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Phylogeny of *Calonectria* based on comparisons of β-tubulin DNA sequences

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Species of *Calonectria* represent an important group of plant pathogenic fungi that cause serious losses to crops in tropical and subtropical climates. Because these fungi are difficult to identify, selected species were subjected to DNA analysis with the aim of producing a more objective identification technique. This was done by amplifying the 5′ end of the β-tubulin gene from isolates representing more than 30 species of *Calonectria*. A neighbour joining analysis was performed on a total data set of 86 isolates, while a representative subset of isolates was analysed by means of maximum parsimony. Both analyses yielded dendrograms with concordant topology. Several clades were strongly supported by bootstrap, confirming the morphological and biological species concepts presently employed. At least two large groupings were evident and both contained small-spored, 1-septate species. The only morphological character that correlated with DNA phylogeny was vesicle shape. These data support *Calonectria* as a monophyletic genus with *Cylindrocladium* anamorphs. Furthermore, this study also emphasises the importance of vesicle morphology in identifying *Cylindrocladium* anamorphs, and hence species of *Calonectria*.

INTRODUCTION

Calonectria is a member of the euascomycete order *Hypocreales* and its species are characterised by the production of *Cylindrocladium* anamorphs (Rossman 1979, Crous & Wingfield 1994). Members of this genus are further defined by their brightly coloured ascomata that change colour when placed in a 3% KOH solution, warty peridial structure and darkened stromatic bases (Rossman 1993, Rossman *et al.* 1999). The *Cylindrocladium* anamorph is the form most frequently encountered in nature, and is also morphologically the most informative. Most species of *Calonectria* are therefore distinguished chiefly on the basis of anamorph morphology.

The conidiophores of species of *Calonectria* terminate in vesicles of characteristic shape (Crous & Wingfield 1994). Conflicting opinions have arisen regarding the use of vesicle morphology as a taxonomic character (Sobers & Alfieri 1972, Hunter & Barnett 1978, Rossman 1983, Peerally 1991). Crous, Phillips & Wingfield (1992) showed that the osmotic potential of the medium influences vesicle shape and that this is a reliable character for distinguishing species, only when studied on osmotically defined media and under standardised conditions of incubation. Consequently, this character has been combined with other morphological features to identify species of *Cylindrocladium* (Crous & Wingfield 1994).

Despite the use of standardised growth conditions, several *Cylindrocladium* species have been described with variable morphological characters. Studies using a greater number of isolates of the same species have revealed intraspecific variation for characters such as conidial size and vesicle shape (Crous & Peerally 1996, Crous, Alfenas & Junghans 1998a). This morphological variation has been the source of much taxonomic confusion in the past, and has resulted in various species being amalgamated (Schoch *et al.* 1999).

Several molecular techniques have been applied to *Calonectria* taxonomy. These include the use of aminopeptidase substrate specificities (Stevens *et al.* 1990), total protein electrophoresis (Crous, Alfenas & Wingfield 1993a), isoenzyme comparisons (El-Gholl *et al.* 1997), DNA hybridisation based techniques (Crous *et al.* 1993b, Victor *et al.* 1997) as well as PCR-based methods (Victor *et al.* 1997). These techniques have been helpful in delimiting several new species (Crous *et al.* 1997a, Victor *et al.* 1997, Crous *et al.* 1999). The first study using DNA sequence comparisons to distinguish species of *Cylindrocladium* was that of Jeng *et al.* (1997) where isolates of *Cy. floridanum* were compared with *Cy. scoparium* using ITS-5.8 S ribosomal DNA. Subsequent analyses demonstrated that there were very few variable characters in this region for *Cylindrocladium* (Schoch *et al.* 1999), and that the use of DNA sequences from alternative genomic regions would be more useful to infer a phylogeny for these taxa.

DNA sequences obtained from the β-tubulin gene have been employed to predict phylogeny for species in the related hypocrealean genus *Gibberella* (O'Donnell, Cigelnik &

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Table 1. Isolates of *Cylindrocladium* studied.

Anamorph	Teleomorph	Accession no.[a]	Collector	Substrate	Origin	Date isolated	Mating strategy[b]	GenBank no.
Cy. angustatum	Unknown	STE-U 2347[c]	*N. E. El-Gholl*	*Rumohrae adiantiformis*	Florida, USA	May 1999	Un	AF207543
Cy. avesiculatum	*Ca.* avesiculata	ATCC 38226[c]	*S. A. Alfieri*	*Ilex vomitoria*	Florida, USA	1971	Ho	AF333392
Cy. candelabrum	*Ca. scoparia*	STE-U 1674[c]	*A. C. Alfenas*	*Eucalyptus* sp.	Bahia, Brazil	Jul. 1990	He	AF210857
		STE-U 1677	*A. C. Alfenas*	*Eucalyptus* sp.	Amazonas, Brazil	1991		AF210858
		STE-U 1951	*A. C. Alfenas*	*Eucalyptus* sp.	Brazil	Jun. 1998		AF210859
		UFV 89	*A. C. Alfenas*	*Eucalyptus* sp.	Brazil	1990		AF320199
Cy. citri	Unknown	CBS 186.36[c]	*H. S. Fawcett*	*Citrus sinensis*	Florida, USA	Jan. 1932	Un	AF333393
Cy. colhounii	*Ca. colhounii*	STE-U 681	*M. J. Wingfield*	Soil	Thailand	Nov. 1993	Ho	AF232852
		STE-U 705	*M. J. Wingfield*	Soil	KwaZulu-Natal, S. Africa	Nov. 1993		AF232854
		STE-U 1237	*P. W. Crous*	*Eucalyptus sp.*	KwaZulu-Natal, S. Africa	Oct. 1995		AF232853
		STE-U 1339	*M. J. Wingfield*	Soil	Indonesia	Mar. 1996		AF232841
Cy. curvisporum	Unknown	STE-U 763[c]	*P. W. Crous*	Soil	Madagascar	Apr. 1994	Un	AF333394
		STE-U 765	*P. W. Crous*	Soil	Madagascar	Apr. 1994		AF333395
Cy. flexuosum	*Ca. clavata*	STE-U 2536[c]	*N. E. El-Gholl*	*Callistemon viminalis*	Florida, USA	Apr. 1978	He	AF333396
Cy. floridanum	*Ca. kyotensis*	ATCC 18834	*T. Terashita*	*Robinia pseudoacacia*	Japan	1968	Ho	AF333397
		ATCC 18882[c]	*R. H. Morrison*	Peach roots	Florida, USA	1967		AF320193
		CBS 413.67	*W .Gerlach*	*Paphiopedilum callosum*	Celle, Germany	Oct. 1967		AF333398
		STE-U 682	*M. J. Wingfield*	Soil	Thailand	Aug. 1993		AF333402
		STE-U 2350	*M. J. Wingfield*	Soil	Hong Kong	1998		AF333401
		IMI 354528	*M. Aragaki*	*Araucaria heterophylla*	Hawaii	1987		AF333399
		IMI 354529	*M. Aragaki*	*Araucaria heterophylla*	Hawaii	1987		AF333400
		UFV 76	*A. C. Alfenas*	*Pinus* sp.	Canada	1990		AF333403
Cy. gracile	Unknown	ATCC 22833	*C. S. Hodges*	*Pinus caribeae*	Brazil	Mar. 1971	Un	AF232850
		IMI 167580	*A. Peerally*	*Camellia sinensis*	Mauritius	1970		AF333404
		PC 551197[c]	Bugnicourt	*Argyreia splendens*	Vietnam	1937		AF232855
		STE-U 623	*M. J. Wingfield*	Soil	Amazonas, Brazil	Apr. 1993		AF333405
		STE-U 1586	*P. W. Crous*	Soil	Amazonas, Brazil	1996		AF232863
Cy. graciloideum	*Ca. gracilipes*	STE-U 1153[c]	*M. J. Wingfield*	Soil	Colombia	Jun. 1996	Ho	AF333406
Cy. hawksworthii	Unknown	MUCL 30866[c]	*A. Peerally*	*Nelumbo necifera*	Mauritius	1990	Un	AF333407
Cy. heptaseptatum	Unknown	FTCC 1002	*N. E. El-Gholl*	*Rumohrae adiantiformis*	Florida, USA	Unknown	Un	AF232866
		FTCC 1003	*N.E. El-Gholl*	*Rumohrae adiantiformis*	Florida, USA	Unknown		AF232867
		STE-U 2344	*N. E. El-Gholl*	*Rumohrae adiantiformis*	Florida, USA	Mar. 1999		AF333408
Cy. insulare	*Ca. insularis*	STE-U 616	*M. J. Wingfield*	Soil	Amazonas, Brazil	Apr. 1993	He	AF210860
		STE-U 768[c]	*P. W. Crous*	Soil	Madagascar	Apr. 1994		AF210861
		STE-U 954	*M. J. Wingfield*	Soil	Veracruz, Mexico	Apr. 1994		AF210862
Cy. leucothoes	Unknown	ATCC 64824[c]	*N. E. El-Gholl*	*Leucothoe axillaris*	Florida, USA	1988	Un	AF308465
		P97.2605	*N. E. El-Gholl*	*Leucothoe* sp.	Florida, USA	1997		AF308466
Cy. macroconidiale	*Ca. macroconidialis*	STE-U 307[c]	*P. W. Crous*	*Eucalyptus grandis*	Mpumalanga, S. Africa	Mar. 1990	Ho	AF232855
		STE-U 413	*P. W. Crous*	Soil	Mpumalanga, S. Africa	May 1990		AF232856
Cy. mexicanum	*Ca. mexicana*	STE-U 927[c]	*M. J. Wingfield*	Soil	Yucatan, Mexico	Apr. 1994	He	AF210863
		STE-U 941	*M. J. Wingfield*	Soil	Holpechén, Mexico	Apr. 1994		AF210864
Cy. multiseptatum	*Ca. multiseptata*	STE-U 1589[c]	*M. J. Wingfield*	*Eucalyptussp.*	Indonesia	Jan. 1997	Ho	AF232865
		STE-U 1602	*M. J. Wingfield*	*Eucalyptus* sp.	Indonesia	Jan. 1997		AF232866
Cy. naviculatum	*Ca. naviculata*	STE-U 627[c]	*M. J. Wingfield*	Soil	Amazonas, Brazil	Apr. 1993	He	AF333409
		STE-U 628	*M. J. Wingfield*	Soil	Amazonas, Brazil	Apr. 1993		AF333410
Cy. ovatum	*Ca. ovata*	UFV 90[c]	*A. C. Alfenas*	Soil	Amazonas, Brazil	1990	He	AF210868
Cy. parasiticum	*Ca. ilicicola*	ATCC 46133	*S. A. Alfieri*	*Cissus rhombifolia*	Florida, USA	1981	Ho	AF333411
		CBS 190.50[c]	*K. B. Boedijn & J. Reitsma*	*Solanum tuberosum*	Java, Indonesia	Feb. 1948		AF333412
		STE-U 723	*M. J. Wingfield*	Soil	Colombia	Jan. 1994		AF333413
Cy. pauciramosum	*Ca. pauciramosa*	STE-U 416	*S. de Buisson*	*Eucalyptus grandis*	N. Province	Jun. 1990	He	AF210869
		STE-U 972[c]	*P. W. Crous*	Soil	W. Cape	Nov. 1994		AF210871
		STE-U 925	*M. J. Wingfield*	Soil	Santa Catarina, Brazil	Apr. 1994		AF210870
Cy. penicilloides	Unknown	CBS 174.55[c]	*M. Ookubu*	*Prunus* sp.	Hatizyo, Japan	Jan. 1952	Un	AF333414
Cy. pseudogracile	*Ca. gracilis*	AR 2677[c]	*A. Y. Rossman*	*Manilkara* sp.	Amazonas, Brazil	Unknown	Ho	AF232858
		STE-U 1588	*P. W. Crous*	Soil	Amazonas, Brazil	1997		AF232864
Cy. pteridis	*Ca. pteridis*	STE-U 2190	*P. W. Crous*	*Eucalyptus* sp.	Amazonas, Brazil	Oct. 1996	He	AF232860
		STE-U 2869	*P. W. Crous*	*Eucalyptus* sp.	Brazil	1997		AF333415
		UFV 43	*J. C. Dianese*	Unknown	Minas Gerais, Brazil	Unknown		AF232859
Cy. quinqueseptatum	*Ca. quinqueseptata*	ATCC 16550	Unknown	*Scolopendrium* sp.	Solomon Islands	1965	He	AF232868
		STE-U 516	*M. J. Wingfield*	*Eucalyptus* sp.	Thailand	Aug. 1992		AF232870
		STE-U 759	*P. W. Crous*	*Eucalyptus* sp.	Madagascar	Jan. 1994		AF232869
Cy. rumohrae	*Ca. rumohrae*	UFV 215[c]	*A. C. Alfenas*	*Rumohrae adiantiformis*	Panama	Jan. 1997	Ho	AF232872
		UFV 218	*A. C. Alfenas*	*Rumohrae adiantiformis*	Panama	Jan. 1997		AF232871
		STE-U 1603	*R. Pieters*	*Adiantum* sp.	The Netherlands	Jan. 1996		AF232873
Cy. scoparium	*Ca. morganii*	ATCC 38227	*S. A. Alfieri*	*Mahonia bealei*	Florida, USA	1970	He	AF210872
		ATCC 46300[c]	*D. M. Benson*	*Leucothoe catesbaei*	North Carolina, USA	1981		AF210873
		STE-U 1720	*N. E. El-Gholl*	*Rosa* sp.	Florida, USA	Jan. 1998		AF210874
		STE-U 1722	*N. E. El-Gholl*	*Dodonea viscosa*	Florida, USA	Jan. 1998		AF210875
Cy. spathiphylli	*Ca. spathiphylli*	ATCC 44730[c]	*S. A. Alfieri*	*Spathiphyllum* sp.	Florida, USA	1982	He	AF333418
		STE-U 1624	*M. J. Wingfield*	Soil	Ecuador	Jun. 1997		AF333419
		STE-U 1641	*M. J. Wingfield*	Soil	Ecuador	Jun. 1997		AF333420
		STE-U 2186	*K. I. Kawowas*	*Heliconia psitacorum*	Florida, USA	1986		AF333421
		STE-U 2188	*A. Thompson*	*Spathiphyllum* sp.	Mpumalanga, S. Africa	Feb. 1998		AF333422
Cy. spathulatum	*Ca. spathulata*	AR 1644	*C. S. Hodges*	*Eucalyptus grandis*	Minas Gerais, Brazil	Unknown	Ho	AF308464
		ATCC 62616[c]	*N. E. El-Gholl*	*Eucalyptus viminalis*	Brazil	1985		AF308463
		STE-U 599	*P. W. Crous*	Soil	Brazil	Jan. 1993	Ho	AF308460
		STE-U 1150	*M. J. Wingfield*	Soil	Colombia	Jan. 1995		AF308461
		STE-U 1484	*P. W. Crous*	Soil	Brazil	Aug. 1998		AF308462
		STE-U 2712	*M. J. Wingfield*	*Eucalyptus grandis*	Colombia	1998		AF308458
Cy. theae	*Ca. indusiata*	ATCC 48895	*N. E. El-Gholl*	*Rhododendron* sp.	Florida, USA	Unknown	Ho	AF232861

Table 1 (cont.)

Anamorph	Teleomorph	Accession no.[a]	Collector	Substrate	Origin	Date isolated	Mating strategy[b]	GenBank no.
		UFV 16	*N. E. El-Gholl*	*Rhododendron* sp.	Minas Gerais, Brazil	Unknown		AF333423
Cy. variabile	*Ca. variabilis*	AR 2675[c]	*F. C. de Albuquerque*	*Didymopanax morototoni*	Pará, Brazil	1990	Ho	AF333424
		UFV 28	*A. C. Alfenas*	*Eucalyptus* sp.	Minas Gerais, Brazil	Unknown		AF333425
Cylindrocladium sp.	*Calonectria* sp.	STE-U 2321	*J. Taylor*	Soil	Madagascar	Dec. 1998	Ho	AF333416
		STE-U 2322	*J. Roux*	Soil	Congo	Dec. 1998		AF333417

[a] ATCC, American Type Culture Collection, Virginia, USA; AR, A. Y. Rossman, United States Department of Agriculture, A.R.S., Beltsville, Maryland, USA; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; IMI, CABI Bioscience, Bakeham Lane, Egham, UK; MUCL, Mycothèque, Laboratoire de Mycologie Systématique et Appliqée, Université Louvain-la-Neuve, Belgium; PC, (P) Laboratoire de Cryptogamie, Paris, France; STE-U, Department of Plant Pathology, Univ. of Stellenbosch, Stellenbosch, South Africa; UFV, (A. C. Alfenas), Department of Plant Pathology, University of Viçosa, Viçosa, Minas Gerais, Brazil.

[b] Ho = homothallic; He = heterothallic; Un = undetermined.

[c] Ex-type cultures.

Nirenberg 1998). Several unlinked loci were used in a study by O'Donnell *et al.* (1998), of which the β-tubulin gene yielded the most variation of all areas sequenced making it useful for determining phylogeny in recently diverged groups. The utility of the β-tubulin gene sequence in determining phylogenetic relationships has also been demonstrated at various taxonomic levels (Schardl *et al.* 1994, Tsai *et al.* 1994, Donaldson *et al.* 1995, Baldauf & Doolittle 1997).

Previous studies of *Calonectria* species confirmed that the gene phylogeny obtained from the 5′ end of β-tubulin is concordant with that obtained from the ITS flanking sequences of the 5.8 S rRNA gene (Crous *et al.* 1999) as well as the HMG box of the *MAT-2* gene (Schoch *et al.* 2000a). The aims of the present study were, therefore, to utilise the DNA sequences of the 5′ end of the β-tubulin gene to obtain a phylogeny for species in *Calonectria*.

MATERIALS AND METHODS

Isolates

Strains were either obtained from culture collections (Table 1) or isolated from infected plant material and soil samples (Crous, Theron & van Zyl 1997b). Cultures are maintained in the culture collection of the Department of Plant Pathology, University of Stellenbosch (STE-U). Ex-type cultures have also been deposited at CABI Bioscience (IMI) and the Centraalbureau voor Schimmelcultures (CBS).

DNA extraction and sequencing

Single conidial isolates (Table 1) were grown on plates of 2% malt extract agar (MEA) (Biolab, Midrand, South Africa). Mycelial mats were cut from the plates using a sterile scalpel and ground to a powder in liquid nitrogen with a mortar and pestle. The buffers and sequencing protocol used were as explained in Schoch *et al.* (1999, 2000b). A 600 base pair (bp) fragment encompassing the first three introns and exons and part of the fourth exon of the β-tubulin gene was amplified with the use of primers T1 (O'Donnell & Cigelnik 1997) and Bt2b (Glass & Donaldson 1995).

Phylogenetic analysis

Sequences were initially aligned using the computer package Malign version 2.7 (Wheeler & Gladstein 1991) and improved manually. Phylogenetic analysis of aligned DNA sequences was performed using the neighbour-joining and heuristic algorithms in PAUP* 4.0 beta version 4 (Swofford 2000) and the resulting trees were printed with the help of Treeview Version 1.6.1 (Page 1996). For the neighbour-joining analysis, the data set containing sequences of 86 isolates was assessed with uncorrected ('*P*') distance methods and ties were broken randomly in PAUP* 4.0. A simple heuristic analysis with the maximum number of trees set to 10000 was also performed. Subsequently, a subset of 30 isolates representing each species was analysed heuristically with 1000 random addition sequences using maximum parsimony. Confidence intervals were determined using 1000 bootstrap replications in all cases. Decay indices were determined with Autodecay. *Fusarium subglutinans* (GenBank: U34417) was chosen as outgroup, because the positions of introns in the β-tubulin gene were comparable to those in the *Calonectria* species of this study. In addition to this, it also proved satisfactory in resolving the relationship of *Calonectria* to other similar genera in the *Hypocreales* (Schoch *et al.* 2000b).

RESULTS

The PCR fragments of the partial β-tubulin gene sequence obtained from the different *Cylindrocladium* species differed in size from 509 to 540 base pairs. The outgroup taxon, *F. circinatum*, had the shortest length (494 bp) (data not shown). The introns had varying numbers of characters. Introns 1–3 respectively had 167, 74 and 112 characters, of which 96, 52 and 78 were informative. The protein coding sequence consisted of 230 total characters with 45 informative sites.

The large number of isolates used in this study necessitated the use of the neighbour-joining analysis method of Saitou & Nei (1987) for an analysis of the complete data set. This data set contained DNA sequence from more than one isolate per species where possible, and consisted of 85 ingroup taxa with 582 total characters of which 316 were parsimony informative. A simple heuristic analysis of the 85 *Calonectria* species, with gaps treated as missing, yielded 2400 trees, and a strict

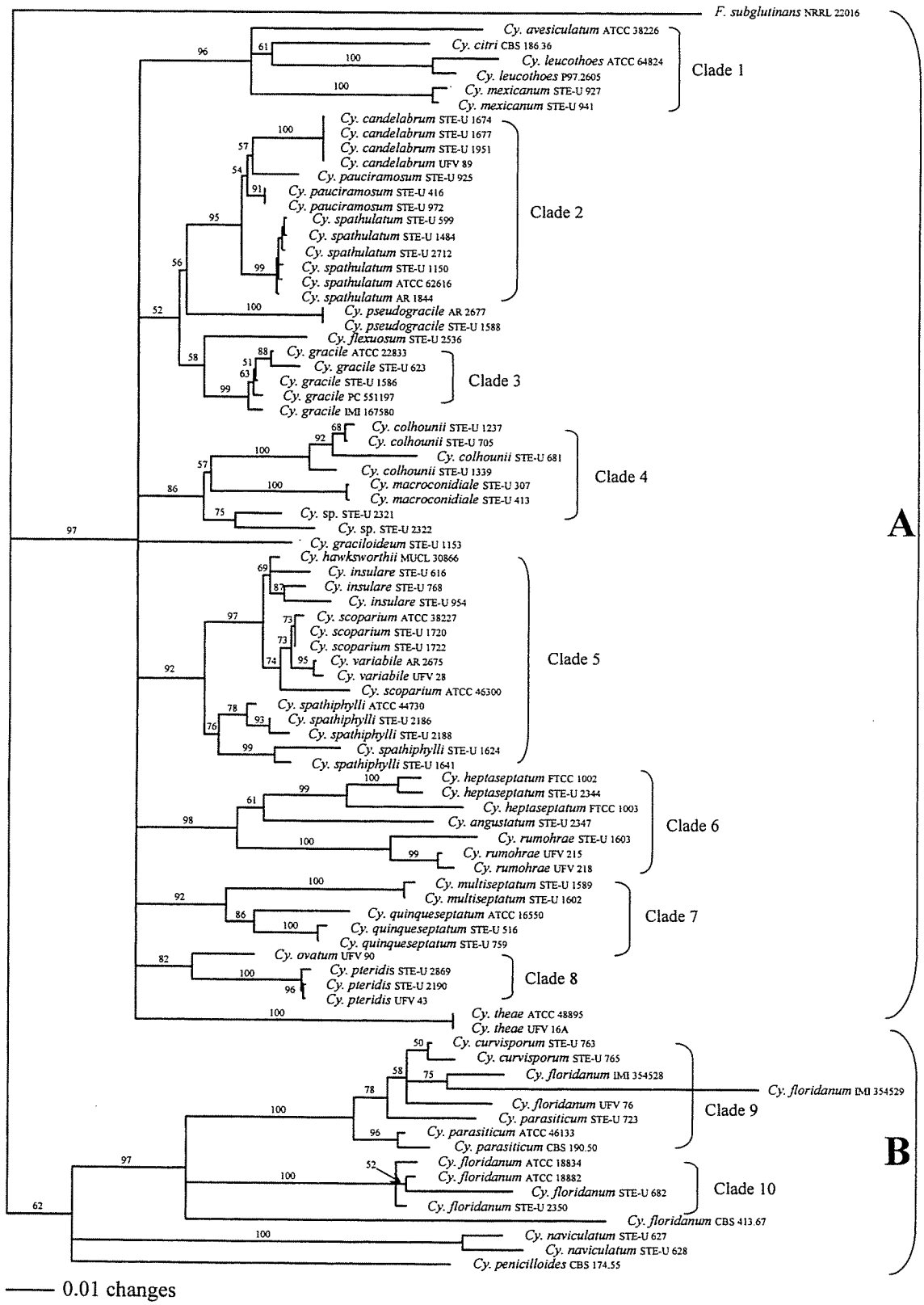

Fig. 1. Neighbour-joining tree of all *Calonectria* isolates studied. Bootstrap values were assessed after 1000 repetitions and values above 50% are shown. A *Fusarium subglutinans* sequence (GenBank: U34417) was used as outgroup.

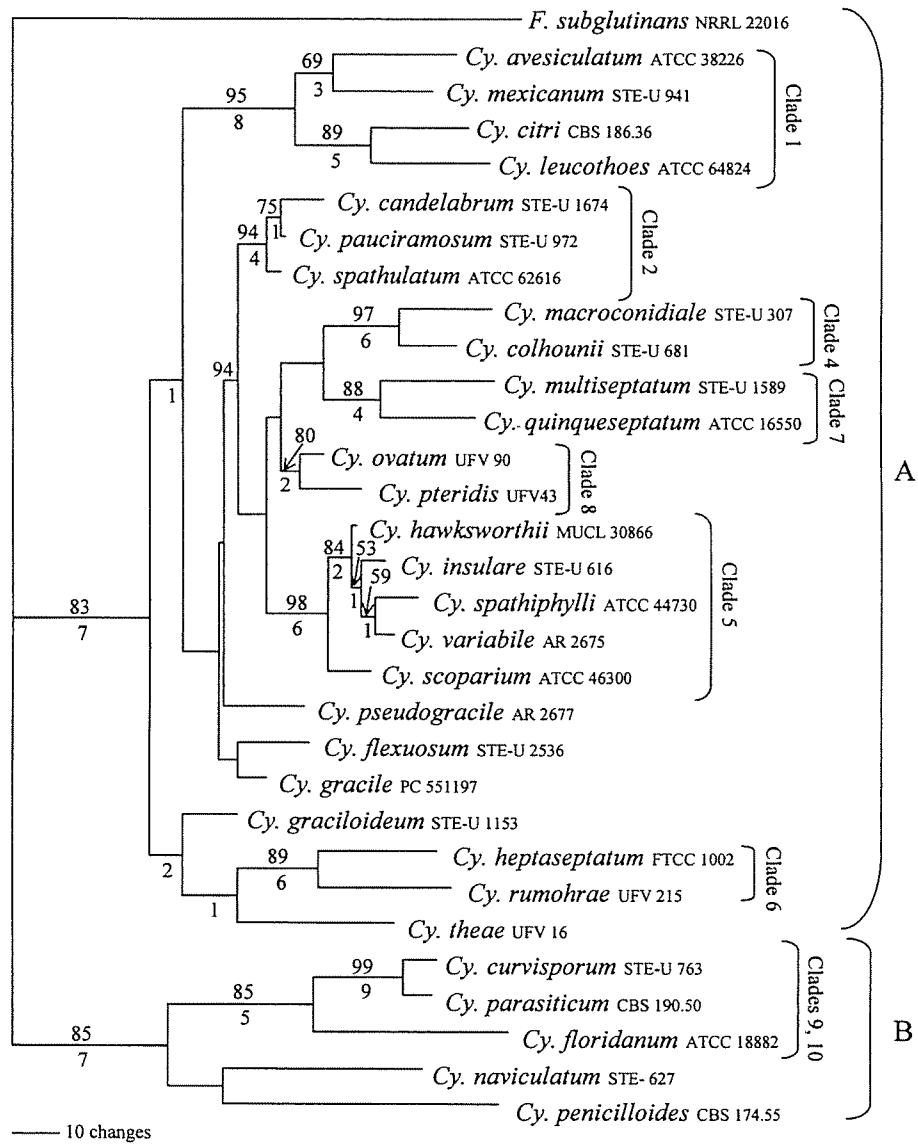

Fig. 2. One of 9 most parsimonious trees obtained from a heuristic analysis of a subset of *Calonectria* isolates (940 steps, CI = 0.576, RI = 0.549, RC = 0.316). Ten steps are indicated by the bar. Clade stability was assessed with 1000 bootstrap replications and values above 50% are shown. Decay indices are shown below the branches. Clades are indicated in accordance to the groupings shown in Fig. 1. A *Fusarium subglutinans* sequence (GenBank: U34417) was used as outgroup.

consensus provided similar clades to those seen in the neighbour-joining tree (Fig. 1). A number of clades within two larger groups were supported by bootstrap (Fig. 1). Group A included the largest number of species, as well as clades 1–8. Group B encompassed a smaller number of species (clades 9 and 10).

In order to test the validity of the proposed relationships seen in the neighbour-joining tree, a smaller data set of 30 taxa (578 characters) containing a single isolate of each species was also analysed by means of maximum parsimony. In order to limit the influence of the high number of indels found in the non-coding regions, only the first character of an insert was coded as a gap and all subsequent characters were coded as missing data. Subsequently, a heuristic search with 1000 random additions yielded 9 trees (Fig. 2). The topology of the tree in Fig. 2 is mainly concordant with that of the neighbour-joining tree in Fig. 1, and shows close relationships for the same morphological species. Two large groups (A and B), which were supported by bootstrap and decay indices, were again evident in this tree. The smaller clades generally conformed to those observed in the neighbour-joining tree (Fig. 1).

DISCUSSION

The present study represents the first DNA phylogeny of the genus *Cylindrocladium*. Previous studies have used smaller subsets of isolates in order to investigate morphologically defined groups, such as those species with multiseptate conidia (Crous *et al.* 1999) and heterothallic species with small, 1-septate conidia (Schoch *et al.* 1999, 2000a). In general these studies have supported morphological species concepts (Crous

et al. 1997b, 1999, Schoch *et al.* 2000a) and have also identified additional genetic groups within morphologically defined taxa (Schoch *et al.* 1999). Results of these and the present study clearly support characters such as conidial morphology (length and septation) and vesicle morphology (shape and width) as primary characters for species identification. Although these characters are shown to be reliable at species level, the morphological complexes that they define are generally not supported based on DNA phylogeny. Furthermore, the mating strategy followed by species (Table 1), also appeared randomly distributed throughout the genus. Thus most clades contained both homo- and heterothallic taxa.

Species of *Calonectria* that have been regarded as closely related based on morphological similarities such as vesicle shape, conidium morphology, phialide morphology, ascus and ascospore morphology, were in most cases shown to be phylogenetically distinct (or distinct from each other). One such example is found with the four species that represent the *Cylindrocladium candelabrum*-complex, namely *Cy. candelabrum*, *Cy. pauciramosum*, *Cy. insulare* and *Cy. mexicanum*. This complex is characterised by small, 1-septate conidia and ellipsoid to obpyriform vesicles (Schoch *et al.* 1999). Of these, only *Cy. candelabrum* and *Cy. pauciramosum* were closely related (clade 2), while *Cy. insulare* and *Cy. mexicanum* clustered in clades 5 and 1, respectively (Figs 1–2). These relationships also emerged when other regions of the genome were compared, namely ITS and the *MAT-2* HMG box (Schoch *et al.* 2000a). Based on the extensive data that are now available, we can conclude that although the morphological features discussed above are valuable at the species level, they do not delineate genetic groups or clades within *Calonectria*.

The only feature that showed some congruence with the various clades illustrated in Figs 1–2 was vesicle shape. Clades that were uniformly representative for this character were clade 2 (spathulate to obpyriform), clades 3, 7, 8 (clavate) and clades 9 and 10 (sphaeropedunculate). The phylogeny presented in this study further suggests that the same vesicle morphology evolved more than once in *Cylindrocladium*, and that it can occur in more than one phylogenetic assemblage.

The β-tubulin phylogeny derived in the present study separated taxa into two groups (A & B), containing several clades strongly supported by bootstrap. Two of the clades (1 & 8) contained taxa that would not normally be considered similar, due to major differences in vesicle morphology. For the remainder (clades 2–7, 9–10), clades were found to represent taxa with similar vesicle morphologies. All other morphological characters, however, were found to be variable.

Clade 2 in the β-tubulin phylogeny includes *Cylindrocladium pauciramosum*, *Cy. candelabrum* and *Cy. spathulatum*. Although the first two species have small, 1-septate conidia, *Cy. spathulatum* is distinct in having larger, multi-septate conidia. In contrast to differences in conidium size and septation, all three species share the same vesicle morphology, and occur commonly in South America, which could indicate a closer evolutionary relationship.

Clade 4 is comprised of three species characterised by clavate vesicles, yellow perithecia, 3-septate conidia and ascospores. *Cy. macroconidiale* is separated from *Cy. colhounii* by its large conidia and predominantly cylindrical phialides (Crous *et al.* 1999), while isolates STE-U 2231 and 2232 are separated from *Cy. colhounii* by their smaller conidial dimensions. *Cy. colhounii* was originally described from Mauritius, *Cy. macroconidiale* from South Africa, and the isolates STE-U 2231 and 2232 also have an African origin. This is presently the only clade in *Calonectria* characterised by yellow perithecia.

The separation of *Cy. scoparium*, *Cy. hawksworthii*, *Cy. insulare* and *Cy. variabile* was not strongly supported by bootstrap in clade 5. Although the first three taxa are morphologically similar (1-septate conidia, heterothallic), *Cy. variabile* is distinct in being homothallic, having multiseptate macroconidia, and regularly forming a microconidial state in culture. *Cy. scoparium* is chiefly known from North America, while *Cy. insulare* and *Cy. hawksworthii* occur on several islands in the Pacific, and *Cy. variabile* is thus far only known from Brazil. In agreement with their phylogeny, isolates of *Cy. scoparium*, *Cy. hawksworthii* and *Cy. insulare* have been found to be sexually compatible (results not shown). It is clear, therefore, that further investigation is required to clarify the relationships of species in this clade, as the phylogenetic, biological, and taxonomic concepts are not in perfect agreement.

Clade 6 represents three species that have clavate vesicles, similar macroconidia, produce micro- as well as megaconidial states, and are all known to be common pathogens of *Rumohrae adiantiformis* in Florida, as well as in South America (Crous *et al.* 1999). Although *Cy. rumohrae* is homothallic and readily produces a teleomorph in culture, no sexual state has thus far been found for either *Cy. heptaseptatum* or *Cy. angustatum* (Crous *et al.* 2000). Species in this clade share the same host and possible centre of origin. *Cylindrocladium multiseptatum* and *Cy. quinqueseptatum* in clade 7 share similar conidium, phialide and vesicle morphology. Both species are also common in Asia (Crous *et al.* 1998b). This similarity in area of origin might, as in clade 6, indicate a linked evolutionary history.

The second group (B) emerging from the β-tubulin phylogeny is comprised of clades 9 and 10 and other outlying species. Clade 9 contains isolates of the *Cy. floridanum* complex, *Cy. parasiticum* and *Cy. curvisporum*. All of these species have sphaeropendunculate vesicles, with differences in conidial shape and septation. Although isolates of *Cy. parasiticum* and *Cy. foridanum* are homothallic and readily produce a teleomorph in culture, no teleomorph has thus far been found for *Cy. curvisporum*. *Cy. parasiticum* and *Cy. floridanum* are well distributed throughout the world, whereas *Cy. curvisporum* is thus far known only from Madagascar. Other than vesicle morphology, no other features clearly link these three taxa. This is presently the only clade in *Calonectria* characterised by sphaeropedunculate vesicles. Isolates identified as *Cy. floridanum* in the present study were shown to be polyphyletic based on sequence data derived from the β-tubulin gene. This finding was further supported by data derived from the ITS and histone regions of rRNA genes (Kang, Schoch & Crous 2001), confirming that these isolates represent more than one species. Isolates IMI 354528 and IMI 354529 were collected from the same locality and host, and represent the same biological species. Some divergence was

observed in the β-tubulin sequence data derived for these two isolates in the present study, which was not depicted in the ITS and histone sequence data (Kang *et al.* 2001). This suggests that a duplicate copy of the β-tubulin gene could have been sequenced for IMI 35429.

Species of *Cy. naviculatum* and *Cy. penicilloides* are basal to clades 9 and 10 and are only weakly supported to form part of group B. Since *Cy. penicilloides* has no known teleomorph and was initially described without any mention of its vesicle morphology (Tubaki 1958), this presents the first instance where its phylogenetic placement could be tested. Although both *Cy. naviculatum* and *Cy. penicilloides* are supported as distinct species, their relationship to other taxa in this complex remains uncertain.

The *Calonectria* phylogeny based on β-tubulin DNA sequence data has confirmed previous taxonomic concepts for this genus (Crous & Wingfield 1994). An ITS-based phylogeny of *Calonectria* and morphologically similar hypocrealean genera with cylindrical macroconidia supported *Calonectria* as a monophyletic group within the *Hypocreales* (Schoch *et al.* 2000b). Furthermore, the genus was also clearly circumscribed as having coloured, warty perithecia (KOH+), with clavate asci devoid of an apical apparatus, and ellipsoid to fusiform, 1-multi-septate ascospores and *Cylindrocladium* anamorphs. The separation of *Calonectria* from *Xenocalonectria* based on the nature of the stipe extention, the absence of a terminal vesicle, 1-septate ellipsoidal ascospores and asci with apical apparati was also supported based on DNA phylogeny (Schoch *et al.* 2000b).

In this study we were able to compare the morphological and biological concepts previously used for species in *Cylindrocladium*, with a phylogenetic species concept. A similar species concept has been applied to isolates in the *Gibberella fujikuroi* complex by O'Donnell *et al.* (1998). The existence of biological species within the confines of morphological species of *Cylindrocladium* was discussed earlier (Schoch *et al.* 1999). The present study has shown that these species are also be phylogenetically distinct. Furthermore, it has clearly indicated as yet undescribed species of *Cylindrocladium*, and has also drawn attention to similarities between some taxa. In general, however, these data corroborate the morphological features presently employed to distinguish these taxa (Crous & Wingfield 1994). The morphological character that has greatest congruence with the DNA based phylogeny presented here was vesicle shape. To a lesser degree, geographic origin also appears to be closely linked to the molecular phylogeny of *Calonectria* species.

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