Flammocladiella anomiae, a new hypocrealean species from France and Bulgaria

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Abstract: *Flammocladiella anomiae* is described and illustrated based on material occurring on ascomata of *Massaria anomia* on *Robinia pseudoacacia* in France and Bulgaria. The placement of this fungus in the genus *Flammocladiella* and its segregation from *F. decora* are based on study of its sexual-asexual morphs, primarily showing differences in ascomatal and conidial dimensions, and phylogenetic comparison of ITS1-5.8S-ITS2 and LSU sequences with those of *F. decora* and species belonging to families in the *Hypocreales*.
Keywords: Ascomycota, *Flammocladiellaceae*, *Hypocreales*, *Massaria*, rDNA, *Robinia pseudoacacia*, taxonomy.

Résumé : *Flammocladiella anomiae* est décrite et illustrée d'après du matériel récolté sur des ascomes de *Massaria anomia* sur *Robinia pseudoacacia* en France et en Bulgarie. Le placement de ce champignon dans le genre *Flammocladiella* et sa séparation de *F. decora* reposent sur les stades sexué/asexué, montrant principalement des différences de dimensions des ascomes et des conidies, et sur la comparaison phylogénétique des séquences ITS1-5.8S-ITS2 et LSU avec celles de *F. decora* et celles d'espèces appartenant à diverses familles parmi les Hypocréales.
Mots-clés : ADN ribosomal, Ascomycota, *Flammocladiellaceae*, Hypocréales, *Massaria*, *Robinia pseudoacacia*, taxinomie.

Introduction

In the continuation of our survey of hypocrealean fungi in temperate area, a species of *Flammocladiella* Crous, L. Lombard & R.K. Schumach. was repeatedly collected on dead stromata of *Massaria anomia* (Fr.) Petr. (*Dothideomycetes*) on its host *Robinia pseudoacacia* L. in France and Bulgaria. The formerly monotypic genus *Flammocladiella* was shown to have an isolated position within the *Hypocreales*, distinct from the *Bionectriaceae*, the most closely related family, justifying the family *Flammocladiellaceae* Crous, L. Lombard & R.K. Schumach. (Crous *et al.*., 2015). *Flammocladiella* was described to accommodate *F. aceris* Crous, L. Lombard & R.K. Schumach., a fungus growing on *Acer platanoides* bark featuring yellowish to yellow-orange ascomata clustered in small groups, with smooth, KOH- wall and ostiolate papillae, associated with a sporodochial asexual morph yielding long cylindrical conidia aggregated in flame-like orange masses. The connection with the underlying ascomata was overlooked. As shown by Lechat & Fournier (2018), *F. aceris* is a synonym of *Nectria decora* (Wallr.) Fuckel, a fungus of uncertain taxonomic position long known to have a fungicolous lifestyle associated with *Massaria* spp. (Beenken, 1997; Gilgen & Senn-Irlet, 2014). This name was recombined as *F. decora* (Wallr.) Lechat & J. Fourn. based on morphological, cultural, ecological and molecular data by Lechat & Fournier (2018).

The fungus occurring on stromata of *M. anomia* mentioned above was first referred to *F. decora* owing to its similar habit and lifestyle, including similar ascospores dimensions and morphology. Three fresh collections from various origins prompted us to carry out cultural and molecular investigations in order to elucidate the degree of relationship between *F. decora* and the fungus on *M. anomia*. In this study we present our results supporting the placement of this fungus in *Flammocladiella* and leading to the segregation of the new species *F. anomiae* distinct from *F. decora*.

Material 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 holotype is deposited in LIP herbarium (University of Lille, France), paratypes collections are deposited in SOMF (Bulgarian Academy of Sciences, Sofia, Bulgaria), and ex-type cultures are deposited in the CBS Collection of the Westerdijk Fungal Biodiversity Institute (Utrecht, The Netherlands) and CIRM (Centre International des Ressources Microbiennes, Marseille, France). Dry clusters of ascomata from the paratype collection were used for genetic analysis. Cultures of living specimens were made on PDA (Potato Dextrose Agar) with 5 mg/l of streptomycin in Petri dishes 9 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 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. 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

Flammocladiella anomiae Lechat & J. Fourn., *sp. nov.* Fig. 3, table 1 – MycoBank MB 833104

Holotype: France, Ariège, Rimont, Las Muros, N 43.013571°, E 1.287768°, alt. 465 m, on ascomata of *Massaria anomia* on a branchlet of *Robinia pseudoacacia*, 6 Dec. 2017, *leg*. J. Fournier, JF17087 (LIP); ex-type culture CBS144256. GenBank ITS and LSU sequences MN597423 and MN597425.

Etymology: The epithet "*anomiae*" refers to the host *Massaria anomia*.

Description:

Ascomata crowded on ostiolar region of immersed ascomata of *Massaria anomia*, seated on a prosenchymatous pseudostroma arising from inside dead ascomata, in groups of 3–20, widely obpyriform to subglobose, (160–)175–240(–250) × (160–)180–220 (–240) µm (Me = 220 × 200 µm, n = 35), orange, appearing pale orange to pale luteous due to a mat of hyaline hyphal elements covering ascomatal wall, collapsing by lateral pinching or not collapsing when dry, not changing colour in 3% KOH or lactic acid. Perithecial apex rounded with a bright orange papilla up to 30–40 µm high, 100 µm diam., composed of a palisade of clavate, thick-walled cells 18–32 × 2.5–3.5 µm, merging with periphyses. Ascomatal surface composed of subisodiametric angular cells 10–12 × 6–10 µm, covered by smooth, hyaline, monilioid hyphal elements 2.5–3.5 µm diam., arising from base, evolving to terminal, subglobose to globose cells reaching 3–6 µm diam. **Ascomatal wall** (excluding hyphal elements) 25–30(–35) µm thick, composed of two regions: outer region 15–20(–25) µm wide, of subglobose to ellipsoidal cells 4–10 × 4–6 µm, with pale orange walls 1–2 µm thick; inner region 8–10 µm wide, of elongate, flattened cells 10–15 × 2–5 µm, with hyaline walls 1–1.5 µm thick. **Asci** (80–)90–110(–120) × (12–)14–16(–20) µm (Me = 100 × 15 µm, n = 20), clavate, shortly stipitate, apex rounded without apical apparatus, containing 8 ascospores obliquely uniseriate or biseriate above and uniseriate below, completely filling each ascus, interspersed with moniliform paraphyses up to 18 µm diam., filled with numerous orange oily droplets. **Ascospores** (22–)26–32(–34) × 6–8 µm (Me = 29 × 7 µm, n = 50), narrowly ellipsoidal, attenuated at ends to fusiform, transversely 3-septate, constricted at septum when mature, hyaline, finely verrucose.

Asexual morph: flammocladiella-like.

Cultural characteristics: After three weeks on PDA, colony reaching 20–25 mm diam., pale yellow, slimy, pink in central area, white at margin. Mycelium composed of smooth septate hyphae 2–3 µm diam. Conidiophores 2.5–3 µm diam., branched with a simple conidiogenous cell producing subcylindrical hyaline conidia, irregularly curved, rounded at tip, attenuated and truncate at base, 1–3-septate, smooth, (30–)37–45(–48) × 2–2.5 µm.

Asexual morph in natural environment: Sporodochia arising from inside dead ascomata of *M. anomia*, covering ostiolar region, white when fresh, forming yellow to bright orange masses frequently becoming flame-shaped when dry. Conidia solitary, appearing fasciculate, flexuous, 60–120(–130) × 2.5–3 µm, truncate at upper tip, attenuated at base, 1–3-sepate when measuring up to 60 µm long, becoming 4–7-septate when exceeding this dimension.

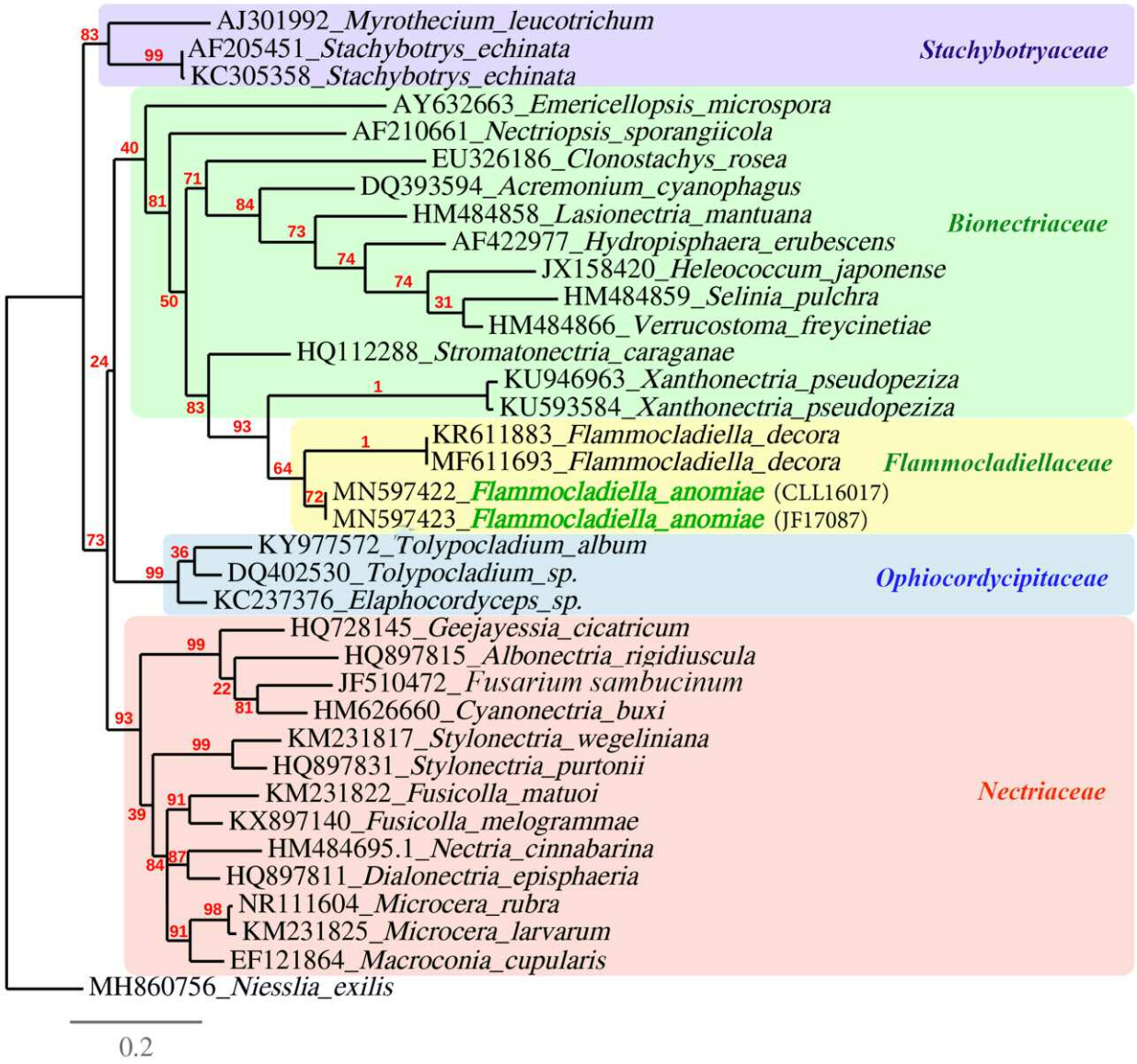

Fig. 1 – Maximum likelihood phylogeny of *Flammocladiella anomiae* (lnL = 5101.86102) inferred by PhymL 3.0, model tS93 from a 580 bp matrix of ITS1-5.8S-ITS2 sequences, rooted with *Niesslia exilis*.

Additional specimens examined: Bulgaria, Eastern Forebalkan, Lovech distr., Troyan municipality, south-east of Golyama Zhelyazna village, near Toplya cave, by main road, N 42°56′54″, E 24°29′13.2″, alt. 485 m, on stromata of *Massaria anomia*, on a thin branch of *Robinia pseudoacacia*, 3 Sep. 2016, *leg*. D. Stoykov, CLL 16017 (LIP), Culture: CBS 142775, Genbank ITS and LSU sequences MN597422 and MN597426; North of Golyama Zhelyazna village, Mikrenska Usoyna forest, Vlaskovska Mahala locality, N 43°00′, E 24°29′40″, alt. 430 m, on stromata of *Massaria anomia* on *Robinia pseudoacacia*, 27 Oct. 2017, *leg*. D. Stoykov (SOMF 30203), Genbank LSU sequence MN597424. France, Côte-d'Or, Bèze, Les Combottes, N 47°28′28.8″, E 5°16′5.66″, alt. 222 m, on stromata of *Massaria anomia* on *Robinia pseudoacacia*, 19 Sep. 2009, *leg*. A. Gardiennet AG09250 (LIP).

Discussion

At first sight, the new species described above is morphologically indistinguishable from *F. decora* in having ascomata associated with *Massaria* spp., of the same colour not changing in 3% KOH or lactic acid and coated by a white hyphal mat, and similar ascospores in shape, septation, ornamentation and dimensions. Slight differences between the new species and *F. decora*, suggesting a different taxonomic status, are its smaller ascomata 220 × 200 µm on average vs 300 × 280 µm, shorter conidia of asexual morph in culture and shorter and less septate conidia of asexual morph in nature (Table 1). As these results are consistent in all the collections we studied, we postulated they may be significant, which was confirmed using the molecular phylogenies.

The segregation of *F. anomia* from *F. decora* that we introduce here is primarily based on the phylogenetic comparison of their ITS and LSU sequences (Figs. 1 and 2), showing that *F. anomiae* is nested in the *Flammocladiaceae* clade on a sister branch to *F. decora*, the two species having 92 and 97% similarity of their ITS and LSU sequences respectively. Our phylogram inferred from ITS sequences (Fig. 1) showed that the closest genus to *Flammocladiella* is the monotypic genus *Xanthonectria* represented by *X. pseudopeziza* (Desm.) Lechat, J. Fourn. & P.-A. Moreau. This species differs in having glabrous ascomata, ascomatal wall 40–65 µm thick and composed

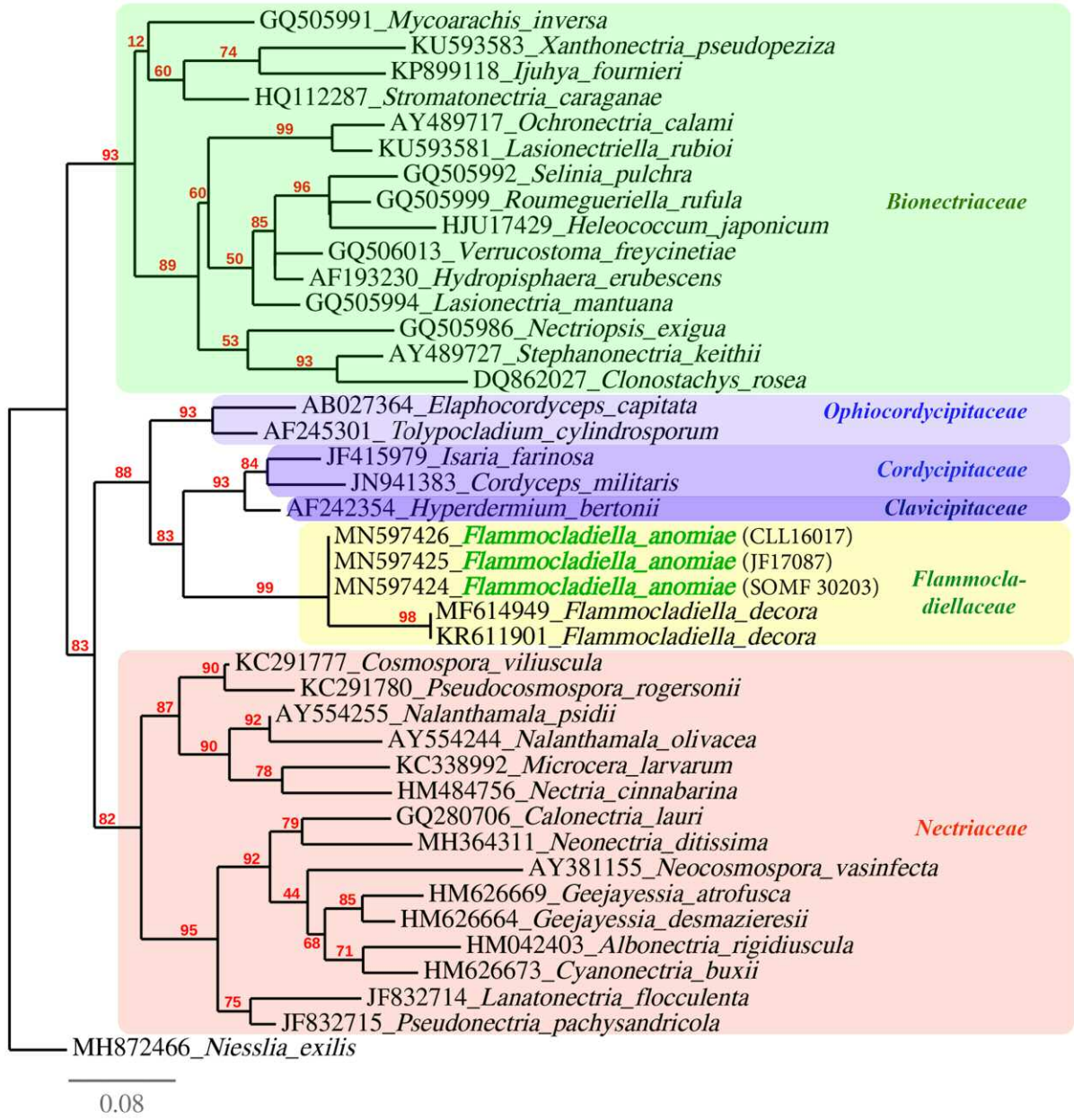

Fig. 2 – Maximum likelihood phylogeny of *Flammocladiella anomiae* (lnL = 3471.64744) inferred by PhymL 3.0, model tS93 from a 840 bp matrix of LSU sequences, rooted with *Niesslia exilis*.

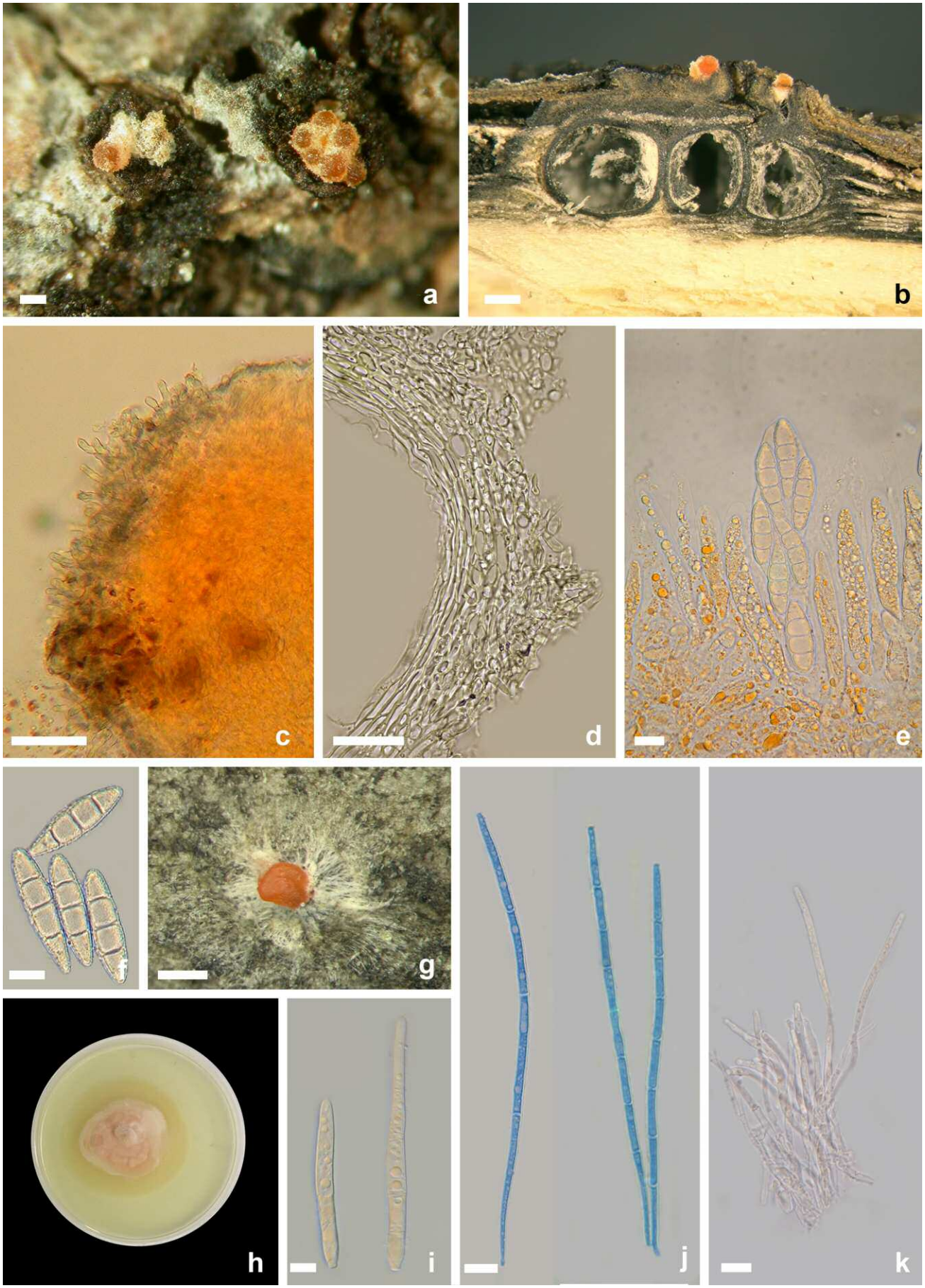

Fig. 3 – a–k: *Flammocladiella anomiae* (JF17087 Holotype) a : Ascomata in natural environment; b: Vertical section of ascomata of *Massaria anomia*, showing white mycelium developing inside and connected with superficial ascomata of *F. anomiae*; c: Ascomatal wall in water showing monilioid, hyphal elements arising from base; d: Vertical section of ascomatal wall in water; e: Asci and ascospores in water showing hamathecium with orange oily droplets; f: Close-up of ascospores in water; g: Sporodochium of asexual morph in natural environment; h: Culture at three weeks; i: Conidia from culture; j: Conidia from nature, in lactic acid with cotton blue; k: Conidiophores and conidia from nature, in lactic acid. Scale bars: a, b, g = 200 µm; c = 100 µm; d = 20 µm; e, f, j, k =10 µm; i = 5 µm.

Table 1 – Comparison of dimensions of ascomata in nature, septation and dimensions of conidia of *F. decora* and *F. anomiae* from natural environment and culture

	Ascomata in nature in µm	Conidia in nature in µm	Conidia in nature septa	Conidia in culture in µm	Conidia in culture septa
F. decora	300 × 280	37–140 × 2.5–3	1–3–6–10	37–65 × 2–2.5	1–3
F. anomiae	220 × 200	60–120 × 2.5–3	1–3–4–7	37–45 × 2–2.5	1–3

of three regions, ascospores up to 60 µm long, 5–7-septate, and belongs to the *Bionectriaceae* (Lechat & Fournier, 2016). In agreement with the results obtained by Crous *et al.*. (2015), our phylogram inferred from LSU sequences (Fig. 2) showed that the *Flammocladiella* clade is clearly distinct from the *Bionectriaceae* and the *Nectriaceae* and has closest affinities with the distantly related families *Clavicipitaceae*, *Cordicypitaceae* and *Ophiocordicypitaceae*, which was discussed by the authors cited above.

Flammocladiella decora as one of its synonyms, primarily *Nectria decora* (Wallr.) Fuckel, has been reported mainly on *Massaria inquinans* (Tode) De Not. or *Massaria* sp. on *Acer* as is the type of the basionym *Sphaeria decora* Wallr. It has also been reported or specimens exist on *M. conspurcata* Sacc. on *Prunus*, *M. ulmi* Fuckel on *Ulmus* and *Massaria* sp. on *Fraxinus* as well as *M. anomia* on *Robinia* (Farr & Rossman, 2019; Rossman, 1983). Given the possible host-specificity of *F. anomiae* for *M. anomia*, most likely the reports and specimens on *M. anomia* and *Massaria* sp. on *Robinia* are *F. anomiae*. According to Voglmayr & Jaklitsch (2011), *Massaria* spp. are highly host-specific and *M. anomia* is the only species known to occur on *Fabaceae*. Further phylogenetic investigations are needed to assess the degree of host-specificity between *Flammocladiella* spp. and their *Massaria* hosts as well as to determine if the specimens on different hosts such as *M. conspurcata* on *Prunus* represent additional new species.

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