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Comparison of RAPD, AFLP, and EF-1α Sequences for the Phylogenetic Analysis of *Fusarium oxysporum* and Its formae speciales in Korea

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Although *Fursarium oxysporum* causes diseases in economically important plant hosts, identification of *F. oxysporum formae speciales* has been difficult due to confusing phenotypic classification systems. To resolve these complexity, we evaluated genetic relationship of nine formae speciales of *F. oxysporum* with random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and translation elongation factor-1 alpha (EF-1α) gene. In addition, the correlation between mycotoxin content of fusaric acid and isolates based on molecular marker data was evaluated using the modified Mantel's test. According to these result, these fusaric acid-producing strains could not identify clearly, and independent of geographic locations and host specificities. However, in the identification of *F. oxysporum* formae speciales, especially, AFLP analysis showed a higher discriminatory power than that of a the RAPD and EF-1α analyses, all three techniques were able to detect genetic variability among *F. oxysporum* formae speciales in this study.

KEYWORDS: Amplified fragment length polymorphism, *Fusarium oxysporum*, Genetic variability, Random amplified polymorphic DNA, Translation elongation factor-1 alpha

Fusarium oxysporum is a common soil-borne fungus that lacks a known sexual stage. These fungi are successful as saprophytes and are able to grow and survive for a long periods on organic matter in soil (Burgess *et al.*, 1981). Most of the interests in this fungus arise their ability to cause diseases in economically important plant hosts, but their near ubiquity in soils worldwide and their ecological activities indicate a much more diverse role in nature. Strains of *F. oxysporum* are known to produce deleterious mycotoxins resulting in toxin contamination of crop products (Booth *et al.*, 1984; Doohan *et al.*, 1998; Nelson *et al.*, 1981). The consumption of mycotoxin-contaminated food and feed products pose an acute risk to human and animal health, as these mycotoxins are carcinogenic and can potentially impair the immune system (Withanage *et al.*, 2001). Recent outbreaks of diseases caused by these fungi pose a great problem for the agricultural industry and are potentially threatening to the global food supply (Clear *et al.*, 2000; McMullen *et al.*, 1997). Although *F. oxysporum* has a wide host range, individual strains are specialized parasites on a limited number of host species (Armstrong *et al.*, 1981). Strains of similar or identical host ranges are assigned to intraspecific groups, called formae speciales (Armstrong *et al.*, 1981; Snyder *et al.*, 1940). These pathogenic strains show a high level of host specificity and are classified into formae speciales on the basis of their ability to cause disease in a particular host or group of hosts (Armstrong *et al.*, 1981). Some of the formae speciales are further divided into subgroups, named races, based on pathogenicity to a set of differential cultivars within the same plant species (Armstrong *et al.*, 1981). Because the hosts of a given formae speciales usually are closely related, it has been assumed that members of a formae speciales are also closely related and may have arisen by descent from a common ancestor. The accurate identification of *Fusarium* species has always been problematic even for expert mycologist. This is because of the contradictory classification systems often proposed by various researchers, primarily based on cultural and morphological characters (Gerlach *et al.*, 1982; Snyder *et al.*, 1945) that could be highly variable depending on the media and cultural conditions. Furthermore, precise determination of *Fusarium* species remains a prerequisite for studying the spread, host infections, and treatment. In addition, degeneration of the cultures and production of mutants may further add to the problems in fungal identification and diagnosis.

Many researchers have developed the phylogentyping methods for identification of related intraspecies. Random amplified polymorphic DNA (RAPD) analysis has many advantages as a means of characterizing genetic variability such as speed, low cost, minimal requirement for DNA, and lack of radioactivity (White *et al.*, 1990). Major polymorphisms in RAPD pattern indicate genetic

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distinctness and can be used to distinguish unrelated groups. RAPD analysis has been used effectively to distinguish between species of *Fusarium* (Voigt *et al.*, 1995; Yli-Mattila *et al.*, 1996). RAPD analysis has also successfully delineated groups within *Fusarium* species including *F. avenaceum* (Yli-Mattila *et al.*, 1996), *F. graminearum* (Vos *et al.*, 1995) and *F. moniliforme* (Voigt *et al.*, 1995), as well as within formae speciales of *F. oxysporum*. More recently, amplified fragment length polymorphism (AFLP) analysis has been used for DNA fingerprinting of microorganisms (Gonzalez *et al.*, 1998). The utility, repeatability, and efficiency of the AFLP technique are leading to broader application of this technique to analysis of fungal populations (Abd-Elsalam *et al.*, 2002a; Majer *et al.*, 1996; Zeller *et al.*, 2000) and *Fusarium* population (Kiprop *et al.*, 2002; Robert *et al.*, 2000; Smouse *et al.*, 1986; Slatkin *et al.*, 1989). O'Donnell indicated that for the genus *Fusarium*, these ribosomal regions contain less interspecific variation than the translation elongation factor-1 alpha (EF-1α) gene (O'Donnell *et al.*, 1998). Due to its high discriminating power at the species level, the EF-1α gene has also been used as a genetic marker for phylogenetic studies, allowing the accurate discrimination of *formae speciales* or strains for specific *Fusarium* species such as *F. oxysporum* and *F. solani* (O'Donnell *et al.*, 2000).

The aims of the present study were therefore: (1) to analyze phylogenetic relationship between of *F. oxysporum* formae speciales, which were isolated in Korea and worldwide by EF-1α region sequencing. (2) to determine whether the ability of these fungi to produce fusaric acid in culture. (3) to develop a reliable and rapid diagnostic tool based on selected RAPD and AFLPs primers for the fast identification of *F. oxysporum* formae speciales.

Materials and Methods

Fungal isolates. List of *F. oxysporum* formae speciales used in this study is shown in Table 1.

Media, Cultural Conditions, and DNA Isolation. For the preparation of total genomic DNA from *F. oxysporum* formae speciales, colonies from potato dextrose agar (PDA: Gibco BRL, CA) medium were transferred to flask containing potato dextrose broth (PDB: Gibco BLR) medium. The flask cultures were incubated at 28°C for 7 d on an orbital shaker, then mycelia were harvested and freeze-dried. The DNA extraction was performed with the DNeasy Plant Mini Kit (QIAGEN, CA) according to the manufacture's protocol.

Fusaric acid isolation and analysis. The method (Smith and Sousadias, 1993) was used for fusaric acid extraction. Fusaric acid was analyzed according to the method (Venter *et al.*, 1998) under reversed-phase conditions using a Waters Nova-pak C18 column (3.9 × 150 mm, 4 μm particles) (Waters, MA). The mobile phase consisted of 40% methanol and 60% of an aqueous solution of 0.62 mmol/*l* Na_2-EDTA and 2% H_3PO_4. The elution time was 10 min. Peaks were detected at a fixed wavelength of 254 nm using a Waters 2487 Dual λ Absorbance Detector. The correlation between mycotoxin content of fusaric acid and isolates based on molecular maker data was evaluated using the modified Mantel's test (Smouse *et al.*, 1986).

Sequencing of EF-1α region. EF-1α was amplified with primers EF-1 and EF-2, which prime within conserved exons (O'Donnell *et al.*, 1998). In addition to the amplification primers, one forward (EF-11) and two reverse internal sequencing primers (EF-21 and EF-22) (O'Donnell *et al.*, 1998) were used with the fluorescent-labeled DyeDeoxy protocol on an automated sequencer (model 377; Perkin-Elmer Applied Biosystems, Foster City, CA).

Phylogenetic analysis. A pairwise alignment was calculated using the Clustal X program (Thompson *et al.*, 1997). Phylogenetic relationships were estimated by MEGA 3 version 3.0 (Sudhir *et al.*, 2004) treating all alignment gaps as missing. A maximum parsimony tree (MP) was inferred using heuristic search, branch-swapping options and tree bisection-reconnection. Confidence values for individual branches were determined by a bootstrap analysis with 100 repeated sampling of the data.

RAPD analysis. RAPD-PCR was carried out in 15 μl of reaction mixture containing: 10 mM Tris-HCl, pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, 0.01% (w/v) gelatin; 60 μM dNTP; 5 pM of primer, approximately 0.1 ng of template DNA for each isolate and 0.75 U of *Taq* DNA polymerase. Twenty decamer primers were randomly chosen among the series OPD (Operon Technologies, Alameda, CA) and tested as primer sequences. All reagents were combined into a pre-mixture, and aliquots were pipetted into 0.5-*ml* tubes before the addition of template DNA to minimize the risk of cross-contamination. Amplification was run in a Perkin-Elmer (Norwalk; CT) Cetus Gene Amp PCR System 9600 programmed for one cycle of 2 min 30 s at 94°C; 45 cycles of 30 s at 94°C, 1 min at 36°C, 2 min at 72°C, with no ramping; one cycle of 5 min at 72°C. RAPD analysis was repeated at least twice for each isolate. Half of the amplification product was loaded in a 1.5% SeaKem LE agarose gel containing 0.5 g of ethidium bromide. Electrophoresis was performed for 2 h 30 min at 3.3 V/cm in 1× TAE running buffer and amplimers were viewed over a UV light source. Gel images were acquired with a Gel Doc 1000 System (Bio-Rad Laboratories; Hercules, CA).

Table 1. List of *Fusarium oxysporum* and its formae speciales used in this study

Taxa	KACC* No.	Origin	Host/Substrate	Characteristics
F. oxysporum	40052	Chuncheon	gladiolus	Dry rot
F. oxysporum	40053	–	cucumber	Fusarium wilt
F. oxysporum	40236	Suwon	carnation	–
F. oxysporum	40237	–	-	–
F. oxysporum	40385	Changwon	carnation	–
F. oxysporum	41078	Hwacheon	greenperilla	Areum^{+}
F. oxysporum	41079	Hongcheon	greenperilla	Manchu^{+}
F. oxysporum	41080	Injae	greenperilla	Areum^{+}, Jinmi^{+}, Manchu^{++}
F. oxysporum	41081	Yangpyung	greenperilla	Yeobsil^{+}, Manchu^{+}, Saeyeobsil^{+}
F. oxysporum	41082	Yeoju	greenperilla	Yeobsil^{+}, Manchu^{+}, Saeyeobsil^{+}
F. oxysporum	41083	Cheongyang	greenperilla	Manchu^{++}, Saeyeobsil^{++}, Jinmi^{+}
F. oxysporum	41084	Kumsan	greenperilla	Manchu^{++}, Saeyeobsil^{++}, Jinmi^{+}
F. oxysporum	41086	Chonan	cymbidum	Virulence^{+}
F. oxysporum	41087	Cheju	cymbidum	Virulence^{+}
F. oxysporum	41088	Namyangju	moth orchid	Virulence^{+++}
F. oxysporum	41089	Kwacheon	cymbidum	Virulence^{++}
F. oxysporum	41090	Hwaseong	cymbidum	Virulence^{+++}
F. oxysporum	41091	Hwaseong	cymbidum	Virulence^{+++}
F. oxysporum f. sp. *cucumerinum*	40525	Germany	cyclamen	–
F. oxysporum f. sp. *cucumerinum*	40527	Florida, USA	–	–
F. oxysporum f. sp. *cyclaminis*	40530	France	cyclamen	–
F. oxysporum f. sp. *dianthi*	40529	–	–	–
F. oxysporum f. sp. *gladioli*	40051	–	gladiolus	Dry rot
F. oxysporum f. sp. *lilii*	40524	Canada	lily	–
F. oxysporum f. sp. *lycopersici*	40032	Korea	tomato	Fusarium wilt
F. oxysporum f. sp. *lycopersici*	40037	Korea	tomato	Fusarium wilt, Virulence^{+++}
F. oxysporum f. sp. *lycopersici*	40038	Korea	tomato	Fusarium wilt, Virulence^{+++}
F. oxysporum f. sp. *lycopersici*	40043	Korea	tomato	Fusarium wilt, Virulence^{+++}
F. oxysporum f. sp. *lycopersici*	40044	Korea	tomato	Fusarium wilt
F. oxysporum f. sp. *lycopersici*	40045	Korea	tomato	Fusarium wilt
F. oxysporum f. sp. *lycopersici*	40046	Korea	tomato	Fusarium wilt
F. oxysporum f. sp. *lycopersici*	40047	Korea	tomato	Fusarium wilt
F. oxysporum f. sp. *lycopersici*	40048	Korea	tomato	Fusarium wilt
F. oxysporum f. sp. *lycopersici*	40238	Japan	tomato	Fusarium wilt
F. oxysporum f. sp. *lycopersici*	40239	Japan	tomato	Fusarium wilt
F. oxysporum f. sp. *lycopersici*	40526	California	tomato	–
F. oxysporum f. sp. *radicis-lycopersici*	40031	Korea	tomato	Fusarium wilt, Crown root rot
F. oxysporum f. sp. *radicis-lycopersici*	40537	Canada	tomato	–
F. oxysporum f. sp. *niveum*	40902	Korea	watermelon	Fusarium wilt
F. oxysporum f. sp. *melonis*	103051	–	–	–
F. oxysporum f. sp. *melonis*	235110	–	–	–
F. oxysporum f. sp. *melonis*	305544	–	–	–

*Korean Agricultural Culture Collection (KACC).

AFLP analysis. The AFLP procedure was carried out as reported by Vos *et al.* (1995) with a few modifications. In brief, two combinations of restriction endonucleases were used. For the combination *Eco*RI/*Mse*I, genomic DNA was incubated for 2 h at 37°C with 2 U of *Mse*I, 5 U of *Eco*RI, 1.2 U of T4 DNA ligase, 50 pmol of *Mse*I adapters and 5 pmol of *Eco*RI adapters. The reaction was cooled to 37°C, supplemented with 15 *μl* of the restriction-ligation buffer containing 5 U of *Eco*RI and incubated at 37°C for an additional 2 h. For adapter ligation, 10 *μl* of the restriction-ligation buffer, containing 50 pmol of *Mse*I adapters, 5 pmol of *Eco*RI adapters, 0.5 mM ATP and 1.2 U of T4 DNA ligase, was added, and the reaction was incubated in 37°C for 3 h. In both cases, a 30-*μl* aliquots of the adapter-ligated DNA was diluted 1 : 10 with distilled water to serve as template in the preselective PCR. The preselective PCR contained 5 *μl* of template, 1 U of AmpliTaq polymerase, 2 *μl* of 10 Taq polymerase buffer, 0.25 mM of each of the four dNTPs, 2.5 mM $MgCl_2$ and 25 ng of *EcoR*I (5'-GACT-GCGTACCAATTC) and *Mse*I (5'-GATGAGTCCT-GATCGG) primers, in a total volume of 20 *μl*. Eight primer pairs were used for the selective amplification. The first amplification cycle was carried out for 30 s at 94°C,

30 s at 65°C and 1 min at 72°C. In each of the following 10 cycles, the annealing temperature was reduced by 1°C. The last 25 cycles were carried out at an annealing temperature of 56°C, and the final extension step was carried out at 72°C for 10 min. Each sample was diluted 1 : 1 with loading buffer, denatured and fractionated on a 6% polyacrylamide sequencing gel in TBE buffer. 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., CA).

Statistical and phylogenetic analysis of RAPD and AFLP. All monomorphic and polymorphic RAPD and AFLP bands were scored by eye and only unambiguously scored bands were used in the analyses. Because RAPDs and AFLPs are dominant markers, they were assumed that each band corresponded to a single character with two alleles, presence (1) and absence (0) of the band, respectively. The following genetic parameters were calculated using a POPGENE computer program (ver. 1.31) developed by Yeh *et al.* (1999). To determine the extent of genetic departure, we calculated the Nei genetic distance (*GD*) and genetic identity (*GI*) for each pairwise combination of accessions (Nei, 1973). The estimation of genetic similarity (*GS*) between genotypes was based on the probability that an amplified fragment from one individual will also be present in another [22]. *GS* was converted to genetic distance (1-*GS*) (Le Thierry *et al.*, 2000). Homogeneity of variance among accessions was tested by Bartlett's statistics. To elucidate the organization of variability within *F. oxysporum*, we examined the genetic variation by partitioning the total genetic diversity (H_T) to within isolates (H_S) using, the genetic diversity statistics of Nei *et al.* (1973). Wright's genetic differentiation among isolates (G_T) was computed for variable loci with FSTAT (Goudet, 1995). Genetic differentiation measured by G_{ST} among isolates was also calculated. Furthermore, gene flow between the pairs of accessions was calculated from G_{ST} values by $Nm = 1/4(1/G_{ST} - 1)$ (Slatkin and Barton, 1989).

Results

Fusaric acid analysis by HPLC. Firstly, to analyze whether fusaric acid is good marker for the identification of *F. oxysporum* formae speciales, the production of fusaric acid was determined using Venter and Steyn (1998). As shown in Table 2, 20 strains of forty-two *F. oxysporum* formae speciales produced fusaric acid and the average concentrations and ranges found were 54.82 μg/g (5.0~208.0). Also, fusaric acid did not find species-specific, host and isolation places. It may be suggested that average concentration and production of fusaric acid is not good for the identification of *F. oxysporum* formae speciales.

Table 2. Identification and concentration of fusaric acid in rice infected with *F. oxysporum* species

Mycotoxin concentrations (μg/g)* detected in rice samples by HPLC					
Isolate no.	formae speciales	Fusaric acid	Isolate no.	formae speciales	Fusaric acid
40052	*F. oxysporum*	8.1	40529	*F. o.* f. sp. *dianthi*	37.9
40053	*F. oxysporum*	–	40051	*F. o.* f. sp. *gladioli*	11.9
40236	*F. oxysporum*	–	40524	*F. o.* f. sp. *lilii*	–
40237	*F. oxysporum*	–	40032	*F. o.* f. sp. *lycopersici*	208.0
40385	*F. oxysporum*	–	40037	*F. o.* f. sp. *lycopersici*	–
41078	*F. oxysporum*	5.0	40038	*F. o.* f. sp. *lycopersici*	–
41079	*F. oxysporum*	–	40043	*F. o.* f. sp. *lycopersici*	–
41080	*F. oxysporum*	–	40044	*F. o.* f. sp. *lycopersici*	–
41081	*F. oxysporum*	–	40045	*F. o.* f. sp. *lycopersici*	23.2
41082	*F. oxysporum*	7.2	40046	*F. o.* f. sp. *lycopersici*	–
41083	*F. oxysporum*	139.6	40047	*F. o.* f. sp. *lycopersici*	27.2
41084	*F. oxysporum*	–	40048	*F. o.* f. sp. *lycopersici*	119.2
41086	*F. oxysporum*	16.4	40238	*F. o.* f. sp. *lycopersici*	60.0
41087	*F. oxysporum*	–	40239	*F. o.* f. sp. *lycopersici*	–
41088	*F. oxysporum*	–	40526	*F. o.* f. sp. *lycopersici*	104.6
41089	*F. oxysporum*	8.4	40031	*F. o.* f. sp. *radicis-lycopersici*	–
41090	*F. oxysporum*	7.0	40537	*F. o.* f. sp. *radicis-lycopersici*	–
41091	*F. oxysporum*	25.3	40902	*F. o.* f. sp. *niveum*	87.9
40525	*F. o.* f. sp. *cucumerinum***	13.6	103051	*F. o.* f. sp. *melonis*	–
40527	*F. o.* f. sp. *cucumerinum*	12.6	235110	*F. o.* f. sp. *melonis*	–
40530	*F. o.* f. sp. *cyclaminis*	173.3	305544	*F. o.* f. sp. *melonis*	–

*(fusaric acid μg/g rice infected with *F. oxysporum*)
***F. o.* (*F. oxysporum*)

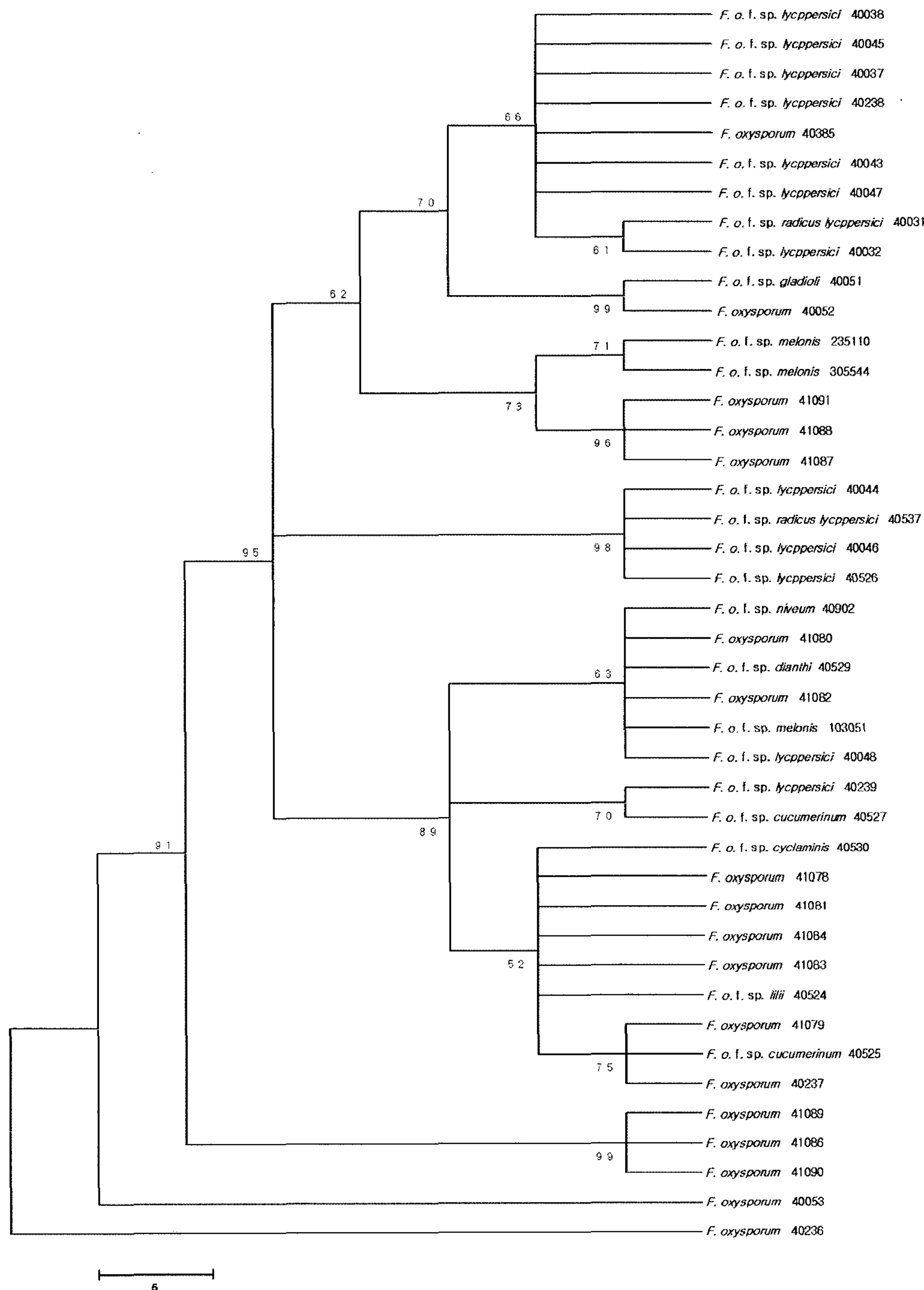

Fig. 1. Phylogenetic relationship among *F. oxysporum* formae speciales assessed by the bootstrap MP method using sequence data of translation elongation factor region.

Phylogenetic analysis of EF-1α region sequencing. The EF-1α gene dataset consisted of 692 nucleotide characters. All of the informative sites were within EF-1α introns. Figure 1 shows a bootstrap consensus tree of *F. oxysporum* formae speciales from maximum parsimony analysis of the EF-1α sequences. The bootstrap consensus tree obtained from neighbor-joining analysis (data not shown) had a topology quite similar to that obtained by parsimony analysis. *F. oxysporum* formae speciales strains were divided four clusters. *F. oxysporum* f. sp. *lycopersici* (40038, 40045, 40037, 40238, 40043, 40047, 40032), *F. oxysporum* (40385, 40052, 41091, 41088, 41087) and *F. oxysporum* f. sp. *melonis* (235110, 305544) form the first clade statistically supported by 62% bootstrap value. The second strongly supported cluster (98%) consists of *F. oxysporum* f. sp. *lycopersici* (40044, 40046, 40526). The third cluster is comprised of *F. oxysporum* (41080, 41082, 41078, 41081, 41084, 41083, 41079, 40237), *F. oxysporum*

f. sp. *niveum*, *F. oxysporum* f. sp. *dianthi*, *melonis* 103051, *F. oxysporum* f. sp. *cucumerinum* (40525, 40527), *F. oxysporum* f. sp. *cyclaminis* and *F. oxysporum* f. sp. *lilii*. Most of the strains except for *F. oxysporum*, *F. oxysporum* f. sp. *lycopersici* belong to this group. This cluster were supported by 89% bootstrap confidence. The fourth strongly supported cluster (98%) consists of *F. oxysporum* (40237, 41086, 41089). Above results show that *F. oxysporum* (40053, 40236) strains separate distinct group respectively.

Table 3. Estimates of genetic diversity of *F. oxysporum* based on RAPD and AFLP markers; total genetic diversity (H_T), genetic diversity within isolates (H_S), and proportion of total genetic diversity partitioned among isolates (G_{ST})

Marker	H_T	H_S	G_{ST}	N_M
RAPD	0.272	0.088	0.678	0.238
AFLP	0.246	0.074	0.700	0.216

RAPD analysis. To obtain reproducible and informative RAPD patterns, special attention was paid to the quality of the DNA template, selection of primers and amplification conditions. From the 20 decamer primers used for a preliminary RAPD analysis, the informative and specific RAPD-profiles of 41 *F. oxysporum* strains

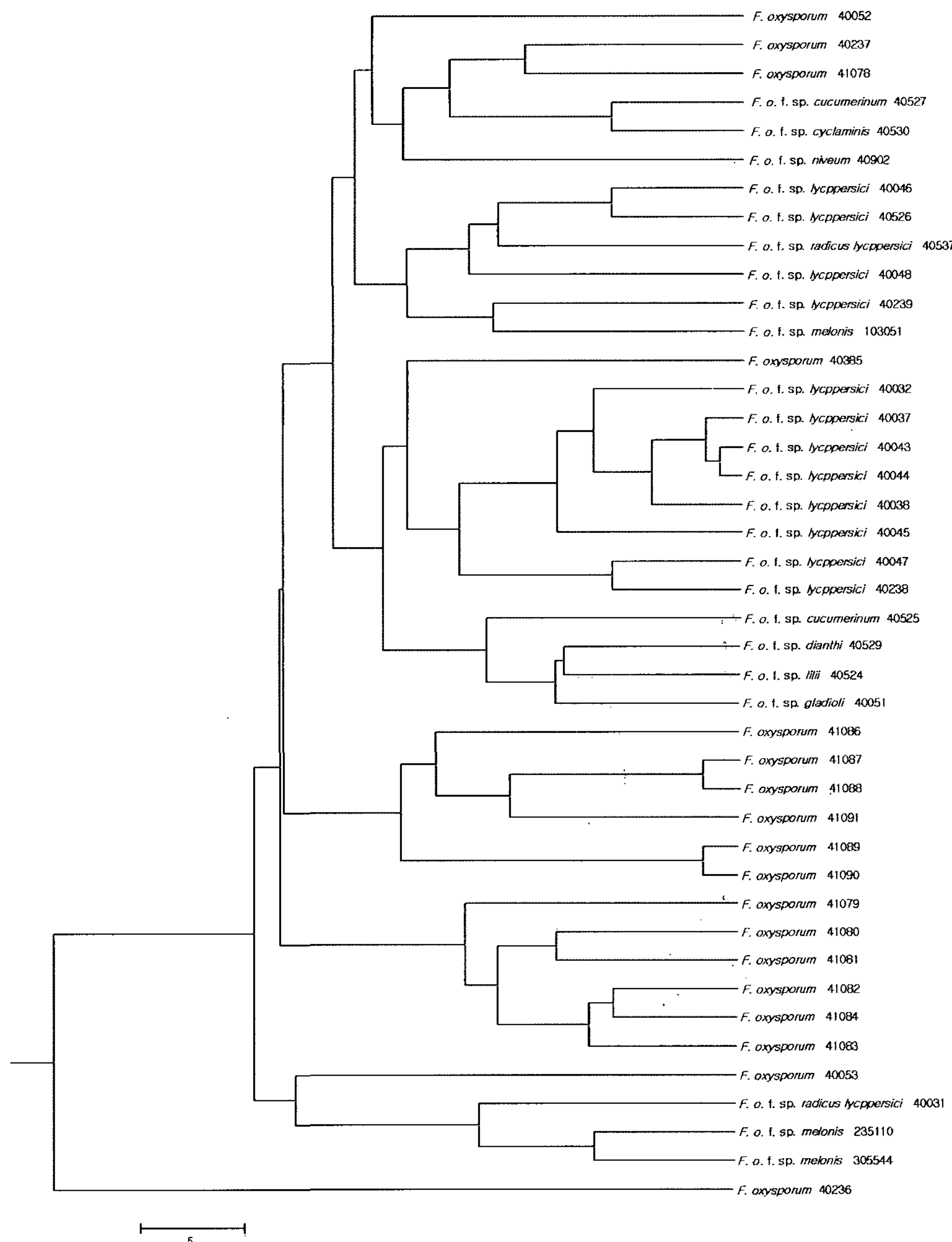

Fig. 2. Combined dendrogram based on the RAPD band patterns of *F. oxysporum* formae speciales.

were produced by ten primers: OPD-1, 2, 3, 7, 11, 13, 15, 16, 18, and 20. Overall, 125 fragments were generated among the tested *F. oxysporum* formae speciales. The lengths of the RAPD fragments were between 700 and 6,000 bps. The number of generated bands was in the range of 3~16 per sample. The analysis of ten primers revealed 125 loci and 124 were polymorphic (99.2%). The G_{ST} value representing an assessment of the proportion of diversity was 0.3678. Total genetic diversity (H_T) is 0.272 (Table 3). The interlocus variation of population genetic diversity (H_S) was 0.088. The average number of individuals exchanged between populations per generation (*Nm*) was calculated 0.238. A similarity matrix based on the proportion of shared fragment (GS) was used to establish the level of relatedness among *F. oxysporum* formae speciales. The estimate of GS ranged from 0.6240 between *F. oxysporum* f. sp. *gladioli* and *F. oxysporum* f. sp. *niveum* to 0.9147 between *F. oxysporum* f. sp. *cucumerinum* and *F. oxysporum* f. sp. *cyclaminis* (data not shown). Clustering of 42 *F. oxysporum* formae speciales populations, using the UPGMA algorithm, was performed based on the matrix of calculated distances (Fig. 2). The phylogenetic tree showed eight distinct groups based on % similarity. Twelve strains of the *F. oxysporum* were clustered two groups. However *F. oxysporum* 40052, 40237, 41078, 40385, 40053 and 40236 created separate clusters. *F. oxysporum* f. sp. *lycopersici* strains were clustered two groups. And duplicate analysis of the random strains revealed no significant differences in banding patterns, although some bands varied in intensity. The results of RAPD analysis were different from the results obtained by the analysis of EF-1α region sequences. The tree also showed genetic differentiation among local accessions for *F. oxysporum* formae speciales.

AFLP analysis. The selective amplification was performed with 8 primer pairs differing in the number of nucleotide extensions at 3'-end. The best results were recorded with primer pairs having +1/+2 additional nucleotides at 3'-terminus (E+AA/M+AG, E+A/M+AG, E+C/M+AG, E+CC/M+AG, E+G/M+AA, E+AG/ M+AA, E+AA/M+CC, and E+AA/M+AG). Primers with one or three nucleotide extensions generated the AFLP patterns of too high or too low complexity. The primers used in this study produced in average of 20~50 fragments per strain.

AFLP fingerprinting of *F. oxysporum* formae speciales with eight primer combinations revealed a total number of 298 unambiguous amplified DNA fragments. The average number of bands per combination was 37.3 bands. The highest level of polymorphism (*Pp*) was 90.94 in *F. oxysporum*, while the lowest value was 19.13 in *F. oxysporum* f. sp. *radicis-lycopersici* (Table 4). Although primers are different in their capacity to reveal polymorphism, those used in this experiment were not significantly different. Intraspecies variability based on the percentage of polymorphic products, the *F. oxysporum* f. sp. *radicis-lycopersici* (0.079) exhibited the lowest genetic diversity (*H*) (Table 4). Across species, the mean of observed alleles (N_A) was 1.513, ranging from 1.191 to 1.909. The effective number of alleles (N_E) was 1.299. Mean gene diversity within populations (*H*) was 0.176. In particular, *F. oxysporum* had the highest expected diversity 0.308 and *F. oxysporum* f. sp. *lycopersici* the lowest 0.079. The genetic frequencies detected with eight primer combinations were calculated and used in estimating phenotypic diversity (*I*) within populations (Table 4). Total of *I* was 0.265 across species, varying from 0.116 for the lowest and 0.463 for the highest. Total genetic diversity value (H_T) was 0.246 for all polymorphic loci (Table 4). The proportion of diversity (G_{ST}) indicated that 0.700 of the total genetic diversity was among species (Table 3). Although the average number of individuals exchanged between populations per generation (*Nm*) was different

Table 4. Measures of genetic variation for AFLP generated among isolates. The number of polymorphic loci (P_A), percentage of polymorphism (P_P), mean number of alleles per locus (A), effective number of alleles per locus (A_E), gene diversity (H), and phenotypic diversity (I)

Species and formae speciales	P_A	P_P	A	A_E	H	I
F. oxysporum	271	90.94	1.909	1.524	0.308	0.463
F. oxysporum f. sp. *cucumerinum*	57	19.13	1.510	1.395	0.219	0.316
F. oxysporum f. sp. *cyclaminis*	N.D*	N.D	N.D	N.D	N.D	N.D
F. oxysporum f. sp. *dianthi*	N.D	N.D	N.D	N.D	N.D	N.D
F. oxysporum f. sp. *gladioli*	N.D	N.D	N.D	N.D	N.D	N.D
F. oxysporum f. sp. *lilii*	N.D	N.D	N.D	N.D	N.D	N.D
F. oxysporum f. sp. *lycopersici*	173	58.05	1.581	1.232	0.146	0.232
F. oxysporum f. sp. *radicis-lycopersici*	57	19.13	1.191	1.135	0.079	0.116
F. oxysporum f. sp. *niveum*	N.D	N.D	N.D	N.D	N.D	N.D
F. oxysporum f. sp. *melonis*	111	37.25	1.373	1.211	0.130	0.197
Mean	133.8	44.9	1.513	1.299	0.176	0.265

*N.D. is not detected.

from each other in the species, an indirect estimate of the mean number of migrants per generation (*Nm* = 0.216) indicated that gene flow was very low among these of *F. oxysporum* formae speciales populations. Genetic distance (*GD*) and genetic identity (*GI*) matrix based on the proportion of shared fragments (*GS*) was used to establish the relationships between the 42 species studied. Values of *GD* were < 0.3888. Values of *GI* were among pairs of accessions ranged from 0.6779 to 0.9143 (data not shown).

The genetic relationships among the populations can be seen in the phylogenetic tree (Fig. 3). Forty two individuals per each population were clustered by UPGMA. The phylogenetic tree showed nine distinct groups based on % similarity. All *F. oxysporum* strains confirmed two clades, except for *F. oxysporum* 41080, 41090, 41091, 40385 and 40236. But this result is slightly different from RAPD result (Fig. 2). In RAPD analysis, *F. oxysporum* 41080, 41090 and 41091 were grouped two major *F. oxysporum* clades. In AFLP analysis, these s trains created separate clusters and *F. oxysporum* f. sp. *lycopersici* strains were claded in the one cluster. Another strains of *F. oxysporum* formae speciales were grouped two distinct clusters, respectively. Above results may be thought that AFLP analysis is more powerful discrimination method than RAPD.

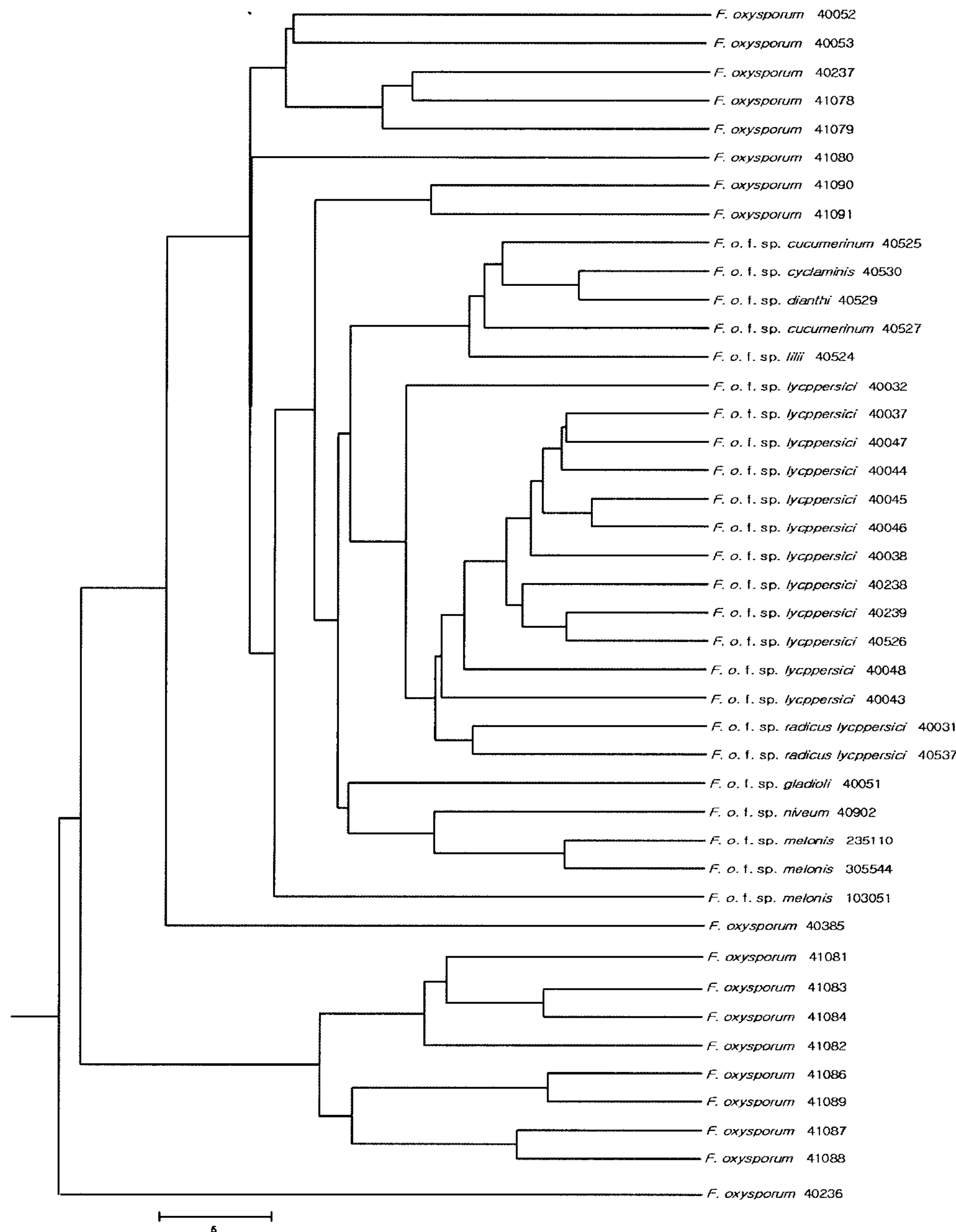

Fig. 3. Combined dendrogram based on the AFLP fragment analysis of *F. oxysporum* formae speciales.

Discussion

All three techniques (RAPD, AFLP and EF-1α analyses) employed in this study resulted in sufficient resolution to detect differences between the genetic profiles of known strains of the same species (*F. oxysporum* formae speciales). While the use of these methods may overcome certain limitations associated with classical taxonomic methods, difference between their lists with regards to the level of polymorphism detected, discriminatory power, effectiveness, and speed of use.

Polymorphism and genetic diversity of *F. oxysporum* were very higher than other strains and showed significant difference. *F. oxysporum* was distributed among several divergent clades (Table 1). Most isolates did not cluster specialized on particular host plants (Figs. 1, 2, and 3). RAPD, EF-1α, region sequencing and AFLP analysis distinguished a combined total of all 42 of the 42 strains tested. Three techniques did not result in identical phylogenic trees for *F. oxysporum* formae speciales. It is interseting to note that these *F. oxysporum* formae speciales were isolated either from the same host or from different hosts. In contract to what is generally assumed, *F. oxysporum* formae speciales are frequently nonmonophyletic. Evidence for a paraphyletic or polyphyletic origin was, presented for the first time by O'Donnell *et al.* (1998). These observations may support the hypothesis that those isolates did not characterized in this study were actually of the same strain. Most isolates did not cluster specialized on particular host plants (Figs. 1, 2, and 3). An assessment of the proportion of diversity present within species indicated that about 70% about AFLP the total genetic diversity was among species (Table 3). Thus, only 30% of genetic variation resided within species. The average number of individuals exchanged between species per generation (*N*m) was estimated to be very low (0.238 for RAPD and 0.216 for AFLP, respectively). Therfore, we cannot rule out that the species is not simple specie or pure strain. Thus *F. oxysporum* used in this study were more distinct taxonomic units. In addiation, Poor correlation between estimates of genetic similarity based on the three different techniques evaluated in this study indicates that these methods may selectively screen for different regions of the genome. To further explore this issue, as well as the close similarity between the strains assayed, a greater number of loci should be analyzed. Then, in an effect to increase the integrity of our analysis, a single dendrogram was constructed that combines the information obtained from all three marker types. Also, it is necessary to identify taxa that additional molecular experiments such as SSR.

F. oxysporum 40236 was claded separately by three methods in this study (Figs. 1, 2, and 3). This isolate has a distinct phylogenetic relationship from other *F. oxysporum* formae speciales. On the basis of these data, we assumed that *F. oxysporum* 40236 was morphologically classified by collectors. However, it is difficult to classify by morphology in this case. As judged by our data, *F. oxysporum* 40236 is not a *F. oxysporum* and belongs to another *F. oxysporum* formae speciales. On the basis of MP analysis, a majority of *F. oxysporum* f. sp. *lycopersici* isolates were clustered a monophytic group which, with few exceptions, was comprised of the same isolates regardless of marker types (Figs. 2 and 3). However, clustering topology of isolates within these groups was not similar when RAPD and AFLP dendrograms were compared. These differences may be attributed to marker sampling error and/or the considerably smaller number of RAPD bands that were evaluated compared with AFLP.

In addition the correlation between RAPD data and mycotoxin content of fusaric acid was low (r = 0.07). The value of AFLP and mycotoxin content of fusaric acid was also low (r = 0.11). The correlations between MP and mycotoxin were some low (r = 0.06). Thus mycotoxin content of fusaric acid in host plant was little taxonomy of *F. oxysporum* (Smouse *et al.*, 1986). According to our result, these fusaric acid producing strains could not identify clearly (Figs. 1, 2, 3), and independent of geographic locations, host.

DNA sequences often give higher resolution than the other molecular markers (Kss *et al.*, 1997). Moreover, comparative studies of nucleotide sequences such as internal transcribed spacer regions (ITS) showed over a wide range of taxonomic levels (White *et al.*, 1990). However, in a recent study, O'Donnell *et al* (1998) indicated that for the genus *Fusarium*, these ribosomal regions contain less interspecific variation than the translation elongation factor 1 alpha gene (EF-1α). The genes for the protein synthesis elongation factors (EF-1α) are being used to investigate phylogenetic relationships in fungi (O'Donnell *et al.*, 2000). Unfortunately, these have so far been no phylogenetic studies based on sequence comparisons in the genus *Fusarium*. DNA sequencing of the EF-1α region was only partly successful in determining the interrelationships of the taxa in study (Fig. 3).

On the basis of the results of this study, RAPD markers were the least polymorphic markers of those evaluated, and consequently had the least resolving power. Though the amount of variability detected with RAPD analysis is dependent upon the selection of appropriate primers, this method has the advantage of being simple to perform and cheap, and does not require a previous knowledge of the genome. Problems with reproducibility have interrupted the use of this technique in the past, due to the low temperature of the hybridization of the primers.

In conclusion, methods like RAPD and AFLP that depend on fingerprinting of complete genome are more discriminative power than the DNA sequencing of the

EF-1α region. Although they play an important role in investigation of phylogenetic relationships among closely related species, important difference between the RAPD and the AFLP techniques lies in the larger number of DNA loci amplified by AFLP. For example, two times as many fragments were generated in the combined AFLP dataset compared to the RAPD dataset. A further advantage of the method is higher reproducibility due to the high stringency conditions of PCR. Both methods suffer from the problem that co-migrating fragments may not be homologous. However, the electrophoresis device equipped with polyacrylamide gel used in the AFLP analysis has an advantage over the agarose gels usually used in electrophoresis of RAPD-fragments and with high probability may overcome the problems of co-migrating bands. In addition to AFLP analysis, in theory, samples loci throughout the entire genome, while gene genealogies examine the evolution of nuclear gene sequences. Clades that are supported by AFLP are likely to reflect independent measures of the evolutionary history of these fungi. Therefore, AFLP analysis could suggest that the AFLP fingerprinting method enabled in a high degree of discrimination and identification of *F. oxysporum* formae speciales and was found to be useful and practical.

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