Mycologia, 96(6), 2004, pp. 1352–1354.

A PCR-RFLP based diagnostic technique to rapidly identify *Seiridium* species causing cypress canker

Paal Krokene[1]

Norwegian Forest Research Institute, N-1432 Ås, Norway

Irene Barnes
Brenda D. Wingfield
Michael J. Wingfield

Department of Genetics, Tree Pathology Co-operative Programme (TPCP), Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, 0002, South Africa

Abstract: *Seiridium cardinale, S. cupressi* and *S. unicorne* represent three distinct species of fungi that cause cankers on *Cupressus* species and the disease collectively known as cypress canker. These fungi cannot be distinguished reliably from each other using morphological characters or ribosomal DNA sequence data. Here we describe a RFLP assay based on digesting *β-tubulin* amplicons with a single endonuclease, HaeIII, which easily can be used to distinguish among these three species. This RFLP assay provides an inexpensive and simple means of identifying *Seiridium* species, which include some of the most serious threats to trees in Cupressaceae.

Key words: *β-tubulin,* cypress canker, diagnostics, HaeIII

Cypress canker is a serious disease that threatens the existence of cypress trees (*Cupressus* spp.) worldwide and particularly in the Mediterranean region (Graniti 1998). Three species of anamorphic fungi in the genus *Seiridium* have been associated with the single disease known as cypress canker, and their taxonomy has been the subject of considerable confusion and debate. Different authors have recognized three, two or a single species based on morphological characters (Boesewinkel 1983, Chou 1989, Swart 1973). Sequence data from ribosomal DNA (ITS1, 5.8S gene and ITS2) failed to distinguish among species and suggested that only one species with variable morphology was responsible for cypress canker (Viljoen et al 1993). Phylogenetic analysis of partial *β-tubulin* and *histone H3* gene sequences, however, suggests that the causal agents of cypress canker belong to three clearly distinguishable species: *S. cardinale, S. cupressi* and *S. unicorne* (Barnes et al 2001). The first two species are aggressive pathogens of different species of Cupressaceae and are important agents of cypress canker (Graniti 1998). *S. unicorne* has a broader host range, is only mildly pathogenic and plays a minor role in cypress canker epidemics (Graniti 1998).

Moricca et al (2000) developed a method to distinguish between *S. cardinale* and *S. cupressi* on the basis of single-strand conformation polymorphism (SSCP) in the ITS2 region. At present there are no reliable methods to distinguish between the highly pathogenic *S. cupressi* and the less pathogenic and morphologically indistinguishable *S. unicorne*.

In this study we present a relatively rapid and inexpensive diagnostic procedure based on restriction fragment length polymorphisms (RFLPs) that can be used to distinguish among isolates of all three causal agents of cypress canker. This procedure makes use of the endonuclease HaeIII and amplicons of the *β-tubulin* gene. *β-tubulin* is a protein-encoding gene with both variable and highly conserved regions and has been widely used in phylogenetic analyses of fungi (e.g., Myburg et al 2002, O'Donnell et al 1998, Thon and Royse 1999).

Thirteen isolates of *Seiridium* from different hosts and geographic locations were used (TABLE I), including authenticated isolates of *S. cardinale* from Italy and Chile, *S. unicorne* from Portugal and South Africa and *S. cupressi* from Greece and New Zealand. We also included two other *Seiridum* species that are not involved in cypress canker, for comparative purposes. These were *S. eucalypti* that grouped closely together with *S. unicorne* in a phylogenetic analysis of partial *β-tubulin* DNA sequences and *S. papillatum* that was more distant and was used as the outgroup in that study (Barnes et al 2001). For information on isolates, culturing conditions and DNA extraction protocols, see Barnes et al (2001).

The *β-tubulin* gene was amplified using the forward primer Bt2a (5′ GGT AAC CAA ATC GGT GCT GCT TTC 3′) and the reverse primer Bt1b (5′ GAC GAG ATC GTT CAT GTT GAA CTC 3′) (Glass and

Accepted for publication June 4, 2004.

[1] Corresponding author. Norwegian Forest Research Institute, Høgskoleveien 8, N-1432 Ås, Norway. Phone: + 47 64 94 90 90. Fax: + 47 64 94 29 80. E-mail: paal.krokene@skogforsk.no

TABLE I. *Seiridium* isolates included in this study

Species	Isolates[a]	Host	Origin	Collector
S. cardinale	CMW 1644	*Cupressus* sp.	Italy	A. Graniti
S. cardinale	CMW 2133	*Cupressus* sp.	Chile	M. Wingfield
S. cardinale	CMW 1645	*Cupressus* sp.	Italy	A. Graniti
S. unicorne	CMW 805	*Cupressus lusitanica*	South Africa	M. Wingfield
S. unicorne	CMW 1648	*Cupressus* sp.	Portugal	A. Graniti
S. unicorne	CMW 1649	*Cupressus* sp.	Portugal	A. Graniti
S. cupressi	CMW 1646	*Cupressus* sp.	Greece	A. Graniti
S. cupressi[b]	CMW 5443	*Cryptomeria japonica*	New Zealand	H. Boesewinkel
S. cupressi	CMW 5596	*Cupressus sempervirens*	South Africa	I. Barnes
S. cupressi[b]	CMW 420	*Cupressus macrocarpa*	New Zealand	S. Chou
S. cupressi	CMW 5282	*Cupressocyparis leylandii*	New Zealand	H. Boesewinkel
S. eucalypti	CMW 5303	*Eucaluyptus delegatensis*	Australia	Z. Q. Yuan
S. papillatum	CMW 5302	*Eucaluyptus delegatensis*	Australia	Z. Q. Yuan

[a] CMW = culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

[b] Originally labelled as *S. unicorne,* but DNA sequence data suggest that they represent *S. cupressi* (Barnes et al. 2001).

Donaldson 1995). PCR was carried out in 25 μL reaction mixtures consisting of DNA template (50–100 ng), Expand High Fidelity PCR System enzyme mix (1.7 units) (Roche Molecular Biochemicals, Alameda, California), Expand HF buffer containing 1.5 mM $MgCl_2$ (supplied with the enzyme) and 0.2 μM of each primer and dNTP. After denaturation at 96 C for 2 min, cycling was carried out for 40 cycles with 30 s at 94 C, 40 s at 55 C and 45 s at 72 C, with a postcycling extension at 72 C for 7 min. An extra 5 s extension period per cycle was added after the first 10 cycles. PCR amplicons were visualized on 1.5% agarose gels stained with ethidium bromide under UV illumination. All unpurified PCR amplicons were digested with the endonuclease HaeIII (Roche) in 20 μL reaction mixtures consisting of 10 μL PCR product, restriction enzyme (5 units), 2 μL 10 × SuRE/Cut Buffer M, and 7.5 μl water. Reaction mixtures were incubated overnight at 37 C, and RFLP bands were visualized on 3% agarose gels stained with ethidium bromide under UV illumination.

The three causal agents of cypress canker had clearly distinguishable RFLP profiles (FIG. 1). The profile generated for *S. cardinale* was distinct from all other *Seiridium* species, which complements the fact that this species morphologically is different from the others in having conidia without or with very short appendages (Boesewinkel 1983). *S. cupressi* and *S. unicorne* morphologically are indistinguishable and had similar profiles but still could be clearly distinguished from each other based on the presence of smaller fragments. Two different RFLP patterns emerged for *S. cupressi* isolates, and these were consistent with the two different subclades that have been recognized for the fungus in phylogenetic analysis of partial *β-tubulin* and *histone H3* gene sequences (Barnes et al 2001). *S. eucalypti* had an identical RFLP profile to *S. unicorne,* but because it only in-

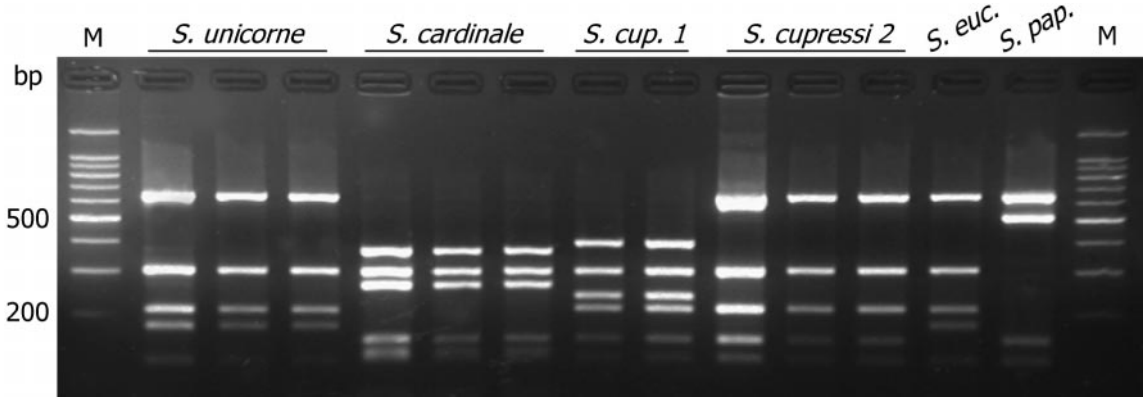

FIG. 1. RFLP profile for 13 different *Seiridium* isolates belonging to five different species. Two subgroups of *S. cupressi* can be distinguished. Lane M; 100 bp ladder marker.

fects *Eucalyptus,* it should not be confused easily with the cypress canker pathogens. *S. papillatum* had a distinct RFLP profile from the other species.

The PCR-RFLP diagnostic technique presented in this study provides an easy route to discriminate among the three morphologically similar fungi that cause the same disease on *Cupressus* spp. Although confusion has arisen in the identification of all three species based on morphology, *S. cardinale* generally can be distinguished from the other two species based on differences in conidial appendages. What is perhaps more important is to be able to distinguish between the highly pathogenic *S. cupressi* and the relatively less pathogenic *S. unicorne,* which are morphologically indistinguishable. The RFLP diagnostic technique clearly will help this process and should contribute to a better understanding of cypress canker and the worldwide distribution of its causal agents.

ACKNOWLEDGMENTS

This research was financed by the Norwegian Forest Research Institute and the Norwegian Research Council under project numbers 152266/730 and 130163/130.

LITERATURE CITED

Barnes I, Roux J, Wingfield MJ, Coetzee MPA, Wingfield BD. 2001. Characterization of *Seiridium* spp. associated with cypress canker based on β-tubulin and histone sequences. Plant Dis 85:317–321.

Boesewinkel HJ. 1983. New records of the tree fungi causing cypress canker in New Zealand, *Seiridium cupressi* (Guba) comb. nov. and *S. cardinale* on *Cupressocyparis* and *S. unicorne* on *Cryptomeria* and *Cupressus*. Trans British Mycol Soc 80:544–547.

Chou CKS. 1989. Morphological and cultural variation of *Seiridium* spp. from cankered Cupressaceae hosts in New Zealand. Eur J For Pathol 19:435–445.

Glass NL, Donaldson GC. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Appl Environ Microbiol 61:1323–1330.

Graniti A. 1998. Cypress canker: a pandemic in progress. Ann Rev Phytopathol 36:91–114.

Moricca S, Børja I, Vendramin CC, Raddi P. 2000. Differentiation of *Seiridium* species associated with virulent cankers on cypress in the Mediterranean region by PCR-SSCP. Plant Pathol 49:774–781.

Myburg H, Gryzenhout M, Wingfield BD, Wingfield MJ. 2002. Beta-tubulin and histone H3 gene sequences distinguish *Cryphonectria cubensis* from South Africa, Asia, and South America. Can J Bot 80:590–596.

O'Donnell K, Cigelnik E, Nirenberg HI. 1998. Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. Mycologia 90:465–493.

Swart HJ. 1973. The fungus causing cypress canker. Trans British Mycol Soc 61:71–82.

Thon MR, Royse DJ. 1999. Partial ß-tubulin gene sequences for evolutionary studies in the Basidiomycotina. Mycologia 91:468–474.

Viljoen CD, Wingfield BD, Wingfield MJ. 1993. Comparison of *Seiridium* isolates associated with cypress canker using sequence data. Exp Mycol 17:323–328.