a fruity type. In most cases, men are not recognized because of their morphological similarity to these varieties when it is not a flowering season.

Both taxa have been regarded as medically and ecologically important plants in Korea. In addition, modern research shown that *G. jasminoides* is cholagogic and promotes the secretion of bile[11]. It has also been shown to have an antibacterial effect on many different kinds of bacteria[11]. However, nothing is known for relationships between var. *radicans* and for. *grandiflora* as well as wild and cultivated *G. jasminoides* in Korea. Therefore, detailed studies, in particular at the DNA level, on genetic diversity of natural populations of both taxa wild *G. jasminoides*, and genetic relationships between wild and cultivated *G. jasminoides* are necessary from the viewpoint of plant evolution.

Wild relatives of cultivated plants represent an interesting system from both agricultural and evolutionary view points[4]. Wild relatives usually store great amounts of genetic variation, which may be of present or future interest for plant improvement programs[2].

In recent years, a number of PCR-based DNA markers, such as RAPD (random amplified polymorphic DNA), SSR (simple sequence repeats) and ISSR (inter simple sequence repeats), have been widely used to investigate genetic diversity and populations genetic structure[7,22]. ISSR primers anneal directly to simple sequence repeats and thus, unlike SSR markers, no prior knowledge of target sequences is required ISSR[10]. Also, the sequences that ISSR targets are abundant throughout the eukaryotic genome and evolve rapidly; consequently ISSR may reveal a much higher number of polymorphic fragments per primer than RAPDs[5]. In addition, studies have indicated that ISSRs

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produce more reliable and reproducible bands compared with RAPDs because of the higher annealing temperature and longer sequence of ISSR primers[20]. Thus, ISSRs have proved to be useful in population genetic studies, especially in detecting closely related individuals[5].

The basic questions are: 1) is it possible to detect the pattern of differentiation and speciation using ISSR makers? and 2) has domestication process eroded the levels of genetic variation of the cultivated species as has been shown in many cultivated species?

Materials and Methods

Plant materials

Seven *G. jasminoides* var. *radicans* populations (three cultivated and four wild or natural populations) and six *G. jasminoides* for. *grandiflora* populations (four cultivated and two wild or natural populations) from Korea were provided for this study (Table 1). Because genus *Gardenia* was only two taxa in East Asia, one natural population of *Rubia akane* Nakai which is belonged the same family *Rubiaceae* was used for the outgroup sample.

In Korea, var. *radicans* and for. *grandiflora* populations trend to occur in small, isolated populations, restricted to a small number of isolated sites. I found only four natural populations for var. *radicans* and two for. *grandiflora* which maintain large population sizes (30 trees >) during five years (2000-2003). More than 30 plants (one leaf per plant) were sampled from each population and their leaves were used for molecular analysis.

Table 1. The code number of populations, localities and type of habitat

Code	Localities	Type of habitat
G. jasminoides var. *radicans*		
Jas-R1	Yeonsan-dong, Yeonje-gu, Busan-ci	Cultivated
Jas-R2	Jang-ansa, Gijang-gun, Gyeongsangnam-do	Cultivated
Jas-R3	Chilam-dong, Jinju-ci, Gyeongsangnam-do	Cultivated
Jas-R4	Sangju-meon, Namhae-gun, Gyeongsangnam-do	Natural
Jas-R5	Sangju-ci, Gyeongsangbuk-do	Natural
Jas-R6	Mt. Giri, Sancheong-gun, Gyeongsangnam-do	Natural
Jas-R7	Mt. Ueolchul, Youngam-gun, Cheonlanam-do	Natural
G. jasminoides for. *grandiflora*		
Jas-G1	Mt. Gerong, Gongju-ci, Chungcheongnam-do	Cultivated
Jas-G2	Mt. Hwangac, Gimcheon-ci, Gyeongsangbuk-do	Cultivated
Jas-G3	Cheolma-meon, Gijang-gun, Gyeongsangnam-do	Cultivated
Jas-G4	Mt. Warong, Sacheon-ci, Gyeongsangnam-do	Cultivated
Jas-G5	Sangbongsao-dong, Jinju-ci, Gyeongsangnam-do	Natural
Jas-G6	Susan-ri, Namhae-gun, Gyeongsangnam-do	Natural

Genomic DNA isolation and ISSR analysis

DNA was extracted using the NucleoSpin Plant (Macherey-Nagel Inc., Valencienner, Germany) according to the manufacturer's protocol. To analyze the DNA of individuals, I selected eight decamer primers that produced ISSR bands both in cultivated and wild taxa in a preliminary test.

Amplification reactions were performed in 0.6 ml tubes containing 25 ul of the reaction buffer; 10 mM Tris-HCl, pH 8.8, 50 mM MgCl2, 100 M each of dATP, dCTP, dGTP, dTTP, 0.2 mM primer, 2.1 units Taq DNA polymerase, and 25 ng of genomic DNA. The amplification products were separated by electrophoresis on 1.5% agarose gels, stained with ethidium bromide, and photographed under UV light using Alpha Image TM (Alpha Innotech Co., U.S.A.).

Data analysis

All monomorphic and polymorphic ISSR bands visible by eye were scored randomly unambiguously scored bands were used in the analyses. Each polymorphic ISSR band was given a score of 1 for presence or 0 for absence. Several standard genetic parameters were estimated using the computer program, POPGENE ver. 1.31[23] and AMOVA[6]. The percentage of polymorphic loci (*Pp*), mean number of alleles per locus (*A*), effective number of alleles per locus (A_E), Nei's[16] gene diversity (*H*), and Shannon's Information index (*I*)[14].

The degree of polymorphism was quantified using phenotypic diversity[3]:

$$H_O = \sum pi \log pi$$

where *pi* is the frequency of a particular phenotype *i*. H_O can be calculated and compared for different populations[19]. Let

$$H_{POP} = 1/n \sum H_O$$

be the average diversity over the *n* different populations and let

$$H_{SP} = \sum p \log p$$

be the diversity of species calculated from the phenotypic frequencies *p* in all the populations considered together[19]. Then the proportion of diversity presented within populations, H_{POP}/H_{SP}, can compared with that of between populations $(H_{SP} - H_{POP})/ H_{SP}$.

The estimation of genetic similarity (GS) between genotypes was based on the probability that an amplified fragment from one individual will also be present in another[17].

Nei's gene diversity formula (H_T, H_S, and G_{ST}) were used to evaluate the distribution of genetic diversity within and among populations[16]. Genetic differentiation measured by G_{ST} among populations was also calculated. Furthermore, gene flow (Nm) between the pairs of populations was calculated from G_{ST} values by $Nm = 0.5(1/G_{ST} - 1)$[15].

Cluster analyses

A phylogenetic tree was constructed by the neighbor-joining (NJ) method[21] based on the genetic distance using the NEIGHBOR program in PHYLIP version 3.57[8].

Results

From the 23 decamer primers used for a preliminary ISSR analysis, eleven primers produced good amplification products both in quality and variability (Table 2). Overall, 88 fragments were generated among the tested *G. jasminoides* array. Invariant monomorphic fragments ranged from 5-12 per primer. Over the 88 fragments, only one locus (ISSR-11-05) was specific to var. *radicans* and only one (ISSR-09-05) for. *grandiflora*.

In a simple measure of intrapopulation variability by the percentage of polymorphic bands, the four cultivated populations of var. *radicans* (Jas-R1, Jas-R2, and Jas-R3) exhibited the lower variation than those of natural populations (Table 3). The same trend is observed at for. *grandiflora*. The natural population Jas-G6 showed the highest (64.8%).

In var. *radicans*, the average observed number of alleles (A) was 1.545 across populations, ranging from 1.500 for the population with the lowest mean number of alleles to 1.614 for the population with the highest mean (Table 4). The mean effective number of alleles (A_E) was similar 1.355. Mean gene diversity within populations (H) was 0.207. The Jas-R5 had the highest gene diversity (0.227), while population Jas-R3 had the lowest (0.181). Shannon's information index (I) was 0.306.

In for. *grandiflora*, A, A_E, H, and I were 1.578, 1.387, 0.222, and 0.328 respectively. Although var. *radicans* showed low

Table 2. List of decamer oligonucleotide utilized as primers, their sequences, and associated polymorphic fragments amplified in the *G. jasminoides*

No. of Primer	Sequence(5′ to 3′)	No. of fragments detected	Pp. bands
ISSR-01	-(AG)8G-	5	4
ISSR-02	-(CT)8G-	8	7
ISSR-03	-(CA)7RG-	12	10
ISSR-04	-(TC)8RA-	11	9
ISSR-05	-GGAGAGGAGAGGAGA-	8	6
ISSR-06	-(GA)8GT-	10	8
ISSR-07	-(GA)8CG-	6	4
ISSR-08	-(GA)8TC-	8	6
ISSR-09	-GCGA(AC)8-	9	7
ISSR-10	-GCGA(CA)8-	5	5
ISSR-11	-CCGG(AC)8-	6	6

Table 3. ISSRs generated among 13 populations of *G. jasminoides* var. *radicans* and *G. jasminoides* for. *grandiflora* used to detect polymorphism among genotypes

Population	Total band	Pp. bands	Polymorphism rate(%)
Jas-R1	84	44	50.0
Jas-R2	85	45	51.1
Jas-R3	83	45	51.1
Jas-R4	86	50	56.8
Jas-R5	87	54	61.4
Jas-R6	86	48	54.6
Jas-R7	87	50	56.8
Mean	85.4	48.0	54.5
Jas-G1	83	45	51.1
Jas-G2	83	47	53.4
Jas-G3	80	49	55.7
Jas-G4	80	52	59.1
Jas-G5	87	55	62.5
Jas-G6	86	57	64.8
Mean	83.1	50.8	57.8

Table 4. Summary of genetic diversity for all loci among 13 populations of *G. jasminoides* var. *radicans* and *G. jasminoides* for. *grandiflora*

Population	*A*	*Ae*	H	*I*	*Ho*
Jas-R1	1.500	1.320	0.187	0.277	1.954
Jas-R2	1.511	1.344	0.199	0.293	1.955
Jas-R3	1.511	1.304	0.181	0.273	1.927
Jas-R4	1.568	1.372	0.215	0.319	1.959
Jas-R5	1.614	1.382	0.227	0.338	1.960
Jas-R6	1.546	1.377	0.215	0.316	1.950
Jas-R7	1.568	1.385	0.222	0.326	1.957
Mean	1.545	1.355	0.207	0.306	1.952
Jas-G1	1.511	1.329	0.191	0.283	1.935
Jas-G2	1.534	1.389	0.217	0.316	1.912
Jas-G3	1.557	1.371	0.213	0.315	1.909
Jas-G4	1.591	1.392	0.226	0.334	1.908
Jas-G5	1.625	1.412	0.237	0.350	1.966
Jas-G6	1.648	1.427	0.250	0.369	1.937
Mean	1.578	1.387	0.222	0.328	1.928

levels of alleles and Shannon's information index than for. *grandiflora* Wilcoxin's signed-rank test, however, there was not significant differences ($p > 0.05$).

The phenotypic frequency of each band was calculated and used in estimating genetic diversity (H_O) within populations (Table 4). Although the mean H_O of var. *radicans* (1.952) was higher than that of for. *grandiflora* (1.928), however there was not shown significant difference (paired *t*-test). The same is observed for other measure of genetic variation such as the diversity of populations (H_{POP}, 1.952 vs. 1.928) and species (H_{SP}, 1.965 vs. 1.959) (Table 5)

An assessment of the proportion of diversity present within populations, H_{POP}/H_{SP}, indicated that about 0.7% the total genetic diversity for var. *radicans* was among populations (Table 5). Thus, the majority of genetic variation (99.3%) resided within populations. It is similar to those of for. *grandiflora*. However, the proportion of diversity present between populations (0.016) of var. *radicans* is two times higher than that (0.007) of for. *grandiflora*.

Total genetic diversity (H_T) was 0.229 for var. *radicans* and 0.258 for for. *grandiflora* (Table 6). The interlocus variation of population genetic diversity (H_S) was low (0.075). The average number of individuals exchanged between populations per generation (*Nm*) was estimated to be moderate (1.34).

A similarity matrix based on the proportion of shared fragments (GS) was used to establish the level of relatedness among var. *radicans* populations and for. *grandiflora* populations (Table 7). The estimate of GS ranged from 0.840 between Jas-R6 and Jas-G2 to 0.980 between Jas-R1 and Jas-R2.

Clustering of 13 *G. jasminoides* populations, using the NJ algorithm, was performed based on the matrix of calculated distances (Fig. 1). The phylogenic tree showed two distinct groups var. *radicans* and for. *grandiflora* populations were well separated each other. Some cultivated and wild populations within each group took same positions in the tree.

Table 5. Partitioning of the genetic diversity into within and among 13 populations of both taxa, *G. jasminoides*

Taxa	*H*pop	*H*sp	*H*pop /*H*sp	(*H*sp-*H*pop) /*H*sp
G. jasminoides var. *radicans*	1.952	1.965	0.993	0.007
G. jasminoides for. *grandiflora*	1.928	1.959	0.984	0.016
Total	1.941	1.984	0.978	0.022

Table 6. Estimates of genetic diversity statistics and 22 polymorphic loci in *G. jasminoides*

Taxa	H_T	H_S	G_{ST}	*Nm*
G. jasminoides var. *radicans*	0.229	0.209	0.125	3.450
G. jasminoides for. *grandiflora*	0.258	0.225	0.129	3.366
Total	0.103	0.075	0.272	1.340

Total genetic diversity (H_T), genetic diversity within populations (H_S), proportion of total genetic diversity partitioned among population (G_{ST}) and gene flow (*Nm*) between the pairs of populations.

Discussion

It is clear that var. *radicans* and for. *grandiflora* were sharply separated by ISSR markers, in spite of the ambiguous morphological definition of the two taxa except the number of petals. This implies that gene flow between two crops is limited although they are recorded crossable with each other.

Table 7. Similarity matrix (above diagonal) of 13 *G. jasminoides* populations based on ISSR using Nei and Li (1979) and genetic distances (below diagonal)

Pop.	Jas-R1	Jas-R2	Jas-R3	Jas-R4	Jas-R5	Jas-R6	Jas-R7	Jas-G1	Jas-G2	Jas-G3	Jas-G4	Jas-G5	Jas-G6
Jas-R1	-	0.980	0.958	0.955	0.952	0.947	0.947	0.885	0.866	0.869	0.857	0.883	0.908
Jas-R2	0.020	-	0.970	0.960	0.951	0.965	0.951	0.885	0.861	0.883	0.872	0.897	0.907
Jas-R3	0.043	0.031	-	0.945	0.945	0.947	0.934	0.871	0.849	0.879	0.874	0.891	0.901
Jas-R4	0.046	0.040	0.057	-	0.975	0.966	0.960	0.875	0.857	0.866	0.865	0.907	0.910
Jas-R5	0.050	0.051	0.057	0.025	-	0.957	0.971	0.881	0.855	0.870	0.867	0.911	0.921
Jas-R6	0.055	0.036	0.055	0.035	0.044	-	0.960	0.859	0.840	0.860	0.854	0.907	0.902
Jas-R7	0.055	0.050	0.068	0.041	0.030	0.041	-	0.881	0.852	0.858	0.872	0.914	0.918
Jas-G1	0.121	0.122	0.139	0.133	0.127	0.152	0.127	-	0.962	0.965	0.972	0.946	0.945
Jas-G2	0.144	0.150	0.164	0.155	0.157	0.174	0.160	0.039	-	0.955	0.946	0.934	0.925
Jas-G3	0.141	0.124	0.129	0.144	0.139	0.151	0.153	0.035	0.047	-	0.970	0.941	0.935
Jas-G4	0.154	0.137	0.135	0.146	0.142	0.158	0.137	0.029	0.056	0.031	-	0.936	0.937
Jas-G5	0.124	0.108	0.115	0.098	0.093	0.098	0.090	0.056	0.068	0.061	0.066	-	0.970

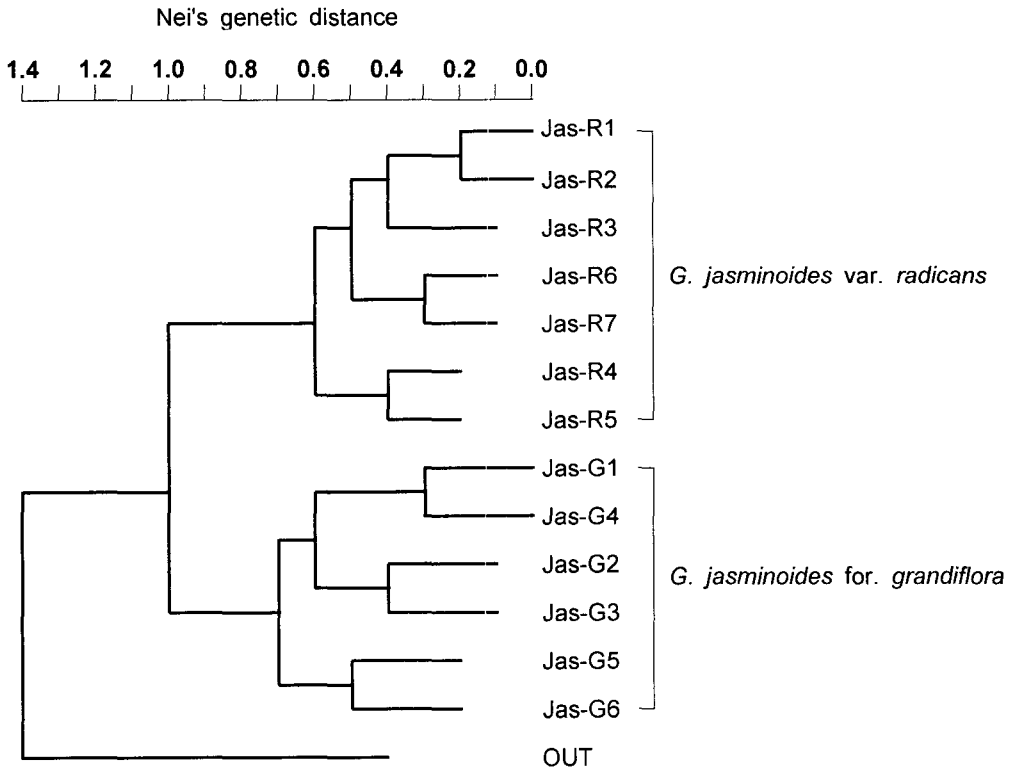

Fig. 1. A neighbor-joining tree for two *G. jasminoides* taxa based on ISSR analysis. OUT: Out group, *Rubia akane*.

The most striking result emerging from this study is the similarity between var. *radicans* and for. *grandiflora*, in terms of genetic diversity and it structure, although var. *radicans* was higher than those of for. *grandiflora*. Because genus *Gardenia* was only two taxa in East Asia, analyses of genetic relationships may suggest hypotheses on the origin and appearance of for. *grandiflora*.

Only one unique which showed in one species locus was found in for. *grandiflora*. Thus, a hypothesis suggests that identical spontaneous mutations in at least same contemporary times would have occurred, leading to the appearance of the. Such an event is statistically highly improbable[9]. Another proposed hypothesis suggests that a recent tree introduced from China or Japan to Korea. However, the existence of several natural populations might invalidate this hypothesis.

To a large extent, the allelic composition of cultivated var. *radicans* and for. *grandiflora* represent subsets of natural populations. For example, cultivated var. *radicans* was found to have fewer total bands (85 vs. 87), lower polymorphism (mean 50.7% vs. 57.4%), and lower genetic diversity (mean 0.189 vs. 0.220) than natural populations. The similar trend is observed at the for. *grandiflora* (Table 3). The comparison of cultivated and wild *G. jasminoides* revealed that the domestication processes via artificial selection have eroded the levels of genetic diversity in cultivated *G. jasminoides*. It is in general accord with the concept that most crops show a reduced level of polymorphisms as compared to their presumed progenitors[4]. Other studies also found that wild species usually maintain higher level of polymorphism compared to cultivated species[1]. But in other species such as barley and common buckwheat, cultivated populations have more genetic variability[18]. In addition, for soybean the domestication process has not eroded the levels of genetic variation[12]. Ultimately high levels of variability of the wild (natural) species are expected because they were not subject to any of the selection pressures of domestication, and the maintenance of higher genetic variability would favor their survival under natural conditions[18].

Although I did not analyze further subdivision of a local population, it may be inferred that ISSR variation that resided mainly within wild *G. jasminoides* populations is maintained in patchily distributed subpopulations or demes, either by random drift of neutral alleles or microenvironmental selection for adaptive alleles. However, no great local differentiation of ISSR variation was observed.

Gene flow between populations was moderate. The val-

ues of estimated *Nm* for var. *radicans* and for. *grandiflora* were 3.450 and 3.366, respectively. These values are large enough to nullify the local differentiation of neutral alleles by random drift[13]. Cultivated fields of *G. jasminoides* and natural populations of wild *G. jasminoides* can be seen side by side. Hence, it can be expected weak or low gene flow from cultivated populations to natural populations. For cultivated *G. jasminoides*, farmers have moved seeds from place to place to forage on good products. Seed dispersal by farmers may be one of the mechanisms of gene flow among cultivated populations.

In a phylogenetic tree based on ISSR markers, the position of the populations in the NJ tree and their geographical position did not almost completely matched in the Korean populations (Fig. 1). Some cultivated and wild populations within each group took same positions in the tree. A few juveniles or seeds of wild populations were transferred to artificial populations directly or indirectly via botanical gardens to artificial populations. It explains the low differentiation among populations and disturbs genetic relationships among populations. In addition, it is relevant to stress the ISSR markers that I used did not allow me to discriminate among all populations. Thus, various marker including ISSR markers may be effective in classifying natural populations of wild *G. jasminoides* as well as cultivated *G. jasminoides* in Korea.

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초록 : ISSR을 이용한 꽃치자와 열매치자의 유전적 관계

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꽃치자(*Gardenia jasminoides* var. *radicans*)와 열매치자(*G. jasminoides* for. *grandiflora*)는 *Gardenia*속으로는 우리나라에 두 분류군밖에 없으며 열매치자는 약용, 식용, 꽃치자는 방향제로 쓰인다. 그런데 꽃이 지고 나면 형태적으로 구분이 거의 되지 않는다. ISSR분석으로 이들 종의 유전적 다양도와 집단구조를 실시하였다. 88개의 DNA 분절에서 한 분류군에만 나타나는 특이밴드가 탐지되었다. 한국의 세 야생 집단은 분리되어 있고 패치 분포를 보이지만 재배종에 비해 높은 유전적 다양성을 유지하고 있었다. 열매치자가 꽃치자보다 유전적 다양성이 높았으며 ISSR 마커로 이들 분류군이 잘 분리되었다. 또한 야생 집단이 재배 집단보다 다양성이 약간 높으나 유의성은 없었다. 이는 재배화과정에서 유전적 다양성의 일부 상실이 있었으나 원위적인 채취와 식재로 야생 집단과 재배 집단의 유전적 교류가 존재하였음을 시사한다.