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AFLP Fingerprinting of *Brassica campestis* L. ssp. *napus* var. *nippo-oleifera* Makino from Korea

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AFLPs (amplified fragment length polymorphisms) were used to estimate the genetic diversity of seven populations of *Brassica campestis* L. ssp. *napus* var. *nippo-oleifera* Makino between naturalized and cultivated populations. The seven Korean populations maintained a high level of genetic diversity. For example, all eight primers were high polymorphic, with an average of 3.2 effective alleles per primer set, and the expected heterozygosity was also high. The majority of genetic variance resided within populations. The combinations of an insect-pollinated, outcrossing breeding system, large population sizes, a high degree of gene flow and a propensity for high fecundity may explain the high level of genetic diversity within cultivated populations. Estimates of genetic similarity on the proportion of shared fragments ranged from 0.952 to 0.999. The high level of gene flow in Korean naturalized populations is mainly caused by seed dispersal via sea tide and the gene flow of cultivated populations may be enhanced in part by artificial pollen dispersal.

Amplified fragment length polymorphism (AFLP) described by Vos et al. (1995) combines the restriction site recognition element of RFLP (restriction fragment length polymorphism) analysis with the exponential amplification aspects of PCR-based marker and provides a less labor intensive, yet more robust compromise between the two basic marker types. Reproducibility, heritability, and intraspecific homology of AFLP have been demonstrated (Mackill et al., 1996), and comparative studies indicate that AFLP offers a high level of utility when compared with other marker systems (Powell et al., 1996; Russell et al., 1997; Teulat et al., 2000).

Wild species provide information regarding the progress of domestication (Doebley, 1989), and it is very important to study natural populations of this species from the viewpoint of crop evolution. *Brassica campestis* L. ssp. *napus* var. *nippo-oleifera* Makino is an important crop and is not listed as an indisputable wild plant in any flora to date. The species is thought to have originated in East Asia and is a particularly rich source of *Brassica* germplasm (Crawford, 1992). It has been suggested that *B. campestis* was domesticated in China which is the current center of diversity (Ladizinsky, 1998). Since most of crops cultivated in Korea are considered to be originated from China, *B. campestis* ssp. *napus* var. *nippo-oleifera* may be also from China. However, there is no concrete evidence available and the origin and genetic diversity information of this species are scarcely reported.

The objective of this study was to assess the amount and structure of genetic diversity within the primary gene pool of naturalized and cultivated populations of *B. campestis* ssp. *napus* var. *nippo-oleifera* using AFLP markers. In addition, our basic question was: Has the domestication process eroded the levels of genetic variation of the cultivated populations as has been shown in most cultivated species (Doebley, 1989)?

Materials and Methods

Plant material

A total of 42 individuals of *B. campestis* ssp. *napus* var. *nippo-oleifera* from five naturalized populations (WPU: Songjung, Haeunde-ku, Pusan, WKE: Kuzora, Keje-kun, Gyeongsangnam-do, WYU: Woelpo, Youngduck-kun, Gyeongsangbuk-do, WNA: Sangju, Namhae-kun, Gyeongsangnam-do, and WKA: Kampo, Youngil-kun, Gyeongsangbuk-do) and two cultivated populations (CPU: Songjung, Haeunde-ku, Pusan and CWO: Wonsa, Sachen-si, Gyeongsangnam-do) in Korea were sampled for analysis (Fig. 1).

Following cold stratification, randomly selected 50 siliques from each population were germinated in a greenhouse. When the seedlings were three weeks old, six individuals from each population were randomly sampled for molecular analysis.

DNA extraction and AFLP analysis

DNA was extracted using the plant DNA Zol Reagent

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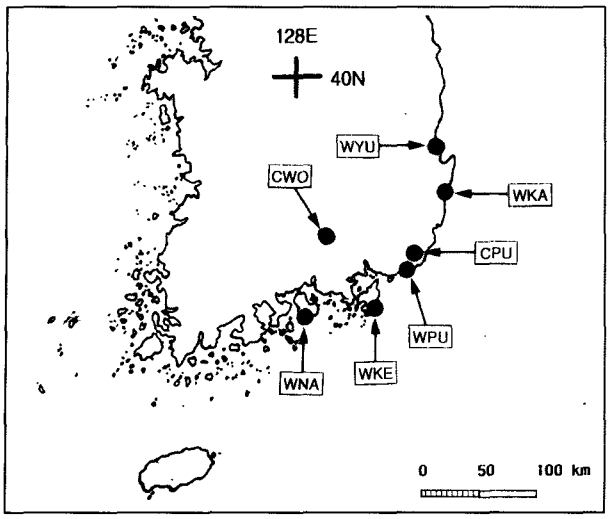

Fig. 1. Locations of populations of *Brassica campestis* L. ssp. *napus* var. *nippo-oleifera* species samples for this study.

(Life Technologies Inc.) according to the manufacturer's protocol. Reactions for AFLP analysis was carried out as described by Vos et al. (1995) with some minor modifications. Briefly, 125 ng of genomic DNA was digested with *Eco*RI and *Mse*I then ligated to *Eco*RI and *Mse*I adapters (AFLP core Reagent Kit, Gibco BRL, Inc.). Amplification was performed using eight primer combinations and gel electrophoresis according to the protocol of AFLP Analysis System I™ (Life Technologies Inc.). The reaction products were analyzed on 8% denaturing polyacrylamide gel. The staining protocol included incubation with silver nitrate solution, developing with sodium carbonate solution, and fixing with acetic acid according to the protocol of the Silver Sequence DNA Sequencing System™ (Promega Co.).

Statistical analysis

All monomorphic and polymorphic AFLP bands visible in at least 95% of the individuals by eye were scored to exclude ambiguous bands in the analysis. A binary data matrix reflecting the presence (1) or absence (0) of each AFLP band was generated for the set of all populations. The degree of polymorphism was quantified using Shannon's index of phenotypic diversity (Bowman et al., 1971):

$$H_O = -\sum pi \ln pi$$

where pi is the frequency of phenotype (King and Schaal, 1989). H_O can be calculated and compared for different populations (Paul et al., 1997). Let:

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

be the average diversity over the n different populations and let:

$$H_{SP} = -\sum p \ln p$$

be the diversity calculated from the phenotypic frequencies p in all the populations considered together (Paul et al., 1997). Then the proportion of diversity present within populations, H_{POP} / H_{SP}, can be compared with those populations, $(H_{SP} - H_{POP}) / H_{SP}$.

Expected heterozygosity H_{EP} for a genetic marker may be calculated from the sum of the squares of allele frequencies.

$$H_{EP} = 1 - \sum pi^2$$

where pi is the allele frequency for the i-th allele (Nei, 1973).

The sum of effective number of alleles (SENA) was calculated by determining the effective number of alleles for each locus (Powell et al., 1996): $SENA = \sum \{(1 / \sum pi^2) - 1\}$.

The genetic similarity (GS) between line i and j estimated using the formula of Dice (1945) as $GS = 2Nij / (Ni + Nj)$, where Nij is the number of common loci between i and j, and Ni and Nj are the number of loci in i and j, respectively. GS was converted to genetic distance (1−GS) (Le Thierry et al., 2000). Differences in molecular variation among populations were tested Bartlett's statistics. Genetic differentiation (F_{ST}) among pairs of the populations and their levels of significance were also calculated. Furthermore, gene flow between the pairs of populations $\{F_{ST} = 1/(4\ Nm + 1)\}$ was calculated from F_{ST} values (Wright, 1951). A phylogenetic tree was constructed by the neighborjoining (NJ) method (Saitou and Nei, 1987) using the NEIGHBOR program in PHYLIP version 3.57 (Felsenstein, 1993).

Results

AFLP fingerprinting of *B. campestis* ssp. *napus* var. *nippo-oleifera* with eight primer combinations revealed a total number of 341 unambiguous amplified DNA fragments (Table 1). On the average, 42.6 bands were scored per primer combination. Three bands out of the 341 bands were found within cultivated populations, while 17 bands were specific to the natural populations. On the average 74.6% polymorphic markers were generated using eight primer pairs (Table 1). In simple measures of intrapopulation variability based on the percentage of polymorphic products, CWO population exhibited the lowest genetic diversity (31.2%). WKE (Kuzora population) showed high variability within populations (46.1%). The percentage of polymorphic bands was significantly different between the naturalized and cultivated populations (F = 5.610). A similarity matrix based on the proportion of shared fragments (GS) was used to establish the level of relatedness among seven populations (Table 5). Estimates of GS ranged from 0.952 to 0.999.

The phenotypic diversity (H_O) calculated for the

Table 1. Informativeness of eight combinations of selective primers used to detect AFLP between *Brassica campestis* L. ssp. *napus* var. *nippo-oleifera* genotypes

Primer		Total		Population													
				WPU		WKE		WYU		WNA		WKA		CPU		CWO	
*Eco*RI	*Mse*I	Band (No.)	*P*	*P*	P_P	*P*	P_P	*P*	P_P	*P*	P_P	*P*	P_P	*P*	P_P	*P*	P_P
ACG	CTC	61	54.1	41.4	80.0	45.8	87.1	30.5	58.1	42.6	78.8	31.7	59.4	27.3	55.6	25.0	50.0
ACT	CAG	55	41.8	29.6	68.2	30.9	73.9	27.8	68.2	32.7	78.3	25.9	63.6	25.5	68.4	20.4	52.4
AGC	CTG	34	58.8	37.5	66.7	38.2	65.0	28.1	50.0	41.2	70.0	29.0	52.9	29.6	61.5	28.6	57.1
AGG	CAC	30	56.7	46.4	86.7	51.7	93.8	37.0	71.4	50.0	88.2	33.3	64.3	31.0	56.3	34.2	66.7
AAC	CAA	50	58.0	42.0	72.4	46.9	82.1	40.8	71.4	46.0	79.3	38.8	67.9	39.6	70.4	37.5	66.7
AAG	CAT	30	53.3	42.9	85.7	53.3	100	44.8	86.7	50.0	93.8	41.4	80.0	28.6	57.1	29.6	61.5
ACA	CAG	44	52.3	44.2	86.4	47.7	91.3	41.9	81.8	47.7	91.3	41.9	81.8	32.4	75.0	28.9	64.7
ACC	CAT	37	56.8	51.4	94.7	54.1	95.2	44.1	83.3	56.8	100	48.6	89.5	45.5	88.2	45.5	88.2
Mean		42.6	54.0	41.9	80.1	46.1	86.1	39.6	71.4	45.9	85.0	36.3	69.9	32.5	66.6	31.2	63.4

P, polymorphic percentage of total bands including monomorphic bands; P_P, polymorphic percentage of only polymorphic bands.

populations ranged from 3.54 to 3.65, with a mean of 3.601 (Table 2). H_O of naturalized populations was higher than those of cultivated populations ($F = 8.12$, paired *t* test). The Korean populations maintained a high level of genetic diversity ($H_{SP} = 3.653$) (Table 3). The same trend was observed at the expected heterozygosity ($H_{EP} = 0.763$) (Table 4).

Since the assessment of the proportion of diversity present within populations (H_{POP} / H_{SP}) indicated that about 2.0% of the total genetic diversity was among populations, and the majority of genetic variation (98.4%) resided within populations (Table 3). The average number of individuals exchanged among populations per generation (*Nm*) was very high (5.0).

In order to estimate the difference in the total amount of polymorphism information for each marker, the effective numbers of alleles (SENA) were calculated (Table 4). Although mean SENA value of the naturalized populations is slightly higher than those of the cultivated populations (CPU and CWO), SENA between populations did not differ from the whole values (Bartlett's test for homogeneity variance). The expected heterozygosity (H_{EP}) calculated for each marker at population level was given for each assay in Table 4. There was no significant difference in the H_{EP} values between both of the two groups (the naturalized and cultivated).

In accordance with the high values of correlation between the matrices of phenetic and genetic distances, N-J analysis of relationships among individuals of *B. campestis* L. ssp. *napus* var. *nippo-oleifera* produced congruent dendrogram (Fig. 3). The analysis of molecularmarkers placed individuals from the same populations in the same cluster.

Discussion

The AFLP analysis of *B. campestis* L. ssp. *napus* var. *nippo-oleifera* in Korea shows similarity between naturalized and cultivated populations, in terms of genetic diversity and structure. Although the genetic diversity parameters of the cultivated group are lower than those of the naturalized groups, *B. campestis* L. ssp. *napus* var. *nippo-oleifera* has relatively high levels of molecular variation as compared to other plant species. For example, AFLP revealed a large number of polymorphic DNA fragments in this study. The levels of genetic diversity in *B. campestis* L. ssp. *napus* var. *nippo-oleifera* are higher than those of other *Brassica* species with very similar life history characteristics (Dos Santos et al., 1994; Thormann et al., 1994). Dos Santos et al. (1994) and Thormann et al. (1994) observed lower genetic diversity using RFLP and RAPD markers within *Brassica* species than our results of *B. campestis* L. ssp. *napus* var. *nippo-oleifera*. The RFLP and RAPD markers generate less polymorphisms than those revealed by AFLPs (Powell et al., 1996; Teulat et al., 2000) and the species were different from each other.

The relatively high level of genetic variation found in

Table 2. Estimates of genetic diversity (H_O) within populations of *Brassica campestis* L. ssp. *napus* var. *nippo-oleifera*

Primer	Population						
	WPU	WKE	WYU	WNA	WKA	CPU	CWO
ACG-CTC	3.988	4.035	4.029	4.057	4.044	3.962	3.992
ACT-CAG	3.890	3.970	3.913	3.978	3.925	3.892	3.926
AGC-CTG	3.356	3.475	3.374	3.448	3.371	3.224	3.229
AGG-CAC	3.224	3.268	3.249	3.289	3.246	3.299	3.304
AAC-CAA	3.823	3.839	3.849	3.860	3.829	3.776	3.813
AAG-CAT	3.230	3.338	3.308	3.324	3.304	3.231	3.196
ACA-CAG	3.678	3.720	3.668	3.746	3.488	3.548	3.567
ACC-CAT	3.420	3.532	3.462	3.524	3.496	3.404	3.408
Mean	3.576	3.647	3.606	3.653	3.588	3.542	3.554

Table 3. Partitioning of the genetic diversity into within and among populations of *Brassica campestis* L. ssp. *napus* var. *nippo-oleifera* for eight primer combinations

Primer	H_{POP}	H_{SP}	H_{POP} / H_{SP}	$(H_{SP} - H_{POP}) / H_{SP}$
ACG-CTC	4.015	4.068	0.987	0.013
ACT-CAG	3.928	3.968	0.990	0.010
AGC-CTG	3.354	3.453	0.971	0.029
AGG-CAC	3.269	3.308	0.988	0.012
AAC-CAA	3.827	3.866	0.990	0.010
AAG-CAT	3.276	3.326	0.985	0.015
ACA-CAG	3.631	3.715	0.977	0.023
ACC-CAT	3.464	3.523	0.983	0.017
Mean	3.595	3.653	0.984	0.016

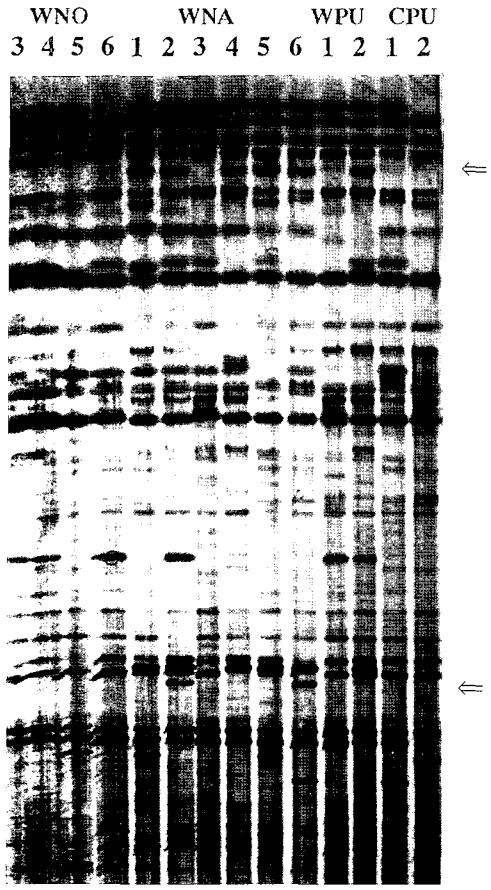

Fig. 2. Fingerprints of the populations studied after PCR with primer combination E:ACA-M:CAG. Code numbers at the top of each lane are given in Fig. 1. The arrows indicate specific bands to the natural populations.

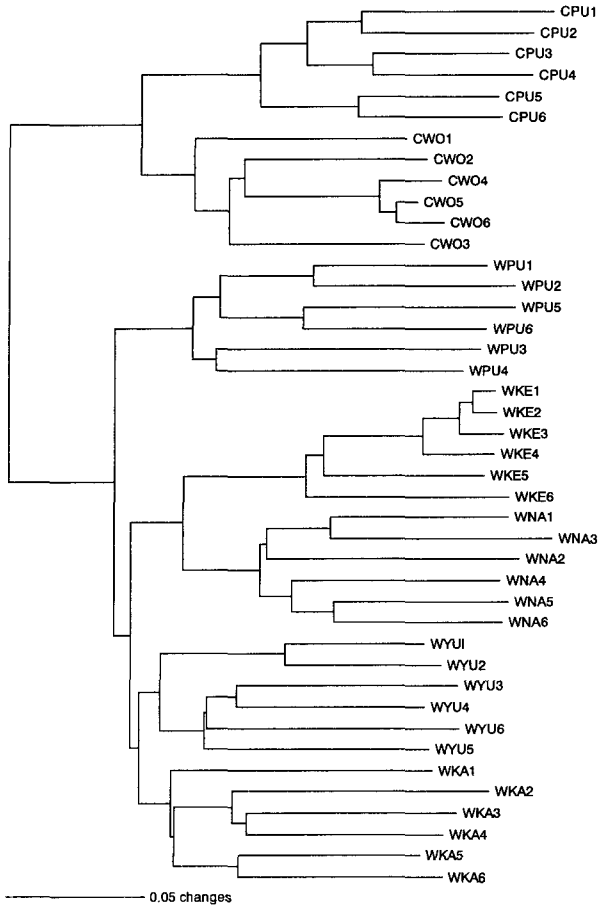

Fig. 3. Dendrogram illustrating genetic relationships among 42 individuals of seven populations of *Brassica campestis* L. ssp. *napus* var. *nippo-oleifera*. Neighbor-joining tree was generated form eight AFLP marker combinations.

the *B. campestis* L. ssp. *napus* var. *nippo-oleifera* populations is consistent with several aspects of its biology. First, the breeding system of the species is an important determinant of variability at both the species and population levels. *Brassica campestis* L. ssp. *napus* var. *nippo-oleifera* is a predominantly outcrossing, and insect-pollinated species. Second, the primary determinant of level of genetic diversity is the effective population size (Barrett and Kohn, 1991). The population size of naturalized *B. campestis* L. ssp. *napus* var. *nippo-oleifera* is large, consisting of 10^4-10^5 individuals; each subpopulation consists of 10^2-10^3 individuals. The gene flow between populations is relatively high ($Nm = 5.0$). These values are large enough to nullify the local differentiation of neutral alleles by random drift (Wright, 1951). Finally, *B. campestis* L. ssp. *napus* var. *nippo-oleifera* has high seed production; an individual plant produces hundreds of seeds in the field or in green house conditions (field observations). Many seeds are deposited in the soil as a seed bank, where they may contribute to later generations (Kang and Yoon, 2000).

We found 2.0% of genetic variation among popula-

Table 4. Sum of effective number of alleles (SENA) and expected heterozygosity (H_{EP}) calculated for seven populations of *Brassica campestis* L. ssp. *napus* var. *nippo-oleifera*

Population	SENA	H_{EP}
WPU	3.070	0.754
WKE	3.456	0.776
WYU	3.272	0.766
WNA	3.450	0.775
WKA	3.292	0.767
CPU	3.003	0.750
CWO	3.045	0.753
Mean	3.227	0.763

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

Population	WPU	WKE	WYU	WNA	WKA	CPU	CWO
WPU	–	0.9866	0.9864	0.9896	0.9865	0.9666	0.9697
WKE	0.0134	–	0.9925	0.9912	0.9866	0.9570	0.9644
WYU	0.0136	0.0075	–	0.9896	0.9985	0.9650	0.9726
WNA	0.0104	0.0088	0.0104	–	0.9911	0.9516	0.9776
WKA	0.0135	0.0134	0.0015	0.0089	–	0.9636	0.9801
CPU	0.0334	0.0430	0.0350	0.0484	0.0364	–	0.9889
CWO	0.0303	0.0356	0.0274	0.0224	0.0199	0.0111	–

tions and 98.4% within populations (Table 3). Hamrick and Godt (1989) used F_{ST} value to indicate the proportion of isozyme diversity residing among populations. They report an average of F_{ST} 22% for perennial herbs compared with 36% for annuals, an average of F_{ST} 23% for sexually reproducing plants compared with 21% for species that reproduces both sexually and asexually, and an average of F_{ST} 20% for animal-pollinated outcrossers compared with 51% for selfers. RAPD-based F_{ST} values are available for 35 plants species (Bussell, 1999). However, the populations of *B. campestis* L. ssp. *napus* var. *nippo-oleifera* appeared to be less for our AFLP differentiated than expected for an insect-pollinated outcrosser. This is somewhat surprising when we consider the fact that the present-day populations of these species are discontinuous and isolated. However, monomorphic bands were included from data analysis to compare with the results of isozyme analysis. It is a major cause for overstating the values. In addition, naturalized populations shared over 95.2% similarities with cultivated populations indicating recent divergence of populations in naturalized and cultivated populations or among total populations. Although there is a significant difference between the gene flow of the two cultivated populations and that of five naturalized populations, gene flow is very high in this study ($Nm = 5.0$). These values are enough to nullify the local differentiation of neutral alleles by random drift (Wright, 1951). In the coastal areas, cultivated fields and naturalized populations of *B. campestis* L. ssp. *napus* var. *nippo-oleifera* can seen side by side (e.g. CPU and WPU in Fig. 1). Hence, gene flows from cultivated populations to naturalized populations can be expected. In addition, we cannot completely rule out the possibility that some naturalized populations escaped from cultivation in the areas where they were planted. We frequently found siliques of *B. campestis* L. ssp. *napus* var. *nippo-oleifera* among flotsams on beaches and also found such seeds can be germinated. The high level of gene flow in *B. campestis* L. ssp. *napus* var. *nippo-oleifera* populations is mainly caused by seed dispersal via sea current. It is a probability that populations of inland were disrupted by seed flow via sea current (Kim and Kim, 2000). However, apiculturists have moved honeybees from place to place to forage on spring flowers including *B. campestis* L. ssp. *napus* var. *nippo-oleifera*. Pollen dispersal by bees may be one of mechanisms of the gene flow among cultivated populations.

Although our data is relatively small and there is a significant difference in the percentage of polymorphism (Table 1), there are no significant differences from the effective allele (SENA) and expected heterozygosity (H_{EP}) (Table 4). Despite the exploitation of ancestry in agronomic species, there is still little understanding of its adaptive value in populations of wild species (Purdy and Bayer, 1995). From this point of view, further studies on *B. campestis* L. ssp. *napus* var. *nippo-oleifera* will add general knowledge on plant population structure, especially of domestication, assess *B. campestis* species in light of their origins, and fill a gap in our knowledge on model colonization.

Using allozyme analysis, the position of the populations in the tree and geographical position in Korean populations almost did not match completely (author's unpublished results). Whatever the phylogenetic consideration, it is relevant to stress that the AFLP fingerprints that we obtained allowed us to discriminate all the Korean populations, even those that cannot be distinguished on the basis of allozyme analysis (Fig. 3). In addition, the high degree of similarity may have caused difficulties in distinguishing genetic differentiation among cultivated populations by allozyme analysis because the isozyme markers generate less polymorphism than AFLP markers (Garcia et al., 2000).

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