43 *Rhinocladiella*

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43.1 INTRODUCTION

43.1.1 Classification and Morphology

The genus *Rhinocladiella* is a dematiaceous (dark-walled) fungus belonging to the mitosporic Herpotrichiellaceae group, family Herpotrichiellaceae, order Chaetothyriales, class Eurotiomycetes, subphylum Pezizomycotina, phylum Ascomycota, and kingdom Fungi. The mitosporic Herpotrichiellaceae group encompasses 13 genera: *Blastacervulus*, *Brycekendrickomyces*, *Cladophialophora*, *Cladoriella*, *Cyphellophora*, *Exophiala*, *Heteroconium*, *Melanchlenus*, *Metulocladosporiella*, *Phaeococcomyces*, *Rhinocladiella*, *Thysanorea*, and *Veronaea* [1].

Currently, the genus *Rhinocladiella* consists of eight recognized species: *Rhinocladiella anceps* (synonyms: *Ramichloridium anceps* and *Veronaea parvispora*), *Rhinocladiella aquaspersa* (obsolete synonym: *Ramichloridium cerophilum*), *Rhinocladiella atrovirens*, *Rhinocladiella basitona* (synonym: *Ramichloridium basitonum*), *Rhinocladiella fasciculata* (basionym: *Ramichloridium fasciculatum*), *Rhinocladiella mackenziei* (obsolete synonyms: *Ramichloridium mackenziei*, *Ramichloridium obovoidea*, and *Ramichloridium obovoiedum*), *Rhinocladiella phaeophora*, and *Rhinocladiella similis*, in addition to 24 unassigned species [1–4]. Of these, *Rhinocladiella aquaspersa*, *Rhinocladiella atrovirens*, and *Rhinocladiella mackenziei* have been shown to infect humans [5]. *Rhinocladiella mackenziei* is a serious human pathogen, causing cerebral phaeohyphomycosis while *Rhinocladiella aquaspersa* is often associated with localized chromoblastomycosis lesions [6–8].

A number of former *Rhinocladiella* species have been transferred to other genera. These include *Rhinocladiella apiculata* (→*Ramichloridium apiculatum*), *Rhinocladiella cellaris* (→*Zasmidium cellare*), *Rhinocladiella compactum* (→*Fonsecaea compacta*), *Rhinocladiella cristaspora* (→*Ardhachandra cristaspora*), *Rhinocladiella elatior* (→*Leptodontidium elatius* var. *elatius*), *Rhinocladiella ellisii* (→*Zasmidium cellare*), *Rhinocladiella mansonii* (synonym: *Exophiala mansonii*), *Rhinocladiella pedrosoi* (→*Fonsecaea pedrosoi*), *Rhinocladiella peruviana* (→*Dicyma peruviana*), *Rhinocladiella schulzeri* (→*Ramichloridium schulzeri*), *Rhinocladiella selenoides* (→*Ardhachandra selenoides*), and *Rhinocladiella spinifera* (→*Exophiala spinifera*) [4].

Rhinocladiella colonies are slow growing, moist in texture, and dark olivaceous brown in color. Submerged hyphae are hyaline to pale olivaceous and smooth; aerial hyphae are more darkly pigmented. Conidial apparatus (consisting of either tips of ascending hyphae or septate conidiophores) is branched and olivaceous brown. Conidiogenous cells are intercalary or terminal, polyblastic, cylindrical to acicular, with a sympodially proliferating and subdenticulate rachis; scars are unthickened and non-pigmented to darkened refractive. Conidia are solitary, hyaline to subhyaline, aseptate, thin walled, smooth, and subglobose, with a slightly pigmented hilum; conidial secession is schizolytic [4].

At the species level, *Rhinocladiella aquaspersa* (obsolete synonyms: *Acrotheca cerophila*, *Cladosporium cerophilum*, and *Ramichloridium cerophilum*) colonies grow slowly, reaching a diameter of 12 mm after 14 days at 24°C on malt extract agar (MEA). Colonies are velvety to hairy, with entire margin, and surface is dark olivaceous gray, with black gelatinous exudate droplets on oatmeal agar (OA). Submerged hyphae (1.5–3 μm wide) are pale olivaceous brown and smooth or slightly rough; aerial hyphae are olivaceous brown, smooth or slightly rough, and somewhat narrower and darker than the submerged hyphae. Conidiophores (2–3 μm × 50 μm) are

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unbranched, arising vertically from creeping aerial hyphae, dark brown, thick walled, smooth or verruculose, hardly tapering toward the apex, with up to three additional septa. Conidiogenous cells are integrated, terminal, proliferating sympodially, rachis short and straight, with crowded, prominent, pigmented unthickened scars, minute, about 0.5 µm in diameter. Conidia (4–11 µm × 2–3 µm) are solitary, fusiform to clavate, thin walled, smooth, 0–1 septate, and subhyaline, with a conspicuous hilum, about 0.5 µm in diameter, slightly raised with an inconspicuous marginal frill. Conidia sometimes produce one to four short secondary conidia [4].

Rhinocladiella atrovirens colonies are restricted, velvety or lanose, olivaceous, and slightly mucoid at the center; reverse is dark olivaceous green to blackish. Conidiophores are short, brown, and thick walled. Conidiogenous cells (9–19 µm × 1.6–2.2 µm) are cylindrical and intercalary or free; denticulate rachis (up to 15 µm long) shows crowded, flat or butt-shaped, unpigmented conidial denticles. Conidia (3.7–5.5 µm × 1.2–1.8 µm) are hyaline, thin and smooth walled, and short cylindrical, with truncate basal scars. Budding cells (3.0–4.3 µm × 1.7–2.5 µm) are hyaline, thin walled, and broadly ellipsoidal. Germinating cells (4.5–6.0 µm) are inflated and spherical to subspherical. An annellidic *Exophiala* synanamorph may be present [4].

Rhinocladiella basitona (synonym: *Ramichloridium basitonum*) colonies are smooth, compact, and slightly raised at the center, flat toward margin, locally with some submerged mycelium, and olivaceous black with black reverse. Hyphae (2 µm wide) are thick walled, olivaceous brown, and septate every 15–20 µm. The conidial apparatus is profusely branched with flexuose cells arising at acute angles, the lower cells often being shorter than the ultimate ones and concolourous with the hyphae. Conidiogenous cells are cylindrical, with the apical part of variable length, producing numerous conidia in sympodial sequence; denticles are truncate, with a slightly darkened scar without a hilum. Conidia (3.5–4.5 µm × 2.2 µm) are hyaline, smooth walled and thin walled, and triangular with a rounded apex and with a clearly discernible basal scar. *Rhinocladiella basitona* differs from *Rhinocladiella anceps* by its basitonously branched conidiophores and triangular conidia [9].

Rhinocladiella fasciculata (basionym*: Ramichloridium fasciculatum*) colonies attain a diameter of 8 mm after 14 days at 24°C on MEA, with entire, smooth, sharp margin; mycelium is velvety, becoming farinose in the center (due to abundant sporulation), olivaceous green to brown; reverse is dark olivaceous. Blackish droplets produced at the center contain masses of *Exophiala* conidia. Submerged hyphae (2–2.5 µm wide) are subhyaline, smooth, and thickwalled; aerial hyphae are pale brown. Conidiophores (220 µm × 2–3 µm) arise vertically from ascending hyphae in loose fascicles and are unbranched or loosely branched at acute angles, cylindrical, smooth, brown, and thick walled at the base, with 0–5 thin additional septa. Conidiogenous cells (30–100 µm long) are terminal, cylindrical, thin walled, smooth, pale brown, and fertile part as wide as the basal part, up to 2 µm wide, proliferating sympodially, giving rise to a rachis (<0.5 µm in diameter) with hardly prominent, slightly pigmented, not thickened scars. Conidia (2.5–6 µm × 2–3 µm) are solitary, smooth, thin walled, subhyaline, and ellipsoidal, with truncate, slightly pigmented hilum (0.5 µm in diameter). Synanamorph forms on torulose hyphae originating from giant cells; compact heads of densely branched hyphae form thin-walled, lateral, subglobose cells, on which conidiogenous cells are formed; conidiogenous cells (12 × 1–1.5 µm) proliferate percurrently, giving rise to tubular annellated zones with inconspicuous annellations. Conidia (2–2.5 µm in diameter) are smooth, thin walled, aseptate, subhyaline, and globose [4].

Rhinocladiella mackenziei (basionym: *Ramichloridium mackenziei*) colonies attain a diameter of 5 mm on MEA after 14 days at 24°C, with entire, smooth, sharp margin; mycelium is densely lanose and raised in the center and olivaceous green to brown; reverse is dark olivaceous. Submerged hyphae (2–3 µm wide) are subhyaline, smooth, and thin-walled; aerial hyphae are pale brown and slightly narrower. Conidiophores (10–25 µm × 2.5–3.5 µm) are slightly or not differentiated from vegetative hyphae, arising laterally from aerial hyphae, with one or two additional septa, often reduced to a discrete or intercalary conidiogenous cell, pale-brown. Conidiogenous cells (5–15 µm × 3–5 µm) are terminal or intercalary, variable in length, occasionally slightly wider than the basal part, pale brown, rachis (0.5 µm in diameter) with slightly prominent, unpigmented, non-thickened scars. Conidia (5–12 µm × 2–5 µm) are golden-brown, thin walled, smooth, ellipsoidal to obovate, and subcylindrical, with darkened, inconspicously thickened, protuberant or truncate hilum (<1 µm in diameter) [4].

Rhinocladiella similis colonies are restricted, mostly dry or initially with some black slime at the centre, velvety, and olivaceous gray with olivaceous black reverse. Budding cells (5 µm × 3 µm) are abundant, pale olivaceous, broadly ellipsoidal, and without capsule in India ink, often inflating and developing into broadly ellipsoidal brown germinating cells (5 µm × 4 µm) that often bear a clearly discernible truncate extension, which bears a very short annellated zone. Hyphae (1.5 µm wide) are pale olivaceous to brown and regularly septate every 20–40 µm. Conidiogenous cells arise at acute angles in a profusely branched conidial apparatus, which is brown, somewhat darker than the sterile hyphae; conidiogeneous cells are cylindrical, 12–20 × 2 µm apically, with an elongating sympodial part bearing conidia on small denticles mainly at the apices of the cells. Conidia (4–7 × 1.5 µm) are subhyaline, noncatenate, cylindrical, and narrowed toward the base, with a small but clearly visible scar. *Rhinocladiella similis* shows preponderantly sympodial conidiogenesis and possesses a profusely branched conidial apparatus of the same texture and pale-brown pigmentation as its mycelium. This feature is the hallmark of *Rhinocladiella* [9].

The main feature to distinguish *Rhinocladiella* from *Ramichloridium* is the presence of *exophiala*-type budding cells in species of *Rhinocladiella*. Although both *Rhinocladiella* and *Veronaea* produce sympodial conidiogenous cells, *Veronaea* is differentiated from *Rhinocladiella*

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by its absence of *exophiala*-type budding cells and its predominantly one-septate conidia (i.e., two-celled conidia) (in comparison with one-celled conidia of *Rhinocladiella*) [4].

43.1.2 Clinical Features and Pathogenesis

Rhinocladiella mackenziei (formerly *Ramichloridium mackenziei*, *Ramichloridium obovoideum*). *Rhinocladiella mackenziei* to the second most common cause (next is *Cladophialophora bantiana*) of central nervous system (CNS) phaeohyphomycosis due to darkly pigmented fungi. *Rhinocladiella mackenziei* infections cause high mortality and are diagnosed exclusively in patients from the Middle East, with more than one-half of the cases occurring in patients with no known underlying immunodeficiency [10–18].

Podnos et al. [19] reported a case of cerebral *Rhinocladiella mackenziei* (*R. obovoideum*) in a 58-year-old Kuwaiti woman, with a history of chronic renal failure requiring hemodialysis. The patient presented with a 3 day history of left frontal headache, blurry vision, dizziness, and right-sided clumsiness. A computed tomography-guided needle biopsy of the parieto-occipital lesion yielded 10 mL of dark caseous fluid, which contained long, branching, septate hyphae. Culture of the biopsy yielded *Rhinocladiella mackenziei* (*R. obovoideum*). Despite treatment with amphotericin B and itraconazole, the patient succumbed to the infection. In a separate study, Kanj et al. [20] described two cases of brain abscesses caused by *Rhinocladiella mackenziei* (*Ramichloridium mackenziei*) in the Middle East. One patient had chronic myelomonocytic leukemia while the other patient was a normal host. Both cases had a fatal outcome despite aggressive antifungal therapy and surgical intervention. Alkhunaizi et al. [18] also described a *Rhinocladiella mackenziei* (*Ramichloridium mackenziei*)-related CNS infection in a patient who underwent living unrelated renal transplantation (LURTX) and who developed hemiplegia and was debilitated.

Rhinocladiella aquaspersa. *Rhinocladiella aquaspersa* is occasional cause of human chromoblastomycosis, which is characterized by the slow development of polymorphic skin lesions (nodules, verrucas, tumores, plaques, and scar tissue). Inside the host, infectious propagules adhere to epithelial cells and differentiate into sclerotic forms, which effectively resist destruction by host effector cells and allow onset of chronic disease.

Arango et al. [21] documented an unusual case of chromoblastomycosis due to *R. aquaspersa* in a 60-year-old male urban resident. The patient presented with darkly pigmented, infiltrative, and crusty lesion in the ear, and the fungus was recovered in culture and identified as *R. aquaspersa* on the basis of the characteristic sporulation. Itraconazole therapy resulted in complete healing. Marques et al. [22] also reported an unusual case of chromoblastomycosis lesions localized in three different sites in a 52-year-old male farm worker from Brazil. Physical examination showed extensive plaques situated on the left leg, left arm, forehead, and left side of the face. Direct examination of biopsies revealed sclerotic cells. A fungus grew in culture and identified as *R. aquaspersa* on the basis of the characteristic conidiation. Badali et al. [23] reported an *R. aquaspersa*-related case of chromoblastomycosis in a 56-year-old male, who presented with warty nodules and lymphatic distribution on the forearm, resembling sporotrichosis. Mycological and histopathological investigation of exudates and biopsy tissue samples demonstrated a granulomatous lesion with muriform cells, the hallmark of chromoblastomycosis. The causative agent was identified as *Rhinocladiella aquaspersa*. A second case of chromoblastomycosis caused by this fungus involved a 62-year-old Brazilian female. In this case, *R. aquaspersa* hyphae instead of muriform cells were observed in tissue.

Dematiaceous (melaninized) fungi responsible for cerebral phaeohyphomycosis (e.g., *Rhinocladiella mackenziei*) are environmental organisms that may enter human host via inhalation [24–27]. These fungi may induce an initial subclinical pulmonary focus before causing CNS infection through hematogenous route [28,29]. Melanin present in their cell walls not only imparts the characteristic dark color to their conidia and hyphae but also acts as virulence factor that plays an important role in the pathogenesis of infections and possibly in their CNS localization [30]. Receptors recognizing melanin or its biochemical byproducts may allow these fungi to cross the blood–brain barrier and enter the brain parenchyma. Melanin may also confer a protective advantage by scavenging free radicals and hypochlorite that are produced by phagocytic cells in their oxidative burst and that would normally kill most organisms. Additionally, melanin may bind to hydrolytic enzymes, thereby preventing their action on the plasma membrane. Hayakawa et al. [31] showed that complement-mediated phagocytosis of the fungus by macrophages represents an important host defense mechanism against *Rhinocladiella aquaspersa*.

43.1.3 Diagnosis

Because dematiaceous fungi are common soil inhabitants and are often considered as laboratory contaminants, their isolations in culture media provide a preliminary indication of their causal roles in phaeohyphomycosis and chromoblastomycosis. Microscopic observation of moniliform hyphae or irregularly swollen hyphae with yeast-like structures (compared to septate, acutely branching, straight-walled hyphae *Aspergillus* species) or muriform cells in tissue biopsy is critical for confirming the involvement of *Rhinocladiella mackenziei* or *Rhinocladiella aquaspersa* in the disease process.

Rhinocladiella mackenziei (classified under *Eurotiomycetes*), the causative agent for severe cerebral phaeohyphomycosis in humans, may be occasionally confused with *Pleurothecium obovoideum* (classified under *Sordariomycetes*) morphologically. However, *P. obovoideum* clusters with sexual species of *Carpoligna* that have *Pleurothecium* anamorphs.

Because of the lengthy time and specialized skills that are needed for macroscopic and microscopic identification of dematiaceous (melanized) fungi, molecular techniques are increasingly utilized for improved speciation and detection

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of these organisms [32–34]. Spatafora et al. [35] undertook a cladistic analysis of 1050 bp of the genes coding for small-subunit ribosomal RNA (SSU rRNA) to clarify the phylogenetic relationship among species of *Exophiala*, *Fonsecaea*, *Phialophora*, *Ramichloridium*, and *Rhinocladiella*. It was noted that the conventional categories of these fungi based on asexual states are not supported by phylogenetic analysis of SSU rRNA sequences. Isolates exhibiting annellidic modes of blastic conidiogenesis (e.g., *Exophiala* spp.) are not monophyletic and are placed as sister taxa to isolates that produce phialides or sympodulae. The results indicated very close relationships between isolates of *Wangiella dermatitidis* and *Exophiala mansonii* as well as between *Rhinocladiella aquaspersa* and *Exophiala jeanselmei*. The etiological agents of chromoblastomycosis form a closely related group (clade), while the agents of phaeohyphomycosis display a broader distribution on the SSU rRNA tree. In a separate study, Zeng and de Hoog [36] exploited the sequence diversity of the internal transcribed spacer (ITS) region of rRNA for reliable identification of dematiaceous fungi.

43.2 METHODS

43.2.1 Sample Preparation

Clinical specimens are examined directly under microscope with a fungal stain. Portions of the samples are inoculated on Sabouraud glucose agar without or with antibiotics. The resulting isolates are identified on the basis of macroscopical and microscopical features. For easy microscopic observation, slide culture technique may be utilized. 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, approx. 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) [4].

For fungal DNA extraction, 50 mL of Sabouraud dextrose (SAB) broth is inoculated by needle with conidia from a 7 day culture in SAB agar and incubated for 72 h at 30°C. The hyphae are recovered on a 0.45 µm-pore-size filter and washed with sterile saline. Aliquots of the fungal hyphae are stored frozen at –70°C until use. Prior to lysis, the hyphae are thawed and suspended in 400 µL of DNA extraction buffer (1 mM EDTA pH 8.0, 1% sodium dodecyl sulfate, 10 mM Tris–HCl pH 7.6, 100 mM NaCl, and 2% Triton X-100). Microcentrifuge tubes (1.5 mL) containing hyphae and buffer are sonicated in a water bath (Branson; model 2210) for 15 min, followed by heating at 100°C for 5 min. Following lysis, DNA is purified using the QIAmp blood kit (Qiagen) and protocols for crude cell lysates supplied by the manufacturer. The purified DNA is stored at 4°C until tested. Alternatively, DNA is extracted with a FastDNA kit (Qbiogene) from mycelium grown for 3–5 days in liquid complete medium or with the UltraClean™ Microbial DNA Isolation Kit (Mo Bio Laboratories) from mycelium taken from fungal colonies on MEA [37].

43.2.2 Detection Procedures

43.2.2.1 Sequence Analysis of ITS Regions

Arzanlou et al. [4] utilized the universal primers ITS1 and ITS4 to amplify the ITS region of the nuclear ribosomal RNA operon, including the 3′ end of the 18S rRNA gene, the first ITS region (ITS1), the 5.8S rRNA gene, the second ITS region (ITS2), and the 5′ end of 28S rRNA gene. Subsequent sequencing analysis allows identification of fungal organisms including *Chaetomium* species.

Procedure

1. Polymerase chain reaction (PCR) mixture (25 µL) is composed of 0.5 U *Taq* polymerase (Bioline), 1× polymerase chain reaction (PCR) buffer, 0.5 mM $MgCl_2$, 0.2 mM of each dNTP, 5 pmol of each primer, and 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 GenBank 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

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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 large-subunit 28S rRNA (LSU) gene may be also amplified with primers LR0R and LR5 followed by sequencing analysis.

43.2.2.2 Sequence Analysis of SSU rRNA Gene

Spatafora et al. [35] utilized primers NS1 and NS4 to amplify a 1150 bp of the SSU rRNA for phylogenetic analysis of dematiaceous fungi including *Exophiala*, *Fonsecaea*, *Phialophora*, *Ramichloridium*, *Rhinocladiella*, and *Wangiella*.

Procedure

1. Culture is grown in potato dextrose liquid media for 1–2 weeks, depending on growth rates. Isolates are confirmed by microscopic examination. Mycelium is collected, frozen in liquid nitrogen, and stored at −70°C prior to DNA extractions.
2. PCR amplification is conducted with 1 cycle of 94°C for 3 min; 40 cycles of 94°C for 1 min, 53°C for 30 s, and 72°C for 1 min; and 1 cycle of 72°C for 5 min.
3. PCR products are purified by using microcentrifuge ultrafiltration cartridges (UFC3 THK 00; Millipore) and concentrated to a final volume of 25 µL. Purified products are sequenced using primers NS1, NS2, and NS4 and SR11R (5′-GGAGCCTGAGAAACGGCTAC-3′) and SR7R.
4. Sequence alignments are analyzed cladistically with the software package PAUP. Informative characters are analyzed by using the tree bisection–reconnection with random sequence addition option; 25 replications are performed. Support for inferred groups is estimated by the bootstrapping technique. Five hundred bootstrap replications are performed for the informative characters alone, using the tree bisection–reconnection with random sequence addition option.

43.3 CONCLUSION

The genus *Rhinocladiella* currently comprises 12 distinct dematiaceous fungal species that are widely distributed in the environments, among which *Rhinocladiella mackenziei* is an uncommon human pathogen, causing fatal cerebral phaeohyphomycosis in the Middle East [38] while *Rhinocladiella aquaspersa* is often associated with localized chromoblastomycosis lesions in various parts of the world. Due to the morphological resemblances among *Rhinocladiella*, *Veronaea*, *Ramichloridium*, and melanized fungi, identification of *Rhinocladiella* spp. on the basis of macroscopic and microscopic characteristics is time-consuming and technically demanding. The use of molecular procedures such as PCR and sequencing analysis of ribosomal rRNA genes and internal transcribed spacer regions provides a rapid and accurate approach for the discrimination of dematiaceous fungi including *Rhinocladiella* species.

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