Differentiation of *Mycosphaerella dearnessii* by Cultural Characters and RAPD Analysis

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ABSTRACT

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Isolates of *Mycosphaerella dearnessii*, the causal agent of brown spot needle blight of pine, were collected from the northern and southern United States and China and analyzed for differences in cultural morphology, growth rate, conidial germination at various temperatures, and randomly applied polymorphic DNA (RAPD) markers. Differences in cultural morphology and conidial germination were detected between the northern U.S. isolates and the other sources, but not between the southern U.S. isolates and Chinese isolates. Molecular polymorphisms (RAPDs) were also detected among 43 isolates from the United States and China. Northern U.S. isolates composed a distinct "northern group," while southern U.S. and Chinese isolates composed a related "southern group." Results of this study support the existence of races within *Mycosphaerella dearnessii*. The southern United States appears to be the origin of the current Chinese population, as suggested by the dendrograms generated using the molecular data.

Additional keywords: brown spot disease of pine, *Lecanosticta acicola, Pinus elliottii, P. palustris, P. sylvestris, P. taeda*.

Brown spot needle blight, or brown spot disease, incited by the ascomycete *Mycosphaerella dearnessii* Barr (syn. *Scirrhia acicola* (Dearn.) Siggers, anamorph = *Lecanosticta acicola* (Thuem) Syd. in Syd. & Petr.) (13) affects many species of pine including slash pine (*Pinus elliottii* Engelm.), longleaf pine (*P. palustris* Mill.), and Scots pine (*P. sylvestris* L.), in North America, South America, Europe, Oceania, and Asia (1,11,17,22,24). Pine trees of all ages may be affected, but the disease is most severe on young trees. On longleaf pine in the southern United States, *M. dearnessii* causes severe growth reduction or death of seedlings in the "grass" stage, while less severe attack may delay the onset of height growth for 2–10 yr (34). Heavy infection in longleaf pine stands can reduce pulpwood yields up to 60% (41). In the northern United States, defoliation of Scots pine caused by brown spot needle blight can render Christmas trees unmarketable (35).

In southeastern China, severe brown spot disease damage occurs on slash pine, loblolly pine (*P. taeda* L.), and Japanese black pine (*P. thunbergii* Parl.), but in contrast to the southern United States, only slight damage occurs on longleaf pine. Brown spot disease was reported near Nanjing, China in the early 1950s, on Japanese black pine, an indigenous Chinese species, but no severe outbreaks occurred until 1978 (24). This was about 5 yr after large-scale introduction of slash pine seed from the United States. According to Li et al (24), China started to import slash pine seeds from the United States in 1973 and many slash pine plantations were established in southeastern China. Since 1978, thousands of acres of young slash pine plantations have been lost due to brown spot disease. The disease has been especially severe in Fujian province, and has now spread throughout several other Chinese provinces (24). The unprecedented severity of brown spot disease on slash pine in China prompted us to carry out further studies of *M. dearnessii*.

Previous studies of virulence, host range, and physiological features of *M. dearnessii* have reported differences between northern and southern U.S. isolates, but not between southern U.S. and Chinese isolates (16,20,23,36). No significant morphological differences have been reported between cultures from various geographic regions. The need for better methods to examine genetic differences among isolates to establish likely colonization histories in different geographic regions is apparent. Randomly amplified polymorphic DNA (RAPD) markers offer one useful approach for genetic studies, especially for organisms with limited morphological characters (5,9,19,21,25,42). In plant pathology, RAPD markers have been used to "track" plant resistance genes (15,27,31), clarify systematics via "fingerprinting" of plant pathogens (2,4,7,18,26,30,32), study pathogen population (3), and examine evolutionary relationships between pathogen races (6). The asexual stage of *M. dearnessii* is a haploid fungus, which provides an additional advantage of using RAPD markers considered to be dominant. We used RAPD analysis, together with other characteristics of *M. dearnessii*, to characterize isolates from different geographic regions. In addition, we sought to gain insight into the possible origin of this pathogen responsible for the epidemics of brown spot disease in China by comparing isolates of *M. dearnessii* from different geographic regions for a large set of molecular genetic markers.

MATERIALS AND METHODS

Fungal isolates. A total of forty-three isolates of *M. dearnessii* were collected from slash pine or Japanese black pine grown in China (15 isolates), from longleaf pine in the southern United States (14 isolates), and from Scots pine or white pine (14 isolates) in the northern United States (Table 1). One isolate of *Mycosphaerella pini*, which colonizes many of the same pine hosts as *M. dearnessii* and is similar in morphology, was chosen as an

"outgroup" species for taxonomic analyses. All isolates were transferred and maintained as single-spore cultures on potato-dextrose agar (PDA) tube slants (39).

Growth rates at different temperatures. Comparisons of growth rates of different isolates were conducted on PDA petri plates (9 cm diameter) at 24, 28, and 32 C using single-spore cultures of 15 isolates, five from each geographic region. Germinating conidia on 1.5% water agar were transferred to PDA plates, with one conidium on each plate. Growth rate was measured as dry mycelial weight, because of slow increase of colony diameter and relatively massive upward growth of the mycelial mass on the medium surface for this fungus. The mycelial weight of each isolate was obtained by cutting out colonies from PDA plates and drying the mycelia at 110 C for 24 h. The correlation between colony diameter and dry weight was also examined by randomly sampling colonies at 5-day intervals during the 35-day growth period at 24 and 28 C. Growth comparisons were conducted by measuring mycelial dry weight of 3 or 4 colonies per isolate at the 35th day of cultures from single spores.

Statistical analyses were performed using the Minitab software package (Minitab, Inc., State College, PA). Analyses of variance (ANOVA) for nested unbalanced designs were conducted to test for differences among geographic regions and isolates, considering isolates to be nested within geographic sources (29). Student's *t* tests were applied for pairwise comparison when significant differences were found (29).

TABLE 1. Geographic origin, *Pinus* host, and original collection date of isolates of *Mycosphaerella dearnessii* used in this study

Isolate	Source Area	Host	Year
FHJ1	Zhejiang, China	*P. thunbergii*	1991
FHJ2[a]	Zhejiang, China	*P. thunbergii*n	1991
FS[a]	Fujie, China	*P. elliottii*	1988
FXJ1	Jiangxi, China	*P. thunbergii*	1992
FXJ4[a]	Jiangxi, China	*P. thunbergii*	1992
FXS2	Jiangxi, China	*P. elliottii*	1992
FXS4	Jiangxi, China	*P. elliottii*	1992
GXC1	Guanxi, China	*P. elliottii*	1992
GXC2	Guanxi, China	*P. elliottii*	1992
GXS1	Guanxi, China	*P. caribaea*	1992
GXS2[a]	Guanxi, China	*P. caribaea*	1992
XGS3	Jiangxi, China	*P. elliottii*	1992
XGS4	Jiangxi, China	*P. elliottii*	1992
XYS2[a]	Jiangsu, China	*P. elliottii*	1992
XYS4	Jiangsu, China	*P. elliottii*	1992
AL21[a]	Alabama, U.S.A.	*P. palustris*	1992
AL34	Alabama, U.S.A.	*P. palustris*	1992
FLL1[a]	Florida, U.S.A.	*P. palustris*	1992
FLL3	Florida, U.S.A.	*P. palustris*	1992
FLL4	Florida, U.S.A.	*P. palustris*	1992
FLS2	Florida, U.S.A.	*P. palustris*	1992
FLS3[a]	Florida, U.S.A.	*P. palustris*	1992
FLS4	Florida, U.S.A.	*P. palustris*	1992
LAL1	Louisiana, U.S.A.	*P. palustris*	1993
LAL2	Louisiana, U.S.A.	*P. palustris*	1993
LAL3[a]	Louisiana, U.S.A.	*P. palustris*	1993
LAL4	Louisiana, U.S.A.	*P. palustris*	1993
MSL1	Mississippi, U.S.A.	*P. palustris*	1989
MSL2[a]	Mississippi, U.S.A.	*P. palustris*	1989
IAS1	Iowa, U.S.A.	*P. sylvestris*	1993
IAS2	Iowa, U.S.A.	*P. sylvestris*	1993
ILS1[a]	Illinois, U.S.A.	*P. sylvestris*	1993
ILS2	Illinois, U.S.A.	*P. sylvestris*	1993
ILS3	Illinois, U.S.A.	*P. sylvestris*	1993
ILS4	Illinois, U.S.A.	*P. sylvestris*	1993
MNS1	Minnesota, U.S.A.	*P. sylvestris*	1993
MNS2	Minnesota, U.S.A.	*P. sylvestris*	1993
WIS1[a]	Wisconsin, U.S.A.	*P. sylvestris*	1989
WIS2[a]	Wisconsin, U.S.A.	*P. sylvestris*	1989
WIS3	Wisconsin, U.S.A.	*P. sylvestris*	1993
WIS4[a]	Wisconsin, U.S.A.	*P. sylvestris*	1993
WIS5[a]	Wisconsin, U.S.A.	*P. sylvestris*	1993
WIW	Wisconsin, U.S.A.	*P. strobus*	1989
M. pini	Wisconsin, U.S.A.	*P. nigra*	1990

[a] Isolates used in germination and growth rate experiments.

Conidial germination at different temperatures. Comparisons of conidial germination at different temperatures were conducted at 24, 28, and 32 C with 15 isolates, five from each geographic source. Percent germination was determined 48 h after conidia were sprayed on water-agar plates. Statistical analyses were similar to those used in the growth comparisons, with the addition that data were transformed (arcsin square root) to normalize variances prior to analyses (29).

Preparation of fungal genomic DNA. Mycelia were grown from a spore suspension of 10^4 spores per milliliter in 3% maltose liquid medium for 72 h, collected by filtration through Miracloth and washed thoroughly with distilled water. Genomic DNAs were prepared with 3% CTAB (hexadecyltrimethylammonium bromide) following the procedure described by Doyle et al (12). Concentrations of DNA were measured with a DNA fluorometer (model TKO100, Hoefer Scientific Instruments, San Francisco, CA) and adjusted to 0.2 ng/µl with Tris-EDTA buffer (33).

Random primers, PCR cycle, gel electrophoresis, and Southern blot. Ten-base oligonucleotide primers (kits E and Y, Operon Technologies, Inc., Alameda, CA) were used for polymerase chain reactions (PCR) in 12.5 µl aliquot (5.94 µl of water, 1.25 µl of 10× reaction buffer, 1.75 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTP, 1.5 µl of primer[4 picmol/µl], 0.06 µl of Taq polymerase [5 unit/µl] and 1 µl of fungal DNA [0.2 ng/µl]). Mineral oil was added to the reaction solution surface to prevent evaporation. A DNA Thermal Cycler 480 (Perkin Elmer Cetus, Norwalk, CT) was programmed for initiation at 94 C for 7 min followed by 40 reaction cycles of 1.5 min. at 94 C for denaturing, 3 min. at 37 C for annealing and 2 min. at 72 C for extension. PCR products were stored at 4 C following reaction prior to gel electrophoresis. Amplicons were electrophoresed in a 1.5% agarose gel with TAE buffer at 60 V for 90 min. Gels were stained for 15 min in ethidium bromide, destained for 15 min in distilled water, and photographed on a DNA Transilluminator (model 3-3000, Fotodyne Inc., New Berlin, WI). Size of the products was estimated relative to a ΦX17/HaeIII standard (Promega, Madison, WI). Each reaction was repeated twice to eliminate "false positives."

Confirmation of sequence similarities between amplicons which comigrated to the same relative position on gels was conducted using Southern blots. DNA was denatured by soaking gels in a solution of 1.5 M NaCl/0.5 M NaOH for 30 min, then trans-

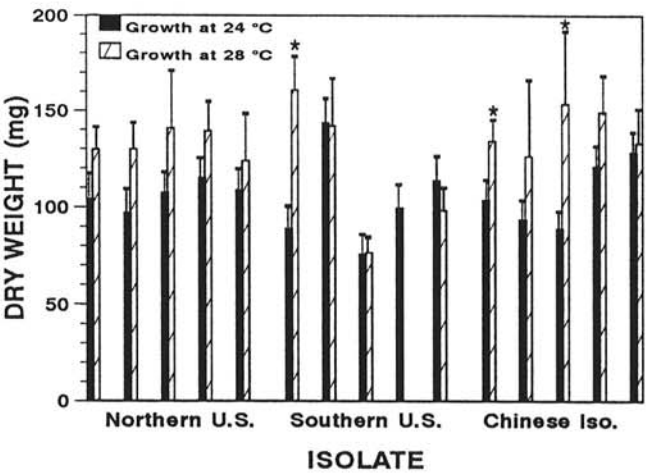

Fig. 1. Dry weight differences among isolates of *Mycosphaerella dearnessii* from different geographic regions grown at 24 and 28 C. Error bar indicates one standard deviation. Isolates that showed significant differences in growth at two temperatures are marked by asterisks ($P < 0.05$); the others are not significantly different.

ferring to a solution of 3 M NaCl/0.5 M Tris (pH 7) for 30 min. Amplicons were transferred from agarose gels to nylon membranes (Blotting Membranes, Bio-Rad Laboratories, Richmond, CA) as described by Sambrook et al (33). Probes were prepared from amplicons extracted from agarose gel using a Prep-A-Gene Kit (Bio-Rad Laboratories), and labeled with ^{32}P using a Random Primed DNA Labeling Kit (Boehringer Mannheim GmbH, Mannheim, Germany). Hybridizations were conducted at 42 C for 16 h following the procedure described by Sambrook et al (33).

Analysis of RAPD data. Amplicons separated in gels were scored as discrete character states (presence/absence) for phenetic analysis using the Fitch-Margoliash method, and Unweighted Pair Group Means Analysis (UPGMA), which were performed by program PHYLIP (Phylogeny Inference Package developed by J. Felsenstein, version 3.5) (14,38). The Fitch-Margoliash method (14) uses the pairwise distance index to construct a dendrogram. A pairwise distance matrix was calculated with the Nei and Li distance index (28): $D_{xy} = 1 - 2N_{xy}/(N_x + N_y)$ in which N_{xy} is the number of fragments shared between a pair of isolates, and N_x and N_y are the numbers of fragments present in isolate x and y, respectively. The same distance matrix was also used to generate a rooted phenetic tree using the UPGMA method (38).

TABLE 2. Summary analysis of variance for mycelial dry weight from isolates representing 5 collections in each of 3 geographic regions (China, southern U.S., and northern U.S.)

Source	df	SS	MS	F
Isolates grown at 24 C for 35 days				
Geographic regions	2	243.7	121.9	0.11
Isolates(regions)	12	13,843.8	1,153.7	2.76[a]
Error	39	16,282.9	417.5	
Total	53	30,370.4		
Isolates grown at 28 C for 35 days				
Geographic regions	2	3,584.7	1,792.4	1.07
Isolates(regions)	11[b]	18,486.3	1,680.6	2.73[c]
Error	36	22,122.0	614.5	
Total	49	44,193.0		

[a] Significant difference, $P < 0.01$.
[b] One isolate failed to grow normally at this temperature.
[c] Significant difference, $P < 0.05$.

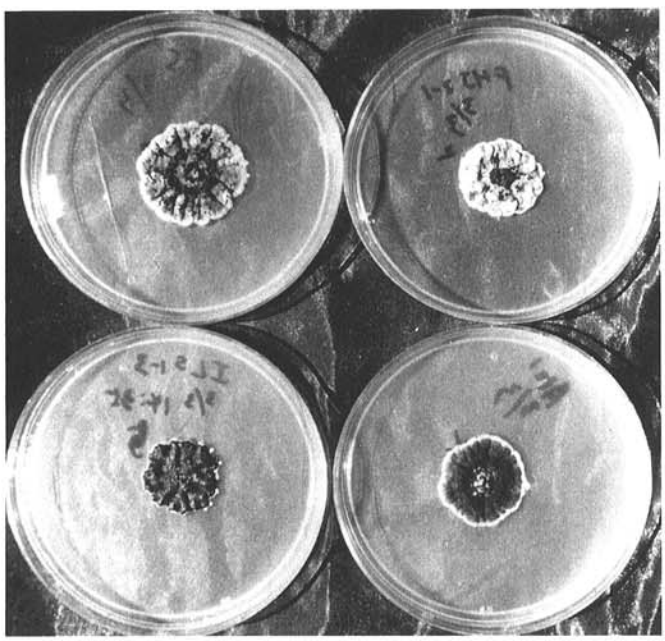

Fig. 2. Colony morphology differences between northern U.S. isolates of *Mycosphaerella dearnessii* and those from other geographic regions. ILS1, a northern isolate (lower left), is slimy and black, while (clockwise from upper left) the Chinese (FS, FHJ2) and southern U.S. (AL21) isolates are dry and gray.

RESULTS

Comparison of growth at different temperatures. Isolates from the northern United States grown at 32 C showed no growth until 15 days and produced fewer colonies on PDA plates than isolates from the southern United States and China (data not shown). During this 15-day period, all isolates grew too slowly to make growth rate measurements. Colony diameter at 24 and 28 C increased linearly during the 35-day culture period. Mycelial dry weight was significantly correlated with colony diameter, with correlation coefficients of $r = 0.958$ at 24 C and $r = 0.967$ at 28 C ($P < 0.05$). Almost all isolates tested grew faster at 28 than at 24 C, but only a few showed statistically significant differences in dry weight at 35 days (Fig. 1). ANOVA of mycelial dry weight data indicated significant differences among isolates within geographic regions, but not between geographic regions (Table 2). Different colony morphologies were also observed among isolates (Fig. 2); after 2 wk of growth on PDA, colonies of isolates from the northern United States were slimy and black due to a confluent layer of conidia. Colonies of isolates from the southern United States and China did not form such a conidium layer and were gray in color.

Comparison of conidial germination at different temperatures. Isolates from the southern U.S. and China germinated better at 24 and 28 C than at 32 C, and generally had a higher germination percentage than isolates from the northern United States (Fig. 3). The conidial germination of northern U.S. isolates decreased rapidly with increasing temperature. At 32 C, only 5.1% of the conidia of northern U.S. isolates germinated, whereas 83.1 and 84.4%, respectively, of the conidia of southern U.S. and China isolates germinated.

RAPD analysis and sequence similarities of amplicons. Twenty of 40 primers tested detected polymorphisms among isolates (Fig. 4) while five primers (E06, E08, E18, Y01, and Y14) revealed no polymorphisms. The other 15 primers either did not yield consistent results, or produced indistinguishable bands under the conditions used. One hundred and sixteen band positions were scored from the twenty 10-base oligonucleotide primers (Table 3). Amplicons from primer Y13 were examined using Southern blots to check sequence similarities between amplicons from different isolates that comigrated at the same relative mobility. Bands 4 and 7 of isolate FHJ1 were used as probes, and hybridized exclusively to comigrated bands from other isolates (Fig. 4).

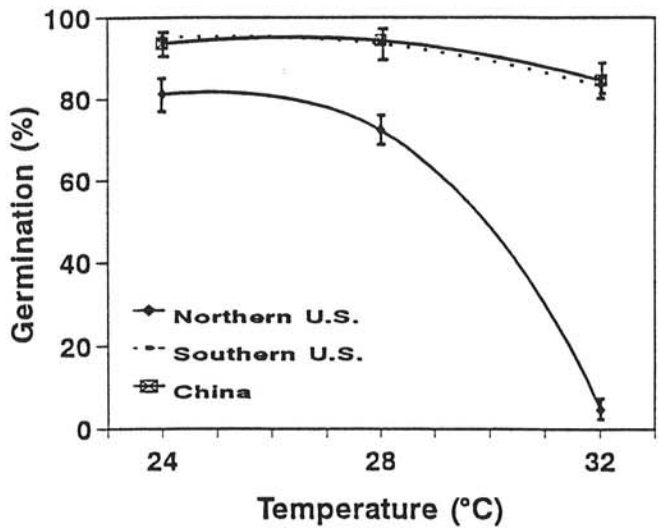

Fig. 3. Conidial germination of *Mycosphaerella dearnessii* isolates at 24, 28 and 32 C. Isolates from the southern United States and southeastern China had a high germination percentage at all temperatures tested, while the germination of northern U.S. isolates decreased rapidly as temperature increased.

Similarities of isolates from different geographic regions. A phenetic tree constructed using the RAPD genetic distance matrix of the Fitch-Margoliash method revealed that all isolates of *M. dearnessii* were clustered into two groups: a "northern group" consisting of all isolates from the north central United States, and a "southern group" consisting of all isolates from the southern U.S. and southeastern China (Fig. 5). Within the southern group, all Chinese isolates clustered together to form a distinct group. One U.S. isolate, MSL1, from Mississippi, provided the connection between the Chinese isolates and the southern U.S. isolates. The outgroup species, *M. pini*, was clearly distinct from *M. dearnessii* indicating that, despite their morphological similarity, *M. pini* and *M. dearnessii* are not closely related.

The UPGMA method generated a similar dendrogram (not shown) to that of the Fitch-Margoliash method with distinctive "southern," "northern," and Chinese groups. Both dendrograms suggest that the Chinese group is closely related to the southern U.S. population but is more distant from the northern U.S. population. In both phenetic trees, only modest differentiation is associated with geographic separation within regions, e.g., isolates GXC1, GXC2, GXS1, and GXS2, and LAL1, LAL2, LAL3, and LAL4. However, no apparent differences were associated with different hosts among the Chinese isolates.

DISCUSSION

In this study, northern U.S. isolates of *M. dearnessii* could be distinguished both from southern U.S. isolates and Chinese isolates based on conidial germination at high temperatures, cultural morphology, and RAPD markers. Physiological and morphological studies of *M. dearnessii* isolates from different hosts and geographic areas have been reported (16,20,23,36). Snow (36) reported no differences in the morphology, physiology or virulence of isolates from loblolly pine and longleaf pine. Kais (20) reported clear differences in physiology and virulence on different pines, but no differences in morphology between northern and southern isolates; he first proposed the possibility of subspecific taxa. The high percentage of germination for the southern U.S. isolates in this paper is consistent with Kais's report (20). Li et al (23) reported that Chinese isolates were similar to one southern U.S. isolate, but different from a northern U.S. isolate in terms of cultural characters and virulence.

The similarities between the southern U.S. isolates and the Chinese isolates and the differences between southern isolates and northern isolates indicated by RAPD analysis are consistent with previous observations (16,20,23). Our analysis of RAPD data for *M. dearnessii* revealed similarities and presumptive evolutionary relationships that could not have been elucidated using physiological or morphological data alone. As shown in the den-

TABLE 3. Nucleotide sequence (5'-3') of the 20 10-mers used in the study and the numbers of bands scored among isolates

Primer	Sequence	No. of Bands
E01	CCCAAGGTCC	1–2
E02	GGTGCGGGAA	2–3
E04	GTGACATGCC	0–6
E07	AGATGCAGCC	1–3
E11	GAGTCTCAGG	1–4
E12	TTATCGCCCC	2–5
E13	CCCGATTCGG	0–5
E14	TGCGGCTGAG	1–3
E15	ACGCACAACC	2–5
E16	GGTGACTGTG	2–4
E20	AACGGTGACC	0–1
Y02	CATCGCCGCA	0–4
Y05	GGCTGCGACA	4–5
Y07	AGAGCCGTCA	1–5
Y10	CAAACGTGGG	1–4
Y13	GGGTCTCGGT	3–5
Y15	AGTCGCCCTT	1–4
Y16	GGGCCAATGT	2–3
Y17	GACGTGGTGA	1–5
Y20	AGCCGTGGAA	2–4

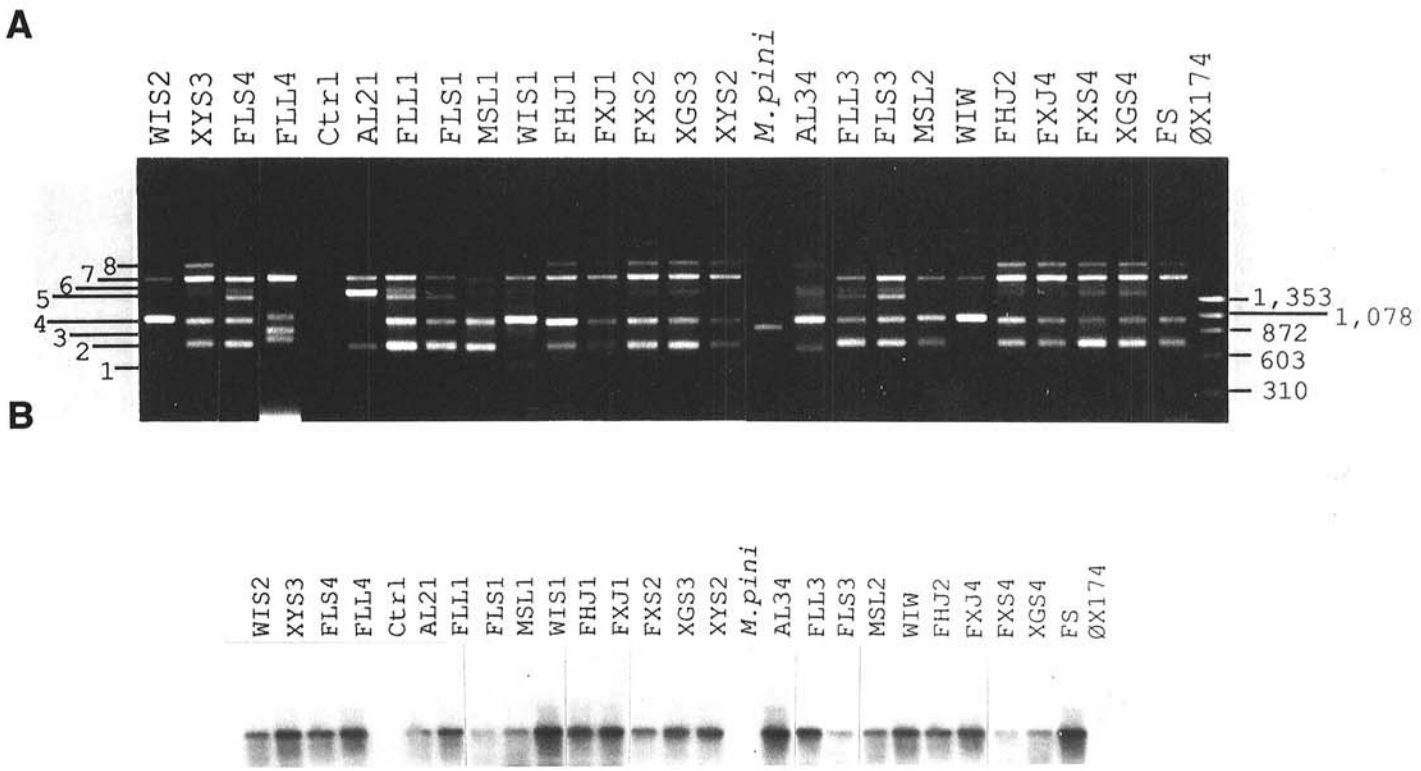

Fig. 4. A, RAPD fragments amplified with primer Y13. Phi X174/HaeIII shown as a standard size marker labeled in basepairs. See table 1 for the geographic regions of each isolate. Reaction without fungal DNA is used as control (Ctrl). **B,** Southern blot of the upper gel probed with band 7 of isolate FHJ1. This probe hybridized exclusively to bands of the same mobility from all isolates.

drogram (Fig. 5), southern U.S. isolates appear to have a slightly greater diversity than Chinese isolates. From the positions of isolates on the dendrogram, it appears that the Chinese isolates could have originated from one or more southern U.S. populations and subsequently evolved into a distinct population, but exactly when and how *M. dearnessii* was introduced to China remains to be answered. Southern and northern isolates in the United States appear quite distinct, probably as a result of geographic isolation and subsequent adaptation to different climatic conditions or hosts.

In China, Li et al (23,24) observed that by the mid-1980s Japanese black pine, an indigenous species in China, was heavily infected in areas isolated from slash pine or other North American pines. In addition, brown spot disease apparently was unknown in some provinces where slash pine was also introduced from the United States. Li et al concluded that Chinese isolates of *M. dearnessii* existed before the era of massive slash pine seed imports and were the cause of brown spot disease in such instances. Based on our RAPD analysis and the work of Li et al (23,24), we conclude that the introduction from the United States of new, highly virulent isolates was not likely to be responsible for the epidemic of brown spot disease in southeast China beginning in 1978. It could be hypothesized that the epidemic of brown spot disease on slash pine in China had one or more possible causes including 1) existing Chinese strains of *M. dearnessii* were highly virulent on newly introduced U.S. seed sources of slash pine; 2) the existence of localized and unique edaphic conditions favored infection of slash pine; and 3) widely planted monocultures created ecosystem conditions conducive to epidemics. This last phenomenon is believed to be responsible for the increase in fusiform rust (*Cronartium fusiforme* Hedgc. & N. Hunt ex Cumm.) epidemics in the southern United States (10). Critical testing of these hypotheses would help clarify the dynamics of epidemics of brown spot disease worldwide.

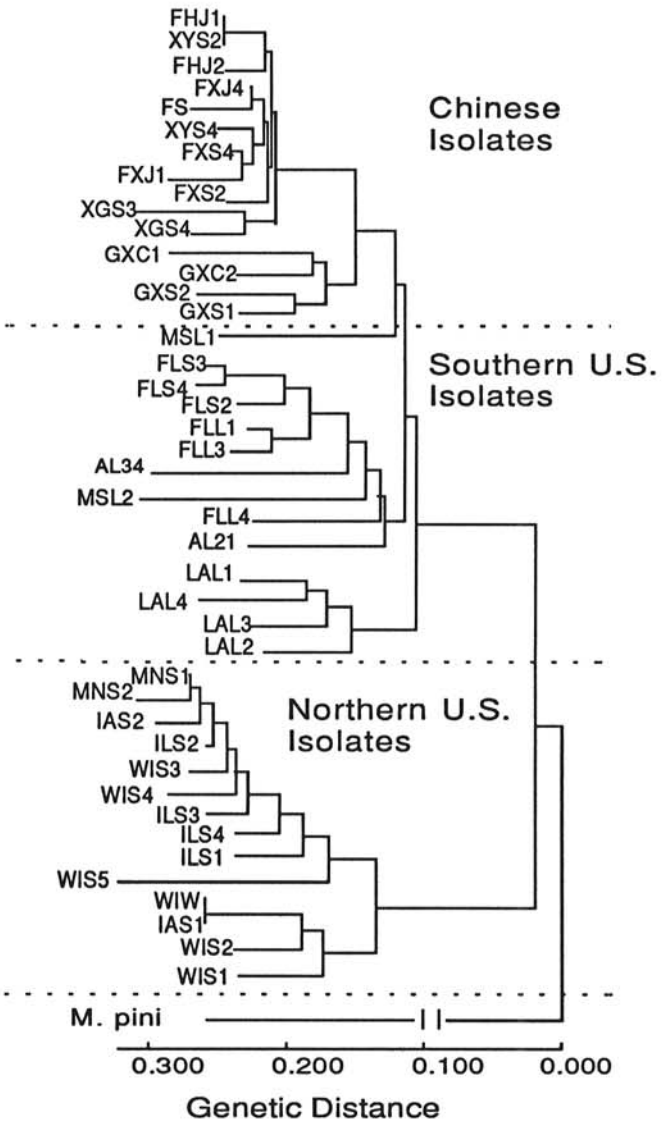

Fig. 5. Fitch-Margoliash phenetic tree based on randomly applied polymorphic DNA (RAPD) data from isolates of *Mycosphaerella dearnessii* generated by PHYLIP. Horizontal lines are proportional to distance values as indicated on the bottom scale. The distance from *M. pini* to its nearest branch is 0.531.

Results from Southern blot analyses suggest that comigrated amplicons of unique primers have high genetic similarities, but we cannot infer identity by descent between U.S. and Chinese isolates. Our results together with previous reports suggest that comigrated RAPD products of the same primer can be assumed to possess a high degree of genetic similarity as long as the variability characterized is intraspecific (9,11). Molecular polymorphisms have been successfully used in progenitor-derivative species studies that trace the population lineages within a species (8,37,40). A study of both northern and southern races of *M. dearnessii* to determine historic worldwide colonization sequences of races remains to be conducted. The differences between northern U.S. isolates and southern U.S. isolates may also be caused by different origins of these two populations. The northern U.S. isolates may be closer to European isolates according to their host species and geographic climatic conditions (11), but similarities and relationships between isolates from these two regions, as well as to isolates from other regions such as Latin America, remain to be examined.

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