Molecular characterization and phylogenetic analyses of *Lophodermella* needle pathogens (Rhytismataceae) on *Pinus* species in the USA and Europe (#57289)

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Molecular characterization and phylogenetic analyses of *Lophodermella* needle pathogens (Rhytismataceae) on *Pinus* species in the USA and Europe

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Increasing prevalence of conifer needle pathogens globally have prompted further studies on pathogen identification and a better understanding of phylogenetic relationships among needle pathogens. Several *Lophodermella* species can be aggressive pathogens causing needle cast in natural pine forests in the USA and Europe. However, their relationships with other Rhytismataceae species have historically been based on similarities of only limited phenotypic characters. Currently, no molecular studies have been completed to elucidate their relationships with other *Lophodermella* needle pathogens. This study collected and sequenced three gene loci, namely: internal transcribed spacer, large ribosomal subunit, and translation elongation factor 1-alpha, from five *Lophodermella* needle pathogens from North America (*L. arcuata, L. concolor, L. montivaga*) and Europe (*L. conjuncta* and *L. sulcigena*) to distinguish phylogeny within Rhytismatacaeae, including *Lophophacidium dooksii*. Phylogenetic analyses of the three loci revealed that all but *Lophodermella conjuncta* that were sampled in this study consistently clustered in a well-supported clade within Rhytismataceae. The multi-gene phylogeny also confirmed consistent nesting of *L. dooksii*, a needle pathogen of *Pinus strobus*, within the clade. Potential synapomorphic characters such as ascomata position and ascospore shape for the distinct clade were also explored. Further, a rhytismataceous species on *P. flexilis* that was morphologically identified as *L. arcuata* was found to be unique based on the sequences at the three loci. This study suggests a potential wider range of host species within the genus and the need for genetic characterization of other *Lophodermella* and *Lophophacidium* species to provide a higher phylogenetic resolution.

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Abstract

Increasing prevalence of conifer needle pathogens globally have prompted further studies on identification and a better understanding of phylogenetic relationships among needle pathogens. Several *Lophodermella* species can be aggressive pathogens causing needle cast in natural pine forests in the USA and Europe. However, their relationships with other Rhytismataceae species have historically been based on similarities of only limited phenotypic characters. Currently, no molecular studies have been completed to elucidate their relationships with other *Lophodermella* needle pathogens. This study collected and sequenced three gene loci, namely: internal transcribed spacer, large ribosomal subunit, and translation elongation factor 1-alpha, from five *Lophodermella* needle pathogens from North America (*L. arcuata, L. concolor, L. montivaga*) and Europe (*L. conjuncta* and *L. sulcigena*) to distinguish phylogeny within Rhytismatacaeae, including *Lophophacidium dooksii*. Phylogenetic analyses revealed that all species but *L. conjuncta* were consistently clustered in a well-supported clade within Rhytismataceae, and also confirmed consistent nesting of *L. dooksii*, a needle pathogen of *Pinus strobus*, within the clade. Potential synapomorphic characters such as ascomata position and ascospore shape for the distinct clade were also explored. Further, a rhytismataceous species on *P. flexilis* that was morphologically identified as *L. arcuata* was found to be genetically unique. This study suggests

a potential wider range of pathogen species within the genus and the need for genetic characterization of other *Lophodermella* and *Lophophacidium* species to provide a higher phylogenetic resolution.

Introduction

Conifer needle diseases are becoming increasingly prevalent due to several factors such as climate change and introduction to new hosts (Woods et al. 2005, Lee et al. 2017, Wyka et al. 2017, Brodde et al. 2018). Native needle pathogens emerge as they move into novel geographic areas while others are increasing in incidence due to faster sporulation enhanced by warmer and wetter conditions (Barnes et al. 2014, Gray et al. 2013, Rodas et al. 2016, Welsh et al. 2014). Recent examples of needle diseases with enhanced severity include Dothistroma needle blight (Woods 2014), Swiss needle cast and Cedar leaf blight (Gray et al. 2013), and white pine needle damage (Wyka et al. 2018, Broders et al. 2015).

In the western region of USA, an increasing prevalence of native *Lophodermella* needle pathogens, which may be attributed to climate change, were observed (Worrall et al. 2012) in dominant and ecologically important natural pine species along the Rocky Mountain Region, *P. contorta* and *P. flexilis* (Lotan and Critchfield 1990, Schoettle 2004). Two needle cast epidemics caused by *L. concolor* and *L. montivaga* were recorded on lodgepole pine (*Pinus contorta*; Worrall et al. 2012) while increased frequency of *L. arcuata* infection was observed in patches of limber pine (*P. flexilis*) stands. Meanwhile, in Europe, heavy infection of *L. sulcigena* and *L. conjuncta* on European mountain pine (*P. mugo*) along the Swiss Alps were recorded in 2018 (Beenken 2019). Despite increasing incidence, there are no wide scale assessments on the impact of *Lophodermella* pathogens in natural pine stands amidst climate change. Past surveys reported minor incidence of *Lophodermella* species such as *L. cerina* and *L. morbida* in the western USA, *L. maureri* in Mexico, and *L. orientalis* in Asia (Darker 1932, Minter 1988b, Minter 1993) but there are no recent surveys nor reports about their increasing incidence in these regions.

Thus far, only nine species belong to *Lophodermella* genus, including *L. arcuata, L. cerina, L. concolor, L. conjuncta, L. maureri, L. montivaga, L. morbida, L. orientalis* and *L. sulcigena* (Mycobank 2019). *Lophodermella* species (Rhytismataceae) are distinguished by their

subhypodermal ascomata, clavate ascospores surrounded by mucilaginous sheath, and wider asci than a closely related genus *Lophodermium* (Darker 1967). While morphometric descriptions are clear in the literature, identification and differentiation among these *Lophodermella* species is challenging. This may be attributed to similarities in early symptoms of the disease, highly variable morphometric features at different developmental stages and mounting medium, secondary fungal invasion, and lack of ideally mature specimens (Worrall et al. 2012). Based on morphological characteristics there have been doubts on disease reports of *L. sulcigena* on *P. radiata, P. halepensis* and *P. contorta* while other diseases still need verification, such as the occurrence of *L. montivaga* on *P. monticola* and *P. flexilis* (Millar 1984).

Molecular characterization could help resolve classification of species closely related to *Lophodermella* such as the case of *Lophophacidium dooksii* on needles of five-needle *Pinus strobus*. The then-undescribed *L. dooksii* was classified under Phacidiaceae due to the lack of morphological characteristics distinctive of Rhytismataceae (Corlett and Shoemaker 1984). However, recent internal transcribed spacer (ITS) phylogenetic studies and morphology suggest *Lophophacidium dooksii* is closely related to *L. arcuata* (Laflamme et al. 2015, Ekanayaka et al. 2019). Following the phylogenetic evidence, Ekanayaka (2019) reclassified *L. dooksii* to Rhytismataceae, but the phylogenetic relationship of *L. dooksii* and *L. arcuata* with other *Lophodermella* species is still unclear.

The lack of molecular information *Lophodermella* spp. makes it difficult to resolve intra- and interspecific phylogenetic relationships. Currently, out of the nine known *Lophodermella* species, only the ITS sequence of *L. arcuata* represents the genus in fungal genetic databases. As emerging pathogens, molecular studies on *Lophodermella* are important in identification and to elucidate of their phylogenetic relationship with other rhytismataceaous species. These will aid in assessing the diversity and impact of emerging or invasive disease threats in conifer forest and will provide insights on fungal biology and evolution of traits. This study aims to fill this gap by analyzing the phylogeny of *Lophodermella* species that cause emerging needle cast diseases in western USA and Europe which include *L. arcuata, L. concolor, L. conjuncta, L. montivaga*, and *L. sulcigena.* We gather and use molecular data from three loci and compare their morphological characters from the resulting phylogeny to identify shared derived characters.

Materials & Methods

Sampling and morphology

Sampling was conducted in known geographic distributions of *L. arcuata, L. concolor, L. montivaga* and *L. dooksii* in the USA. Similarly, *L. sulcigena* and *L. concolor* samples were collected from their known distributions in Europe. Needles from 32 *P. contorta* trees from natural stands infected with *L. montivaga* and/or *L. concolor* were collected in June 2018 across 12 sites within Gunnison National Forest, Colorado, USA (Table 1). *Lophodermella arcuata* on *P. flexilis* stands were collected from Rocky Mountain National Park, Colorado, USA in June 2018 and June 2019 while the eastern white pine (*P. strobus*) needles symptomatic of *L. dooksii* were collected from natural stands in Maine, USA in May 2019. Needles of the *P. mugo* infected with *L. sulcigena* and *L. conjuncta* were collected in the Swiss and Austrian Alps in 2018 (Table 1). Needles were placed into separate paper bags and stored at 4°C until DNA extraction.

Morphology of the fungal pathogens from randomly selected fresh symptomatic needles was characterized for fungal identification (Fig 1). Midsections of ascomata were cut using a razor blade and mounted in 3% potassium hydroxide (KOH). Measurements of fruiting structures were taken from mounted materials. Morphological traits common among species based on published descriptions were compared (Table 2; Corlett and Shoemaker 1984, Darker 1932, Millar and Minter 1966, 1978, Minter and Millar 1993a, Worrall et al. 2012).

DNA extraction and sequencing

Cultures from single-spore isolations of *L. montivaga*, *L. concolor* and *L. arcuata* were attempted but did not yield pure cultures, as these are thought to be potentially obligate fungi. Similar to previous observations (Darker 1932), mature spores isolated did not germinate and development of germ tubes in a few spores became arrested. Therefore, to be able to extract adequate amounts of quality DNA, fruiting bodies from three to five symptomatic needles from each tree were used for DNA extraction. DNA was extracted using a CTAB method with slight modifications in tissue grinding (Cubero et al. 1999). To prepare the samples, hysterothecia were cut into 1 mm long pieces and placed in 2 mL centrifuge tubes with one 5 mm glass bead and two 2.3 mm metal beads. To grind the samples, the tubes were submerged in liquid nitrogen

before grinding using FastPrep (MP Biomedicals) for 30 seconds at speed 4 or 5. This previous process was repeated three times prior to the CTAB DNA extraction procedure developed by Cubero et al. (1999). DNA quantification and purity were assessed using NanoDrop 1000 Spectrophotometer (Thermo Scientific). For *L. sulcigena* and *L. conjuncta*, single fruiting bodies (ca. 3-4 mm long pieces) each were prepared out of dry pine needles. DNA was extracted from the lyophilized and ground fruit bodies using the KingFisher/Flex Purification System (ThermoFisher Scientific) according to the manufacturer's protocol and the chemicals for automated DNA extraction from fungal samples with Kingfisher 96/Flex supplied by LGC Genomics GmbH (Berlin).

DNA was amplified at the following loci: internal transcribed spacer region and 5.8S ribosomal RNA (ITS), large subunit ribosomal nucleic acid (LSU), and translation elongation factor (TEF1α). Primers used include ITS1 and ITS4 (White et al. 1990), LROR and LR5 or LR6 (Vilaglys and Hester 1990), and EF1-983F and EFgr (Rehner 2001). The ITS locus was amplified at optimal annealing temperatures between 50 – 55 ºC with 30 cycles while TEF1α and LSU were amplified at 56ºC annealing temperature with 35 cycles (Tanney and Seifert 2017).

PCR products were purified using ExoSAP-IT (Affymetrix™). All purified amplicons were sent to Eurofins Genomics LLC for sequencing. Additionally, cloning of PCR products for each locus was performed on *L. concolor* and *L. montivaga* samples using pGEM® T-Easy Vector Systems (Promega) to confirm that a sequenced amplicon was of single species. Three to seven clones were sequenced for each sample and found to be 99.81 to 100% identical to the sequence of its corresponding original PCR product. Sequences were matched using NCBI Nucleotide Basic Local Assignment Search Tool (BLASTn) for fungal identification and were accessioned in NCBI GenBank (Table 1). Sequence data were trimmed and manually checked using Geneious version R9.0.5 (Biomatters, Auckland, New Zealand) and subsequently aligned using MUSCLE (Edgar 2004). A consensus tree of the concatenated dataset was stored in TreeBase (Submission ID 26836).

Phylogenetic analyses for each locus were constructed using Bayesian inference (MrBayes; Huelsenbeck and Ronquist 2001) and maximum likelihood methods (PhyML; Guindon et al. 2010) as modules in Geneious v. R9.0.5. Optimal substitution models for each dataset generated using DT-ModSel (Minin et al. 2003) were as follows: *SYM + G* for ITS, *TrNef + G* for TEF1α, *TrN + I + G* for LSU, and *SYM + I + G* for the concatenated dataset. For models of evolution that are not available in either MrBayes or PhyML modules, the next best complex models were applied. Bayesian tree was analyzed by running Markov Chain Monte Carlo (MCMC) for up to 1,100,000 generations with four heated chains. Maximum likelihood tree was analyzed using 200 bootstraps. Bayesian and maximum likelihood trees were generated with support thresholds of 80% and 50%, respectively, with a 20% burn-in. The phylogenies were rooted to *Chalara* spp. (*Chalara* sp. MFLU 18-1812 and *Chalara* sp. MFLU 15-3167) following Ekanayaka et al. (2019). To evaluate the congruence of the three loci dataset, partition homogeneity test was conducted using PAUP version 4.0a (Barker and Lutzoni 2002). Three loci dataset was combined using Sequence Matrix (Vaidya et al. 2011). Published sequences of known related species in GenBank database were included in the phylogenetic analysis (Table 3).

Character mapping

Morphological characters were selected based on ~~the presence~~ in literature and their use for taxonomic classification of Rhytismataceae. Characters were coded based on published descriptions (Table 4; Darker 1932, 1967, Minter 1988a, Minter and Millar 1993a, 1993b, 1993c, Tanney and Seifert 2017, MycoBank 2019, Fungi and Lichens of Great Britain and Ireland 2019) and then mapped on the ITS dataset phylogeny which had a more comprehensive set of Rhytismataceae species. All morphological characters were coded as unordered and mapped with parsimony ancestral trace reconstruction using Mesquite v.3.6 (Maddison and Maddison 2018).

Results

Molecular and Phylogenetic Analyses

PCR amplification produced a single band for each sample per locus. Chromatograms for most forward and reverse sequences did not show multiple peaks at base calls, indicating uniform amplicons. Amplicons of the ITS, TEF1α and LSU yielded products of 596, 819 and 1091 base pairs, respectively. Of the 40 samples of *Lophodermella* species and *L. dooksii* at the ITS, a total

of nine genotypes were found with 83 polymorphic (segregating) sites and 64 parsimony informative sites were observed. At the TEF1α, the 37 samples of *Lophodermella* species and *L. dooksii* had eight genotypes, and 77 of the 105 polymorphic sites were considered informative. Sequences of the 35 *Lophodermella* species and *L. dooksii* samples at the LSU resulted to nine haplotypes with 106 total polymorphic sites and 62 parsimony informative sites. BLAST results of sequences are presented in Table S1.

Several *Lophodermella* species and *L. dooksii* clustered in a well-supported clade (hereinafter referred to as the LOD clade) at the ITS, LSU, TEF1α phylogenies and concatenated dataset. This clade composed of haplotypes of *L. montivaga, L. concolor, L. arcuata, L. sulcigena, Lophodermella* sp. and *L. dooksii* in the ITS phylogeny was well-supported in the Bayesian phylogeny with a 0.96 posterior probability (PP), excluding *L. conjuncta* (Fig. S1). Similarly, for the LSU phylogeny, both Bayesian and ML phylogenies produced the same ~~clade~~ well-supported clade (1.0 PP and 98.8 bootstrap support (BS); Fig. S2). *Lophodermella conjuncta* remained distinct from the clade representing all other *Lophodermella* species at the LSU phylogeny. At the TEF1α region, LOD clade had high support at 1.0 PP and 98.1 BS, (Fig. S3), but did not include both *L. concolor* and *L. conjuncta*. Partition homogeneity tests of the three datasets resulted in a p-value = 0.99, indicating congruent ITS, LSU and TEF1α topologies. A concatenated phylogeny showed all *Lophodermella* species, except *L. conjuncta*, that were sampled in this study, as well as *L. dooksii*, belonged to a well-supported clade with 0.99 PP and 96.9 BS support values (Fig 2).

Morphology and Phylogeny of Lophodermella on P. flexilis

Based on the phylogenetic analyses, two separate *Lophodermella* species were collected from *P. flexilis* in the Rocky Mountain Region. Using the concatenated dataset, *L. arcuata* from Rocky Mountain National Park (RMNP_LU1 and RMNP_LU16) clustered with *L. arcuata* AY465518.1 from NCBI GenBank with 1.0 PP and 100 BS, whereas RMNP_01 clustered with *Lophophacidium dooksii* samples (Tables 1 and 3) with 0.98 PP and 87 BS (Figure 1). Similarly, RMNP_01 and *L. dooksii* (MB5, Table 1) clustered at ITS (0.98 PP and 91.3 BS; Fig. S1) and TEF1α (0.96 PP, 67.7 BS; Fig. S3), indicating that RMNP_01 may represent a new species, distinct from *L. arcuata*. Morphologically, sample RMNP_01 had subhypodermal hysterothecia

measuring 0.48 – 0.6 x 0.16 – 0.168 mm and were tanned at mesophyll and hypodermis. Asci were broadly saccate measuring 96 – 130 x 12 – 14 µm. Ascospores were clavate, measuring 58 – 76 µm long and 3.8 – 4 µm wide. Ascospores were also covered with mucilaginous sheath (10 µm wide, Fig 3). These fit the morphometric traits of *L. arcuata* (Table 2). Further, both *Lophodermella* sp. and *L. arcuata* were found on *P. flexilis*.

Shared characteristics of Lophodermella clade

Five traits were used in this study due to the unavailability of morphological data or unclear morphological distinctions of other species within Rhytismataceae (Table 4). The first four morphological characteristics included were those described by Darker (1967) as key characteristics of species within *Lophodermella*. These included ascomata shape and position, asci shape and ascospore shape. Host was included as an ecological trait. The only character conserved within the LOD clade composed of the five *Lophodermella* species and *L. dooksii* was subhypodermal ascomata position in a median transverse section (Fig 4A). All of the *Lophodermella* species sampled in this study occur on pine hosts. The shape of ascomata or hysterothecia, asci and ascospores differed within the LOD clade. *Lophodermella* hysterothecia were mostly elliptical and elongated while hysterothecia of *Lophophacidium dooksii* were linear. *Lophodermella* had clavate ascospores while ascospores of *L. dooksii* were fusiform to oval. All species in the clade, except *L. concolor*, had broadly saccate to clavate asci. While all morphological characters obtained an individual retention index (RI) ≥ 0.50, only ascomata position and ascospore shape had consistency index (CI) ≥ 0.50, which may imply synapomorphy of the two characters (Fig. 4).

Discussion

This study revealed a well-supported clade consisting of several *Lophodermella* species including *L. montivaga, L. concolor, L. arcuata, L. sulcigena,* and *Lophodermella* sp. within Rhytismataceae. *Lophodermella conjuncta*, however, was consistently placed outside of this clade. In all phylogenies, *Lophophacidium dooksii* consistently clustered within the LOD clade. Despite highly similar morphological characteristics, this study showed that *Lophodermella* pathogens are molecularly distinct from each other and may represent more species and more ~~molecular~~ genetic diversity than previously thought. This study also identified shared

characteristics within the LOD clade ~~and explored on morphological characters~~ that could be useful in taxon classification.

Molecular and Phylogenetic Analyses of Lophodermella

A concatenated dataset of the three loci clearly separated *L. montivaga* and *L. concolor* that both infect *P. contorta* and distinguished the *Lophodermella* species from other closely related fungi. *Lophodermella montivaga*, *L. concolor, L. arcuata, L. sulcigena, Lophodermella* sp. and *Lophophacidium dooksii* formed the LOD clade, which were distinct from species within the genera *Lophodermium* (Ortiz-Garcia et al. 2003) and *Spathularia-Cudonia* (Ge et al. 2014). However, at TEF1α, *L. concolor* was excluded from the LOD clade, but was placed in the clade at the LSU and ITS. This could be attributed to a fewer number of sequenced Rhytismataceae species resulting in low phylogenetic resolution or other genetic loci may best represent the species phylogeny. While additional sequences at each locus would likely improve phylogenetic resolution, whole-genome sequencing would provide greater advantage in phylogenetic reconstruction as well as gain deeper evolutionary perspectives on rhytismataceous needle pathogens.

Exclusion of *L. conjuncta* in the LOD clade may suggest polyphyly of the genus. This is the first report of the potential polyphyly of *Lophodermella* within Rhytismataceae. Polyphyletic genera are commonly observed within Rhytismatales partly due to the use of distinctive yet non-synapomorphic characters for generic-level classification (Lantz et al., 2011). *Lophodermium* is an example of a polyphyletic genus that appears in the radiate, bilateral and *Picea*-associated clades (2011). Reorganization of *Lophodermium* was not possible due to the wide diversity of species in the group (Darker 1967). Monophyletic genera also exist within Rhytismataceae that includes *Cudonia* and *Terriera* (Lantz et al., 2011). However, since *Lophodermella* is an understudied genus where molecular studies are rare, this present study does not disregard potential changes in the phylogenetic arrangement and polyphyly, as more species are genetically investigated. Increased sampling of species within the two genera provided further evidence of *Cudonia* as a monophyletic genus but suggested that *Spathularia* was polyphyletic (Ge et al. 2014). Thus, further investigation of *Lophodermella* species ~~with no~~ molecular information still needs to be conducted to confirm these phylogenetic arrangements.

285

286 The present study supported a close relationship of *L. montivaga* and *L. sulcigena* compared to
287 the other species within the LOD clade. Darker (1932) speculated that *L. sulcigena* from Europe
288 may be identical to *L. montivaga* due to morphological similarities. Though morphological
289 distinctions between the two species are poorly defined, this present study provided molecular
290 evidence that *L. montivaga* and *L. sulcigena* are two distinct species. Another previous
291 speculation was the possibility that *L. arcuata* is a variety of either *L. montivaga* or *L. sulcigena*
292 owing to its resemblance to both species and its limited occurrence (Darker 1932). However,
293 symptom and ascocarp development in both species were different and thus were maintained as
294 two different species (Millar 1984). Genetic evidence gave support that *L. arcuata* is distinct
295 from *L. sulcigena* and *L. montivaga*.
296
297 Consistent nesting of *Lophophacidium dooksii* in a *Lophodermella* clade was observed in all
298 phylogenies, which concurs with a previous molecular study (Laflamme et al. 2015). Results
299 herein showed that *L. dooksii* is more closely related to *Lophodermella* sp. (from *P. flexilis*) than
300 to *L. montivaga* and *L. arcuata*, and provides more evidence for the transfer of the species from
301 Phacidiaceae to Rhytismataceae as proposed by Ekanayaka et al. (2019). Interestingly, *L. dooksii*
302 was synonymous to *Canavirgella banfieldii*, a species classified under Rhytismataceae, but the
303 former taxonomic name was given priority due to its earlier publication (Laflamme et al. 2015).
304 In other studies, use of multiple loci supported the placement of *Cudonia* and *Spathularia* from
305 Geoglossaceae to Rhytismataceae (Gernandt et al. 2001, Lantz et al. 2011, Ge et al. 2014), which
306 these results also support (Fig. S1-3).
307
308 *Phylogeny of* Lophodermella *sp. from P. flexilis*
309 Individual phylogenies in this study could not confirm the species identity of the *Lophodermella*
310 sp. from limber pine collected at RMNP as it did not cluster together with *L. arcuata* samples.
311 Genetic data suggests *Lophodermella* sp. may represent a separate species distinct from *L.*
312 *arcuata* despite significant morphological similarities and host association. Since needle samples
313 with this potentially new species were only collected from one tree, we did not attempt to
314 formally name the species but temporarily named at the genus level as *Lophodermella*. Further
315 investigation needs to be conducted to differentiate this species with other *Lophodermella*

316 species described in literature and to define the population diversity of *L. arcuata*. Further,
317 results from this study also suggest that undescribed cryptic *Lophodermella* species exist on pine
318 hosts.
319
320 *Morphological and Lifestyle Traits of the* Lophodermella *clade*
321 Classification of Rhytismataceae genera has been challenged by the limited morphological
322 features for characterization. Darker (1967) revised the genera within the previous
323 Hypodermataceae based on the characteristics of their ascomata or hysterothecia, asci, and
324 pycnidia or a combination of these characters. Spore shape, septation and color were secondary
325 characters to delimit the genera (Darker 1967). Further, Lantz et al. (2011) described ascomata
326 and spores as unreliable characters for genus delimitation in Rhytismatales but found that a
327 combination with other traits was potentially useful. Aside from symptom and ascocarp
328 development, morphometric traits such as size of ascus, ascospores and ascomata are still used as
329 distinctive but problematic characters across *Lophodermella* species. For example, while
330 ascospore size was identified as a reliable criterion, measurements of spores varied depending on
331 the freshness of specimen and thus cannot easily be used for identification of *Lophodermella*
332 species (Millar 1984). This study showed that, at the genus level, subhypodermal ascomata and
333 ascospore shape may be used as diagnostic characters for delimitation of genus *Lophodermella.*
334 Interestingly, aside from subhypodermal hysterothecia, all species within the LOD clade
335 produced a tanned hypodermis. Further, despite low consistency, the strong retention of asci
336 shape may also suggest its role in taxa distinction.
337
338 Difficulty in obtaining pure cultures of *L. montivaga*, *L. concolor* and *L. dooksii* can also
339 potentially limit further characterization of other traits such as growth and development, and
340 pathogenicity. Similar to other studies, we were not able to grow in culture the *Lophodermella*
341 species sampled in this study, suggesting an obligate lifestyle. Use of agar cultures including
342 pine extract agar did not yield successful cultures of *Lophodermella* (Millar 1984). Some studies
343 also described *L. dooksii* and *Bifusella linearis* as obligate fungal pathogens after unsuccessful
344 attempts of obtaining cultures or only obtaining short-lived cultures (Broders et al. 2015, Merrill
345 et al. 1996). In contrast, previous studies were able to isolate pure cultures of *L. sulcigena* on
346 malt agar (Jalkanen 1985, Kowalski and Krygier 1996). Similarly, a number of studies

documented several *Lophodermium* species (e.g., Decker et al. 2001, Wilson et al. 1994) growing in 2% malt extract agar. *Elytroderma deformans* needed an acidic pine decoction agar substrate or an addition of pine needle extracts to significantly grow in culture (Laurent 1962, Legge 1964).

Most *Lophodermella* species appear to be either specific to and distributed according to a single host species (i.e., *L. maureri* on *P. ayacahuite* in Mexico and *L. orientalis* on *P. kesiya* in Asia) or to a group of host species within a *Pinus* classification with similar number of needles (i.e., *L. sulcigena* and *L. conjuncta* on two-needle pines of subsection Pinus in Europe, and *L. concolor* on two-needle pines of subgenus Pinus in western North America; Millar 1984, Gernandt et al. 2005). Further, *L. arcuata* and *L. maureri* are the only two *Lophodermella* species on five-needle pines of subsection Strobus. In contrast, *L. cerina* was reported to have a broader host range occurring on two- to three-needle *Pinus* species in sections Trifoliae and Pinus (subgenus *Pinus;* Millar 1984, Gernandt et al. 2005). *Lophodermella montivaga* was also documented on two- to five-needle Haploxylon and Diploxylon pines. In this study, genetic information was used to verify the association of *Lophodermella* species with a known host. It allowed us to identify additional species on *P. flexilis* that would have otherwise been classified as *L. arcuata* based on its morphology and host association. Thus, it can serve as a tool to assess the extent of these fungal species across different hosts in different geographic regions.

Conclusion

This study sequenced and characterized emerging *Lophodermella* needle cast pathogens on *Pinus* in North America and Europe. It highlights a distinct clade composed of *Lophodermella* species and *Lophophacidium dooksii* within Rhytismataceae. Further, this study also observed a *Lophodermella* species on *P. flexilis* that is morphologically similar yet genetically distinct from *L. arcuata,* which suggests presence of undescribed cryptic *Lophodermella* species. Further investigations of *Lophodermella* species using advanced molecular tools can also help answer genetic, evolutionary and ecological inquiries such as on population structure, pathogenicity, host specialization, hybridization, and other biological inferences.

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Figure Legend:

Figure Ascomata of *Lophodermella concolor* (a) and *L. montivaga* (b) on *Pinus contorta* from Gunnison National Forest, Colorado, USA; *Lophodermella* sp. (c) and *Lophodermella arcuata* (d) on *P. flexilis* from Rocky Mountain National Park, Colorado, USA; *Lophophacidium dooksii* on *P. strobus* from Massabesic, Maine, USA (e); and *L. conjuncta* (f) and *L. sulcigena* (g) on *P. mugo* from Austria and Switzerland.

Figure 2 - Bayesian phylogeny depicting phylogenetic relationships of *Lophodermella montivaga* and *L. concolor* within the *Lophodermella* clade based on three gene regions including the internal transcribed spacer (ITS), large ribosomal subunit (LSU) and translation elongation factor 1-alpha (TEF1α). Bayesian posterior probabilities (PP) greater than 0.80 and bootstrap (BS) support values from maximum likelihood analysis greater than 50 are shown above and below node, respectively. Species in bold are samples derived from this study. Numbers correspond to genotypes after concatenation.

Figure 3 - Morphological characters of *Lophodermella* sp. on *Pinus flexilis* collected from Rocky Mountain National Park, Colorado, USA. Subhypodermal hysterothecia with tanned mesophyll and hypodermis (a, d), clavate ascospores with gelatinous sheath (b) and broadly saccate asci (c). Magnification used: 10x and 20x, respectively, for photos of the hysterothecia (a and d); 40x for photos of ascospore and asci (b and c).

Figure 4 - Morphological characters mapped onto ITS phylogenetic tree with the parsimony ancestral reconstruction method using Mesquite v.3.6 with retention indices ≥ 0.50, ascomata position (a) and ascospore shape (b).

Table 1(on next page)

Sample information

Table 1. Collection information, GenBank accession and haplotype numbers for each *Lophodermella* species and *Lophophacidium dooksii* for the three loci, namely: internal transcribed spacer region and 5.8S ribosomal RNA (ITS), large ribosomal subunit (LSU) and translation elongation factor (TEF1).

Table 1. Collection information, GenBank accession and haplotype numbers for each *Lophodermella* species and *Lophophacidium dooksii* for the three loci, namely: internal transcribed spacer region and 5.8S ribosomal RNA (ITS), large ribosomal subunit (LSU) and translation elongation factor (TEF1-α).

Sample ID	Location	Host	Collection Date	Collectors	GenBank Accession Number; (Genotype)		
					ITS	LSU	TEF1-α
***Lophodermella concolor* (Dearn.) Darker**							
CS6C	Cold Spring Campground, Gunnison National Forest, Colorado, USA	*Pinus contorta*	12 June 2018	JE Stewart, JP Ata, KS Burns, SB Marchetti, JJ Worrall	MN937619; (1)	MN937581; (1)	MN937651; (1)
CS9C	Cold Spring Campground, Gunnison National Forest, Colorado,	*Pinus contorta*	12 June 2018	JE Stewart, JP Ata, KS Burns, SB Marchetti, JJ Worrall	MN937612; (1)	MN937579; (1)	MN937650; (1)

	USA						
FS6C	Fisherman Trail, Gunnison National Forest, Colorado, USA	*Pinus contorta*	12 June 2018	JE Stewart, JP Ata, KS Burns, SB Marchetti, JJ Worrall	MN937618; (1)	MN937582; (1)	MN937647; (1)
FS8C	Fisherman Trail, Gunnison National Forest, Colorado, USA	*Pinus contorta*	12 June 2018	JE Stewart, JP Ata, KS Burns, SB Marchetti, JJ Worrall	MN937610; (2)	MN937580; (1)	MN937653; (1)
PT2C	Pitkin, Gunnison National Forest, Colorado, USA	*Pinus contorta*	14 June 2018	JE Stewart, JP Ata, KS Burns, SB Marchetti, JJ Worrall	MN937616; (1)	MN937577; (1)	MN937646; (1)
PT3C	Pitkin, Gunnison National Forest, Colorado, USA	*Pinus contorta*	14 June 2018	JE Stewart, JP Ata, KS Burns, SB Marchetti,	MN937614; (1)	MN937583; (1)	MN937652; (1)

				JJ Worrall			
SR3C	Slate River, Gunnison National Forest, Colorado, USA	*Pinus contorta*	13 June 2018	JE Stewart, JP Ata, KS Burns, SB Marchetti, JJ Worrall	MN937617; (1)	MN937578; (1)	MN937649; (1)
SR6C	Slate River, Gunnison National Forest, Colorado, USA	*Pinus contorta*	13 June 2018	JE Stewart, JP Ata, KS Burns, SB Marchetti, JJ Worrall	MN937613; (1)	MN937584; (1)	MN937648; (1)
LP7C	Lodgepole Campground, Gunnison National Forest, Colorado, USA	*Pinus contorta*	12 June 2018	JE Stewart, JP Ata, KS Burns, SB Marchetti, JJ Worrall	MN937621; (1)	MN937588; (3)	MN937654; (1)
LV7C	Lakeview Campground,	*Pinus contorta*	14 June 2018	JE Stewart, JP Ata, KS Burns,	MN937620; (1)	MN937575; (1)	MN937657; (1)

	Gunnison National Forest, Colorado, USA			SB Marchetti, JJ Worrall			
LV8C	Lakeview Campground, Gunnison National Forest, Colorado, USA	*Pinus contorta*	12 June 2018	JE Stewart, JP Ata, KS Burns, SB Marchetti, JJ Worrall	MN937615; (1)	MN937576; (2)	MN937655; (1)
OJ11C	Oh Be Joyful, Gunnison National Forest, Colorado, USA	*Pinus contorta*	13 June 2018	JE Stewart, JP Ata, KS Burns, SB Marchetti, JJ Worrall	MN937611; (1)	MN937574; (1)	MN937656; (1)
***Lophodermella montivaga* Petrak**							
CU1M	Cumberland, Gunnison National Forest, Colorado,	*Pinus contorta*	14 June 2018	JE Stewart, JP Ata, KS Burns, SB Marchetti,	MN937633; (1)	MN937586; (1)	MN937669; (1)

	USA			JJ Worrall			
LVP2M	Lakeview Campground, Gunnison National Forest, Colorado, USA	*Pinus contorta*	14 June 2018	JE Stewart, JP Ata, KS Burns, SB Marchetti, JJ Worrall	MN937634; (1)	MT906358; (1)	-
LVP3M	Lakeview Campground, Gunnison National Forest, Colorado, USA	*Pinus contorta*	14 June 2018	JE Stewart, JP Ata, KS Burns, SB Marchetti, JJ Worrall	MN937635; (1)	MN937598; (1)	MN937672; (1)
NC2M	North Cumberland, Gunnison National Forest, Colorado, USA	*Pinus contorta*	14 June 2018	JE Stewart, JP Ata, KS Burns, SB Marchetti, JJ Worrall	MN937625; (1)	MN937592; (1)	MN937667; (1)

NC6M	North Cumberland, Gunnison National Forest, Colorado, USA	*Pinus contorta*	14 June 2018	JE Stewart, JP Ata, KS Burns, SB Marchetti, JJ Worrall	MN937626; (1)	MN937601; (1)	MN937674; (1)
NC8M	North Cumberland, Gunnison National Forest, Colorado, USA	*Pinus contorta*	14 June 2018	JE Stewart, JP Ata, KS Burns, SB Marchetti, JJ Worrall	MN937627; (1)	MN937593; (1)	MN937671; (1)
NC9M	North Cumberland, Gunnison National Forest, Colorado, USA	*Pinus contorta*	14 June 2018	JE Stewart, JP Ata, KS Burns, SB Marchetti, JJ Worrall	MN937636; (1)	-	MN937668; (1)
NC10M	North	*Pinus*	14 June 2018	JE Stewart, JP	MN937637;	-	MT919224;

	Cumberland, Gunnison National Forest, Colorado, USA	*contorta*		Ata, KS Burns, SB Marchetti, JJ Worrall	(1)		(1)
OJ3M	Oh Be Joyful, Gunnison National Forest, Colorado, USA	*Pinus contorta*	13 June 2018	JE Stewart, JP Ata, KS Burns, SB Marchetti, JJ Worrall	MN937641; (1)	-	MT919226; (1)
PT6M	Pitkin, Gunnison National Forest, Colorado, USA	*Pinus contorta*	14 June 2018	JE Stewart, JP Ata, KS Burns, SB Marchetti, JJ Worrall	MN937640; (2)	MN937594; (1)	MN937661; (1)
PT8M	Pitkin, Gunnison National Forest, Colorado, USA	*Pinus contorta*	14 June 2018	JE Stewart, JP Ata, KS Burns, SB Marchetti, JJ Worrall	MN937628; (1)	MN937602; (1)	MN937660; (1)

PT9M	Pitkin, Gunnison National Forest, Colorado, USA	*Pinus contorta*	14 June 2018	JE Stewart, JP Ata, KS Burns, SB Marchetti, JJ Worrall	MN937642; (1)	MN937587; (1)	-
PT10M	Pitkin, Gunnison National Forest, Colorado, USA	*Pinus contorta*	14 June 2018	JE Stewart, JP Ata, KS Burns, SB Marchetti, JJ Worrall	MN937622; (1)	MN937591; (1)	MN937670; (1)
PT11M	Pitkin, Gunnison National Forest, Colorado, USA	*Pinus contorta*	14 June 2018	JE Stewart, JP Ata, KS Burns, SB Marchetti, JJ Worrall	MN937630; (1)	MN937595; (1)	MN937663; (1)
SR9M	Slate River, Gunnison National Forest, Colorado, USA	*Pinus contorta*	13 June 2018	JE Stewart, JP Ata, KS Burns, SB Marchetti, JJ Worrall	MN937643; (3)	-	MN937659; (1)

TC1M	Tincup, Gunnison National Forest, Colorado, USA	*Pinus contorta*	14 June 2018	JE Stewart, JP Ata, KS Burns, SB Marchetti, JJ Worrall	MN937631; (1)	MN937596; (1)	-
TC3M	Tincup, Gunnison National Forest, Colorado, USA	*Pinus contorta*	14 June 2018	JE Stewart, JP Ata, KS Burns, SB Marchetti, JJ Worrall	MN937632; (1)	MN937597; (1)	MN937666; (1)
TC9M	Tincup, Gunnison National Forest, Colorado, USA	*Pinus contorta*	14 June 2018	JE Stewart, JP Ata, KS Burns, SB Marchetti, JJ Worrall	MN937629; (1)	MN937599; (1)	MN937673; (1)
TL8M	Taylor Park, Gunnison National Forest, Colorado, USA	*Pinus contorta*	21 August 2018	SB Marchetti	MN937638; (1)	MN937600; (1)	MN937662; (1)

TL9M	Taylor Park, Gunnison National Forest, Colorado, USA	*Pinus contorta*	21 August 2018	SB Marchetti	MN937639; (2)	-	MT919225; (1)
***Lophodermella* sp.**							
RMNP_01	Rocky Mountain National Park, Colorado, USA	*Pinus flexilis*	05 July 2018	KS Burns	MN937645	MN937590	MN937665
***Lophodermella arcuata* (Darker) Darker**							
RMNP_LU1	Rocky Mountain National Park, Colorado, USA	*Pinus flexilis*	24 July 2019	KS Burns	MN937644; (1)	MN937585; (1)	MN937658; (1)
RMNP_LU16	Rocky Mountain National Park, Colorado, USA	*Pinus flexilis*	24 July 2019	KS Burns	MT906333; (1)	MT906359; (1)	MT919227; (2)
***Lophophacidium dooksii* Corlett and Shoemaker**							

MB5	Massabesic Experimental Forest, Maine, USA	*Pinus strobus*	03 May 2019	IA Munck, JE Stewart, JP Ata, A Bergdahl, W Searles	MN937623	MN937589	MN937664
***Lophodermella sulcigena* (Rostr.) Höhn.**							
PH18_0656	Canton Ticino, Passo del Lucomagno, Switzerland	*Pinus mugo*	10 July 2018	G Moretti	MN937624	MN937604	MN937675
***Lophodermella conjuncta* (Darker) Darker**							
PH18_0655	Canton of Grisons, Lenzerheide, Switzerland	*Pinus mugo*	18 April 2018	M Vanoni	MN937607; (1)	MN937605; (1)	MN937677; (1)
PHP19_0986	Canton Bern, Kandersteg,	*Pinus mugo*	18 June 2018	J Meyer, L Beenken	MN937609; (2)	MN937606; (1)	MN937676; (1)

	Oeschi-Forest, Switzerland						
PHP19_0987	Tyrol, Scharnitz, Karwendel Valley Austria	*Pinus mugo*	11 June 2018	T Cech, L. Beenken	MN937608; (3)	MN937603; (1)	MN937678; (1)

Table 2(on next page)

Morphological characterization

Characteristics of *Lophodermella* species and *Lophophacidium dooksii* based on published descriptions

1 **Table 2.** Characteristics of *Lophodermella* species and *Lophophacidium dooksii* based on published descriptions

Features	*Lophodermella concolor* (Dearn.) Darker	*Lophodermella montivaga* Petrak	*Lophodermella arcuata* (Darker) Darker	*Lophophacidium dooksii* Corlett & Shoemaker	*Lophodermella sulcigena* (Rostr.) Höhn.	*Lophodermella conjuncta* (Darker) Darker
Ascomata						
Size (mm)	0.4-0.8 X 0.28-0.44	0.75-8 X 0.28-0.4	0.38-3.13 X 0.25-0.45	(4.5-) 13-22 X 0.28-0.4	2-20 X 0.30-0.45	0.50-4.0 X 0.20-0.30
Depth (µm)	200-280	220-250	210-260	180-280	200-250	140-180
Opening	longitudinal split along stomata	longitudinal split	Longitudinal split along stomata	Vertical row of cells	longitudinal split	longitudinal split
Paraphyses						
Size (µm)	About as long as the asci	Up to 150 x *ca* 1	120-135 x 0.5-1	(80-) 90-120 X 1.5-2.0	100– 120 X 1	135-150 X 1.0-2.0
Gelatinous sheath/Mucous coat	Present	Present	Present	Present	Present	Absent
Septation	Present	Present	Inconspicuous	Present	Present	Present
Asci						
Size (µm)	120-225 X 15-17	120-160 X 12-15	110-160 X 14-20	(70-) 85-110 (-120) X 14-18 (-20)	110-140 X 13-15	(100)110–160 X 15–16
Opening mechanism	No obvious pre-formed apical apparatus (small apical	No obvious pre-formed apical apparatus (small apical hole or split after spores are	No obvious pre-formed opening mechanism (small apical hole or split after	Unitunicate	No obvious pre-formed apical apparatus	No obvious pre-formed apical apparatus

	hole or split after spores are released)	released)	spores are released)			
Number of spores	8	8	8	8	4–8	8
Ascospore						
Size (µm)	45-60 X (4) 6-8	40-50 X 3-4	40-50-(95) x 4-6	22-32 X 6-7.5	27-40 (65) X 4-5 (6)	(65) 75-90 (100)X 2.5-3.5
Mucilaginous/gelatinous sheath	Present	Present	Present	Lacking	Present	Present
Hosts	*Pinus banksiana, P. contorta*, *P. contorta* var. *murrayana*	*Pinus attenuata, P. contorta*	*Pinus albicaulis, P. flexilis, P. lambertiana, P. monticola*	*Pinus strobus*	*Pinus sylvestris, P. mugo, P. nigra* var. *maritima*, *P. contorta.*	*Pinus mugo*, *P. nigra* var. *maritima*, *P. sylvestris.*
References	Darker 1932, Minter and Millar 1993c, Funk 1985, Worrall et al. 2012	Darker 1932, Minter and Millar 1993c, Worrall et al. 2012	Darker 1932, Minter and Millar 1993a	Corlett and Shoemaker 1984, Merill et al. 1996	Darker 1932, Millar and Minter 1978, Beenken 2019	Darker 1932, Millar and Minter 1966, Beenken 2019

2

Table 3(on next page)

Sequences used in the study

Sequences downloaded from NCBI GenBank and used in phylogenies.

Table 3. Sequences downloaded from NCBI GenBank and used in phylogenies.

Species	Gene Region	Isolate/Strain/Voucher ID	GenBank Accession Number
Bifusella camelliae	LSU	HOU561	KF797450.1
Bifusella linearis	ITS	BPI843543	AY465527.1
Bifusella linearis	ITS	EBJul30-15	KT000195.1
Chalara sp.	ITS	MFLU 18-1812	MK584986.1
Chalara sp.	LSU	MFLU-18-1812	MK592006.1
Chalara sp.	ITS	MFLU 15-3167	MK584995.1
Chalara sp.	LSU	MFLU 15-3167	MK591953.1
Chalara sp.	TEF1α	MFLU 15-3167	MK348529.1
Coccomyces mucronatus	ITS	R73	GU138732.1
Coccomyces strobi	LSU	DAOMC251589	MH457157.1
Coccomyces strobi	TEF1α	AFTOL-ID1250	DQ471099.2
Colpoma quercinum	ITS	--	U92306.1
Colpoma quercinum	LSU	Lantz 368 (UPS)	HM140513.1
Cudonia confusa	TEF1α	C315	KC833384.1
Cudonia sichuanensis	ITS	C328	KC833122.1
Cudonia sichuanensis	LSU	C328	KC833220.1
Cudonia sichuanensis	TEF1α	C328	KC833386.1
Elytroderma deformans	ITS	--	AF203469.1
Fungal Endophyte	ITS	3277	DQ979552.1
Fungal Endophyte	LSU	3277	DQ79426.1
Fungal Endophyte	ITS	5744	DQ979779.1
Hypoderma campanulatum	LSU	ICMP: 17383	HM140517.1

Hypoderma hederae	LSU	Lantz & Minter 421 (UPS)	HM140522.1
Hypoderma minterii	ITS	275B/ BJTC201203	JX232416.1
Hypoderma rubi	ITS	PRJ R902	JF683416.1
Lirula macrospora	ITS	LM-CASTCBS	AF462441.1
Lirula macrospora	LSU	13	HQ902152.1
Lophodermella arcuata	ITS	BPI842080	AY465518.1
Lophodermium australe	ITS	1	EU934074.1
Lophodermium conigenum	ITS	A08	LC033959.1
Lophodermium culmigenum	LSU	Lantz 442 (UPS)	HM140540.1
Lophodermium molitoris	ITS	CV1_3a	KM106803.1
Lophodermium nitidum	LSU	Lantz 435 (UPS)	HM140547.1
Lophodermium sphaeroides	LSU	Lantz 382 (UPS)	HM140556.1
Lophodermium picea	ITS	P1	KX009045.1
Lophodermium pinastri	ITS	Yinggao	AY422490.1
Lophodermium sp. (*Lophodermium resinosum*)	ITS	JBT-2017a	KY485127.1
Lophodermium sp. (*Lophodermium resinosum*)	LSU	JBT-2017a/ NB-770-1/ DAOMC 251482	KY485135.1/ NG_060349.1
Lophodermium resinosum	TEF1α	DAOMC 251482	KY702582.1
Lophophacidium dooksii	ITS	737873	KF889651.1
Lophophacidium dooksii	ITS	B13N3	KF889704.1
Meloderma desmazieresii	ITS	--	AF203470.1
Rhytisma acerinum	ITS	Hou et al. 203	GQ253100.1
Rhytisma punctatum	ITS	WA-1	MH507272.1

Rhytisma salicinum	ITS	BPI843550	AY465516.1
Spathularia flavida subsp. *rufa*	ITS	H336	KC833071.1
Spathularia flavida subsp. *rufa*	LSU	H336	KC833228.1
Spathularia flavida subsp. *rufa*	TEF1α	H336	KC833395.1
Spatularia velutipes	TEF1α	S3/ JC32	KC833431.1
Terriera minor	LSU	Lantz & Minter 418 (UPS)	HM140569.1
Therrya fuckelii	ITS	CBS 377.58	JF683416.1
Therrya pini	ITS	CBS 177.56	MH857568.1
Therrya pini	LSU	CBS 177.56	MH869111.1
Tryblidiopsis pinastri	ITS	CBS 445.71	JF793678.1
Tryblidiopsis pinastri	LSU	CBS 445.71	MH871979.1
Tryblidiopsis pinastri	TEF1α	AFTOL-ID 1319	DQ471106.1

2

Table 4(on next page)

Character states of Lophodermella species

Character and character states used for phylogenetic reconstructions of *Lophodermella* species.

1 **Table 4.** Character and character states used for phylogenetic reconstructions of *Lophodermella* species.

No.	Character	Character States
1	Ascomata: Shape	0 non-linear or -elliptical, 1 mostly linear, nervisequious, dark brown to black, 2 mostly elliptical to elongate, concolorous to black
2	Ascomata: Position on substrate/host tissue (median transverse section)	0 external/superficial, 1 subcuticular, 2 intraepidermal, 3 subepidermal, 4 subhypodermal
3	Asci: Shape	0 more or less broadly saccate to clavate, 1 narrowly clavate or cylindrical
4	Ascospores: Shape	0 acicular, 1 filiform, 2 clavate, 3 cylindrical, 4 fusiform to oval, 5 rod-shaped, 6 double spindle-shaped, 7 ellipsoid to fusiod
5	Ecological character: Host	0 non-pine, 1 pine

2

Figure 1

Pictures of Lophodemella fruiting bodies.

Ascomata of *Lophodermella concolor* (a) and *L. montivaga* (b) on *Pinus contorta* from Gunnison National Forest, Colorado, USA; *Lophodermella* sp. (c) and *Lophodermella arcuata* (d) on *P. flexilis* from Rocky Mountain National Park, Colorado, USA; *Lophophacidium dooksii* on *P. strobus* from Massabesic, Maine, USA (e); and *L. conjuncta* (f) and *L. sulcigena* (g) on *P. mugo* from Austria and Switzerland.

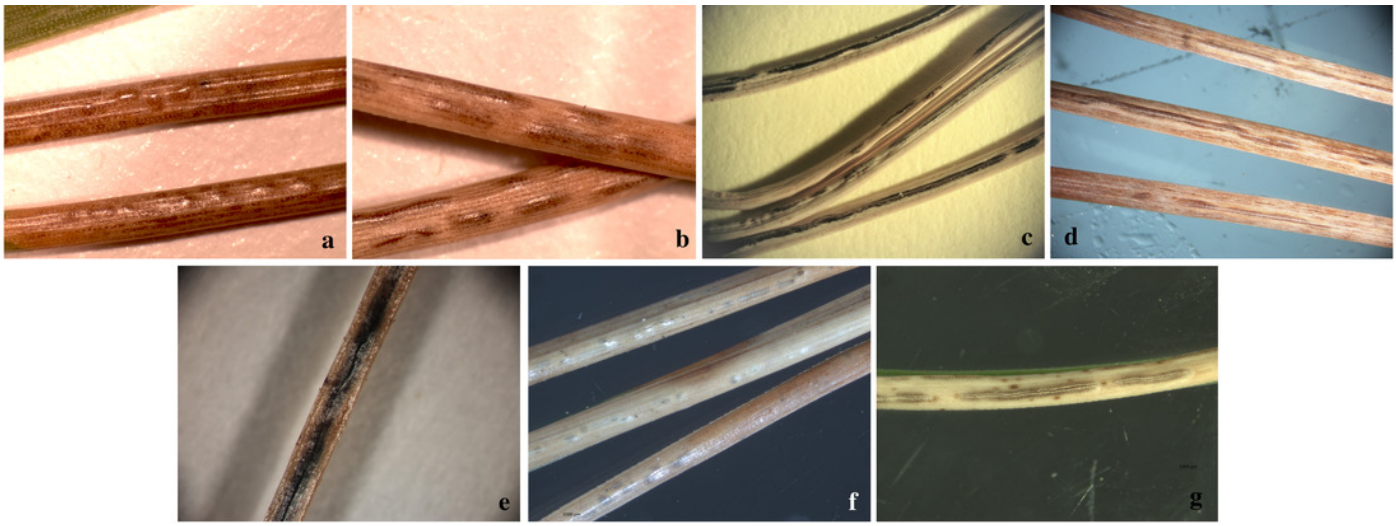


Figure 2

Phylogeny of Lophodermella species

Bayesian phylogeny depicting phylogenetic relationships of *Lophodermella montivaga* and *L. concolor* within the *Lophodermella* clade based on three gene regions including the internal transcribed spacer (ITS), large ribosomal subunit (LSU) and translation elongation factor 1-alpha (TEF1□). Bayesian posterior probabilities (PP) greater than 0.80 and bootstrap (BS) support values from maximum likelihood analysis greater than 50 are shown above and below node, respectively. Species in bold are samples derived from this study. Numbers correspond to genotypes after concatenation.

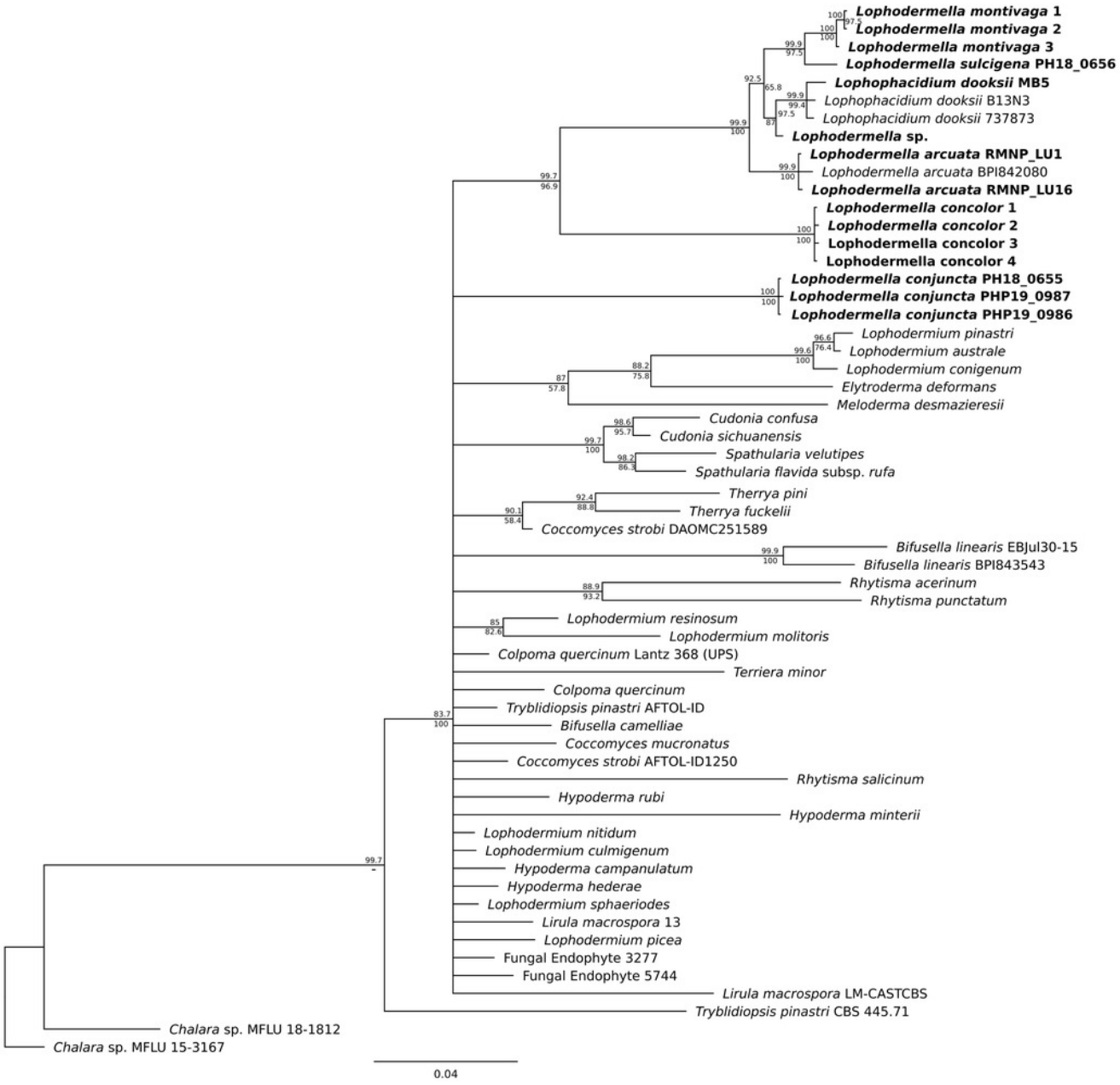

Lophodermella montivaga 1
Lophodermella montivaga 2
Lophodermella montivaga 3
Lophodermella sulcigena PH18_0656
Lophophacidium dooksii MB5
Lophophacidium dooksii B13N3
Lophophacidium dooksii 737873
Lophodermella sp.
Lophodermella arcuata RMNP_LU1
Lophodermella arcuata BPI842080
Lophodermella arcuata RMNP_LU16
Lophodermella concolor 1
Lophodermella concolor 2
Lophodermella concolor 3
Lophodermella concolor 4
Lophodermella conjuncta PH18_0655
Lophodermella conjuncta PHP19_0987
Lophodermella conjuncta PHP19_0986
Lophodermium pinastri
Lophodermium australe
Lophodermium conigenum
Elytroderma deformans
Meloderma desmazieresii
Cudonia confusa
Cudonia sichuanensis
Spathularia velutipes
Spathularia flavida subsp. rufa
Therrya pini
Therrya fuckelii
Coccomyces strobi DAOMC251589
Bifusella linearis EBJul30-15
Bifusella linearis BPI843543
Rhytisma acerinum
Rhytisma punctatum
Lophodermium resinosum
Lophodermium molitoris
Colpoma quercinum Lantz 368 (UPS)
Terriera minor
Colpoma quercinum
Tryblidiopsis pinastri AFTOL-ID
Bifusella camelliae
Coccomyces mucronatus
Coccomyces strobi AFTOL-ID1250
Rhytisma salicinum
Hypoderma rubi
Hypoderma minterii
Lophodermium nitidum
Lophodermium culmigenum
Hypoderma campanulatum
Hypoderma hederae
Lophodermium sphaeriodes
Lirula macrospora 13
Lophodermium picea
Fungal Endophyte 3277
Fungal Endophyte 5744
Lirula macrospora LM-CASTCBS
Tryblidiopsis pinastri CBS 445.71
Chalara sp. MFLU 18-1812
Chalara sp. MFLU 15-3167
0.04

Figure 3

Morphological characters of *Lophodermella* sp. on *Pinus flexilis* collected from Rocky Mountain National Park, Colorado, USA.

Morphological characters of *Lophodermella* sp. on *Pinus flexilis* collected from Rocky Mountain National Park, Colorado, USA. Subhypodermal hysterothecia with tanned mesophyll and hypodermis (a, d), clavate ascospores with gelatinous sheath (b) and broadly saccate asci (c). Magnification used: 10x and 20x, respectively, for photos of the hysterothecia (a and d); 40x for photos of ascospore and asci (b and c).

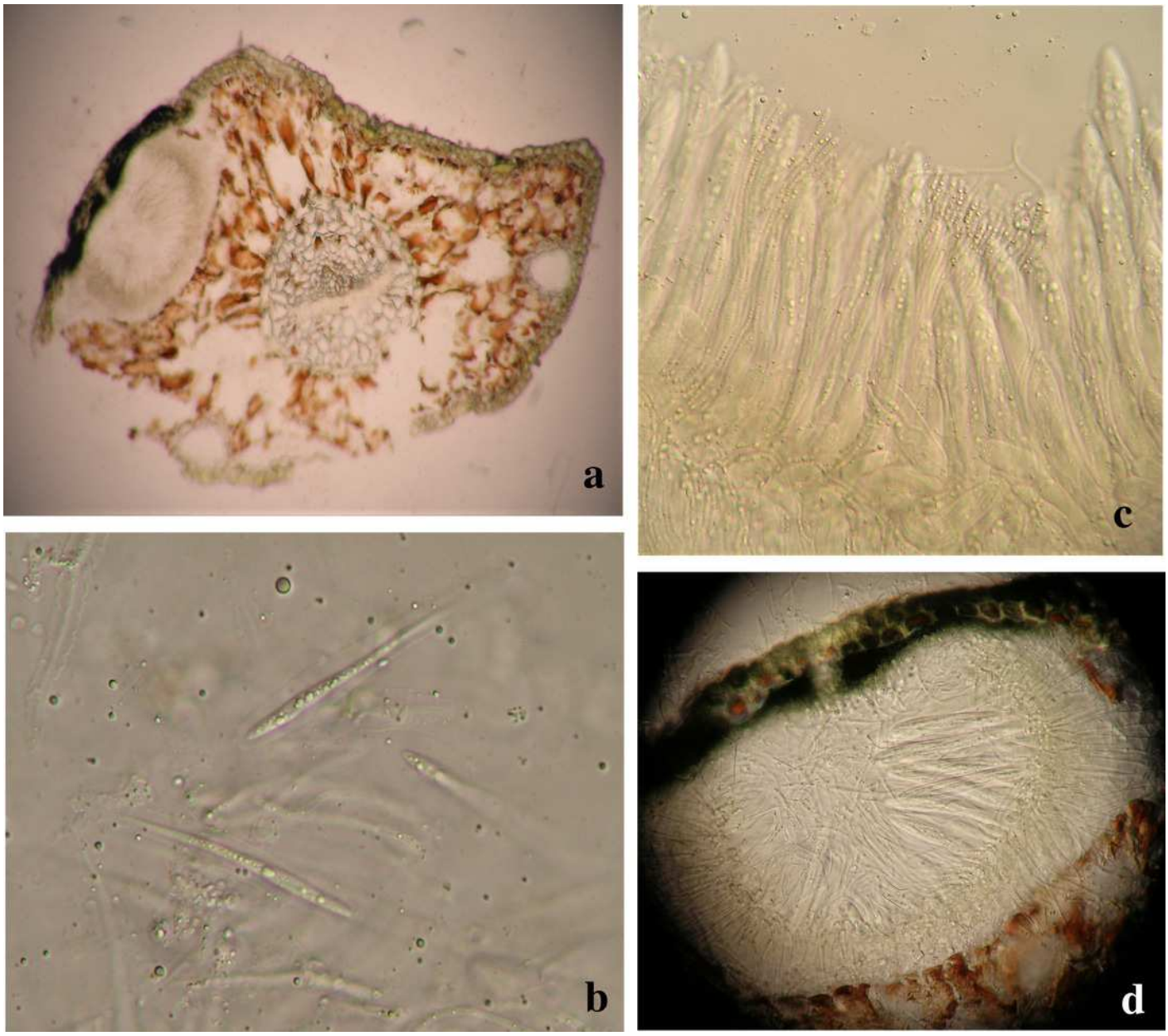
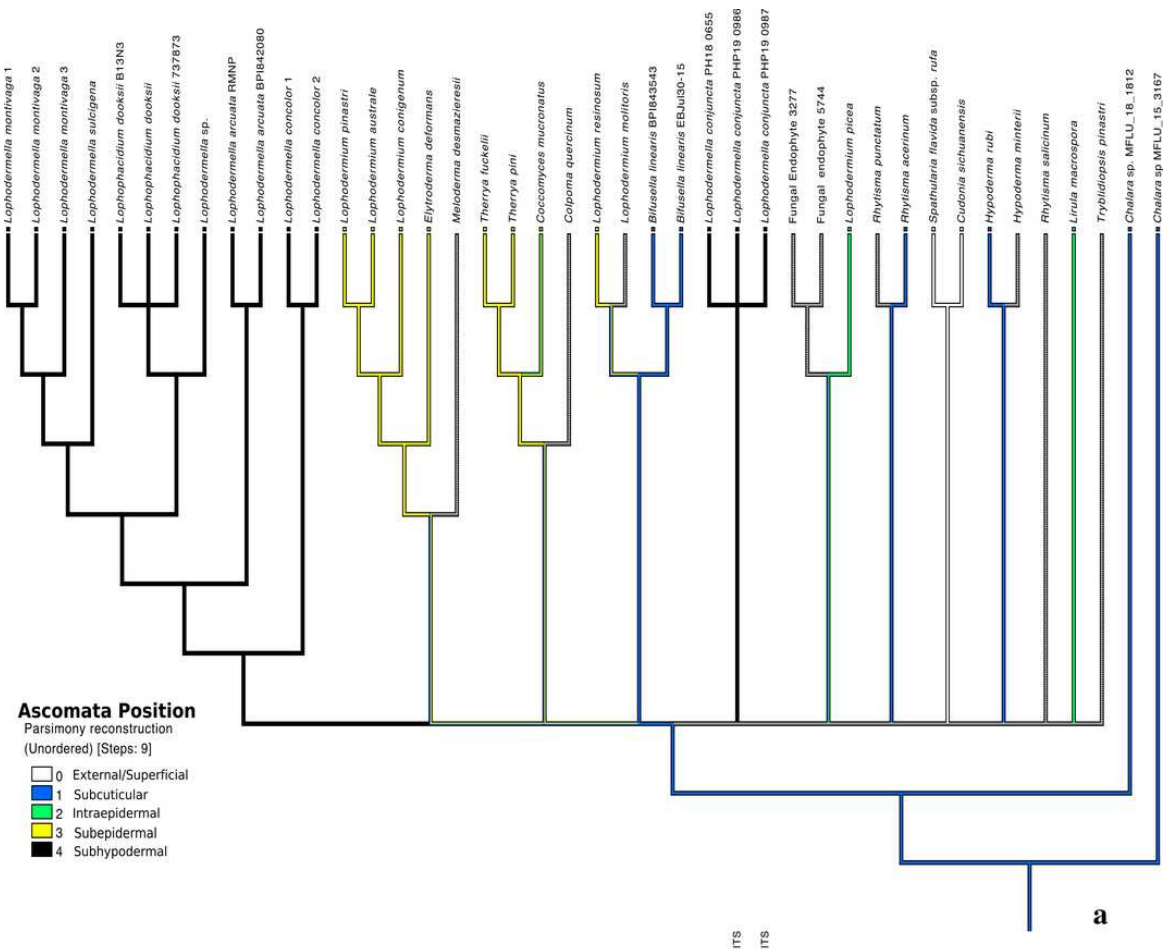
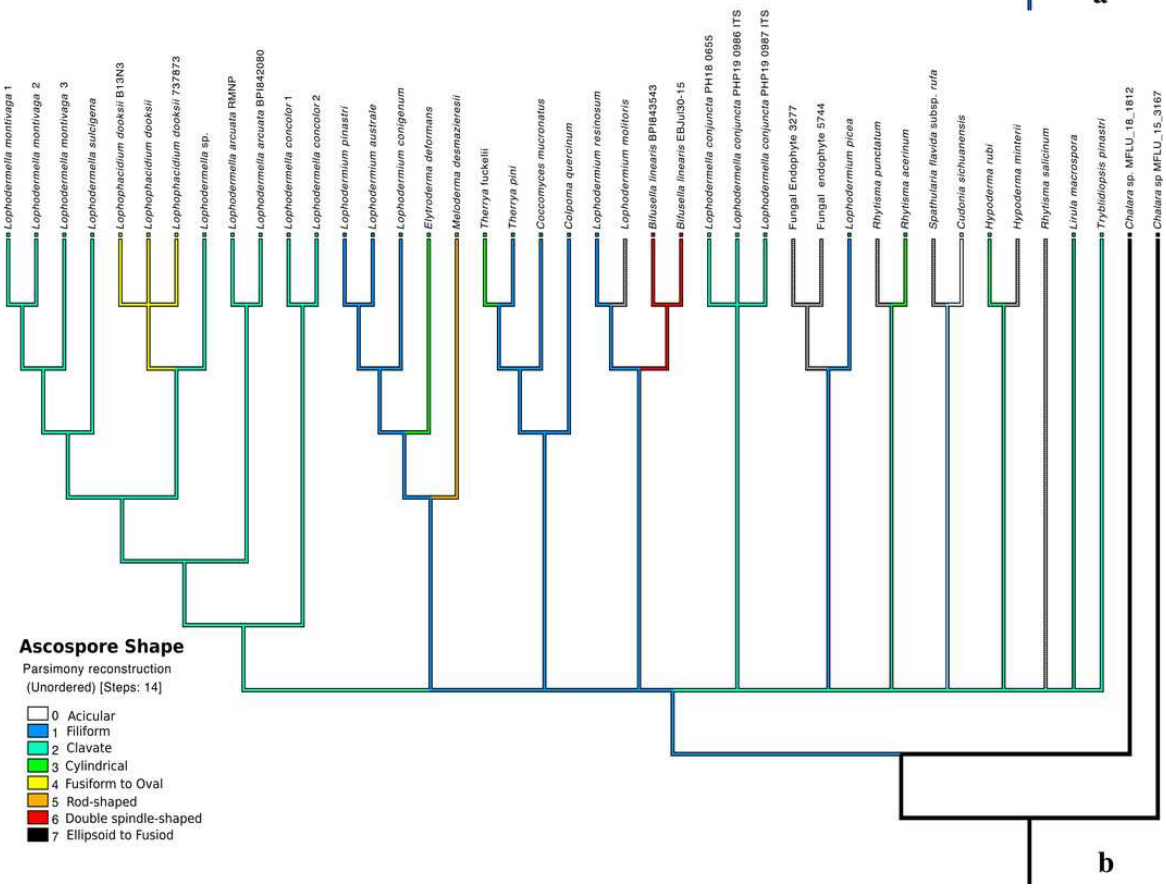

a
c
b
d

Figure 4

Morphological characters mapped onto ITS phylogenetic tree

Morphological characters mapped onto ITS phylogenetic tree with the parsimony ancestral reconstruction method using Mesquite v.3.6 with retention indices ≥ 0.50, ascomata position (a) and ascospore shape (b).


Lophodermella monthvaga 1
Lophodermella monthvaga 2
Lophodermella monthvaga 3
Lophodermella sulcigena
Lophophacidium dooksii B13N3
Lophophacidium dooksii
Lophophacidium dooksii 737873
Lophodermella sp.
Lophodermella arcuata RMNP
Lophodermella arcuata BPI842080
Lophodermella concolor 1
Lophodermella concolor 2
Lophodermium pinastri
Lophodermium australe
Lophodermium conigenum
Elytroderma deformans
Meloderma desmazieresii
Therrya fuckelii
Therrya pini
Coccomyces mucronatus
Colpoma quercinum
Lophodermium resinosum
Lophodermium molitoris
Bifuselia linearis BPI843543
Bifuselia linearis EBJul30-15
Lophodermella conjuncta PH18 0655
Lophodermella conjuncta PHP19 0986
Lophodermella conjuncta PHP19 0987
Fungal Endophyte 3277
Fungal endophyte 5744
Lophodermium picea
Rhytisma punctatum
Rhytisma acerinum
Spathularia flavida subsp. rufa
Cudonia sichuanensis
Hypoderma rubi
Hypoderma minterii
Rhytisma salicinum
Linula macrospora
Tryblidiopsis pinastri
Chalara sp. MFLU_18_1812
Chalara sp MFLU_15_3167
Ascomata Position
Parsimony reconstruction
(Unordered) [Steps: 9]
0 External/Superficial
1 Subcuticular
2 Intraepidermal
3 Subepidermal
4 Subhypodermal
a


Lophodermella monthvaga 1
Lophodermella monthvaga 2
Lophodermella monthvaga 3
Lophodermella sulcigena
Lophophacidium dooksii B13N3
Lophophacidium dooksii
Lophophacidium dooksii 737873
Lophodermella sp.
Lophodermella arcuata RMNP
Lophodermella arcuata BPI842080
Lophodermella concolor 1
Lophodermella concolor 2
Lophodermium pinastri
Lophodermium australe
Lophodermium conigenum
Elytroderma deformans
Meloderma desmazieresii
Therrya fuckelii
Therrya pini
Coccomyces mucronatus
Colpoma quercinum
Lophodermium resinosum
Lophodermium molitoris
Bifuselia linearis BPI843543
Bifuselia linearis EBJul30-15
Lophodermella conjuncta PH18 0655
Lophodermella conjuncta PHP19 0986 ITS
Lophodermella conjuncta PHP19 0987 ITS
Fungal Endophyte 3277
Fungal endophyte 5744
Lophodermium picea
Rhytisma punctatum
Rhytisma acerinum
Spathularia flavida subsp. rufa
Cudonia sichuanensis
Hypoderma rubi
Hypoderma minterii
Rhytisma salicinum
Linula macrospora
Tryblidiopsis pinastri
Chalara sp. MFLU_18_1812
Chalara sp MFLU_15_3167
Ascospore Shape
Parsimony reconstruction
(Unordered) [Steps: 14]
0 Acicular
1 Filiform
2 Clavate
3 Cylindrical
4 Fusiform to Oval
5 Rod-shaped
6 Double spindle-shaped
7 Ellipsoid to Fusiod
b