Article

Lopadostoma fagi (Lopadostomataceae) on *Fagus sylvatica* from Italy

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Abstract

A new record of *Lopadostoma fagi* is described in Italy from *Fagus sylvatica* based on morphological and molecular data. It has effused-pulvinate stromata immersed in the host tissue, with a narrow, black, carbonized encasement. The ectostromatic disc is visible as a clypeus and surrounded by the reddish brown bark surface. In the combined phylogenetic analysis of ITS, LSU andRPB2 sequence data, the strain derived from the specimen clustered with other *L. fagi* strains with high bootstrap support. A morphological description with detailed photographs for *L. fagi* is provided in this study.

Introduction

Lopadostoma (Nitschke) Traverso (*Anthostoma* subgen. *Lopadostoma* Nitschke) was accepted in the Xylariaceae by Læssøe (1994). However the libertella–like asexual morphs of *Lopadostoma* are more or less similar to those of Diatrypaceae with scolecosporus conidia produced on sympodially proliferating conidiogenesis cells (Ju et al. 1993). Rappaz (1992, 1993) placed *Anthostoma decipiens* (DC.) Nitschke in Diatrypaceae, which was accepted by Lu and Hyde (2000). The unclear distinction between Xylariaceae and Diatrypaceae has been addressed by several authors (Rogers 1994, Stadler et al. 2013). Recent molecular data have resulted in several changes to the placement of *Lopadostoma* and *Creosphaeria* Theiss., which formed a monophyletic clade between Xylariaceae and Diatrypaceae (Jaklitsch et al. 2014, Daranagama et al. 2015, Maharachchikumbura et al. 2015). Thus Senanayake et al. (2015) introduced a new family Lopadostomataceae, in the order Xylariales, to accommodate *Lopadostoma* and *Creosphaeria*, based on the phylogeny and also the morphology of the libertella–like asexual morphs of these two genera.

The morphology of *Lopadostoma* has been comprehensively studied in Jaklitsch et al. (2014) who accepted 12 species. The sexual morph of *Lopadostoma* species usually possess pustulate-effuse stromata, immersed in the host which later become erumpent through the bark and appear as blackened areas containing several perithecia, which are clustered in a valsoid arrangement with convergent ostiolar necks. Asci are usually unitunicate, cylindrical, with J+ apical apparatus, with eight uniseriate, dark coloured ascospores, with a straight germ slit (Jaklitsch et al. 2104). The asexual morph of *Lopadostoma* as reported in Ju et al. (1993) and Jaklitsch et al. (2014) as libertella-like with falcate, unicellular, hyaline conidia.

In this study we examine the phylogenetic placement of our fresh collection with all the known species of *Lopadostoma*, as well as genera like *Anthostomella* and *Creosphaeria* with similar morphology. We have performed a multigene analysis using ITS, LSU and RPB2 sequence data to determine the phylogenetic placement of this species, in which we identified our collection as *Lopadostoma fagi* Jaklitsch, J. Fourn. & Voglmayr. A detailed morphological description is provided for the species.

Materials and Method

Sampling and morphology

A fresh specimen was obtained from Italy on *Fagus sylvatica* L. Morphological examination and microphotography was carried out as described by Daranagama et al. (2015a, b). Axenic cultures were developed and maintained in Oat Agar (OA) medium for observation and DNA extraction as described by Daranagama et al. (2015a, b).

DNA extraction, PCR and sequencing

DNA was extracted and the ITS, LSU and RPB2 genes were amplified following the protocols outlined by Daranagama et al. (2015). PCR products were visualized in 1% agarose gel electrophoresis, stained with Goldview (Geneshun Biotech, China) with D2000 DNA ladder (Realtimes Biotech, Beijing, China). All the PCR products were purified according to the company protocols and DNA sequencing was performed using the same primers in an Applied Biosystem 3730 DNA analyzer at Sinogenomax Company, Beijing, China.

Sequence alignment and phylogenetic analyses

Raw sequences were assembled with Contig Express 2003 (Invitrogen, Carlsbad, CA). The assembled consensus sequences were initially aligned with ClustalW and optimized with MAFFT v. 7 using default settings (Katoh & Standley 2013) (http://mafft.cbrc.jp/alignment/server/) and adjusted manually where necessary. The initial identities of the newly generated sequences were determined by analyzing them with all available type-derived and authentic sequences of *Lopadostoma* (Jaklitsch et al. 2014). The familial placement was determined by analyzing with strains belong to Lopadostomataceae, Diatrypaceae and Xylariaceae (Table 1).

Evolutionary models for phylogenetic analyses were selected independently for each locus using MrModeltest 2.3 (Nylander 2004) under the Akaike Information Criterion (AIC). ML phylogenetic trees were constructed using RAxML 7.4.2 Black Box (Stamatakis 2006, Stamatakis et al. 2008) available in the CIPRES Science Gateway platform (Miller et al. 2010). For the combined dataset all free modal parameters were obtained using RAxML with ML estimate of 25 per site rate categories. The RAxML software accommodated the GTR model of nucleotide substitution with the additional options of modeling rate heterogeneity (Γ) and proportion invariable sites (I).

Phylogenetic trees and data files were viewed in MEGA 5 (Tamura et al. 2011) and Fig tree v1.4 (Rambaut and Drummond 2008). Maximum Likelihood values (equal to or above 50) are indicated above or below nodes. All the sequences generated in this study were deposited in GenBank (Table 1). Faces of fungi numbers are as explained in Jayasiri et al. (2015).

Table 1 GenBank accession numbers and their strain numbers.

| Species	Strain number	ITS	RPB2	LSU
Creosphaeria sassafras	CM AT–018	DQ631934	DQ631964	DQ840056
Cryptosphaeria eunomia	olrim954	AY787693	–	–
Daldinia concentrica	CBS 113277	AY616683	–	–
Diatrype bullata	UCDDCh400	DQ006946	–	–
Diatrype disciformis	AFTOL-ID 927	–	DQ470915	DQ470964
Diatrypella verruciformis	UCROK1467	JX144793	–	–
Eutypa armeniacae	ATCC 28120	DQ006948	–	–
Eutypa lata	CBS 208.87	DQ006927	KF453595	–
Hypoxylon fragiforme	MUCL 51264	KM186294	KM186296	KM186295
Lopadostoma fagi	**MFLUCC 15-0008**	**KU820972**	**KU820973**	**KU956001**
Rhopalostroma lekae	MFLUCC 13–0123	KJ472428	KJ472427	KJ472429
Sordaria fimicola	CBS 723.96	AY681188	DQ368647	AF132330
Xylaria hypoxylon	CBS122620	AM993141	KM186302	KM186301

The other GB numbers used in this study were followed as mentioned in Jaklitsch et al. (2014).

Results

Molecular Phylogenetic Analysis
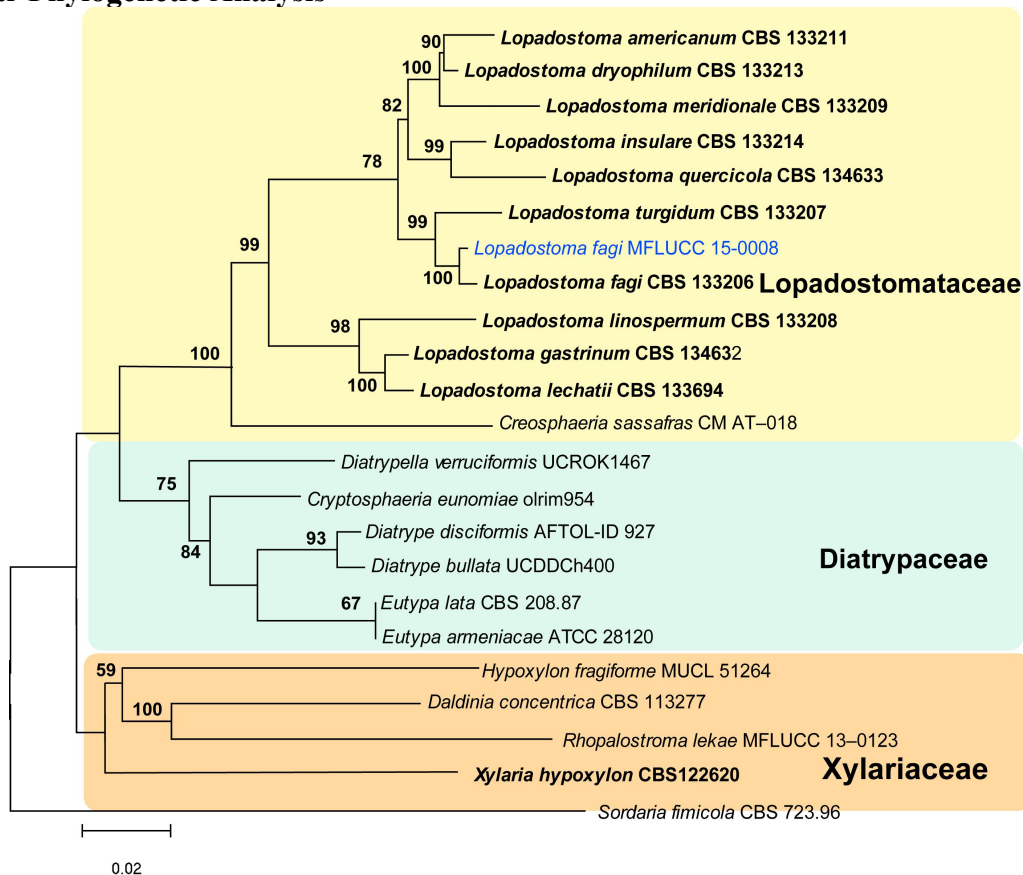
Fig.1. Phylogram inferred from likelihood analysis using combined ITS/LSU/RPB2 sequence data. Strain/culture numbers are given following the taxon names. The new sequences generated in this study are in blue. Strain/culture designations are given following the taxon names. Holo-, neo- or epitype strains/specimens are formatted in **bold**. The bootstrap support values from likelihood analysis >50% from 1000 RAxML replicates are shown above or below the branches. The tree is rooted with *Sordaria fimicola*.
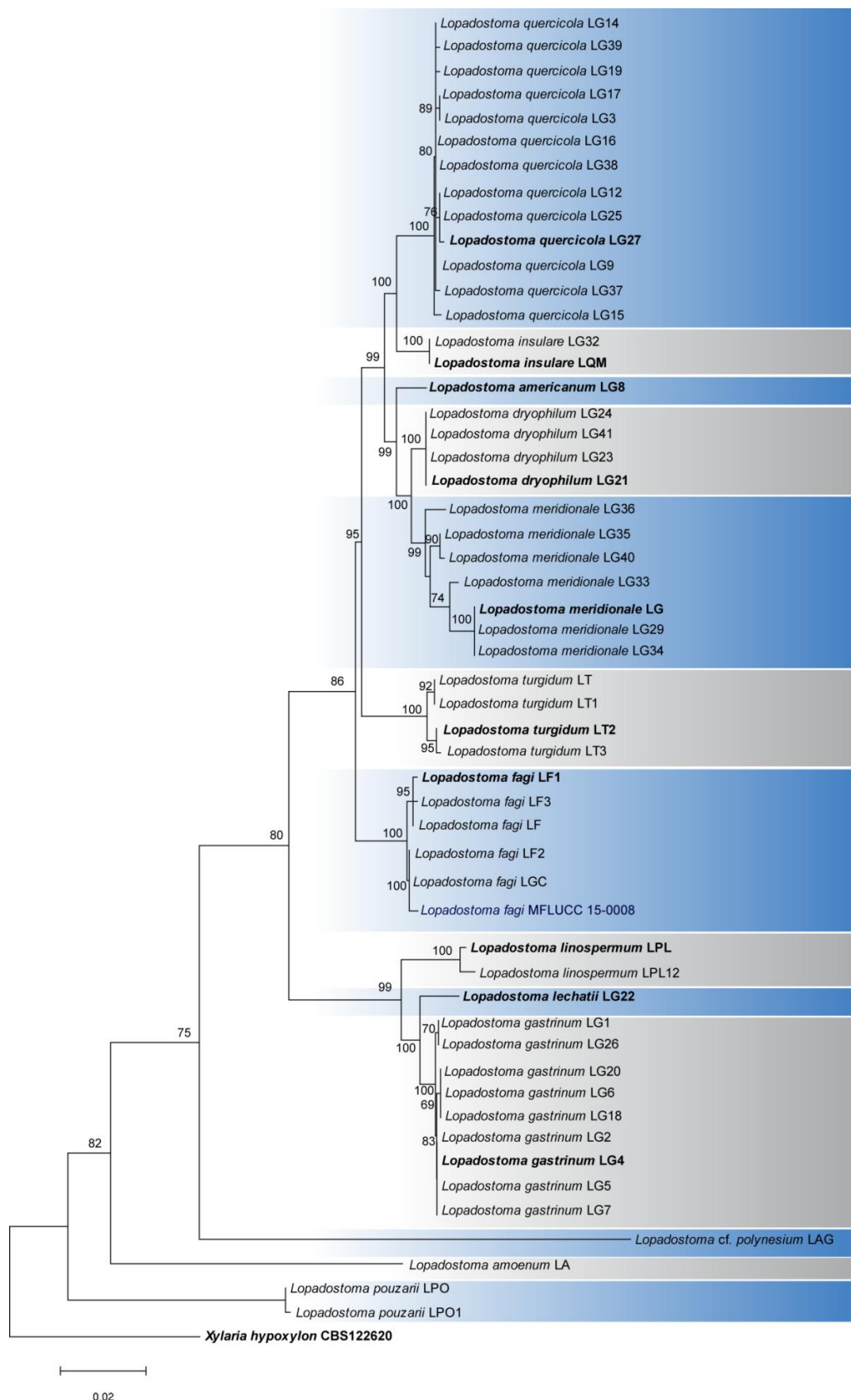
Fig.2. Phylogram inferred from Maximum likelihood analysis using combined ITS–LSU–RPB2 sequence data of *Lopadostoma* species. Strain/culture numbers are given following the taxon names. The new sequences generated in this study are in blue. Strain/culture designations are given following the taxon names. Holo-, neo- or epitype strains/specimens are formatted in **bold**. The bootstrap support values from likelihood analysis >50% from 1000 RAxML replicates are shown above or below the branches. The tree is rooted with *Xylaria hypoxylon*.

The combined alignment of ITS, LSU and RPB2consisted of 2507 total characters with 23 isolates including the out group taxon. All characters were assessed to be unordered and equally weighed. Gaps were treated as missing data. Resolutions of the terminal clades in the multi-locus tree are better than any individual dataset (data not presented). The topology of the resulted ML tree inferred from RAxML is presented as Fig. 1. The phylogenetic data presented in Fig. 1 resolve three monophyletic groups which indicate separate families; Diatrypaceae, Lopadostomataceae and Xylariaceae. Our strain is clearly unrelated to the Xylariaceae or Diatrypaceae families. The latter forms a monophyletic clade as a sister group to Lopadostomataceae. However, in the ML analysis (Fig. 1) this relationship did not obtain branch support above 50 %.

In this analysis *Lopadostoma* forms a monophyletic clade with high bootstrap support. *Creosphaeria sassafras* is placed singly on a branch sister (100%) to *Lopadostoma*. Our strain with high bootstrap support, clustered within Lopadostomataceae (Fig. 1). The ML phylogenetic tree for the genus *Lopadostoma* was reconstructed using combined ITS-LSU-RPB2 sequence data and the resulted tree is presented as Fig. 2. Molecular data suggests that our strain is related to the *L. fagi* strain from previous studies with high bootstrap support (Fig. 2), which has similar morphological characters. Therefore we have identified this species as *Lopadostoma fagi*.

Taxonomy

Lopadostoma fagi Jaklitsch, J. Fourn. & Voglmayr, in Jaklitsch et al. Persoonia, Mol. Phyl. Evol. Fungi 32: 63 (2014)   Fig. 3, 4
*Facesoffungi number*: FoF 01897
Saprobic on dead twigs and branches of *Fagus sylvatica* L. **Sexual morph**: *Stromata* 700–1500 × 300–800 μm ($\bar{x}$ = 1200 × 500 μm, n = 30), effused–pulvinate, immersed in the host and erumpent from bark, bluntly conical, surrounded by a narrow, black, carbonized encasement, appearing as a black line, ectostromatic disc visible as a clypeus and surrounded by reddish brown bark surface, convex, raised, dark grey, entostroma dark, usually black, multi-peritheciate. *Ostiole* papillate with inconspicuous ostiolar openings. *Perithecia* 200–800 × 150–680 μm ($\bar{x}$ = 400 × 520 μm, n = 30), 2–5 clustered in valsoid groups, mono-distichous, subglobose to flask-shaped, at the periphery inclined toward the center, tissue between perithecia loosely arranged, composed of white fungal tissue, mixed with light coloured bark cells, tissue beneath the perithecia compact, black, with short convergent ostiolar necks. *Paraphyses* are numerous, long, apically free, 1–3.5 μm wide ($\bar{x}$ = 3 μm, n = 30), rarely branched. *Asci* 79.8–103.5 × 4.6–6.1 μm ($\bar{x}$ = 91 × 5.3 μm, n = 30), unitunicate, 8-spored, cylindrical, pedicellate, ellipsoidal-discoid, inconspicuous apical apparatus typically 2–3.5 μm wide. *Ascospores* 7.5–8.7 × 3.2–4.2 μm ($\bar{x}$ = 7.9 × 3.9 μm, n = 30), uniseriate, oblong to narrowly ellipsoid, symmetrical to slightly inequilateral, unicellular, lacking a dwarf cell, at first hyaline, turning pale brown and dark brown at maturity, smooth-walled, with a straight germ slit across the entire spore length present, when immature with 2 large guttules.

Culture characteristics – Colonies on Difco OA very slow growing, reaching the edge of 9 cm Petri-dishes in 3–3 ½ months at 25–27 °C, at first whitish, velvety, azonate, with slightly lobed margins, developing sporulating regions as light yellow mycelial masses in culture; later, reverse turning light orange.

Specimen examined – Italy, Province of Forlì-Cesena, Campigna - Santa Sofia, dead branch of *Fagus sylvatica* L (Fagaceae), 19 September 2014, E. Camporesi, (MFLU 15-2600, KUN), living culture (MFLUCC 15-0008, KIBCC).

Notes – *Lopadostoma fagi* is reported as a common species in corticated branches of *Fagus sylvatica* from Europe (Jaklitsch et al. 2014). This specimen was collected from Italy on the same host. According to the morphological description of *L. fagi* provided in Jaklitsch et al. (2014) it is characterized in having distinctly papillate ostioles and smaller, narrower ascospores with a straight, full length germ slit. Similar morphological traits were observed in our collection. However, we observed 2–5 perithecia, in stroma more or less in a valsoid configuration and rarely distichous, even though according to Jaklitsch et al. (2014) it is mentioned as (3–) 6–8 in a cluster
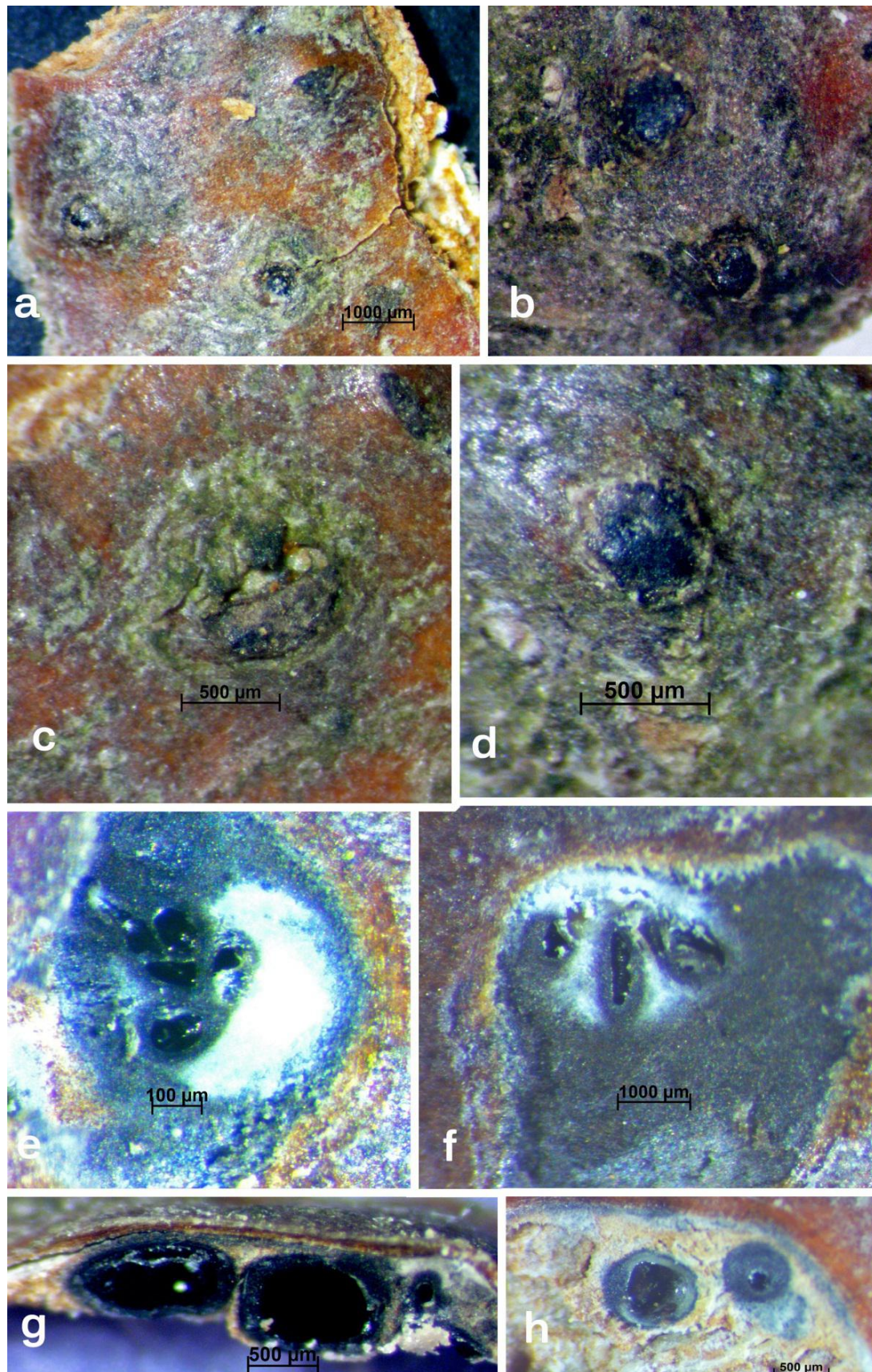
Fig. 3 – *Lopadostoma fagi*. a-c. Surface of stromata on host, d. ectostromatic disc, e, h. transverse sections of stromata, f, g. vertical sections of stromata. Scale bars: a = 1000 μm, b–d = 500 μm, e = 100 μm, f = 1000 μm, g, h = 500 μm
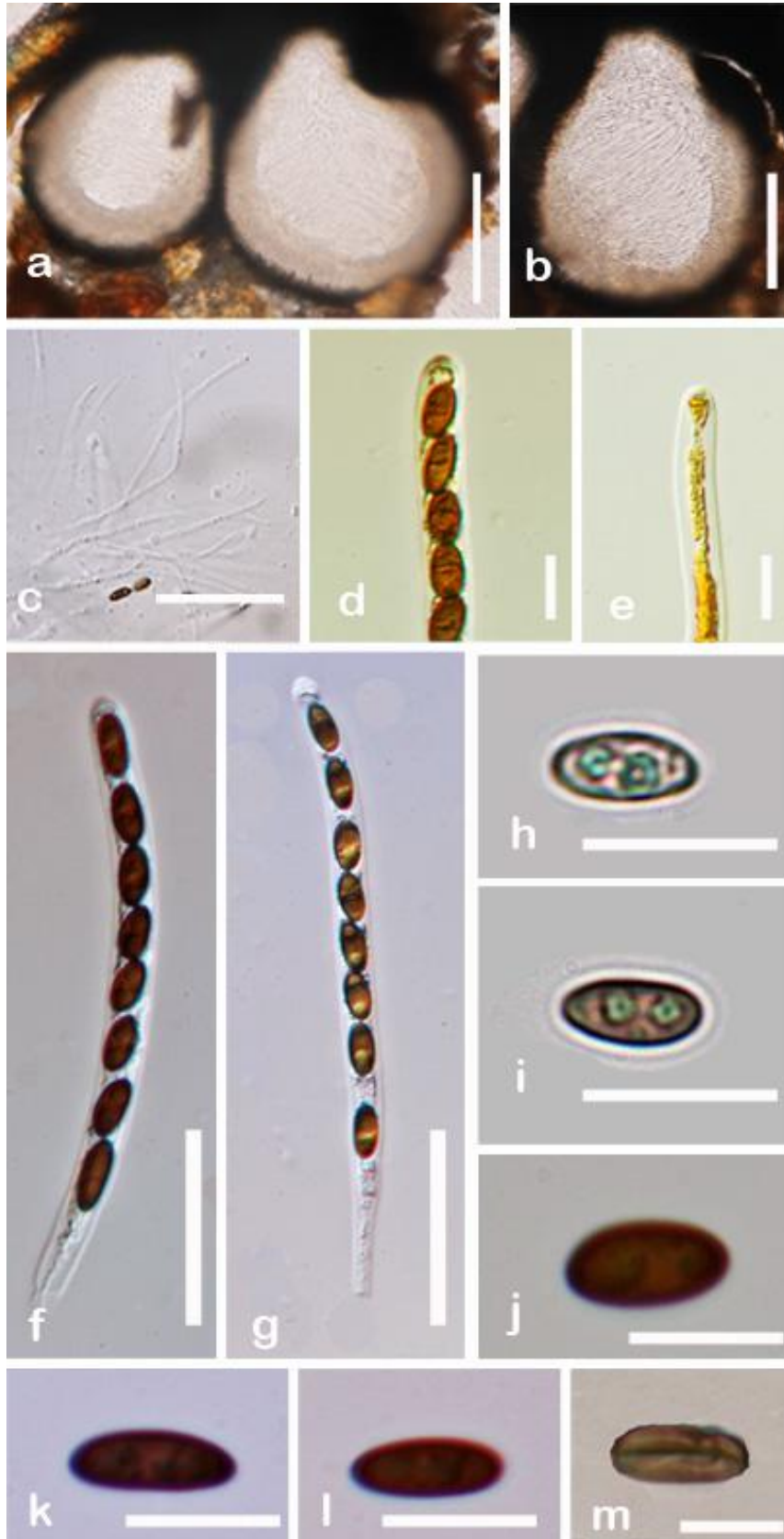
Fig. 4 – *Lopadostoma fagi*. a, b. Vertical sections of stromata, c. Paraphyses, d, e. Asci with inconspicuous apical apparatus (d-in Melzer's reagent, e-in Lugol solution), f, g. Mature asci, h-i. Immature ascospores, j-l. Mature ascospores, m. Ascospore with straight germ slit. Scale bars: a, b = 300 μm, f = 30 μm, d-e = 10 μm, f, g = 30 μm, h-m = 10 μm.

and monostichous. In addition there are slight variations in the measurements of perithecia, asci and ascospores which can be due to the geographical variations and environmental conditions. The slow germination of ascospores (3 weeks on MEA and 4–5 weeks on OA) later resulted in whitish colonies is similar to those observed by Jaklitsch et al. (2014) who mentioned as 1–4 weeks for initial germination of ascospores on MEA. Due to these morphological similarities between our collection and the previously studied specimens, we have identified this species as *L. fagi*, which is also confirmed by the molecular studies.

Discussion

Lopadostoma comprised several unrelated species which were previously introduced by Martin (1969, 1976) until the genus was revised by Rappaz (1995) and recently by Jaklitsch et al. (2014). The latter study limited the genus to 12 species and provided molecular data for ten species. Therefore the genus now comprises only species with pustular stromatic development in the host. *Lopadostoma* clearly forms a highly supported monophyletic clade in the phylogenetic analysis which is now placed in *Lopadostomataceae* (Senanayake et al. 2015).

Most of the species in *Lopadostoma* exhibit overlapping characters which makes it difficult to identify them separately. These variations are caused by changes of climatic conditions during stroma development and maturation as well as based on the host factors (Jaklitsch et al. 2014). Our collection has similar morphological characters of stromata, asci and ascospores with *L. fagi*. Except those slight variations we observed in asci and ascospores measurements this collection is typical of *L. fagi*. However, according to Jaklitsch et al. (2014) the qualitative characters are more informative than quantitative characters due to the considerable intraspecific variation.

Therefore molecular data is useful in identification at the species level. Particularly protein-coding genes are superior and useful than other genes such as ITS or LSU (Stadler et al. 2013, Jaklitsch et al. 2014, Daranagama et al. 2015a) since they can provide better resolution at terminal clades and higher statistical supports. The use of RPB2 gene sequences in this study as well as previous studies in combination of ITS and LSU results in better phylogenetic placements, which is also correlated with the morphology and host preference.

The chemical profiles of species of *Lopadostoma* have not been studied to date. Since the Xylariales are rich in production of secondary metabolites (Stadler and Hellwig 2005, Stadler et al. 2013) the analysis of *Lopadostoma* species for their secondary metabolites will be useful for a better taxonomic classification.

Acknowledgments

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