Thyronectria abieticola (*Hypocreales*), a new species from France on *Abies alba*

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Ascomycete.org, 10 (1) : 55–61
Mise en ligne le 25/02/2018
10.25664/ART-228

Abstract: A new species of *Thyronectria* growing on corticated branches and twigs of *Abies alba* is described from France. It is characterized by morphology of sexual and asexual morphs, by ITS rDNA data and by ecology. Ascomata are stromatic, blackish, not obviously changing colour in 3% KOH, covered by a greenish scurf and they have hyaline to very pale brown, one-septate, smooth ascospores 10–12.3 × 5.2–6.2 µm, not budding in the ascus to form ascoconidia. Microscopic observation of the ascomatal wall reveals versicoloured regions that change colour in 3% KOH and lactic acid. The phylogenetic analysis based on ITS sequences sets it within *Thyronectria* and apart from the morphologically most similar species. It is supposed to be host-specific to *Abies alba* based on three collections exclusively made on this host.
Keywords: Ascomycota, *Nectriaceae*, ribosomal DNA, taxonomy.

Résumé : une espèce nouvelle de *Thyronectria* venant sur branches et brindilles cortiquées d'*Abies alba* est décrite de France. Elle est caractérisée par la morphologie de ses stades sexué et asexué, par les données moléculaires et par son écologie. Les ascomes sont stromatiques, noirâtres, ne changent pas de couleur dans la potasse à 3 % de façon évidente, sont couverts d'un revêtement écailleux verdâtre et possèdent des ascospores hyalines à brun très pâle, uniseptées, lisses, 10–12.3 × 5.2–6.2 µm, ne formant pas d'ascoconidies par bourgeonnement. L'observation microscopique de la paroi des ascomes révèle la présence de couches de couleurs variables, qui changent de teinte en présence de potasse à 3 % ou d'acide lactique. L'analyse phylogénétique de sa séquence ITS la place dans *Thyronectria* et la distingue des espèces morphologiquement les plus proches. Son affinité supposée pour *Abies alba* est fondée sur trois récoltes faites exclusivement sur cet hôte.
Mots-clés : ADN ribosomal, Ascomycota, *Nectriaceae*, taxinomie.

Introduction

During fieldwork in the east-central part of France, a distinctive hypocrealean fungus characterized by dark-coloured ascomata covered by a greenish scurf and seated on a stroma erumpent from bark was repeatedly encountered on corticated branches of fallen *Abies alba* Mill. in Jura, Haute-Saône and Saône-et-Loire departments. As this combination of characters is suggestive of the newly re-instated genus *Thyronectria* Sacc. (Jaklitsch & Voglmayr, 2014), we carried out a detailed morphological study of this fungus, along with single-spore isolation on PDA and sequencing of the material obtained in culture.

Thyronectria as currently conceived accommodates species formerly placed in the *Thyridiaceae* J.Z. Yue & O.E. Erikss. by Rossman *et al.* (1999), along with species placed in *Pleonectria* Sacc. by Hirooka *et al.* (2012). Based on a multigene phylogenetic study and the observation of apical paraphyses in *T. patavina* Sacc., the type species of *Thyronectria*, as well as in several species of *Pleonectria*, Jaklitsch & Voglmayr (2014) re-defined *Thyronectria* and showed that *Mattirolia* Berl. & Bres. and *Thyronectroidea* Seaver were earlier synonyms of *Thyronectria*. Their phylogenetic results were confirmed by those published by Lombard *et al.* (2015). More recently, *Allantonectria* Earle was likewise demonstrated to be a synonym of *Thyronectria* by Voglmayr *et al.* (2016). As a result, *Thyronectria* currently encompasses stromatic nectriaceous fungi with KOH+ to KOH- perithecia usually becoming cupulate upon drying and covered by yellow to greenish scurf, a hamathecium composed of apical paraphyses originating from an apical cushion and growing downwards, and variously shaped, coloured, septate and ornamented ascospores sometimes budding in the ascus to produce ascoconidia. For a detailed definition of the genus and its nomenclatural and taxonomic history, the reader is referred to Jaklitsch & Voglmayr (2014). Since this date, in the context of this new definition of *Thyronectria*, two new species were described from Spain by Checa *et al.* (2015), three new species from China by Zeng & Zhuang (2016) and one from Ukraine by Voglmayr *et al.* (2016).

We present here morphological and molecular evidence supporting the status of the material collected in France on *Abies alba* as an undescribed species of *Thyronectria*, and we formally describe it here as *T. abieticola*.

Materials and methods

Specimens were examined using the method described by Rossman *et al.* (1999). Microscopic observations and measurements were made in water. The holotype specimen and paratypes are deposited in LIP herbarium (Lille) and living cultures were deposited in the CBS 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., 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

Thyronectria abieticola Lechat, Gardiennet & J. Fourn., *sp. nov.* – MycoBank MB 824039 – Figs. 1–4

Diagnosis: Differs from *T. ilicicola* and *T. sinopica*, the most closely resembling species regarding ascospores and conidial morphology and dimensions, by superficially blackish ascomata not obviously changing colour in 3% KOH, covered with a greenish scurf and occurrence on a coniferous host.

Holotype: France, Saône-et-Loire, La Grande Verrière, on corticated branch and twigs of fallen *Abies alba*, 19 Nov. 2011, *leg*. Jean-Pierre Dechaume, CLL11034 (LIP), culture ex-type CBS 128932; additional culture deposited at CIRM (UMR-1163-Biodiversité et Biotechnologie Fongiques, INRA/Aix-Marseille Université, ESIL Polytech, Marseille, France): BRFM 1582; ITS rDNA GenBank ex-holotype sequence: KX897141.

Etymology: The specific epithet refers to the host *Abies alba* (*Pinaceae*).

Additional specimens (paratypes): France, Jura, Moissey, massif de la Serre, on bark of fallen trunk of *Abies alba*, 30 Oct. 2014, *leg*. A. Gardiennet, AG14204 (LIP); Haute-Saône, Gray, forêt domaniale des Hauts Bois, on corticated twigs of *Abies alba*, 23 Nov. 2016, *leg*. A. Gardiennet, AG16147 (LIP).

Ascomata superficial on a well-developed hypostroma, appearing in small groups of (2–)5–12 through cracks of bark, slightly erumpent above bark surface, globose to subglobose, 280–360 µm high, 280–380 µm diam., cupulate or not when dry, superficially black, unevenly covered by a greenish scurf turning into thicker scales when moistened and often forming an areolate pattern, ostiolate, with a minute, central, black papilla. **Hypostromata** up to 2.5 mm diam., superficially blackish, brownish yellow in section, not changing colour in 3% KOH, pseudoparenchymatous, composed of thick-walled angular to prismatic orange to brownish yellow cells, 8–20 × 6–10 µm with wall 2–2.5(–3) µm thick. **Ascomatal wall** 32–58 µm thick, of three regions: outer region 5–15 µm thick, dull brown, composed of 1–3 layers of angular to strongly flattened thick-walled cells 8–18 × 4–7 µm, wall 2–2.5 µm thick, not changing colour in 3% KOH, evolving to an amorphous brown layer composed of collapsed and occluded cells peeling into scales forming the superficial scurf; scales bearing greenish-yellow waxy granules releasing a fugacious yellow pigment in 3% KOH; median region up to 32 µm thick and purple in upper half, decreasing to 10–14 µm and brownish grey in lower half, composed of thick-walled angular to flattened cells 9–16 × 4.5–10 µm, wall 2–2.5 µm thick, turning greenish grey or bluish grey in 3% KOH and red in lactic acid; inner region 10–20 µm thick, intergrading with the median region, of similar but slightly smaller cells with wall 1–1.5 µm thick, subhyaline to orange brown, turning yellow in 3% KOH and lactic acid. **Ostiolar papilla** composed of palisadic, orange brown cells becoming hyaline, narrower and thin-walled toward the ostiolar canal, merging with periphyses 1.2–2 µm diam. **Apical paraphyses** numerous, descending to the bases of asci, filamentous to narrowly moniliform, branched and sparingly anastomosed, 2–4.5 µm diam., up to 6 µm diam. in lower part. **Asci** cylindrical to clavate, 65–80 (–90) × 8–11 µm, apex simple, 8-spored. **Ascospores** (9.9–)10.1–12.3(–13.3) × (4.9–)5.2–6.2(–6.9) µm (Me = 11.1 × 5.7 µm, n = 60), irregularly biseriate when immature, becoming obliquely uniseriate at maturity, ellipsoid with broadly rounded ends, 1-septate, at first hyaline, becoming pale brownish at maturity, smooth-walled, not to barely constricted at the septum, not budding inside or outside the asci; ascomatal content pale orange due to the faintly pigmented ascospores. Asexual morph on the natural substrate not seen.

Culture characteristics (Fig. 3):

Colony circular, reaching 7–8 cm diam. after two weeks at 25°C, greenish yellow, becoming irregularly brownish at margin; reverse yellowish cream in centre and dark brown at margin. Colony surface cottony with aerial mycelium yellowish white, producing sporodochial conidial masses after 2 weeks. **Hyphae** bearing lateral phialidic pegs enteroblastic, monophialidic, flask-shaped, 2.5–8.5(–10.5) µm long, 2–3.5 µm wide at base. **Conidia** formed abundantly on slimy heads, narrowly ellipsoidal to cylindrical, sometimes swollen at ends, hyaline, containing 1–2 droplets, smooth, non-septate, (3.5–)8.0–10(–12) × (1.8–)2.5–3(–3.7) (n= 50). **Pycnidia** produced after one month, pale orange, subglobose, 250–320 µm diam.; peridium composed of globose, subglobose to angular, thick-walled cells 4.5–17 × 5–11 µm, not changing colour in 3% KOH; conidiophores branched, smooth, hyaline, 8–11 × 2–8 µm arising from hyphae 2.5–4 µm wide. **Conidiogenous cells** phialidic, subulate, (10–)12–19(–21) × (1.5–)2–2.5(–2.8) µm (Me= 16 × 2.2, n= 20). **Conidia** oblong to ellipsoidal, sometimes attenuated at base (3.5–)4.0–5.5(–6) × (1.5–)2–2.5(–3) µm (Me= 5 × 22, n= 40), aseptate, hyaline, smooth, forming an amber apical slimy mass.

Results and discussion

The fungus described above is characterized by gregarious, blackish, soft-textured perithecial ascomata coated with a greenish scurf, seated on a hypostroma erumpent from bark, a hamathecium composed of apical paraphyses, unitunicate, cylindrical asci containing eight two-celled ascospores and a pycnidial asexual morph in culture composed of branched, phialidic conidiophores. This set of characters fits well in the genus *Thyronectria* as newly delimited by Jaklitsch & Voglmayr (2014), which is supported by the ML phylogeny based on rDNA ITS sequences showing the new taxon nested within the *Thyronectria* clade (Fig. 4). Based on its two-celled, broadly ellipsoid, hyaline to pale brown, smooth ascospores 10.1–12.3× 5.2–6.2 µm not budding in the ascus to produce ascoconidia, this fungus keys out close to *T. ilicicola* (Hirooka, Rossman & P. Chaverri) Jaklitsch & Voglmayr and *T. sinopica* (Fr.) Jaklitsch & Voglmayr (Jaklitsch & Voglmayr, 2014). Both *T. ilicicola* and *T. sinopica* differ from our new taxon by their bay to scarlet ascomata coated with a yellow scurf and turning darker red in 3% KOH (Hirooka *et al.*, 2012). Moreover, the former is restricted to *Ilex aquifolium* (*Aquifoliaceae*) and the latter to *Hedera helix* (*Araliaceae*), unlike our new taxon which is consistently associated with *Abies alba*. For these reasons, the fungus described above was determined to represent a previously undescribed species of *Thyronectria* and we propose the new taxon *T. abieticola* to accommodate it.

The black ascomata of *T. abieticola* do not exhibit a visible discolouration in 3% KOH or lactic acid under the stereo microscope, but microscopic observations of ascomatal wall sections shows otherwise. The purple to brownish grey median region turns greenish grey in 3% KOH and red in lactic acid, while the subhyaline to orange brown inner region turns yellow in both 3% KOH and lactic acid (Fig. 2 f, g). The versicoloured ascomatal wall of *T. abieticola* and its discolourations in 3% KOH and lactic acid appear specific and provides an additional good morphological marker to distinguish it from other *Thyronectria* species.

A BLAST search revealed a maximum ITS sequence similarity of 97.2% with *T. virens* Harkn., 97.1% with *T. zanthoxyli* (Peck) Ellis & Everh. and *T. rhodochlora* (Mont.) Seeler. *Thyronectria rhodochlora*, *T. virens* and *T. zanthoxyli* are clearly different from *T. abieticola* by their muriform ascospores. Comparison of ITS sequences is useful to support the generic placement of our new taxon in *Thyronectria* but a more accurate definition of its phylogenetic affinities with other members of the genus would be better addressed by a multigene phylogenetic study like that performed by Jaklitsch & Voglmayr (2014).

Host specificity for a plant species, genus or family is widespread within *Thyronectria*, this is why *T. abieticola*, occurring on *Abies alba*,

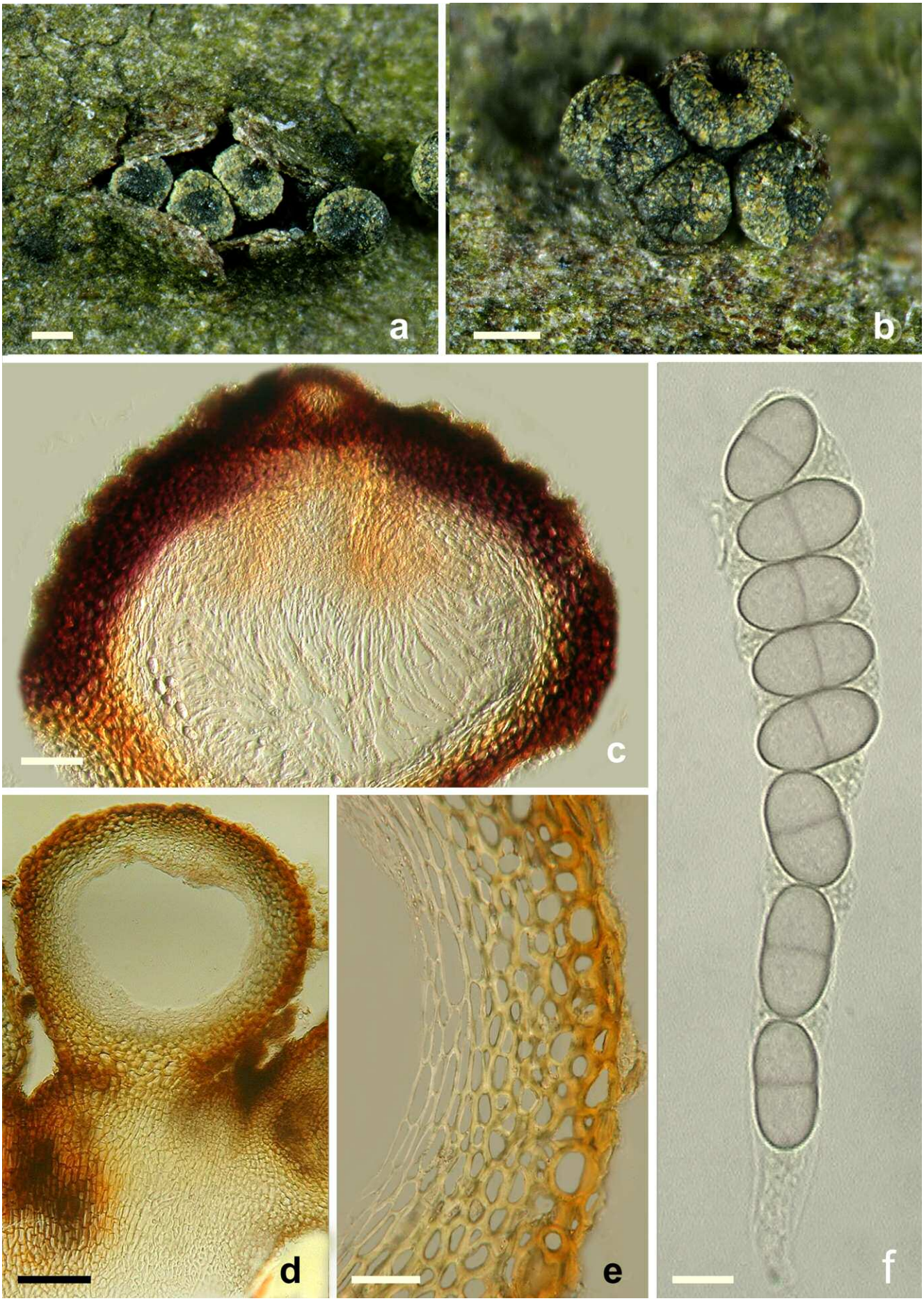

Fig. 1 – a–f: *Thyronectria abieticola* (Holotype CLL11034). a–b: Ascomata on the substratum. c-f: Micromorphology observed in water; c: Vertical section of a perithecium showing apical paraphyses. d: Vertical section of a perithecium showing the pseudoparenchymatous hypostroma. e: Section through the lateral ascomatal wall. f: 8-spored ascus. Scale bars: a, b = 200 µm; c = 50 µm; d = 100 µm; e = 20 µm; f = 5 µm.

Ascomatal wall of this fungus in vertical section is variously coloured when observed in water, specialy the middle region which appears orange brown to purple according to selected perithecium, while changing of colour in 3% KOH or lactic acid is constant in all ascomata (Fig. 2 below).

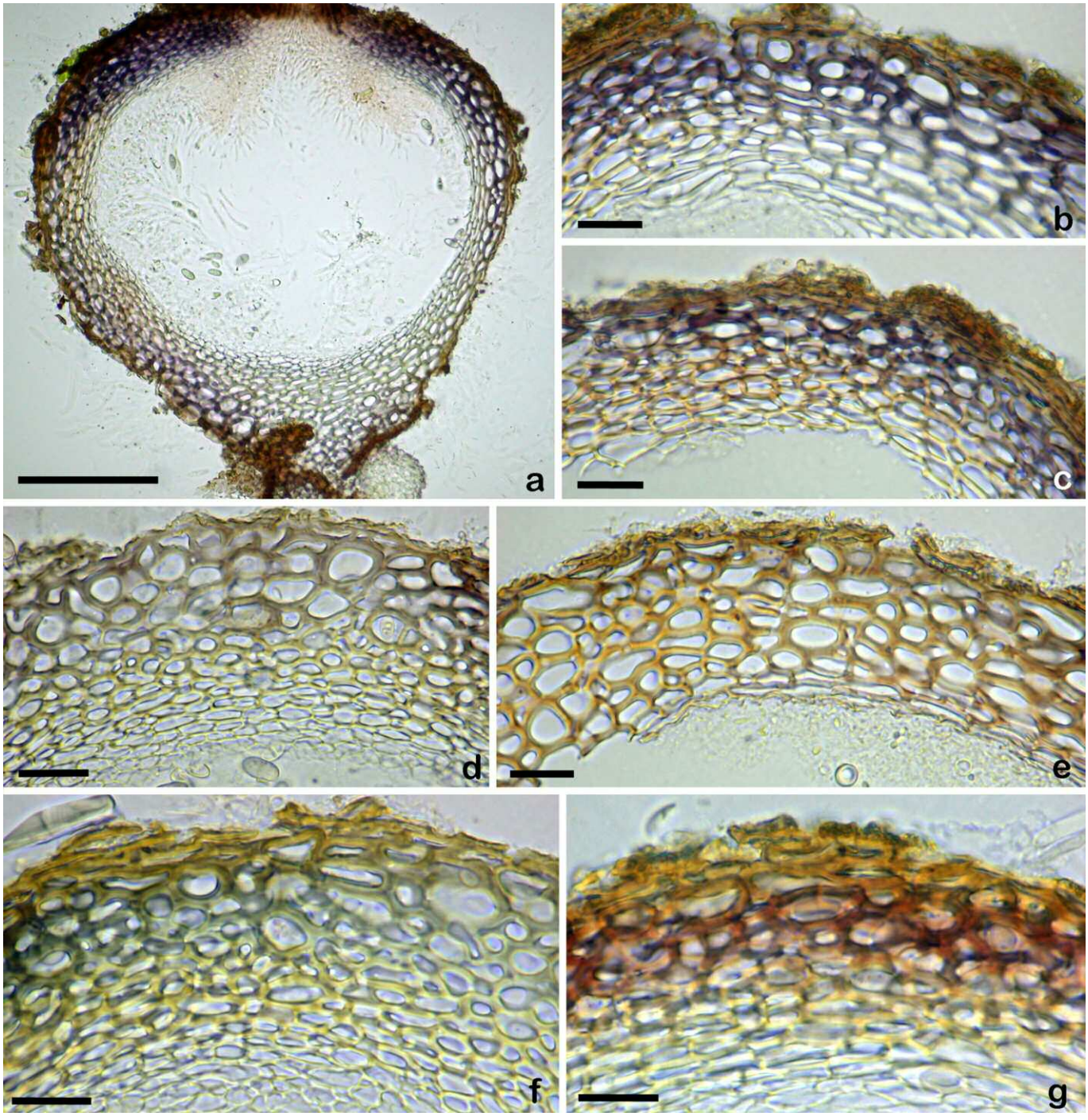

Fig. 2 – *Thyronectria abieticola* (paratype AG14204). a: Ascoma in vertical section. b–g: Ascomatal wall in section showing the variously coloured regions; b, c from upper half; d–g from lower half. a–e in water, f in 3% KOH and g in lactic acid. Scale bars: a= 100 µm; b-g= 20 µm.

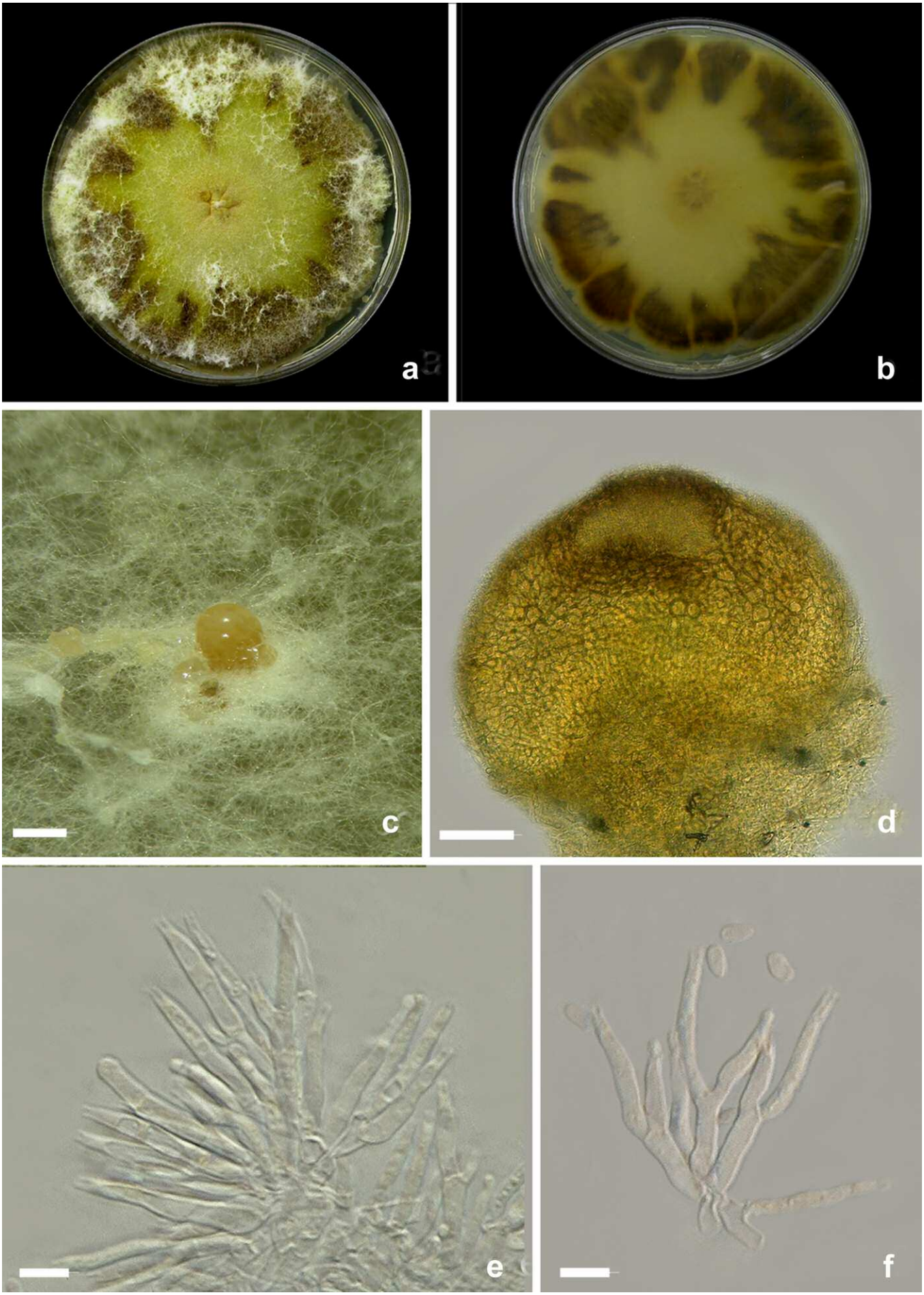

Fig. 3 – a–f: Culture of *Thyronectria abieticola* (Holotype CLL11034). a: Colony after two weeks. b: Reverse of the culture. c: Pycnidium in culture. d: Pycnidium, close-up in water. e–f: Conidiophores, conidiogenous cells and conidia from pycnidium. Scale bars: c= 200 µm; d= 50 µm; e-f= 5 µm

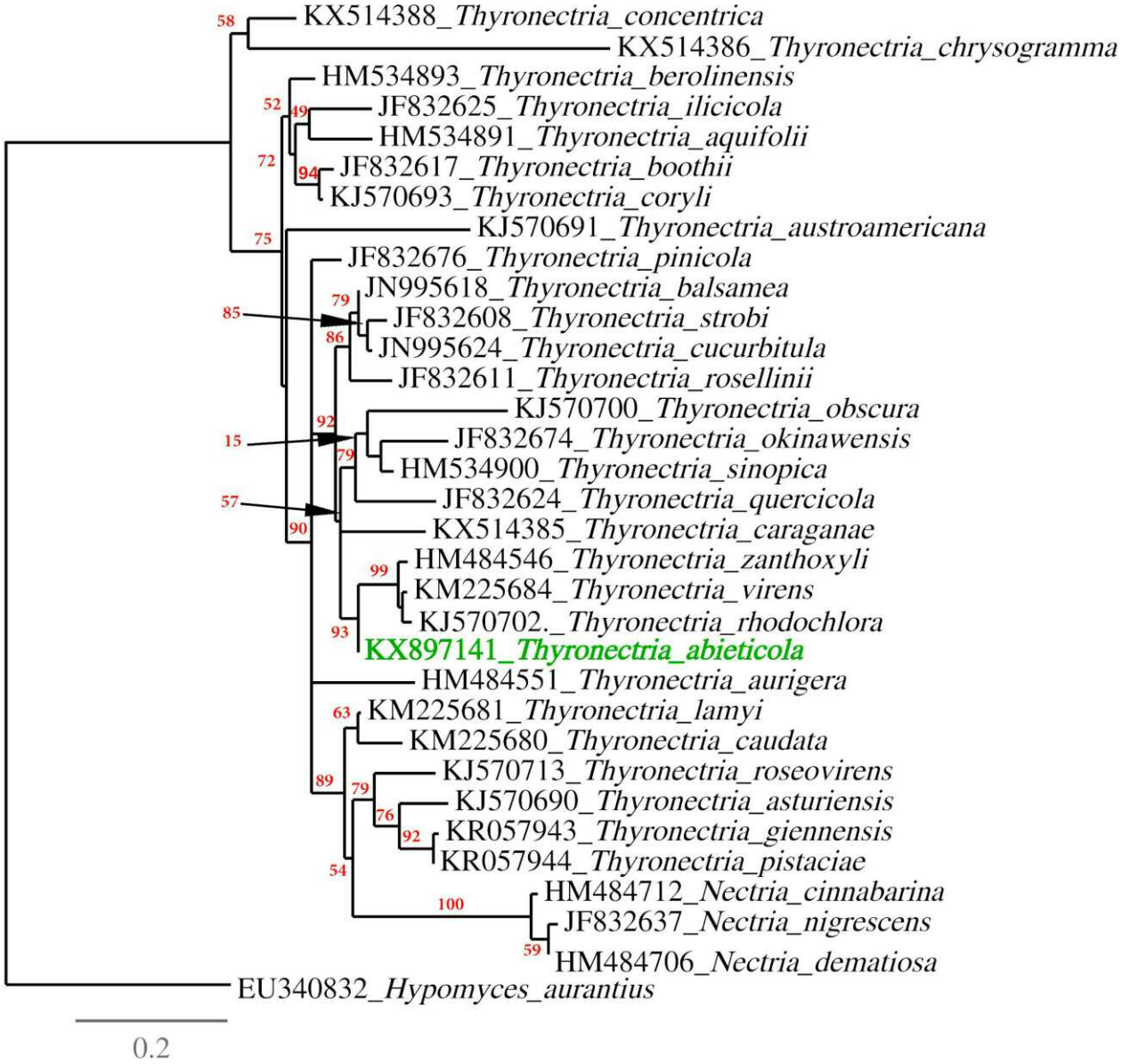

Fig. 4 – Maximum likelihood phylogeny (-lnL = 2552.79116) of *Thyronectria abieticola* inferred by PhyML 3.0, model HKY85 from a 585 bp matrix of ITS1-5.8S-ITS2 rDNA sequences, rooted with *Hypomyces aurantius*. ML bootstrap support is given above or below the branches.

should be compared in first place with other known species occurring on *Abies*, viz. *T. balsamea* (Cooke & Peck) Seeler in North America and *T. rosellinii* (Carestia) Jaklitsch & Voglmayr in Asia, Europe and North America. *Thyronectria balsamea* essentially differs from *T. abieticola* in having long, muriform ascospores while *T. rosellinii* has filiform and mutiseptate ascospores, and ascospores of both species produce bacillar ascoconidia. Six further species known to occur on coniferous hosts (Jaklitsch & Voglmayr, 2014; Checa *et al.*, 2015; Zeng & Zhuang, 2016) can likewise be easily distinguished from *T. abieticola* primarily by ascospore morphology. *Thyronectria boothii* (Hirooka, Rossman & P. Chaverri) Jaklitsch & Voglmayr, known from Europe on *Picea abies* and *T. pinicola* (Kirschst.) Jaklitsch & Voglmayr, known from Asia, Europe and North America on *Pinus* spp. feature long-cylindrical to long-fusiform muriform ascospores budding to produce ascoconidia. *Thyronectria cucurbitula* (Tode) Jaklitsch & Voglmayr, known from Europe and North America on *Pinus* spp., *T. strobi* (Hirooka, Rossman & P. Chaverri) Jaklitsch & Voglmayr, known from Europe and North America primarily on *Pinus strobus* and *T. sinensis* Z.Q. Zeng & W.Y. Zhuang, known from China on *Pinus* sp. all differ from *T. abieticola* by long-fusiform to long-filiform multiseptate ascospores budding to produce ascoconidia. Finally, *T. orientalis* Z.Q. Zeng & W.Y. Zhuang, known from China possibly on *Pinus* sp. features ellipsoid to subfusiform muriform ascospores, which sets it apart from *T. abieticola*. Although several species of *Thyronectria* were reported as fungicolous (Jaklitsch & Voglmayr, 2014), we did not see *T. abieticola* associated with a fungus in any of our collections.

Acknowledgements

The authors gratefully acknowledge Dr. Walter Jaklitsch and Dr. Hermann Voglmayr (University of Vienna, Austria) for their presubmission reviews and their valuable suggestions to improve this paper. Jean-Pierre Dechaume (La Grande Verrière, France) is warmly thanked for the material he collected.

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