Molecular Identification and Phylogenetic Grouping of *Diaporthe phaseolorum* and *Phomopsis longicolla* Isolates from Soybean

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Accepted for publication 30 August 1998.

ABSTRACT

Zhang, A. W., Riccioni, L., Pedersen, W. L., Kollipara, K. P., and Hartman, G. L. 1998. Molecular identification and phylogenetic grouping of *Diaporthe phaseolorum* and *Phomopsis longicolla* isolates from soybean. *Phytopathology* 88:1306-1314.

Diaporthe phaseolorum and *Phomopsis longicolla* isolates from soybean were examined using traditional mycological characteristics and molecular methods. Cultural characteristics including types of fruiting bodies and conidia were assessed for isolates collected from soybean stems and seeds. Cultures were identified as *P. longicolla*, *D. phaseolorum* var. *caulivora*, *D. phaseolorum* var. *meridionalis*, or *D. phaseolorum* var. *sojae*. Molecular markers for these groups were developed and analyzed using polymerase chain reaction restriction fragment length polymorphisms (PCR-RFLP) and DNA sequencing in the internal transcribed spacer (ITS) and the 5.8S ribosomal DNA. The ITS₄ and ITS₅ primers amplified PCR products for all isolates studied. Gel electrophoresis of undigested PCR products and DNA sequencing produced various fragment lengths including 604 bp for *P. longicolla*, 602 and 603 bp for *D. phaseolorum* var. *caulivora*, 603 bp for *D. phaseolorum* var. *meridionalis*, and from 597 to 609 bp for *D. phaseolorum* var. *sojae*. Digestion of these PCR products with enzymes *AluI*, *HhaI*, *MseI*, *RsaI*, and *ScrFI* resulted in distinct bands for identification

of *P. longicolla* and the varieties of *D. phaseolorum*. All *P. longicolla*, *D. phaseolorum* var. *caulivora*, and *D. phaseolorum* var. *meridionalis* isolates were distinguished using *AluI* and *HhaI* with *RsaI* or *ScrFI*. The banding patterns of *D. phaseolorum* var. *sojae* isolates were complex and were separated into 11 subgroups after digestion with *AluI*, *HhaI*, *MseI*, *RsaI*, and *ScrFI*. Phylogenetic analysis of 20 isolates of *D. phaseolorum* and *P. longicolla* based on the DNA sequence of the ITS region resolved six clades termed A, B, C, D, E, and F. Clade A included all sequenced *D. phaseolorum* var. *caulivora* isolates, two from Italy and one from the United States. Isolates in clade B were exclusively associated with *D. phaseolorum* var. *meridionalis*. Clades A and B formed a well-supported monophyletic group. Isolates in clades C, D, E, and F were morphologically defined as isolates of *P. longicolla*, *D. phaseolorum* var. *sojae*, and *Diaporthe* spp. The ITS sequences similarity of seven geographically diverse *P. longicolla* isolates illustrated that *P. longicolla* isolates have a similar genetic background, with some affiliations to some *D. phaseolorum* var. *sojae* isolates. Morphological characteristics of the isolates along with the terminal clades of the ITS phylogeny suggest that *P. longicolla* is an individual species, *D. phaseolorum* var. *caulivora* and *D. phaseolorum* var. *meridionalis* are varieties of *D. phaseolorum*, and *D. phaseolorum* var. *sojae* is either several varieties of *D. phaseolorum* or possibly several distinct species.

Phomopsis seed decay, pod and stem blight, and stem canker of soybean (*Glycine max* (L.) Merr.) are three diseases caused by the *Diaporthe/Phomopsis* complex (29,39). The fungi in this complex are endemic to soybean production areas throughout the world and can cause reductions in stands, yield, and seed quality (1,3,4,31). The causal organisms are *Phomopsis longicolla* T. W. Hobbs (teleomorph unknown) and three varieties of *Diaporthe phaseolorum* (Cooke & Ellis) Sacc. (*D. phaseolorum*) (anamorph *P. phaseoli* (Desmaz.) Sacc.): *D. phaseolorum* var. *caulivora* K. L. Athow and R. M. Caldwell, *D. phaseolorum* var. *meridionalis* Fernandez, and *D. phaseolorum* var. *sojae* (S. G. Lehman) Wehmeyer (16,29,30,39).

P. longicolla differs from *D. phaseolorum* var. *caulivora*, *D. phaseolorum* var. *meridionalis*, and *D. phaseolorum* var. *sojae* in its culture characteristics, morphology (does not have a known teleomorph), and causation (primary cause of seed decay) (20,24,29). *D. phaseolorum* var. *sojae* has a wider host range and is more vari-

able in cultural and other characteristics. It is recognized as a variety of *D. phaseolorum*, because it has both α and β conidia, pycnidia with short or no pycnidial beaks, and typical perithecia. *D. phaseolorum* var. *sojae* is accepted as the main causal agent of pod and stem blight (25,26). The incitant of northern soybean stem canker, *D. phaseolorum* var. *caulivora*, was first distinguished and named based on its pathogenicity, homothallism, presence of perithecia, and absence of pycnidia. The causal agent of southern soybean stem canker, *D. phaseolorum* var. *meridionalis*, is closely related to *D. phaseolorum* var. *caulivora*, but differs in morphology, heat tolerance, and epidemiology (present only the southern United States) (4,19,22).

Some plant-pathogenic groups of fungi, like those in the soybean *Diaporthe/Phomopsis* complex, have historically been difficult to identify. The identification of *Diaporthe/Phomopsis* isolates from soybeans has been based on morphological characteristics including color and colony appearance, size and shape of stromata, the ratio and size of α and β conidia, and the arrangement and shape of perithecia (29,39). The causal organism often has been identified by the disease it causes on soybeans. The identification of these organisms has been difficult because the morphological characteristics often are too variable to establish the appropriate taxonomic rank (30). Except for random amplified polymorphic DNA (RAPD) analysis that was used to confirm the existence of at least three different subspecific taxa of *D. phaseolorum* on soybean (16), there has been no other research on the molecular classification of soybean isolates making up the *Diaporthe/Phomopsis* complex. It is important to understand the phylogenetic relationship of these fungi

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Publication no. P-1998-1019-02R

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in the *Diaporthe/Phomopsis* complex to understand more about their diversity and genetic relationship.

The ribosomal RNA genes in fungi vary among species within a genus or among populations (42). Ribosomal RNA are tandemly repeated with 60 to 220 copies per haploid genome in fungi. Each repeat unit contains a copy each of the 18S, 5.8S, and 28S RNAs, and two internal transcribed spacers (ITS₁ and ITS₂) (9,35).

Polymerase chain reaction (PCR) coupled with restriction fragment length polymorphism (RFLP) analysis in the ITS region has been used in fungal taxonomy and phylogenetic studies (6,8,11,27). Molecular differences among *Phomopsis* spp. have been analyzed by sequencing the amplified ITS region of isolates from different hosts, and it was proposed that the host-based species concept of *Phomopsis* was not reliable (34).

The objectives of our research were to (i) infer the phylogeny of the *Diaporthe/Phomopsis* complex on soybean based on the nucleotide sequence divergence in the ITS regions of the ribosomal DNA (rDNA); and (ii) evaluate the potential of the ITS region as genetic markers for species delimitation by comparing sequences, PCR-RFLP banding patterns, and morphological characteristics of the *Diaporthe/Phomopsis* isolates collected from soybean.

MATERIALS AND METHODS

Fungal isolates and morphological identification. Isolates of *D. phaseolorum* and *P. longicolla* were obtained from soybean seeds and stems collected from Argentina, Brazil, People's Republic of China, Italy, Korea, and the United States (Table 1). All isolates were single spored and maintained on potato dextrose agar (PDA) in petri dishes stored at 4°C.

All isolates were grown on PDA and incubated under 12-h-per-day fluorescent light at 22 to 25°C for at least 10 days before being identified based on cultural characteristics (20,29,39). Some isolates were grown on autoclaved soybean stems on water agar, and others were inoculated onto soybean plants in the greenhouse to induce fruiting bodies to facilitate identification.

All isolates were tested for pathogenicity on soybean. Soybean seeds of cv. Williams 82 were planted in 10-cm-diameter pots in a soil/sand (1:1) mix and thinned to three plants per pot. Nine plants (three pots per replication) with fully expanded unifoliate leaves (12- to 15-day-old seedlings) were inoculated by spraying a blended mycelial suspension that consisted of 1 g wet weight of cultures grown on potato dextrose broth (PDB) for 2 to 3 days mixed in 100 ml of double-distilled H₂O. Nine plants were sprayed with distilled water as a control. All plants were placed in a moist chamber for 3 days and then transferred to the greenhouse bench. Plants were sprayed with 2% paraquat (Gramoxone; Zeneca Corp., Wilmington, DE) 15 days after inoculation to induce plant senescence and the production of fungal fruiting structures on soybean stems. Stems without any symptoms were excised and placed in a moist seed-germination chamber for 2 weeks to observe if fungal fruiting bodies formed. Selected isolates also were inoculated on 15-day-old seedlings in trays (80 by 40 cm²) with 20 seedlings per tray. A total of 10 seedlings (five plants per two repetitions) were inoculated by placing a mycelial PDA plug (5 mm²) upside down on one cotyledon that was prewounded 2 to 3 mm from the stem with a razor blade. A PDA plug without mycelia was used as a control. The plants were covered with a plastic dome for 3 days to maintain high humidity and then uncovered. The number of dead plants and the length of stem lesions were recorded 10 days after inoculation.

DNA extraction. Pieces of agar from the margins of actively growing colonies were transferred to 100 ml of PDB. *P. longicolla* isolates were grown at 24°C for 48 h and *D. phaseolorum* isolates were grown for 96 to 120 h. Mycelia were collected by squeezing the decanted cultures through one layer of Miracloth (Calbiochem, La Jolla, CA). Total genomic DNA was extracted from 1 g of mycelia using a previously described protocol (5).

PCR amplification and enzymatic digests. The ITS region of the nuclear rDNA from *P. longicolla*, *D. phaseolorum* var. *caulivora*, *D. phaseolorum* var. *meridionalis*, and *D. phaseolorum* var. *sojae* isolates were amplified using primers ITS₄ and ITS₅. PCRs were performed with a Perkin-Elmer DNA thermal cycler (Perkin-Elmer Cetus, Emeryville, CA) with a reaction mix of 50 mM KCl; 2.5 mM MgCl₂; 10 mM Tris-HCl at pH 8.3; 0.2 mM each of dTTP, dATP, dGTP, and dCTP; 50 pmol of the primers; 2.5 units of *Taq* polymerase (Gibco BRL Corp., Grand Island, MD); and 25 ng of genomic DNA in a final volume of 50 µl. Reactions were run for 1 cycle at 96°C for 3 min and 30 cycles each at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. The amplification efficiency was checked by agarose gel electrophoresis using 5 µl of the PCR products (42).

PCR products from the ITS region were digested with restriction enzymes *AluI*, *MseI*, *HhaI*, *RsaI*, and *SerFI*, according to the manufacturer's recommendations (Gibco BRL Corp. and New England Biolabs, Beverly, MA), using 7 µl of the PCR product (approximately 200 to 500 ng of DNA), 1.5 µl of buffer (10×), and 6.5 µl of double-distilled H₂O at 37°C for 2 to 4 h. Enzyme-digested PCR products were size-fractionated on a mixed agarose gel of 1% ultra pure agarose (Gibco BRL Corp.) and 1% 3:1 high resolution blended agarose (Amresco, Solon, OH) at 3.5 V/cm, stained with ethidium bromide, visualized on a UV transilluminator, and photographed. A 1-kb double-stranded DNA ladder (Gibco BRL Corp.) was used as size marker.

DNA sequencing. Seven isolates of *P. longicolla*, three isolates of *D. phaseolorum* var. *caulivora*, five isolates of *D. phaseolorum* var. *meridionalis*, eleven isolates of *D. phaseolorum* var. *sojae*, and one isolate of *Diaporthe* were chosen to represent the various DNA groups based on the restriction banding patterns (Table 1). PCR products from the ITS regions were size-fractionated on a 1% agarose gel in Tris-acetate-EDTA buffer. DNA was purified from agarose blocks using the Gel Purification Kit 50 (Qiagen Inc., Chatsworth, CA). Purified DNA was cloned and sequenced as previously described (43). Sequences were checked between complementary strands for reading errors, and ambiguities were resolved if possible. Complete sequences were aligned by multiple alignment within the species using SeqEd (version 1.0.3; Applied Biosystems Division, Foster City, CA) to correct misread residues.

Data analyses. RFLP banding patterns of *Diaporthe/Phomopsis* isolates representing different RFLP groups were compared and analyzed by gel electrophoresis and restriction maps created from the DNA sequences of the ITS and the 5.8S rDNA by using computer program DNASTAR's LaserGene software (DNASTAR Inc., Madison, WI).

DNA sequences of the ITS₁ and ITS₂ regions and the 5.8S rDNA from all the taxa were aligned using the CLUSTAL W program by setting the opening penalties and extension penalties of five and three, respectively, and by using the weighted toggle transitions in the multiple-alignment parameter option (21). This alignment was further examined and slightly optimized manually.

Maximum parsimony analysis of the aligned sequences was conducted using the PAUP program (version 3.1, Illinois Natural History Survey, Champaign). The sequence of *Gaeumannomyces graminis* (Sacc.) Arx and Oliver (GenBank accession number U17213) was included in the alignment and was used as an outgroup, while the *D. phaseolorum* and *P. longicolla* isolates were considered as an ingroup in the phylogenetic analysis. Alignment gaps were treated as missing data. The distance matrix was computed to estimate the genetic distance among the isolates and to identify the isolates with identical sequences ("0" value point in the distance matrix). The Heuristic search procedure was performed with tree bisection-reconnection (TBR) branch swapping, MULPARS, steepest descent, collapse, and ACCTRAN optimization options to identify the most parsimonious tree. Bootstrap analysis (15) with 500 replicates was performed to measure the degree of support for each branch in the strict consensus tree. The decay values (12) were calculated up to five steps longer than the short-

est tree to determine the robustness of clades found in the most parsimonious trees. The search was limited to five additional steps because of computational constraints (14). The homoplasy measurements including consistency index (CI) (23), retention index (RI),

and rescaled consistency (RC) (13) were estimated. The *g₁* statistic, a measure of skewness of tree-length distribution (18), was computed by generating 10,000 random parsimonious trees using the random tree option of the PAUP program.

TABLE 1. Isolates of *Phomopsis longicolla* (PL), *Diaporthe phaseolorum* var. *caulivora* (DPC), *D. phaseolorum* var. *meridionalis* (DPM), and *D. phaseolorum* var. *sojae* (DPS) used for polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) sequencing and morphological observations

Code ^a	Species	Geographic location	Source	NCBI accession ^b
AK76*	PL	Arkansas, United States	J. Rupe	AF000206
HO101*	PL	Chunbuk, Korea	E. W. Park	AF000211
3113	PL	Illinois, United States	G. L. Hartman	U97658
3113-1	PL	Illinois, United States	G. L. Hartman	AF000209
3114-3	PL	Illinois, United States	G. L. Hartman	AF000208
IL3281*	PL	Illinois, United States	G. L. Hartman	
95-VS-24*	PL	Illinois, United States	G. L. Hartman	
IO117*	PL	Iowa, United States	G. L. Hartman	
628*	PL	Italy	A. Porta-Puglia	AF000207
628II	PL	Italy	A. Porta-Puglia	AF000210
632*	PL	Italy	A. Porta-Puglia	
717-F*	PL	Italy	A. Porta-Puglia	
MO628*	PL	Missouri, United States	G. L. Hartman	
MO125*	PL	Missouri, United States	G. L. Hartman	
NEB94*	PL	Nebraska, United States	G. L. Hartman	
600*	PL	Ohio, United States	G. L. Hartman	
95-VS-43*	DPC	Illinois, United States	G. L. Hartman	AF000212
713*	DPC	Italy	A. Porta-Puglia	AF000567
713'* ^c	DPC	Italy	A. Porta-Puglia	
720*	DPC	Italy	A. Porta-Puglia	AF000563
721*	DPC	Italy	A. Porta-Puglia	
DPCA*	DPC	Italy	A. Porta-Puglia	
DPCB*	DPC	Italy	A. Porta-Puglia	
DPCC*	DPC	Italy	A. Porta-Puglia	
DPCS1**	DPC	Italy	A. Porta-Puglia	
DPCS2**	DPC	Italy	A. Porta-Puglia	
DPCS3**	DPC	Illinois, United States	G. L. Hartman	
479**	DPC	Illinois, United States	G. L. Hartman	
497A**	DPC	Illinois, United States	G. L. Hartman	
AG1**	DPM	Argentina	G. L. Hartman	
AG2**	DPM	Argentina	G. L. Hartman	
AG3**	DPM	Argentina	G. L. Hartman	
84-15*	DPM	Arkansas, United States	J. Rupe	
90-48*	DPM	Arkansas, United States	J. Rupe	AF000564
SDP174*	DPM	Arkansas, United States	J. Rupe	
STC-2*	DPM	Arkansas, United States	J. Rupe	AF000566
STC-2'*	DPM	Arkansas, United States	J. Rupe	AF001015
STC-3	DPM	Arkansas, United States	J. Rupe	AF001016
STC-5*	DPM	Arkansas, United States	J. Rupe	
STC-7*	DPM	Arkansas, United States	J. Rupe	
STC-8*	DPM	Arkansas, United States	J. Rupe	
PMSP1*	DPM	Brazil	D. S. Joccoud Fo.	AF000565
PMSP2*	DPM	Brazil	D. S. Joccoud Fo.	
PMSP4**	DPM	Brazil	D. S. Joccoud Fo.	
PMSP5**	DPM	Brazil	D. S. Joccoud Fo.	
624*	DPM	Florida, United States	R. Schultheiss	
AK25A*	DPS	Arkansas, United States	J. Rupe	AF001025
PSSP2*	DPS	Brazil	D. S. Joccoud Fo.	
PSSP4*	DPS	Brazil	D. S. Joccoud Fo.	
PSSP5*	DPS	Brazil	D. S. Joccoud Fo.	AF001020
ID102*	DPS	Chunbuk, Korea	E. W. Park	AF001024
KB103*	DPS	Chunbuk, Korea	E. W. Park	
SW-93-3*	DPS	Chunbuk, Korea	E. W. Park	
SW-93-5*	DPS	Chunbuk, Korea	E. W. Park	
SW-93-13*	DPS	Chunbuk, Korea	E. W. Park	AF001018
473-3*	DPS	Italy	A. Porta-Puglia	
473-4*	DPS	Italy	A. Porta-Puglia	AF001021
714-4*	DPS	Italy	A. Porta-Puglia	AF001028
714-5*	DPS	Italy	A. Porta-Puglia	
793-A*	DPS	Italy	A. Porta-Puglia	AF001014
793-B*	DPS	Italy	A. Porta-Puglia	AF001019
FAO125*	DPS	Italy	A. Porta-Puglia	AF001017
FAO126*	DPS	Italy	A. Porta-Puglia	AF001022
PRDPS*	DPS	Puerto Rico	G. L. Hartman	AF001023
SW-93-10*	<i>Diaporthe</i> sp.	Chunbuk, Korea	E. W. Park	AF001026
SW-93-11*	<i>Diaporthe</i> sp.	Chunbuk, Korea	E. W. Park	AF001027

^a * = isolates demonstrated in PCR-RFLP gel pictures, ** = isolates were used for PCR-RFLP, but not demonstrated in PCR-RFLP gel pictures.

^b Accession numbers of sequences submitted to GenBank, National Center of Biotech. Institute.

^c Duplicate of isolate 713.

Phylogenetic analysis was also performed by the maximum likelihood method (15) using the fastDNAmI program (33). Most parsimonious trees were constructed with a transition/transversion ratio of 2.0 default, jumble or random sequence addition order, and global branch swapping options. Distance matrices were also computed and were used to estimate the distance trees using DNA-DIST and NEIGHBOR programs of the PHYLIP (version 3.5; University of Washington, Seattle).

RESULTS

Morphological and cultural characteristics. Morphological observations of *D. phaseolorum* and *P. longicolla* isolates were used for the initial classification (Table 1). All *P. longicolla* isolates produced white mycelia with occasional greenish-yellow surface areas turning light brown with age. Pycnidia usually were in clus-

ters, had long beaks, and exuded only α conidia. Black stroma usually were large and widespread, but in some cases were circular, small in diameter (2 to 5 mm), and few in number.

D. phaseolorum var. *meridionalis* isolates produced lanose colonies that initially were white-tufted and, 10 to 15 days later, turned tan to buff. Most isolates readily produced pycnidia with α conidia, and a few others either produced perithecia with age or did not produce any fruiting bodies. The underside of cultures was generally tan to buff with brown, irregular-shaped stromas (2 to 10 mm) sometimes embedded in the medium. All isolates produced perithecia on soybean stems incubated on water agar.

D. phaseolorum var. *caulivora* isolates had white, closely appressed mycelia. A few isolates turned light tan with age. As cultures aged, immersed perithecia formed with the tips of their beaks emerged from the medium surface. Stroma were infrequent and small (1 to 2 mm), circular, and spread throughout the medium.

Fig. 1. Restriction banding patterns of polymerase chain reaction-amplified internal transcribed spacer of nuclear ribosomal DNA of *Phomopsis longicolla* (PL), *Diaporthe phaseolorum* var. *caulivora* (DPC), *D. phaseolorum* var. *meridionalis* (DPM), and *D. phaseolorum* var. *sojae* (DPS) using five restriction enzymes.

One isolate, DPCA, formed larger, irregular brown stroma immersed in the medium. Most of the isolates produced perithecia on soybean stems incubated on water agar.

Of the isolates identified as *D. phaseolorum* var. *sojae*, only isolate AK25A produced perithecia on PDA and on soybean stems incubated on water agar. The remaining isolates produced pycnidia and were variable in cultural characteristics. The mycelia were generally floccose and ropelike, but differed in color. Isolates FAO126, ID102, PSSP4, and PSSP5 were white; SW-93-5 was light gray; 473-4 and SW-93-13 were gray brown; 793-A was light brown; 793-B and FAO125 had yellow tufts; and PR *D. phaseolorum* var. *sojae* was dark brown. Pycnidia were beakless, solitary, and small or aggregated in large conidiomata. The stroma were usually small (2 to 5 mm) and scattered, but were circular and larger (5 to 10 mm) in isolates FAO125 and very large and irregular in isolates FAO126, ID102, and SW-93-5. All isolates produced α and β conidia.

Isolates SW-93-10 and SW-93-11 were defined as *Diaporthe* spp. They produced white colonies; scattered, isolated pycnidia with abundant α and β conidia; perithecia with long protruding beaks; and nearly colorless undersides with large black *P. longicolla*-like stromata. These isolates did not produce stem blight or stem canker, but perithecia formed on detached stems in a moist chamber and resembled the perithecia produced in PDA.

All *P. longicolla* and *D. phaseolorum* var. *sojae* isolates infected stems when soybean plants were spray-inoculated and then treated with paraquat. Pycnidia of *P. longicolla* isolates covered 70 to 90% of the stem surface, while pycnidia of *D. phaseolorum* var. *sojae* isolates covered 20 to 90% of the stem surface. After soybean plants were spray-inoculated and then treated with paraquat, *D. phaseolorum* var. *caulivora* and *D. phaseolorum* var. *meridionalis* did not produce any fruiting bodies, while *D. phaseolorum* var. *meridionalis* isolates produced pycnidia and perithecia only on detached stems under moist incubation. All *D. phaseolorum* var. *caulivora* and *D. phaseolorum* var. *meridionalis* isolates produced 0.5- to 2-mm lesions on stems after inoculation on wounded cotyledons. *D. phaseolorum* var. *caulivora* isolates killed 40% of the plants after 15 days.

PCR-RFLP. The ITS₄ and ITS₅ primers amplified PCR products for all isolates studied. Gel electrophoresis of undigested PCR products and DNA sequencing produced various fragment lengths including 604 bp for *P. longicolla*, 602 and 603 bp for *D. phaseolorum* var. *caulivora*, 603 bp for *D. phaseolorum* var. *meridionalis*, and from 597 to 609 bp for *D. phaseolorum* var. *sojae*. Digestion of these PCR products with enzymes *Alu*I, *Hha*I, *Mse*I, *Rsa*I, and *Scr*FI resulted in distinct bands for identification of *P. longicolla* and varieties of *D. phaseolorum*. All *P. longicolla*, *D. phaseolorum* var. *caulivora*, and *D. phaseolorum* var. *meridionalis* isolates were distinguished using *Alu*I and *Hha*I with *Rsa*I or *Scr*FI. Enzyme *Hha*I separated *D. phaseolorum* var. *caulivora* into two groups. Isolate AK25A had typical *D. phaseolorum* var. *sojae* morphological characteristics and was classified as one RFLP subgroup along with isolate PSSP2 (Figs. 1 and 2). Estimated restriction fragment lengths using the DNA sequences of the ITS and the 5.8S region of rDNA for selected RFLP marker bands of *Diaporthe/Phomopsis* isolates representing each RFLP groups are listed (Table 2). With RFLP marker bands, *Diaporthe* isolates SW-93-10 and SW-93-11 were separated into one RFLP group. The banding patterns of *D. phaseolorum* var. *sojae* isolates were complex and could be separated into 11 subgroups after they were digested with the five enzymes. Among these *D. phaseolorum* var. *sojae* isolates, three isolates appeared in *D. phaseolorum* var. *sojae* subgroups I and K, and only one or two isolates occurred in the other *D. phaseolorum* var. *sojae* subgroups.

Phylogenetic analysis. Aligned DNA sequences of the ITS region of all 27 isolates had 632 characters. After checking the pairwise distance matrix created by 27 isolates, six of the seven isolates of *P. longicolla* from Italy, Korea, and the United States and one of the five from *D. phaseolorum* var. *meridionalis* (also from the United States) were excluded from the analysis because of 100% sequence similarity. The pairwise distance analysis of 20 isolates had percentage mean sequence divergence of 0 for *P. longicolla*, 0.002 to 0.183 for *D. phaseolorum* var. *caulivora*, 0.033 to 0.23 for *D. phaseolorum* var. *meridionalis*, and 0.017 to 0.58 for *D. phaseolorum* var. *sojae* (Table 3). Analysis of the entire sequence based on equally weighted character states resulted in five most parsimonious trees with equal lengths of 306 steps. A strict consensus tree was created from the most parsimonious trees using bootstrap and decay values (Fig. 3). The CI and RI of these trees were 0.529 and 0.690, respectively, when uninformative characters were excluded. The bootstrap values ranged from 57 to 100% for the consensus clades. When tree length (number of evolutionary steps) of the most parsimonious trees was increased from 306 steps to 311 steps by one-step increments, the number of trees increased from 5 to more than 17,200 (Table 4). The CI, RI, and rescaled CI had inverse linear correlation to the increase in tree length. The g1 statistic calcu-

Fig. 2. Restriction banding patterns of polymerase chain reaction-amplified internal transcribed spacer of nuclear ribosomal DNA of *Diaporthe phaseolorum* var. *caulivora* (DPC), *D. phaseolorum* var. *sojae* (DPS), and *Diaporthe* spp. using five restriction enzymes.

lated from 10,000 random trees was -0.6389. One of the five most parsimonious trees with the number of transitions and transversion swappings for each branch is represented (Fig. 4).

The evolutionary tree constructed by the maximum likelihood method and distance trees estimated by the neighbor joining analysis (data not shown) were very similar in their topologies and were completely congruent with the phylogeny inferred by the maximum parsimony analysis (Figs. 3 and 4).

Twenty sequences of *P. longicolla* and *D. phaseolorum* isolates were defined as clades A, B, C, D, E, and F (Fig. 3). Clade A consists of *D. phaseolorum* var. *caulivora* isolates, two collected from Italy and one from the United States. Clade B was exclusively

associated with *D. phaseolorum* var. *meridionalis*. *D. phaseolorum* var. *caulivora* and *D. phaseolorum* var. *meridionalis* isolates together formed a monophyletic group with a bootstrap value of 93 and 100 and decay values of +3 and $\geq +5$, respectively. Isolates in clades C, D, E, and F were morphologically defined as *P. longicolla*, *D. phaseolorum* var. *sojae*, and *Diaporthe* sp. Clade C contained two subclades. Subclade C₁ included *D. phaseolorum* var. *sojae* isolates 473-4 and SW-93-13 and was strongly supported by a bootstrap value of 100 and $\geq +5$ decay value; subclade C₂ included *D. phaseolorum* var. *sojae* isolates FAO125 and PR *D. phaseolorum* var. *sojae*. *Diaporthe* isolate SW-93-10 alone formed clade D. Clade E had two *D. phaseolorum* var. *sojae*

TABLE 2. DNA fragment sizes produced by five restriction enzymes from polymerase chain reaction restriction fragment length polymorphisms (PCR-RFLP) of the internal transcribed spacer (ITS₁ and ITS₂) and the 5.8S regions of ribosomal DNA for *Phomopsis longicolla* (PL), *Diaporthe phaseolorum* var. *caulivora* (DPC), *D. phaseolorum* var. *meridionalis* (DPM), and *D. phaseolorum* var. *sojae* (DPS) isolates from soybean

Fungus	Sub-group ^a	Isolate	Restriction enzyme ^b				
			<i>AluI</i>	<i>RsaI</i>	<i>HhaI</i>	<i>MseI</i>	<i>SacFI</i>
PL	...	AK76, HO101, 3113, 3113-1, 3114-3, IL3281, 95-VS-24, IO117, 628, 628II, 632, 717-F, MO628, MO125, NEB94, 600*	458*, 146*	394*, 210*	260*, 228*, 61, 46, 9	526*, 57, 21	254*, 147*, 107*, 96
DPM	...	AG1, AG2, AG3, 84-15, 90-48, SDP174, STC-2, STC-2', STC-3, STC-5, STC-7, STC-8, PMSP1, PMSP2, PMSP4, PMSP5, 624	603*	603*	228*, 135*, 122, 63, 46, 9	484*, 57, 41, 21	265*, 96*, 69*, 66, 63, 43
DPC	A	DPCA, DPDS1, DPDS2, DPDS3, 497A, 479, 95-VS-43, 713, 713', 720, 721	281*, 176*, 146*	603*	260*, 226*, 62, 46, 9	484*, 57, 41, 21	254*, 96*, 77, 70, 63, 43
	B	DPCB, DPCC	281*, 176*, 145*	602*	228*, 134*, 122, 63, 46, 9	484*, 57, 40, 21	254*, 96*, 76, 70, 63, 43
DPS	A	473-3, 473-4	339*, 146*, 124*	609*	228*, 134*, 129, 63, 46, 9	490*, 57, 41, 21	250*, 96*, 79, 70, 69, 43
	B	714-4	459*, 144*	603*	227*, 132*, 126, 63, 46, 9	306*, 181*, 58, 38, 20	330*, 95, 70, 65, 43
	C	793-a	459*, 144*	393*, 210*	260*, 224*, 63, 46, 9	306*, 181*, 58, 38, 20	332*, 95*, 70*, 63, 43
	D	793-b	597*	597*	225*, 131*, 126*, 62, 44, 9	509*, 57, 21	253*, 200*, 144*
	E	AK25A, PSSP2	459*, 145*	604*	272*, 261*, 62, 9	525*, 58, 21	255*, 145*, 95*, 67*, 42
	F	FAO125	459*, 144*	481*, 122*	261*, 179*, 90*, 63, 9	247*, 181*, 58, 56, 41, 20	332*, 95*, 70*, 63, 43
	G	FAO126	460*, 146*	606*	260*, 229*, 63, 45, 9	528*, 58, 20	250*, 154*, 95*, 64, 43
	H	PRDPS	456*, 146*	602*	260*, 225*, 62, 46, 9	301*, 181*, 59, 41, 20	315*, 95*, 79*, 70, 43
	I	ID102, PSSP4, PSSP5	457*, 144*	601*	260*, 226*, 45, 61, 9	523*, 58, 20	255*, 143*, 108*, 95
	J	SW-93-3	335*, 146*, 124*	605*	228*, 131*, 130, 62, 45, 9	488*, 58, 39, 20	267*, 96*, 70*, 65, 64, 43
	K	KB103, SW-93-13, SW-93-5	459*, 145*	604*	272*, 261*, 62, 9	488*, 58, 39, 20	200*, 150*, 95*, 64, 52, 43
<i>Diaporthe</i> spp.	...	SW-93-10, SW-93-11	460*, 146*	606*	225*, 136*, 128, 62, 46, 9	308*, 181*, 58, 39, 20	255*, 97*, 69*, 66, 64, 44, 11

^a Subgroups of DPC and DPS.

^b * = marker bands used for molecular grouping.

TABLE 3. Pairwise divergence between 20 *Diaporthe phaseolorum* and *Phomopsis longicolla* isolates from soybean and *Gaeumannomyces graminis* computed by comparing their unambiguously aligned sequences^a

	Isolate code number																				
Isolate	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1. 95-VS-43	...	0.002	0.003	0.033	0.032	0.030	0.038	0.038	0.078	0.059	0.054	0.040	0.042	0.048	0.064	0.045	0.057	0.020	0.039	0.054	0.183
2. 713	1	...	0.002	0.032	0.030	0.028	0.037	0.037	0.076	0.057	0.052	0.039	0.040	0.047	0.062	0.044	0.055	0.018	0.037	0.052	0.181
3. 720	2	1	...	0.033	0.032	0.030	0.038	0.038	0.078	0.059	0.054	0.040	0.042	0.048	0.064	0.045	0.057	0.020	0.039	0.054	0.179
4. 90-48	20	19	20	...	0.002	0.003	0.005	0.050	0.079	0.067	0.066	0.049	0.055	0.055	0.077	0.054	0.065	0.037	0.044	0.067	0.172
5. STC-2	19	18	19	1	...	0.002	0.007	0.048	0.079	0.065	0.064	0.047	0.054	0.053	0.075	0.052	0.065	0.035	0.042	0.065	0.170
6. STC-3	18	17	18	2	1	...	0.008	0.050	0.076	0.062	0.061	0.045	0.052	0.055	0.072	0.051	0.062	0.033	0.039	0.062	0.168
7. STC-2'	23	22	23	3	4	5	...	0.053	0.079	0.069	0.061	0.054	0.060	0.057	0.080	0.057	0.067	0.042	0.049	0.070	0.175
8. SW-93-13	23	22	23	30	29	30	32	...	0.096	0.079	0.074	0.053	0.064	0.012	0.077	0.052	0.077	0.045	0.055	0.067	0.183
9. 793-B	46	45	46	47	47	45	47	57	...	0.057	0.057	0.098	0.083	0.098	0.071	0.099	0.062	0.078	0.091	0.064	0.188
10. PSSP5	35	34	35	40	39	37	41	47	34	...	0.020	0.073	0.066	0.077	0.032	0.067	0.018	0.057	0.057	0.027	0.179
11. ID102	32	31	32	39	38	36	36	44	34	12	...	0.073	0.056	0.071	0.035	0.066	0.018	0.057	0.061	0.017	0.185
12. FAO126	24	23	24	29	28	27	32	32	58	43	43	...	0.049	0.055	0.082	0.038	0.073	0.050	0.052	0.074	0.186
13. 793-A	25	24	25	33	32	31	36	38	49	39	33	29	...	0.064	0.062	0.052	0.061	0.043	0.039	0.052	0.192
14. 473-4	29	28	29	33	32	33	34	7	58	46	42	33	38	...	0.080	0.050	0.074	0.052	0.057	0.069	0.186
15. FAO126	38	37	38	46	45	43	48	46	42	19	21	49	37	48	...	0.067	0.035	0.057	0.061	0.020	0.185
16. PRDPS	27	26	27	32	31	30	34	31	58	40	39	23	31	30	40	...	0.064	0.044	0.047	0.057	0.191
17. AK25A	34	33	34	39	39	37	40	46	37	11	11	43	36	44	21	38	...	0.059	0.059	0.023	0.187
18. SW-93-10	12	11	12	22	21	20	25	27	46	34	34	30	26	31	34	26	35	...	0.030	0.054	0.169
19. 714-4	23	22	23	26	25	23	29	33	54	34	36	31	23	34	36	28	35	18	...	0.054	0.172
20. AK76	32	31	32	40	39	37	42	40	38	16	10	44	31	41	12	34	14	32	32	...	0.183
21. <i>G. graminis</i>	99	98	97	93	92	91	95	99	101	97	100	100	104	101	100	103	101	92	93	99	...

^a The absolute distance values are shown in the left side of the diagonal and the percent mean distance values are shown in the right side of the diagonal.

isolates. Clade F contained the *P. longicolla* isolate AK76 and some *D. phaseolorum* var. *sojae* isolates.

DISCUSSION

The taxonomic status and nomenclature of the *Diaporthe/Phomopsis* complex on soybean has been reviewed (30). *D. phaseolorum* and *P. phaseoli* were recognized, respectively, as the valid binomials for the teleomorph and anamorph belonging to this complex, while *P. longicolla* was accepted as a separate entity (30). The previous classification of *D. phaseolorum* at the variety level was considered to be unsatisfactory because of the considerable variability in morphology, physiology, and host specificity. Instead, it was proposed that the forma specialis concept should be adopted for intraspecific designations (30). This nomenclature was not accepted (16,20), and the correct usage of names is still an issue. Morphological observations in our study demonstrated that isolates within *P. longicolla*, *D. phaseolorum* var. *caulivora*, and *D. phaseolorum* var. *meridionalis* have distinguishable characteristics, although morphological identification is often time consuming and not always defined. *P. longicolla* isolates had black, large, and widely dispersed stroma in agar media. This is an important characteristic to identify isolates of *P. longicolla*, but occasionally we observed some isolates with small (2 to 5 mm) and few pycnidia. *D. phaseolorum* var. *sojae* isolates varied in cultural characteristics, confirming what has been reported (16,25,29,30). This variation may be explained by its heterothallic nature (41).

To use molecular characteristics for systematic studies, the appropriate molecular markers need to resolve the desired taxonomic level (16,28,32,38,42), showing neither excessive variation nor homogeneity at the rank of interest (7). The ITS region of rDNA has been used to differentiate species and also to differentiate subgroups within species (10,17,28,37). The ITS region includes a 5.8S subunit that provided an appropriate length of DNA for observing variation of the *Diaporthe/Phomopsis* complex in our study. No single enzyme distinguished all *P. longicolla*, *D. phaseolorum* var. *caulivora*, *D. phaseolorum* var. *meridionalis*, and *D. phaseolorum* var. *sojae* isolates; however, three enzymes clearly distinguished *P. longicolla*, *D. phaseolorum* var. *meridionalis*, and *D. phaseolorum* var. *caulivora* isolates from each other.

Of the *Diaporthe* and *Phomopsis* isolates used in this experiment, each *P. longicolla* and *D. phaseolorum* var. *meridionalis* isolate originated from three different countries, while *D. phaseolorum* var. *caulivora* isolates come from two countries (Table 1). The isolates of *P. longicolla*, *D. phaseolorum* var. *caulivora*, and *D. phaseolorum* var. *meridionalis* have their own uniform PCR-RFLP banding patterns; and the *D. phaseolorum* var. *caulivora* isolates produced two *Hha*I digestion patterns. However, isolates within *D. phaseolorum* var. *sojae* and *Diaporthe* spp. were separated into 12 groups. The PCR-RFLP groups generated in our study corresponded morphologically to delineated species of *P. longicolla*, *D. phaseolorum* var. *meridionalis*, and *D. phaseolorum* var. *caulivora* regardless of the isolates' geographic origins. Based on the PCR-RFLP markers summarized (Table 2; Figs. 1 and 2),

Fig. 3. Strict consensus of the five most parsimonious trees with equal length of 306 steps obtained from equally weighted parsimonious analysis using the branch-and-bound option of the PAUP program of the entire unambiguously aligned internal transcribed spacers region from 20 isolates of *Diaporthe phaseolorum* and *Phomopsis longicolla*. The numbers above the branches indicate the number of times a monophyletic group occurred in 500 bootstrap replicates. The numbers shown below the branches indicate the additional number of steps over 306 (the total number of steps in the shortest tree) needed to collapse that branch (decay values). Decay analysis with tree length $\geq$ five steps longer than the most parsimonious trees could not be performed owing to the computational constraints. A, B, C₁, C₂, D, E, and F are names of subclades. G-gram = *Gaeumannomyces graminis*, PL = *Phomopsis longicolla*, DPC = *Diaporthe phaseolorum* var. *caulivora*, DPM = *D. phaseolorum* var. *meridionalis*, DPS = *D. phaseolorum* var. *sojae*, and DP = *Diaporthe phaseolorum*.

Diaporthe and *Phomopsis* isolates can be identified based on molecular characterization.

Sequence variation of the ITS region has been used to study intra- and interspecific divergence. For example, the ITS₁ sequences among northern hemisphere species of *Armillaria* were identical, although these species could be differentiated based on interfertility tests, morphology, and other molecular criteria (2). In contrast, ITS sequences were used to separate species among intersterility groups in *Pleurotus* spp. (40). The differences in ITS sequence divergence among these taxonomic groups of fungi may reflect different rates of ITS sequence evolution, the time frame in which population divergence and speciation occurred, and the time elapsed since speciation. Genetic diversity within *Phomopsis* and *Diaporthe* spp. and the validity of the host-based species concept that has prevailed were assayed and tested using the ITS region of rDNA (34). The results of this study disagree with the classification system that identifies *Phomopsis* spp. based on their hosts. However, isolates used in that study (34) were not morphologically identified to species, and there is insufficient data to determine whether the terminal clades in the ITS phylogeny included one or more species. In our study, four taxonomic groups of *P. longicolla* and *D. phaseolorum* from only soybean isolates were classified to species and variety by traditional morphological methods and their phylogenetic relationships based on DNA sequencing.

Phylogenetic analysis of 21 ITS sequences representing each PCR-RFLP banding pattern yielded five equally parsimonious trees. The number of most parsimonious trees obtained can be attributed to the number of terminal units analyzed (14) and to the homoplasy of the ITS sequence data (36). Most of the alternative topologies among the most parsimonious trees occurred at terminal and subterminal clades, particularly in branches supported by relatively few characters. Trees with more than 311 steps could not be examined because of the limitation of the memory capacity in the PAUP program tree buffer. The g1 statistic calculated from 10,000 random trees was -0.6389, indicating that the tree length distribution was significantly skewed to the left (negative value), suggesting a strong phylogenetic signal in the data.

The sequence alignment result showed that Italian *D. phaseolorum* var. *caulivora* varied in the ITS region. This indicates that there are genetic differences among the *D. phaseolorum* var. *caulivora* isolates from one area. The same occurred within the *D. phaseolorum* var. *meridionalis* isolates in clade B; even though all isolates originated from one area in the United States, the sequences varied. It seems possible that *D. phaseolorum* var. *caulivora* and *D. phaseolorum* var. *meridionalis*, which both cause soybean stem canker, together with some *D. phaseolorum* var. *sojae* may have originated from a common ancestor. An unusual aspect of stem canker is that *D. phaseolorum* var. *caulivora* and *D. phaseolorum* var. *meridionalis* colonize all soybean cultivars, but disease only develops on susceptible cultivars (3). Selection pressure due to deployment of resistant host cultivars may have increased genetic diversity within isolates of *D. phaseolorum* var. *caulivora* and *D. phaseolorum* var. *meridionalis*.

P. longicolla isolate AK76 showed a close relationship to *D. phaseolorum* var. *sojae* isolate AK25A and formed a moderately supported subclade (59% bootstrap value and +2 decay index)

TABLE 4. Decay analysis of DNA sequences of the internal transcribed spacer region of 20 *Diaporthe phaseolorum* and *Phomopsis longicolla* isolates using Heuristic search of the PAUP program

Tree length in no. of steps	Total no. of trees found	Consistency index	Retention index	Rescaled consistency
306	5	0.529	0.690	0.469
≤307	41	0.526	0.687	0.465
≤308	203	0.675	0.684	0.462
≤309	1,654	0.673	0.680	0.458
≤310	7,409	0.671	0.677	0.454
≤311	>17,200	0.669	0.674	0.451

with the isolate FAO126. This isolate was classified as *D. phaseolorum* var. *sojae*, even though it showed similar cultural characteristics to *P. longicolla* isolates including absence of a *Diaporthe* stage, white mycelium, α conidia, extensive black stroma, few β conidia, and pycnidia with long beaks. *D. phaseolorum* var. *sojae* appeared to be a polyphyletic taxon, having some isolates associated with the *D. phaseolorum* var. *caulivora* and some with the *D. phaseolorum* var. *meridionalis* clades. However, this association was poorly supported by <50% bootstrap values. Instead of the three *D. phaseolorum* varieties being closely related to each other, the phylogenetic analysis of the ITS sequences indicate that the *P. longicolla* and some *D. phaseolorum* var. *sojae* have a closer relationship. Similar conclusions were made in another study (16) using the morphology of soybean *Diaporthe* and *Phomopsis* isolates and RAPD analysis. *D. phaseolorum* var. *sojae* contains greater genetic variability than any other species or varieties studied (Fig. 3).

The similarity of ITS sequences of the seven *P. longicolla* isolates representing different geographic distributions illustrates a uniform genetic background among *P. longicolla* isolates. Viewed as the prevalent causal organism of *Phomopsis* seed decay (3), *P. longicolla* has been distributed worldwide with the dissemination of soybean seeds. *P. longicolla* does not have a known teleomorph (20,24,25), which may account for its apparent genetic uniformity.

Previous studies based on morphological observations offered many criteria to identify and classify various isolates of the *Diaporthe/Phomopsis* complex. By using PCR-RFLP, we were able to separate *P. longicolla*, *D. phaseolorum* var. *caulivora*, *D. phaseolorum*

Fig. 4. One of the five most parsimonious trees with a length of 306 steps from equally weighted parsimony analysis of the entire unambiguously aligned internal transcribed spacers region from 20 isolates of *Diaporthe phaseolorum* and *Phomopsis longicolla*. The pairs of numbers separated by a hyphen refer to the number of transitions-transversions supporting that branch. Gg = *Gaeumannomyces graminis*, PL = *Phomopsis longicolla*, DPC = *Diaporthe phaseolorum* var. *caulivora*, DPM = *D. phaseolorum* var. *meridionalis*, DPS = *D. phaseolorum* var. *sojae*, and DP = *Diaporthe phaseolorum*.

var. *meridionalis*, and *D. phaseolorum* var. *sojae* isolates faster. This method could be used for routine identification of these fungi. Isolates associated with terminal clades in the ITS phylogeny suggests that *P. longicolla* is a single species. *D. phaseolorum* var. *caulivora* and *D. phaseolorum* var. *meridionalis* are two varieties of *D. phaseolorum*, while *D. phaseolorum* var. *sojae* may be divided into several varieties or even distinct species.

ACKNOWLEDGMENTS

The participation of L. Riccioni was supported by the minister per le. Project No. 451, Area 10. We thank W. D. Chen, J. B. Sinclair, and L. Vaillancourt for their critical suggestions and for reviewing this manuscript; G. Concaf and R. L. Ma for skilled technical assistance; and D. S. Joccound Fo., A. Porta-Puglia, J. Rupe, R. Schultheiss, and E. W. Park for *Phomopsis* and *Diaporthe* isolates.

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