Development of a PCR-RFLP Based Detection Method for the Oak Pathogens Diplodia corticola and D. quercivora

Tyler J. Dreaden, John M. Davis, and Jason A. Smith, School of Forest Resources and Conservation, University of Florida, Gainesville, FL; and Michael J. Wingfield, Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, South Africa

Accepted for publication 14 February 2014. Published 29 April 2014.

ABSTRACT


The canker pathogens Diplodia corticola and D. quercivora were first reported affecting oaks in the United States in 2010 and 2014, respectively. A rapid and cost-effective method to identify isolates of both fungal species was needed to facilitate survey and detection efforts. This was achieved by developing selective primers that generate a PCR product followed by a restriction enzyme digestion with MseI to differentiate D. corticola from D. quercivora amplicons.

INTRODUCTION

Diplodia corticola A. J. L. Phillips, Alves et Luque is a well-known canker pathogen of oak (Quercus spp.) that is contributing to the decline of oaks in the Mediterranean region (2). Recently, the pathogen has been affecting Quercus spp. in California, Vitis vinifera in California and Texas (9,13,12), and live oak (Q. virginiana Mill.) in Florida (4). D. quercivora Linaldeddu & A. J. L. Phillips is closely related to D. corticola and causes similar cankers on oaks and has only been found in Tunisia and Florida (8,5).

In 2010, live oak trees in a landscape in Florida were found with numerous branch cankers that were caused by D. corticola (4). In 2011, after recovering additional morphologically similar Diplodia isolates, DNA sequencing revealed that they fell into two groups, D. corticola (4) and a fungus that was later described as D. quercivora (5,8). The two species can be distinguished based on morphology and by comparing DNA sequences (8); however, these methods are time consuming and increase the cost of differentiating isolates.

The goal of this study was to develop a method to rapidly and cost-effectively screen and identify suspect isolates from oak as either D. corticola or D. quercivora based on DNA, without the need for sequencing. Here we describe the development of an assay that involves PCR with taxa-specific primers and restriction enzyme digestion to amplify and differentiate DNA from D. corticola and D. quercivora.

PRIMER DESIGN AND EVALUATION

One isolate each of D. corticola and D. quercivora recovered from Q. virginiana in Florida (isolate PL1010 accession is KF500478; isolate PL1345 accession is KF500479).

D. corticola was also recovered in the Mediterranean region (3,4) with numerous branch cankers that were caused by D. corticola (4). In 2011, after recovering additional morphologically similar Diplodia isolates, DNA sequencing revealed that they fell into two groups, D. corticola (4) and a fungus that was later described as D. quercivora (5,8). The two species can be distinguished based on morphology and by comparing DNA sequences (8); however, these methods are time consuming and increase the cost of differentiating isolates.

The goal of this study was to develop a method to rapidly and cost-effectively screen and identify suspect isolates from oak as either D. corticola or D. quercivora based on DNA, without the need for sequencing. Here we describe the development of an assay that involves PCR with taxa-specific primers and restriction enzyme digestion to amplify and differentiate DNA from D. corticola and D. quercivora.

Corresponding author: Tyler J. Dreaden. Email: tdreaden@ufl.edu

doi:10.1094/PHP-RS-13-0122
© 2014 The American Phytopathological Society
DNA samples used in this study was checked by amplifying a portion of the 18S small subunit (SSU) rDNA, using primers NS1/NS4 to confirm they contained amplifiable DNA (14). PCR consisted of 2.5 µl of 10x ImmoBuffer, 2.5 µl of 2 mM deoxynucleotide triphosphates (Bioline USA Inc., Taunton, MA), 1.5 µl of 50 mM MgCl₂, 1 µl of 10 mM DcDq1 primer, 1 µl of 10 mM DcDq1a primer, 1 µl of 10 mM DcDq3 primer, 0.125 µl immolase DNA polymerase (Bioline USA Inc.), 14.375 µl H₂O, 1 µl of 10 mM DcDq1a primer, 1 µl of 10 mM DcDq3 primer, 0.125 µl deoxynucleotide triphosphates (Bioline USA Inc.), 14.375 µl H₂O, and 1 µl of DNA template. PCR conditions for DcDq1 (CGAGTGCTACGACCGAGGA) / DcDq1a (CGAGTGCTACGACCGAGGA) / DcDq3 (GAACCCACTTCCGGAGGT) were optimized by testing a range (52-68°C) of annealing temperatures. The final thermocycler conditions consisted of 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 45 s.

RESTRICTION ENZYME DIGESTION

Geneious Pro 5.6.6 was used to identify restriction enzyme digestion sites that could distinguish PCR amplicons, using primers DcDq1/DcDq1a/DcDq3, of D. corticola from D. quercivora. An MseI site was found that produces different sized fragments that could readily separate D. corticola from D. quercivora (Table 2). The restriction enzyme digestion consisted of 0.25 µl MseI 10,000 units/ml, 10 µl PCR amplicons in PCR buffer, incubation at 37°C for 8 h and then viewed on 2% (w/v) agarose gels (Fig. 3). D. corticola and D. quercivora could be distinguished by the absence of the 173 bp band and presence of the 83/88 bp band in D. quercivora for all DNA samples with at least 1 ng of DNA.

When digested amplicons were stained by mixing SYBR Green with loading buffer as described above, the resulting gel revealed bands that were less clear (Fig. 3) when compared to post staining with SYBR Green (data not shown) or staining with ethidium bromide (Fig. 3). But, D. corticola and D. quercivora were differentiated with all three staining methods tested. Under these non-optimal restriction digestion conditions (digestion in PCR buffer), signs of incomplete digestion were observed (Fig. 3). This could likely be eliminated by using greater quantities of primers DcDq1/DcDq1a/DcDq3.
enzyme, less DNA, or by purifying the PCR product and digesting it using the recommended buffer. However, the small levels of incomplete digestion did not affect the ability to differentiate the two species, so no extra steps are required for their identification.

The use of a restriction enzyme digestion of the D. corticola/D. quercivora amplicons eliminates the need for DNA sequencing; rather, polymorphism at an enzyme recognition site (5’-TTAA-3’) is detected. The complete sequence of the LSU from D. quercivora holotype (CBS 133852) is not available to us, but we infer the holotype is near-identical to D. quercivora PL1345 (from this study), since the two isolates have only 2 bp differences in the EF1 and ITS loci (5). Additionally, this region of the LSU is highly conserved with only 1 bp difference between D. corticola PL1010 and D. quercivora PL1345.

SURVEY OF D. CORTICOLA/D. QUERCIVORA ISOLATES

A survey of fungal isolates from symptomatic live oaks was conducted which included 9 isolates from 6 counties in Florida (Table 3). The samples were first cultured on artificial media (4,5). DNA was extracted from the cultures and the quality confirmed, as described above. PCR and the MseI digestion was then performed, as described, using D. corticola/D. quercivora positive controls and a no-template negative control present for all PCR runs. The unknown isolates were then identified by the distinctive bands (Table 2) when the MseI digested PCR amplicons were fractionized on an agarose gel. The species identities are given in Table 3.

SUMMARY AND CONCLUSIONS

We have developed the first assay for the rapid and accurate detection of the globally invasive oak pathogens D. corticola and D. quercivora. Our results provide a solution to the problem of distinguishing among these two pathogen species, which incite similar disease symptoms on live oaks. Previously, identifying and differentiating these pathogens required isolation on artificial medium followed by DNA extraction, PCR, sequencing, and sequence analysis or time-consuming morphological comparisons. Using the method, PCR followed by restriction

![TABLE 2](image)

**TABLE 2** Size (in bp) of *D. corticola* and *D. quercivora* taxon-specific PCR amplicons after digestion with MseI (differences are italicized).

<table>
<thead>
<tr>
<th><em>D. corticola</em> isolate PL1010</th>
<th><em>D. quercivora</em> isolate PL1345</th>
</tr>
</thead>
<tbody>
<tr>
<td>173</td>
<td>88</td>
</tr>
<tr>
<td>361</td>
<td>361</td>
</tr>
<tr>
<td>354</td>
<td>354</td>
</tr>
<tr>
<td>145</td>
<td>145</td>
</tr>
</tbody>
</table>

![TABLE 3](image)

**TABLE 3** Isolates used in the survey of symptomatic live oaks.

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Date collected</th>
<th>Florida county</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>PL1346</td>
<td>8/29/11</td>
<td>Alachua</td>
<td><em>D. quercivora</em></td>
</tr>
<tr>
<td>PL1347</td>
<td>9/13/11</td>
<td>Marion</td>
<td><em>D. quercivora</em></td>
</tr>
<tr>
<td>PL1476</td>
<td>4/23/12</td>
<td>Levy</td>
<td><em>D. corticola</em></td>
</tr>
<tr>
<td>PL1477</td>
<td>4/23/12</td>
<td>Levy</td>
<td><em>D. corticola</em></td>
</tr>
<tr>
<td>PL1478</td>
<td>8/29/12</td>
<td>Washington</td>
<td><em>D. corticola</em></td>
</tr>
<tr>
<td>PL1480</td>
<td>7/3/12</td>
<td>Duval</td>
<td><em>D. corticola</em></td>
</tr>
<tr>
<td>PL1518</td>
<td>9/19/12</td>
<td>Polk</td>
<td><em>D. corticola</em></td>
</tr>
<tr>
<td>PL1519</td>
<td>9/19/12</td>
<td>Polk</td>
<td><em>D. corticola</em></td>
</tr>
<tr>
<td>PL1520</td>
<td>9/19/12</td>
<td>Polk</td>
<td><em>D. corticola</em></td>
</tr>
</tbody>
</table>

![FIGURE 2](image)

**FIGURE 2** Gel (1% w/v agarose) image from *D. corticola/D. quercivora* taxon-specific PCR assay showing the 1,039 bp amplicon in duplicate (using primers DcDq1, DcDq1a, and DcDq3) with DNA from:

![FIGURE 3](image)

**FIGURE 3** Gel (2% w/v agarose) images of MseI restriction enzyme digestion of DcDq1, DcDq1a, and DcDq3 PCR amplicons from Figure 2, using DNA from *D. corticola* (13. CBS112549, 14. UCROK1246, 15. UCROK488, and 17. PL1010 1 ng) and *D. quercivora* (16. PL1265 and 19. PL1345 1 ng). *D. quercivora* is differentiated from *D. corticola* by the absence of the 173 bp band and presence of the 83/88 bp band in the *D. quercivora* digestions. The top gel was stained using ethidium bromide (the marker starts at 800 bp and decrease in 100 bp increments), the bottom gel was stained by mixing SYBR Green with the loading buffer (the marker starts at 650 bp and decreases in 50 bp increments).
digestion with MseI is sufficient. This method could potentially be applied directly to symptomatic plant tissues, eliminating the need for cultures. However, this has not been assessed experimentally yet.

Since *D. quercivora* is a potentially destructive pathogen (8), which has only been recently discovered in the United States (5), agencies and plant disease diagnostic clinics should monitor its distribution and evaluate its contribution to oak damage and mortality. As a result of this detection method and the subsequent survey, *D. corticola* and *D. quercivora* has now been confirmed from live oak in Duval, Highlands, Levy, Polk, and Washington; and Alachua, Duval, and Marion counties, respectively, in Florida. *D. quercivora* and *D. corticola* can be distinguished from each other based on morphology (8), but this is not ideal because of their many similarities. Although it is not known whether these pathogens have been misidentified previously, *Diplodia* isolates in fungal collections should be evaluated to determine if these species are present. Such surveys would provide improved information regarding the origin and distribution of these new pathogens. The protocols developed in this study will make it possible to shorten the length of time required for such work without the need for DNA sequencing.

**ACKNOWLEDGMENTS**

This project was supported by a grant from the USDA-Forest Service (Region 8). We thank Adam Black, Dr. Tuan Duong, and Dr. Wilhelm DeBeer for their assistance.

---

**LITERATURE CITED**