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ORIGINAL ARTICLE

Lophodermium pini-mugonis sp. nov. on needles of *Pinus mugo* from the Alps based on morphological and molecular data

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Abstract *Lophodermium pini-mugonis*, collected on needles of *Pinus mugo* from German Alps, is described as a species new to science. It is characterized by subcuticular ascomata with a wrinkled surface and a somewhat untidy outline, a complex structure of lip cells, and ellipsoidal conidia. An analysis of the internal transcribed spaces of rDNA showed that *Lophodermium pini-mugonis* is, sister to *Lophodermium autumnale* and distantly related to other *Lophodermium* species on pines. The hypothesis of cospeciation of *Lophodermium* species with members of the Pinaceae is discussed.

Keywords Cospeciation · ITS · Rhytismatales · Taxonomy

Introduction

Species of *Lophodermium* Chev. on *Pinus* spp. are familiar to forest pathologists in many parts of the world (Minter 1981). Some *Lophodermium* species on needles of pines are pathogenic fungi, such as *L. seditiosum* Minter, Staley & Millar, causing serious needle-cast of pines in North America, Europe, and Northeast China (Minter 1981; Minter et al. 1978; He et al. 1985). The longstanding taxonomical problems of *Lophodermium* species on pines were resolved by Minter (Minter et al. 1978; Minter and Millar 1980; Minter 1981), who contributed a monograph on species of *Lophodermium* on pines worldwide, in which 15 species are recognized. After Minter's study, many new taxa of *Lophodermium* on needles of *Pinus* spp. have been proposed (He et al. 1986; Lin and Tang 1988; Hou and Liu 1990; Liu and Qiu 1995; Hou et al. 1996; Lin et al. 1993, 1995a, b, Sokolski et al. 2004; Hou and Piepenbring 2006). In the present paper, we describe a further species of *Lophodermium* on *Pinus mugo* Turra from Oberstdorf in the German Alps based on morphological and molecular data.

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Materials and methods

Morphological studies

Sections of different thickness of ascomata were made by hand using a razor blade. Microscopic preparations were made in water, Melzer's reagent, 5% (w/v) KOH, or 0.1% (w/v) cotton blue in lactic acid. For observation of ascomatal outlines in vertical section, sections were mounted in lactic acid or cotton blue with pretreatment in water. Gelatinous sheaths surrounding ascospores and paraphyses were observed in water or cotton blue. Ascospore contents were drawn based on observations in water mounts. Measurements were made from 20 ascospores and asci for each specimen using material mounted in 5% KOH or Melzer's reagent.

Molecular methods

Three new sequences were obtained from ascomata of *Lophodermium pini-mugonis*, *Lophodermium autumnale*, and *Lophodermium nitens* (Table 1). Ascomata with host

Table 1 Species used in the molecular analysis and their respective GenBank accession numbers

Species	Genera of host	Voucher specimen	GenBank accession No.
Elytroderma deformans (Weir) Darker	*Pinus*		AF203469
Lirula macrospore (R. Hartig) Darker	*Picea*		AF462440
Lophodermium pini-mugonis	*Pinus*	Hou et al. 568 (M)	
Lophodermium autumnale Darker	*Abies*	C. L. Hou 475c (AAUF)	
Lophodermium baculiferum Mayr	*Pinus*		AY100655
Lophodermium conigenum (Brunaud) Hilitzer	*Pinus*		AF473559
Lophodermium conigenum (Brunaud) Hilitzer	*Pinus*		AY422489
Lophodermium indianum Suj. Singh & Minter	*Pinus*		AY100642
Lophodermium molitoris Minter	*Pinus*		AY247752
Lophodermium nitens Darker	*Pinus*	C. L. Hou 486b (AAUF)	
Lophodermium piceae (Fuckel) Höhn.	*Abies*		AY775683
Lophodermium pinastri (Schrad.) Chev.	*Pinus*		AY100649
Lophodermium seditiosum Minter, Staley & Millar	*Pinus*		AY77570
Lophodermium australe Dearn.	*Pinus*		AY100647
Lophodermium macci Sokolski & Bérubé	*Pinus*		AF540561

tissue was crushed by shaking the samples for 3 min at 30 Hz (Mixer Mill MM 301; Retsch, Haan, Germany) in a 1.5-ml tube together with one tungsten carbide ball 3 mm in diameter. Total genomic DNA was extracted from ascomata using the PeqLab E.Z.N.A.® Fungal DNA kit following the manufacturer's protocol. The ITS rDNA region was amplified with PCR using the primers ITS1f and ITS4 (White et al. 1990, Gardes and Bruns 1993). PCR was performed in 25-μl reactions, plate 1.0 μl, primer1/2 (25 μM each)1.0 μl, 2 × MasterMix 12.5 μl, add ddH_2O to 25 μl, under the following parameters: 94°C for 40 s, 45°C for 55 s, 72°C for 1 min, for a total of 30 cycles, and a final extension step at 72°C for 7 min. The PCR products were sent to Invitrogen Biotechnology (Beijing, China) for purifying, sequencing, and editing. The other ITS rDNA sequences included in this study have been downloaded from GenBank (Table 1).

Phylogenetic analyses

DNA sequences were aligned with the program Clustal X (Thompson et al. 1997). Further manual alignment was done in Se-Al v.2.03a (Rambaut 2000). The following phylogenetic analyses were run on Macintosh computers. Sequences of the ITS rDNA were analyzed for all 15 taxa using maximum parsimony performed in PAUP* 4.0b10 (Swofford et al. 1996). Maximum parsimony analysis was conducted using heuristic searches with 1,000 replicates of random-addition sequence, tree bisection reconnection (TBR) branch swapping, and no maxtree limit. All characters were equally weighted and unordered. Gaps were treated as missing data to minimize homology assumptions.

Results

The morphology indicates that the specimen collected in the Bavarian Alps represents a new species of *Lophodermium* (Figs. 1 and 2). The length of the ITS alignment was 540 bp, including 123 variable positions, 118 of which were phylogenetically informative. The 15 ITS region sequences ranged in length from 424 to 540 bp, due mainly to the presence of 15, 18, 72, 79 bp insertions in species of *Lirula macrospora*, *Lophodermium piceae, Lophodermium pini-mugonis*, and *Lophodermium autumnale*, respectively. The maximum parsimony analysis of sequences resulted in an equally most parsimonious tree that had a length (TL) of 498 steps, consistency index (CI) of 0.7048, retention index (RI) of 0.5727, and homoplasy index (HI) of 0.2952. The tree topology of maximum parsimony analysis was shown as Fig. 3. After deletion of the insertions, a maximum parsimony analysis was executed with 431 bp (TL=417, CI=0.6475, RI=0.5702, HI=0.3525), 116 characters were phylogenetically informative. The tree topology of maximum parsimony analysis was the same as the one with insertions (Fig. 3).

Lophodermium pini-mugonis C.L. Hou & M. Piepenbr., sp. nov. Figs. 1 and 3.

Ascomata 450–500×800–1,200(–1,400) μm, subcuticularia, elliptica; paraphyses filiformes, apicibus leniter curvatis; asci 90–135×9–14 μm, cylindrici; ascosporae 60–90×1.5–2 μm, cylindricae, filiformes. Conidiomata abaxialia, 80–150×100–250 μm, 30–40 μm alta; cellulae conidiogenae 6–15×2–3 μm; conidia ellipsoidea, 2–3.5×0.8–1.2 μm.

Holotype On needle of *Pinus mugo* (Pinaceae), Oberstdorf, Bavarian Alps, Germany, alt. ca. 1,400 m, 14 Oct. 2003, C. L. Hou, R. Kirschner and M. Piepenbring (M).

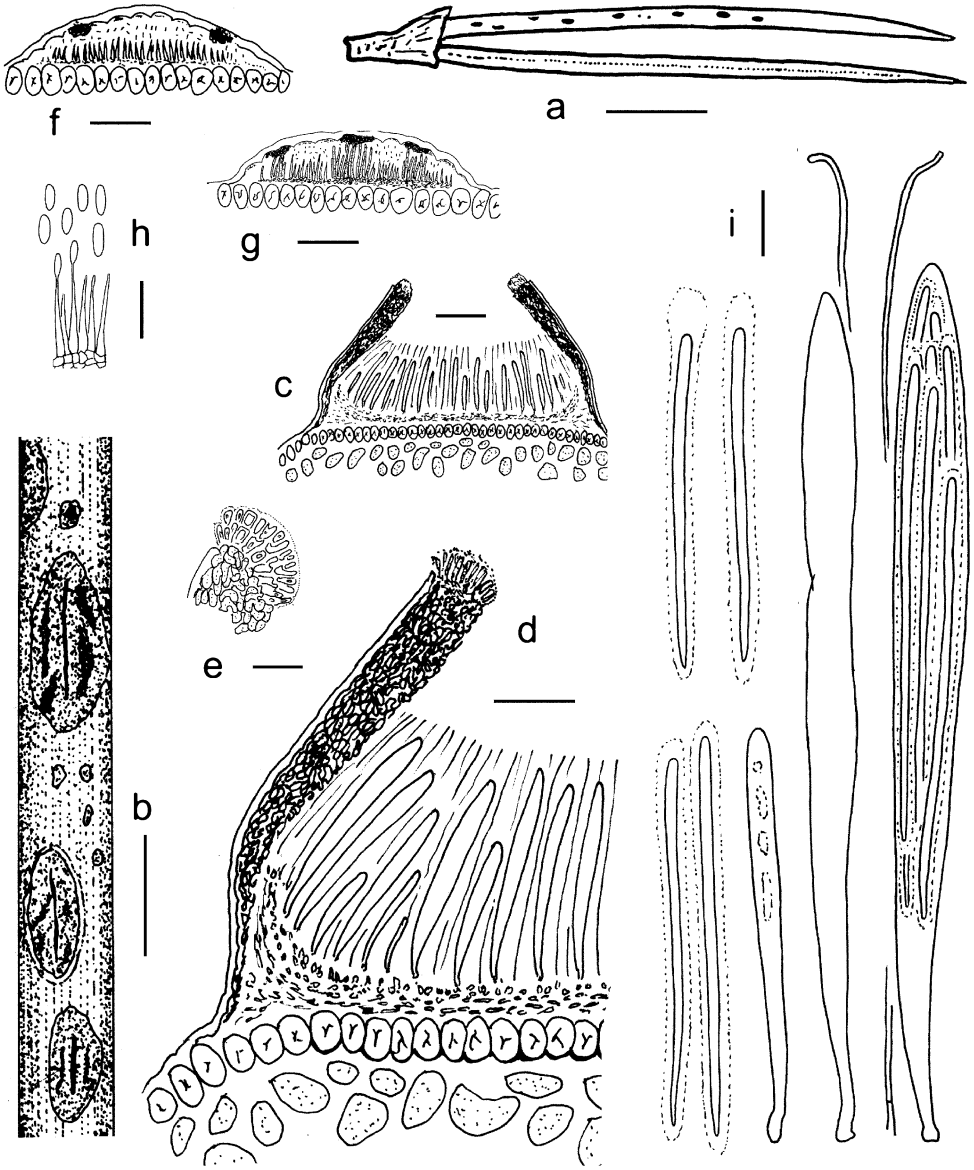

Fig. 1 *Lophodermium pini-mugonis* on *Pinus mugo*. a. Needles bearing ascomata. Scale bar=1 cm. b. Ascomata observed under a dissecting microscope. Scale bar=1 mm. c. Ascoma in vertical section. Scale bar=100 μm. d. Detailed structure of an ascoma in vertical section. Scale bar=50 μm. Fig. e. Detailed structure of lip cells. f-g. Conidiomata in vertical section. Scale bar=20 μm. h. Conidiogenous cells and conidia. Scale bar=5 μm. i. Paraphyses, a young ascus, a mature ascus with ascospores, an ascus after the liberation of the ascospores, and discharged ascospores with gelatinous sheaths. Scale bar=10 μm

Etymology The name of the species refers to the host.

Ascomata on the abaxial surfaces of needles. In surface view, ascomata 400–500×800–1,200(–1,400) μm, elliptical, grayish black, shiny, wrinkled, with somewhat irregular outline, the narrow central part of ascomata near the opening raising above the substrate surface, opening by a single longitudinal split, lips hyaline. In median vertical section, ascomata subcuticular. Covering stroma up to 45–60 μm thick near the centre of ascomata, becoming thinner towards the edges, consisting of an outer layer of host cuticle and an inner layer of thick-walled, dark brown textura epidermoidea to textura angularis. Lip cells 10–22×2–3 μm, hyaline, branched and septate, embedded in a gelatinous matrix. Basal stroma absent, but sometimes the lower side of epidermal cells under the hymenium slightly tinted. Subhymenium 10–15 μm thick, hyaline, composed of hyphae and textura intricata. Paraphyses, 100–150 μm long, filiform, septate or not, <1 μm wide, apex simple, slightly curved. Asci ripening sequentially, 9–14×90–135 μm, clavate-cylindric, with a conspicuous stalk up to 30 μm long, thin-walled, J-, without circumapical thickening, discharging spores through a small apical hole, 8-spored. Ascospores arranged in a fascicle, 60–90×1.5–2 μm, filiform,

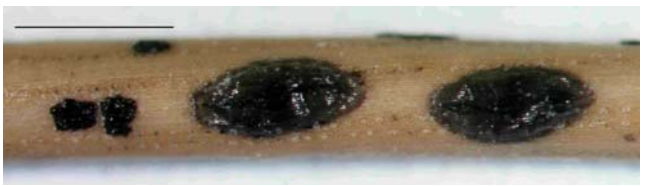

Fig. 2 Photograph of *Lophodermium pini-mugonis*. Ascomata and conidiomata on a needle of *Pinus mugo*. Scale bar=1 mm

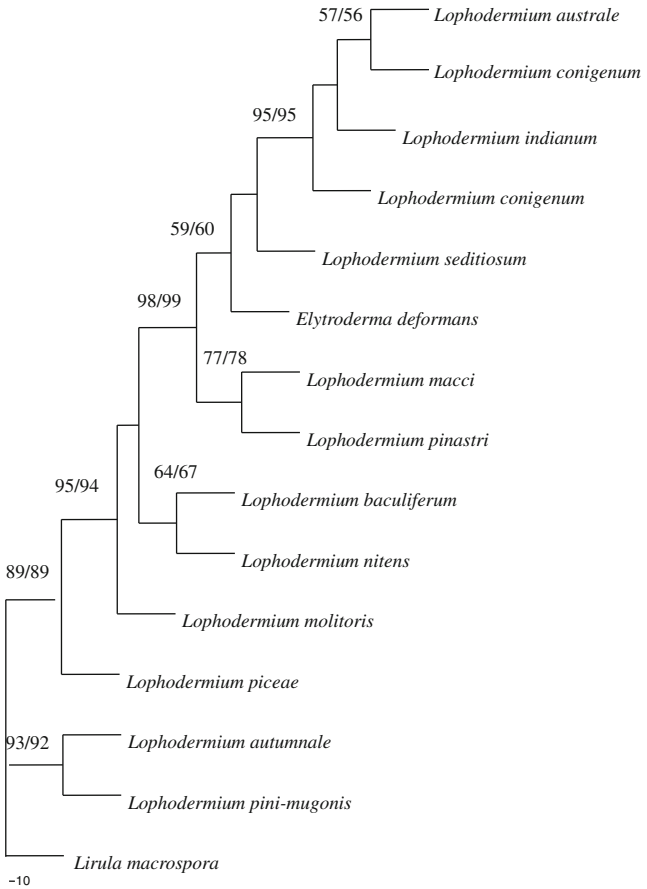

Fig. 3 Phylogenetic hypothesis derived from maximum parsimony analysis of the rDNA internal transcribed spacers and 5.8 S rDNA sequences of *Lophodermium pini-mugonis* and related species, using *Lirula macrospora* as an outgroup. Bootstrap values of more than 50 % from 1000 replications are shown on the respective branches. The bootstrap values under the bias were from the maximum parsimony analysis of ITS sequence without insertions

slightly tapering towards the base, hyaline, aseptate, with gelatinous sheaths 1–2 μm thick, sometimes with a gelatinous cap.

Conidiomata on the abaxial side. In surface of view conidiomata 80–150×100–250 μm, brown to black, shiny, elliptical or slightly irregular, opening by one to several small ostioles or by irregular splits. In vertical section, conidiomata subcuticular, 30–40 μm deep, upper wall composed of host cuticle and unclear fungal cells, brown but dark brown near the opening. Basal wall 4–5 μm thick, composed of hyaline, angular cells. Sometimes a column of filiform, multi-septate, brown elements beneath ostioles, extending to the upper wall of the opening. Conidiogenous cells 6–15×2–3 μm, hyaline, tapering to the apex. Conidia hyaline, ellipsoidal, 2–3.5×0.8–1.2 μm. Zone line present only when adjacent to *L. conigenum* (Brunaud) Hilitzer.

Ecology Lophodermium pini-mugonis was found on needles attached to twigs.

Distribution This species is only known from the type collection.

Specimen studied for comparison Lophodermium autumnale Darker on *Abies* sp. China, Yunnan province, Lijiang Laojunshan, alt. ca. 3,600 m, 25 July 2007, C. L Hou 475c (AAUF).

Discussion

The depth of embedding of ascomata in the host tissue is very important for the identification of the species in the family of Rhytismataceae (Minter 1981; Cannon and Minter 1986; Johnston 2001; Hou 2004). *Lophodermium pini-mugonis* is characterized by the subcuticular ascomata. Five *Lophodermium* species are known on pines with subcuticular ascomata, i.e. *L. anhuiense* Y. R. Lin, *L. confluens* Y. R. Lin, C. L. Hou and W. F. Zheng, *L. kumaunicum* Minter and M. P. Sharma, *L. nitens* Darker, and *L. pini-sibiricae* C. L. Hou and S. Q. Liu. *L. confluens* differs from *L. pini-mugonis* by confluent ascomata and by occurring on living needles (Lin et al. 1995a, b). *L. kumaunicum* is easily recognized by its rather pointed ascomata with lower walls scalloped, concave in vertical section (Cannon and Minter 1986). *Lophodermium pini-mugonis* is more similar to *L. anhuiense*, *L. pini-sibiricae*, and *L. nitens*. However, all three species have ascomata with a tidy, smooth surface and a conspicuous black basal layer, lip cells are lacking or lip cells disappearing at maturity, with abundant black zone lines, and occur on haploxylon pines. *L. pini-mugonis* has a wrinkled and grayish black ascomatal surface and a conspicuous lips and lip cells. Black zone lines are present only when adjacent to another *Lophodermium* species, *L. conigenum.* Conidia are ellipsoidal rather than rod shaped, and *L. pini-mugonis* grows on diploxylon pines.

Three rhytismataceous species on *Abies* or *Picea*, *Lophodermium autumnale*, *L. piceae,* and *Lirula macrospore*, as well as one species *Lophodermium pini-mugonis* on *Pinus*, have insertions of variable in length within ITS1, which are not observed in the others on needles of *Pinus*. The significance of the insertion for these species is unclear.

The maximum parsimony analysis of ITS sequence of 15 species showed that *Lophodermium pini-mugonis* and *Lophodermium autumnale* formed a well-supported clade with a bootstrap value of 93%. In order to avoid the effects of the insertions on the tree topology, we executed a maximum parsimony analysis of ITS sequence of 15 species without insertions. The result showed that the tree topology of maximum parsimony analysis was the same as the one with the insertions but with a bootstrap value of

92%. Therefore, the large insertions are not the reason that two species were placed on adjacent branches. A close relationship is also evident by morphological features, wrinkled and grayish black surface of ascomata, similar structure of covering stroma, and similar shapes of asci and ascospores. The wrinkled, grayish black ascomatal surface of ascomata of *L. pini-mugonis* is reminiscent of *L. autumnale* Darker (Darker 1932). However, *L. autumnale* usually follows on nervisequent species on *Abies* spp, such as *Lirula nervisequia* (DC.) Darker, and has 0–3(–4) septate ascospores, conidiomata are unknown.

The ITS sequence analysis by Ortiz-García et al. (2003) showed that *Lophodermium* species from needles of pines formed a monophyletic sister group to *Lophodermium* species from more distant hosts from the southern hemisphere, but not to *L. piceae* from *Picea*. The partial LSU sequence analysis by Hou (2004) indicated that species of Rhytismatales on needles of *Picea* and *Abies* are distantly related to species on needles of other coniferous species, including Cupressaceae and Taxodiaceae. These findings are inconsistent with the hypothesis that speciation has occurred in the fungi in parallel with their host but show conspicuous host jumps. In this study, the ITS sequence analysis is in conflict with former studies, because *L. pini-mugonis* appears to be closely related to species on *Abies* and *Picea* rather than to species on *Pinus*. When additional DNA sequences for more species than exist at present become available, multigene analyses might be helpful for study of cospeciation in *Lophodermium* on needles of Pinaceae.

Mature ascomata of *Lophodermium* species on pines usually can be found in zones at lower altitudes from March to July. Those of *L. pini-mugonis* and *L. conigenum*, however, were found conspicuously late, in September/October, probably due to the colder climate in the Alps.

Lophodermium pini-mugonis is a rare species and its ascomata often grow together with *L. conigenum*.

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