A French collection of *Pseudoplectania nigrella* (*Sarcosomataceae*, *Pezizales*) is closely related to endophytic fungi

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Abstract: The author describes and illustrates a collection of ebony cup fungi, *Pseudoplectania nigrella* (Pers.) Fuckel, harvested in Maritime Alps near the Valley of Marvels (Mercantour National Park, France). Phylogenetic analysis confirmed close relationships with endophytic fungi, shedding light on possibly neglected aspects of *P. nigrella* lifestyle.
Keywords: Ascomycota, ITS, saprotroph, endophyte.

Résumé : L'auteur décrit et illustre une récolte de *Pseudoplectania nigrella* (Pers.) Fuckel dans les Alpes-Maritimes, aux abords de la vallée des Merveilles (Parc National du Mercantour, France). L'analyse phylogénétique suggère une parenté avec des champignons endophytes et invite à reconsidérer le caractère exclusivement saprotrophe de cette espèce.
Mots-clés : Ascomycota, ITS, saprotrophe, endophyte.

Introduction

Last spring we harvested a set of blackish apothecia growing on a mossy soil covered with dead spruce needles, at the edge of a riparian forest in Maritime Alps (altitude: 1300 m; France). Macro- and microscopic examination confirmed these fungi belong to the genus *Pseudoplectania* Fuckel.

Pseudoplectania (Latin for "false *Plectania*") was coined by Fuckel (1870) to distinguish species producing spherical ascospores, namely *P. nigrella* and *P. fulgens* (Pers.) Fuckel [1], from similar-looking species producing ellipsoid ascospores, characteristic for the genus *Plectania* Fuckel. This concept of two independent genera is further supported by studies on ascus and ascospore ultrastructure (Bellemère *et al.*, 1990), ascospore cell wall (Li & Kimbrough, 1995), and by molecular data (Harrington *et al.*, 1999; Perry *et al.*, 2007). However, other authors, such as Paden (1983), consider instead a sole genus (*Plectania s.l.*) containing a section *Sphaerosporae* Paden for species forming spherical spores. This view is supported by the occurrence of spherical spore primordia in true *Plectania* species (Korf, 1973), and by the discovery of *Conoplea* anamorphs in the two groups (Paden, 1983). Recent phylogenetic studies confirmed *Plectania* and *Pseudoplectania* are sister clades (Carbone *et al.*, 2013). We therefore adopt the concept of two independent genera in the present work.

To date, *Pseudoplectania* comprises ten to twelve species (Carbone *et al.*, 2013; Glejdura *et al.*, 2015), including some recently described taxa, such as *P. ryvardenii* Iturr., Mardones & H. Urbina (Iturriaga *et al.*, 2012), *P. tasmanica* M. Carbone, Agnello & P. Alvarado and *P. affinis* M. Carbone, Agnello & P. Alvarado (Carbone *et al.*, 2014). In Europe, *P. lignicola* Glejdura, V. Kučera, Lizoň & Kunca was recently described from Slovakian and Czech collections (Glejdura *et al.*, 2015).

Among these species, *P. nigrella* has gained particular attention after a potent antimicrobial peptide, the so-called plectasin, was isolated from this species (Mygind *et al.*, 2005). Plectasin structure is similar to defensins found in spiders, scorpions, dragonflies and mussels (Mygind *et al.*, 2005). This peptide binds an essential bacterial cell wall precursor (Schneider *et al.*, 2010), thereby impairing cell wall biosynthesis. Plectasin and its derivative have been successfully used in treating infections caused by multi-resistant bacteria (Andes *et al.*, 2009).

Material and methods

Morphological study — Microscopic observations were carried out on fresh material using an Olympus BH2 microscope equipped for Differential Interferential Contrast (DIC), with 20× NA 0.80 and 60× NA 1.4 oil immersion objectives. Water mounts were used for the observation of the pigmentation and for measurements. Sections were obtained with a Labonord 100 Plus vibratome and stained with Congo red SDS. Lugol's solution was used to evaluate ascus amyloidity. Photomicrographs were aquired using a Canon EOS 7D Mark II DSLR camera. Measurements were performed on microphotographs using a computer. Mean values were calculated from 14 independent measurements. Extreme values are indicated in brackets.

Amplification and sequencing — Internal transcribed spacer (ITS) sequences were amplified directly from *P. nigrella* ascoma using Phire Plant Direct PCR Master Mix (Thermo Fisher Scientific, Waltham, USA) following manufacturer's instructions, using primer ITS1-F/ITS4 (White *et al.*, 1990; Gardes & Bruns, 1993). Plectasin was amplified using primers PL-F (5'-ATGCAATTTACCACCATCCT-3') and PL-R (5'-CTAGTAACACTTGCAAACAA-3'). The reaction consisted of 40 cycles of amplification comprising 5 s of denaturation at 95 °C, 5 s of annealing at 54 °C and 20 s of elongation at 72°C. The amplicons were separated by agarose gel electrophoresis, purified using a MinElute Gel Extraction Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions, and cloned into pGEM-T vector (Promega, USA). After blue-white selection, positives clones were sequenced (Source BioScience, Nottingham, UK).

Phylogenetic analyses — *P. nigrella* ITS sequence was aligned with the most similar sequences scored by the UNITE (https://unite.ut.ee/) massBLASTer software (Nilsson *et al.*, 2018) using ProbCons v1.12 (Do *et al.*, 2005) with default parameters (consistency reps: 2; iterative refinement reps: 100). After alignment, poorly aligned regions and/or containing gaps were removed with Gblocks v0.91b (Castresana, 2000) using the following parameters: a minimum block length of 10 after gap cleaning, no gap positions allowed in the final alignment, rejection of all segments with contiguous nonconserved positions bigger than 4, and at least 85% sequences for a flank position. A phylogenetic tree was then reconstructed using the maximum likelihood method implemented in the PhyML program v3.1/3.0 aLRT (Guindon *et al.*, 2010). The default substitution model was selected assuming an estimated proportion of invariant sites (of 0.483) and 4 gamma-distributed rate categories to account for rate heterogeneity across sites. The gamma shape parameter was estimated directly from the data (gamma = 0.766). Reliability for internal branch was assessed using the bootstrapping method (100 bootstrap replicates). The entire pipeline was run using the online phylogenetic tool suite https://www.phylogeny.fr/ (Dereeper *et al.*, 2008, 2010). Graphical representation and edition of the phylogenetic tree were performed

[1] *P. fulgens* is now treated as a member of genus *Caloscypha* (Boudier, 1885).

with the interactive Tree Of Life (iTOL) tool version 4 (https://itol.embl.de) (Letunic & Bork, 2019).

Axenic culture of *P. nigrella* samples — Fungal isolate H11.20 (Genbank: MF347770) was maintained on potato dextrose agar (PDA) plates at room temperature in the dark. Mycelium was propagated to fresh plates using agar plugs.

Sequence data availability — *P. nigrella* ITS sequence has been deposited to GenBank under the accession number MN398980. ITS sequences obtained from basidiomycete controls are available upon request.

Taxonomy

Pseudoplectania nigrella (Pers.) Fuckel, *Jahrbücher des Nassauischen Vereins für Naturkunde,* 23–24: 324 (1870).

Homotypic synonyms: *Peziza nigrella* Pers., *Synopsis methodica fungorum*: 648 (1801); *Lachnea nigrella* (Pers.) Gillet, *Champignons de France. Les Discomycètes*, 3: 78 (1880); *Plectania nigrella* (Pers.) P. Karst., *Acta Societatis pro Fauna et Flora Fennica*, 2 (6): 119 (1885); *Otidella nigrella* (Pers.) J. Schröter, *Kryptogamen-Flora von Schlesien,* 3.1 (33–40): 48 (1885); *Crouania nigrella* (Pers.) Hazsl., *Math. Term. Köz.*, 21: 261 (1886); *Scypharia nigrella* (Pers.) Quél., *Enchiridion Fungorum*: 283 (1886); *Sepultaria nigrella* (Pers.) Lambotte, *Mém. Soc. Roy. Sci. Liège*: 301 (1887); *Sphaerospora nigrella* (Pers.) Massee, *British Fungus-Flora*, 4: 296 (1895)

Other synonyms: *Helvella hemisphaerica* Wulfen, *Schriften Berlin. Ges. Naturf. Freunde,* 8: 141 (1788); *Peziza epibrya* Sauter, *Hedwigia*, 20 (9): 133 (1881), *nom. illegit*.

Description

Apothecia (Fig. 1) up to 3 cm, entirely black and matt excepting the shiny hymenium, first deeply cupulate, opening at maturity, sessile. **Outer surface** tomentose, somewhat wrinkled, with an eroded margin. **Asci** cylindrical with a long tapered base, aporynchous, octospored, inamyloid. **Ascospores** spherical, (10.6–) 11.4 ± 0.3 µm in diameter, smooth, hyaline, thick-walled (0.5–0.8 µm). Sterile elements of two types: **paraphyses** filiform, septate, slightly expanded apically, sometimes branched near the top, with an amorphous brownish pigment; **hymenial hairs** not septate and larger. **Ectal excipulum** of *textura angularis*, with some thick-walled, brown-pigmented cells in the most external layer. **Medullary excipulum** of *textura intricata*. **Hairs** brownish, often coiled but occasionally straight, septate, thick-walled (0.5–1.0 µm).

Studied material: France. Alpes-Maritimes, Saint-Martin-Vésubie, valley of Madone de Fenestre, alt. 1300 m, May 2019, at the edge of the riparian forest around the river Vésubie, on a mossy soil covered with dead spruce needles (pers. herb. EE00038).

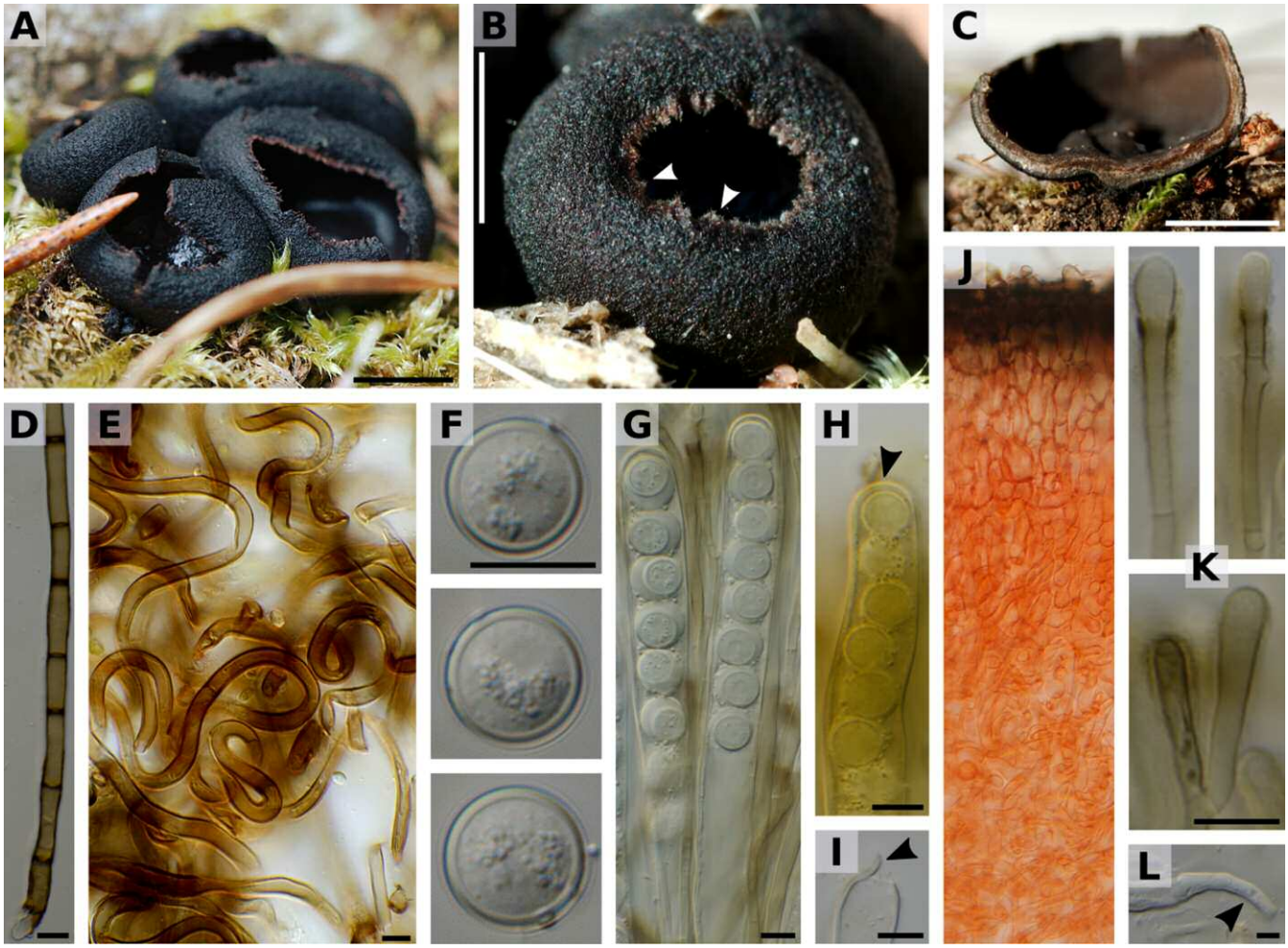


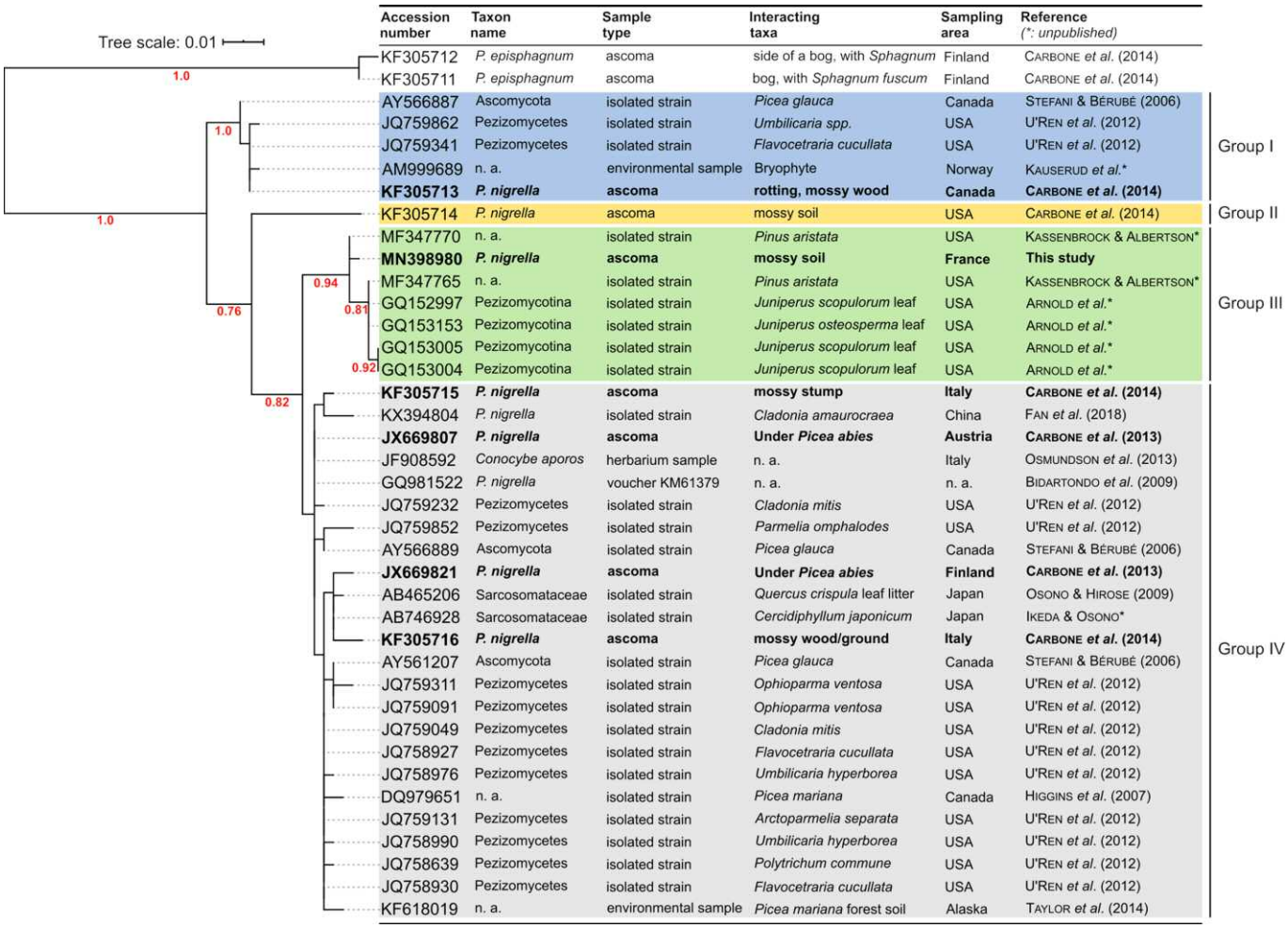
Figure 1 – Macro- and microscopic features of the *P. nigrella* sample from Maritime Alps. (A) Growth habit. (B) Detailed view showing tomentose margin. (C) Section through cup-shaped apothecia showing absence of stipe. (D–E) Hairs, either straight (D) or coiled (E). (F) Ascospores from spore print. (G) Asci and ascospores. (H) Ascus stained with Lugol reagent, showing absence of detectable amyloid compounds. (I) Ascus opercule. (J) Section through the apothecium after staining with Congo Red SDS, showing ectal excipulum of *textura angularis*, and medullary excipulum of *textura intricata*. (K) Paraphyses with brown pigment. (L) Tapered ascus basis. Scale bar = 1 cm (A–C) or 10 µm (D–L).

Phylogenetic analysis

We generated a phylogenetic tree of *P. nigrella* ITS rRNA variation using data gained from our *P. nigrella* ascomata (Genbank: MN398980) and 36 related sequences available in public databases, in addition to 2 sequences corresponding to the similar-looking species *Pseudoplectania episphagnum* (J. Favre) M. Carbone, Agnello & P. Alvarado (Fig. 2). Consistent with results obtained by Carbone *et al.* (2014), sequences split into two main groups with strong bootstrap support (P = 1.0) that confirmed the discrimination of *P. nigrella* and *P. episphagnum* as independent taxa. Within the *P. nigrella* group, we identified four major subgroups, labelled as groups I, II, III and IV. Group I (Fig. 2, blue) consisted of one European and four North American samples and included a *P. nigrella* ascoma harvested on rotting, mossy wood (GenBank: KF305713; Carbone *et al*., 2014). This group could represent *Pseudoplectania lignicola* (Carbone, pers. comm.). Group II (Fig. 2, yellow) contained a single ascoma from the USA. Groups I and II were identical to those identified by Carbone *et al.* (2014). Our *P. nigrella* sample was part of group III (Fig. 2, green) together with two North American fungal endophytes isolated from *Pinus aristata* needles [GenBank: MF347770 (voucher strain H11.20) and MF347765 (voucher strain B7.14)]; Kassenbrock & Albertson, unpublished). Group III further contained four samples isolates from *Juniperus* leaves (GenBank: GQ152997, GQ153004, GQ153005 and GQ153153; Arnold *et al*., unpublished). Thus, our data confirm and expand the corresponding group identified by Carbone *et al.*, 2014. Finally, group IV (Fig. 2, grey) contained 24 sequences, including four European collections of *P. nigrella* ascoma from mossy stump or ground (Carbone *et al*., 2013, 2014) and a problematic herbarium sample annotated as *Conocybe aporos* Kits van Wav. (current name: *Pholiotina aporos* (Kits van Wav.) Clémençon) (GenBank: JF908592; Osmundson *et al*., 2013), whose sequence may result from a contamination during sample processing. Taken together, these data suggest that the current definition of *P. nigrella* comprises at least four distinct lineages supported by ascoma collections. In addition, three of these groups contain endophytic and/or endolichenic strains, suggesting *P. nigrella* lifestyle may not be exclusively saprotrophic.

Molecular analysis of *Pinus aristata*-associated voucher strain H11.20

In an attempt to strengthen our conclusions in the absence of ascomata to confirm the identity of the foliar endophytes related to our collection, we assessed the occurrence of plectasin in the *P. aristata*-associated voucher strain H11.20 (GenBank: MF347770; Kassenbrock & Albertson, unpublished) (Fig. 3). Indeed, plectasin is considered a hallmark of *Pseudoplectania* (Mygind *et al*., 2005). To this end, we designed primers to amplify the 300 bp plectasin precursor sequence. First, we established a simple, high-throughput protocol for rapid fungal DNA amplification from tiny (1 mm^2) mycelium clumps based on Phire Plant Direct PCR Master Mix (Thermo Fisher Scientific, Waltham, USA). We assessed efficiency and reproducibility of this method by re-amplifying ITS region of strain H11.20 as well as those of three well-characterized basidiomycete species (*Gymnopus confluens* (Pers.) Antonín, Halling & Noordel., *Boletus edulis* Bull.



Accession number	Taxon name	Sample type	Interacting taxa	Sampling area	Reference (*: unpublished)	
KF305712	*P. episphagnum*	ascoma	side of a bog, with *Sphagnum*	Finland	Carbone *et al.* (2014)	
KF305711	*P. episphagnum*	ascoma	bog, with *Sphagnum fuscum*	Finland	Carbone *et al.* (2014)	
AY566887	Ascomycota	isolated strain	*Picea glauca*	Canada	Stefani & Bérubé (2006)	
JQ759862	Pezizomycetes	isolated strain	*Umbilicaria spp.*	USA	U'Ren *et al.* (2012)	
JQ759341	Pezizomycetes	isolated strain	*Flavocetraria cucullata*	USA	U'Ren *et al.* (2012)	Group I
AM999689	n. a.	environmental sample	Bryophyte	Norway	Kauserud *et al.**	
KF305713	***P. nigrella***	**ascoma**	**rotting, mossy wood**	**Canada**	**Carbone *et al.* (2014)**	
KF305714	*P. nigrella*	ascoma	mossy soil	USA	Carbone *et al.* (2014)	Group II
MF347770	n. a.	isolated strain	*Pinus aristata*	USA	Kassenbrock & Albertson*	
MN398980	***P. nigrella***	**ascoma**	**mossy soil**	**France**	**This study**	
MF347765	n. a.	isolated strain	*Pinus aristata*	USA	Kassenbrock & Albertson*	
GQ152997	Pezizomycotina	isolated strain	*Juniperus scopulorum* leaf	USA	Arnold *et al.**	Group III
GQ153153	Pezizomycotina	isolated strain	*Juniperus osteosperma* leaf	USA	Arnold *et al.**	
GQ153005	Pezizomycotina	isolated strain	*Juniperus scopulorum* leaf	USA	Arnold *et al.**	
GQ153004	Pezizomycotina	isolated strain	*Juniperus scopulorum* leaf	USA	Arnold *et al.**	
KF305715	***P. nigrella***	**ascoma**	**mossy stump**	**Italy**	**Carbone *et al.* (2014)**	
KX394804	*P. nigrella*	isolated strain	*Cladonia amaurocraea*	China	Fan *et al.* (2018)	
JX669807	***P. nigrella***	**ascoma**	**Under *Picea abies***	**Austria**	**Carbone *et al.* (2013)**	
JF908592	*Conocybe aporos*	herbarium sample	n. a.	Italy	Osmundson *et al.* (2013)	
GQ981522	*P. nigrella*	voucher KM61379	n. a.	n. a.	Bidartondo *et al.* (2009)	
JQ759232	Pezizomycetes	isolated strain	*Cladonia mitis*	USA	U'Ren *et al.* (2012)	
JQ759852	Pezizomycetes	isolated strain	*Parmelia omphalodes*	USA	U'Ren *et al.* (2012)	
AY566889	Ascomycota	isolated strain	*Picea glauca*	Canada	Stefani & Bérubé (2006)	
JX669821	***P. nigrella***	**ascoma**	**Under *Picea abies***	**Finland**	**Carbone *et al.* (2013)**	
AB465206	Sarcosomataceae	isolated strain	*Quercus crispula* leaf litter	Japan	Osono & Hirose (2009)	
AB746928	Sarcosomataceae	isolated strain	*Cercidiphyllum japonicum*	Japan	Ikeda & Osono*	
KF305716	***P. nigrella***	**ascoma**	**mossy wood/ground**	**Italy**	**Carbone *et al.* (2014)**	Group IV
AY561207	Ascomycota	isolated strain	*Picea glauca*	Canada	Stefani & Bérubé (2006)	
JQ759311	Pezizomycetes	isolated strain	*Ophioparma ventosa*	USA	U'Ren *et al.* (2012)	
JQ759091	Pezizomycetes	isolated strain	*Ophioparma ventosa*	USA	U'Ren *et al.* (2012)	
JQ759049	Pezizomycetes	isolated strain	*Cladonia mitis*	USA	U'Ren *et al.* (2012)	
JQ758927	Pezizomycetes	isolated strain	*Flavocetraria cucullata*	USA	U'Ren *et al.* (2012)	
JQ758976	Pezizomycetes	isolated strain	*Umbilicaria hyperborea*	USA	U'Ren *et al.* (2012)	
DQ979651	n. a.	isolated strain	*Picea mariana*	Canada	Higgins *et al.* (2007)	
JQ759131	Pezizomycetes	isolated strain	*Arctoparmelia separata*	USA	U'Ren *et al.* (2012)	
JQ758990	Pezizomycetes	isolated strain	*Umbilicaria hyperborea*	USA	U'Ren *et al.* (2012)	
JQ758639	Pezizomycetes	isolated strain	*Polytrichum commune*	USA	U'Ren *et al.* (2012)	
JQ758930	Pezizomycetes	isolated strain	*Flavocetraria cucullata*	USA	U'Ren *et al.* (2012)	
KF618019	n. a.	environmental sample	*Picea mariana* forest soil	Alaska	Taylor *et al.* (2014)	

Figure 2 – Phylogenetic tree of *Pseudoplectania nigrella* and *P. episphagnum* ITS rRNA variation. Lines highlighted in bold represent sequences obtained from ascoma. Blue, yellow, green and grey subgroups consist of at least one *P. nigrella* sample identified based on morphological data, clustering with endophytic or endolichenic fungal strains. (*) Unpublished work. n.a. : not available.

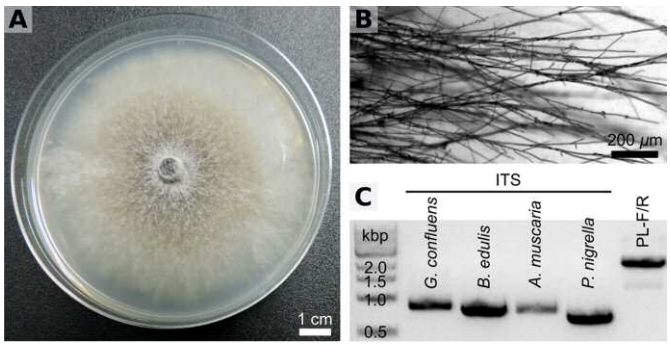


Figure 3 – Molecular analysis of voucher strain H11.20. (A) Growth habit of voucher strain H11.20. (B) Magnified view of hyphae from strain H11.20. (C) Agarose gel electrophoresis of amplicons corresponding to ITS sequences of *Gymnopus confluens*, *Boletus edulis*, *Amanita muscaria* (used a controls), and *P. nigrella*. The last well shows the 2-kb amplicon obtained upon PCR amplification with PL-F/PL-R primers on fungal isolate H11.20.

and *Amanita muscaria* (L.) Lam.) (Fig. 3C). We obtained a single amplicon for all samples tested, which we then confirmed by sequencing. Hence, direct amplification on mycelium clumps is a reliable, faster alternative to genomic DNA extraction followed by PCR.

PCR reaction using PL-F/PL-R primers resulted in a single amplicon of approximately 2 kb (Fig. 3C), whose sequence could not be associated with plectasin. A similar amplicon was obtained from our *P. nigrella* ascoma. Sequences shared more than 99% identity at the nucleotide level (Fig. S1). In addition, the 2-kb amplicon contained an open reading frame encoding a putative Rpn2-like proteasome subunit (Fig. S1). This sequence showed more than 80% amino acid identity with proteins from several ascomycete lineages, including *Sphaerosporella* (Svrček) Svrček & Kubicka, *Pyronema* Carus, *Tuber* F.H. Wigg., *Terfezia* (Tul. & C. Tul.) Tul. & C. Tul., *Choiromyces* Vittad. and *Morchella* Pers., suggesting it is of fungal origin and does not result from contamination. In light of these results, investigations are being initiated to obtain fruiting bodies and confirm the identity of the H11.20 strain based on morphological criteria.

Discussion

Ecological features (mossy soil with spruce needles, near spruces at the subalpine level), as well as the sessile ascoma, unequivocally support the identification of our collection as *P. nigrella*. Microscopically, our determination is further supported by the occurrence of coiled hairs and straight paraphysis tips, although these characters also occur in other *Pseudoplectania* species such as *Pseudoplectania episphagnum*. Therefore, we second Carbone's (2013) statement that macroscopic, ecological and geographical data are of major importance for the identification of *Pseudoplectania* species. Molecular data confirmed that our sample differ from the sister species *P. episphagnum*, which is exclusively associated with *Sphagnum* on bogs and consists in shortly stipitate apothecia (Carbone *et al*., 2014). Our work therefore further supports discrimination between these two taxa.

Phylogenetic analysis of ribosomal sequences confirmed the close relationships between *P. nigrella* collections and several endophytic or endolichenic fungi. Interestingly, most *P. nigrella*-related endophytic samples were collected from acicular (needle-like) leaves, which is reminiscent of typical *P. nigrella* ecological features. Besides, careful surface-sterilization of leaves and thalli prior to cultivation excludes surface contamination with *P. nigrella* spores. Altogether, these findings support the hypothesis that *P. nigrella* may undergo a partially endophytic lifestyle. In agreement with this hypothesis, most endophytic or endolichenic species reported to date belong to the Ascomycota division, and includes members of *Pezizomycetes* (Higgins *et al*., 2007). However, unequivocal characterization of *P. nigrella*-related fungal endophytes at the species level is required to confirm this observation.

In a preliminary attempt to test our hypothesis, we designed primers to amplify the coding sequence of plectasin, a defensin-like gene described as a hallmark of *Pseudoplectania* species (Mygind *et al*., 2005). We could amplify plectasin neither from the H11.20 voucher strain nor from our *P. nigrella* sample. Instead, both samples led to an almost identical 2-kb amplicon encoding a putative Rpn2-like proteasome subunit. PCR-based methods are sensitive to silent mutations which may impair primer binding and therefore trigger false negatives. Furthermore, in the absence of a genome sequence, primer binding specificity cannot be assessed, which may in turn lead to off-target amplifications.

In the absence of ITS sequence corresponding to the voucher strain used by Mygind *et al.* (2005), one can hypothesize that their sample may belong to a distinct *P. nigrella* subgroup. Whether plectasin sequence is conserved at the nucleotide level between these subgroups remains to be detemined.

The 5′ end of the plectasin coding sequence encodes a signal peptide which may undergo different selection pressure compared to the 3′ end encoding the defensin domain. For instance, studies of plectasin-related defensins from *Pyronema omphalodes* (Bull.) Fuckel (GenBank: CCX33670) showed low conservation of the signal peptide even at the protein level, while the defensin domain was almost identical (Traeger *et al*., 2013). As a result, failure to amplify plectasin coding sequence may not be informative. Therefore, robust methods to produce fruiting bodies from axenic cultures will be established. Molecular data obtained from unequivocally identified *Pseudoplectania* samples, including *P. nigrella*-related foliar endophytes, will broaden our understanding of their lifestyle. They will also provide insights in the relationships between *Pseudoplectania* and other genera within *Sarcosomataceae*.

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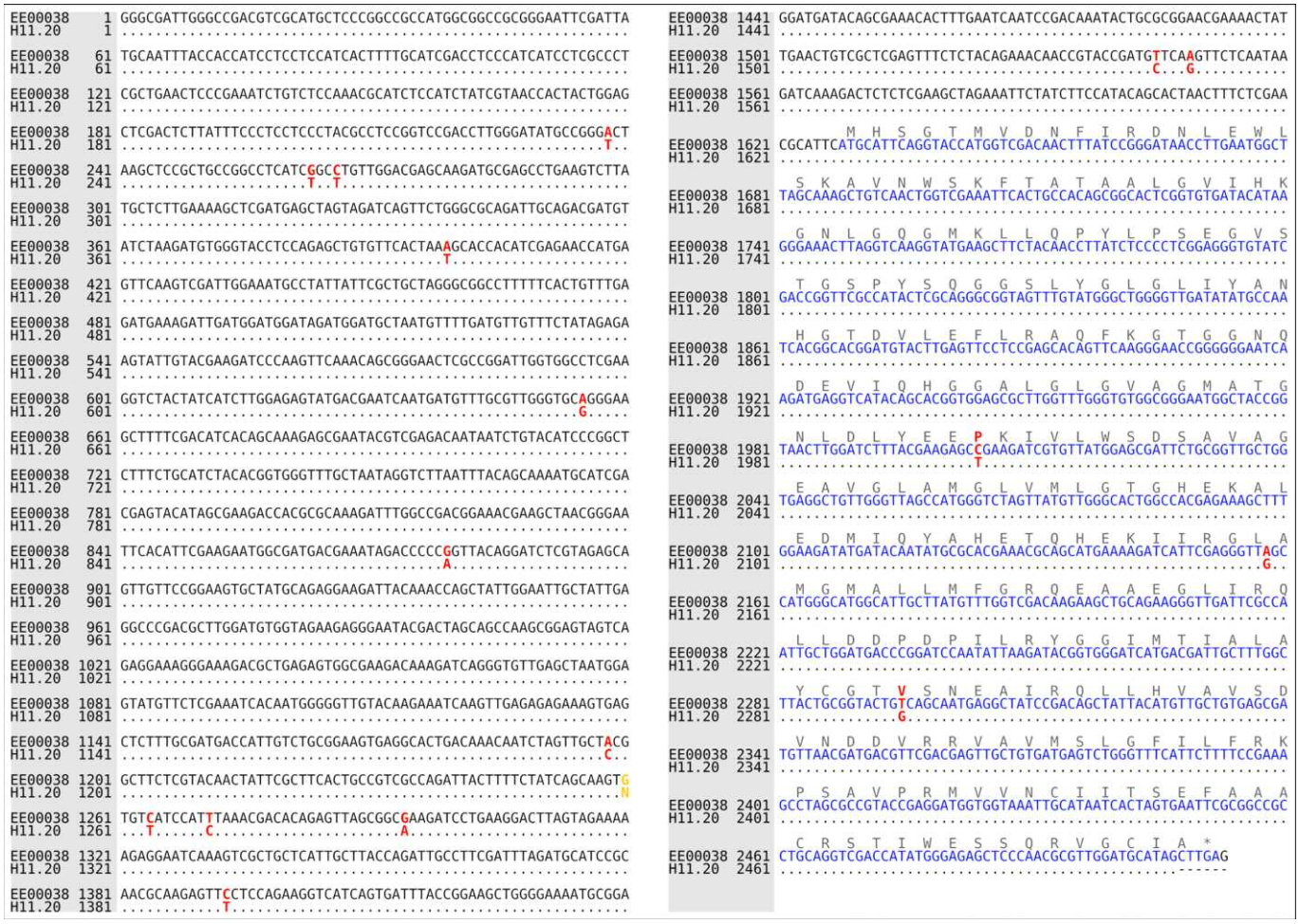

Figure S1 – Alignment of the 2-kb amplicons obtained with PL-F/PL-R primer pairs. Dots indicate consensus. Differences are highlighted in red. Ambiguous bases are indicated in yellow. The Rpn2-like coding sequence is indicated in blue. Residues corresponding to the EE00038 sequence are indicated in grey.

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