Two new species of *Stylonectria* (*Nectriaceae*) from the French Alps

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Abstract: Two new species of *Stylonectria* are described and illustrated from specimens collected in the French Alps. Ascomatal morphology, fungicolous lifestyle, and phylogenetic comparison of their ITS sequences with species in six similar genera of *Nectriaceae* having a fusarium-like asexual morph support their placement in *Stylonectria*. Based on morphological divergences of both sexual and asexual morphs, as well as the ITS sequences from known *Stylonectria* species, *S. alpina* and *S. tuedensis* are proposed as new species.
Keywords: *Ascomycota*, *Diplodia*, *Eutypella*, fungicolous *Hypocreales*, ribosomal DNA, *Sorbus*, taxonomy.

Résumé : deux nouvelles espèces de *Stylonectria* sont décrites et illustrées à partir de matériel récolté dans les Alpes françaises. La morphologie des ascomes, l'habitat fongicole et la comparaison phylogénétique de leurs séquences ITS avec celles d'espèces appartenant à six genres semblables de *Nectriaceae* ayant un stade asexué de type fusarium soutiennent leur placement dans les *Stylonectria*. En se fondant sur les divergences morphologiques de leurs formes sexuées et asexuées, ainsi que de leurs séquences ITS par rapport à celles des espèces connues de *Stylonectria*, *S. alpina* et *S. tuedensis* sont proposées comme nouvelles espèces.
Mots-clés : ADN ribosomal, *Ascomycota*, *Diplodia*, *Eutypella*, Hypocréales fongicoles, *Sorbus*, taxinomie.

Introduction

An inventorial survey of *Ascomycota* in the French Alps, initiated by Ascomycete.org in Vanoise National Park in August 2020, led to the discovery of two new species of *Stylonectria* Höhn. (*Hypocreales*) occurring on dead stromata of pyrenomycetes. One collection was made on *Diplodia* sp. on *Sorbus chamaemespilus* (L.) Crantz and the other on *Eutypella* sp. on *Sorbus aucuparia* L. Both collections were studied morphologically, then successfully cultured and sequenced. We present here the morphological, cultural, and molecular data supporting the placement of both collections in *Stylonectria* as the new species *S. alpina* and *S. tuedensis*.

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 lactic cotton blue. The holotype specimens were deposited in LIP herbarium (University of Lille, France). Cultures of the 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 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. Chromatograms were checked searching for putative reading errors and manually corrected. Nucleotide sequences were deposited in GenBank. Sequences generated in this study and those obtained from GenBank were aligned under Clustal W (Thomson *et al.*, 1994). The evolutionary history conducted using MEGA version 6 (Tamura *et al.*, 2013) was inferred by using the maximum likelihood method based on the Tamura-Nei model (Tamura & Nei, 1993).

Taxonomy

Stylonectria alpina Lechat & J. Fourn., *sp. nov.* Fig. 2
Mycobank: MB 838505

Diagnosis: Similar to *S. norvegica* from which it differs by having larger ascomata 345 × 315 µm vs. 290 × 250 µm on average, larger macroconidia (10–)12–18(–20) × 3.5–4.5 µm vs. (8.5–)10–13(–14) × 1.8–2.5 µm, and occurrence on *Diplodia* sp.

Holotype: France, Savoie, Bramans, refuge du Petit Mont Cenis, 45.211864° N 6.888616° E, 2200 m asl., on dead stromata of *Diplodia* sp. on *Sorbus chamaemespilus*, 2 Sep. 2020, *leg*. M. Hairaud & B. Capoen, CLL20026 (LIP CLL20026), ITS Genbank sequence: MW479392.

Etymology: The epithet *alpina* refers to the occurrence of this species in the alpine zone.

Ascomata aggregated in groups of 5–20(–30) on dead stromata of host, subglobose to obpyriform, (280–)320–370(–390) µm high, (280–)300–330(–340) µm diam., partially immersed in host tissues, yellow, orange-red to dark red, not collapsing or laterally pinched upon drying, becoming purple in 3% KOH and yellow in lactic acid. **Ascomatal apex** with a broad, flat disc, concolorous to slightly darker than ascomatal wall, (160–)180–250(–280) µm diam., composed of narrowly clavate, thick-walled cells 10–20 × 2.5–3.5(–4) µm, with yellow to orange wall, not changing colour in 3% KOH or lactic acid. **Ascomatal surface** smooth to slightly roughened, composed of globose to ellipsoidal, angular, thick-walled cells. **Ascomatal wall** in vertical section 40–45 µm thick, composed of two regions; outer region 20–30 µm thick, composed of globose, subglobose to ellipsoidal, thick-walled cells (4–)5–10 × (4–)5–6(–8) µm, with orange walls up to 3 µm thick; inner region 10–15 µm thick, of hyaline, compressed, elongate cells (8–)10–15 × 5–7 µm apically merging with periphyses. **Asci** cylindrical, shortly stipitate, 75–85 × 6.5–8 µm (Me = 80 × 7 µm, n = 20), apex flat to slightly rounded, with a faint apical ring-like thickening, containing 8 uniseriate ascospores, interspersed with deliquescing, filamentous to narrowly moniliform paraphyses. **Ascospores** ellipsoidal, widely rounded at ends, 1-septate, (8.5–)9–10(–11) × 5–5.8(–6) µm (Me = 9.5 × 5.5, n = 50), hyaline, smooth, multigutulate.

Cultural characteristics: After two weeks at 25°C on Difco PDA, colony 2–2.5 cm diam., floccose, pale yellow in centre, with a few shades of orange, producing a fusarium-like asexual morph, sporulating at white to cream, slimy margin. No microconidia produced.

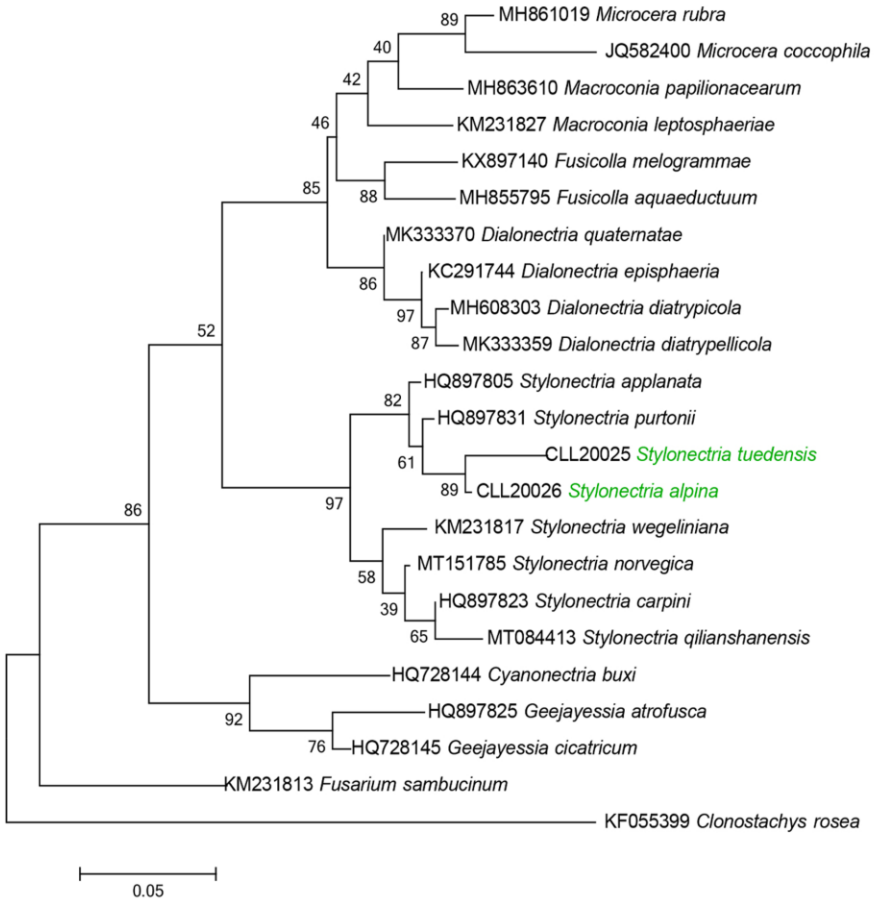

Fig. 1 – Maximum likelihood phylogram (-lnL = 3307.45) inferred from ITS gene sequences of species having fusarium-like asexual morphs rooted with *Clonostachys rosea*.

Macroconidia long-fusiform, falcate, acute at both ends, 1-septate, (10–)12–18(–20) × 3.5–4.5 µm, smooth, hyaline. Unfortunately, the culture was no longer viable.

Stylonectria tuedensis Lechat & J. Fourn., *sp. nov.* Fig. 3
Mycobank: MB 838506

Diagnosis: Differs from *S. wegeliniana*, the only other known species with ornamented ascospores, by having smaller ascomata, smaller ascospores 10–12(–13) × 4.5–5.5 µm vs. 15–18 × 7.5–9 µm, and wider, 1–3-septate macroconidia.

Holotype: France, Savoie, Les Allues, Plan de Tuéda, 45.355549° N 6.596539° E,1740 m asl., on dead stromata of *Eutypella* sp. associated with *Ascocoryne* sp. asexual morph on *Sorbus aucuparia* L., 24 Aug. 2020, *leg*. A. Mombert, CLL20025 (LIP CLL20025), ITS GenBank sequence: MW479391.

Etymology: the epithet *tuedensis* refers to the Tueda Nature Reserve where this species was collected.

Ascomata aggregated in groups of (5–)10–50(–80) on stromata of host, subglobose to obpyriform, first orange, turning dark red at maturity, partially immersed between ostioles of host, (240–)260–300(–340) µm high, (180–)200–260(–280) µm diam., orange-red to dark red, not collapsing when dry, becoming purple in 3% KOH and yellow in lactic acid. **Ascomatal apex** with a flat disc, concolourous to much darker than ascomatal wall, (100–)110–160(–180) µm diam., composed of thick-walled, narrowly clavate cells with orange wall 15–25 × 2–3 µm, with a tiny, hyaline central papilla. **Ascomatal surface** smooth to slightly roughened, composed of cells of undefined shape forming a *textura epidermoidea*. **Ascomatal wall** in vertical section 25–35(–40) µm thick, composed of two regions; outer region 20–25 µm thick, composed of globose to ellipsoidal, thick-walled cells (4–)8–12 × (4–)5–7(–10) µm, with orange wall 1.5–2 µm thick; inner region 8–10(–15) µm thick, of hyaline, compressed, elongate cells (7–)10–18 × (4–)5–8 µm, apically merging with periphyses. **Asci** cylindrical, short-stipitate (65–)70–80 (85) × 5–7 µm (Me = 75 × 6 µm, n = 20), apex flat to slightly rounded, with a faint ring-like apical thickening, containing 8 uniseriate ascospores; filamentose to narrowly moniliform paraphyses inserted between asci. **Ascospores** (9–)10–12(–13) × (4.5–)5–5.5 µm (Me = 11 × 5.3 µm, n = 50), ellipsoidal, equally 1-septate, hyaline, becoming pale brown, thick-walled when mature, smooth to faintly spinulose, ornamentation often inconspicuous.

Cultural characteristics: After two weeks at 25°C on Difco PDA, colony 2–2.5 cm diam., floccose, pale orange in centre, white to cream in middle area, sporulating at slimy margin, producing a fusarium-like asexual morph. No microconidia produced. Macroconidia long-fusiform, falcate, acute at both ends, (0–)1–3-septate, (14–)18–30(–35) × 3.5–4.5(–5) µm, smooth, hyaline. The culture was no longer viable.

Discussion

Based on a molecular phylogenetic re-evaluation of cosmospora-like nectriaceaous fungi by Gräfenhan *et al.* (2011), the genus *Stylonectria*, introduced by Höhnel (1915), currently accommodates some fungicolous species previously assigned to *Cosmospora* Rabenh. (Rossman *et al.*, 1999), *Dialonectria* (Sacc.) Cooke, and *Nectria* Fr. (Booth, 1959; Samuels, 1976). Gräfenhan *et al.* (2011) recognized four species in *Stylonectria*, including *S. applanata* Höhn., the type species, *S. carpini* Gräfenhan, *S. purtonii* (Grev.) Gräfenhan, and *S. wegeliniana* (Rehm) Gräfenhan, Voglmayr & Jaklitsch. They are characterised by a fungicolous lifestyle on old stromata of black pyrenomycetes, ascomata with a wall of two regions changing

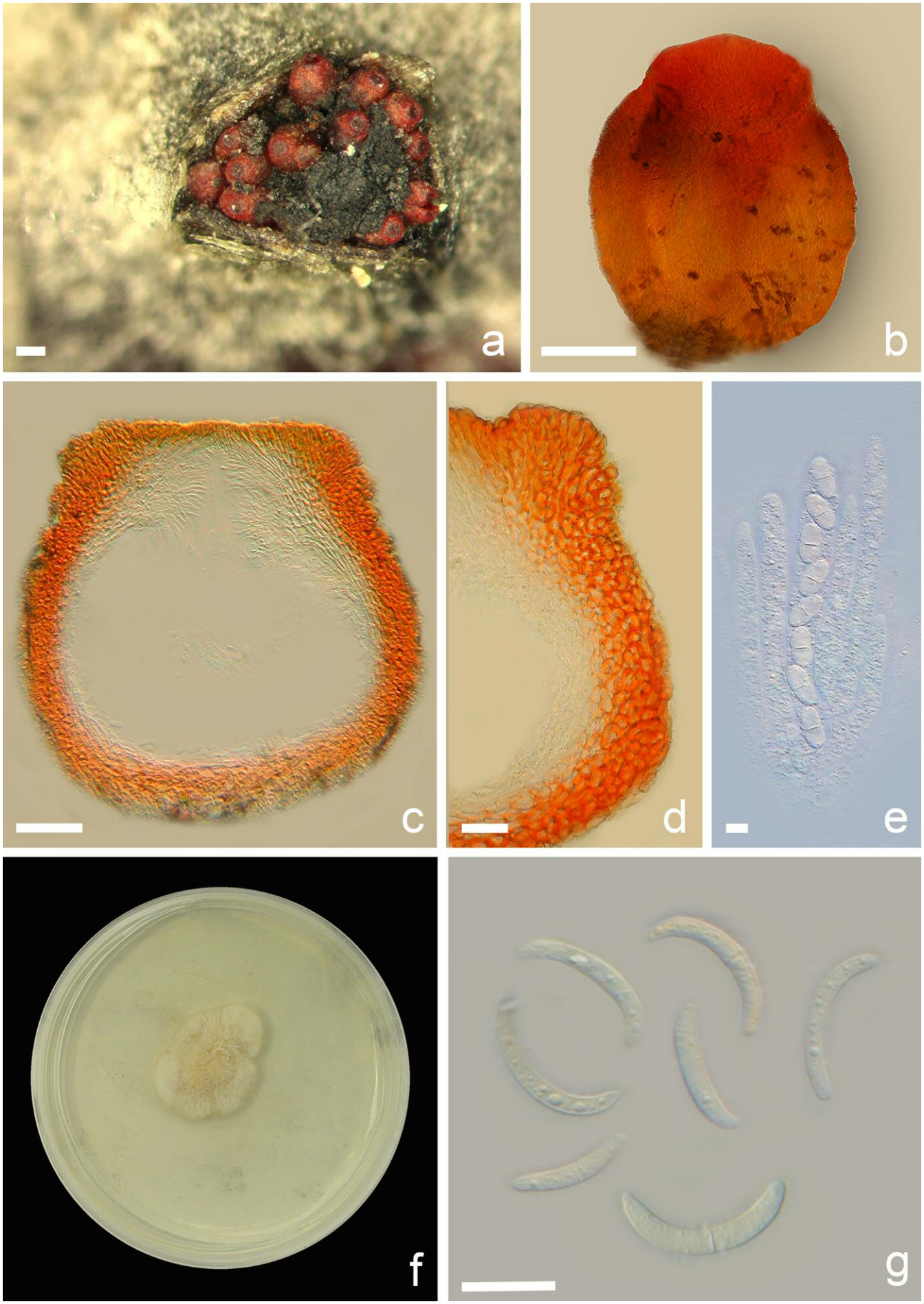

Fig. 2 – a-g: *Stylonectria alpina* (CLL20026 Holotype); a: Ascomata in natural environment; b: Perithecium in water; c: Vertical section through a perithecium; d: Close-up of lateral ascomatal wall in vertical section; e: Asci and ascospores in lactic cotton blue; f: Culture at one week; g: Conidia in lactic acid. Scale bars: a = 200 µm; b = 100 µm; c = 50 µm; d = 20 µm; e = 5 µm; g = 10 µm.

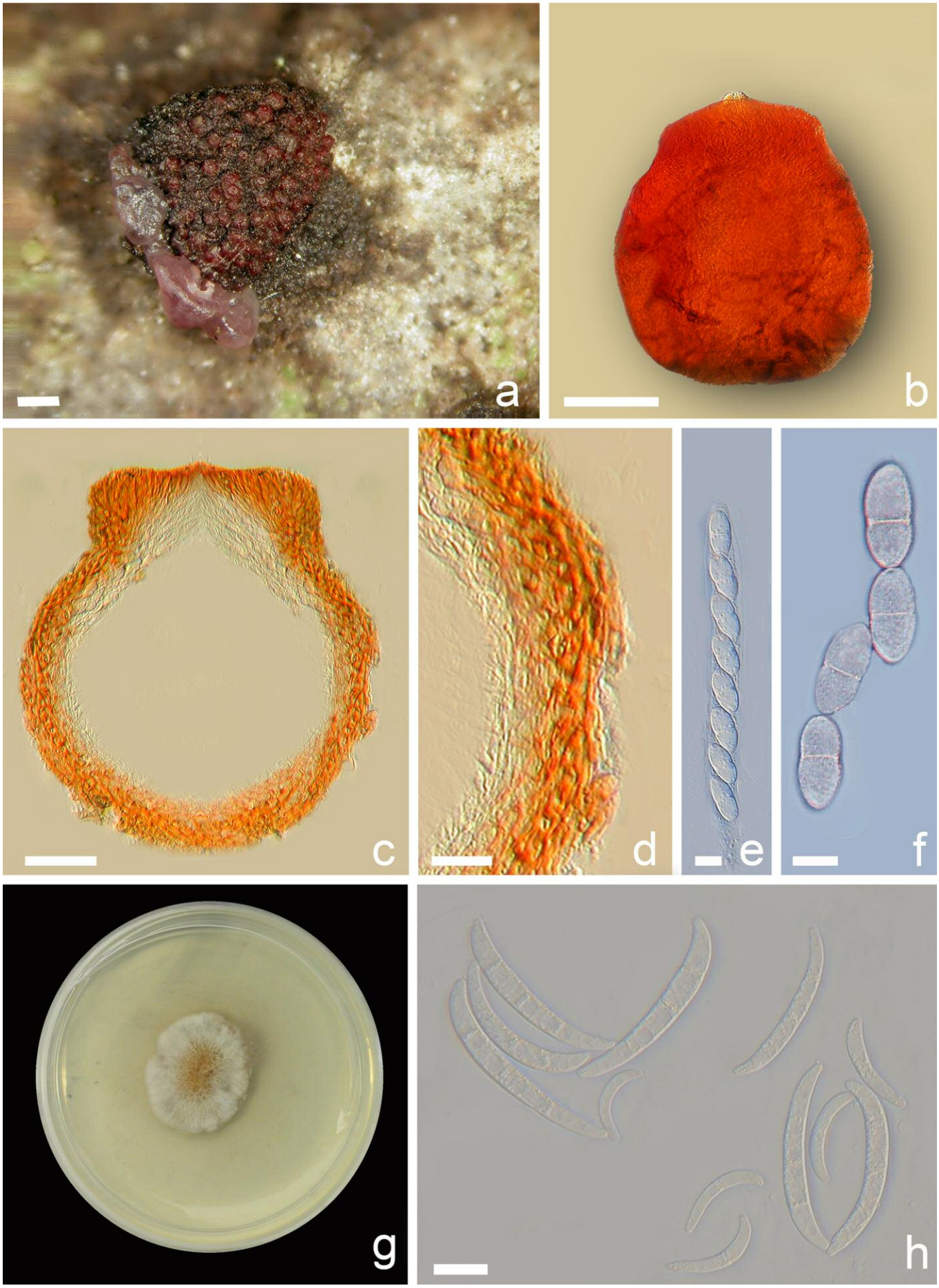

Fig. 3 – a-g: *Stylonectria tuedensis* (CLL20025 Holotype); a: Ascomata in natural environment associated with asexual morph of *Ascocoryne* sp.; b: Perithecium in water; c: Vertical section through perithecium; d: Close-up of lateral ascomatal wall in vertical section; e: Asci and ascospores in lactic cotton blue; f: Close-up of ascospores in lactic cotton blue; g: Culture at one week; h: Conidia from culture. Scale bars: a = 500 µm; b = 100 µm; c = 50 µm; d = 20 µm; e, f = 5 µm; h = 10 µm.

colour in 3% KOH or lactic acid, and a conspicuous flattened apical disc, as well as a fusarium-like asexual morph. They were shown to cluster on a well-supported clade distant from other related genera. Recently, two new species were introduced, the first by Lechat *et al*. (2015) as *S. norvegica* Lechat, J. Fourn. & Nordén and the second by Zeng *et al.* (2020) as *S. qilianshanensis* Z.Q. Zeng & W.Y. Zhuang.

The new species described above match well this ecological and morphological definition and our phylogenetic analysis of ITS sequences (Fig. 1) shows that the two new species are indeed nested in the *Stylonectria* clade but distantly related to the known species. The closest species to our two fungi is *S. purtonii*, which is mainly distinguished by narrower ascospores 8–11 × 3.5–4.5 µm and macroconidia 20–24 × 1.5-2 µm, and the production of microconidia in culture, according to Booth (1959) and Samuels (1976). Moreover, *S. alpina* and *S. tuedensis* differ from *S. purtonii* in having respectively only 96 and 93% similarity of their ITS sequences.

Stylonectria alpina is morphologically similar to *S. norvegica* but differs in having larger ascomata (280–)320–370(–390) µm high, (280–)300–330(–340) µm diam. vs. 250–330(–350) µm high, 200–300 µm diam., larger macroconidia (10–)12–18(–20) × 3.5–4.5 µm vs. (8.5–)10–13(–14) × 1.8–2.5 µm, and by its occurrence on *Diplodia* sp. while *S. norvegica* occurs on *Diatrypella* sp. (Lechat *et al*., 2015); moreover, both species have only 95% similarity of their ITS sequences.

Stylonectria tuedensis and *S. wegeliniana* are the only species known to have ornamented ascospores, but the former differs in having smaller ascomata (240–)260–300(–340) µm high, (180–)200–260(–280) µm diam. vs. 300–450 µm high, 250–350 µm diam., smaller ascospores 10–12(–13) × 4.5–5.5 µm vs. 15–18 × 7.5–9 µm, producing only macroconidia 1–3-septate in culture, while *S. wegeliniana* produces 1-celled microconidia and 1-septate macroconidia (Jaklitsch, comm. pers.), both species having only 91% similarity of their ITS sequences.

The combination of molecular data and morphological, as well as cultural characters therefore clearly supports the recognition of *S. alpina* and *S. tuedensis* as distinct species, bringing the number of known species to eight, suggesting that *Stylonectria* is more diverse than previously assumed.

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Authors' contributions

Christian Lechat was responsible for the study conception, morphological studies, *in vitro* cultures, phylogenetic analyses, figures and plates design, registrations to MycoBank and GenBank. The first draft of the manuscript was written by Christian Lechat and was critically reviewed by Jacques Fournier who proposed an enhanced version. Michel Hairaud and Andgelo Mombert provided voucher specimens and made preliminary morphological observations. All authors read and approved the final manuscript.

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