Clonostachys spinulosispora (*Hypocreales*, *Bionectriaceae*), a new species on palm from French Guiana

Christian LECHAT
Jacques FOURNIER

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Abstract: *Clonostachys spinulosispora* is introduced as a new species of *Clonostachys* (syn. *Bionectria*), based on material collected in French Guiana on dead palm leaves of *Astrocaryum vulgare*. The placement of the new species in *Clonostachys* is supported by morphological characters of the asexual morph obtained in culture and the bionectria-like sexual morph of the specimen in nature as well as analysis of ITS sequences. Its segregation from other known species of *Clonostachys* is primarily based on its striking ascospore ornamentation.
Keywords: Ascomycota, bionectria-like, ribosomal DNA, taxonomy.

Résumé : *Clonostachys spinulosispora* est présentée comme une nouvelle espèce de *Clonostachys* (syn. *Bionectria*), sur la base de matériel récolté sur palme d'*Astrocaryum vulgare* en Guyane française. Le placement de la nouvelle espèce dans le genre *Clonostachys* repose sur les caractères morphologiques de la forme asexuée obtenue en culture et de la forme sexuée de type bionectria observable dans la nature, ainsi que sur l'analyse des séquences ITS. Elle se distingue principalement des autres espèces connues de *Clonostachys* par la remarquable ornementation de ses ascospores.
Mots-clés : ADN ribosomal, Ascomycota, *Bionectria*, taxinomie.

Introduction

During a field trip carried out over two weeks in June 2012 in French Guiana, an unknown bionectriaceous species was collected on dead palm leaves of *Astrocaryum vulgare* Mart. (*Arecaceae*). The genus *Clonostachys* Corda (syn. *Bionectria* Speg.) was selected as the correct name for this genus (Rossman *et al.*, 2013). This genus is distinguished from other genera in the *Bionectriaceae* by a sexual morph of ascomata seated on a pseudoparenchymatous stroma or arising directly on the substrate, white, pale yellow, orange to dark brownish-orange, not changing colour in 3% KOH or lactic acid, not collapsing or laterally pinched when dry, warted or smooth; ascomatal wall composed of 1–3 regions with outer region composed of subglobose to globose, thick-walled cells; ascospores smooth, spinulose, striate or warted, and an asexual morph of penicillate conidiophores bearing yellow to salmon conidia *en masse*, as defined by Rossman *et al.* (1999) and Schroers (2001). The new species is placed in *Clonostachys* based on morphological traits of its sexual-asexual morphs and phylogenetic comparison of ITS sequences with 27 *Clonostachys* species and *Verrucostoma freycinetiae* Hirooka, Tak. Kobay. & P. Chaverri as outgroup. Based on these characteristics, phylogenetic analysis and comparison with known *Clonostachys* species, the specimens described herein are determined to represent a previously undescribed species.

Materials and methods

Specimens were 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 and the paratype are deposited at LIP herbarium (University of Lille) and cultures at the CBS Cultures 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 9 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 (Santander, Spain): Total DNA was extracted from dry specimens 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 centrifugated 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 cold ethanol 70%, centrifugated 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. 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).

Taxonomy

Clonostachys spinulosispora Lechat & J. Fourn., *sp. nov.* – Figs. 1-2 – MB 827070

Diagnosis: Differs from all known species of *Clonostachys* in having conspicuously spinulose ascospores with spines up to 2 µm long, and by its occurrence on palm leaf of *Astrocaryum vulgare* in a tropical environment.

Holotype MBT 382984: French Guiana, Régina, Nouragues Natural Reserve, Inselberg camp, primary rainforest, on aerial, dead palm leaf of *Astrocaryum vulgare* Mart. (*Arecaceae*), 16 Jun. 2012, *leg.* C. Lechat CLLG12001(LIP), ex-holotype culture CBS133762. GenBank ITS: MH634702.

Etymology: Epithet derived from Latin *spinulosus* = spiny, for the ascospore wall ornamentation.

Ascomata solitary, superficial, non-stromatic, smooth, matt, scattered on substrate with smooth, hyaline, septate basal hyphae, globose, (320–)350–400(–420) µm diam. (Me = 380 µm, n = 20), white to pale yellow or pale orange, collapsing by laterally pinching or not collapsing when dry, easily removed from substrate, not changing colour in 3% KOH or lactic acid. **Perithecial apex** with minute, con-

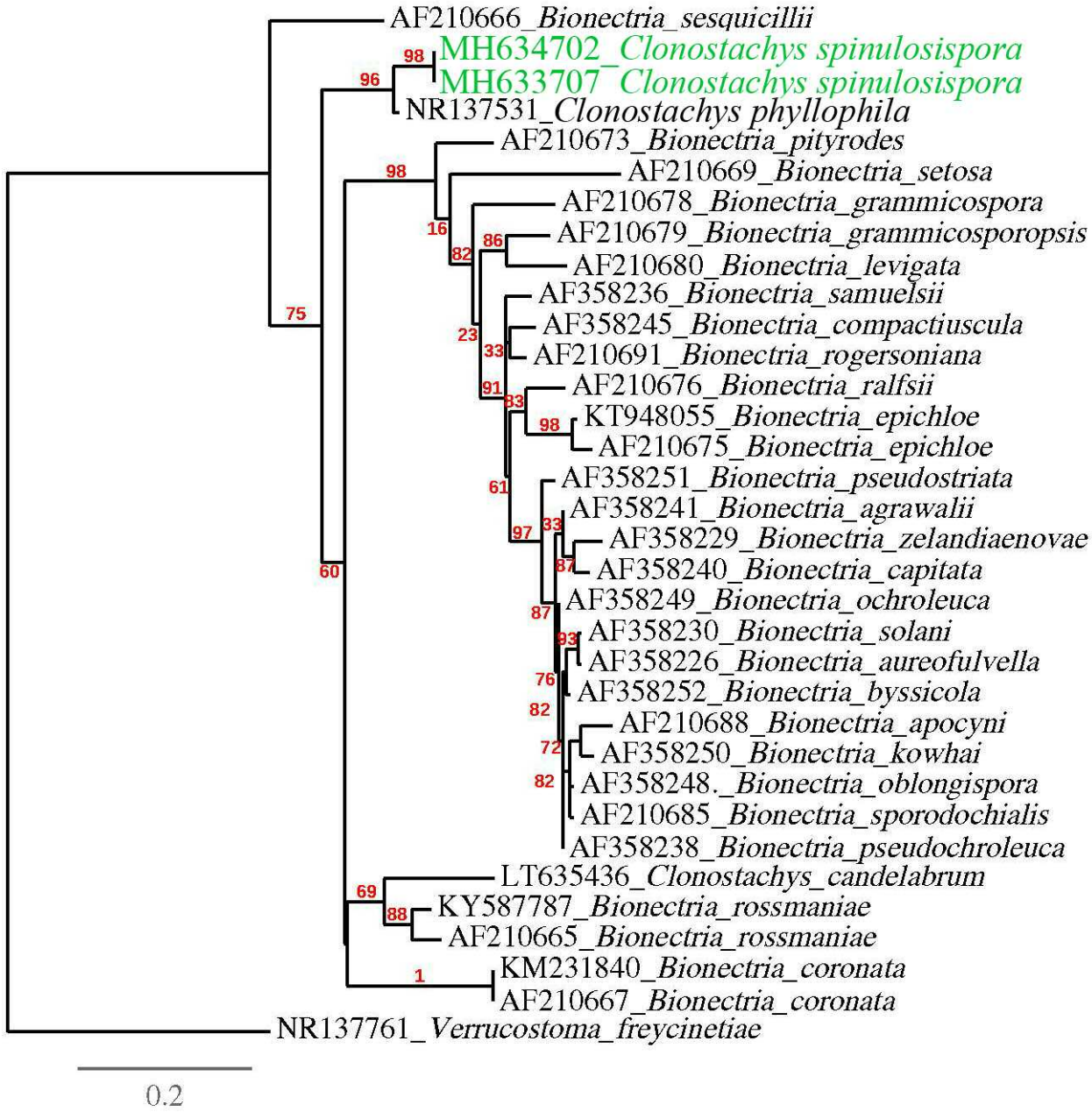

Fig. 1 – Maximum likelihood phylogeny (-lnL = 2848.34809) of *Clonostachys spinulosispora* inferred by PhyML 3.0, model HKY85 from a 538 bp matrix of ITS sequences, rooted with *Verrucostoma freycinetiae*.

colourous, pointed papilla 50–60 µm diam., composed of cylindrical cells 15–25 µm long, 2–3 µm wide with wall 1 µm thick, slightly enlarged at apex, hyaline to very pale yellow. **Ascomatal wall** 45–50 µm thick, composed of two regions: outer region 35–45 µm wide, of subglobose to ellipsoidal thick-walled cells 8–15 × 5–10 µm, with pale orange walls 1.5–2.5 µm thick; inner region 10–18 µm wide, of elongate, flattened thick-walled cells 9–15 × 3–9.5 µm, with hyaline walls 1.5–2.5 µm thick. **Ascomatal surface** composed of subglobose to ellipsoidal, subangular cells up to 20 µm in greatest dimension. **Asci** (45–)55–65(–70) × (11–)12–18(–20) µm (Me = 62.5 × 16 µm, n = 20), clavate, apex rounded to slightly flattened, with a ring-like apical thickening, containing 8 biseriate ascospores or biseriate above and uniseriate below, completely filling each ascus. **Ascospores** (20–)22–26(–28) × (6–)7–8(–9) µm (Me = 24 × 7.5 µm, n = 30), narrowly ellipsoidal with attenuated ends to fusiform, equally 1-septate, slightly constricted at septum, hyaline, conspicuously spinulose with spines up to 2 µm long.

Culture characteristics: After one week on PDA at 25°C, colony 40–45 mm diam., centre pale yellow, median area pale orange due to released conidia and white at fimbriate margin, producing a yellow colouration in medium. Mycelium composed of septate, hyaline, smooth hyphae 2.5–3 µm diam. Conidiophores monomorphic, penicillate, arising from aerial hyphae, macronematous, flexuous, hyaline, stipe and lateral branches 11–35 long, 2.5–3 µm diam., bearing subulate conidiogenous cells 7–12 µm long, 2–3 µm diam. Conidia hyaline, aseptate, narrowly ellipsoidal to subcylindrical with rounded apex, attenuated at base with a median, apiculate hilum, smooth-walled, 4.5–6.5 × 2.3–3 µm.

Additional specimen examined (paratype): French Guiana, Régina, Nouragues Natural Reserve, piste de la Pinotière Perchée, on palm leaf of *Astrocaryum vulgare*, 19 Jun. 2012, *leg*. C. Lechat, CLLG12028(LIP). GenBank ITS: MH633707.

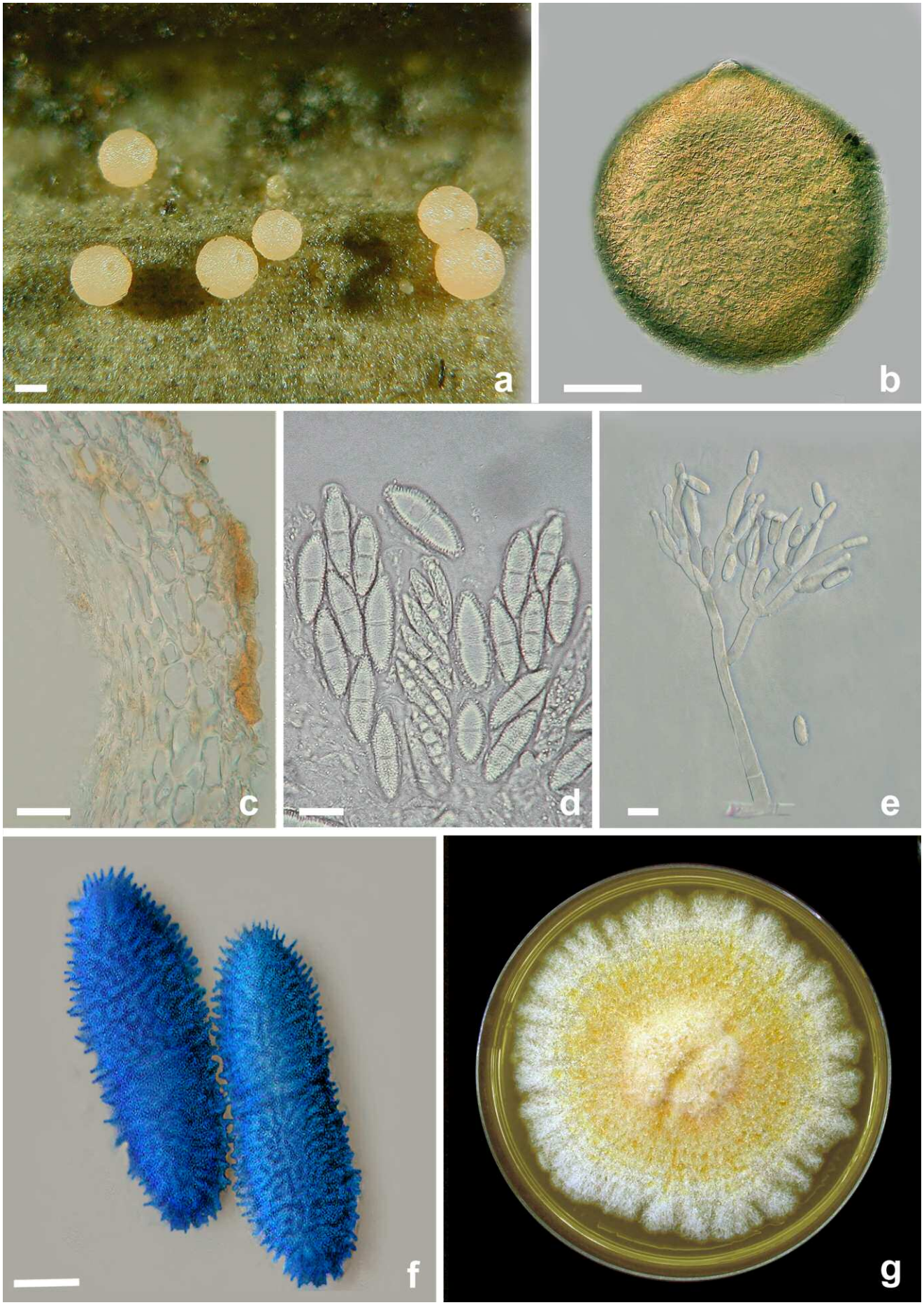

Fig. 2 – a-g: *Clonostachys spinulosipora* (CLLG12001, Holotype); a: Habit of ascomata on the substrate; b: Close-up of a perithecium in side view in water showing a minute papilla. c: Lateral ascomatal wall in vertical section in water; d: Asci and ascospores in water; e: Conidiophores and conidia in water. f: Close-up of two ascospores in lactic cotton blue, not heated. g: Culture at three weeks. Scale bars: a = 200 µm; b = 100 µm; c = 20 µm; d = 10 µm; e, f = 5 µm.

Discussion

Clonostachys spinulosispora is unambiguously placed in the genus *Clonostachys* based on morphological features of its sexual and asexual morphs and phylogenetic analysis of ITS sequences. The comparison with the *Clonostachys* species reported in the literature (Hirooka & Kobayashi, 2007; Luo & Zhuang, 2010; Rossman *et al*., 1999; Schroers, 2001; Schroers *et al*., 1999) shows its distinctiveness. Only two species of *Clonostachys* (as *Bionectria*) occurring on palm are known: *C. pseudochroleuca* Schroers and *C. verrucispora* Schroers (Schroers, 2001), but they differ from the new taxon in having warted ascospores less than 18 µm long, while *C. spinulosispora* has larger, spinulose ascospores up to 26 µm long, with unusually long spines (Fig. 2). The phylogenetic analysis carried out in the present study (Fig. 1), comparing *C. spinulosispora* with 27 species of *Clonostachys* (often listed as *Bionectria*) places our fungus in a distant subclade along with *C. phyllophila* Schroers, whose sexual morph is unknown. Morphologically, *C. phyllophila* differs from *C. spinulosispora* in having conidia with a laterally displaced hilum, arranged in chains and forming white columns, while conidia of *C. spinulosispora* have a median hilum and do not form columns, and our phylogenetic analysis showed only 97% similarity of their ITS sequences, which supports their distinctiveness. The segregation of the new taxon *C. spinulosispora* from other known species of *Clonostachys* is warranted by the combination of the above characters. Finally, *Clonostachys spinulosispora* is the only known species of *Clonostachys* having such an ascospore ornamentation, which warrants its status as a new taxon.

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1: C. Lechat – 64 route de Chizé, 79360 Villiers-en-Bois, France – lechat@ascofrance.fr

2: J. Fournier – Las Muros, 09420 Rimont, France – jacques.fournier@club-internet.fr