Confirmation of Two Distinct Populations of *Sphaeropsis sapinea* in the North Central United States Using RAPDs

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ABSTRACT

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Sphaeropsis shoot blight and canker, caused by *Sphaeropsis sapinea*, occurs on coniferous hosts worldwide and has resulted in considerable damage in the north central United States. Differences in morphology among isolates from this region suggest the existence of two subgroups, referred to as A and B morphotypes. However, these morphological differences are not consistent. Random amplified polymorphic DNA markers (RAPDs) were obtained for 16 A and 16 B morphotype isolates of *S. sapinea* collected in Michigan, Minnesota, and Wisconsin from *Pinus banksiana, P. nigra, P. resinosa, P. strobus*, and *P. sylvestris*. Relationships among isolates were determined using both cluster analyses and parsimony methods. All analyses placed the A morphotype isolates in one group and the B morphotype isolates in another group. Accurate identification of isolate morphotype will be useful in future pathological studies. These techniques may also help resolve relationships among *S. sapinea* isolates from their numerous hosts throughout the world.

Additional keywords: Diplodia pinea.

Sphaeropsis sapinea (Fr.:Fr) Dyko & Sutton in Sutton (syn. *Diplodia pinea* (Desmaz.) J. Kickx fil) is the cause of Sphaeropsis shoot blight and canker of *Cedrus, Juniperus, Picea, Pseudotsuga*, and more than 30 species of *Pinus* (4,23). The disease occurs worldwide and has been associated with significant economic damage in exotic pine plantations in New Zealand, Australia, and South Africa (3,33). It also occurs in the north central United States on Austrian pine (*Pinus nigra* Arnold), Scots pine (*P. sylvestris* L.), red pine (*P. resinosa* Aiton), and jack pine (*P. banksiana* Lamb.) (11,13,15,30). Although the disease has been recognized on several hosts in the region since at least the 1930s (10,30), severe damage has been reported in nurseries and plantations only since the 1970s (15). These epidemics prompted several more recent examinations of the biology of *S. sapinea*, including investigation of variability within the pathogen population.

Palmer et al. (14) recognized two groups of isolates within *S. sapinea*, which they designated type A and type B. Isolates of the A type have been obtained from many coniferous hosts throughout the world, whereas B type isolates have been obtained only from red and jack pines in the north central United States (28). We refer to these groupings as morphotypes because the differentiation is made primarily on morphological criteria and the taxonomic significance of the groupings is unknown (9). A morphotype isolates grow more quickly on agar media than B morphotype isolates and produce abundant white to gray-green aerial mycelium. Mycelium of B morphotype isolates is dark gray and closely appressed to the agar surface (14). Wang et al. (29) reported that conidia of A morphotype isolates are smooth and slightly larger than the pitted conidia of the B morphotype isolates. However, a high degree of variability in the spore surface was subsequently reported (25).

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Overlapping host ranges within the north central United States and possible differences in virulence between the morphotypes (14) necessitate development of an accurate method to distinguish morphotypes as a prerequisite to further pathological studies. The polymerase chain reaction procedure that detects polymorphisms in DNA, random amplified polymorphic DNAs (RAPDs), has been shown to resolve relationships at the population and species level while avoiding the disadvantages of restriction fragment length polymorphisms (RFLPs) (31,32). RAPD markers have been used to distinguish fungal races, pathotypes, strains, and isolates (2,6–8,12,21). We used RAPDs coupled with cluster and parsimony analysis techniques to confirm the existence of two distinct entities within *S. sapinea* in the north central United States.

MATERIALS AND METHODS

Fungal isolates. The *S. sapinea* isolates used in this study were selected to represent A and B morphotypes collected from *Pinus* species in the north central United States (Table 1). In addition to the indication of morphotype determined by M. Palmer for some of the isolates she provided, differences in growth rate and production of aerial mycelium were used for preliminary categorization of each isolate used in this study as either A or B morphotype. However, the degree of variability exhibited by some isolates made this determination only tentative. Each culture originated from a single conidium. Four isolates of *Diplodia mutila* (teleomorph *Botryosphaeria stevensii*) from *Juniperus scopulorum* (27) were used as an outgroup in cluster and parsimony analyses. This species was chosen because traditional taxonomy places it close to *S. sapinea* (22).

Genomic DNA extraction. Isolates were grown in 0.5-ml potato-dextrose broth (PDB) in 1.5-ml microfuge tubes for 7 days at room temperature. The cultures were centrifuged at 8,000 × *g* for 5 min, and the PDB was aspirated. The pellets were washed twice with 10 mM Tris–1 mM EDTA. DNA was extracted by a

procedure used for plant tissue (5). The mycelium was ground in 250 µl of extraction buffer (100 mM Tris, pH 8.0, 50 mM EDTA, 500 mM NaCl, 10 mM β-mercaptoethanol) with a Kontes pestle (Vineland, NJ). Another 250 µl of extraction buffer and 33 µl of 20% sodium dodecyl sulfate were added, and the mixture was vortexed thoroughly and incubated at 65°C for 10 min. One hundred sixty µl of 5 M potassium acetate was added to the mixture, which was then vortexed briefly and centrifuged at 8,000 × *g* for 10 min. Four hundred fifty µl of supernatant was transferred to a new 1.5-ml microfuge tube, and 225 µl of isopropanol was added. This was vortexed briefly and centrifuged at 8,000 × *g* for 10 min. The supernatant was aspirated, and the DNA pellet was washed with 70% ethanol, then dried in a Speed-Vac (Savant Instruments Inc., Farmingdale, NY). The DNA pellet was resuspended in 100 µl of distilled water. DNA concentrations were estimated with a mini-flourimeter (Model TKO 100, Hoefer Scientific Instruments, San Francisco) and diluted to 5 to 10 ng/µl if necessary. The DNA was stored at –20°C.

Amplification conditions. Preliminary tests showed that a variation of the standard DNA amplification using long primers (>17 nucleotides) increased reproducibility of the banding patterns. DNA amplification took place in two steps, the first allowing low-stringency annealing of primers and the second allowing high-stringency annealing (31). The low-stringency reaction took place in a 10-µl reaction mixture consisting of 0.25 U Taq DNA polymerase, 1× buffer, 3.75 mM $MgCl_2$, 0.25 mM each dNTP (all purchased from Promega Corp., Madison, WI), 20 µM primer, and 5 to 10 ng of DNA. Primer sequences were as follows: DS2: 5′-CTGCGACTGAATCTTGCAG; DS3: 5′-GCGAAATGTGTCCTTTGATG; DS4: 5′-GTACCCTCAATCTCGTG; DS5: 5′-TTGAGAAGCTGTAGAAGG; DS9: 5′-GAGATCTATGTTGCACC; DS10: 5′-GGAGGATTTGCTAACTGAG; DS16: 5′-GGGGATCCCAGTCATGTACCGTTGATCG; DS19: 5′-CAGGTCAGCACCTTTCCATCC. The reaction mixture was overlaid with mineral oil, and the following temperature profile was used in a Perkin-Elmer Thermal Cycler 480 (Norwalk, CT): 2 cycles of 5 min at 94°C, 5 min at 40°C, and 5 min at 72°C. Ten cycles of high-stringency annealing followed: 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C. After this reaction, 90 µl of solution containing 1 U Taq DNA polymerase, 1× buffer, 1.5 mM $MgCl_2$, and 0.125 mM each dNTP was added to each tube. Amplification with high-stringency annealing was continued for 25 cycles. DNA fragments were separated on a 0.7% agarose gel (SeaKem LE, FMC BioProducts, Rockland, ME) with Tris-boric acid-EDTA buffer (TBE) and photographed after staining with ethidium bromide. Each isolate was assayed three times by growing a culture, extracting DNA, amplifying the DNA with each primer, and separating the fragments.

TABLE 1. Fungal isolates used for random amplified polymorphic DNA (RAPD) analysis

Fungus	Isolate no.	Morphotype[a]	Host	Origin
Sphaeropsis sapinea	92-12	A	*Pinus nigra*	Milwaukee, WI
	92-14	A	*P. nigra*	Dane Co., WI
	92-19	A	*P. resinosa*	Wood Co., WI
	92-27	A	*P. resinosa*	Wood Co., WI
	92-41	A	*P. resinosa*	Buffalo Co., WI
	92-43	A	*P. sylvestris*	Waushara Co., WI
	92-46	A	*P. nigra*	La Crosse Co., WI
	92-60	A	*P. resinosa*	Sheboygan Co., WI
	92-66	A	*P. sylvestris*	Kalamazoo Co., MI
	120	A	*P. resinosa*	Gogebic Co., MI
	128	A	*P. resinosa*	Grant Co., WI
	173	A	*P. strobus*	Wisconsin
	221	A	*P. resinosa*	St. Louis Co., MN
	239	A	*P. resinosa*	Douglas Co., WI
	411	A	*P. resinosa*	Clearwater Co., MN
	470	A	*P. resinosa*	Wadena Co., MN
	92-1	B	*P. resinosa*	Wood Co., WI
	92-2	B	*P. resinosa*	Wood Co., WI
	92-8	B	*P. banksiana*	Wood Co., WI
	92-9	B	*P. banksiana*	Wood Co., WI
	92-56	B	*P. banksiana*	Clark Co., WI
	100	B	*P. banksiana*	Jackson Co., WI
	113	B	*P. banksiana*	Michigan
	124	B	*P. banksiana*	Jackson Co., WI
	131	B	*P. banksiana*	Michigan
	215	B	*P. resinosa*	Douglas Co., WI
	457	B	*P. resinosa*	Jackson Co., WI
	459	B	*P. banksiana*	Douglas Co., WI
	462	B	*P. resinosa*	Clearwater Co., MN
	465	B	*P. resinosa*	Douglas Co., WI
	466	B	*P. resinosa*	Douglas Co., WI
	474	B	*P. resinosa*	Wadena Co., MN
Diplodia mutila	94-1	NA	*Juniperus scopulorum*	Republic Co., KS
	94-3	NA	*J. scopulorum*	Riley Co., KS
	94-4	NA	*J. scopulorum*	Reno Co., KS
	94-5	NA	*J. scopulorum*	Sedgwick Co.,KS

[a] NA = not applicable.

Southern blots. Co-migrating RAPD fragments that occurred in both morphotypes or in at least one morphotype and the outgroup were used as probes for Southern analyses (17) to determine whether the co-migrating fragments were homologous. The Flash/Prime-it Labeling and Detection kit or the Illuminator kit (Stratagene, La Jolla, CA) was used for this analysis; probe labeling, hybridizations, blot washes at high stringency, and detection were performed according to the manufacturer's directions.

Data analysis. Each isolate was scored for the presence or absence of each amplification product (Fig. 1). Co-migrating fragments that were shown by Southern analysis to be nonhomologous were scored as distinct fragments. Only amplification products that were present throughout each of three repetitions for at least one isolate were scored.

Relationships among isolates were determined by two methods. The first involved calculation of a simple matching coefficient (S_{sm}) or Jacquard's coefficient (S_J) for each pair of isolates. The simple matching coefficient was calculated by the formula described by Sneath and Sokal (20):

$$S_{sm} = m/(m + u)$$

where m is the number of bands found in common between two isolates and u is the total number of bands unique to each isolate. The formula for Jacquard's coefficient is:

$$S_J = a/n - d$$

where a is the number of matched 1's, n is the total sample size, and d is the total number of matched 0's. This coefficient does not count common negative data. Dendrograms were constructed after cluster analysis of the similarity coefficients by unweighted pair-group method using arithmetic averages (UPGMA) and single linkage (20). These calculations were performed with the programs SIMQUAL and SAHN of the software package NTSYS-pc version 1.80 (16).

For the second method, the binary data matrix was used to construct cladograms using the computer program Phylogenetic Analysis Using Parsimony (PAUP version 3.1.1) (26). The data were analyzed using both Wagner parsimony, which assumes that all characters change from one state to another with equal probability, and Dollo parsimony, which only allows characters to change to the derived state (priming site present) once but allows unlimited reversals. The true situation with RAPD data falls between these two scenarios, with a priming site being more likely to be lost than gained. The heuristic algorithm with branch-swapping (tree bisection and reconnection) was used to generate a set of most parsimonious trees. A 50% majority-rule consensus

tree was generated by 200 repetitions of the bootstrap algorithm to determine branch strengths.

RESULTS

Amplification products. The eight RAPD primers produced 43 scorable DNA fragments. Twenty of these fragments were polymorphic between the A morphotype and the B morphotype. Nine fragments were produced only by the outgroup isolates (Fig. 1). All primers except DS4 yielded a banding pattern that differentiated the two morphotypes.

Southern analyses. Seven DNA fragments were tested for homology. A 1,000-bp fragment generated by primer DS9 that occurs in all *S. sapinea* isolates was homologous within each morphotype, but not between the morphotypes. Two *D. mutila* fragments co-migrated with *S. sapinea* fragments but were not homologous to them.

One probe hybridized to three fragments of different sizes produced by one primer. Amplification of the larger of the three fragments (1.8 kb) yielded all three fragments, indicating that the smaller fragments were sub-sequences of the largest fragment. No data from this primer were used in the analyses.

Data analyses. Both cluster analysis and parsimony yielded similar results. The simple matching coefficients of similarity indicate that A morphotype isolates are more similar to each other (>85% similarity) than to B morphotype isolates (<59% similarity), and B morphotype isolates are more similar to each other (>78%) than to A morphotype isolates (data not shown). Thus, the dendrogram generated by UPGMA placed all isolates of each morphotype into two distinct groups (Fig. 2). The dendrogram generated by the single-linkage method differed from the UPGMA dendrogram mainly by linking the B morphotype group to the outgroup (Fig. 2). Using Jacquard's coefficient in combination with UPGMA or single-linkage did not significantly change the structure of the tree, although the similarity values were slightly lower (data not shown).

Isolate	Primer							
	2	3	4	5	9	10	16	19
92-12	1111	0100101	1	100010	0100010	00010	0001010	110000
92-14	1111	0100101	1	100010	0010010	00010	0001100	110000
92-19	1111	0100100	1	100010	0100010	00010	0001010	110000
92-27	1111	0100101	1	100010	0010010	00010	0001100	110000
92-41	1111	0100101	1	100010	0010010	00010	1001100	110000
92-43	1111	0100101	1	100010	0010010	00010	0001100	110000
92-46	1111	0100101	1	100010	0010010	00010	0001100	110000
92-60	1111	0100101	1	100010	0010010	00010	0001100	110000
92-66	1111	0100101	1	100010	0010010	10010	0001100	110000
120	1111	0100101	1	100010	0010010	00010	0001000	110000
128	1111	0100101	1	100000	0010010	10010	0001010	110000
173	1111	0100100	1	100010	0010010	00010	0001100	110000
221	1111	0100101	1	100010	0010010	10010	0001100	110000
239	1111	0100101	1	100010	0010010	00010	0001100	110000
411	1111	0100101	1	100010	0010010	10110	0001010	110000
470	1111	0100101	1	100010	0010010	10010	0001100	110000
92-1	0011	0000010	1	001000	0001001	01000	0100101	011000
92-2	0011	0000010	1	001000	0001001	11000	0100101	011000
92-8	0011	0000010	1	001000	0001001	11000	0100101	011000
92-9	0001	0000010	1	001000	0001001	11000	0100100	010100
92-56	0011	0000010	1	001000	0001001	01000	0100100	010100
100	0001	0000010	1	001000	0001001	11000	0100100	010100
113	0111	0000010	1	011000	0101001	01000	0100101	011000
124	0101	0000010	1	001000	0001001	01000	0100100	010100
131	0101	0000010	1	101000	0001001	11000	0100100	010100
215	0111	0000010	1	001000	0001001	01000	0100101	011000
457	0111	0010011	1	001000	0001001	11000	0000101	001000
459	0101	0000110	1	001100	0001001	11000	0100100	010100
462	0101	0000010	1	001000	0001001	01000	0100100	010100
465	0001	0000010	1	001000	0001001	11000	0100100	011100
466	0011	0100010	1	001000	0001001	11000	0100100	010100
474	0001	0000010	1	001000	0001001	11000	0100100	010100
94-1	0010	1001000	0	000101	0000001	00001	0010000	010011
94-3	0010	0001000	0	000111	0000101	00001	1010000	010011
94-4	0010	0001000	0	000111	1000101	00001	1010000	010011
94-5	0010	1001000	0	000111	1000001	00001	0000000	010011

Fig. 1. Binary presence–absence data matrix for RAPD fragments of *Sphaeropsis sapinea* and *Diplodia mutila* isolates. The presence or absence of a RAPD marker is specified by 1 or 0, respectively. Prefix of primer name has been omitted.

Similar results were obtained using PAUP. Twelve equally parsimonious trees with lengths of 66 steps were generated using Wagner parsimony (consistency index = 0.65; retention index = 0.94), all with the A morphotype isolates in one clade and the B morphotype isolates in a separate clade. Bootstrap analysis with 200 repetitions shows that the A morphotype clade is present in 100% of the trees and the B morphotype clade in 96% of the trees (Fig. 3). Dollo parsimony yielded a longer tree (91 steps), but the topology was not significantly different from that in Figure 3 (data not shown).

DISCUSSION

Relatively high morphological variability has been reported by many who have examined the fungi currently referred to as *S. sapinea*. Despite reported differences in spore surface characteristics and size, cultural morphology and growth rates, isozymes, and spore germination and germ-tube growth on fungicide-amended medium (1,14,24,25), use of these characters to clearly distinguish groups of isolates within the species has been less than satisfactory. Although we initially used differences in growth

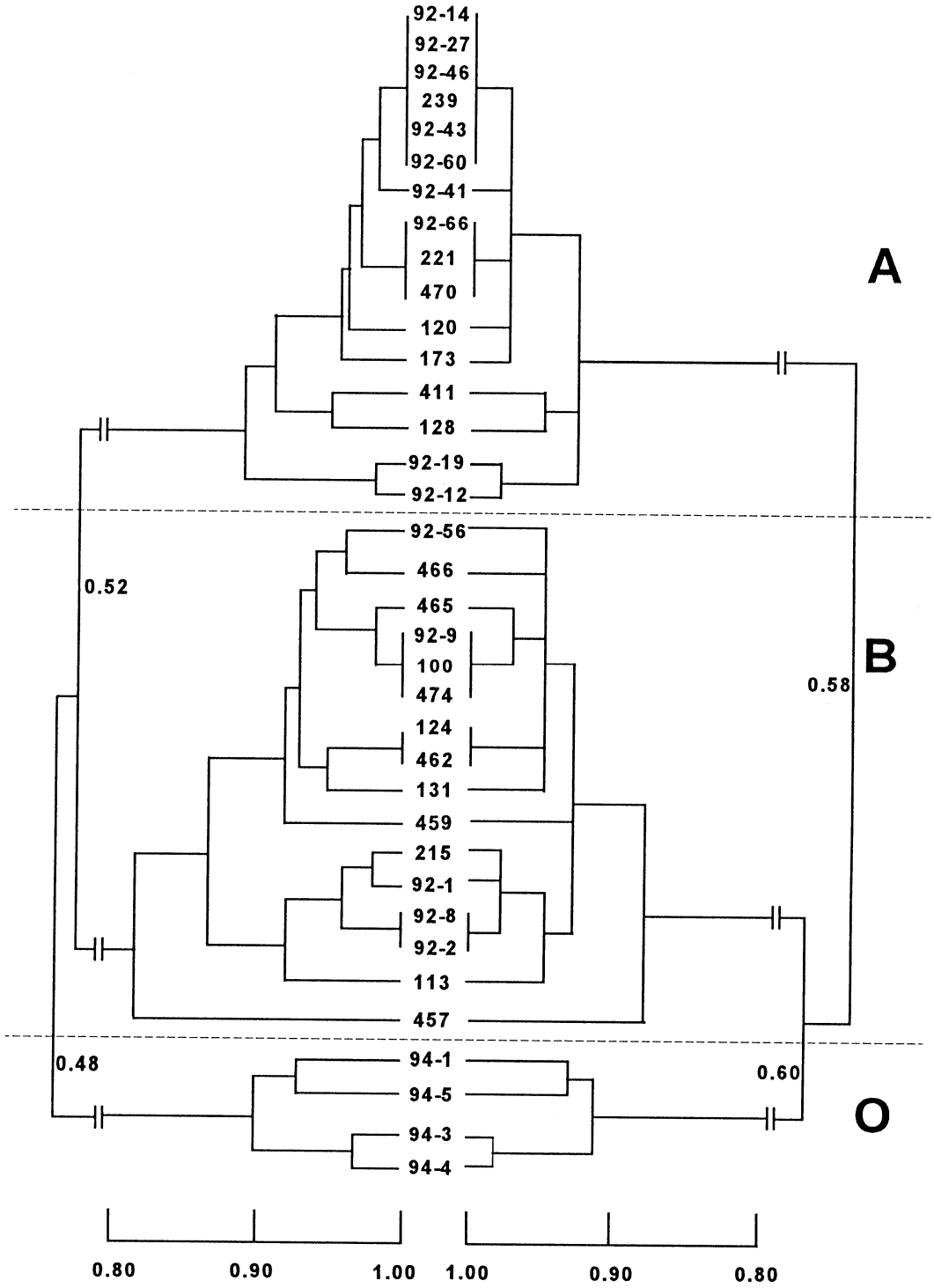

Fig. 2. Dendrograms generated by UPGMA (left) and single linkage (right) using the simple matching coefficient of similarity in the program NTSYS-pc based on 43 RAPD fragments. Dotted lines separate **A,** *Sphaeropsis sapinea* A morphotype; **B,** *S. sapinea* B morphotype; and **O,** the outgroup *Diplodia mutila*.

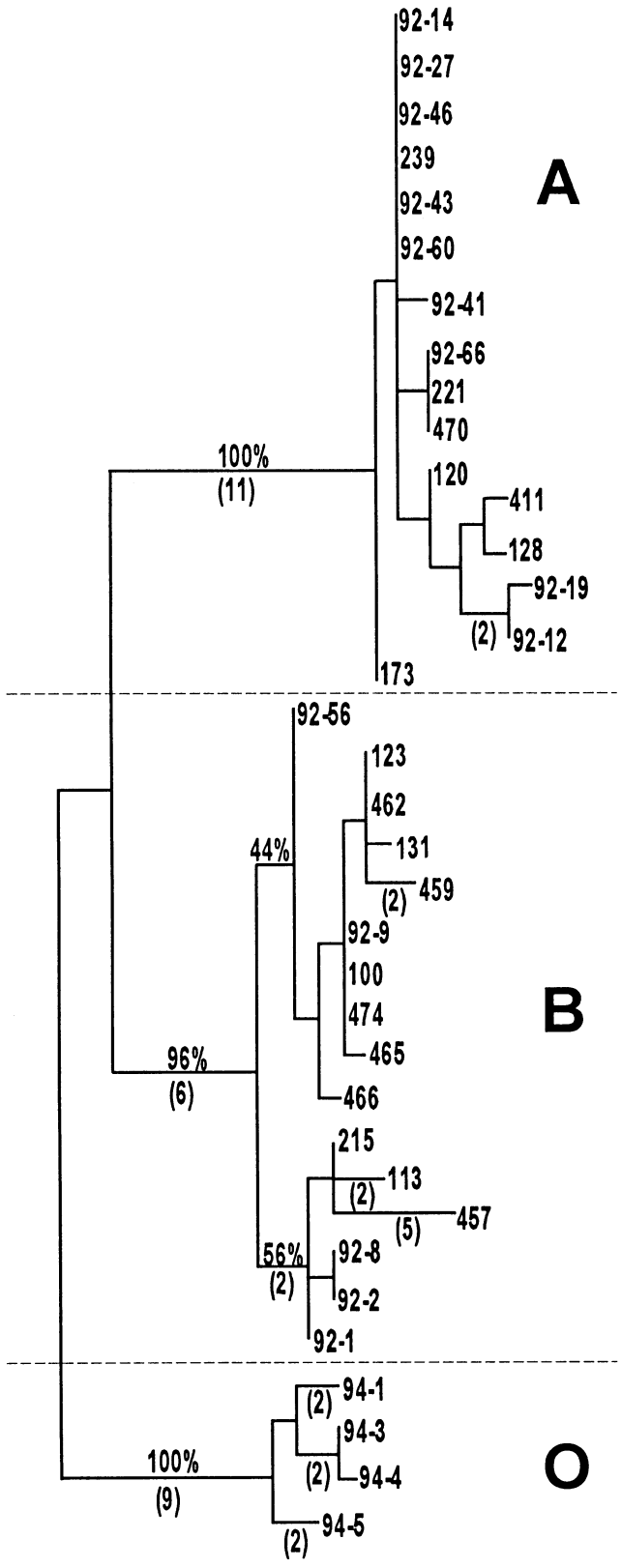

Fig. 3. Cladogram of 50% majority-rule consensus of 12 most parsimonious trees showing the relationships of *Sphaeropsis sapinea* and *Diplodia mutila* isolates based on RAPD fragments. Relationships were determined using the heuristic search in PAUP version 3.1.1 (Wagner parsimony). Branch strengths were tested by 200 repetitions of the bootstrap algorithm with branch swapping (number above the line). Branch lengths greater than 1 are given below the line. Dotted lines separate **A,** *Sphaeropsis sapinea* A morphotype; **B,** *S. sapinea* B morphotype; and **O,** the outgroup *Diplodia mutila*.

rate and cultural morphology to categorize isolates we used in this study, we also observed a range in these characteristics among isolates of a particular morphotype and for individual isolates after successive transfers and storage. Swart et al. (24) concluded, after examination of variability in South African isolates, that there may be more than two distinct types or that variation may occur as a continuum without any distinct types or strains.

Our results indicate, however, that isolates from pine hosts in the north central United States comprise two very distinct groups that can be differentiated using RAPD markers. Several different methods of data analysis of the RAPD bands were performed to determine the effects of various character weighting and grouping schemes on the output. All data analyses grouped the A morphotype isolates separately from the B morphotype isolates and clearly differentiated isolates that had been difficult to morphologically characterize. Grouping by geographic location was not evident. Subsequent analyses using RAPD markers may clarify the relationships among *S. sapinea* isolates and morphologically similar fungi from other parts of the world and other hosts. Preliminary experiments involving a set of *S. sapinea* isolates from different parts of the United States, New Zealand, and South Africa show that the methodology presented here could easily be applied to this problem (18).

Although there is potential to use RAPD data for phylogenetic studies, Southern analyses indicate that care must be used in selecting data to be used for cladistic analyses. As shown by Smith et al. (19), RAPD products may be the result of types of genotypic variation that violate the assumptions of cladistics, mainly that characters are independent and homologous. In our study, Southern analysis found three occurrences of co-migrating nonhomologous DNA fragments that were subsequently scored as separate markers and one occurrence of related fragments of different size in a single reaction. These were found to be the result of multiple priming sites in a 1.8-kb DNA fragment and were excluded from data analyses. The necessity of performing Southern analyses on RAPD products makes the method less attractive for phylogenetic analyses, but the RAPD markers that are independent characters still provide a valuable tool for morphotype identification.

The pathological implications of the existence of discrete groups within *S. sapinea* to forests and plantations in the north central United States are uncertain. Our results do confirm the occurrence of two distinct morphotypes of the fungus on red pine. The suggestion of differences between morphotypes in virulence on red and jack pines was made following experiments that included only one isolate of each type and that yielded results lacking statistically significant differences (14). Additional work is necessary, using a representative sample of isolates of each morphotype, to clarify the host ranges and wound requirements for infection. In addition, it might be judicious to reevaluate other past research on the biology of *S. sapinea* and confirm conclusions by experimentation with isolates of known morphotype. After more complete characterization of the biology of these groups, it will be possible to review disease management recommendations and alter them as necessary to minimize the impact of Sphaeropsis shoot blight and canker on forest values.

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