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PCR-RFLP and Sequence Analysis of the rDNA ITS Region in the *Fusarium* spp.

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To investigate the genetic relationship among 12 species belonging to the *Fusarium* section *Martiella, Dlaminia, Gibbosum, Arthrosporiella, Liseola* and *Elegans*, the internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA) were amplified with primer pITS1 and pITS4 using the polymerase chain reaction (PCR). After the amplified products were digested with 7 restriction enzymes, restriction fragment length polymorphism (RFLP) patterns were analyzed. The partial nucleotide sequences of the ITS region were determined and compared. Little variation was observed in the size of the amplified product having sizes of 550bp or 570bp. Based on the RFLP analysis, the 12 species studied were divided into 5 RFLP types. In particular, strains belonging to the section *Martiella* were separated into three RFLP types. Interestingly, the RFLP type of *F. solani* f. sp. *piperis* was identical with that of isolates belonging to the section *Elegans*. In the dendrogram derived from RFLP analysis of the ITS region, the *Fusarium* spp. examined were divided into two major groups. In general, section *Martiella* excluding *F. solani* f. sp. *piperis* showed relatively low similarity with the other section. The dendrogram based on the sequencing analysis of the ITS2 region also gave the same results as that of the RFLP analysis. As expected, 5.8S, a coding region, was highly conserved, whereas the ITS2 region was more variable and informative. The difference in the ITS2 region between the length of *F. solani* and its formae speciales excluding *F. solani* f. sp. *piperis* and that of other species was caused by the insertion/deletion of nucleotides in positions 143-148 and 179-192.

Key words: rDNA, internal transcribed spacer (ITS), PCR, restriction fragment length polymorphism, *Fusarium*, sequencing analysis

The genus *Fusarium* is a widely-distributed phytopathogen (7) found in a broad range of hosts (8). It brings about wilts and root rot disease by producing secondary metabolites, T2-toxin, its derivatives and mycotoxin such as zearalenone and trichothecene, causing great economic loss of crops.

The current fungal taxonomic system have been identified by macroconidia and microconidia in the asexual stage, morphological character of chlamydospore, host range, and secondary metabolites (23). However, the plasticity and intergradation of the phenotypic traits offered difficulty in identifying the filamentous fungi (4, 14, 15). For these reasons, the molecular biological method has been recently introduced in *Fusarium* systematics and the molecular variation at the DNA level has been studied in many works.

The ribosomal DNA (rDNA) repeat unit contains genic and nongenic or spacer regions. Each repeat unit consists of a copy of 18S-5.8S and 28S-like rDNA and two spacers, the internal transcribed spacer(ITS) and intergenic spacer(IGS) (17). The rDNA genes have been employed to analyze major evolutionary events because it is highly conserved, whereas the rDNA internal transcribed spacer (ITS1 and ITS2) was more variable so that it has been used for the investigation of the species-level relationship (2, 21). Thus, the ITS region has been used in classifying many other fungi species because of its systematic and taxonomic usefulness (3).

A few studies using molecular techniques for identifying species of the genus *Fusarium* have been made so far, which has been restricted within some species such as the section *Elegans* (6, 12) and the section *Liseola* (13, 24). These studies showed that nucleotide sequences of ITS regions were useful for identifying the *Fusarium* species. Nakamura *et al.* (16) reported that PCR-RFLP analysis is more useful in detecting DNA polymorphism in objective sequences than through sequencing analysis.

In this study, to compare genetic similarity among *Fusarium* spp., we amplified the ITS region of the rDNA repeat unit by using PCR, digested with 7 restriction endonucleases and sequenced its ITS2. The aims of this

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study were to determine phylogenetic relationships and usefulness of the ITS region as a genetic marker within these species.

Materials and Methods

Fungal species and culture condition

The species name, source and section of the *Fusarium* spp. used in this study are given in Table 1.

All species were maintained at 4°C on potato dextrose agar (PDA) (Difco). Mycelium for DNA extraction was grown in 250 ml of potato dextrose broth (PDB) (Difco) in a rotary shaker (Vision Bio Tech) at 180 rpm for 24 h at 28°C. After vacuum filtration, the mycelium was lyophilized, ground with sea sand and stored at -20°C.

DNA extraction

Genomic DNA for PCR was extracted using a modified method of Kim *et al.* (6). Prepared mycelium (approximately 0.5 g) was suspended in CTAB extraction buffer (0.7 M NaCl, 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1% 2-mercaptoethanol, 1% CTAB) and extracted with phenol/chloroform/isoamylalcohol [25:24:1] and chloroform/isoamylalcohol [24:1]. RNA was degraded by treatment with RNase (50 μg/ml) for 30 min. at 37°C. DNA was then precipitated by adding 2.5 volumes of absolute ethanol and pelleted by centrifugation for 15 min at 12,000 rpm. The pellet was washed with 70 % ethanol, air dried and resuspended in 1 mM TE buffer (10 mM Tris-HCl, 1 mM EDTA (pH 8.0)). DNA concentration and purity were measured using a spectrophotometer (Shimadzu UV-120) at 260 nm and 280 nm.

Polymerase chain reaction

The priming sites for all PCR and sequencing are indicated in Fig. 1. The ITS region was amplified with the primer ITS1 (5'-TCCGTTGGTGAACCAGCG G-3') (24) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (25). Amplification was performed in 100 μl of reaction mixture containing 50 pmol of both primers, 2.5 units *Taq* DNA polymerase (Takara), 200 μM of each dNTP, 10 μl of 10 × PCR buffer and 0.2 μg of template DNA. The mixture was overlaid with 30 μl of mineral oil and subjected to PCR in a ThermojeT cycler (Japan).

An initial denaturation step for 3 min at 95°C was followed by 30 cycles of annealing for 40 sec at 58°C, extension for 40 sec at 72°C and denaturation for 40 sec at 94°C before a final extension step for 5 min at 72°C. The PCR product was run on 1.4% agarose gels, stained with ethidium bromide (EtBr) and visualized under a UV transilluminator.

Enzyme digestions

The reaction mixture was precipitated by adding 0.1 volume of 3 M sodium acetate and 2.5 volumes of absolute ethanol to remove excess dNTPs and primers, and resuspended in sterilized distilled water. Amplified products were digested for 4 h at an appropriate temperature with the following 15 restriction enzymes : *Eco*RI, *Hin*cII, *Pst*I, *Sma*I (Takara), *Sph*I, *Hin*fI, *Hae*III, *Bgl*II, *Hin*dIII, *Sac*I, *Sal*I, *Stu*I, *Xho*I, *Xba*I and *Msp*I (Bioneer). The digested DNA fragments were separated on 1.5% agarose gels. Gels were stained with ethidium bromide and photographed under a UV transilluminator.

The partial sequencing of ITS region

For sequencing of the patial of 5.8S and 28S, and completed ITS2, internal primer ITS3 (5'-GCATCGAT-

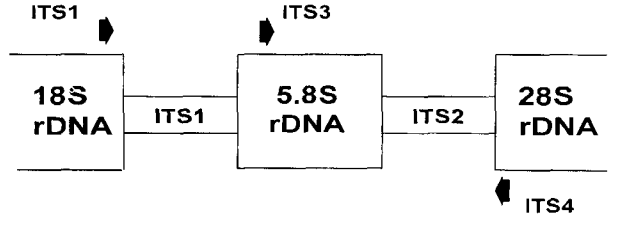

Fig. 1. Localization of PCR and sequencing primers used for the amplification and sequencing reaction of the ITS region from the total DNA extract of the *Fusarium* species.

Table 1. List of *Fusarium* spp. used in this study

Species	Isolate number	Source	Section
Fusarium solani	7475	Australia	*Martiella*
Fusarium solani f. sp. *mori*	840046	MAFF[a]	*Martiella*
Fusarium solani f. sp. *phaseoli*	305607	MAFF	*Martiella*
Fusarium solani f. sp. *piperis*	236575	MAFF	*Martiella*
Fusarium solani f. sp. *pisi*	840047	MAFF	*Martiella*
Fusarium nygamai	7132	Australia	*Dlaminia*
Fusarium oxysporum f. sp. *conglutinans*	744001	MAFF	*Elegans*
Fusarium oxysporum f. sp. *fragariae*	744009	MAFF	*Elegans*
Fusarium oxysporum f. sp. *raphani*		Korea	*Elegans*
Fusarium equiseti	236724	MAFF	*Gibbosum*
Fusarium pallidoroseum	236666	MAFF	*Arthrosporiella*
Fusarium proliferatum	6787	Australia	*Liseola*

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GAGAACGCAGC-3') (25) were used additionally and the priming sites were indicated in Fig. 1. Amplified product on agarose gel was cut and purified with EASYTRAP™ (Takara Shuzo Co., Ltd., Japan) DNA elution kit. PCR-direct sequencing for the ITS region was carried out using the PCR sequencing kit (Bioneer). PCR conditions included a predenaturation for 3 min at 95°C, 40 cycles of denaturation for 30 sec at 95°C, annealing for 30 sec at 56°C and extension for 1 min at 72°C. After the thermal cycling program was completed, the reaction mixture was added to 4 μl of DNA sequencing stop solution and heated for 2 min at 90°C before loading 2.0-3.0 μl of each reaction on a 6% polyacrylamide (acrylamide : bisacrylamide = 19:1) sequencing gel for approximately 3 hrs. The gel was stained using a DNA silver staining kit (Bioneer) and exposed for 1 h to APC film (Promega). Sequ-ences were visually aligned with published sequences of the ITS region of *F. sambucinum* (16) in order to define the gene-spacer junctions.

RFLP analysis

The molecular size of each fragment was estimated using a standard curve of migration versus the log of the molecular size of 25/100bp ladder. Fragments which migrated the same distance during agarose gel electrophoresis were considered to be fragments in common. A data matrix was constructed from the restriction analysis and the presence or absence of a restriction site was indicated as 1 or 0, respectively.

The computer program NTSYS-PC (version 1.70) was used to analyze the relationship among the *Fusarium* spp. studied. Similarity matrix was generated using the program Qualitative. This matrix was subjected to the unweighted pair group method with arithmetical averages (UPGMA) (23). Cluster analysis was performed on the similarity matrix with the SAHN program using UPGMA and a dendrogram was produced with the TREE program.

Sequences analysis

All analyses were carried out using programmes within DNASIS (version 2.1). The phylogenetic tree was obtained from a multiple align plot of ITS sequences among the *Fusarium* species studied. Identity (%) among the *Fusarium* species was indicated on the tree. The sequences from all species examined were submitted to GeneBank with accession numbers (Table 2)

Results

Amplification of the ITS region

Amplification with primers ITS1 and ITS4 resulted in approximately 550 bp or 570 bp of fragment. It was approximately 550 bp in all the strains of section *Elegans*, *Gibbosum*, and *Arthrosporiella* and 570 bp in section *Dlaminia* and *Liseola*. However, *F. solani* and 3 *F .solani* f. sp. yielded 570 bp in size differing from the 550 bp of *F. solani* f. sp. *piperis*.

RFLP Analysis

Seven of the 15 restriction endonuclease tested, *Eco*R I, *Sph*I, *Hinc*II, *Pst*I, *Sma*I, *Hin*fI, *Hae*III, *Bgl*II, *Hin*dIII, *Sac*I, *Sal*I, *Stu*I, *Xho*I, *Xba*I and *Msp*I, had restriction sites in the ITS region of the *Fusarium* species. Restriction fragments were obtained with the enzymes *Eco*RI, *Sph*I, *Pst*I, *Sma*I, *Hin*fI, *Hae*III, and *Msp*I (Fig. 2). According to restriction patterns generated by the seven enzymes, five RFLP groups were easily defined. The score of restriction fragment size distributions are shown in Table 2.

Digestion of the ITS amplification products with *Eco*RI resulted in two banding patterns with one cutting site in this region of DNA except *F. solani* f. sp. *phaseoli*. Digestion of the ITS region with *Sph*I showed two banding patterns with one cutting site in all of species, giving rise to fragment of 320 bp and 230 or 250 bp. Digestion with *Pst*I divided into two patterns. Three among 12 species, *F. solani* f. sp. *phaseoli*, *F. nygamai* and *F. proliferatum*, had one restriction site and the remaining 9 species did not

Table 2. Restriction fragment size (in base pairs) of *Fusarium* ITS region digested with *Eco*RI, *Sph*I, *Pst*I, *Hae*III, *Hin*fI, *Msp*I and *Sma*I

Strains	*Eco*RI	*Sph*I	*Pst*I	*Hae*III	*Hin*fI	*Msp*I	*Sma*I	ITS Type	total size	Accession number
F. solani	320,250	320,250	570	230,130,110,100	280,270	360,210	350,230	II	570	AF 129104
F. solani. f. sp. *mori*	320,250	320,250	570	230,130,110,100	280,270	360,240	350,230	II	570	AF 129105
F. solani. f. sp. *phaseoli*	570	320,250	420,150	230,130,110,100	290,280	360,180	350,230	V	570	AF 130140
F. solani. f. sp. *piperis*	300,250	320,230	550	340,120,90	280,200,100	450,110	550	I	550	AF 130141
F. solani. f. sp. *pisi*	320,250	320,250	570	230,130,110,100	280,270	360,210	350,230	II	570	AF 130142
F. nygamai	320,250	320,250	420,150	280,120,100,90	290,280	260,200,110	350,230	III	570	AF 162897
F. oxy. f. sp. *conglutinans*	300,250	320,230	550	340,120,90	280,200,100	450,110	550	I	550	AF 162898
F. oxy. f. sp. *fragariae*	300,250	320,230	550	340,120,90	280,200,100	450,110	550	I	550	AF 162899
F. oxy. f. sp. *raphani*	300,250	320,230	550	340,120,90	280,200,100	450,110	550	I	550	AF 162900
F. equseti	300,250	320,230	550	340,120,90	280,200,100	550	550	IV	550	AF 162901
F. palidoroseum	300,250	320,230	550	340,120,90	280,200,100	550	550	IV	550	AF 162902
F. proliferatum	320,250	320,250	420,150	280,120,100,90	290,280	260,200,110	350,230	III	570	AF 162903

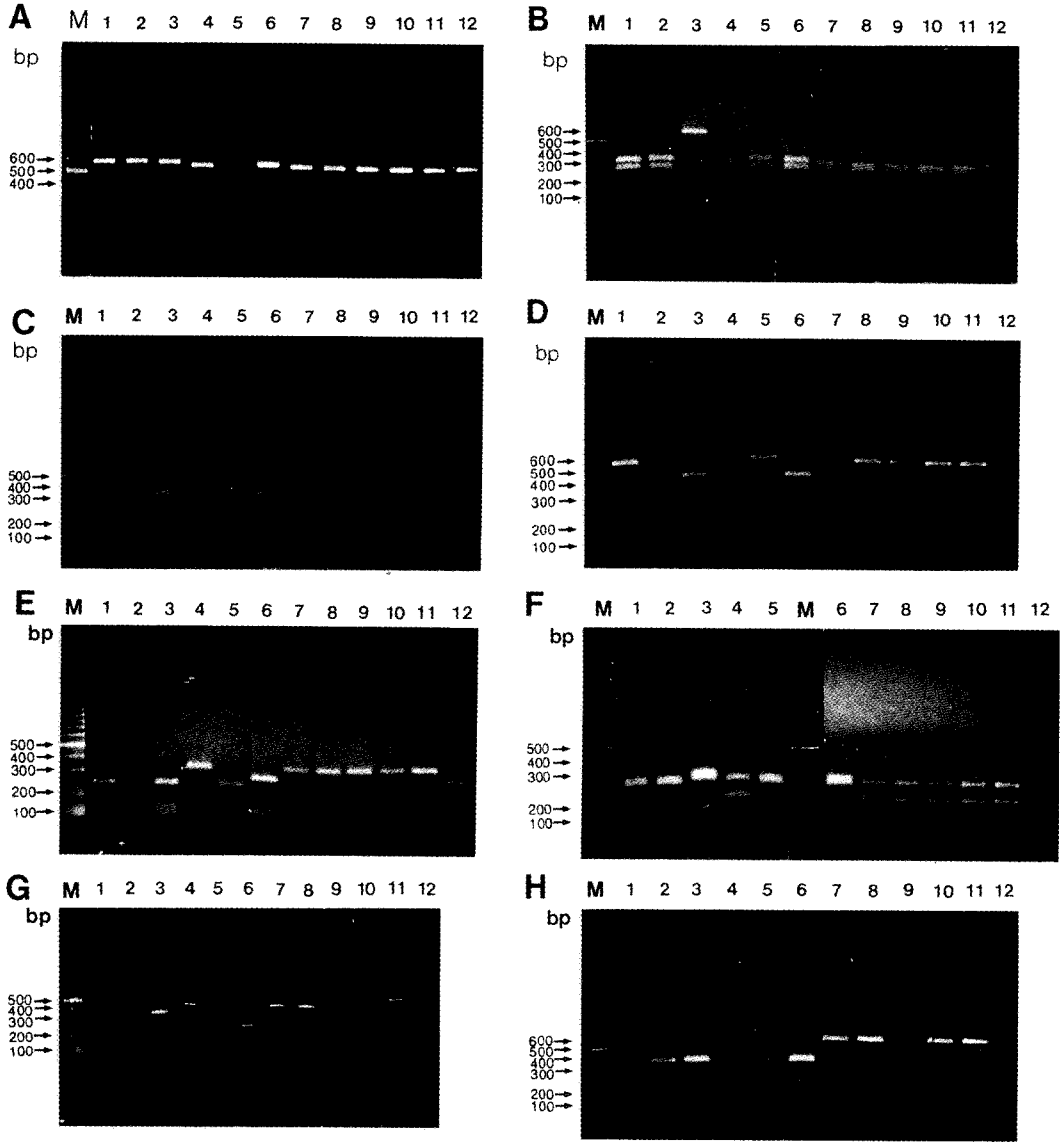

Fig. 2. Agarose gels showing : (A) amplification of the ITS region (ITS1, ITS2 and 5.8S); and restriction patterns of PCR-amplified rDNA digested with *Eco*RI (B), *Sph*I (C), *Pst*I (D), *Hae*III (E), *Hin*fI (F), *Msp*I (G) and *Sma*I (H). M. DNA size marker of 25/100pb ladder, 1. *F. solani*, 2. *F. solani* f. sp. *mori*, 3. *F. solani* f. sp. *phaseoli*, 4. *F. solani* f. sp. *piperis*, 6. *F. solani* f. sp. *pisi*, 6. *F. nygamai*, 7. *F. oxysporum* f. sp. *conglutinans*, 8. *F. oxysporum* f. sp. *fragariae*, 9. *F. oxysporum* f. sp. *raphani*, 10. *F. equiseti*, 11. *F. palidoroseum*, 12. *F. proliferatum*.

cleave. Digestion with *Hae*III resulted in two banding patterns with two or three cleavage sites. Digestion with *Hin*fI exhibited two banding patterns producing two or three fragments. The restriction patterns with the most variation in both fragment sizes and restriction sites were generated by *Msp*I. Digestion with *Sma*I showed two distinct types ; one with an endonuclease restriction site giving rise to two fragments of 350 and 230 bp, and with an non-restriction site.

Banding patterns exhibited by the strains belonging to section *Martiella* could be clearly grouped in three other RFLP groups designated as I, II, and V. According to Table 2, *F. solani* f. sp. *piperis* had an identical RFLP type, Type I, with that of the section *Elegans*. Based on restriction fragments, the genetic similarity matrix was obtained (Table 3). The similarity among 12 strains ranged from the highest, 100%, to the lowest, 28.6%. By the UPGMA method, we constructed a dendrogram based on Table 3 (Fig. 3).

The cluster analysis separated the 12 species into two major groups. One group included the section *Martiella* except *F. solani* f. sp. *piperis*, *F. nygamai* (section *Dlaminia)* and *F. proliferatum* (section *Liseola)* with about 54.3% similarity between *Martiella* and the other two section. The second group included section *Elegans*, *Gibbosum* and *Arthrosporiella* with 91.4% similarity between *Ele-*

Table 3. Genetic similarity coefficient matrix among 12 strains in the genus *Fusarium* was calculated from RFLP patterns digested with 7 enzymes *Eco*RI, *Sph*I, *Pst*I, *Sma*I, *Hin*fI, *Hae*III, and *Msp*I

	1	2	3	4	5	6	7	8	9	10	11	12
1	1.000											
2	1.000	1.000										
3	0.714	0.714	1.000									
4	0.343	0.343	0.286	1.000								
5	1.000	1.000	0.714	0.343	1.000							
6	0.543	0.543	0.600	0.457	0.543	1.000						
7	0.343	0.343	0.286	1.000	0.343	0.457	1.000					
8	0.343	0.343	0.286	1.000	0.343	0.457	1.000	1.000				
9	0.343	0.343	0.286	1.000	0.343	0.457	1.000	1.000	1.000			
10	0.371	0.371	0.314	0.914	0.371	0.429	0.914	0.914	0.914	1.000		
11	0.371	0.371	0.314	0.914	0.371	0.429	0.914	0.914	0.914	1.000	1.000	
12	0.543	0.543	0.600	0.457	0.543	1.000	0.457	0.457	0.457	0.429	0.429	1.000

Number : 1. *F. solani*, 2. *F. solani* f. sp. *mori*, 3. *F. solani* f. sp. *phaseoli*, 4. *F. solani* f. sp. *piperis*, 5. *F. solani* f. sp. *pisi*, 6. *F. nygamai*, 7. *F. oxysporum* f. sp. *conglutinans*, 8. *F. oxysporum* f. sp. *fragariae*, 9. *F. oxysporum* f. sp. *raphani*, 10. *F. equiseti*, 11. *F. pallidoroseum*, 12. *F. proliferatum*

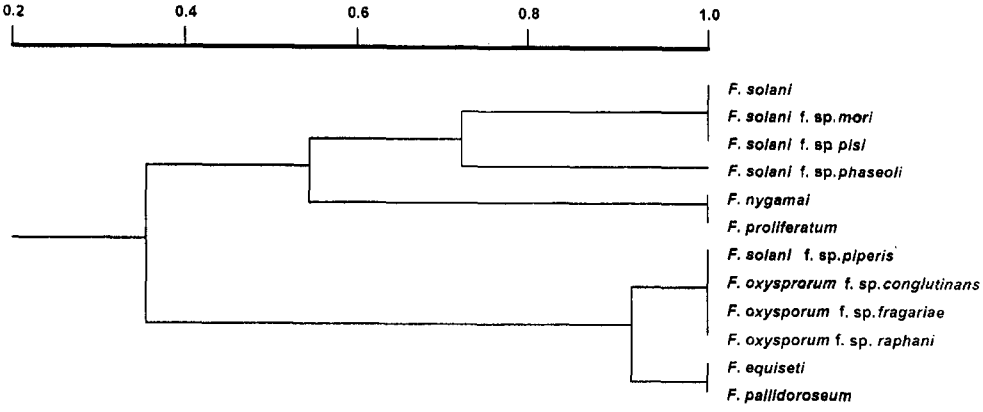

Fig. 3. UPGMA dendrogram showing relationships among the 12 strains of *Fusarium* based on restriction site data.

gans and the other section. Interestingly, *F. solani* f. sp. *piperis* was clustered with section *Elegans* in the second group (100% similarity), even though it is classified to section *Martiella.*

Sequencing of the ITS2 region

The size of the ITS2 region was 170-174 bp in 570 bp PCR products and 146-149 bp in 550 bp. The partial 5.8S rDNA gene was identical 128 bp in length from all strains, except that *F. solani* f. sp. *phaseoli* showed one nucleotide deletion in position 24.

The multi-plot, shown in Fig. 4, was obtained by aligning the sequences of strains with that of *F. sambucinum* as the reference species to confirm the gene junction. The length difference in the ITS2 sequences among strains was about 25 bp, caused by the deletion and insertion of the nucleotide at position 143-148 and 178-192. Hot spot, the region which displayed highly frequent base changes, was located on the position 168-193 at the ITS2 region.

The strains belonging to the section *Martiella* had a higher rate of base substitution than those belonging to other sections in the ITS2 region. The phylogenetic tree (Fig. 5) obtained from multi-plot had the same grouping pattern as the dendrogram from PCR-RFLP.

Resulting from sequencing analysis, the species with the identical RFLP type was distinguished from others with a high similarity. The similarity percentage was shown on the phylogenetic tree.

Discussion

According to the result of RFLP, twelve strains examined were divided into five RFLP type. Of the 5 RFLP types, three types, that is I, II, and V appeared to section *Martiella. F. solani* f. sp. *phaseoli* was different from other strains having a *Pst*I restriction site and the absence of the *Eco*RI restriction site. This result correlated with the report that *F. solani* f. sp. *phaseoli* was a distinct species within *F. solani* complex (18). O'Donnell (18) reported that the *F. solani* f. sp. *phaseoli* isolates from soybeans could be distinguished easily from the *F. solani* complex shown by the presence of a unique *Pst*I restriction site within the ITS2 region.

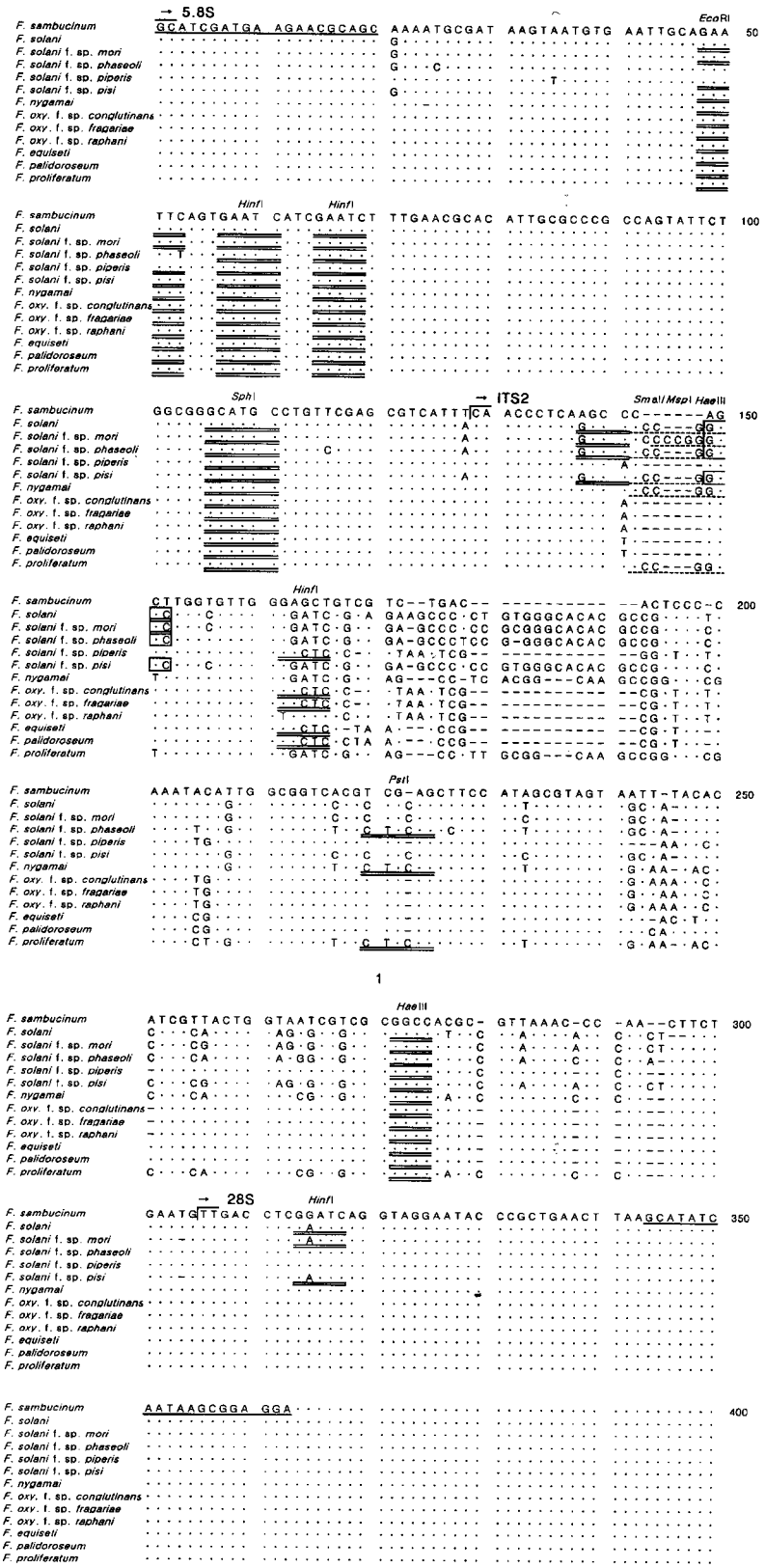

Fig. 4. Nucleotide sequence alignment of the partial 5.8S rRNA gene, ITS2 and the 5' end of the 28S rRNA gene. Identity to the reference sequence of *F. sambucinum* (EMBL accession number X65481) is indicated by dots. Alignment gaps (insertion or deletion) are indicated by dashes. Oligonucleotide priming sites and restriction sites are indicated by underlines.

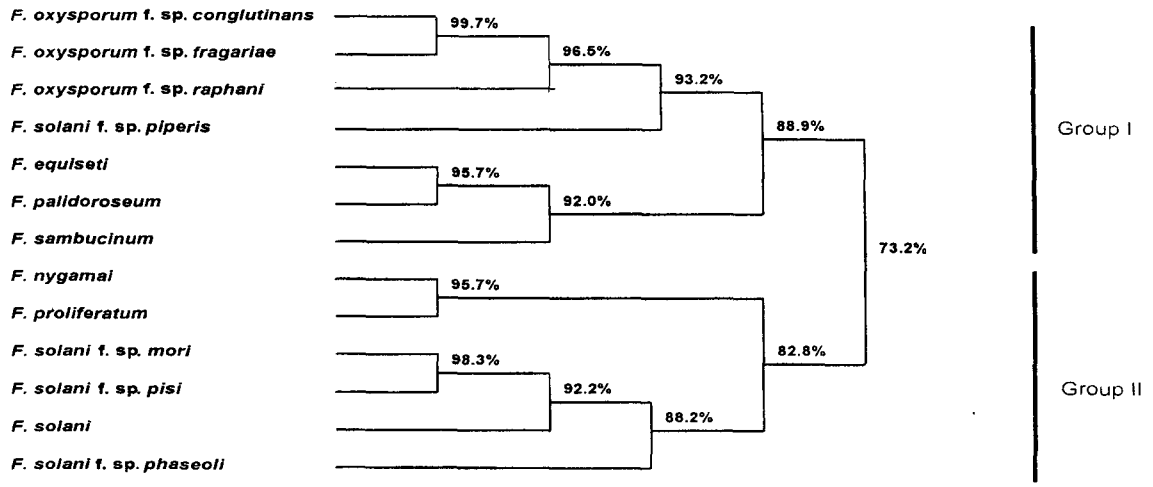

Fig. 5. The phylogenetic tree based on the nucleotide sequence of the ITS2 region in the genus *Fusarium.*

Isolates of this species were easily identified by their morphologically distinct macroconidia, blueish colonies with abundant sporodochia and the rare production of microconidia (11, 20, 26). Interestingly, *F. nygamai* (section *Dlaminia*) and *F. proliferatum* (section *Liseola*) had a *Pst*I restriction site. Interestingly, *F. solani* f. sp. *piperis* having an identical RFLP type with the species belonging to section *Elegans* was separately clustered with the strains belonging to the same section. The ITS2 sequences for *F. solani* f. sp. *piperis* was deleted/inserted at positions 143-148 and 178-192 and this appearance was similar to that for strains with 550 bp in total ITS length. Booth (1) reported that the similarity between section *Elegans* and section *Martiella* displayed rather examples of parallel evolution than closely related species.

Therefore, the result of this study suggests that *F. solani* f. sp. *piperis* is a potential example for parallel evolution between the section *Elegans* and section *Martiella.*

According to the phylogenetic tree by ITS-RFLP, 12 strains were divided into two major groups. The first group included strains belonging to section *Martiella* except for *F. solani* f. sp. *piperis*, *Dlaminia* and *Liseola*. Strains including the first group was subdivided into two minor groups : *Martiella* was separated from *Dlaminia* and *Liseola* with low similarity, 54.3%. The second group included the section *Elegans*, *F. solani* f. sp. *piperis* belonging to the section *Martiella*, *Gibbosum* and *Arthrosporiella*. This group was subdivided into two minor groups : section *Elegans* and *F. solani* f. sp. *piperis* belonging to section *Martiella* and the other section including the same major group which showed high similarity, 91.4%. Further study of high similarity among species belonging to the section *Elegans* and *F. solani* f. sp. *piperis* is required.

In the result of partial ITS2 sequencing, strains were placed into group matching with those determined by phylogenetic analysis of ITS-RFLP. A clear grouping of species according to ITS2 sequence divergence was matched, close to the traditional classification based on morphological criteria.

The previous report for sequence analyzing of large subunit of rRNA gene among species belonging to genus *Fusairum* showed that section *Elegans* was very closely genetically related with section *Liseola* (2, 5). However in this study, section *Elegans* was distinguished from section *Liseola* having a different RFLP type and located on a different group in the dendgrogram, thus it is thought that RFLP analysis of the ITS region is a useful method for identifying between the two sections, *Elegans* and *Liseola.* In recent years, it has been described that species such as *F. nygamai*, *F. napiforme*, *F. dlaminii*, and *F. beomiforme* possessed all characters of both sections, *Elegans* and *Liseola.* Because these species could not be included appropriately within these two secions, Kwasna *et al.* (10) included these species into a new section, *Dlaminia.*

Waalwijk *et al.*(24) reported that when the sequencing analysis of the ITS1 and ITS2 region of strains belonging to section *Dlaminia* and *Liseola*, *F. nygamai* and *F. proliferatum* were located on the same group in the dendrogram for the ITS2 region. Also, in their report, *F. oxysporum* was clustered in a different group on the dendrogram for ITS2 from *F. nygamai* and *F. proliferatum.* The report of Waalwijk *et al.* (24) was consistent with those of our results. *F. nygamai* belonging to section *Dlaminia* was closely related to strains belonging to section *Liseola* than strains belonging to *Elegans*. Similar results also have been reported as the distinguishing markers using isozyme patterns (9) and electrophoretic karyotype (13). But the exact phylogeny among these three section still remains questionable; therefore, additional species and comparison with other regions of rDNA are needed.

The species-specific banding patterns of restriction endonuclease-digested rDNA amplified by PCR suggest that

this procedure may provide a reliable taxonomical tool for the identification of the *Fusarium* species.

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