19 *Ramichloridium*

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19.1 INTRODUCTION

Ramichloridium is an anamorph genus that comprises multiple species of saprobes, human and, plant pathogens. Morphologically, these organisms possess erect, dark, branched or unbranched conidiophores and predominantly aseptate conidia produced on a sympodially proliferating rachis. Currently, *Ramichloridium schulzeri* is the main *Ramichloridium* species involved in human disease, and other previous human pathogens within the genus, that is, *Ramichloridium mackenziei, Ramichloridium basitonum*, and *Ramichloridium musae* have been now reclassified as *Rhinocladiella mackenziei, Rhinocladiella basitonum*, and *Periconiella musae,* respectively.

19.1.1 Classification and Morphology

The genus *Ramichloridium* is a dematiaceous (dark-walled) fungus belonging to the mitosporic Capnodiales group, order Capnodiales, class Dothideomycetes, subphylum Pezizomycotina, phylum Ascomycota, kingdom Fungi. Currently, the genus *Ramichloridium* consists of nine recognized species: *Ramichloridium apiculatum* (the type species of the genus), *Ramichloridium australiense*, *Ramichloridium biverticillatum*, *Ramichloridium brasilianum*, *Ramichloridium epichloes*, *Ramichloridium indicum* (basionym: *Chloridium apiculatum*), *Ramichloridium pini*, *Ramichloridium schulzeri* (obsolete synonyms: *Acrotheca acuta*, *Chloridium schulzeri*, *Pleurophragmium acutum*, *Psilobotrys schulzeri*, *Rhinocladiella schulzeri* and *Rhinotrichum multisporum*), and *Ramichloridium strelitziae*, in addition to five unassigned species [1–4]. No teleomorph has been linked to the species of *Ramichloridium* so far.

Other former species in the genus include *Ramichloridium anceps* (formerly *Veronaea parvispora*) (→*Rhinocladiella anceps*), *Ramichloridium basitonum* (→*Rhinocladiella basitona*), *Ramichloridium cerophilum* (→*Rhinocladiella aquaspersa*), *Ramichloridium fasciculatum* (→*Rhinocladiella fasciculata*), *Ramichloridium mackenziei* (formerly *Ramichloridium obovoidea* and *Ramichloridium obovoiedum*) (→*Rhinocladiella mackenziei*), *Ramichloridium musae* (formerly *Veronaea musae*) (→*Periconiella musae*), and *Ramichloridium subulatum* (→*Radulidium subulatum*) (http://www.indexfungorum.org/) [5–9].

Ramichloridium colonies are flat to raised with entire margin; surface is olivaceous-green to olivaceous-black. Mycelium consists of submerged and aerial hyphae: submerged hyphae are hyaline to subhyaline and thin walled whereas aerial hyphae are smooth or verrucose. Conidiophores are straight, unbranched, rarely branched, thick walled, dark brown (darker than the subtending hyphae), and continuous or with several additional thin septa. Conidiogenous cells are integrated, terminal, polyblastic, smooth, thick walled, golden-brown, apical part subhyaline, with sympodial proliferation, straight or flexuose, geniculate or nodose, with conspicuous conidiogenous loci. Scars are crowded or scattered, unthickened, unpigmented to faintly pigmented, or slightly prominent denticles. Conidia are solitary, with 0–1 septum, subhyaline to pale brown, smooth to coarsely verrucose, thin walled, obovate, obconical or globose to ellipsoidal, fusiform, with a slightly pigmented hilum; conidial secession is schizolytic. The type species of the genus is *R. apiculatum* [7,8].

At the species level, *Ramichloridium apiculatum* (basionym: *Chloridium apiculatum*) colonies reach a diameter of 35 mm after 14 days at 24°C on malt extract plates (MEA), with minimum growth temperature of above 6°C, optimum temperature at 24°C, maximum temperature at 30°C. Colonies are raised, velvety, dense, with entire margin; surface is olivaceous-green, reverse is olivaceous-black, often with a diffusing citron-yellow pigment. Submerged hyphae (1–2.5 μm wide) are hyaline to subhyaline, thin walled; aerial

hyphae are slightly darker, smooth walled. Conidiophores (up to 100 µm long) generally arise at right angles from creeping aerial hyphae, straight, unbranched, thick walled, dark brown, continuous or with 1–3 additional thin septa; intercalary cells are 10–28 µm long. Conidiogenous cells (25–47 × 2–3.5 µm) are integrated, terminal, smooth, thick walled, golden-brown, straight, cylindrical, proliferating sympodially, resulting in a straight rachis with conspicuous conidiogenous loci. Scars (less than 1 µm in diameter) are prominent, crowded, slightly pigmented. Conidia (3–7.5 × 2–4 µm) are solitary, obovate to obconical, pale brown, finely verrucose. Hilum (about 1 µm in diameter) is conspicuous, slightly pigmented [7,8].

Ramichloridium australiense colonies are slow growing, reaching a diameter of 8 mm after 14 days at 24°C on MEA, with entire, smooth margin. Mycelium is flat, olivaceous-gray, becoming granular, with gelatinous droplets at the margin developing with age; reverse is pale olivaceous-gray. Submerged hyphae (1–2 µm wide) are hyaline, smooth, thin walled; aerial hyphae are pale brown, warted. Conidiophores arise vertically and clearly differentiate from creeping aerial hyphae, up to 400 µm tall, with several additional thin septa. Intercalary cells (8–40 µm × 2–5 µm) arise from the broadest part at the base tapering toward the apex, are subhyaline, later become pale brown and warted in the lower part. Subtending hyphae are thick walled, warted. Conidiogenous cells (10–18 µm long) are integrated, terminal, proliferating sympodially, giving rise to a short rachis with conspicuous conidiogenous loci. Scars (about 1 µm in diameter) are slightly thickened and darkened. Conidia (10–23 µm × 2.5–3 µm) are solitary, aseptate, thin walled, smooth, subhyaline, subcylindrical to obclavate, with a truncated base and a slightly darkened and thickened hilum (1.5–2 µm in diameter), rarely fusing at the basal part [7,8].

Ramichloridium biverticillatum (basionym: *Periconiella musae*) is named after its biverticillate conidiophores. Colonies are slow growing, reaching a diameter of 16 mm after 14 days at 24°C on MEA, with entire, smooth, sharp margin, compact, and velvety. Surface is vinaceous-buff to olivaceous-buff; reverse is buff. Submerged hyphae (1–2 µm wide) are smooth, hyaline, thin walled; aerial hyphae are subhyaline, smooth, slightly darker. Conidiophores (2–3 µm × 250 µm) arise vertically from creeping aerial hyphae, pale brown, profusely branched, biverticillate, with up to three levels of main branches, which taper distally. Conidiogenous cells (15–50 µm long) are terminally integrated, cylindrical, variable in length, rachis straight or geniculate, pale brown, as wide as the basal part, elongating sympodially, forming a rachis with crowded, slightly darkened and thickened minute scars (<0.5 µm wide). Conidia (2–5 × 1.5–2.5 µm) are solitary, aseptate, hyaline to subhyaline, dacryoid to pyriform, smooth, thin walled, with an inconspicuous hilum. *Ramichloridium biverticillatum* is a new name based on *Periconiella musae*. It is differentiated from *Periconiella musae* by having profusely branched conidiophores, and smaller conidia (2–5 µm × 1.5–2.5 µm) than those of *Periconiella musae* (5–11 µm × 2–3 µm) [7,8].

Ramichloridium brasilianum colonies are slow-growing, reaching a diameter of 6 mm after 14 days at 24°C on MEA, and appear velvety to hairy, with entire margin; surface is dark olivaceous-gray; black gelatinous exudate droplets produced on oatmeal agar (OA). Submerged hyphae (1.5–2 µm wide) are pale olivaceous, smooth or slightly rough; aerial hyphae are olivaceous, smooth or rough, narrower and darker than the submerged hyphae. Conidiophores (2–2.5 µm × 70 µm) are unbranched, arising vertically from creeping aerial hyphae, straight or flexuose, dark brown, with up to 10 additional septa, thick walled, and cylindrical. Conidiogenous cells (10–30 µm long) are integrated, terminal, proliferating sympodially, giving rise to a long, straight rachis with crowded, slightly darkened minute scars (about 0.5 µm in diameter). Conidia (4–8.5 µm × 2–3 µm) are solitary, obovoid to fusiform with the widest part below the middle, thin walled, verruculose, aseptate, pale brown, slightly rounded at the apex, truncated at the base, with a slightly thickened and darkened hilum (1–1.5 µm in diameter) [7,8].

Ramichloridium indicum (basionym: *Chloridium indicum*; synonyms: *Veronaea indica* and *Veronaea verrucosa*) colonies reach a diameter of 35 mm after 14 days at 24°C on MEA. Colonies are velvety, rather compact, slightly elevated, with entire, smooth, whitish margin, dark olivaceous-green in the central part. Submerged hyphae (1–2.5 µm wide) are smooth, thin walled, hyaline, with thin septa; aerial hyphae (2–2.5 µm wide) are coarsely verrucose, olivaceous-green, thick walled, with thin septa. Conidiophores (250 µm × 2–4 µm) arise vertically from creeping hyphae at right angles, are straight, unbranched, thick walled, smooth, dark brown, with up to 10 thin septa, often with inflated basal cells. Conidiogenous cells (up to 165 µm long) are terminally integrated, smooth, dark brown, sympodially proliferating, rachis straight or flexuose, geniculate or nodose, subhyaline; scars (about 0.5 µm in diameter) are thickened and darkened, clustered at nodes. Conidia (5–10 µm × 4–9 µm) are solitary, with 0–1 septum, not constricted at the septum, subhyaline to pale brown, smooth or coarsely verrucose, rather thin walled, broadly ellipsoidal to globose, with truncated base; hilum (about 1 µm in diameter) is conspicuous, slightly darkened, not thickened [7,8].

Ramichloridium schulzeri colonies grow moderately rapidly, and appear compact, flat; submerged hyphae are pale orange; aerial hyphae are powdery, brownish; reverse is pink to orange. Conidiophores (up to 250 µm long) are erect, straight, unbranched, thick walled, reddish-brown, gradually becoming paler toward the apex, elongating sympodially during conidiogenesis, with scattered, pimple-shaped conidium bearing denticles, which have unpigmented scars. Conidia (6.5–37 µm × 3–4 µm) are subhyaline, smooth walled or slightly rough walled, ellipsoidal, obovoidal or fusiform, usually with an acuminate base and unpigmented scars. *Ramichloridium schulzeri*, including its varieties, is phylogenetically as well as morphologically distinct from the other genera in the *Ramichloridium* complex [7,8].

Ramichloridium strelitziae (named after its host, *Strelitzia*) colonies are slow growing, reaching a diameter of 5 mm after 14 days at 24°C on MEA, with entire margin; aerial mycelium is compact, raised, dense, olivaceous-gray; reverse is olivaceous-black. Submerged hyphae (2–2.5 µm wide) are smooth, hyaline, thin walled; aerial hyphae are pale brown, verrucose. Conidiophores (40 µm × 2 µm) arise vertically from creeping aerial hyphae, clearly differentiated from the vegetative hyphae, subhyaline, later becoming pale brown, thick walled, smooth, or verruculose, with 1–3 additional septa. Conidiogenous cells (10–35 µm long) are integrated, terminal, cylindrical, subhyaline, later turning pale brown, fertile part as wide as the basal part, proliferating sympodially, forming a straight rachis with slightly thickened and darkened, circular, somewhat protruding scars (about 0.5 µm in diameter). Conidia (3–5.5 µm × 1–2.5 µm) are solitary, aseptate, smooth or verruculose, subhyaline, oblong, ellipsoidal to clavate, with truncated base and unthickened, nonpigmented hilum [7,8].

Phylogenetically, the genus *Ramichloridium* is related to the genera *Periconiella*, *Rhinocladiella* and *Veronaea*. The main morphological feature to distinguish *Ramichloridium* from *Rhinocladiella* is the presence of exophiala-type budding cells in the species of *Rhinocladiella* [3] (see also Chapter 43 of this book). Although both *Ramichloridium* and *Veronaea* lack exophiala-type budding cells, *Veronaea* is differentiated from *Ramichloridium* by having predominantly 1-septate conidia (i.e., two-celled conidia) (in comparison with one-celled or aspetate conidia of *Ramichloridium*). The largely aseptate conidia are also found in *Rhinocladiella* [10]. *Periconiella* differs from *Veronaea* chiefly by its dark brown, apically branched conidiophores [11].

Members of the genus *Ramichloridium* are largely soil saprobes. However, *Ramichloridium pini* is a known plant pathogen, causing a needle disease on *Pinus contorta* [3], and *Ramichloridium schulzeri* may be occasionally involved in human infection.

19.1.2 Clinical Features

The genus *Ramichloridium* represents 1 of 70 dematiaceous filamentous fungal genera that are largely saprophytes, but have the potential to cause phaeohyphomycosis, chromoblastomycosis, and eumycetoma in humans, especially those with suppressed immune functions [12–16]. *Ramichloridium schulzeri* was reported in a case of "golden tongue" involving a 54-year-old woman with acute lymphocytic leukemia. The patient was neutropenic, with an erosive lesion on the left side of the tongue, which extended over the dorsum of the tongue and appeared golden orange. Microscopic examination of surface scrapings revealed branching septate mycelia, and culture of a biopsy specimen of the tongue grew a few colonies of a fungus. The fungus was golden orange on Sabouraud's glucose agar and brown-gray on corn-meal agar and was identified as *Ramichloridium schulzeri*. The lesion was resolved after the patient underwent a week therapy with amphotericin B [17].

19.1.3 Diagnosis

The genus *Ramichloridium* is characterized by the presence of erect, dark, differentiated, branched or unbranched conidiophores and predominantly aseptate conidia produced on a sympodially proliferating rachis [2].

Morphologically and phylogenetically, *Ramichloridium* demonstrates close relation to *Rhinocladiella* and *Veronaea*. *Ramichloridium* and *Rhinocladiella* are separated mainly on the basis of (i) macronematous conidiophores in *Ramichloridium* versus micronematous conidiophores in *Rhinocladiella* and (ii) presence of a yellow or an orange diffusible pigment in *Ramichloridium* and absence in *Rhinocladiella* [3].

Other useful features for differentiation among *Ramichloridium*, *Rhinocladiella*, and *Veronaea* include (i) the absence of exophiala-type budding cells in *Ramichloridium* and *Veronaea* and its presence in *Rhinocladiella*; (ii) production of largely two-celled conidia (one-septate) by *Veronaea* and one-celled conidia (aseptate) by *Ramichloridium* and *Rhinocladiella* [10].

Nonetheless, identification of *Ramichloridium* from *Rhinocladiella* and *Veronaea* on the basis of macroscopic and microscopic characteristics are not only time consuming, but also technically challenging. For these reasons, molecular techniques targeting the sequence diversity of the internal transcribed spacer (ITS) region of ribosomal RNA (rRNA), as well as small subunit (SSU) and large subunit (LSU) rRNA, have been developed and applied for improved differentiation of *Ramichloridium* from *Rhinocladiella* and *Veronaea* [18–20].

19.2 METHODS

19.2.1 Sample Preparation

Biopsy samples are examined under microscope for mycotic elements using various stains. Samples are also cultured on inhibitory mold agar, modified Sabouraud agars, or potato dextrose agar (PDA). Colony colors (surface and reverse) are assessed after 2–4 weeks on different media at 25°C in the dark. Isolates are cultured on 2% MEA by obtaining single conidial colonies. Colonies are subcultured onto fresh MEA, OA, PDA, and synthetic nutrient-poor agar (SNA) and incubated at 25°C under continuous near-ultraviolet light to promote sporulation [6,8].

Microscopic structures are observed on tease or tape preparations and slide cultures for up to 21 days. Slide cultures are set up in Petri dishes containing 2 mL of sterile water, into which a U-shaped glass rod is placed, extending above the water surface. A block of freshly growing fungal colony, about 1 cm^2, is placed onto a sterile microscope slide, covered with a somewhat larger, sterile glass cover slip and incubated in the moist chamber. Fungal sporulation is monitored over time, and when optimal, images are captured by means of a Nikon camera system (Digital Sight DS-5M, Nikon Corporation, Japan). Structures are mounted in lactic acid and 30 measurements (×1000 magnification) are determined [10].

Genomic DNA is isolated from fungal mycelium grown on MEA, using the UltraClean™ Microbial DNA Isolation Kit (Mo Bio Laboratories) according to the manufacturer's protocols. Alternatively, genomic DNA is extracted following the cetyltrimethylammonium bromide (CTAB)-based protocol [6,8].

19.2.2 Detection Procedures

Arzanlou et al. [10] utilized the universal primers ITS1 and ITS4 [21] to amplify the ITS region of the nuclear rRNA operon, including: the 3′ end of the 18S rRNA gene, the first internal transcribed spacer region (ITS1), the 5.8S rRNA gene, the second internal transcribed spacer region (ITS2) and the 5′ end of 28S rRNA gene. Subsequent sequencing analysis allows identification of fungal organisms including *Chaetomium* species.

Procedure

1. PCR mixture (25 µL) is composed of 0.5 U *Taq* polymerase (Bioline), 1× PCR buffer, 0.5 mM $MgCl_2$, 0.2 mM of each dNTP, 5 pmol of each primer, approximately 10–15 ng of fungal genomic DNA.
2. Amplification is performed on a GeneAmp PCR System 9700 (Applied Biosystems) with primary denaturation at 96°C for 5 min; 36 cycles of 96°C for 30 s, 52°C for 30 s, and 72°C for 60 s; a final extension at 72°C for 7 min.
3. The amplicons are sequenced using BigDye Terminator v. 3.1 (Applied Biosystems,) or DYEnamicET Terminator (Amersham Biosciences) Cycle Sequencing Kits and analyzed on an ABI Prism 3700 (Applied Biosystems).
4. Newly generated sequences are subjected to a Blast search of the NCBI databases, sequences with high similarity are downloaded from GenBank and comparisons are made on the basis of the alignment of the obtained sequences.
5. Phylogenetic analysis is performed with PAUP (Phylogenetic Analysis Using Parsimony) v. 4.0b10 using the neighbor-joining algorithm with the uncorrected ("p"), the Kimura 2-parameter and the HKY85 substitution models. Alignment gaps longer than 10 bases are coded as single events for the phylogenetic analyses; the remaining gaps are treated as missing data. Any ties are broken randomly when encountered.
6. Phylogenetic relationships are also inferred with the parsimony algorithm using the heuristic search option with simple taxon additions and tree bisection and reconstruction (TBR) as the branch-swapping algorithm; alignment gaps are treated as a fifth character state and all characters are unordered and of equal weight. Branches of zero length are collapsed and all multiple, equally parsimonious trees are saved.
7. Other measures calculated include tree length, consistency index, retention index, and rescaled consistency index (TL, CI, RI, and RC, respectively). The robustness of the obtained trees is evaluated by 1000 bootstrap replications. Bayesian analysis is performed. The best nucleotide substitution model is determined using MrModeltest v. 2.2. MrBayes v. 3.1.2 is used to perform phylogenetic analyses, using a general time-reversible (GTR) substitution model with inverse gamma rates, dirichlet base frequencies and the temp value set to 0.5.

Note. Part of the LSU 28S rRNA gene may be also amplified with primers LR0R and LR5 followed by sequencing analysis.

19.3 CONCLUSION

The genus *Ramichloridium* consists of nine dematiaceous fungal species of which *Ramichloridium schulzeri* is involved in human infection. Due to the fact that *Ramichloridium* spp. closely resembles *Rhinocladiella* (which contains a significant human pathogenic species *Rhinocladiella mackenziei*) and *Veronaea* morphologically, it is important to determine these organisms to species level. As phenotypical methods for identification of *Ramichloridium*, *Rhinocladiella*, and *Veronaea* lack desired speed, accuracy, and convenience, molecular techniques such as PCR and sequencing analysis of the ITS region of rRNA, as well as SSU and LSU rRNA, offer a valuable alternative for improved differentiation of these organisms.

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