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

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ABSTRACT

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 Rhytismataceae, including *Lophophacidium dooksii*. Phylogenetic analyses of the three loci revealed that all but *L. 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.

Subjects Ecology, Evolutionary Studies, Mycology, Plant Science, Forestry
Keywords Pine, Needle, Phylogeny, Pathogen, Morphology, Molecular

Submitted 2 February 2021
Accepted 20 April 2021
Published 10 June 2021

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Academic editor
Simon Shamoun

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DOI **10.7717/peerj.11435**

INTRODUCTION

Conifer needle diseases are becoming increasingly prevalent due to several factors such as climate change and introduction to new hosts (*Woods, Coates & Hamann, 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, Lewis & Woods, 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, Marchetti & Mask, 2012*) in *Pinus contorta* and *P. flexilis*. These two pine hosts are naturally dominant and ecologically important species along the Rocky Mountain Region (*Lotan & Critchfield, 1990*; *Schoettle, 2004*). Two needle cast epidemics caused by *L. concolor* and *L. montivaga* were recorded on *P. contorta* (*Worrall, Marchetti & Mask, 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 short outbreaks or minor incidence of *Lophodermella* species such as *L. cerina* in southern USA, *L. morbida* in the western USA, *L. maureri* in Mexico, and *L. orientalis* in Asia (*Czabator et al., 1971*; *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. maureri, L. montivaga* and *L. morbida* in North America, *L. conjuncta* and *L. sulcigena* in Europe, and *L. orientalis* in Asia (*MycoBank Database, 2016*). *Lophodermella* species (Rhytismataceae) are distinguished by their subhypodermal ascomata, clavate ascospores surrounded by mucilaginous sheath, and wider asci than the 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, Marchetti & Mask, 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*. In 1984, the newly described *L. dooksii* was classified under Phacidiaceae due to the lack of morphological characteristics distinctive of Rhytismataceae (*Corlett & 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 on *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 (i.e., NCBI-nr, UNITE, DNA Data Bank of Japan). As emerging pathogens, molecular studies on *Lophodermella* are important for pathogen identification. These will elucidate phylogenetic relationship of *Lophodermella* with other rhytismataceaous species. These will also 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 three-loci 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 test monophyly of this genus by including other genera within Rhytismataceae and by using molecular phylogenies to guide the identification of shared and unique traits among *Lophodermella* species for genus and species delineation.

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 and August 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 July 2019 while the eastern white pine (*P. strobus*) needles symptomatic of *L. dooksii* were collected from natural stands in Maine, USA in May 2019. Collections were approved by the USDA Forest Service, Forest Health Protection. 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 & Shoemaker, 1984*; *Darker, 1932*; *Millar & Minter, 1980*; *Millar & Minter, 1978*; *Minter & Millar, 1993a*; *Worrall, Marchetti & Mask, 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

Table 1 Collection information, GenBank accession and genotype numbers for each *Lophodermella* species and *Lophophacidium dooksii* for the three loci, namely: internal transcribed spacer region 1, 5.8S ribosomal RNA and internal transcribed spacer region 2 (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	CS, GNF, CO, USA	*Pinus contorta*	12 June 2018	JE Stewart, JP Ata, KS Burns, SB Marchetti, JJ Worrall	MN937619; (1)	MN937581; (1)	MN937651; (1)
CS9C	CS, GNF, CO, USA	*P. contorta*	12 June 2018	”	MN937612; (1)	MN937579; (1)	MN937650; (1)
FS6C	FS, GNF, CO, USA	*P. contorta*	12 June 2018	”	MN937618; (1)	MN937582; (1)	MN937647; (1)
FS8C	FS, GNF, CO, USA	*P. contorta*	12 June 2018	”	MN937610; (2)	MN937580; (1)	MN937653; (1)
LP7C	LP, GNF, CO, USA	*P. contorta*	12 June 2018	”	MN937621; (1)	MN937588; (3)	MN937654; (1)
LV7C	LV, GNF, CO, USA	*P. contorta*	14 June 2018	”	MN937620; (1)	MN937575; (1)	MN937657; (1)
LV8C	LV, GNF, CO, USA	*P. contorta*	12 June 2018	”	MN937615; (1)	MN937576; (2)	MN937655; (1)
PT2C	PT, GNF, CO, USA	*P. contorta*	14 June 2018	”	MN937616; (1)	MN937577; (1)	MN937646; (1)
PT3C	PT, GNF, CO, USA	*P. contorta*	14 June 2018	”	MN937614; (1)	MN937583; (1)	MN937652; (1)
SR3C	SR, GNF, CO, USA	*P. contorta*	13 June 2018	”	MN937617; (1)	MN937578; (1)	MN937649; (1)
SR6C	SR, GNF, CO, USA	*P. contorta*	13 June 2018	”	MN937613; (1)	MN937584; (1)	MN937648; (1)
OJ11C	OJ, GNF, CO, USA	*P. contorta*	13 June 2018	”	MN937611; (1)	MN937574; (1)	MN937656; (1)
***Lophodermella montivaga* Petrak**							
CU1M	CU, GNF, CO, USA	*P. contorta*	14 June 2018	”	MN937633; (1)	MN937586; (1)	MN937669; (1)
LVP2M	LV, GNF, CO, USA	*P. contorta*	14 June 2018	”	MN937634; (1)	MT906358; (1)	–
LVP3M	LV, GNF, CO, USA	*P. contorta*	14 June 2018	”	MN937635; (1)	MN937598; (1)	MN937672; (1)
NC2M	NC, GNF, CO, USA	*P. contorta*	14 June 2018	”	MN937625; (1)	MN937592; (1)	MN937667; (1)
NC6M	NC, GNF, CO, USA	*P. contorta*	14 June 2018	”	MN937626; (1)	MN937601; (1)	MN937674; (1)
NC8M	NC, GNF, CO, USA	*P. contorta*	14 June 2018	”	MN937627; (1)	MN937593; (1)	MN937671; (1)
NC9M	NC, GNF, CO, USA	*Pinus contorta*	14 June 2018	”	MN937636; (1)	–	MN937668; (1)

(*continued on next page*)

Table 1 (*continued*)

Sample ID	Location	Host	Collection Date	Collectors	GenBank Accession Number; (Genotype)		
					ITS	LSU	TEF1-α
NC10M	NC, GNF, CO, USA	*P. contorta*	14 June 2018	”	MN937637; (1)	–	MT919224; (1)
OJ3M	OJ, GNF, CO, USA	*P. contorta*	13 June 2018	”	MN937641; (1)	–	MT919226; (1)
PT6M	PT, GNF, CO, USA	*P. contorta*	14 June 2018	”	MN937640; (2)	MN937594; (1)	MN937661; (1)
PT8M	PT, GNF, CO, USA	*P. contorta*	14 June 2018	”	MN937628; (1)	MN937602; (1)	MN937660; (1)
PT9M	PT, GNF, CO, USA	*P. contorta*	14 June 2018	”	MN937642; (1)	MN937587; (1)	–
PT10M	PT, GNF, CO, USA	*P. contorta*	14 June 2018	”	MN937622; (1)	MN937591; (1)	MN937670; (1)
PT11M	PT, GNF, CO, USA	*P. contorta*	14 June 2018	”	MN937630; (1)	MN937595; (1)	MN937663; (1)
SR9M	SR, GNF, CO, USA	*P. contorta*	13 June 2018	”	MN937643; (3)	–	MN937659; (1)
TC1M	TC, GNF, CO, USA	*P. contorta*	14 June 2018	”	MN937631; (1)	MN937596; (1)	–
TC3M	TC, GNF, CO, USA	*P. contorta*	14 June 2018	”	MN937632; (1)	MN937597; (1)	MN937666; (1)
TC9M	TC, GNF, CO, USA	*P. contorta*	14 June 2018	”	MN937629; (1)	MN937599; (1)	MN937673; (1)
TL8M	TL, GNF, CO, USA	*P. contorta*	21 August 2018	SB Marchetti	MN937638; (1)	MN937600; (1)	MN937662; (1)
TL9M	TL, GNF, CO, USA	*P. contorta*	21 August 2018	SB Marchetti	MN937639; (2)	–	MT919225; (1)
***Lophodermella* sp.**							
RMNP_01	RMNP, CO, USA	*Pinus flexilis*	05 July 2018	KS Burns	MN937645	MN937590	MN937665
***Lophodermella arcuata* (Darker) Darker**							
RMNP_LU1	RMNP, CO, USA	*P. flexilis*	24 July 2019	KS Burns	MN937644; (1)	MN937585; (1)	MN937658; (1)
RMNP_LU16	RMNP, CO, USA	*P. flexilis*	24 July 2019	KS Burns	MT906333; (1)	MT906359; (1)	MT919227; (2)
***Lophophacidium dooksii* Corlett and Shoemaker**							
MB5	Massabesic Experimental Forest, ME, 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, CH	*Pinus mugo*	10 July 2018	G Moretti	MN937624	MN937604	MN937675

(*continued on next page*)

Table 1 (*continued*)

Sample ID	Location	Host	Collection Date	Collectors	GenBank Accession Number; (Genotype)		
					ITS	LSU	TEF1-α
***Lophodermella conjuncta* (Darker) Darker**							
PH18_0655	Canton Grisons, Lenzerheide, CH	*P. mugo*	18 April 2018	M Vanoni	MN937607; (1)	MN937605; (1)	MN937677; (1)
PHP19_0986	Canton Bern, Kandersteg, Oeschi-Forest, CH	*P. mugo*	18 June 2018	J Meyer, L Beenken	MN937609; (2)	MN937606; (1)	MN937676; (1)
PHP19_0987	Tyrol, Scharnitz, Karwendel Valley, AT	*P. mugo*	11 June 2018	T Cech, L. Beenken	MN937608; (3)	MN937603; (1)	MN937678; (1)

Notes.

CS, Cold Springs Campground; CU, Cumberland; FS, Fisherman Trail; LP, Lodgepole Campground; LV, Lakeview Campground; NC, North Cumberland; OJ, Oh Be Joyful; PT, Pitkin; SR, Slate River; TC, Tincup; TL, Taylor Park; GNF, Gunnison National Forest; RMNP, Rocky Mountain National Park; CO, Colorado; ME, Maine; USA, United States of America; CH, Switzerland; AT, Austria.

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 one mm long pieces and placed in two mL centrifuge tubes with one five 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 s 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). Meanwhile, the DNA extraction of *L. sulcigena* and *L. conjuncta* samples was performed in Europe. Single fruiting bodies (ca. 3–4 mm long pieces) 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 1, 5.8S ribosomal RNA and internal transcribed spacer region 2 (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 (*Vilgalys & Hester, 1990*), and EF1-983F and EFgr (*Rehner, 2001*). The ITS locus was amplified at optimal annealing temperatures between 50–55 °C with 30 cycles while TEF1α and LSU were amplified at 56° C annealing temperature with 35 cycles and other cycle parameters following *Tanney & Seifert (2017)*. Amplification of each locus was performed in a 25-µL PCR reaction mixture of 1× standard Taq reaction buffer, 0.2 mM of each dNTP, 0.4 µM of forward and reverse primer set, 0.625 units Taq polymerase, and 40 ng template DNA. For ITS amplification, the cycle parameters included initial denaturation at 94 °C for 2 mins, followed by 30

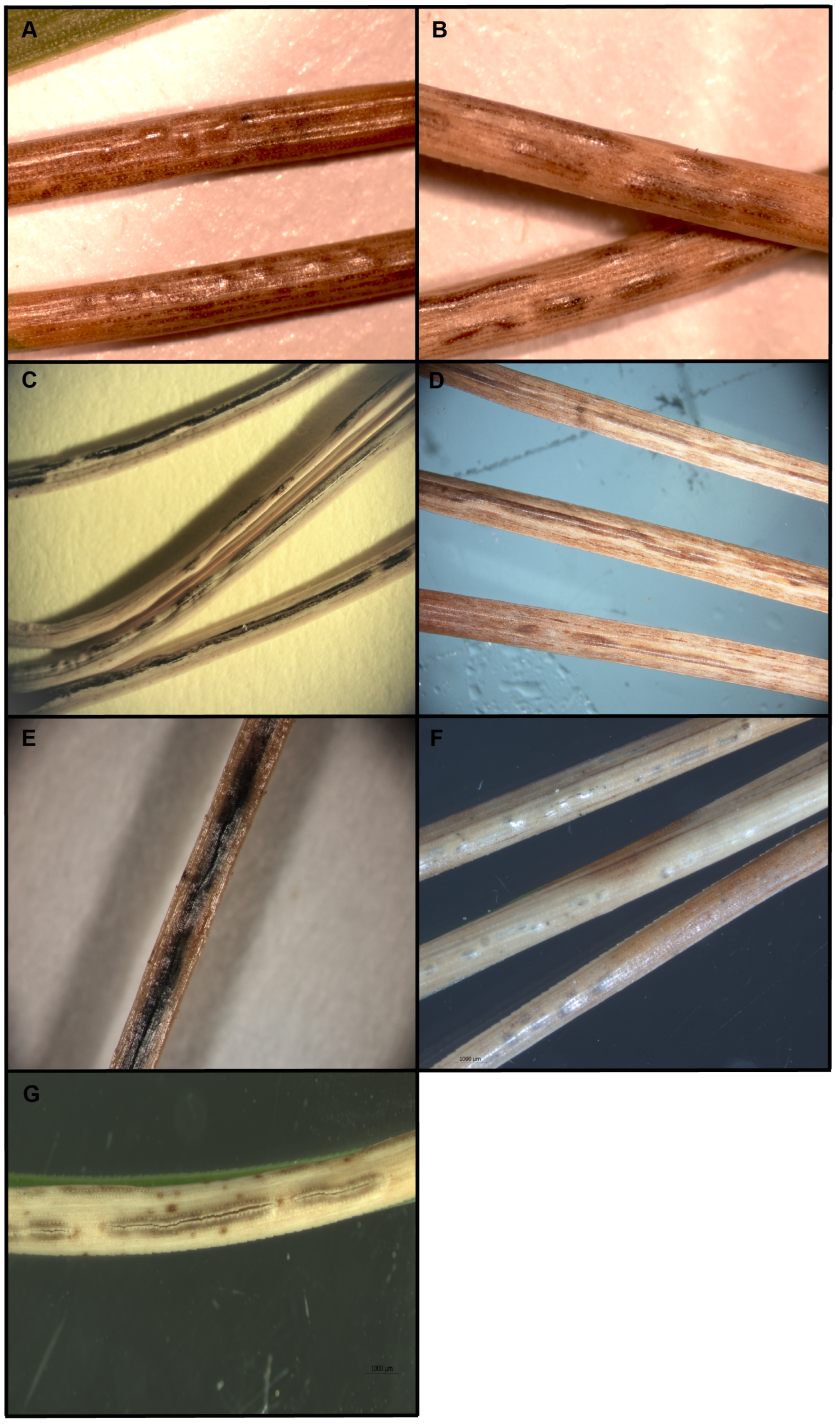

Figure 1 Ascomata of *Lophodermella concolor* (A) and *L. montivaga* (B) on *Pinus contorta* from Gunnison National Forest, Colorado, USA; *Lophodermella* sp. (C) and *Lophodermella arcuata* (D) on *P. flexilis* from Rocky Mountain National Park, Colorado, USA; *Lophophacidium dooksii* on *P. strobus* from Massabesic, Maine, USA (E); and *L. conjuncta* (F) and *L. sulcigena* (G) on *P. mugo* from Austria and Switzerland.

Full-size DOI: 10.7717/peerj.11435/fig-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	*Lophodermella sulcigena* (Rostr.) Höhn	*Lophodermella conjuncta* (Darker) Darker
(A). *Ascomata (hysterothecia)*					
Size (mm)	0.4–0. 8 × 0.28–0.44	0.75–8 × 0.28–0.4	0.38–3.13 × 0.25–0.45	2–20 × 0.30–0.45	0.50–4.0 × 0.20–0.30
Depth (µm)	200–280	220–250	210–260	200–250	140–180
Opening	longitudinal split along stomata	longitudinal split	Longitudinal split along stomata	longitudinal split	longitudinal split
Paraphyses					
Size (µm)	About as long as the asci	Up to 150 ×*ca* 1	120–135 × 0.5–1	100–120 × 1	135–150 × 1.0–2.0
Gelatinous sheath/ Mucous coat	Present	Present	Present	Present	Absent
Septation	Present	Present	Inconspicuous	Present	Present
Asci					
Size (µm)	120–225 × 15–17	120–160 × 12–15	110–160 × 14–20	110–140 × 13–15	(100)110–160 × 15–16
Opening mechanism	No obvious pre-formed apical apparatus (small apical hole or split after spores are released)	No obvious pre-formed apical apparatus (small apical hole or split after spores are released)	No obvious pre-formed opening mechanism (small apical hole or split after spores are released)	No obvious pre-formed apical apparatus	No obvious pre-formed apical apparatus
Number of spores	8	8	8	4–8	8
Ascospore					
Size (µm)	45–60 × (4) 6–8	40–50 × 3–4	40–50-(95) ×4–6	27–40 (65) ×4-5 (6)	(65) 75–90 (100) ×2.5–3.5
Mucilaginous/ gelatinous sheath	Present	Present	Present	Present	Present
Hosts (number of needles)	*Pinus banksiana* (2), *P. contorta* (2), *P. contorta* var. *murrayana* (2), *P. sylvestris* (2)	*Pinus attenuata* (3), *P. contorta* (2), *P. sylvestris* (2), *P. ponderosa* (3), *P. radiata* (3), *P. flexilis* (5), *P. monticola* (5)	*Pinus albicaulis* (5), *P. flexilis* (5), *P. lambertiana* (5), *P. monticola* (5)	*Pinus sylvestris* (2), *P. mugo* (2), *P. nigra* var. *maritima* (2)	*Pinus mugo* (2), *P. nigra* var. *Maritima* (2), *P. sylvestris* (2).
Distribution	Western USA, Canada	Western USA	Western USA	Europe	Europe
Conidiomata	Not observed	Not observed	Not observed	Unknown	Unknown
References	(*Darker, 1932*; *Millar, 1984*; *Minter & Millar, 1993b*; *Funk, 1985*; *Worrall, Marchetti & Mask, 2012*)	(*Darker, 1932*; *Millar, 1984*; *Minter & Millar, 1993c*; *Worrall, Marchetti & Mask, 2012*)	(*Darker, 1932*; *Minter & Millar, 1993a*)	(*Darker, 1932*; *Millar, 1984*; *Millar & Minter, 1978*, *Beenken, 2019*)	(*Darker, 1932*; *Millar, 1984*; *Millar & Minter, 1980*, *Beenken, 2019*)
(B). *Ascomata (hysterothecia)*					
Size (mm)	0.6–2. 75 × 0.3–0.63	300–2,500 × 250–550	1–6 (22)	0.5–2 × 0.4–0.8	(4.5−) 13–22× 0.28–0.4
Depth (µm)	*ca* 280	–	350–370	–	180–280
Opening	longitudinal split along stomata	Longitudinal split	–	longitudinal split along stomata	Vertical row of cells
Paraphyses					
Size (µm)	180- 200× 1–3	2.5–3.5 (width)	120–140× 2–3.5	2–3 (width)	(80−) 90–120× 1.5–2.0
Gelatinous sheath/Mucous coat	Present (inconspicuous)	Present	–	Present	Present
Septation	Present	Present	Present	Present	Present

(*continued on next page*)

Table 2 (*continued*)

Features	*Lophodermella concolor* (Dearn.) Darker	*Lophodermella montivaga* Petrak	*Lophodermella arcuata* (Darker) Darker	*Lophodermella sulcigena* (Rostr.) Höhn	*Lophodermella conjuncta* (Darker) Darker
Asci					
Size (µm)	160–225 × 17–21	55–80 × 8–13	95–162	110–150 × 14–18	(70−) 85–110 (−120) - ×14–18 (−20)
Opening mechanism	No obvious pre-formed apical apparatus (small apical hole or split after spores are released)	Opening by a large apical hole	–	No obvious pre-formed apical apparatus (small apical hole or split after spores are released)	Unitunicate
Number of spores	8	8	8	8	8
Ascospore					
Size (µm)	68- 78 × 3–3.5	30- 50 × 2.5–3.5	23–53 × 2.5–3.5	30–65 × 2.5–3.5	22–32 × 6–7.5
Mucilaginous/gelatinous sheath	Present	Present	Present	Present	Lacking
Hosts (number of needles)	*Pinus contorta* (2), *P. elliottii var. elliottii* (3), *P. ponderosa* (3), *P. taeda* (3), *P. sylvestris* (2)	*Pinus ayacahuite* (5)	*Pinus ponderosa* (3), *P. attenuata* (3)	*Pinus kesiya* (3, sometimes 2 or 4)	*Pinus strobus* (5)
Distribution	Western USA	Mexico	Western USA	Asia	Canada, USA
Conidiomata	Not observed (present in *P. contorta*)	Not observed	Present	Only fresh collected specimens	Not reported
References	(*Darker, 1932*; *Millar, 1984*; *Minter & Millar, 1993d*)	(*Minter, 1988b*)	(*Staley & Bynum, 1972*)	(*Minter, 1993*)	(*Corlett & Shoemaker, 1984*; *Merrill, Wenner & Dreisbach, 1996*)

cycles of denaturation at 94 °C for 40 s, optimal annealing temperature for 40 s, extension at 72 °C for 1 min, and final extension at 72 °C for 5 mins.

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 at least three randomly selected *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 locus per sample and found to be 99.81 to 100% identical to the sequence of its corresponding original PCR product. Sequences were compared to NCBI sequence database using the Nucleotide Basic Local Assignment Search Tool (BLASTn) 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*). Polymorphic sites were determined using DnaSP (*Rozas et al., 2003*).

Phylogenetic analyses for each locus were constructed using Bayesian inference (MrBayes; *Huelsenbeck & 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 TEF1 α, *TrN + I + G* for LSU, and *SYM + I + G* for the concatenated

Table 3 Character and character states used for phylogenetic reconstructions of *Lophodermella* species.

No.	Character	Character States
(A). Characters to assess genus delineation		
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
(B). Characters to assess species delineation		
1	Ascomata: length	0 hysterothecia $\geq$ 1 mm, 1 hysterothecia short
2	Ascomata: color	0 brown, 1 concolorous
3	Ascomata: fusion	0 not fused, 1 fused
4	Ascospores: shape, size	0 short (23–60 µm) clavate, 1 elongate clavate (68–90 µm), 2 fusiform to oval, 3 cylindrical, 4 ellipsoid to fusoid
5	Asci: number of spores	4 four-spored, 8 eight-spored
6	Host: number of pine needles	2 two-needle pine, 3 three-needle pine, 5 five-needle pine

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 1,000 bootstraps. Bayesian and maximum likelihood trees were generated with support thresholds of 80% with a 20% burn-in and 50%, respectively. 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 & Lutzoni, 2002*). This resulted in a *p*-value = 0.99, indicating congruence among the ITS, LSU and TEF1α datasets. Tree topologies from individual loci were also compared using the reciprocal 70% bootstrap approach (*Mason-Gamer & Kellogg, 1996*). Similarly, results also revealed no significant incongruence between the three datasets. Thus, the three loci dataset was combined using Sequence Matrix (*Vaidya, Lohman & Meier, 2011*). The alignment and consensus tree of the concatenated dataset were stored in TreeBase (Submission ID 26836). Published sequences of known related species in GenBank database were included in the phylogenetic analysis (Table S1). The rhytismataceous species were selected based on similarity to *Lophodermella* sequences and availability in NCBI database.

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 3; *Darker, 1932*; *Darker, 1967*; *Minter, 1988a*; *Minter & Millar, 1993a*; *Minter & Millar, 1993b*; *Minter & Millar, 1993c*; *Tanney & Seifert, 2017*,

MycoBank Database, 2016; *Fungi and Lichens of Great Britain and Ireland, 2019*) and then mapped on the Bayesian ITS dataset phylogeny which had a more comprehensive set of Rhytismataceae species in well-supported clades. To assess distinct morphological characters among *Lophodermella* species, key characters were selected based on *Darker (1932)* and *Hunt & Ziller (1978)*. These were then mapped on a separate Bayesian ITS phylogeny (*GTR+I+G* model) that was limited to *Lophodermella* species and two outgroups (*Elytroderma deformans* and *Chalara* sp.). All morphological characters were coded as unordered and mapped with parsimony ancestral trace reconstruction using Mesquite v.3.6 (*Maddison & Maddison, 2018*).

RESULTS

Molecular and phylogenetic analyses

PCR amplification produced a single band for each sample per locus. Chromatograms for forward and reverse sequences did not show multiple peaks at base calls, indicating uniform amplicons. Amplicons of the ITS, TEF1α and LSU yielded products that ranged from 347 to 543, 678 to 811 and 790 to 1,077 base pairs, respectively. Of the 40 samples of *Lophodermella* spp. 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 TEF1 α, 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* spp and *L. dooksii* samples at the LSU resulted in nine genotypes with 106 total polymorphic sites and 62 parsimony informative sites. BLAST results of sequences are presented in Table S2 .

Several *Lophodermella* spp. and *L. dooksii* clustered in a well-supported clade (hereinafter referred to as the LOD clade) at the ITS, LSU and TEF1α phylogenies. This clade composed of genotypes 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 97.9 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 94.4 BS, (Fig. S3), but did not include both *L. concolor* and *L. conjuncta*. Similar to the ITS and LSU phylogenies, the 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 75.5 BS support values (Fig. 2). Distance matrix is shown in Table S3.

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 with 0.98 PP (Fig. 1). Similarly, RMNP_01 and *L.*

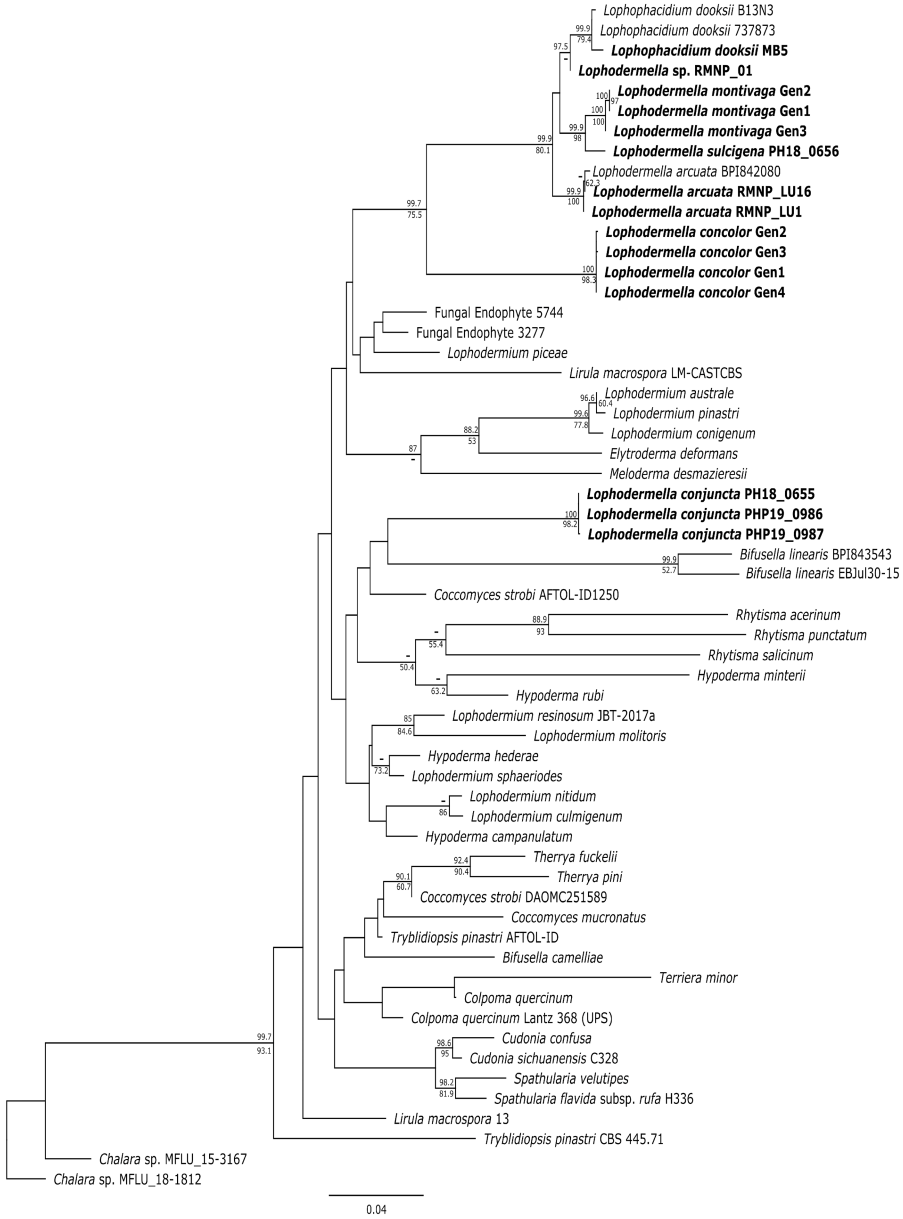

Figure 2 Maximum likelihood phylogeny depicting phylogenetic relationships of *Lophodermella* species within Rhytismataceae based on three gene regions including the internal transcribed spacer (ITS), large ribosomal subunit (LSU) and translation elongation factor 1-alpha (TEF1a). Bayesian posterior 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.

Full-size DOI: 10.7717/peerj.11435/fig-2

dooksii (MB5) were found into a cluster with 0.98 PP and 89.9 BS, and 0.96 PP and 71.2 BS at the ITS (Fig. S1) and TEF1α (Fig. S3) trees respectively, 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× 0.16–0.168 mm and were tanned

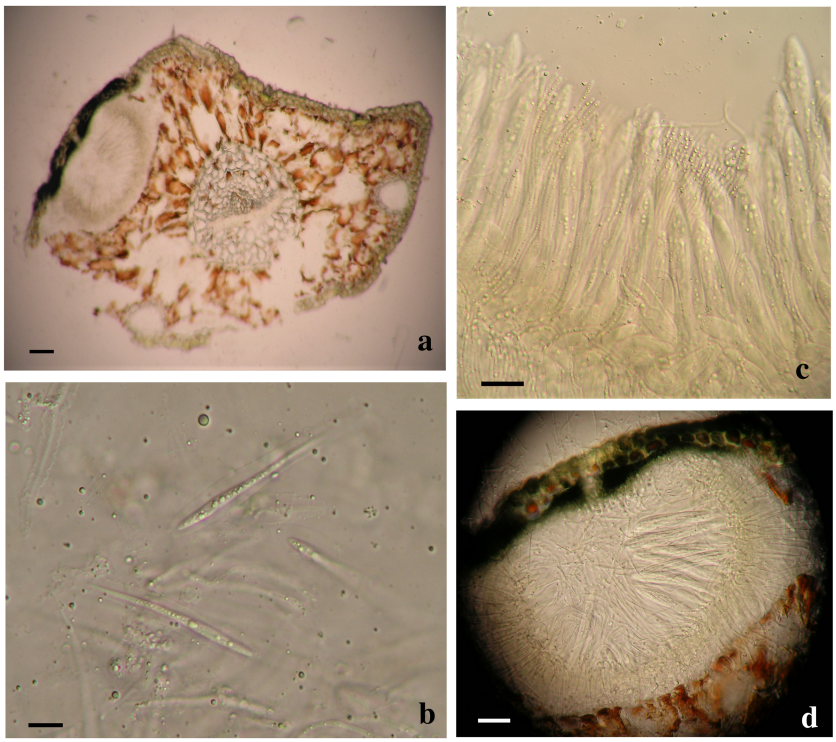

Figure 3 Morphological characters of *Lophodermella.* sp. on *Pinus flexilis* collected from Rocky Mountain National Park, Colorado, USA. Subhypodermal hysterothecia with tanned mesophyll and hypodermis (A, B), clavate ascospores with gelatinous sheath (B) and broadly saccate asci (C). Size bars A, C and D 20 mm; B 10 mm.

Full-size DOI: 10.7717/peerj.11435/fig-3

at mesophyll and hypodermis. Asci were broadly saccate measuring 96–130× 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* in similar geographic location.

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 3, Table S4). 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. 4). 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. To measure homoplasy and fit of characters, individual consistency (CI) and retention indices (RI) were measured. While all morphological characters obtained an RI $\geq$ 0.50, only ascomata position and ascospore shape had CI $\geq$ 0.50, which may imply synapomorphy of the two characters (Fig. 4).

Distinct characters were observed across *Lophodermella* spp., which may be useful for species identification and delineation (Fig. 5, Table S5). Short and concolorous hysterothecia were distinct in *L. concolor* while elongated clavate ascospore and fused hysterothecia were distinct in *L. conjuncta*. The fusiform to oval ascospore was unique to *Lophophacidium dooksii.* Meanwhile, *L. montivaga*, *Lophodermella* sp. (RMNP_01) and *L. sulcigena* only differed at their host occurrence. Hysterothecia of *L. arcuata* was reported to be concolorous when dry as opposed to that of *Lophodermella* sp. (RMNP_01) which remains dark brown. All of the six characters for species delineation generated a mean CI and RI of 0.95 and 0.92, respectively.

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 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, in the TEF1α phylogeny, *L. concolor* was excluded from the LOD clade, but was placed in the clade at the LSU and ITS phylogenies. 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.,*

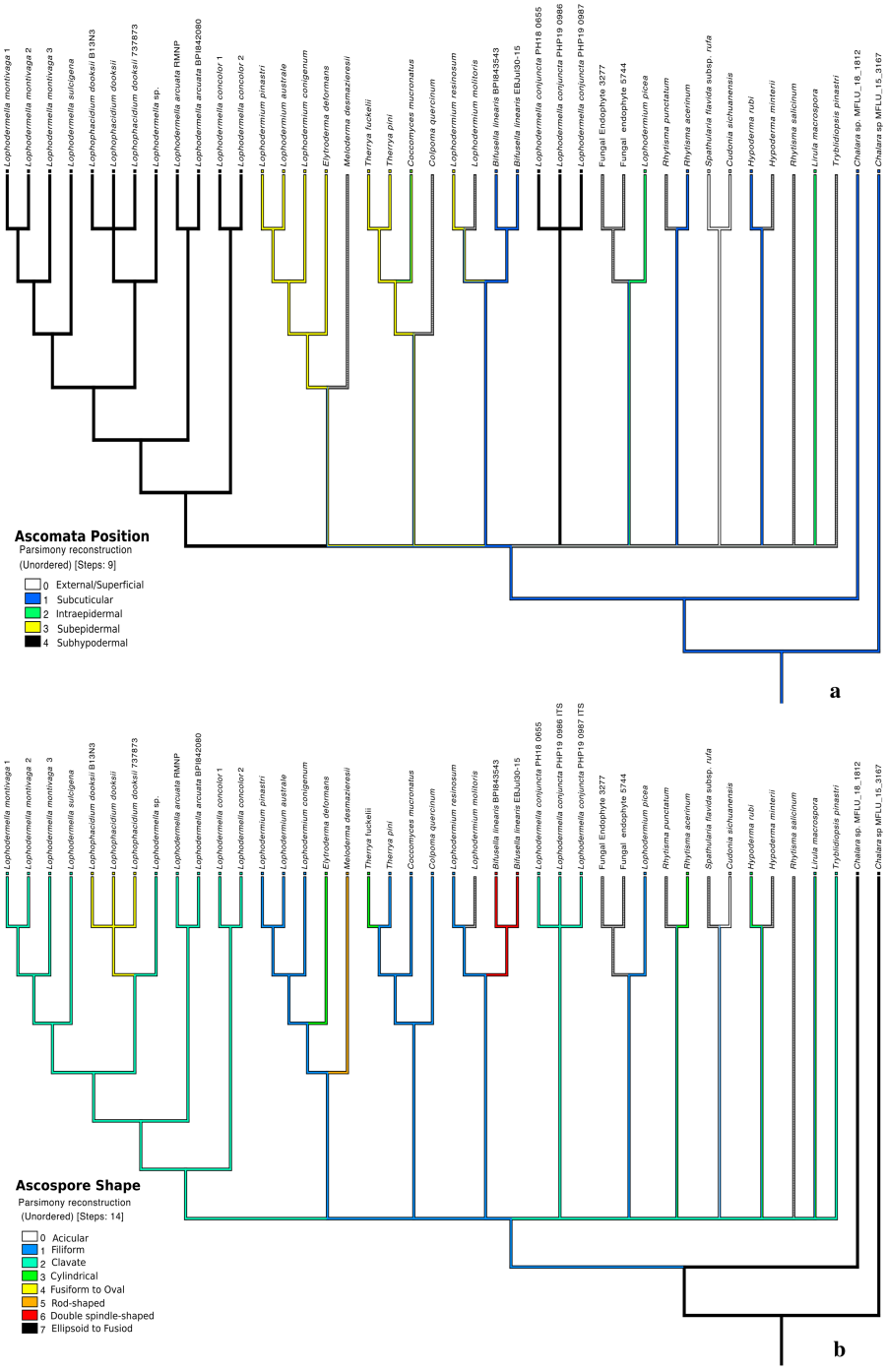

Figure 4 **Morphological characters mapped onto ITS phylogenetic tree with the parsimony ancestral reconstruction method using Mesquite v.3.6 with retention indices ≥0.50, ascomata position (A) and ascospore shape (B).**

Full-size DOI: 10.7717/peerj.11435/fig-4

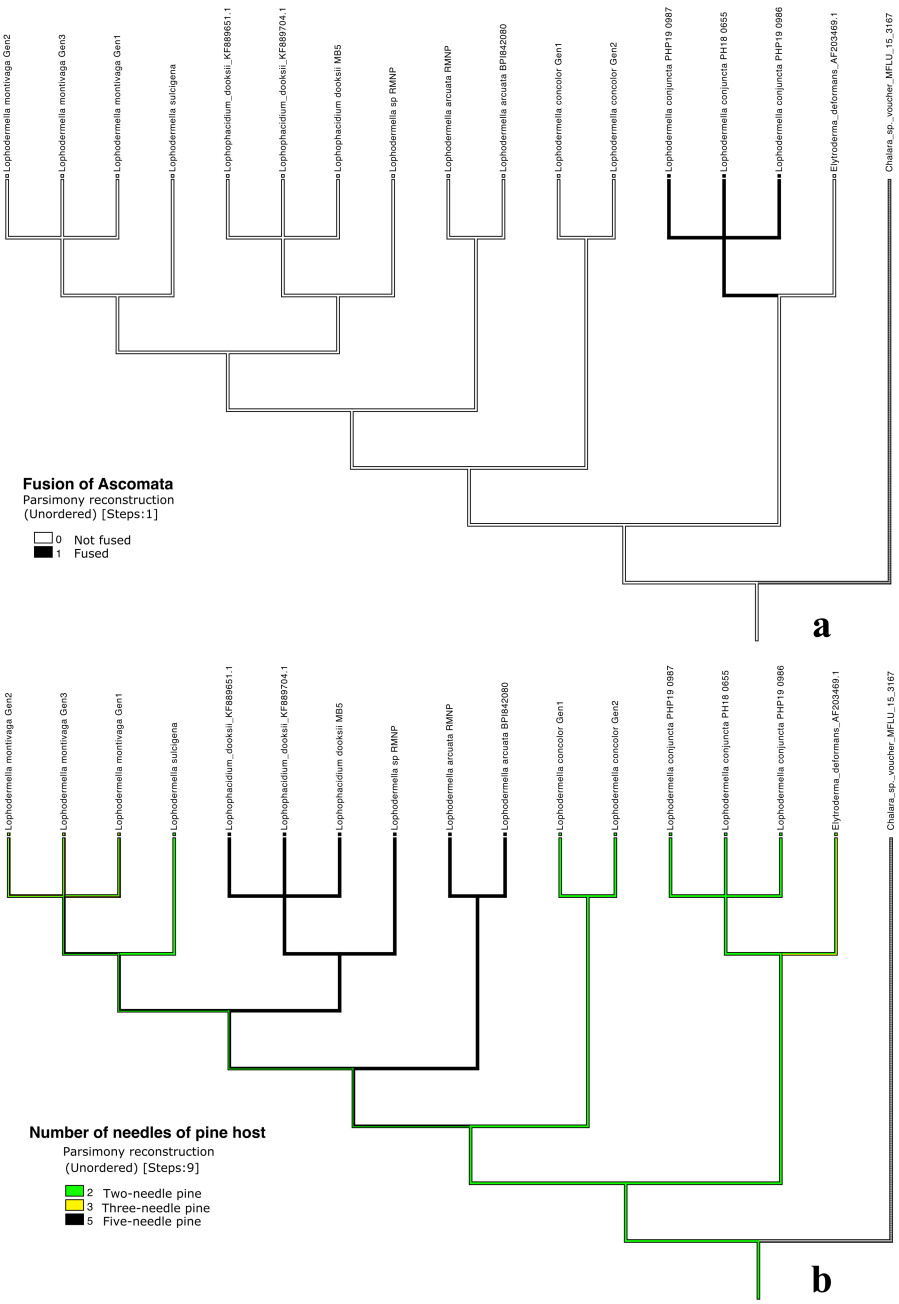

Figure 5 Morphological characters mapped onto Bayesian ITS phylogenetic tree with the parsimony ancestral reconstruction method using Mesquite v.3.6. Fusion of ascomata (A) and number of needles of pine host (B).

Full-size DOI: 10.7717/peerj.11435/fig-5

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, this present study does not disregard potential changes in the phylogenetic arrangement and polyphyly as more *Lophodermella* species will be 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*). It may also be possible that *L. conjuncta* belong to a separate genus that shares close morphological and phylogenetic relationship with *Lophodermella*. Thus, further investigation of other *Lophodermella* species which so far have no available sequence data still needs to be conducted to confirm these phylogenetic arrangements.

The present study supported a close relationship of *L. montivaga* and *L. sulcigena* compared to the other species within the LOD clade. *Darker (1932)* speculated that *L. sulcigena* from Europe may be identical to *L. montivaga* due to morphological similarities. Despite the overlapping morphological distinctions between the two species, this present study provided molecular evidence that *L. montivaga* and *L. sulcigena* are two distinct species. Another previous speculation was the possibility that *L. arcuata* is a variety of either *L. montivaga* or *L. sulcigena* owing to its resemblance to both species and its limited occurrence (*Darker, 1932*). However, symptom and ascocarp development in both species were different and thus were maintained as two different species (*Millar, 1984*). Genetic evidence gave support that *L. arcuata* is distinct from *L. sulcigena* and *L. montivaga*.

Consistent nesting of *Lophophacidium dooksii* in a *Lophodermella* clade was observed in all phylogenies, which concurs with a previous molecular study (*Laflamme et al., 2015*). Results herein showed that *L. dooksii* is more closely related to *Lophodermella* sp. (from *P. flexilis*) than to *L. montivaga* and *L. arcuata*, and provides more evidence for the transfer of the species from Phacidiaceae to Rhytismataceae as proposed by *Ekanayaka et al. (2019)*. We did not attempt to reclassify the taxon to *Lophodermella* since we did not have large sample size and type specimen to conduct further validations. Interestingly, *L. dooksii* was synonymous to *Canavirgella banfieldii*, a species classified under Rhytismataceae, but the former taxonomic name was given priority due to its earlier publication (*Laflamme et al., 2015*). In other studies, use of multiple loci supported the placement of *Cudonia* and *Spathularia* from Geoglossaceae to Rhytismataceae (*Gernandt et al., 2001*; *Lantz et al., 2011*; *Ge et al., 2014*), which these results also support (Figs. S1–S3).

Phylogeny of *Lophodermella* sp. from P. *flexilis*

Individual phylogenies in this study could not confirm the species identity of the *Lophodermella* sp. from *P. flexilis* collected at RMNP as it did not cluster together with *L. arcuata* samples. Aside from morphometric features, initial examination identified RMNP_01 sample as *L. arcuata* due to its occurrence on *P. flexilis* in Colorado. *Minter & Millar (1993a)*, *Minter & Millar (1993b)*; considered host preference and geographic distribution as criteria for identification of *L. arcuata* due to the consistent reports on this species being the only member of the genus occurring on five-needle pines in North America. However, genetic data suggests *Lophodermella* sp. may represent a separate species distinct from *L. arcuata*. Since needle samples with this potentially new species were only collected from one tree, we did not attempt to formally name the species but temporarily named at the genus level as *Lophodermella*. Further investigation needs to be conducted

to differentiate this species with other *Lophodermella* species described in literature and to define the population diversity of *L. arcuata*. Further, results from this study also suggest that undescribed cryptic *Lophodermella* species exist on pine hosts.

Morphological and Lifestyle Traits of the Lophodermella clade

Classification of Rhytismataceae genera has been challenged by the limited morphological features for characterization. *Darker (1967)* revised the genera within the previous Hypodermataceae based on the characteristics of their ascomata or hysterothecia, asci, and pycnidia or a combination of these characters. Spore shape, septation and color were secondary characters to delimit the genera (*Darker, 1967*). Further, *Lantz et al. (2011)* described ascomata and spores as unreliable characters for genus delimitation in Rhytismatales but found that a combination with other traits was potentially useful. This study showed that, at the genus level, subhypodermal ascomata and ascospore shape may be used as diagnostic characters for delimitation of genus *Lophodermella.* This is congruent to the dichotomous key produced by *Darker (1967)* to delimit this genus. Despite its inclusion in the LOD clade, *Lophophacidium dooksii* did not have clavate ascospores but rather had ascospores with fusiform to oval shape. Interestingly, aside from subhypodermal hysterothecia, all species within the LOD clade produced a tanned hypodermis. Furthermore, despite low consistency, the strong retention of asci shape may also suggest its role in taxa distinction.

Within *Lophodermella* genus, morphometric traits such as size of ascospores and hysterothecia are still used as distinctive characters. This study showed that a combination of morphological and ecological characters may be used to distinguish *Lophodermella* species, particularly ascospore and hysterothecia length, hysterothecia color, and the number of needles on pine host. However, these characters may also become problematic in practice. For example, while ascospore size was identified as a reliable criterion, measurements of spores varied depending on the freshness of specimen and thus cannot easily be used for identification of *Lophodermella* species (*Millar, 1984*). Further, concolorous hysterothecia as key character may be misleading as some species can also produce conspicuous hysterothecia (*Millar, 1984*).

Difficulty in obtaining pure cultures of *L. montivaga*, *L. concolor* and *L. dooksii* can also potentially limit further characterization of other traits such as physiology and pathogenicity. Similar to other studies, we were not able to grow in culture the *Lophodermella* species sampled in this study, suggesting an obligate lifestyle. Use of agar cultures including pine extract agar did not yield successful cultures of *Lophodermella* (*Millar, 1984*). Some studies also described *L. dooksii* and *Bifusella linearis* as obligate fungal pathogens after unsuccessful attempts of obtaining cultures or only obtaining short-lived cultures (*Broders et al., 2015*; *Merrill, Wenner & Dreisbach, 1996*). In contrast, previous studies were able to isolate pure cultures of *L. sulcigena* on malt agar (*Jalkanen, 1985*; *Kowalski & Krygier, 1996*). Similarly, a number of studies documented several *Lophodermium* species (e.g., *Decker, Hsiang & Peterson, 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*). Consequently, while environmental DNA may be available, the absence of pure cultures of many *Lophodermella* species limit further molecular research that require a high pure DNA concentration.

Most *Lophodermella* species appear to be either specific to a single host species distributed in a certain geographic region (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*). Furthermore, *L. arcuata* and *L. maureri* are the only two *Lophodermella* species on five-needle pines of subsection Strobus while *L. morbida* only occurs exclusively on three-needle pines under section Trifoliae. 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.

ACKNOWLEDGEMENTS

The authors also thank Aaron Bergdahl and Wayne Searles of Maine Forest Service, and Jessica Cancelliere of New York State Department of Environmental Conservation for their assistance in *Lophophacidium dooksii* sample collection. The authors are also grateful to the USFS Northern Research Station for access to the Massabesic Experimental Forest. Dr. Jorge Ibarra Caballero assisted in DNA extraction and sample collection in Colorado. The authors also thank Dr. Thomas L Cech (Vienna) for his assistance in the European *Lophodermella* collection. The molecular work on the European specimens was carried out in the WSL plant protection laboratory (Birmensdorf, CH)—many thanks to its staff for the helpful assistance.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

This study is funded by USDA Forest Service Forest Health Protection Special Technology Development Program (STDP-R2-2017-3). The European Lophodermella species were studied within the project "Spread of pine diseases in Switzerland" under the direction of Joana Meyer (Zurich), financed by the Swiss Federal Office of Environment. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures

The following grant information was disclosed by the authors:
USDA Forest Service Forest Health Protection Special Technology Development Program: STDP-R2-2017-3.
Swiss Federal Office of Environment.

Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Jessa P. Ata conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Kelly S. Burns and Jane E. Stewart conceived and designed the experiments, performed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- Suzanne Marchetti performed the experiments, analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.
- Isabel Munck performed the experiments, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Ludwig Beenken performed the experiments, analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.
- James J. Worrall conceived and designed the experiments, authored or reviewed drafts of the paper, and approved the final draft.

Field Study Permissions

The following information was supplied relating to field study approvals (i.e., approving body and any reference numbers):

Collections were approved by the USDA Forest Service Forest Health Protection.

Data Availability

The following information was supplied regarding data availability:

The ITS, LSU, and TEF sequences are available at GenBank: ITS (MN937610–MN937641), LSU (MT906358; MN937875–MN937598), TEF (MT919221–MT919227; MN937646–MN937678).

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.11435#supplemental-information.

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