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Comparison of *Seiridium* Isolates Associated with Cypress Canker using Sequence Data

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VILJOEN, C. D., WINGFIELD, B. D., AND WINGFIELD, M. J. 1993. Comparison of *Seiridium* isolates associated with cypress canker using sequence data. *Experimental Mycology* 17, 000–000. Three species of *Seiridium* have been associated with cypress canker. These include *Seiridium cardinale*, *Seiridium unicorne*, and *Seiridium cupressi* and are distinguished based on conidial appendage morphology. Some authorities believe that *S. cupressi* is conspecific with *S. unicorne* as these two species possess appendaged conidia while *S. cardinale* does not. Others are of the view that only one species of *Seiridium* with variable morphology is associated with cypress canker. In this study the variable first internal transcribed spacer region of the ribosomal RNA genes, from isolates of *Seiridium* associated with cypress canker, was sequenced and compared. Results suggest that species of *Seiridium* associated with cypress canker are closely related. Moreover sequence data support the view that *S. cupressi* and *S. unicorne* are synonyms of *S. cardinale*.

INDEX DESCRIPTORS: *Seiridium;* cypress canker; ITS1; DNA sequence; PCR; phylogeny; ribosomal RNA genes.

Cypress canker associated with species of *Seiridium* is a serious disease of Cupressaceae in various parts of the world (Graniti, 1986). A remarkable aspect of this disease is that three different species of *Seiridium* are known as causal agents of cypress canker. *Seiridium cardinale* (Wagner) Sutton & Gibson, 1972), *Seiridium unicorne* (Cooke & Ellis) Sutton), and *Seiridium cupressi* (Guba) Boesewinkel) (Graniti, 1986).

Species of *Seiridium* associated with cypress canker are separated based on the presence or absence of apical and basal conidial appendages. *S. cardinale* is characterized by having conidia with very short (1 μm) appendages (Graniti, 1986). In contrast *S. unicorne* and *S. cupressi* have conidia with apical and basal appendages up to 13 μm in length. The latter species has been separated based on the fact that appendages in *S. unicorne* are frequently perpendicular to the long axis of the conidium whereas those of *S. cardinale* are rarely perpendicular (Boesewinkel, 1983).

The taxonomy of *Seiridium* species associated with cypress canker is controversial and has been the subject of considerable debate. Swart (1973) suggested that only one species, of variable morphology, is associated with this disease. In contrast, Boesewinkel (1983) described *S. cupressi* and thus provided justification for separating this species from the similarly appendaged *S. unicorne*. This was despite Sutton's (1980) acceptance of only two species, *S. cardinale* and *S. unicorne*. Graniti (1986) reaffirmed that three distinct species of *Seiridium* are associated with cypress canker.

Chou (1989) reexamined the taxonomy of *Seiridium* species associated with cypress canker and concluded that there was no justification for the separation of *S. unicorne* and *S. cupressi*. He did, however, maintain *S. unicorne* and *S. cardinale* as distinct species.

In our studies of cypress canker in Southern Africa, we have experienced consider-

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able difficulty in distinguishing between species. For example, both appendaged and nonappendaged species of *Seiridium* have been found associated with cankers on adjacent or even the same tree. These have been identified as *S. cardinale* or *S. unicorne*. To add to the confusion, we have also found both conidial forms in single acervuli on cankers and this has led us to doubt the validity of distinguishing two species associated with cypress canker based on conidial morphology.

A comparison of the sequences from the ribosomal RNA gene operon has proved successful in determining phylogenetic relationships of different organisms (Bowman *et al.*, 1992; Kurtzman, 1992; Marchant, 1991). Specifically, the first internal transcribed spacer (ITS1) region is known to be highly variable among organisms from the same species (Chambers *et al.*, 1986; Nazar *et al.*, 1988; Yeh and Lee, 1990). This region is situated between the small subunit ribosomal RNA gene and the 5.8S ribosomal RNA gene (Garber *et al.*, 1988). The aim of this study was to compare the putative three species of *Seiridium* associated with cypress canker based on comparisons of sequence data for the ITS1 region of the ribosomal RNA genes.

MATERIALS AND METHODS

Twelve isolates of *Seiridium* associated with cypress canker in various parts of the world were included in this study (Table 1). These included authenticated species of *S. cardinale*, *S. unicorne*, and *S. cupressi* from Italy, Greece, and Portugal, respectively, supplied by Dr. A. Graniti (Dipartimento di Patologia Vegetale, University of Bari, Bari, Italy). Authenticated isolates of *S. unicorne* and *S. cardinale* from New Zealand were supplied By Dr. C. K. S. Chou (Forest Research Institute, Rotorua, New Zealand) with the knowledge that he does not distinguish between *S. unicorne* and *S. cupressi*. Isolates from Southern Africa were from cankers on *Cupressus* spp. in various parts of the country and were identified based on the presence or absence of conidial appendages. Of the latter set, a single isolate was not provided a species epithet because it originated from a spore mass from a single acervulus containing both appendaged and nonappendaged conidia. Where conidia were present in the latter isolate, they were morphologically more typical of those of *S. cupressi* than *S. unicorne*.

TABLE 1
Isolates of *Seiridium* and *Pestalotiopsis*, and Their Sources

Isolate	Species	Origin
1	*S. cardinale*	Italy
2	*S. cardinale*	New Zealand
3	*S. cardinale*	South Africa
4	*S. cardinale*	South Africa
5	*S. cardinale*	South Africa
6	*S. cupressi*	Greece
7	*S. unicorne*	Portugal
8	*S. unicorne*	New Zealand
9	*S. unicorne*	South Africa
10	*S. unicorne*	Lesotho
11	*S. unicorne*	South Africa
12	*Seiridium* sp.	South Africa
13	*P. guepinii*	CBS 361.61

Note. Isolates of *Seiridium* from Italy, Greece, and Portugal were supplied by Dr. A. Graniti, those from New Zealand by Dr. C. K. S. Chou, and all other cultures are from the culture collection of the junior author.

Seiridium species belong to the Coelomycetes of the order Blastromatineae with appendaged, septate conidia in which one or a number of conidial cells are darkened (Sutton, 1980). Other than *Seiridium*, this group of fungi includes genera such as *Pestalotiopsis* (Steyaert) Sutton) and *Pestalotia* (de Not.) Sutton). *Pestalotiopsis guepinii* (Steyaert) Sutton) (CBS 361.61) obtained from the Centraalbureau voor Schimmelcultures, Baarn, was included in this study to serve as an outgroup in the phylogenetic analysis.

Cultures were grown on cellophane discs placed on malt-extract agar (20 g/liter malt-

extract, 20 g/liter agar). Once the mycelia had covered the discs, they were lifted from the agar, freeze-dried, and stored at −70°C. Nucleic acids were isolated using a modified procedure of Chirgwin *et al.* (1979). The freeze-dried mycelia were transferred to 1.5-ml Eppendorf tubes, and 100 μl of 4 *M* guanidinium thiocyanate buffer (4 *M* guanidinium thiocyanate, 30 m*M* sodium lauryl sarcosinate, 24 m*M* trisodium citrate) was added. The lyophilized mycelia were homogenized using an Eppendorf pestle and guanidinium thiocyanate buffer was added to a final volume of 1.5 ml, mixed, and placed on ice for 15 min. The cellular debris was removed by centrifugation for 10 min at 14000 rpm. DNA was precipitated from the supernatant using 0.1 vol 3 *M* sodium acetate and 0.6 vol isopropanol. This precipitate was collected by centrifugation for 20 min at 14000 rpm, washed with 70% ethanol, and resuspended in sterile dH_2O.

Phenol/chloroform extractions were performed to remove the remaining contaminating protein and the nucleic acids were again precipitated, from the aqueous phase, washed, and resuspended. The integrity and concentration of the isolated nucleic acids were assessed by agarose electrophoresis.

The ITS1 region was amplified using the polymerase chain reaction (PCR) (Saiki, 1988). The primer pair PITS1 and PITS2 was used in amplification reactions (White *et al.*, 1990). The sequences of PITS1 and PITS2 are 5'-TCCGTAGGTGAACCTGCGG-3' and 5'-GCTGCGTTCTTCATCGATGC-3', respectively. Reactions were carried out in a Hybaid Omnigene temperature cycler (Hybaid, Middlesex, UK) for 35 cycles using Promega *Taq* polymerase, the supplied 10× buffer, and a 25 m*M* $MgCl_2$ stock solution (Promega Corp., Madison, WI). A final concentration of 5.5 m*M* $MgCl_2$ was used in a 100-μl reaction. An initial denaturation step of 5 min at 96°C was performed and subsequent cycles were, 1 min at 92°C, 10 s at 58°C, and 15 s at 70°C. This was followed by a 5-min final extension step at 70°C.

The amplified DNA fragments were visualized on a 1.5% (w/v) agarose gel to assess the amplification and then purified using the Magic PCR Preps (Promega Corp.). The fmol DNA Sequencing System (Promega Corp.) was used to sequence the PCR products. The PITS2 primer product was used for sequencing. Sequences were visually aligned and phylogenetic comparisons made using PAUP (phylogenetic analysis using parsimony) (Swofford, 1985) and DNABOOT analysis (bootstrap confidence intervals on DNA parsimony) (Felsenstein, 1988). With PAUP, the branch and bound option was used to find the most parsimonious tree.

RESULTS

In all the amplification reactions, a single DNA fragment was obtained as assessed by gel electrophoresis. The PCR fragment obtained for all isolates was similar in size, approximately 200 bp.

The optimum template and primer concentrations used in sequencing reactions were empirically determined. It was, however, found that when using the fmol Sequencing System a final primer concentration of 0.2 OD/ml was used in PCR reactions. Excess of primer resulted in an increase of nonspecific termination.

For each of the 13 isolates sequenced in this study, at least 183 bases were read (Fig. 1). The PAUP analysis produced one tree after the extensive branch and bound option was used (Fig. 2). The *Pestalotiopsis* outgroup formed a branch related to but apart from the *Seiridium* isolates. The unidentified S. African isolate was the most distantly related to other isolates of *Seiridium* but still clusters strongly within this group. *S. cardinale* from New Zealand was more closely related to *S. unicorne*, also from New Zealand, than to other *S. cardinale* isolates. Similarly, *S. unicorne* from S. Africa clustered closer to a *S. cardinale*,

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S. cardinale    (1)   GACGCT-CAGATTACAATAAAATAACAAGAGTTGAATGGT---CCACCGGC
S. cardinale    (2)   ......-.....................................---.......
S. cardinale    (3)   ......-.....................................---.......
S. cardinale    (4)   ..G.-.-.....................................---.......
S. cardinale    (5)   ..G...-..........C.........................---.......

S. cupressi     (6)   ......-.....................................---.......

S. unicorne     (7)   ......-.....................................---.......
S. unicorne     (8)   ......-.....................................---.......
S. unicorne     (9)   ..G.-.-.....................................---.......
S. unicorne     (10)  ....-.-.....................................C..---.......
S. unicorne     (11)  ..G...-..........C.........................---.......

Seiridium sp.   (12)  ......-...................G.....CC.....---.......

P. guepinii     (13)  .C..G.T....GA.....NNNA..........---....AGT.......
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(1)   AG-TCGAC-CA-CCAGACCG-TTCCA-GGTAGGCCAGCCCGGATCGCTCCAG-GTAGGCTGTTCCA
(2)   ..-.....-..-.........-.....-.....................-.....-............
(3)   ..-.....-..-.........-.....-.....................-.....-............
(4)   ..-.....-..-.........-.....-.....................-.....-............
(5)   ..-.....-..-.........-..G..-.....................-.....-............

(6)   ..-.....-..-.........-.....-.....................-.....-............

(7)   ..-.....-..-.........-.....-.....T...............-.....-............
(8)   .C-.....-..A.........-T....-.....................-.....-............
(9)   .CG.....-..A.........T....-.....................-.....-............
(10)  ..-.....C..-.........-.....-.....................-.....-............
(11)  ..-.G...-..-.....G..-..G..-.....G..TG...........-.....-............

(12)  ..-.....CC.-.....-..-.....-..CCT....-...CT...T.....CA...........C

(13)  ..-----------..-.----G..A..G....A...-.------.---.....-.-...-.AAGG.-
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(1)   GGTAGGTACA-GGTAGCTTCTCC-GA-GGCAACAAAGGTAAAGTT-CACATGGGTTTTGGGAGTT
(2)   ..........-.........G.C..-.............-....-.......-...........
(3)   ..........-.........G.C..-.............-....-...................
(4)   ..........-.........G.-..-.........-.....-G.....................
(5)   ..A...A...-.........G.G..-.............-....G.....................

(6)   .........-G.........G.C..-.............C....-...........-.......

(7)   ....---C..-.........G.C..-..NC.T.......-....-.......-...........
(8)   .........-.........G.C..-.........T..-....-...........-C.......
(9)   .........-.........G.C..-.............-....-...........-NNNNNNN
(10)  .........-.........G.C..-.............-....-...........-.......
(11)  .........-.........G.C..T.............-....G....-.....-.......

(12)  T......C..A.........G.C..-......N..NN.T.....-.......-...NNNNNNNN

(13)  .C..-----..G.........G.C..-........T..-T.....-.........--.......
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FIG. 1. One hundred eighty-three bases of aligned sequence of the ITS1 region from 12 *Seiridium* isolates and one *Pestalotsiopsis* isolate. N indicates an unknown base; a dash indicates a deletion in the sequence. A dot indicates a base homologous and identical to the corresponding base in *S. cardinale* (1) sequence.

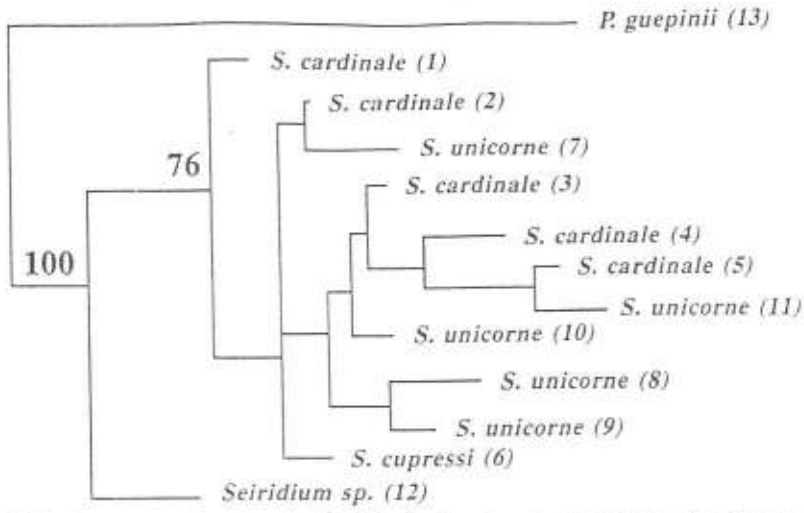

FIG. 2. Dendrogram produced using PAUP showing the phylogenetic relationship between the strains of *Seiridium* associated with cypress canker. Where appropriate the bootstrap confidence intervals are shown.

also from S. Africa, than other *S. unicorne* isolates. *S. cupressi* formed a subcluster by itself and was not more closely related to *S. cardinale* than to *S. unicorne*.

The bootstrap analysis produced the same result with minor differences to the PAUP analysis with regard to the other *Seiridium* isolates (Fig. 2). The *Seiridium* isolates grouped together with a confidence interval of 76%; the unidentified *Seiridium* isolate was the most distantly related to the other *Seiridium* isolates but still grouped together with a confidence interval of 100%. However, the grouping of *Seiridium* species was at a much lower percentage, between 12 and 97%. *S. cardinale* from South Africa was most closely linked to a *S. unicorne*, also from South Africa, with a confidence interval of 97%. No *S. cardinale* species were directly linked to *S. cardinale* isolates; the same is true for *S. unicorne*.

DISCUSSION

Results of this study have shown that species of *Seiridium* associated with cypress canker form one closely related phylogenetic group. Indeed, isolates that had been assigned to *S. unicorne* were in some cases more similar to *S. cardinale* then *S. unicorne*. *S. cupressi* was equally more closely related to *S. cardinale* than *S. unicorne*. The low percentage confidence intervals within the *Seiridium* group with the bootstrap analysis reaffirm the ambiguity of relationships between the three species of *Seiridium*. Despite morphological differences between these species, there is no justification for their separation based on these data. Furthermore, we conclude that a single species of *Seiridium*, of variable morphology, is associated with cypress canker.

The use of an outgroup in this study was important in order to give relevance to the extent of relatedness of isolates used. The outgroup therefore acts as a rule or measure to determine the scale of relatedness. The distance between *P. guepinii*, the outgroup, and the other isolates used in this study confirms the variable nature of the ITS1 region between species. Based on this we would have expected to find a greater hypothetical distance between the different *Seiridium* species than is observed. Therefore, the high degree of homology found

within this highly variable region, for the isolates associated with cypress canker, indicates that these isolates are more closely related than would be expected at the species level.

Our results suggest that the presence or absence of conidial appendages and morphological differences in appendage form are unreliable characteristics in *Seiridium*. They are therefore, not suitable characteristics for species delimitation in isolates of *Seiridium* associated with cypress canker. Furthermore, their taxonomic value in other species of *Seiridium* and indeed Blastomatineae (Sutton, 1980) deserves further study. These structures are likely to play a part in conidial dispersal and factors affecting their presence or absence and morphology should also be investigated.

Based on this study, there appears to be little justification for separating isolates of *Seiridium* associated with cypress canker into different species. The results are, therefore, consistent with our contention that it would be most unusual to find different species of fungi associated with the same disease, on the same tree or even in some cases associated with the same canker. We therefore, support the view of Chou (1989) that *S. cupressi* should be reduced to synonymy with *S. unicorne*. We also support the suggestion of Swart (1973) that only one species, of variable morphology, is associated with cypress canker. The causal agent of cypress canker should therefore be known as *S. cardinale*.

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REFERENCES

BOESEWINKEL, H. J. 1983. New records of three fungi causing canker in New Zealand, *Seiridium cupressi* (Guba) comb. nov. and *S. cardinale* on Cupressocyparis and *S. unicorne* on Cryptomeria and Cupressus. *Trans. Br. Mycol. Soc.* 80: 544–547.

BOWMAN, B. H., TAYLOR, J. W., BROWNLEE, A. G., SHI-DA LU, J. L., AND WHITE, T. J. 1992. Molecular evolution of fungi: Relationships of the Basidiomycetes, Ascomycetes, and Chytridiomycetes. *Mol. Biol. Evol.* 9: 285–296.

CHAMBERS, C., DUTTA, S. K., AND CROUCH, R. J. 1986. *Neurospora crassa* ribosomal DNA: Sequence of internal transcribed spacer and comparison with *N. intermedia* and *N. sitophila*. *Gene* 44: 159–164.

CHIRGWIN, J. M., PRZBYLA, A. E., MACDONALD, R. J., AND RUTTER, W. J. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 18: 5294–5299.

CHOU, C. K. S. 1989. Morphological and cultural variation of *Seiridium* spp. from cankered *Cupressaceae* hosts in New Zealand. *Eur. J. For. Pathol.* 19: 435–445.

FELSENSTEIN, J. 1988. *DNABOOT-Bootstrap Confidence Intervals on DNA Parsimony 3.1*. University of Washington.

GARBER, R. C., TURGEON, B. G., SELKER, E. U., AND YODER, O. C. 1988. Organization of ribosomal RNA genes in the fungus *Cochliobolus heterostrophus*. *Curr. Genet.* 14: 573–582.

GRANITI, A. 1986. Seiridium cardinale and other cypress cankers. *EPPO Bull.* 16: 479–486.

KURTZMAN, C. P. 1992. rRNA sequence comparisons for assessing phylogenetic relationships among yeasts. *Int. J. Syst. Bacteriol.* 42: 1–6.

MARCHANT, A. 1991. New molecular genetic techniques for the inference of evolutionary relationships. *Biol. Vestn.* 2: 1–22.

NAZAR, R. N., WENG, W. M., AND ABRAHAMSON, J. L. A. 1988. Nucleotide sequence of the 18S–25S ribosomal RNA intergenic region from a thermophile. *Thermomyces lanuginosus*. *J. Bacteriol. Chem.* 262: 7523–7527.

SAIKI, R. K., GELFAND, D. A., STOFFEL, S., SCHARF, S. J., HIGUCHI, R., HORN, G. T., MULLIS, K. B., AND ERLICH, H. A. 1988. Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239: 487–491.

SUTTON, B. C. 1969. Forest microfungi. III. The heterogeneity of *Pestalotia* de Not. section sexoculatae Klebahn sensu Guba. *Can. J. Bot.* 48: 2083–2094.

SUTTON, B. C. 1980. *The Coelomycetes*. Commonwealth Mycological Institute, Kew.

SWART, H. J. 1973. The fungus causing cypress canker. *Trans. Br. Mycol. Soc.* 61: 71–82.

SWOFFORD, D. L. 1985. *PAUP Phylogenetic Analysis using Parsimony Version 2.4.1*. Champaign, IL.

WHITE, T. J., BRUNS, T., LEE, S., AND TAYLOR, J. 1990. Amplification and direct sequence of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: A Guide to Methods and Applications* (A. M. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, Eds.), pp. 315–322. Academic Press, San Diego.

YEH, L., AND LEE, J. C. 1990. Structural analysis of the internal transcribed spacer 2 of the precursor ribosomal RNA from *Saccharomyces cerevisiae*. *J. Mol. Biol.* 211: 699–712.