

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 lateral 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. (1993a) were able to pair heterothallic isolates of *C. scoparium* (pyriform to ellipsoid vesicles) and *C. candelabrum* Viegas (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. Et-GHOLL et al. (1993) described an additional species in this complex, namely *C. ovatum* Et-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

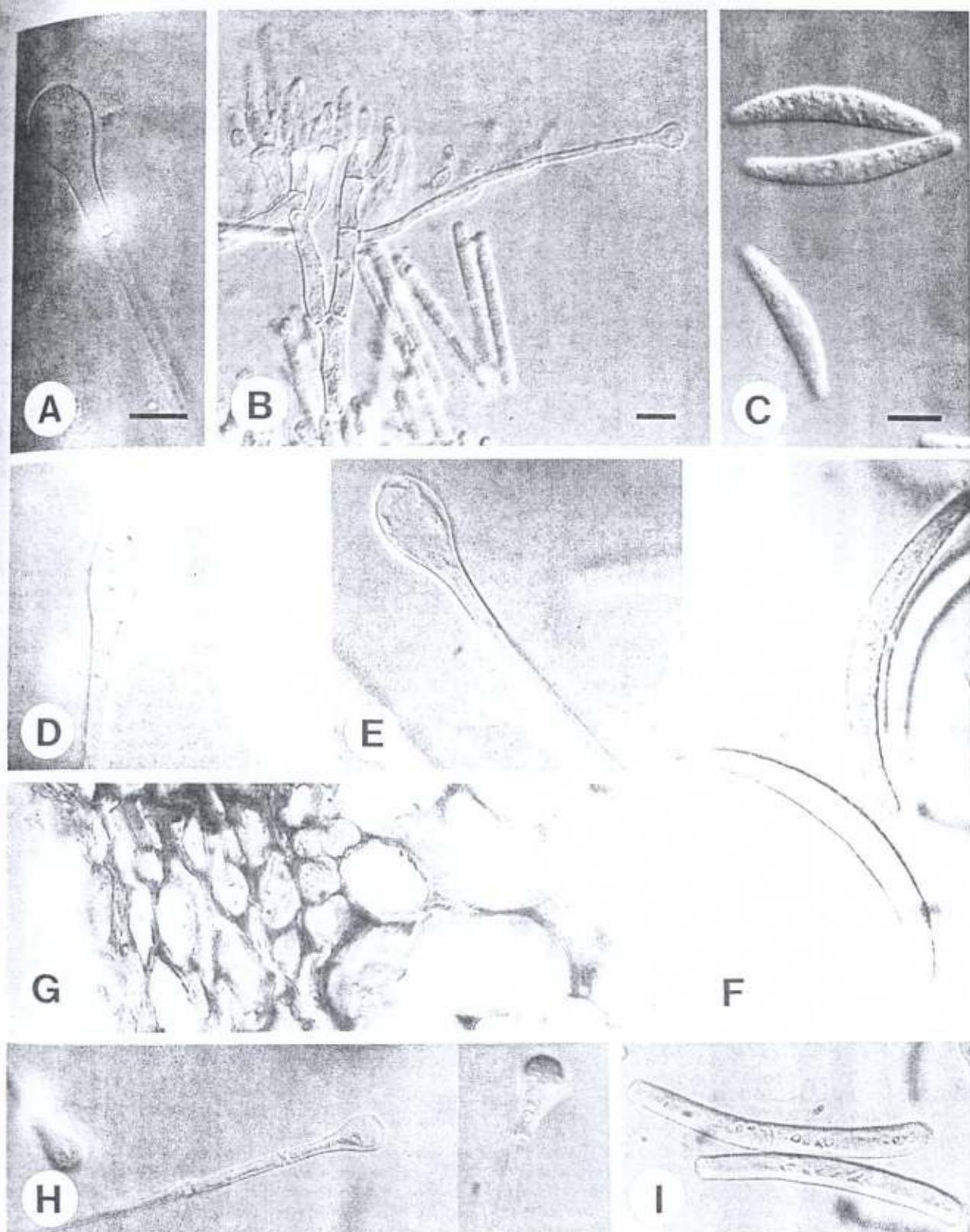


Fig. 1. *Cylindrocladium* and *Calonectria* species. A-C. *Ca. kyotensis*. A. Sphaeropedunculate vesicle. B. Lateral branch forming on a penicillate conidiophore. C. Ascospores of *Ca. kyotensis*. D. Obpyriform vesicle of *C. candelabrum*. E-G. *C. ovata*. E. Ovoid vesicle. F. Fusiform ascospores. G.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

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; KISILER 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; BIDCHKA 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., 1993a, 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. morgani* (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 H₂O) after 6 d (CROUS and WINGFIELD, 1993). Colony colour and chlamydsore formation (rated from inverted plates) was determined as explained by CROUS et al. (1993a). 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. (1993a). There were two sets of mating tem-

Table 1. Isolates of *Cylindrocladium* species compared in this study

Species	Accession number	Host	Location	Collector	Date isolated	Reference
<i>Cylindrocladium floridanum</i>	ATCC 18834 ^{1,2}	<i>Robinia pseudoacacia</i>	Japan	TERASHITA	1963	TERASHITA 1968
	ATCC 18882 ^{1,2}	<i>Prunus persica</i>	Florida, USA		1965	SOBERS and SEYMOUR, 1967
	CBS 413.67 ^{1,2}	<i>Paphiopedilum callosum</i>	Germany	W. GERLACH	1967	GERLACH, 1968
	ATCC 42971	<i>Rumohra adiantiformis</i>	Florida, USA	S. A. ALFIERI	1980	Present study
	UFV 76 ^{1,2}	<i>Pinus</i> sp.	Canada	A. C. ALFENAS	1990	Present study
	UFV 77 ¹	<i>Pinus</i> sp.	Canada	A. C. ALFENAS	1990	Present study
	UFV 78 ¹	<i>Pinus</i> sp.	Canada	A. C. ALFENAS	1990	Present study
	STE-U 499 ¹	Spruce	Canada	S. GREIFENHAGEN	1992	Present study
	STE-U 500 ¹	Spruce	Canada	S. GREIFENHAGEN	1992	Present study
	STE-U 519	White spruce	N. Ontario, Canada	J. JUZWIK	1992	Present study
	STE-U 520 ¹	White spruce	N. Ontario, Canada	J. JUZWIK	1992	Present study
	STE-U 521 ¹	Black spruce	N. Ontario, Canada	J. JUZWIK	1992	Present study
	STE-U 522 ¹	White spruce	S. Ontario, Canada	J. JUZWIK	1992	Present study
	STE-U 624 ^{1,2}	Soil	Amazonas, Brazil	M. J. WINGFIELD	1993	Present study
	STE-U 655 ^{1,2}	White spruce	Wisconsin, USA	A. C. ALFENAS	1993	Present study
	STE-U 682 ^{1,2}	Soil	Thailand	M. J. WINGFIELD	1993	Present study
	STE-U 684 ^{1,2}	Soil	Hong Kong	M. J. WINGFIELD	1993	Present study
	P88.5641 ¹	<i>Rumohra adiantiformis</i>	Florida, USA	N. E. EL-GHOLL	1988	Present study
	P85.2179 ¹	<i>Pinus palustris</i>	Florida, USA	N. E. EL-GHOLL	1985	Present study
	PPRI 4140 ¹	<i>Prunus persica</i>	Florida, USA	A. C. ALFENAS	1990	Present study
	PPRI 4167	-	-	A. C. ALFENAS	1990	Present study
ATCC 58925	<i>Trifolium pratense</i>	Florida, USA	D. BELL	-	Present study	
PPRI 4169	-	Florida, USA	A. C. ALFENAS	1990	Present study	
ATCC 22677 ^{1,2}	<i>Prunus persica</i>	Georgia, USA	D. J. WEAVER	-	Present study	
AR 2573	Conifer roots	Ontario, Canada	J. JUZWIK	1984	Present study	
AR 2574	Conifer roots	Ontario, Canada	J. JUZWIK	1984	Present study	
AR 2578	Conifer roots	Ontario, Canada	J. JUZWIK	1984	Present study	
AR 2579	Conifer roots	Ontario, Canada	J. JUZWIK	1984	Present study	
AR 2581	Conifer roots	Ontario, Canada	J. JUZWIK	1984	Present study	
AR 1785 ¹	<i>Mimosa meansii</i>	Amazonas, Brazil	R. P. KOH	1978	Present study	
<i>C. scoparium</i>	ATCC 46300 ^{1,2}	<i>Leucothoe catesbaei</i>	Carolina, USA	D. M. BENSON	1981	Present study
	ATCC 38227 ^{1,2}	<i>Mahonia bealei</i>	Florida, USA	S. A. ALFIERI	1970	ALFIERI et al., 1970
<i>C. candelabrum</i>	PPRI 4202	<i>Eucalyptus</i> sp.	Natal, R.S.A.	M. J. WINGFIELD	-	CROUS et al., 1993a
	PPRI 4199	<i>Eucalyptus</i> sp.	E. Gauteng, R.S.A.	P. W. GROUS	1990	CROUS et al., 1993a
	PPRI 4146	<i>Eucalyptus</i> sp.	Brazil	A. C. ALFENAS	-	CROUS et al., 1993a
	PPRI 4163 ^{1,2}	<i>Eucalyptus</i> sp.	Brazil	A. C. ALFENAS	-	CROUS and WINGFIELD, 1994
	PPRI 4153 ^{1,2}	<i>Eucalyptus</i> sp.	Brazil	A. C. ALFENAS	-	CROUS and WINGFIELD, 1994
STE-U 654 ¹	White spruce	Wisconsin, USA	A. C. ALFENAS	1993	Present study	
<i>C. naviculatum</i>	STE-U 627 ^{1,2}	Soil	Amazonas, Brazil	M. J. WINGFIELD	1993	Present study
	STE-U 628	Soil	Amazonas, Brazil	M. J. WINGFIELD	1993	Present study
<i>C. ovatum</i>	UFV 91	<i>Eucalyptus</i> sp.	Amazonas, Brazil	N. E. EL-GHOLL	1990	Present study
	UFV 90	<i>Eucalyptus</i> sp.	Amazonas, Brazil	N. E. EL-GHOLL	1990	Present study
	ATCC 76225	<i>Eucalyptus</i> sp.	Amazonas, Brazil	N. E. EL-GHOLL	1990	Present study
	STE-U 618	Soil	Amazonas, Brazil	M. J. WINGFIELD	1993	Present study
	STE-U 619	Soil	Amazonas, Brazil	M. J. WINGFIELD	1993	Present study
	UFV 24	<i>Eucalyptus</i> sp.	Amazonas, Brazil	J. C. DIANESE	1993	Present study
	UnB 1026 ^{1,2}	<i>Eucalyptus</i> sp.	Amazonas, Brazil	J. C. DIANESE	1993	Present study
	UnB 1028	<i>Eucalyptus</i> sp.	Amazonas, Brazil	J. C. DIANESE	1993	Present study
	PPRI 4162 ^{1,2}	<i>Eucalyptus</i> sp.	Amazonas, Brazil	J. C. DIANESE	1993	Present study
<i>C. curvisporum</i>	STE-U 763 ^{1,2,3}	Soil	Tamatave, Madagascar	P. W. CROUS	1994	Present study

¹ Isolates selected for RAPD analysis² Isolates selected for AT-DNA analysis³ A new species of *Cylindrocladium*

peratures (15 and 25 °C), and triplicate plates for each combination at each temperature. Plates were sealed in plastic bags and examined twice each month for perithecial development.

Fungal DNA isolation

One isolate, representative of *C. naviculatum* and the two mating type strains of *C. ovatum*, *C. scoparium* and *C. candelabrum*, together with 21 strains of *C. floridanum* were selected for RAPD analysis. For RFLP analysis 17 representative strains of the five species studied were selected according to their RAPD profiles. Isolation of the fungal DNA was carried out as described previously by CROUS et al. (1993b), except that cetyltrimethylammonium bromide (CTAB) (Sigma Chemical Co.; St. Louis, USA) and 5 M NaCl were added to the mycelial mixture to a final concentration of 1% (v/v) and 0.7 M, respectively, after the ground mycelia were lysed for 16 hr at 65 °C, and incubated for a further 1 hr at 65 °C (FANG et al., 1992). Proteins were subsequently denatured and the DNA precipitated as described previously in CROUS et al. (1993b).

RAPD analysis

The 28 strains selected for this analysis included the respective mating types of *C. candelabrum*, *C. scoparium*, *C. ovatum*, 21 strains of *C. floridanum*, and one of *C. naviculatum* (Table 1). Primers used in this study were purchased from Operon Technologies (Kit E) (Operon Technologies Inc., Alameda, CA94501, U.S.A.). In preliminary tests, 20 Operon primers (OPE-01 to OPE-20) were used to detect RAPD polymorphisms in *C. naviculatum* (STE-U 627) and a *Cylindrocladium* sp. isolated in Madagascar (STE-U 763). Five primer sequences were selected for use with all isolates: OPE-02, 5'GGTGGGGAA3'; OPE-03, 5'CCAGATGCAG3'; OPE-04, 5'GTGACATGCC3'; OPE-14, 5'TCGGGCTGAG3'; OPE-20, 5'AACGGAGACC3'.

Amplification reactions were performed in volumes of 50 µl containing 75 ng of genomic template DNA, dATP, dCTP, dGTP and dTTP (200 µM final concentration), 50 pmol oligonucleotide primer and 1 unit of Taq polymerase (Boehringer Mannheim Ltd, South Africa), in 5 mM MgCl₂. Each reaction was overlaid with 60 µl of mineral oil to prevent evaporation. Amplification was performed in a Biometra Trio-Thermoblock DNA Thermal Cycler. Except for the first cycle where denaturation was carried out for 120 s at 96 °C, the rest of the cycles were as follows: denaturation at 92 °C for 30 s, annealing at 37 °C for 30 s, and polymerisation at 72 °C for 60 s. The fastest available transitions between temperatures were used. These cycles were repeated 40 times. Amplified DNA fragments were separated in 1.4% (w/v) MP agarose gels (Boehringer Mannheim, South Africa), with 0.5 µl ethidium bromide (10 mg/ml) using 1 × TAE buffer (SAMBROOK et al., 1989) at a constant voltage of 15 V/cm, and visualized by illumination under uv light. Phage Lambda DNA digested with the restriction enzymes *EcoRI* and *HindIII* was used as a molecular weight standard.

AT-DNA polymorphisms

Total genomic DNA was subjected to digestion for 16 hr with the restriction enzymes *HaeIII*, and *MspI*, according to the re-

commendations of the suppliers (Boehringer Mannheim, Johannesburg, South Africa). The DNA fragments were resolved in 1% (w/v) agarose gels using 0.5 × TBE Buffer (SAMBROOK et al., 1989). Phage lambda DNA digested with the restriction enzymes (*EcoRI* and *HindIII*) was used as a molecular weight standard.

Analyses of RAPD and AT-DNA data

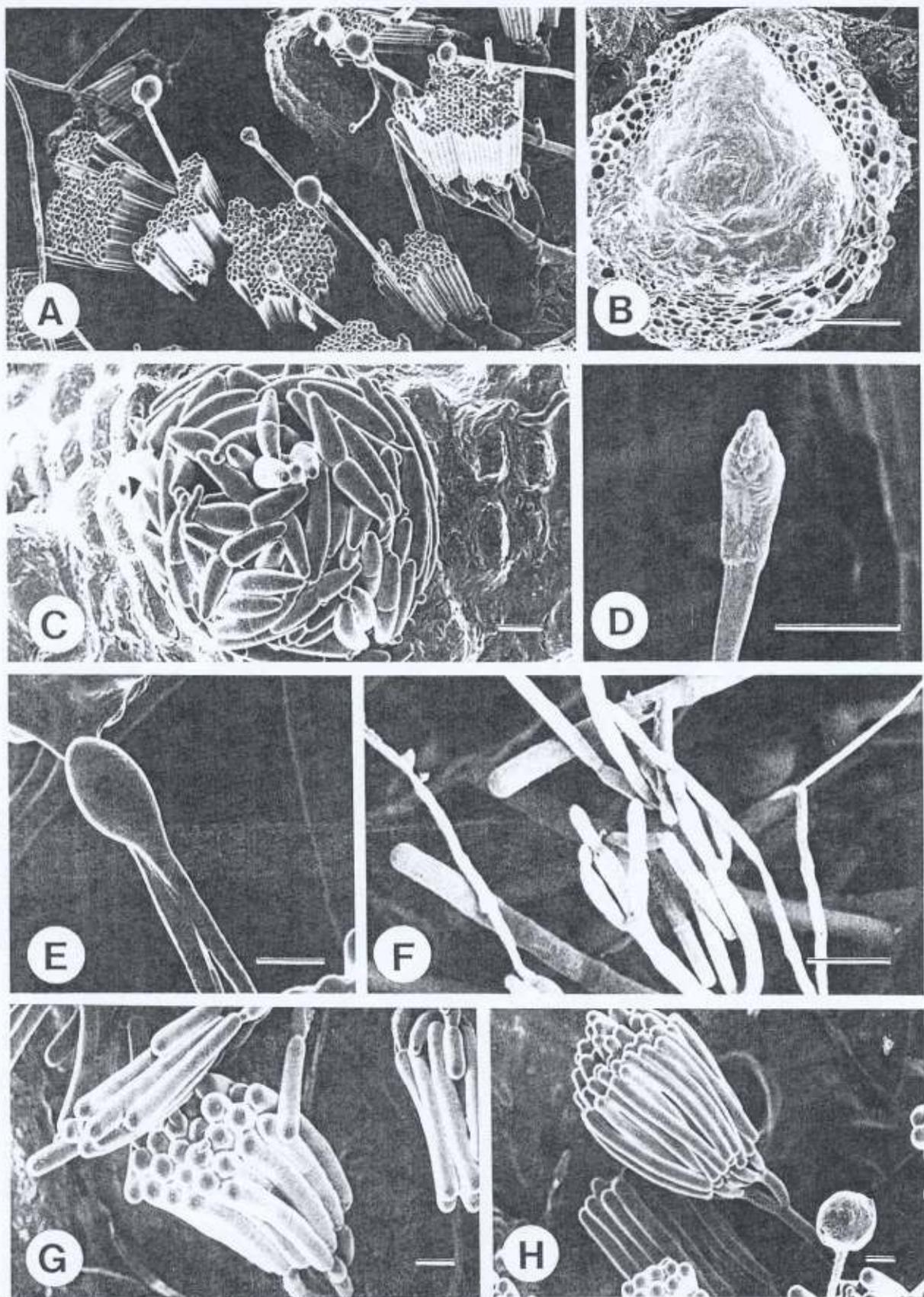
The DNA profiles produced by AT-DNA polymorphisms were analysed using the F-value equation of NEI and LI (1979), RAPD profiles were analysed using the F-value equation as described by GABRIEL et al. (1988), and recommended for RAPD analysis by GODWIN and ANNIS (1991). These F-values were multiplied by 100 to obtain the percentage similarity (% similarity) existing between two variables. Although the % similarity values were calculated for each pair of strains with each primer and restriction enzyme, only the combined % similarity values (HAUSNER et al., 1993) for each pair with all five primers as well as both restriction enzymes are given, since the data sets were consistent with each other. These data were then converted to an index of genetic dissimilarity (100% similarity). Analyses were performed using the CLUSTER procedure of the SAS Institute Inc. (1989). Dissimilarity values were clustered with the average linkage method, with the RSQUARE option specified. A phenogram was constructed using the TREE procedure. The closeness of the relationship between two variables are reported as correlation coefficients (*r*) (SNEDECOR and COCHRAN, 1980).

Results

Morphological characteristics

Based on the species concepts of CROUS and WINGFIELD (1994), most isolates could be identified as either *C. scoparium* (pyriform to ellipsoid vesicles), *C. ovatum* (ovate vesicles), *C. candelabrum* (obpyriform to ellipsoid vesicles), *C. floridanum* (sphaeropedunculate vesicles) or *C. naviculatum* (naviculate vesicles) (Fig. 2A–H). Isolates of all these species had 1-septate conidia, except some strains of *C. ovatum* that developed up to three conidial septa, and also formed a microconidial state (AL-GHOLLI et al., 1993). Conidia of *C. scoparium*, *C. floridanum* and *C. candelabrum* were similar in size, ranging from 33–66 µm in length, and 3–5 µm in width. Although conidia of *C. naviculatum* also fell within this range, they were only 3–4 µm wide, while those of *C. ovatum* could be up to 80 µm long and 6 µm wide (CROUS and WINGFIELD 1994). One strain isolated from a Brazilian soil sample, (STE-U 624), had vesicles which were mostly spathulate to obpyriform or ovate to sphaeropedunculate. Due to its distinct vesicle morphology this isolate could, therefore, not be accommodated in any of the species under consideration. Isolate STE-U 763 was obtained from soil collected in Madagascar. Vesicles were sphaeropedunculate, similar to those of *C. floridanum*. However, conidia were 1(–3)-septate, and

Fig. 2. *Cylindrocladium* and *Calonectria* species. A–C. *Ca. kyotensis*. A. Penicillate conidiophores with stipe extensions terminating in sphaeropedunculate vesicles. B. Vertical section through a perithecium. C. One-septate ascospores exuding from an ostiole. D. Naviculate vesicle of *C. naviculatum*. E–F. *Ca. ovata*. E. Ovoid vesicle. F. Macroconidia with a microconidiophore and microconidia in the foreground. G–H. *C. curvisporum*. G. Curved conidia. H. Terminal sphaeropedunculate vesicle and curved conidia (all bars = 10 µm, except bar for B = 100 µm).



45–70 × 4–6 µm, thus also tending to be slightly larger than those of *C. floridanum* (Figs. 1H, I; 2G, H; 4).

Culture characteristics

Other than *C. naviculatum* which formed chlamydo-spores in moderate numbers, strains of all other species formed extensive chlamydo-spores on MEA. Cardinal temperature requirements for growth of the various species compared were similar to those given by CROUS and WINGFIELD (1994). This feature could not, however, be used to distinguish isolates at species level.

Scanning electron microscopy

Using Cryo-SEM, differences in vesicle shape were clearly visible amongst the five different species. Although vesicle of *C. floridanum* were similar to those of the Malagassy isolate (STE-U 763), conidia were straight, and not curved as observed with the latter isolate (Fig. 2; G, H).

Effect of osmotic potential on linear growth

Growth of isolates on media with different osmotic potentials (KCl medium) did not provide a means to distinguish between *C. floridanum*, *C. ovatum*, *C. candelabrum* and *C. scoparium*. Optimum growth for these species occurred at –4.5 MPa, and similar declining growth curves were observed on media with lower osmotic potentials. However, *C. naviculatum* (STE-U 627, 628) had a radial growth of 10 mm at –4.5 MPa, in contrast to isolates of other species that only reached 5–7 mm on this medium.

Mating studies

The ex-type culture of *Ca. kyotensis* (ATCC 18834), as well as those of its recognized synonyms (ATCC 18882 and CBS 413.67), were confirmed to be homothallic, producing perithecia with ascospores when mated with themselves. Previous studies (A. C. Alfenas, Univ. of Viçosa, MG, Brazil, personal communication) found several Canadian isolates (UFV 76–78) to be heterothallic, pro-

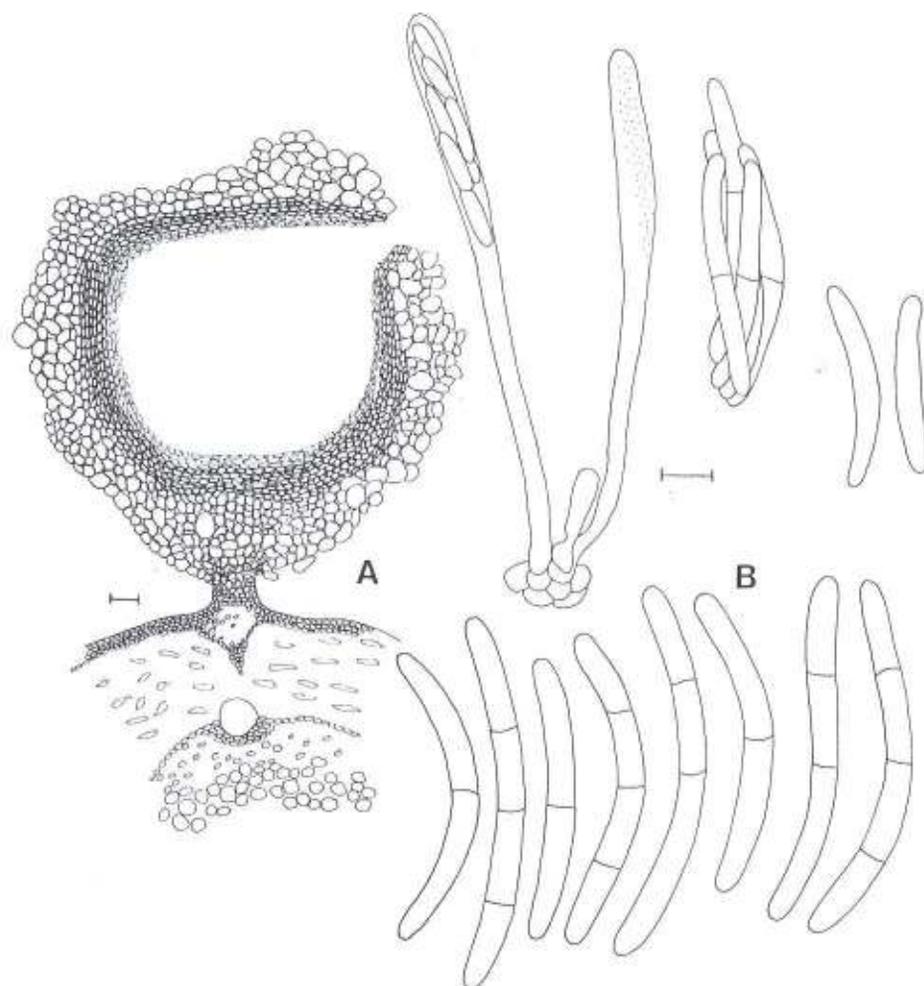


Fig. 3. A–B. *Calonectria ovata*. A. Vertical section through a perithecium. B. Asci with fusiform to cylindrical, 1(–3)-septate ascospores (bar = 10 µm).

ducing perithecia morphologically similar to *Ca. kyotensis*. In this study, however, these isolates could not be induced to form perithecia with viable progeny. Other than for homothallic strains of *Ca. kyotensis*, no matings done at 15°C under nuv light were successful. Characterized opposite mating type strains of respectively *C. scoparium* and *C. candelabrum* (CROUS et al., 1993 a) that were included in this study produced protoperithecia only. The two single conidial isolates of *C. naviculatum* (STE-U 627, 628) also produced protoperithecia. Of the 703 possible combinations, mature perithecia with viable ascospores were obtained after 8–12 wks when *C. ovatum* isolates (PPRI 4162 × UNB 1026) were paired (Fig. 3A, B). No other matings gave rise to perithecia with viable progeny.

RAPD analysis

Total DNA of the 28 strains selected for RAPD analysis were randomly amplified with primers OPE-02, 03, 04, 14 and 20, respectively. All five primers tested produced specific profiles consisting of between 6–20 bands for each of the strains examined. The size of the amplified DNA sequences detected varied from 100 to 3500 bp. The reproducibility of the bands obtained was tested to determine whether these amplification polymorphisms were useful as genetic markers. The results showed that the bands are highly reproducible. In order to confirm that the observed bands were amplified DNA and not primer artefacts (INNES et al., 1990), genomic DNA was excluded as a control reaction for each primer. No primer artefacts were detected for the primers tested.

Two major RAPD-profiles were observed amongst isolates of *C. floridanum* (Figs. 5, 6; lanes 10–29). The first group (group I) (Figs. 5, 6; lanes 10–15) included the representative ex-type strain of *Ca. kyotensis* (ATCC 18834) together with its synonyms (SOBERS, 1972) *Ca. uniseptata* (CBS 413.67) and *Ca. floridana* (ATCC 18882). All of these isolates showed a high degree of similarity (78–97%) with each other, and clustered together at $r = 0.999$ (Fig. 9). The second group within *C. floridanum* (group II) (Figs. 5, 6; lanes 17–26), included most of the Canadian isolates as well as one isolate from Brazil (AR 1785) and two isolates from North America (ATCC 22677 and STE-U 655) (Table 1).

The profiles obtained for the two mating type strains of each heterothallic species, *C. candelabrum*, *C. scoparium* and *C. ovatum*, indicated similarity of 77–100% at intra-species level and clustered as individual biological species at $r = 0.999$ (Figs. 5, 6, 9; lanes 4–9). In agreement with its general morphology, *C. naviculatum* proved to be distinct from the four other species based on its unique RAPD profiles (Figs. 5, 6; lane 2). Isolate STE-U 654 (obpyriform vesicles) showed 53–62% similarity ($r = 0.97$) with two isolates of *C. candelabrum* (PPRI 4163, 4153) (Figs. 5, 6, 9; lanes 6, 7, 27). Isolates STE-U 624, 682 and 684 of *C. floridanum* exhibited profiles distinct from all other isolates examined. Although, *C. floridanum* (STE-U 624) was found to have a variable vesicle morphology, *C. floridanum* (STE-U 682 and 684) could not be distin-

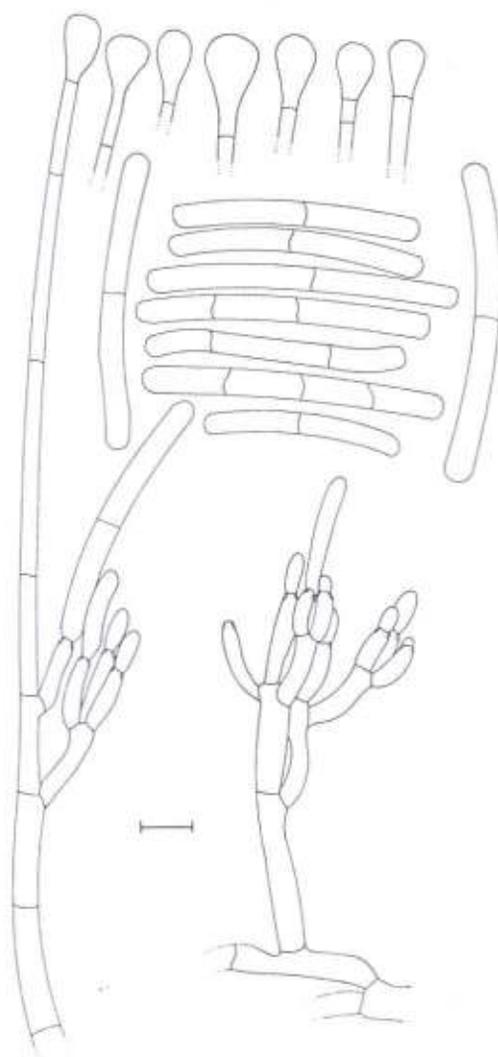


Fig. 4. Penicillate conidiophores of *Cylindrocladium curvisporum* with sphaeropedunculate vesicles and curved, 1(-3)-septate conidia (bar \triangleq 10 μ m).

guished from *Ca. kyotensis* (ATCC 18834) based on general morphology. In agreement with its distinct morphological features, the Malagassy isolate (STE-U 763) also had unique RAPD profiles for all primers tested, suggesting that it is a distinct species with curved, 1(-3)-septate conidia (Figs. 1H, I; 2G, H; 4; 5; 6, lane 3).

AT-DNA polymorphisms

Total DNA of 17 isolates representing the five species under consideration was subjected to digestion with the restriction enzymes *MspI* and *HaeIII*. The two different mating type strains of the respective species, PPRI 4162 and UnB 1026 (*C. ovatum*); PPRI 4163 and PPRI 4153 (*C. candelabrum*); ATCC 46300 and ATCC 38227 (*C. scoparium*) as well as the three homothallic isolates of *Ca. kyotensis*, ATCC 18882, 18834 and CBS 413.67 (results not shown), produced the same profiles with both restric-

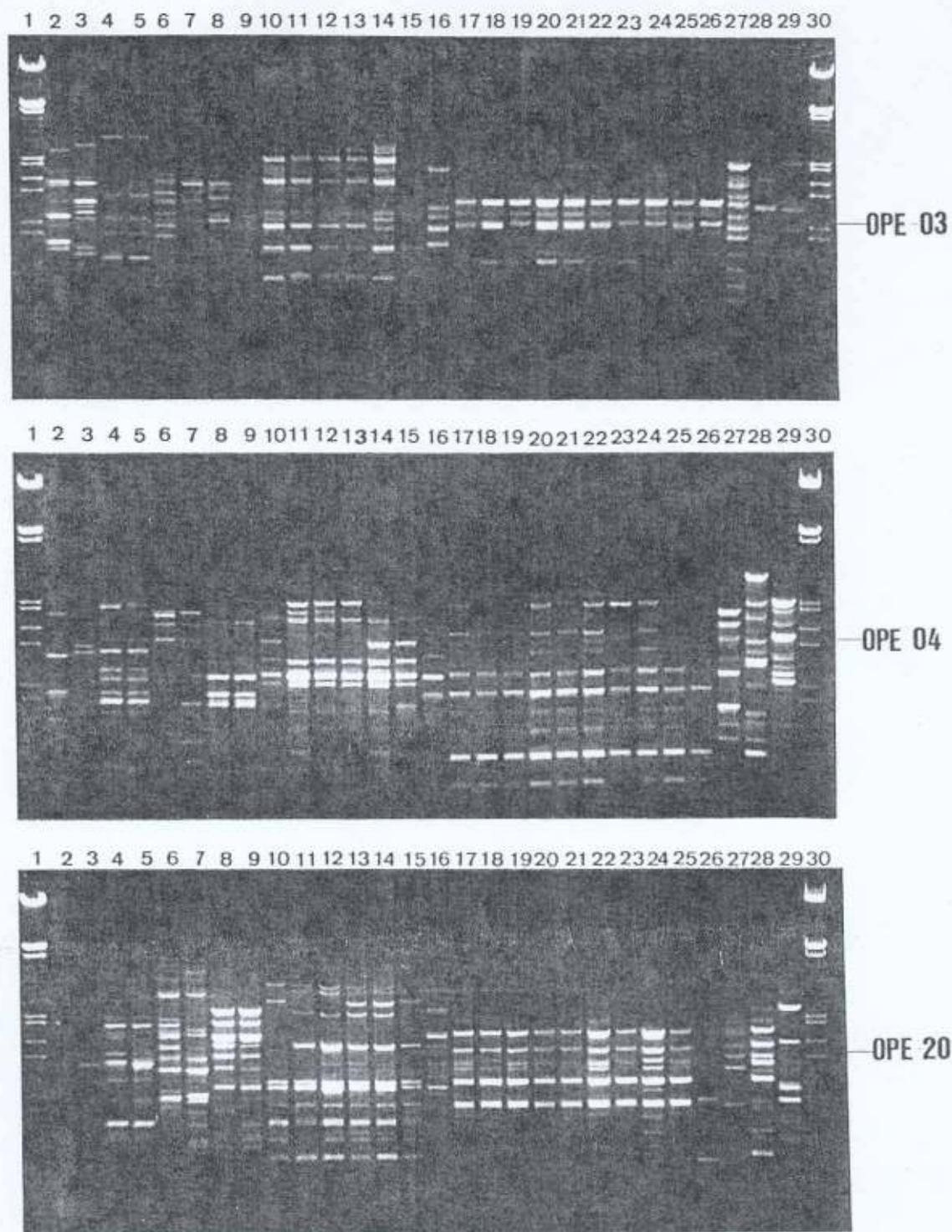
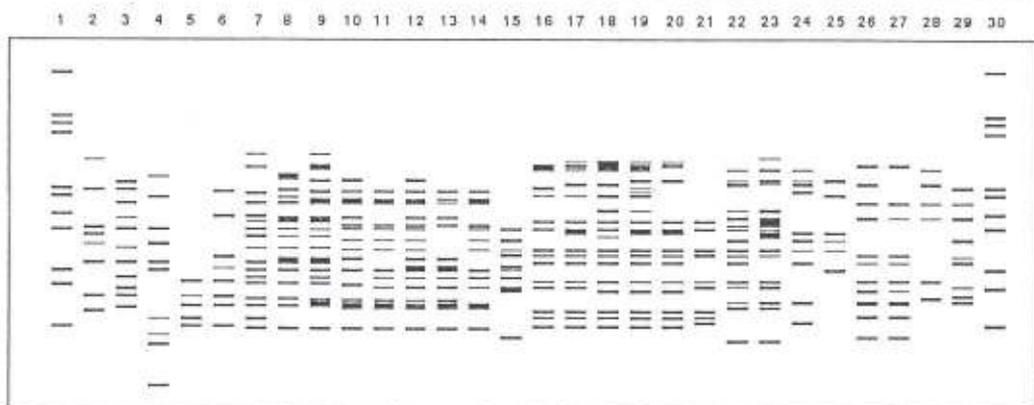
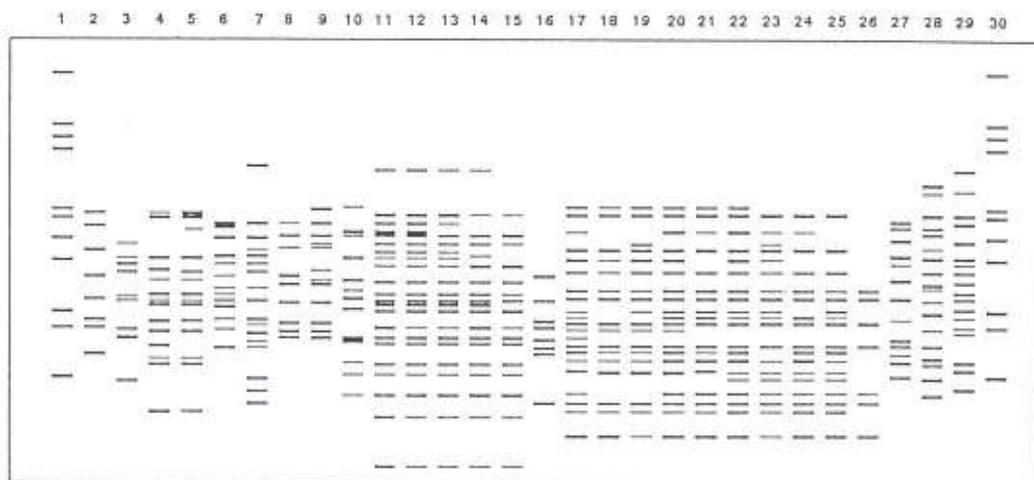
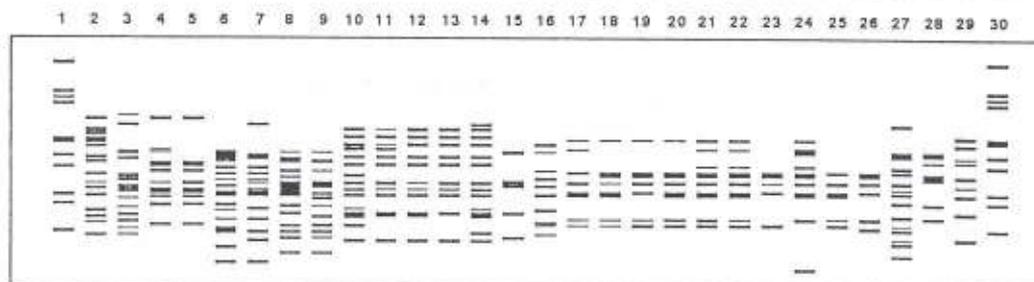
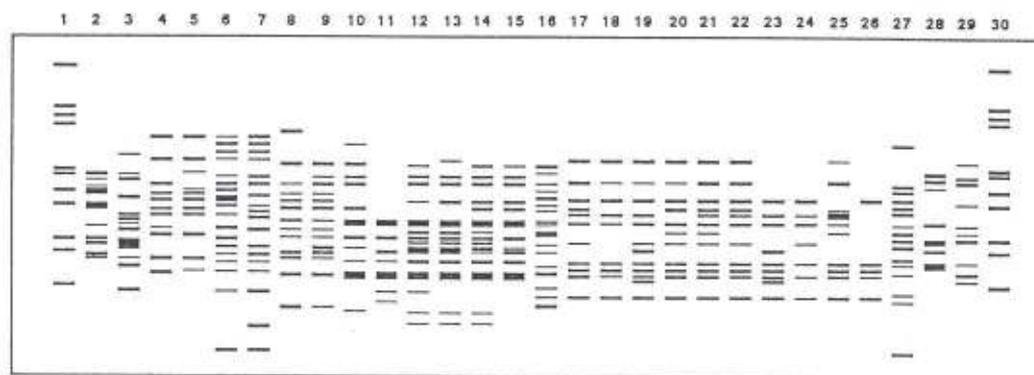


Fig. 5. RAPD profiles generated using the primers OPE-03, 04, and 20. Lanes 1 and 30 are the molecular weight marker phage lambda DNA digested with the restriction enzymes *EcoRI* and *HindIII*. Lane 2: *Cylindrocladium naviculatum* STE-U 627. Lane 3: *C. curvisporum* STE-U 763. Lanes 4–5: *Calonectria ovata* PPRI 4162 and UnB 1026. Lanes 6–7: *Calonectria morgani* PPRI 4163 and PPRI 4153. Lanes 8–9: *Calonectria scoparia* ATCC 46300 and ATCC 38227. Lanes 10–29: *Cylindrocladium floridanum* ATCC 18882, ATCC 18834, CBS 413.67, PPRI 4140, P 85.2179, P 88.5641, STE-U 624, STE-U 499, STE-U 500, UFV 76, UFV 77, UFV 78, STE-U 520, STE-U 522, ATCC 22677, AR 1785, STE-U 655, STE-U 654, STE-U 682 and STE-U 684.



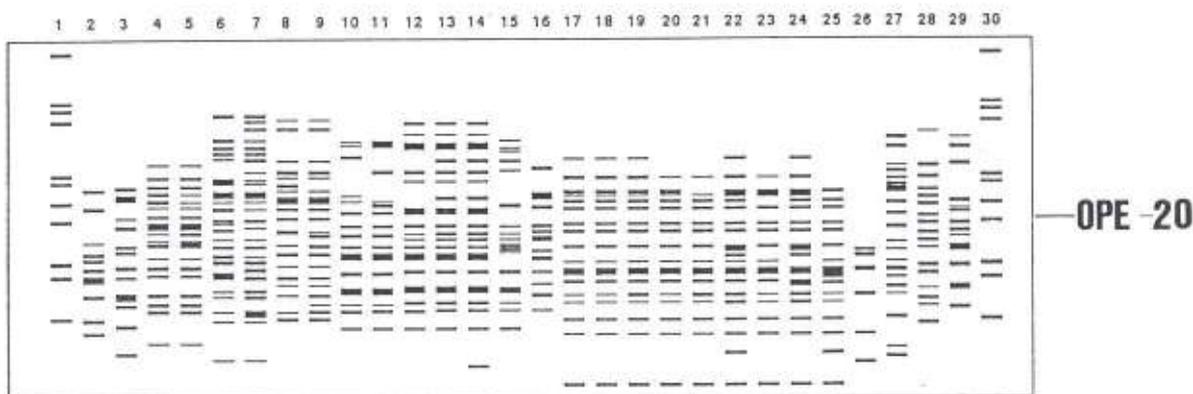


Fig. 6. Schematic representation of the RAPD profiles generated by the primers OPE-02, 03, 04, 14 and 20 and used for cluster analysis. Details for all five primers is as in Fig. 6; (=) major bands, (-) minor bands.

tion enzymes. Therefore, the *MspI* and *HaeIII* AT-DNA profiles of only one representative type strain of each of these apparent biological species, as well as ATCC 18834 and six isolates of *C. floridanum* are shown in Figs. 7, and 8. Two distinct groups could be detected amongst the isolates of *C. floridanum* (Figs. 7, 8; lanes 10–14). Data analysis conducted on combined AT-DNA profiles of isolates PPRI 4163 and 4153 (*C. candelabrum*), as well as isolate STE-U 654 (*Cylindrocladium* sp.), showed a similarity of 95–100%, which is in agreement with the RAPD results. Furthermore, AT-DNA profiles of *C. floridanum*-like isolates STE-U 624, 682 and 684 were distinct from those of *C. floridanum* group I and II. Interspecific similarity between the five species ranged between 0–28%. In the two groups of *C. floridanum* high intraspecific (70–100% and 53–72%), and low interspecific similarity coefficients (0–13%), were observed.

Discussion

RAPD analysis was found to supplement morphological observations, as well as to enhance the importance of seemingly insignificant morphological features. These findings were further supported by the AT-DNA profiles of the various *Cylindrocladium* spp., thereby confirming the species concepts as proposed by CROUS and WINGFIELD (1994). Using these molecular techniques, clear distinctions could be made between *C. naviculatum*, *C. floridanum*, *C. scoparium*, *C. candelabrum* and *C. ovatum*.

In an earlier study on the influence of osmotic potential of the growth medium on vesicle morphology and radial growth, *C. scoparium* was shown to have a higher optimal radial growth at lower osmotic potentials (-8.9 MPa), than *C. floridanum* (-6.9 MPa) (CROUS et al., 1992). Results of the present study, where more isolates were included, have shown that radial growth on media with different osmotic potentials cannot be used to distinguish *C. floridanum*, *C. scoparium*, *C. candelabrum* and *C. ovatum*. Similarly, these species were also found to have similar culture characteristics and temperature requirements for growth.

Cylindrocladium scoparium and *C. candelabrum*

CROUS et al. (1993 a) reported that although these two species are morphologically similar, they could be distinguished based upon vesicle morphology (pyriform for *C. scoparium*, obpyriform for *C. candelabrum*), and the presence of distinct mating populations. Furthermore, ascospores of *Ca. morgani* were (24–)37(-49) × (4–)7(-8) µm, and thus smaller than those of *Ca. scoparia*, which were (28–)41(-68) × (4–)5(-7) µm (CROUS et al., 1993 a). Using RAPD analysis, two different mating types of *C. candelabrum* were found to share 77% similarity ($r = 0.98$) and those of *C. scoparium* 92% ($r = 0.999$). In contrast they only shared 27–38% interspecific similarity and clustered at $r = 0.27$, thus substantiating the view of CROUS et al. (1993 a) that they represent distinct species (Fig. 9). These observations were also confirmed by the AT-DNA polymorphism data (Fig. 10). Furthermore, isolate STE-U 654 (*Cylindrocladium* sp. with obpyriform vesicles) from North America shared 53–62% similarity ($r = 0.97$) with *C. candelabrum* (Figs. 9, 10). These data suggest that this is the first record of *C. candelabrum* from North America. Until recently all isolates of *C. candelabrum* have been treated as *C. scoparium* (CROUS et al., 1993 a), and subsequently *C. candelabrum* has only been confirmed from countries such as Australia, Brazil, India, Kenya, Madagascar, Mauritius and South Africa (CROUS and WINGFIELD, 1994; CROUS and SWART, 1995).

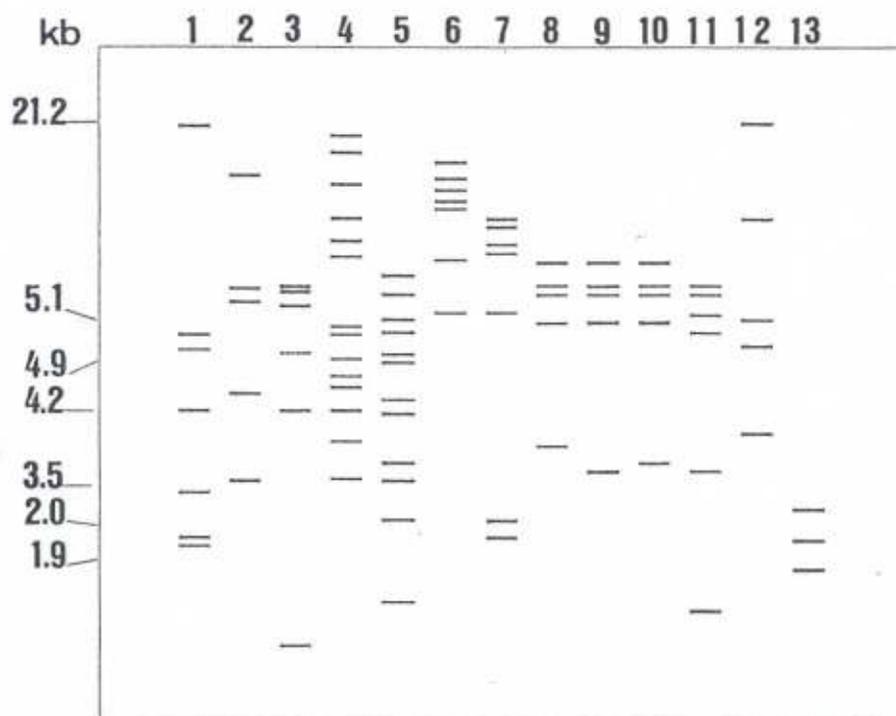
Cylindrocladium ovatum

This species is characterized by ovate vesicles, 1(-3)-septate conidia and the presence of a microconidial state. All known collections of this species have been obtained in the Amazonas Province of Brazil. Among these are several strains that lack the ability to form microconidia, or that have not been observed to form 3-septate conidia. However, a comparison of their α -isoesterase banding patterns (results not given), showed them to share a high degree of homology.

In mating studies, two isolates (PPRI 4162 × UNB 1026), readily produced *Calonectria* perithecia with viable



A



B

Fig. 7. Actual (A) and schematic (B) representation of total DNA digested by the restriction enzyme *Hae*III and separated on a 1% agarose gel. Lane 1 is phage lambda DNA digested with the restriction enzymes *Eco*RI and *Hind*III. Lane 2: *Cylindrocladium naviculatum* STE-U 627. Lane 3: *C. curvisporum* STE-U 763. Lane 4: *Calonectria ovata* PPRI 4162. Lane 5: *Ca. morganii* PPRI 4163. Lane 6: *Ca. scoparia* ATCC 46300. Lane 7: *Ca. kyotensis* ATCC 18834. Lanes 8–13: *C. floridanum* UFV 76, ATCC 22677, STE-U 655, STE-U 624, STE-U 682 and STE-U 684. Bands shown in B were used in the cluster analysis.

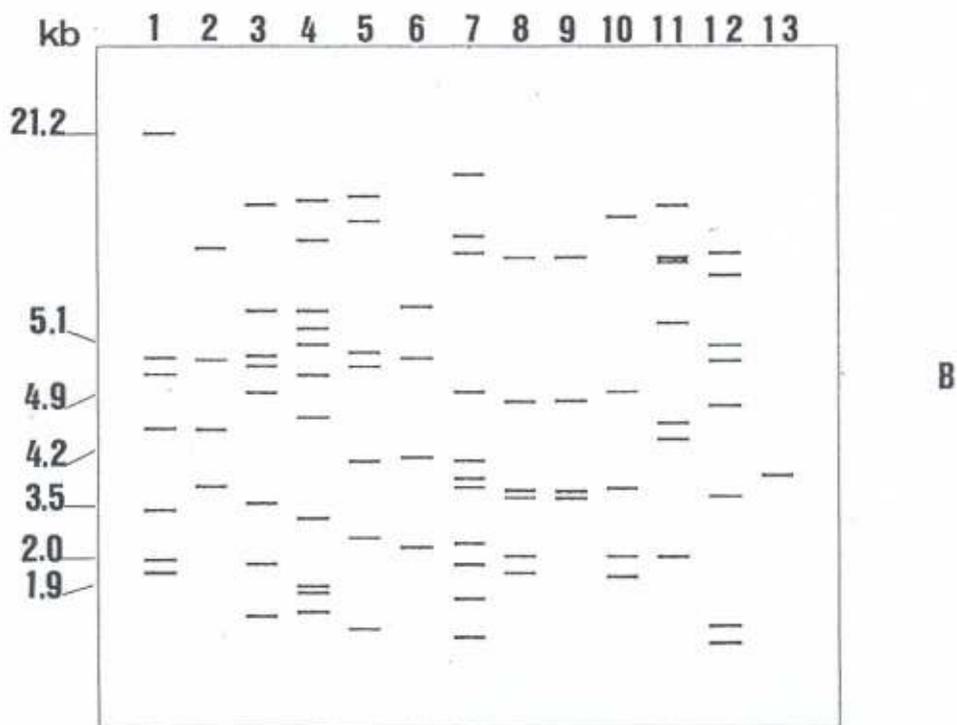
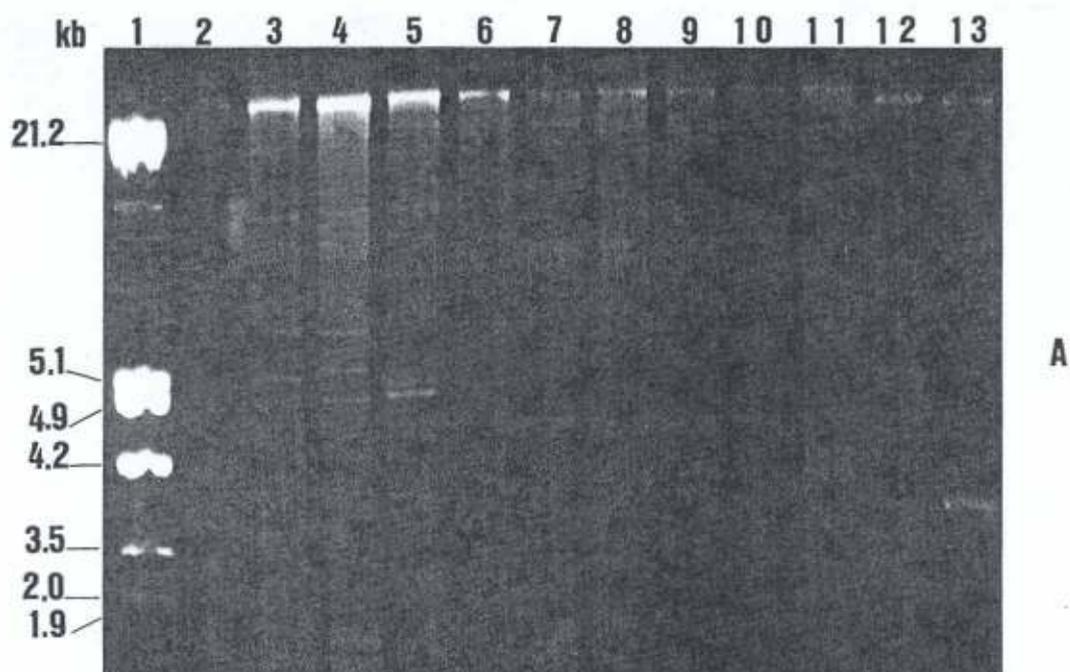


Fig. 8. Actual (A) and schematic (B) representation of total DNA digested with the restriction enzyme (*MspI*) and separated on a 1% agarose gel. Bands shown in B were used in the cluster analysis. Details are as in Fig. 7.

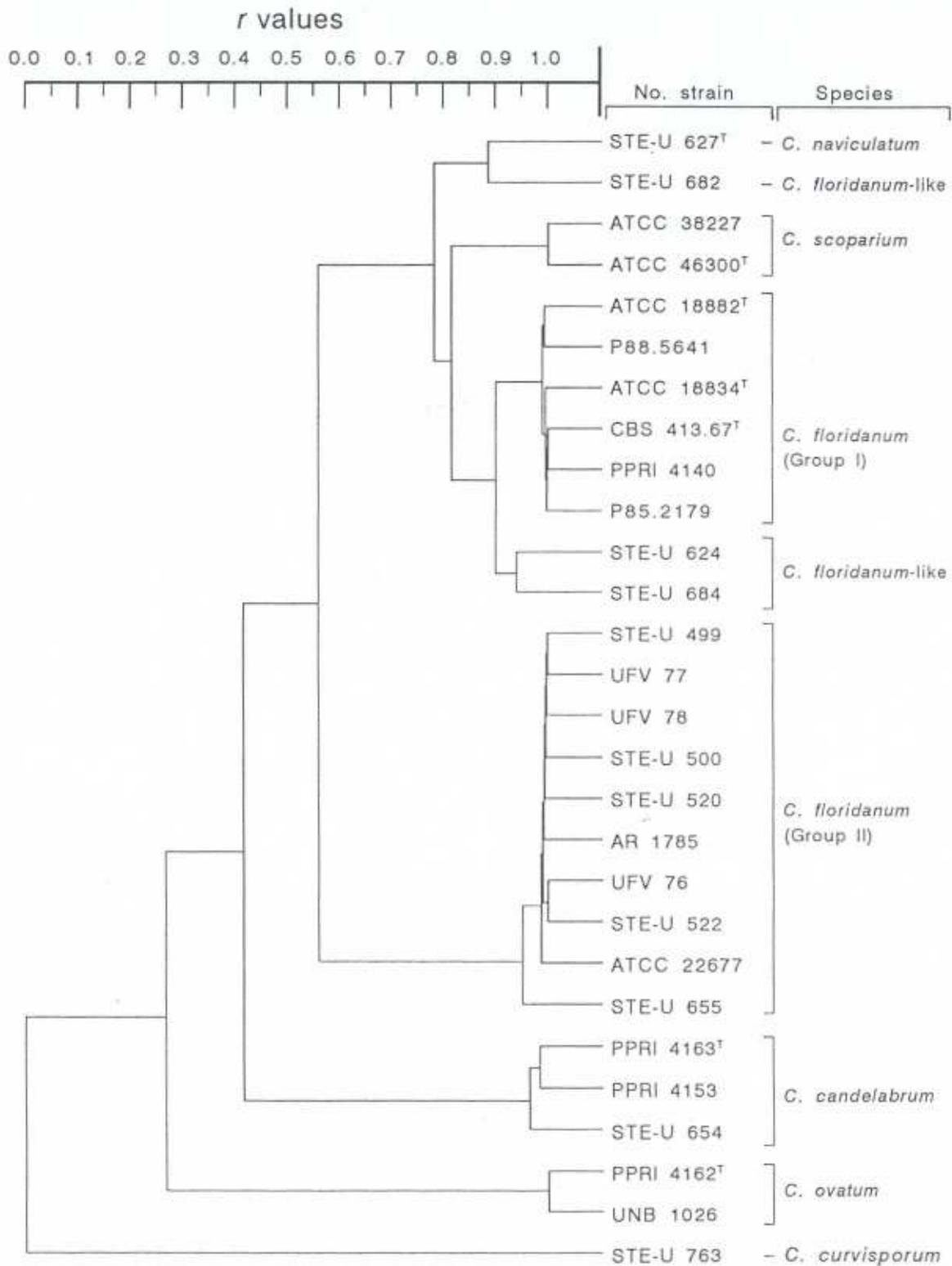


Fig. 9. A phenogram constructed from the dissimilarity matrix (100 - % similarity) calculated from the combined similarity matrix in Table 2 obtained from the RAPD profiles. With the CLUSTER procedure of SAS Institute Inc., a TREE was constructed using the average linkage method. The mean correlation coefficient *r* was used to draw the phenogram.

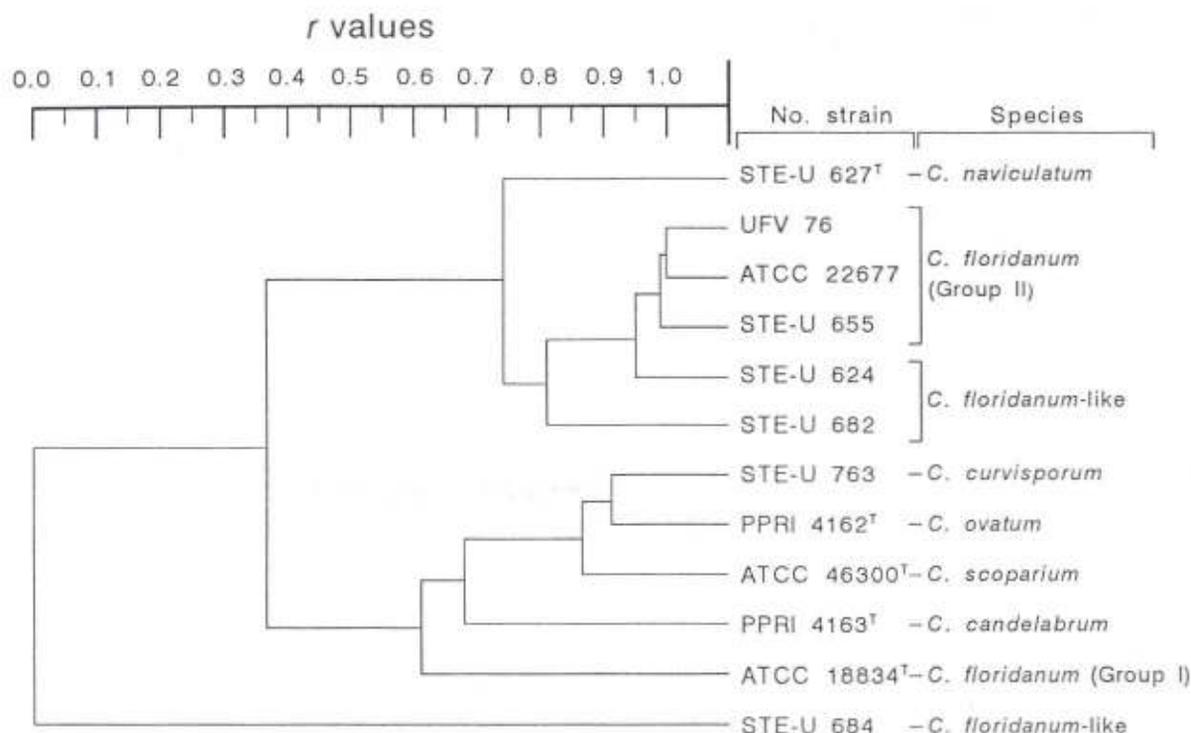


Fig. 10. A phenogram constructed from the dissimilarity matrix (100 - % similarity) calculated from the combined similarity matrix in Table 3 obtained from the AT-DNA profiles. With the CLUSTER procedure of SAS Institute Inc., a TREE was constructed using the average linkage method. The mean correlation coefficient r was used to draw the phenogram.

progeny. Ascospores were found to be 1(-3)-septate, (35-)60(-70) \times (4-)5(-6) μm , thus being larger than those of *Ca. morgani* (24(-37)(-49) \times (4-)7(-8) μm and *Ca. kyotensis* (18-)35(-48) \times (4-)5(-7) μm , but similar in range to that of *C. scoparia* (28(-41)(-68) \times (4-)5(-7) μm . However, *C. candelabrum* has obpyriform vesicles, and 1-septate conidia (33-)45(-66) \times (3-)4(-5) μm , while *C. ovatum* has ovate vesicles, and 1(-3)-septate conidia, (-36-)65(-80) \times (4-)5(-6) μm . Based on their distinct morphology, as well as the fact that the two mating strains shared 99% intraspecific similarity ($r = 0.999$), 18-47% interspecific similarity, and clustered at $r = 0.27$ with the other taxa discussed above, a new epithet must be provided for the *Calonectria* teleomorph of *C. ovatum*:

Calonectria ovata Victor & Crous sp. nov. Figs. 1F, G; 3

Perithecia rubri-brunnea, superficialia, solitaria vel 2-3 aggregata, globosa ad ovoidea, 350-550 \times 350-450 μm , ostiolo papillato, crocea. Parietis perithecii bistratus; stratum exterius ex textura globulosa constante, 40-70 μm latum; stratum interius ex textura angulari constante, 20-40 μm latum; stratum hymenii ex prismatica constante, 10-15 μm latum; basis perithecii 40-60 μm lata ex cellulis angularibus, basi denigratis. Asci hyalini, clavati, 70-120 \times 10-25 μm , instipitem longum tenuem gradatim angustati, 8-sporei. Ascosporeae hyalinae, cylindricae ad fusiformes, obtusae, falcatae ad sigmoideae, 1(-3)-sep-

tatae ad septum non constrictae, guttulate, (35-)60(-70) \times (4-)5(-6) μm .

HOLOTYPE: Brazil, Amazonas, on *Eucalyptus*, 1993, leg. J. Dianese, (PPRI 4162 (UFV 55) \times UNB 1026), PREM 51726 (specimen).

Perithecia red-brown, superficial on substrate, solitary or in clusters of 2-3, globose to ovoid, 350-550 \times 350-450 μm , with papillate ostiole, orange, turning blood-red in 3% KOH. Perithecial wall consisting of two layers: outside layer of *textura globulosa*, 40-70 μm wide; inner layer of *texture angularis*, 20-40 μm wide; hymenium layer of *texture prismatica*, hyaline, 10-15 μm wide; perithecial base 40-60 μm wide, consisting of angular cells, blackened around the point of attachment. Asci hyaline, clavate, 70-120 \times 10-25 μm , tapering to a long thin stalk, containing eight ascospores. Ascospores hyaline, cylindrical to fusiform with rounded ends, falcate to sigmoid, 1(-3)-septate, not constricted at septa, guttulate (35-)60(-70) \times (4-)5(-6) μm .

Cylindrocladium floricidum

Using nuclear DNA fingerprinting, WANG et al. (1993) reported three groups present amongst 26 isolates of *C. floricidum* and *C. scoparium*, which they had obtained from nurseries in Ontario, Minnesota and Wisconsin. Two of these groups were found to be within *C. floricidum*, with the third group representing *C. scoparium*.

Results obtained using RAPD and AT-DNA analyses in the present study confirmed the results of WANG *et al.* (1993), and showed the isolates in group II of *C. floridanum* (Figs. 5–8) to be distinct from the ex-type strains (ATCC 18882, 18834 and CBS 413.67) in group I. Interspecific similarity of 12–45% was observed between these two groups, and they clustered together at low *r*-values ($r = 0.56$ for RAPD, and $r = 0.37$ for AT-DNA analysis) (Figs. 9, 10). Furthermore, although *Ca. kyotensis* is homothallic, earlier mating studies with some isolates present in group II suggested that these isolates may be heterothallic. In an examination of their vesicle morphologies, it was found that these isolates tended to have vesicles that were less rounded than the sphaeropedunculate vesicles of the type strain of *C. floridanum*. This difference was very slight, however, and could, therefore, not be used to distinguish these two groups unequivocally.

In the present study, RFLP profiles of AT-DNA digested with the restriction enzymes *Hae*III and *Msp*I (Figs. 7, 8; lanes 7–10) could be used to distinguish the two *C. floridanum* groups. These groups were also defined using RAPD analysis, and shared only 0–9% interspecific similarity ($r = 0.37$) with the type strain in group I (Tables 2, 3). Results obtained with RAPD and RFLP analyses of the representative strains of *C. scoparium* furthermore showed the latter species to be distinct from both groups of *C. floridanum* (Figs. 9, 10).

The low similarity coefficients shared between the *C. floridanum* isolates of group I and II suggested that these geographically isolated groups had been present in their specific regions (Table 1) long enough to have developed separately, and that little or no genetic exchange has occurred between them in recent time. The % similarity values obtained from RAPD analysis compared favourably to results obtained for other genera, where similarity coefficients were 71–100% and 20–24% for intra- and inter-group comparisons, respectively (GOODWIN and ANNIS, 1991; STRONGMAN and MACKAY, 1993).

Isolates STE-U 682 and STE-U 684 of *C. floridanum* were morphologically similar to the type strain of *Ca. kyotensis*. Based on their RAPD and RFLP profiles, however, these strains were distinct from each other, as well as from *Ca. kyotensis*, and showed only 40–51% similarity with the type strain of *Ca. kyotensis* and its two synonyms (Tables 2, 3; Figs. 5–10). These data suggest that they represent distinct species, and that further studies comparing them to other *Cylindrocladium* species may be necessary.

Isolate STE-U 624 (*C. floridanum*) was isolated from soil collected in Brazil. In agreement with its distinct vesicle morphology, the highest % similarity was with one isolate of *C. floridanum* (ATCC 18882) (58%; Tables 2, 3; Figs. 5–8). These results suggested that this strain could possibly be representative of yet another group within *Ca. kyotensis*.

Cylindrocladium isolate STE-U 763 represented one of several strains recently obtained from soil samples collected in Madagascar. Although it had sphaeropedunculate vesicles similar to that of *C. floridanum*, it was distinct in having curved 1(-3)-septate conidia, (45-)60(-70) × (4-)5(-6) μm. Furthermore, its highest % similarity was

34% with *C. candelabrum* (Tables 2, 3; Figs. 5–8; lane 3). Based on these distinct morphological and molecular differences, this isolate is described as a new species of *Cylindrocladium* below:

***Cylindrocladium curvisporum* Crous & Victor sp. nov.**
Figs. 1H, I; 2G, H; 4

Macroconidiophora. Stipes septatus, hyalinus, in vesiculam sphaeropedunculatam (5-)8(-10) μm diam. terminans. Conidiophori rami: rami primarii non septati, vel rate 1 septati, (13-)20(-35) × (4-)5(-6) μm; rami secundarii non septati, (10-)15(-19) × 5(-6) μm; rami tertiarii rari, non septati, (10-)15(-19) × 5(-6) μm. Phialides elongatae, doliiformes ad reniformes, hyalinae, non septatae, (10-)12(-15) × (3-)4(-4.5) μm. Conidia cylindrica, hyalina, recta vel curvata, apicibus obtusis, 1(-3)-septata, (45-)60(-70) × (4-)5(-6) μm.

HOLOTYPE: Madagascar, Tamatave, soil, April 1994, leg. P. W. Crous, PREM 51751.

Macroconidiophores. Stipe septate, hyaline, terminating in a sphaeropedunculate vesicle (5-)8(-10) μm diam.; (110-)130(-150) μm long. Conidiophore branches: primary branches non-septate or rarely 1-septate, (13-)20(-35) × (4-)5(-6) μm; secondary branches non-septate, (10-)15(-19) × 5(-6) μm; tertiary branches rare, non-septate, (10-)15(-19) × 5(-6) μm. Phialides elongate, doliiform to reniform, hyaline, non-septate, (10-)12(-15) × (3-)4(-4.5) μm. Conidia cylindrical, hyaline, straight or curved with rounded ends, 1(-3)-septate, (45-)60(-70) × (4-)5(-6) μm. Colony colour (reverse) 13'b, ochreous (Rayner, 1970). Chlamydozoospores in medium numbers, with medium to extensive sporulation on aerial mycelium. Cardinal temperature requirements for growth minimum above 10 °C, optimum 25 °C, maximum below 35 °C.

Although no teleomorph developed in the present study, protoperithecia were observed on CLA, and it is possible that this strain, as with *C. ovatum*, would produce a teleomorph if the right mating types were collected.

The results of this study have clearly shown that there is more variation within well-known species of *Cylindrocladium* than was previously thought. *C. floridanum* proved to be a more diverse species with the presence of at least two main groups as indicated by RAPD and AT-DNA profiles. Based on the genetic divergence between groups I and II within *C. floridanum* their geographic separation and slight differences in vesicle morphology, they could be considered to represent sibling species.

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