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Analysis of Gene Diversity and Phenetic Relationship of Water Dropwort Species in Korea Using RAPD (OPB Primers) Markers

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Water dropworts, *Oenanthe javanica* and *O. javanica* var. *japonica* are called “minari” in Korea and are eaten as a vegetable. *Cicuta virosa* is common European water hemlock and has toxic properties, such as cicutoxin. Molecular variations of water dropwort species in Korea were investigated using random amplified polymorphic DNA (RAPD). The six populations were studied with 10 primers (Operon, OPB) for RAPD analysis. The 72 DNA fragments (bands) were found among six populations. Among these 72 bands, 61 (84.7%) bands were polymorphic. The typical populations of *Cicuta virosa* in Korea were small, isolated, and patchily distributed for natural populations and they maintained a high level of genetic diversity. However, when cultivated populations of *O. javanica* var. *japonica* were large and widely grown in rice paddies as vegetables, they maintained a lower genetic diversity than those of *C. virosa* and wild populations of *O. javanica*. Although the diversity indices of wild populations were shown to be higher than those of cultivated populations, no significant difference for measures of genetic variability was shown. Total genetic diversity value (H_T) was 0.342. The interlocus variation in the within-population genetic diversity (H_S) was 0.201. The proportion of total genetic variation due to differences among populations (G_{ST}) range was 0.414, indicating that 41.4% of the total variation was among populations. In conclusion, the RAPD technique was a useful method for discrimination between *C. virosa* and *O. javanica*. In addition, RAPD-OPB markers could further distinguish the strains from different food sources.

Key words : *Cicuta virosa*, genetic diversity, *Oenanthe javanica* var. *japonica*, RAPD, water dropwort

Introduction

Oenanthe is derived from the Greek oinos (=wine) and anthos (=flower), from the wine-like scent of the flowers. The name Water Hemlock is, though incorrectly, often popularly applied to several species of Oenanthe, the genus of the water dropwort, which of all the British umbelliferous plants are the most poisonous [3]. The poisonous members are hemlock (*Conium maculatum*), cowbane (*Cicuta virosa*), and hemlock water dropwort [17]. Species within the Umbelliferae (also called Apiaceae) family are divided into the Cicuta and Oenanthe genera. *Oenanthe javanica* (Blume) DeCanolle var. *japonica* Honda, commonly Java water dropwort, water celery, water dropwort, Chinese celery, and Japanese (flat leaf) parsley, is a plant of the genus Oenanthe originating from East Asia [21]. In Korea, the plant is called minari and is eaten a vegetable. The award-winning 2020 drama film Minari is named for the vegetable. *C. virosa* is common European water hemlock. *C. virosa* has the same toxic properties as cicutoxin, one of the most potent convulsant. Cicutoxin is a potentgamma- aminobutyric acid (GABA) antagonist that induces recurrent seizures and exerts cholinergic effects. While the root of the cowbane (*Cicuta virosa*) is single and conical in form, that of *Oenanthe javanica* consists of clusters of fleshy tubers similar to those of the cowbane. *C. virosa* has also occasionally been eaten in mistake, either for wild celery or water parsnip, with very serious results, great agony, sickness, convulsions, or even death resulting.

Random Amplified Polymorphic DNA (RAPD) markers are DNA fragments from PCR amplification of random segments of genomic DNA with single primer of arbitrary nucleotide sequence. Unlike traditional PCR analysis, RAPD (pronounced "rapid") does not require any specific knowledge

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of the DNA sequence of the target organism: the identical 10-mer primers will or will not amplify a segment of DNA, depending on positions that are complementary to the primers' sequence [19].

The discovery that PCR with random primers can be used to amplify a set of randomly distributed loci in any genome facilitated the development of genetic markers for a variety of purposes [5, 18]. The simplicity and applicability of the RAPD technique have captivated many scientists' interests. Perhaps the main reason for the success of RAPD analysis is the gain of a large number of genetic markers that require small amounts of DNA without the requirement for cloning, sequencing or any other form of the molecular characterization of the genome of the species in question.

Cultivar identification, parentage determination, genetic relationship evaluation, and estimation of population genetic variability are some examples of the multiple uses of the RAPD technique [2, 12].

In this paper, I provided genetic information and RAPD markers that can distinguish between edible water parsley (*O. javanica*) and toxic water parsley (*C. virosa*).

Materials and Methods

DNA extract

One *Cicuta virosa* L. population in Korea was collected from natural site (Table 1). Three wild or natural populations of *Oenanthe javanica* (Blume) DeCanolle populations and two cultivated populations of *Oenanthe javanica* (Blume) DeCanolle var. *japonica* Honda were provided for this study.

DNA was extracted using the plant DNA Zol Reagent (Life Technologies Inc., Grand Island, New York, U.S.A.) according to the manufacturer's protocol. In the Plant DNAzol® procedure, plant samples are pulverized in liquid nitrogen or homogenized, and genomic DNA is extracted from the homogenate with Plant DNAzol®. Following extraction, plant debris is removed by centrifugation and DNA is precipitated from the supernatant with ethanol. The resulting DNA pellet is washed with ethanol and solubilized.

RAPD analysis

Ten arbitrarily chosen primers (Operon, OPB, USA) were used (Table 2). The polymerase chain reaction (PCR) is a relatively simple technique that amplifies a DNA template to produce specific DNA fragments in vitro. The amplification profile was 28 cycles of 94℃ for 30 sec, 42℃ for 60 sec, 72℃ for 60 sec, preceded by an initial denaturation at 94℃ for 90 sec and followed by a final extension at 72℃ for 5 min using the Them cycler (Takara, TP600, Japan). The amplification products were separated by electrophoresis on 2.0% agarose gels, stained with SYBR® Green, and photographed under UV light using Alpha Image TM (Alpha Innotech Co., USA). All the reactions were repeated twice and only reproducible bands were scored for analyses (Table 2).

Statistical analysis

Polymorphism information content (PIC) value was calculated using the formula PIC, 1 - ∑ pi2, where pi is the frequency of the ith allele [15].

The following genetic parameters were calculated using a POPGENE computer program (ver. 1.31) developed by Yeh et al. [20]: the percentage of polymorphic loci (Pp), mean numbers of alleles per locus (*A*), effective number of alleles per locus (*Ae*) gene diversity (*H*), and Shannon's index of phenotypic diversity [9].

The phenotypic frequency of each band was calculated and used in estimating total genetic diversity (H_T), genetic diversity within five populations (it means five populations) (H_S) proportion of total genetic diversity partitioned among populations (G_{ST}), and gene flow (*Nm*) [9].

The estimation of genetic identity between genotypes and genetic distances among populations were based on the probability that an amplified fragment from one individual will also be present in another [10, 16]. A phenetic relationship was constructed by the neighbor-joining (NJ) method [13]

Table 1. Code and locations of genus Oenanthe in this study

Species	Code	Characteristics	Locality of population
Cicuta virosa	T-1	Taxic water dropwort	Gimje-si, Korea
Oenanthe javanica	W-1	Wild water dropwort	Gimje-si, Korea
	W-2	Wild water dropwort	Jinju-ci, Korea
	W-3	Wild water dropwort	Busan-si, Korea
O. javanica var. *japonica*	C-1	Cultivated water dropwort	Cheongdo-gun, Korea
	C-2	Cultivated water dropwort	Yangsan-si, Korea

Table 2. List of decamer oligonucleotide utilized as OPB primers, their sequences, and associated polymorphic fragments amplified in genus Oenanthe

No. of Primer	Sequence (5' to 3')	No. of fragments detected	Pp. of bands	PIC
OPB-01	GTTTCGCTCC	2	0	0.500
OPB-02	TGATCCCTGG	1	0	0.000
OPB-03	CATCCCCCTG	1	0	0.000
OPB-04	GGACTGGATT	5	3	0.787
OPB-05	TGCGCCCTTC	13	13	0.920
OPB-06	TGCTCTGCCC	14	13	0.818
OPB-07	GGTGACGCAG	7	6	0.854
OPB-08	GTCCACACGG	13	13	0.818
OPB-09	TGGGGGACTC	9	7	0.881
OPB-10	CTGCTGGGAC	7	6	0.908
Total	-	72	61	0.649

using the MEGAX [8].

Results

The six populations were studied with 10 oligonucleotide primers (OPB1-OPB10) for RAPD analysis. From the 10 decamer primers used for a preliminary RAPD analysis, they produced good amplification products both in quality and variability. The number of bands generated by each primer varied from 1 (OPB-01, OPB-02, OPB-03) to 13 (OPB-05, OPB-06, OPB-08), with an average of 7.2 bands per primer (Table 2). 72 DNA fragments (bands) were found among 6 populations. Among these 72 bands, 61 (84.7%) bands were polymorphic. OPB-06-01 locus can be recognized as unique locus of *C. virosa*. OPB-08-12 and OPB-08-13 loci were found specific to *O. javanica* and *O. javanica* var. *japonica*. Thus these loci can be used distinguish the origin of the genera.

Polymorphism information content (PIC) for RAPD primers ranged from 0.0 to 0.908 with an average of 0.649 per primer (Table 2).

In a simple measure of intra-species variability by the percentage of polymorphic bands, the population T-1 showed the highest (56.9%). The population C-2 exhibited the lowest variation (44.4%) (Table 3). Other cultivated C-1 was also low variation (45.8%). Mean number of alleles per locus (*A*) ranged from 1.444 to 1.569 with a total of 1.847 for the total species. The effective number of alleles per locus (*Ae*) ranged from 1.277 to 1.426 with a total of 1.616 for species. The phenotypic frequency of each band was calculated and used in estimating genetic diversity (*H*) within populations. The mean *H* was 0.342 across species. In particular, T-1 had high expected diversity and C-2, the low. Shannon's index of phenotypic diversity (*I*) of the T-1 (0.341) was highest of all populations and the W-2 was the second. The typical population of *C. virosa* in Korea was small, isolated, and patchily distributed for natural populations and it maintained a high level of genetic diversity. Whereas, cultivated population of *O. javanica* var. *japonica* were large and wide grown in rice paddies for vegetables, they maintained a lower genetic diversity than those of *C. virosa* and wild populations of *O. javanica*. Although the diversity indices of wild populations were shown higher than those of cultivated populations, there did not show significant difference for measures of genetic variability (chi-square test).

Table 3. Measures of genetic variability for RAPD generated among genus Oenanthe

Code	N_P	P_P	*A*	*Ae*	*H*	*I*
T-1	41	56.9	1.569	1.426	0.235	0.341
W-1	38	52.8	1.528	1.350	0.201	0.297
W-2	36	50.0	1.500	1.376	0.208	0.301
W-3	37	51.4	1.154	1.364	0.205	0.300
C-1	33	45.8	1.458	1.338	0.189	0.274
C-2	32	44.4	1.444	1.277	0.165	0.245
Total	61	84.7	1.847	1.616	0.342	0.457

N_P: The number of polymorphic loci, P_p: The percentage of polymorphic loci, *A*: Mean numbers of alleles per locus, A_e: Effective number of alleles per locus, *H*: Gene diversity, *I*: Shannon's index of phenotypic diversity.

Total genetic diversity values (H_T) was 0.342 (Table 4). The interlocus variation in the within-population genetic diversity (H_S) was low (0.201). On a per-locus basis, the proportion of total genetic variation due to differences among populations (G_{ST}) ranged was 0.414, indicating that 41.4% of the total variation was among populations. An assessment of the proportion of diversity present within species, 58.6%

Table 4. Estimates of genetic diversity of genus Oenanthe in Korea

Locus	H_T	H_S	G_{ST}	*Nm*
OPB4	0.333	0.216	0.349	3.567
OPB5	0.395	0.187	0.510	0.497
OPB6	0.370	0.203	0.427	1.348
OPB7	0.436	0.266	0.413	3.127
OPB8	0.467	0.304	0.350	2.040
OPB9	0.409	0.302	0.320	3.936
OPB10	0.365	0.204	0.402	0.726
Mean	0.342	0.201	0.414	0.707

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*)

of genetic variation resided within populations. The estimate of gene flow, based on G_{ST}, was slightly high among Korean populations of water dropwort (*Nm* = 0.707). The *Nm* value varies for each of the six primers, but there was no significant difference in the overall genetic diversity scale (chi-square = 2.407, $p>0.05$).

A similarity matrix based on the proportion of shared fragments (GS) was used to evaluate relatedness among species. The estimate of GS ranged from 0.719 to 0.976 (Table 5). W-2 and W-3 populations were most similarity.

RAPD bands from the 10 primers were used for cluster analysis. Cluster analysis of RAPD markers showed that *C. virosa* and *O. javanica* were obviously classified into two different groups (Fig. 1). Wild species (*O. javanica*) and cultivated species (*O. javanica* var. *japonica*) were clustered one clade. Consequently, the RAPD technique was a useful method for discrimination of *C. virosa* and *O. javanica*.

Discussion

RAPD marker diversity of wild species, *O. javanica* was higher than that of cultivated species, *O. javanica* var. *japonica* (Table 3). It is in general accord with the concept that most crops show a reduced level of polymorphisms as compared to their presumed progenitors [4]. This is in concordance with the many results obtained using RAPD [1, 11]. Namely, the domestication processes via artificial selection have eroded the levels of genetic diversity in cultivated crops. Cultivated populations of *O. javanica* were maintained a lower degree of polymorphy than do their natural counterparts by allyzyme analysis [6]. Although the several diversity indices of wild populations were shown higher than those of cultivated populations, there did not show significant difference ($p<0.05$). Ji et al. [7] reported that water domestication was short duration. However, they excluded Korea from the geograplic distribution of common water dropwort (*O. javanica*), including Southeast Asia, China, and Japan. For a more detailed analysis, a study on spatial distribution is needed.

Table 5. Similarity matrix (above diagonal) of 6 populations based on OPB and genetic distances (below diagonal)

Pop.	T-1	W-1	W-2	W-3	C-1	C-2
T-1	-	0.719	0.772	0.784	0.728	0.771
W-1	0.330	-	0.869	0.861	0.703	0.741
W-2	0.259	0.140	-	0.976	0.706	0.803
W-3	0.244	0.149	0.024	-	0.730	0.812
C-1	0.317	0.353	0.348	0.315	-	0.834
C-2	0.261	0.300	0.220	0.208	0.179	-

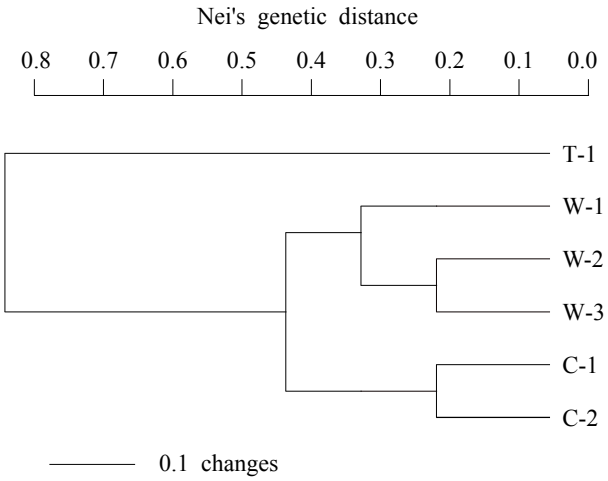

Fig. 1. A neighbor-joining tree for 6 populations of genus Oenanthe in Korea based on PAPD analysis with OPB primers.

Cultivated and wild species are well cut anywhere along the water. On the other hand, *C. virosa* is an endangered species in Korea (Class II). *C. virosa* is similar in form to cultivated (*O. javanica* var. *japonica*) or wild species (*O. javanica*). There is a problem with treating wild species and cultivated species as variants. In Korea, *O. javanica* and *O. javanica* var. *japonica* have been eaten as eatable vegetables. However, *C. virosa* is dangerous to eat because of their toxicity. Namely, *C. virosa* is known one of poisonous species of Cicuta. The genus contain a toxin named cicutoxin which causes central nervous system stimulatory effects including seizures following ingestion [14]. It is difficult to distinguish cultivated water parsley from poisonous water parsley in form, not only for the general public but also for experts. It is necessary to distinguish between these two spe-

cies in molecular biology.

The results of this study could be used for exploiting the genetic diversity in the two species (*C. virosa* and *O. javanica*) in taxonomy programs and in sampling and managing germplasm collections. It was possible to identify accessions, particularly those of divergent origins, by RAPD. Although I did not analyze further subdivision of a local population, I may infer that RAPD variation that resided mainly within *C. virosa* species is maintained in patchily distributed subpopulations or demes, either by random drift of neutral alleles or micro- environmental selection for adaptive alleles.

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The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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초록 : 한국 미나리 집단에 대한 RAPD (OPB 프라이머)에 의한 유전적 다양성과 표현형 관계

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*Oenanthe javanica*와 *O. javanica* var. *japonica* 한국 내 식용미나리이다. *Cicuta virosa*는 독미나리로 시큐톡신을 함유하고 있다. 미나리에 대한 RAPD 마크에 의한 분자적 변이를 조사하였다. RAPD 분석을 위해 10개 올리고프라이머(오페론, OPB)로 6개 집단을 분석하였다. 6개 집단에서 72개 DNA 분절(밴드)을 찾았다. 72 밴드 중 61개(84.7%) 밴드는 다형현상을 나타내었다. 한국 내 독미나리 자연 집단은 작고 격리되어 패치 분포를 이루고 있지만 높은 유전적 다양도를 가지고 있었다. 반면에, 재배종 미나리(*Oenanthe javanica* var. *japonica*) 집단은 채소용으로 논에서 광범위한 분포를 나타내지만 독미나리와 야생종 미나리에 비해 낮은 유전적 다양도를 나타내었다. 비록 야생집단이 재배집단에 비해 다양도가 높지만 유의한 차이는 없었다. 전체 유전적 다양도(H_T)는 0.342였다. 집단 내 유전적 다양도(H_S)는 0.201이였다. 유전자 좌위에 근거한 집단 간 분화에서 전체 유전적 다양도의 비율(G_{ST})은 0.414이므로 전체 변이의 41.4%는 집단 간에 있었다. 결론으로 RAPD기법은 독미나리와 식용미나리의 동정에 유용하였다. 또, RAPD의 OPB 마크는 미나리의 다른 자원과 식품 자원을 식별하는 데 도움이 될 수 있는 분자 마크임을 보여주었다.