Fouragea gyrophorica sp. nov. from China, with morphological and phylogenetic evidence

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Abstract. A new species *Fouragea gyrophorica* is described from China. It is most similar to *F. vegae*, but differs by its sparsely to densely branched apothecia, and it often has an obvious prothallus, and the presence of gyrophoric acid detected by TLC. This is the first report that *Fouragea* contains lichen substances. An analysis of its relationships based on molecular phylogeny is given. A world key to the species of *Fouragea* is also presented.

Key words. Lichenized fungi, taxonomy, Opegraphaceae, foliicolous.

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The genus *Opegrapha* Ach. is widely distributed, and about 300 species have been reported in the world, including seventy lichenicolous species (Coppins et al. 2021; Diederich et al. 2018; Kirk et al. 2008). Most species live on the bark of forest trees in warm temperate and tropical regions, but a few live on leaves, rocks and soil (Cannon et al. 2021; Lücking 2008). In 1880, Trevisan established the genus *Fouragea* for Montagne's *Opegrapha filicina*, which is distinguished from the common, corticolous species, especially by its thin ascocarps (due to the foliicolous lifestyle), basally without dark excipulum, and by the photobiont *Phycopeltis* instead of *Trentepohlia*. However, some lichenologists assumed those biological characters were not sufficient to consider it as a separate genus and they still described this species in *Opegrapha*. (Santesson 1952). In 2011, the genus *Fouragea* Trevis. was proposed by Ertz & Tehler again to accommodate some species of foliicolous *Opegrapha* (Ertz & Tehler 2011; Lücking 2008). Further support of this was provided by molecular evidence, which showed that the foliicolous *O. filicina* and *O. viridistellata* Sérus., Lücking & Sparrius, formed an independent lineage at the base of the Opegraphaceae (Ertz et al. 2009; Ertz & Tehler 2011). Thus, *Fouragea* was reinstated for foliicolous *Opegrapha* species based on the phylogenetic results, as was suggested in Ertz & Tehler 2011 (Frisch et al. 2014). However, the foliicolous species of *Opegrapha* with more than 7-septate ascospores and those with goniocystangia (Sérusiaux 1985) still need to be further studied with molecular data.

The genus *Fouragea* belongs to the family Opegraphaceae (Ascomycota, Arthoniomycetes, Arthoniales), and the type species is *Fouragea filicina* (Mont.) Trevis. There are six species known in the genus, which are mainly distributed in neotropical and paleotropical areas (Ertz 2020; Frisch et al. 2014; Lücking et al. 2016). *Fouragea* has been regarded as lacking lichen substances (Ertz 2020). The humid tropical and subtropical forests in South China harbor a rich diversity of foliicolous lichens, but these areas have been poorly studied. Until now, only *F. filicina* was reported from China (as *Opegrapha filicina* in Wei & Jiang 1991). During our study, one species new to science was discovered and we here describe it using a combination of phenotypic and molecular data.

Material & methods

Specimens. The specimens were collected in Hainan Province, China, and are preserved in the

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Fungarium-Lichenarium of the Institute of Microbiology, Chinese Academy of Sciences (HMAS-L). The morphological studies were observed and photographed by stereomicroscope (MZ101) and light microscope (Zeiss Axioscope 2, Zeiss Axio Imager A2). The lichen products were detected by standardized thin layer chromatography (TLC) using solvent systems B and C (Elix 2014; Orange et al. 2001) and comparison with authentic samples.

DNA extraction, amplification and sequencing. Genomic DNA was extracted from the specimens using Plant Direct PCR kit. The nuclear ribosomal large subunit (nuLSU) was amplified using the primers LIC15R/LR6 (Miadlikowska et al. 2002; Vilgalys & Hester 1990), and the mitochondrial ribosomal small subunit (mtSSU) was amplified using primers mrSSU1/mrSSU3R (Zoller et al.1999). Reactions were carried out in 25 μL reaction system containing 2 μL each primer solution (10 μM), 1 μL genomic DNA, 7 μL ddH2O, and 13 μL 2 × Taq PCR MasterMix®. PCR conditions of nuLSU comprised: initial denaturation for 15 min at 95°C, followed by 45 cycles of 45 s at 95°C, 45 s at 53°C, 1 min at 72°C, and a final extension for 7 min at 72°C (Frisch et al. 2014). PCR conditions of mtSSU comprised: initial denaturation for 3 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 52°C, 1 min at 72°C , and a final extension for 7 min at 72°C. The PCR products were checked on 1% agarose electrophoresis gels stained with ethidium bromide.

Phylogenetic analysis. DNA sequences were manually assembled with SeqMan implemented in Lasergene v7.1, with consideration of gaps and ambiguous sequences (Swindell & Plasterer 1997). The six newly generated mtSSU and nuLSU sequences were submitted to GenBank and analyzed with related sequences of the Opegraphaceae which obtained from GenBank by Basic Local Alignment Search Tool (BLAST). In addition, other genera in Opegraphaceae having molecular sequence data available, including *Combea* De Not., *Dictyographa* Müll.Arg., *Dolichocarpus* R.Sant., *Ingaderia* Darb., *Llimonaea* Egea & Torrente, *Nyungwea* Sérus., Eb.Fisch. & Killmann, *Opegrapha*, *Paraingaderia* Ertz & Tehler, *Paralecanographa* Ertz & Tehler, *Paraschismatomma* Ertz & Tehler, *Pentagenella* Darb., *Schizopelte* Th.Fr., and *Sparria* Ertz & Tehler, were all included in the phylogenetic analysis (**Table 1**).

The sequences were aligned by using the online website MAFFT V.7.505 (Katoh et al. 2019). The software BioEdit v7.2 was used to check aligned matrices (Hall 1999). To assess any potential conflicts in the gene tree topologies for those two loci, single-locus matrices were analyzed using maximum likelihood (ML) in RAxML v.8.2.12 (Stamatakis 2014). Conflicts were evaluated manually by comparing the topology and position of each of the major clades in relation to their topology and position in the concatenated analysis, only when no significant conflict was detected, the two markers nuLSU and mtSSU were combined (Jiang et al. 2020). *Lecanactis abietina* (Ach.) Körb. and *L. borbonica* Ertz & Tehler belonging to Roccellaceae, the related family with Opegraphaceae, were chosen as outgroup (Ertz 2020).

The maximum likelihood (ML) analysis involving 1000 bootstrap pseudoreplicates was generated in RAxML-HPC v.8.2.12 (Stamatakis 2014). The best-fit substitution model was selected using jModelTest 2 (Darriba et al. 2012). In the maximum likelihood analyse, the GTR + I + G model was selected as the best model accordding to Akaike Information Criterion (AIC). Bayesian analyses were performed by using MrBayes v.3.2.7 (Ronquist et al. 2012). Appropriate nucleotide substitution models and parameters were determined by Modeltest v. 3.7 (Posada & Crandall 1998). Posterior probabilities (PP) were performed by Markov Chain Monte Carlo sampling (MCMC) (Huelsenbeck & Ronquist 2001; Rannala & Yang 1996; Zhaxybayeva & Gogarten 2002). Six simultaneous Markov chains were run for 1,500,000 generations, and trees were sampled every 100th generation. The run was automatically stopped when the average standard deviation of split frequencies reached below 0.01 (Maharachchikumbura et al. 2015). The value of burn-in was set to discard 25% of trees when calculating the posterior probabilities. Bayesian posterior probabilities were obtained from the 50% majority rule consensus of the trees kept. The phylogenetic tree was visualized and edited by iTOl V.6.0 (Letunic & Bork 2021). The bootstrap support value above 70% and posterior probabilities above 95% were considered threshold values (Jiang et al. 2021).

Table 1. Species and DNA sequences used in the phylogenetic analyses. Newly generated sequences are indicated in bold.

Species	Voucher	GenBank No.		Country
		nuLSU	mtSSU	
Combea mollusca	Tehler7725 (S)	EF081383	AY571384	South Africa
Dictyographa arabica	Ertz 11678 (BR)	HQ454570	–	Galapagos Islands
D. varians	Tehler 9346 (S)	HQ454576	–	Yemen, Socotra Island
Dolichocarpus chilensis	Tehler 8373 (S)	HQ454528	–	Chile
Fouragea filicina	Ertz 7994 (BR)	EU704095	EU704067	Rwanda
***F. gyrophorica* sp. nov.**	**HMAS-L 0147815**	**OP751508**	**OP751543**	**China**
***F. gyrophorica* sp. nov.**	**HMAS-L 0147794**	**OP751509**	**OP751544**	**China**
***F. gyrophorica* sp. nov.**	**HMAS-L 0147810**	**OP751510**	**OP751545**	**China**
F. vegae	Ertz 21823 (BR)	MT944352	–	Mayotte
F. viridistellata	Ertz 4795 (BR)	EU704104	EU704076	Reunion
Ingaderia pulcherrima	Tehler 9886 (S)	HQ454539	–	Chile
Lecanactis abietina	Ertz 5068 (BR)	AY548812	AY548813	Belgium
L. borbonica	Ertz 4780 (BR)	EU704092	EU704060	La Reúnion
Llimonaea flexuosa	Ertz 13880 (BR)	HQ454569	–	Canary Islands
Nyungwea anguinella	Ertz 10027 (BR)	EU704086	EU704054	Gabon
N. pallida	Frisch11/Ug24 (UPS)	KJ851066	KJ851023	Uganda
Opegrapha lithyrga	Ertz 8784 (BR)	EU704096	EU704068	Belgium
O. niveoatra	Ertz 7529 (BR)	EU704098	EU704070	Belgium
O. niveoatra	PRA-JV23582	–	OK465622	Czech Republic
O. vermicellifera	Ertz 7562 (BR)	EU704105	EU704077	Belgium
O. vulgata	Ertz 7564 (BR)	EU704108	EU704080	Belgium
Paraingaderia placodioidea	Tehler 9315 (S)	HQ454632	–	Yemen, Socotra Island
Paralecanographa grumulosa	Tehler 9809 (S)	HQ454542	–	Great Britain, Gibraltar
Paraschismatomma ochroleucum	Tehler 9090 (S)	HQ454617	–	Mexico
Pentagenella gracillima	Tehler 8366 (S)	HQ454534	–	Chile
P. gracillima	Tehler 9897 (S)	HQ454536	–	Chile
Schizopelte crustosa	Tehler 9114 (S)	HQ454563	–	Mexico
S. parishii	Tehler 9099 (S)	HQ454532	–	Mexico
Sparria endlicheri	Ertz 14067 (BR)	HQ454512	–	Belgium

Results and Discussion

The dataset included 3 LSU sequences, and 3 mtSSU sequences newly generated in this study. The LSU and mtSSU sequences were analyzed separately and subsequently compared with the two-marker tree based on a concatenated alignment with 1644 bp (**Supplementary File S1**). No different relationships were revealed by the separate analyses for the LSU and mtSSU datasets, all with reciprocal posterior probabilities (PP) of 0.99; therefore, these two markers can be combined. The phylogenetic tree constructed from ML based on the nuLSU and mtSSU sequences is shown in **Fig. 1**. The result showed that the genus *Fouragea* formed an independent clade. Thus, the genus *Opegrapha* s.str. and *Fouragea* are two well-supported lineages, and it is consistent with those results in other publications (Ertz 2020; Ertz et al. 2009; Ertz & Tehler 2011; Frisch et al. 2014).

Within the well-supported (92/1.00) monophyletic four-species clade of *Fouragea*, the species *F. gyrophorica* X.D.Xue & S.H.Jiang formed an independent clade, sister to *F. viridistellata* (Sérus., Lücking & Sparrius) Ertz & Frisch, *F. vegae* (R.Sant.) Ertz and *F. filicina* (Mont.) Trevis. Besides, this species contains gyrophoric acid, but other *Fouragea* species have no secondary metabolites. Combining other morphological characters, it can be recognized as a new species.

Taxonomy

Fouragea gyrophorica X.D.Xue & S.H.Jiang, *sp. nov*. **Fig. 2**

Fungal Names FN571223

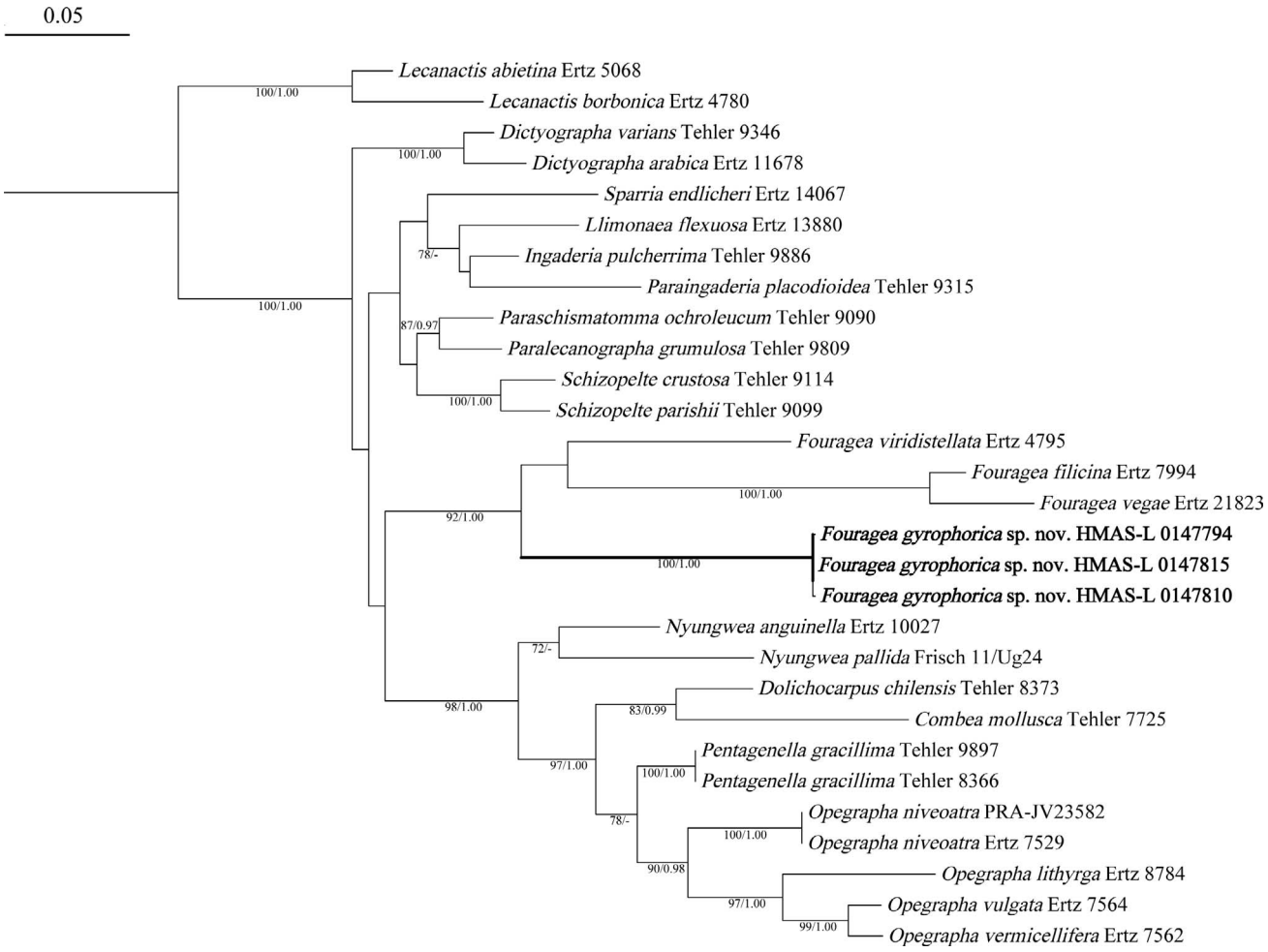

Figure 1. Maximum likelihood phylogenetic tree generated from the analysis of the concatenated nuLSU + mtSSU sequences of the *Opegraphaceae*, and closely related species downloaded from GenBank. The newly sequenced samples of *Fouragea gyrophorica* is in bold. The numbers at the nodes indicate bootstrap values (BS ≥70%) / Bayesian Posterior Probabilities (BPP ≥0.95). Genetic distance scale = 0.05 changes per site.

The new species is most similar to Fouragea vegae, but differs in its sparsely to densely branched apothecia, obvious prothallus and gyrophoric acid detected by TLC.

Type: CHINA. Hainan: Ledong County, Guyu Forest Trestle Road, Jianfengling National Forest Park Rainforest, 108°55′45″E, 18°44′56″N, alt. 654 m, on leaves, 10 Dec. 2019, *Z.T. Yao HN20192696* (holotype, HMAS-L 0147815).

Description. Thallus foliicolous, crustose, thin, greyish green to yellowish green, usually continuous; prothallus visible, pale brown to dark brown. Photobiont *Phycopeltis*, cell oblong-rectangular, 6–10 × 4–7 μm, arranged irregularly. Ascomata adnate to sessile, lirellate, short to elongate, sparsely to densely branched, 0.2–1.1 mm in length, 0.06–0.15 mm in width; disk slit-like, narrow, margin black; hypothecium hyaline to pale brown, 7.5–12.5 μm high, K/I–, hymenium colorless, I+ orange-red, K/I+ pale blue; paraphyses branched and anastomosing. Asci 8-spored, clavate, 22.5–37.5 × 7.5–11 μm, I–, K/I+ faintly blue. Ascospores small, colorless, narrowly fusiform, round and blunt at the end, with slightly expanded cells in the middle and upper parts, 3-septate, 8–16 × 1.4–3.5 μm, slightly constricted and easily broken at the septum, with a gelatinous sheath, 0.8–1.6 μm thick. Pycnidia not seen.

Etymology. The epithet refers to the thallus containing gyrophoric acid.

Chemistry. Thallus K–, C+ red, KC+ red, P–; Gyrophoric acid detected by TLC (**Supplementary Files S2 and S3**).

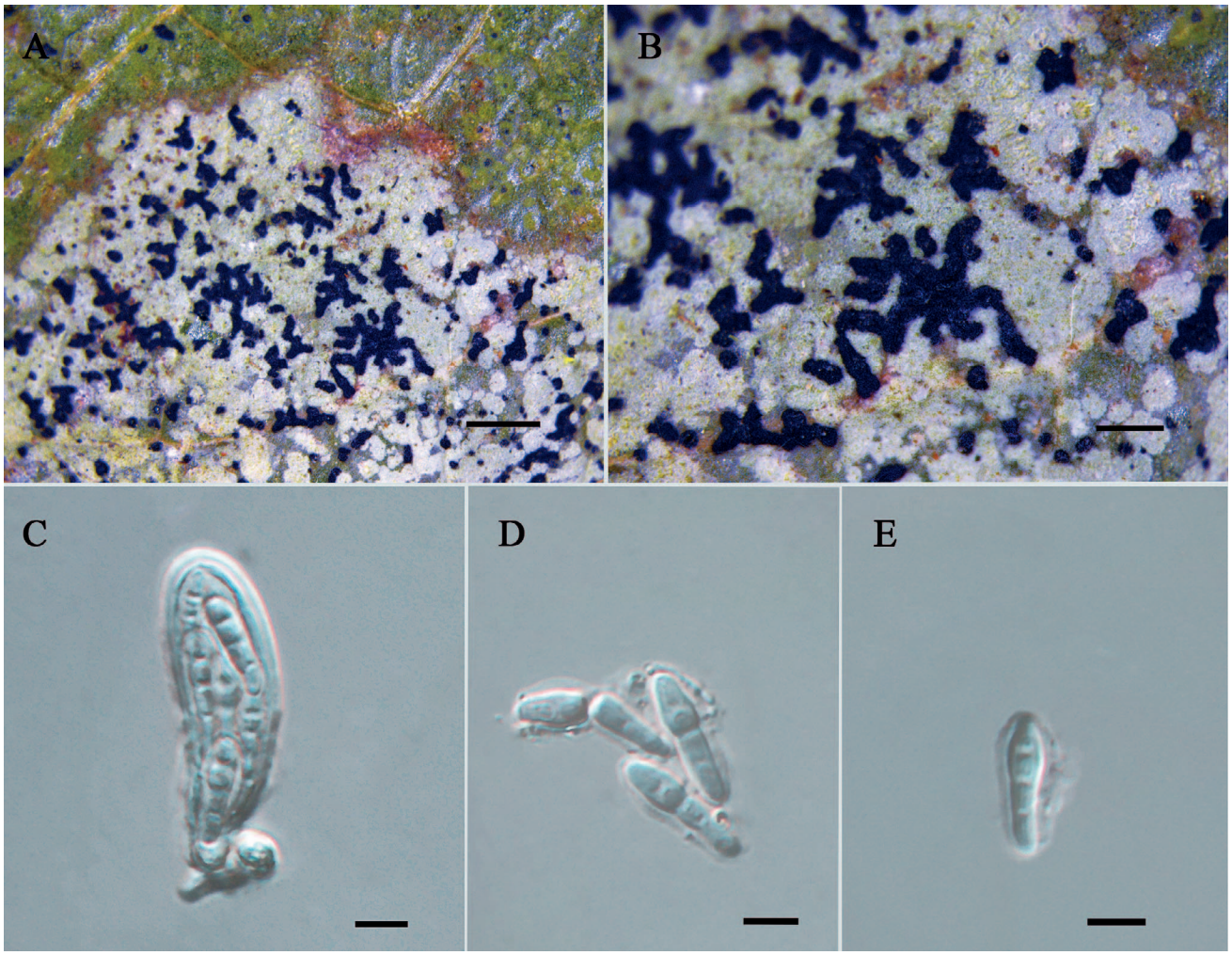

Figure 2. *Fouragea gyrophorica* sp. nov. (holotype HMAS-L 0147815): **A.** Thallus with ascomata. **B.** Apothecia in detail. **C.** Asci. **D–E.** Ascospores. Scale bars: A–B = 1 mm; C–E = 5 µm. Online pdf in color.

Habitat and distribution. The new species grows on leaves in wet tropical forest in Hainan. At present, it is known only from the type locality.

Additional specimens examined. CHINA. HAINAN: Ledong County, Guyu Forest Trestle Road, Jianfengling National Forest Park Rainforest, 108°55′45″E, 18°44′56″N, alt. 654 m, on leaves, 10 Dec. 2019, *Z.T. Yao HN20192674* (HMAS-L 0147794), *Z.T. Yao HN20192683* (HMAS-L 0147810), *Z.T. Yao HN20192669* (HMAS-L 0152319).

Remarks. For the thallus morphology and ascospore size, this new species is most similar to *Fouragea vegae* (Ertz 2020; Lücking 2008; Santesson 1952). However, *F. gyrophorica* can be easily distinguished from *F. vegae* when it has more densely branched apothecia, and it often has obvious prothallus. *Fouragea virisdistellata* was related with this new species, but the latter usually has stellate apothecia covered by a thin thalline layer and 4-septate ascospores (Sérusiaux et al. 2008). *Fouragea filicina* is another similar species, but its asci and ascospores are larger, and ascospores often 5-septate, the thallus surface usually with numerous black spots (Lücking 2008; Santesson 1952). Importantly, the new species *F. gyrophorica* can be distinguished from other *Fouragea* species by gyrophoric acid (**Supplementary Files S2 and S3**). It should be noted that this is the first report about *Fouragea* containing secondary metabolites. Combining morphological and phylogenetic analysis, there is sufficient evidence to verify the species is new to science.

Key to species of Fouragea known in the world (updated from Ertz 2020)

1a. Ascospores 3-septate 2
1b. Ascospores 4–5-septate 3
2a. Lirellae mostly unbranched; no lichen substances detected ..... ***F. vegae***
2b. Lirellae sparsely to densely branched; gyrophoric acid present ..... ***F. gyrophorica***
3a. Lirellae covered by a thin layer of thalline tissue, sparsely to densely branched; ascospores usually 4-septate, 14–23 × 4–7 µm ..... ***F. viridistellata***
3b. Lirellae not covered by a layer of thalline tissue, mostly unbranched or with a few short branches, ascospores usually 5-septate, 16–27 × 2–6 µm 4
4a. Thallus green to dark green, with numerous, black dots ..... ***F. filicina***
4b. Thallus yellowish to brownish green or pale greenish grey to white, without black dots 5
5a. Thallus pale greenish white to white, dispersed into irregular patches ..... ***F. alba***
5b. Thallus pale yellowish to brownish green or pale greenish grey, continuous 6
6a. Ascomata 0.08–0.15 mm wide; ascospores 2–3 µm wide ..... ***F. heliabravoa***
6b. Ascomata 0.15–0.25 mm wide; ascospores 4–6 µm wide ..... ***F. tuxtlensis***

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Supplementary documents online:

Supplementary File S1. Concatenated two-locus (nuLSU, mtSSU) dataset.

Supplementary File S2. The lichen substances detected by TLC using solvent system B.

Supplementary File S3. The lichen substances detected by TLC using solvent system C.