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A comparison of generic concepts in *Calonectria* and *Nectria* with anamorphs in *Cylindrocladium* and *Cylindrocladiella*

Cylindrocladium and Cylindrocladiella have been considered as a single genus by numerous authors. We dispute this fact because Cylindrocladium has Calonectria teleomorphs, whereas Cylindrocladiella has Nectria teleomorphs. In addition to the differences in their teleomorph states, the anamorphs are also morphologically distinct and probably not closely related. The tendency to amalgamate Cylindrocladium and Cylindrocladiella has chiefly resulted from interpreting the stipitate conidiophores as a unifying characteristic. It must, however, be expected that similar themes in morphology will result from adaptation to analogous environments, and therefore convergence. We conclude that separation of taxa based on single characteristics should be avoided. Rather, consideration of as many characteristics of the holomorph as possible is essential. In the fungi discussed here, teleomorph characteristics tend to be conserved and uniform, whereas anamorphs are more variable, often providing excellent characters for species delimitation.

Ninety years after the establishment of the hyphomycete genus *Cylindrocladium* Morgan,[1] Boesewinkel transferred several small-spored species to a new and separate genus, *Cylindrocladiella* Boesewewinkel.[2] The latter genus currently comprises six species,[3] whereas *Cylindrocladium* includes more than 20 species.[4] Subsequent to the establishment of *Cylindrocladiella* as a genus distinct from *Cylindrocladium*, there has been controversy as to the validity of this separation, with some authors accepting *Cylindrocladiella*[3,5] and others choosing not to do so.[6,7] In the most recent monographic study of this group of fungi, Peerally[8] formulated arguments against accepting the generic status of *Cylindrocladiella*. During the course of the past three years, our correspondence and discussions with mycologists in many parts of the world has underlined the fact that a great deal of uncertainty exists as to the generic status of *Cylindrocladiella*. We are of the opinion that *Cylindrocladium* and *Cylindrocladiella* are distinct genera and believe that this view needs to be clearly presented. The aim of this study is, therefore, to outline the differences between *Cylindrocladium* and *Cylindrocladiella*, and to present information on trends in the morphology of species in these two genera.

Materials and methods

Isolates studied

Single-conidial isolates of 20 species of *Cylindrocladium* (*C.*) and six species of *Cylindrocladiella* (*Ca.*) were studied. These included *C. avesiculatum* Gill, Alfieri & Sobers (ATCC 38226), *C. candelabrum* Viegas (PPRI 4153), *C. citri* (Fawcett & Klotz) Boedijn & Reitsma (CBS 186.36), *C. clavatum* Hodges & May (PPRI 3994), *C. colhounii* Peerally var. *colhounii* (PPRI 4182), and var. *macroconidialis* Crous, Wingfield & Alfenas (PPRI 4000), *C. floridanum* Sobers & Seymour (ATCC 42971), *C. gracile* (Bug.) Boesew. (PC 551197), *C. hawksworthii* Peerally (MUCL 30866), *C. heptaseptatum* Sobers, Alfieri & Knauss (ATCC 42972), *C. ilicicola* (Hawley) Boedijn & Reitsma (PPRI 4151), *C. pteridis* Wolf (PPRI 4157), *C. quinqueseptatum* Boedijn & Reitsma (ATCC 16550), *C. reteaudii* (Bugn.) Boesew. (ATCC 64824), *C. scoparium* Morgan (ATCC 46300), *C. spathiphylli* Schoulties, El-Gholl & Alfieri (ATCC 44730), *C. spathulatum* El-Gholl, Kimbrough, Barnard, Alfieri & Schoulties (ATCC 62616), *C. theae* (Petch) Sub. (PPRI 4188) and three new *Cylindrocladium* spp. (PPRI 4175),[9] (PPRI 4213)[10] and (ATCC 76225).[11] The *Cylindrocladiella* species were *Ca. camelliae* (Venkataramani et Venkata Ram) Boesew. (PPRI 3990), *Ca. elegans* Crous & Wingfield (PPRI 4050), *Ca. infestans* Boesewinkel (ATCC 44816), *Ca. lageniformis* Crous, Wingfield & Alfenas (PPRI 4449), *Ca. novae-zelandiae* (Boesew.) Boesew. (ATCC 44815) and *Ca. parva* (Anderson) Boesew. (PPRI 50929).

Microscopy

Light microscopy. Single-conidial isolates of the respective species were plated onto carnation-leaf agar (CLA),[12,13] incubated at 25°C under near-ultraviolet light, and examined after 7 days. Only material occurring on the leaves was examined. Mounts were prepared in lactophenol cotton blue. All measurements were made under the (100×) oil-immersion objective. Stipes examined were all on conidiophores with at least one primary, and one secondary branch bearing phialides. Stipe length was measured from the basal septum to the vesicle tip (for *Cylindrocladiella* spp.), and from the highest primary branch to vesicle tip for *Cylindrocladium* spp.

Scanning electron microscopy. Scanning electron microscopy was used to observe conidium development and phialide morphology in a *Cylindrocladium* sp. (ATCC 76225), and *Ca. camelliae* (PPRI 3990). Specimens were either prepared for cryo scanning electron microscopy using an Oxford CT 1500B cryo-transfer system, and viewed using a Joel JSM 6100 scanning electron microscope, or fixed in 2.5% glutaraldehyde and 1.5% osmium tetroxide in a 0.1 M phosphate buffer, dehydrated in a graded acetone series and critical-point dried. Specimens were coated with gold–palladium and examined using a Jeol 6400 scanning electron microscope.

Culture characteristics

Chlamydospore production and the presence of odours produced by the various species were rated on malt-extract agar (MEA) (20 g Oxoid malt extract, 15 g Difco agar, 1 000 ml H_2O) plates incubated at 25°C for 6 days in the dark. Production of chlamydospores and microsclerotia were rated for the density of thickened, pigmented hyphae present as viewed from the underside of plates. Isolates were also inoculated into 500-ml Erlenmeyer flasks containing malt-extract broth, and rated for the presence or absence of extracellular polysaccharides after 7 days of incubation at 25°C in the dark.

Time-lapse photomicrography

Conidium development and arrangement in a *Cylindrocladium* sp. (ATCC 76225) and *Ca. camelliae* were compared on MEA using the modified plate-culture technique.[14] Plates were inoculated and incubated at 25°C in the dark for 1 day. A rectangular block of the colony was subsequently removed

with a sterile scalpel, the groove partly covered with a cover slip, and the plate was then mounted on a light microscope stage for examination.

Results and discussion

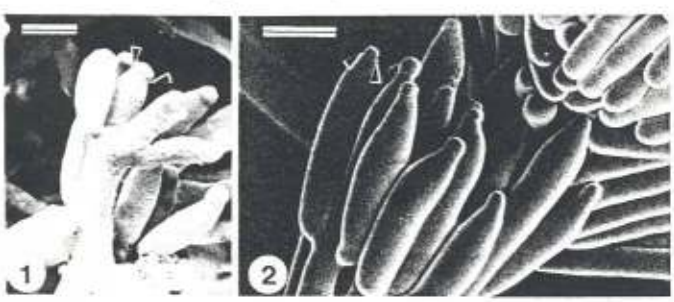
Teleomorph

Cylindrocladium spp. have teleomorphs in *Calonectria*, while those of *Cylindrocladiella* are disposed in *Nectria*.[2] Rossman[15] characterized *Calonectria* by stating that the perithecia have an outer layer which consists of large globose cells, giving it a warty appearance. Perithecia also become dark red when mounted in 3% KOH. Boesewinkel[2] stated that the asci of *Calonectria* spp. are very characteristic, being club-shaped, and having their ascospores aggregated in the upper two-thirds of the ascus. Furthermore, ascospores are mostly fusiform, seldom ellipsoid, and 1–6-septate. In *Nectria* teleomorphs of *Cylindrocladiella* spp., perithecia are smooth, and have cylindrical asci with 1-septate ascospores evenly distributed throughout the ascus. Although *N. camelliae* (Shipton) Boesewinkel is the only teleomorph presently known for the genus *Cylindrocladiella*, other *Nectria* states have also been observed for *Ca. parva* and *Ca. lageniformis*.

Anamorph

Conidiophore type. Penicillate conidiophores with phialides at the apices of conidiophore branches are common to both *Cylindrocladium* and *Cylindrocladiella*. The stipes from these conidiophores extend above the phialides, and terminate in thin-walled vesicles with shapes characteristic for the species. The penicillate conidiophores of *Cylindrocladium* spp. can have tertiary and quaternary branches, whereas only primary and secondary branches occur in species of *Cylindrocladiella*. Furthermore, *Cylindrocladiella* spp. can have conidiophores resembling those of *Verticillium* spp.,[21] a characteristic for which Boesewinkel used the term 'subverticillate'.[2] Species can either have subverticillate conidiophores ending in two or three phialides (*Ca. camelliae*), or can branch at more than one level (*Ca. infestans*). In contrast, *Cylindrocladium* spp. have only penicillate conidiophores.

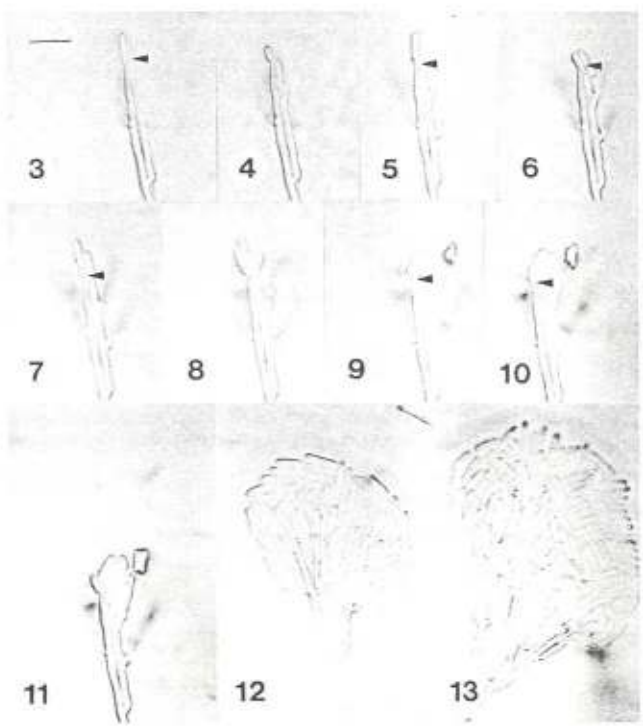
Phialide shape, collarettes and conidium arrangement. Both *Cylindrocladium* and *Cylindrocladiella* have conidia that are produced on hyaline, determinate, monophialides (*sensu* Sutton[16]), with the first conidium developing holoblastically, and subsequent conidia produced enteroblastically. Although collarettes have been noted for both genera,[2,17] their phialide apices are distinctly different. In *Cylindrocladium* the apices of the phialides are wider than those of *Cylindrocladiella*, also having inconspicuous collarettes situated below the phialide apex, with a distinct outward taper (Fig. 1). In *Cylindrocladiella*, phialide apices are narrow, collarettes extend above the phialide apex, and taper distinctly towards the inside. (Fig. 2).



Figs 1, 2. Phialides with collarettes of a *Cylindrocladium* sp. (ATCC 76225) and *Ca. camelliae* (bars = 5 μm). Fig. 1. *Cylindrocladium* phialides with divergent collarettes. Fig. 2. *Cylindrocladiella* phialides with convergent collarettes.

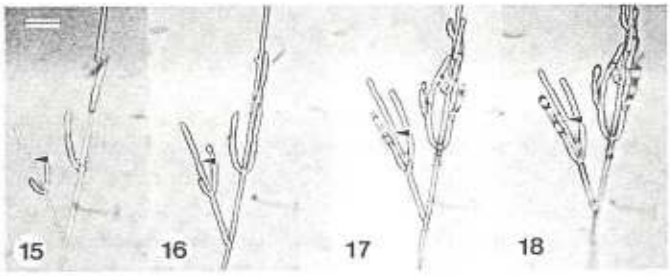
Onofri and Zucconi[18] have recently shown that the parallel arrangement of conidia in *Chaetopsina* Rambelli is the result of mucilaginous, slightly asymmetric conidia which can accumulate on the surface of the collarettes at the phialide apex. Conidia in both *Cylindrocladium* and *Cylindrocladiella* are cylindrical to slightly asymmetrical, covered with an irregular layer of mucous similar to *Chaetopsina*. Conidia are arranged in parallel clusters in *Cylindrocladium*, but in rounded spore masses in *Cylindrocladiella*. Several factors are thought to affect the arrangement of these conidia, including the width of the phialide apex, and the length and taper of the collarettes. It also appears that *Cylindrocladiella* spp. accumulate more mucous around their spores than is true of *Cylindrocladium* spp. Furthermore, the smaller conidia of *Cylindrocladiella* spp. are also better suited to accumulate in rounded clusters than are the larger conidia of *Cylindrocladium* spp.

Using time-lapse photomicrography, the first conidium in *Cylindrocladiella* could be seen to form holoblastically. The second, enteroblastic conidium then lifts the first conidium, which is pushed to the side of the second conidium, and rests below the phialide apex and between the other phialides. Conidia then accumulate, and form a rounded cluster (Figs 3–14). In *Cylindrocladium* spp. the second conidium also lifts the first, which is then pushed onto the collarette or remains above the phialide apex. The conidia generally do not slide as far down into the phialides as they do in *Cylindrocladiella*. This is presumably due to the angle and position of the collarette in *Cylindrocladium*, as well as the larger conidia, which in turn increases the contact area between the conidia (Figs 15–20). The same phenomenon has also been observed in *Chaetopsina fulva* Rambelli, where the slightly asymmetrical



Figs 3–13. Time-lapse photomicroscopic study of conidium development in *Ca. camelliae* (bar = 15 μm). Fig. 3. Mature conidium (0:00). Fig. 4. Second conidium pushing the first off to the side (0:30). Fig. 5. Third conidium forming (1:30). Fig. 6. Third conidium pushing the second off onto the side (2:00). Fig. 7. Fourth conidium forming (3:00). Fig. 8. Fourth conidium pushing others onto the side (4:30). Fig. 9. Fifth conidium forming (4:30). Fig. 10. Fifth conidium pushing others onto the side (5:30). Fig. 11. Initial stage of rounded conidial cluster (6:00). Figs 12, 13. Rounded cluster of conidia at (7:00) and (8:00).

Fig. 14. Scanning electron micrograph of a rounded conidial cluster in *Ca. camelliae* (bar = 10 μm).



Figs 15–18. Time-lapse photomicrographic study of conidium development in *Cylindrocladium* (bar = 26 μm). Fig. 15. Mature conidium (0:00). Fig. 16. Second conidium forming (1:00). Fig. 17. Second conidium lifting the first, which runs off onto its side (5:30). Fig. 18. Second conidium lifting the first conidium to the position where the parallel cluster will form (6:30).

collarettes facilitate a lateral movement of the cylindrical conidia.[18]

Stipe characteristics, and correlation with conidium length. One of the most obvious differences between *Cylindrocladium* and *Cylindrocladiella* is the nature of the stipe, which is thick-walled and non-septate in *Cylindrocladiella* (basal septum just above the whorl of phialides), and thin-walled and septate in all *Cylindrocladium* spp. other than *C. avesiculatum*. In the latter species the stipe is septate, thick-walled, ending in a clavate, thin-walled vesicle, or accumulating to a thick-walled apex devoid of a vesicle. In *Cylindrocladium* spp. there is a significant (P = 0.05) correlation between average stipe and conidium length (r = 0.822) (Fig. 21). This suggests that the height the stipe extends above the conidia is related to conidial

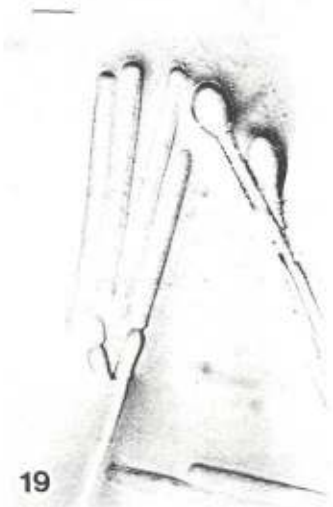


Fig. 19. Initial stages in the formation of a parallel conidial cluster in *Cylindrocladium* (bar = 10 μm).

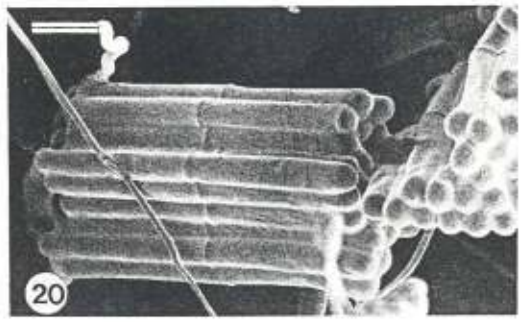
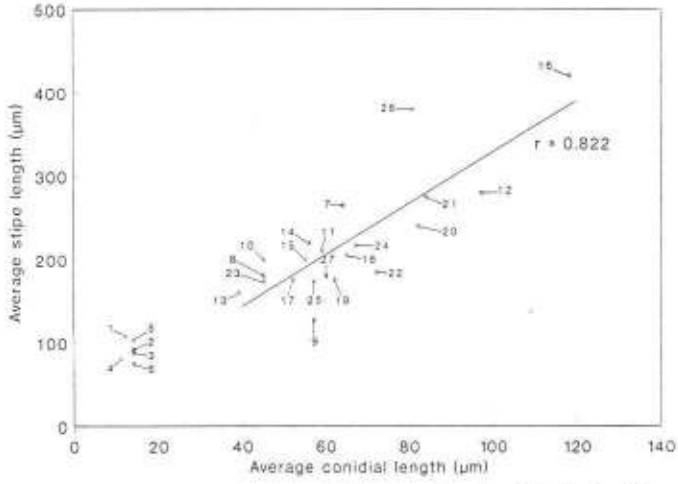


Fig. 20. Scanning electron micrograph of a parallel conidial cluster in *Cylindrocladium* (bar = 10 μm).

size of species, and that stipe length is an important taxonomic characteristic. This correlation would further suggest that the stipe could play a role in either supporting the conidia, or in luring insects or mites for spore dispersal. No such relationship was found between conidial and stipe length in *Cylindrocladiella*. The thick-walled and non-septate stipes in *Cylindrocladiella* might well have a different function to that in *Cylindrocladium*.

Microconidial states. Although microconidia are more common in genera such as *Fusarium* Link and *Cylindrocarpon* Wollenw., microconidial states do occur in *Cylindrocladium*. They are, however, absent in *Cylindrocladiella*. Microconidial states are known for *C. pteridis*, *C. heptaseptatum*, *C. ilicicola*, *C. quinqueseptatum*, and three new *Cylindrocladium* spp.[4,9,10,11,19,20] Stipes of conidiophores in microconidial states are always septate, and frequently terminate in an apical vesicle, usually with the same morphology as that of the macrovesicle. Microconidial states are not formed by all isolates of a species, and their taxonomic value is therefore limited.

Correlation between phialide class and conidium length. Phialides of *Cylindrocladium* spp. were found to vary in shape depending on the conidium size. This characteristic is, however, not found in *Cylindrocladiella*, and is probably due to the fact that conidia in species of this genus are more similar in



Figs 21. Average stipe and conidium lengths of *Cylindrocladiella* and *Cylindrocladium* spp. as determined on carnation-leaf agar after 7 days at 25°C under near-ultraviolet light. Species: 1, *Ca. camelliae*; 2, *Ca. elegans*; 3, *Ca. infestans*; 4, *Ca. lageniformis*; 5, *Ca. novae-zelandiae*; 6, *Ca. parva*; 7, *C. avesiculatum*; 8, *C. candelabrum*; 9, *C. citri*; 10, *C. clavatum*; 11, *C. colhounii* var. *colhounii*; 12, *C. colhounii* var. *macroconidialis*; 13, *C. floridanum*; 14, *C. gracile*; 15, *C. hawksworthii*; 16, *C. heptaseptatum*; 17, *C. ilicicola*; 18, *C. ovatum*; 19, *C. parasiticum*; 20, *C. pteridis*; 21, *C. quinqueseptatum*; 22, *C. reteaudii*; 23, *C. scoparium*; 24, *C. spathiphylli*; 25, *C. spathulatum*; 26, *C. theae*; 27, *C. variabile*.

size than those of *Cylindrocladium*. *Cylindrocladium* spp. with small conidia usually have doliiform to reniform phialides (*C. candelabrum, C. clavatum, C. floridanum and C. scoparium*), while those of intermediate length (*C. ilicicola and C. spathiphylli*) have more elongate doliiform to reniform phialides. Species with large conidia (*C. colhounii* var. *macroconidialis, C. heptaseptatum, C. quinqueseptatum and C. theae*), have allantoid to cylindrical phialides. An exception in this regard is *C. pteridis*, which has large conidia, and we would therefore expect it to have allantoid to cylindrical phialides, rather than its elongate doliiform to reniform phialides.

Phialide shape is a useful taxonomic character in *Cylindrocladium*, and gives an indication as to whether conidia will be small, medium, or large. *Cylindrocladiella* spp. have doliiform to reniform or cymbiform phialides if they have small conidia, and doliiform to reniform phialides if they have larger conidia. The cymbiform phialide shape can also be correlated with the presence of subverticillate conidiophores, which are absent in *Cylindrocladium*.

Conidium morphology. Species of *Cylindrocladium* have conidia which are 1–9-septate. In *Cylindrocladiella*, conidia that have more than one septum are considered as abnormal,[3] and isolates which produced a small number of multi-septate conidia lost this ability with subculturing. Although *Cylindrocladium* spp. have a wide range of conidial forms as argued by Peerally,[8] only one species, *C. brasiliensis* Peerally, has been reported to have conidia in the vicinity of 30 µm.[21] However, when this fungus was cultivated on CLA, conidia were found to be similar in size to those of other collections of *C. scoparium*.[22] Furthermore, species of *Cylindrocladiella* hardly ever have conidia larger than 20 µm, making them comparable only with the microconidial states produced by *Cylindrocladium* spp.

Chlamydospore production, arrangement and culture characteristics. Chlamydospores are primarily arranged in chains in *Cylindrocladiella*, and in clusters in *Cylindrocladium*.[2] However, with time, microsclerotia (a cluster of chlamydospores) also form in *Cylindrocladiella*,[3] but are usually more prominent in *Cylindrocladium*.

Species of *Cylindrocladiella* tend to produce copious amounts of slime when grown on MEA or potato-dextrose agar, as well as in broths of these media. This is in contrast to *Cylindrocladium* spp., which do not produce slime in culture. Furthermore, *Cylindrocladiella* spp. produce a very prominent odour in culture,[23] which is very faint in cultures of *Cylindrocladium* spp. Culture characters therefore indicate distinct differences between these two genera.

Conclusions

The argument of Peerally[8] that Boesewinkel[2] erected the genus *Cylindrocladiella* purely on the basis of smaller conidia is clearly unfounded. Comparisons made in this study show that there are many distinct morphological differences between these two genera. Furthermore, their teleomorphs are also distinct, which is indicative of differences at least at the generic level. To synonymize the anamorphs under one epithet solely on the basis of their both having stipes with terminal vesicles and cylindrical conidia is unreasonable. We therefore strongly suggest that they should be recognized as two separate genera.

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