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Recombination in *Calonectria morganii* and phylogeny with other heterothallic small-spored *Calonectria* species

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Abstract: The *Calonectria morganii* cultures studied were isolated from several hosts in the USA. Isolates were mated in all combinations, and one successful mating was selected to establish whether recombination occurred. RAPD and mating type data of parental isolates and progeny confirmed *C. morganii* to have a heterothallic mating system. Furthermore, to infer the phylogenetic relationships of *C. morganii* with morphologically similar *Calonectria* spp., DNA sequences of the ribosomal 5.8S RNA gene and the flanking internal transcribed (ITS) spacers, as well as part of the high mobility group (HMG) box (forming part of the *MAT2* mating type gene) and the β-tubulin gene, were analyzed. Maximum parsimony yielded concordant trees for all three data sets. These data supported the morphological and biological species concepts proposed for *C. morganii* and other *Calonectria* species with, similar, small-spored *Cylindrocladium* anamorphs.

Key Words: β-tubulin, *Cylindrocladium*, ITS, *MAT2*, rRNA

INTRODUCTION

Cylindrocladium scoparium Morgan is the type species of the anamorph genus *Cylindrocladium* Morgan (Morgan 1892). Members of this genus have *Calonectria* De Not. teleomorphs, are ubiquitous plant pathogens and have been isolated mainly in tropical and subtropical regions of the world. *Cylindrocladium scoparium* (teleomorph *Calonectria morganii* Crous, Alfenas & M. J. Wingf.) is the form most frequently encountered and has reportedly been associated with a wide range of disease symptoms, including damping off, root rot, cutting rot, stem cankers, leaf-spot and seedling blight (Cordell and Rowan 1975). Although this species has been reported from over 30 plant families (Booth and Gibson 1973, French and Menge 1978, Peerally 1991, Waipara et al 1996), recent data (Schoch et al 1999) suggest that many of these records were incorrectly ascribed to *C. scoparium*.

The main taxonomic criteria used for the identification of these species are conidial and ascospore size and septation, vesicle shape and diameter of the anamorph, and perithecial morphology of the teleomorph. This explains the preference for using the anamorph in species designations in this and previous studies. Although the reliability of the terminal vesicle as a criterion for species identification has been questioned by some workers (Hunter and Barnett 1978, Rossman 1983), Crous et al (1992) showed that it is useful when studied under controlled conditions on carnation-leaf agar (CLA) (Fisher et al 1982).

However, uncertainty still exists regarding the identification of *C. scoparium*, and it has frequently been confused with other species with 1-septate, small conidia. These include *C. ovatum* El-Gholl, Alfenas, Crous & T.S. Schub. (ovoid vesicles), *C. floridanum* Sobers & C.P. Seym. (sphaeropedunculate vesicles) and *C. candelabrum* Viégas (obpyriform vesicles). Victor et al (1997) compared isolates of these taxa and showed that they represent different species. The latter study also confirmed the existence of genetically distinct groups among isolates of *C. floridanum*, which was initially reported by Jeng et al (1997). A similar situation has also been found to exist in other species complexes such as *C. gracile* (Crous et al 1995, 1997a, b) and *C. candelabrum* (Schoch et al 1999).

Cylindrocladium scoparium has been reported from various areas worldwide, including Africa (Doidge

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1950, Darvas et al 1978, Botha and Crous 1992), South America (Palmucci et al 1996, Tozetto and Ribeiro 1996), Europe (Overmeyer et al 1996, Polizzi and Azzaro 1996), Asia (Mohanan and Sharma 1985, Srinivasan and Gunasekaran 1995) and New Zealand (Waipara et al 1996). However, the presence of *C. scoparium* has only been confirmed from North America and Brazil (Crous et al 1993a), and many of the isolates discussed in the previously mentioned reports have proven to be the newly described *C. pauciramosum* C. L. Schoch & Crous, which forms part of the *C. candelabrum* species complex (Schoch et al 1999).

The low mating frequency reported in previous studies of *C. scoparium* (Crous et al 1993a) and related species (Victor et al 1997) have complicated studies in these fungi by limiting the use of mating testers for species identification. Overmeyer et al (1996) reported only a single mating between mating type tester strains obtained from the American Type Culture Collection (ATCC). Furthermore, no successful matings were obtained with any of the additional 32 strains isolated from various hosts in Germany. High success rates were recently obtained for matings done with *C. ovatum* (Crous et al 1998) and species in the *C. candelabrum* species complex (Schoch et al 1999). These results confirmed that these species have biallelic, heterothallic mating systems. A similar mating system was originally described for *C. scoparium* (Crous et al 1993a). Results obtained by Overmeyer et al (1996) indicated a different scenario, because only one parent was reported to contribute to the genetic makeup of progeny. However, as so few matings with *C. scoparium* have proven successful in the past (Crous et al 1993a, Overmeyer et al 1996, Victor et al 1997), it was decided to also employ molecular techniques to provide more information on whether recombination occurred or not.

Random amplified polymorphic DNA (RAPD) is a technique applied to answer various genetically oriented questions. Previous studies have applied RAPD data in order to show recombination, albeit between agricultural crops (Echt et al 1992) or fungal pathogens (Nicholson et al 1995, Campbell et al 1999). This technique was therefore chosen to assess whether recombination occurred during matings of *C. scoparium*.

The phylogenetic relatedness of various *Cylindrocladium* species as suggested by morphological features is still largely uncertain. Several molecular characters have previously been used to analyze relationships between *Cylindrocladium* spp. These include protein profiles (Crous et al 1993b), RAPD (Victor et al 1997) and restriction fragment length polymorphisms (RFLP) (Crous et al 1997b). Previous results by Jeng et al (1997) showed that isolates of *C. scoparium* and *C. floridanum* could be distinguished by DNA sequence analysis of the 5.8S ribosomal RNA gene and flanking internally transcribed spacers (ITS). More recently data obtained from mating studies were combined with the analysis of ITS sequences in the *C. candelabrum* species complex (Schoch et al 1999), emphasizing the low number of informative characters available in the DNA sequence data of the ITS region. In a study aimed at differentiating species in the *Gibberella fujikuroi* (Sawada) Wollenw. species complex, O'Donnell et al (1998) employed sequence data of the nuclear 28S rDNA, mitochondrial small subunit (SSU) and β-tubulin gene. From these data, it was shown that the β-tubulin gene yielded the most variation of all areas sequenced, making it useful for inferring phylogeny in newly diverged groups. Degenerate primers based on conserved regions in the HMG (high mobility group) box in the *mt a-1* mating type gene of *Neurospora crassa* Shear & B.O. Dodge have successfully been employed to amplify partial *MAT2* sequences from other species in the pyrenomycetes (Arie et al 1997, Turgeon 1998, Witthuhn 1999).

Based on the clear advantages of these techniques to differentiate closely related species, the aim of the present study was to use these sequences to infer the phylogeny of *C. scoparium* and other small-spored, heterothallic *Cylindrocladium* species.

MATERIALS AND METHODS

Cylindrocladium scoparium strains studied were either isolated from symptomatic material, or obtained from the American Type Culture Collection (ATCC 46300 and ATCC 38227) (TABLE I). All isolates were identified using the methods reported by Crous et al (1997b) and Schoch et al (1999).

Isolates were mated in all possible combinations. This was achieved by removing 3-mm diam agar plugs from the periphery of actively growing cultures and placing them on carnation leaf agar plates as described by Crous et al (1997a). Two different isolates were placed in a Petri dish with carnation leaves between them. Plates were subsequently incubated for 2 mo at 22 C as explained in Schoch et al (1999). Successful matings were regarded as those isolate combinations that produced perithecia with fertile, exuding ascospores. Perithecia were harvested, ascospores obtained and cultured on 2% malt extract agar (MEA) (Biolab, Midrand, South Africa). Ascospores were obtained from a successful mating of STE-U 1720 and STE-U 1722. Fifteen isolates were randomly selected, cultured and mated with each of the parental isolates in order to determine their mating type and subsequently used in the RAPD analysis.

TABLE I. *Cylindrocladium* isolates used in this study

Species	Original No.	Collector	Host	Origin
C. scoparium	STE-U 496	A.C. Alfenas	Unknown	USA
	STE-U 497	A.C. Alfenas	Unknown	USA
	STE-U 654	A.C. Alfenas	Unknown	USA
	STE-U 655	A.C. Alfenas	Unknown	USA
	STE-U 1720	N.E. El-Gholl	*Rosa* sp.	Florida, USA
	STE-U 1721	N.E. El-Gholl	*Conocarpus erectus*	Florida, USA
	STE-U 1722	N.E. El-Gholl	*Dodonea viscosa*	Florida, USA
	STE-U 1723	N.E. El-Gholl	*Nandina domestica*	Florida, USA
	ATCC 38227	S.A. Alfieri	*Mahonia bealei*	Florida, USA
	ATCC 46300	D.M. Benson	*Leucothoe catesbaei*	North Carolina, USA
C. pauciramosum	STE-U 416	S. de Buisson	*Eucalyptus grandis*	N. Prov., R.S.A.
	STE-U 925	M.J. Wingfield	Soil	Santa Catarina, Brazil
	STE-U 972	P.W. Crous	*Eucalyptus grandis*	Western Cape, R.S.A.
C. candelabrum	STE-U 1677	A.C. Alfenas	*Eucalyptus* sp.	Amazonas, Brazil
	STE-U 1675	A.C. Alfenas	*Eucalyptus* sp.	Bahia, Brazil
	STE-U 1951	A.C. Alfenas	Soil	Brazil
C. insulare	STE-U 616	M.J. Wingfield	Soil	Amazonas, Brazil
	STE-U 768	P.W. Crous	Soil	Tamatave, Madagascar
	STE-U 954	M.J. Wingfield	Soil	Veracruz, Mexico
C. mexicanum	STE-U 927	M.J. Wingfield	Soil	Yucatan, Mexico
	STE-U 941	M.J. Wingfield	Soil	Campeche, Mexico
C. ovatum	UFV 90	A.C. Alfenas	Soil	Brazil
	STE-U 2232	P.W. Crous	*Eucalyptus* sp.	Brazil
C. multiseptatum	STE-U 1589	M.J. Wingfield	*Eucalyptus* sp.	Indonesia
	STE-U 1602	M.J. Wingfield	*Eucalyptus* sp.	Indonesia

DNA analysis.—Single conidial and ascospore isolates were grown on MEA plates and plugs transferred into 500-mL Erlenmeyer flasks containing 100 mL liquid MEA broth. Flasks were shaken at 25 C and 125 rpm for approx 7 d. Mycelia were collected by filtration (Whatman no. 1 filter paper) and DNA was extracted as described by Crous et al (1993b).

RAPD analysis. PCR reactions (25 µL total volume) contained 1.5 units Biotaq (Bioline, London, UK) with the buffer as recommended by the manufacturer, 1 mm deoxynucleoside triphosphates, 4 mm $MgCl_2$, 0.5 µM primer oligonucleotide and approximately 10 to 30 ng of fungal genomic DNA. Reactions were performed on a Rapidcycler (Idaho Technology, USA). RAPD reaction conditions consisted of the following: an initial denaturation for 30 s at 96 C, followed by 40 cycles of 30 s at 96 C, 30 s at 38 C and 30 s at 72 C, then a final elongation step of 2 min at 72 C.

Amplified DNA fragments were separated on 1.6% (w/v) CE agarose gels (Boehringer Mannheim, South Africa), with ethidium bromide (1 µg/mL) using 0.5 X TBE buffer and run at a constant voltage of 60 V. Fragments were visualized and photographed under ultraviolet light. Thirteen decameric oligonucleotides (OPE 02, 03, 04, 07, 09, 10, 11, 13, 15, 16, 17, OPM 06, OPY 20, Operon Technologies Inc., USA) were screened. Two primers, OPE 17 and OPE 16 (CTA CTG CCG T) were selected for further analysis after they yielded polymorphic bands separating both parental isolates in repetitive runs.

DNA fingerprints were evaluated by visual inspection of the photographs of the gels. The data were scored on the presence or absence of fragments within each individual sample. Possible recombination observed in the parental isolates could be seen in progeny as determined by the cosegregation of bands that were polymorphic in the parents.

PCR amplifications. The strategy of Arie et al (1997) was followed, using two degenerate primers based on the *Neurospora crassa mat a-1* HMG box, NcHMG1 (CCY CGY CCY CCY AAY GCN TAY AT) and NCHMG2 (CGN GGR TTR TAR CGR TAR TNR GG). Although several *Calonectria* species were tested with the degenerate primers, the clearest band was obtained from a homothallic species, *Calonectria colhounii* Peerally. Fragments with an approximate size of 300 bp [based on known sequences from *Neurospora crassa* (Staben and Yanofsky 1990)] were subsequently cut from the gel with a scalpel. DNA was recovered from the agarose matrix using Wizard PCR Preps (Promega Corporation, Madison, Wisconsin), then sequenced. The amino acid translation obtained from the sequence was compared to the *N. crassa mt a-1* HMG sequence obtained from GenBank (M54787) (Staben and Yanofsky 1990) in order to confirm its identity. This sequence was used to design two internal PCR primers, ColHMG1 (CCA GAT GCT GAA

GCA GCT CAA CC) and ColHMG2 (GCT TCT TGA TGA GCT CAG CC). Fragments of approx 170 bp were amplified and sequenced with these primers. A range of different species in the genus *Calonectria* from both mating types were tested for specific PCR amplification of a *MAT2* HMG box fragment using primers ColHMG1 and ColHMG2 under the following conditions: an initial denaturation for 2 min at 96 C, followed by 35 cycles of 15 s at 96 C, 30 s at 55 C and 35 s at 75 C. A final elongation step of 4 min at 75 C was included. PCR amplifications were performed on a Rapidcycler (Idaho Technology, USA).

A 600-bp fragment of the β-tubulin gene was amplified with primers T1 (O'Donnell and Cigelnik 1997) and Bt2b (Glass and Donaldson 1995). Amplification and visualization conditions were the same as for the HMG box.

The ITS1 and ITS2 internally transcribed spacers as well as the 5.8S ribosomal RNA gene were amplified, yielding a fragment consisting of 537 bp. DNA was amplified using the primers ITS1 and ITS4 (White et al 1990). Amplification and visualization conditions were the same as for the HMG box.

Sequencing analysis. Initially both mating types of each species were tested for amplification with the primers ColHMG1 and ColHMG2. After the *MAT2* mating types were identified as those isolates yielding a fragment of approx 300 bp, two isolates belonging to this mating type were used for further comparisons. Additional isolates belonging to the opposite mating type (based on the absence of the *MAT2* sequence) were used for the β-tubulin and ITS data sets. DNA was extracted as described by Crous et al (1993b) and PCR performed as mentioned previously. PCR products were purified using Wizard PCR Preps (Promega Corporation, Madison, Wisconsin). Both strands of each PCR product were sequenced. All reactions were run on an ABI Prism 377 DNA Sequencer (Perkin-Elmer, Norwalk, Connecticut) using conditions as described by Schoch et al (1999). Sequences were aligned with the computer package Malign version 2.7 (Wheeler and Gladstein 1991) and improved manually. Phylogenetic analysis of aligned DNA sequences was performed using PAUP* Version 4.0b1 (Swofford 1998) and printed with the help of Treeview Version 1.5 (Page 1996). The sequences of *Fusarium subglutinans* (Wollenw. & Reinking) Nelson et al (NRRL 22016) deposited by O'Donnell et al (1998), were obtained (GenBank accession numbers ITS: U34559, β-tubulin: U34417), and used as outgroups in the ITS and β-tubulin data sets. A sequence of *Fusarium oxysporum* Shltdl.: Fr. (O-17) obtained from GenBank (AB005040) was used as outgroup for the partial *MAT2* HMG data set. The ITS data set included sequences from previous studies lodged at GenBank: STE-U 971 (AF059280), STE-U 1675 (AF059281), STE-U 768 (AF059282) and STE-U 941 (AF059283). All sequences obtained in this study were also deposited at GenBank as a phylogenetic group with alignment gaps included. The accession numbers of newly obtained sequences are as follows: ITS, AF210876–AF210890; β-tubulin, AF210857–AF210875; HMG box, AF210845–AF210855. Unweighted parsimony analysis was performed using the branch and bound search option on all data sets. Gaps were treated as a fifth character, but in order to remove ambiguities only the first position was coded as such. Subsequent gap positions were coded as missing data. Confidence intervals for nodes were determined using 1000 bootstrap replications and the branch and bound search option for the β-tubulin and combined data sets. Due to the high number of possible trees the bootstrap values of the ITS data set were determined by means of a heuristic search with 1000 random additions and 1000 bootstrap replications. Data sets were also assessed by using neighbor-joining with uncorrected pair frequencies and maximum-likelihood distance methods in PAUP* version 4.0b1.

RESULTS

Sexual compatibility.—Crous et al (1993a) and Overmeyer et al (1996) previously reported that mating compatibility was low for *C. scoparium.* This was also true in the present study. From a total of ten isolates (TABLE I), including the reference isolates obtained from ATCC, only five isolates (STE-U 1720, STE-U 1722, STE-U 1723, ATCC 38227, ATCC 46300) could be crossed successfully. In the case of successful crosses fertile perithecia appeared after two to three weeks. Successful crosses were: STE-U 1720 × STE-U 1722, STE-U 1720 × STE-U 1723, STE-U 1720 × ATCC 46300 and ATCC 38227 × ATCC 46300.

A successful mating between isolates STE-U 1720 and STE-U 1722 was selected for further study. Perithecia were found to be pale yellow to light orange in this cross. Isolate STE-U 1720 also successfully mated with the reference isolate ATCC 46300 and this cross yielded perithecia ranging from pale yellow to orange-brown in color. Viable progeny confirmed that these isolates belonged to the same biological species, namely *Ca. morganii.* After several unsuccessful attempts, a fertile cross was obtained between the two reference isolates (ATCC 46300 and ATCC 38227). Perithecial color in this instance was orange to red-brown as reported previously (Crous et al 1993a, Overmeyer et al 1996).

Only isolates STE-U 1720 and ATCC 46300 produced protoperithecia when plated alone on CLA, indicating that isolates STE-U 1722 and ATCC 38227 were possibly female sterile, and unable to function as hermaphrodites. Ascospores were recovered from the mating between isolate STE-U 1720 and STE-U 1722. Out of the 15 ascospores obtained from this mating, 8 crossed with STE-U 1720 and 7 with STE-U 1722. The mating type composition of these ascospores was thus 8:7, which approaches the 1:1 ratio expected for Mendelian segregation.

RAPD analysis.—Fifteen randomly chosen ascospores were used in the RAPD study. Primers were screened against the two parental isolates in order to find polymorphic bands between them. Most primers yielded

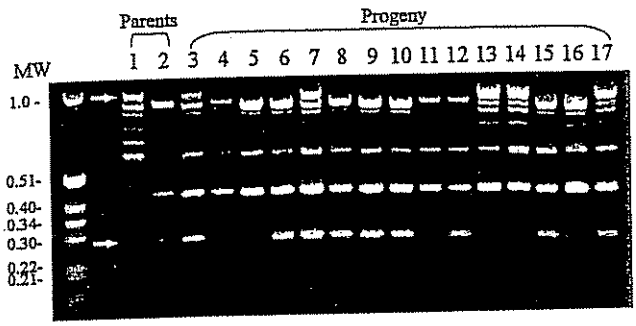

FIG. 1. Electropherogram showing RAPD profiles obtained with primer OPE 17. The two unique polymorphic bands are indicated (arrows). A lambda DNA size marker (kb) is also shown. Amplification products from parental isolates were loaded in lane 1 (STE-U 1720) and 2 (STE-U 1722). Products from ascospore progeny (A1–A15) are shown in lanes 3–17.

profiles that appeared to be highly monomorphic. Only one primer showed clear polymorphic bands between the two parents, OPE 17 (FIG. 1). The markers shown in FIG. 1 cosegregated in three of the fifteen progeny (lanes 3, 7 and 17). Additional polymorphic bands in parent 1 (STE-U 1720) also cosegregated with the indicated polymorphic band (see arrow, FIG. 1) in parent 2 (STE-U 1722), further supporting the hypothesis that genetic material was derived from both parents. These data suggest that the ascospore progeny are the result of a true heterothallic cross. A band at approx 0.45 kb was also found to be present in the one parent and all of the progeny.

Phylogeny.—Three regions of the genome were used for phylogenetic comparisons. The *Cylindrocladium* specific primers obtained from the *MAT2* HMG box of an isolate of *C. colhounii* yielded products from several other *Cylindrocladium* species. Partial HMG box sequences from the *MAT2* mating types of the small-spored heterothallic species, *C. scoparium, C. candelabrum, C. insulare, C. pauciramosum* and *C. ovatum* were also obtained. Where possible two isolates from disparate geographic areas were used for each species, in order to sample for intraspecific variation. In addition, the ITS ribosomal region and part of the β-tubulin gene were amplified and used for comparisons. Sequences of the opposite mating type for each species were also included in the β-tubulin and ITS data sets. Two isolates from the multiseptate, large-spored species, *C. multiseptatum* Crous & M.J. Wingf., were included in order to investigate intrageneric phylogeny (see FIGS. 2–4). The ITS data set consisted of 484 nucleotide characters, of which 13 were parsimony informative, while the β-tubulin data set contained 111 parsimony informative sites out of 538 nucleotide characters.

The portion of the β-tubulin gene sequenced possessed three introns containing 93 informative sites. In contrast only 15% of the informative sites were in the coding regions. Third position substitutions within exons were predominant (65%), while only 16% and 19%, respectively, were in the first and second positions. Trees obtained from only the β-tubulin gene coding regions could not distinguish between the species *C. insulare* and *C. scoparium* as well as *C. pauciramosum* and *C. candelabrum* with any meaningful bootstrap support (results not shown). However, these species were still concordant with a tree from the total β-tubulin data set (FIG. 2).

A partition-homogeneity analysis performed with PAUP* version 4.0b1 revealed an underlying similarity in the phylogenies ($P = 0.84$, where $P \leq 0.05$ would indicate significant incongruence) obtained with the ITS and β-tubulin data sets. An additional analysis found the same for the tree introns of the β-tubulin data set ($P = 0.56$). The disparity in the number of informative characters is reflected in the bootstrap values revealed in FIG. 2. Nodes generally had lower support in the ITS data set than in the β-tubulin data set. A closer relationship of the Brazilian isolate of *C. pauciramosum* (STE-U 925), with the apparent sibling species *C. candelabrum* is also evident from the β-tubulin data set, although with low bootstrap support. Both these taxa were shown to be distinct biological species (Schoch et al 1999), but a closer relationship between isolates from similar geographical origins is suggested from these data.

The topology of the tree based on the *MAT2* HMG box sequences (FIG. 3) is concordant with the results discussed above. Although isolates of *C. ovatum* were shown to be distinct from other isolates, relationships between this species and other species were not well supported by bootstrap in any data set. The *MAT2* HMG box data set consisted of 171 nucleotide characters, of which 27 were parsimony informative. A branch and bound search yielded 13 trees. As with the ITS data set, it was not possible to distinguish between isolates of *C. scoparium* and *C. insulare.* However, both of these species could be separated using the β-tubulin data.

A final analysis was done with the combined ITS and β-tubulin data sets (FIG. 4) consisting of 1022 nucleotide characters with 124 being parsimony informative. A branch and bound search on this data set yielded eight most parsimonious trees. This analysis reconfirmed the topology seen in the earlier dendrograms. Compared to the β-tubulin data set, higher bootstrap support for a separation of *C. scoparium* and *C. insulare* was observed. Most other branches were also supported by higher bootstrap values.

Isolates of *C. multiseptatum* were shown to be dis-

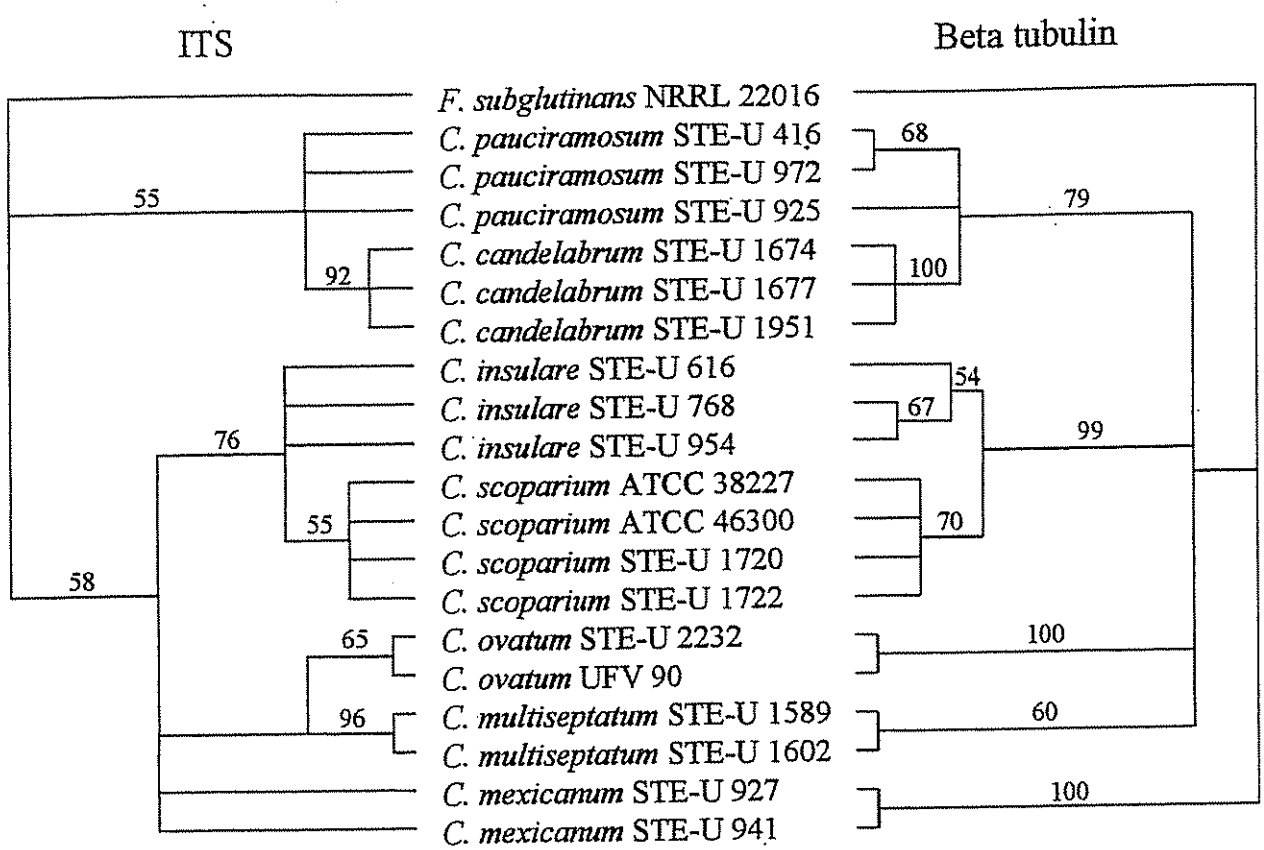

FIG. 2. Concordance of two selected most parsimonious trees generated from aligned sequences of the 5.8S gene and flanking ITS regions (done with a heuristic search with 1000 random addition sequences, 186 trees, 86 steps, CI = 0.965, RI = 0.941, RC = 0.908) as well as the β-tubulin gene (with a branch and bound search, 27 trees, 320 steps CI = 0.844, RI = 0.854, RC = 0.721) in PAUP* version 4.0b1. Clade stability was assessed with 1000 bootstrap replications and values above 50% are shown.

tant from the other small-spored species (FIG. 4) in agreement with the difference in morphology. However, isolates from *C. mexicanum*, the fourth species described within the *C. candelabrum* species complex, also grouped distantly compared to the other small-spored species. Neighbor-joining and maximum-likelihood trees for all data sets (results not shown) were concordant with those obtained through maximum parsimony.

DISCUSSION

These results support the view that *C. scoparium* has a heterothallic mating system. Furthermore, sequence data from all three genomic regions also support *C. scoparium* as a morphological and biological species, distinct from other morphologically similar small-spored *Cylindrocladium* spp.

Results of the mating study are in direct contrast with those previously obtained (Overmeyer et al 1996), where a system involving genetic material from only one parent was suggested in *C. scoparium*. Using RAPD markers, recombinant profiles obtained from both the parental isolates (STE-U 1720 and STE-U 1722) were observed in the F_1 generation. A phylogenetic analysis of RAPD data obtained by Overmeyer et al (1996), however, showed all progeny to group with one parent. In addition to this, no backcross was reported with strain ATCC 38227. However, F_1 isolates were reported to intercross, indicating the existence of both mating types in the sample used. The absence of protoperithecia reported by Overmeyer et al (1996), and also observed in this study, indicate that isolate ATCC 38227 has lost the ability to act as a hermaphrodite in a cross. This fact, combined with the low fertility observed in our study, could explain why Overmeyer et al (1996) were unsuccessful in backcrossing ascospore progeny with ATCC 38227. Matings conducted in the present study showed that ascospore progeny could mate with either of the parental isolates. The mating type ratio observed also supported Mendelian segregation. However, since one of the parents (STE-U 1722) also appeared to be female sterile, we expect that a larger sample will produce a number of ascospores that are also female sterile and unable to mate with this par-

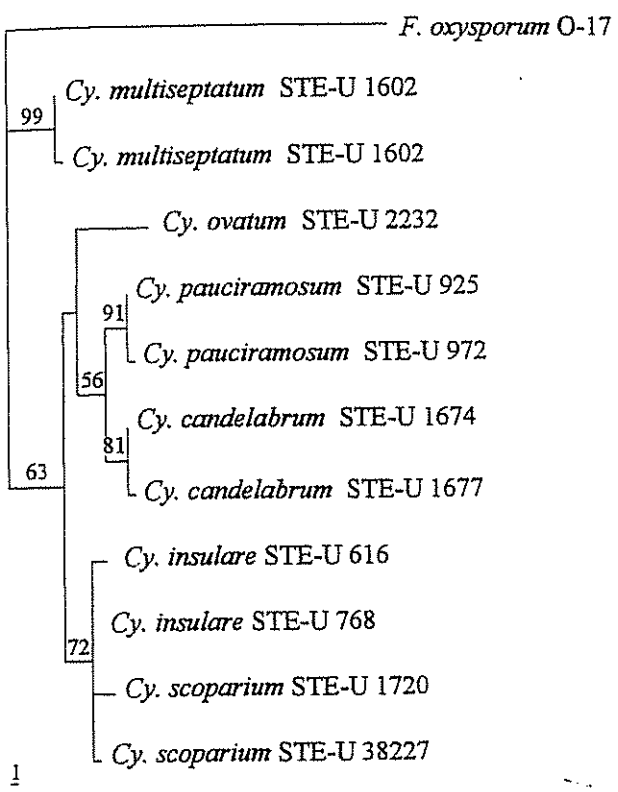

FIG. 3. One of 13 most parsimonious trees (48 steps CI = 0.917, RI = 0.920, RC = 0.843) generated by the branch and bound algorithm in PAUP* version 4.0b1 based on sequences of the *MAT2* HMG box. Clade stability was assessed with 1000 bootstrap replications (values above 50% are shown) and *F. oxysporum* was used as outgroup. Bar = 1 bp substitution.

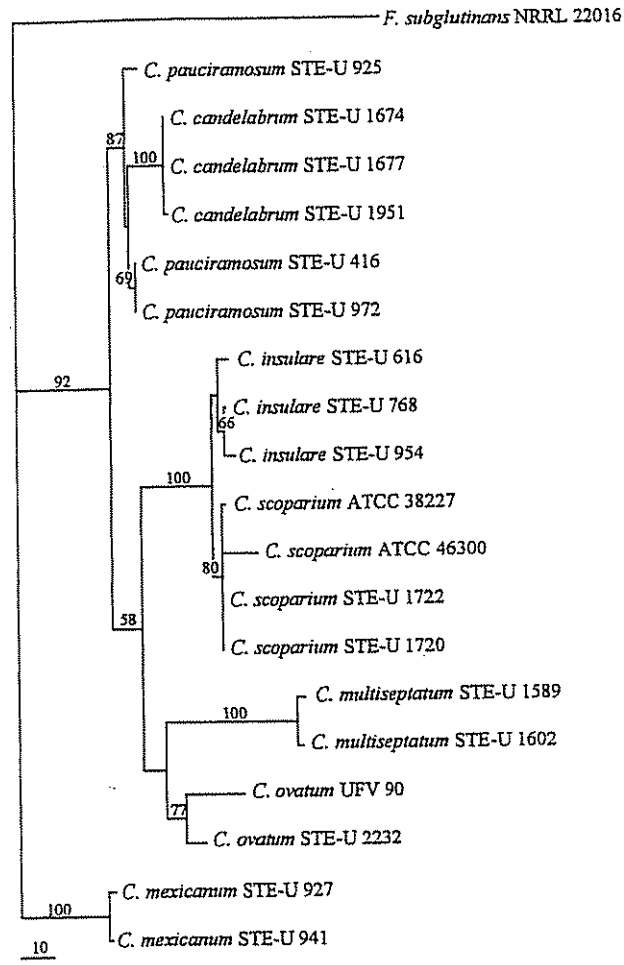

FIG. 4. One of 8 most parsimonious trees (407 steps CI = 0.867, RI = 0.863, RC = 0.748) generated with a branch and bound algorithm in PAUP* version 4.0b1 from aligned sequences of combined data set of the 5.8S rRNA gene and flanking ITS regions as well as the β-tubulin gene. Clade stability was assessed with 1000 bootstrap replications and values above 50% are shown. *F. subglutinans* was used as outgroup. Bar = 10 bp substitutions.

ent, even though they are of the opposite mating type.

RAPD results obtained from 15 ascospores in the present study indicate that both parents contributed to the genetic make-up of the progeny. The designation of all isolates as either *MAT1* or *MAT2*, using DNA sequence data, their novel RAPD profiles as well as their mating behavior with tester strains, is further proof that a heterothallic, possibly biallelic, mating system exists in *C. scoparium*.

In order to determine the phylogenetic relationships between other heterothallic, small-spored *Cylindrocladium* species and *C. scoparium*, several genomic DNA regions were sequenced and analyzed. From our results, it is clear that, in spite of their similar morphology, these species can be differentiated based on DNA phylogeny. Although only a segment of 170 bp was obtained from the HMG box, trees were similar in topology compared to those obtained from β-tubulin and ITS sequences.

These results further indicate that *C. scoparium* is very closely related to *C. insulare*. Only one area of the genome tested, β-tubulin, could distinguish isolates of these two species. Although this was not supported by high bootstrap support, a combined data set of both ITS and β-tubulin sequences gave much better support for their separation (FIG. 4). The variation in perithecial color observed between crosses of these isolates and those involving the ATCC reference isolates support this observation. This finding also underlines the fact that in some heterothallic species of *Calonectria* variation can occur regarding perithecial color, thus reducing the usefulness of this feature for species identification (Crous and Wingfield 1994).

The β-tubulin-based tree grouped isolate STE-U 925 of *C. pauciramosum* with isolates of *C. candelabrum*. All of these isolates were collected in Brazil. In other studies where β-tubulin sequences were ob-

tained from a wider range of *C. pauciramosum* isolates (Schoch et al unpubl), clusters correlated with geographical origin, but also confirmed a closer relationship among various South American species, and between *C. pauciramosum* and *C. candelabrum* in particular. The high similarity shown between these two species indicates that they probably are sibling species.

Other than rDNA ITS sequences, DNA sequences obtained from genes such as β-tubulin and *MAT2* appear to be more variable and yielded much higher resolution for interspecies differentiation. However, more information is needed regarding intraspecies variation and phylogenetic relationships as well as population dynamics between some of the closely related species in *Cylindrocladium,* before these results can be seen as comprehensive.

Differing characters found for other *Cylindrocladium* species, such as optimum growth temperature (Crous and Wingfield 1994, Schoch et al 1999), fungicide profiles (Jayasinghe and Wijesundera 1995) and pathogenicity (Alfieri et al 1972, Blum et al 1992, Crous et al 1993c) highlight the need for accurate identification of even seemingly closely related species. The fact that *C. scoparium* is regularly confused with morphologically similar species further underlines this requirement. This is exemplified by recent new reports of one of the species in the *C. candelabrum* species complex, *C. pauciramosum* from Italy (Polizzi and Crous 1999) and Florida (Koike et al 1999). The apparent lack of resolution in morphological characters in this genus necessitates the use of sexual compatibility (where applicable) as well as molecular characters in order to identify morphologically similar species.

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