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Genetic Variation in *Cylindrocladium floridanum* and other Morphologically Similar *Cylindrocladium* Species

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Summary

Species of *Cylindrocladium* (C.) are anamorphs of the genus *Calonectria* (*Ca.*), and are important pathogens of numerous crops worldwide. *C. floridanum* is characterized by short, 1-septate, straight, cylindrical conidia and sphaeropedunculate vesicles. Isolates of *Ca. kyotensis* (anam. *C. floridanum*), *Ca. candelabra* (anam. *C. scoparium*), *Ca. morganii* (anam. *C. candelabrum*), *C. ovatum* and *C. naviculatum* were compared based on morphology, sexual compatibility, radial growth on media with different osmotic potentials, RAPD markers and A + T-rich DNA (AT-DNA) polymorphisms. In CLUSTER analyses of the data using the average linkage method, all five species clustered separately. RAPD profiles of ex-type cultures of the two acknowledged synonyms of *Ca. kyotensis* (*Ca. floridana*, *Ca. uniseptata*) shared 78–97% similarity, supporting their conspecificity. Strains of the opposite mating type of the respective heterothallic species studied shared high similarity coefficients of 77% for *Ca. candelabra*, and 92% for *Ca. morganii*. Two opposite mating types of *C. ovatum* with 99% similarity mated to produce a new teleomorph, described here as *Calonectria ovata*. Based on their RAPD and AT-DNA profiles, two major groups could be distinguished within *Ca. kyotensis*. Group two (including most of the Canadian isolates) shared only 12–45% similarity with the ex-type strains (group one) and clustered with mean correlation coefficients of $r = 0.56$ (RAPD analysis) and $r = 0.37$ (AT-DNA analysis), respectively. An isolate similar to *Ca. kyotensis* but with curved conidia had distinct RAPD and AT-DNA profiles, and shared less than 35% similarity ($r = 0.00$) with any of the species studied. These findings suggest that strains with curved conidia and sphaeropedunculate vesicles represent an undescribed taxon, and the name *Cylindrocladium curvisporum* is thus proposed for them.

Key words: AT-DNA and RAPD polymorphisms – *Calonectria ovata* – *Cylindrocladium curvisporum* – Systematics

Introduction

Calonectria kyotensis Terash. is well known as an important root pathogen of a wide range of crops (Sobers and Seymour, 1967; Kuhlman et al., 1980; Sharma et al., 1984; Boesewinkel, 1986; Juzwik and Testa, 1991; Crous and Wingfield, 1993). The anamorph, *Cylindrocladium floridanum* Sobers & C. P. Seym., is distinguished from *C. scoparium* Morgan by its sphaeropedunculate vesicles (Sobers and Seymour, 1967), and laterial stipes originating from secondary and tertiary conidiophore branches (Fig. 1A, B) (Morrison and French, 1969). *Terashita* (1968) described *Ca. kyotensis* (Fig. 1C) as the teleomorph of *C. floridanum*), and Sobers (1972) subsequently reduced *Ca. floridana* Sobers and *Ca. uniseptata* Gerlach to synonymy with *Ca. kyotensis*.

Crous et al. (1993 a) were able to pair heterothallic isolates of *C. scoparium* (pyriform to ellipsoid vesicles) and *C. candelabrum* Viégas (obpyriform to ellipsoid vesicles) (Fig. 1D), and thus described the teleomorphs *Ca. morganii* Crous et al. and *Ca. scoparia* Ribeiro et al. for these anamorphs. El-Gholl et al. (1993) described an additional species in this complex, namely *C. ovatum* El-Gholl et al., which is characterized by ovate vesicles and 1(–3)-septate conidia (Fig. 1E–G). *Ca. kyotensis* (ATCC 18834), the teleomorph of *C. floridanum*, is known to be homothallic. Despite this, several heterothallic strains resembling *C. floridanum* have recently been isolated and received from Canada, while another *C. floridanum*-like strain with curved conidia was recently isolated from soil

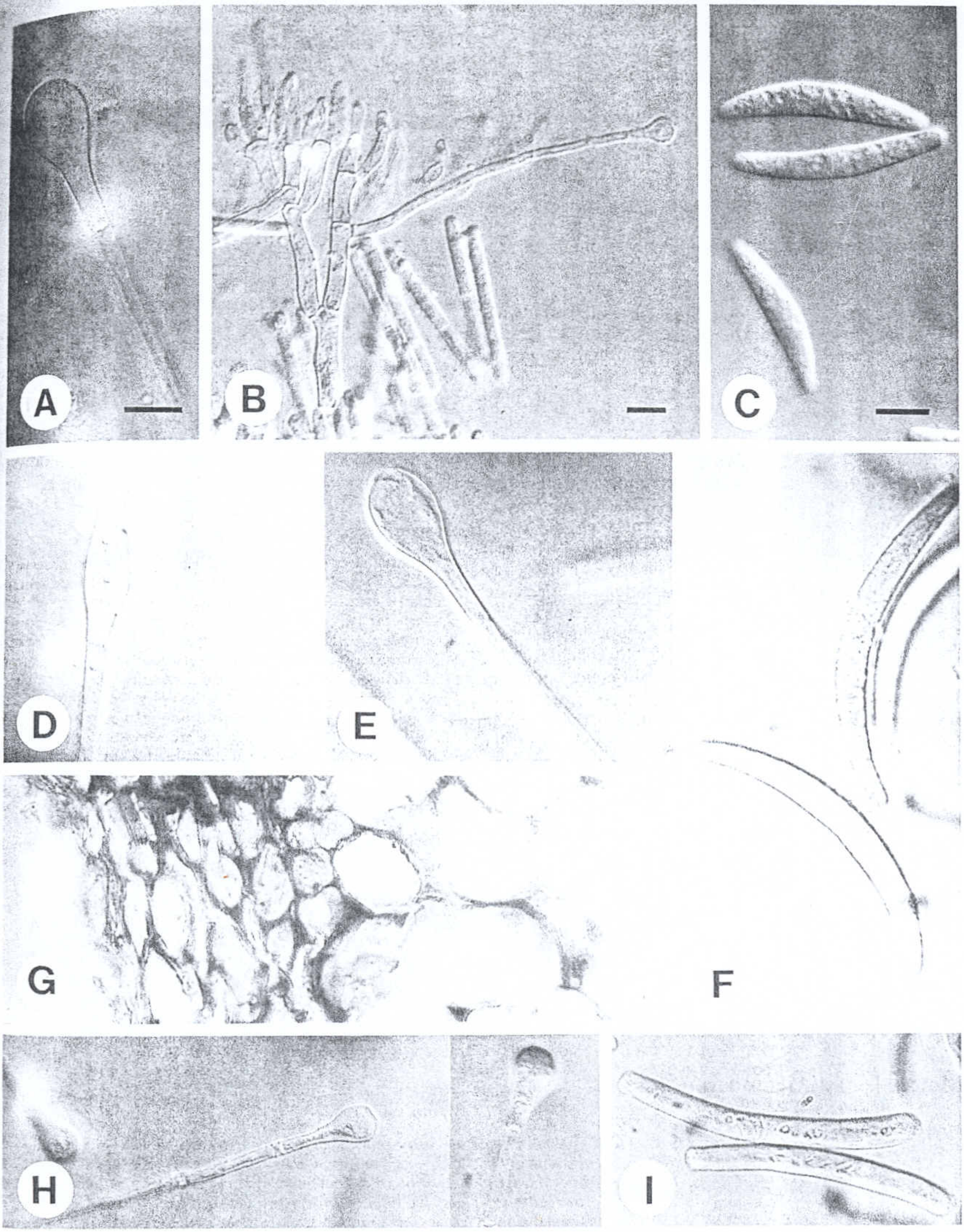

Fig. 1. *Cylindrocladium* and *Calonectria* species. A–C. *Ca. kyotensis*. A. Sphaeropedunculate vesicle. B. Lateral branch forming on a penicillate conidiophore. C. Ascospores of *Ca. kyotensis*. D. Obpyriform vesicle of *C. candelabrum*. E–G. *Ca. ovata*. E. Ovoid vesicle. F. Fusiform ascospores. G. Transverse section through a perithecium showing the various wall layers. H–I. *C. curvisporum*. H. Sphaeropedunculate vesicles. I. Curved 1-septate conidia (bars ≙ 10 µm).

in Madagascar (Fig. 1H, I). This suggests that *C. floridanum* is heterogeneous, and that the various components of this group must be characterized.

The separation of *C. scoparium* and *C. floridanum* is problematic, as many cultures do not sporulate (Stevens et al., 1990), and the vesicles in these species share similar morphology (Crous and Wingfield, 1994). Due to the similarity in conidium and vesicle morphology, Rossman (1983) and Samuels et al. (1991) suggested that *C. floridanum* be treated as a synonym of *C. scoparium*.

Molecular and biochemical techniques have been used extensively to resolve taxonomic problems and to identify species within fungal genera such as *Fusarium* Link: Fr. (Coddington et al., 1987; Kistler et al., 1991), *Phytophthora* de Bary (Förster and Coffey, 1991; Hwang et al., 1991), *Septoria* Fr. (McDonald and Martinez 1990), *Colletotrichum* Corda (Freeman et al., 1993), as well as *Phomopsis* (Sacc.) Bubák (Meijer et al., 1994). Random amplified polymorphic DNA (RAPD) has previously been used in filamentous fungi to distinguish biotypes, races, vegetative compatibility groups (Crowhurst et al., 1991; Guthrie et al., 1992; Leung et al., 1992; Meijer et al., 1994), to assess genomic variability (Goodwin and Annis, 1991), and to identify species (Reeves and Ball, 1991; Chalmers et al., 1992; Guthrie et al., 1992; Bidochka et al., 1994; Zimand et al., 1994). Several biochemical and molecular techniques have also recently been employed to supplement morphological criteria in distinguishing *Cylindrocladium* spp. (Stevens et al., 1990; Crous et al., 1993 a, b, c; 1995). Using aminopeptidase substrate specificities, Stevens et al. (1990) concluded that *C. floridanum* and *C. scoparium* were different, but closely related taxa. El-Gholl et al. (1993) confirmed these results by means of α-isoesterase banding patterns, while Crous et al. (1992) showed that radial growth on media amended with KCl provided an additional method to distinguish these taxa.

Evidence at hand strongly suggests that *Ca. kyotensis* and strains of its anamorph *C. floridanum* represent a number of distinct taxa. Morphological characteristics alone are clearly insufficient to resolve this taxonomic dilemma. The aim of this study was to characterize and determine the genetic variation within and between isolates of *Ca. kyotensis* and morphologically similar species, using morphology, culture characteristics, radial growth on media with different osmotic potentials, sexual compatibility, RAPD markers, as well as A+T-rich DNA (AT-DNA) polymorphisms.

Materials and Methods

Morphological characteristics

Ex-type and authenticated cultures of *Ca. kyotensis* (anam. *C. floridanum*) (ATCC 18834), *Ca. candelabra* (anam. *C. scoparium*) (ATCC 46300), *Ca. morganii* (anam. *C. candelabrum*) (PPRI 4163) and *C. ovatum* (ATCC 76225) were examined along other authenticated isolates of each species (Crous and Wingfield, 1994), and several previously unidentified strains. The ex-type culture of *C. naviculatum* Crous & M. J. Wingf. (STE-U 627) was also included in this study to serve as an outgroup (Table 1).

Strains derived from single conidia were plated onto carnation-leaf agar (CLA) (Fisher et al., 1982; Crous et al., 1992), incubated at 25 °C under near-ultraviolet (nuv) light and examined after 7 days (d). Vesicles examined were all on stipes of conidiophores with at least one primary, and one secondary branch bearing phialides. Vesicles that showed signs of proliferation were ignored. Vesicle width was measured at the widest point and stipe length measured from above the highest primary branch to the vesicle tip. Fifty examples of each structure were measured, averages determined and extremes given in parentheses.

Culture characteristics

Maximum radial growth of species in culture was determined on malt-extract agar (MEA) (20 g Oxoid malt extract, 15 g Difco agar, 1000 ml H_2O) after 6 d (Crous and Wingfield, 1993). Colony colour and chlamydospore formation (rated from inverted plates) was determined as explained by Crous et al. (1993 a). Colour designations used were those of Rayner (1970). Cardinal temperatures for growth were determined on MEA plates at 5, 8, 10, 15, 20, 25, 30, 33 and 35 °C in triplicate. Radial growth was assessed after 6 d as explained in Crous and Wingfield (1994).

Scanning electron microscopy

Scanning electron microscopy (SEM) was used to observe differences in vesicle and conidium morphology of representative strains of each of the five species, as well as an isolate from Madagascar (STE-U 763) with curved conidia. Specimens were flash frozen (−212 °C) in liquid nitrogen under vacuum for cryo-SEM, transferred to the preparation chamber, and then to the SEM chamber where the frozen samples were sublimated (−80 °C) to remove ice particles. Samples were sputter coated with gold palladium in the preparation chamber for 75 s under 1.2 KV at −170 °C. Specimens were viewed under 5 KV at −188 °C with a Jeol JSM 6100 scanning electron microscope.

Effect of osmotic potential on linear growth

Potato dextrose agar (PDA) with an osmotic potential of −0.4 MPA (−1 MPa = −10 bars) was prepared as described by Nelson et al. (1983). PDA containing KCl (KCl medium), which lowers the osmotic potential of the medium (Fisher et al., 1983), was prepared as explained in Crous et al. (1992). KCl medium with osmotic potentials of −3.6, −4.5, −5.5 and −8.9 MPa (before inoculation) were inoculated with agar plugs (3 mm diam.) taken from the periphery of actively growing colonies. Radial growth of each isolate was assessed on triplicate plates after 6 d at 25 °C in the dark. Average growth rate was obtained from the mean of four colony radials on each of the three plates (Crous et al., 1992).

Mating studies

Thirty-eight single-conidial isolates (Table 1) were paired with each other in all possible combinations. Pairings were done on plates of CLA containing three pieces of carnation leaf. Single-conidial isolates were grown on MEA for 7 d at 25 °C in the dark. Agar discs (3 mm diam.) from the periphery of the actively growing colonies were used for inoculation. Two isolates were placed on opposite sides of each piece of carnation leaf as explained by Crous et al. (1993 a). There were two sets of mating tem-