Genetic Diversity in *Sclerotium* (*Athelia*) *rolfsii* and Related Species

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ABSTRACT

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Pairings among 119 isolates of *Sclerotium rolfsii*, 11 of *S. delphinii*, and two of *S. coffeicola* on potato-dextrose agar to establish mycelial compatibility groups (MCGs) revealed 49, 3, and 2 MCGs, respectively, in a worldwide collection. Within an MCG, isolates were often from the same host and geographical area; however, widely diverse isolates also were grouped within the same MCG. The host of origin of the isolate was not correlated with the MCG except in *S. delphinii*. Many MCGs were comprised of only one isolate. Variation in nuclear rDNA [internal transcribed spacer (ITS) regions] was examined following restriction enzyme digests. Restriction fragment length polymorphisms (RFLPs) were obtained with *Alu*I, *Hpa*II, *Rsa*I, and *Mbo*I and could distinguish amongst the three *Sclerotium* spp. and three *Athelia* spp. (an outgroup). Combined banding patterns for the four enzymes were used to characterize intraspecific variation in the three *Sclerotium* spp. There were 12 subspecific groupings in *S. rolfsii*, one in *S. delphinii*, and two in *S. coffeicola*; some of these groupings correlated with their MCG. However, isolates within an MCG could show different ITS-RFLP patterns and certain patterns were also dispersed among different MCGs. The total sum of digested fragment sizes exceeded the undigested polymerase chain reaction product in several *S. rolfsii* isolates. This length discrepancy was not due to artifacts or incomplete digestion and, therefore, must have resulted from variation among rDNA copies in the presence of restriction sites. Furthermore, segregation of two *Mbo*I restriction patterns in the ITS region among 29 single-basidiospore strains, derived from four parental field isolates of *S. rolfsii* with one pattern, suggested the presence of two distinct rDNA types in the field isolates. The rDNA types may reflect a heterokaryotic nuclear condition in field isolates of *S. rolfsii*. Restriction maps and phylogenetic analyses supported a close affinity of the three *Sclerotium* spp., which may be more appropriately designated as subspecific varieties of *S. rolfsii*.

Additional keywords: barrage, heterokaryon, vegetative compatibility.

Sclerotium rolfsii Sacc. (teleomorph: *Athelia rolfsii* (Curzi) Tu & Kimbrough) is a soilborne plant pathogenic fungus which is prevalent in warm temperate and subtropical regions of the world (2,25). The pathogen has a host range of over 500 plant species, mostly comprised of dicotyledonous plants and a few monocotyledonous species (2). Symptoms include crown and root rot, stem canker, and damping-off, and the resulting disease is southern wilt or blight, or southern stem rot (2,26).

Cultures of *S. rolfsii* originating from different geographical areas and hosts frequently display variation in morphological characteristics (27,28), but the species is readily identifiable by its sclerotial size, color, and structure (31). Two related species, *S. delphinii* and *S. coffeicola*, which are reported to occur on ornamental bulbs (delphinium, iris, and lily) and coffee, respectively, produce larger sclerotia that are lighter brown to orange in color (2,38). It is not clear whether these differences in host occurrence and morphology of sclerotia are sufficient to warrant a separate species designation for these isolates.

Within *S. rolfsii*, field isolates also may exhibit mycelial incompatibility by forming barrage zones when paired in culture (28), which has led to the establishment of mycelial compatibility groups (MCGs) within the species (26,28). These field isolates are presumed to be heterokaryons based on an analysis of single-basidiospore progeny derived from them (26,27,29,30). Other than these reported differences in morphology and MCG, there have been no studies to characterize the extent of diversity within *S. rolfsii* and its related species.

The objectives of this research were to (i) determine the extent of MCGs in a worldwide collection of isolates; (ii) characterize the degree of inter- and intraspecific variation in nuclear rDNA [internal transcribed spacer (ITS) regions]; and (iii) develop restriction maps and conduct phylogenetic analyses to determine the relationships among the three *Sclerotium* spp.

MATERIALS AND METHODS

Fungal isolates and culture maintenance. The isolate designation, host, area, year of isolation, and source of 133 *Sclerotium* and four *Athelia* isolates included in this study are listed in Table 1. In addition, 29 single-basidiospore strains obtained earlier (27,28) from four field isolates of *S. rolfsii* (2672, DP18-8, 1126, and 1059) were included. All cultures were maintained on potato-dextrose agar (PDA) (Difco Laboratories, Detroit) in 100 × 15-mm petri dishes or in test tube slants and stored at room temperature (23 to 26°C). For long-term storage, isolates were either placed under sterile mineral oil in test tube slants or on PDA at room temperature.

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Mycelial compatibility groups. Twenty-five tester isolates, each representative of an MCG identified in an earlier study (28), were used to identify additional MCGs among 47 isolates in this study. The unknown isolates were paired against each tester isolate on PDA. Mycelial plugs (6-mm diameter) taken from the edge of 5- to 7-day-old colonies were placed approximately 25 to 35 mm apart in 100 × 15-mm petri dishes and incubated at 24 to 28°C. Three isolates were usually paired on one dish and the test was repeated two or three times. The pairings were examined macroscopically after 5 to 14 days for the presence of an antagonism (barrage or aversion) zone in the region of mycelial contact (27,28). Slow-growing isolates were initiated 1 to 2 weeks before pairing with fast-growing isolates.

DNA isolation. Isolates representing the three *Sclerotium* spp. (109 isolates) and three *Athelia* spp. (four isolates) were selected (Table 1). Initially, mycelia from 5- to 7-day-old potato-dextrose broth cultures were used either fresh or frozen (liquid nitrogen) for genomic DNA extraction using several standard extraction protocols (20,23,35). Because of varying degrees of polysaccharide contamination, a Stratagene DNA extraction kit (200600; Strategene Inc., La Jolla, CA) was selected for rapid DNA extraction with minimal polysaccharide contamination, according to the following modified protocol. Mycelium was grown on 4 × 6-cm pieces of autoclaved cellophane (type 195-Putsd-2) over PDA in 100 × 15-mm petri dishes. A 6-mm-diameter plug taken from the margin of a 3- to 8-day-old culture of each isolate was placed on

TABLE 1. Host, area of isolation, source, and mycelial compatibility group (MCG) designation of isolates of *Sclerotium rolfsii* and related species included in this study[a]

Isolate	Host of origin	Area and year of isolation	Source	MCG
Sclerotium rolfsii				
112	Wheat	Arkansas, 1978	D. Slack	13
114, 116	Sweet potato	Louisiana	C. Clark	5
115	Tomato	Louisiana	C. Clark	5
120	Blue lupine	Georgia, 1977	D. Bell	14
121	Hyacinth-bean	Georgia, 1977	D. Bell	3
122	Peanut	Georgia, 1978	D. Bell	8
123	Asparagus	Georgia, 1978	D. Bell	15
124	Sunflower	Georgia, 1978	D. Bell	8
125, 126	Peanut	North Carolina	L. Lucas	7
128	Tobacco	North Carolina	L. Lucas	1
131	Creeping bugleweed	Kansas, 1980	F. Crowe	16
133	Tomato	Maryland	R. Lumsden	17
134	Peanut	Alabama, 1979	P. Backman	8
135, 136	Iris	Washington, 1979	G. Chastagner	6
*138[b]	Annual bluegrass-bentgrass	Chile, 1981	L. Leach	21
140	Peanut	Texas, 1980	S. Lyda	2
141	Sorghum	Texas, 1983	G. Odvody	3
*143	Annual bluegrass-bentgrass	North Carolina	L. Lucas	...[c]
*144, *145, *146	Bentgrass	Australia, 1980	T. Siviour	18, 19, 20
*150	Bean	North Carolina, 1981	S. Gurkin	32
153	...	North Carolina	...	33
*155	Cantaloupe	North Carolina, 1981	S. Gurkin	5
*156	Tobacco	North Carolina, 1982	S. Gurkin	34
*157 (ATCC 62665)	Soybean	North Carolina, 1982	S. Gurkin	...
*158	Creeping bugleweed	North Carolina, 1982	S. Gurkin	35
162	Soybean	Arkansas, 1982	M. Hirrel	36
*163	Soybean	Arkansas, 1982	M. Hirrel	37
*166	Carrot	North Carolina, 1983	Z. Punja	28
171	Soybean	Arkansas, 1983	M. Hirrell	38
*176	Goosegrass	North Carolina, 1982	S. Gurkin	33
180	Crowfoot grass	North Carolina, 1983	S. Gurkin	39
182	Purple nutsedge	North Carolina, 1983	S. Gurkin	40
*185	Tomato	Brazil	P. B. Adams	41
*190	Bean	North Carolina, 1984	S. F. Jenkins	5
*191	Pepper	North Carolina, 1984	S. F. Jenkins	42
*192 (ATCC 62669)	Pepper	North Carolina, 1984	S. F. Jenkins	5
194	Apple	North Carolina, 1984	S. F. Jenkins	43
195	Bean	North Carolina, 1984	J. Cordoso	44
*197	Cucumber	Mississippi, 1984	B. Graves	29
*198	Pea	North Carolina, 1984	S. F. Jenkins	...
202	Star of Bethlehem	California, 1985	D. Woods	45
*1001, *1123, 1128, 1129, 2394	Bean	California, 1977-78	K. Kimble	2
1002, 1112, 1113	Sugar beet	California, 1977	Z. Punja	4
*1003	Sunflower	California, 1978	Z. Punja	1
1008	Apple	California, 1979	L. Leach	8
1058	Creeping bugleweed	California, 1975	P. Hiatt	6
*1059	Onion	California, 1971	E. Butler	1
*1094	Apple	California, 1974	P. Hiatt	10
1111	Sugar beet	California, 1977	Z. Punja	1
1114	Sugar beet	California, 1977	Z. Punja	11

(continued on next page)

[a] ATCC = American Type Culture Collection, Rockville, MD; IMI = Imperial Mycological Institute (currently, Commonwealth Mycological Institute, Kew, England); FP = Forest Products Laboratory (currently, Center for Forest Mycology Research, Madison, WI); CBS = Centraalbureau voor Schimmelcultures, Baarn, the Netherlands; DAOM = Plant Research Institute, Department of Agriculture, Mycology (currently, Canadian Collection of Fungus Cultures, Ottawa, Canada).

[b] * = Isolates included in molecular analysis.

[c] ... = Denotes missing data.

the cellophane and incubated at 24 to 28°C for 5 to 7 days. The plug was removed and the mycelium was peeled off the cellophane, cut into four to six pieces with a razor blade, and homogenized in 10 ml of buffer A [50 mM Tris HCl (pH 8.2); 20 mM EDTA; 2% sodium dodecyl sulfate] using a hand-held Dounce homogenizer (Bellco Biotechnology, Vineland, NJ). Proteinase K (500 µg/ml; Sigma Chemical Co., St. Louis) was added to the homogenate and incubated in a shaking water bath at 55°C for 2 hr or overnight at 37°C. One-third volume of saturated NaCl (6 M) was added to the suspension and centrifuged at 5,300 × *g* for 20 min at room temperature. The supernatant was incubated with RNase A (30 to 50 µg/ml) at 37°C for 30 min before ethanol precipitation at –20°C overnight. The precipitate was centrifuged at 5,300 × *g* for 15 min. The resulting pellet was washed with 70% ethanol, air-dried, and resuspended in 100 to 400 µl of Tris-EDTA buffer [10 mM Tris Cl (pH 8.0); 1 mM EDTA (pH 8.0)] and stored at 4°C. Extremely viscous pellets were further extracted with a phenol (Tris-EDTA saturated phenol)/Sevag (Sevag = 24:1 chloroform/isoamylalcohol) mixture (1:1, vol/vol) to reduce polysaccharide contamination. The supernatant was reprecipitated with 0.3 M NaOAc (pH 5.2) and 95% ethanol, stored at –20°C overnight, and centrifuged at 13,000 × *g*. DNA samples with residual polysaccharide were diluted (1:5) in Tris-EDTA buffer and then spun in a microcentrifuge (6,200 × *g*) at room temperature for 2 to 3 min prior to polymerase chain reaction (PCR) amplification.

PCR amplification and enzymatic digests. Universal primer pairs, ITS1-ITS4, ITS1-ITS2, and ITS3-ITS4 (41) were used to

TABLE 1. *(continued from preceding page)*

Isolate	Host of origin	Area and year of isolation	Source	MCG
*1120	Bean	California, 1977	K. Kimble	1
*1125, *1126 (ATCC 44902)	Annual bluegrass-bentgrass	California, 1978-79	Z. Punja	1
*1127, 1130, *1134	Annual bluegrass-bentgrass	California, 1978-79	Z. Punja	1
DP18-1, *DP18-2, *DP18-6	Annual bluegrass-bentgrass	California, 1978-79	Z. Punja	1
DP18-7, *DP18-8, *DP18-9	Annual bluegrass-bentgrass	California, 1978-79	Z. Punja	1
*DP18-11, *DP18-apron	Annual bluegrass-bentgrass	California, 1978-79	Z. Punja	1
*1135	Garlic	California, 1979	E. Butler	2
1136	Bean	California, 1981	D. Hall	4
*1137	Sugar beet	California, 1981	K. Kimble	1
*1138	Cantaloupe	California, 1981	K. Kimble	12
*1140	Tomato	Mexico, 1982	K. Kimble	1
1143	Tomato	California, 1982	P. Somerville	46
*1163	Tomato	California, 1967	E. Butler	2
*1376, 2315	Annual bluegrass-bentgrass	California, 1978-79	P. Hiatt	3
*2313, *2672 (ATCC 44904), 2823	Annual bluegrass-bentgrass	California, 1978-79	P. Hiatt	1
2279, 2479, *3078	Annual bluegrass	Maryland, 1979	N. O'Neill	8
*AS-1, *AS-6	Alstroemeria	Alberta, 1993	K. Chang	26
*Beet 1	Beet	California, 1978	Z. Punja	2
*3080	Bean	Indonesia, 1993	Z. Punja	47
*3081	Peanut	Indonesia, 1993	Z. Punja	48
*3082	Wheat	Nepal, 1993	J. Dubin	49
*3083	Peanut	Bangladesh	K. Benozir	31
*3084	Eggplant	Bangladesh	K. Benozir	31
*3085, *3086	Delphinium	Colorado, 1992	A. Schwend	29
Ga carrot	Carrot	Georgia, 1983	Z. Punja	1
Ga-pepper10	Pepper	Georgia, 1983	Z. Punja	1
*Ga onion13	Onion	Georgia, 1983	Z. Punja	3
*OK121	Peanut	Oklahoma, 1992	K. Conway	36
*OK128, OK164, *OK233, OK315	Peanut	Oklahoma, 1992	K. Conway	27
*ChetA (ATCC 26325)	Potato	Israel, 1972	I. Chet	22
*ChetR (ATCC 26326)	Sugar beet	Israel, 1972	I. Chet	23
*WM902, *WM904, *WM906, *WM909	Peanut	Georgia, 1991	T. Brenneman	28
*WM908, *WM917	Peanut	Georgia, 1991	T. Brenneman	30
*WM913	Peanut	Georgia, 1991	T. Brenneman	29
ATCC 15201	...	...	...	24
ATCC 15209	Ficus	...	...	25
Sclerotium delphinii				
ATCC 15200	Iris	New Brunswick	ATCC	...
*196	Creeping bugleweed	North Carolina, 1984	S. F. Jenkins	1d
*1057 (ATCC 62671)	Ajuga	California, 1975	P. Hiatt	9 (2d)
*W-10, *W-11	Lily	Washington, 1991	K. Riley	3d
*W-20, *W-21, *W-22	Iris	Washington, 1991	K. Riley	3d
*W-23, *W-30, *W-31	Iris	Washington, 1991	K. Riley	3d
*4010 (ATCC)	...	...	ATCC	3d
Sclerotium coffeicola				
*IMI 338651	...	..., 1990	IMI	1c
*ATCC 16056	Coffee	Costa Rica	ATCC	2c
Athelia bombacina				
*867	...	...	J. Andrews	...
Athelia epiphylla FP				
*FPI 33892-T	...	..., 1975	M. J. Larsen	...
Athelia epiphylla CBS				
*CBS 419.72	Birch	...	J. Stalpers	...
Athelia pellicularis				
*741 (DAOM)	Pine	Alberta, 1953	V. J. Norden	...

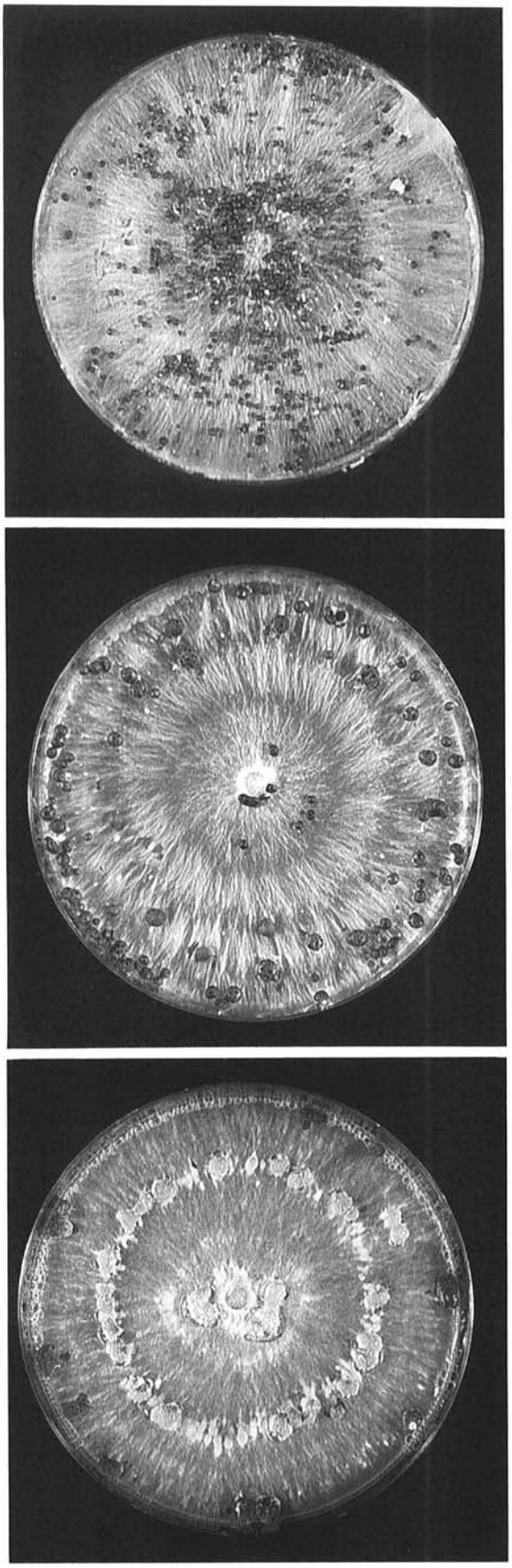

Fig. 1. Comparative morphology of *Sclerotium rolfsii* (top dish), *S. delphinii* (middle dish), and *S. coffeicola* (bottom dish). Isolates were grown on potato-dextrose agar for 17 days (*S. rolfsii* and *S. delphinii*) or 30 days (*S. coffeicola*).

amplify the ITS regions and the 5.8S gene of nuclear ribosomal DNA. PCR reaction volumes of 25, 50, or 100 µl contained 200 µM each of the four deoxynucleotides (Perkin-Elmer Corp., Norwalk, CT), 1.5 mM $MgCl_2$, 1 µM of each primer, 1× reaction buffer [10 mM Tris HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100 (Promega Corp., Madison, WI)], approximately 20 to 60 ng of DNA template, and 50 µl of light paraffin oil to overlay the total volume. Reactions were incubated at 94°C for 5 to 10 min prior to adding 0.25 to 0.5 units of *Taq* DNA polymerase (Promega Corp.). Negative controls were included with all PCR amplifications to test for the presence of contaminants in the reagents. Forty cycles of the following temperature/time profile were performed using a thermal cycler [Ericomp (Ericomp Inc., San Diego, CA)]: 1 min at 94°C for DNA denaturation, 2 min at 55°C for primer annealing, and 3 min at 72°C for primer extension with a final extension of 10 min at 72°C. PCR conditions were optimized to increase primer specificity using 30 cycles [Perkin-Elmer 480 thermal cycler (Perkin-Elmer Corp.)] of the following profile: 1 min at 94°C for DNA denaturation, 1.5 min at either 55 or 58°C for primer annealing, and 2 min at 72°C for primer extension with a final extension of 10 min at 72°C. In addition, three different sources of polymerase enzymes [*Taq* DNA polymerase (Promega Corp.; Gibco BRL, Burlington, Ontario, Canada) and Ultima (Perkin-Elmer Corp.)] were compared to assess fidelity of results. Amplification products (1 to 3 µl) were visualized on a 2% (wt/vol) agarose [ultrapure (Gibco BRL)], ethidium-bromide stained (0.5 µg/ml) gel with a UV light source. PCR products were either excised from 1.0% (wt/vol) low-melting-point agarose and purified using Magic PCR Preps kit (Promega Corp.) or used directly for restriction enzyme digests.

Approximately 200 to 500 ng of PCR product was digested according to manufacturer's recommendations (Gibco BRL and Promega Corp.) with one of the following enzymes: *Alu*I, *Ava*I, *Ava*II, *Cfo*I, *Dde*I, *Hae*III, *Hin*fI, *Hpa*I, *Mbo*I, *Rsa*I, *Sau*3AI, and *Taq*I. Enzyme-digested PCR products were size-fractionated on a 2% (wt/vol) 3:1 wide range/standard agarose gel (Sigma) or on a 2% (wt/vol) composite agarose gel of 0.75% (wt/vol) NuSeive GTC agarose (FMC BioProducts, Rockland, ME) and 1.25% (wt/vol) agarose [ultrapure (Gibco BRL)] at 3.5 V/cm, stained with ethidium bromide, and visualized over a UV source and photographed [Kodak ROYAL pan film: RPF 4141 (Eastman Kodak Co., Rochester, NY)]. A 1-kilobase (kb) and/or 100-base pair (bp) dsDNA ladder (Gibco BRL) was used as a size marker.

Restriction fragment length polymorphisms (RFLPs) resulting from *Alu*I, *Hpa*II, *Rsa*I, and *Mbo*I digests of the three amplified regions (ITSI, ITSII, and ITS combined) (41) were used to construct restriction site maps for six isolates of *S. rolfsii*, two of *S. delphinii*, and two of *S. coffeicola*, which were chosen to represent different ITS-RFLP patterns.

Higher resolution of small and comigrating bands (< 100 bp) was achieved following electrophoresis on nondenaturing polyacrylamide 8% (wt/vol) or 12% (wt/vol) minigels (36). Polyacrylamide gels were electrophoresced at 8 V/cm in 1× Tris-borate-EDTA and stained in 1× Tris-borate-EDTA buffer with ethidium bromide (5 µg/ml) for approximately 40 min at room temperature. Bands were visualized and photographed as previously described.

Data analysis. Restriction fragment lengths were calculated using an National Institutes of Health computer program (32) from scanned gel photo images [Microtek ScanMaker II (Microtek, Inc., Chicopee, MA)] of 2% agarose gels. Regression analysis of 100-bp ladder standards and/or 1-kb ladder standards (Gibco BRL) was determined and used to calculate final lengths (bp) for all samples. Sequence data (G. C. Adams, Jr. and B. Kropp, *unpublished data*) for one isolate each of *S. rolfsii* and *S. delphinii*, and for three *Athelia* spp. were analyzed for restriction cut sites (*Alu*I, *Hpa*II, *Rsa*I, and *Mbo*I) using GeneWorks (12).

The restriction site maps were analyzed for the presence (1) or absence (0) of *Alu*I, *Hpa*II, *Rsa*I, and *Mbo*I sites. The results were

combined to produce a binomial data set that was used to infer phylogenetic relationships among isolates. Two phylogenetic inference methods contained in two separate computer analysis packages were employed: parsimony analysis in PAUP (Phylogeny Analysis Using Parsimony package) (39) and the parsimony algorithm of PHYLIP (Phylogeny Inference Package) (9). Trees rerooted by the outgroup (*Athelia* spp.) were generated using PAUP parsimony analyses with the Heuristic search option (1,000 bootstrap replications comprised of 10 sets of 100 replications), which resulted in a final 50% majority-rule consensus tree. In PHYLIP, 1,000 bootstrap replications of the initial data set were invoked using the SEQBOOT option and subsequently applied to Wagner parsimony (MIX option). A 50% majority-rule consensus tree (rerooted by the outgroup) was generated using the CONSENSE option.

RESULTS

Mycelial compatibility groups. The three *Sclerotium* spp. could be distinguished by their morphological characteristics, such as the number and size of sclerotia formed on PDA (Fig. 1), and conformed to previous morphological criteria used to distinguish them (2,38). Most pairings of the *S. rolfsii* and *S. delphinii* unknowns with testers were readily scored for the presence (1) or absence (0) of an aversion zone within 7 to 10 days. Hyphae of compatible isolates intermingled or produced a knitted ridge. The contact zone of hyphae was difficult to discern (Fig. 2A) and sclerotia formed randomly over the colony surface of both isolates. Incompatible pairings were typified by thinning hyphae with a distinct barrage or aversion zone of varying widths (Fig. 2B).

Twenty-four MCGs were found in addition to the 25 MCGs

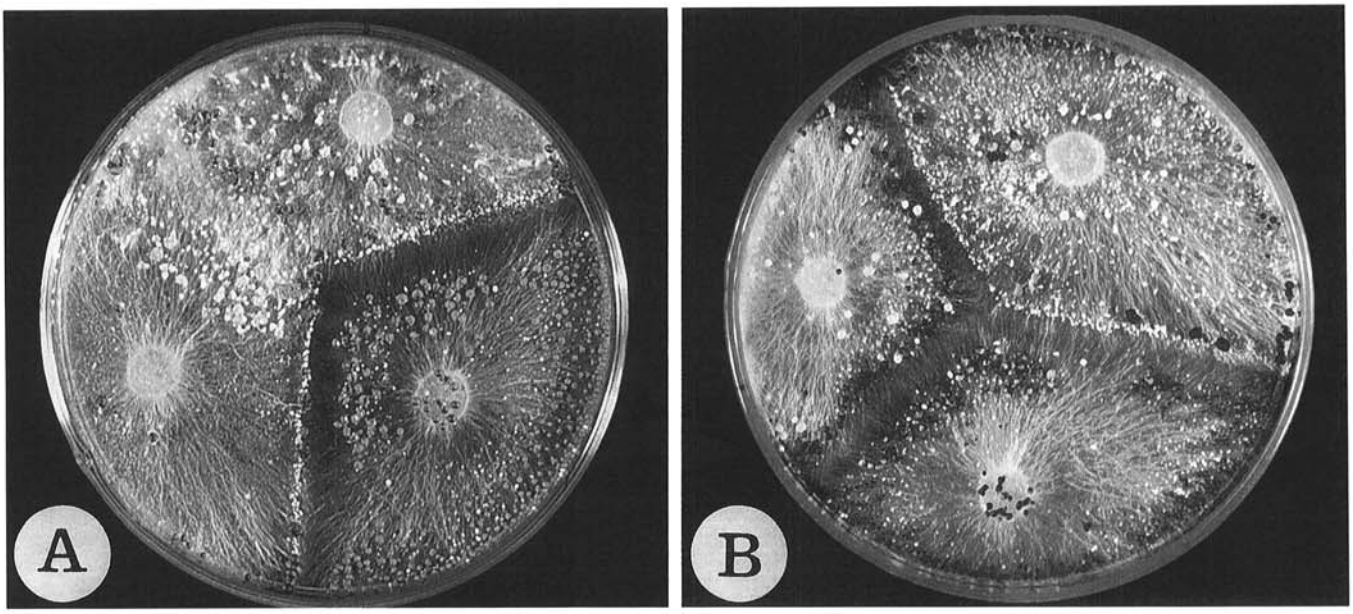

Fig. 2. Aversion (barrage) zone formation between isolates of *Sclerotium rolfsii*. **A,** Comparison of a compatible reaction (top and left colonies) with incompatible reactions (right colony); **B,** Isolates from three mycelial compatibility groups paired against each other, showing all incompatible reactions. Photos were taken after 12 days of growth on potato-dextrose agar.

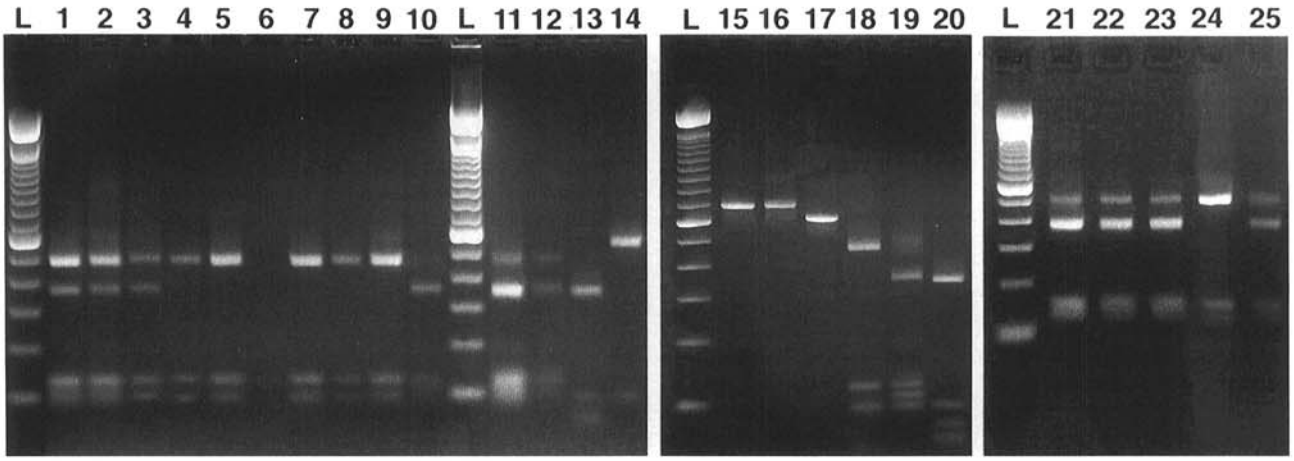

Fig. 3. Internal transcribed spacer-restriction fragment length polymorphism (ITS-RFLP) *Alu*I patterns for *Sclerotium rolfsii*, *S. delphinii*, and *S. coffeicola*. Lane L = 100-bp DNA ladder (BRL); lanes 1 to 20 = polymerase chain reaction (PCR) annealing temperature of 55°C and extension time of 3 min; lanes 1 to 3 = pattern A2 for *S. rolfsii* 3080, 158, and 1094; lanes 4, 5, 7 to 9, and 18 = pattern A1 for *S. rolfsii* 138, 3082, AS-6, 1140, ChetR, and OK121; lane 10 = pattern A3 for *S. rolfsii* 3081; lanes 11 and 12 = pattern A2 for *S. delphinii* isolates 196 and W10; lanes 13 and 14 = pattern A4 and A5 for *S. coffeicola* IMI and ATCC; lanes 15 to 17 = ITS amplified products for *S. rolfsii* 138, *S. delphinii* 196, and *S. coffeicola* IMI; lane 18 = pattern A2 for *S. delphinii* 196; lane 19 = pattern A4 for *S. coffeicola* IMI; lanes 21 to 25 = PCR annealing temperature of either 55 or 58°C, and extension time of 2 min and either Promega or BRL *Taq* DNA polymerase; lane 21 = *S. delphinii* W10 (Promega *Taq*, annealing temperature of 55°C); lanes 22 and 23 = *S. delphinii* 196 (BRL *Taq*) annealing temperature of 55 and 58°C, respectively; lane 24 = *S. rolfsii* 3085 (BRL *Taq*, annealing temperature of 55°C); lane 25 = *S. delphinii* 4010 (BRL *Taq* and annealing temperature of 58°C).

previously described by Punja and Grogan (28), giving a total of 49 MCGs for the 119 *S. rolfsii* isolates tested (Table 1). Isolates from one host and area, e.g., annual bluegrass isolates from California or peanut isolates from Oklahoma or Georgia, were often in the same MCG. Conversely, isolates from one geographical region, e.g., California or North Carolina, frequently belonged to several MCGs (Table 1). An MCG could also contain isolates from widely different geographical regions, e.g., MCG 1 and MCG 8. Thirty-three MCGs contained a single isolate. Among 11 *S. delphinii* isolates, three MCGs were found, and all isolates from Washington were in one MCG (Table 1). Isolate 1057, suspected to be *S. delphinii* because of its large sclerotia, was incompatible with all *S. rolfsii* testers.

PCR amplification of ITS region. Initially, primer pair ITS1-ITS4 did not yield a PCR product for all isolates of *S. rolfsii*, possibly because of variability in template concentration and/or DNA quality due to polysaccharides. However, when less than 100 ng of template was used, most PCR reactions were successful and no PCR product was obtained in the negative controls. Gel electrophoresis of unpurified PCR products from *S. rolfsii* and *S. delphinii* always yielded a uniform band of approximately 720 bp, while *S. coffeicola* (Imperial Mycological Institute [IMI], currently Commonwealth Mycological Institute, Kew, England) and *S. coffeicola* (American Type Culture Collection [ATCC], Rockville, MD) had a band of approximately 635 and 685 bp, respectively (data not shown). Isolates of *A. pellicularis* and *A. epiphylla* yielded a band of about 680 bp and in *A. bombacina* the band was about 730 bp.

RFLPs in ITS-rDNA. *Field isolates.* In a small subset of isolates (three *S. rolfsii*, one *S. delphinii*, and one *S. coffeicola*) in which DNA was initially amplified and digested with 12 restriction enzymes, only four enzymes (*Alu*I, *Hpa*II, *Rsa*I, and *Mbo*I)

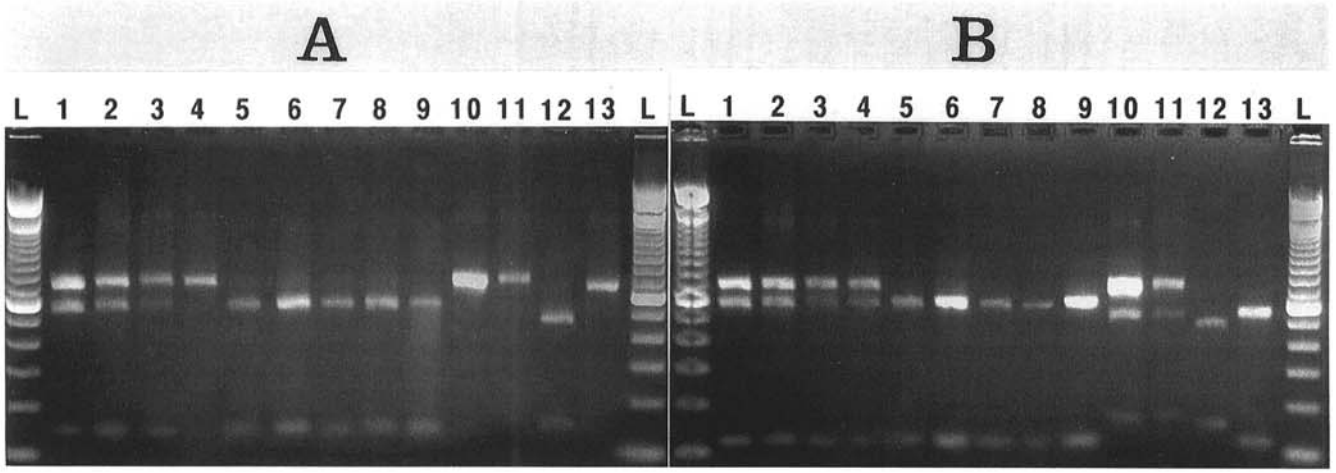

Fig. 4. A, Internal transcribed spacer-restriction fragment length polymorphism (ITS-RFLP) *Hpa*II patterns for *Sclerotium rolfsii*, *S. delphinii*, and *S. coffeicola*. Lane L = 100-bp DNA ladder (BRL); lanes 1 to 3 = pattern H3 for *S. rolfsii* isolates 3080, 158, and 1094, respectively; lane 4 = pattern H1 for *S. rolfsii* 3082; lanes 5 to 9 = pattern H2 for *S. rolfsii* 138, 3082, ChetR, 1140, and AS-6; lanes 10 and 11 = pattern H1 for *S. delphinii* 196 and W10; lanes 12 and 13 = pattern H4 and H5 of *S. coffeicola* isolates IMI and ATCC. **B,** ITS-RFLP *Rsa*I patterns of *S. rolfsii*, *S. delphinii*, and *S. coffeicola*. Lane L = 100-bp DNA ladder (BRL); lanes 1 to 4 = pattern R1 for *S. rolfsii* isolates 3080, 158, 1094, 3081; lanes 5 to 9 = pattern R2 for *S. rolfsii* isolates 138, 3082, ChetR, 1140, and AS-6; lanes 10 and 11 = pattern R3 for *S. delphinii* 196 and W10; lanes 12 and 13 = pattern R4 and R5 for *S. coffeicola* IMI and ATCC.

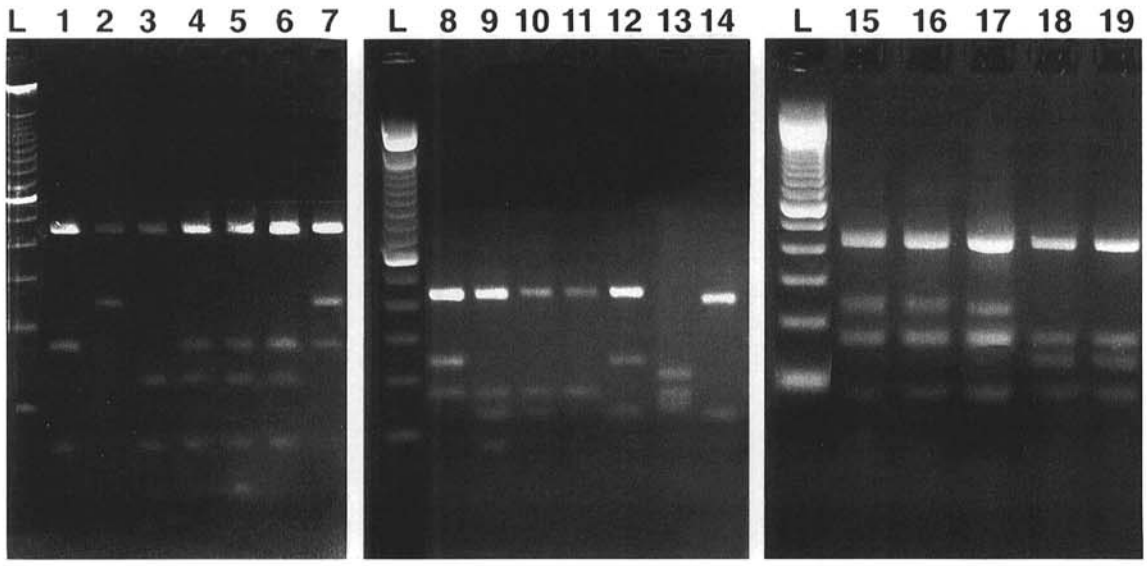

Fig. 5. Internal transcribed spacer-restriction fragment length polymorphism (ITS-RFLP) *Mbo*I patterns of *Sclerotium rolfsii*, *S. delphinii*, and *S. coffeicola*. Lane L = 100-bp DNA ladder (BRL); lanes 1 and 11 = pattern M1 for *S. rolfsii* 185 and 138; lanes 2, 7, and 8 = pattern M2 for *S. rolfsii* 1140, DP18-2, and 3080; lane 3 = pattern M5 for *S. rolfsii* ChetA; lanes 4 to 6, 9, and 10 = pattern M3 for *S. rolfsii* ChetR, WM71, 3084, 158, and 1094; lane 12 = pattern M4 for *S. rolfsii* 3082; lanes 13 and 14 = pattern M6 and M7 for *S. coffeicola* IMI and ATCC; lanes 15 to 19 = polymerase chain reaction annealing temperature of 58°C, 2 min extension, and *Taq* DNA polymerase as indicated: lanes 15 to 17 = pattern M2 for *S. delphinii* W10 [BRL and Ultima (Perkin-Elmer) *Taq*, respectively]; lanes 18 and 19 = pattern M3 for *S. rolfsii* 158 (BRL and Promega *Taq*, respectively).

TABLE 2. Estimated restriction fragment sizes (bp) following digestion of polymerase chain reaction-amplified internal transcribed spacer (primer pair ITS1-ITS4) from three *Sclerotium* and three *Athelia* species with four restriction enzymes[a]

	Internal transcribed spacer-restriction fragment length polymorphism (ITS-RFLP) banding patterns[b]										
	Sclerotium							*Athelia*			
						coffeicola			*epiphylla*		
Enzyme	*rolfsii*					*delphinii*	IMI	ATCC	*bombacina*	CBS	FP	*pellicularis*
*Alu*I	A1	A2	A3			A2	A4	A5	A6	A7	A7	A8
										680	680	
								585				
												535
	495	495				(495)						
		385	385			385						
							380					
									360			
									205			
	130	130	130			130						
		110*[c]	110*			110*			110			110
	95	95	95			95	95	95				
							70					
							45**[d]					
									35*			35*
*Hpa*II	H1	H2	H3			H1	H4	H5	H6	H7	H8	H9
	720		720			720						
								685				
		580	580									
									510			
											560	
							490					
										485		
												365
												295
									210			
										195		
							145					
		140	140									
											120	
*Rsa*I	R1	R2				R3	R4	R5	R6	R7	R7	R7
									725			
	720					720						
										680	680	680
	595	595										
								560				
						555						
							480					
						165						
							155					
	125	125						125				
*Mbo*I	M1	M2	M3	M4	M5	M2	M6	M7	M8	M9	M10	M11
	445	445	445	445	445	445			445			
								440				
									260		260	
												255
		240		240		240				240	240	240
							220					
	165	165	165			165	165					
										160	160	
												155
							150			150	150	
								140				
			130	130	130							
										105	105	
							75	75				
	75	75	75	75	75	75						
			30*	30*	30*							
	25*	25*		25*		25*			25*	25*	25*	25*

[a] ATCC = American Type Culture Collection, Rockville, MD; IMI = Imperial Mycological Institute (currently, Commonwealth Mycological Institute, Kew, England); FP = Forest Products Laboratory (currently, Center for Forest Mycology Research, Madison, WI); CBS = Centraalbureau voor Schimmelcultures, Baarn, the Netherlands.

[b] Represents sizes +/– 5 bp.

[c] * = Base pair fragments which were resolved using polyacrylamide gels.

[d] ** = Presence of two bands of similar base pair size.

could differentiate the three species. Subsequently, 67 *S. rolfsii*, 11 *S. delphinii*, and two *S. coffeicola* isolates were digested with *Alu*I, *Hpa*II, and *Rsa*I. The resulting banding patterns (Figs. 3, 4, and 5) clearly differentiated *S. coffeicola* from *S. rolfsii* and *S. delphinii*, but only *Rsa*I distinguished all isolates of *S. rolfsii* from *S. delphinii* (Fig. 4B). The different enzymes gave distinct banding pattern types (ITS-RFLP patterns), resulting in five patterns each for *Alu*I, *Hpa*II, and *Rsa*I, and seven patterns for *Mbo*I (Table 2). In the *Athelia* spp., three patterns for *Alu*I, two for *Rsa*I, and four each for *Hpa*II and *Mbo*I were observed (Table 2, Fig. 6). Several repeated base pair measurements were made for the bands in each pattern (Table 2). In several instances, the sum of fragment sizes in some patterns in *S. rolfsii* (e.g., A2, H3, and R1) and *S. delphinii* (e.g., A2, R3, and M2) exceeded size estimates for the undigested PCR products, and not all fragments were accounted for in *Mbo*I patterns M1 and M5 (Table 2). *Alu*I banding patterns of *S. delphinii* isolates consistently showed a very faint band of 495 bp and a more intense 385 bp band, while *S. rolfsii* isolates had bands of equal intensity of 495 and 385 bp (Fig. 5). By combining the *Alu*I, *Hpa*II, *Rsa*I, and *Mbo*I ITS-RFLP patterns for each isolate, ITS-RFLP groups were established (Table 3). There were 12 subspecific groupings in *S. rolfsii*, one in *S. delphinii*, and two in *S. coffeicola*.

Single-basidiospore (SB) strains. The undigested PCR product in four *S. rolfsii* field isolates and 29 SB strains was a uniform band of approximately 720 bp. No banding pattern difference was observed with 11 of the 12 enzymes tested. However, with *Mbo*I, two patterns were detected in the SB strains that showed a segregation from the parental pattern, which was approximately 445, 240, 165, 75, and 25 bp (Fig. 6B). In the 29 SB strains, 19 had a pattern of 445 and 240 bp, and eight had a pattern of 445, 165, and 75 bp, which was equivalent to ITS-RFLP pattern M1 (Fig. 6B). One SB strain each in the D1 and D2 series had a banding pattern that was identical to that of the parent isolate (M2).

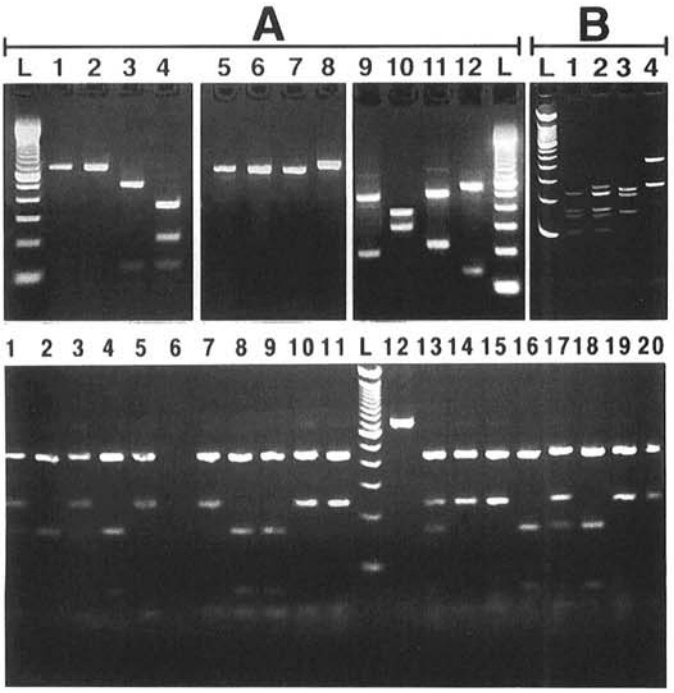

Fig. 6. Top panel. **A,** Internal transcribed spacer-restriction fragment length polymorphism (ITS-RFLP) patterns in *Athelia* spp. using *Alu*I, *Hpa*II, *Rsa*I, and *Mbo*I. Section A is 2% agarose gel: lane L = 100-bp DNA ladder (BRL); lanes 1 to 4 = *Alu*I patterns A7, A7, A8, and A6 for *A. epiphylla* CBS and FP, *A. pellicularis* and *A. bombacina*, respectively; lanes 5 to 7 = *Rsa*I ITS pattern R7 (uncut) for *A. epiphylla* CBS and FP, and *A. pellicularis*, respectively; lane 8 = *Rsa*I pattern R6 (no sites) of *A. bombacina*; lanes 9 to 12 = *Hpa*II pattern H7, H8, H9, and H6 for *A. epiphylla* CBS and FP, *A. pellicularis* and *A. bombacina*, respectively. Section B is 8% polyacrylamide gel: lane L = 100-bp DNA ladder (BRL); lanes 1 to 4 = pattern M9, M10, M11, and M8 of *A. epiphylla* CBS and FP, *A. pellicularis*, and *A. bombacina*, respectively. Lower panel. ITS-RFLP *Mbo*I patterns of *Sclerotium rolfsii* field isolates (M2) and segregating patterns for single-basidiospore (SB) strains. Lane L = 100-bp DNA ladder (BRL); lanes 1, 13, and 17 = *S. rolfsii* parent isolates 2672 (D1-SB progeny), DP18-8 (D2-SB progeny), and 1126 (D3-SB progeny), respectively; lanes 2 to 5, and 7 to 11 = SB strains D1-1, -2, -3, -4, -7, -8, -9, and -10; lane 12 = ITS amplified product for DP18-8; lanes 14 to 16 = SB strains D2-3, -6, and -9; lanes 18 to 20 = SB strains D3-1, -5, and -6.

Restriction maps. The amplified PCR product for *S. rolfsii* and *S. delphinii* using the ITS1-ITS2 (ITSI) and ITS3-ITS4 (ITSII) primer pairs was approximately 300 and 440 bp in size, respectively. In *S. coffeicola* (IMI) the product was approximately 280 and 375 bp in length, and in *S. coffeicola* (ATCC) about 290 and 425 bp. Since segregation patterns were observed in SB strains, it was apparent that field isolates could contain more than one type of ITS-rDNA sequence. Thus, restriction maps are presented in two scenarios, where appropriate, to show the presence of possibly two ribosomal digest patterns referred to as ITS-RFLP subtypes (Fig. 7). A conserved *Mbo*I restriction site upstream of the ITS2 priming site was used to align restriction maps. This *Mbo*I site was confirmed from sequence data (G. C. Adams, Jr. and B. Kropp, *unpublished data*), and was also present in all *Athelia* spp. Not all restriction sites could be mapped to an exact location. However, establishing whether certain restriction sites resided in either the ITSI or ITSII region was useful to confirm base pair calculations based on RFLP data and to generate a data set of unique restriction sites among isolates to include in the phylogenetic analysis. In general, fragment sizes and presence and location of restriction sites corresponded with sequence data, with only minor differences.

Phylogenetic analysis. The presence or absence of restriction sites depicted in the ITS restriction maps (Fig. 7) was scored for all isolates of *Sclerotium* spp. and *Athelia* spp. and resulted in an 81 individual by 56 character binary matrix. Individuals with identical patterns for all four enzymes were grouped, reducing the matrix to 19 ITS patterns and 56 characters. Since more than one type of ribosomal sequence could occur in some isolates, two restriction site scenarios (rDNA subtypes) were represented in the binary data set, where applicable. Each rDNA subtype was represented by the same number of characters, but with a binary code that reflected enzyme site differences. To be consistent, binary sets for restriction sites with only one subtype were duplicated. For example, the restriction map of *Alu*I ITS-RFLP pattern A1 showed only one possible rDNA type since the sum of fragments equaled the uncut PCR-ITS product. A total of eight *Alu*I sites was observed among the 19 ITS patterns included; therefore, the binary matrix representing these sites was duplicated in the data set for a total of 16 characters. In contrast, the sum of fragments for ITS-RFLP pattern A2, which exceeded the uncut PCR-ITS fragment (720 bp), was represented by two sets of eight binary characters, each showing different restriction site scenarios, for a total of 16 characters. The data matrix (not shown) can be made available upon request.

Nonbootstrapped parsimony using the Heuristic search option (PAUP) resulted in five trees of minimum length equal to 69 while nonbootstrapped Wagner parsimony (PHYLIP) resulted in 26 trees. Majority-rule consensus trees inferred from Wagner parsimony (1,000 bootstraps; rerooted by the outgroup) and from Heuristic search (1,000 bootstraps; rerooted by the outgroup) supported similar clades and subgroups (Fig. 8). A distinct clade with a high bootstrap value of 95% consisted of two subgroups: one subgroup, comprised of *S. rolfsii* ITS-RFLP groups (I to XII) and *S. delphinii* (XIV), was supported by a bootstrap value of 76 and 78% in the Wagner and Heuristic parsimony, respectively; the other subgroup consisted of a single isolate of *S. coffeicola* (XVI). Within the former subgroup, the relationships among *S. rolfsii* and *S. delphinii* isolates were either not resolved

(Heuristic) or were marginally resolved (bootstrap ≤ 48%) (Wagner) (Fig. 8). The association of *S. coffeicola* (XV) with *A. epiphylla* (XVIII and XIX) was supported 98% in both parsimony analyses; however, *S. coffeicola* (XV) branched separately from *S. rolfsii* and *S. delphinii* (one subgroup) and *S. coffeicola* (XVI) (second subgroup). The inferred relationship between the two *A. epiphylla* isolates (XIX and XVIII) was supported by a high bootstrap value of 85% (Heuristic) and 88% (Wagner). In addition, *A. bombacina* (XVII) and *A. pellicularis* (XX) were grouped together (98% and 100%) in Heuristic and Wagner parsimony analyses, respectively. Removing the outgroup in the Heuristic analysis did not change the relationship among *S. rolfsii* and *S. delphinii* isolates, but did group both *S. coffeicola* isolates with a bootstrap value of 79% (data not shown).

DISCUSSION

Mycelial pairings of isolates of *S. rolfsii* and *S. delphinii* revealed the presence of at least 52 MCGs in a worldwide collection of isolates, extending the 25 groups described previously (28). MCGs are described among field isolates of *Sclerotinia sclerotiorum* (18) and among populations of wood-decaying basidiomycetes (8,13,22,33,34). Isolates from the same MCG or vegetative compatibility group (VCG) are presumed to be more genetically similar than isolates from different groups (19,22). Many loci and alleles are involved in the incompatibility, and mating-type loci and cytoplasmic differences also can contribute to vegetative incompatibility reactions (14,22,40). To date, the molecular basis for the recognition and lytic reactions associated with vegetative incompatibility has not been elucidated.

It has been reported that a wide diversity of fungal compatibility groups can occur within a limited area, e.g., within a host or in a geographically isolated region; or, conversely, VCG distribution may be limited to one group or to a specific area (4,22,37). There was some correlation between MCG in *S. rolfsii* and host of origin of isolates and geographical area. For example, in MCG 27 and 28, all members were from the same geographical area. Isolates from the same host and geographical area that also belonged to the same MCG could be clonally derived. MCGs were also found to contain isolates from different hosts and areas, and a given geographical area (e.g., California) could have a diversity of MCGs present (7 groups for 38 isolates).

The manner in which new MCGs arise in *S. rolfsii* is not known. Carlile (6) suggested that a single VCG could predominate in an area if a fungal isolate colonized a new area or host and then spread vegetatively. Over time, if genetic diversity occurs among individuals, e.g., through mutation, new MCGs can result as adapted individuals are distinguished from nonclonal individuals (40). The recovery of a particular MCG of *S. rolfsii* in widely different geographical areas and on different hosts may have resulted from spread because of agricultural practices. Predominant MCGs may have spread through dissemination of planting materials, soil, or seed. Unique MCGs found in certain regions (e.g., single-member MCG 18, 19, and 20, each comprised of an isolate from Australia) could have resulted from a recent colonization and/or subsequent geographical isolation. There may also have been certain selection pressures brought about by new hosts and ecological niches that gave rise to new strains, represented by new MCGs, which were better adapted to the area. A large number of VCGs within small geographical areas may be indicative of sexually reproducing populations (19,22). In *S. rolfsii*, the importance of sexual reproduction as a mechanism for generating new MCGs has not been established.

In *S. delphinii*, which is reported to have a host range that predominantly includes the bulbs of rhizomatous plants (iris and lily) and is restricted to the temperate regions of North America and Eurasia (2,38), only three MCGs were found, with nine isolates from iris in Washington belonging to one group. The low number of MCGs could suggest that this species was introduced relatively recently or not yet had the opportunity to spread to different ar-

TABLE 3. Internal transcribed spacer-restriction fragment length polymorphism (ITS-RFLP) groupings of *Sclerotium* spp. and *Athelia* spp. based on four restriction enzyme digest patterns and isolate and mycelial compatibility group (MCG) distribution[a]

Group Number	ITS-RFLP[b]	Isolates in each group	MCG
S. rolfsii			
I	A1, H2, R2, M[c]	138, 144, 145, 150, 155, 163, 185, 1003, AS-1, AS-6, 3085, 3086	1, 5, 18, 19, 21, 26, 29, 30, 37, 41
II	A2, H3, R1, M2	143, 166, 190, 191, 192, 198, 3080, OK121, OK128, OK233, OK315, WM902, WM904, WM906, WM908, WM909, WM917	5, 27, 28, 42, 47
III	A1, H2, R2, M2	146, 156, 1120, 1123, 1059, 1125, 1126, 1127, 1134, DP18-2, DP18-6, DP18-8, DP18-9, DP18-11, 1135, 1137, 1140, 1163, 2313, 2672	1, 2, 34
IV	A1, H2, R2, M3	176, 1138, 1376, 3083, 3084, Chet R, WM71, WM913	3, 12, 23, 29, 31, 33
V	A2, H3, R1, M1	158, 1094	10
VI	A1, H3, R1, M3	157, GA13	3
VII	A1, H2, R1, M2	1001	2
VIII	A1, H3, R2, M1	197	29
IX	A2, H1, R1, M3	3078	8
X	A3, H1, R1, M1	3081	48
XI	A1, H2, R2, M4	3082	49
XII	A1, H2, R2, M5	Chet A	22
S. delphinii			
XIV	A2, H1, R3, M2	W10, W11, W20, W21, W22, W23, W30, W31, 196, 1057, 4010	9, 1d, 2d, 3d
S. coffeicola			
XV	A4, H4, R4, M6	IMI	1c
XVI	A5, H5, R5, M7	ATCC	2c
Athelia spp.			
XVII	A7, H6, R6 , M9	*A. bombacina* 867	
XVIII	A6, H7, R6 , M10	*A. epiphylla* FP	
XIX	A6, H8, R6 , M9	*A. epiphylla* CBS	
XX	A8, H9, R6 , M8	*A. pellicularis* 741	

[a] ATCC = American Type Culture Collection, Rockville, MD; IMI = Imperial Mycological Institute (currently, Commonwealth Mycological Institute, Kew, England).

[b] Individuals with identical RFLPs for four enzymes were grouped together.

[c] A = *Alu*I, R = *Rsa*I, H = *Hpa*II, and M = *Mbo*I.

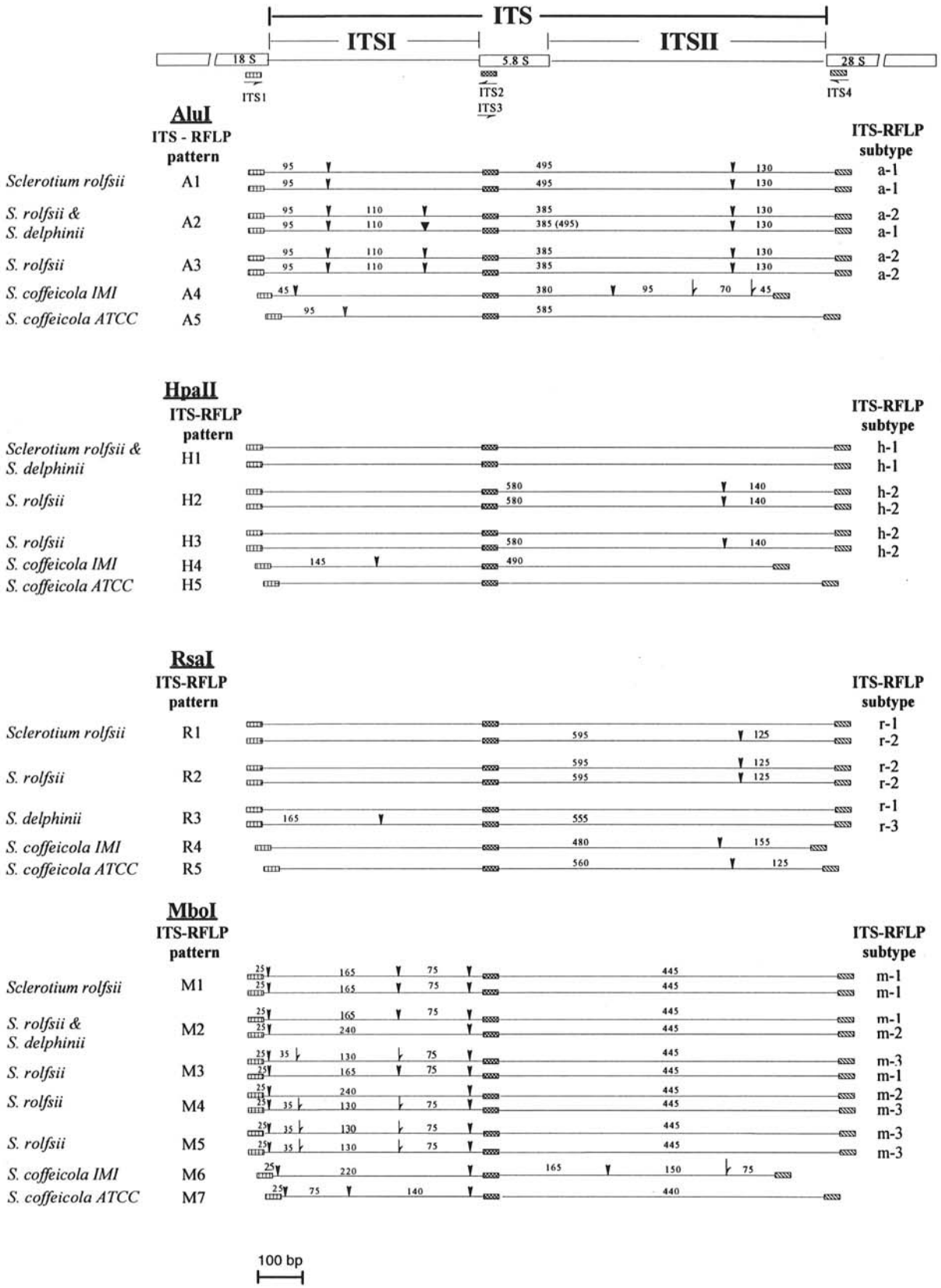

Fig. 7. Restriction maps of internal transcribed spacer (ITS) region for *Sclerotium rolfsii*, *S. delphinii*, and *S. coffeicola*, amplified with primer pair ITS1-ITS4. Restriction maps for *Athelia* spp. were based on ITS-restriction fragment length polymorphism (RFLP) data and sequence data provided by G. C. Adams, Jr. and B. Kropp (*unpublished data*) and analyzed using GeneWorks. **A,** *Alu*I sites; **B,** *Hpa*II sites; **C,** *Rsa*I sites; **D,** *Mbo*I sites. ▼ = restriction site; ▼ = restriction site not present in all tandem repeats of *S. delphinii* isolates; ⊦ = exact location of site unknown; ⊞ = ITS1 primer (18 bp); ⊠ = ITS2 and ITS3 primers (20 bp); ▨ = ITS4 primer (20 bp).

eas, although a greater number of isolates needs to be examined to more accurately estimate MCG diversity. In *S. coffeicola*, which occurs in the Western Hemisphere, two MCGs were found.

A comparison of amplified ITS products, ITS-RFLP patterns, and restriction maps showed that *S. rolfsii* and *S. delphinii* were closely related. The banding patterns in *S. delphinii* were identical for all isolates, and also were represented by some isolates of *S. rolfsii*. Intraspecific variation in patterns was observed within *S. rolfsii*, which was used to obtain 12 ITS-RFLP groupings. Inter- and intraspecific variation within the ITS regions have been reported in several fungi, and are mostly due to deletion or insertion events in the ITSI and ITSII regions (5,7,16,24). From sequencing of the ITS region, it has been found that unique individuals are not necessarily correlated to host, nor are they restricted in geographical range (1,18,21,24).

In *S. rolfsii*, isolates with the same ITS-RFLP pattern were predominant in an area and MCG. For example, most MCG 1 isolates had the group III pattern; many were obtained in California from turf and some were collected over a time period from 1967 to 1981. Thus, clonally derived isolates within a MCG appeared to share ITS restriction site similarity. In contrast, members within one MCG which are subject to different selection, mutation, and drift (15) could possess the same vegetative compatibility alleles but have different ITS sequences, e.g., MCG 29 contained isolates with patterns I, IV, and VIII. A specific ITS-RFLP pattern was found in MCG 5, 27, 28, and 43 for isolates from North Carolina, Oklahoma, and Georgia. The relationship of these isolates to an isolate from Indonesia on bean, also with ITS-pattern II but in a unique MCG, is unknown. Similarly, ITS pattern III predominantly found in MCG 1 also occurred in MCG 2 and MCG 34 for isolates from California and North Carolina, respectively. Isolates with the same ITS-RFLP pattern, but in different MCGs, may have been exposed to selection pressures that would have resulted in genetic changes in the alleles that govern MCG. Deletions or point mutations in any of the MCG alleles would be sufficient to change MCG identities (22).

Two field isolates of *S. rolfsii* collected about 10 meters apart from two hosts (bean and peanut) in Indonesia differed in both ITS pattern and MCG. Although sexual reproduction in *S. rolfsii* is reported infrequently in nature, it could have a role in tropical regions or climates. Aerial infections of hosts, such as corn and cowpea, have been reported and may have resulted from basidiospore inoculum (25). Further investigations of isolates which exhibit a large diversity in MCG types within a localized area may elucidate the role of sexual reproduction in population diversity in *S. rolfsii*.

The total sum of digested fragment sizes exceeded the undigested PCR product in several cases in *S. rolfsii*. This length discrepancy was not because of artifacts or incomplete digestion, and, therefore, must have resulted from variation among rDNA copies in the presence of restriction sites or in size. Gardes et al. (10) reported that dikaryotic field isolates of *Laccaria bicolor* contained mixed rDNA types, which gave rise to amplified products of different base pair lengths. However, the ITS amplified product in *S. rolfsii* and *S. delphinii* was uniform in size. The two *Mbo*I digest patterns among the SB strains which segregated from the parental RFLP pattern further indicated two distinct rDNA types in *S. rolfsii*. Previous morphological studies of these SB strains suggest the presence of two distinct nuclear types, i.e., a heterokaryotic condition (26,27,30). Two SB strains which exhibited the parental *Mbo*I RFLP pattern presumably originated from dikaryotic spores. The association of two nuclear types with segregation of random amplified polymorphic DNA (RAPD) markers was recently demonstrated in *Suillus granulatus* (17).

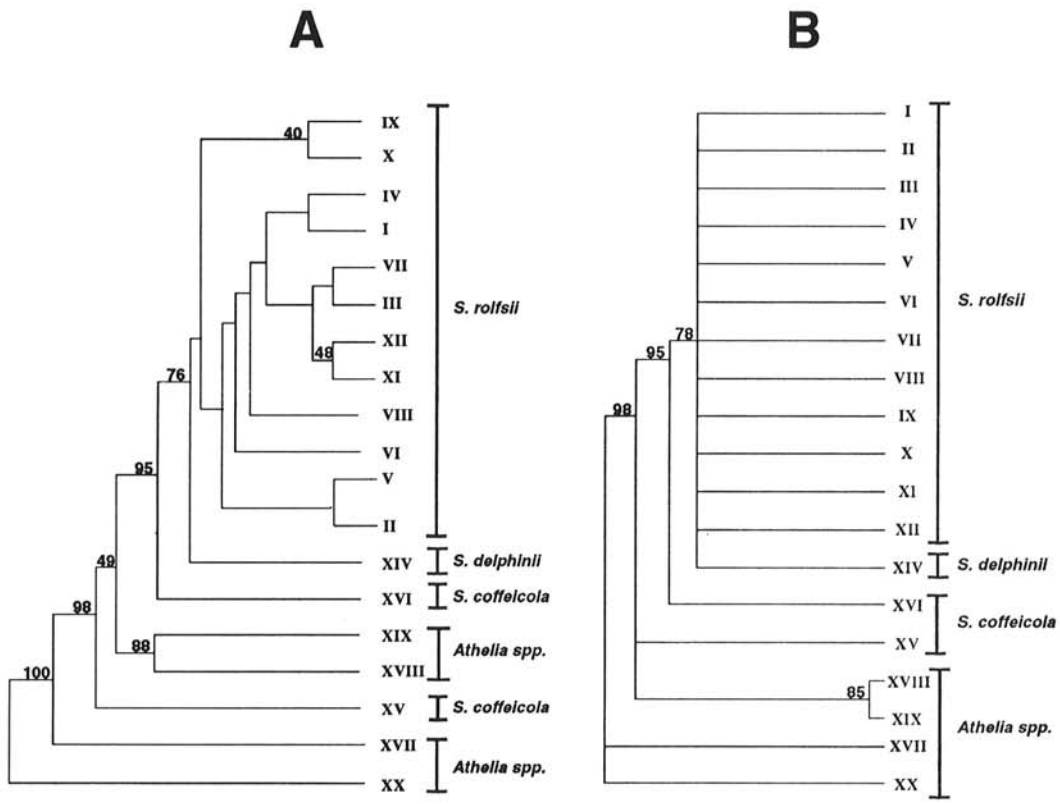

Fig. 8. Inferred phylogenetic relationships of three *Sclerotium* and three *Athelia* species based on *Alu*I, *Hpa*II, *Rsa*I, and *Mbo*I restriction sites of internal transcribed spacer (ITS) region. Binary data matrix included 19 ITS-restriction fragment length polymorphism (RFLP) patterns (Table 3), representing 69 *S. rolfsii* (I to XII), 11 *S. delphinii* (XIV), two *S. coffeicola* (XV and XVI), and three *Athelia* spp. (outgroup) (XVII to XX) and 60 restriction site characters. **A,** Rerooted by the outgroup (*Athelia* spp.), majority-rule consensus tree generated by Wagner parsimony (PHYLIP). Bootstrap values (based on 1,000 replications) of the internal branches are indicated for values ≥ 40%. **B,** Rerooted by the outgroup (*Athelia* spp.), majority-rule consensus tree generated with Heuristic parsimony search (PAUP). Initial Maxtrees option set at 100. Bootstrap values (based on 1,000 replications) of the internal branches are indicated.

However, the inference of rDNA type with nuclear type in *S. rolfsii* needs to be confirmed.

The ITS-RFLP patterns and sequence information of the ITS region (G. C. Adams, Jr. and B. Kropp, *unpublished data*) supported the close relationship of *S. delphinii* to *S. rolfsii*; many patterns were identical in both species. Differences in sclerotial morphology and host range are currently used to differentiate these two species (38), and *S. delphinii* may have recently diverged from *S. rolfsii* through polymorphisms within genes that confer host preference and sclerotial size. Although the host range of *S. delphinii* is reported to be narrower than *S. rolfsii*, it occurs on several dicotyledonous hosts (2). Based on the similarity of the two species, we supported a variety designation of *S. delphinii* as *S. rolfsii* var. *delphinii* (Welch) (3) and *S. rolfsii* as *S. rolfsii* var. *rolfsii*. The ITS-RFLP data further supported the distinction of *S. rolfsii* var. *rolfsii* and *S. rolfsii* var. *delphinii* from that of *S. rolfsii* var. *coffeicola*, although more isolates would be required to determine the full extent of genetic diversity within this latter variety. Since neither of the latter two varieties have been induced to form the sexual state, the teleomorph association remains unknown. The ITS-RFLP patterns in combination with MCG could be used to identify specific isolates of *S. rolfsii* and *S. delphinii* and may be useful in monitoring the subsequent spread of the these isolates.

PAUP-HEURISTIC and PHYLIP-WAGNER parsimony trees based on ITS restriction site data distinguished the three varieties of *Sclerotium*. Since most isolates of *S. rolfsii* var. *rolfsii* clustered together along multiple branches and more than one RFLP pattern occurred within a single branch, a close evolutionary relationship among the different isolates was inferred. However, low bootstrap values did not support branching orders or clades in relation to ITS-patterns of *S. rolfsii*. Thus, the inability to resolve *S. rolfsii* var. *rolfsii* isolates exhibiting low intraspecific variation may have indicated a recent divergence among these populations. No variation was observed among *S. delphinii* isolates, but intraspecific variability was observed between the two *S. coffeicola* isolates which originated from different areas. Low intraspecific ITS variation (e.g., < 2%) had been observed in other basidiomycetes, such as *L. bicolor* (11). The phylogenetic relationships of the *Sclerotium* spp. were inferred from the limited data set presently available and could be further examined with independent character sets, such as restriction sites of other genes or nucleotide sequences. Inclusion of more enzymes to examine the ITS region would be important to increase the confidence in the inferred relationships.

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