Characterization of *Sphaeropsis sapinea* from the West Central United States by Means of Random Amplified Polymorphic DNA Marker Analysis

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ABSTRACT

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Two morphotypes (A and B) of the conifer pathogen *Sphaeropsis sapinea* recently have been confirmed as distinct populations by analyses of random amplified polymorphic DNA (RAPD) markers. Because much of the research on Sphaeropsis shoot blight and canker has been conducted in the west central United States, a study was undertaken to determine the morphotype(s) of *S. sapinea* encountered in this region. RAPD markers were obtained for 42 isolates of *S. sapinea* collected in Iowa, Nebraska, Oklahoma, and South Dakota from *Picea pungens*, *Pinus contorta*, *P. nigra*, *P. ponderosa*, *P. resinosa*, *P. sylvestris*, and *Pseudotsuga menziesii*. Relationships among these isolates, and eight other previously characterized isolates, were determined by cluster analysis. All 42 west central region isolates were placed in a single group with the previously characterized A morphotype isolates. This result facilitates interpretation of past research in that region and extrapolation to other areas where the A morphotype of *S. sapinea* is present.

Additional keyword: *Diplodia pinea*

Sphaeropsis sapinea (Fr.:Fr) Dyko & Sutton in Sutton is the cause of Sphaeropsis shoot blight and canker of *Cedrus* Trew, *Juniperus* L., *Picea* A. Dietr., *Pseudotsuga* Carrière, and over 30 species of *Pinus* L. (7,23). The disease occurs worldwide and has been associated with significant economic damage in exotic pine plantations in New Zealand, Australia, and South Africa (6,31). It also occurs generally in the central and eastern United States on many introduced and native coniferous hosts (29).

Some of the most severe damage from Sphaeropsis shoot blight and canker has occurred in the west central United States. The pathogen (identified at the time as *S. ellisii* Sacc.) was first noted on dead twig or canker samples of *P. strobus* L. in Iowa and *P. sylvestris* L. in Kansas by Hedgcock in 1932 (9). Soon after that it was identified (as *S. ellisii* or *Diplodia pinea* (Desmaz.) J. Kickx fil) in Kansas, Nebraska, Oklahoma, and South Dakota on additional hosts including *P. edulis* Engelm., *P. mugo* Turra (reported as *P. montana* Mill.), *P. nigra* Arnold, *P. ponderosa* Douglas ex Lawson & C. Lawson, and *Pseudotsuga menziesii* (Mirb.) Franco (reported as *P. taxifolia* (Lamb.) Britton) (11,20,26–29). Chronic occurrence and periodic epidemics affecting nursery seedlings and trees in shelterbelt and ornamental settings in the west central region stimulated research on epidemiology and management of the disease (4,16–19). Investigation of variability within the pathogen population, however, was initiated elsewhere.

Palmer et al. (15) recognized two groups, designated as A type and B type, among isolates of *S. sapinea* from Michigan, Minnesota, and Wisconsin in the north central United States. Isolates of the A type have been obtained from many coniferous hosts throughout the world, whereas B type isolates have been obtained from *Pinus banksiana* and *P. resinosa* Aiton and only from the north central United States (14, 15,21,24,25). These groupings are referred to as morphotypes because the differentiation is made primarily upon morphological criteria and the taxonomic significance of the groupings is unknown (8). Isolates of the A morphotype grow more quickly on potato dextrose agar medium 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 (15). Recently, we have confirmed that isolates of these morphotypes constitute two distinct populations of *S. sapinea* in the north central United States by analyses of random amplified polymorphic DNA (RAPD) markers (21).

Demonstration of differences between morphotypes of *S. sapinea* in aggressiveness and response to host water stress (1–3) indicates the need to determine the morphotype(s) of the pathogen encountered in current research. Similarly, the ability to interpret and extrapolate from conclusions of past research in the west central United States is currently limited by the lack of morphotype identification. Therefore, we have used RAPD markers coupled with a cluster analysis technique to characterize isolates of *Sphaeropsis sapinea* obtained from several coniferous hosts in the west central United States.

MATERIALS AND METHODS

Fungal isolates. The *S. sapinea* isolates used in this study were selected to represent the geographic and host range of the pathogen in the west central United States (Table 1). Isolates 94-29 through 94-40 were provided from a culture collection at the USDA Forest Service, Rocky Mountain Forest and Range Experiment Station, National Agroforestry Center, Lincoln, NE. These isolates originated during the period of active research on this pathogen at that facility during the 1960s through the 1980s. Other isolates were obtained from symptomatic host material collected by the authors or cooperators in 1994 and 1995. Among the locations from which isolates originated were the following: Lincoln, NE, and the University of Nebraska Horning State Farm, Cass County, NE, sites of previous research on *S. sapinea* by Glenn W. Peterson and others; the Black Hills region of western South Dakota in which a shoot blight and canker epidemic during 1979 to 1982 has been described (10); the USDA Forest Service Bessey Tree Nursery located at the Nebraska National Forest, Halsey, NE; and the South Dakota Division of Forestry Big Sioux Nursery, Watertown, SD. Hosts included *Picea pungens* Engelm., *Pinus contorta* Loudon, *P. nigra*, *P. ponderosa*, *P. resinosa*, *P. sylvestris* L., and *Pseudotsuga menziesii*. Although present at several locations, *Pinus banksiana* trees were not symptomatic and pycnidia of the pathogen were not observed on shoots or cones. Thus, no isolates were obtained from this species. When multiple isolates from the same host species at a particular location were present in the collection, the choice of isolates was arbitrary. Eight additional isolates from pine hosts in Michigan, Wisconsin, or Minnesota were in-

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cluded for comparison (Table 1). These eight isolates were previously characterized based on analyses of RAPD markers (21). Each isolate originated from a single conidium.

Genomic DNA extraction and amplification. Procedures, materials (including primers), and equipment were described previously (21). Eight 17 to 21 nucleotide primers were used. DNA amplification took place in two steps, the first allowing low stringency annealing of primers and the second allowing high stringency annealing. DNA fragments were separated on agarose gels and photographed after staining with ethidium bromide. Usually, each isolate was assayed three times by growing a culture, extracting DNA, amplifying the DNA with each primer, and separating the fragments. However, for some isolate and primer combinations only two assays were performed.

Data analysis. Each isolate was scored for the presence or absence of each amplification product. Only amplification products that were present throughout all repetitions for at least one isolate were scored. Because nearly all RAPD patterns of the west central region isolates matched those previously established for A morphotype isolates (21), no Southern blots were done to test fragment homology.

Relationships among isolates were determined by calculation of a simple matching coefficient (S_{sm}) for each pair of isolates. The simple matching coefficient was calculated by the formula described by Sneath and Sokal (22): $S_{sm} = m/(m + u)$, 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. A dendrogram was constructed after cluster analysis of the similarity coefficients by the unweighted pair-group method using arithmetic averages (UPG MA) (22). These calculations were performed with the programs SIMQUAL and SAHN of the software package NTSYS-pc version 1.80 (Exeter Software, Setauket, NY).

Table 1. *Sphaeropsis sapinea* isolates used for random amplified polymorphic DNA (RAPD) marker analysis

Isolate no.[a]	Morphotype	Host	Origin
94-29 (245)	A	*Pinus sylvestris*	Lincoln, NE
94-30 (248)	A	*P. nigra*	Lincoln, NE
94-31 (294)	A	*P. nigra*	Lincoln, NE
94-32 (305)	A	*P. ponderosa*	Crawford, NE
94-33 (306)	A	*P. ponderosa*	Crawford, NE
94-34 (307)	A	*P. ponderosa*	Crawford, NE
94-35 (309)	A	*P. ponderosa*	Custer, SD
94-36 (310)	A	*P. ponderosa*	Custer, SD
94-38 (319)	A	*P. ponderosa*	Pennington, SD
94-39 (325)	A	*P. nigra*	Norman, OK
94-40 (326)	A	*P. nigra*	Norman, OK
94-46	A	*Picea pungens*	Halsey, NE
94-48	A	*Picea pungens*	Halsey, NE
94-50	A	*Pseudotsuga menziesii*	Watertown, SD
94-53	A	*Pinus contorta*	Halsey, NE
94-54	A	*P. nigra*	Halsey, NE
94-56	A	*P. ponderosa*	Yankton, SD
94-58	A	*P. sylvestris*	Cass Co., NE
94-62	A	*P. nigra*	Cass Co., NE
94-66	A	*P. sylvestris*	Cass Co., NE
94-71	A	*P. ponderosa*	Cass Co., NE
94-73	A	*P. ponderosa*	Cass Co., NE
94-80	A	*P. sylvestris*	Watertown, SD
94-82	A	*P. nigra*	Jasper Co., IA
94-86	A	*P. resinosa*	Cass Co., NE
94-88	A	*P. resinosa*	Cass Co., NE
94-90	A	*P. ponderosa*	Watertown, SD
94-92	A	*P. ponderosa*	Watertown, SD
94-93	A	*P. nigra*	Watertown, SD
94-94	A	*P. nigra*	Adair Co., IA
94-95	A	*P. nigra*	Polk Co., IA
94-96	A	*P. ponderosa*	Watertown, SD
94-100	A	*P. sylvestris*	Watertown, SD
94-111	A	*P. ponderosa*	Yankton, SD
94-113	A	*P. sylvestris*	Halsey, NE
94-115	A	*P. nigra*	Halsey, NE
95-67	A	*P. nigra*	Comanche Co., OK
95-68	A	*P. nigra*	Payne Co., OK
95-69	A	*P. nigra*	Pottawatomie Co., OK
95-70	A	*P. nigra*	Custer Co., OK
95-72	A	*P. nigra*	Seminole Co., OK
95-74	A	*P. nigra*	Canadian Co., OK
239	A	*P. resinosa*	Douglas Co., WI
411	A	*P. resinosa*	Clearwater Co., MN
92-19	A	*P. resinosa*	Wood Co., WI
92-66	A	*P. sylvestris*	Kalamazoo Co., MI
113	B	*P. banksiana*	Gogebic Co., MI
124	B	*P. banksiana*	Jackson Co., WI
215	B	*P. resinosa*	Douglas Co., WI
474	B	*P. resinosa*	Wadena Co., MN

[a] Authors' isolate numbers and, in parentheses, isolate numbers from the culture collection of the USDA Forest Service, Rocky Mountain Forest and Range Experiment Station, National Agroforestry Center.

RESULTS

Amplification products. The eight RAPD primers produced 38 scorable DNA fragments. Sixteen fragments were poly-

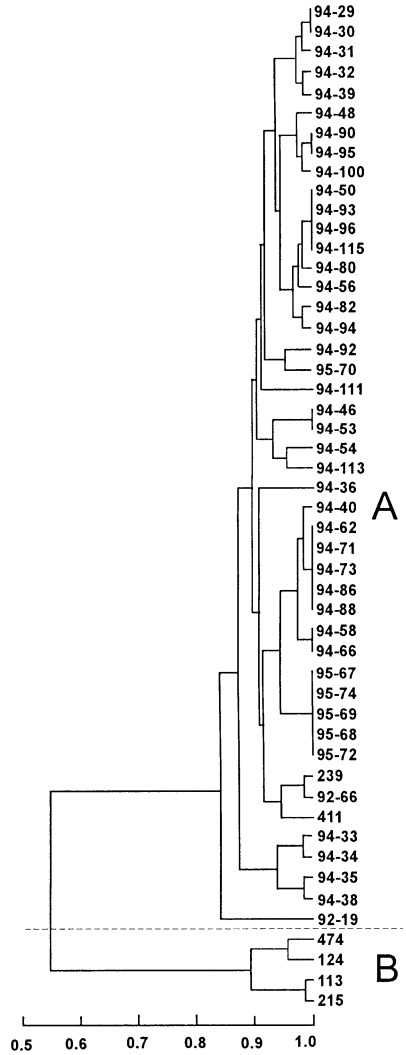

Fig. 1. Dendrogram generated by the unweighted pair-group method using arithmetic averages (UPGMA), with the simple matching coefficient of similarity in the program NTSYS-pc based on 38 random amplified polymorphic DNA fragments. Dotted line separates **(A)** west central U.S. isolates of *Sphaeropsis sapinea* grouped with four previously characterized A morphotype isolates and **(B)** *S. sapinea* B morphotype isolates.

morphic among isolates of a group comprising the west central region isolates and the four previously characterized A morphotype isolates. Twenty-two fragments were polymorphic between this group and the B morphotype isolates. Nine fragments were produced only by the B morphotype isolates. All primers except one (DS4) yielded a banding pattern that differentiated the two morphotypes.

Data analyses. The dendrogram generated by UPGMA placed all isolates from the west central United States in one group with the A morphotype isolates from the north central United States (Fig. 1). The simple matching coefficients of similarity indicated at least 84% similarity among isolates that constituted this group, and 88% similarity among the B morphotype isolates. The simple matching coefficients of similarity indicate 54% similarity between these two groups.

Subgroups often comprised highly similar isolates that had originated from diverse hosts (Fig. 1). This similarity sometimes occurred among isolates from a limited area of collection. For example, five of the seven isolates obtained from four different host species at the Horning State Farm, Cass Co., NE, were monomorphic for all the RAPD markers, with the remaining two isolates very similar to the first five. Different host species at widely separated sites also yielded highly similar isolates. For example, another subgroup comprised isolates 94-48, 94-90, 94-95, and 94-100. These isolates had been obtained from *Picea pungens*, *Pinus ponderosa*, *P. nigra*, and *P. sylvestris* located in three different states.

DISCUSSION

Results of this study are consistent with our previous conclusion that isolates of *S. sapinea* from coniferous hosts can be differentiated into distinct groups by analysis of RAPD markers (21). Unlike collections from several pine hosts in Michigan, Minnesota, Wisconsin, and Michigan that included isolates of both A and B morphotypes, this sample of isolates from the west central United States consisted of only the A morphotype. Similarity among A morphotype isolates should facilitate interpretation of conclusions from previous studies in that region and their application to current research and disease management. Results also indicate the potential for further research on host and environmental influences on the distribution and prevalence of each of these two *S. sapinea* morphotypes.

Results of amplification and data analysis are very similar to those obtained for *S. sapinea* isolates from pine hosts in Michigan, Minnesota, and Wisconsin (21). Twenty-two fragments were polymorphic between the A and B isolates, compared with 20 in the previous study. Similarity (from simple matching coefficients) within the A group in each study was high (85 and 88%) and similarity between the A and B groups in each study was comparable (54 and 52%). These consistencies indicate the potential to use these methods for further clarification of relationships among *S. sapinea* isolates throughout the broad geographic and host range of the pathogen.

Lack of host specialization among the A morphotype isolates might help to explain the very broad host range of *S. sapinea*. In the sample of isolates examined, there was no indication of grouping by host. Rather, several different species (of up to three genera) sometimes were affected by groups of highly similar A morphotype isolates.

In contrast to this nonspecific occurrence of the A morphotype, failure to find B isolates might suggest specialization or limitation of the latter morphotype. Host specialization has been suggested by the previous reports of B morphotype isolates only from *P. banksiana,* or from *P. resinosa* where *P. banksiana* also occurred (14,15, 21,25). The lack of B morphotype isolates in our sample was not, however, due to absence of *P. banksiana* from all collection sites. This species was present at several locations, but was not observed to be affected. The B morphotype might be rare or absent due to unfavorable climate or other geographical limitations. In addition, substantial field resistance of *P. banksiana* to A morphotype isolates might be indicated by absence of shoot blight and canker on this host at our collection locations, and lack of any literature report of *S. sapinea* on this host in the west central United States.

Prevalence of disease associated with the A morphotype isolates might reflect not just their general occurrence, but also response of this morphotype to host condition. Sources of physiological stress, including drought, have been associated with damage by *S. sapinea* to pines (5,12–14, 23,26,30). Recently, differential response of A and B morphotypes to low host leaf water potential has been demonstrated (1, 2). In controlled experiments and a 3-year field study, colonization of wounded, inoculated red pine shoots by A morphotype isolates was enhanced in trees under even moderate drought stress (as low as approximately –1.8 MPa). Colonization by B morphotype isolates was not influenced by host water potential. Isolates of the A morphotype of *S. sapinea* may be particularly able to exploit the effects of harsh sites and periodically severe droughts that occur in the west central United States, especially on coniferous hosts planted outside their natural range.

Occurrence of only a single morphotype among stored isolates from previous investigators and our new collections simplifies the interpretation of past research results. Work in the west central United States elucidated important aspects of the disease cycle including inoculum dispersal, infection biology, phenological effects on host susceptibility, and chemical control (4, 16–19). This research was probably based on observation of the A morphotype only. Future investigations should clarify similarities and differences in fundamental characteristics and distributions of A and B morphotypes. If necessary, disease management recommendations might then be modified to better estimate risks of severe disease and to minimize the impact of Sphaeropsis shoot blight and canker on trees and forest values.

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