Identification of Gremmeniella abietina Races with Random Amplified Polymorphic DNA Markers

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Seven random amplified polymorphic DNA (RAPD) markers amplified from four oligonucleotides (10-mers) by the polymerase chain reaction were used to distinguish between the North American and European races of Gremmeniella abietina, the causal agent of Scleroderris canker of conifers. Forty-three isolates of the pathogen from 11 different host species originating from 11 countries, states, and provinces were tested; race designation was consistent with results from immunogenic and soluble-protein assays. By using RAPD markers, it was possible to identify G. abietina races by DNA amplifications directly from fruiting bodies, thus eliminating the need to culture the fungus, as is necessary with immunogenic and soluble-protein assays. Two isolates which had been previously classified as intermediate were clearly identified as belonging to either one of the two races by using RAPD markers. No interracial hybrids were detected in our survey. Patterns of amplification products from the European race in North America were identical to patterns of European isolates, further substantiating that this is an introduced race to the North American continent.

Scleroderris canker, caused by the ascomycete Gremmeniella abietina (Lagerb.) Morelet [= Scleroderris lagerbergii Gremmen, = Ascochyta abietina (Lagerb.) Schlapher-Bernhard; anamorph, Brunchorstia pinea (Karst.) Hoehn], has been recognized as a serious conifer disease in Europe for more than a century (3). It was identified in 1965 in North America (9), where it caused considerable damage, especially in nurseries and to small trees in plantations. In the 1970s, a severe epidemic developed in the state of New York (12), but the symptoms associated with this new epidemic differed from the classical symptoms by their presence in the tops of large trees, by the general lack of stem cankers, and by the almost complete absence of the sexual stage of the fungus. It was observed that these symptoms resembled the symptoms caused by this pathogen in Europe (5, 12). Growth rates, conidium size, and immunogenic reactions established that two races of the fungus were present in North America and that the new race was serologically identical to the race present in Europe. The new race was therefore called the European (EU) race, while the other race was named the North American (NA) race (4, 5).

As a result of these findings, and because the EU race was reported to have a broader host range than the NA race (3, 13), quarantines were established to contain the EU race of this pathogen and prevent its spread to states and provinces where it was not already present (1). As an alternative to the serological assay, a soluble-protein assay was developed to provide characteristic protein banding patterns of the two races. This method was generally slightly faster and less tedious than the serological assay, and it also allowed the differentiation between other taxa of Gremmeniella spp. (12). However, this technique still necessitated culturing the fungus (which requires approximately 1 month), and problems were encountered with protein variability within race and even within sample (2, 10, 11). For example, it was found that the protein banding patterns could vary with the age of the cultures. Also, there were difficulties with samples that could not be assigned to either race (9a).

The presence of putative hybrids has been reported (2, 6, 10, 14, 15, 17). However, on the basis of present techniques, it was impossible to confirm or disprove the hypothesis that the two races hybridize in nature.

To overcome these limitations, we have used the random amplified polymorphic DNA (RAPD) technique to provide a diagnostic tool for the identification of the races of this pathogen. This diagnosis is rapid, reproducible, and uses minimal amounts of tissue, thereby allowing identification of the pathogen without culturing. This article describes our use of selected RAPD markers to differentiate between races of G. abietina from the mycelium as well as directly from fruiting bodies.

MATERIALS AND METHODS

Samplers. A total of 43 isolates collected from 11 conifer species in the northeastern United States, Canada, and Western Europe were studied (Table 1). The samples were cultured for 1 month on dialysis membranes that were placed on petri dishes containing an agar medium consisting of 25 ml of Campbell's V-8 juice, 15 g of Bacto Agar, 7.5 g of malt extract, and 475 ml of water dispensed at 25 ml per petri dish; the mycelium was either used immediately or lyophilized and stored at −20°C until needed. Samples for amplifications from fructifications (pycnidia and cryptopycnidia) were obtained as part of an annual survey of G. abietina (courtesy of Solange Simard, Ministère des Forêts du Québec) or were collected from the Laurentian Park (courtesy of Gaston Laflamme, Laurentian Forestry Centre). In both cases, the infected branches were stored at room temperature in paper bags until needed.

DNA extractions. DNA was extracted by a modified cetyltrimethylammonium bromide protocol (19). Approximately 10 mg of lyophilized mycelium was ground in liquid nitrogen with a mortar and pestle. Seven hundred microliters of extraction buffer [700 mM NaCl, 50 mM Tris-HCl (pH 8), 10...
mM EDTA, 1% (β-2-mercaptoethanol, 1% cetyl-trimethylammonium bromide) was added to each mycelium sample, and the mixtures were incubated at 65°C for 1 h. The mixtures were emulsified by adding an equal volume of chloroform-isooamyl alcohol (24:1), vortexing, and centrifuging for 5 min at 12,000 × g. The upper phases were precipitated with 75 μl of ammonium acetate (7.5 mM) and 600 μl of cold absolute ethanol and centrifuged for 5 min at 10,000 × g. The resulting pellets were washed with 70% ethanol and then dried and resuspended in 50 μl of TE8 (10 mM Tris-HCl [pH 8], 1 mM EDTA). DNAs were stored at 4°C until needed. A 1:10 dilution of each extraction was used for the amplifications.

For extractions from fruiting bodies, one pycnidium or cryptocystidium was ground with a disposable polypropylene pellet pestle in a 1.5-ml Eppendorf tube containing 50 μl of extraction buffer and approximately 10 mg of diatomaceous earth (Sigma Chemical Co., St. Louis, Mo.); 350 μl of extraction buffer was added, and the extraction was performed as already described. A 1:10 dilution was used for these amplifications.

**DNA amplifications.** Amplifications, modified from the method of Williams et al. (18), were performed in volumes of 25 μl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl₂, 0.0001% gelatin, 100 μM each deoxynucleoside triphosphate (Pharmacia, Uppsala, Sweden), 0.2 μM oligonucleotides (10-mer Kit A; Operon Technologies, Alameda, Calif.), 2 μl (approximately 10 ng) of genomic DNA, and 0.5 U of Taq DNA polymerase (Boehringer Mannheim Biochemical, Mannheim, Germany) overlaid with a drop of mineral oil. Amplifications were performed in a DNA thermal cycler (model 480; Perkin-Elmer Cetus, Norwalk, Conn.) programmed for a denaturation step at 94°C for 3 min, followed by 1 cycle at 35°C for 4 min and 72°C for 2 min, and then 45 cycles of 94°C for 1 min, 35°C for 1 min, and 72°C for 2 min. The amplifications ended with a 10-min extension at 72°C. Amplification products were separated by electrophoresis on 1.2 or 1.5% agarose gels by using TAE buffer (40 mM Tris-acetate–1 mM EDTA [pH 8.0]) and visualized by UV fluorescence after ethidium bromide staining.

**Data analysis.** We selected five isolates (NA isolates 4093, 4338, and 4648; EU isolates 3459 and 4131) to screen a total of 20 oligonucleotides for RAPD polymorphisms. Oligonucleotides that produced reproducible polymorphisms interracially but not intraracially were then retained to screen the remainder of our collection. Diagnostic polymorphisms were identified by the name of the oligonucleotide and the size of the DNA amplification product. Amplification of polymorphic primer combinations were repeated at least twice to ensure reproducibility.

### RESULTS

Four primers (OPA3, OPA4, OPA7, and OPA11) produced seven amplification products that were monomorphic intraracially but polymorphic interracially. Amplifications with primer OPA3 resulted in the production of a 1,700-bp DNA fragment in all of the NA samples tested but in none of the EU isolates. However, amplifications with the same primer produced a DNA fragment (750 bp) in all EU isolates which was absent in NA isolates (Fig. 1A; Table 2). Similarly, amplifications with primer OPA4 resulted in a 2,100-bp product in all NA isolates but in none of the EU isolates (Table 2). Two other primers allowed separation of the EU and NA races: amplifications with primer OPA11 produced a 1,200-bp fragment only in NA isolates and a 2,300-bp DNA fragment only in EU isolates (Fig. 1B; Table 2), and OPA7 produced two DNA fragments (1,800 and 850 bp) that were present in EU isolates but absent in NA isolates (Fig. 2; Table 2). The remainder of the primers tested produced polymorphisms both inter- and intraracially and were therefore not considered useful for diagnosis purposes. However, such primers would be useful to study intraracial variability and population structure.

Although some primers were essentially monomorphic intraracially, others showed intraracial polymorphisms. For example, amplifications with primer OPA7 produced two genotypes in the EU race from North America, but eight genotypes were found in the NA race (Fig. 2). Nei’s measure of gene diversity (8) (the probability that two individuals
sampled at random (differ in type) was 0.167 in EU isolates from North America and 0.894 in NA isolates.

Patterns of amplification from pycnidia and cryptopycnidia were clear and consistent with amplifications from mycelial DNA (Fig. 3).

All of our race assignments were consistent with those previously given by using the soluble-protein assay. However, two samples, 1499p and US0015, which until now could not be assigned to a race, were clearly identified as belonging to the NA and EU races, respectively (Fig. 1). No apparent hybrids were detected in our collections.

**TABLE 2. RAPD markers diagnostic of NA and EU races of *G. abietina***

<table>
<thead>
<tr>
<th>Primer*</th>
<th>Length of fragment (bp)</th>
<th>Race identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPA3</td>
<td>1,700</td>
<td>NA</td>
</tr>
<tr>
<td>OPA3</td>
<td>750</td>
<td>EU</td>
</tr>
<tr>
<td>OPA4</td>
<td>2,100</td>
<td>NA</td>
</tr>
<tr>
<td>OPA7</td>
<td>1,800</td>
<td>EU</td>
</tr>
<tr>
<td>OPA7</td>
<td>850</td>
<td>EU</td>
</tr>
<tr>
<td>OPA11</td>
<td>1,200</td>
<td>NA</td>
</tr>
<tr>
<td>OPA11</td>
<td>2,300</td>
<td>EU</td>
</tr>
</tbody>
</table>

* Primers were 10-mers obtained from Operon Technologies. Primer sequences are as follows: OPA3, 5'-AGTCAGCCAC-3'; OPA4, 5'-AATCGG CTG-3'; OPA7, 5'-GAAACGGGTG-3'; OPA11, 5'-CAATCGCCGT-3'.

**FIG. 1.** Amplification products of *G. abietina* DNA from NA and EU races by using oligonucleotide primers OPA3 (A) and OPA11 (B). Arrows indicate diagnostic DNA fragments that are monomorphic intraracially but polymorphic interracially.

**FIG. 2.** Amplification products of *G. abietina* DNA from NA and EU races by using oligonucleotide primer OPA7. Arrows indicate diagnostic DNA fragments that are monomorphic intraracially but polymorphic interracially.

**DISCUSSION**

The results clearly indicate the potential for using DNA amplification techniques to quickly assign *G. abietina* to either of its two most important races found in North America. This technique offers several advantages over the conventional diagnostic tools, serological and soluble-protein assays. The RAPD technique is quick, reliable, insensitive to the age of the tissue sampled, and requires only minute amounts of material for identification. Since DNA can be extracted directly from fructifications, it is now possible to identify races of *G. abietina* without having to culture the fungus. This will greatly facilitate surveys of the frequency and distribution of these two races in North America and allow unequivocal identification of interracial hybrids.

Our results support findings from previous surveys based on serological and soluble protein assays concerning the probable common origin of the EU race present in North America and the race present in Europe (4, 5). Even though isolates originated from different countries and continents, the patterns of amplification products were consistent intraracially for the EU race for most of the primers studied but clearly different from the NA race.

In recent surveys of Canada and the United States, both...
races were found in New York, Vermont, New Hampshire, Maine, Québec, Ontario, and New Brunswick, while only the NA race was present in Newfoundland and only the NA race was present in Michigan, Minnesota, and Wisconsin (7, 15, 16). However, the serology assay did not provide information concerning intraracial variability. It is interesting to note, as was reported for protein profiles (10), that very low levels of polymorphism were detected among representatives of the EU race in North America but some primers revealed high levels of intraracial polymorphism in the NA race (e.g., OPA7 Fig. 2). Although more data need to be gathered to compare genetic diversity in the European and North American populations of the EU race, the preliminary results reported here are consistent with the hypothesis of a fairly recent introduction of the EU race to North America (5) and the resulting founder effect (i.e., colonization by few individuals) and reduction of genetic variability. Putative interracial hybrids showing symptoms characteristic of both races have been reported previously by using serological and soluble-protein assays (2, 6, 10, 14, 15, 17). For example, one isolate found in the upper crown of a tree (typical of the EU race) also produced abundant apothecia (typical of the NA race) (15). In our limited collection, two isolates classified as intermediate by using either the serology (6) or soluble-protein assay (9a) were clearly assigned to a race by each of our seven RAPD markers. Since we chose our markers for their intraracial monomorphism, true hybrids should be easily detected with our assay, resulting in the presence of some markers diagnostic of both races in a single isolate. Nevertheless, a more exhaustive survey of the G. abietina populations with RAPD markers, especially in areas of heavy infection with both races, might detect the presence of interracial hybrids. A significant improvement reported here is the possibility to assign races directly from fruiting bodies, thereby eliminating the tedious step of culturing the fungus prior to the assay. Furthermore, amplifications of DNA extracted from either conidia or cryptocaryomycia yielded the same patterns of amplified DNA fragments. This could be important since different types of fruiting bodies are often present on different samples. The technique described here can be further refined by sequencing diagnostic DNA fragments and synthesizing race-specific primers which would amplify a single diagnostic DNA fragment (sequence-tagged sites) instead of complex patterns of amplification products. This improvement would allow identification of G. abietina races from any infected tissue even prior to sporulation and severe epidemic development.

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REFERENCES


