Three new holomorphic species of *Volutella* (*Nectriaceae*, *Hypocreales*) from Saül (French Guiana)

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Abstract: Three new species of *Volutella* are described and illustrated based on specimens collected in French Guiana. These species are placed in *Volutella* based on morphological characteristics of the sexual morph, asexual morph obtained in culture and phylogenetic comparison of ITS and LSU sequences with known species of *Volutella*, leading us to propose *V. minutissima*, *V. saulensis* and *V. thonneliana* as new species.
Keywords: *Ascomycota*, ribosomal DNA, taxonomy.

Résumé : trois nouvelles espèces de *Volutella* sont décrites et illustrées à partir de spécimens récoltés en Guyane française. Ces espèces sont placées dans le genre *Volutella* sur la base des caractéristiques morphologiques des formes sexuées, des formes asexuées obtenues en cultures, ainsi que de la comparaison phylogénétique des séquences ITS et LSU avec celles d'espèces connues de *Volutella*, ce qui nous amène à proposer *V. minutissima*, *V. saulensis* et *V. thonneliana* comme nouvelles espèces.
Mots-clés : ADN ribosomal, *Ascomycota*, taxinomie.

Introduction

During a collection trip to French Guiana, within the framework of the Atlas de la Biodiversité Communale (ABC) de Saül, three hypocrealean fungi were collected, two on bark of *Sterculia pruriens* (Aubl.) K. Schum. and one on dead leaves of palm. The three species were successfully cultured and produced a volutella-like asexual morph. These cultures were sequenced. Phylogenetic analyses (Fig. 1) of combined ITS and LSU sequences compared to those of known species with a volutella-like asexual morph (Table 1) among the genera *Volutella* Fr., *Coccinonectria* L. Lombard & Crous and *Pseudonectria* Seaver, supported their placement in *Volutella*.

Materials and methods

Dry specimens were rehydrated and 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 unheated lactic cotton blue. The holotypes are deposited in LIP herbarium (University of Lille, France), ex-type cultures are deposited at CIRM-CF (Centre International des Ressources Microbiennes, Marseille, France). Cultures of living specimens were made on PDA (Potato Dextrose Agar) with 5 mg/l of streptomycin in Petri dishes 5 cm diam. incubated at 25°C. DNA extraction, amplification, and sequencing were performed by ALVALAB (Oviedo, Spain): Total DNA was extracted from pure cultures blending a portion of mycelium 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 pellets were washed in 70% cold ethanol, centrifuged again for 2 min and dried. They were 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.

The protocol used at CIRM for the extraction and amplification of LSU marker of *V. saulensis* was as follows: After growth of cultures on PDA medium at 25°C for 7–10 days, genomic DNA was extracted from a portion of fresh mycelium using the Nucleospin plan II kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. For the cell lysis step, the mycelium was fragmented

Table 1 – Genera, species and GenBank accession numbers of sequences used in the phylogenetic analyses. In bold: sequences generated for this study.

Species	ITS	LSU	Species	ITS	LSU
Chaetopsina fulva	NR_145061	MH869087	*Volutella citrinella*	MK357063	HQ843772
Chaetopsina pinicola	NR_137823	NG_058865	*Volutella consors*	JQ693162	JF937571
Clonostachys pityrodes	MH864280	MH875729	*Volutella consors*	HM008927	MH878487
Coccinonectria pachysandricola	KM231775	MH876441	*Volutella delonicis*	NR_171101	NG_073864
Coccinonectria rusci	KM231773	MH875479	*Volutella lini*	JQ693169	–
Pseudonectria buxi	KT225535	MH877719	*Volutella lini*	JQ647452	–
Pseudonectria foliicola	NR_164229	MW465903	*Volutella minutissima*	**ON209633**	**ON209644**
Volutella aeria	KU746708	KU746753	*Volutella ramkumari*	JQ647453	–
Volutella aeria	MZ400595	KU746754	*Volutella saulensis*	**ON453969**	**ON453967**
Volutella ciliata	JQ693166	MH875955	*Volutella thailandensis*	MH388368	MH376742
Volutella ciliata	MH855701	MH867220	*Volutella thonneliana*	**ON181663**	**ON181657**

using FastPrep-24™ 5G Benchtop Homogenizer in a lysing Matrix A tube containing the lysis buffer PL1 and RNAse. The sample thus obtained was purified following the Nucleospin plant II protocol (steps 3 to 7). ITS5 and ITS4 primers (White *et al.*, 1990; Gardes & Bruns, 1993) were used for PCR amplification of the ITS1-5.8S rRNA-ITS2 gene and sequencing reaction and LR5 – LR0R (Vilgalys & Hester, 1990) for the nuclear large subunit, using High Fidelity PCR master mix and primers (0.3 µM) (Roche, France). DNA amplification was then performed in a Mastercycler Nexus GSX1 (Eppendorf, Montesson, France) using the following sequence: 1 cycle at 94°C for 2 min; 10 cycles of 94°C for 30 s/55°C for 90 s/72°C for 1 min; 20 cycles of 94°C for 90 s/ 51°C for 90 s/72°C for 1 min; then 1 cycle at 72°C for 7 min. The PCR products were checked on FlashGel™ DNA System (Lonza, Schwitzerland), and sequenced by GENEWIZ (Leipzig, Germany). Chromatograms were checked searching for putative reading errors, and these were manually corrected.

Phylogenetic 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).

Nomenclature follows MycoBank (Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands).

Taxonomy

Volutella minutissima Lechat & J. Fourn., *sp. nov.* Fig. 2
Mycobank : MB844466

Diagnosis: Differs in having glabrous ascomata the smallest of the known *Volutella* species 150–170 µm high, 120–150 µm wide, and synnematal sporodochia.

Holotype: French Guiana, Saül, Roche-Bateau trail, 3.62056° N, -53.199899° E, on dead bark of *Sterculia pruriens* (Aubl.) K. Schum., 30 Mar. 2021, *leg*. C. Lechat, LIP CLLG21106, ex-holotype culture: BRFM3396; Genbank sequences: ITS = ON209633, LSU = ON209644

Etymology: the epithet *minutissima* refers to the minute ascomata.

Ascomata scattered on host or in groups of 2–8, minute, superficial with base slightly immersed in substratum, obpyriform, 150–170 µm high, 120–150 µm wide (Me = 170 × 135 µm, n = 8), reddish orange to red, smooth, glabrous, not collapsing when dry, with reddish brown to nearly black, rounded, shiny apex, not changing colour in 3% KOH, turning yellow in lactic acid. **Apex** 60–80 µm diam at base, made of tightly aggregated cylindrical orange cells 18–30 × 2–3 µm, with dark brown wall, becoming hyaline at tip, 1–2-septate. **Ascomatal surface** composed of cells of undefined shape, forming a *textura epidermoidea*. **Ascomatal wall** in vertical section of a single region 12–15 µm thick, composed of globose to ellipsoidal, thick-walled cells, with brownish orange wall, becoming flattened, hyaline towards interior. **Asci** narrowly clavate, short-stipitate, 38–46 × 5–7 µm, apex simple, with eight ascospores irregularly biseriate in upper part and uniseriate below. **Paraphyses** moniliform, up to 10 µm wide at base, inserted between asci. **Ascospores** long ellipsoidal, (8–)9(–10) × 2.4–2.8 µm (Me = 9 × 2.6 µm, n = 30), 1-septate, smooth-walled, hyaline.

Culture characteristics: After 10 days at 25°C on Difco PDA: colonies 4–4.5 cm, white to pale yellow, producing white synnemata bearing a pale yellow to pale orange conidial mass, surrounded by long, hyaline setae. Synnemata 200–400 µm high, 60–80 µm diam., made of hyphal, parallel, septate elements 2–3 µm wide. Setae arising from hyphal elements of stipe 400–800 µm long, 3–5 µm diam., thick-walled, with wall 1–1.5 µm thick, tapering to a round tip, multiseptate, hyaline. Conidiophores 28–45 × 3–3.5 µm, hyaline, branched, ultimate branches bearing subulate phialides 16–25 × 2–3 µm with a flared collarette. Conidia ellipsoidal 4.5–6(–7) × 2.5–2.8(–3) µm, non-septate, smooth, hyaline.

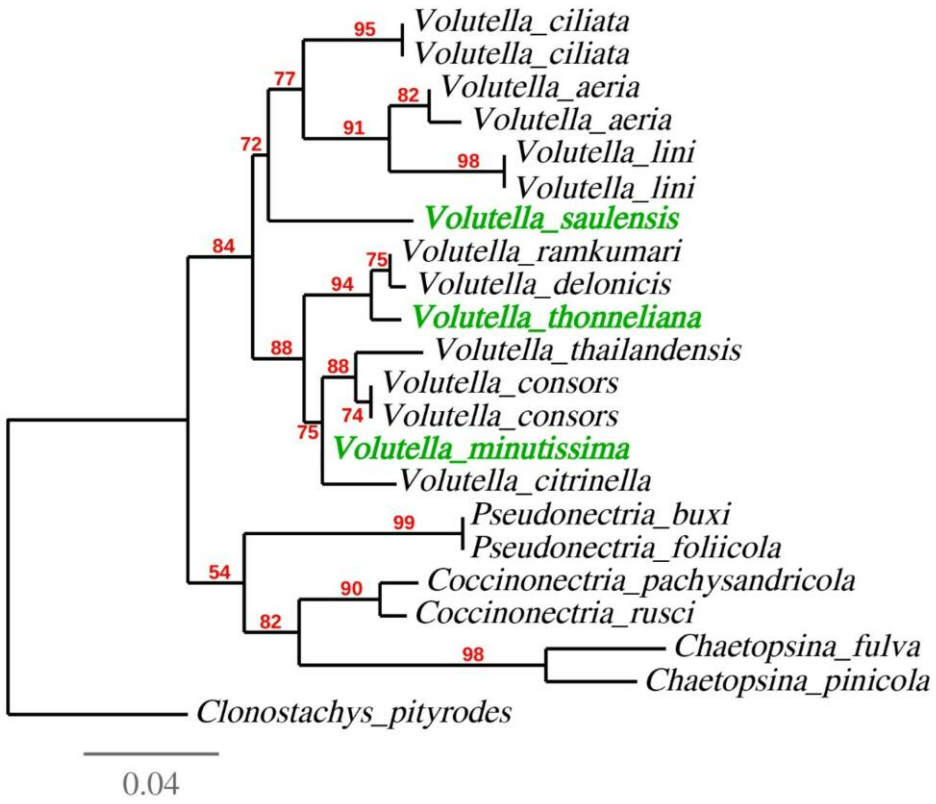

Fig. 1 – Maximum likelihood phylogeny (-lnL = 1296.91788) inferred from combined ITS + LSU gene sequences of *Volutella* species, rooted with *Clonostachys pityrodes* Schroers (*Bionectriaceae*).

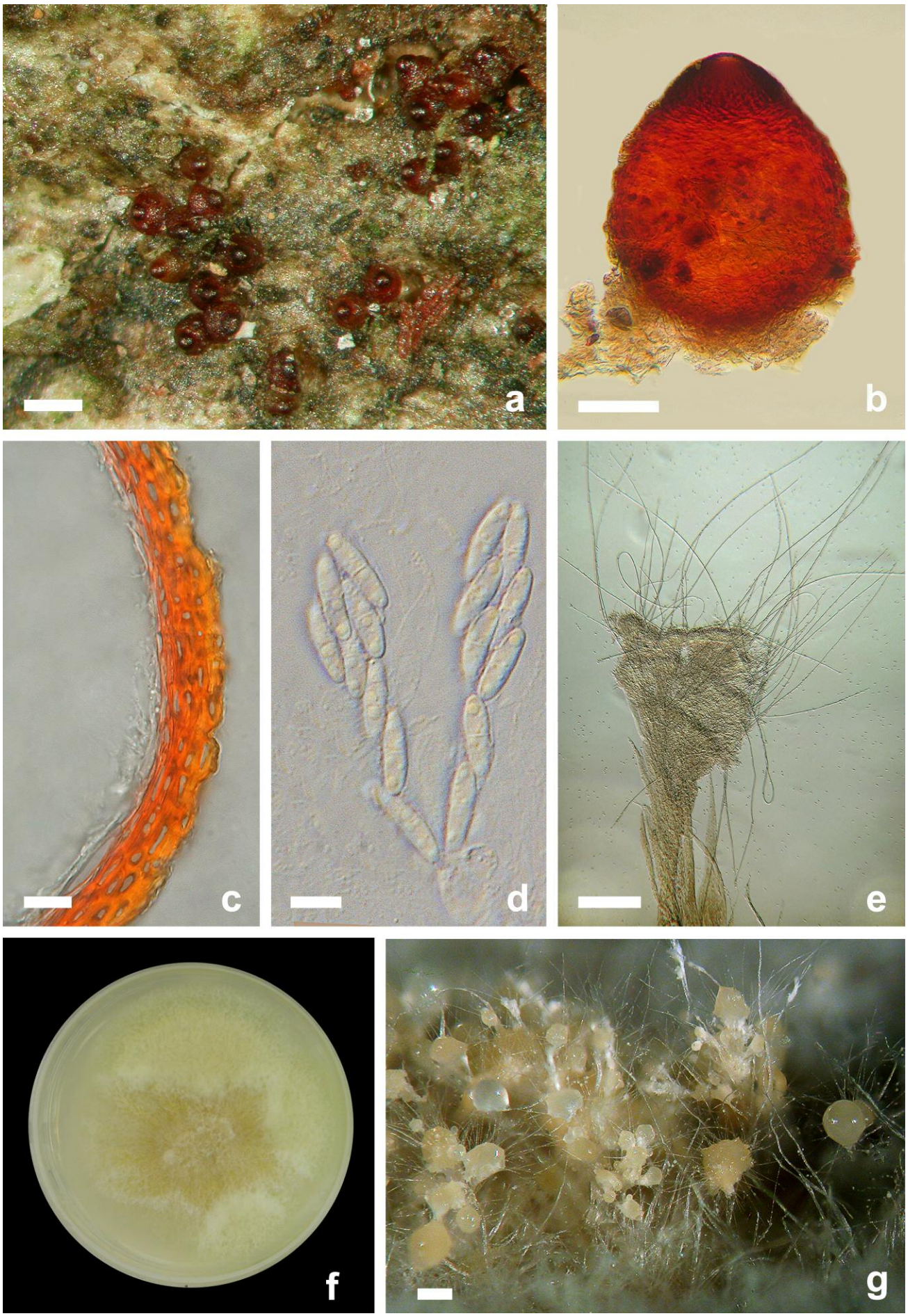

Fig. 2 – a-g: *Volutella minutissima* (Holotype LIP CLLG21106). a: Dry ascomata on the substrate. b: Close-up of ascoma in side view in water. c: Vertical section through the lateral ascomatal wall. d: Asci and ascospores in water. e: Synnematal sporodochium from culture, in water. f: Culture at three weeks. Synnemata from culture. Scale bars: a, g = 200 µm; b = 50 µm; c = 10 µm; d = 5 µm; e = 100 µm.

Volutella saulensis Lechat & J. Fourn., *sp. nov.* Fig. 3
Mycobank: MB 844492

Diagnosis: Differs from known perithecial *Volutella* species by having agglutinated hairs arranged in a crown of triangular fascicles on the upper third of ascomata, and verrucose ascospores.

Holotype: French Guiana, Saül, Roche-Bateau trail, 3.62056° N, -53.199899° E, on dead bark of *Sterculia pruriens* (Aubl.) K. Schum., 30 Mar. 2021, *leg*. C. Lechat, LIP CLLG21099-d, ex-holotype culture: BRFM3418; Genbank sequences: ITS = ON453969, LSU = ON453967.

Etymology: the epithet *saulensis* refers to Saül, the locality where this fungus was collected.

Ascomata solitary, sparse, scattered on substrate, non-stromatic, globose to subglobose, 240–270 µm high, 230–250 µm wide (Me = 255 × 240 µm, n = 10), not collapsing when dry, not changing colour in 3% KOH, turning yellow in lactic acid. **Apex** conical 25–30 µm high, 55–65 µm diam. at base, smooth, composed of cylindrical to narrowly clavate cells 15–20 × 2–3 µm, with light brown wall. **Ascomatal surface** composed of cells of undefined shape, forming a *textura epidermoidea*, totally obscured by thick-walled, yellow, hyphal elements 3–4 µm diam., arising from base of ascomata, proliferating and agglutinating at free ends to form a crown of triangular fascicles on upper third of ascomata. **Ascomatal wall** in vertical section of a single region 12–18 µm thick, made of ellipsoidal, thick-walled cells 4–8 × 1.5–2.5 µm, with yellow to pale orange wall 1.5–2 m thick, becoming hyaline and flattened towards interior. **Asci** clavate, stipitate, 55–60 × 7–9 µm, apex simple, rounded, with eight ascospores irregularly biseriate in upper part and uniseriate below. **Paraphyses** moniliform, inserted between asci, up to 12 µm diam. at base. **Ascospores** (8.5–)9–10(–11) × 3–3.5(–4) µm (Me = 9.6 × 3.3 µm, n = 30), 1-septate, hyaline, verrucose.

Culture characteristics: After 10 days at 25°C on Difco PDA: colonies 3.5–4 cm, white to pale yellow, producing white, sessile sporodochia, bearing a white to pale yellow conidial mass, surrounded by long, hyaline setae. Setae 350–500 µm long, 3–3.5(–4) µm diam., thick-walled, with wall 1 µm thick, multi-septate, tapering to a rounded tip, hyaline. Conidiophores 30–45 × 3–3.5 µm, hyaline, branched, ultimate branches bearing 4–7 subulate phialides 16–20 × 2.5–3 µm with a flared collarette. Conidia ellipsoidal to subcylindrical, 4–8 × 3–4 µm, hyaline, smooth.

Volutella thonneliana Lechat & J. Fourn., *sp. nov.* Fig. 4
Mycobank: MB844467

Diagnosis: Differs from known perithecial *Volutella* species by having ascomata not changing colour in 3% KOH or lactic acid and covered by thick-walled, unbranched hairs, and its occurrence on palm leaves.

Holotype: French Guiana, Saül, trail to Monts La Fumée, 3.637173° N, -53.204727° E, ca. 260 m, on unidentified decayed palm leaves, 4 Apr. 2021, *leg*. C. Lechat, LIP CLLG21167, ex-holotype culture: BRFM3397; Genbank sequences: ITS = ON181663, LSU = ON181657.

Etymology: The specific epithet "*thonneliana*" refers to Audrey Thonnel (Parc National Amazonien de Guyane) to whom the authors dedicate this species in appreciation of her management of the ABC of Saül and her friendly and efficient collaboration in the field in 2021.

Ascomata non-stromatic, superficial, solitary or in groups of 2–4, scattered by several hundred on each leaf, subglobose 170–190 µm high, 150–170 µm wide (Me = 180 × 160 µm, n = 10), not collapsing upon drying or rarely, laterally pinched when dry, pale orange, not changing colour in 3% KOH or lactic acid, smooth. **Apex** conical 15–25 µm high, 30–40 µm diam. at base, composed of cylindrical to slightly clavate, thin-walled, pale yellow to hyaline cells 10–22 × 2–2.5 µm. **Ascomatal surface** composed of cells of undefined shape, forming a *textura epidermoidea*, with hyaline to pale yellow, erect, thick-walled, unbranched hairs, 20–45 µm long, 3.5–5 µm diam., 2–4-septate, thick-walled, rounded at tip, smooth, arising from cells of ascomatal wall on upper two thirds of ascomata, except ostiolar region. **Ascomatal wall** in vertical section 10–12 µm thick, of a single region made of globose to ellipsoidal, thick-walled cells 4–8 × 2.5–4 µm, with orange wall 2–2.5 m thick, becoming hyaline, flattened towards interior. **Asci** evanescent, clavate, short-stipitate, 45–55 × 6–8 µm, apex simple, rounded, with eight ascospores irregularly biseriate in upper part and uniseriate below. **Paraphyses** moniliform, inserted between asci, up to 15 µm diam. at base. **Ascospores** long ellipsoidal to fusiform (12–)13–15(–16) × 2.5–3(–3.5) µm (Me = 14.1 × 2.8 µm, n = 30), 1-septate, slightly constricted at septum, with two droplets in each cell, hyaline to pale brownish yellow when mature, smooth-walled.

Culture characteristics: After 10 days at 25°C on Difco PDA: colonies 4–4.5 cm, white to pale yellow, producing white synnemata bearing a pale yellow to pale orange conidial mass, surrounded by long, hyaline setae. Synnemata 200–340 µm high, 60–80 µm diam., made of parallel, septate hyphal elements 2–2.5 µm wide. Setae 220–270 µm long, 4–6 µm diam., thick-walled, with wall 1.5–2.5 µm thick, tapering to an acute end, non-septate, hyaline. Conidiophores 32–48(–55) × 2.8–3.2 µm, hyaline, branched, ultimate branches bearing subulate phialides 8–14 × 2–3 µm, narrowed at tip, without collarette. Conidia subcylindrical, attenuated at base, 5.5–8(–9) × 1.5–2.5 µm, non-septate, smooth, hyaline.

Discussion

Volutella was introduced by Fries (1832) for four anamorphic species characterised by a synnematous asexual morph with sporodochia apically bearing slimy conidial masses, frequently associated with long hyaline setae: *V. carnea* Fr., *V. ciliata* (Alb. & Schwein.) Fr. (type species), *V. pallens* (Nees & T. Nees) Fr. and *V. volvata* Tode. Numerous species of *Volutella* were described since then, and over 150 taxa are reported in Index Fungorum (www.indexfungorum.org), but only four of these were known as sexual morphs: *V. asiana* (J. Luo, X.M. Zhang & W.Y. Zhuang) L. Lombard & Crous, *V. ciliata*, *V. citrinella* (Cooke & Massee) Seifert and *V. consors* (Ellis & Everh.) Seifert, Gräfenhan & Schroers. Sexual morphs of *V. ciliata* and *V. asiana* were recently described by Luo & Zhuang (2012) as *Volutellonectria*, but as pointed out by Crous *et al.* (2015), the name *Volutellonectria* is confusing in nomenclatural terms and should be replaced by *Volutella*, which has priority by date. The three species described above are characterised by non-stromatic ascomata with wall less than 20 µm thick of a single region and volutella-like asexual morphs. Their placement in *Volutella* is confirmed by the phylogenetic analyses of their ITS and LSU sequences (Fig. 1). Among the three new species, *V. minutissima* (Fig. 2) is distinct in having the smallest ascomata in the genus, which are glabrous, not changing colour in 3% KOH, but turning yellow in lactic acid. *Volutella saulensis* (Fig. 3) differs by having agglutinated hairs arranged in a crown of triangular teeth on the upper third of ascomata and verrucose ascospores, while *V. thonneliana* is characterised by ascomata with erect, thick-walled hairs on upper two thirds of ascomatal wall, and morphologically resembles some *Coccinonectria* and *Pseudonectria* species, but differs from them by having ascomata not changing colour in 3% KOH or lactic acid. Our phylogenetic analysis (Fig. 1) showed that the species of *Volutella* form a monophyletic clade distinct from the clades *Coccinonectria* and *Pseudonectria*, as previously demonstrated by Crous *et al.* (2015). *Volutella minutissima* is nested on a sister branch to *V. citrinella*, and, although the two species are morphologically similar, *V. citrinella* differs by having larger ascospores (9–)9.8–11.7(–12.6) × 2.7–3(–3.5) µm vs. (8–)9(–10) × 2.4–2.8 µm, longer conidia in culture 6–12 × 2–2.5 µm vs. 4.5–6(–7) × 2.5–2.8(–3) µm (Gräfenhan *et al.*, 2011), and only 96% and 97% similarity of their ITS and LSU sequences respectively. *Volutella saulensis* is nested on an isolated branch near *V. ciliata*, which primarily differs by having larger ascospores and smaller conidia, with only 90% similarity of their ITS or LSU sequences. *Volutella thonneliana* is nested

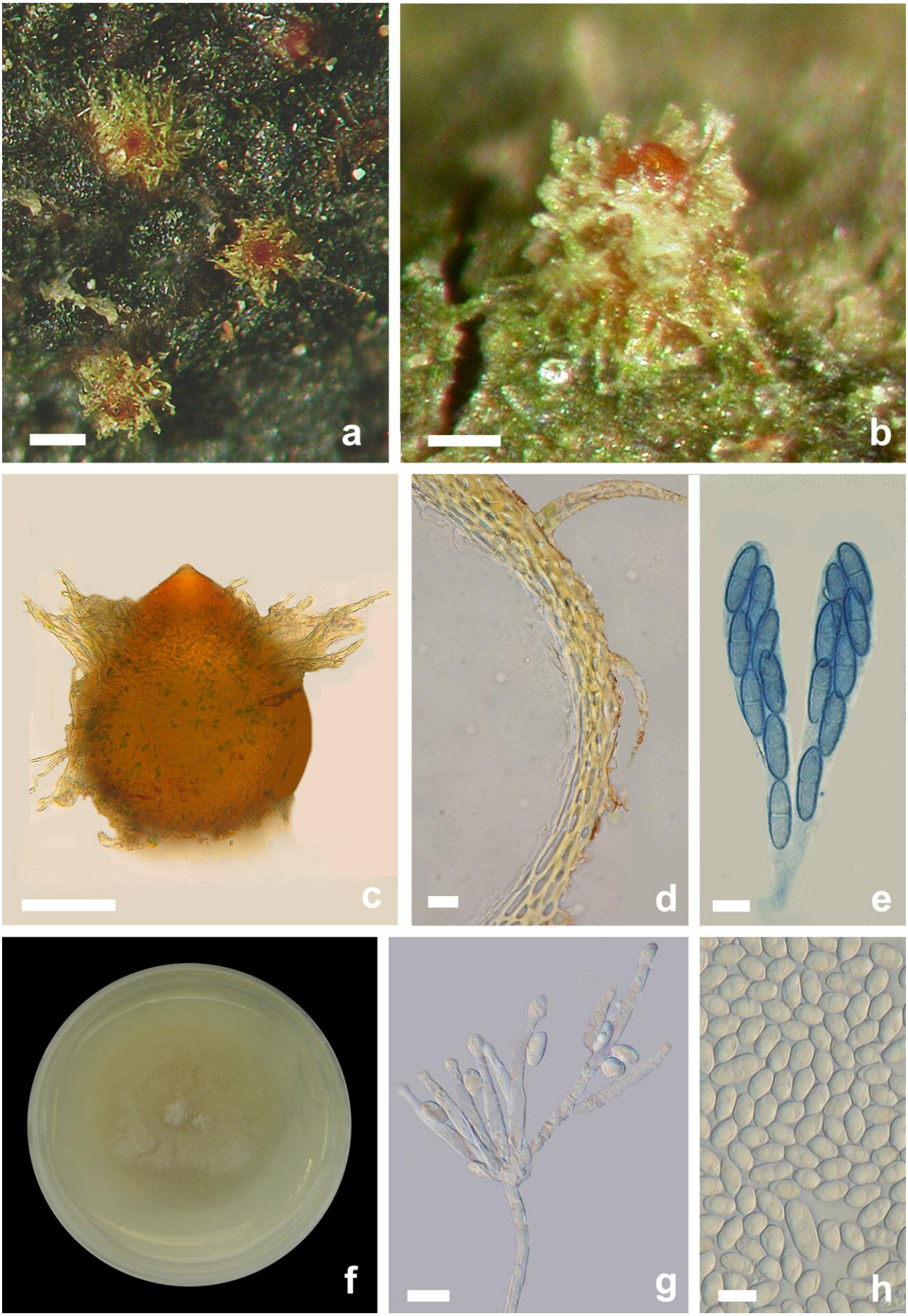

Fig. 3 – a–h: *Volutella saulensis* (Holotype LIP CLLG21099-d). a, b: Dry ascomata on the substrate. c: Close-up of ascoma in side view in water. d: Vertical section through the lateral ascomatal wall in water. e: Asci and ascospores in cotton blue. f: Culture at three weeks. Conidiophore and conidia from culture, in water. h: Conidia in lactic acid. Scale bars: a = 200 µm; b-c = 100 µm; d, g = 10 µm; e, h = 5 µm.

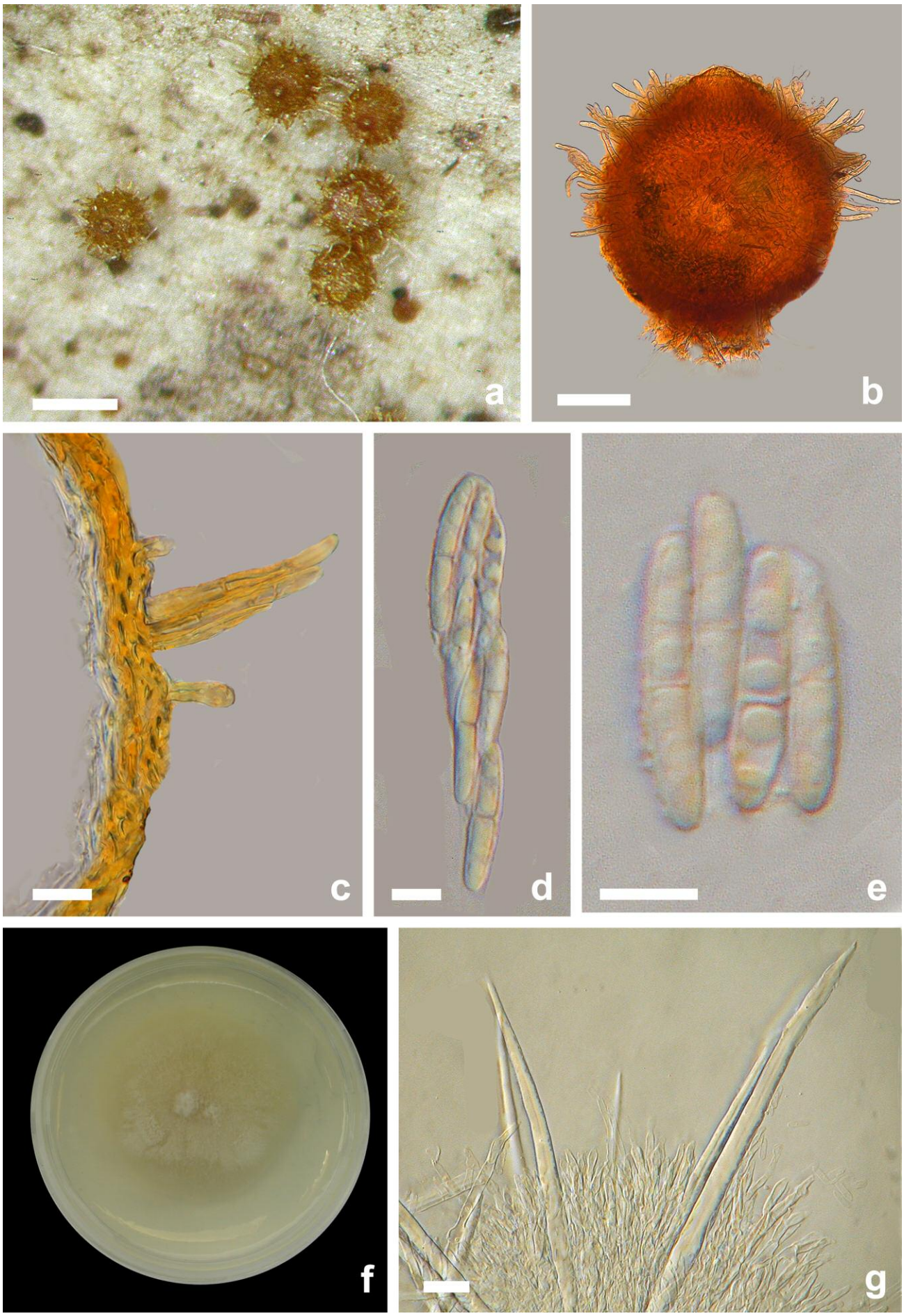

Fig. 4 – a–g: *Volutella thonneliana* (Holotype LIP CLLG21167). a: Dry ascomata on the substrate. b: Close-up of ascoma in side view. c: Vertical section through the lateral ascomatal wall. d: Asci and ascospores. e: Close-up of ascospores. f: Culture at three weeks. g: Conidiophores and setae from culture (b–-e, g in water). Scale bars: a = 200 µm; b = 50 µm; c, g = 10 µm; d–e = 5 µm.

on a sister branch to *V. ramkumari* A.K. Sarbhoy, which differs by having significantly longer setae surrounding conidiophores 300–600 µm vs. 220–270 µm, narrower conidia 6–7 × 1.2 µm vs. 5.5–8(–9) × 1.5–2.5 µm (Sarbhoy, 1967) and only 97% similarity of their ITS sequences. Unfortunately, there is no ITS or LSU sequence available in Genbank for *V. asiana*, so this species was not included in our phylogenetic analysis. However, this species morphologically differs from our new species by having ascomata turning dark red in 3% KOH and orange-yellow in lactic acid, as well as longer conidia with a median displaced hilum (Luo *et al*., 2012). Based on the morphological characteristics of the sexual-asexual morphs and phylogenetic analyses of their ITS and LSU sequences, *V. minutissima*, *V. saulensis* and *V. thonneliana* are proposed as new species, raising the number of known *Volutella* sexual morphs to seven.

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Author's contributions

Christian Lechat was responsible for the conception of the study, morphological studies, cultures, phylogenetic analyses, design of figures and plates and writing a first draft. Jacques Fournier critically reviewed the first draft and proposed an improved version and took care of the registration at MycoBank. Delphine Chaduli and Anne Favel managed the culture collection in which the cultures were deposited, re-cultured and sequenced *V. saulensis* and took care of the registrations at GenBank. All authors except CL read and approved the final manuscript.

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