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The *Cylindrocladium candelabrum* species complex includes four distinct mating populations

Conrad L. Schoch
Pedro W. Crous[1]
Department of Plant Pathology, University of Stellenbosch, P. Bag X1, Matieland 7602, South Africa

Brenda D. Wingfield
Department of Genetics, University of Pretoria, Pretoria 0002, South Africa

Michael J. Wingfield
Tree Pathology Co-operative Programme, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria 0002, South Africa

Abstract: *Cylindrocladium candelabrum*-like isolates were collected from a wide variety of geographic locations and compared based on their morphology, sexual compatibility and the nucleotide sequences of their rDNA ITS regions. All isolates included in this study mated to produce *Calonectria* teleomorphs with viable progeny. Four distinct mating populations were identified, each representing a genetically isolated, biallelic, heterothallic population. Several representative isolates of each mating population, reflecting geographic diversity, were chosen for sequence comparisons. The internal transcribed spacer (ITS) regions 1 and 2 that flank the 5.8S rDNA gene, as well as the gene itself, were sequenced and compared. All isolates representing the same group yielded similar sequences, but small, consistent differences were found between the groups. Based on these results we recognise *Calonectria scoparia* (anamorph *Cylindrocladium candelabrum*), and describe three new species, namely *Calonectria pauciramosa* (anamorph *Cylindrocladium pauciramosum*), *Calonectria insularis* (anamorph *Cylindrocladium insularae*) and *Calonectria mexicana* (anamorph *Cylindrocladium mexicanum*).

Key Words: *Calonectria,* ITS sequence analysis, mating studies, systematics

INTRODUCTION

Cylindrocladium scoparium Morgan, the type species of *Cylindrocladium* Morgan (*Cy.*) (Morgan 1892), has been associated with a wide range of plant disease problems in over 30 families throughout the world (Booth and Gibson 1973, French and Menge 1978, Peerally 1991, Waipara et al 1996). This species is, however, the most commonly incorrectly identified taxon in the genus. After several years of extensive collection by the authors, *Cy. scoparium* s. s. has been confirmed from only North America, but has possibly also been introduced into Europe (Overmeyer et al 1996).

Cylindrocladium scoparium, still incorrectly treated by many researchers as synonymous with *Cy. floridanum* Sobers & C. P. Seymour, has been the subject of much controversy. Victor et al (1997) used morphology, sexual compatibility, RAPD markers and A+T-rich total DNA polymorphisms to compare *Cy. scoparium* (teleomorph *Calonectria morganii* Crous et al), *Cy. candelabrum* Viégas (teleomorph *Ca. scoparia* Peerally), *Cy. ovatum* El Gholl et al (teleomorph *Ca. ovata* D. Victor and Crous) and *Cy. floridanum* (teleomorph *Ca. kyotensis* Terash.). This study showed that these species represent distinct taxa. Furthermore, evidence was presented to show that more than one species possibly exists in the *Cy. floridanum* complex. Additionally, based on DNA fingerprinting with human minisatellite DNA as a probe, Jeng et al (1997) showed the presence of three groups of isolates in collections of *Cy. floridanum* from Canada and the USA.

Among the small-spored species of *Cylindrocladium, Cy. scoparium* has also commonly been confused with taxa such as *Cy. ovatum* and *Cy. candelabrum.* All three of the latter species are heterothallic. In a recent study Crous et al (1998) confirmed the biallelic, heterothallic nature of *Cy. ovatum.* In earlier studies, however, very low mating percentages were obtained for *Cy. candelabrum* and *Cy. scoparium* (Crous et al 1993a, Overmeyer et al 1996), suggesting that further research was required to elucidate their mating systems.

Cylindrocladium candelabrum, which was originally described from leaves of a *Luma* sp. in Brazil, was characterized by Viégas (1946) as having narrowly ellipsoidal vesicles and 1-septate conidia, 40–88 × 5–6 µm. Crous et al (1993a) reexamined the type specimen (IACM 440), and found it to be almost completely devoid of material, but the few conidia that

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[1] Corresponding author, E-mail: pwc@maties.sun.ac.za

were observed were 46–70 × 3.5–5 µm, and the vesicles were ellipsoidal to narrowly obpyriform. A neotype (PREM 51045) was subsequently designated, and two isolates PPRI 4153 and 4163 identified as the two mating tester strains. The species concept of *Cy. candelabrum* was complicated by Peerally (1991) who considered it synonymous with *Cy. ellipticum* Alfieri, Seymour & Sobers. The latter species was later shown to be a synonym of *Cy. scoparium* (Crous et al 1993a). To readily distinguish these species, *Cy. scoparium* was circumscribed as having ellipsoidal to pyriform vesicles (widest above the middle), while those of *Cy. candelabrum* were ellipsoidal to obpyriform (widest below the middle). However, a high degree of plasticity was observed amongst *Cy. candelabrum*-like isolates. This was particularly true in their vesicle shape, conidiophore branching pattern and conidial dimensions. Due to the low mating type frequency of isolates in previous studies, no clear indication was obtained on the nature and relevance of this variation amongst *Cy. candelabrum* isolates, and the species was accepted as being highly variable.

Molecular tools have become increasingly useful in providing additional evidence that has supported the interpretation of morphological variation. Several techniques including protein profiles (Crous et al 1993a), RAPD (Victor et al 1997) and RFLP (Crous et al 1997b), have been applied to the taxonomy of *Cylindrocladium* spp. The nucleotide sequences of the ribosomal DNA (rDNA) region contain intermittent functional and nonfunctional regions (Furlong et al 1983). The more conserved rDNA genes allow for comparisons between higher taxa. For example, Rehner and Samuels (1995) compared the nucleotide sequences of the 28S rDNA gene from a wide range of hypocrealean taxa, including *Cy. scoparium* and *Cy. floridanum*. More variable areas are provided by intergenic regions such as the internal transcribed spacers (ITS1 and ITS2) that flank the 5.8S rDNA gene. Various researchers have used these sequences to resolve intra- and interspecies phylogenies (Nazar et al 1991, Sreenivasprasad et al 1994, Bryan et al 1995, Jeng et al 1996, Witthuhn et al 1998).

Recently Jeng et al (1997) published ITS1, ITS2 and 5.8S rDNA sequences of *Cy. scoparium* and *Cy. floridanum*. In these comparisons, one 6-bp deletion and three point mutations were found in the ITS2 region. This indicated the potential of this region to be used as a tool to differentiate between morphologically similar *Cy. candelabrum*-like species. Accordingly, the present study was undertaken to investigate the application of a biological species concept as well as a phylogenetic species concept to isolates provisionally accommodated in the *Cy. candelabrum* species complex. Using these data, it was possible to evaluate the value of morphological characters in *Cylindrocladium*.

MATERIALS AND METHODS

Isolates.—*Cylindrocladium candelabrum* isolates were either obtained from symptomatic material or they were baited from soil samples. Soil samples were collected and treated as explained in Crous et al (1997a). Type specimens were lodged at the National Collection of Fungi in Pretoria (PREM), and ex-type cultures maintained in the culture collection of the Department of Plant Pathology, University of Stellenbosch, South Africa (STE-U).

Sexual compatibility.—One hundred single conidial *Cy. candelabrum*-like isolates (listed under the results), originating from various geographic locations were mated in all possible combinations. This was achieved by removing 3 mm diam agar plugs from the periphery of actively growing cultures and placing them on CLA plates as described by Crous et al (1993a). Two different isolates were placed in a Petri dish with carnation leaves between them. Following this, plates were packed in stacks of 10, sealed in plastic bags and incubated on the laboratory bench at 22 C. Protoperithecia appeared after 2 wk and successful matings were determined after 2 mo of incubation. Successful matings were regarded as those isolate combinations that produced perithecia with fertile, extruding ascospores. Mating groups were subsequently distinguished and strains that resulted in prolific matings were selected from each group. For each mating group identified, ascospores were obtained from two matings, involving four separate isolates. Seven single ascospores were sub-cultured for each mating group, and these were crossed in all possible combinations in order to reconfirm the biallelic, heterothallic nature of each mating population. Two isolates of opposing mating type were selected as tester strains from these isolates, and these were subsequently mated with the tester strains of the other groups to reconfirm that no mating was occurring between groups.

Sequence comparisons.—Four isolates, two isolates per mating type, of each mating group (species), representing a wide geographic distribution (TABLE I), were selected for sequencing. Single conidial isolates were grown on MEA plates and plugs were transferred into 500 mL Erlenmeyer flasks containing 100 mL liquid MEA broth. Flasks were shaken at 25 C and 125 rpm for approximately 7 d. Mycelium was collected by filtration (Whatman no. 1 filter paper) and DNA was extracted as described by Crous et al (1993b). Both strands of the ITS1 and ITS2 intergenic spacers as well as the 5.8S ribosomal gene were sequenced and compared. Sequences were deposited at GenBank (AF059280–AF059283). DNA was amplified using the primers ITS1 (5′-dTCCGTAGGTGAACCTGCGG) and ITS4 (5′-dTCCTCCGCTTATTGATATGC) (White et al 1990). The region amplified was the 5.8S ribosomal gene and the two internal transcribed spacers (ITS1 and ITS2) flanking the gene. PCR amplifications were performed on a Hybaid Omnigene Temperature Cycler (Hybaid, Middlesex, UK). Reactions comprised of 1 µL Expand High Fidelity DNA polymer-