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Phylogenetics of *Lophodermium* from pine

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Abstract: *Lophodermium* comprises ascomycetous fungi that are both needle-cast pathogens and asymptomatic endophytes on a diversity of plant hosts. It is distinguished from other genera in the family Rhytismataceae by its filiform ascospores and ascocarps that open by a longitudinal slit. Nucleotide sequences of the internal transcribed spacer (ITS) region of nuclear ribosomal DNA were used to infer phylogenetic relationships within *Lophodermium*. Twenty-nine sequences from approximately 11 species of *Lophodermium* were analyzed together with eight sequences from isolates thought to represent six other genera of Rhytismataceae: *Elytroderma*, *Lirula*, *Meloderma*, *Terriera*, *Tryblidiopsis* and *Colpoma*. Two putative *Meloderma desmazieresii* isolates occurred within the *Lophodermium* clade but separate from one another, one grouped with *L. indianum* and the other with *L. nitens*. An isolate of *Elytroderma deformans* also occurred within the *Lophodermium* clade but on a solitary branch. The occurrence of these genera within the *Lophodermium* clade might be due to problems in generic concepts in Rhytismataceae, such as emphasis on spore morphology to delimit genera, to difficulty of isolating Rhytismataceae needle pathogens from material that also is colonized by *Lophodermium* or to a combination of both factors. We also evaluated the congruence of host distribution and several morphological characters on the ITS phylogeny. *Lophodermium* species from pine hosts formed a monophyletic sister group to *Lophodermium* species from more distant hosts from the southern hemisphere, but not to *L. piceae* from *Picea*. The ITS topology indicated that *Lophodermium* does not show strict cospeciation with pines at deeper branches, although several closely related isolates have closely related hosts. Pathogenic species occupy derived positions in the pine clade, suggesting that pathogenicity has evolved from endophytism. A new combination is proposed, *Terriera minor* (Tehon) P.R. Johnst.

Key words: endophyte, evolution, ITS region, pathogen, phylogeny, Rhytismataceae

INTRODUCTION

Fungal endophytes live all or most of their lives internally and asymptomatically in plant tissue (Stone and Petrini 1997, Saikkonen et al 1998) and have been found in all woody plants that have been examined (Carroll and Carroll 1978, Petrini et al 1982, Chapela and Boddy 1988, Espinosa-García and Langenheim 1990, Hata and Futai 1995, Stone et al 2000). Endophytic fungi of woody hosts are a highly diverse assemblage, primarily of ascomycetes, representing a range of symbiotic interactions with their host plants, from commensals and mutualists to latent pathogens. Most phylogenetic studies of aboveground endophytes have focused on grass symbionts (i.e., Schardl et al 1991, Glenn et al 1996, Reddy et al 1998, but see Gernandt et al 1997). Although both have been termed ''endophytes'', endophytes of grasses are limited to a relatively small group of genera in the Clavicipitaceae, while endophytic fungi of

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woody hosts are biologically and ecologically more diverse (Stone et al 2000). Phylogenetic studies of woody plant endophytes are needed to evaluate degrees of specialization to habitats and hosts, endophyte diversity and distribution patterns, and the evolutionary pathways leading to this widespread association.

Lophodermium Chevall. is a large and complex genus in the family Rhytismataceae (Rhytismatales, Ascomycota). This genus offers a great opportunity to study the evolutionary history of the important and widespread symbiotic relationships among endophytes and woody plants. Approximately 103 species have been described on different plant groups (Kirk et al 2001). *Lophodermium* is conspicuous on senescent and abscised conifer needles and recognizable in culture by its anamorphic states; it is among the most common endophytes isolated from *Pinus*, *Abies* and *Picea* (Stone et al 2000). Although more than 20 species of *Lophodermium* are known to colonize needles of coniferous trees and shrubs, only one of these, *L. seditiosum*, is a major pathogen, causing needle cast of pines in forest nurseries and plantations (Minter and Millar 1980, Sinclair et al 1987). In most *Lophodermium* species from pine, ascocarps usually are found on recently abscised needles. These fungi colonize healthy needles, where they reside as endophytes within the asymptomatic host tissue; ascocarps typically mature after needle abscission (Deckert et al 2001). *Lophodermium* species have been described from several other plant families and are especially diverse on Poaceae and Ericales (Cannon and Minter 1983, Johnston 1989, 1992, 2001, Ellis and Ellis 1997).

Lophodermium is characterized by apothecia covered with a clypeus, which opens in a single longitudinal slit, and hyaline, filiform, nonseptate ascospores. Delimitation of *Lophodermium* species in pines is based on characters observed in mid-point cross sections of mature ascocarps. These characters include the position of the ascocarp in relation to the host's epidermis and hypodermis, the number and position of displaced epidermal cells, the presence and pigmentation of distinctive ''lip'' cells along the slit zone and the degree of pigmentation of the subhymenial lower wall associated with the presence of a perimeter line. Characters related to the external appearance include the presence of zone lines or perimeter lines, the size and shape of the ascocarp and its position in relation to the stomatal rows (Darker 1967, Minter 1981).

Several authors have emphasized the artificial nature of the separation of many genera in Rhytismataceae, including *Lophodermium* (Darker 1967, Johnston 1989, 1990, Spooner 1991). Early classifications based generic delimitation primarily on ascospore shape. More recent classifications include characteristics of the ascocarp, asci and ascospores, as well as the anamorph. For example, Johnston (1988, 1989) delineated six different groups with 21 *Lophodermium* species from New Zealand based on distinctive developmental features of the ascocarps. The same developmental features that defined some of these groups were shared by species in different genera within the family, including *Hypoderma*, *Meloderma* and *Coccomyces*.

A comprehensive monograph of *Lophodermium* is a difficult task, considering its putative artificial constitution, its complexity and the number of species ascribed to the genus. In this study we used internal transcribed spacer (ITS) region sequences from nuclear ribosomal (nr) DNA to infer phylogenetic relationships for *Lophodermium* species with different host preferences and geographic distributions. The ITS region has been used widely to resolve intraspecific and interspecific phylogenetic relationships, providing insights into the utility of conventional morphological characters and species delimitation in fungi (Lee and Taylor 1992, Carbone and Kohn 1993, Harrington and Potter 1997, Johnston and Jones 1997, Jacobs and Rehner 1998). Monophyly of the genus is tested by the inclusion of six other genera in the family Rhytismataceae. The range of interactions between *Lophodermium* and its hosts also provides an opportunity to study levels of specificity and to trace the transition between pathogenicity and endophytism. Finally, by mapping morphological and developmental characters used for species delimitation onto the molecular phylogeny, we investigate levels of convergent evolution in this group and whether certain morphological characters can be attributed to common descent or rather to anatomical similarities in their hosts.

MATERIALS AND METHODS

Fungal isolates.—The phylogenetic analysis was based on 29 isolates representing 11 described species, as many as five additional undescribed *Lophodermium* species from Mexican pines (see below) and two *Leptostroma* species presumed to be anamorphs of *Lophodermium* (TABLE I). Several species were represented by individuals from multiple hosts or populations. Three additional ITS sequences (*L. nitens* AF426057, *L. nitens* AF426061 and *Meloderma desmazieresii* AF426056) from a previously published study (Deckert et al 2002) obtained from GenBank were included, although they were missing the first 40 bp of ITS1. Isolates of *Elytroderma deformans*, *Lirula macrospora*, *Meloderma desmazieresii*, *Colpoma quercinum* and *Tryblidiopsis pinastri* were obtained from culture collections. All five of these genera occurred in a Rhytismataceae clade with *Lo-*

TABLE I. Strains included in this study

Taxon	Host	Culture[e]	Origin	Genbank No.
Leptostroma decipiens Petr.	*Pinus ponderosa* Douglas ex C. Lawson	ma4n7m1	Oregon, USA	AY100653
Leptostroma decipiens 2	*Pinus ponderosa*	ja4n5ts	Oregon, USA	AY100654
Leptostroma sp.	*Pinus ayacahuite* Ehrenb. ex Schltdl.	loduchcon	Durango, Mexico	AY100652
Lophodermium actinothyrium Fuckel	Poaceae	ICMP 14599	Argentina	AY100663
Lophodermium agathidis Minter & Hettige	*Agathis australis* (D. Don) Steud.	ICMP 13976	New Zealand	AY100661
Lophodermium agathidis 2	*Metrosideros fulgens* Gaertn.	ICMP 14958	New Zealand	AY100662
Lophodermium australe Dearn.	*Pinus palustris* Mill.	loau	Florida, USA	U92308
Lophodermium australe 2	*Pinus pseudostrobus* Lindl.	lopaus	Oaxaca, Mexico	AY100647
Lophodermium baculiferum Mayr	*Pinus ponderosa*	loba1	Oregon, USA	AY100655
Lophodermium baculiferum 2	*Pinus contorta* Douglas	loba2	Oregon, USA	AY100658
Lophodermium baculiferum 3	*Pinus montezumae* Lamb.	mon2zem	Morelos, Mexico	AY100656
Lophodermium baculiferum 4	*Pinus montezumae*	monbacp	Nuevo León, Mexico	AY100657
Lophodermium conigenum (Brunaud) Hilitzer	*Pinus radiata* D. Don	ICMP 13978	New Zealand	AY100645
Lophodermium conigenum 2	*Pinus radiata*	ICMP 13979	New Zealand	AY100646
Lophodermium indianum Suj. Singh & Minter	*Pinus greggii* Engelm. ex Parl.	loingr	Nuevo León, México	AY100642
Lophodermium indianum 2	*Pinus hartwegii* Lindl.	haroax	Oaxaca, Mexico	AY100641
Lophodermium molitoris Minter	*Pinus taeda* L.	CBS 597.84	North Carolina, USA	AY100659
Lophodermium nitens Darker	*Pinus ayacahuite*	6iayani	Nuevo León, Mexico	AY100640
Lophodermium nitens[b]	*Pinus strobus*	NS4-2	Nova Scotia, Canada	AF426057
Lophodermium nitens[b]	*Pinus strobus*	BRMC1-7	Ontario, Canada	AF426061
Lophodermium piceae (Fuckel) Höhn.	*Picea abies* (L.) Karsten	93011	Waadt, Germany	AF203471
Lophodermium pinastri (Schrad.) Chevall.	*Pinus sylvestris* L.	BBA L230	Lüneburg, Germany	AY100650
Lophodermium pinastri 2	*Pinus ponderosa*	loppon	Oregon, USA	AY100649
Lophodermium seditiosum Minter, Staley & Millar	*Pinus sylvestris*	BBA L225	Lüneburg, Germany	AF203468
Lophodermium sp 1	*Pinus ayacahuite*	ayazem2	Morelos, Mexico	AY100643
Lophodermium sp 2	*Pinus douglasiana* Martínez	lodou	Oaxaca, Mexico	AY100644
Lophodermium sp 3	*Pinus montezumae*	mon1zem	Morelos, Mexico	AY100648
Lophodermium sp 4	*Pinus chiapensis*(Martínez) Andresen	chia1ss	Oaxaca, Mexico	AY100651
Lophodermium sp 5	*Pinus montezumae*	montoco5	Chiapas, Mexico	AY100660
Elytroderma deformans (Weir) Darker[a]	*Pinus ponderosa*	CBS 181.68	Montana, USA	AF203469
Lirula macrospora (R. Hartig) Darker	*Picea abies*	CBS 592.84	As, Norway	AF203472
Meloderma desmazieresii (Duby) Darker[a,c]	*Pinus ayacahuite* Ehrenb.	CBS 612.84	Mexico, Mexico	AF203470
Meloderma desmazieresii[b,c]	*Pinus strobus*	MD3	Ontario, Canada	AF426056
Terriera minor[d]	*Pseudopanax chathamicum* T. Kirk	ICMP 13974	New Zealand	AY100664
Terriera minor 2	*Nothofagus menziesii* Oerst.	ICMP 13973	New Zealand	AY100665
Tryblidiopsis pinastri (Pers.) P. Karst.	*Pinus* sp.	CBS 234	Sweden	U92307
Colpoma quercinum (Pers.) Wallr.	*Quercus* sp.	BBA C313	Münden, Germany	U92306

[a] These isolates may be misidentified (see text).

[b] Two *L. nitens* strains and one *M. desmazieresii* strain are from Deckert et al (2002).

[c] This spelling of the epithet is adopted as a correction of an error in use of a termination (ICBN 2000, Art. 60.11, Rec. 60C.1(b)).

[d] *Terriera minor* (Tehon) P. R. Johnst. comb. nov. Basionym: *Clithris minor* Tehon, Botanical Gazette 65: 554, 1918.

[e] BBA = Institut für Forstpflanzenkrankheiten, Hann Münden, Germany; CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; ICMP = International Collection Of Microorganisms From Plants, Landcare Research, Auckland, New Zealand.

phodermium in a phylogenetic analysis of ascomycete small-subunit nrDNA sequences (Gernandt et al 2001). *Elytroderma deformans*, *L. macrospora* and *M. desmazieresii* share characteristics with some species in *Lophodermium*, including host substrate, ascocarp structure and development, and anamorph morphology (Darker 1967, Cannon and Minter 1983, Johnston 1988, 1989). *Terriera minor* differs from *Lophodermium* in structure of the ascomatal primordium and in the ascospores lacking a gelatinous sheath (Johnston 2001, as *Lophodermium minus*). We included these four species to test the monophyly of *Lophodermium*, while *Tryblidiopsis pinastri* and *Colpoma quercinum*, two morphologically more distinct members of Rhytismataceae, were used to root the phylogenetic trees (Nixon and Carpenter 1993). *Tryblidiopsis pinastri* and *C. quercinum* occur on dead stems and branches and have distinctive erumpent ascomata.

Three *Lophodermium* anamorphs were obtained from surface-sterilized asymptomatic needles maintained on potato-dextrose agar (Difco), supplemented with 0.1% malt extract and 0.15% yeast extract (PDMYA). Needles were surface sterilized by successive immersion in 70% ethanol for 1 min, 4% sodium hypochlorite for 10 min, 70% ethanol for 1 min and two rinses in sterile water. Two of these cultures were designated as the *Leptostroma decipiens* anamorph of *Lophodermium baculiferum*, whereas the third was undetermined (*Leptostroma* sp.). The remaining 17 specimens from pine were obtained from cultures recovered from ascospores expelled from apothecia on needle fragments suspended above the agar surface (these isolates were not knowingly derived from single spores). Fifteen isolates from pines were collected within the natural distribution ranges of their hosts, and two *L. conigenum* isolates were collected from introduced *Pinus radiata* in New Zealand. Four *Lophodermium* cultures were obtained from public culture collections (TABLE I). During the course of this study, as many as five undescribed *Lophodermium* species from Mexican pines were discovered. Ortiz-García, Salas-Lizana and Alvarez-Buylla are preparing formal descriptions of these species for publication.

DNA extraction and amplification.—A modification of the procedure of Doyle and Doyle (1987) was used to extract DNA from fungal cultures. Samples were ground in 1.5 mL tubes with sand and 1 mL of 60 C 2× CTAB supplemented with 2% sodium bisulfite, then incubated at 37 C for 1–2 h. Two chloroform : isoamyl alcohol (24:1) extractions were performed, DNA was precipitated in cold isopropanol, and the pellet was washed with 70% ethanol and dissolved in TE (10 mM Tris-HCL pH 7–8, 0.1 mM EDTA). PCR reactions were performed in 100 μL volumes with 1.5 U Replitherm™ DNA polymerase (Epicentre Technologies, Madison, Wisconsin), 1× Replitherm™ buffer, 1.5 mM $MgCl_2$, 4% DMSO, 2% BSA, 200 μmol each dNTP, 1.5 μmol each primer and approximately 50 ng sample DNA. The primers used to amplify the ITS region were ITS1F: 5′ CTT GGT CAT TTA GAG GAA GTA A 3′ (White et al 1990) and, NL6A: 5′ CAA GTG CTT CCC TTT CAA CA 3′ (Egger 1995). These primers anneal to the 18S small- and 28S large-subunit nuclear ribosomal RNA genes, respectively. PCR conditions were 40 cycles at 94 C, denaturation for 45 s, 55 C annealing for 45 s and 72 C extension for 90 s. Reactions were terminated after a final extension at 72 C for 9 min. Products were purified with QIAquick gel extraction kits (Qiagen, Chatsworth, California). Cycle sequencing with dye terminator chemistry was performed using an ABI model 373A fluorescent sequencer (Applied Biosystems, Foster City, California). Products were sequenced in both 5′ to 3′ and 3′ to 5′ directions.

Sequence analyses.—Sequence alignments were performed using CLUSTALW (Thompson et al 1994) with a gap weight = 40, gap length weight = 5, and adjusted manually by visual inspection in Genetic Data Environment (Smith et al 1994). Phylogenetic analyses were conducted with PAUP* version 4.0b10 (Swofford 2002) using the parsimony criterion. Heuristic searches were performed with these options: 500 replicates of random-addition sequence, tree bisection reconnection (TBR) branch swapping and no maxtree limit. Gaps were treated as missing data to minimize homology assumptions. All characters were equally weighted and unordered. Support for branching topologies was evaluated with 500 replicates of bootstrapping (Felsenstein 1985). A maxtree limit of 10 000, random-addition sequence with 10 replicates and TBR branch swapping were used in the bootstrap analysis. To examine the number of additional steps required to collapse each clade, Bremer support/decay indices (Bremer 1988, 1994) were calculated by using the command "keep trees" for trees 1–4 steps longer and by the constraint consensus analysis for each clade with longer steps (Morgan 1997). In both methods, the above heuristic search parameters were used.

Morphological character analysis.—Nineteen primarily qualitative morphological characters were scored for most of the isolates (TABLE II and TABLE III). Characters were chosen based on availability and on traditional use for taxonomic assignment of *Lophodermium* species in pines and were scored based on comparison of isolates with published descriptions (Darker 1967, Johnston 1989, 1992, 2001, Minter 1981, Minter and Hettige 1983). All morphological characters were coded as unordered and were optimized onto the trees obtained from the ITS region analysis by using unweighted parsimony with multistate taxa treated as polymorphic. The molecular data matrix is available in TreeBASE (M1360).

RESULTS

Sequence analysis.—The PCR products resulting from using the fungal specific primers included partial sequences from the 18S rRNA gene and 28S rRNA gene and an approximately 225 bp intron at the 3′ end of the small subunit for all strains, except *L. pinastri* 2, *L. nitens*, *L. baculiferum* 3, *L. agathidis* 2 and *Elytroderma deformans*. Due to alignment difficulties and questionable homology, these insertions were excluded from the analyses along with the 18S and 28S rRNA genes. Boundaries of the ITS1, 5.8S rDNA

TABLE II. Characters and character states used for phylogenetic reconstructions

No.	Characters and States
1	Asomata: position subcuticular (0); partly subepidermal and subcuticular (1); subepidermal (2); partly subcuticular, subepidermal, and subhypodermal (3); laterally subhypodermal (4)
2	Ascomata: opening nonlinear (0); linear (1)
3	Ascomata: linear opening along epidermal cells (0); along stomata (1)
4	Ascomata: shape elliptical to oblong (0); elliptical with acute apex (1); linear (2); irregular, erumpent (3)
5	Subhymenial tissue: pigmentation absent (0); slight in basal wall (1); strong in basal wall (2)
6	Modified cells at ascomata opening: absent (0); present (1)
7	lip cells: colorless (0), grey (1); black (2); green/blue (3); red (4)
8	Perimeter line: absent (0); present (1)
9	Black zone lines: absent (0); present (1)
10	Ascospores: shape filiform (0); not filiform (1)
11	Ascospores: septa absent (0); present (1)
12	Paraphyses: simple (0); swollen (1); hooked (2); branched (3)
13	Conidiomata: absent (0); present (1)
14	Conidiomata: opening mostly irregular tears (0); one ostiole (1); more than one ostiole (2)
15	Host: not pine (0); pine (1)
16	Substrate for reproduction: needles or leaves (0); needles or leaves and cones (1); bark (2)
17	Epidermal cell displacement: none (0); centrally and together on basal wall (1); scattered on basal wall (2)
18	Ascus apices: acute (0); papillate (1); cylindrical truncate (2)
19	Conidia: shape cylindrical (0); elliptical (1); falcate or lunate (2)

gene and ITS2 were based on comparison with published sequences (Lee and Taylor 1992, Morales et al 1993, Jasalavich et al 1995). The length of the ITS alignment was 484 bp, including 201 variable positions, 162 of which were phylogenetically informative. The 34 complete ITS region sequences ranged in length from 424 to 455 bp. Most length variation was in ITS1 (range 122–150 bp, mean = 129.5 ± 8.4), due mainly to the presence of an 18 bp insertion present in both isolates of *T. minor* and partially shared with outgroup and *L. piceae* in 13 positions. The 5.8S rDNA gene was 158 bp in all species. The ITS2 varied between 143 and 158 bp (mean = 149.1 ± 4.9) and had a higher G + C content (59%) than ITS1 and 5.8S (46–47%), a pattern that also is characteristic of angiosperms, some genera in the family Pinaceae and other groups of fungi (Baldwin et al 1995, Kuhls et al 1997, O'Donnell et al 1998, Liston et al 1999).

The uncorrected intraspecific percentage of nucleotide differences was 5.6% between *L. pinastri* isolates, 2.9% between *L. agathidis* isolates, 1.3% between *T. minor* isolates, 0.9% between *L. indianum* isolates, 0.4% between *L. australe* isolates, 0% between *L. conigenum* isolates, and ranged from 0% to 7.6% between *L. baculiferum* isolates and from 0.2% to 3.8% between *L. nitens* isolates. Intrageneric nucleotide differences were as high as 19.5% between *L. agathidis* and *L. conigenum*.

Phylogenetic analysis.—The heuristic search recovered eight equally most-parsimonious trees (FIG. 1). Twenty-one clades had bootstrap support greater than 50%, 14 of which were greater than 90%. Decay indices ranged from 0 in the two branches uniting closely related species at the tips of the tree that collapsed in the strict-consensus tree (not shown) to 19 steps on the branch uniting two isolates of *L. agathidis*. A *Lophodermium* clade was resolved in the strict-consensus tree but received low bootstrap and decay support: (61% bootstrap, decay index = 2).

A subset of the *Lophodermium* clade comprising 28 isolates from pine needles, hereafter referred to as the pine clade, received bootstrap support of 83% and a decay index of four (FIG. 1). Two isolates, both identified as *Meloderma desmazieresii*, occurred in the pine clade but in separate parts of the tree. The *M. desmazieresii* sequence of CBS culture 612.84 from *Pinus ayacahuite* had only a single nucleotide difference from *L. nitens* NS4-2 isolated from *P. strobus* (Deckert et al 2002), and the *M. desmazieresii* sequence from *P. strobus* had only a single nucleotide difference from an undescribed *Lophodermium* species from *P. ayacahuite*. Furthermore, *Elytroderma deformans* CBS181.68 occupied a solitary branch within the *Lophodermium* clade. The *E. deformans* strain did not match any *Lophodermium* isolate sequenced (8.0–13.7% nucleotide divergence from other members of the pine clade). The occurrence of these three se-

TABLE III. Character states for 23 species

Taxon	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
actino	2	1	0	0	1	1	0	0	0	0	0	2	0	?	0	0	0	0	?
agathi	0	1	0	0	1	1	1	0	1	0	0	?	1	1	0	0	2	0	1
austra	3	1	0	2	0	1	0,1	0	0	0	0	0	1	0	1	0	2	0	0
baculi	2	1	1	0	2	0	?	0	1	0	0	2	1	2	1	0	0	1	0
conigi	1	1	0	1	2	1	1,2	1	1	0	0	1,2	1	2	1	0	2	0	0
decipi	2	1	1	0	2	0	?	0	1	0	0	2	1	2	1	0	0	1	0
indian	3	1	0	0	2	1	1	1	1	0	0	1	0	?	1	0	2	0	?
minus	2	1	0	0	1	0	?	0	0	0	1	3	1	?	0	0	0	0	0
molito	1	1	0	0	1,2	1	1	1	1	0	0	0	1	0	1	0	1	1	0
nitens	0	1	0	0	1	0	?	1	1	0	0	1,2	1	0	1	0	0	1	0
picea	2	1	0	0	1	0	?	1	1	0	0	1	1	?	0	0	0	0	?
pinast	1	1	0	0	1	1	4	1	1	0	0	0	1	2	1	1	1	0	0
sediti	2	1	0	1	1	1	3	1	1	0	0	0	1	1	1	1	0	0	0
Losp.1	1	1	0	1	2	1	0	1	0	0	0	1	1	1	1	0	1	0	0
Losp.2	4	1	0	0	0	1	1	0	0	0	0	0	?	?	1	0	0	0	?
Losp.3	2	1	0	0	1	1	3	1	1	0	0	2	1	?	1	0	1	1	0
Losp.4	1	1	0	1	2	1	0	1	?	0	0	?	?	?	1	0	1	?	?
Losp.5	2	1	0	0	1	0	?	1	0	0	0	?	1	?	1	0	1	?	?
E.defo	4	1	0	2	0	0	?	0	0	1	1	1	1	?	1	0	2	0	?
L.macr	0	1	1	0	1	0	?	1	1	0	0	1	1	1,2	0	0	0	2	1
M.desm	1	1	0	0	2	1	?	1	0	1	0	1	1	?	1	0	2	0	0
T.pina	?	0	?	3	1	0	?	0	0	1	1	0,1	1	?	1	2	?	2	2
C.quer	?	0	?	3	?	0	?	0	0	0	0	2	?	?	0	2	?	0	?
CI on combined tree	0.40	1.0	0.33	0.50	0.43	0.25	0.70	0.17	0.17	1.0	0.50	0.60	0.33	0.43	0.5	0.67	0.25	0.4	0.67

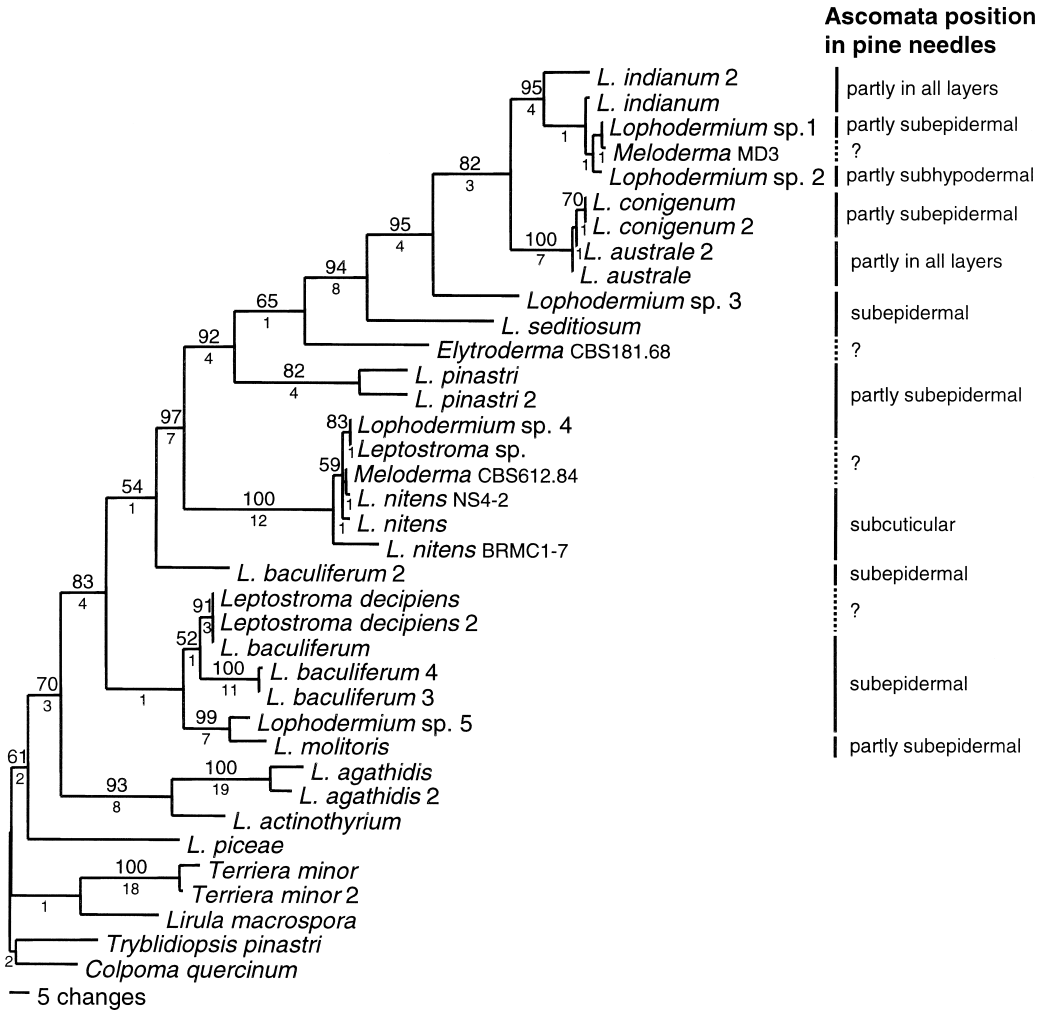

FIG. 1. One of the eight most-parsimonious trees obtained for *Lophodermium* isolates and related genera. Trees are based on 162 informative characters, length (L) = 528 steps, consistency index (CI) = 0.577, CI excluding uninformative characters (CIe) = 0.541, retention index (RI) = 0.816, and rescaled CI (RC) = 0.471. Branch lengths are proportional to the number of character state changes. Numbers above branches are bootstrap values greater than 50%; numbers below branches are decay indices. The position of the ascomata in pine needles (character 1, TABLE III) is indicated at the right for *Lophodermium* species with pine hosts.

quences in the *Lophodermium* clade suggests either human error or the need to synonymize one or both of these genera under *Lophodermium* (see below). Regardless, these three sequences are considered representatives of *Lophodermium* in the following description of the results.

At the base of the pine clade, *L. molitoris* and *Lophodermium* sp. 5 were in a tenuous sister relationship to three *L. baculiferum* isolates paraphyletic to two *Leptostroma decipiens* isolates (FIG. 1). A fourth *L. baculiferum* isolate was in an intermediate position between this group and the remaining species of *Lophodermium* from pines. The remainder of the pine clade included *L. nitens*, *L. pinastri*, *L. indianum*, *L. conigenum*, *L. australe*, several undescribed species from Mexican pines, and the *Meloderma* and *Elytroderma* sequences. The highly similar *L. conigenum* and *L. australe* sequences formed a clade, with the *L. australe* sequences paraphyletic to the two *L. conigenum* sequences. Two undescribed *Lophodermium* isolates from *P. ayacahuite* and *P. douglasiana*, a *Meloderma* sequence from *P. strobus*, and two (paraphyletic) *L. indianum* isolates together formed a well-supported clade.

Other *Lophodermium* species were from a phylogenetically diverse assemblage of hosts and were paraphyletic with respect to the pine clade. Two *L. agathidis* isolates from *Agathis* (Araucariaceae) and *Metrosideros* (Myrtaceae), respectively, in New Zealand were monophyletic and in a sister position to *L. actinothyrium* isolated from Poaceae in Argentina. *Lophodermium piceae* was in a sister position to all other

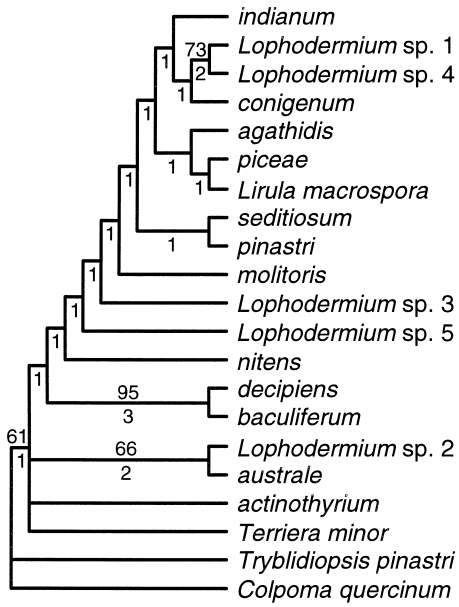

FIG. 2. Strict consensus of eight equally most-parsimonious trees based on 18 morphological characters (L = 70 steps, CI = 0.59, CIe = 0.58, RI = 0.64 and RC = 0.38). Numbers above branches are bootstrap values greater than 50%; numbers below branches are decay indices.

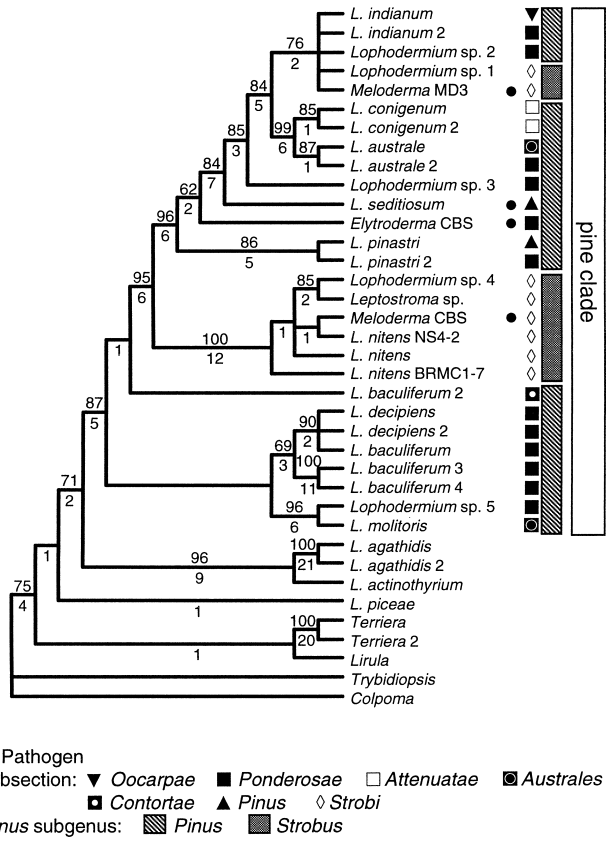

FIG. 3. Strict-consensus tree of 42 equally parsimonious combined ITS and morphology trees for *Lophodermium* isolates and related genera (180 informative characters, L = 630 steps, CI = 0.557, CIe = 0.525, RI = 0.794, RC = 0.442). Host classification is from Price et al (1998).

Lophodermium sequences. The branch at the base of the *Lophodermium* clade had low bootstrap support (61%). Of the remaining genera, two monophyletic *Terriera* sequences formed a weakly supported sister relationship to *Lirula macrospora* and *Tryblidiopsis pinastri* formed a weakly supported sister relationship to *Colpoma quercinum*.

Morphological and combined analyses.—Preliminary parsimony analyses indicated that inclusion of three taxa with a moderate to high amount of missing morphological data (*Leptostroma* sp., *Meloderma*, and *Elytroderma*) resulted in unresolved trees when analyzed apart from ITS (not shown). Deleting these three taxa reduced the number of informative characters from 19 to 18 (character 10, ascospore shape, was no longer informative) but resulted in a more resolved tree (FIG. 2). Nevertheless, only four branches received bootstrap values greater than 50%, of which only two agreed in topology with the ITS tree, one uniting *Lophodermium baculiferum* and its anamorph, *Leptostroma decipiens* (95% bootstrap) and the other uniting *Tryblidiopsis* and *Colpoma* (61% bootstrap).

Character states from TABLES II and III were appended to the corresponding taxa in the ITS matrix, except for the *Leptostroma* sp., *Elytroderma* and *Meloderma* exemplars, which were scored as missing. Parsimony analysis of the combined morphological and ITS data (503 characters) resulted in 42 most-parsimonious trees. The character consistency index (CI), branch resolution and branch support of the strict-consensus tree (FIG. 3) were slightly lower in the combined dataset than with ITS alone. Morphological character states traditionally used for species delimitation of *Lophodermium* species on pine hosts (TABLE II) generally had high levels of homoplasy on the combined tree. The mean CI for all characters mapped onto the combined tree was 0.49 (SD = 0.24). The variable morphological characters with the highest CI values ≥(0.50) were: ascomata opening, ascomata shape, lip cell color, ascospore septa, paraphyses shape and conidium shape (TABLE III). Two ecological characters had CI values ≥0.50: host and substrate of fructification. Ascomata position had relatively low consistency (CI = 0.40; see distribution in FIG. 1).

Host associations.—Some species of *Lophodermium* from pine were from distantly related hosts and others were found on the same, or closely related hosts. Although a phylogeny of all pine species was not available, the subgeneric and subsectional classifications of pines (Price et al 1998) are indicated in FIG.

3. Based on our incomplete sampling, species or clades on closely related hosts (within the same pine subsection) included *L. conigenum* (*P. radiata*), the clade including *Lophodermium* sp. 4, *Leptostroma* sp., *Meloderma* from CBS, and three *L. nitens* isolates (*P. ayacahuite*, *P. chiapensis* and *P. strobus*), and the clade of two *L. decipiens* isolates (*P. ponderosa*) and three *L. baculiferum* isolates (*P. ponderosa* and *P. montezumae*). *Lophodermium* species on intermediately related hosts (different subsection, same section) included *L. indianum* (*P. greggii* and *P. hartwegii*) and *L. australe* (*P. pseudostrobus* and *P. palustris*). *Lophodermium* species from more distantly related pine hosts include *L. pinastri* (*P. ponderosa* and *P. sylvestris*) and *L. baculiferum* 2 (*P. contorta*), which did not group with the other three *L. baculiferum* isolates from *P. ponderosa* and *P. montezumae*.

DISCUSSION

Generic segregation and morphological characters.—When Darker (1967) erected the genus *Meloderma*, he pointed out similarities in ascus shape and in the position of the developing ascocarp in relation to the host tissue between *Meloderma desmazieresii* and *Lophodermium* species from pines. More recent studies have described similarities in ascocarp development related to the internal differentiation of the upper wall of unopened ascocarps between *M. desmazieresii* and *Lophodermium* from pines (Johnston 1988), anamorphs (Minter 1980) and ascospore discharge mechanisms (Minter and Cannon 1984). Both *Meloderma* and *Elytroderma* have been segregated from *Lophodermium* species mainly based on ascospore morphology. Species of *Meloderma* have short, cylindrical ascospores versus the filiform ascospores in *Lophodermium*. *Elytroderma* is distinguished by its large, clavate, 1-septate ascospores.

In the ITS results reported here, *Elytroderma deformans* from *P. ponderosa* and two strains of *Meloderma desmazieresii* from *P. ayacahuite* and *P. strobus* occurred in the *Lophodermium* pine clade but in distinct parts of the tree. If this placement is not a result of human error (see below), then it suggests that ascus shape and position of the developing ascocarp should be weighted more heavily than ascospore shape in the delineation of *Lophodermium* because *Elytroderma* and *Meloderma* have similar ascocarp development patterns. Characters related to the patterns of development of the sterile tissues of the ascocarps have proven to be more useful than spore shape in defining other groups within the Rhytismataceae (Johnston 1988, 1989). The taxonomic significance of these characters remains uncertain because ascocarp development is poorly understood in most species. Furthermore, different *Lophodermium* species that share closely related hosts may have similar ascocarp developmental patterns, particularly if the anatomy and disposition of the available tissues in the hosts constrain ascocarp development. However, the possibility that ascocarp development is a reliable criterion for the delimitation of genera comprising natural groups within the Rhytismataceae should stimulate further ontogenetic studies.

The occurrence of both *Elytroderma* and *Meloderma* in the *Lophodermium* pine clade further suggests that ascospore shape, such as filiform versus clavate or septate, is a reliable character for species, but not generic, delimitation. Studies of other ascomycetes have reported convergence in ascospore and conidium shape (e.g., Gaudet et al 1989, Wingfield et al 1994). The inconsistent placement of the two *M. desmazieresii* sequences might be the result of parallel modifications in ascospore shape (from filiform to cylindrical). If so, *M. desmazieresii* might be polytypic and hitherto undetected differences might separate these two strains into separate species. This is supported by the geographical separation of the strains; the CBS isolate of *M. desmazieresii* was obtained from *P. ayachahuite* at a pinetum in Texcoco, central Mexico, and the Deckert et al isolate was obtained from *P. strobus* in eastern Canada. If, as the ITS results suggest, ascospore shape is not a reliable character for genus-level differences, *M. desmazieresii* might not be monotypic and actually represent *Lophodermium* species with reduced ascospores.

An alternate explanation is that the *Elytroderma* and *Meloderma* sequences used in this study are misidentified *Lophodermium*. If so, the presumed *Elytroderma deformans* CBS181.68 sequence occurs on a solitary branch within the *Lophodermium* clade and does not match any *Lophodermium* species yet sequenced. The CBS *Meloderma* isolate from *P. ayacahuite* in central Mexico has a high sequence similarity with *L. nitens* strains throughout North America (Deckert et al 2002; this study, FIGS. 1 and 3). Although the *Meloderma* strain from Deckert et al was different from our sequence, it still occurred in the *Lophodermium* pine clade, in a sister relationship to *Lophodermium* sp. 1. Given the disagreement between the two *Meloderma* sequences and the limitation of having only a single *Elytroderma* sequence, the possibility that the *Elytroderma* and at least one of the two *Meloderma* isolates might have been contaminated by *Lophodermium*, either during isolation or subsequent handling, must be considered. We have no details regarding the care used in the isolation of the CBS cultures, but the *Meloderma* from *P. strobus* was isolated after positive identification of the ascocarp and under careful observation of the ascospores (Deckert

et al 2002). Small-subunit sequences based on the two CBS isolates in question have been included in a previous study as *Elytroderma* and *Meloderma* (Gernandt et al 2001). The uncertain identities of these cultures suggest caution when relying on cultures and sequences from third parties, particularly when these cultures are sterile and their identity cannot be rechecked. Despite these uncertainties, we hypothesize that *Meloderma* and *Elytroderma* are members of *Lophodermium*, although this interpretation deserves further study.

Species delineation.—Morphological characters traditionally used for species delimitation showed different degrees of phylogenetic value (TABLE III). Most characters were homoplastic with respect to the molecular phylogenetic reconstruction. The depth of embedding of the ascocarp, a key character for distinguishing pine inhabiting *Lophodermium* species (Minter 1981), showed much plasticity on the ITS tree (FIG. 1). There might be multiple sources of incongruence between the phylogenetic hypotheses and the morphological characters used for species delimitation. One possibility is that closely related species or even the same species can colonize hosts with different needle anatomies, resulting in differences in the location of the ascocarp in relation to the number of pine cell layers in the needle. This could be the case for the clade that comprises *L. indianum* and two undescribed species of *Lophodermium* (sp. 1 and sp. 2). *Pinus hartwegii* and *P. greggii* have 2–3 layers of hypodermal cells, while *P. ayacahuite* has only one (Farjon and Styles 1997). This anatomical difference limits the sites available for ascocarp insertion in *P. ayacahuite* and similar soft pines and thus constrains the potential morphological characters related to the embedding of the ascocarp.

Most *Lophodermium* taxa with unique combinations of morphological characters also have unique molecular sequences. The five putative novel species from Mexico have unique ITS sequences. Only *Leptostroma* sp. from *P. ayacahuite* has an identical nucleotide sequence to that of *Lophodermium* sp. 4 from *P. chiapensis*, suggesting that they are conspecific. Nevertheless, some morphologically different species have identical or almost-identical sequences, while molecular variations are comparable to interspecific differences for *L. pinastri* (5.6%) and *L. baculiferum* (as high as 7.6%). These exceptions can be explained in part by dependence of morphological characters used for species delimitation, such as depth of embedding of the ascocarp, on the anatomy of the hosts and partly by reproductive isolation preceding morphological divergence. In the former instance, different species of *Lophodermium* in anatomically similar hosts may be morphologically similar and the same species in anatomically different hosts may be morphologically different. In the latter instance, ecological differences independent from host anatomy can accompany morphological differences. Minter and Millar (1980) studied the biology and ecology of a group of *Lophodermium* species inhabiting *Pinus sylvestris*: *L. seditiosum*, *L. pinastri* and *L. conigenum*. They reported marked differences in their habitat use, fructification and sporulation periods, as well as in their pathogenicity. The phylogenetic separation of these species in the ITS tree corroborates their species status and suggests that such ecological differences might be used to supplement morphological evidence in delineating species.

Host specificity.—Some *Lophodermium* species represented by more than one isolate had noticeably divergent ITS sequences (FIG. 1), with higher divergence found in isolates from different host species. This was the case for the *Lophodermium baculiferum* species complex, including the anamorph, *Leptostroma decipiens*. ITS intraspecific nucleotide divergence in this complex correlated with the phylogenetic relationships of the hosts. All individuals isolated from *Pinus ponderosa* and originating from a small geographic range in Oregon had identical sequences. Isolates of the same morphospecies taken from *P. montezumae* across a relatively extensive geographic range in Mexico also had little nucleotide variation (0.24%). *Lophodermium baculiferum* isolates from different pine species had higher nucleotide divergence. Isolates from hosts in the same subsection had a mean nucleotide divergence of 4.2%, whereas isolates from different subsections had a mean nucleotide divergence of 6.1%. *Pinus ponderosa* and *P. montezumae* are closely related members of subsection *Ponderosae* (Price et al 1998), and their *L. baculiferum* isolates have intermediate levels of divergence, despite the geographical isolation of their hosts. In contrast, *L. baculiferum* isolated from *P. contortae* in subsection *Contortae* (Price et al 1998) displayed higher nucleotide divergence, despite the overlapping geographic distributions of *P. ponderosa* and *P. contorta* along the West Coast. The successive increase in nucleotide divergence for *L. baculiferum* isolates in relation to phylogenetic divergence in their hosts is consistent with host-mediated speciation.

Levels of intraspecific variation among *L. baculiferum* isolates were comparatively high (up to 6.1%). As in previous studies (Carbone and Kohn 1993, Morales et al 1993, Ko et al 1997), divergence among isolates from different hosts may be high enough to justify splitting them into separate species (or sub-

species). This is consistent with the idea that speciation has occurred in the fungi in parallel with host speciation. Mating studies and reciprocal inoculations might be appropriate to investigate whether molecular divergence and host specificity justify the segregation of additional species in this group. Molecular and morphological studies of additional *L. baculiferum* isolates would be useful to decide whether the current taxonomy is appropriate.

Although some *Lophodermium* species, such as *L. baculiferum*, may be undergoing host-mediated speciation, others show evidence of host switching. The *L. indianum*, *Lophodermium* sp. 1, *Meloderma* MD3 and *Lophodermium* sp. 2 clade has low sequence divergence, despite having a host distribution that includes three subsections and both subgenera of *Pinus* (FIGS. 1 and 3). Species of *L. pinastri* occur on two different sections of hard pines, although high sequence divergence among the strains could be explained by inadequate taxonomic sampling or by ancient host tracking followed by extinction on more closely related hosts. The *L. agathidis* isolates collected exclusively from New Zealand but from different hosts also offer an example of a generalist species capable of host switching. *Lophodermium agathidis* has a wide host and geographic range through tropical and subtropical regions (Johnston 2001). Despite the phylogenetic distance of the hosts (a conifer and an angiosperm), ITS nucleotide divergence between the two individuals of *L. agathidis* (2.9%) were within the range of other conspecifics in this study.

Cryptic speciation may be occurring in similar taxa on different hosts, and therefore differences in habitat and behavior may indicate genetic discontinuities. Chemical and morphological characters of the pines hosts, such as needle anatomy and secondary metabolites, may influence fungal colonization. There is evidence that specific host-fungus relationships occur between *Leptostroma* anamorphs and their hosts (Sieber-Canavesi et al 1991), and this is reflected by the fact that host preference and substrate often provide a guide for identifying *Lophodermium* species. Similar needle anatomy and secondary compounds might permit host switching and colonization, especially between closely related pines in sympatry (Espinosa-García et al 1993). Horizontal transfer in *Lophodermium* by broadcasting of ascospores may increase gene flow and preclude specialization in some species, but host tracking seems to be occurring in others.

Evolution of pathogenicity.—The phylogenetic results provide evidence of at least one instance of pathogenicity evolving from endophytism within the studied pine-inhabiting fungi. *Lophodermium seditiosum*, which occurs in a derived part of the pine clade, attacks two- and three-needle pines, causing needle death during their first growing season and sometimes killing seedlings (Hansen and Lewis 1997). This species has the widest host range within the genus *Pinus*, reported from more than 40 different host species. Evidence for endophytism in several *Lophodermium* species included in this study has been reported (Minter et al 1978, Minter and Millar 1980, Suske and Acker 1989, Wilson et al 1994).

Elytroderma deformans and *Meloderma desmazieresii* are considered serious pathogens of several pine species. *Elytroderma deformans* causes ''witches broom'' and severe defoliation (Weir 1916, Childs et al 1971), and *Meloderma desmazieresii* causes a needle blight (Hansen and Lewis 1997). If any of the three sequences representing these two genera are confirmed to be correct, then their position nested within endophytic species of *Lophodermium* further would support the idea that pathogenicity has evolved from endophytism in this group. Furthermore, because of their separation in the ITS tree from *L. seditiosum*, confirmation of either *Meloderma* sequence would reveal a second derivation of pathogenism from endophytism. Other studies also have shown evidence of multiple origins of pathogenic fungi from nonpathogenic relatives (Bowman and Taylor 1993, Bowman et al 1996).

Indirect evidence supports the view that most *Lophodermium* species are endophytes; their fruiting bodies frequently are associated with recently senescent or dead tissue, and viable infections commonly are found in living tissues of asymptomatic hosts (Carroll and Carroll 1978, Hata and Futai 1993, 1996, Stone et al 2000). Other representatives of Rhytismataceae, including *Colpoma quercinum* and *Tryblidiopsis pinastri*, also are considered endophytes. They colonize living branches and play an important role in natural decay and shedding of dead branches (Livsey 1993, Livsey and Minter 1994, Kowalski and Kehr 1996). Delaying reproduction until needle senescence might represent a less adaptive strategy compared to pathogenic fungi that aggressively colonize host tissue after infection, allowing them to reproduce within a year after infection, but severe needle pathogens deplete substrate availability for subsequent infections because only current-season needles are available commonly for colonization (Sinclair et al 1987). In contrast, endophytes can colonize a wider range of needle age classes and therefore their populations should not be as vulnerable as pathogen populations in years when environmental conditions are unfavorable for ascospore dispersal and colonization. The ability to occupy different, often longer-lived host substrates may permit pathogen-

ic fungi to ameliorate such environmental variability; *Lophodermium seditiosum* can be found fruiting on pine cones, and *E. deformans* also invades shoots and twigs.

The molecular approach used in this study helped to interpret relationships between the genus *Lophodermium* and selected species in the family Rhytismataceae by providing phylogenetic resolution that we were unable to obtain with morphological characters traditionally used in taxonomic studies, but many questions remain. A better understanding of the biological and evolutionary relationships among *Lophodermium* and related genera might be guided by considering host associations, which are useful in studying cospeciation, host switching and the evolution of endophytism and pathogenism. Ideally, future studies of such patterns will involve formal comparison of robust, fully resolved phylogenies for plant hosts and Rhytismataceae parasites, backed by improved species delineation and clearer understanding of the ecological role of each species. Inclusion of other members of *Lophodermium* and species of allied genera also will be required before a decision can be made on the most appropriate way to classify this diverse genus.

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