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First report of *Myrothecium roridum* from a gymnosperm

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Abstract: Although *Myrothecium roridum* has been reported as a pathogen and an endophyte with a wide host range among Angiosperms, it has never before been reported from a gymnosperm host. Reports of this fungus are also much more common in Asia than in North America where *M. roridum* is infrequently found on introduced plants in the warmer, southernmost parts of the United States. Thus, it was surprising on three levels to isolate endophytic *M. roridum* from a North American native gymnosperm, *Pinus albicaulis*, at high elevation in Crater Lake National Park (CLNP) in Oregon. In PDA culture, its olive green conidia were 6-8 µm long (mean of 6.8) × 2 µm wide, and cylindrical with rounded ends. Conidia formed dark green to black masses on sessile sporodochia in concentric zones. The sequence identity of the ITS1, ITS2 and the 5.8S rRNA of CLNP isolate RV10#75 (GenBank accession

GQ152603) with deposited sequences of *M. roridum* in the National Center for Biotechnology Information database was 99.82%. The conservation implications of this finding are discussed.

Key words: conifer, endophyte, gymnosperm, Hypocreales, pine, *Pinus*

Introduction: *Myrothecium roridum* Tode ex Fr. has been reported as a pathogen causing "serious losses in cotton (India), coffee (Guatemala), *Antirrhinum* (U.S.A.), and *Viola* (Canada)" (Fitton and Holiday 1970) via leaf spots, cankers and a variety of rots (Farr *et al.* 2009). However, species of *Myrothecium* can also be endophytes (Tejesvi *et al.* 2006), and endophytic isolates of *M. roridum* and *Myrothecium* sp. can produce biologically active trichothecenes that can potentially work against pathogens such as the rice blast fungus, *Pyricularia oryzae* Cavara (Wang *et al.* 2007), and *Sclerotinia sclerotiorum* (Lib.) de Bary (Xie *et al.* 2008). *Myrothecium roridum* has been described as plurivorous (Fitton and Holiday 1970), and although a total of 263 fungus-host records include 119 host genera, all reported hosts are Angiosperms (Farr *et al.* 2009). Another species of *Myrothecium* that was only reported as *M. cf. indicum*, was found in phloem of *Pinus sylvestris* L. infested by *Tomicus piniperda* L. (Jankowiak and Kurek 2006). However, *M. roridum* itself has never been reported from a gymnosperm (Farr *et al.* 2009; Fitton and Holliday 1970; Hansen and Lewis 1997; Tulloch 1972).

Myrothecium roridum is thought to be "possibly more serious in warmer regions" (Fitton and Holliday 1970). In line with this, an optimum temperature for its conidial germination has been reported as 28°C (Fitton and Holliday 1970). Furthermore, records of *M. roridum* in the United States reflect this tendency. Of 52 records, 42 were from the following southern states: FL, CA, NC, MS, TX, LA and GA (Farr *et al.* 2009). *Myrothecium roridum* may also be more common in Asia in that there are 70 records from India, Malaysia, and Papua New Guinea alone (Farr *et al.* 2009).

Whitebark pine, or *Pinus albicaulis* Engelm. is a high-elevation species in western North America that is threatened by white pine blister rust (Schoettle and Sniezko 2007). Endophytes can potentially mediate resistance to white pine blister rust (Ganley *et al.* 2008), and as part of a whitebark pine restoration project at Crater Lake National Park in Oregon, endophytes were isolated from needles of *P. albicaulis*. Two isolates of *M. roridum* were obtained from different trees that represent the first world record of this fungus from a gymnosperm.

Materials and Methods:

Isolation and morphological identification

A total of ten trees were sampled in Crater Lake National Park in the following manner: ten healthy needles from each of eight trees, 20 from a ninth tree, and 30 from a tenth tree. The 130 needles were surface-sterilized (Ganley and Newcombe 2006) and plated on PDA. Endophytes were isolated as they emerged from needles. Twenty five conidia of *M. roridum* CLNP RV10#75 were measured for width and length with 1000x magnification of a Zeiss Axioskop 2 microscope (Carl Zeiss, Inc., New York, USA). We tested conidial germination at three temperatures: 12, 19, and 28 degrees C. Percent germination was calculated from 300 conidial observations at each temperature. The culture of *M. roridum* CLNP RV10#75 is kept at -80°C in the culture collection of the Forest Pathology and Plant Symbiosis Laboratory of the University of Idaho, Moscow ID 83844-1133, USA.

DNA extraction and ITS1-5.8S-ITS2 analysis
One hundred and sixty mg of 7-day pure CLNP RV10#75 mycelium was scraped for a total genomic DNA isolation using the DNeasy® Plant Mini extraction kit of QIAGEN (QIAGEN Inc., California, USA). The methods of DNA extraction, ITS1-5.8S-ITS2 amplification and sequencing were performed based on Shipunov *et al*. (2008). Both forward and reverse sequences were assembled, aligned and manually edited with the program Sequencher 4.5 (Gene Codes, Ann Arbor, Michigan, USA). The 551 bp of partial 18S-ITS1-5.8s-ITS2-partial 28S sequence of the isolate CLNP RV10#75 was submitted to GenBank as accession number GQ152603.

Results and Discussion: As an endophyte in needles of *Pinus albicaulis*, CLNP RV 10#75 was one of two isolates of *M. roridum* from a total of 80 isolates that were selected for further characterization including sequencing. The other isolate that proved to be *M. roridum*, CLNP RV 2 #65, was virtually identical to CLNP RV 10#75. These two isolates of *M. roridum* were from needles of different trees. All morphological characteristics of the CLNP isolate RV10#75 were the same as the description of *Myrothecium roridum* (Ellis 1971, Tulloch 1972). On PDA, the white colony of *M. roridum* CLNP isolate RV10#75 slowly grew to 19 mm in diameter at 7 days, 22 mm at 10 days and 34 mm at 14 days (Fig. 1) with a brown color observed on the reverse (Fig. 2). After culturing for 10 days, the CLNP isolate RV10#75 produced sessile sporodochia with a white mycelial base of packed conidiophores, and with a green color from conidia on top. Later on the green conidia became dark olivaceous green to black (Fig. 3), and these formed slimy masses on the sporodochia. Figs. 4 and 5 show the young hyphal tip growth from slide culture, and hyphal roping in older mycelium, respectively. Microscopic examination of asexual structures showed no setae at the base of sporodochia. Conidia were produced on phialidic conidiogenous cells. The size of cylindrical conidia with rounded ends was generally 6-8 µm (mean of 6.8) × 2 µm with considerable variation in pigmentation (Fig. 6). *Myrothecium cf. indicum* (now regarded as a synonym of *M. leucotrichum* (Peck) M.C. Tulloch – Tulloch 1972) was previously reported from a gymnosperm (Jankowiak and Kurek 2006), but *M. leucotrichum* has longer conidia (8-11 × 1.5-2 µm) and sporodochia with setae (Ellis 1971; Tulloch 1972). The results of ITS sequence-based BLAST search strongly supported the conclusion that we had already reached on the basis of morphology; the CLNP isolate RV10#75 is *M. roridum* as it showed 99.82% sequence identity (i.e., 550/551 base positions) with *M. roridum* ATCC strain '52801' (GenBank AY254155) and with endophytic *M. roridum* from China (GenBank FJ231214). Notably, unmatched base pairs of the CLNP isolate RV10#75 ITS sequence were at different positions from these two most closely related strains of *M. roridum* in GenBank implying that the difference is not from mis-incorporation of nucleotide during PCR reaction. The ITS sequence alignment of the CLNP isolate RV10#75 and *M. leucotrichum* (GenBank AJ30200) was also performed, given the association of the latter with a gymnosperm. A result of pair-wise alignment showed only 95.83% identity. Thus, CLNP isolate RV10#75 was identified as *M. roridum*, and not *M. leucotrichum*, both on the basis of morphology and ITS sequences.

Conidial germination of *M. roridum* CLNP RV10#75 was minimal at 12°C and close to 100% at both 19°C and 28°C (Fig. 7). This broad optimum for conidial germination may be distinct from that of tropical isolates of *M. roridum* (Fitton and Holliday 1970). More research will be needed to see if *M. roridum* CLNP RV10#75 is as locally adapted to environmental conditions at CLNP as the other endophytes of *Pinus albicaulis* that we have obtained there.

Once it was clear that we had isolated *M. roridum* from a gymnosperm (i.e., *P. albicaulis* at CLNP) we not only checked for previous associations with gymnosperms in Farr *et al.* (2009), Fitton and Holliday (1970), and Hansen and Lewis (1997), but we also checked recent, sequence-based studies of endophyte diversity in gymnosperms (Arnold *et al.* 2007; Ganley and Newcombe 2006). We found no evidence that *M. roridum* has ever been found as a pathogen or as an endophyte in a gymnosperm.

We found *M. roridum* in two of ten trees as a result of quite minimal sampling; additional sampling will determine its abundance at CLNP. The implications for management in this much-visited national park appear on the surface to be contingent upon the origin of the *M. roridum* population there. If an isolated but natural population at CLNP, *M. roridum* would potentially be as protected as all other native organisms in the park. If, on the other hand, *M. roridum* proves to be an alien introduction at CLNP, precautions might be needed to avoid its dispersal to other native plant communities and ecosystems in the region.

Unfortunately, origins, or native ranges, of fungi such as *M. roridum*, are difficult to prove. One way to possibly explain the occurrence of a fungus with a primarily Asian distribution in whitebark pine at CLNP is to consider the Pleistocene migration of whitebark pine itself from Asia into North America via Beringia (Liston *et al.* 2007). However, if its origin remains unknown, management options may hinge on whether or not *M. roridum* mediates resistance to white pine blister rust (Ganley *et al.* 2008), given that the latter is the main threat to whitebark pine communities and the main obstacle in whitebark pine restoration (Schoettle and Sniezko 2007). Since *M. roridum* produces two trichothecenes that significantly affect the rice blast pathogen (Wang *et al.* 2007) and another *Myrothecium* sp. produces inhibitors that are effective against *S. sclerotiorum* (Xie *et al.* 2008), it is at least possible that *M. roridum* will aid whitebark pine restoration at CLNP by acting against white pine blister rust. Further research is necessary.

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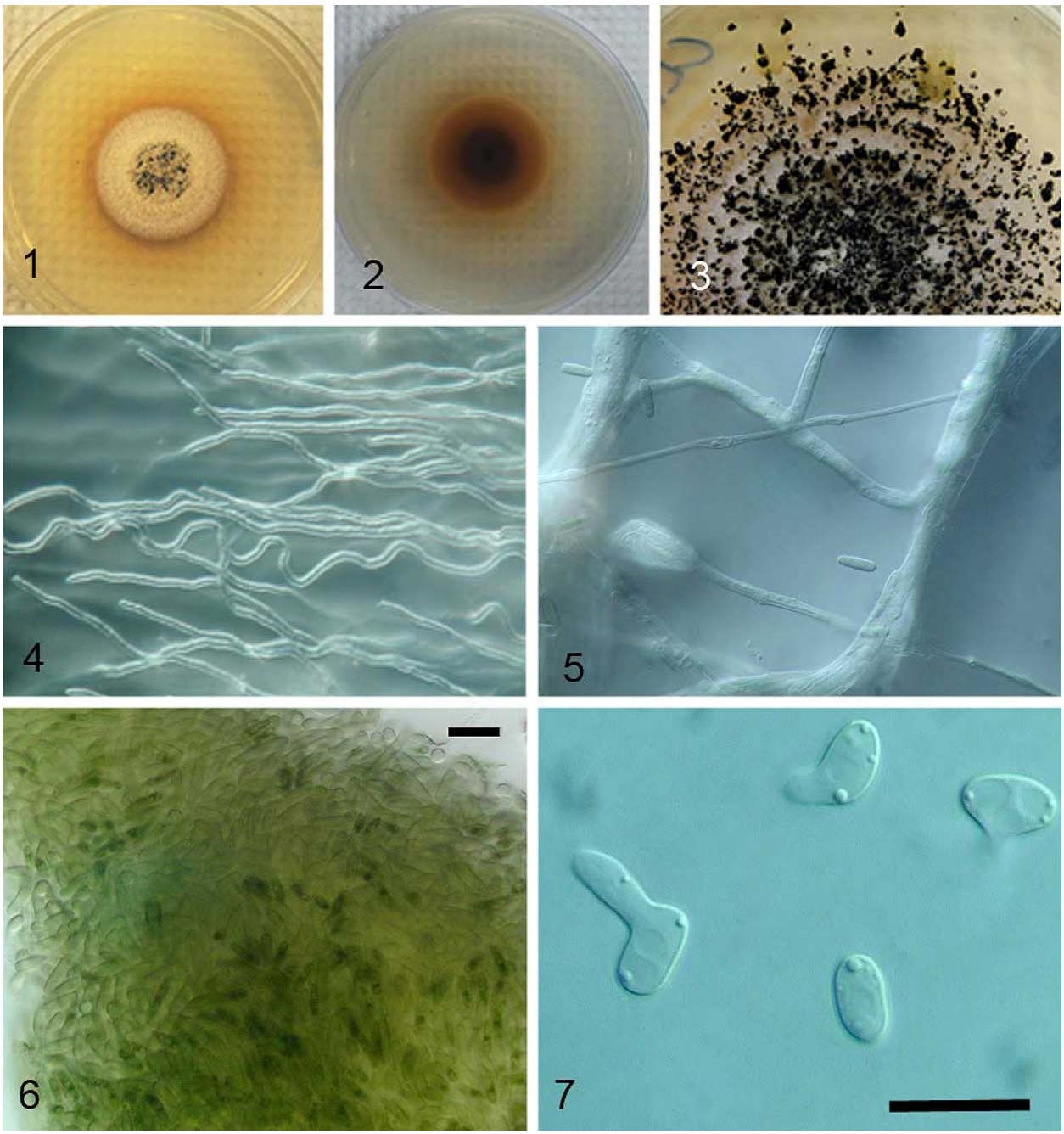

Figs 1-3. The culture of *Myrothecium roridum* CLNP isolate RV10#75 on PDA. Fig.1. 14-day-old culture. Fig. 2. The brown color of the colony reverse. Fig. 3. One-month-old culture with a concentric ring pattern of white mycelium and dark green sporodochia. Figs. 4, 5. Microscopic hyphal morphology of CLNP isolate RV10#75. Fig. 4. Undulating hyphae from slide culture. Fig. 5. Hyphal ropes and single hyphae. Figs. 6, 7. Microscopic morphology of CLNP isolate RV10#75 conidia. Fig. 6. Pigmentation among conidia from a single sporodochium. Scale bar equals to 10 µm. Fig. 7. Conidial germination at 28°C.