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Genetic Diversity and Population Structure of *Spiraea prunifolia* for. *simpliciflora* by Inter-Simple Sequence Repeats

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85 individual *Spiraea prunifolia* for. *simpliciflora* (*Rosaceae*) were sampled to examine the genetic diversity and population structure of *S. prunifolia* for. *simpliciflora* populations. Inter-simple sequence repeats (ISSR) produced 65 polymorphic loci and identified 78 ISSR genotypes. Three multilocus genotypes were shared by more than one plant within a population. Total genetic diversity values (H_T) and interlocus variation in the within-population genetic diversity (H_S) were 0.293 and 0.183, respectively. On a per-locus basis, the proportion of total genetic variation due to differences among populations (G_{ST}) was 0.373. This indicated that about 37.3% of the total variation was among populations. ISSR markers are very effective in classifying natural population levels of *S. prunifolia* for. *simpliciflora* in Korea. In addition, insights into the relative gene diversity among and within populations of *S. prunifolia* for. *simpliciflora* would be useful in plant breeding and also for the development of strategies for *ex situ* conservation of plant genetic resources.

Key words : Inter-simple sequence repeats (ISSR), genetic diversity, *Spiraea prunifolia* for. *simpliciflora*

Introduction

Spiraea (also known as meadowsweet) is a genus with 80-100 species of shrubs in the subfamily Spiraeoideae (*Rosaceae*) [10]. They are native to the temperate Northern Hemisphere, with the greatest diversity in eastern Asia [15]. *Spiraea* contains methyl salicylate and other salicylates, compounds with similar medicinal properties of aspirin. Unlike aspirin, meadowsweet is effective in treating stomach disorders in minute amounts. The salicylates in this plant are a highly effective analgesic, anti-inflammatory, and fever reducer, without the side affects attributed to aspirin [25].

Spiraea prunifolia Siebold & Zucc. forma *simpliciflora* Nakai occurs in mountains of Korea. This species is also reported from China and Taiwan [15]. It occupies specialized habitats in subalpine communities and forest at lower elevation mountain sites. When this species is represented potential clones or clusters, pure population sizes range from 1 to 10 or more plants [9]. Thus, Korea Forest Service has listed the species as the protected species since 2000.

Inter-simple sequence repeats (ISSR) amplification is a technique which can rapidly differentiate between closely related individuals [27]. The ISSR technique involves anchoring of designed primers to a subset of simple sequence repeats (SSRs) and amplify the region between two closely spaced, oppositely oriented SSRs [4]. SSRs, also called microsatellites, are randomly arranged arrays of short repeats comprising a few nucleotides [5,20], and there is estimated to be a total of 5×10^3 to 3×10^5 microsatellites per plant genome [1]. ISSR within a species is also useful for detecting genetic polymorphisms and can be a highly variable region of DNA [18]. ISSR have the advantage over randomly amplified polymorphic DNA (RAPD) in that the primers are longer, allowing for more stringent annealing temperatures [23]. These higher temperatures apparently provide a higher reproducibility of bands than in RAPD [10,24]. Tsumura et al. [21] found that most of their ISSR bands (96%) segregated according to Mendelian expectations.

This study was carried out to examine seven populations of *S. prunifolia* for. *simpliciflora* in order to evaluate genetic diversity and population structure in this species.

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Materials and Methods

DNA extraction and ISSR procedure

85 individuals of *S. prunifolia* for. *simpliciflora* were sampled from seven natural populations (Fig. 1). The distance between the selected individuals was about 25 m to avoid including individuals emanating from the same lineage because this species can reproduce by means of stems or roots. Individuals were labeled by removing a portion of the fresh

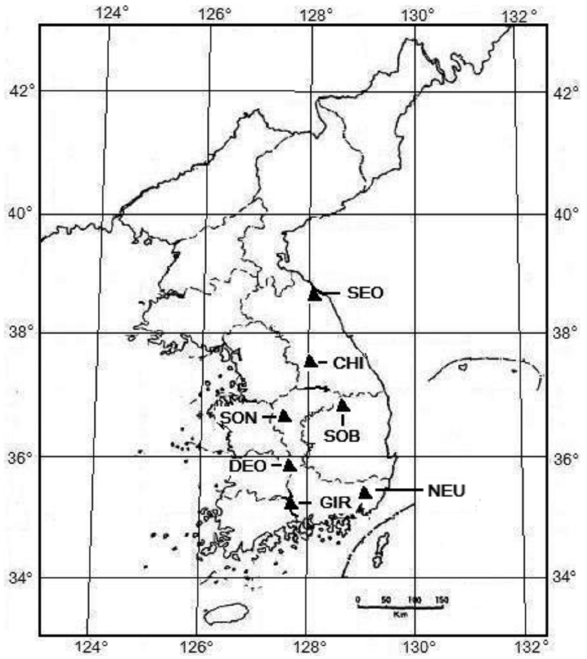

Fig. 1. The collection sites of *S. prunifolia* for. *simpliciflora* in Korea. SEO: Mt. Seorak, Seorak-dong, Sokcho-shi, Gangwon-do, CHI: Mt. Chiak, Socho-myeon, Wonju-shi, Gyeonggi-do, SON: Mt. Sonkni, Naesongni-myeon, Boeun-gun, Chungcheongbuk-do, SOB: Mt. Sobak. Punggi-eup, Yeongju-shi, Gyeongsangbuk-do, DEO: Mt. Deogyu, Gyebuk-myeon, Jangsu-gun, Jeollabuk-do, GIR: Mt. Giri, Samjang-myeon, Sancheong-gun, Gyeongsangnam-do, NEU: Mt. Neundong, Miryang-shi, Gyeongsangnam-do.

leaf. DNA was extracted with the plant DNA Zol Kit (Life Technologies Inc., Grand Island, New York, USA) according to the manufacturer's protocol.

Fifteen arbitrarily chosen primers of Bioneer Technoligies (Korea) were used. All the reactions were repeated twice and only reproducible bands were scored for analyses.

Amplification reactions were performed in total 50 μl mixed solution containing 2.5 μl of the reaction buffer, 10 mM Tris-HCl (pH 8.8), 1.25 mM dNTP, 5.0 pM primer, 2.5 units Taq DNA polymerase, and 25 ng of genomic DNA. Initial denaturation was carried out for 1 min at 94°C, followed by 35 cycles of 50 sec at 94°C, 30 sec at 45°C, 2 min at 72°C, and a final 10 min extension at 72°C.

A 100 bp ladder DNA marker (Pharmacia) was used in the end of for the estimation of fragment size. 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., USA).

Statistical analyses

All ISSR bands were scored by eye and only unambiguously scored bands were used in the analyses. Because ISSRs are dominant markers, they were assumed that each band corresponded to a single character with two alleles, presence (1) and absence (0) of the band, respectively. Loci were named based on the primer and observed band size.

The following genetic parameters were calculated using a POPGENE computer program (ver. 1.31) developed by Yeh et al. [26]: allele frequencies, the percentage of polymorphic loci (P_p), mean numbers of alleles per locus (A), effective number of alleles per locus (A_E), gene diversity (H), and Shannon's index of phenotypic diversity [12].

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 [11]. GS was converted to genetic distance (1-GS). Homogeneity of variance among accessions was tested by Bartlett's statistics.

The Mantel test was examined the correlation between the matrix of genetic distance and spatial distance within a site. By use of allele frequencies, the probability that each genotype could arise independently was calculated following Parks and Werth [13].

A phenetic relationship was constructed by the neighbor-joining (NJ) method [17] using the NEIGHBOR program in PHYLIP version 3.57 [2].

Results

From the fifteen decamer primers used for a preliminary ISSR analysis, nine primers of them produced good amplification products both in quality and variability (Table 1). For the 85 samples, these nine primers produced 76 scorable bands, 65 of which were polymorphic (85.5%).

Seven of 85 were composed of plants with identical ISSR type. However, 91.8% of sampled individuals contain plants of more that one ISSR type, apparently representing independent fertilization events. The spatial relationships of the shared and unique genotypes in both populations (DEO and DEU) are shown in Fig. 2. Three multilocus genotypes, DO7 in DEO population, DE1 and DE8 in DEU population, were shared by more than one plant within a population. The genotypes did not had individuals from other populations. The highest number of individuals sharing a

Table 1. Lists of decamer oligonucleotides utilized as primers, their sequences, and associated fragments amplified in *S. prunifolia* for. *simpliciflora*

No. of primer	Sequence (5′→3′)	No. of fragments	No. of polymorphic bands
ISSR01	-$(AG)_8$G-	7	5
ISSR02	-$(CT)_8$G-	10	10
ISSR03	-$(CA)_8$G-	8	7
ISSR04	-$(TC)_8$RA-	11	9
ISSR05	-G$(GA)_4$GGAGA-	9	8
ISSR06	-$(GA)_8$GY-	8	7
ISSR08	-$(GA)_8$TC-	10	8
ISSR10	-GCGA$(CA)_8$-	8	8
ISSR11	CCGG$(AC)_8$	5	3
Total	-	76	65

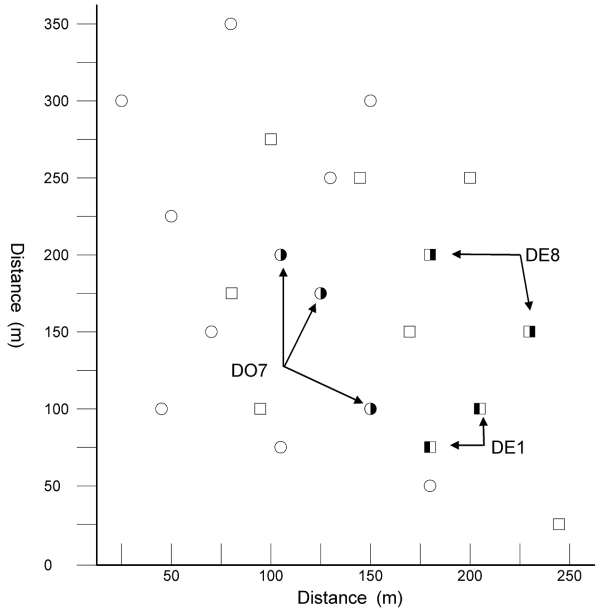

Fig. 2. Spatial distribution in two populations of *S. prunifolia* for. *simpliciflora*. Circles represent single plants of population DEO and split circles do plants with the shared genotype in this population. Squares represent single plants of population DEU and split squares do plants with the shared genotypes in this population. Plants with shared are designated with arrows.

genotype at a population was three. All individuals of the five remainder populations had only unique genotypes in their populations and omitted their spatial relationships and distributions.

Across populations, the average number of alleles per locus (A) was 1.513, ranging from 1.461 to 1.579 (Table 2). The effective numbers of alleles per locus (A_E) at the species (A_{ES}) and the population levels (A_{EP}) were 1.519 and 1.317, respectively. Mean genetic diversity within populations was 0.183. In particular, the population GIR had the highest expected diversity (0.226); population DEU, the lowest (0.141). Mean Shannon's information index (I) was 0.272, ranging from 0.217 to 0.330.

Table 2. Measures of genetic variation for *S. prunifolia* for. *simpliciflora*

Pop.	Np	Pp	A	A_E	H	I
GIR	44	57.9	1.579	1.397	0.226	0.330
DEO	35	46.1	1.461	1.231	0.141	0.217
NEU	39	51.3	1.513	1.248	0.149	0.229
SOB	41	54.0	1.540	1.369	0.212	0.311
SON	38	50.0	1.500	1.260	0.155	0.237
CHI	40	52.6	1.526	1.372	0.210	0.306
SEO	36	47.4	1.474	1.345	0.191	0.277
Mean	39.0	51.3	1.513	1.317	0.183	0.272
Species	65	85.5	1.855	1.519	0.294	0.437

The number of polymorphic loci (Np), percentage of polymorphism (Pp), mean number of alleles per locus (A), effective number of alleles per locus (A_E), gene diversity (H), and Shannon's information index (I)

Total genetic diversity values (H_T) and interlocus variation in the within-population genetic diversity (H_S) were 0.293 and 0.183, respectively (Table 3). On a per-locus basis,

Table 3. Estimates of genetic diversity of *S. prunifolia* for. *simpliciflora*. Total genetic diversity (H_T), genetic diversity within populations (H_S), the proportion of total genetic diversity partitioned among populations (G_{ST}), and gene flow (Nm)

Locus	Sample Size	H_T	H_S	G_{ST}	Nm
ISSR01-03	85	0.081	0.076	0.059	7.944
ISSR01-04	85	0.446	0.265	0.407	0.729
ISSR01-05	85	0.461	0.154	0.667	0.250
ISSR01-06	85	0.182	0.059	0.674	0.242
ISSR01-07	85	0.292	0.264	0.096	4.712
ISSR02-01	85	0.462	0.288	0.377	0.826
ISSR02-02	85	0.210	0.139	0.339	0.976
ISSR02-03	85	0.4929	0.380	0.228	1.689
ISSR02-04	85	0.325	0.261	0.199	2.013
ISSR02-05	85	0.496	0.248	0.500	0.500
ISSR02-06	85	0.489	0.189	0.614	0.315
ISSR02-07	85	0.488	0.192	0.607	0.324
ISSR02-08	85	0.140	0.097	0.307	1.129
ISSR02-09	85	0.356	0.317	0.109	4.102
ISSR02-10	85	0.467	0.286	0.387	0.793
ISSR03-02	85	0.493	0.380	0.228	1.689
ISSR03-03	85	0.349	0.298	0.145	2.947
ISSR03-04	85	0.492	0.216	0.561	0.391
ISSR03-05	85	0.492	0.204	0.584	0.356
ISSR03-06	85	0.488	0.192	0.607	0.324

Table 3. Continue

Locus	Sample Size	H_T	H_S	G_{ST}	Nm
ISSR03-07	85	0.233	0.202	0.136	3.185
ISSR03-08	85	0.356	0.310	0.130	3.350
ISSR04-01	85	0.418	0.175	0.581	0.360
ISSR04-02	85	0.039	0.036	0.061	7.747
ISSR04-03	85	0.345	0.271	0.214	1.834
ISSR04-05	85	0.376	0.162	0.571	0.376
ISSR04-06	85	0.079	0.059	0.258	1.437
ISSR04-08	85	0.489	0.275	0.437	0.644
ISSR04-09	85	0.203	0.161	0.211	1.876
ISSR04-10	85	0.459	0.264	0.426	0.675
ISSR04-11	85	0.490	0.210	0.571	0.375
ISSR05-01	85	0.500	0.131	0.738	0.178
ISSR05-02	85	0.434	0.163	0.624	0.302
ISSR05-03	85	0.466	0.232	0.503	0.495
ISSR05-04	85	0.110	0.069	0.372	0.846
ISSR05-06	85	0.489	0.400	0.182	2.241
ISSR05-07	85	0.198	0.163	0.175	2.366
ISSR05-08	85	0.417	0.322	0.228	1.694
ISSR05-09	85	0.499	0.153	0.694	0.221
ISSR07-01	85	0.331	0.111	0.666	0.251
ISSR07-02	85	0.403	0.260	0.355	0.908
ISSR07-04	85	0.464	0.387	0.167	2.497
ISSR07-05	85	0.481	0.200	0.584	0.357
ISSR07-06	85	0.079	0.059	0.258	1.437
ISSR07-07	85	0.239	0.137	0.426	0.674
ISSR07-08	85	0.079	0.059	0.258	1.437
ISSR08-02	85	0.488	0.475	0.027	17.741
ISSR08-03	85	0.076	0.057	0.248	1.520
ISSR08-04	85	0.480	0.389	0.188	2.156
ISSR08-05	85	0.481	0.200	0.584	0.357
ISSR08-06	85	0.079	0.059	0.258	1.437
ISSR08-07	85	0.260	0.126	0.518	0.465
ISSR08-08	85	0.079	0.059	0.258	1.437
ISSR08-09	85	0.474	0.428	0.097	4.662
ISSR09-01	85	0.488	0.416	0.147	2.899
ISSR09-02	85	0.214	0.127	0.408	0.727
ISSR09-03	85	0.086	0.062	0.284	1.261
ISSR09-04	85	0.478	0.384	0.197	2.044
ISSR09-05	85	0.481	0.200	0.584	0.357
ISSR09-06	85	0.079	0.059	0.258	1.437
ISSR09-07	85	0.311	0.181	0.416	0.701
ISSR09-08	85	0.080	0.059	0.258	1.437
ISSR10-02	85	0.488	0.483	0.009	53.207
ISSR10-03	85	0.238	0.233	0.020	25.103
ISSR10-05	85	0.497	0.459	0.076	6.041
Mean	85	0.293	0.183	0.373	0.839

the proportion of total genetic variation due to differences among populations (G_{ST}) was 0.373. This indicated that about 37.3% of the total variation was among populations. These values indicate that most of the genetic diversity of *S. prunifolia* for. *simpliciflora* is found within populations and there is a little among-population differentiation. The estimate of gene flow, based on G_{ST}, was slightly low among Korean populations of *S. prunifolia* for. *simpliciflora* (N_m=0.839).

Table 4. Genetic identity (upper diagonal) among seven populations of *S. prunifolia* for. *simpliciflora* and genetic distances (low diagonal) based on ISSR analysis

Pop.	GIR	DEO	NEU	SOB	SON	CHI	SEO
GIR	-	0.994	0.828	0.876	0.870	0.878	0.816
DEO	0.006	-	0.830	0.875	0.867	0.874	0.815
NEU	0.189	0.187	-	0.899	0.717	0.724	0.736
SOB	0.132	0.134	0.107	-	0.820	0.815	0.830
SON	0.139	0.143	0.332	0.198	-	0.983	0.849
CHI	0.130	0.135	0.323	0.205	0.017	-	0.850
SEO	0.203	0.205	0.307	0.186	0.164	0.163	-

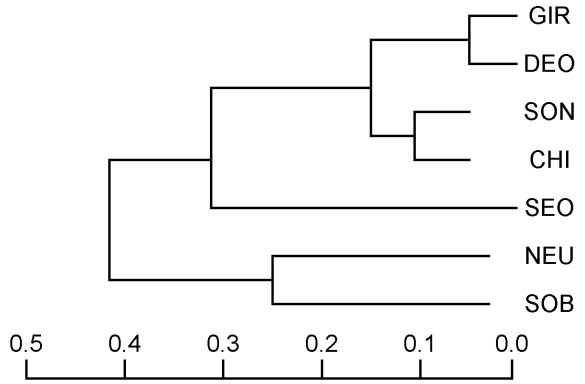

Fig. 3. A phenogram showing the relationships among seven populations based on data of genetic distance obtained by ISSR.

Values of genetic distance (D) were $<$0.332 (Table 4). Genetic identity values among pairs of populations ranged from 0.717 to 0.994.

The Mantel test was used to test for correlations between the matrix of genetic diversity and spatial distance. Genetic diversity correlated with spatial distance in *S. prunifolia* for. *simpliciflora* populations.

Clustering of accessions was performed based on the matrix of calculated distances using the NJ algorithm (Fig. 3). In dendrogram, all populations were well separated from each other. Thus, ISSR markers are very effective in classifying natural population levels of *S. prunifolia* for. *simpliciflora* in Korea.

Discussion

Seven *S. prunifolia* for. *simpliciflora* populations with 85 individuals included 78 genotypes, 73 of which were restricted

to a single plant. Three multilocus genotypes were shared by more than one plant within a population (Fig. 2). Because clustering of *S. prunifolia* for. *simpliciflora* is considered mainly vegetation spreading, it is expected that the shared genotypes among individuals at a long distance is the result of siblines by random mating.

This unique genotypes resulted from outcrossing. The probability of two different individuals having the same genotype will increase with higher rates of inbreeding. Hence the likelihood of a parent producing an identical offspring should increase the probability of identical genotypes from independent sexual events. Therefore, the probability of shared genotypes arising from same parents is a conservative estimate and the true values would be high.

At natural populations, animals may be responsible for long distance dispersal events. It is expected the pollen is disperse only a short distance from the parent plant, producing a patchy distribution. Clutivated *S. prunifolia* for. *simpliciflora* are probably dispersed mainly through vegetation reproduction. The plant clusters are an extreme example of this type of distribution. However, none of the disjunct plants sampled less than 25 meter apart had identical genotypes. The Mantel test supported this by showing correlation between genetic distance and spatial distribution. The data are consistent with the dispersal of seed being an important factor in the population structure of this species. It is possible that some of these disjunct identical genotypes were formed by neighboring individuals.

The differences among populations (G_{ST}) was 0.373. This value showed a little genetic differentiation among populations of *S. prunifolia* for. *simpliciflora*. The most of interpopulational genetic differentiation in species of *S. prunifolia* for. *simpliciflora* was assumed to be the product of low rates of gene flow (0.839).

In ISSR analysis, *S. prunifolia* for. *simpliciflora* maintains a higher than average level of genetic diversity compared with other plant species [6], although there is the difference in methodology (e.g., ISSR is a dominant marker and allozyme is a co-dominant marker) that may preclude meaningful comparisons. For example, its genetic diversity of 0.183 is higher than that for temperate-zone species (0.146), species with a sexual reproduction mode (0.151), and those with a long-lived woody habit (0.177) [6]. The same trends are observed at other genetic parameters such A and A_E. Thus, genetic diversity in *S. prunifolia* for. *simpliciflora* is higher than that of most plant on land. The mechanism for maintaining this high level of diversity in *S. prunifolia* for. *simpliciflora*, a supposedly outcrossing plant, requires examination. Before addressing the source of variation, it is needed to ask whether ISSR data can be used to test the assumptions that *S. prunifolia* for. *simpliciflora* is indeed self-incompatibility. Although ISSR loci can potentially distinguish many individuals, they unfortunately do not measure true heterozygosity due to their generally dominant inheritance. It is helpful to study with codominant isozyme markers [3]. For this reason, the level of inbreeding cannot be determined with ISSR data alone. Based on the results of other studies, inbreeding is expected. There are mechanisms for maintaining polymorphisms such as variable selection over space, which may maintain levels of polymorphisms identical to random mating [7]. However, no data exist to test this hypothesis in *S. prunifolia* for. *simpliciflora*. Alternatively, some ISSR bands may not behave in a Mendelian manner. For example Tsumura et al [21] found 3 of the 77 bands studied departed from Mendelian expectations. This suggests a high mutation rate from generation to generation. It is therefore possible that high mutation rates of ISSR loci in *S. prunifolia* for. *simpliciflora* may account for the genetic diversity observed. The simple sequence repeats, which are the basis for the primer site of ISSRs, are known to have a high rate of gaining and losing repeat units due to DNA slippage [19].

The genetic diversity of plant populations concerns natural resource managers. The NEU population had a lower genetic diversity than the others. Several reasons may account for this including sampling size. However, this population does stand out in two ways that need further investigation. The NEU population is isolated from the Taeback Mountains. The NEU population may be less diverse because of fewer individuals contributing to the gene pool. Population size has been correlated with genetic diversity for several plant species, with smaller populations maintaining less genetic diversity [22].

Typical populations of *S. prunifolia* for. *simpliciflora* are small and distributed in patches. Until recently, much of the Korean forest has been disturbed by the cutting of trees [8]. The main concern of persistence of *S. prunifolia* for. *simpliciflora* is continued habitat destruction and fragmentation such as a medicine and road construction. Consequently, natural *S. prunifolia* for. *simpliciflora* populations have suffered individuals' loss, reduction of populations and fragmentation of remaining populations by human activities such as

over-gathering medicinal plants. Thus insights into the relative gene diversity among and within wild populations of *S. prunifolia* for. *simpliciflora* would be useful in plant breeding and also for the development of strategies for *ex situ* conservation of plant genetic resources [13,16]. The mean 37.3% genetic differentiation coefficient of *S. prunifolia* for. *simpliciflora* from ISSR. Based on the available data such as H and G_{ST} values (Tables 2 and 3) several populations of each group should be preserved, especially those with high variation. These populations could be used as a source of genetic diversity for the restoration of genetically poor populations.

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초록 : 조팝나무의 유전적 다양성과 집단구조 분석을 위한 ISSR 분석

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조팝나무는 목본이며 약용으로 매우 중요하며 우리나라 산림청 지정 보호수종이다. 이 속내 7집단에서 85개체에 대해 ISSR (inter simple sequence repeats) 마커로 이들 집단에 대한 유전적 변이와 집단구조를 조사하였다. 65개의 다형성 좌위와 78개 ISSR 유전자형을 얻었다. 덕유산 집단과 능동산 집단에는 1개체 이상 공유하는 유전자형이 포함되어 있었다. 전체 유전적 다양도는 종수준과 집단수준에서 각각 0.293과 0.183이였다. 집단의 분화(G_{ST})는 0.373으로 나타났다. 따라서 전체 변이의 37.3%는 집단 간에 있었다. ISSR 마커로 한국 내 조팝나무 집단의 분화는 잘 분리되어 ISSR로 조팝나무 집단 연구에 유익하며 유전적 다양도와 집단구조의 통찰은 종보전에 대한 기초 정보로 활용할 수 있을 것으로 사료된다.