

# Genetic Diversity of *Perilla* and Related Weedy Types in Korea Determined by AFLP Analyses

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## ABSTRACT

*Perilla frutescens* (L.) Britton var. *frutescens* is extensively cultivated on a large scale throughout the Korea, whereas var. *crispa* is not. The weedy types of both var. *frutescens* and var. *crispa* are often found along roadsides, waste lands, and around farmers' fields. Although *Perilla* is one of the important leafy vegetable and oil crops in Korea, systematic analyses on its genetic structure have been limited and are needed for future breeding progress. The objective of this study was to determine genetic diversity and relatedness in Korean accessions of *Perilla*. Field surveys and amplified fragment length polymorphism (AFLP) analyses were conducted to determine genetic diversity. Analyses of 30 *Perilla* accessions by seven AFLP primer combinations identified a total of 121 fragments, of which 72 (60%) were polymorphic at the species level. Shanon's index of diversity  $H_s$  of the AFLP variations for cultivated type of var. *frutescens*, weedy type of var. *frutescens*, and weedy type of var. *crispa* were 0.63, 2.00, and 1.75, respectively. The weedy type of var. *frutescens* exhibited the highest variation. Most of the AFLP variation (78%) resided within populations. In the phylogenetic tree, two major clusters were recognized: (i) cultivated type of var. *frutescens* and weedy type of var. *frutescens* and (ii) weedy type of var. *crispa*. Although the wild species of *P. frutescens* has not been identified, the weedy *Perilla* is the key taxon for our understanding of the origin of cultivated var. *frutescens*.

**P**ERILLA FRUTESCENS (Labiatae) is a self-fertilizing annual species which is widely cultivated in East Asia. The species includes two types that are characterized on the basis of their morphology and dual uses. One, *P. frutescens* var. *frutescens*, is an oil crop. The common names are Ren in Chinese, Dlggae in Korean, and Egoma in Japanese. The other, *P. frutescens* var. *crispa*, is a medicine or a vegetable crop in China. The common names are Zisu in Chinese, Cha-jo-ki in Korean, and Shiso in Japanese. In addition to those two types, weedy plants of both *Perilla* crops are commonly found in such habitats as roadsides, waste lands, and around farmers' fields in East Asia (Nitta and Ohnishi, 1999). *Perilla frutescens* var. *frutescens* generally is taller, larger in seed size (above 2 mm), and has soft seeds, green leaves and stems, and nonwrinkled leaves with a fragrance specific to var. *frutescens*. *Perilla frutescens* var. *crispa* is smaller in plant height and seed size (below 2 mm), and has hard seeds, red or green coloration in the leaves

and stem, wrinkly or nonwrinkly leaves, and a fragrance specific to var. *crispa*.

Today *P. frutescens* var. *frutescens* is extensively cultivated and used in Korea (Nitta, 2001), although cultivated var. *frutescens* probably originated in China (Makino, 1961). In Korea, var. *frutescens* is used not only as an oil crop but also as a vegetable crop. Recently, accompanied by increased meat consumption and development of various cooking methods of fresh leaves, var. *frutescens* became one of the most important crops in Korea. However, modern breeding methods and systematic introduction of germplasm have not been practiced widely on this crop in Korea. No cultivars have been developed solely for production as oil or vegetable crop.

The success of any breeding or genetic conservation program is dependent on understanding the amount and distribution of the genetic variation present in the gene pool. In plants, the AFLP technique provides useful information regarding genetic diversity and genetic relationships among cultivated species and its wild relatives. It has been used to establish genetic relationships in many species (Maughan et al., 1996; Sharma et al., 1996; Paul et al., 1997; Mace et al., 1999; Le Thierry d'Ennequin et al., 2000). The objective of this study was to determine genetic diversity and relatedness in Korean accessions of *Perilla*.

## MATERIALS AND METHODS

A field survey for cultivation and utilization of *Perilla* and weedy types was conducted in Korea from 27 Oct. to 14 Nov. 1998 (Fig. 1). In the present study, we tentatively classified the *Perilla* samples as being of the cultivated or weedy type according to the morphological characters and the conditions where they were collected (cultivated or not). A subset of each collection was deposited in the Genetic Resources Centre, Rural Development and Administration of Korea, Suwon, Korea, for permanent seed preservation.

The materials for AFLP analysis consisted of 30 accessions (14 of var. *frutescens*, nine of the weedy type of var. *frutescens*, and seven of the weedy type of var. *crispa*), which were selected to cover almost all regions in Korea (Table 1). Total DNA was extracted from leaf tissue of individual plants of each accession by means of the Plant DNAzol Reagent protocol (Gibco BRL Inc., Grand Island, NY). The genomic DNA from five plants of each accession was pooled for analysis.

The AFLP analysis was performed according to Zabeau and Vos (1993) and Vos et al. (1995) with some minor modifications. The genomic DNA (125 ng) was digested with *EcoRI* and *MseI* (AFLP core Reagent Kit, Gibco BRL Inc., Grand Island, NY) in a final volume of 12.5  $\mu$ L. After inactivation at 70°C for 15 min, ligation with the adapters was performed. The adapter ligation solution of 12 and 0.5  $\mu$ L of T4 ligase (AFLP core Reagent Kit) was added to the digested DNA.

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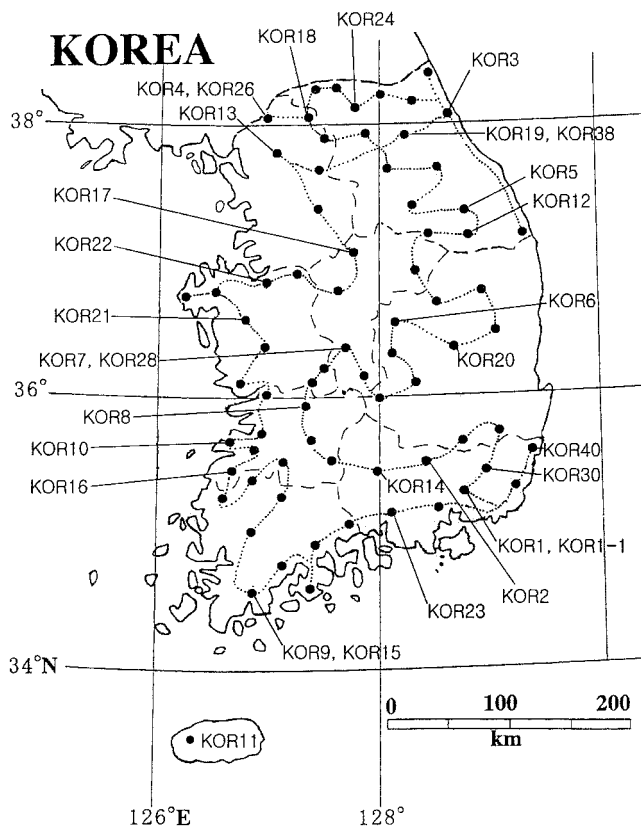


Fig. 1. Routes of the field survey for *Perilla* in Korea. The places with accession number are those used for AFLP analyses. See Table 1 for the accession numbers. ●: location visited and where a sample was collected.

The resulting reaction mixture was incubated at 20°C for 2 h. This was followed by a preamplification step with the primers that were complementary to the adapters with one additional selective nucleotide on its 3' end, which was performed in a

total volume of 25.5  $\mu$ L containing 2.5  $\mu$ L of ligation mixture (diluted 10 times in TE). The polymerase chain reaction (PCR) was performed in 20 cycles of denaturation at 94°C for 30 s, followed by annealing at 56°C for 60 s, ending with extension at 72°C for 60 s. Amplification reactions were performed as 1 cycle of denaturation at 94°C for 60 s, followed by annealing at 65°C for 60 s, ending with extension at 72°C for 90 s, and then followed by 10 cycles of the conditions listed above, except with a 1°C lower annealing temperature each cycle, and finally 23 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 60 s. Seven primer combinations (Table 2) were used by choosing the most effective combinations out of five primers associated with *EcoRI* (E+AAGG; E+AAGC; E+ACTG; E+ACCG; E+ACAT) and seven primers associated with *MseI* (M+CAA; M+CTT; M+CTC; M+CTG; M+CAT; M+CTA; M+CAC). Amplification products were electrophoresed in 13% (w/v) denaturing polyacrylamide gels at 120 V for 15 h in 1 $\times$  Tris-Glycine buffer. Bands were detected by silver nitrate with a kit of the silver sequence DNA staining reagents (Promega Corp., Madison, WI).

The degree of AFLP polymorphisms was quantified by Shannon's information index for phenotypic diversity (Browman et al., 1971):

$$H_s = - \sum P_i \ln P_i,$$

where  $H_s$  is the phenotypic diversity value of a group S and  $P_i$  is the frequency of the  $i$ th AFLP phenotype (band) in a specific group S. The phenotypic diversity value was calculated and compared for different groups as described by Wachira et al. (1995). Let  $H_{avg} = 1/n \sum H_s$  be the average diversity over  $n$  groups, and let  $H_T = - \sum P \ln P$  be the phenotypic diversity calculated from all groups considered together, where  $P$  is the frequency of a given AFLP phenotype averaged over all groups. Then the within-group diversity,  $H_{avg}/H_T$ , can be compared with the between-group diversity component,  $(H_T - H_{avg})/H_T$ .

For each accession, a binary matrix reflecting presence (1) or absence (0) of a specific AFLP-band was generated. Only heavy bands, ranging approximately from 100 to 600 base

Table 1. *Perilla* samples used for AFLP analysis.

Accession no.	Village, town, or city	Prefecture	Type
KOR1	Miryang-shi	Kyongsangnam-do	Cultivated type of var. <i>frutescens</i>
KOR2	Hapchon-gun	Kyongsangnam-do	Cultivated type of var. <i>frutescens</i>
KOR3	Yang-yang-gun	Kangwon-do	Cultivated type of var. <i>frutescens</i>
KOR4	Yonchon-gun	Kyonggi-do	Cultivated type of var. <i>frutescens</i>
KOR5	Chongson-gun	Kangwon-do	Cultivated type of var. <i>frutescens</i>
KOR6	Yechon-gun	Kyongsangbuk-do	Cultivated type of var. <i>frutescens</i>
KOR7	Okchon-gun	Chungchongbuk-do	Cultivated type of var. <i>frutescens</i>
KOR8	Chinan-gun	Chollabuk-do	Cultivated type of var. <i>frutescens</i>
KOR9	Kangjin-gun	Chollanam-do	Cultivated type of var. <i>frutescens</i>
KOR10	Tae-an-gun	Chungchongnam-do	Cultivated type of var. <i>frutescens</i>
KOR11	Cheju-shi	Cheju-do	Cultivated type of var. <i>frutescens</i>
KOR12	Chongson-gun	Kangwon-do	Cultivated type of var. <i>frutescens</i>
KOR40	Ulsan-shi	Kyongsangnam-do	Cultivated type of var. <i>frutescens</i>
KOR1-1	Miryang-shi	Kyongsangnam-do	Cultivated type of var. <i>frutescens</i>
KOR13	Yangpyong-gun	Kyonggi-do	Weedy type of var. <i>frutescens</i>
KOR14	Kochang-gun	Kyongsangnam-do	Weedy type of var. <i>frutescens</i>
KOR15	Kangjin-gun	Chollanam-do	Weedy type of var. <i>frutescens</i>
KOR16	Kochang-gun	Chollabuk-do	Weedy type of var. <i>frutescens</i>
KOR17	Umsong-gun	Chungchongbuk-do	Weedy type of var. <i>frutescens</i>
KOR24	Hwachon-gun	Kangwon-do	Weedy type of var. <i>frutescens</i>
KOR26	Yonchon-gun	Kyonggi-do	Weedy type of var. <i>frutescens</i>
KOR28	Okchon-gun	Chungchongbuk-do	Weedy type of var. <i>frutescens</i>
KOR30	Miryang-shi	Kyongsangnam-do	Weedy type of var. <i>frutescens</i>
KOR18	Pochon-gun	Kyonggi-do	Weedy type of var. <i>crispa</i>
KOR19	Hongchon-gun	Kangwon-do	Weedy type of var. <i>crispa</i>
KOR20	Andong-shi	Kyongsangbuk-do	Weedy type of var. <i>crispa</i>
KOR21	Chong-yang-gun	Chungchongnam-do	Weedy type of var. <i>crispa</i>
KOR22	Tangjin-gun	Chungchongnam-do	Weedy type of var. <i>crispa</i>
KOR23	Sachon-shi	Kyongsangnam-do	Weedy type of var. <i>crispa</i>
KOR38	Hongchon-gun	Kangwon-do	Weedy type of var. <i>crispa</i>

**Table 2.** Number of AFLP fragments generated with seven primer pairs among *Perilla* and related weedy types.

Primer combination	Total number of fragments	Number of polymorphic bands detected			Total	Percentage of polymorphic fragments
		Cultivated type of var. <i>frutescens</i>	Weedy type of var. <i>frutescens</i>	Weedy type of var. <i>crispa</i>		
1. E-AAGG+M-CAA	19.0	5.0	8.0	6.0	10.0	52.6
2. E-AAGC+M-CTT	15.0	1.0	4.0	6.0	9.0	60.0
3. E-ACTG+M-CTC	22.0	6.0	17.0	12.0	17.0	78.3
4. E-ACCG+M-CTG	15.0	1.0	6.0	5.0	10.0	66.7
5. E-ACAT+M-CAT	15.0	4.0	4.0	5.0	6.0	41.2
6. E-AAGG+M-CTA	19.0	4.0	8.0	7.0	9.0	50.0
7. E-ACAT+M-CAC	16.0	4.0	6.0	7.0	11.0	68.8
<b>Total</b>	<b>121.0</b>	<b>25.0</b>	<b>53.0</b>	<b>48.0</b>	<b>72.0</b>	<b>59.7</b>
<b>Avg.</b>	<b>17.3</b>	<b>3.6</b>	<b>7.6</b>	<b>6.9</b>	<b>10.3</b>	
<b>Percent polymorphism</b>		<b>20.7</b>	<b>43.8</b>	<b>39.7</b>	<b>59.5</b>	

pairs (bp), were scored and faint bands were ignored. The genetic relationships among *Perilla* and related weedy types were constructed by the neighbor joining method (Saitou and Nei, 1987) using the NTSYS-pc program (Rohlf, 1989).

## RESULTS AND DISCUSSION

We found that var. *frutescens* is extensively cultivated for production of leaf and seed on large scale. In addition,

many weedy plants of var. *frutescens* were commonly found along roadsides, in wastelands, and the borders of cultivated fields (Fig. 2a). In most cases, farmers recognized weedy type plants of var. *frutescens* as an escaped inedible form of var. *frutescens*. The weedy type of var. *frutescens* has green leaves and stems and the same low fragrance as var. *frutescens*, but its seeds are smaller (<2 mm) and harder than those of



**Fig. 2.** The weedy types of (a) *Perilla* var. *frutescens* and of (b) var. *crispa*, which were found in Korea.

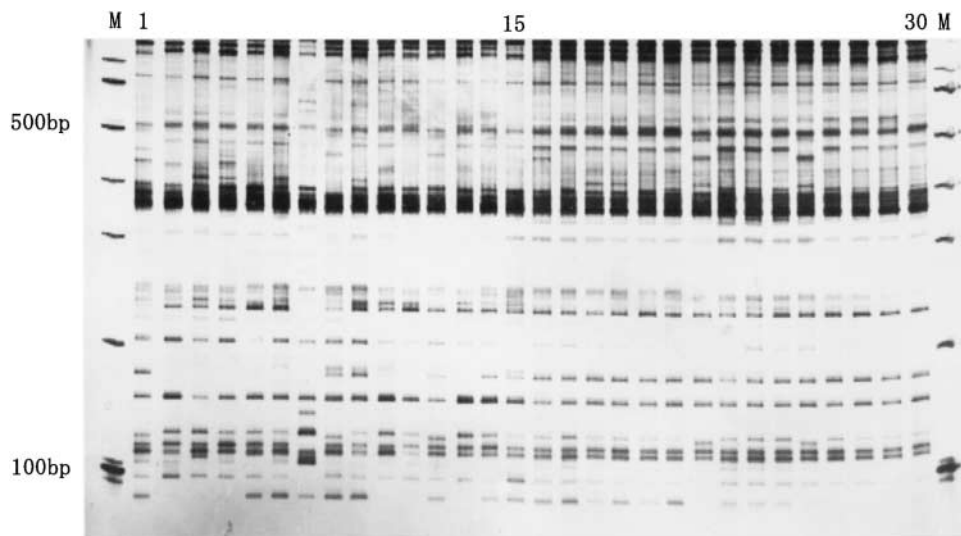


Fig. 3. Example of an AFLP profile with the primer pair E-AAGC+M-CTT. Lanes 1 to 15 are weedy type samples of var. *frutescens* and var. *crispa* and lanes 16 to 30 are cultivated type samples of var. *frutescens*.

cultivated var. *frutescens*. On the other hand, cultivation of var. *crispa* was not found. Its weedy type was found only around farmhouses (Fig. 2b). The accessions of cultivated var. *frutescens* in Korea had four different seed coat colors: white, gray, brown, and dark brown. Each seed sample collected in Korea was often the mixture of two or three seed colors. Cultivated var. *frutescens* was larger in seed size (>2 mm), and had either soft or hard seeds. The weedy types of var. *frutescens* and of var. *crispa* were uniform in seed color, always dark brown, and had small, hard seeds.

Analysis of 30 accessions of *P. frutescens* with seven AFLP primer combinations detected a total of 121 fragments, of which 72 (60%) were polymorphic at the species level; i.e., some samples of the species had a particular fragment, whereas others did not (Table 2). All seven primer combinations generated polymorphic fragments (Fig. 3). The number of fragments detected by each primer combination ranged from 15 in the primer combination E-AAGC/M-CTT, E-ACCG/M-CTG, and E-ACAT/M-CAT to 22 in E-ACTG/M-CTC (Table 2). The number of polymorphic fragments for each primer combination varied from six for E-ACAT/M-CAT to 17 for E-ACTG/M-CTC with an average of 10.3 (Table 2). The percentage of polymorphic fragments ranged from 41.2% (E-ACAT/M-CAT) to 78.3% (E-ACTG/M-CTC).

Phenotypic diversity ( $H_s$ ) calculated for each of the

seven primer combinations ranged from 0.92 to 4.53, with an average of 1.95 (Table 3). The estimated diversity was highly dependent on the primer combinations. The average phenotypic diversity values were 0.63, 2.00, and 1.75 for cultivated and weedy types of var. *frutescens*, and the weedy type of var. *crispa*, respectively (Table 3). The weedy type of var. *frutescens* was the most variable, whereas the cultivated var. *frutescens* the least. Partitioning the phenotypic diversity into within-group and between-group components showed that most (78%) of the AFLP variation was within-group variation (Table 3).

The phylogenetic tree constructed by the neighbor joining method showed two major clusters (Fig. 4). One cluster was the cultivated and weedy type of var. *frutescens* of var. *frutescens* and the other cluster was the weedy type of var. *crispa*. Two samples (KOR18, KOR30) of weedy types of var. *frutescens* and of var. *crispa* are outliers from the two main clusters. Several accessions of weedy types of var. *frutescens* and of var. *crispa* could not be discriminated by the AFLP analyses.

Most accessions of cultivated and weedy types of var. *frutescens* and weedy type of var. *crispa* were easily separated by AFLPs as expected from distinct morphological characters of the two *Perilla* crops and their weedy types (Lee and Ohnishi, 2001). The weedy type of var. *frutescens* was more polymorphic and had a greater  $H_s$  value than the cultivated var. *frutescens* and

Table 3. Estimates of phenotypic diversity ( $H_s$ ) among *Perilla* and related weedy types.

Primer combination	Cultivated type of var. <i>frutescens</i>	Weedy type of var. <i>frutescens</i>	Weedy type of var. <i>crispa</i>	$H_T$	$H_{avg}/H_T^\dagger$	$(H_T - H_{avg})/H_T$
1. E-AAGG+M-CAA	1.20	2.12	1.54	2.15	0.75	0.25
2. E-AAGC+M-CTT	0.13	1.07	1.35	1.41	0.60	0.40
3. E-ACTG+M-CTC	0.84	4.88	2.86	4.53	0.63	0.37
4. E-ACCG+M-CTG	0.07	1.59	1.86	1.39	0.84	0.16
5. E-ACAT+M-CAT	0.79	1.25	1.43	1.24	0.93	0.07
6. E-AAGG+M-CTA	1.08	2.28	1.86	2.02	0.86	0.14
7. E-ACAT+M-CAC	0.27	0.80	1.32	0.92	0.87	0.13
Avg.	0.63	2.00	1.75	1.95	0.78	0.22

$^\dagger H_{avg}/H_T$  = within-group variation;  $(H_T - H_{avg})/H_T$  = between group variation.

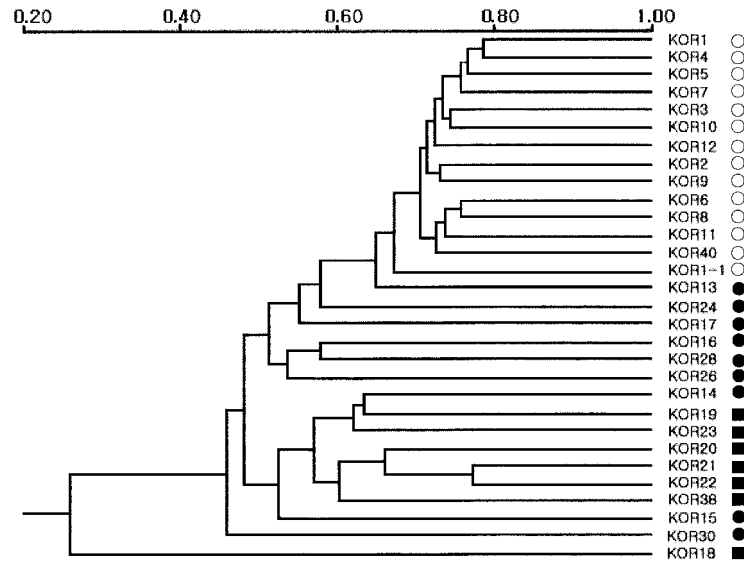


Fig. 4. Neighbor joining tree based on AFLP markers. ○: Cultivated type of *var. frutescens*; ●: Weedy type of *var. frutescens*; ■: Weedy type of *var. crispa*.

the weedy type of *var. crispa* (Tables 2, 3). In addition, in the field survey, many weedy samples of *var. frutescens* were found. Today, *var. frutescens* is extensively cultivated in Korea (Nitta, 2001). These results suggest that Korea may be a secondary center of diversity of *P. frutescens* in East Asia. China was suggested as the center of primary diversity by other investigators because of the ancient history of *Perilla* cultivation (Li, 1969) and very high genetic and morphological variations in Chinese accessions (Makino, 1961, p. 1060; Zeven and de Wet, 1982; Nitta, 2001). The cultivated *var. frutescens* is significantly different from the weedy type of *var. frutescens* in such morphological characters as seed size, seed hardness, seed color, flowering time, and leaf and stem color at the seedling stage (Lee and Ohnishi, 2001). The cultivated type of *var. frutescens* showed more diverse morphological variation than the weedy type of *var. frutescens* in spite of less AFLP variation. These results indicated that many variations in these morphological characters might have been found and selected by farmers, and have been rapidly and explosively diffused, or these characters might vary under cultivation. In addition, genetic variation (as evidenced by AFLPs) has been lost because of selection and random drift.

Nitta and Ohnishi (1999) reported that the weedy type of *var. frutescens* has large and hard seeds. However, some cultivated samples in Korea also had large hard seeds (the weedy type according to Nitta and Ohnishi, 1999) but were actually cultivated in fields. These samples (KOR5, KOR8, KOR11, KOR12, and KOR40) were included in the cultivated type cluster of *var. frutescens* in the AFLP analysis (Fig. 4). Therefore, these samples are the cultivated type, although they have hard seeds. The weedy type of *var. frutescens* and weedy type of *var. crispa* could be discriminated by morphological characters such as the color of leaves and stems and plant fragrance (Lee and Ohnishi, 2001). However, two samples (KOR14, KOR15) of the weedy type of *var. frutescens* were clustered with the weedy type of *var.*

*crispa* on the basis of AFLP analysis (Fig. 4). This might indicate the possibility of intervarietal crosses among the *Perilla* species and related weedy types, which requires further studies. The outcrossing rate in *Perilla* species is not known. Additionally, two samples (KOR18, KOR30) of weedy types of *var. frutescens* and of *var. crispa* are outliers from the two main clusters. Although the wild species of *P. frutescens* has not yet been identified, the our results may provide some evidence that the weedy *Perilla* is the key taxon for our understanding of the origin of cultivated *var. frutescens* and of *var. crispa*. The taxonomic position of the weedy samples and the origin of *Perilla* species needs to be clarified by future studies.

The geographic locations of the samples were not related to their position in the phylogenetic tree (Fig. 4). A similar result was obtained by random amplified polymorphic DNA (RAPD) analyses by Nitta and Ohnishi (1999). This implies that the diffusion of *Perilla* crops and their weedy types from China to Korea and Japan may be through multiple routes which are neither directional nor gradual.

Today, in Korea, the collection of genetic resources is important to prevent the genetic erosion of weedy types of *var. frutescens* and of *var. crispa*. The assessment of genetic relationships among genetic resources of *Perilla* crops and their weedy types by AFLP analysis in this study will be helpful to the *Perilla* breeding program in Korea. The present study has demonstrated the utility of AFLP analysis in the study of genetic diversity in *Perilla* crops and their weedy types.

#### ACKNOWLEDGMENTS

We are very grateful to the anonymous farmers who kindly provided us their seeds and answered our questions during our field survey in Korea. This is contribution No. 116 from Plant Germ-Plasm Institute, Faculty of Agriculture, Kyoto University.

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