New insights into *Stilbocrea* (*Hypocreales*, *Bionectriaceae*): recognition of *S. colubrensis*, a new species from Martinique (French West Indies), and observations on lifestyle and synnematous asexual morphs of *S. gracilipes* and *S. macrostoma*

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Abstract: *Stilbocrea colubrensis* sp. nov. is described and illustrated based on a collection on *Bambusa vulgaris* in Martinique. The placement of this species in *Stilbocrea* is supported by morphological characters and analysis of ITS and LSU sequences. This species differs from known species of *Stilbocrea* in having ascomata immersed in a prosenchymatous stroma composed of verrucose hyphae. In addition, morphological characteristics of synnemata of *S. gracilipes* and *S. macrostoma* are illustrated and discussed and *S. macrostoma* is shown to have only one type of synnemata.
Keywords: Ascomycota, *Bambusa*, ribosomal DNA, taxonomy.

Résumé : *Stilbocrea colubrensis* sp. nov. est décrite et illustrée d'après une récolte sur *Bambusa vulgaris* en Martinique. Son placement dans le genre *Stilbocrea* s'appuie les caractères morphologiques et l'analyse des séquences ITS et LSU. Cette espèce diffère des espèces de *Stilbocrea* connues par des ascomes immergés dans un stroma prosenchymateux composé d'hyphes verruqueuses. Par ailleurs, les caractères morphologiques des synnemata de *S. gracilipes* et *S. macrostoma* sont illustrés et commentés et il est montré que *S. macrostoma* n'est associé qu'à un seul type de synnemata.
Mots-clés : ADN ribosomal, Ascomycota, *Bambusa*, taxinomie.

Introduction

In the continuity of the research program on the fungal diversity of Lesser Antilles (Courtecuisse, 2006), an intriguing hypocrealean fungus was collected on *Bambusa vulgaris* Schrad. ex J.C. Wendl (*Poaceae*) in Martinique. Based on its pale yellow to pale orange ascomata not changing colour in 3% KOH or lactic acid, this fungus was assigned to *Bionectriaceae* Samuels & Rossman; its generic placement in *Stilbocrea* was suggested by ascomata embedded in a white effused hyphal stroma, verrucose ascospores and synnematous asexual morph. This was confirmed by a phylogenetic analysis of both ITS and LSU sequences (Figs. 1 and 2), showing this fungus in the *Stilbocrea* clade, clearly setting it apart from other species. As our fungus did not match any of the five known *Stilbocrea* species (Rossman *et al.*., 1999; Seifert, 1985; de Beer *et al.*., 2013; Voglmayr & Jaklitsch, 2019), especially in having ascomata embedded in verrucose hyphae, we propose *Stilbocrea colubrensis* as a new species to accommodate it. We document in this paper the morphological and molecular results supporting this new species and we discuss the affinities of *Stilbocrea* with the morphologically similar genus *Protocreopsis* Doi.

In addition, several fresh collections of *S. macrostoma* (Berk. & M.A. Curtis) Höhn., the type species, enabled us to observe, illustrate and discuss the morphology of the two types of synnemata described by Seifert (1985) for this species, and to elucidate its obscure relationship with *S. gracilipes* (Tul. & C. Tul.) Samuels & Seifert.

Materials and methods

The specimen was examined using the method described by Rossman *et al.*. (1999). Microscopic observations and measurements were made in water and the ascospore ornamentation was observed in lactic cotton blue not heated. The holotype specimen is deposited in LIP herbarium (University of Lille) and cultures in the CBS collection of the Westerdijk Fungal Biodiversity Institute (Utrecht, the Netherlands). Cultures of the living specimen were made on PDA (Potato Dextrose Agar) with 5 mg/l of streptomycin in Petri dishes 5 cm diam. A mass of ascospores and asci was removed from a perithecium with a fine needle and placed in a drop of sterile water that was stirred with a needle to distribute the elements on the slide. A part of the drop containing ascospores was placed on PDA using a sterile micropipette, then the Petri dish was incubated at 25 °C. DNA extraction, amplification, and sequencing were performed by ALVALAB (Oviedo, Spain): Total DNA was extracted from pure culture blending a portion of it by using a micropestle in 600 µL CTAB buffer (CTAB 2%, NaCl 1.4 M, EDTA pH 8.0 20 mM, Tris-HCl pH 8.0 100 mM). The resulting mixture was incubated for 15 min at 65 °C. A similar volume of chloroform: isoamylalcohol (24:1) was added and carefully mixed with the samples until their emulsion. It was then centrifuged for 10 min at 13.000 g, and the DNA in the supernatant was precipitated with a volume of isopropanol. After a new centrifugation of 15 min at the same speed, the pellet was washed in 70% cold ethanol, centrifuged again for 2 min and dried. It was finally resuspended in 200 µL ddH_2O. PCR amplification was performed with the primers ITS1F and ITS4 (White *et al.*., 1990; Gardes & Bruns, 1993) for ITS, while LR0R and LR5 (Vilgalys & Hester, 1990) were used to amplify the 28S nLSU region. PCR reactions were performed under a program consisting of a hot start at 95 °C for 5 min, followed by 35 cycles at 94 °C, 54 °C and 72 °C (45, 30 and 45 s respectively) and a final 72 °C step 10 min. PCR products were checked in 1% agarose gels, and positive reactions were sequenced with primer ITS4. Chromatograms were checked searching for putative reading errors, and these were corrected.

Analyses were performed online at www.phylogeny.lirmm.fr (Dereeper *et al.*., 2008). Maximum likelihood phylogenetic analyses were performed with PhyML 3.0 aLRT (Zwickl, 2006), using the GTR + I + Γ model of evolution. Branch support was assessed using the non-parametric version of the approximate likelihood-ratio test, implemented in PhyML SH-aLRT (Anisimova & Gascuel, 2006).

Specimens of *S. gracilipes* and *S. macrostoma* examined during this study: French West Indies, Martinique, Le Prêcheur, Anse Couleuvre, on a dead, unidentified, dicotyledonous twig, mixed with *S. macrostoma*, 30 Jul. 2016, *leg*. C. Lechat CLLM16011 (LIP); *ibid*. CLLM16015 (LIP). French Guiana, Saül, Gros Arbres trail, both *S. macrostoma* and *S. gracilipes* on a dead unidentified twig, 24 Aug. 2018, *leg*. C. Lechat CLLG18033 (LIP); *ibid*., *S. gracilipes* on bark of unidentified tree, 25 Aug. 2018, *leg*. C. Lechat CLLG18044-b (LIP); *ibid*., *S. macrostoma* on a dead unidentified twig, 26 Aug. 2018, *leg*. C. Lechat CLLG18056 (LIP).

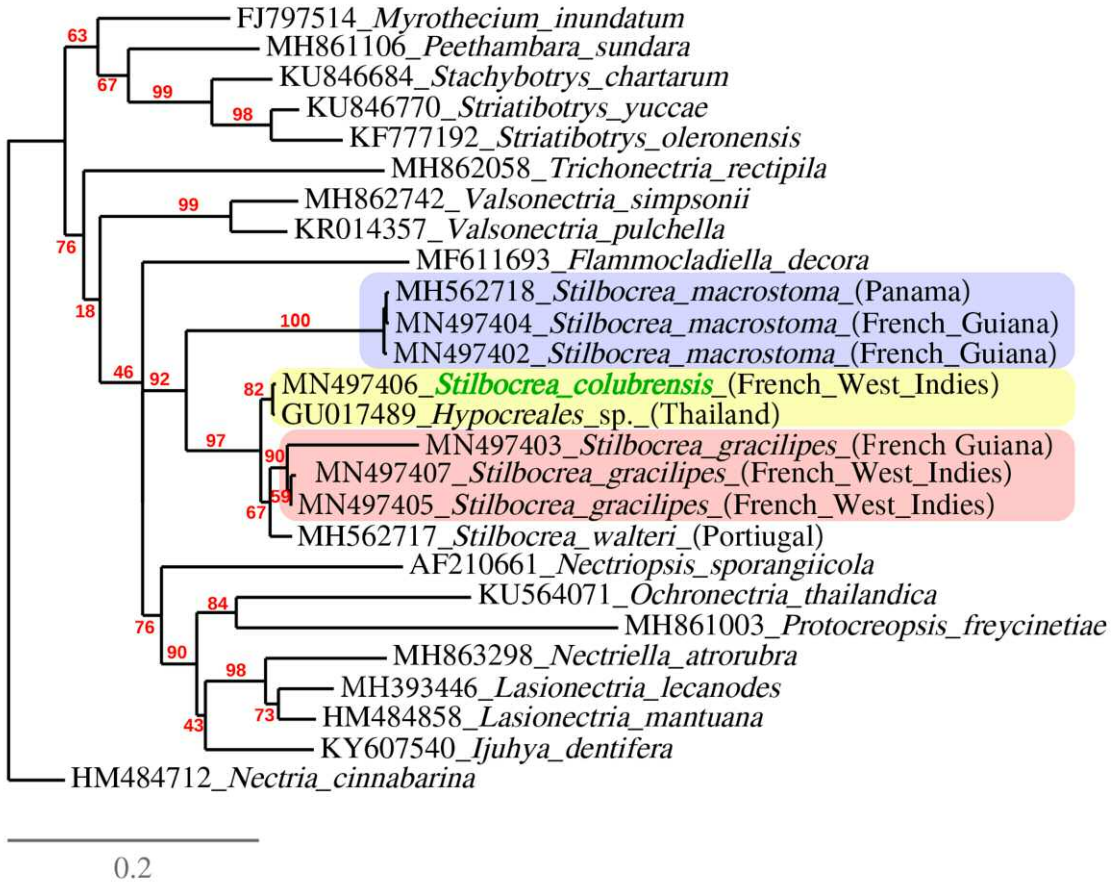

Fig. 1 – Maximum likelihood phylogeny (-lnL = 4787.19560) of *Stilbocrea colubrensis* within *Bionectriaceae* inferred by PhyML 3.0, model HKY85 from a 680 bp matrix, based on ITS sequences, rooted with *Nectria cinnabarina*.

Taxonomy:

Stilbocrea colubrensis Lechat & J. Fourn., *sp. nov.* Fig. 3
Mycobank: MB832695

Diagnosis: Differs from known species of *Stilbocrea* in having ascomata immersed in a hyphal stroma composed of verrucose hyphae and by its occurrence on bamboo.

Holotype: French West Indies, Martinique, Le Prêcheur, Anse Couleuvre, coastal mesophilic rainforest, on a dead culm of *Bambusa vulgaris* fallen across the river Couleuvre, 28 Jul. 2016, *leg*. C. Lechat and J. Fournier, CLLM16003 (LIP); ex-type culture CBS141857; GenBank ITS and LSU sequences: MN497406 and MN497409.

Etymology: The epithet "*colubrensis*" derives from the Latin name "*colubra*", meaning grass-snake, and referring to the river Couleuvre and to Anse Couleuvre, the locality where this species was collected.

Ascomata in groups of (3–)5–20, immersed in cottony mycelium, globose, (250–)270–300(–330) µm diam, pale yellow to pale orange, not changing colour in 3% KOH or lactic acid, collapsing cupulate when dry with orange papilla visible between white hyphal elements of mycelium. **Mycelium** surrounding ascomata white, composed of branched, septate, thick-walled, verrucose hyphae 2–3 µm wide, of indefinite length, with wall 1–1.5 µm thick, coiled and rounded at free ends. **Ascomatal wall** 30–35(–40) µm thick, composed of two regions: outer region 20–25 µm wide, of globose to ellipsoidal cells 5–8 × 3–5 µm, with pale yellow walls 1–1.5 µm thick; inner region 10–15 µm wide, of elongate, flattened cells 5–12 × 2–3 µm with hyaline walls 0.5–1.5 µm thick. **Asci** (80–)85–90(–95) × (8–)9–12.5(–15) µm (Me = 87 × 11 µm, n=20), short stipitate, subcylindrical to narrowly clavate, without ring, with eight obliquely uniseriate ascospores. **Ascospores** (11–)12–13.5(–14) × (3.5–)4–5(–5.5) µm (Me = 13 × 4.5 µm, n = 30), ellipsoidal to widely fusiform, hyaline, equally 1-septate, septum thin and often inconspicuous, not constricted, verrucose. **Synnemata** on natural substrate sparse, white, up to 1500 µm tall, slender, unbranched, 50–80 µm diam., bulbous at base, emerging from mycelium, with pale yellow swollen head, producing narrowly ellipsoidal, smooth, hyaline conidia 6–8(–9) × 2–2.5 µm.

Cultural characteristics: Colony after two weeks on PDA, 38–45 mm diam, pale salmon, slimy in center, pale cream to white at margin, producing acremonium-like sporodochia; reverse pale yellow. Floccose aerial mycelium radiating from middle area to margin, composed of smooth, branched, septate, thin-walled hyphae 2–3.5 µm wide, rounded at free ends. Conidiophores arising from aerial hyphae, macronematous, mononematous, hyaline, smooth, 10–18(–20) long, 2–2.5 µm diam. Conidiogenous cells monophialidic, terminal, cylindrical, 8–14 µm long, 1.5–2.5 µm wide with a minute, not flared collarette. Conidia grouped at tip of phialides to form a mucous head, aseptate, narrowly ellipsoidal to subcylindrical with rounded ends, without abscission scar, smooth, hyaline, 4–7(–8) × 2–2.5 µm (Me = 6 × 2.3 µm, n = 30). No synnemata produced in culture after six weeks.

Discussion

Stilbocrea colubrensis is characterized by pale yellow to pale orange ascomata embedded in cottony mycelium composed of white, verrucose hyphae, verrucose ascospores and a synnematous asexual morph (Fig. 3). It meets the key feature of the *Bionectriaceae* by hav-

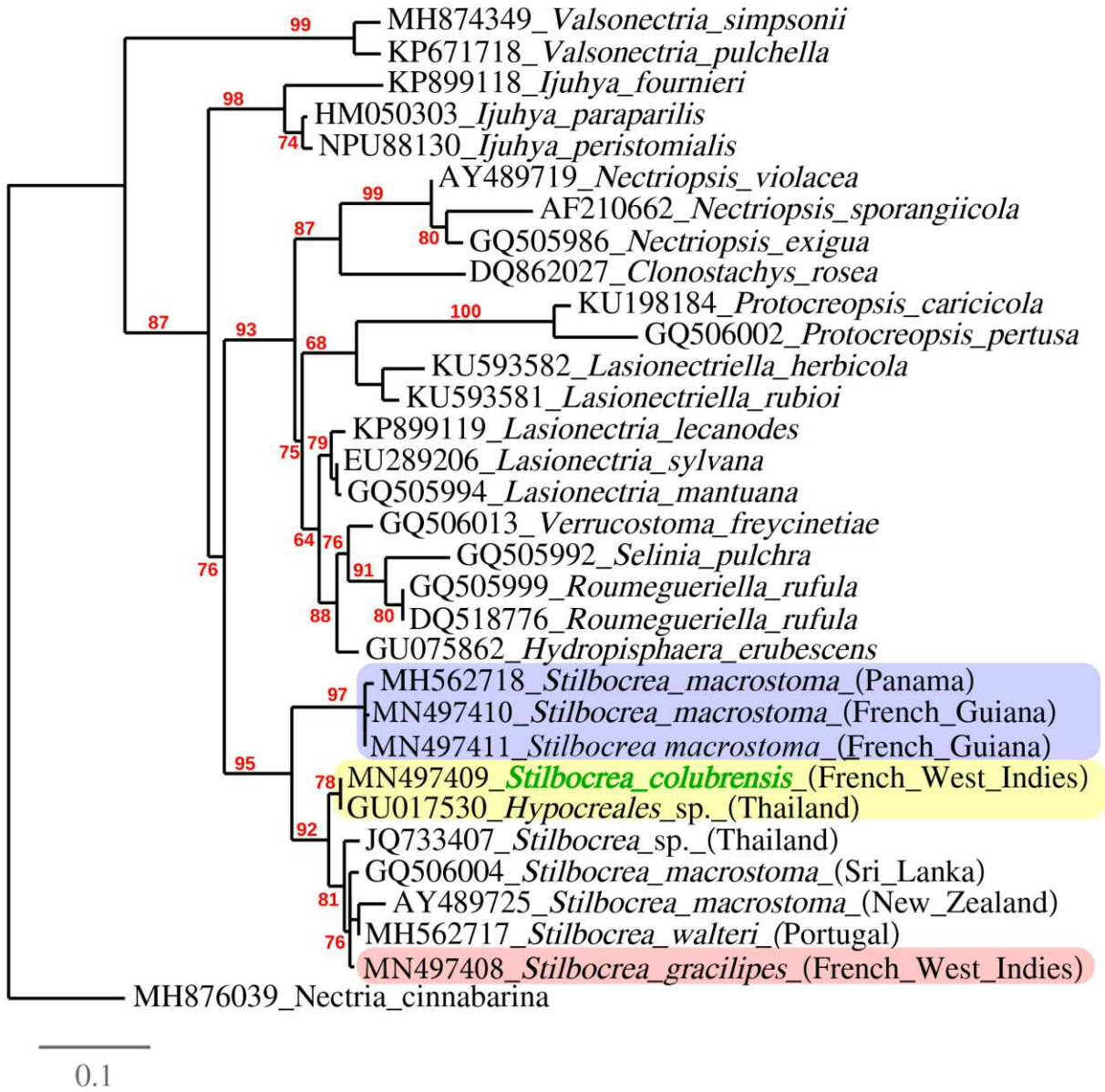

Fig. 2 – Maximum likelihood phylogeny (-lnL = 2883.13802) of *Stilbocrea colubrensis* within *Bionectriaceae* inferred by PhyML 3.0, model HKY85 from a 835 bp matrix, based on LSU sequences, rooted with *Nectria cinnabarina*.

ing pallid ascomata not changing colour in 3% KOH or lactic acid (Rossman *et al.*., 1999). Known bionectriaceous genera featuring gregarious ascomata embedded in a hyphal stroma are *Protocreopsis* and *Stilbocrea*.

Overall morphology of *Protocreopsis* is similar to that of *Stilbocrea* but the former genus is distinguished by lacking synnematous asexual morph, habitat on monocotyledonous leaves or debris and mostly striate ascospores, while the latter has synnemata of the asexual morph present on the stroma and/or on the substrate, habitat on woody substrates or decaying ascomycetous stromata, and verrucose ascospores (Rossman *et al.*., 1999; Lechat & Fournier, 2015). However, a single species currently placed in *Stilbocrea*, *S. impressa* (Mont.) Samuels, is known to lack a synnematous asexual morph and to have an acremonium-like asexual morph in culture (Rossman *et al.*., 1999). This feature shared with *Protocreopsis* makes the boundaries between both genera ill-defined when considered on a morphological basis only. By lack of available ITS or LSU sequences, *S. impressa* was not included in our phylogenetic analysis and its status remains unresolved.

Although different from *Protocreopsis* and *Stilbocrea* by immersed-erumpent stromata, the genus *Valsonectria* Speg. was likewise considered because some species share with them pallid ascomata immersed in a pseudoparenchymatous or prosenchymatous stroma and an acremonium-like asexual morph (Rossman *et al.*., 1999). *Valsonectria simpsonii* Samuels & Seifert (Samuels & Seifert, 1997), known from France on living branch of *Eleagnus pungens*, is particularly reminiscent of *Stilbocrea* in having a synnematous asexual morph in nature and in culture, evolving to acremonium-like in degenerated cultures. In their discussion, Samuels & Seifert (1997) underlined the close morphological similarity of *V. simpsonii* with *S. macrostoma* (as *Nectria macrostoma* Berk. & M.A. Curtis). However, *V. simpsonii*, which is the *Valsonectria* species most closely resembling *S. colubrensis*, is different in having smooth, wider, thick-walled stromatal hyphae 5–6 µm wide, thinner ascomatal wall 15 µm thick and more narrowly ellipsoid, coarsely striate ascospores 10.5–13 × 3.5–4.5 µm. Moreover, *V. simpsonii* clusters with *V. pulchella* Speg. in a separate clade distant from *Stilbocrea* (Figs. 1 and 2).

The placement of our fungus in *Stilbocrea* is well supported by our phylogenetic analyses of ITS and LSU sequences. The combination of its morphological characters sets it apart from the other known species of *Stilbocrea*, the most similar species being *S. macrostoma*, from which *S. colubrensis* mainly differs by roughened stromatal hyphae. The segregation of this new species is confirmed by our ITS and LSU phylogenetic analyses showing respectively 85.9 and 97.5% similarity between both species. Surprisingly, *S. colubrensis* has 100% similarity with an unidentified endophytic isolate (as *Hypocreales* sp.) from Thailand (Sakayaroj *et al.*., 2010), which suggests a pantropical distribution for this fungus.

The two most widespread *Stilbocrea* species in the tropics are *S. gracilipes* and *S. macrostoma*; these can be readily distinguished by the colour of their stromata and synnemata, and by the degree of immersion of ascomata in the stroma (Seifert, 1985; Rossman *et al.*., 1999). Based on a well-documented study describing the asexual

Table 1 – Comparative table of synnemata and conidia characteristics of *S. gracilipes* and *S. macrostoma*, according to Seifert (1985) and data recorded in the present study

	Synnemata	Conidia shape		Conidia dimensions µm	
		Seifert (1985)	this study	Seifert (1985)	this study
Stilbocrea gracilipes	grey, grey brown to blackish, granulose in upper part	ellipsoid to ovoid, moderately thick-walled	ellipsoid to subcylindrical moderately thick-walled	4–7(–8) × 2–3(–3.5) in nature	4–8(–9) × 2.5–3 in nature
				3–6 × 1.5–2.5 in culture	4.5–7 × 1.5–2 in culture
Stilbocrea macrostoma	white, orange with pink tones, smooth	ellipsoid to subcylindrical thin-walled	ellipsoid to subcylindrical thin-walled	3–6(–13) × 1–2(–2.5) in nature	3–8(–10) × 2–2.5 in nature
				5–6 × 1.2–2 in culture	4–7 × 1.5–2.5 in culture

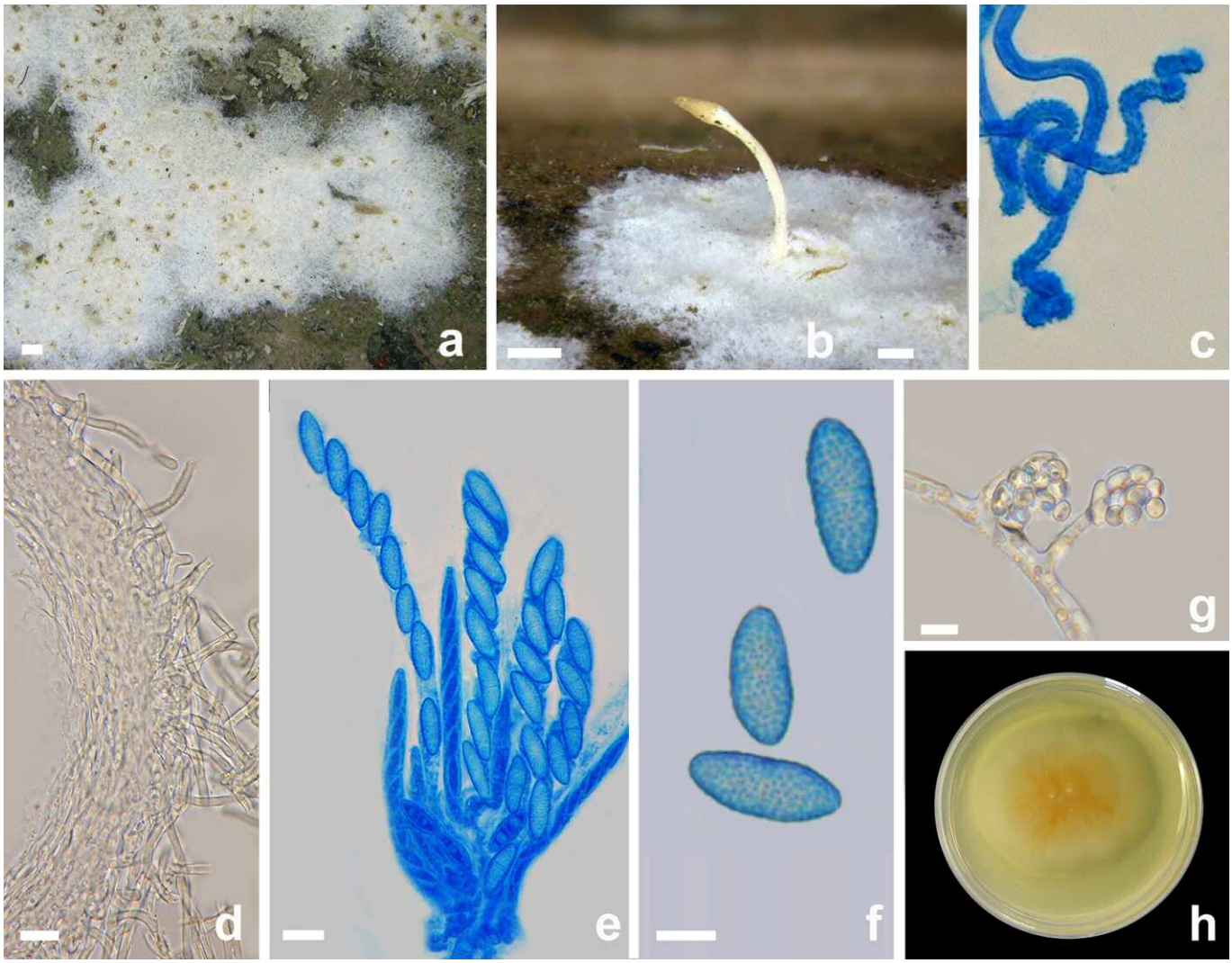

Fig. 3 – a-h: *Stilbocrea colubrensis* (Holotype CLLM16003); a: Ascomata on the substrate; b: Synnema in natural environment; c: Mycelial, verrucose hyphae surrounding ascomata, in lactic cotton blue; d: Lateral ascomatal wall in vertical section; e: Asci and ascospores in lactic acid cotton blue; f: Close-up of ascospores in lactic cotton blue showing a verrucose wall; g: Conidiophores and conidia from culture; h: Culture at two weeks. Scale bars: a, b = 500 µm; c, f, g = 5 µm; d, e = 10 µm.

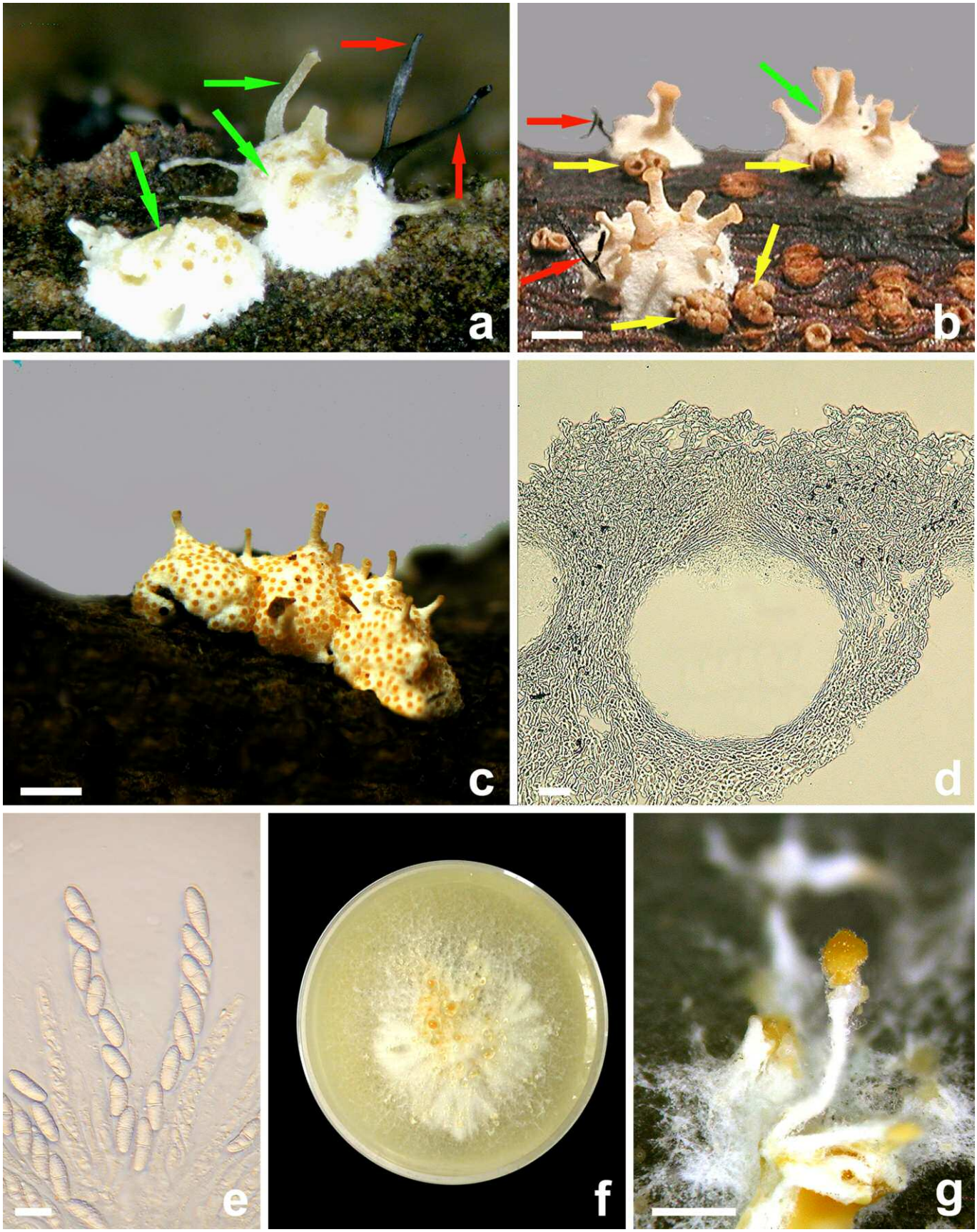

Fig. 4 – a–g: *Stilbocrea macrostoma*; a, b: Young stromata and synnemata (green arrows) developing on synnemata (red arrows) and ascomata (yellow arrows) of S. gracilipes; c: Mature stromata in natural environment; d: Vertical section through perithecium and prosenchymatous stroma; e: Asci and ascospores in water; f: Culture at three weeks; g: Synnemata from culture. Scale bars: a–c, g = 1 mm; d = 20 µm; e = 10 µm.

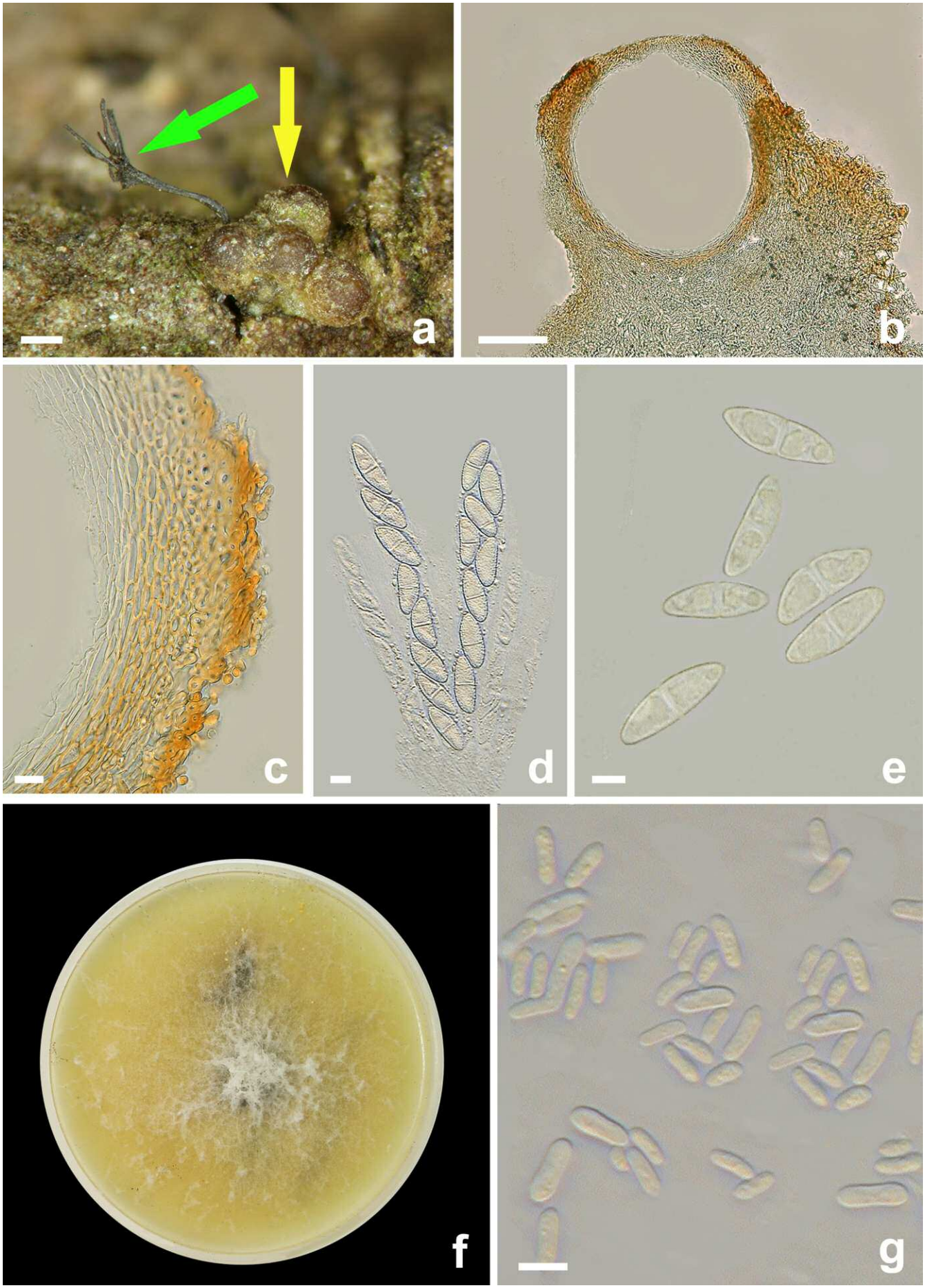

Fig. 5 – a–g: *Stilbocrea gracilipes*; a: Ascomata (yellow arrow) and synnemata (green arrow) in natural environment; b: Vertical section through ascoma and prosenchymatous stroma; c: Vertical section through lateral ascomatal wall; d: Asci and ascospores in water; e: Ascospores appearing smooth in KOH; f: Culture at three weeks; g: Conidia of acremonium-like asexual morph from culture. Scale bars: a = 200 µm; b = 100 µm; c = 10 µm; d, e, g = 5 µm.

Key to known species of *Stilbocrea*

1– Only known as asexual morph, with globose to subglobose conidia ..... ***S. aterrima***
1– Holomorphic species; asexual morph with ellipsoid, oblong or allantoid conidia **2**
2– Stromatal wall changing colour in KOH and LA ..... ***S. walteri***
2– Stromatal wall not changing colour in KOH and LA **3**
3– Synnemata absent; ascospores 15–22 × 7– 10 µm ..... ***S. impressa***
3– Synnemata present; ascospores smaller, not over 15 µm long and 6 µm wide **4**
4– Stromata dark grey to reddish brown, with ascomata strongly exposed to superficial ..... ***S. gracilipes***
4– Stromata white, orange or grey, with ascomata fully immersed **5**
5– Stromata white, orange or grey, compact, composed of smooth hyphae; on dicot hosts or fungicolous ..... ***S. macrostoma***
5– Stromata white, cottony, composed of loose, verrucose hyphae; on bamboo ..... ***S. colubrensis***

and sexual morphs of *S. macrostoma*, Seifert (1985) noticed the presence of two different types of synnemata associated in nature with the stromata: A-synnemata white, orange or orange-pink and B-synnemata grey, black or grey brown, producing slightly different conidia (Table 1). He also pointed out that B-synnemata resemble those of *S. gracilipes* and that in culture B-synnemata may appear associated with colonies of *S. macrostoma* after 4–6 weeks, which was interpreted as proof supporting the presence of two different types of synnemata associated with *S. macrostoma*.

Examination of six collections of fresh material revealed that these two types of synnemata represent two different species of *Stilbocrea*, as already noticed by Guu *et al.*. (2010). According to our observations, white to orange-pink synnemata are associated with *S. macrostoma*, while black or greyish brown synnemata belong to *S. gracilipes*, as proved by morphology of conidia from nature and culture (Table 1) as well as phylogenetic analyses of ITS and LSU sequences from culture (Figs. 1 and 2). Both species are almost always found growing together, with stromata of *S. macrostoma*, partially to totally spreading over those of *S. gracilipes* (Fig. 4), in accordance with its frequently reported fungicolous lifestyle. When the stromata of the latter are completely overlain by *S. macrostoma*, only the black synnemata remain visible and can then be mistaken for a second type of synnemata associated with *S. macrostoma*. In this context, the presence of black synnemata occurring in cultures of *S. macrostoma* can be viewed as a contamination by ascospores or conidia of *S. gracilipes* due to their closely linked ways of growing; this could also explain the surprising placement of two specimens of *S. macrostoma*: GQ506004 and AY489725 from Sri Lanka and New Zealand respectively, in the *S. gracilipes* clade as showed in our phylogenetic analysis of LSU sequences (Fig. 2). These particular lifestyles and growth patterns have been confusing in the past, but obviously *S. macrostoma* possesses a single type of synnemata.

Based on multigene phylogenetic analysis, Voglmayr & Jaklitsch (2019) recently expanded the definition of *Stilbocrea* by introducing *S. walteri* Voglmayr & Jaklitsch, a corticolous species from Portugal. This fungus resembles *S. gracilipes* in having dark-coloured stromata with exposed ascomatal contours, but deviates from typical *Stilbocrea* species in lacking synnemata and in that its medium peridial layer, composed of dark olive green cells, turns red brown in KOH and olivaceous to umber brown in LA. The ascomatal wall reaction with KOH and LA is typical for *Nectriaceae* and is supposedly absent in *Bionectriaceae*, clearly setting *S. walteri* apart from its relatives. However, it should be noted that the typical KOH reaction in *Nectriaceae* is red to purple and that reaction to LA is yellow (Rossman *et al.*., 1999), which supports the placement of *S. walteri* outside the *Nectriaceae* and rather shows it as an atypical *Bionectriaceae*. Their multigene phylogeny shows that strains deposited as *S. macrostoma* do not form one well-supported clade, which suggests the possibility of cryptic or misidentified species and the necessity of further investigations.

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