

Genetic Variation in *Fusarium oxysporum* f. sp. *fragariae* Populations Based on RAPD and rDNA RFLP Analyses

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Fusarium oxysporum f. sp. *fragariae* is a fungal pathogen causing strawberry wilt disease. The random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphisms (RFLPs) of intergenic spacer (IGS) region of rDNA were used to identify genetic variation among 22 *F. oxysporum* f. sp. *fragariae* isolates. All isolates could be distinguished from each other by RAPD analysis and RFLP of 2.6 kb amplified with primer CNS1 and CNL12 for IGS region of rDNA. Cluster analysis using UPGMA showed eight distinct clusters based on the banding patterns obtained from RAPD and rDNA RFLP. These results indicate that *F. oxysporum* f. sp. *fragariae* isolates are genetically distinct from each other. There was a high level genetic variation among *F. oxysporum* f. sp. *fragariae*.

Keywords : *Fusarium oxysporum* f. sp. *fragariae*, genetic variation, RAPD, rDNA, RFLP

Strawberry (*Fragariae* X *ananassa* Duch.) is an economically important crop. In recent years, strawberry production has shown worldwide increase (FAO Statistical Databases). However, *Fusarium oxysporum* Schlechtend.: Fr. f. sp. *fragariae* Winks and Williams, which causes strawberry Fusarium wilt disease, has dramatically decreased commercial strawberry production (Winks and Williams, 1965). The forma specialis causing wilt on strawberry has been designated as f. sp. *fragariae* (Wilhelm, 1984). Isolates that cause the disease in members of a particular family, genus, (or) species are formae speciales.

Recently, molecular methods such as the PCR have been described to resolve genetic variation among isolates within or between formae speciales of *F. oxysporum* (McDonald, 1997). Especially, random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphisms (RFLPs) of rDNA have been widely used for assessing genetic diversity, genome mapping, and molecular diagno-

stics of many fungal species (Annamalai et al., 1995; Manulis et al., 1994).

Another strategy to detect genetic variation is the use of defined PCR amplified fragments as substrate for RFLP or sequence analysis. The ribosomal DNA (rDNA) regions have often been chosen for taxonomic and phylogenetic studies, because sequence data are available and contain both variable and conserved regions; despite the discrimination at the genus, species, or intraspecific level. The rDNA repeat includes both highly conserved genes and more variable spacer regions.

The spacer regions, including the internal transcribed spacer (ITS) and the intergenic spacer (IGS), have been used to examine relationships among more closely related taxa by analysis of RFLP analysis (Appel and Gordon 1995; Edel et al., 1995). The rDNA region analyses have been used successfully to differentiate other *Fusarium* species such as *F. avenaceum*, *F. arthroporioides*, and *F. tricinctum* (Yli-Mattila et al., 2002); and within the *Fusarium* formae speciales such as *F. oxysporum* f. sp. *cyclaminis* (Woudt et al., 1995), *F. oxysporum* f. sp. *dianthi* (Manicom et al., 1990), *F. oxysporum* f. sp. *gladioli* (Mes et al., 1994), and *F. oxysporum* f. sp. *phaseoli* (Woo et al., 1996).

In this study, RAPD markers and RFLPs of IGS region of rDNA were used to ascertain the affinities and variation among *F. oxysporum* f. sp. *fragariae* isolates of infected strawberry. This study evaluated the genetic variation that could be useful for further resistance-breeding of strawberry against this pathogen.

Materials and Methods

Isolates. Twenty-two isolates of *F. oxysporum* f. sp. *fragariae* were collected from different major strawberry-cultivating areas in Korea (Table 1). After pathogenicity test on strawberry (cultivar: Dochiodome), they were used for this investigation. Individual isolates were selected through the subculture of a single conidium.

Genomic DNA extraction. Isolates were cultured on potato

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Table 1. The isolates of *Fusarium oxysporum* f. sp. *fragariae* used in this study

| No. | Isolate code | Strawberry cultivar | Collection site | Collection year |
|-----|--------------|---------------------|---------------------|-----------------|
| 1 | Fo1 | Nyoho | Nonsan, Chungnam | 1995 |
| 2 | Fo3 | Nyoho | Nonsan, Chungnam | 1995 |
| 3 | Fo4 | Nyoho | Buyeo, Chungnam | 1995 |
| 4 | Fo6 | Hokowase | Gongju, Chungnam | 1995 |
| 5 | F011 | Hokowase | Hongseong, Chungnam | 1995 |
| 6 | FF801 | Hokowase | Damyang, Jeonnam | 1996 |
| 7 | FF802 | Hokowase | Damyang, Jeonnam | 1996 |
| 8 | FF406 | Hokowase | Damyang, Jeonnam | 1996 |
| 9 | FF408 | Hokowase | Nonsan, Chungnam | 1996 |
| 10 | FF409 | Hokowase | Nonsan, Chungnam | 1996 |
| 11 | Fo13 | Hokowase | Hongseong, Chungnam | 1996 |
| 12 | Fo23 | Reiko | Nonsan, Chungnam | 1996 |
| 13 | Fo30 | Nyoho | Nonsan, Chungnam | 1996 |
| 14 | Fo40 | Redpearl | Iksan, Jeonbuk | 1996 |
| 15 | Fo41 | Redpearl | Iksan, Jeonbuk | 1996 |
| 16 | Fo42 | Akihime | Nonsan, Chungnam | 2001 |
| 17 | Fo45 | Akihime | Daedeok-gu, Daejeon | 2001 |
| 18 | Fo47 | Dochiodome | Nonsan, Chungnam | 2001 |
| 19 | Fo48 | Dochiodome | Nonsan, Chungnam | 2001 |
| 20 | Fo50 | Johong | Gangseo-gu, Busan | 2001 |
| 21 | Fo54 | Dochiodome | Gongju, Chungnam | 2001 |
| 22 | Fo56 | Akihime | Yeongi, Chungnam | 2001 |

dextrose agar. Three to four mycelial plugs (4 mm diam) were transferred to flasks containing 150 ml of potato dextrose broth and incubated at 25°C on an orbital shaker (125 rpm) for 4 days. Mycelia from cultures were collected onto Miracloth by vacuum filtration, washed with sterile distilled water, lyophilized, and ground in liquid nitrogen. Total genomic DNA was extracted from the powdered sample of mycelium by using the method described previously by Lee and Taylor (1990). The DNA was resuspended in 50 µl of TE buffer and stored at -20°C.

RAPD analysis. Fifteen arbitrarily chosen decamer primers (OPA-01, 02, 03, 04, 05, 06, 07, 08, 09, 10, 11, 12, 13, 14, 15) from Operon primer kit (Operon Technology Inc, USA) were tested for PCR amplifications of *F. o. f. sp. fragariae* DNA. The preliminary amplification determined the optimal concentration of the component in the PCR reaction mixture. Amplifications were performed in a total volume of 20 µl containing 20 ng genomic DNA, 50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris/HCl (pH 8.3), 200 µM dATP, dCTP, dGTP and dTTP, 10 picomoles of primer, and 1 U of *Taq* DNA polymerase (Promega Corp. Madison, WI, USA).

Each reaction was overlaid with one drop of mineral oil. PCR was carried out in PTC 100 programmable thermal cycler (MJ Research, Water Town, MA, USA). The program included an initial denaturation at 94°C for 5 min, 45 cycles with denaturation at 94°C for 2 min, annealing 34°C for 1 min, an extension step at 72°C for 2 min, and a final extension at 72°C for 15 min. Negative

controls (no template DNA) were used for each set of experiments to test for the presence of nonspecific reaction. All experiments were repeated at least three times. The PCR products were electrophoresed on 1.2% agarose gel by using 0.5 × TBE buffer, stained with ethidium bromide and visualized in a UV transilluminator.

rDNA RFLP analysis. The IGS region of the rDNA was amplified by using the primer CNL12 (5'-CTGAACGCCTC-TAAGTCAG-3') and CNS1 (5'-GAGACAAGCATATGACTA-CTG-3') (Appel and Gordon, 1996). PCR amplifications were performed in a total volume of 30 µl reaction mixture containing 20 ng genomic DNA, 50 mM KCl, 2.5 mM MgCl₂, 10 mM Tris/HCl (pH 8.3), 200 µM dNTP (N = A, C, G and T), 0.5 µM each of the CNS1 and CNL12 primers and 1 U of *Taq* DNA polymerase.

Each reaction was overlaid with one drop of mineral oil. Amplification was carried out in a PTC 100 programmable thermal cycler. Program included an initial denaturation step of 94°C, for 3 min, followed by 35 amplification cycles of denaturation, annealing, and extension. Temperature and times for these steps in the first 13 cycles were 95°C for 35s; 58°C for 55s; and 72°C for 45s. Cycles 14-26 and 27-35 used the same parameters, except that the 72°C extension steps were increased from 2 min to 3 min within the two sets of cycles, respectively.

After 35 cycles were completed, the samples were incubated for an additional 10 min at 72°C. Negative controls (no template DNA) were used for each set of experiments to test for the presence of nonspecific reaction. In separate reactions, 10 µl aliquots of the IGS PCR products were digested with the restriction enzymes, *Ava* II, *Hinf* I, *Eco*R I, and *Eco*R V; according to the manufacturer's instructions (Boehringer Mannheim, Germany). The digested products were separated on 2% agarose gel in 0.5 × TBE buffer. The gels were stained with ethidium bromide and visualized in a UV transilluminator.

Data analysis. Each profile was compared on the basis of the presence (1) versus absence (0) of amplified band. The data generated from RAPD and RFLP for each isolate were pooled. A genetic similarity matrix was calculated based on the method of simple matching coefficient and the values were used to generate a similarity matrix (Sneath and Sokal, 1973). The resulting matrix was analyzed for hierarchical clustering of the isolates by the unweighted pair-group with arithmetic averages (UPGMA). All calculations were conducted by using the computer program NTSYS pc (Rohlf, 1990).

Results

RAPD analysis. Genomic DNA isolated from 22 isolates of *F. oxysporum* f. sp. *fragariae* was subjected to RAPD-PCR analysis with 15 random decamer primers. In the preliminary experiments, 9 out of the 15 primers tested produced distinct and reproducible band profiles, and polymorphisms produced by seven primers, except OPA-07 and OPA-11, were relatively low (data not shown). Two of the nine primers were used for a comparative analysis of the 22 isolates of *F. oxysporum* f. sp. *fragariae*.

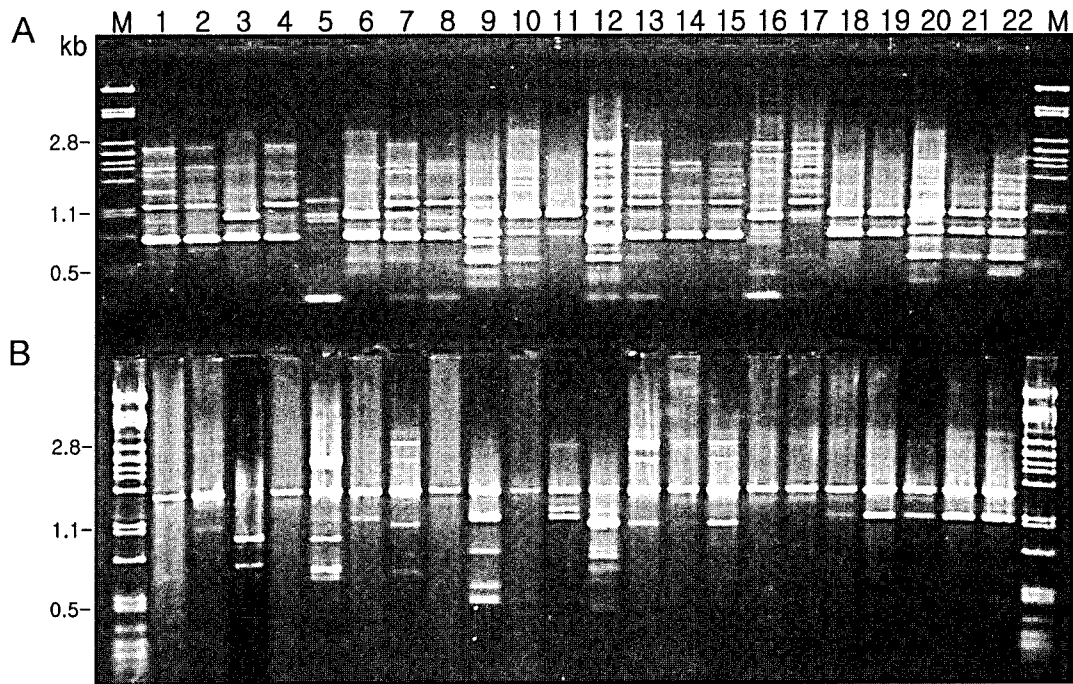


Fig. 1. RAPD patterns on 1.2% agarose gel of amplified fragment generated from 22 isolates of *Fusarium oxysporum* f. sp. *fragariae* with random primer (A) OPA-07 and (B) OPA-11. Lane M, DNA marker (DNA digested with *Pst*I), lane 1, Fo1; 2, Fo3; 3, Fo4; 4, Fo6; 5, Fo11; 6, FF801; 7, FF802; 8, FF406; 9, FF408; 10, FF409; 11, Fo13; 12, Fo23; 13, Fo30; 14, Fo40; 15, Fo41; 16, Fo42; 17, Fo45; 18, Fo47; 19, Fo48; 20, Fo50; 21, Fo54; and 22, Fo56.

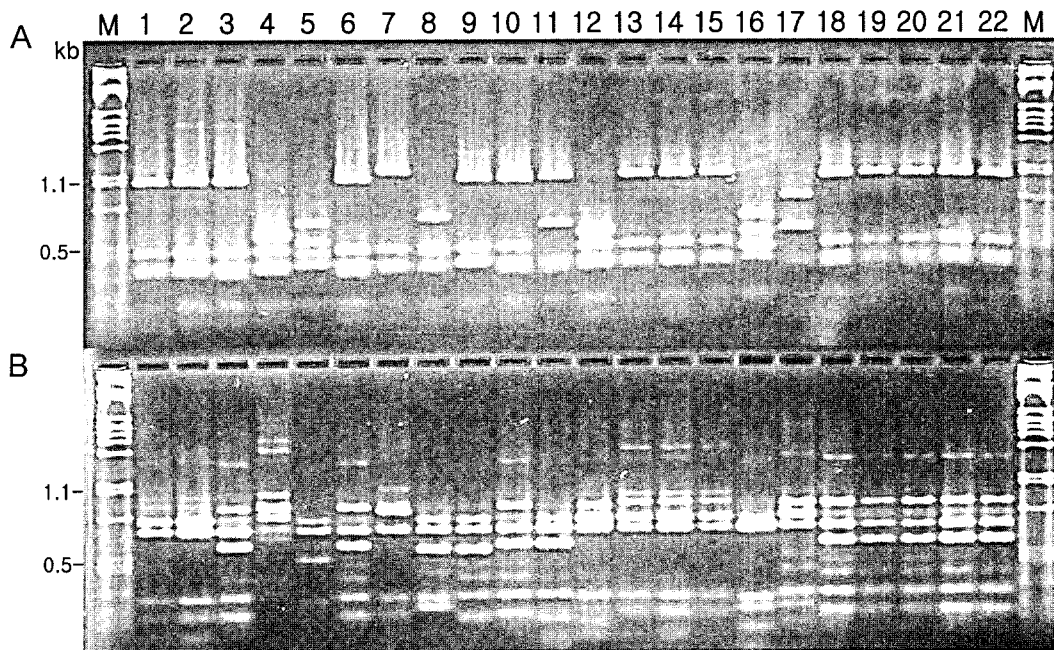


Fig. 2. Electrophoretic patterns on 2% agarose gel RFLPs of the amplified intergenic spacer (IGS) region from 22 isolates of *Fusarium oxysporum* f. sp. *fragariae* digested with (A) *Av*all and (B) *H*infI. Lane M, DNA marker (DNA digested with *Pst*I), lane 1, Fo1; 2, Fo3; 3, Fo4; 4, Fo6; 5, Fo11; 6, FF801; 7, FF802; 8, FF406; 9, FF408; 10, FF409; 11, Fo13; 12, Fo23; 13, Fo30; 14, Fo40; 15, Fo41; 16, Fo42; 17, Fo45; 18, Fo47; 19, Fo48; 20, Fo50; 21, Fo54; and 22, Fo56.

The primers, including OPA-07 (5'-GAAACGGGTG-3') and OPA-11 (5'-CAATCGCCGT-3'), generated polymorphic band in all isolates (Fig. 1). Amplified fragments were ranged from 150 bp to 2.4 kb. RAPD assays of the 22 isolates with two primers yielded 175 bands which were polymorphic. The number of DNA fragments, amplified and scored per isolate for individual primer, ranged from 2 to 8. These data show that RAPD is a convenient method for distinguishing the isolates of *F. oxysporum* f. sp. *fragariae* and also reveal a high degree of genetic variation among the isolates.

rDNA RFLP analysis. A single double-stranded product of approximately 2.6 kb, representing IGS, was amplified from all 22 isolates by using primers CNS1 and CNL12. Amplified IGSs were digested with four endonucleases: *Ava* II, *Hinf* I, *Eco*R I, and *Eco*R V. Digested DNA fragments of amplified IGS region with *Ava* II and *Hinf* I were made of 2 to 7 bands per isolate (Fig. 2). No restriction patterns were observed by *Eco*R I and *Eco*R V digestion. However, there was no correlation between the isolates. The restriction analysis yielded a specific

restriction pattern for each isolate ranging approximately from 200 bp to 1.2 kb. *Ava* II digestion of the IGS region produced more or less similar restriction fragment pattern in all 22 isolates, but some fragments differed in size among the isolates.

On the other hand, *Hinf* I digestion showed quite a difference in the restriction patterns among all 22 isolates. However, RFLP patterns for each isolate were different from each other by each endonuclease. This result suggests that they are genetically dissimilar. The banding patterns of rDNA obtained by *Ava* II and *Hinf* I digestions were supported to a large extent by those obtained from RAPD analysis. Enzymatic digestions of IGS revealed RFLPs reflecting variation between the isolates.

A similarity matrix on simple matching coefficients was calculated from the combined data based on the RAPD and rDNA RFLP of all 22 isolates. The matrix was used to construct a dendrogram using the UPGMA option of NTSYS for establishing to analyze the level of relatedness among the 22 isolates. The dendrogram shows that hierarchical clustering separated the isolates into eight

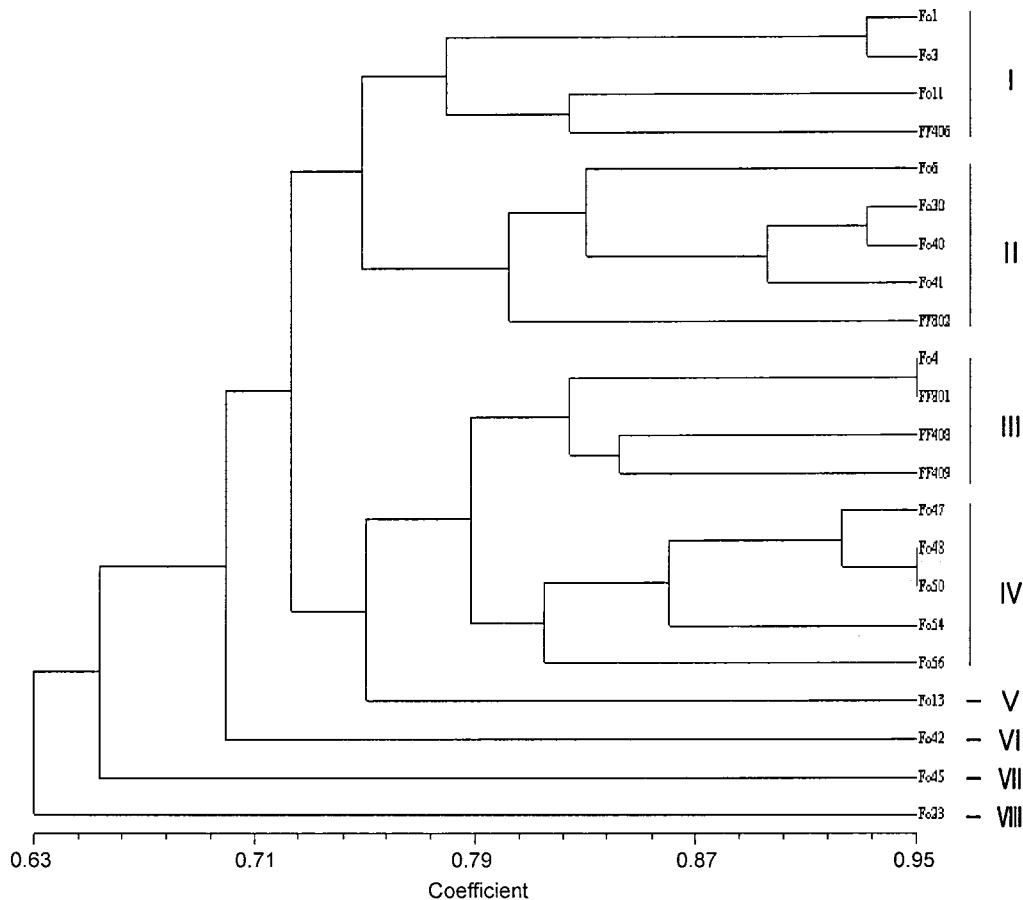


Fig. 3. Dendrogram showing relationship among the 22 isolates of *Fusarium oxysporum* f. sp. *fragariae*. Genetic similarities were obtained by RAPD, and RFLPs of IGS of rDNA.

groups according to their similarity coefficients (Fig. 3).

The similarity coefficients among isolates ranged from 0.63 to 0.95, where 0.95 is identical. The similarities among the isolates tested ranged from 65% to 78%. Cluster analysis using cutoff of similarity ≥ 93 revealed eight genetically distinct groups, designated I to VIII. Groups I and III included four isolates of Fo1, Fo3, Fo11, and FF406, and four isolates of Fo4, FF801, FF408, FF409, respectively. Groups II and IV occupied five isolates of Fo6, Fo30, Fo40, Fo41, and FF802, and five isolates of Fo47, Fo48, Fo50, Fo54, and Fo56, respectively. The groups V, VI, VII, and VIII included one isolate of Fo13, Fo42, Fo45, and Fo23, respectively.

Although the 22 isolates were collected from different regions in Korea, they could be clearly differentiated. Our results suggest a high level genetic variation among isolates of the same forma specialis. In general, no correlation was observed between the isolates in the groups and their geographical origin, although distinct banding pattern between isolates distinguished the isolates. The Korean isolates of *F. oxysporum* f. sp. *fragariae* showed a high degree of genetic variation.

Discussion

Genetic characterization of *F. oxysporum* f. sp. *fragariae* is essential for the efficient management of Fusarium wilt through use of resistant cultivars in strawberry-growing areas. This study demonstrated that RAPD-PCR and RFLP of the IGS region of rDNA provided valuable information as to the degree of variability and level of genetic relatedness among 22 isolates of *F. oxysporum* f. sp. *fragariae*.

The strawberry wilt was detected in Korea and demonstrated by Kim et al. (1982). Thirty two isolates of *F. oxysporum* f. sp. *fragariae* were separated into two groups based on the analyses of vegetative compatibility groups (VCGs), isozyme polymorphisms, and pathogenicity (Hyun et al., 1996). Hyun and Park (1996) have shown that RAPD profiles separated 24 isolates of *F. oxysporum* f. sp. *fragariae* into two distinct groups. These studies are not focused on rDNA RFLP analysis to discriminate the variation among these formae speciales in addition to RAPD analysis.

Several studies have reported that RFLPs of rDNA are also useful in examining variation in formae speciales of *Fusarium oxysporum* (Appel and Gordon, 1995; Jacobson and Gordon, 1990; Kistler et al., 1987). However, an understanding of occurrence, distribution, and genetic relatedness of such pathogenic variants is a prerequisite for developing effective and efficient disease management; but only a few surveys have been conducted on this devastating

pathogen. The genetic affinities between these formae speciales have not yet been clearly understood.

In our study, we used the RAPD and RFLPs of IGS region of rDNA analyses to evaluate genetic variation among *F. oxysporum* f. sp. *fragariae* isolates. In previous studies, RAPD analysis has been used successfully to delineate groups within formae speciales of *F. oxysporum*. One group was delineated with *F. oxysporum* f. sp. *albedinis* (Tantaoui et al., 1996), two groups within f. sp. *dianthi* (Manulis et al., 1994), and two to three groups within f. sp. *pisi* (Grajal-Martin et al., 1993).

Our results indicate that there is a high level of genetic variation among the isolates of *F. oxysporum* f. sp. *fragariae* in Korea. Cluster analysis separated the *F. oxysporum* f. sp. *fragariae* isolates into eight distinct groups. Comparison of more variable DNA regions might allow discrimination of formae speciales that are grouped in the same species. For example, the IGS region that separated nuclear rDNA repeat units, as well as some mitochondrial DNA regions were shown to be variable enough to allow differentiation of closely related *Fusarium* strains (Jacobson and Gordon, 1990).

In this study, the RFLP of IGS region was used to examine genetic relationships among the isolates of *F. oxysporum* f. sp. *fragariae*. The size of the IGS region of rDNA, amplified from all isolates, was approximately 2.6 kb. Cai et al. (2003) have reported that a single fragment of approximately 2.6 kb was amplified from the 60 isolates of *F. oxysporum* f. sp. *lycopersici* with CNL12 and CNS1. Yli-Mattila et al. (2002) have shown that the length of the IGS region was approximately 2.6 kb in the isolates of *F. avenaceum*, *F. arthrosporioides* and *F. tricinctum*.

Similar results were reported in 56 isolates of pathogenic and nonpathogenic *F. oxysporum* (Appel and Gordon, 1995). These results support our findings. Kistler et al. (1987) have shown that differences were detected in the size of the amplified IGS region (2.3-3.1 kb) which could suggest that deletion and/or addition mutations may have occurred in this region of rDNA. In our study, the RFLP analysis showed variation among the IGS region. This suggests that it might be possible to delimit the 22 isolates of *F. oxysporum* f. sp. *fragariae* from each other.

Woo et al. (1996) have reported that there was a good correspondence of RFLP and RAPD banding patterns among 20 isolates of pathogenic strains of *F. oxysporum* f. sp. *phaseoli*. These results were correlated with the vegetative compatibility and showed the isolates to be genetically similar. Manicom and Baayen (1993) have reported that the RFLP technique showed that two major coincident groups of VCG and RFLP exist between the population of *F. oxysporum* f. sp. *dianthi* in Israel. In our study the RAPD and RFLPs of rDNA IGS region generated

distinct banding patterns, but there was no correlation between the isolates; instead, dissimilarities were observed among them.

Our investigations confirm the high level of genetic variability in *F. oxysporum* f. sp. *fragariae*, pointing out the dissimilarity between these isolates obtained from different geographical locations in Korea. This study demonstrates the reliability of the RAPD and RFLP of rDNA as tools for addressing questions on geographical distribution of population of this pathogen.

In conclusion, isolates of *F. oxysporum* f. sp. *fragariae* are different from each other and have existing high level of genetic variation. These observations could be helpful for further breeding studies of strawberry cultivars resistant to this destructive pathogen.

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