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Genetic and Phylogenetic Relationships of Genus *Hemerocallis* in Korea Using ISSR

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Genus *Hemerocallis* is a herbaceous species and some species among their taxa are very important herbal medicines. We evaluated representative samples of the eight taxa in Korea with inter simple sequence repeats (ISSR) markers to estimate phylogenetic relationships within taxa of this genus. The studied taxa were *Hemerocallis fulva* L., *H. fulva* for. *kwanso*, *H. dumortieri* Morren, *H. coreana* Nakai, *H. hongdoensis* M.G.Chung & S.S.Kang, *H. middendorffi* Trautv. et Mayer, *H. thunbergii* Baker, *H. minor* Miller. In addition, we investigated the genetic variation and structure of Korean populations of these taxa. The mean genetic diversity was 0.098 across species, varying from 0.068 to 0.123. A low level of genetic variation was found in populations of *Hemerocallis* species. Specially, gene diversity for *H. minor* was maintained the highest among genus *Hemerocallis*. An indirect estimate of the number of migrants per generation (Nm=0.218) indicated that gene flow was not extensive among Korean populations of *Hemerocallis* species. The phylogenic tree showed distinct three clades. One includes *H. fulva*, *H. fulva* for. *kwanso* and *H. middendorffi*. Another includes three *Hemerocallis* species, *H. dumortieri*, *H. thunbergii* and *H. minor*. The *H. coreana* and *H. hongdoensis* were shown as the sister group to the second clades. Although the size of sampling was not large enough for eight Korean *Hemerocallis* species, the analyses of ISSRs will certainly provide an enhanced view on the phylogeny of species.

Key words : Genus *Hemerocallis*, inter simple sequence repeats, phylogenetic relationships

Introduction

Genus *Hemerocallis*, a genus of the family *Liliaceae* consists of 30 species [16]. They are diploid species (2n=22) and its varieties are 2n=33 [14]. Genus *Hemerocallis* is a herbaceous species and mainly distributes in northeastern Asia [19]. Most species of the genus are economically important for its leaves, flowers, and roots, which historically were used in Korea for medicine, chronic rheumatism, a heart simulant, and jaundice [9]. Most species of the genus are also cultivated as garden products in Korea [14]. For example, young leaves and budding flowers of *H. fulva* L. are commonly called "Neomnamul" and "Geumchimchae", respectively. In addition, the dry roots of *H. fulva* is called "Woenchochae" and they are used as a Chinese medicine.

The genus *Hemerocallis* is comprised of about eight or ten taxa (seven species, two variety, and one form) in Korea [14]. The taxonomy of *Hemerocallis* has processed mainly through morphological characteristics [16,17,19] and random amplified polymorphic DNA (RAPD) markers [9]. However morphological characteristics are restricted by their resolving power mainly because of the small number of variables available.

Morphological variations in leaf shape, stem length, and flower type were different among *Hemerocallis dumortieri* Morr., *H. fulva* var. *kwanso* Regel., and *H. thunbergii* Baker [9]. However, no differences was observed among lines of *H. fulva* [9].

During the last decade several novel DNA-markers have emerged, which have been rapidly used as common routine laboratory tools available for genome analysis. Since their introduction, RAPD markers have become very popular and have been used for a variety of purposes in plant genetic analysis, mainly because of the easy of analysis [5,20]. In addition, inter-simple sequence repeats (ISSR) markers are a potentially useful tool for species discrimination [1,15]. Especially ISSR assay has been useful in determining genetic relationships among closely related species [7]. ISSR analysis is quick, robust, requires minimal preliminary work [10]. Cultivar identification, parentage determination, genetic relationship evaluation, and estimation of population genetic variability are some examples of the multiple uses of the ISSR technique [1,10].

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The aims of this study were; 1) to estimate how much total genetic diversity is maintained in the *Hemerocallis* species, 2) to describe how genetic variation is distributed within and among species, and to elucidate the suitability and efficiency of the ISSR analyses in assessing the phenetic relationships among the related species in Korea.

Thus, it is indispensably important issue to establish the distribution system on the basis of discrimination of the herbal medicines and quality control. The first step in this process is to develop efficient markers.

Materials and Methods

Sampling procedure and DNA extraction

All of the eight taxa of genus *Hemerocallis, H. fulva* L., *H. fulva* for. *kwanso, H. dumortieri* Morren, *H. coreana* Nakai, *H. hongdoensis* M.G.Chung & S.S.Kang, *H. middendorffi* Trautv. et Mayer, *H. thunbergii* Baker, and *H. minor* Miller were collected from natural populations in Korea (Table 1). To analyze the proportion of genetic diversity among and within taxa, twenty plants were randomly collected from each taxon.

The genomic DNA of the 180 samples including outgroup (*Hosta capitata*) was extracted from fresh leaves (1.2 g). DNA was extracted using the plant DNA Zol Kit (Life Technologies Inc., Grand Island, New York, USA) according to the manufacturer's protocol. The DNA concentration of each sample was determined spectrometrically and was electrophoresed on a 0.8 % agarose gel to confirm quality.

ISSR analysis

Eleven arbitrarily chosen primers of Bioneer Technoligies (Korea) were used. All the reactions were repeated twice and only reproducible bands were scored for analyses. From the eleven decamer primers used for a preliminary ISSR analysis, six primers of them produced good amplification products both in quality and variability.

Amplification reactions were performed in 0.6 ml tubes containing 2.5 μl of the reaction buffer, 10 mM Tris-HCl (pH 8.8), 1.25 mM each of dATP, dCTP, dGTP, dTTP, 5.0 pM primer, 2.5 units Taq DNA polymerase, and 25 ng of genomic DNA. 2.5 μl (500 μg/ml) of 100 bp ladder DNA marker (Pharmacia, Piscataway, NJ) was used in the end of the gel 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.

The following genetic parameters were calculated using a POPGENE computer program (ver. 1.31) developed by Yeh et al. [23]: the percentage of polymorphic loci (P_p), mean numbers of alleles per locus (A), effective number of alleles per locus (A_E) and gene diversity (H) [18].

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

A phenetic relationship was constructed by the neighbor-joining (NJ) method [21] using the NEIGHBOR program in PHYLIP version 3.57 [6].

Results

From the eleven decamer primers used for a preliminary

Table 1. Codes and population locations of genus *Hemerocallis*

Codes	Scientific name	Localities
FUL	*H. fulva* L.	Sucheong-dong, Osan-si Gyeonggi-do
FFK	*H. fulva* for. *kwanso* Regel	Sohol-eup, Pocheon-si, Gyeonggi-do
DUM	*H. dumortieri* Morren	Cheongha-myeon, Buk-gu, Pohang-si, Gyeongsangbuk-do
COR	*H. coreana* Nakai	Cheongha-myeon, Buk-gu, Pohang-si, Gyeongsangbuk-do
HON	*H. hongdoensis* M.G.Chung & S.S.Kang	Hongdo-ri, Heuksan-myeon, Sinan-gun, Hongdo, Jeollanam-do
MID	*H. middendorffi* Trautv. et Mayer	Sohol-eup, Pocheon-si, Gyeonggi-do
THU	*H. thunbergii* Baker	Ongyong-myeon, Kwangyang-si, Jeollanam-do
MIN	*H. minor* Miller	Seorak-myeon, Gapyeng-gun, Gyeonggi-do

ISSR analysis, six primers of them produced good amplification products for eight taxa of genus *Hemerocallis* in quality and variability, while the remaining primers (ISSR-01, ISSR-02, ISSR-03, ISSR-07, ISSR-08, ISSR-09, ISSR-10, ISSR-12) did not amplified or showed smear banding patterns (Table 2). For eight taxa of genus *Hemerocallis,* the primers ISSR-04 and ISSR-05 revealed the seven and nine distinct fragments, respectively. Both primers, ISSR-06 and ISSR-11 revealed eleven PCR fragments. The primer ISSR-11 was shown the highest percentage of polymorphic fragments, while the lowest percentage was generated by the primer ISSR-04. Overall, 56 reliable ISSR markers were produced with sizes ranging from 600 to 2,100 bp. A total of 32 (57.1%) of these bands were polymorphic. The remaining fragments were monomorphic in all taxa.

In a simple measure of intraspecies variability by the percentage of polymorphic bands, *H. dumortieri* and *H. coreana* exhibited the lowest variation (21.1%). *H. minor* showed the highest (31.6%) (Table 3). A mean of 26.4% of the loci was polymorphic within taxa.

Table 2. List of decamer oligonucleotides utilized as primers, their sequences, and associated polymorphic fragments amplified in genus *Hemerocallis*

No. of primer	Sequence (5′→3′)	No. of fragments
ISSR-04	$(TC)_8RA$	7
ISSR-05	$G(GA)_4GGAGA$	9
ISSR-06	$(GA)_8GY$	11
ISSR-08	$(GA)_8TC$	8
ISSR-10	$GCGA(CA)_8$	10
ISSR-11	$CCGG(AC)_8$	11
Total	-	56

Table 3. Measures of genetic variation for genus *Hemerocallis*

Taxa	*Np*	*Pp*	*A*	A_E	*H*	*I*
FUL	11	29.0	1.290	1.212	0.117	0.170
FFK	11	29.0	1.290	1.204	0.115	0.168
DUM	8	21.1	1.211	1.109	0.068	0.104
COR	8	21.1	1.211	1.130	0.075	0.112
HON	11	29.0	1.290	1.177	0.106	0.158
MID	9	23.7	1.238	1.166	0.095	0.138
THU	10	26.3	1.263	1.138	0.085	0.130
MIN	12	31.6	1.316	1.215	0.123	0.181
Mean	10.0	26.4	1.264	1.169	0.098	0.145
Total	32	57.1	1.842	1.560	0.324	0.479

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*)

Mean number of alleles per locus (*A*) ranged from 1.211 to 1.316 with a mean of 1.264. *H. minor* showed the highest and *H. dumortieri* and *H. coreana* did the lowest. The effective number of alleles per locus (A_E) ranged from 1.109 to 1.215 with a mean of 1.169.

The phenotypic frequency of each band was calculated and used in estimating genetic diversity (*H*) within taxa. As the typical populations of wild *Hemerocallis* were small, isolated, and patchily distributed for natural populations, they maintained a low level of genetic diversity for polymorphic primers. The total *H* was 0.098 across species. Shannon's index of phenotypic diversity (*I*) of *H. minor* (0.181) was highest of all taxa and *H. fulva* was the second (0.170).

Total genetic diversity values (H_T) varied between 0.069 for *H. thunbergii* and 0.176 *H. dumortieri,* for an average over all polymorphic loci of 0.323 (Table 4). Interlocus variation in the within-population genetic diversity (H_S) was low (0.098). On a per-locus basis, the proportion of total genetic variation due to differences among populations (G_{ST}) ranged from 0.256 for *H. hongdoensis* to 0.671 for *H. minor,* with a mean of 0.697. This indicated that about 69.7% of the total variation was among populations. The estimate of gene flow, based on G_{ST}, was slightly high among populations (Nm=0.218).

A similarity matrix based on the proportion of shared fragments (GS) was used to evaluate relatedness among species (Table 5). The genetic similarities between species ranged from a minimum value of 0.568 between *H. fulva* and *H. hongdoensis* and the maximum value of 0.991 between *H. fulva* and *H. fulva* for. *kwanso*. Values of genetic distance (D) were <0.567.

Fig. 1 showed the amplified products of the primer ISSR-06. *H. coreana* and *H. hongdoensis* were not shared with

Table 4. Estimates of genetic diversity of genus *Hemerocallis*

Populations	H_T (SD)	H_S (SD)	G_{ST}	*Nm*
FUL	0.110 (0.034)	0.076 (0.020)	0.314	1.093
FFK	0.118 (0.038)	0.049 (0.010)	0.585	0.355
DUM	0.176 (0.026)	0.039 (0.010)	0.497	0.506
COR	0.069 (0.022)	0.050 (0.012)	0.279	1.295
HON	0.102 (0.030)	0.076 (0.020)	0.256	1.450
MID	0.099 (0.034)	0.055 (0.016)	0.440	0.636
THU	0.095 (0.032)	0.032 (0.005)	0.669	0.247
MIN	0.133 (0.042)	0.044 (0.010)	0.671	0.245
Total	0.323 (0.028)	0.098 (0.009)	0.697	0.218

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

Table 5. Genetic identity (upper diagonal) among eight taxa of genus *Hemerocallis* and genetic distances (low diagonal) based on ISSR analysis

Taxa	FUL	FFK	DUM	COR	HON	MID	THU	MIN
FUL	-	0.991	0.947	0.651	0.568	0.631	0.662	0.713
FFK	0.009	-	0.932	0.657	0.588	0.666	0.682	0.722
DUM	0.055	0.071	-	0.658	0.587	0.609	0.646	0.671
COR	0.430	0.420	0.418	-	0.668	0.686	0.769	0.789
HON	0.566	0.530	0.533	0.404	-	0.756	0.665	0.689
MID	0.461	0.406	0.496	0.378	0.280	-	0.775	0.746
THU	0.412	0.383	0.438	0.263	0.408	0.254	-	0.913
MIN	0.338	0.326	0.399	0.237	0.372	0.293	0.091	-

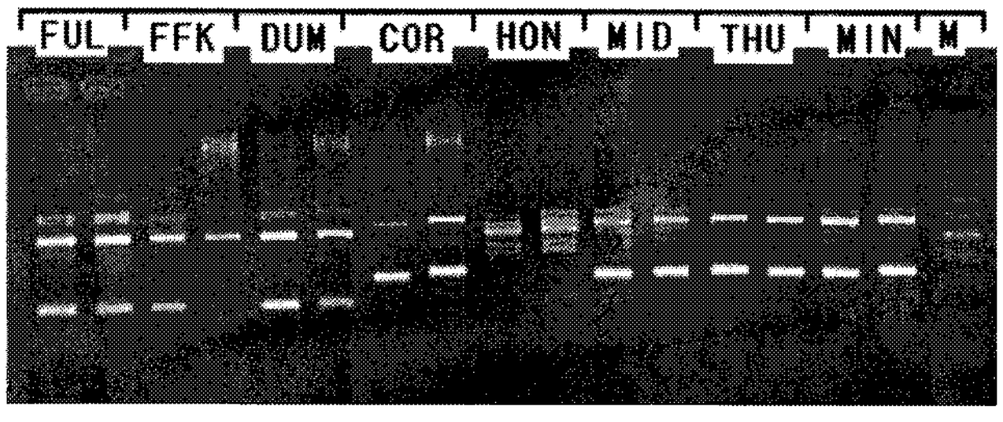

Fig. 1. ISSR profiles of genus *Hemerocallis* by ISSR-06 primer.

other species. Results of Fig. 2 also seemed to demonstrate differences in the degree of shared bands among species.

Clustering of accessions using the NJ algorithm was performed based on the matrix of calculated distances (Fig. 3).

Three main clades were recognized: (1) *H. fulva, H. fulva* for. *kwanso,* and *H. middendorffi;* (2) *H. dumortieri, H. thunbergii,* and *H. minor;* (3) *H. coreana* and *H. hongdoensis.* Korean and Chinese accessions were well separated as distinct groups. The tree also showed genetic differentiation between wild and cultivated accessions for Korean species.

Discussion

In ISSR analysis, the level of genetic diversity of eight species belonging to genus *Hemerocallis* maintained lower than the average of other plant species, although there is the difference in methodology (e.g., dominant marker and co-dominant marker) that may preclude meaningful comparisons. For example, its genetic diversity of 0.098 is lower than that of temperate-zone species (0.146), dicots (0.136), species with a sexual reproduction mode (0.151), and those with a long-lived herbaceous (0.205) [8]. The mean percentage of polymorphic loci at the species level is 26.4%, which is also lower than that of species with temperature-zone distributions (48.5%), dicots (44.8%), and species

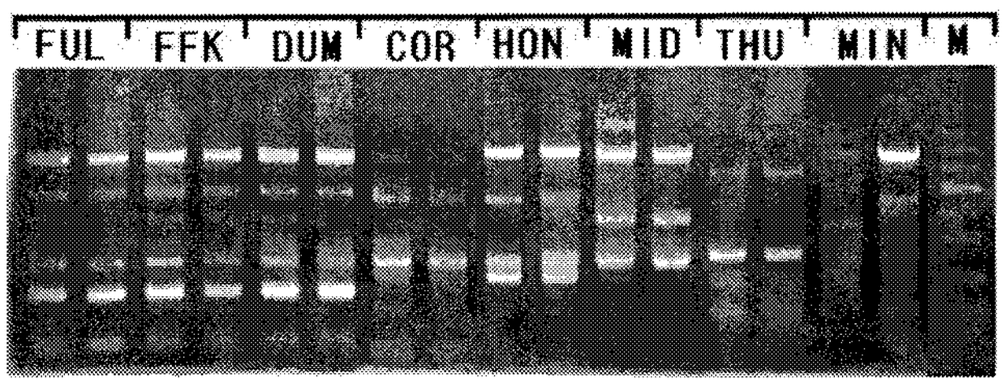

Fig. 2. ISSR profiles of genus *Hemerocallis* by ISSR-11 primer.

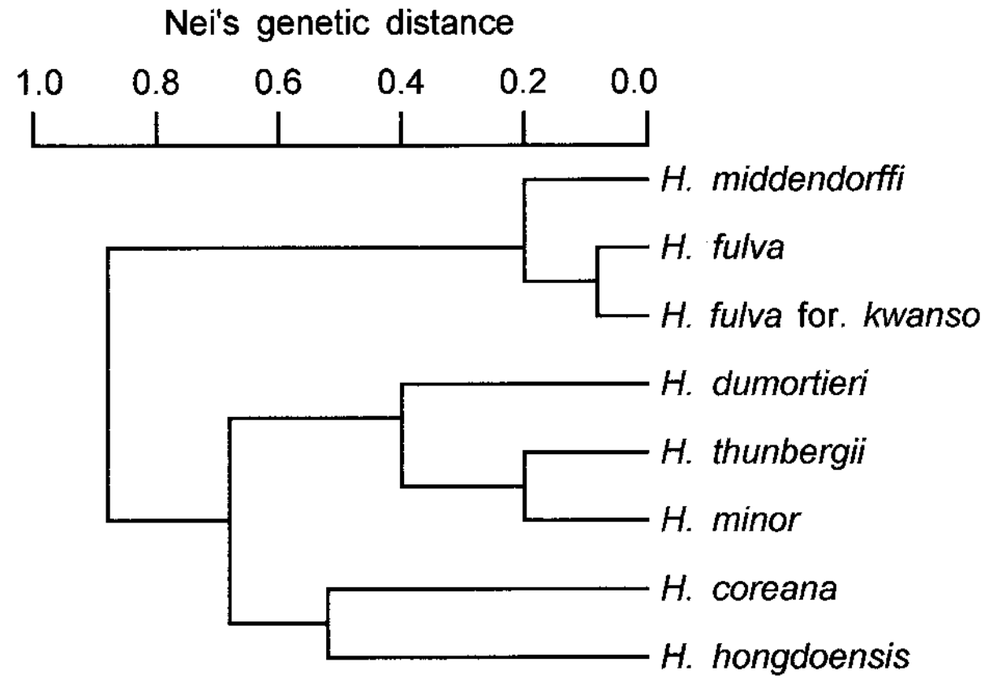

Fig. 3. A phenogram showing the relationships among eight species of genus *Hemerocallis* based on data of genetic distance obtained by ISSR.

with a sexual reproduction mode (51.6%) and long-lived perennial herbaceous (39.6%) [8].

Genetic diversity in some species of genus *Hemerocallis* has been examined with allozymes [2,3,4,11,12] and RAPD (ranmomly amplified polymorphic DNA) [9]. Populations of the three species, *H. thunbergii, H. middendorffii,* and *H. exaltata* from Japan maintain high levels of allozyme variation: mean expected heterozygosities were 0.288, 0.209, and 0.169, respectively. In this study, mean heterozygosities for eight species was 0.098 (Table 2). In RAPD analysis, the percentage of polymorphic loci in *H. fulva, H. fulva* for. *kwanso,* and *H. dumortieri,* is 34% [9]. The percentage of polymorphic loci at the genus level for genus *Hemerocallis* is 57.1% (Table 2).

Chromosome numbers are mostly 2n=22. *H. dumortieri, H. coreana, H. hongdoensis, H. middendorffi, H. thunbergii,* and *H. minor* are all 2n=22. While *H. fulva* and *H. fulva* var. *kwanso* has 2n=33 [14]. This is in agreement with the results of this study (Fig. 3).

In the study with RAPD, *H. fulva* var. *kwanso* was different in shape of leaf and type of flower from *H. fulva. H. dumortieri* and *H. middendorffii* were clearly different from *H. fulva* in shape of leaf, the length of stem, and type of flower

[9]. *H. fulva* var. *kwanso* and *H. thunbergii* were grouped into small clades, while *H. dumortieri* and *H. thunbergii* have distinct relationships. This results by RAPD is not in agreement with our results obtained by ISSR.

Considering that genus *Hemerocallis* consists of 30 species in the world, it is surprising that about eight or ten taxa of those species exists in only the South Korea. There was a significant positive correlation between genetic and geographic distance among populations and it was highly likely due to habitat differentiation (r=0.561; p<0.001). Yasumoto and Yahra [22] reported that post-pollination reproductive isolation between diurnally and nocturnally flowering daylilies, *H. fulva* and *H. citrina*. In, *H. fulva* and *H. citrina*, flower longevity was only half a day and the flowering time overlapped only 2 hr in the evening. However, there is no isolation against hybridization when *H. citrina* is a pollen recipient, and the resulting hybrids showed high fertility [22]. Indirect estimates of the number of migrants per generation (Nm=0.218) indicate that gene flow have not been extensive in genus *Hemerocallis*. This is not surprising when we consider the fact that no specialized seed dispersal mechanism is known, flowers are visited by bees, and the present-day populations of the species are discontinuous and isolated [11].

Alternative interpretations can be made for the pattern we observed. First, the reproductive isolation was weakened under secondary contact on Hongdo Island (*H. hongdoensis*). Second, the reproductive isolation among species on inland is in a primary condition, and the stronger isolation is developed among Korean populations of several species in genus *Hemerocallis*. *H. hongdoensis* is considered as a by-product of local adaption in different habitats.

Although the size of sampling was not large enough for genus *Hemerocallis*, the analyses of ISSRs will certainly provide an enhanced view on the phylogeny of species. In summary, ISSR amplifications is a simple and effective method,that can be used in the identification and intraspecific differentiation of genus *Hemerocallis* with a high degree of discrimination and reproducibility. In addition, additional molecular experiments such as AFLP (amplified fragment length polymorphism), microsatellites, and ITS (nuclear ribosomal DNA internal transcribed spacer sequences) are necessary to identify species.

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초록 : ISSR에 의한 한국 내 원추리속 식물의 유전적 및 계통학적 연구

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원추리속(Genus *Hemerocallis*) 식물은 초본이며 일부 종은 약용으로 매우 중요하다. 이 속내 8개 분류군에 대해 ISSR (inter simple sequence repeats) 마커로 계통학적 관계를 분석하였다. 조사한 식물은 원추리(*Hemerocallis fulva*), 왕원추리(*H. fulva* for. *kwanso*), 각시원추리(*H. dumortieri*), 골잎원추리(*H. coreana*), 홍도원추리(*H. hongdoensis*), 큰원추리(*H. middendorffi*), 노랑원추리(*H. thunbergii*), 애기원추리(*H. minor*)이다. 또한 이들 분류군에 대한 유전적 변이와 구조를 조사하였다. 종간 유전적 다양도는 0.068~0.123이며 평균 유전적 다양도는 0.098로 전반적으로 낮았다. 애기원추리가 가장 높은 값을 나타내었다. 세대 당 이주하는 개체수는 매우 적었다(*Nm*=0.218). 원추리속 종은 계통도에서 세 분지군으로 나누어졌다. 한 그룹은 *H. fulva, H. fulva* for. *kwanso, H. middendorffi*였다. 다른 그룹은 *Hemerocallis, H. dumortieri, H. thunbergii, H. minor*였다. 나머지는 *H. coreana*와 *H. hongdoensis*로 두 번째 그룹과 자매군을 형성하였다. 비록 종 내 적은 개체수로 분석하였지만 원추리속 식물종이 ISSR 마커로 잘 분리되었다.