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Phylogeny of Korean Isolates of *Phytophthora* Species Based on Sequence Analysis of Internal Transcribed Spacer of Ribosomal DNA

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The internal transcribed spacer regions (ITS I, 5.8S and ITS II) of the ribosomal DNAs were amplified from Korean isolates of *Phytophthora* spp. and sequenced to characterize them. Sequences from 33 isolates previously identified as *P. boehmeriae*, *P. cactorum*, *P. cambivora*, *P. capsici*, *P. cinnamomi*, *P. citricola*, *P. citrophthora*, *P. cryptogea*, *P. drechsleri*, *P. erythroseptica*, *P. infestans*, *P. megasperma*, *P. melonis*, *P. nicotianae*, *P. palmivora* and *P. sojae* were compared with published sequences, and a phylogenetic tree was produced. All isolates belonging to 10 species, *P. cactorum*, *P. cambivora*, *P. capsici*, *P. cinnamomi*, *P. citricola*, *P. infestans*, *P. nicotianae*, *P. palmivora* and *P. sojae* were clearly clustered into published isolates of each species above 97% bootstrap value. Cucurbits isolates of *Phytophthora* previously identified as either *P. melonis* or *P. drechsleri* showed distinct evolutionary lineages from the *P. cryptogea*-*P. drechsleri* complex group, indicating that *P. melonis* is a valid species. A Korean isolate of *P. megasperma* was closely related to isolates of *P. cryptogea*-*P. drechsleri* complex group, *P. erythroseptica*, and *P. megasperma* group F. However, Korean isolates of *P. erythroseptica* showed distant genetic relationship with published isolates of *P. erythroseptica* (CBS 956.87). It is probable that the two Korean isolates could be genetically different from foreign isolates or misidentified. A grouping of species according to ITS sequence divergence matched, to some degree, the broad classification based on type of papilla. However, a separation of semi-papillate species and papillate species was not evident in this study.

Keywords : *Phytophthora*, ribosomal DNA, phylogeny.

Introduction

The genus *Phytophthora* is one of the most important plant pathogens attacking almost all plant groups. Since de Bary established the genus with *P. infestans* as the type species in 1876, 95 species and five varieties have been reported worldwide (Erwin and Ribeiro, 1996). From a survey of *Phytophthora* diseases on plants in Korea from 1996 to 1999, 990 isolates were collected from 66 host plants and classified into 17 species, such as *P. boehmeriae*, *P. cactorum*, *P. cambivora*, *P. capsici*, *P. cinnamomi*, *P. citricola*, *P. citrophthora*, *P. cryptogea*, *P. drechsleri*, *P. erythroseptica*, *P. infestans*, *P. macrospora*, *P. megasperma*, *P. melonis*, *P. nicotianae*, *P. palmivora* and *P. sojae* (Jee, 1999).

Keys for identification of the *Phytophthora* relied heavily on morphological and cultural traits. However, morphological characteristics are highly variable among isolates within species and overlapping features exist between species, which often lead identification difficult (Erwin and Ribeiro, 1996). Consequently, many researchers combined morphological traits with molecular characters in the taxonomy and phylogenic study of *Phytophthora* (Cooke and Duncan, 1997; Lee and Taylor, 1992; Forster et al., 1990).

Ribosomal DNA (rDNA) in fungi is organized in clusters of tandem repeats with several hundred copies per genome. The gene is conserved and contains sequence components possessing different evolutionary rates, which are phylogenetically and taxonomically informative for the study of genetic relatedness at generic or species level (Bruns et al., 1991). rDNA was used to delineate species and clarify evolutionary relationship of *Phytophthora* spp. (Hong et al., 1999; Hong et al., 1998; Cooke and Duncan, 1997; Crawford et al., 1996; Forster et al., 1995; Lee and Taylor, 1992). Cooke and Duncan (1997) reported that the resultant grouping of species based on internal transcribed spacer (ITS) I and ITS II sequences of the rDNA generally agreed with the grouping established by classic morphological criteria. Crawford et al. (1996) confirmed recent reclassification as biological species of *P. medicaginis*, *P. trifolii* and *P. sojae* from *P. megasperma* species complex on the basis of rDNA sequence analysis.

Korean isolates of *Phytophthora* were also investigated by PCR-RFLP of ITS region and small subunit (SSU) of rDNA (Hong et al., 1999; 1998). Ninety-five Korean isolates of 16 species were divided into 13 genetic groups. Two isolates of *P. erythroseptica* showed the same band pattern with those of *P. sojae*, and the isolates of *P. crypto-*

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gea, *P. drechsleri* and *P. megasperma* formed one complex group which were not compatible with the delineation of the species.

In this study, we employed analysis of ITS region to address several issues concerning *Phytophthora* taxonomy of Korean isolates. The aims of this study were (1) to provide an evolutionary placement for Korean isolates of *P. erythroseptica* and *P. sojae*, (2) to determine whether current identification of *P. megasperma*, KACC 40401, is valid, (3) to reveal evolutionary relationships among *P. cryptogea*, *P. drechsleri* and *P. melonis*, (4) to examine phylogenetic relationships among *Phytophthora* spp.

Materials and Methods

Fungal isolates and extraction of genomic DNA. Thirty-one isolates of *Phytophthora* spp. used in this study were selected from 95 isolates of the former study (Hong et al., 1999). Three isolates of *P. drechsleri*, *P. erythroseptica* and *P. melonis* were obtained from ATCC, CBS and Taiwan, respectively. Detailed information for the isolates including 14 reference isolates is presented in Table 1

Extraction of genomic DNA from the isolates was conducted according to the procedure described by Hong et al (1998) which basically followed by Lee and Taylor (1990).

Amplification of the ITS of rDNA. Primers, ITS1 (5'-TCC-GTA-GGT-GAA-CCG-CGG-3') and ITS4 (5'-TCC-TCC-GCT-TAT-TGA-TAT-GC-3'), designed by White et al. (1990), were used for the amplification of ITS region from genomic DNA of the isolates. PCRs were conducted in 100 μl reaction volumes Each reaction tubes contained 1 μl of template DNA (≒ 100 ng), 10 μl of 10X buffer [50 mM KCl, 100 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 15 mM $MgCl_2$], 4 μl of each 2.5 mM dNTP, 0.75 μl of each 100 uM primers, 0.5 μl of *Taq* polymerase (5 unit/μl) and 83 μl of ddH_2O. Two drops of mineral oil were placed on the top of each reaction mixture. The thermal cycling parameter was denaturation for one min at 95°C, annealing for one min at 58°C, and polymerization for two min at 72°C. Thirty-five cycles

Table 1. Isolates of *Phytophthora* used in this study

Species	Isolate No.	RFLP type[a]	Host	Alternative source[b] and reference	GenBank accession no.
P. boehmeriae	KACC[c] 40173	*Pbh*	*Ailanthus altissima*	P-96118 (Kim & Kim, 1993)	AF228076
P. cactorum	KACC 40176	*Pcc*	*Malus pumila* var. *dulcissima*	Pb-09 (Jee et al., 1997b)	AF228077
P. cactorum	KACC 40174	*Pcc*	*Pyrus serotina*	P-9776	AF087480
P. cactorum[d]	**UQ1318**		***Panax quinquefolius* (U.S.A.)**	**(Crawford et al., 1996)**	**L41357**
P. cambivora	KACC 40160	*Pcm*	*Malus pumila* var. *dulcissima*	P-9780	
P. cambivora	KACC 40159	*Pcm*	*Malus pumila* var. *dulcissima*	Pb-06 (Jee et al. 1997b)	AF087479
P. cambivora					**AJ007040**
P. capsici	KACC 40157	*Pcp*	*Capsicum annuum*	Pa-11	AF228078
P. capsici	KACC 40177	*Pcp*	*Lycopersicon esculentum*	P-9512	AF228079
P. capsici	**87**		*Capsicum annuum*	**(Ristaino et al., 1998)**	**AF007021/00428**[e]
P. cinnamomi	KACC 40182	*Pcn*	*Larix leptolepis*	P-9796	AF087478
P. cinnamomi	**UQ734**		***Allocasuarina* sp. (Australia)**	**(Crawford et al., 1996)**	**L41374**
P. citricola	KACC 40184	*Pctrc*	*Zizyphus jujuba* var. *inermis*	P-97101 (Jee et al., 1998b)	AF228080
P. citricola	**DAR34210**		**Soil (Australia)**	**(Crawford et al., 1996)**	**L41375**
P. citrophthora	KACC 40186	*Pctrp*	*Citrus sinensis*	P-9715 (Song et al., 1997)	AF228081
P. citrophthora	KACC 40188	*Pctrp*	*Malus pumila* var. *dulcissima*	Pb-40	
P. cryptogea	KACC 40469	*Pcr-dr1*	*Gerbera jamesonii*	P-9620 (Jee et al., 1996)	AF087476
P. cryptogea	KACC 40161	*Pcr-dr2*	*Gerbera jamesonii*	P-9536 (Jee et al., 1996)	
P. cryptogea	KACC 40162	*Pcr-dr3*	*Gerbera jamesonii*	P-9672	AF087477
P. cryptogea	KACC 40189	*Pcr-dr4*	*Brassica campestris* ssp. *pekinensis*	P-9724	AF087475
P. cryptogea	**UQ754**		**Soil (Australia)**	**(Crawford et al., 1996)**	**L41376**
P. drechsleri	KACC F4	*Pcr-dr1*	*Lycopersicon esculentum*	P-9614 (Jee et al., 1998c)	AF087471
P. drechsleri	KACC 40463	*Pcr-dr1*	*Lactuca sativa*	P-9801	
P. drechsleri	KACC 40191	*Pcr-dr2*	*Angelica gigas*	P-9519	
P. drechsleri	KACC 40467	*Pcr-dr2*	*Lycium chinense*	P-97105	AF087472
P. drechsleri	KACC 40193	*Pml*	*Cucumis sativus*	P-9617	AF087473
P. drechsleri	KACC 40192	*Pml*	*Cucumis melo*	P-9532	
P. drechsleri	ATCC 58424		*Cucumis sativa*	KACC 40709	AF228095
P. drechsleri	**IMI 122165**		***Westringua* sp. (Australia)**	**(Crawford et al., 1996)**	**L41377**

Table 1. Continued

Species	Isolate No.	RFLP type[a]	Host	Alternative source[b] and reference	GenBank accession no.
P. erythroseptica	KACC 40449	*Per-so*	*Astragalus membranaceus*	P-9766	AF087474
P. erythroseptica	KACC40200	*Per-so*	*Pueraria lobata*	P-96117 (Kim & Kim, 1993)	
P. erythroseptica	CBS 956.87		*Solanum tuberosum* (U.S.A)	KACC 40704	AF228082
P. infestans	KACC 40707	*Pin*	Solanum tuberosum	inf-6	AF228083
P. infestans	KACC 40706	*Pin*	Lycopersicon esculentum	inf-1	AF228084
P. infestans	**89/AF1**		***Solanum tuberosum* (Wales)**	**(Cooke & Duncan, 1997)**	**Y08669/70**[e]
P. megasperma	KACC 40401	*Pcr-dr5*	*Lycopersicon esculentum*	P-9608 (Jee et al., 1998c)	
P. megasperma	**Pm12**		***Cicer arietinum* (Spain)**		**L41380**
P. megasperma	**EP1**		***Asparagus officinalis* (U.S.A.)**		**L41381**
P. megasperma	**P1330**		***Pseudotsuga menziesii* (U.S.A)**		**L41382**
P. melonis	KACC 40444	*Pml*	*Cucumis melo* (Taiwan)	Ahn, P. J.	AF228094
P. nicotianae	KACC 40403	*Pnc*	*Epiphyllum trucatum*	P-9516 (Jee et al., 1998a)	AF228085
P. nicotianae	KACC 40407	*Pnc*	*Lilium longiflorum*	P-9695 (Jee et al., 1998a)	AF228086
P. nicotianae	**IMI354395**		***Banksia* sp. (Australia)**	**(Crawford et al., 1996)**	**L41383**
P. palmivora	KACC 40167	*Ppl*	*Chrysalidocarpus lutescens*	P-9601 (Jee et al., 1997a)	AF228087
P. palmivora	KACC 40409	*Ppl*	*Cymbidium* sp	P-9741	AF228088
P. palmivora	**P80**		***Cocos* sp. (Jamaica)**	**(Crawford et al., 1996)**	**L41384**
P sojae	KACC 40412	*Per-so*	*Glycine max*	P-9662 (Jee et al., 1998d)	AF228089
P. sojae	KACC 40468	*Per-so*	*Glycine max*	P-98145	
P. sojae[f]			*Glycine max*	A. Drenth	

[a] Band patterns generated by PCR-RFLP analysis of ITS region and small subunit of rDNA (Hong et al., 1999).
[b] The isolate number used by Jee, Hyeong-Jin, Plant Pathology Division, NIAST.
[c] Accession number of Korean Agricultural Culture Collection (KACC), Molecular Genetics Division, NIAST.
[d] Nucleotide sequence data of the isolates written with bold were obtained from GenBank.
[e] The left accession no. corresponds to ITS I and the right accession no. corresponds to ITS . The nucleotide sequences of 5.8S were replaced by those of Korean isolates for alignment.
[f] Nucleotide sequence data of the isolate were obtained by personal communication from Andre Drenth, CRC for Tropical Plant Pathology, Queensland Univ., Australia.

were conducted and the first denaturation and the last polymerization time were extended to 4 min and 8 min, respectively. The success of amplification was monitored by 1% agarose gel electrophoresis.

Cloning and sequencing of the ITS regions. The PCR products were separated on a 0.8% agarose gel, and then the bands of expected size were purified with a QIAquick gel extraction kit (Qiagen GmbH, Germany). Purified DNA were ligated with pGEM-T easy vector (Promega Co, U.S.A). Ligated plasmids were then transformed into *Escherichia coli* DH5αF' cells, and transformants were selected by blue-white screening procedure (Sambrook et al. 1989). Plasmids carrying the ITS regions were isolated from liquid cultures of the transformants using QIAquick plasmid minikit (Qiagene GmbH, Germany). Purified plasmids were sequenced using on an ABI 377 automatic sequencer (Perkin Elmer. U.S.A.).

Phylogenetic analysis. The sequences of the ITS regions were first aligned using CLUSTAL W software (Thompson et al., 1994). The nucleotide similarity values were calculated from the alignment. An evolutionary distance matrix was generated as described by Jukes & Cantor (1969). Evolutionary trees for the data sets were inferred by the neighbor-joining method of Saitou & Nei (1987) using MEGA program (Kumar et al., 1993). The stability of relationships was assessed by performing bootstrap analysis of the neighbor-joining data based on 1,000 resamplings.

Nucleotide sequence accession numbers. The complete ITS region sequences of the *Phytophthora* spp. determined in this study have been submitted to the GenBank at the NCBI. The accession numbers were shown in Table 1.

Results

The primers, ITS1 and ITS4, amplified a single fragment from genomic DNAs of all 35 isolates. The fragments carried part of 18S and 28S, and full of ITS I, 5.8S and ITS II, and ranged in size from 840 bp (*P. capsici*) to 920 bp (*P. cambivora*). ITS I lengths varied as much as 60 bp, from 174 bp for *P. capsici* to 234 bp for *P. cambivora*. Less variation was shown in ITS II. The ITS II of all isolates except *P. boehmeriae* (459 bp) ranged in size from 414 bp (*P. palmivora*) to 439 bp (*P. cambivora*). Lengths of the 18S, 5.8S and 28S rDNAs amplified were not much variant, and were 29 bp, 159-161 bp and 58-60 bp, respectively. The detailed

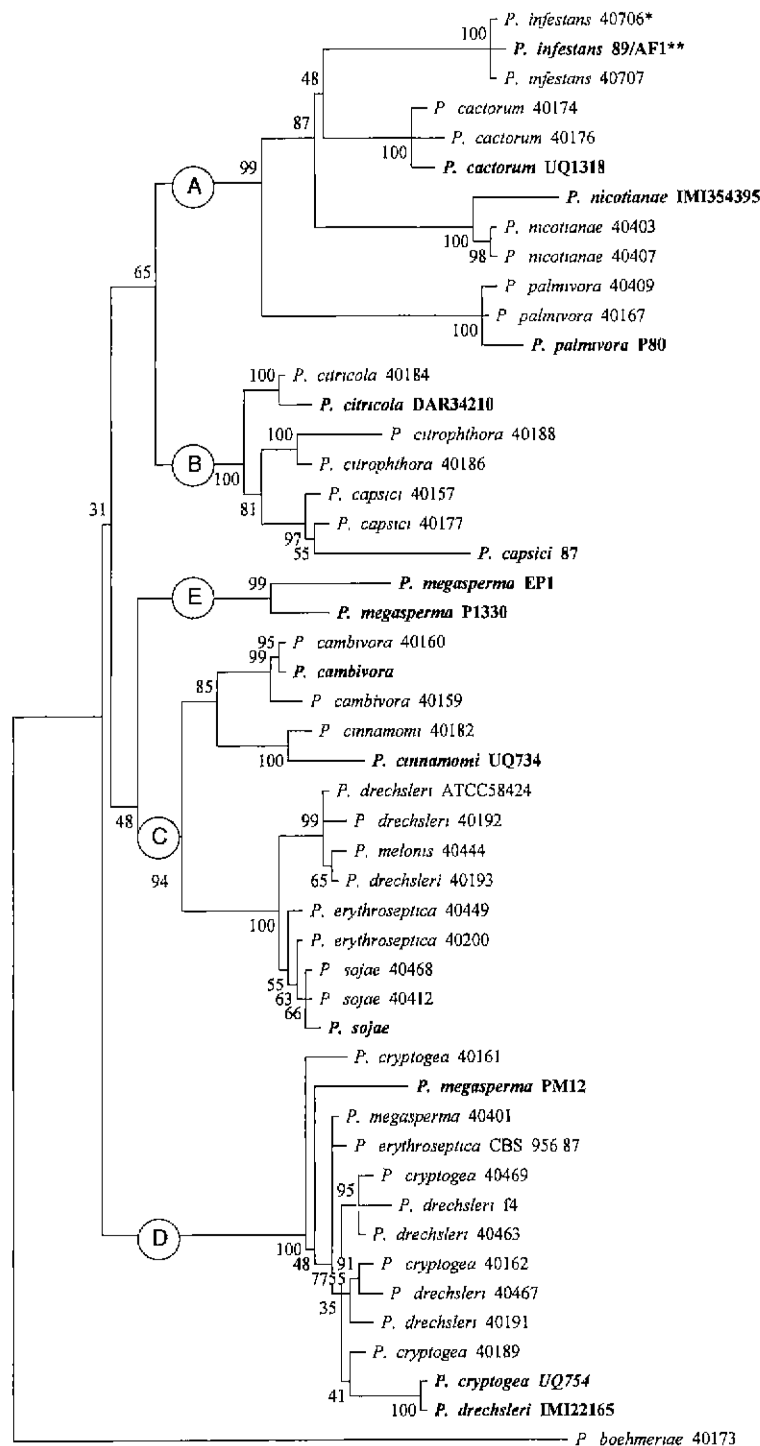

Fig. 1. Phylogenetic tree indicating the relationships among Korean isolates and published isolates and among *Phytophthora* spp. based on sequences of ITS regions. An evolutionary distance matrix was generated as described by Jukes and Cantor and the branching pattern was generated by neighbor-joining method. The numbers at the nodes indicate the bootstrap value of 1000 resampled data set. *Numbers of five ciphers which begin with 4 are KACC accession no. **The sequence data of isolates written with bold were obtained from GenBank or personal communication.

information about the sequences are in the GenBank (Table 1).

Primer sequences of each fragment were removed for alignment. Nucleotide sequences of the 35 isolates examined in this study and 14 sequences already published by other researchers were aligned using CLUSTAL W. Multi-

ple alignment revealed very little divergence close to the ends of the 18S and 28S genes and within the 5.8S gene. More sequence variation was seen in ITS I with only a few short regions showing complete homology across all species examined, whereas in ITS II the sequences were more conserved than in ITS I.

Forty-nine isolates of 16 species generated five distinct molecular groups (Fig. 1). The papillate and semi-papillate *Phytophthora* spp. formed two separate groups (group A and B). *P. infestans, P. cactorum, P. nicotianae* and *P. palmivora* composed group A, and *P. citricola, P. citrophthora,* and *P. capsici* composed group B. The non-papillate species formed three groups (group C, D and E). The group C included *P. cambivora, P. cinamomi, P. melonis, P. sojae,* and two isolates that were previously identified as *P. erythroseptica*. Ten isolates of *P. cryptogea-P. drechsleri* complex group, two isolates of *P. megasperma* and an isolate of *P. erythroseptica* were included in group D. Only two isolates of *P. megasperma* from asparagus and douglas fir in U.S.A were in group E.

Korean isolates of *P. cactorum, P. cambivora, P. capsici, P. cinnamomi, P. citricola, P. infestans, P. melonis, P. nicotianae* and *P. palmivora* clustered with published isolates of each species above 97% bootstrap value. However, two Korean isolates of *P. erythroseptica* showed very close relationship with two Korean isolates and an Australian isolate of *P. sojae* with 97.8%-99.5% sequence homology (data not shown), but an isolate of *P. erythroseptica* (CBS 956.87) (Mills et al., 1991) was located in distinct group D, showing 76.4% and 75.5% sequence similarity with the two Korean isolates. Two Korean isolates and an Australian isolate of *P. sojae* were clustered into a group on the level of 99.8% sequence homology. Four isolates of *P. megasperma* were separated into group D and E. KACC 40401 identified as *P. megasperma* (Jee et al., 1998c) were clustered into group D, showing 98%, 95-96.9% and 92.4% sequence homology with *P. erythroseptica* (CBS 956.87), isolates of *P. cryptogea-P. drechsleri* complex group (Hong et al., 1999), and *P. megasperma* PM12 from chickpea (Crawford et al., 1996), respectively. Four cucurbits isolates of *Phytophthora*, which were formerly classified into *P. drechsleri* or *P. melonis* (Mills et al., 1991), were distantly related with *P. cryptogea-P. drechsleri* complex group (69.3-79%).

Discussion

Genetic diversity of Korean isolates of *Phytophthora* spp. was previously investigated by PCR-RFLP of rDNA (Hong et al., 1999). Each of all papillate and semi-papillate species has shown its unique band pattern. However, many nonpapillate species such as *P. cryptoga, P. drechsleri, P. erythroseptica, P. melonis, P. megasperma* and *P. sojae*, were problematic in their taxonomy. This study could give some solutions for these problems (Fig. 1). Identification of Korean isolates of *P. sojae* was verified by their homogeniety with Australian isolate of *P. sojae*. However, two Korean isolates of *P. erythroseptica* showed distant relationship with a reliable isolate of *P. erythroseptica* (CBS 956.87). It was probable that the two Korean isolates could be genetically different from the *P. erythroseptica* or misidentified. *P. melonis* (cucurbits isolate of *P. drechsleri*) and *P. cryptogea-P. drechsleri* complex group (Hong et al., 1999) showed separate evolutionary lineages. It provides a basis for delineation of cucurbits isolates within *P. cryptogea/P. drechsleri* species complex. But, some of the problems were not solved. A Korean isolate of *P. megasperma* was closely related to *P. erythroseptica* (CBS 956.87), *P. megasperma* PM12 from chickpea and *P. cryptogea-P. drechsleri* complex group isolates. The identification of the isolate is still unclear. *P. cyptogea and P. drechsleri* were not differentiated on this study.

KACC 40200 from *Pueraria lobata* (Kim and Kim, 1993) and KACC 40449 from *Astragalus membranaceus* (Jee, 1998) were identified as *P. erythroseptica* in that they were nonpapillate shape of sporangia, amphigynous, elongated and cylindrical antheridia, and aplerotic oospores. However, the two Korean isolates of *P. erythroseptica* in group C showed distinct relationship with the reliable isolate of *P. erythroseptica* (CBS 956.87) in group D (Fig. 1). Several isolates of *P. erythroseptica* including CBS 956.87 were clustered into *P. cryptogea/P. drechsleri* A, B, C, D and E group which are included in group D in this study, by isozyme analysis (Mills et al., 1991) and by sequence analysis of ITS I (Forster et al., 1995). Therefore, it was probable that two Korean isolates could be misidentified. This is further supported by the fact that the isolates were no growth at 7°C, although minimum temperature for growth of *P. erythroseptica* is 2.5°C.

What species are the two isolates identified as? They showed similar morphological characters with original descriptions of the species, but had larger antheridia and more aplerotic oogonia than CBS 956.87. From this study, the isolates were located in group C, showing close relationship with *P. sojae* and *P. melonis*. The group also included *P. vignae* from Forster et al. (1995). However, the isolates were easily distinguished from the species, *P. sojae, P. melonis* and *P. vignae* by morphology and cultural characters. They were isolated only from Leguminosae, and growth occurred at neither 7°C nor 35°C. More molecular characterizations as well as morphological characteristics are needed for identification of these isolates.

P. sojae is easily differentiated from the other *Phytophthora* spp. in that it does not grow on PDA and is host-specific to soybean. Identification of Korean isolates of *P.*

sojae were verified with the fact that the sequences of the isolates showed 99.8% homology with that of Australian isolate of *P. sojae*.

P. melonis, formerly included in *P. cryptogea/P. drechsleri* species complex (Mills et al., 1991), showed distinct relationship with the other isolates of the species complex, although the other isolates of the species complex were clustered into a group with high similarity, over 95% (Fig. 1). It provides a basis for delineation of Cucurbites isolates within *P. cryptogea/P. drechsleri* species complex. For taxonomy of *P. cryptoge/P. drechsleri* species complex except *P. melonis*, analysis of more isolates from world wide origin are needed.

Morphological species complex, *P. megasperma*, were one of the most problematic species in *Phytophthora* taxonomy. It is homothalic, producing oogonia with predominantly paragynous antheridia and nonpapillate sporangia. A mitochondrial DNA analysis of worldwide origin generated nine distinct molecular groups, some of which were not closely related to each other (Forster and coffey, 1993). In this study, isolates of *P. megasperma* interspersed in group D and E (Fig. 1). KACC 40401 (KACC F3020) which was homothalic, and produced predominantly amphigynous and rarely paragynous antheridia (Jee et al., 1998c), showed closed relationship with PM12 isolate (Crawford, 1996) which corresponds with *P. megasperma* F group (Forster and Coffey, 1993). The isolates also showed closed relationship with *P. erythroseptica* (CBS 956.87) and isolates of *P. cryptogea-P. drechsleri* complex group. Based on this result, it was difficult to determine species. It is thought that analysis of intergenic spacer (IGS) of rDNA, more variable region than ITS, would be useful for solution of this problem.

In other Korean isolates of *P. cactorum, P. cambivora, P. capsici, P. cinnamomi, P. citricola, P. infestans, P. nicotianae,* and *P. palmivora,* their homogeneities with published foregin isolates were verified (Fig. 1). In *P. citrophthora* and *P. boehmeriae,* there were no published sequence data to compare with. However, evolutionary placement (group B in this study) of isolates of *P. citrophthora* were the same as results of Forster et al. (1995).

A grouping of species according to ITS sequence divergence matched, to some degree, the broad classification based on type of papilla. However, a separation of semipapillate species and papillate species was not evident. In grouping of the species, results of this study are similar with those in the previous study (Cooke et al., 1997; Crawford et al., 1996; Forster et al., 1995). This study added *P. nicotianae* into the group of Forster et al. (1995) which included *P. megasperma E, P. palmivora, P. cactorum.* and *P. infestans*.

The major problems in *Phytophthora* taxonomy are on apaillate species. Current classification systems in apapillate *Phytophthora* spp. includes form and size of sporangia, mating system, the form of antheridial attachment and size of oospore within oogonia. A high level of variation in these characters within and between species were frequently observed, thus making classification based on these criteria unreliable. Recent genetic analyses have suggested a solution for taxonomy of *P. megasperma* and *P. cryptogea/P. drechsleri* species complex. Nucleotide sequences of rDNA have also provided some informations for taxonomy of Korean isolates of *Phytophthora* spp. But, KACC 40200 and KACC 40449 formerly identified as *P. erythroseptica,* and KACC 40401, formerly identified as *P. megsperma,* were not verified on their identification. It is thought that analysis of IGS of rDNA, more variable region than ITS, as well as morphological characteristics would be helpful for their characterization.

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