Morphological plasticity in Cladosporium sphaerospermum

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Key words
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INTRODUCTION

Cladosporium is one of the largest genera of hyphomycetes, with more than 772 names (Braun et al. 2003, Dugan et al. 2004). Cladosporium species are common, widespread fungi, including endophytic, fungalicious, human pathogenic, phytopathogenic and saprobic species (Crous et al. 2007b). Saprobic species occur on various senescing and dead leaves and stems of herbaceous and woody plants, are secondary invaders of necrotic leaf spots, and are frequently isolated from air, soil, foodstuffs, paint, textiles and other organic matter (Riesen & Sieber 1985, Brown et al. 1998, El-Morsy 2000). Furthermore, some Cladosporium species, such as C. bruhnei, are common contaminants in clinical laboratories and cause allergic lung mycoses (de Hoog et al. 2000, Schubert et al. 2007b).

Because the genus Cladosporium is very heterogeneous, David (1997) attempted to circumscribe Cladosporium based on scanning electron microscopic examinations of the scar and hilum structure in Cladosporum and Heterosporum. In so doing, David (1997) demonstrated that the structures of the conidigenous loci and hila in Heterosporum fully agree with those of Cladosporum, proving that Heterosporum was a synonym of Cladosporium. Furthermore, he introduced the term ‘coronate’ for the Cladosporum scar type, which is characterised by having a central convex part (dome), surrounded by a raised periclinal rim (Schubert et al. 2007b, Zalar et al. 2007). Several workers have employed DNA sequence data to prove that Cladosporum s.str. is a sister clade to Mycosphaerella s.str. (Braun et al. 2003, Crous et al. 2007a, b, Schubert et al. 2007a, b), having teleomorphs in Davidiella. Schoch et al. (2006) employed DNA sequence data of four loci (SSU nrDNA, LSU nrDNA, EF-1α, RPB2), revealing species of Davidiella to cluster in a separate family (Davidiellaceae) from species of Mycosphaerellaceae (Mycosphaerellaceae), with both families residing in the Capnodiales (Dothideomycetes).

A cladosporioid hyphomycete, deposited in the patent collection of the United States Department of Agriculture Northern Regional Research Lab (now National Centre for Agricultural Utilization Research) as NRRL 8131 (= ATCC 38493), was referenced in U.S. Patent 4.086.268 as Cladosporium lignicolus (sic, without author). The patent does not state the original substrate, but wood is a logical inference from the specific epithet. Ho et al. (1999) described NRRL 8131 from culture, and provided several photomicrographs illustrating the fungus, including 0–1 transversely septeate, obclavate conidia with subrostrate apices (‘alternarioid’ conidia in their description). A comparison of the latter description and illustrations with the original diagnosis of C. ligncola revealed obvious discrepancies and called into question the correctness of the identification of the NRRL strain. To help ascertain the identity of NRRL 8131, type material of C. ligncola has been re-examined and compared with the North American strain.

We were originally attracted to re-examination of NRRL 8131 not only by the discrepancies referenced above, but by the unique conidial morphology: the ‘alternarioid’ conidia resembled the beaked conidia of Alternaria spp. (Simmons 2007). Scanning electron microscopy (SEM) examination of conidogenous loci and conidial hila in the NRRL strain of ‘C. ligncola’ confirmed generic affinity, and sequence analyses of the actin, translation elongation factor 1-α and the internal transcribed spacer region (ACT, EF, ITS) were used to assign the strain to species.

MATERIALS AND METHODS

Materials examined
Several specimens deposited at PRM (Herbarium, Department of Mycology, National Museum, Prague, Czech Republic) under C. ligncola were examined: on rotten wood, Czechia, near...
| Anamorph          | e | Teleomorph                | Accession number | Host Country | Collector        | Source                  | GenBank numbers |
|-------------------|---|---------------------------|------------------|--------------|------------------|-------------------------|-----------------|
| C. antarcticum    |   | Davidiella atrolineata    | CBS 609.12       | Antarctica   | C. Miller        | –                       | EF679444, EF679444, EF679445 |
|                   |   | C. bruhnei                | CBS 815.34       | Belgium      | –                | –                       | EF679377, EF679378, EF679379 |
|                   |   | C. cladosporoides complex| –                 | –            | –                | –                       | –               |
|                   |   | C. cladosporoides complex| –                 | –            | –                | –                       | –               |
|                   |   | C. herbaroides            | –                 | –            | –                | –                       | –               |
|                   |   | C. herbarum               | –                 | –            | –                | –                       | –               |
|                   |   | C. iridis                 | –                 | –            | –                | –                       | –               |
|                   |   | C. macrocarpum            | –                 | –            | –                | –                       | –               |
|                   |   | C. macrocarpum            | –                 | –            | –                | –                       | –               |
|                   |   | C. ossifragi              | –                 | –            | –                | –                       | –               |
|                   |   | C. pseudiridis            | –                 | –            | –                | –                       | –               |
|                   |   | C. ramotenellum           | –                 | –            | –                | –                       | –               |
|                   |   | C. sinuosum               | –                 | –            | –                | –                       | –               |

**Table 1** Cladosporium isolates used for sequence analysis.
DNA isolation and phylogenetic analysis

Fungal colonies were established on agar plates, and genomic DNA was isolated as described in Gams et al. (2007). Partial gene sequences were determined as described by Crous et al. (2006) for actin (ACT), translation elongation factor 1-α (EF), and of the nuclear rDNA operon spanning the 3’ end of the 18S rRNA gene, the first internal transcribed spacer, the 5.8S rRNA gene, the second internal transcribed spacer and the 5’ end of the 28S rRNA gene (ITS). The nucleotide sequences were generated using both forward and reverse PCR primers to ensure good quality sequences over the entire length of the amplicon. Sequence data obtained from Schubert et al. (2007b) and Zalar et al. (2007) were used as reference data for the alignments (Table 1). Subsequent sequence alignment and phylogenetic analysis followed the methods of Crous et al. (2006). Gaps longer than 10 bases were coded as single events for the phylogenetic analyses; the remaining gaps were treated as new character states. Sequence data were deposited in GenBank (Table 1) and the alignment and tree in TreeBASE (www.treebase.org).

Morphology

Strain NRRL 8131 (= CBS 117728) was grown on 2 % potato-dextrose agar (PDA), synthetic nutrient-poor agar (SNA), and 2 % malt extract agar (MEA) (Gams et al. 2007), and incubated under continuous near-ultraviolet light at 25 °C to promote sporulation or in the dark for assessing colony characters (Schubert et al. 2007b, Zalar et al. 2007), and suspensions of conidia were preserved in glycerol for long term storage at -80 °C and in liquid nitrogen. Subcultures on MEA plates were used for scanning electron microscopy, and SNA and MEA slide cultures or plates for light microscopy. Microscopic observations were made from colonies cultivated for 7 d under continuous near-ultraviolet light at 25 °C on SNA and MEA. Preparations were mounted in Shear’s solution (Gams et al. 2007) or 85 % lactic acid. To study conidial development and branching patterns, squares of transparent adhesive tape (Titan Ultra Clear Tape, Conglom Inc., Toronto, Canada) were placed on conidiophores growing in the zone between the colony margin and 2 cm inwards, and mounted between two drops of Shear’s solution under a glass cover slip. Conidial terminology follows that of Schubert et al. (2007b). Colonies were cultivated on PDA, SNA and MEA for 14 d at 25 °C in the dark, after which the surface and reverse colours were rated using the charts of Rayner (1970). Linear growth was determined on MEA and SNA plates by inoculating three plates of each medium and incubating them for 14 d at 25 °C.
### Table 2

| Parameter                              | ITS¹ | ACT¹ | EF¹ | Combined |
|----------------------------------------|------|------|-----|----------|
| Number of alignment positions          | 495  | 219  | 380 | 1094     |
| Number of parsimony informative characters | 37   | 99   | 177 | 313      |
| Number of variable and parsimony-uninformative characters | 100  | 30   | 54  | 184      |
| Number of constant characters          | 358  | 90   | 149 | 597      |

¹ ACT: partial actin gene; EF: partial elongation factor 1-α gene; ITS: internal transcribed spacer region.

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SEM was conducted by mounting small (2–3 mm³) sections of MEA agar with fungal growth onto SEM stubs, subjecting the mounts to osmium tetroxide vapours for 2 h, and gold-coating the specimens with a Technics Hummer V sputter coater. Specimens were observed and photographed with a Hitachi S-570 scanning electron microscope.

### RESULTS

#### DNA phylogeny

The manually adjusted concatenated alignment contained 44 sequences (including the outgroup sequence) and the three loci were represented by a total of 1094 characters including alignment gaps which were used in the analysis (Table 2). The result of the partition homogeneity test ($P = 0.685$) indicated that the loci were congruent and the sequence data could therefore be analysed as a concatenated alignment. A single most parsimonious tree (TL = 1253 steps; CI = 0.663; RI = 0.871; RC = 0.578), which is shown in Fig. 1, was obtained from the parsimony analysis of the combined genes. Phylogenetic analyses using the concatenated alignment as well as the individual loci (data not shown) all conclusively demonstrated that CBS 117728 clustered with *C. sphaerospermum* in accordance with combined ITS, ACT and EF sequences, and that it was the sister taxon of MRC 10263 (= CPC 14016) (Fig. 1). Neighbour-joining analysis using three substitution models (uncorrected ‘p’, Kimura 2-parameter and HKY85) on the sequence data yielded trees with identical topologies (data not shown).

#### Taxonomy

In PRM 657823, only a trimmatostroma-like hyphomycete was found. The three other specimens, including the type material (PRM 155424 holotype, PRM 657824, and PRM 657821) exclusively contained *C. herbarum*, well-characterised by having nodulose conidiophores and verrucose conidia (Schubert et al. 2007b). The type of *C. lignicola* was also examined by Hughes (1958), who reduced it to synonymy with *C. herbarum*, a treatment that was confirmed during the course of the present study. The examination of NRRL 8131 ‘*C. lignicola*’ clearly showed that this fungus is quite distinct from...
the true *C. lignicola* (= *C. herbarum*) by having non-nodulose conidiophores and almost smooth to verruculose, often short rostrate conidia (Fig. 2, 3). The general habit of this fungus is obviously cladosporioid, including conspicuous, somewhat protuberant conidiogenous loci. The periclinal rim is evident, but the central dome is rather low and not very conspicuous when viewed by light microscopy (Fig. 2). SEM studies confirmed that scar structure conforms to the coronate *Cladosporium* type (Fig. 3).

![Fig. 2](image_url)

**Fig. 2** Light micrographs of *Cladosporium sphaerospermum* NRRL 8131. a–h. Conidiophores at various stages of development, showing their characteristic branching patterns, ramoconidia, secondary ramoconidia, intercalary conidia, and small, terminal conidia (all on SNA); i. conidiophore with alternarioid secondary ramoconium (arrow), formed on MEA; j, k. secondary ramoconidia and intercalary conidia (note older intercalary conidia, which become dark brown and globose). — Scale bars = 10 µm.
Description — On MEA or SNA, hyphae 1–5 µm wide, sparingly to richly branched (angles between 45 and 90°), sometimes anastomosing, loosely to densely septate, thin-walled, almost smooth to distinctly rough-walled, subhyaline in narrow hyphae to medium dark olivaceous-brown, subcylindrical to irregular in outline by swellings and constrictions at septa. Conidiophores little differentiated, micronematous, barely discernable and distinguishable from ordinary hyphae, becoming macronematous on SNA after 14 d (15–)31–125(–250) × (2.5–)3–4.5(–5.5) µm, predominantly unbranched. Conidiogenous cells integrated, terminal and intercalary, 10–25 × (2.5–)3–4(–5) µm, with a single or up to three conidiogenous loci, 1–1.5 µm diam, coronate, but central dome low and not very conspicuous when viewed by light microscopy. Conidia catenate, in simple or usually branched chains, subglobose, ellipsoid-ovoid, obovoid, broadly fusiform, limoniform, straight to somewhat curved; terminal conidia with a single basal hilum and intercalary conidia with two hila on MEA (3–)4–10(–13) × (2–)3–4(–5) µm, 0(–1)-septate, length becoming shorter towards the apex; on SNA (2.5–)3.5–8(–10.5) × 2.5–4.5(–5) µm (mean 5.9 (std dev 1.4) × 3.4 (std dev 0.4), n = 50), length/width 1.1–3.8 (mean = 1.8); secondary rami conidia on MEA with 3–5 hila, (6–)10–20(–25) × 3–5 µm; on SNA with 2–4 hila, 8.5–20 × 2.5–4.5 µm (mean 12.5 (std dev 2.7) × 2.9 (std dev 0.4), n = 25), 0–1-septate, sometimes alternarioid, obclavate, subrostrate (the alternarioid ones seldom observed when cultivated on SNA after 7 d, but readily observed on PDA and MEA); rami conidia (‘true rami conidia’ sensu Crous et al. 2007b) on SNA (11–)13–34(–43) × (2.5–)3–4 µm; rami conidia and small terminal conidia in general subhyaline to pale olivaceous or olivaceous-brown, thin-walled, almost smooth to distinctly verruculose, hila conspicuous, 0.75–1.25 µm diam, coronate, often at the end of protuberant, short, terminal projections, 1–2 µm long or even longer in secondary rami conidia with beak-like ends.

**Fig. 3** Scanning electron micrographs of *Cladosporium sphaerospermum* NRRL 8131. a, b. Branching chains of conidia, showing conidiogenous loci with disjunctors (arrows); c. apex of conidiophore with conidiogenous scar in profile (arrow); d. two conidiogenous loci at apex of a secondary rami conidium, the upper (arrow) clearly coronate; e. two conidiogenous loci at apex of a conidiophore, the one facing the viewer is clearly coronate (arrow); f. two conidiogenous loci (arrows) at apex of a secondary rami conidium are coronate. — Scale bars: a–c = 2.5 µm, d = 1 µm, e = 5 µm, f = 1.25 µm.
Cultural characteristics — On MEA, colonies attaining 45 mm diam at 25 °C after 14 d; dark olivaceous, powdery, velvety, reverse dark olivaceous-grey. Colonies on PDA attaining 50 mm diam at 25 °C after 14 d, olivaceous-grey in centre, iron-grey in outer region, reverse iron-grey. On SNA, colonies attaining 30 mm diam at 25 °C after 14 d, semi-translucent and olivaceous-grey to iron-grey, with wide translucent margin.

Specimen examined. USA (no additional data known), isolated from wood, CBS-H 20086 (HAL 1846 F), dried culture ex ATCC 38493, cultures ATCC 38493 = CBS 117728 = CPC 12098 = NRRL 8131.

Notes — NRRL 8131 differs from previously known isolates of C. sphaerospermum in having mostly unbranched, micro- nematous conidiophores, only becoming macronematous with age, and frequently substrate, occasionally ‘alternarioid’ conidia. Also, conidia on MEA and SNA exceed in size conidia of other isolates. Conidial length/width (mean 1.8, n = 50) on SNA exceeds that from the standard description (range = 1.1–1.5, Zalar et al. 2007), with 32 % of conidia of NRRL 8131 falling within the range from Zalar et al. (2007).

DISCUSSION

Re-examination of type material of C. lignicola and a putatively North American strain NRRL 8131, originally referred to the latter species, demonstrated clearly that the two fungi are distinct. The type of C. lignicola was formerly examined by Hughes (1958), who reduced it to synonymy with C. herbarum, a treatment confirmed during the course of the present investigations.

The North American strain (NRRL 8131) is C. sphaerospermum, but differs in morphology from previously known isolates of that species. It is easily distinguishable from C. herbarum (including C. lignicola) and all other know species of Cladosporium s.str., by having obclavate, short rostrate, sometimes ‘alternarioid’ conidia. Individual conidia often conformed to the spherical shape generally typical of isolates of C. sphaerospermum, but such conidia of NRRL 8131 could be somewhat larger than the upper limits of 4(–7) × 3.5(–4.5) µm given for C. sphaerospermum in Zalar et al. (2007). Furthermore, the conidiophores are at first consistently micronematous, much later they may become more macronematous, and they are usually unbranched. The conidiophores in other isolates of C. sphaerospermum are often branched in vivo as well as in vitro. However, not only did NRRL 8131 cluster with strains of C. sphaerospermum (Fig. 1), but the neotype of C. sphaerospermum (CBS 193.54) occasionally displayed substrate ‘beaks’ on ramosconidia (e.g., fig. 5G in Zalar et al. 2007). Because our sequence data conclusively displayed subrostrate ‘beaks’ on ramoconidia (e.g., fig. 5G in Zalar et al. 2007), with 32 % of conidia of NRRL 8131 falling within the range from Zalar et al. (2007).

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