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Molecular and Morphological Identification of *Colletotrichum* Species of Clinical Interest

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***Colletotrichum* species have caused human infections in recent years. Because of the difficulties in recognizing them in vitro, we have designed a quick and unambiguous molecular test, based on the amplification of a specific fragment of the internal transcribed spacer 1 region, to distinguish any *Colletotrichum* isolate from other fungi, including the common pathogenic species. Analysis of the sequences of the ribosomal DNA (rDNA) fragment showed sufficient variability to clearly separate the five species of *Colletotrichum* that are of clinical interest, i.e., *Colletotrichum coccodes*, *C. crassipes*, *C. dematium*, *C. gloeosporioides*, and *C. graminicola*. Sequencing of the D1-D2 region of the large-subunit rDNA gene also supported these results. Additionally, we reviewed the most suitable morphological characteristics for the in vitro identification of these increasingly important opportunistic fungi.**

Hyphomycetes are the most common pathogenic moulds, but coelomycetes can also be found in human infections. These infections mainly occur in immunosuppressed hosts, although healthy individuals are also occasionally affected when the epithelial barrier is disturbed. Coelomycetes are asexual fungi that produce their fertile hyphae in specialized structures called conidiomata. They mainly produce two types of conidiomata: (i) pycnidia, which are more or less spherical fruit bodies with an opening only at the apex and a layer of the conidiogenous cells lining the inner cavity wall, and (ii) acervuli, which are cup-shaped fruiting bodies whose fertile hyphae form a palisade on the surface of the conidiomata. In a recent review of infections by coelomycetes, 11 genera were reported, but those most commonly found in clinical specimens are *Colletotrichum*, *Nattrassia*, and *Phoma* (24).

The genus *Colletotrichum* is one of the most important genera of plant pathogens. It has a worldwide distribution but is found mainly in subtropical and tropical regions (1). *Colletotrichum* species cause economically significant diseases of plants (generally referred to as anthracnoses) that affect cereals and grasses, legumes, vegetables, and perennial crops, including fruit trees (1). The key morphological features which identify the genus are its acervular conidiomata, often with setae (dark-pigmented, unbranched, thick-walled sterile hyphae usually pointed at the tip), producing elongated slimy conidia, and the presence of appressoria (thick-walled swellings at the end of a hypha or germ tube useful for attaching the fungus to the host surface before penetration of the tissue). The genus *Colletotrichum* encompasses numerous species, and the key criterion for their identification is based mainly on determining the plant host. In the most recent review of the genus, Sutton (23) accepted 39 species but indicated that the separation or recognition of some of these species is unclear.

Five species of *Colletotrichum* have been reported to cause infections in humans. They are *Colletotrichum coccodes*, *C. crassipes*, *C. dematium*, *C. gloeosporioides*, and *C. graminicola* (3, 4). The predominant infection is keratitis following traumatic implantation (5, 27), but subcutaneous and systemic infections among immunosuppressed patients have also been reported recently (3, 7, 14). A recent article described three cases of phaeohyphomycosis caused by *Colletotrichum*, involving patients who were undergoing chemotherapy for hematological malignancies (18). A sixth species, *C. acutatum*, recently caused a disseminated infection in a sea turtle (13).

When members of the genus *Colletotrichum* grow in culture, they do not usually produce their typical conidiomata, but they can be recognized easily by the presence of appressoria. However, in clinical strains, even such structures can be absent, making their identification difficult. Prompt diagnosis of the species involved in a *Colletotrichum* infection may be necessary to assess the correct treatment, because antifungal susceptibility depends on the species, e.g., while itraconazole was not active in vitro against *C. coccodes* and *C. dematium*, it was active against some isolates of *C. gloeosporioides* (7). We report a diagnostic molecular test that is suitable for quick and unambiguous recognition of any fungal strain belonging to *Colletotrichum*. We also review the most suitable morphological characteristics and compare the sequences of a specific fragment of the internal transcribed spacer 1 (ITS1) region of reference strains of the clinically important *Colletotrichum* species in order to determine whether sufficient variability exists for identification to species level. We also tested the D1-D2 domain of the large-subunit (LSU) ribosomal DNA (rDNA) gene to prove the consistency of the clades obtained by analyzing the ITS1 region.

MATERIALS AND METHODS

Strains. Twenty strains of *Colletotrichum* species of clinical interest were included in the study (Table 1). For eight of these strains, a partial ITS region and/or the D1-D2 region of the LSU rDNA was sequenced. Another 10 strains from our collection, belonging to several contaminant and pathogenic fungi, were also tested to demonstrate the specificity of the designed primers. These fungi were *Aspergillus fumigatus* (FMR 7763), *Aspergillus flavus* (FMR 7775),

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TABLE 1. Strains of *Colletotrichum* studied genetically and/or morphologically

Species	Strain	Source of isolate	Gene sequence	Accession no.
C. coccodes	IMI[a] 136601	Root of *Lycopersicon esculentum*		
	CBS[b] 122.25	Stem of *Solanum tuberosum*		
	CBS 125.57	*S. tuberosum*		
	CBS 527.77	Root of *L. esculentum*		
	CBS 528.77	Agricultural soil		
C. crassipes	IMI 302450	*Cocos nucifera*	D1-D2	AJ565909
	CBS 169.59	*Oncidium excavatum*	D1-D2	AJ565913
	CBS 159.75	Stored grains	D1-D2	AJ565919
	CBS 109355	Human: phaeohyphomycotic cyst	ITS1 (partial)	AJ565918
			D1-D2	AJ565920
C. dematium	CBS 167.49	Stem of *Funkia sieboldiana*	ITS1 (partial)	AJ565916
			D1-D2	AJ565910
	CBS 170.59	Stump of *Brassica* sp.		
	CBS 714.95	*Limonium* sp.		
C. dematium (*C. truncatum*)	CBS 351.73	*Beta vulgaris*	ITS1 (partial)	AJ565917
			D1-D2	AJ565911
C. gloeosporioides	CBS 147.28	Fruit of *Citrus paradisi*		
	CBS 160.50	Fruit of *C. paradisi*		
	CBS 465.83	Needle and twig of *Araucaria excelsa*		
	CBS 572.97	*Digitalis lanata*		
	CBS 573.97	*Mangifera indica*		
	CBS 102275	Human: subcutaneous hyalohypho mycosis	ITS1 (partial)	AJ565915
			D1-D2	AJ565914
C. graminicola	CBS 305.69	*Brachypodium sylvaticum*	D1-D2	AJ565912

[a] CABI Bioscience, Egham, United Kingdom.
[b] CBS, Utrecht, The Netherlands.

Penicillium sp. (FMR 6569), *Trichophyton mentagrophytes* (FMR 6873), *Microsporum canis* (FMR 7418), *Saccharomyces cerevisiae* (FMR C121), *Chaetomium globosum* (FMR C158), *Cryptococcus neoformans* (FMR C123), *Scedosporium apiospermum* (FMR C72), and *Fusarium solani* (FMR C851). All of the strains were cultured on potato dextrose agar (PDA; Pronadisa, Madrid, Spain) at 25°C. The main morphological features of all *Colletotrichum* strains included in the study were examined on PDA and on potato carrot agar (20 g of potatoes, 20 g of carrots, 18 g of agar, 1,000 ml of tap water; home made) cultures incubated at 25°C under 12 h of darkness alternating with 12 h of near-UV light and in slide culture preparations under the same conditions (22).

Primer design. Two primers, Col-F (forward; 5′-AACCCTTTGTGAACATA CCT-3′) and Col-R (reverse; 5′-CCACTCAGAAGAAACGTCGTT-3′), were designed after comparing numerous *Colletotrichum* ITS1-5.8S-ITS2 sequences in the GenBank database. The sequences were aligned with ClustalX (25), followed by manual adjustments with a text editor. The primer set was complementary to a highly conserved region of ITS1.

PCR assays. DNA was extracted and purified directly from fungal colonies using the Fast DNA kit (Bio101, Vista, Calif.). Fungal suspensions were vortexed with a FastPrep FP120 instrument (Thermo Savant, Holbrook, N.Y.) to disrupt the fungal cells. The reaction mixture for the PCR comprised 0.1 to 10 ng of DNA template in a total volume of 50 μl, with final concentrations of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 2.5 mM $MgCl_2$ (10× Perkin-Elmer buffer II plus $MgCl_2$ solution [Roche Molecular Systems, Branchburg, N.J.]), a 1 μM concentration of each primer, 1.5 U of AmpliTaq DNA polymerase (Roche), and a 100 μM concentration of each deoxynucleoside triphospate (Promega, Madison, Wis.). The PCR conditions were denaturation for 5 min at 94°C followed by a touchdown procedure consisting of 30 s at 95°C and then annealing for 1 min at decreasing temperatures from 60 to 55°C during the first seven cycles (with 2°C decremental steps in cycles 2 to 6 and a 1°C decremental step in cycle 7). The final step was an extension at 72°C for 7 min. A total of 36 cycles were performed. Next, 5 μl of the PCR product was electrophoresed in a 2% agarose gel in the presence of ethidium bromide and visualized under UV light. A 100-bp DNA ladder marker (Invitrogen, Barcelona, Spain) was used as the size standard. The products were purified using the GFX PCR DNA purification kit (Pharmacia Biotech, Cerdanyola, Spain) and stored at −20°C until they were used in sequencing.

D1-D2 rDNA amplification. The D1-D2 regions of the LSU rDNAs from the strains in Table 1 were amplified using the NL1 and NL4 primers (17). The PCR conditions were predenaturation at 94°C for 5 min; 30 cycles at 95°C for 30 s, 51°C for 1 min, and 72°C for 1 min; and a final incubation at 72°C for 7 min. The final products were electrophoresed and purified as described above.

DNA sequencing and phylogenetic analysis. The protocol for sequencing was that of the *Taq* DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems, Gouda, The Netherlands). The reactions were performed using the newly designed primers (Col-F and Col-R) and the NL1 and NL4 primers, and they were run on a model 310 DNA sequencer (Applied Biosystems). The consensus sequences were obtained using the Autoassembler program (Applied Biosystems). The sequences were aligned and adjusted as described above.

Phylogenetic analyses of the Col fragments and the D1-D2 domains were performed by the neighbor-joining method (19) with the MEGA version 2.1 computer program (11). The trees were constructed using the Kimura two-parameter distance model (10) with the pairwise deletion of gaps option. The robustness of branches was assessed by bootstrap analysis with 1,000 replicates.

RESULTS

Morphological study. All the strains of *Colletotrichum* tested grew very quickly, usually covering the whole surface of the petri dish in 10 days, and they showed profuse sporulation on potato carrot agar. This medium was better than PDA for observing the main microscopic features of the fungi. Although acervular conidiomata were not present, all strains produced conidiogenous cells directly on the agar surface and/or throughout the aerial mycelium of the colony. The conidiogenous cells were hyaline, cylindrical, or tapered and measured up to 20 by 3 to 4 μm. Setae were produced by most isolates, except two *C. gloeosporioides* isolates (CBS 465.83 and CBS 102275). They were septate, dark brown, thick walled, acicular, and up to 200 μm long. Neither conidiogenous cells nor setae were useful characters to add differentiation among species. Useful characters were the presence or absence of sclerotia and the morphologies of conidia and of appressoria (Fig. 1 and Table 2); however, appressoria may be absent in some strains

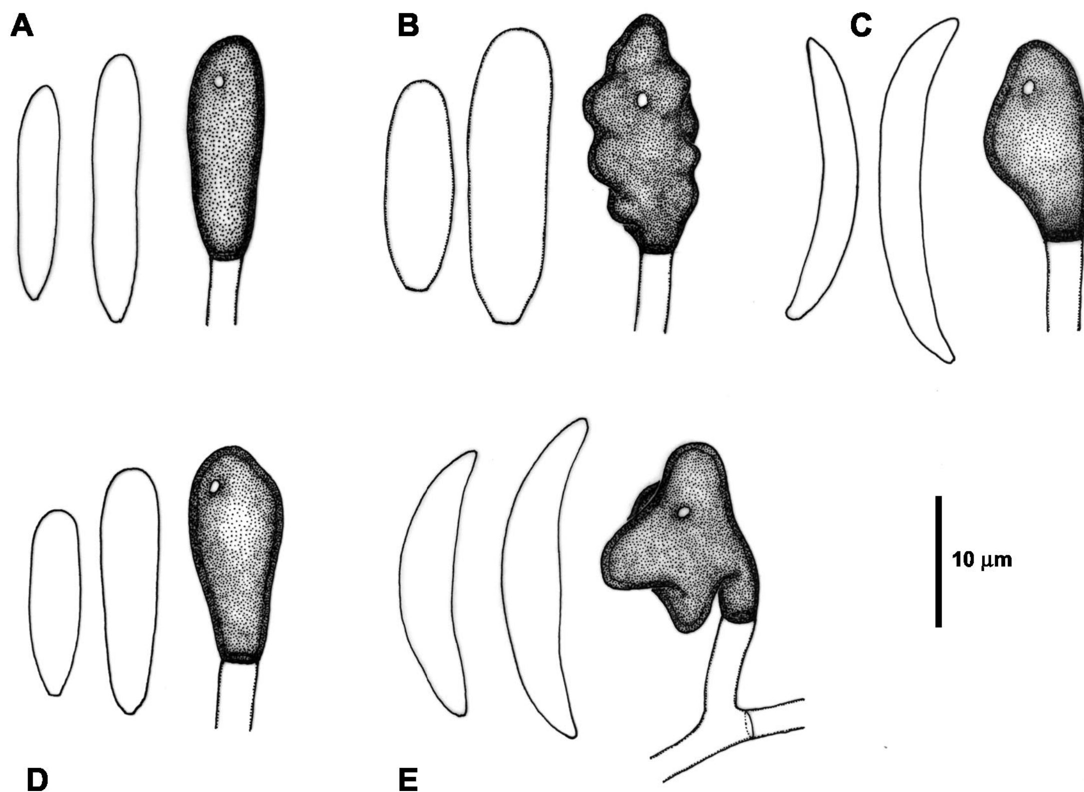

FIG. 1. Conidia and appressoria of *Colletotrichum* species of clinical interest. (A) *C. coccodes*; (B) *C. crassipes*; (C) *C. dematium*; (D) *C. gloeosporioides*; (E) *C. graminicola*.

of *C. gloeosporioides*. *C. coccodes*, *C. dematium*, and *C. graminicola* developed sclerotia within 5 to 7 days. These species differed in having conidia which were straight and fusiform (*C. coccodes*) or falcate (*C. dematium* and *C. graminicola*). The conidia of *C. dematium* were narrower (3 to 4 μm) and olive grey in mass compared with those of *C. graminicola*, which were 4 to 6 μm wide and salmon orange in mass. The appressoria of *C. dematium* had entire (smooth) margins, while those of *C. graminicola* were very irregular. *C. crassipes* and *C. gloeosporioides* did not develop sclerotia and had straight cylindrical conidia that were 6 to 8 μm wide in the former and 4 to 6 μm wide in the latter. The morphologies of the appressoria differed: they were crenate or deeply lobed in *C. crassipes* and entire or slightly lobed in *C. gloeosporioides*. One strain of *C. gloeosporioides* (CBS 465.83) failed to produce appressoria.

Molecular study. The amplicons from 20 strains of *Colletotrichum*, obtained using the newly designed primers Col-F and Col-R, were ~130 to 157 bp in length. The specificities of these primers were confirmed by the unsuccessful amplification of DNAs from the variety of human pathogenic fungi listed above and exposed under the previously described conditions.

Figure 2 shows a distance tree of the region corresponding to the Col products of 4 of our strains and 11 strains from GenBank. Two main clades were observed. One of these comprised the sequences of *C. coccodes* (Z32930, Z32931, and Z32933), the sequence of *C. crassipes* (AJ565918), and two

TABLE 2. Most important morphological features for separating five *Colletotrichum* species of clinical interest

Species	Sclerotia	Appressoria	Conidia
C. coccodes	Present (globose)	Margin entire	Straight; fusiform; abruptly tapered to each end; 16–22 by 3–4 μm
C. crassipes	Absent	Margin crenate or deeply lobed	Straight; cylindrical; apex obtuse; base truncate; 11–20 by 6–8 μm
C. dematium	Present (conical)	Margin entire or slightly irregularly lobed	Falcate; fusiform; gradually tapered to each end; 19–25 by 2.5–3.5 μm
C. gloeosporioides	Absent	Margin entire; sometimes lobed	Straight; cylindrical; apex obtuse; base truncate; 6–25 by 4–6 μm
C. graminicola	Present (irregular)	Margin very irregular	Falcate; fusiform; gradually tapered to the apex and base; 24–28 by 4–6 μm

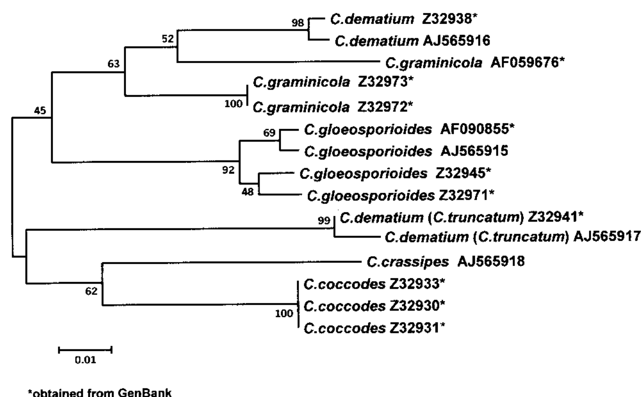

FIG. 2. Unrooted neighbor-joining tree of 15 sequences of Col products of rDNA from *Colletotrichum* species. The branch lengths are proportional to genetic distance, which is indicated by the bar. Bootstrap values (percentages of 1,000 replications) are indicated at the internodes.

sequences of *C. dematium* (Z32941 and AJ565917). The other comprised the sequences of *C. gloeosporioides* (Z32945, Z32971, AF090855, and AJ565915) and *C. graminicola* (Z32972 and Z32973) and the other two sequences of *C. dematium* (Z32938 and AJ565916). All strains of each species tested, except those of *C. dematium*, formed a cluster that received high statistical support. The four sequences of *C. dematium* were placed in two clusters that were far away from each other. Each of them comprised one of the two sequences from GenBank and others sequenced by us. The results were similar in the phylogenetic tree obtained by analyzing the sequences of the moderately variable D1-D2 domain of the LSU rDNA gene region (Fig. 3). In that case, the species tested were also clearly separated.

DISCUSSION

Because of its economic importance as a plant pathogen, *Colletotrichum* has received a lot of attention from numerous authors, and several molecular methods have been developed to detect it in plant tissue (2, 8, 15) or to determine phylogenetic relationships (6, 9, 20, 21). However, the taxonomy of *Colletotrichum* is still unclear. At least 11 generic synonyms have been reported for *Colletotrichum*, and ~900 species have been included in the genus (23). For *C. gloeosporioides* alone, the most common species of the genus, ~600 synonyms have been cited (26). When *Colletotrichum* species parasitize plants in vivo, they form intra- or subepidermal, acervular, or occasionally stromatic conidiomata with a wall of isodiametric or elongate cells generally covered or surrounded by stiff pigmented setae. In culture, as mentioned, these structures are not produced, and for an unskilled laboratorian a clinical isolate of *Colletotrichum* can be confused with an undetermined hyphomycete because the conidia of *Colletotrichum* are very different from those of any known opportunistic or pathogenic species. Fertile structures (perithecia, asci, and ascospores) of the sexual state (*Glomerella*) of some species of *Colletotrichum*, such as *C. gloeosporioides* and *C. graminicola*, can sometimes be found in vitro, but this only occurs in very old cultures and under suitable conditions (16). These structures are therefore not helpful in identification of *Colletotrichum* species. The most important features are those detailed in Table 2. Because of the difficulties involved in recognizing isolates of *Colletotrichum*, especially somewhat atypical strains, and in identifying them to the species level, the simple molecular technique that we have developed offers an alternative method for confirming and identifying clinical strains. To quality control the molecular method, we tested several strains belonging to different opportunistic or pathogenic fungal species and some representatives of common laboratory contaminants, all of them hyphomycetes.

The differences shown between the two *C. dematium* ITS1 sequences (Z32938 and Z32941) provided by GenBank (Fig. 2) have been reported by Sreenivasaprasad et al. (21). These authors considered that the sequence Z32941 belonged to *C. truncatum*. This species was considered valid by Sutton (23), but only as a form within *C. dematium* by von Arx (26). Our study demonstrated that one of the two strains that we received from the Centraalbureau voor Schimmelcultures (CBS) as *C. dematium* (CBS 351.73) also belonged to *C. truncatum.* The morphological differences between these taxa are very subtle. According to Sutton (23), they differ in the size of the conidia, but we were not able to differentiate them morphologically. The only clinical isolate of *C. dematium* so far reported (12) is not available, and from the description of the case, it is very difficult to ascertain to which of the two species, *C. dematium* or *C. truncatum*, it actually belongs.

For diagnostic purposes, the sequencing method developed here showed its objective value by confirming that the strain CBS 109355, recently isolated from a subcutaneous infection in Brazil and identified according to its morphological features as *C. crassipes* (3), does indeed belong to that species. This strain had previously been misidentified as *C. gloeosporioides* (L. G. M. Castro, E. Miki Ito, R. S. Nunes, E. M. Heins-Vaccari, C. da Silva Lacaz, and J. Guarro, Proc. 14th Int. Soc. Hum. Anim. Mycol., p. 264, 2000). In the D1-D2 phylogenetic tree (Fig. 3), the sequence of this strain was in the same branch as the other two reference strains of *C. crassipes* (CBS 159.75 and IMI 302450) and received high bootstrap support (96%). The identification of a previously described clinical strain from Brazil as *C. gloeosporioides* was also confirmed here (Fig. 2 and 3). The sequence of that strain (CBS 102275) nested with the other sequences of the species obtained from GenBank. In the

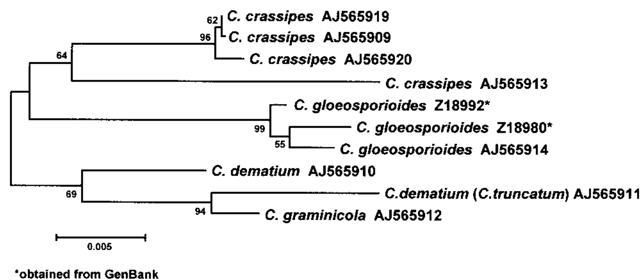

FIG. 3. Unrooted neighbor-joining tree of 10 sequences of D1-D2 regions of LSU rDNAs from *Colletotrichum* species. The branch lengths are proportional to genetic distance, which is indicated by the bar. Bootstrap values (percentages of 1,000 replications) are indicated at the internodes.

two trees, the clades received high bootstrap support (92 and 99%, respectively).

In summary, this study shows that molecular techniques are very useful and important tools. They can be used to complement identification based only on morphological criteria, which in the diagnosis of mycoses is still the most common technique.

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