Systematic appraisal of species complexes within *Cylindrocladiella*

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Twenty-four strains of six *Cylindrocladiella* spp. were compared with respect to their morphology, nuclear ribosomal DNA (nuclear rDNA) polymorphisms, and restriction fragment length polymorphisms of A + T-rich DNA (AT-DNA). Two distinct DNA profiles were observed for the ten strains of *C. camelliae/peruviana*-complex studied. One profile corresponded with the type strain of *C. peruviana*, which was earlier regarded as a synonym of *C. camelliae*. Based on Southern analyses and AT-DNA, *C. parva*, *C. elegans* and *C. lageniformis* were confirmed to be distinct species with little intraspecific variation. Although *C. infestans* was shown to have some variation among the four strains studied, they could not clearly be distinguished as separate taxa. Based on general morphology, nuclear rDNA and AT-DNA polymorphisms, seven species of *Cylindrocladiella* are recognized.

Species of *Cylindrocladium* and *Cylindrocladiella* are widely distributed throughout the world and are pathogenic to a wide range of angiosperm and gymnosperm hosts in areas of high humidity (Crous, Phillips & Wingfield, 1991; Peerally, 1991). Several species of these genera are notorious pathogens in nurseries and *Eucalyptus* plantations and are associated with damping-off, root rot, leaf spot, seedling blight, cutting rot, stem cankers as well as several other diseases (Crous *et al.*, 1991; Peerally, 1991; Crous, Phillips & Wingfield, 1993).

Since Morgan (1892) erected *Cylindrocladium*, more than 20 distinct species have been described (Boedijn & Reitsma, 1950; Crous *et al.*, 1991; Peerally, 1991; Crous & Wingfield, 1994; Crous *et al.*, 1994*a*; Crous, Korf & Van Zyl, 1995; Victor *et al.*, 1997). Boesewinkel (1982) placed several small-spored *Cylindrocladium* species in a new genus, *Cylindrocladiella*. Peerally (1991) questioned the utility of this separation. Subsequently, the generic status of *Cylindrocladium* and *Cylindrocladiella*, as well as their similarities to other hyphomycete genera has been reviewed (Samuels *et al.*, 1991; Crous & Wingfield, 1993; Crous, Wingfield & Lennox, 1994*b*). Known teleomorphs of respective *Cylindrocladiella* and *Cylindrocladium* spp. are classified in different genera, thus enhancing the importance of seemingly insignificant morphological differences in the anamorphs (Crous *et al.*, 1994*b*; Watanabe, 1994).

Total protein and isozyme electrophoresis profiles have been used to distinguish species of *Cylindorocladium* and *Cylindrocladiella* (Zumpetta, 1976; Moreira, 1989; Alfenas *et al.*, 1991; Crous *et al.*, 1993*a*; El-Gholl *et al.*, 1993). However, when using isozymes, many allelic variations do not result in differences in the electrophoretic mobility of proteins, and hence cannot be detected by isozyme electrophoresis (Murphy *et al.*, 1990). Protein variation can also be affected by culture conditions (Panabières *et al.*, 1989; Leung, Nelson & Leach, 1993). The value and importance of studying restriction fragment length polymorphisms (RFLPs) in fungal systematics has been reviewed by several authors (Taylor, 1986; Metzenberg, 1991; Kohn, 1992; Freeman, Pham & Rodriquez, 1993; Leung *et al.*, 1993; Maclean *et al.*, 1993). Restriction enzyme digests of total DNA have resulted in the separation of repetitive sequences which may originate from either mitochondrial DNA (mtDNA) or the ribosomal repeat unit (rDNA) of nuclear DNA (Taylor, 1986; Coddington *et al.*, 1987; Panabiéres *et al.*, 1989; Crous *et al.*, 1993*a*; Varga *et al.*, 1993; Louw *et al.*, 1995).

Based on classical taxonomy, Boesewinkel (1982) recognized five species in *Cylindrocladiella*. Because of the plasticity of these fungi on different media and under various environmental conditions, previous studies have shown that standardization of incubation conditions and growth media is essential to distinguish species in this complex (Peerally, 1991; Crous, Phillips & Wingfield, 1992). In a recent reassessment of *Cylindrocladiella* (Crous & Wingfield, 1993), two additional species were established and *C. peruviana* (Bat., J. L. Bezerra & M. M. P. Herrera) Boesew. was reduced to synonymy with *C. camelliae* (Venkataram. & C. S. V. Ram) Boesew. In the present study, we examined 24 *Cylindrocladiella* strains. Using classical taxonomy, morphological variation was observed among strains identified as *C. camelliae*, *C. infestans* Boesew. and *C. lageniformis* Crous, M. J. Wingf. & Alfenas. The aim of the present study was therefore to reassess all species in *Cylindrocladiella* based on their

morphology, and the similarity of their DNA banding patterns.

MATERIALS AND METHODS

Morphological characteristics

A strain of *C. cameliae* (PPRI 3990), as well as ex-type strains of *C. elegans* Crous & M. J. Wingf. (PPRI 4050), *C. infestans* (ATCC 44816), *C. lageniformis* (PPRI 4449), *C. novae-zelandiae* (Boesew.) Boesew. (ATCC 44815), *C. parva* (P. J. Anderson) Boesew. (ATCC 28272), *C. peruviana* (IMUR 1843) and *Nectria camelliae* (Shipton) Boesew. (ATCC 38571) were examined along with several other previously unidentified strains (Table 1). All strains used in this study were placed in the culture collection of the Department of Plant Pathology at the University of Stellenbosch (STE-U).

Single-conidium isolates were plated onto carnation-leaf agar (CLA) (Fisher *et al*., 1982; Crous *et al*., 1992), incubated at 25 °C under nuv light, and examined after 7 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 the basal septum to the vesicle tip. Thirty examples of each structure were measured, averages determined, and extremes given in parentheses.

Fungal DNA extraction

This procedure was carried out as described previously by Crous *et al*. (1993*a*), except that cetyltrimethylammonium bromide (CTAB) (Sigma Chemical Co., St Louis, U.S.A.) and 5 M NaCl were added to the mycelial mixture to a final concentration of 2% (v/v) and 0·9 M, respectively, after the ground mycelia were lysed for 16 h at 65°, and incubated for a further 1 h at 65°. Proteins were subsequently denaturated and the DNA precipitated as described previously. Total genomic DNA of two isolates (STE-U 540 and PPRI 3990) was fractionated by CsCl/bisbenzimide density gradient centrifugation (Garber & Yoder, 1983), resulting in two bands. The upper band, which was less prominent than the lower band and reported to contain the mitochondrial genome (Vincent *et al*., 1986; Anderson, Petsche & Smith, 1987; Hwang *et al*., 1991), was collected and purified for comparison with total genomic digests.

Nuclear rDNA analysis

Total DNA (*ca* 10 µg) of each of 10 representative isolates was subjected to 3 h restriction digestion with the restriction enzymes *Eco*R I, *Xho* I, *Pvu* I, *Stu* I, and double digestion with *Eco*R I/*Xho* I and *Eco*R I/*Hin*d III, respectively, according to the recommendations of the suppliers (Boehringer Mannheim, Johannesburg, South Africa). The DNA was separated on horizontal 1% agarose gels and transferred to Hybond-N nylon membranes (Amersham, Johannesburg, South Africa) according to standard procedures (Sambrook *et al*., 1989). The *Neurospora crassa* ribosomal DNA (rDNA) was purified from plasmid pMF2 (Russel *et al*., 1984) as a 6·3 kb *Pst* I fragment and labelled with [α-^{32}P]dATP (Amersham) as described by Feinberg & Vogelstein (1983). Southern hybridizations and

Table 1. *Cylindrocladiella* isolates used.

	Accession number	Host	Location	Collector	Date isolated	References
C. camelliae	PPRI 3990	*Eucalyptus grandis*	South Africa	P. W. Crous	1990	Crous *et al*., 1993*b*
	PPRI 3993	*E. grandis*	South Africa	P. W. Crous	1990	Crous & Wingfield, 1993
C. elegans	PPRI 4050	Leaf litter	South Africa	P. W. Crous	1989	Crous & Wingfield, 1993
	STE-U 518	Leaf litter	South Africa	P. W. Crous	1992	Present study
	PPRI 4210	*Eucalyptus* sp.	South Africa	P. W. Crous	1989	Crous & Wingfield, 1993
C. infestans	ATCC 44816	*Pinus pinea*	New Zealand	H. J. Boesewinkel	1982	Boesewinkel, 1982
	IMI 299376	*Arenga pinnata*	Indonesia	K. B. Boedijn & J. Reitsma	1949	Boesewinkel, 1982
	ATCC 38571	*Pinus pinea*	Australia	W. A. Shipton	1973	Boesewinkel, 1982
	PPRI 4450	*Eucalyptus* sp.	Brazil	A. C. Alfenas	1992	Crous & Wingfield, 1993
C. lageniformis	PPRI 4449	*Eucalyptus* sp.	Brazil	A. C. Alfenas	1992	Crous & Wingfield, 1993
	STE-U 576	*Vitus vinifera*	South Africa	S. Ferreira	1993	Present study
	STE-U 523	Leaf litter	South Africa	P. W. Crous	1992	Present study
C. novae-zelandiae	ATCC 44815	*Rhododendron indicum*	New Zealand	H. J. Boesewinkel	1981	Boesewinkel, 1982
C. parva	ATCC 28272	*Telopea speciosissima*	New Zealand	H. J. Boesewinkel	1974	Boesewinkel, 1982
	PPRI 3999	*Pinus radiata*	South Africa	P. W. Crous	1990	Crous & Wingfield, 1993
	STE-U 502	*Prunus* sp.	South Africa	S. Lamprecht	1991	Present study
C. peruviana	IMUR 1843	Ants	Brazil	M. P. Herrera	1963	Boesewinkel, 1982
	PPRI 3991	*Acacia mearnsii*	South Africa	P. W. Crous	1990	Crous & Wingfield, 1993
	STE-U 398	*Eucalyptus* sp.	South Africa	P. W. Crous	1990	Present only
	STE-U 451	*Eucalyptus* sp.	South Africa	P. W. Crous	1990	Present study
	STE-U 540	–	Japan	T. Terashita	1969	Present study
	STE-U 541	–	Japan	T. Terashita	1968	Present study
	STE-U 524	Leaf litter	South Africa	P. W. Crous	1992	Present study
	UFV 194	*Eucalyptus* sp.	Brazil	A. C. Alfenas	1993	Present study

stringency washes were performed at 60° using the method described by Sambrook *et al*. (1989).

AT-DNA analysis

Total genomic DNA of isolates listed in Table 1 was subjected to digestion for 16 h with the restriction enzyme *Msp* I, according to the recommendations of the suppliers (Boehringer Mannheim). The DNA was separated on a Hoefer Scientific Instruments horizontal electrophoresis unit (HE 99) using a 1% (w/v) agarose gel. The gels were run at 6 V cm^{-1} in a 0·5 × TBE buffer (pH 8) (Sambrook *et al*., 1989). After separation, the DNA was stained with 20 µl ethidium bromide (10 mg ml^{-1}) in 600 ml 0·5 × TBE (pH 8) for 1 h and destained in 0·5 × TBE (pH 8) for at least 2 h. The DNA banding patterns on the agarose gels were photographed using type 55 Polaroid film. Phage lambda DNA digested with the restriction enzymes *Eco*R I and *Hin*d III was used as a molecular weight standard.

RESULTS AND DISCUSSION

Although morphological and cultural characteristics have proven to be important in defining different *Cylindrocladiella* spp. (Figs 1–12), a high degree of morphological variation was present among strains of some species, thereby hampering the use of these characteristics in identification. In contrast, nuclear rDNA and AT-DNA polymorphisms have previously been used to differentiate between closely related fungal species, and were also able to show intraspecific variation (Coddington *et al*., 1987; Moody & Tyler, 1990; Vilgalys & Hester, 1990; Föster & Coffey, 1992; Freeman *et al*., 1993; Croft & Varga, 1994; Barroso, Blesa & Labarere, 1995; Crous *et al*., 1995; Louw *et al*., 1995; Crous, Theron & Van Zyl, 1997).

Restriction enzymes such as *Hae* III and *Msp* I recognize 4 bp sequences that are GC-rich and have subsequently been used to analyse repetitive elements in mitochondrial DNA as well as nuclear DNA that are AT-rich (Coddington *et al*., 1987; Förster & Coffey, 1992, 1993; Hausner, Reid & Klassen, 1993). AT-rich sequences appear as distinct bands against the nuclear DNA background (Förster & Coffey, 1993). In the present study we found that the separation of the nuclear DNA (lower band) from the AT-DNA (upper band) using CsCl/bisbenzimide density centrifugation (Garber & Yoder, 1983; Vincent *et al*., 1986), and digestion of the latter with the restriction enzyme *Msp* I resulted in similar banding patterns to total genomic digests. We therefore refer to this DNA as AT-DNA (Moody & Tyler, 1990; Freeman *et al*., 1993). Using nuclear rDNA and AT-DNA polymorphisms in conjunction with morphological and culture criteria, seven species of *Cylindrocladiella* were distinguished (Figs 13, 14). However, when these techniques or features were separated and a sole criterion was used, species such as *C. peruviana* and *C. infestans* appeared heterogeneous (Figs 13, 14).

Cylindrocladiella novae-zelandiae

This species is known from one collection only (Boesewinkel, 1981), and is distinguished from other species by its slow growth rate, sparse chlamydospore formation, conidium dimensions, and irregularly lanceolate vesicles. The representative isolate of *C. novae-zelandiae* (ATCC 44815) could furthermore be distinguished from the other species by its distinct nuclear rDNA (Fig. 13), and AT-DNA banding patterns (Fig. 14).

Cylindrocladiella elegans

Crous & Wingfield (1993) reported that strains of *C. elegans* frequently have ellipsoid vesicles resembling those of *C. camelliae*; they can be distinguished, however, from the latter by their colony colour, temperature requirements for growth and conidium dimensions. The AT-DNA profiles *C. elegans* could be distinguished from *C. camelliae*, but showed some similarity to *Nectria camelliae* (ATCC 38571) (Fig. 14). Based on its nuclear rDNA profiles, however, this species was easily distinguished from others in the genus (Fig. 13 A, B, D).

During the present study, several strains of *C. elegans* that have been stored under sterile water or on autoclaved soil showed a loss of fertility, culture colour and chlamydospore formation when revived. The nuclear rDNA and AT-DNA profiles of atypical *C. elegans* strains remained stable, and were similar to those of sporulating strains.

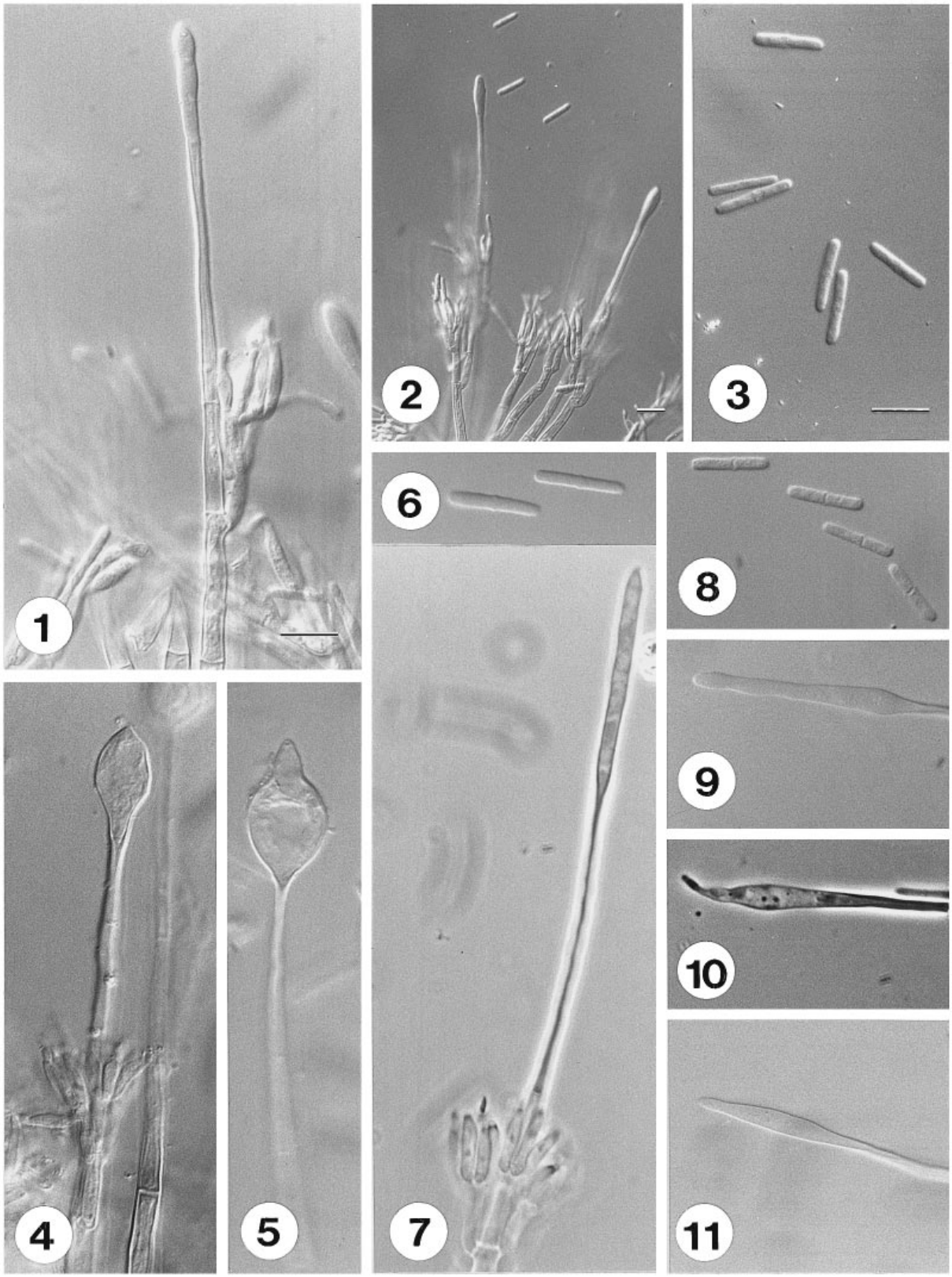
Cylindrocladiella camelliae and *C. peruviana*

Cylindrocladiella peruviana is morphologically similar to *C. camelliae*, both having ellipsoid to lanceolate vesicles, similar conidial dimensions and temperature requirements for growth (Crous & Wingfield, 1993). Peerally (1991) regarded *C. peruviana* as synonymous with *C. camelliae*. Examining dried specimens (IMI 170223, IMI 309038) derived from the original type strain (IMUR 1843), Crous & Wingfield (1993) concurred with the synonymy as proposed by Peerally (1991).

In the present study, an examination of the ex-type strain of *C. peruviana* (IMUR 1843) found some differences in vesicle morphology to isolates of *C. camelliae* (PPRI 3990, 3993). Although vesicles of *C. camelliae* tended to be slightly narrower (25–)35–45(–50) × 3–4 µm than those of *C. peruviana* (15–)25–40(–60) × (3–)4–5 µm, the most obvious difference was in their taper. Vesicles of *C. camelliae* were widest in the middle, whereas those of *C. peruviana* were widest in the lower third of the vesicle (Figs 9, 10, 12). A comparison of the AT-DNA of strains of *C. camelliae* (PPRI 3990, 3993) showed a very low similarity with those of *C. peruviana* (IMUR 1843, PPRI 3991, STE-U 398, 451, 524, 540, 541, UFV 194) (Fig. 14). Although some variation was found among the AT-DNA of strains of *C. peruviana*, the two species were also easily distinguished based upon their nuclear rDNA polymorphisms (Fig. 13 A, D–F).

Cylindrocladiella parva and *C. lageniformis*

Cylindrocladiella parva is characterized by being a fast-growing, low-temperature species with pyriform vesicles and penicillate conidiophores. *C. lageniformis* (PPRI 4449) is distinguished from *C. parva* in having lageniform to ovoid vesicles and penicillate as well as subverticillate conidiophores (Crous & Wingfield, 1993). Two recently collected South African strains



Figs 1–11. Conidiophores, vesicles and conidia of *Cylindrocladiella* spp. **Figs 1–3.** Penicillate conidiophores and conidia of *C. infestans* (PPRI 4450). **Figs 4, 5.** Variation in vesicle morphology of *C. lageniformis* (STE-U 576, PPRI 4449). **Figs 6, 7.** Conidia and penicillate conidiophore of *C. infestans* (ATCC 44816). **Fig. 8.** Conidia of *C. infestans* (ATCC 38571). **Fig. 9.** Lanceolate vesicle of *C. peruviana* (IMUR 1843). **Fig. 10.** Ellipsoid vesicle of *C. camelliae* (IMI 47717). **Fig. 11.** Ellipsoid vesicle of *C. elegans* (PPRI 4050). Bar = 10 µm, figs 4–11 the same scale as fig. 3.

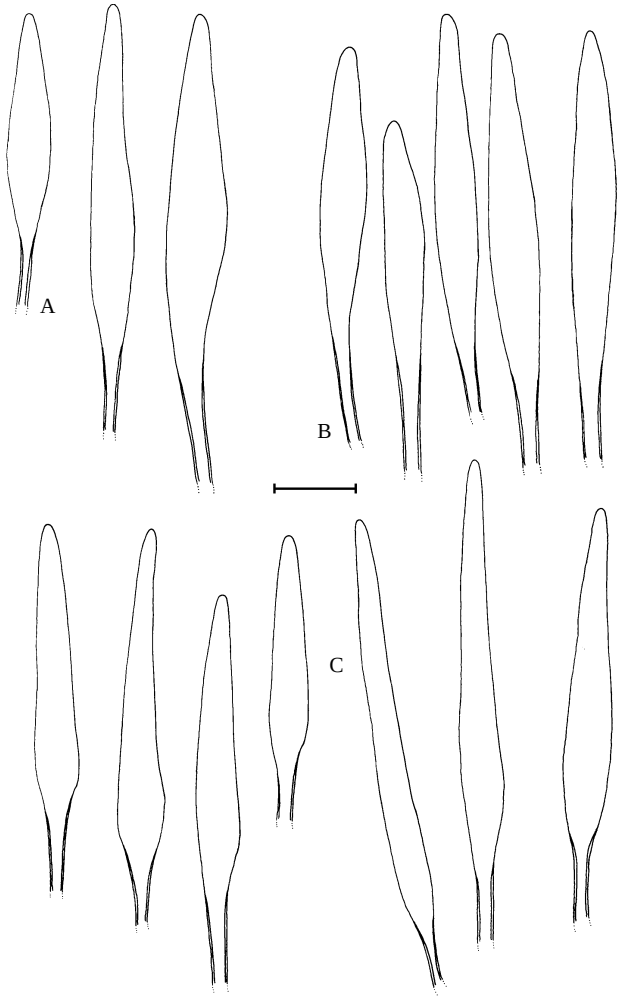


Fig. 12 A–C. Vesicles of *Cylindrocladiella* spp. in culture. (A) *C. camelliae* on potato dextrose agar (IMI 47717 ex-type). *C. camelliae* (B) on CLA (PPRI 3990). (C) *C. peruviana* on CLA (IMUR 1843 ex-type). Bar = 10 μm.

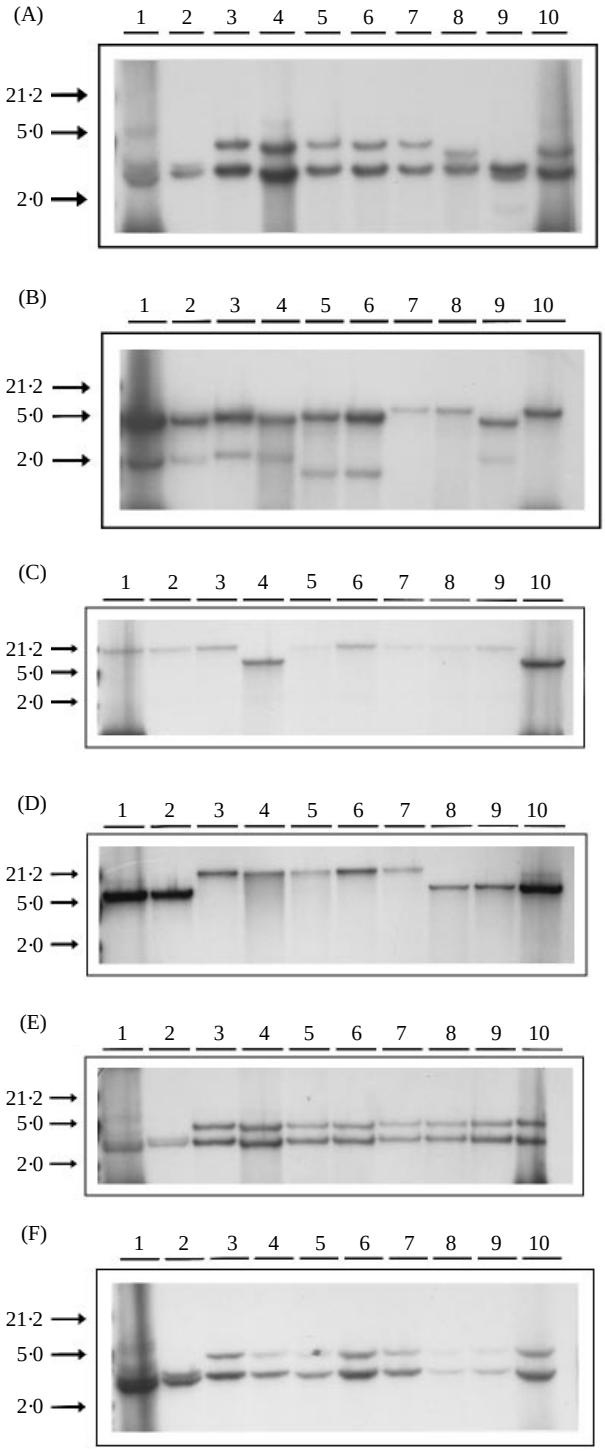


Fig. 13 A–F. Ribosomal DNA hybridization patterns for (A) *Eco*R I and *Xho*I, (B) *Stu*I, (C) *Pvu*I, (D) *Xho* I (E) *Eco*R I and (F) *Eco*R I/*Hin*d III digested nuclear rDNA of strains of *Cylindrocladiella* spp. Lanes 1, 2: *C. peruviana* (IMUR 1843, UFV 194). Lane 3: *C. camelliae* (PPRI 3990). Lane 4. *C. parva* (ATCC 28272). Lanes 5–7: *C. infestans* (ATCC 38571, PPRI 4450, ATCC 44816). Lane 8: *C. elegans* (PPRI 4050). Lane 9: *C. lageniformis* PPRI 4440. Lane 10: *C. novae-zelandiae* (ATCC 44815). Size markers are lambda DNA digested with *Eco*R I and *Hin*d III.

(STE-U 576, 523; Table 1), could not be allocated to either *C. parva* or *C. lageniformis* based on their general morphology. The presence of subverticillate conidiophores and general vesicle morphology suggested they belong to *C. lageniformis*. However, vesicles were never lageniform, but rather ovoid to pyriform suggesting a similarity with *C. parva*.

A comparison of the nuclear rDNA and AT-DNA profiles of the type strains of *C. parva* (ATCC 28272) and *C. lageniformis* (PPRI 4449) showed them to be distinct species (Figs 13 A, C, D, 14). The AT-DNA profiles of the two intermediate South African strains (STE-U 576, 523) were found to be similar with that of *C. lageniformis* (results not shown). These findings suggest that *C. lageniformis* is more variable in its vesicle morphology than initially accepted, and that its distribution is not only confined to Brazil.

Nectria camelliae *and its anamorph* Cylindrocladiella infestans

In his examination of the type strain of *Nectria camelliae* (ATCC 38571), Boesewinkel (1982) found that the anamorph was distinct from that of *C. camelliae* after which the teleomorph was originally named by Shipton (1979). Boesewinkel (1982) state that the type strain of *N. camelliae* produced an anamorph and teleomorph similar to those of a strain collected by him from wood and bark of a *Pinus* sp. in New Zealand. He subsequently described the anamorph

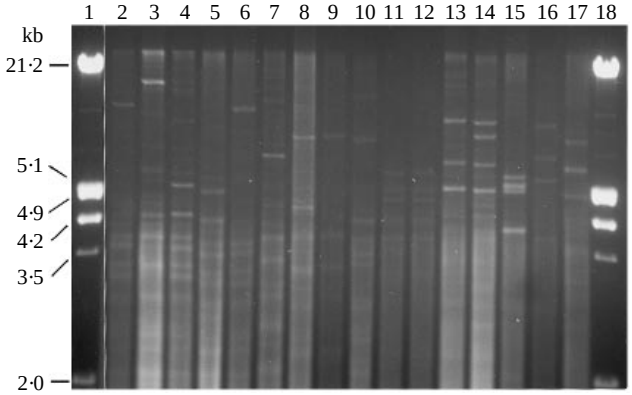


Fig. 14. Representation of total genomic DNA digested by *Msp* I and separated on a 1% agarose gel. Lanes 1 and 18 are phage lambda DNA digested with the restriction enzymes *Eco*R I and *Hin*d III. Lanes 2–6: *C. peruviana* (IMUR 1843 ex-type, STE-U 540, STE-U 395, STE-U 451, UFV 194). Lane 7: *C. camelliae* (PPRI 3990). Lanes 8, 9: *C. elegans* (PPRI 4050 ex-type, PPRI 4210). Lane 10: *Nectria camelliae* (ATCC 38571 ex-type). Lanes 11, 12: *C. infestans* (ATCC 44816 ex-type, IMI 299376). Lanes 13, 14: *C. parva* (ATCC 28272 ex-type, PPRI 3999). Lane 15: *C. lageniformis* (PPRI 4449 ex-type). Lane 16: *C. novae-zelandiae* (ATCC 44815 ex-type). Lane 17: *C. infestans* (PPRI 4450).

obtained from that collection as *C. infestans*, stating that its teleomorph was the same as *N. camelliae*. Boesewinkel (1982) further characterized *C. infestans* as having subverticillate as well as penicillate conidiophores, lanceolate to cylindrical vesicles 4–5 µm diam., and 1-septate conidia (7·5–)12–18(–22·5) × (1·5–)2·5–3·5(–5) µm. The latter characteristics were also accepted for this species by Peerally (1991) as well as by Crous & Wingfield (1993).

A comparison of the nuclear rDNA profiles of isolates from the *N. camelliae*-complex (ATCC 44816, 38571, PPRI 4450) found them to be similar for all restriction enzymes, except isolate ATCC 44816, which differed with *Stu* I (Fig. 13B). Based on their AT-DNA profiles, however, these isolates were found to be even more variable (Fig. 14). They were similar, however, regarding their temperature requirements for growth, vesicle morphology and culture characteristics. Conidia of *N. camelliae* (ATCC 38571) were up to 15 µm long, whereas those of *C. infestans* (ATCC 44816) were up to 25 µm long (Shipton, 1979; Boesewinkel, 1982). Results of the present study were inconclusive in trying to characterize the variation within *N. camelliae*. More isolates would have to be collected, and possibly more sensitive techniques employed to determine the acceptable variation within this species.

CONCLUSIONS

The present study showed that the six *Cylindrocladiella* species identified by Crous & Wingfield (1993) on the basis of morphology and cultural characteristics could also be distinguished based on their DNA banding patterns (Figs 13, 14). Although *C. peruviana* is very similar to *C. camelliae* and has been treated as a synonym (Crous & Wingfield, 1993), differences were observed in vesicle taper between strains of these two species. These differences were also confirmed via their nuclear rDNA and AT-DNA banding patterns, suggesting that they could very well be two distinct species. Some variation in the DNA profiles was observed among isolates of *N. camelliae*. More isolates would however have to be collected to further characterise this species, as the techniques and criteria used in this study could not unequivocally distinguish or group strains in this species complex. Nevertheless, the DNA profiles obtained with these techniques proved to be a useful adjunct to other taxonomic criteria. Furthermore, it also helped to define subtle morphological or developmental properties that are characteristic of these species and that have been overlooked in the past.

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