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The classification and comparison of genetic diversity of genus *Malus* using RAPD

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Genus *Malus* is a long-lived woody species primarily distributed throughout Asia. Many species of this genus are regarded as agriculturally and ecologically important. The phynetics and genetic diversity among eight species of genus *Malus* were reconstructed using the random amplified polymorphic DNA (RAPD) markers. In a simple measure of intraspecies variability by the percentage of polymorphic bands, the *M. micromalus* exhibited the lowest variation (34.7%). The *M. pumila* showed the highest (50.0%). Mean number of alleles per locus (*A*) ranged from 1.347 to 1.500 with a mean of 1.437. The phenotypic frequency of each band was calculated and used in estimating genetic diversity (*H*) within species. The mean of *H* was 0.190 across species, varying from 0.155 to 0.220. In particular, two cultivated species, *M. pumila* and *M. asiatica,* had high expected diversity, 0.314 and 0.307, respectively. On a per locus basis, the proportion of total genetic variation due to differences among species ranged from 0.388 to 0.472 with a mean of 0.423, indicating that 42.3% of the total variation was found among species. The phylogenetic tree showed three distinct clades. One includes *M. sieversii, M. pumila,* and *M. asiatica.* Another includes three *M. baccata* taxa. The other includes *M. sieboldii, M. floribunsa,* and *M. micromalus.* One variety and one form of *M. sieboldii* were well separated each other. RAPD markers are useful in germ-plasm classification of genus *Malus* and evolutionary studies.

Key words – Genus *Malus*, RAPD, Phylogenetic relationships

Introduction

Malus (Miller), a genus of the family Rosaceae consists of diploid species (2n=34, 51) and is mainly distributed in northeastern Asia [7]. Especially, *Malus pumila* Miller of the genus is economically important for its fruits (apples).

Apples are certainly among the earliest fruits to be gathered by people, and their domestication is probably preceeded by a long period of unintentional planting via rubbish dispersal. It is difficult to determine exactly when the apple was first domesticated, but the Greeks and Romans were growing apples at least 2,500 years ago [10]. The Romans spread the apple across Europe during their invasions and it was dispersed to the New World by European settlers during the 16th century.

Malus asiatica Nakai is distributed in northeastern China, Korea, and Japan. Asian regions such as China, Korea, Japan and Russia are well known for giving various *Malus* species [7]. The genus *Malus* is comprised of about eight taxa (six species, one variety, and one form) in Korea. The taxonomy of *Malus* has been processed mainly through morphological characteristics [15] and allozymes [4,21]. However, morphological characteristics are restricted their resolving powers because of the small number of available characters. Allozyme analysis is cost-effective and can be applied without extensive technical development and allozyme exhibit Mendelian inheritance. Nevertheless, there are several reasons to apply other types of markers. For instance, attempts to measure gene flow at small spatial scale by allozyme alleles are frequently frustrated by the limited variability of allozymes [2]. The development of molecular makers has provided powerful tools that may overcome such limitations. Random amplified polymorphic DNA (RAPD) analysis is quick, robust and requires minimal preliminary work [1]. Efficient methods to clarify the taxonomic status of several species are much needed [11].

The aims of this study were; 1) to estimate how much total genetic diversity is maintained in the *Malus* species, 2) to describe how genetic variation is distributed within and among species, and 3) to elucidate the suitability and efficiency of the RAPD analyses assess the phylogenetic relationships among the related *Malus* species in Korea.

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Materials and Methods

Plant materials

The plant materials were used the molecular studies of 88 samples representing nine species of *Malus*. *Chaenomeles speciosa* (Sweet) Nakai and *Pyrus pyrifolia* (Burm. fil) Nakai var. *culta* (Makino) Nakai were used as outgroups to compare the phylogenic relationships. Eight species of the genus *Malus*, *M. asiatica*, *M. baccata*, *M. baccata* var. *mandshurica*, *M. baccata* for. *Minor*, *M. floribunda*, *M. micromalus*, *M. pumila*, and *M. sieboldii*, were collected from populations in Korea (Table 1). Wild apple species, *M. sieversii* was obtained from the Korea Forest Research Institute. *M. pumila* and *M. asiatica* are cultivated species and other species are wild (natural) species. One young leaf per mature tree (5 yr) was sampled. To analyze the proportion of genetic diversity among and within taxa, 100 plants were randomly collected from each taxon.

DNA extraction and RAPD analysis

The genomic DNA of all samples including the outgroup was extracted from fresh leaves using the plant DNA Zol Kit (Life Technologies Inc., Grand Island, New York, U.S.A.) 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.

Forty arbitrarily chosen 10-mer primers, the kits C and D (OPC-01 to 20 and OPD-01 to 20) of Operon Technoligies (Alameda, Co.) were used. All the reactions were repeated twice and only reproducible bands were scored for analyses (Table 2).

Table 1. Distribution of selected species of genus *Malus* in this study and their chromosome numbers (adopted from Way et al., 1991)

Species	Chromosome number (2n)	Distribution
M. sieversii	?	North-wast China
M. pumila	34, 51, 68	Europe
M. asiatica	34	North and north-east China, Korea
M. baccata	34, 68	North and north-east China, Korea
M. baccata var. *mandshurica*	34	North-east China, Korea
M. baccata for. *minor*	34	Korea
M. sieboldii	34	Korea
M. floribunda	34	Korea, Japan
M. micromalus	34	Korea (endemic to Jeju Island)

Amplification reactions were conducted under standardized conditions in a 25 μl reaction volume containing 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. A 100 bp ladder DNA marker (Pharmacia) was used 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 RAPD bands were scored by eye and only unambiguously scored bands were used in the analyses. Because RAPDs are dominant markers, it was assumed that each band corresponded to a single character with two alleles, presence (1) or absence (0) of the band.

The following genetic parameters were calculated using a POPGENE computer program (ver. 1.31) developed by Yeh et al. [24]: 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) [17].

To elucidate the organization of the variation in *Malus* taxa, genetic variation was examined by partitioning of the total genetic diversity (H_T) to within (H_S) and among (D_{ST}) taxa components using Nei's genetic diversity statistics [16]. A measure of differentiation among populations, relative to the total diversity was calculated at each locus as $G_{ST} = D_{ST}/H_T$. Furthermore, gene flow (Nm) between the pairs of populations was calculated from G_{ST} values by $Nm = 0.5(1/G_{ST} - 1)$.

To elucidate the extent of genetic departure of populations from each other, Nei's genetic identity (I) and genetic distance (D) were calculated for each pairwise combination of populations [16].

The degree of polymorphism was quantified using Shannon's index of phenotypic diversity [3]:

$$H_O = - p_i \log p_i$$

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

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

be the average diversity over the different populations and let

$H_{SP} = - \; p \log p$

be the diversity calculated from the phenotypic frequencies p in all populations considered together. Then the proportion of diversity present within populations, H_{POP}/H_{SP}, can be compared with that of between populations (G_{ST}), $(H_{SP} - H_{POP})/H_{SP}$.

A phenetic relationship was constructed by the neighborjoining (NJ) method [20] using the NEIGHBOR program in PHYLIP version 3.57 [5].

Results

From the 40 decamer primers used for a preliminary RAPD analysis, seventeen primers of them produced good amplification products both in quality and variability (Table 2). Overall, 98 fragments were generated among the tested *Malus* array. The fragments ranged from 4-9 per primer (Fig. 1).

In a simple measure of intraspecies variability by the percentage of polymorphic bands, *M. pumila* showed the highest (50.0%) (Table 3). *M. micromalus* exhibited the lowest

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

No. of primer	Sequence(5'->3')	No. of fragments
OPC01	TTCGAGCCAG	6
OPC02	GTGAGGCGTC	8
OPC03	GGGGGTCTTT	6
OPC05	GATGACCGCC	4
OPC06	GAACGGACTC	7
OPC08	GTCCCGACGA	4
OPC10	TGTCTGGGTG	5
OPC13	AAGCCTCGTC	6
OPC14	TGCGTGCTTC	3
OPC17	TTCCCCCCAG	7
OPC20	ACTTCGCCAC	6
OPD01	ACCGCGAACG	4
OPD02	CGACCCAACC	8
OPD05	TGAGCGGACA	9
OPD07	TTGGCACGGG	3
OPD11	AGCGCCATTG	5
OPD16	AGGGCGTAAG	7
Total	-	98

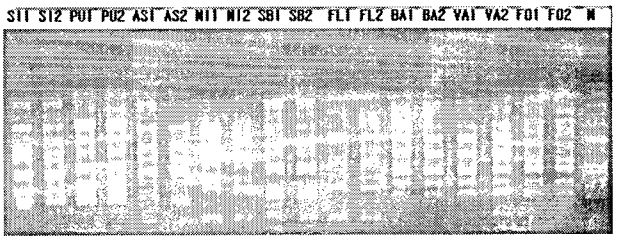

Fig. 1. RAPD profiles by OPD05 for nine *Malus* taxa. SI1 and SI2: *M. sieversii*, PU1 and PU2: *M. pumila*, AS1 and AS2: *M. asiatica*, MI1 and MI2: *M. micromalus*, SB1 and SB2: *M. sieboldii*, FL1 and FL2: *M. floribunda*, BA1 and BA2: *M. baccata*, VA1 and VA2: *M. baccata* var. *mandshurica*, FO1 and FO2: *M. baccata* for. *Minor*. M: marker.

variation (34.7%). Mean number of alleles per locus (*A*) ranged from 1.347 to 1.500 with a mean of 1.437. The effective number of alleles per locus (*Ae*) ranged from 1.290 to 1.405.

The phenotypic frequency of each band was calculated and used in estimating genetic diversity (*H*) within species. Although the typical species except two cultivated species (*M. pumila* and *M. asiatica*) were small, isolated, and patchily distributed for natural populations, they maintained a high level of genetic diversity for seventeen primers. The mean of *H* was 0.190 across species, varying from 0.155 to 0.220. In particular, both cultivated species, *M. pumila* and *M. asiatica*, had high expected diversity, 0.220 and 0.216, respectively. Isolated endemic species, *M. micromalus* had the lowest (0.155).

Shannon's index of phenotypic diversity (*I*) of *M. pumila* (0.314) was the highest among all species and *M. asiatica*

Table 3. Measures of genetic variation for genus *Malus*. 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*)

Taxa	*Np*	*Pp*	*A*	A_E	*H*	*I*
M. sieversii	45	45.9	1.459	1.377	0.205	0.292
M. pumila	49	50.0	1.500	1.404	0.220	0.314
M. asiatica	47	48.0	1.480	1.405	0.216	0.307
M. baccata	45	45.9	1.459	1.337	0.186	0.269
M. baccata var. *mandshurica*	43	43.9	1.439	1.353	0.190	0.272
M. baccata for. *minor*	41	41.8	1.418	1.362	0.191	0.271
M. sieboldii	42	42.9	1.429	1.366	0.193	0.274
M. floribunda	39	38.8	1.398	1.319	0.175	0.250
M. micromalus	34	34.7	1.347	1.290	0.155	0.220
Mean	42.8	43.5	1.437	1.357	0.190	0.274
Total (genus level)	83	84.7	1.847	1.581	0.326	0.479

was the second (0.220).

Total genetic diversity (H_T) varied between 0.144 for *M. micromalus* and 0.207 for *M. pumila* (Table 4). The interlocus variation of genetic diversity (H_S) was low (0.134). On a per locus basis, the proportion of total genetic variation due to differences among species (G_{ST}) ranged from 0.101 for *M. micromalus* to 0.307 for *M. baccata* for. *Minor* with a mean of 0.240, indicating that 24% of the total variation was found among species. An assessment of the proportion of diversity present within species, 76% of genetic variation resided within taxa. The *Nm* was estimated to be moderate (1.482).

An assessment of the proportion of diversity present within species, H_{POP}/H_{SP}, indicated that about 57.7% the total genetic diversity was among species. Thus, about 42.3% of genetic variation resided within genus (Table 5).

Genetic identity (*I*) based on the proportion of shared fragments was used to evaluate relatedness among species.

Table 4. Estimates of genetic diversity of *Malus* taxa. 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*)

	H_T	H_S	G_{ST}	*Nm*
M. sieversii	0.195	0.140	0.285	1.258
M. pumila	0.207	0.149	0.280	1.289
M. asiatica	0.200	0.144	0.281	1.282
M. baccata	0.173	0.129	0.252	1.482
M. baccata var. *mandshurica*	0.184	0.135	0.266	1.382
M. baccata for. *minor*	0.177	0.123	0.307	1.129
M. sieboldii	0.189	0.151	0.201	1.994
M. floribunda	0.166	0.137	0.175	2.360
M. micromalus	0.144	0.101	0.101	1.163
Mean	0.182	0.134	0.240	1.482
Total (genus level)	0.328	0.192	0.414	0.708

Table 5. Partitioning of the genetic diversity into within and among *Malus* taxa by RAPD

Taxa	H_{SP}	H_{POP}	H_{POP}/H_{SP}	$(H_{SP}-H_{POP})/H_{SP}$
M. sieversii	2.767	1.567	0.566	0.434
M. pumila	2.758	1.457	0.528	0.472
M. asiatica	2.723	1.558	0.572	0.428
M. baccata	2.658	1.536	0.578	0.422
M. baccata var. *mandshurica*	2.608	1.543	0.592	0.408
M. baccata for. *minor*	2.670	1.530	0.573	0.427
M. sieboldii	2.537	1.518	0.598	0.402
M. floribunda	2.441	1.495	0.612	0.388
M. micromalus	2.498	0.246	0.577	0.423
Mean	2.629	1.383	0.577	0.423

Table 6. Genetic identity (upper diagonal) of *Malus* taxa and genetic distances (low diagonal) based on RAPD analysis

Taxa	M-1	M-2	M-3	M-4	M-5	M-6	M-7	M-8	M-9
M-1	-	0.985	0.907	0.806	0.826	0.793	0.741	0.756	0.703
M-2	0.016	-	0.895	0.799	0.818	0.788	0.744	0.759	0.702
M-3	0.097	0.111	-	0.857	0.795	0.783	0.787	0.890	0.754
M-4	0.216	0.224	0.154	-	0.869	0.865	0.817	0.782	0.788
M-5	0.192	0.201	0.229	0.140	-	0.925	0.814	0.788	0.732
M-6	0.232	0.238	0.245	0.145	0.078	-	0.081	0.791	0.748
M-7	0.300	0.295	0.240	0.203	0.206	0.222	-	0.916	0.884
M-8	0.280	0.276	0.236	0.245	0.238	0.235	0.088	-	0.897
M-9	0.352	0.354	0.283	0.238	0.313	0.290	0.124	0.108	-

M-1: *M. sieversii*, M-2: *M. pumila*, M-3: *M. asiatica*, M-4: *M. baccata*, M-5: *M. baccata* for. *Minor*, M-6: *M. baccata* var. *mandshurica*, M-7: *M. sieboldii*, M-8: *M. floribunda*, M-9: *M. micromalus*.

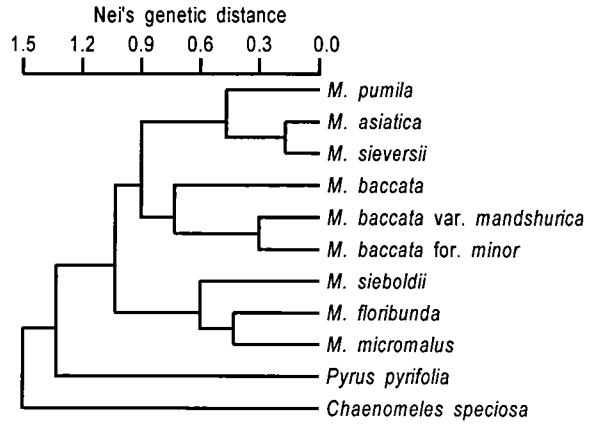

Fig. 2. A phenogram showing the relationships among nine *Malus* taxa and two outgroups based on data of genetic distance obtained by RAPD.

The estimate of *I* ranged from 0.016 to 0.354 (Table 6).

Clustering of populations, using the NJ algorithm, was performed based on the matrix of calculated distances (Fig. 2). The phylogenic tree showed three distinct clades. One includes *M. sieversii*, *M. pumila*, and *M. asiatica*. Another includes three *M. baccata* taxa. The other includes *M. sieboldii*, *M. floribunsa*, and *M. micromalus*. One variety and one form of *M. sieboldii* were well separated each other. The tree also showed genetic differentiation among Korean species. One endemic species, *M. micromalus* is only isolated Jeju Island in the South Korea and is separated from *M. sieboldii* and *M. floribunsa*.

Discussion

In apple, as with other fruit tree species, two main fac-

tors have been identified that modulate tree architecture and overall tree size [9]. The first one is the genotype, which affects branching density (e.g., related to frequency of latent buds or to as the physiological abortion of young growing points, referred to as the extinction phenomenon), proportion of short vs. long shoots (e.g., type I tree characterized by high branching density and short branches vs. type IV cultivars characterized by scare branching and longer branches), and flowering pattern (e.g., laternal vs. terminal flowering). A second factor is the root system, which has been used as an efficient although empirical means to control tree size, with variable results on flowering (i.e., dependent on the genotype) [23].

In RAPD analysis, eight species and one variety belonging to genus *Malus* maintain a moderate or higher than average level of genetic diversity compared with other plant species, although there is difference in methodology (e.g., dominant marker and co-dominant marker) that may preclude meaningful comparisons. For example, its genetic diversity of 0.190 is higher than that for temperate-zone species (0.146), dicots (0.136), species with a sexual reproduction mode (0.151), and those with a long-lived woody habit (0.177) [6].

Geographic range has been shown to be strongly associated with the level of variation maintained both within populations and at the species level. Widely distributed species tend to maintain more variation than more narrowly distributed species level [6]. For all Korean *Malus* taxa where the number of alleles per polymorphic loci was calculated, relatively widespread species (*M. sieboldii, M. floribunsa,* and *M. baccata*) except apple species had more alleles than restricted species (*M. micromalus, M. baccata* var. *mandshurica, M. baccata* for. *Minor*).

The comparison of cultivated apple (*M. pumila* and *M. asiatica*) and wild species of genus *Malus* revealed that the domestication processes via artificial selection do not have eroded the levels of genetic diversity in cultivated apples. It is not in general accord with the concept that most crops show a reduced level of polymorphisms as compared to their presumed progenitors. Many studies 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 species have more genetic variability [18]. In addition, for soybean the domestication process has not eroded the levels of genetic variation [12]. It is accord that domesticated apples were hybridized with many wild species as they were spread by humans [7].

The genus of apples, *Malus,* belongs to the subfamily *Pomoideae* of the Rosaceae family. Another important fruit tree peer (*Pyrus*), also belongs to the same subfamily. There are over 30 primary species of apple and most can be readily hybridized [13,22]. The cultivated apple is probably the result of interspecific hybridization and is most appropriately called *Malus* x *domestica* [14]. Its primary wild ancestor is *Malus sieversii* whose range is centered at the border between western China and the former Soviet Union [8]. Apples are the main forest tree there and display the full range of colors, forms and tastes found in domesticated apples across the world [9]. Other species of *Malus* which contributed to the genetic background of the apple include: *M. orintalis* of Caucasia, *M. sylvestris* from Europe, *M. baccata* from Siberia, *M. mandshurica* from Manchuria (China) and *M. prunifolia* from China [7].

At present, the phenetic positions of these species shown in Fig. 1 seem to be agreed with results of morphological and distribution data. *M. baccata* var. *mandshurica* which has many hairs in leaves and petioles is sister to *M. baccata* for. *minor* which is hairless and is denticulate in leaf margin. One of the most striking features in this paper apple species are at least less similar to *M. sieboldii, M. floribunsa,* and *M. micromalus*. If RAPD data can be used identify the origin of hybrids, three species could be rule out the candidates of ancestors or rink species to reveal the history of several hybrid species.

In the present study, only *M. sieversii* was found to be closely to *M. pumila* and *M. asiatica*. Additional analysis of other species of genus *Malus* will hopefully further clarify the relationships among the *Malus* taxa.

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초록 : RAPD를 이용한 능금속 식물종의 계통관계와 유전적 다양성

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능금속(*Malus*) 식물은 다년생 목본으로 국내에는 약 8종이 있다. 이 속에 있는 사과(*M. pumila*)는 경제적 중요 작물로 그 기원은 서중국의 야생종 *M. sieversii*일 것으로 추정되고 있다. 우리나라에 자생하는 *Malus*내 모든 분류군과 중국의 *M. sieversii*를 RAPD로 분석하였다. 재배종이 높은 다양성을 나타낸 반면 제주아그배나무가 가장 낮은 다양성을 나타내었다. 재배종이 야생종보다 유전적 다양도가 더 높게 나타나 재배화 과정에서 여러 종과 교잡이 일어나 많은 유전자가 침투된 것이라는 보고를 뒷받침한다. 이 속은 *M. sieversii*를 포함한 사과나무, 능금나무가 같은 분지군을 형성하였고, 개아그배나무 또는 제주아그배나무(*Malus micromalus*), 아그배나무(*Malus sieboldii*), 꽃아그배나무(*Malus floribunda*)가 같은 분지군, 야광나무(*Malus baccata*), 개야광나무(*Malus baccata* for. *minor*), 털야광나무(*Malus baccata* var. *mandshurica*)가 같은 분지군을 형성하였다. 한국내 재배종 사과와 능금이 국내 자생종 능금속에서 진화나 분지한 것은 아닌 것으로 판명되었고 오히려 중국 야생종이 그 기원의 하나일 가능성이 시사된다.