Molecular Differentiation of *Fusarium solani* f. sp. *glycines* from Other *F. solani* Based on Mitochondrial Small Subunit rDNA Sequences

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ABSTRACT

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Fusarium solani is a soilborne plant pathogen that infects many different hosts. Within the species, there is some specialization, and a number of forma specialis have been described based on host affiliation. One of these, *F. solani* f. sp. *glycines*, infects soybean and causes sudden death syndrome. To differentiate between *F. solani* f. sp. *glycines* and other *F. solani* isolates, a partial sequence of the mitochondrial small subunit (mtSSU) rRNA gene was amplified by polymerase chain reaction and sequenced from 14 *F. solani* f. sp. *glycines* and 24 *F. solani* isolates from various plant hosts. All *F. solani* f. sp. *glycines* isolates had identical sequences. A single, unique insertion of cytosine occurred in all *F. solani* isolates but not in any of the *F. solani* f. sp. *glycines* isolates. Two major lineages, distinguished by sequence divergence and the presence or absence of multiple insertions, occurred in *F. solani* isolates. Cladistic analysis produced a single most-parsimonious tree with three major clades. The first clade contained all *F. solani* f. sp. *glycines* isolates. A second clade grouped together all of the *F. solani* isolates that had only a single nucleotide insertion difference from the first clade. Genetic distance between these two clades was 0.016. A third clade was formed by five *F. solani* isolates that had multiple insertions. Isolates in the third clade had a genetic distance of 0.040 from the first and second clades. Based on the sequence data, it is likely that *F. solani* f. sp. *glycines* has a shorter evolutionary history than other *F. solani* isolates that have either single or multiple nucleotide insertions. The differences in nucleotide insertions in part of the mtSSU rRNA gene between *F. solani* f. sp. *glycines* and other *F. solani* isolates provide a direct and reliable way to distinguish isolates of *F. solani*.

Sudden death syndrome (SDS) is an economically important soybean disease (11,38). The symptoms of SDS include root rot and vascular discoloration of roots and stems. The most conspicuous symptoms of SDS occur on leaves, beginning with chlorotic mottling and proceeding to interveinal chlorosis, necrosis, and defoliation. The causal organism, *Fusarium solani* (Mart.) Sacc. f. sp. *glycines* (30), is a soilborne fungus first identified in 1989 (31,32). Previously, nomenclature designated *F. solani* form A for SDS-causing *F. solani* and form B for the *F. solani* seedling and root rot pathogen that did not cause SDS (30). In addition, *F. solani* f. sp. *glycines* SDS-causing isolates were reported to form chlamydospores (21) similar to some other *F. solani* isolates (6) and were reported to produce mostly macroconidia rather than microconidia (30,31).

In the past, identification of *F. solani* f. sp. *glycines* has relied on morphological and cultural characteristics. However, in culture, isolates often differ in phenotypic traits, such as colony morphology, pigment production, and sporulation. For example, blue pigmentation in cultures of some *F. solani* f. sp. *glycines* isolates varied from light to dark, and some of the isolates did not produce the blue pigment (30,33). However, some of the *F. solani* non–SDS-causing isolates appeared blue (25). The morphologically based classification system has not provided an accurate tool for the identification of *F. solani* f. sp. *glycines* and has not resolved the relationship of isolates within the *F. solani* complex.

A molecular approach based on discrete DNA sequence data offers considerable promise in the establishment of an objective, phylogenetically based system for the classification of *Fusarium* species (4,9,26). O'Donnell and Gray (27) analyzed the phylogenetic relationship of several species within the *F. solani* complex based on nuclear ribosomal DNA sequences. In their study, SDS-causing isolates were grouped with *F. solani* f. sp. *phaseoli*. Results from random amplified polymorphic DNA (RAPD) analysis (1) showed that *F. solani* SDS-causing isolates formed a cluster that represented a biological meaningful subgroup within *F. solani* f. sp. *phaseoli*, and it was suggested that the subgroup represents a separate form species. Understanding the molecular relationship between *F. solani* f. sp. *glycines* that causes SDS and other *F. solani* will provide insight in the specific identification and detection of *F. solani* f. sp. *glycines*.

In many organisms, mitochondrial DNA has a higher rate of evolution than nuclear DNA (2). It has been reported that the base substitutions per nucleotide are roughly 16-fold greater in the mitochondrial small subunit (mtSSU) rRNA gene than in the nuclear small subunit rRNA gene in 10 members of mushrooms in the order Boletales (3). Mitochondrial DNA may serve as a useful alternative to differentiate closely related species for phylogenetic analysis of fungi other than nuclear genes (3).

Restriction enzyme fragment length polymorphisms (RFLPs) of mitochondrial DNA have been used for biological variation studies in *Aspergillus* spp. (17), *Cochliobolus heterostrophus* (8), strains

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Names are necessary to report factually on available data; however, the United States Department of Agriculture (USDA) neither guarantees nor warrants 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.

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of *Neurospora* spp. (5), *F. oxysporum* (13,14,16), and *Glomerella* (*Colletotrichum*) spp. (37). Sequence analysis of the mtSSU rDNA has been used in fungal identification, specific primer development, and phylogenetic studies (19,28). However, extensive studies in the mtSSU rDNA of *F. solani* f. sp. *glycines* have not been reported. The objectives of our study were to (i) analyze the molecular differences and relatedness between *F. solani* f. sp. *glycines* and other *F. solani* isolates based on mtSSU rDNA sequences and (ii) evaluate the potential use of the mtSSU rDNA region for differentiation of *F. solani* f. sp. *glycines* from other *F. solani* isolates. Preliminary results were recently reported (22).

MATERIALS AND METHODS

Fungal isolates and culture morphology. The host affiliation, geographic origins, and sources of the fungal isolates used for polymerase chain reaction (PCR) amplification and sequencing of the partial mtSSU rDNA in this study are listed in Table 1. In addition to 14 *F. solani* f. sp. *glycines* isolates, 24 *F. solani* non–SDS-causing isolates from 9 hosts were used. All isolates were maintained either on 2% water agar or potato dextrose agar (PDA) at 4°C or air-dried and kept at room temperature.

For cultural and morphological studies, isolates were grown on PDA plates, sealed with Parafilm, incubated at 23 to 25°C in the dark, and examined after 5 to 20 days. After inoculation, colony color was determined based on a mycological color chart (29). Growth rates were determined by measuring the colony diameter after inoculation in triplicate PDA plates at 5 days. To examine the conidial types of the isolates, 2- to 3-week-old cultures were either directly examined with an Olympus BX60 microscope (Leco Co, Chicago) at 400, 600, or 1,000×, or stained with 0.15 μg/ml of 4′-6-diamidino-2-phenylindole (Sigma-Aldrich Chemical Co., St. Louis) as described previously (21).

Pathogenicity tests and cultural filtrate toxicity assay. Soybean seeds of susceptible cv. Great Lakes 3202 were sown in Ray Leach Cone-Tainers (Stuewe & Sons, Inc., Corvallis, OR) and inoculated with fungus-infested sorghum grains. Infested sorghum grain (3 cm^3) was placed 2 to 3 cm below a soybean seed in each Cone-Tainer. Noninfested sorghum grain was used as a control. Cone-Tainers were placed in a growth chamber for a 12-h photoperiod with a light intensity of 300 μm^{-2} s^{-1} at day and night temperatures of 28 and 22°C, respectively. Seven plants were inoculated with each fungal isolate. Treatments (isolates) were arranged in a randomized complete block design with two replications. Foliar symptoms were recorded 28 days after planting with a 1 to 5 disease severity scale (10).

Cell-free culture filtrates were prepared from *F. solani* f. sp. *glycines* and other *F. solani* non–SDS-causing isolates. A total of 10 plugs (4 mm in diameter) from colony margins on 2% water agar plates were placed into 100 ml of modified Septoria medium (34) and incubated at 25°C for 12 days. Cultures were filtered through Whatman No. 1 filter paper (Whatman Inc., Clifton, NJ) and sterile 0.22-μm membranes (Millipore Corp., Bedford, MA). Seedlings (3 weeks old) of cv. Great Lakes 3202 were cut and placed in test

TABLE 1. Fungal isolates used for polymerase chain reaction-amplification and sequencing of the partial mitochondrial small subunit ribosomal DNA in this study

Isolate	Host/substrate	Geographic origin	Source or contributor	GenBank Accession no.
Fusarium solani f. sp. *glycines*				
Mont-1	*Glycines max*	Illinois	P. Stevens	AF124995
98-7	*G. max*	Illinois	S. Li	AF124996
B6-8v	*G. max*	Illinois	L. Gray	AF124997
VR309-1	*G. max*	Illinois	L. Gray	AF124998
St 1-1	*G. max*	Illinois	L. Gray	AF124999
AR269	*G. max*	Arkansas	J. Rupe	AF125000
17-1	*G. max*	Arkansas	J. Rupe	AF125001
Pcmo1	*G. max*	Arkansas	J. Rupe	AF125002
NRRL[a] 22823	*G. max*	Indiana	L. Gray	AF125003
W1	*G. max*	Wisconsin	L. Achenbach	AF125004
W4	*G. max*	Wisconsin	L. Achenbach	AF125005
FSA-1	*G. max*	Mississippi	C. Nickell	AF125006
K-1	*G. max*	Kansas	L. Gray	AF125007
BH-F2-13	*G. max*	Iowa	X. Yang	AF125008
F. solani				
91-10-1-G	*G. max*	North Dakota	B. Nelson	AF125009
95-115-3-A	*G. max*	North Dakota	B. Nelson	AF125010
95-32-1	*G. max*	North Dakota	B. Nelson	AF125011
95-133-1	*G. max*	North Dakota	B. Nelson	AF125012
95-41-1	*G. max*	North Dakota	B. Nelson	AF125013
95-51-1	*G. max*	North Dakota	B. Nelson	AF125014
95-109-1	*G. max*	North Dakota	B. Nelson	AF125015
NRRL 22820[b]	*G. max*	Indiana	T. Abney	AF125016
T8	*Pisum sativum*	New York	H. Van Etten	AF125017
6-36	*P. sativum*	New York	H. Van Etten	AF125030
966	*P. sativum*	Washington	P. Nelson	AF125018
937	*P. sativum*	Washington	P. Nelson	AF125019
F46	*P. sativum*	Washington	J. Kraft	AF125020
D5	*Medicago sativum*	Wisconsin	C. Grau	AF125021
16-alfalfa	*M. sativum*	New York	P. Nelson	AF125022
NRRL 22382	*Phaseolus vulgaris*	Germany	H. Nirenberg	AF125023
W-8-1-3R-I	*P. vulgaris*	Washington	J. Kraft	AF125028
Fsph-2[a]	*P. vulgaris*	Washington	J. Kraft	AF125029
3-bean	*P. vulgaris*	Idaho	C. Strausbaugh	AF125024
71-tomato	*Lycopersicum esculentum*	California	A. Thornton	AF125025
72-Pumpkin	*Cucurbita pepo*	California	A. Thornton	AF125027
46-cucurbit	*Cucurbita* sp.	California	A. Thornton	AF125031
1-potato	*Solanum tuberosum*	Idaho	C. Strausbaugh	AF125026
5-lupine	*Lupinus* spp.	Australia	E. Dann	AF125032

[a] Northern Regional Research Lab, Peoria, Illinois.
[b] *Nectria haematococca* MPVI.

tubes containing 25 ml of fungal culture filtrates at 1:50 dilution (vol/vol) with sterile distilled water. Disease severity of foliar symptoms was recorded daily from 3 to 10 days after stem immersion to examine whether any of culture filtrates caused soybean SDS-like foliar symptoms (23). The experiment was repeated at least once.

DNA extraction and PCR amplification. Fungal isolates were grown on PDA at 23 to 25°C for 10 to 15 days. Total genomic DNA was extracted from cultures on agar plates (20,35). PCR amplification of the mtSSU rDNA region was performed using the NMS1 and NMS2 primers, which were designed to amplify a portion of the mtSSU rRNA gene in ascomycetes (19). PCR was performed in a 50-µl mixture that contained 0.2 mM each of dATP, dCTP, dGTP, and dTTP; 50 mM KCl; 10 mM Tris-HCL at pH 8.3; 2.0 mM $MgCl_2$; 50 pmol of primers; 50 ng of genomic DNA, and 1 U of *Taq* DNA polymerase (Perkin-Elmer Corp. Norwalk, CT). A negative control that excluded the DNA template was included in every experiment to test for reagent contamination. Amplification was performed with a GeneAmp PCR System 2400 DNA thermal cycler (Perkin-Elmer Cetus, Emeryville, CA) programmed as follows: 1 cycle at 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, 50°C for 60 s, and 72°C for 90 s. A 10-min extension at 72°C was conducted after 30 cycles. PCR products were examined by electrophoresis of 5-µl aliquots on a 1.5 or 2% agarose gel, stained with ethidium bromide, and visualized with a UV transilluminator (Fotodyne Inc., Hartland, WI).

DNA sequencing. In all, 38 isolates, including 14 *F. solani* f. sp. *glycines* and 24 other *F. solani* isolates, were sequenced for the NMS1- and NMS2-amplified mtSSU rDNA region. Prior to sequencing, PCR products were purified by filtration though Ultrafree-MC low-protein binding regenerated cellulose membrane filter units (NMWL) (Millipore) according to the manufacture's instructions.

Approximately 30 to 50 ng of the purified PCR products was subjected to sequencing using the Dye Terminator Cycle Sequencing kit with an Applied Biosystem 373A sequencer (Perkin-Elmer Corp.). The conditions for the cycle-sequencing reactions were as follows: 1 min at 95°C for initial denaturation, followed by 25 cycles of 15 s at 95°C, 5 s at 45°C, and 4 min at 60°C. Primers NMS1 and NMS2 were used as sequencing primers for the first and second strand, respectively.

Sequence data analyses. DNA sequences were edited using Sequencher (Gene Codes Corp., Ann Arbor, MI) and aligned using the multiple sequences alignment program CLUSTAL W (36). All sequence data analyses were performed by the program PAUP*4.0b1 (Sinauer Associates Inc., Sunderland, MA). A matrix of genetic distances was generated using Kimura's two-parameter model (15). The mtSSU rDNA sequence of *Nectria cinnabarina* (provided by K. O'Donnell) was used as an outgroup in the data analysis. For neighbor-joining, the unweighted pair group method with arithmetic averages (UPGMA) and maximum likelihood analyses tree searches were done using all available characters, and gaps were either treated as missing data (748 characters for analysis) or excluded (617 characters for analysis). In all of the trees, the transition/transversion ratio was equal to 2. To assess the confidence level of each internal tree branch, 1,000 bootstrap replicates (7) were performed in the neighbor-joining and UPGMA analyses. To execute an exhaustive search for the best tree using maximum likelihood criterion, all 14 *F. solani* f. sp. *glycines* isolates with identical sequences were represented by a single taxon, and one sequence was used to represent all identical 18 *F. solani* non–SDS-causing isolates with a single nucleotide insertion in the analysis. The total number of taxa for the exhaustive search under this combination was nine.

Maximum parsimony analysis was conducted first by the exhaustive search option after combining isolates with identical sequences, followed by the heuristic search option for individual isolate sequences. Alignment gaps were treated in two ways, either as a fifth character state or as missing data. Uninformative characters (12) were excluded from the analysis; all characters were unordered and unweighted. The heuristic search option was performed using the tree bisection-reconnection branch swapping algorithm with 1,000 random addition sequences. Confidence levels of internal branches were assessed by 1,000 bootstrap replications.

RESULTS

Cultural and morphological characteristics. *F. solani* f. sp. *glycines* isolates grew slowly on PDA and generally formed appressed or semiappressed colonies that appeared reddish light to dark blue. Repeated subculturing or prolonged storage of cultures on PDA resulted in reduced sporulation and an increase in aerial mycelium. The coloration of individual *F. solani* non–SDS-causing isolates ranged from white to gray, yellow, pink-orange, blue, or purple. Most of the isolates produced aerial mycelium.

F. solani f. sp. *glycines* isolates produced macroconidia that were mostly sickle shaped with four cells, but three-, five-, and six-celled macroconidia were occasionally found. In contrast, *F. solani* non–SDS-causing isolates predominately produced microconidia. However, there were some exceptions. For example, *F. solani* f. sp. *phaseoli* isolate NRRL 22382 and a soybean non–SDS-causing isolate, 95-133-1, produced both micro- and macroconidia.

Pathogenicity and toxicity. All *F. solani* f. sp. *glycines* isolates caused SDS foliar symptoms beginning 10 to 12 days after inoculation. Disease severity on foliage ranged from 65 to 90% of the leaf area affected (Table 2). Other *F. solani* isolates tested did not cause SDS-like foliar symptoms, although some caused root rot or atypical foliar symptoms, such as yellow spots or chlorosis of leaf margins. *F. solani* f. sp. *glycines* isolates caused significantly ($P < 0.01$) higher disease severity on foliage, greater root lesion length, and a higher percentage of root lesions than other *F. solani* isolates.

Cuttings of soybean seedlings with their stems immersed in culture filtrates prepared from *F. solani* f. sp. *glycines* isolates developed SDS-like foliar symptoms but not when immersed in filtrates of other *F. solani* isolates. Disease severity on foliage ranged from 55 to 86% of the leaf area affected when using filtrate from *F. solani* f. sp. *glycines* compared with 0 to 8% for filtrate from *F. solani* (Table 2).

Genetic divergence and phylogenetic analyses. All isolates studied gave strong amplification of a single PCR product in the mtSSU rDNA region using primers NMS1 and NMS2. Variations in the length of the amplified DNA fragment among isolates were observed through both agarose gel electrophoresis and DNA sequencing of the PCR products. All 14 isolates of *F. solani* f. sp. *glycines* had identical length (626 bp) of the amplified fragment. All *F. solani* non–SDS-causing isolates had a longer amplified product than *F. solani* f. sp. *glycines* isolates: 19 were 627 bp, with a single insertion of cytosine between the nucleotide positions 90 and 91 of the *F. solani* f. sp. *glycines* sequence. This single nucleotide insertion was present in all studied non–SDS-causing isolates but absent in the SDS-causing isolates. Five *F. solani* non–SDS-causing isolates had multiple insertions in two regions of the

TABLE 2. Range of foliar disease severity of soybean plants root-inoculated with *Fusarium solani* f. sp. *glycine* or *F. solani* and stems of cut soybean seedlings immersed in cell-free culture filtrates of *F. solani* f. sp. *glycine* or *F. solani*

		Foliar disease severity (%)	
Fusarium sp.[a]	No. tested	Root-inoculated[b]	Stem cutting[c]
F. solani f. sp. *glycine*	45	65–90	55–86
F. solani	50	0–10	0–8

[a] The *F. solani* f. sp. *glycine* isolates caused soybean sudden death syndrome (SDS); the *F. solani* isolates did not cause SDS.
[b] Inoculated with infested sorghum grain (10).
[c] Li et al. (23).

amplified fragment: between nucleotide positions 77 and 100 and between nucleotide positions 474 and 502 of the *F. solani* f. sp. *glycines* sequences (Fig. 1). The amplified fragment length of these five non–SDS-causing isolates ranged from 719 to 744 bp.

Pairwise genetic distances among all studied isolates based on the Kimura's two-parameter model are presented in Table 3. All 14 *F. solani* f. sp. *glycines* isolates had identical DNA sequences in the amplified mtSSU region. All *F. solani* non–SDS-causing isolates with a single insertion, except an isolate from pumpkin (72-pumpkin), also shared an identical DNA sequence of the amplified region among themselves and had a genetic distance of 0.016 from the sequence of *F. solani* f. sp. *glycines*. In contrast, the average genetic distance between *F. solani* non–SDS-causing isolates with a single insertion and those with multiple insertions was 0.040, which is the same as the average genetic distance value (0.040) between *F. solani* f. sp. *glycines* and *F. solani* isolates with multiple insertions.

When gaps were treated as a fifth character state, there was a total of 748 nucleotide characters; 133 were parsimony-informative and used in the maximum parsimony analysis. The most parsimonious tree resulted from the heuristic search with 1,000 random addition sequences, and the tree bisection-reconnection branch-swapping algorithm had a tree length of 214 and a consistency index of 0.8832 (Fig. 2). The same most parsimonious tree was the result of an exhaustive search after combining all isolates with identical sequences (data not shown). The next shortest tree had 215 steps, a length shared by two trees that had a consistency index of 0.8791. Three trees had a tree length of 216 and a consistency index of 0.8750. All five of these trees clearly demonstrated the three major clades. The first clade was composed of all *F. solani* f. sp. *glycines* isolates. The second clade contained all *F. solani* non–SDS-causing isolates possessing only a single extra nucleotide when compared with all *F. solani* f. sp. *glycines*. These two groups formed sister clades to each other. The third clade was formed by the five *F. solani* non–SDS-causing isolates with multiple insertions. The differences among the five shortest trees were on the arrangement of the isolates in the third clade, which had weak bootstrap values among branches within the clade (Fig. 2). There was no significant difference in tree topology when gaps were treated as missing data (tree not shown). However, bootstrap analysis indicated that

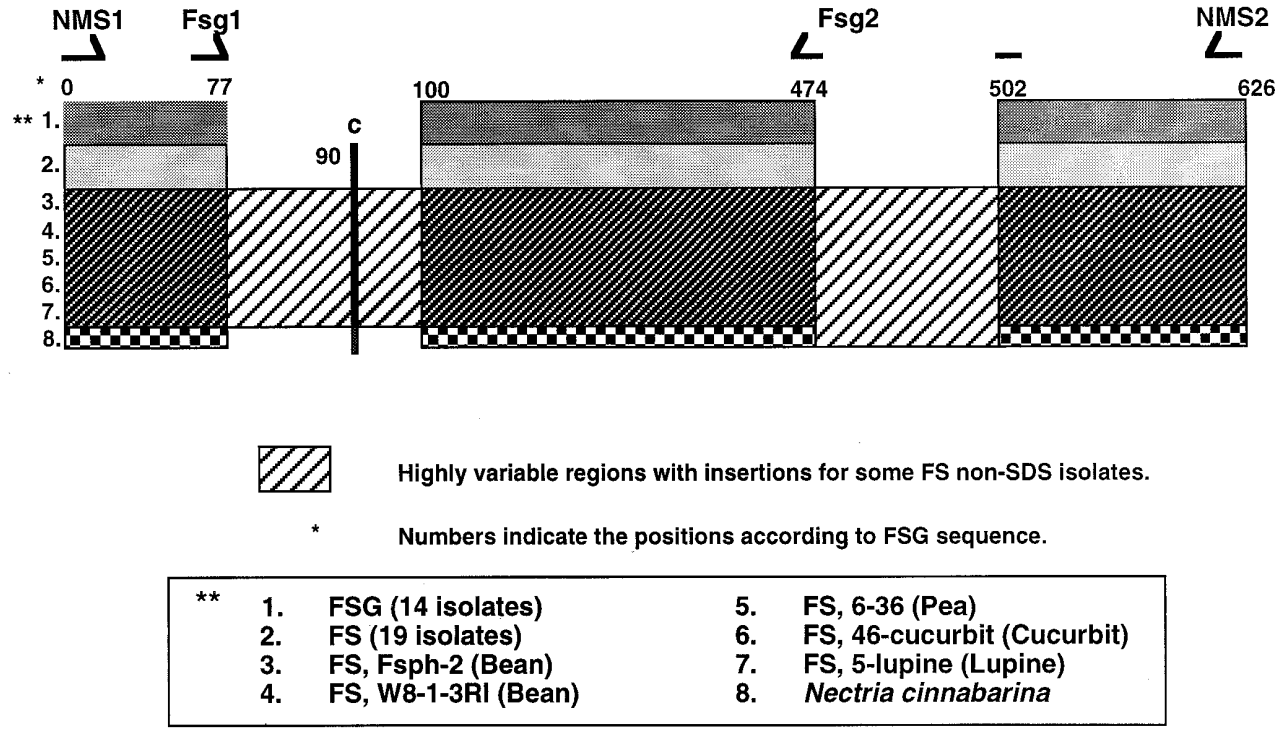

Fig. 1. Schematic representation of the NMS1/NMS2 polymerase chain reaction-amplified DNA fragment. All *Fusarium solani* f. sp. *glycines* (FSG) isolates had identical sequences in this region. A single insertion of cytosine was present in all *F. solani* (FS) non–sudden death syndrome (SDS)-causing isolates sequenced but absent in all FSG isolates. Two highly variable regions with multiple insertions were shown in some FS non–SDS-causing isolates.

TABLE 3. Genetic distances between *Fusarium solani* f. sp. *glycines* (FSG) and other *F. solani* (FS) isolates, estimated using Kimura's two-parameter model

Isolate/host	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. FSG (14 isolates)/soybean	…	0.016	0.016	0.016	0.016	0.016	0.016	0.015	0.029	0.045	0.048	0.043	0.036	0.048
2. FS (8 isolates)/soybean	…	…	0.000	0.000	0.000	0.000	0.000	0.005	0.031	0.050	0.051	0.045	0.038	0.050
3. FS (4 isolates)/pea	…	…	…	0.000	0.000	0.000	0.000	0.005	0.031	0.050	0.051	0.045	0.038	0.050
4. FS (2 isolates)/alfalfa	…	…	…	…	0.000	0.000	0.000	0.005	0.031	0.050	0.051	0.045	0.038	0.050
5. FS (2 isolates)/bean	…	…	…	…	…	0.000	0.000	0.005	0.031	0.050	0.051	0.045	0.038	0.050
6. FS (1-potato)/potato	…	…	…	…	…	…	0.000	0.005	0.031	0.050	0.051	0.045	0.038	0.050
7. FS 71-tomato/tomato	…	…	…	…	…	…	…	0.005	0.031	0.050	0.051	0.045	0.038	0.050
8. FS 72-pumpkin/pumpkin	…	…	…	…	…	…	…	…	0.026	0.045	0.046	0.039	0.033	0.045
9. FS Fsph-2/bean	…	…	…	…	…	…	…	…	…	0.047	0.041	0.028	0.020	0.094
10. FS W8-1-3RI/bean	…	…	…	…	…	…	…	…	…	…	0.038	0.051	0.042	0.103
11. FS 6-36/pea	…	…	…	…	…	…	…	…	…	…	…	0.049	0.039	0.096
12. FS 46-cucurbit/cucurbit	…	…	…	…	…	…	…	…	…	…	…	…	0.014	0.085
13. FS 5-lupine/lupine	…	…	…	…	…	…	…	…	…	…	…	…	…	0.075
14. *Nectria cinnabarina*	…	…	…	…	…	…	…	…	…	…	…	…	…	…

the branches were weaker in statistical support in this latter tree than in the one that resulted from treating gaps as the fifth character state.

Gaps were either treated as missing data or excluded from the analysis when the sequences were subjected to neighbor-joining, UPGMA and maximum-likelihood analyses. All these methods produced trees that shared topology similar to the maximum parsimonious tree in having the same three distinct clades and an identical arrangement of a clade containing all *F. solani* f. sp. *glycines* and its sister clade containing all *F. solani* non–SDS-causing isolates with a single nucleotide insertion. The positions of the five *F. solani* non–SDS-causing isolates with multiple insertions within the third clade varied depending on the analysis method. Bootstrap analysis with 1,000 replicates indicated strong support for the three major clades that resulted from neighbor-joining analysis in which gapped characters were excluded (Fig. 3). The same tree topology formed when gaps were treated as missing data in the analysis. The difference between the neighbor-joining tree and the most parsimonious tree was in the position of the isolate Fsph-2. This isolate was a sister taxon to the Lupine-Cucurbit subclade in the neighbor-joining tree, but it was grouped with isolates W8-1-3RI and 6-36 in the most parsimonious tree. For UPGMA, Fsph-2 was grouped with isolate 5-lupine, and the branches within the clade were weakly supported (data not shown). The best maximum-likelihood tree (data not shown) has the same topology as the neighbor-joining tree when gapped characters were excluded from the analysis (617 characters remained). When gaps were treated as missing data, the resolution of the third clade was improved, but an isolate from cucurbit (46-cucurbit) was grouped with isolates from bean (Fsph-2) and pea (6-36), while an isolate from lupine (5-lupine) was a sister taxon to this group. In the most parsimonious tree, isolates 46-cucurbit and 5-lupine formed a subgroup (Fig. 2) with a significant bootstrap value (100%).

DISCUSSION

In this study, biological characterizations, including culture morphology, pathogenicity, and culture filtrate toxicity tests, were used to compare isolates of *F. solani* f. sp. *glycines* and other *F. solani* that did not cause SDS in soybean. In general, most of the *F. solani* f. sp. *glycines* isolates produced blue pigment on PDA, but some of the *F. solani* isolates appeared blue and were similar to *F. solani* f. sp. *glycines* isolates, although they did not cause SDS symptoms in both pathogenicity and culture filtrate toxicity tests. *F. solani* f. sp. *glycines* isolates caused greater foliar disease symptoms than other *F. solani* isolates when plants were inoculated or cuttings were placed in cell-free culture filtrates. The toxin or toxins responsible for SDS symptoms found in cell-free culture filtrates of *F. solani* f. sp. *glycines* cultures is unique to these isolates but not to other non–SDS-causing *F. solani* isolates.

From the molecular approach, our genetic and phylogenetic analyses strongly support the existence of three distinct clades among the *F. solani* isolates used in our study. All *F. solani* f. sp. *glycines* isolates (n = 14) from soybeans had identical sequences of the amplified DNA fragment from the mtSSU rDNA region. This may mean either that *F. solani* f. sp. *glycines* is more recently devel-

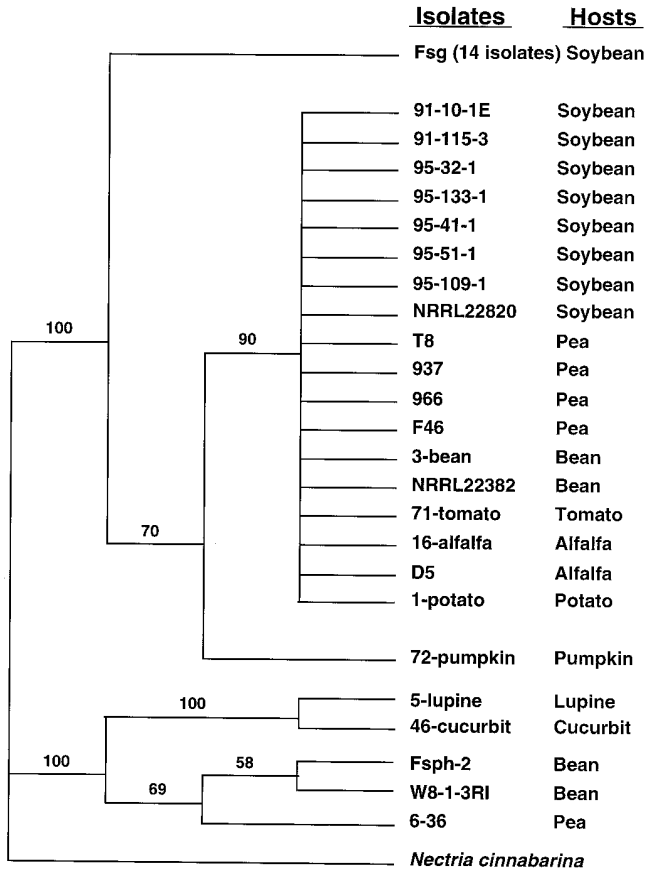

Fig. 2. Maximum parsimony analysis of all sequenced *Fusarium solani* f. sp. *glycines* (Fsg) and *F. solani* non–sudden death syndrome-causing isolates based on the partial sequences of the mitochondrial small subunit rRNA gene. *Nectria cinnabarina* was used as an outgroup to root the tree. A single most parsimonious tree was obtained from a heuristic random-sequence addition search, with gaps coded as a fifth character state. Tree length = 214; consistency index = 0.8832; and retention index = 0.9471. Bootstrap values >50% that resulted from 1,000 bootstrap replicates are shown above branches.

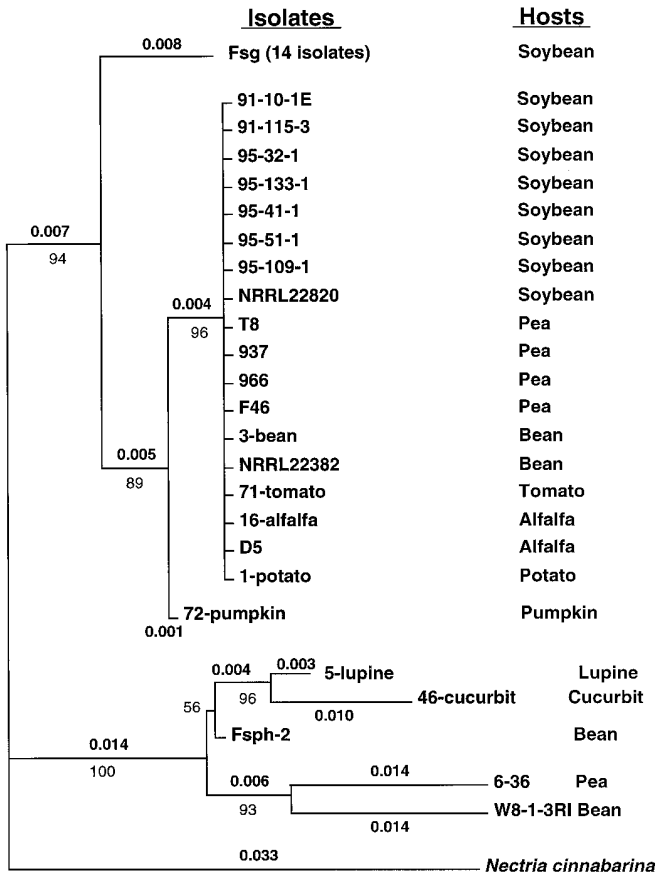

Fig. 3. Neighbor-joining analysis of all sequenced *Fusarium solani* f. sp. *glycines* (Fsg) and *F. solani* non–sudden death syndrome-causing isolates based on the partial sequences of the mitochondrial small subunit rRNA gene. Bootstrap values >50% that resulted from 1,000 replicates are shown under branches, while values of branch length are shown over branches, except for isolates 46-cucurbit and W8-1-3RI. Total number of characters applied was 617.

oped or that the amplified region is highly conserved in the mitochondrial genome of *F. solani*. It was intriguing to find that there were two groups of *F. solani* non–SDS-causing isolates, in view of the sequence divergence and the presence and absence of multiple insertions in the amplified fragment. The first group had only a single nucleotide insertion when compared to the sequence of *F. solani* f. sp. *glycines*. This insertion also was found in the second group of *F. solani* non–SDS-causing isolates, which, in contrast, also had multiple insertions in two regions of the amplified fragments (Fig. 1). The genetic distance value between *F. solani* non–SDS-causing isolates with single insertion and *F. solani* f. sp. *glycines* was much smaller (0.016) than that between *F. solani* non–SDS-causing isolates with single and multiple insertions (0.040). In fact, this latter distance was identical to that observed between *F. solani* non–SDS-causing isolates with multiple insertions and *F. solani* f. sp. *glycines*. In view of the sequence similarity and the absence of multiple insertions, the most parsimonious explanation is that both *F. solani* f. sp. *glycines* and *F. solani* non–SDS-causing isolates with a single insertion evolved from a recent common ancestor closely related to *F. solani* non–SDS-causing isolates with multiple insertions. According to this scheme, sequences of the multiple insertions might, in fact, have existed in the ancestral species of *F. solani* and were subsequently lost through the course of evolution that led to the common ancestor of *F. solani* f. sp. *glycines* and *F. solani* non–SDS-causing isolates with single nucleotide insertion. Such a relationship among the three groups (*F. solani* f. sp. *glycines*, *F. solani* non–SDS-causing isolates with single insertion, and *F. solani* non–SDS-causing isolates with multiple insertions) is consistent in all analyses in the present study (Figs. 2 and 3). Furthermore, the sequences of the insertions between nucleotide positions 77 and 100 of *F. solani* f. sp. *glycines* were highly similar among the five *F. solani* non–SDS-causing isolates. In contrast, the sequences of the insertions between nucleotide positions 474 and 502 of *F. solani* f. sp. *glycines* varied both in length and character states among these five isolates.

The finding that 18 of the 19 *F. solani* non–SDS-causing isolates with a single insertion studied, from various hosts, had identical sequences for the amplified fragment indicated that these isolates may have evolved before their host specialization. The one that showed a difference was an isolate from pumpkin (72-pumpkin). It would be interesting to study more isolates from pumpkin to see if they are host specific and also closely related to the common *F. solani* non–SDS-causing isolates. Although the five isolates with multiple insertions formed a strongly supported clade (bootstrap value = 100%; Figs. 2 and 3), their relative position within the clade differed depending on the method of analysis used. Most informative characters for resolving the relationship among these five isolates were contributed by the sequences of the insertions. Gaps could not be treated as a new character state when we set the optimality criterion to maximum likelihood or distance in PAUP; therefore, the number of informative characters in the regions with insertions were reduced. From the maximum parsimony analysis in which gaps were treated as a new character state, isolates from lupine (5-lupine) and cucurbit (46-cucurbit) were closely related (bootstrap value of these group = 100%) because they shared more synapomorphic characters. This relationship also was resolved in the maximum-likelihood and neighbor-joining analyses. However, the relationship with other isolates in this clade was poorly resolved in all methods of analysis. More informative characters are needed to improve the resolution within this clade. The results were not significantly improved when the gaps were either treated as missing data or excluded from the analysis.

The variations in both length and sequence of the amplified fragment of the five isolates with multiple insertions suggests that these isolates have a longer evolutionary history than the *F. solani* f. sp. *glycines* and *F. solani* non–SDS-causing isolates with a single nucleotide insertion.

Isolates of *F. solani* from beans and soybeans have been reported to be similar based on nuclear rDNA sequence (27). However, extensive studies comparing biological and pathological characteristics on these two groups of *F. solani* have shown how they differ and why *F. solani* f. sp. *glycines* should not be grouped with *F. solani* f. sp. *phaseoli* (30). In our study, four *F. solani* f. sp. *phaseoli* isolates were sequenced. Two of them (3-bean and NRRL 22382) had identical sequences and were grouped with the *F. solani* non–SDS-causing isolates that had a single nucleotide insertion. The other two isolates (Fsph-2 and W8-1-3RI) had multiple insertions. However, one isolate, received as *F. solani* f. sp. *phaseoli*, had an identical sequence with *F. solani* f. sp. *glycines* in the amplified mtSSU region (data not shown). This isolate also caused SDS foliar symptoms in pathogenicity tests. Further experiments are being conducted to determine if this isolate is indeed *F. solani* f. sp. *glycines.* More isolates need to be analyzed to determine the phylogenetic relationship between *F. solani* f. sp. *glycines* and *F. solani* f. sp. *phaseoli*.

Characterization of the population structure of fungal pathogens is important for understanding an organism's biology and for developing disease-control strategies (24). Phylogenetic relationships among individuals are one of the components of population structure (18). As other genes besides the mtSSU rRNA gene are sequenced within the *F. solani* complex, a better phylogenetic and population analysis will be possible and may provide more insight as to the phylogenetic relationship of isolates in this complex. The information obtained in this current study will aid in the molecular identification and detection of *F. solani* f. sp. *glycines*. Based on our sequence data, a pair of primers (Fsg1 and Fsg2; Fig. 1) was developed (22), and their application for the specific detection of *F. solani* f. sp. *glycines* from field samples is in progress.

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