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ORIGINAL ARTICLE

Sordaria fimicola-like ascomycete isolated from *Pinus coulteri* needles in Slovakia

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Abstract
This is the first report of *Sordaria fimicola*-like ascomycete which was encountered during a diversity study of injured tissues of coulter pine in Slovakia. The fungus was identified as *Sordaria fimicola* by morphological analyses. Sequence analysis of internal transcribed spacer region (ITS) showed that the fungus is highly related to the ITS sequences of several *S. fimicola* isolates documenting wide ecological valence and geographical distribution of *S. fimicola*-like ascomycetes.

Keywords Morphological study · Phylogenetic analysis · *Pinus coulteri* · *Sordariaceous* species

Introduction

Genus *Sordaria* belongs to Sordariaceae (Sordariomycetes, Sordariales) which are a family of perithecial fungi inhabiting herbivore dung or decaying plant parts, rarely coniferous needles. This family with dark, usually ostiolate, globose or flask-shaped solitary black large ascomata and unitunicate, cylindrical asci colonise whole stems and xylem of trees at dry sites (Petrini and Fisher 1988, 1990; Fisher et al. 1992, 1993). They all have a short life cycle, usually 7–12 days, and are easily grown in culture. Dettman et al. (2001) characterized the members of the filamentous ascomycete family Sordariaceae based on cylindrical unitunicate asci produced within darkly pigmented flask-shaped ascocarps (perithecia) with or without prominent ostioles. The genera placed within this family are differentiated mainly by ascospore morphology and ornamentation. According to Bell (1983), Alexepoulos et al. (1996), García et al. (2004) and Lumbsch and Huhndorf (2007) perithecia of this family elongated at one end contain asci with eight ascospores in a linear arrangement. Ascospores are obovoid or subglobose, single celled, smooth-walled, dark brown to black, pitted, reticulate or striate and show wide variation of differnet kinds of appendages or sheaths. Each of which has a single, usually basally situated germination pore that sometimes projects slightly. Spores are surrounded by gelatinous sheath which is sometimes thick and conspicuous to even in some cases difficult to detect or they have wall ornamentations, but lack gelatinous appendages.

In this paper, we have identified *Sordaria fimicola*-like ascomycete (Roberge ex Desm.) Ces. & De Not. obtained from necrotic needles of *Pinus coulteri* D. Don in Slovakia by cultural, morphological methods and sequence data of the ITS region of rDNA.

Material and methods

From spring to autumn 2015–2016, needles of *Pinus coulteri* with blight symptoms were collected in the geographic location Arborétum Mlyňany SAS (48°19′10.99′′N, 18°22′8′′E., altitude 165–217 m a.s.l., northen moderate climatic zone with four seasons, average daily temperature of 10.6 °C, average annual atmospheric precipitation of 541 mm). Altogether 15 trees were studied. The age of evaluated trees was between 20 and 25 years. Samples were taken from some sections of trees with damaged needles. The needles parts cut from the diseased pine plants were surface-sterilized by immersion in sodium hypochlorite solution (1% available chlorine) for 15 min, later dipped in sterilized distilled water, dried with

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sterilized-blotting paper and placed on fresh Petri dish (PD) including on nutritive 3% PDA medium (Merck, Darmstadt, Germany). Petri dishes were kept in test chamber with constant temperature and humidity (24 °C and 45% humidity in dark conditions) in a versatile environmental test chamber MLR-351H - Sanyo) (Sanyo Electric Co., Ltd., Osaka, Japan). Occurrence of fungus *Sordaria fimicola* was characterized through macro- and microscopic characters. Morphometric measurements of fungal structures were made from each PD sample with the occurrence of fungus. The samples of biological material were deposited in herbarium at the Institute of Forest Ecology of the Slovak Academy of Sciences, Department of Phytopathology and mycology in Nitra. Study of fungal structures was performed with a light clinical microscope BX41 (Olympus Tokyo, Japan) under a 400× and 1000× magnification. Measurements were made through the medium of QuickPhotomicro 2.2 programme (PROMICRA, s.r.o., Prague, Czech Republic). The identification of fungus was made according to its anamorph and teleomorph morphology. Fungus was identified to the level of the genus using the taxonomic guide of Petrini and Fischer (1988); Fischer et al. (1992; 1993); Weber (2002); Asgari et al. (2007) and the morphometric values were compared with previously published data for the taxa (Alexepoulos et al. 1996; Doveri 2004; Crous et al. 2009).

For molecular identification a total genomic DNA of C1 isolate was isolated from fresh mycelia grown on PDA plates using microwave treatment and subsequent Triton X-100 lysis (Goodwin and Lee 1993). Both DNA concentrations and quality were checked using gel electrophoresis in 1% agarose gel (Sambrook et al. 1989). The ITS1–5.8S-ITS2 ribosomal DNA regions ITS region of rDNA operon was amplified using primer pair ITS1 and ITS4 and conditions specified by White et al. (1990). The PCR amplified ITS product was purified using Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, USA), cloned using InsTAclone™ PCR Cloning Kit (ThermoFisher Scientific, Colorado, USA) and sequenced from both sides using universal M13 sequencing primers at GATC-Biotech (Konstanz, Germany). DNA sequences were assembled using DNA Baser software (Heracle BioSoft SRL, Arges, Mioveni, Romania) and submitted to the GenBank database under accession No. KY930619. DNA sequences were compared to the GenBank database using BLASTn algorithm (Altschul et al. 1990). The sequences showing the highest similarity were downloaded and multiple sequence alignment was built using MEGA6 software (Tamura et al. 2013). The

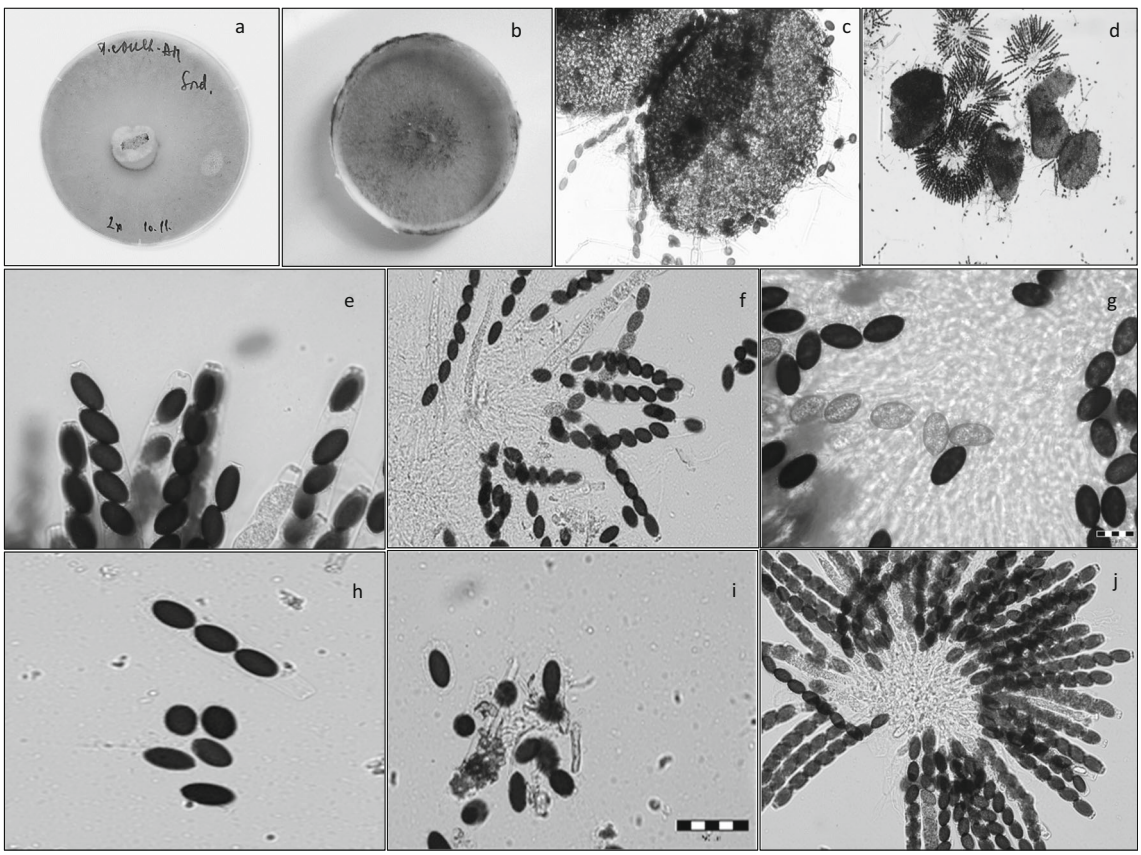

Fig. 1 *Sordaria fimicola*-like ascomycete on *Pinus coulteri*. **a** colony on PDA after 1 week on *P. coulteri*; **b** abundant perithecia at the agar surface; **c, d** ascomata with setae; **e** end of ascus; **f** 8-spored asci; **g** immature and mature ascospores with granular contents; **h** mature ascospores; **i** ascospore gelatinous sheath; **j** rosettes of asci. Scale bars: **g** = 20 μm, ***i*** = 50 μm

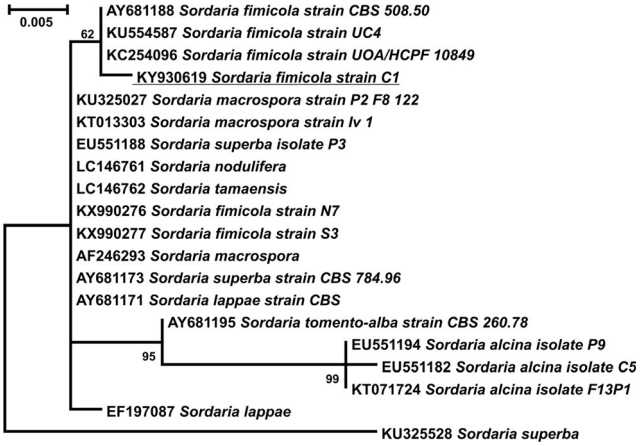

Fig. 2 Unrooted phylogenetic tree documenting relatedness of C1 strain of *Sordaria fimicola* (underlined) to other members of *Sordaria* genus based on ITS sequences similarity. The tree was constructed using Neighbor Joining method. Numbers at nodes are bootsrap values after 1000 replications

phylogenetic relatedness of sequences was inferred using the Neighbor Joining method based on the Kimura 2-parameter model implemented in MEGA6.

Results

Pine sapwood samples showing necrosis symptoms, discolouration, brown spots and blight symptoms are often colonised by different species of fungi. The interesting fungus on needles of the *Pinus* species among others was *Sordaria fimicola*-like ascomycete C1. Culture characteristics of this fungus in our experiments isolated from necrotic needles of *P. coulteri* cultivated on PDA medium: Colonies on PDA agar growing rapidly attaining a diameter of 7.0–7.5 cm within 2 weeks at 24 °C were at first white (Fig. 1a), thin, with abundant aerial mycelium which was submerged, consisting of a thin layer of abundant perithecia at the agar surface (Fig. 1b). Mycelium was composed of hyaline, branched, smooth-walled, septate, 3.0–3.5 μm wide hyphae. Black, flask-shaped perithecia (Fig. 1c, d) size 385–450 × 260–350 μm were basically ovoid, but elongated at one end, superficial, non-stromatic, glabrous or sparsely covered with flexuous colourless hairs. Perithecial cylindrical and papillose neck, which elongating to mature dark brown to nearly black asci was 80 (120) μm in size. Setae which occur relatively scarce were hyaline, smooth-walled, strait with globose or subglobose apices, 39(48) × 3–5 μm. The C1 isolate formed 8-spored asci with truncate apex and small apical rings 6–26 μm in size. Asci were thin-walled, fasciculate, unitunicate, aseptate, cylindrical to clavate, sometimes with a non-amyloid apical structures, 200(230) × 20 μm, with a well developed apical structure (Fig. 1e). The asci elongate into the ostiole one at a time to release the ascospores. Ascospores are in one row (uniseriate), one-celled, linearly arranged (Fig. 1f), first olivaceous green (Fig. 1g) to pale brown coloured, later dark brown pigmented at maturity (Fig. 1h), broadly ovoid to ellipsoidal, sometime subglobose, smooth-walled with granular contents without guttules. One of the asci stretches and pushes through the ostiolar opening, while its base remains attached to the perithecial wall 50 μm in size. Asci were 24–25 × 12(13)-14 μm in size, with a colourless basal germ slit 1–2 μm wide which is longitudinal on the flat side, extending over the entire length of the spore. Ascospores were surrounded by a hyaline, thin, later disappearing gelatinous 3–6 μm thick sheath (Fig. 1i). The asci formed rosettes without paraphyses (Fig. 1j).

Based on morphology traits the C1 isolate was identified as *S. fimicola*. Molecular analysis of ITS region showed that C1 isolate is highly related to the *S. fimicola* ITS sequences with

Table 1 Comparison of morphometric measurements of ascospore size of the fungi *Sordaria fimicola*, *Sordaria macrospora* and *Coniochaeta malacotricha*

Sordaria fimicola		*Sordaria macrospora*		*Coniochaeta malacotricha*	
authors	ascospore size (μm)	authors	ascospore size (μm)	authors	ascospore size (μm)
Lundqvist 1972	(17-)18–24 × (9.5-)10–13	Walley and Harvey 1965	31.2 × 17.9	Arx and Müller 1954	9–12 × 6–7
Watanabe 1989	15–20 × 10–12.5	Fields 1970	31 × 17	Mahoney and Favre 1981	8–13 × 6–9 × 5–6
Bell 1983	14–15 × 9–16, [(17)18–24 × 9.5)10–13]	Bell 1983	28–38 × 16–19	Checa et al. 1988	11–12 × 9–10
Mungai et al. 2012	15.5–18.5 × 9.5-11.5	Crous et al. 2009	25–35 × 17–22	Chlebicki 1991	12–13.5 × 9.6–10 × 5.6
Kavak 2012	20–22 × 12–13	Ivanová et al. 2016	20(22)28 × 13(14)-20	Lumley et al. 2000	10–12.5 × 5.5–7
Ivanová 2015	17–22 × 10–11			Cannon and Kirk 2007	10.5–13 × 9–10.5 × 6–7.5
Sádlíková 2015	19.5–23 × 14			Asgari et al. 2007	10–14 × 9–13 × 6-8
Our experiment	24–25 × 12(13)14				

Table 2 Distinguishing characters between *Sordaria macrospora* and *Coniochaeta malacotricha* identified on different hosts with signs of *Sordaria fimicola*-like ascomycete on *Pinus coulteri*

Causal agent	*S. fimicola*-like ascomycete	*S. macrospora*	*S. macrospora*	*C. malacotricha*	*C. malacotricha*	*C. malacotricha*
Host plant	*Pinus coulteri*	*Pinus nigra*		*Pinus sylvestris*	*Pinus sylvestris*	*Pinus* sp., *Picea* sp
Ascomata (μm)	Perithecium obpyriform, 385–450 × 260–350, neck 80(120)	Perithecium pyriform 370–400(500) × 250–3-00, neck 35–60(150)	Perithecium superficial, pear-shaped, 400–700, ostiolum 30 × 12	Immature perithecia (without asci and spores)	Perithecium egg-shaped or conical, gregarious, 230–300 diam., 320–400 high	Ascocarp pyriform to subglobose 270–290 × 200–23-5
Setae (μm)	Hyaline, scarce, smooth-walled 39(48) × 3–5	Brown or hyaline, straight, smooth-walled 55(68) × 2	On the outher surface often with soft setae	Dark brown, pointed 30–60 × 3.5–4, wall 0.8–1 thick	Densely covered with black, shining hairs 50–60 × 3	Densely covered aculeate setae
Paraphyses	Absent	Absent	Absent	Absent	Paraphyses indistinct	–
Asci (μm)	8-spored, uniseriate, cylindrical, 150–230 × 19–23	8-spored, cylindrical, unitunicate, 160–175 × 20	Unitunicate, 8-spored cylindrical, uniseriate with a non-amyloid apical thickening	Absent	Cylindrical, 8-spored, 80–92 × 11–12	–
Ascospores:						
Size (μm)	24–25 × 12(13)14	20(22)28 × 13(14)-20	25–35 × 17–22	Absent	12–13.5 × 9.6–10 × 5.6	11–12 × 9–10
Color	Brown	Brown	Brown	–	Brown with dark brown	
Shape	1-celled, ellipsoidal, aseptate	Uniseriate, 1-celled, granular contents	Ellipsoidal, smooth	–	Circular or elliptical (Mill-stone shaped) flat	Broadly ellipsoidal to subcircular
Germ slit	Visible	Visible	A colourless basal germ pore	–	Visible	Germ slit completely encircling the spore
Mucous sheath	Gelatinous sheath 1–2 μm in size	Surrounded by a gelatinous sheath	Surrounded by a gelatinous sheath	Not sheen	Not sheen	–
Guttules/oil drops	–	–	–	2 or some tiny guttules	Large refractive oil drop	–
Colonies on PDA	White, aerial mycelium	Pale white, abundant aerial mycelium	Fast growing, at first white with abundant aerial mycelium	Colonies with pale colours, nearly white to pale salmon-coloured, sparse, no aerial mycelium	–	–
Hyphae (μm)	Hyaline, branched, smooth-walled, septate, 3.0–3.5	Hyaline, 4.0–5.0	Hyaline, thin-walled, septate, irregularly branched	1.5–3.5(−4), hyaline	–	–
Conidia (μm)	–	–	–	Smaller, ovoid, apiculate (2.5–)3–4(−5) × 1–2	–	–
Chlamydosp.	–	Lacking	–	Absent		–
Authors	Our experiment	Ivanová et al. 2016	Crous et al. 2009	Weber 2002	Chlebicki 1991	Checa et al. 1988

Table 3 Comparison of the morphological characteristics of species *Sordaria fimicola* identified on different hosts with signs of *Sordaria fimicola*-like ascomycete isolated on *P. coulteri*

Host	*Pinus coulteri*	*Acer palmatum*	*Datura inoxia*		*Prunus javasakura, P. lannesiana*	
Causal agent	***S. fimicola*-like ascomycete**	***S. fimicola***	***S. fimicola***	***S. fimicola***	***S. fimicola***	***S. fimicola***
Colonies on PDA	White, aerial mycelium	–	Aerially, superficially mycelium, fast growing, whitish to grey, later dark grey and black	–	–	–
Hyphae	Hyaline, septate, branched, smooth-walled, 3.0–3.5 μm	Hyaline, 2 μm wide	Hyaline, dichotomous branched	–	–	–
Ascomata (μm)	Perithecium densely aggregated, superficial, obpyriform, 385–450 × 260–350	Perithecial, solitary subglobose to pyriform 370 × 320	Perithecium dense aggregated, light, later dark brown and black, obpyriform and elevate, average 3–5 × 0.-8–1.1 m-m	Perithecium, semi-immersed to superficial, 550–620- high, dark brown, scattered, or gregarious, ovoid to pyriform	Perithecium 291.5–550 × (185-)200–425	Mostly densely aggregated, superficial, obpyriform 360–420 × 240–325, glabrus
Neck (μm)	Cylindrical, papillose 80(120)	100–160	–	Conical or subcylindrical 110–120 × 120–150	90–125 × 60–100	Cylindrical, papilose, 120–240 × 100
Setae (μm)	Flexuous, colourless, 39(48) × 3–5	Brown or hyaline, straight setae 80–100 × 6	–	Hyphoid hairs sparsely covered ascomata	Hairs	Flexous colourless hairs
Paraphyses	Absent	Absent	–	Moniliform, septate with segments 4.5–12.5 μm broad, abundant, containing hyaline vacuoles	–	Not observed
Asci (μm)	8-spored, uniseriate, cylindrical, 150–230 × 19–23	150(165) × 15.8 8-spored, cylindric, fasciculate, unitunicate with a truncate apex and small apical rings	Long cylindrical, hyaline, had a hole on tip, 220–310 × 17–22	8-spored, cylindrical, 111–163 × 10.5–14, flattened at apex, short stipitate, with a lobate stipe and prominent apical apparatus	127.5–202.5 × 13.7–17.5(22.5)	Cylindrical, short-stipitate, 8-spored, spores in a single row, apical ring 3.5–4 μm, 2 μm high, subapical chamber wide 7 μm, truncate apex 9 μm wide, (155-)170–215 × 14–17,
Ascospores (μm)	Brown, ellipsoidal, aseptate, 1-celled, 21(23)27 × 12–15	Green to brown, one-celled, ellipsoidal, smooth-walled, germ pore 17–22 × 10–11, granular contents	Smooth ellipsoidal, green at the beginning, dark green and black in later, 20–22 × 12–13	15.5–18.5 × 9.5–11.5, dark brown, ovoid, obliquely to vertically uniseriate, ovoid, smooth ellipsoidal, occasionally ovoid, slightly pointed and apiculate at base, basal single germ pore	15–20 × 10–12.5	Aseptate, binucleate, dark brown, ellipsoidal to obovoid, basal germ pore, without visible inner structure (17-)18–24 × (9.5-)10–13
Mucous/ gelatinous sheath	Visible gelatinous sheath 3–6 μm in size	Visible gelatinous sheath	Gelatinous sheath	Gelatinous sheath usually invaginated at the apiculum	–	Gelatinous sheath surrounding the spore except for a basal invagination
author	Our experiment	Ivanová 2015	Kavak 2012	Mungai et al. 2012	Watanabe 1989	Lundqvist 1972

similarities as high as 99% at nucleotide level. Multiple sequence alignments placed ITS sequence of C1 isolate to the separate cluster of *S. fimicola* ITS sequences isolated from different sources worldwide (Fig. 2.). The sequence deposited under accession number AY681188 originated from dung from Canada, the sequence KU554587 from grapevine from Italy, and the sequence KC254096 from human clinical material from Greece. The data document wide ecological valence and geographical distribution of *S. fimicola*-like ascomycetes.

Discussion

Obtained features about fungus *S. fimicola* distinguish this fungus from *Coniochaeta malacotricha* of which perithecia are egg-shaped or conical and gregarious (Chlebicki 1991), pyriform to subglobose (Checa et al. 1988) or immature without sac and spores (Weber 2002), and from *S. macrospora* which perithecia pyriform, black, setose, solitary with a central ostiole (Bell 1983; Petrini and Fisher 1988; Engh et al. 2007; Crous et al. 2009; Ivanová et al. 2016).

The genus *Sordaria* is characterized by cylindrical asci containing eight uniseriately arranged, single celled dark ascospores, each of which has a single (usually basally situated) germination pore that sometimes projects slightly. Each obovoid/subglobose ascospore is surrounded by a gelatinous sheath which is difficult to detect unless by Congo red (Bell 1983). According to Crous et al. (2009) asci of *S. macrospora* containing 8 ascospores, which are uniseriate, brown, ellipsoidal, smooth, surrounded by a gelatinous sheath, with a colourless basal germ pore and gelatinous layer around the ascospores was visible only in water. This hyaline, gelatinous sheath, ranging from narrow, irregular and indistinct to prominent is present in some species of *Coniochaeta*, absent in others and not noted in most of them (Mahoney and Favre 1981).

In Table 1 we expose ascospore size and shape which are important taxonomic and valuable criteria for distinguishing species, although there is a considerable variation within species. *S. fimicola* differs from *S. macrospora* in having smaller spores, ellipsoidal rather than broadly ellipsoidal and smaller perithecia and asci (Doveri 2004). According to Petrini and Fischer (Petrini and Fisher 1988) and Engh et al. (2007) ascospores are mostly dark, ascus bears distinctive apical pore. No conidia present. Our results are different in size and shape of ascospores, which are in *C. malacotricha* mill-stone shaped, broadly elliptical in face view, narrowly elliptical in side view (Asgari et al. 2007), broadly ellipsoidal to subcircular (typically millstone shaped) (Checa et al. 1988) or millstone shaped, from oblong to oblong-elliptical and the side view elliptical, dark brown, strongly flattened with a prominent germ slit completely encircling the spore (Cannon and Kirk 2007). The results achieved by other authors lead to *C. malacotricha*, except that the ascospores of those species have guttules.

Based on teleomorph and anamorph morphology, the related fungus was identified as *S. fimicola* as described by the *S. fimicola*-like isolates formed 15 % of cultivated isolates. Important finding is that this fungus was identified for the first time associated with the damaged needles of *Pinus coulteri* in Slovakia. Distinguishing characters between the types of *S. macrospora* and *C. malacotricha* identified on different coniferous host trees with symptoms of a fungus *S. fimicola*-like ascomycete isolated on *P. coulteri* are in Table 2. Comparison of the morphological characteristics of *S. fimicola* identified on different hosts with signs of *S. fimicola*-like ascomycete isolated on *P. coulteri* is in Table 3.

Phylogenetic analysis of ITS sequences of C1 isolate confirmed that it is highly related to the *S. fimicola* with similarities over 99%. The true phylogenetic placement of C1 isolate will require another experiments as ITS sequence of C1 isolate was located outside group of *S. fimicola* sequences.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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