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# Genetic diversity of *Millettia japonica* in Korea as revealed by ISSR analysis

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# ISSR 분석으로 살펴본 애기등의 유전적 다양성

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**ABSTRACT:** This study employed inter-simple-sequence repeat (ISSR) to assess genetic variation among 189 individuals representing 10 populations (nine in Korea and one in Japan) of *Millettia japonica,* which has recently been lifted from the endangered species of Korea. The calculated Shannon's information index value ($I = 0.2689$) of the species was appreciable and was higher than other endangered leguminous woody taxa. Gochang ($I = 0.2968$), Namhae ($I = 0.2951$), and Mt. Toham ($I = 0.2823$) populations showed relatively high genetic diversity, whereas the Kyushu (in Japan) population ($I = 0.2487$) exhibited the lowest. The results of an analysis of molecular variance indicated that 86.49% of the diversity was attributed to within populations, and 13.51% to differences among populations, suggesting that *M. japonica* populations do not have significant geographic differentiation and that the gene flow between populations exists to some extent ($Nm = 1.8446$). Continuous habitat monitoring should be conducted to conserve genetic diversity of *M. japonica*, particularly for those populations with relatively high genetic diversity. Selection of many individuals from the populations in Gochang, Namhae, and Mt. Toham is thought to be an appropriate strategy for *ex situ* conservation of *M. japonica* in Korea.

**Keywords:** *Millettia japonica*, endangered species, conservation, ISSR, genetic diversity

**적 요:** 최근 환경부 멸종위기종 급에서 해제된 애기등(*Millettia japonica*) 집단의 유전적 다양성 분석을 위해 10개 집단(한국 9집단, 일본 1집단) 189개체에 대한 ISSR (Inter-Simple-Sequence-Repeat) 분석을 수행하였다. 조사된 애기등의 유전적 다양성은 같은 과내의 멸종위기종보다 더 높은 것으로 조사되었다(Shannon's information index: $I = 0.2689$). 집단별 유전적 다양성은 전북 고창($I = 0.2968$) 집단과 경남 남해($I = 0.2951$), 경북 토함산($I = 0.2823$) 집단이 높았으며, 일본 큐슈($I = 0.2487$) 집단이 가장 낮았다. 애기등 10개 집단이 공유하는 유전변이의 양은 전체 유전변이의 86.49%로 나타났고, 전체의 13.51%가 집단간 유전적 차이에 의한 것으로 나타났다. 또한 조사된 애기등 집단간 교류를 나타내는 *Nm* 값(1.8446)이 비교적 높은 것으로 나타났으며, 이에 따라 집단간 유전적 분화가 크게 일어나지 않았음을 알 수 있었다. 본 연구 결과 애기등의 유전자원 보존을 위해서는 유전다양도가 높은 집단을 중심으로 지속적인 자생지 모니터링이 요구되며, 현지외 보존을 위해서는 더 높은 유전적 다양도를 지닌 전북 고창, 경남 남해, 경북 토함산 집단에서 다수 개체를 선발하는 보존 전략이 적절할 것으로 판단된다.

**주요어:** 애기등, 멸종위기종, 보전, ISSR, 유전다양도

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*Millettia japonica* (Siebold & Zucc.) A. Gray is a deciduous leguminous vine classified under the *Callerya* group of tribe Millettieae (Hu et al., 2002; Hu and Chang, 2003). The *Callerya* group includes *Callerya* Endlicher, *Wisteria* Nuttall, *Afgekia* Craib, and *Millettia japonica*, which have plastid genomes lacking the inverted repeat region (Wojciechowski et al., 1999). This molecular feature is consistent with morphological similarities among these taxa (Lavin, 1987).

*M. japonica* has limited distribution in the southern part of Korea (with the exception of Jeju Island) and Japan (S. Honshu, Kyushu, Shikoku). In Korea, a small number of populations occur in Gyeongsangnam-do (GN), Gyeongsangbuk-do (GB), Jeollanam-do (JN) and Jeollabuk-do (JB). According to the Ministry of Environment and Forest Service survey, most populations of *M. japonica* in GN (Geoje, Tongyeong), GB (Gyeongju), JN (Jindo, Shinan) and JB (Mt. Moak and Gochang) are located near or close to seaside areas. An additional population was recently discovered on Mt. Toham in Gyeongju city, and this was confirmed as the northernmost population in Korea (Fig.1).

The Korean Ministry of Environment designated *M. japonica* as a protected wild plant in 1998 and as a vulnerable species in 2001, yet its population size has decreased significantly due to natural and/or anthropogenic threats. Faced with extinction, *M. japonica* status was uplifted to second-grade endangered species in 2005. *M. japonica* populations were detected to be sufficient in 2012; thus, the Ministry of Environment released *M. japonica* from the endangered species list. However, *M. japonica* remains a rare plant in Korea and requires ongoing management and various conservation efforts including a population genetic study. Despite conservation importance of this species, intraspecific information such as genetic diversity and genetic differentiation among populations remains largely unknown.


**Fig. 1.** The locations of the sampled *Millettia japonica* populations.

Inter simple sequence repeat (ISSR) primer sequences consist of a di- or tri-nucleotide simple sequence repeat [e.g. $(CA)_7$, $(AGT)_5$] usually with a 3'-anchoring sequence of 1–3 nucleotides for amplifying the regions between two microsatellite repeats (Wolfe and Liston, 1998; refer Table 2). Despite limitations as co-dominant markers, ISSR analyses have been successfully used to assay the levels of clonal diversity and genetic variation within and among populations of endangered plant taxa (Fang, 1997; Xiao et al., 2004; Ge et al., 2005; Chung et al., 2006; Liu et al., 2013). Among the approaches to evaluate the genetic diversity of populations, ISSR markers are more reproducible (Semagn et al, 2006) with more polymorphic bands produced (Reddy et al., 2002) compared with those of random amplified polymorphic DNA.

We employed ISSR markers to examine the levels and distribution of genetic variability within and among populations of *M. japonica*, which can serve as basic information for developing efficient conservation strategies of this species in Korea.

## Materials and Methods

### Plant material

Fresh leaves of 189 individuals were collected from 10 natural populations of *M. japonica* in Korea and Japan. Samples from national parks were collected with permission and support from the relevant authorities. Each population was represented by about 20 plants as shown in Fig. 1 and Table 1. A smaller number of individual was sampled from the Mt. Toham population due to its limited population size. All individuals sampled within each population were separated by at least 5 m to avoid collecting shoots connected by a stolon. The leaf samples were stored at −70°C until required for genetic analysis. Voucher specimens of one representative accession from each population were deposited in the Herbarium of Hallym University.

### DNA extraction and polymerase chain reaction (PCR) amplification

Total genomic DNA was extracted from frozen leaves using Extract-N-Amp™ Plantkits (Sigma, St. Louis, MO, USA) following the manufacturer's manual. Ten of 79 ISSR primer sets obtained from the University of British Columbia (Biotechnology Laboratory, University of British Columbia,

**Table 1.** Localities, sample size (*Ns*) and population size (*N*) of *Millettia japonica* populations examined in this study.

| Taxa | *Locality | Population abbreviation | GPS code | Ns | N |
|---|---|---|---|---|---|
| *M. japonica* | Jindo, JN, Korea | MJ 01 | N34° 21' 46.3 E126° 10' 16.2 | 20 | >1000 |
| *M. japonica* | Shinan, JN, Korea | MJ 02 | N34° 46' 50.2 E126° 06' 12.9 | 20 | >1000 |
| *M. japonica* | Gochang, JB, Korea | MJ 03 | N35° 28' 50.7 E126° 33' 55.0 | 20 | >1000 |
| *M. japonica* | Mt. Moak, JB, Korea | MJ 04 | N35° 43' 39.8 E127° 03' 13.0 | 20 | >1000 |
| *M. japonica* | Mt. Toham, GB, Korea | MJ 05 | N35° 48' 28.4 E129° 19' 52.6 | 11 | <100 |
| *M. japonica* | Namhae, GN, Korea | MJ 06 | N34° 45' 40.3 E127° 57' 20.8 | 20 | 500 |
| *M. japonica* | Geoje, GN, Korea | MJ 07 | N34° 46' 26.0 E127° 57' 48.9 | 20 | 500 |
| *M. japonica* | Tongyeong, GN, Korea | MJ 08 | N34° 48' 01.2 E128° 23' 47.6 | 19 | 500 |
| *M. japonica* | Yeosu, JN, Korea | MJ 09 | N34° 24' 25 E128° 46' 47 | 20 | 500 |
| *M. japonica* | Fukuoka, Kyushu, Japan | MJ 10 | N33° 49' 52.1 E130° 47' 33.3 | 19 | <100 |

*GB: Gyeongsangbuk-do, GG: Gyeonggi-do, GN: Gyeongsangnam-do, JB: Jeollabuk-do, JN: Jeollanam-do

**Table 2.** Sequences of ISSR primers, number of bands scored, and polymorphic information content (PIC) for each ISSR primer.

| Primer | Sequence 5' - 3' | Amplified bands | | PIC |
|---|---|---|---|---|
| | | total | polymorphic(%) | |
| UBC810 | $(GA)_8T$ | 16 | 16(100) | 0.323 |
| UBC814 | $(CT)_8A$ | 8 | 8(100) | 0.260 |
| UBC815 | $(CT)_8G$ | 26 | 25(96) | 0.260 |
| UBC825 | $(AC)_8T$ | 22 | 22(100) | 0.339 |
| UBC827 | $(AC)_8G$ | 15 | 14(93) | 0.422 |
| UBC844 | $(CT)_8RC$ | 18 | 18(100) | 0.338 |
| UBC847 | $(CA)_8RG$ | 30 | 30(100) | 0.312 |
| UBC850 | $(GT)_8YC$ | 24 | 24(100) | 0.288 |
| UBC855 | $(AC)_8YT$ | 21 | 19(90) | 0.455 |
| UBC878 | $(GGAT)_4$ | 27 | 27(100) | 0.223 |
| | Total/Average | 207 | 203(98) | 0.322 |

Y is a single letter abbreviation for mixed base positions for C and T; and R is a single letter abbreviation for mixed base positions for A and G.

http://www.biotech.ubc.ca/services/naps/primers/Primers.pdf) produced reliable and reproducible bands for data scoring, and these were selected for evaluating genetic diversity (Table 2). PCR was performed in a total volume of 20 μl containing 10 ng template DNA, 2.5 μL of 10× Ex taq buffer, 2 μL of 2.5 nM dNTPs, 1 μL of 10 pmol primers, 0.1 μL of *Taq* polymerase and distilled water up to the final volume. The PCR was carried out using a GeneAMP PCR system 9700 thermocycler programmed with the following temperature profile consisting of an initial predenaturation step at 95°C for 5 min, followed by 30 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 55°C, extension for 1 min at 72°C, and a final extension step of 10 min at 72°C.

## Data analysis

Data were scored as 1 for the presence (dominant type) and 0 for the absence (recessive type) of a DNA band in each locus and then used for further analyses. Polymorphic information content (PIC) was calculated by applying the formula (Bostein et al., 1980): $PIC_i = 1 - \sum P^2_{ij}$, where P*ij*, is the frequency of the *j*th allele for the *i*th marker. The POPGENE 32 program (Yeh et al., 1999) was used to analyze the statistical measures of the observed number of marker alleles (*na*), effective number of alleles (*ne*), Nei's genetic diversity (*h*) (Nei, 1973), Shannon's information index (*I*) (Shanon and Weaver, 1949), number of polymorphic loci (*np*), and percentage of polymorphic loci (*fp*). Gene flow (expressed as *Nm*) was estimated from $G_{ST}$values [$Nm = 0.5\ (1 - G_{ST})/G_{ST}$; McDermott and McDonald 1993]. The $G_{ST}$ values generated by POPGENE were given for the whole population and expressed as the gene diversity of the single population subtracted from the gene diversity of the total population divided by the gene diversity of the total population ($G_{ST} = 1 - H_S/H_T$). Analysis of molecular variance (AMOVA) was performed, using Arlequin v3.1 (Excoffier et al., 1992) to determine the degree of genetic diversity distribution ($F_{ST}$) within and among populations. A dendrogram was constructed using the unweighted pair group method average (UPGMA) clustering procedure based on Nei's genetic distance (Nei, 1978) implemented in POPGENE 32 (Yeh et al., 1999). To examine the correlation between genetic and geographic distances among populations Mantel tests (10000 randomizations) were conducted using the computer program IBD (isolation by distance) ver. 1.52 (Bohonak, 2002). A principal components analysis (PCA) was performed using NTSYSpc software ver. 2.21 (Rohlf, 1998) to demonstrate Dice grouping. The Dice similarity coefficient was calculated from the equation Dice*ij* = 2a*i* (2a*ij* + b*i* + c*j*) (Dice, 1945).

# Results

## Genetic diversity

Polymorphisms were found in 203 of the 207 loci analyzed from the 10 primers. The average percentage of polymorphic bands was 98%. The size range of the amplified products was 200-1900 bp, and the number of products per locus varied from eight in UBC 814 to 30 in UBC 847 (Table 2). The PIC values for the ISSR primers used in the analysis were estimated. The PIC values were the highest (0.455) in UBC 855, whereas UBC 878 revealed the lowest (0.223) PIC value (Table 2). The average PIC value for the amplification products was 0.322.

The genetic diversity indices of *Millettia japonica* estimated using the ISSR markers are shown in Table 3. With an overall average of 57.80% as a whole, the percentage of polymorphic loci (*fp*) of the MJ 03 (64.02%) and MJ 06 (61.68%) showed relatively high values, whereas MJ 05 (53.27%) exhibited the lowest value (Table 3).

The calculated gene diversity (*h*) of *M. japonica* populations was the highest for MJ 03 (0.1933) and the lowest value for MJ 10 (0.1584), respectively (Table 3). A recently discovered small (*N*: <100) wild population (Mt. Toham population, MJ 05) of the species exhibited relatively high gene diversity values (*h*: 0.1880). The Shannon information index (*I*) was the highest (0.2968) for MJ 03 and the lowest (0.2487) for MJ 10 (in average 0.2689).

## Genetic differentiation

The AMOVA analysis of the 10 *M. japonica* populations showed an $F_{ST}$ value of 0.1351, indicating 13.51% of the among population variation and 86.49% of the within population variation (Table 4). The average total genetic diversity of *M. japonica* ($H_T$) was 0.2177, mean within-population genetic diversity ($H_S$) was 0.1712, whereas the proportion of total genetic diversity found among the population ($G_{ST}$) was 0.2133, and the gene flow (*Nm*) estimated from the $G_{ST}$ value was 1.8446. In the bioplot distribution from the two-dimensional graphs obtained by the PCA, the individuals of each population were mixed with individuals from other populations, indicating low genetic differentiation among populations (Fig. 2). Nei's genetic distance (Nei, 1978) between MJ 05 and MJ 07 showed a maximum value of 0.0732, and the distance between MJ 01 and MJ 02 showed a minimum value of 0.0245. The average distance value of all *M. japonica* populations was 0.0484 (data not shown).

A cluster analysis (UPGMA) was used to generate a dendrogram based on Nei's genetic distance among the 10 populations studied to represent the genetic relationship among populations (Fig. 3). The clustering pattern was generally not correlated with geographic distribution. For example, the

**Table 4.** Analysis of molecular variance (AMOVA) at the ISSR loci in 10 populations of *Millettia japonica* ($F_{ST}$ : 0.1351, P-value < 0.001).

| Source of variation | df | Sum of squares | Variance component | Percentage of variation |
|---|---|---|---|---|
| Among populations | 9 | 697.849 | 3.06955 | 13.51 |
| Within populations | 179 | 3517.749 | 19.65223 | 86.49 |
| Total | 188 | 4215.598 | 22.72178 | |

**Table 3.** Genetic variability measures of 10 populations of *Millettia japonica*. Refer to Table 1 for population abbreviations.

| Population | ss | *na* (mean±s.d) | *ne* (mean±s.d) | *h* | *I* | *np* | *fp* |
|---|---|---|---|---|---|---|---|
| MJ 01 | 20 | 1.5794±0.4948 | 1.2842±0.3314 | 0.1753 | 0.2709 | 124 | 57.94 |
| MJ 02 | 20 | 1.5607±0.4975 | 1.2532±0.3169 | 0.1589 | 0.2490 | 120 | 56.07 |
| MJ 03 | 20 | 1.6402±0.4811 | 1.3222±0.3583 | 0.1933 | 0.2968 | 137 | 64.02 |
| MJ 04 | 20 | 1.5841±0.4940 | 1.2737±0.3131 | 0.1723 | 0.2682 | 125 | 58.41 |
| MJ 05 | 11 | 1.5327±0.5001 | 1.3176±0.3613 | 0.1880 | 0.2823 | 114 | 53.27 |
| MJ 06 | 20 | 1.6168±0.4873 | 1.3224±0.3579 | 0.1931 | 0.2951 | 132 | 61.68 |
| MJ 07 | 20 | 1.5467±0.4990 | 1.2723±0.3504 | 0.1634 | 0.2514 | 117 | 54.67 |
| MJ 08 | 19 | 1.5654±0.4969 | 1.2679±0.3337 | 0.1645 | 0.2555 | 121 | 56.54 |
| MJ 09 | 20 | 1.5794±0.4948 | 1.2982±0.3606 | 0.1773 | 0.2712 | 124 | 57.94 |
| MJ 10 | 19 | 1.5748±0.4955 | 1.2546±0.3233 | 0.1584 | 0.2487 | 123 | 57.48 |

ss = sample size (number of individuals)
*na* = Observed number of alleles (mean and standard variation)
*ne* = effective number of alleles (mean and standard variation)
*h* = Nei's (1973) gene diversity (mean)
*I* = Shannon's (1949) Information index (mean)
*np* = number of polymorphic loci
*fp* = percentage of polymorphic loci.

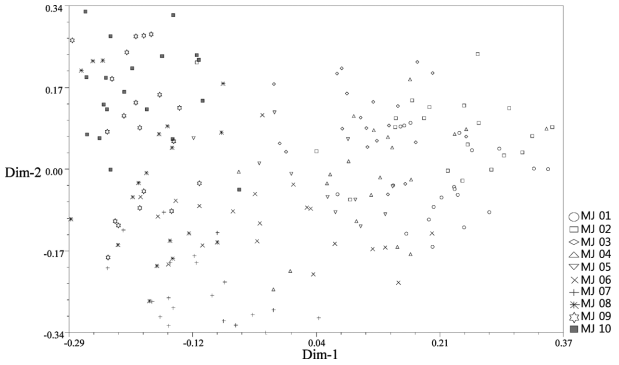


**Fig. 2.** Two-dimensional graph from the principal component analysis using Dice similarity coefficients for the 10 populations of *Millettia japonica*. Refer to Table 1 for abbreviations.

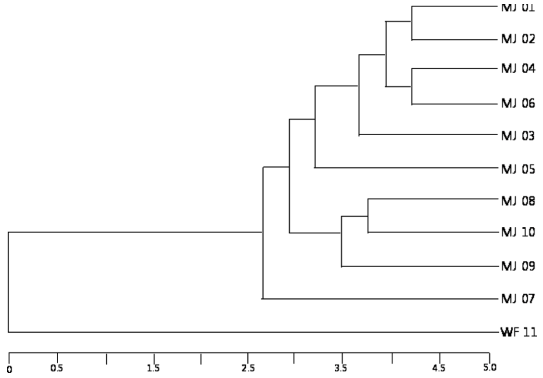


**Fig. 3.** UPGMA tree representing the genetic relationships among 10 populations of Millettia japonica based on Nei's genetic distance measures (Nei, 1978). Refer to Table 1 for population name abbreviations.

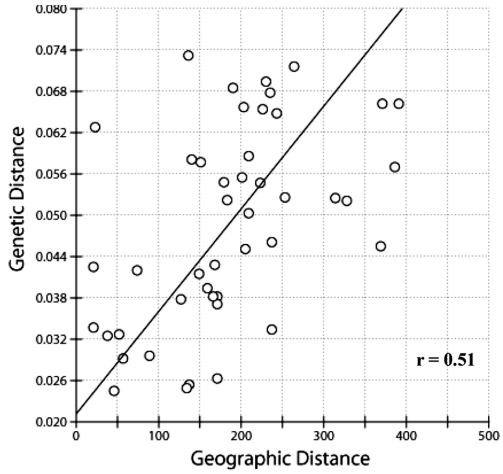


**Fig. 4.** Correlation between the genetic distance (Nei, 1978) and geographic distance (km) of *Millettia japonica* populations as revealed by the Mantel test.

Tongyeong population (MJ 08) was grouped with the Japanese population (MJ 10) rather than its neighboring population MJ 07 (Geoje population). The results from the Mantel test also indicated that the genetic distance was not significantly correlated with geographic distance among populations (r = 0.51; Fig. 4).

## Discussion

### Genetic diversity and genetic differentiation

The genetic diversity among rare plants is low due to their limited distribution (Godt et al., 1997). Considering the narrow distribution of *M. japonica*, the observed level of genetic variation within the Korean population is appreciable. The calculated diversity index of the species (Shannon's information index, $I = 0.2689$) was slightly lower than other critically endangered species such as *Thuja sutchuenensis* Franch. ($I = 0.295$; Liu et al., 2013), yet the value was significantly higher than two endangered leguminous species *Ammopiptanthus mongolicus* (Maxim.) Cheng f. and *A. nanus* (M. Pop.) Cheng f. ($I = 0.1832$ and 0.1025, respectively; Ge et al., 2005). The level of genetic variability observed in *M. japonica* is similar to that of *Oplopanax elatus* (Lee et al., 2002), and *Rubus coreanus* (Hong et al., 2003), which reproduce both sexually and vegetatively. Ha (2010) reported that *M. japonica* has both sexual (seed) and asexual (stolon) modes of the reproduction, but a specific pollination mechanism was not addressed. The appreciable level of genetic diversity observed in *M. japonica* seems to be due to its mixed reproduction mode.

The AMOVA analysis based on the observed genetic variation showed that 13.51% of the total variation resulted from differentiation among populations (Table 4). The $F_{ST}$ value of *M. japonica* (0.1351), which was lower than 0.25, is considered to be low genetic differentiation among populations (Hartle and Clark, 1997). An effective gene flow per generation ($Nm = 1.8446$) higher than one successful migrant per generation indicates active gene flow occurring among populations as shown in the mixed bioplot distribution in the graphs generated by PCA analysis (Fig. 2) and the lack of correlation between genetic distance and geographic distance (Figs. 3 and 4). These results indicate that frequent gene flow hindered genetic differentiation among the Korean populations of *M. japonica* by genetic drift. It is also possible that this species has not had much time to differentiate since its colonization in Korea. However, no studies have yet examined the genetic diversity among the Japanese populations of *M. japonica*. Comprehensive studies encompassing more populations from Japan are essential to critically address current levels and the origin of *M. japonica* population genetic structure.

### Conservation implications

Knowledge of the current levels and distribution of genetic diversity is important for designing conservation strategies for endangered species (Falk and Holsinger, 1991; Hamrick and Godt, 1996). Measuring and maintaining genetic variability provides essential background information for effective conservation (Avise and Hamrick, 1996). This study showed low genetic differentiation among all populations of *M. japonica*; hence, an effective strategy for conservation is to select and manage populations with higher genetic diversity. Our results indicate that MJ 03 (Gochang, JN), MJ 06 (Namhae, GN), and MJ 05 (Mt. Toham, GB) had higher genetic diversity than that of the seven other populations. MJ 05, a recently discovered population in the northern limit line of Korea is smaller than other populations. Species with smaller populations, which can be typical of endangered species, are vulnerable to loss of genetic variation (Ellstrand and Elam, 1993). However, the MJ 05 population showed a slightly higher genetic diversity than others despite its small size. Thus, the northernmost population MJ 05 together with MJ 03 and MJ 06 should be preferentially considered for conservation. Located in a provincial park (MJ 03) and a national park (MJ 05), these populations have been relatively well protected. However, MJ 06, which is distributed outside a protected area is prone to interference and should be consistently monitored. For *ex situ* conservation to maintain the genetic diversity of *M. japonica*, it is suggested that selecting individuals from the populations with higher genetic variability (i.e., MJ 03, 05, 06) is an effective strategy.

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