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Phylogenetic Relationships of the Soybean Sudden Death Syndrome Pathogen *Fusarium solani* f. sp. *phaseoli* Inferred from rDNA Sequence Data and PCR Primers for Its Identification

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Phylogenetic relationships of several species within the *Fusarium solani*-complex were investigated using characters from the nuclear ribosomal DNA. Genetic variation within 24 isolates, including 5 soybean sudden death syndrome (SDS) strains, was assessed using rDNA sequence data and restriction fragment length polymorphic markers. By these techniques, the causal agent of soybean SDS was identified as *F. solani* f. sp. *phaseoli*. In separate cladistic analyses, *Plectosphaerella cucumerina* and *Nectria cinnabarina* or *F. ventricosum* were used for rooting purposes. Monophyly of the *F. solani*-complex was strongly supported by bootstrap and decay analyses. Parsimony analysis indicates that this complex is composed of a number of phylogenetically distinct species, including *Neocosmospora vasinfecta*, *F. solani* f. sp. *phaseoli*, and biological species designated as MPI, MPV, and MPVI of *N. haematococca*. The results demonstrate complete congruence between biological and phylogenetic species within the *N. haematococca*-complex. In addition, DNA sequence data were used to design a PCR primer pair which could specifically amplify DNA from isolates of the SDS pathogen from infected plants.

Additional keywords: Glycine max, Phaseolus vulgaris, Nectria haematococca, Neocosmospora vasinfecta, species concepts.

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Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrents the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

Fusarium solani (Mart.) Appel & Wollenw. emend Snyd. & Hans. [meiotic state or teleomorph = *Nectria haematococca* Berk & Br.] is a polytypic species complex (VanEtten and Kistler 1988) responsible for a diverse range of biologically distinct plant diseases (Farr et al. 1989), mycotoxicoses, and superficial and fatal systemic infections of animals, including humans (Marasas et al. 1984). Traditional classification and identification of species within this complex and of other fusaria has been based on shared morphological traits of the anamorph, colony morphology of single-spore isolates cultured on special media, host range, secondary metabolite profile (Thrane 1990), and to a lesser extent microanatomy of the teleomorph (Booth 1971).

Given the paucity, plasticity, and intergradation of phenotypic traits, it is not surprising that morphological species concepts employed in taxonomic treatments of *Fusarium* (Booth 1971; Gerlach and Nirenberg 1982; Nelson et al. 1983) are incongruent, especially in the treatment of sections *Martiella* and *Ventricosum* (*F. solani sensu* Snyder and Hansen, 1953). For this reason, a molecular systematic approach based on discrete DNA sequence data offers considerable promise in the establishment of an objective, phylogenetically based system of classification of the fusaria (Guadet et al. 1989; Bruns et al. 1991). Furthermore, the same nucleotide sequence data can be used to design DNA-based probes for the detection, and rapid and accurate identification of unknown isolates.

In prior molecular systematic studies of *Fusarium*, Guadet et al. (1989) and O'Donnell (1993) used partial nuclear large rDNA gene sequences for phylogenetic reconstruction. However, taxon sampling within the *F. solani*-complex was limited in these studies. In the present study, DNA sequence data from the nuclear rDNA internal transcribed spacer (ITS) region and D1 and D2 domains of the nuclear large subunit 28S rDNA were analyzed cladistically using maximum parsimony in order to evaluate the validity of Snyder and Hansen's (1953) polytypic species concept as currently applied to *F. solani* (Nelson et al. 1983; Burgess et al. 1988).

In this report, we used molecular data to study the two pathogenic forms of *F. solani* described on soybeans: "FSA"

for those that cause SDS and "FSB" for those that cause seedling disease and root rot (Abney et al. 1993). Results reported here indicate the "FSA" SDS-inducing strains are a phylogenetically distinct species which Snyder and Hansen (1953) treated as *F. solani* f. sp. *phaseoli* (Burkh.) Snyd. & Hans. and the "FSB" strains are an unrelated fungus, *Plectosphaerella cucumerina* (Lindf.) W. Gams. Further evidence is presented demonstrating that a teleomorph previously reported as *N. haematococca* for the SDS pathogen (Abney et al. 1993) is *P. cucumerina*. DNA sequence data was used to design a PCR primer pair which specifically amplifies DNA from the causal agent of sudden death syndrome (SDS) of soybeans (*Glycine max* (L.) Merr.) from both axenically grown mycelium and infected soybean plants.

RESULTS

Phylogenetics.

The ITS and 28S rDNA primer pairs successfully amplified a single PCR product of approximately 600-bp from the genomic DNA of each plant and fungal species tested. As expected (O'Donnell 1992), sequence analysis revealed that the rDNA genic spacer organization exhibited the following conserved features: the ITS1 is flanked at its 5′ end by the 3′ end of the nuclear small 18S rDNA while the nuclear large 28S rDNA is 3′ to the ITS2 and the ITS1 and ITS2 flank the 5.8S gene (Fig. 1).

Direct sequencing of the double-stranded PCR products revealed that the *F. solani* strains fell into 5 discrete sequence

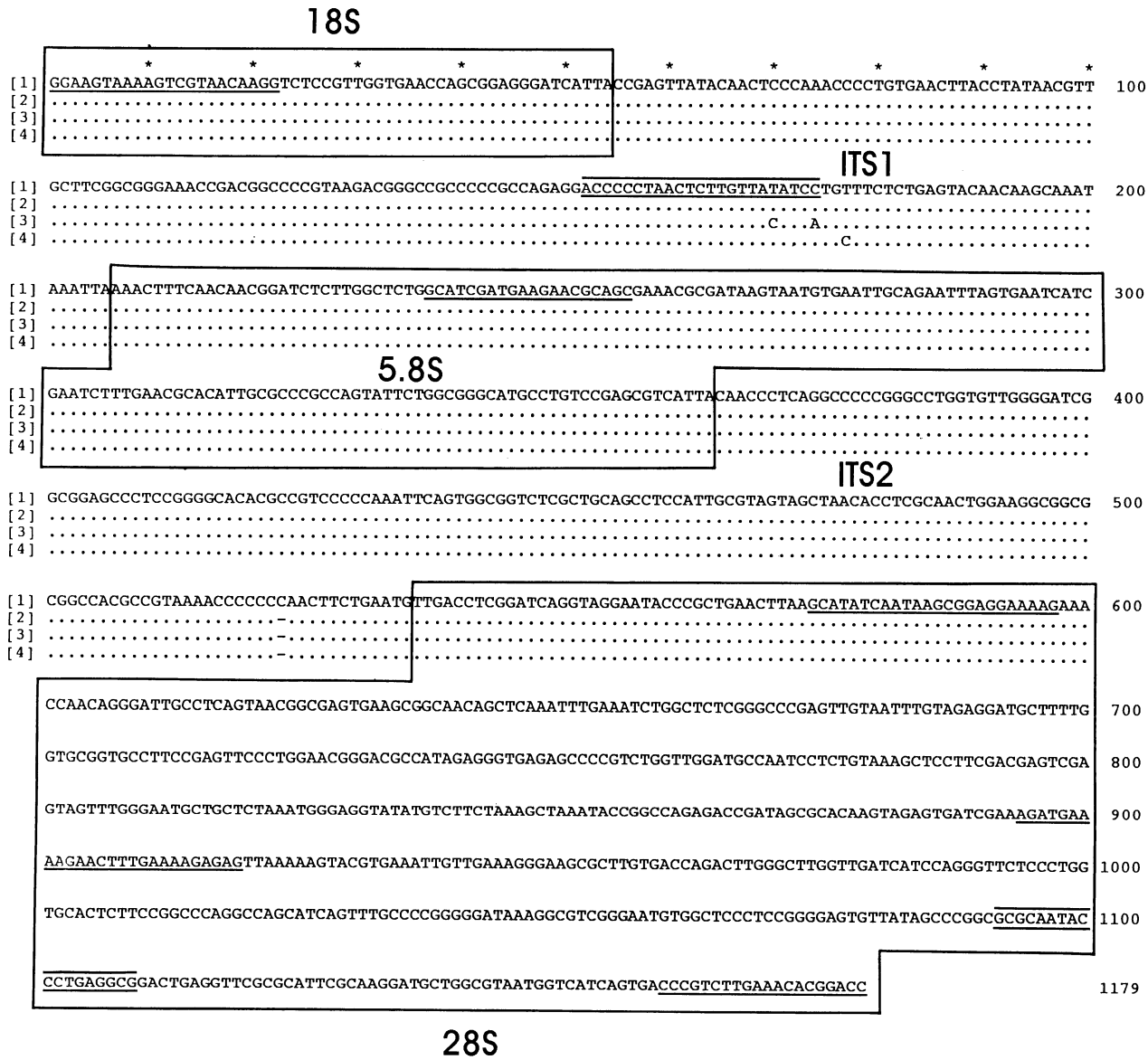

Fig. 1. Complete nucleotide sequence of the noncoding strand of the 13 *Fusarium solani* f. sp. *phaseoli* isolates amplified with primer pair ITS5/NL4. The 13 strains fell into 4 [1-4] sequence classes [Table 1]. Matches (.) and gaps (–) are shown below the reference SDS strain [#1 rDNA class]. Numbering of the sequence begins with the 5′- most nucleotide sequenced. Blocks delineate the three genic regions: the 18S, 5.8S, and 28S rDNA genes. PCR and sequencing primer sites are underlined. The sequence of the forward (FspF) and complement of the reverse (FspR) species-specific PCR primers are over and underlined.

groups (7 including *Neocosmospora vasinfecta* and *F. ventricosum*). The sequences of the 5 SDS-causing isolates, including 4 "FSA" strains (Abney et al. 1993), were virtually identical to the 8 *F. solani* f. sp. *phaseoli* isolates obtained from three continents (Figs. 1 and 2, Table 1). Isolates of this species are easily identified by their morphologically distinct macroconidia, blueish colonies with abundant sporodochia, and the rare production of microconidia (Wollenweber 1943; Sakurai and Matuo 1960; Matuo and Snyder 1973). Our results indicate that the "FSB" perithecial-forming isolates identified by Abney et al. (1993) as the SDS pathogen teleomorph *Nectria haematococca* are actually *Plectosphaerella cucumerina*. The "FSB" isolates of Abney et al. (1993) share identical teleomorphic and anamorphic features with *P. cucumerina*. Both produce slender brown, flask-shaped ascomata and one-septate, multiguttulate phialoconidia. In addition, identical DNA sequence data was obtained from 15 *P. cucumerina* reference strains (K. O'Donnell, unpublished) obtained from 5 major culture collections (IMI, England; DAOM, Canada; ATCC, USA; BBA, Germany; and CBS, The Netherlands) and the "FSB" isolates, strongly suggesting the strains are conspecific.

Congruence of the morphological and molecular data indicates that the casual agent of SDS of soybeans is *F. solani* f. *phaseoli* (Snyder and Hansen 1953) (= *F. solani* (Mart.) Sacc. var. *solani* (Gerlach and Nirenberg 1982), or *F. solani* (Mart.)

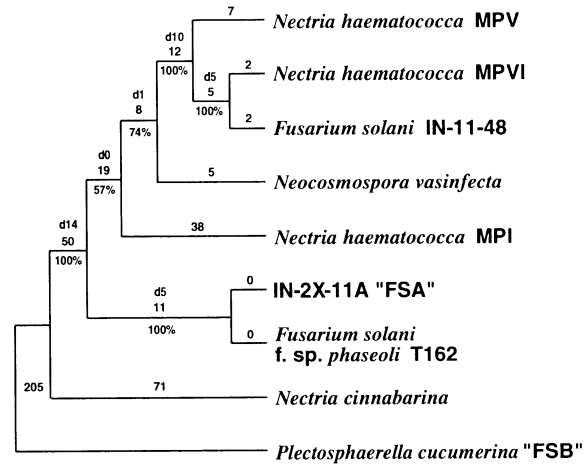

Fig. 2. Maximum parsimony analysis of phylogenetic relationships among selected taxa within *Fusarium solani*-complex based on rDNA sequence data. *Plectosphaerella cucumerina* and *Nectria cinnabarina* were used as outgroups to root the tree. The branch-and-bound option of PAUP 3.1.1 found two equally parsimonous trees (435 steps, consistency index = 0.899). Clade stability was assessed by 1000 heuristic bootstrap replication, percentages (indicated by "%") and decay indices (indicated by d "#", up to 14 steps). Edge lengths are also indicated on the cladogram. Gaps were treated as missing data.

Table 1. Strains studied

NRRL no.	Fsp rDNA type[a]	Geographic origin	Isolate no(s).[b] (depositor/collector)	Substrate	Received as/ identified as[c]
22292	1	Illinois	Mont-1 (L. Gray)	*Glycine max*	Fs/Fsp
22823	1	Indiana	IN-11-60-4 (T. S. Abney)	*Glycine max*	Fs/Fsp
22825	1	Indiana	IN-F2X-11A (T. S. Abney)	*Glycine max*	Fs/Fsp
22489	1	...	T-501 = FSA 'Poinset' (H. VanEtten, K. Roy)	*Glycine max*	Fs/Fsp
22490	1	...	T-502 = FSA 'Lu' (H. VanEtten, K. Roy)	*Glycine max*	Fs/Fsp
22158	2	New York	ATCC 60860 = FBI-S (D. A. Smith)	*Phaseolus vulgaris*	Fsp
22276	2	...	ATCC 38466 = T-162 (H. VanEtten)	*Phaseolus vulgaris*	Fsp
22678	3	California	S-712 = 18W (P. Nelson, S. N. Smith)	*Phaseolus vulgaris*	Fs/Fsp
22732	3	Germany	BBA 64384 (H. Nirenberg)	*Phaseolus vulgaris*	Fsm3/Fsp
22743	3	Brazil	BBA 68441 (H. Nirenberg)	*Phaseolus vulgaris*	Fsm3/Fsp
22744	3	Brazil	BBA 68442 (H. Nirenberg)	*Phaseolus vulgaris*	Fsm3/Fsp
22275	4	...	ATCC 42361 = SUF 386 (T. Matuo)	*Phaseolus vulgaris*	Fsp
22411	4	United States	BBA 64394 = CBS 265.50 (H. Nirenberg)	*Phaseolus vulgaris*	Fsp
22819	...	Indiana	IN-11P-12 (T. S. Abney)	*Glycine max*	Fs/Pc
22821	...	Indiana	IN-2X-11B (T. S. Abney)	*Glycine max*	Fs/Pc
22822	...	Indiana	IN-11-12 (T. S. Abney)	*Glycine max*	Fs/Pc
22824	...	Indiana	IN-11-50-2 (T. S. Abney)	*Glycine max*	Fs/Pc
22820	...	Indiana	IN-11-48 (T. S. Abney)	*Glycine max*	Fs/Nh-MPVI
22098	...	Greece	LE-NhI-5 (L. Epstein)	*Cucurbita* sp.	Nh-MPI
22141	...	New Zealand	PGB-153 (B. Hawthorne)	*Cucurbita* sp.	Nh-MPV
22278	...	...	ATCC 38467 = T-8 (H. VanEtten)	*Pisum sativum*	Nh-MPVI
22166	...	Illinois	ATCC 62199 (L. M. Carris)	*Heteroderma glycines*	Ncos
20484	...	...	MUCL 19(G. Hennebert)	...	Ncin
20846	...	Germany	CBS 748.79 = BBA 62452 (W. Gerlach)	wheat field soil	Fvent

[a] See Figure 1 for DNA sequence comparison of rDNA polymorphisms in *Fusarium solani* f.sp. phaseoli.

[b] ATCC = American Type Culture Collection, Rockville, MD; BBA = Biologische Bundenanstalt für Land- und Forstwirtschaft, Berlin, Germany; CBS = Centraalbureau voor Schimmelcultures, Baarn, The Netherlands; MUCL = Mycotheque de l'Universite Catholique de Louvain, Louvain-la-Neuve, Belgium; H. VanEtten, Department of Plant Pathology, University of Arizona, Tucson, Arizona; P. Nelson, Department of Plant Pathology, Pennsylvania State University, University Park, PA; T. S. Abney, Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN;L. Epstein, Department of Plant Pathology, University of California, Berkeley, CA; B. Hawthorne, CRI Plant Protection, Private Bag, Auckland, New Zealand.

[c] Fs = *Fusarium solani* (Mart.) Appel & Wollenw. emend. Snyd. & Hans.; Fsp = *Fusarium solani* f. phaseoli (Burkh.) Snyd. & Hans.; Nh-MPVI = *Nectria haematococca* Berk. & Br. MPVI, *Fusarium solani* f. *pisi* (Jones) Snyder & Hansen; Fsm3 = *Fusarium solani* (Mart.) var. *martii* (Appel & Wollenw.) Wollenw. f. 3 Snyder; Pc = *Plectosphaerella cucumerina* (Lindf.) W. Gams; Nh-MPI = *Nectria haematococca* Berk. & Br. MPI, *Fusarium solani* f. *cucurbitae* Snyd. & Hans. (race 1); Nh-MPV = *Nectria haematococca* Berk. & Br. MPV, *Fusarium solani* f. *cucurbitae* Snyd. & Hans. (race 2); Ncos = *Neocosmospora vasinfecta* E. F. Smith; Ncin = *Nectria cinnabarina* (Tode:Fr.) Fr.; Fven = *Fusarium ventricosum* Appel & Wollenw.

var. *Martii* (Appel & Wollenw. *sub species*) Wollenw. *forma* 3 Snyder (Wollenweber and Reinking 1935)). Phylogenetic reconstruction suggests that *F. solani* f. sp. *phaseoli*, including the SDS pathogen, is a distinct species within the *F. solani*-complex (Fig. 2). Two separate searches were implemented by the branch-and-bound option of PAUP 3.1.1 (Swofford 1993), testing two different outgroups as a root for the rDNA gene tree. In the first search, employing *Plectosphaerella cucumerina* and *Nectria cinnabarina* as outgroups, PAUP found two equally parsimonous trees of 435 steps (Fig. 2 = tree #1; consistency index = 0.889). Tree #2 (not shown) was identical in topology to tree #1 except that *N. haematococca* MPI,

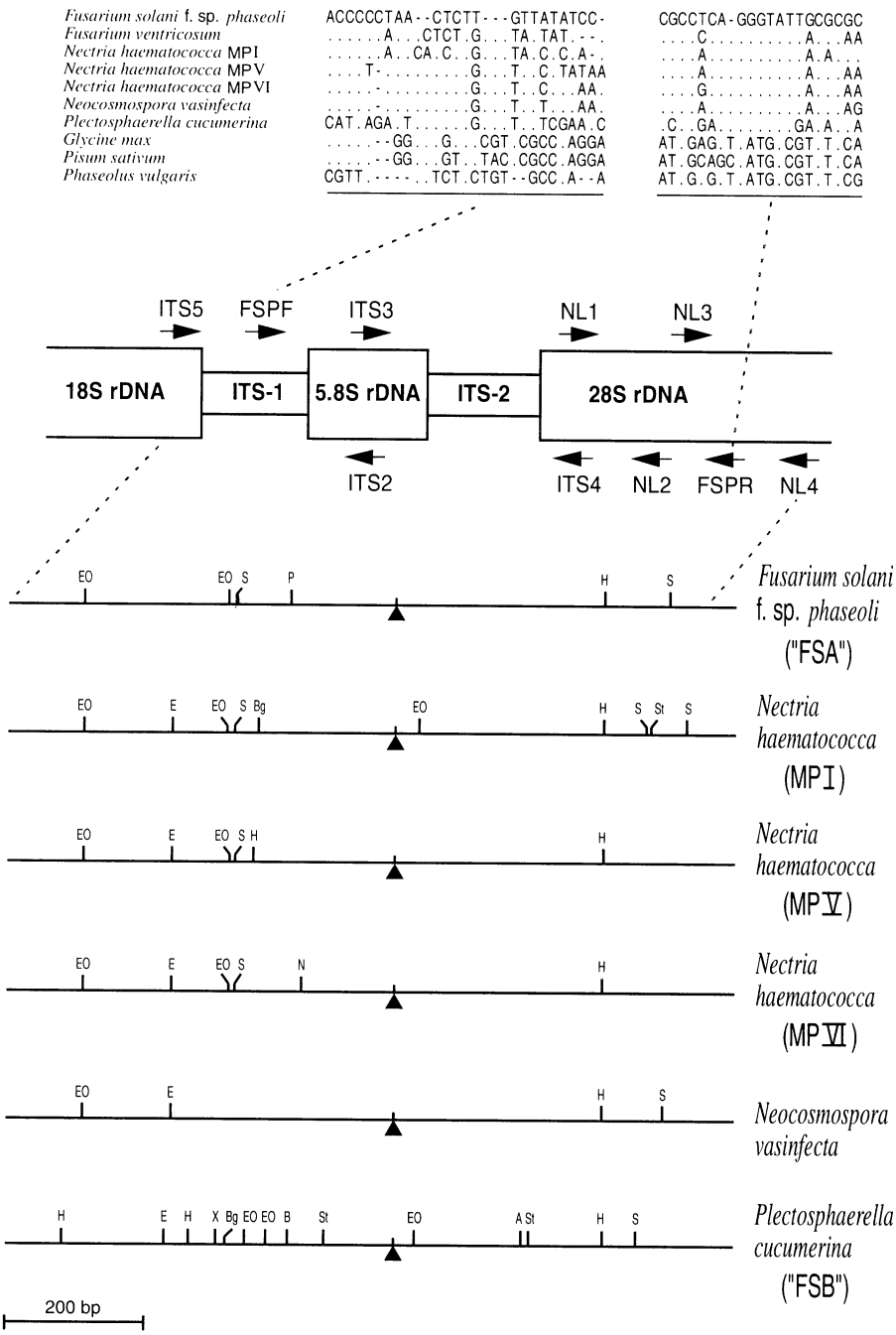

Fig. 3. Design of *Fusarium solani* f. sp. *phaseoli* species-specific PCR primer pair. The approximate location of the forward primer (FspF located in ITS1) and the reverse primer (FspR located in D2 region of 28S rDNA) are shown in relation to the rDNA region amplified with ITS5/NL4. Primer sequences are given (5′-to-3′) with matches (.) and mismatches to the DNAs of 7 fungal and 3 plants shown below each. Alignment gaps are indicated by (–). The forward primer is based on the antisense strand while the reverse primer is based on the coding strand. Species-specific hexanucleotide restriction maps of the ITS-28S rDNA regions of the six taxa amplified by the PCR primer pair ITS5/NL4 are aligned below the gene map. The triangle below each map indicates the position of the NL1 and ITS4 primers. EO, *Eco*O109I; S, *Sma*I; P, *Pst*I; H, *Hae*II; E, *Eco*RI; Bg, *Bgl*I; St, *Sty*I; N, *Nru*I; X, *Xho*I; B, *Bam*HI; A, *Acc*I.

instead of *F. solani* f. sp. *phaseoli*, was a sister group to the rest of the *F. solani* clade. Clade stablity, as assessed by bootstrap analysis (1,000 replications) and decay indices (= d, up to 14 steps), revealed that monophyly of the ingroup was strongly supported (100% bootstrap interval; d = 14). Furthermore, monophyly of other terminal groups is strongly supported by the bootstrap, and these include: *F. solani* f. sp. *phaseoli* (100%, d = 5), *N. haematococca* MPV—*N. haematococca* MPVI (100%, d = 10), and *N. haematococca* MPVI—*F. solani* IN-11-48 (from Abney et al. 1993) (100%, d = 5). Lower bootstrap values are correlated with lower decay indices (ex., 74% and d = 1 for *Neocosmospora vasinfecta*; 57% and d = 0 for *N. haematococca* MPI). *Nectria* is paraphyletic with *Neocosmospora vasinfecta* derived within the *F. solani* clade. Because the outgroups contributed 276 steps (i.e., 63.4%) of the tree length, a second search was run in which we tested *F. ventricosum* (Gerlach and Nirenberg 1982) as a possible less homoplaseous outgroup to root the tree. The branch-and-bound option of PAUP 3.1.1 found a single most parsimonous tree of 174 steps, 63 steps of which are contributed by the outgroup (consistency index = 0.891). This tree is identical in topology to tree #1 (Fig. 2) except that *N. haematococca* MPI and *N. vasinfecta* exchanged positions in the *F. ventricosum* outgroup-rooted tree. In both the *F. ventricosum* and *P. cucumerina–N. cinnabarina* outgroup rooted trees, high bootstrap values (Hillis and Bull 1993) are correlated with high decay indices. Lastly, we used PAUP 3.1.1 and MacClade to determine the change in tree length by constraining *P. cucumerina* to the *F. solani* f. sp. *phaseoli* clade. The constrained tree (not shown) was 32 additional steps (tree length = 467 steps).

The *F. solani* f. sp. *phaseoli* isolates from soybeans, which were all collected in the South Central United States, exhibited a unique single base pair insertion within the ITS2 (Fig. 1) absent from French bean isolates of this species. Taxon-specific hexanucleotide restriction maps of six taxa are shown in Figure 3. The *F. solani* f. sp. *phaseoli* isolates can be distinguished easily from the taxa shown by the presence of a unique *Pst*I restriction site within the ITS2 and the absence of an otherwise conserved *Eco*RI site within the 5.8S rDNA gene.

Primer design and detection of the SDS pathogen.

Based on the aligned fungal and plant rDNA sequences (Fig. 3), we designed and tested one *F. solani* f. sp. *phaseoli*-specific PCR primer pair (FspF/FspR), to amplify DNA from various species within the *F. solani*-complex, fusaria within *Gibberella* (O'Donnell 1993), and three plant species: soybean, French bean, and garden pea. In experiments carried out on fungal genomic DNA, the FspF/FspR primer pair was able to specifically amplify a PCR product of 957 to 958 bp only from the *F. solani* f. sp. *phaseoli* strains (Fig. 4) as predicted from the rDNA sequence data (Figs. 1 and 3).

To determine whether the *F. solani* f. sp. *phaseoli*-specific PCR primers could detect the SDS pathogen in diseased plants, we tested the primer set on inoculated soybeans and on uninoculated control plants. In duplicate experiments, in which 4 infected and 4 control plants were tested 3 and 5 days after inoculation, the FspF/FspR primer pair was able to detect *F. solani* f. sp. *phaseoli* in all 16 infected plants assayed but not in the uninoculated negative controls (Fig. 5). While the SDS-specific primers were able to specifically amplify the target fungal DNA from DNA-extracted root tissue of infected plants, the amplification product was always fainter than from auxenically grown mycelium and much fainter than from the amplification obtained with the ITS5/NL4 primer pair which was run as a positive control (Fig. 4, lane 13).

DISCUSSION

Phylogenetics.

Results reported here indicate the causal agent of soybean sudden death syndrome (SDS) is *F. solani* f. sp. *phaseoli*, a phylogenetically distinct species within the *F. solani*-complex. This species is morphologically distinct (Matuo and Snyder 1973; Gerlach and Nirenberg 1982) and includes isolates from both soybean and French bean. Not suprisingly, isolates of *F. solani* f. sp. *phaseoli* exhibit an identical pathology on each host plant (Melgar et al. 1994). Results of this study indicate that the SDS-causing strains isolated from soybeans within the South Central United States may represent a distinct clade within this species since they share a unique single base pair substitution within the ITS2. Molecular systematic and morphological analysis of the four Abney et al. (1993) "FSB" strains, including the homothallic isolate (IN-2X-11B = NRRL 22821) reported to have given rise to the "FSA" SDS-causing strains and 15 reference strains of *Plec-*

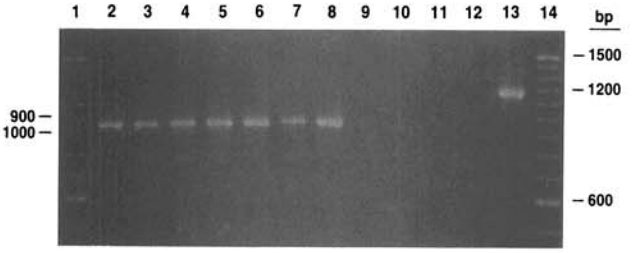

Fig. 4. Specific amplification of *Fusarium solani* f. sp. *phaseoli* DNA with the FspF/FspR PCR primer pair. One hundred-base pair molecular weight marker (Gibco BRL; Gaithersburg, MD) (lanes 1 and 14); products of PCR using DNA from *F. solani* f. sp. *phaseoli* isolates (lanes 2 to 8); *Nectria haematococca* MPI (lane 9); *N. haematococca* MPV (lane 10); *N. haematococca* MPVI (lane 11); *Plectosphaerella cucumerina* (lane 12); ITS5/NL4 positive control PCR product from *F. solani* f. sp. *phaseoli* (lane 13). The 957- to 958-bp fragment was amplified only from *F. solani* f.sp. *phaseoli* (lanes 2 to 8). No DNA negative controls were run for all isolates (not shown) and no PCR products were amplified.

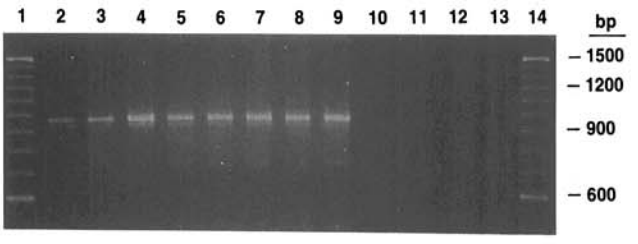

Fig. 5. Detection of *F. solani* f. sp. *phaseoli* in soybean plants 3 days (lanes 2 to 5) and 5 days (lanes 6 to 9) after inoculation, using the FspF/FspR PCR primer pair. Uninfected control plants (lanes 0 to 13), 100-bp molecular weight marker (lanes 1 and 14).

tosphaerella cucumerina, indicate the "FSB" strains are *P. cucumerina* and not *Nectria haematococca* as reported (Abney et al. 1993). Although Abney et al. (1993) noted that perithecia of "all three *Nectria* isolates...were brown at maturity," they did not note that a key morphological feature used to distinguish *P. cucumerina* from *N. haematococca* (Domsch et al. 1980) is that mature perithecia are brown in the former and red in the latter. Therefore, a teleomorph for *F. solani* f. sp. *phaseoli* remains to be discovered. Abney et al. (1993) described a third morphologically distinct strain isolated from diseased soybeans (IN-11-48 = NRRL 22820). Phylogenetic analysis indicates this isolate is related most closely to *N. haematococca* MPVI and mating experiments are being conducted to determine whether this isolate and *N. haematococca* MPVI (VanEtten 1978) are the same biological species (H. D. VanEtten, personal communication).

The preliminary phylogenetic analysis revealed that Snyder and Hansen's (1953) polytypic concept of *F. solani*, subsequently adopted in some taxonomic treatments of *Fusarium* (Nelson et al. 1983; Burgess et al. 1988), is artificial (O'Donnell et al. 1993). Results presented here demonstrate that *F. solani* f. sp. *phaseoli*, *N. haematococca* MPI, MPV, MPVI and *Neocosmospora vasinfecta* represent phylogenetically distinct species (Cracraft 1989; Davis and Nixon 1992) within a monophyletic lineage strongly supported by two measures of clade stability: bootstrap analysis (100%) and decay indices (d = 14). These findings are consistent with the results of two previous studies. Using biochemical, genetic, and physiological data, VanEtten and Kistler (1988) concluded that *N. haematococca* MPI and MPVI are distinct species in a biological sense. Similarly, Crowhurst et al. (1991) used RAPDs to demonstrate divergent genome organization in *N. haematococca* f. sp. *cucurbitae* MPI (race 1) and MPV (race 2).

Although *Neocosmospora vasinfecta* has never been connected to *Fusarium*, its *Fusarium*-like microconidial state (Domsch et al. 1980) and ability to produce naphthazarines (Kern 1978) are synapomorphies indicating a common ancestry with other members of the *F. solani* clade. Autapomorphies such as nonseptate ascospores and absence of fusiform macroconidia have apparently obscured the correct phylogenetic position of *N. vasinfecta*. The present phylogenetic reconstruction demonstrates that *Nectria* is an artificial genus since *Neocosmospora* is derived within it. In a previous study (O'Donnell 1993), *Gibberella* was also shown to be derived within *Nectria*. Under the current nomenclatural system, a paraphyletic taxon such as *Nectria* will most likely be eliminated by recognizing less-inclusive monophyletic segregate genera (de Queiroz and Gauthier 1994). Unfortunately, this will result in a nomenclatural change (e.g., *Neocosmospora* may replace *Nectria* as the teleomorph of the *F. solani* clade).

Results from the present study demonstrate that the current polytypic morphological (Snyder and Hansen 1953) and biological species concepts (Matuo and Snyder 1973) applied to *F. solani/N. haematococca* do not accurately describe the phyletic diversity that exists within this large species complex. Snyder and Hansen's concept was based on the practical need to identify isolates using morphology exclusively, but does not take into account that morphological and molecular evolution are decoupled among some species within this clade (Larson 1989), and that morphological evolution of some species has been static. One important finding of this study is the concordant groupings of biological and phylogenetic species within the *N. haematococca*-complex. Since this congruence has also been observed within the *Gibberella fujikuroi*-complex of *Fusarium* (O'Donnell and Ciglenik, unpublished), it strongly suggests that both species concepts represent fundamental taxonomic units (Vilgalys and Sun 1994). However, the biological species concept is inadequate for describing cladistic diversity and for routine identification purposes for several reasons: Approximately one-half of the species within the *F. solani* clade are know only as asexual anamorphs, there are at least 20 undescribed heterothallic and 6 homothallic species (O'Donnell, Cigelnik, Samuels, Nirenberg, unpublished), it takes up to a month or more to run a mating experiment, and many field isolates fail to mate with tester strains (Matuo and Snyder 1973). A phylogenetic species concept (Cracraft 1989; Davis and Nixon 1992) obviates the problems mentioned above and provides the theoretical framework for recording the phyletic diversity within the *F. solani* clade and other fusaria.

PCR-based detection.

In the present study, ribosomal DNA spacer and genic sequences have been exploited as in numerous studies directed at PCR-based identification of fungal isolates (reviewed by Henson and French 1993; Moukhamedov et al. 1994; Gardes and Bruns 1993). The FspF/FspR primer pair reported here provides a rapid and sensitive molecular tool for specifically amplifying a 957 to 958-bp ribosomal DNA fragment from the etiological agent of soybean sudden death syndrome (SDS), *F. solani* f. sp. *phaseoli*. Because the optimal annealing temperature was several degrees below the Tm, the PCR primer pair worked equally well on four strains from French bean which have two mismatches in the 3′ end of the FspF primer (Fell 1993). The SDS pathogen-specific primer set reported here may be useful in describing qualitative and quantitative (Simon et al. 1992) aspects of this soilborne pathogen in fields prior to planting. Since oligonucleotide probes are sensitive enough to detect a 1-bp substitution (Gardes et al. 1991), it should be possible to use nuclear rDNA polymorphisms identified in this study together with those identified from other unlinked loci to study the population structure of *F. solani* f. sp. *phaseoli* in greater detail. The general utility of PCR-based detection and identification of other fusaria, however, may be limited due to the large number of species within the *F. solani*-complex and within *Fusarium*. Instead, DNA-based methods for the identification of *Fusarium* are likely to be based on a dot blot assay, using nonradioactively labeled species-specific oligonucleotides as hybridization probes (Lee et al. 1993; Bruns and Gardes 1993). PCR-based identification of the fusaria, including phytopathogenic and medically important isolates (Mardiak et al. 1993), will require a large DNA sequence database derived from loci variable enough to distinguish the species of interest. Fortunately, the rDNA region examined in this study offers many advantages of an ideal target locus for the identification of species (Bruns and Gardes 1993). Furthermore, the same database used to design species-specific probes can be used to develop an objective, phylogenetically based classification and to refine the phylogenetic species concept within *Fusarium*.

MATERIALS AND METHODS

Fungal strains, plants, and growth conditions.

The 24 single-spore isolates of the fungi studied (Table 1) are stored in liquid nitrogen and/or by lyophilization in the ARS Culture Collection (NCAUR, Peoria, IL). Mycelium for DNA extraction was grown in broth culture, harvested by filtration and lyophilized overnight as described in O'Donnell (1992). Soybean (*Glycine max* (L.) Merr.) cultivar Spencer, French bean (*Phaseolus vulgaris* L.) cultivar Top Crop, and garden pea (*Pisum sativum* L.) Plant Introduction 206.832 plants were grown in a steamed soil-sand mix (1:1, v/v) in 10-cm clay pots in a greenhouse for two weeks.

Plant inoculation.

F. solani f. sp. *phaseoli* SDS isolate Mont-1 (NRRL 22292) was grown in sterile sand-cornmeal medium (3 sand:1 corn meal, v/v) at 25°C for 10 days. The infested medium was used to inoculate 2-week-old Spencer soybean plants which had been grown in 15-cm clay pots (five plants/pot) of heat-treated soil-sand mix (1:1, v/v) in a greenhouse. Plants were inoculated by removing the soil from around the crown area of each plant and approximately 1 g of the infected sand-cornmeal inoculum was added to the area around each plant. The inoculation site was covered with soil-sand and the plants were grown in the greenhouse as previously described (Gray and Hepburn 1992).

Isolation of DNA from infected plants and isolation of pathogen.

At days 3 and 5 after inoculation, 4 inoculated and 4 control plants were removed. The crown area and roots of each plant were washed under running tap water to remove any adhering soil or mycelium. The plant roots were blotted on sterile paper towels, crown tissue, and lateral roots were cross-sectioned with a razor blade, and approximately 80 to 100 mg of plant tissue was extracted as reported above. Samples from each experimental and control plant were plated onto the surface of potato-dextrose agar (Difco, Detriot, MI) containing 60 µg of tetracycline per ml. Plates were incubated at 25°C for 5 days and observed for growth of *F. solani* f. sp. *phaseoli*.

DNA extraction and PCR.

Fungal DNA was extracted using a modification of the SDS protocol of Raeder and Broda (1985) as described previously (O'Donnell 1992). Plant material (0.5 g fresh weight) was frozen in dry ice, ground to a fine powder, and extracted for DNA using the microwave Miniprep of Goodwin and Lee (1993) which included GeneClean II (Bio 101, LaJolla, CA) as an additional purification step. Locations of the PCR and sequencing primers are indicated in Figure 1. The internal transcribed spacer (ITS) region and D1 and D2 domains at the 5′ end of the nuclear large 28S rDNA gene were amplified symmetrically with the primer pair ITS5/ITS4 (White et al. 1990) and NL1/NL4 (O'Donnell 1992), respectively, or with ITS5/NL4. The ITS5/NL4 PCR product is 1,178 to 1,179 bp in *Fusarium solani* f. sp. *phaseoli* as determined by DNA sequencing (Fig. 1).

DNA sequencing.

Following PCR amplification, primers and deoxynucleotide triphosphates were removed with GeneClean II. Double-stranded DNA templates were sequenced completely on both strands with the Applied Biosystems (Foster City, CA) non-radioactive Taq dyedeoxy terminator cycle sequencing kit in a Perkin-Elmer thermal cycler programmed using maximum ramp times as follows: 25 cycles at 96°C for 30 s and 50 or 60°C for 4.25 min, followed by a 4°C soak. Sequencing reactions were extracted once with about 50 µl of chloroform to remove the mineral oil overlay and once with an equal volume of phenol/chloroform/water (68:14:18) to remove most of the excess primers and unincorporated dyedeoxy terminators. Samples were further purified by gel filtration through 2-ml spin columns (5 Prime->3 Prime; Boulder, CO) containing super-fine G50 sephadex (Pharmacia; Piscataway, NJ) equilibrated in ddH_2O. All sequencing reactions were run on an Applied Biosystems 373A DNA sequencer using a 6% gel mix (Bio-Rad; Richmond, CA) in 1× TBE buffer. To sequence the plant DNA templates completely on both strands, 2 internal sequencing primers were designed for the ITS region [P-ITS3 5′-GCATCGATGAAGAACGTAGC and its reverse complement P-ITS2] and 2 for the nuclear large 28S rDNA [P-NL3 5′-ATGAAAAGGACTTTGAAAAGAGAG and its reverse complement P-NL2].

Sequence analysis, phylogenetics, and species-specific probe design.

DNA sequences were visually aligned with QEdit Version 2.15, a DOS text editor software program (SemWare; Marietta, GA), and analyzed using MacVector 3.5 (International Biotechnologies Inc.; New Haven, CT), PAUP 3.1.1 (Swofford 1993), and MacClade Version 3 (Maddison and Maddison 1992). Maximum parsimony trees were inferred using the branch-and-bound option of PAUP (Swofford 1993). Support for the phylogenetic groupings was measured by 1,000 bootstrap replications (Hillis and Bull 1993) and decay indices (Rehner and Samuels 1994) up to 14 steps. The *F. solani* f. sp. *phaseoli* SDS species-specific PCR primer pair was designed on the basis of the sequence alignment (Fig. 3) and used the following thermal cycle program in which maximum ramp times were employed: 35 to 40 cycles at 94°C for 35 s, 60°C for 55 s, and 72°C for 2 min, followed by a 10-min extension at 72°C, and a 4°C soak.

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