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Ramichloridium strelitziae associated with sooty blotch and flyspeck on *Ravenala madagascariensis* in China

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Abstract — The first report of *Ramichloridium strelitziae* from China is documented. In a survey of host plants for SBFS fungi, we isolated the fungus from colonies on stems of *Ravenala madagascariensis* collected from Haikou, Hainan, China. It is distinguished from the other species in the genus by morphological characters and phylogenetic analysis based on ITS sequences.

Key words — microfungi, *Capnodiales*, taxonomy, phylogeny, traveller's palm

Introduction

Sooty blotch and flyspeck (SBFS) are epiphytes that colonize the waxy cuticle of a wide range of plants in humid regions worldwide. SBFS fungi can result in a black or sooty appearance leading to cosmetic damage that can cause significant economic losses (Batzer et al. 2005).

For approximately 70 years, SBFS was viewed as two distinct diseases, each caused by a single species of fungus. Recently, this concept was revised by combining morphological characterization with genetic analysis. Presently, it is evident that SBFS includes a spectrum of intergraded mycelial types and comprises more than 80 species (Batzer et al. 2008, Frank et al. 2010, Gleason et al. 2011, Li et al. 2012, Yang et al. 2010).

In this study, we identified one isolate that was described as the first record of *R. strelitziae* on stems of *Ravenala madagascariensis* Sonn. (*Strelitziaceae*; traveller's palm) from China based on morphological comparison and phylogenetic relationships.

= Joint first authors

Materials & methods

Isolates

Ravenala madagascariensis stems with flyspeck signs (Gleason et al. 2011) were found in Jinniuling Park in Haikou city, Hainan Province (20°00′26″N 110°20′31″E), China, in October 2011. Thalli on the stem were transferred directly from colonies to potato dextrose agar (PDA) slants in a sterile environment and cultured at 25°C for 1 month in darkness (Sun et al. 2003). Hyphal tips were then transferred to malt extract agar (MEA) plates. The isolates were allowed to grow onto an adjacent, sterile cover slip that had been partially inserted into the agar surface at a 60° angle in order to measure and observe fungal structures (Li et al. 2011). Microscopic examination was made after 14 d of incubation. Thirty measurements per relevant microscopic structure were gathered where possible. Colony descriptions (surface and reverse) were made after 2 weeks of growth on MEA plates at 25±1°C in the dark. The isolate acronym HKLRJ-4 was a temporary laboratory number. Representative dried culture and plant specimens were deposited in the Fungal Herbarium of Northwest A&F University (HMUABO), Yangling, Shaanxi Province, China.

DNA extraction, PCR, and phylogenetic analysis

Genomic DNA for polymerase chain reaction (PCR) was obtained according to the protocol of Li et al. (2011). The primer pair ITS1-F and ITS4 was used to amplify the internal transcribed spacer (ITS) region of nuclear ribosomal DNA. The PCR reactions were carried out with Taq polymerase, 1× PCR buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 μM of each primer, and 2 μl of template DNA, and was made up to a total volume of 25 μl with sterile water. Reactions were performed on a Bio-Rad PCR System S1000TM Thermal Cycler. The amplification conditions were: initial denaturation at 94°C for 90 min followed by 35 cycles of denaturation at 94°C for 35 s, annealing at 52°C for 60 s, extension at 72°C for 1 min, and a final extension step at 72°C for 10 min. The PCR products were sequenced by Sangon Biotech Co., Shanghai, China.

The ITS nucleotide sequence generated in this study was added to other sequences with high homology as the result of a BLAST search. *Cladosporium bruhnei* was used as the outgroup taxon. Sequences were imported into BioEdit 5.0.9.1 (Hall 1999). Preliminary alignments were performed using CLUSTAL-X (Thompson et al. 1997), then manually adjusted. Phylogenetic analysis of aligned DNA sequences was carried out with PAUP version 4.0b 10 for 32-bit Microsoft Windows (Swofford 2003). Heuristic searches were performed with 1,000 random sequence additions. Clade stability was assessed by 1,000 bootstrap replications. Other measures calculated for parsimony analysis included tree length, consistency index (CI), retention index (RI), rescaled consistency index (RC) and homoplasy index (HI).

The ITS sequence described in this study is deposited in GenBank as JX502176, and the alignment and representative tree were deposited in TreeBase (http://purl.org/phylo/treebase/phylows/study/TB2:S13803).

Results

Phylogenetic analyses

The ITS alignment contained 29 taxa (including the outgroup) and 520 characters including alignment gaps. Of these characters, 266 were constant,

62 were variable and parsimony-uninformative, and 192 were parsimony-informative. One of the 10 equally most parsimonious trees saved from the maximum parsimony analysis is shown in Fig. 1. From the most parsimonious tree, two major clades were resolved. One clade, with 100% bootstrap support, contained fifteen species in *Ramichloridium* sensu stricto, *Uwebraunia*, *Dissoconium*, and *Pseudoveronaea* (all in *Dissoconiaceae*). The other major clade

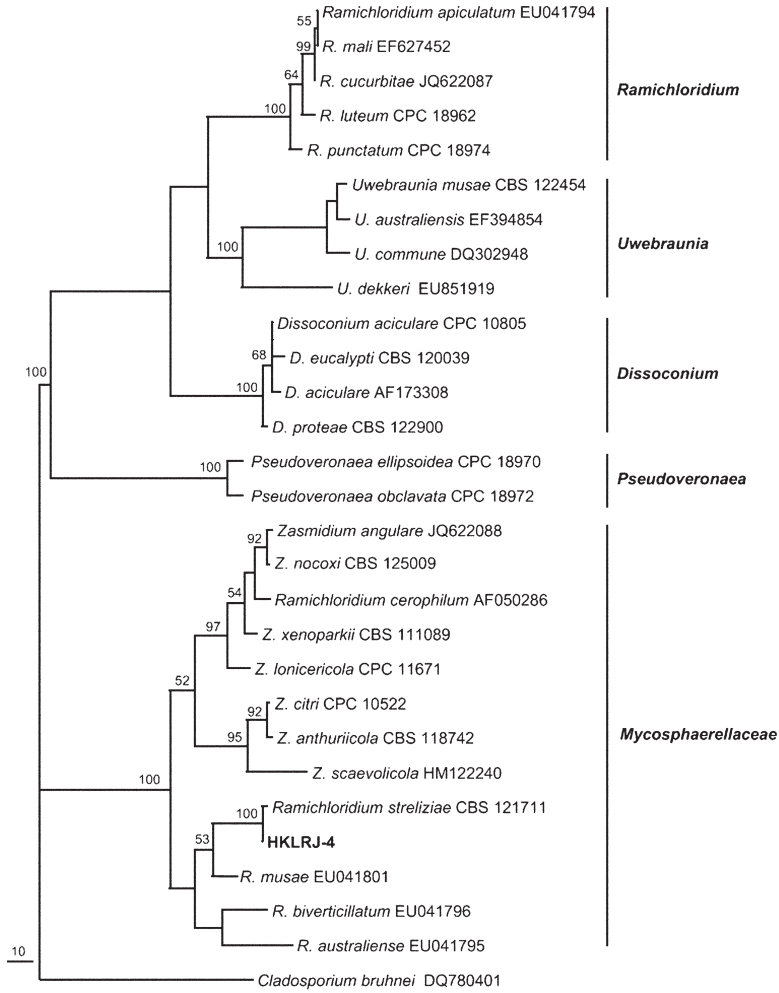

Fig. 1. One of 10 equally parsimonious trees determined from ITS sequences. Bootstrap support values (>50%) based on 1000 replicates are shown at the node. (CI = 0.6611, RI = 0.8657, RC = 0.5723, HI = 0.3389). The scale bar shows 10 changes. The tree is rooted to *Cladosporium bruhnei* and the new sequence is presented in bold.

had a bootstrap value of 100% including twelve species in *Ramichloridium* sensu lato and *Zasmidium* (all in *Mycosphaerellaceae*). Our isolate (HKLRJ-4) grouped with *Ramichloridium strelitziae* (*Mycosphaerellaceae*) with 100% bootstrap support, indicating that they might represent the same species.

Morphology and culture characteristics

Ramichloridium strelitziae Arzanlou, W. Gams & Crous, Stud. Mycol. 58: 74. 2007. Fig. 2

Mycelium consisting of smooth, thin-walled, septate, verrucose, pale brown hyphae, 2–3 µm wide. Conidiophores erect, solitary, arising from creeping aerial hyphae, subhyaline, later becoming pale brown, thick-walled, 1–3(–4)-septate, smooth or verruculose, 2 µm wide, up to 43 µm long. Conidiogenous cells terminally integrated, (8–)11–38 µm long, subhyaline, later turning pale brown, fertile part as wide as the basal part, sympodially proliferating, forming a straight rachis; Scars thickened and darkened, approx. 0.5 µm diam. Conidia (3.5–)4–5 × (1–)2(–3) µm, solitary, aseptate, subhyaline, smooth or verruculose, oblong, ellipsoidal to clavate, base truncate with unthickened, non-pigmented hilum.

Cultural characteristics — Colony diameter after 14 d on MEA at 24°C reached 6 mm diam with entire margin and compact, raised, dense aerial mycelium, surface olivaceous-grey, reverse olivaceous-black.

Host characteristics — On stem surface, shiny, black, sclerotium-like bodies, round to oval (120–220 µm diam) with no visible mycelial mat, densely arranged (3–5/mm^2). The flyspeck symptom on *Ravenala madagascariensis* did not physically damage the plants, but greatly reduced their ornamental value.

Based on phylogenetic analysis and morphological characters of the anamorph, we identified isolate HKLRJ-4 as *Ramichloridium strelitziae*.

Specimen examined: **China. Hainan Province**, Haikou City, Jinniuling Park, 20°00′26″N 110°20′31″E, on stems of *Ravenala madagascariensis*, Oct 2011, Li WH (HMUABO HKLRJ-4; GenBank, JX502176).

Discussion

The modern circumscription of *Ramichloridium* is still heterogenous, containing species in two different families (*Dissoconiaceae* and *Mycosphaerellaceae*), and only species clustering in *Capnodiales* were considered to be true *Ramichloridium* (Arzanlou et al. 2007). According to Li et al. (2012) and Arzanlou et al. (2007), *R. apiculatum* (the generic type), *R. cucurbitae*, *R. indicum*, *R. luteum*, *R. mali*, and *R. punctatum* (*Ramichloridium* s. str.) clustered with *Dissoconium* species (all now placed in *Dissoconiaceae*), and other *Ramichloridium* s. lat. species grouped in *Mycosphaerellaceae*. Our strain clustered among the mycosphaerellacean species with *R. strelitziae*.

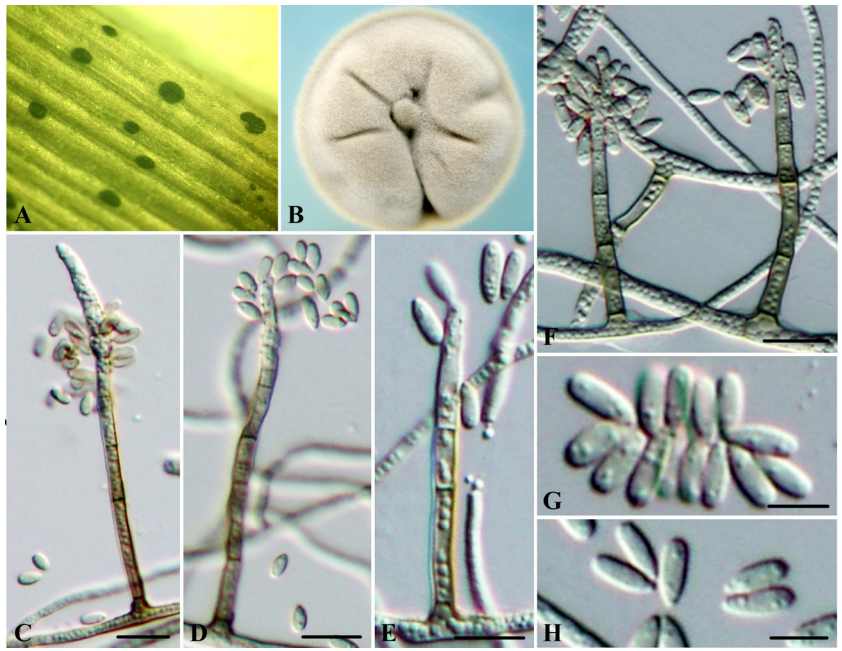

Fig. 2. *Ramichloridium strelitziae* (HKLRJ-4). A. Signs on stem of *Ravenala madagascariensis* with close-up view. B. Colony on MEA after 14 days. C–F. Conidiophores. G–H. Conidia. Bars: C–F = 10μm; G–H = 5μm.

Arzanlou et al. (2007) reported *Ramichloridium strelitziae* from leaves of *Strelitzia nicolai* in South Africa. The species has also been found in bathrooms and washing machines and is able to degrade surfactants, soap, and shampoo (Hamada & Abe 2010). Our results show that *R. strelitziae* may also be involved in the sooty blotch and flyspeck complex.

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