Two new species of *Clonostachys* (*Bionectriaceae*, *Hypocreales*) from Saül (French Guiana)

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Abstract: *Clonostachys pilosella* and *C. pnagiana* are described and illustrated as new species, based on collections from Saül in French Guiana. The asexual morphs of these fungi were obtained in culture and cultures were sequenced. The two new species are placed in *Clonostachys*, based on ascomata not changing colour in 3% KOH or lactic acid, clonostachys-like asexual morphs and phylogenetic comparison of LSU sequences with 18 known *Clonostachys* species.
Keywords: Ascomycota, *Bionectria*, ribosomal DNA, taxonomy.

Résumé : *Clonostachys pilosella* et *C. pnagiana* sont décrites et illustrées comme nouvelles espèces, fondées sur des récoltes faites à Saül en Guyane française. Les formes asexuées de ces champignons ont été obtenues en culture et les cultures ont été séquencées. Les deux espèces nouvelles sont placées dans le genre *Clonostachys*, fondées sur les ascomes ne changeant pas de couleur dans le KOH à 3 % ou dans l'acide lactique, la forme asexuée de type clonostachys et la comparaison des séquences LSU avec 18 espèces connues de *Clonostachys*.
Mots-clés : ADN ribosomal, Ascomycota, *Bionectria*, taxinomie.

Introduction

In the continuity of our inventorial survey of fungi in Saül in French Guiana carried out in the context of the ABC project initiated by the Parc National Amazonien de Guyane (Gardiennet *et al*., 2019, Lechat & Fournier, 2018; 2019a; 2019b; 2019c; Lechat *et al*., 2019), two unknown bionectriaceous species were collected. Morphological characteristics of sexual-asexual morphs as well as phylogenetic analysis of LSU sequences revealed that they belong to *Clonostachys* Corda. We present herein our results leading to the recognition of *C. pilosella* and *C. pnagiana* as new species. As we pointed out in our previous papers, many unknown species remain to be discovered in the Guianese forests. This study supports this hypothesis with the two new species described below, added to the two new species previously described from French Guiana, namely *C. saulensis* Lechat & J. Fourn. and *C. spinulosispora* Lechat & J. Fourn. (Lechat & Fournier, 2018; Lechat *et al*., 2019).

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. The holotypes are deposited in LIP herbarium (University of Lille, France), and ex-type cultures are deposited in the Collection of the CIRM (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 them 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 LR0R and LR5 (Vilgalys & Hester, 1990) 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. 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

Clonostachys pilosella Lechat & J. Fourn., *sp. nov.* Fig. 2
Mycobank: MB835065

Holotype: French Guiana, Saül, Gros Arbres trail, on bark of an unidentified tree, 18 Jun. 2019, *leg*. C. Lechat, CLLG19028 (LIP), ex-type culture BRFM 3113, GenBank LSU sequence: MT248415.

Diagnosis: Differs from all known *Clonostachys* species with striate ascospores in having fasciculate hairs around upper part of ascomata and fusiform ascospores 15–17 × 3.5–4 µm.

Etymology: Epithet derived from the diminutive of Latin *pilosus* = hairy, for the unusual fasciculate hairs around upper part of ascomata.

Ascomata solitary to gregarious, non-stromatic, globose, 180–220 µm diam., smooth, pale yellow to pale orange becoming orange and laterally pinched when dry, erumpent, semi-immersed and with the base eventually remaining immersed in host tissues. **Ascomatal surface** composed of globose to subglobose thick-walled cells 15–22 × 12–15 µm with orange wall 2–2.8 m thick, with fasciculate, thick-walled, hyaline, septate hairs 20–55 × 2–3 µm, rounded and enlarged at free ends, up to 5 µm wide, arising from cells of ascomatal wall to form a crown of triangular teeth around upper part of ascomata. **Perithecial apex** rounded with a minute papilla composed of cylindrical to narrowly clavate cells 10–15 × 2–2.5 µm, with pale yellow wall 1 µm thick, merging with periphyses. **Ascomatal wall** 30–40(–50) µm thick, of two regions, containing sparse, orange oily droplets; outer region 20–30 µm thick, composed of subglobose to globose cells 4.5–7 × 4–6 µm, with pale yellow walls 1–1.5 µm thick; inner region 15–20 µm thick, composed of subglobose cells 4.5–9 × 3.5–5 µm with hyaline wall 1.5–2(–3) µm thick. **Asci** unitunicate, clavate, stipitate (60–)65–75(–80) × 8.5–10 µm (Me = 72 × 9.5 µm, n = 30), with 8 ascospores biseriately overlapping or irreg-

ularly disposed in upper part, uniseriate in lower part, apex simple. No **paraphyses** observed. **Ascospores** (14–)15–17(–19) × 3.5–4 µm (Me = 16.5 × 3.8 µm, n = 30), fusiform, slightly curved, equally 1-septate, slightly constricted or not at septum, hyaline, striate.

Cultural characteristics: After two weeks on PDA at 25°C, colony 40–45 mm diam., centre pale brown, median area whitish orange, whitish at margin, producing a pale yellow colouration in medium. Aerial mycelium composed of septate, hyaline, smooth hyphae 2–3 µm diam. Conidiophores 90–140 µm high, 5–8 µm diam., branched, hyaline, septate, arising from aerial hyphae, each branch bearing 3–5 subulate conidiogenous cells 14–26 µm long, 5–6 µm diam, with a small collarette. Conidia hyaline, aseptate, ellipsoidal, smooth-walled, without a visible hilum (13–)18–25(–28) × 8–10 µm.

Clonostachys pnagiana Lechat & J. Fourn., *sp. nov.* Fig. 3
Mycobank: MB835066

Holotype: French Guiana, Saül trail leading to mountain La Fumée, on bark of unidentified tree, 19 Jun. 2019, *leg*. C. Lechat CLLG19041 (LIP), ex-holotype culture BRFM 3057. GenBank LSU sequences: MT248416

Diagnosis: Similar to *C. grammicospora* (Ferd. & Winge) Schroers & Samuels in having light orange to light brownish orange strongly warted ascomata, but differs in having significantly larger, fusiform, verrucose ascospores (22–)25–28(–30) × 6–8 µm vs. (8.2–)10.6–12.6(–17.6) × (3–)3.8–4.6(–6.2) µm, ellipsoid and striate in *C. grammicospora*, and smaller conidia in culture.

Etymology: Epithet "*pnagiana*" refers to the acronym of Parc National Amazonien de Guyane where this species was collected.

Ascomata scattered on substrate, superficial, seated in groups of 3–25 on a minute pseudoparenchymatous hypostroma, strongly warted, globose, (260–)290–320(–340) µm diam. (Me = 300 µm, n = 20), papillate, with warts around upper margin of ascomata, pale orange to light brownish orange, matt, collapsing by laterally pinching when dry, not changing colour in 3% KOH or lactic acid. Papilla conical, 60–80 µm diam., composed of cylindrical to narrowly clavate cells 6–30 µm long, 2–2.5 µm wide, with pale yellow wall. **Ascomatal wall** 45–55 µm thick, composed of two regions containing numerous oily droplets: outer region, 30–40 µm wide excluding warts, of subglobose to widely ellipsoidal thin-walled cells 8–18 × 8–15 µm, with hyaline to very pale yellow walls 1 µm thick, agglutinating to form warts up to 100 µm high; inner region 10–15 µm wide, of elongate, flattened, thick-walled cells 8–14 × 3–5 µm, with hyaline walls 2–2.5 µm thick. **Asci** 80–95 × (13)14–16(–17) µm (Me = 90 × 15 µm, n=30), clavate, apex simple, rounded to slightly flattened, containing 8 biseriate ascospores or biseriately overlapping above and uniseriate below. Filamentous **paraphyses** interspersed between the asci 2–2.5 µm diam. **Ascospores** (22–)25–28(–30) × 6–8 µm (Me = 26.5 × 7 µm, n=30), fusiform, acute at ends, equally 1-septate, slightly constricted at septum, hyaline, verrucose.

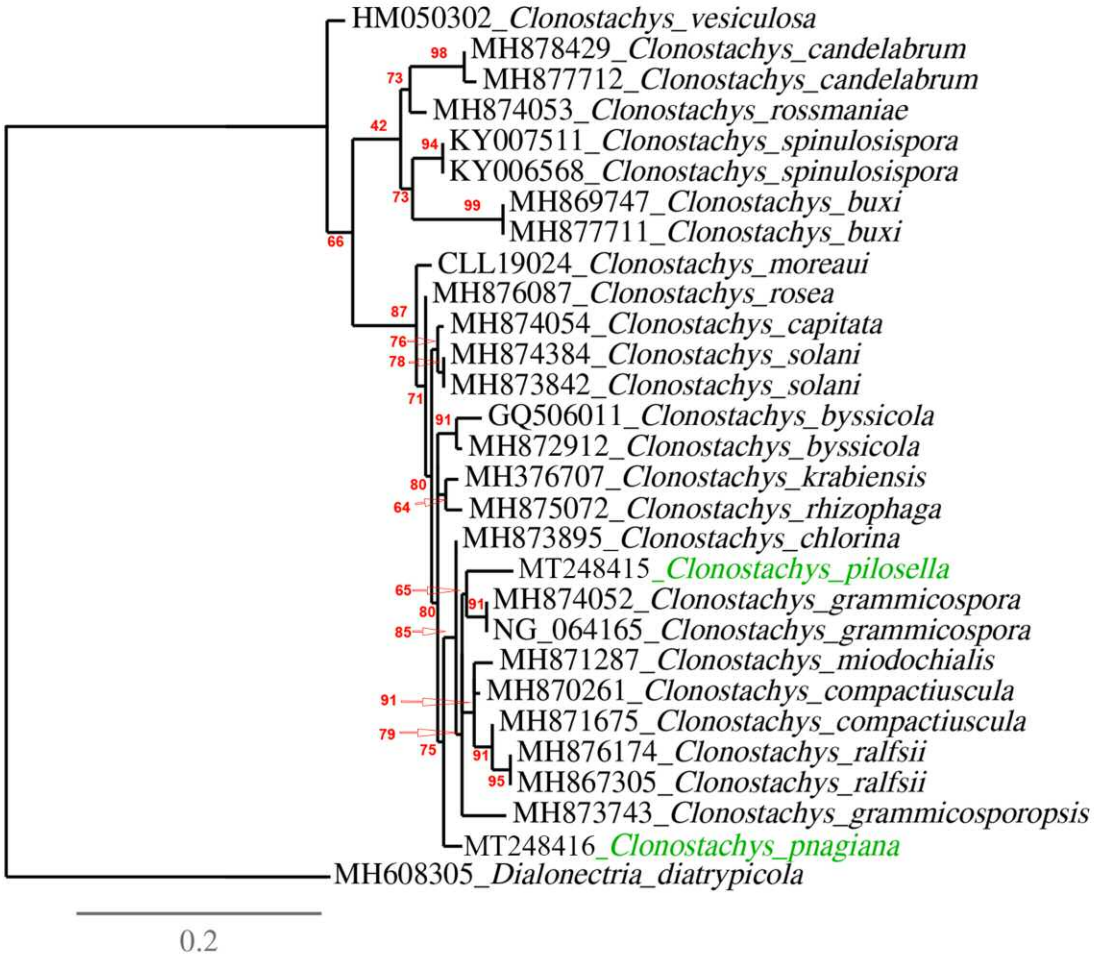

Fig. 1 – Maximum likelihood phylogeny (-lnL = 2079.82127) of *Clonostachys* spp. inferred by PhyML 3.0, model HKY85 from a 890 bp matrix of 28S rRNA sequences, rooted with *Dialonectria diatrypicola*.

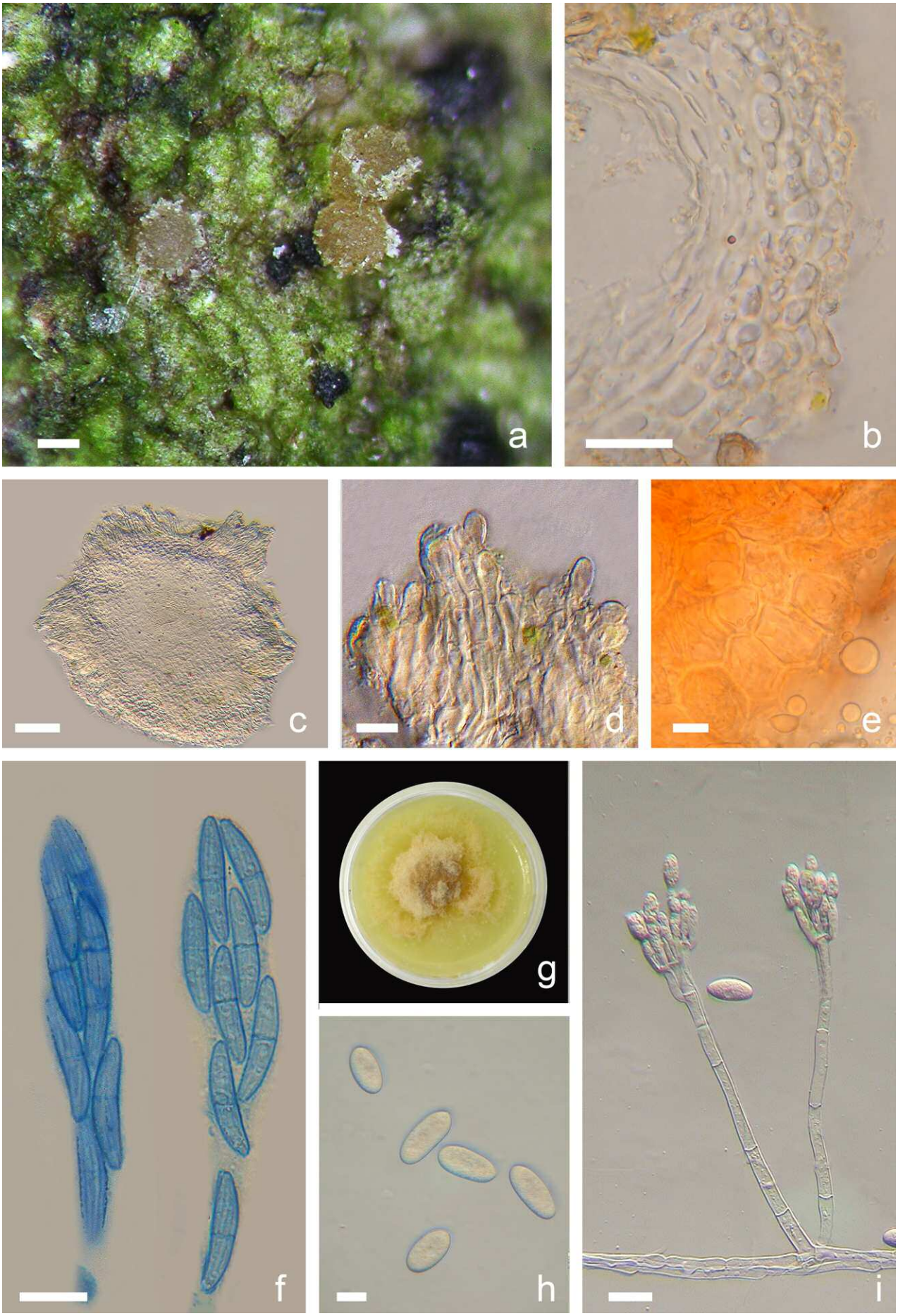

Fig. 2 – a-g: *Clonostachys pilosella* (CLLG19028, Holotype); a: Habit of ascomata on the substrate; b: Lateral ascomatal wall in vertical section in water; c: Perithecium in top view in water showing fasciculate hairs; d: Close-up of fasciculate hairs, in water; e: Cells of ascomatal surface; f: Asci and ascospores in lactic acid cotton blue not heated; g: Culture at three weeks; h: Conidia from culture, in water; i: Conidiophores and conidia in water. Scale bars: a = 100 µm; b, i = 20 µm; c = 50 µm; d, e, f, h = 10 µm.

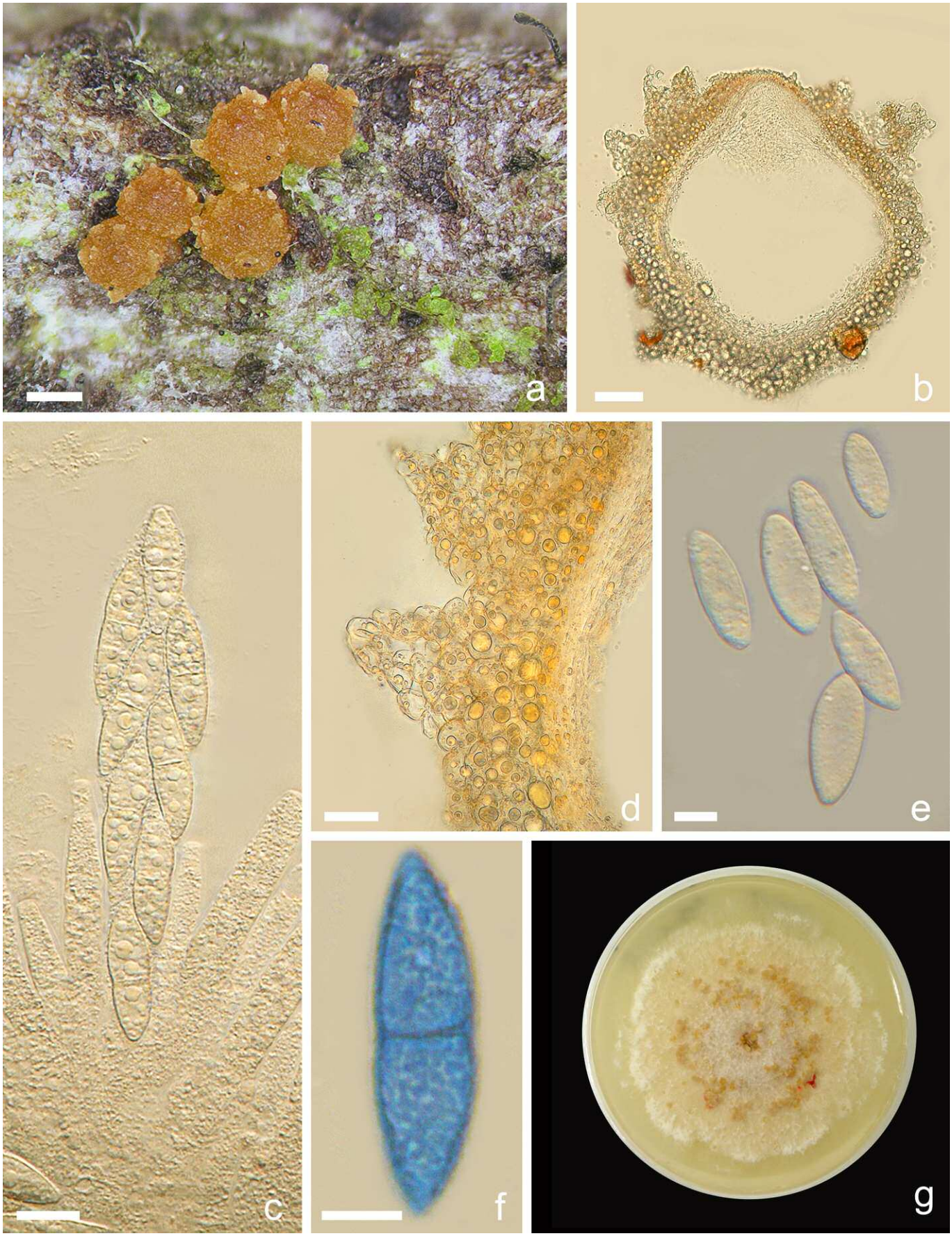

Fig. 3 – a-g: *Clonostachys pnagiana* (CLLG19041, Holotype); a: Habit of ascomata on the substrate; b: Vertical section through a perithecium. c: Asci and ascospores in water; d: Lateral ascomatal wall in vertical section in water; e: Conidia from culture, in water. f: Close-up of ascospore in lactic cotton blue, not heated. g: Culture at three weeks. Scale bars: a = 200 µm; b = 50 µm; c = 10 µm; d = 20 µm; e, f = 5 µm.

Culture characteristics: After three weeks on PDA at 25°C, colony 40–45 mm diam., centre pale brown, median area pale yellow, white at fimbriate margin, not producing colouration in medium. Mycelium composed of septate, hyaline, smooth hyphae 2–3 µm diam. Conidiophores branched, hyaline, septate, 3–4 µm diam, arising from aerial hyphae, each branch bearing 3–5 terminal, subulate conidiogenous cells 15–22 µm long, 2.5–3 µm diam at base, 2 µm diam at tip, with an inconspicuous collarette. Conidia hyaline to pale orange, aseptate, narrowly ellipsoidal to subcylindrical, attenuated at tip, with a slightly, laterally displaced hilum, smooth-walled, (12–) 15–22(–25) × (4.5–)5–7(–8) µm.

Results and discussion

Within the *Bionectriaceae*, characterised by ascomata with a KOH-wall, *Clonostachys* is unambiguously defined by its verticillate to penicillate chlonostachys-like asexual morphs (Rossman *et al*., 1999; Schroers, 2001). The genus name *Clonostachys* was retained over the former genus name *Bionectria* Speg. (Rossman *et al*., 2013). The two species described above match well some morphological characteristics of *Clonostachys* illustrated by Lechat & Fournier (2018; 2020, this paper), Lechat *et al.* (2019), and their placement in this genus is well supported by our phylogenetic analysis of LSU sequences (Fig. 1).

Clonostachys pilosella is primarily characterized by pallid, non-stromatic ascomata bearing fasciculate thick-walled hairs forming a crown around the upper part of ascomata. This is a most unusual character in *Clonostachys*, so far only known in *C. buxi* (J.C. Schmidt ex Link) Schroers which occurs on dead leaves of *Buxus sempervirens* in Europe and differs by having much smaller, smooth-walled aseptate ascospores 10.4–12 × 3–3.4 µm (Schroers, 2001). Our phylogenetic analysis (Fig. 1) shows that *C. pilosella* is distant from *C. buxi* and nested on a sister branch to *C. grammicospora* Schroers & Samuels, whose type is known from Saül, collected by G.J. Samuels (Schroers, 2001). *Clonostachys grammicospora* differs from *C. pilosella* by orange, scaly to warted ascomata lacking fasciculate hairs and smaller ellipsoid ascospores (8.2–)10.6–12.6(–17.6) × (3–)3.8–4.6 (–6.2) µm; moreover, both species differ by only 98% similarity of their LSU sequences.

Clonostachys pnagiana is characterized by orange, stromatic, strongly warted ascomata, with numerous orange oily droplets in ascomatal wall and hymenium, and large, fusiform, verrucose ascospores (22–)25–28(–30) × 6–8 µm. A similar ascomatal morphology associated with striate ascospores can be encountered in *C. grammicospora*, *C. grammicosporopsis* Schroers & Samuels and *C. subquaternata* Schroers & Samuels (Schroers, 2001). They all feature smaller ascospores than *C. pnagiana*, respectively (8.2–)10.6–12.6(–17.6) × (3–)3.8–4.6(–6.2) µm, (9–)12.6–15(–18.4) × (3.6–) 4.6–5.6(–7.4) µm and (10–)15.4–18.6(–26) × (3.6–)5.2–6.6(–9.6) µm (Schroers, 2001). Our phylogenetic tree shows that *C. pnagiana* is nested on an isolated branch and a BLAST search shows that the closest hit is *C. chlorina* Schroers, from which it differs by 99% similarity of their LSU sequences. *Clonostachys chlorina* is known from Brazil as asexual morph only. It can be distinguished from *C. pnagiana* by a yellowish green reverse of colonies and smaller and more broadly ellipsoid conidia in culture, (6.2–)7–7.4(–8.8) × (3.2–)3.6–4.2(–4.4) µm (Schroers, 2001).

For *Clonostachys pilosella* we considered a possible association with algae or lichens, suggested by the image of ascomata *in situ* that we provide above. This cannot be absolutely ruled out, as bark of recently dead branches is frequently colonized by such organisms. However, a thorough observation of the whole colony shows that ascomata mainly occur on bare bark without visible connection with other organisms, which rather suggests a typical colonization of bark tissues.

Comparison of morphological characteristics of sexual-asexual morphs of both collections with known *Clonostachys* species, as well as molecular analyses, therefore lead us to propose *C. pilosella* Lechat & J. Fourn. and *C. pnagiana* Lechat & J. Fourn. as new species.

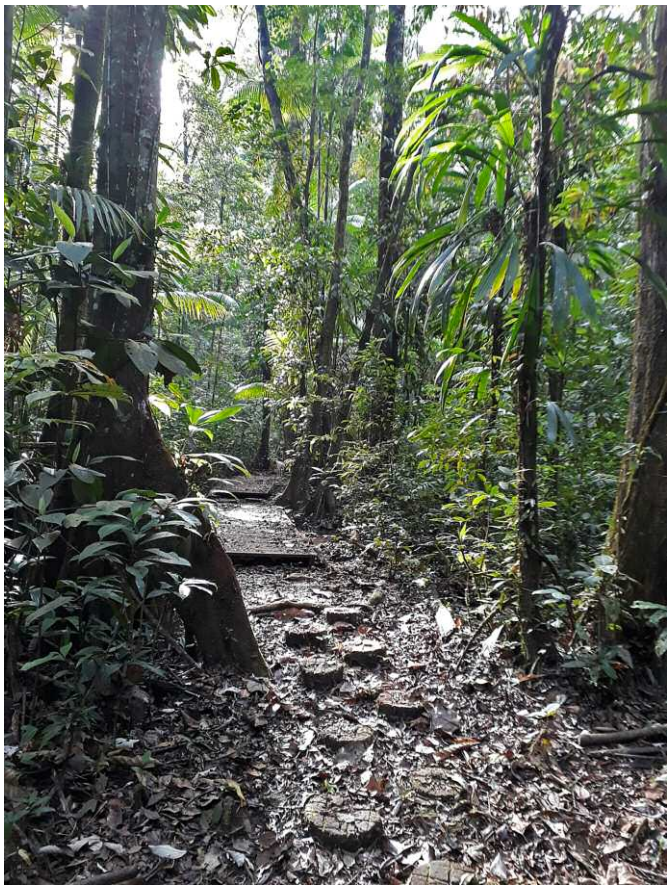

Fig. 4 – Forest trail around Saül (photo A. Gardiennet).

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