Note

Snow mold fungus *Racodium therryanum* is phylogenetically *Herpotrichia juniperi*

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ABSTRACT

Racodium therryanum is a snow mold causing snow blight and seed rot in conifers. The sexual stage of *R. therryanum* has not been found, however, it has been speculated that *Herpotrichia juniperi*, which causes brown felt blight, is the sexual stage of *R. therryanum*. In this study, we conducted phylogenetic analysis using 28S large subunit (LSU) rDNA, 18S small subunit (SSU) rDNA, translation elongation factor 1-alpha (*tef1*), and RNA polymerase II second largest subunit (*rpb2*). Four isolates were identified as *R. therryanum* based on their morphological characteristics; together with two strains of *H. juniperi*, they composed a clade supported by high bootstrap and Bayesian posterior probability values. Therefore, we conclude that the snow mold previously described as *R. therryanum* is phylogenetically *H. juniperi*.

Keywords: Melanommataceae, phylogeny, snow blight

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Racodium therryanum is a snow mold fungus that causes snow blight (Racodium snow blight) and seed rot in conifers and some broadleaf tree species (Cheng & Igarashi, 1987; Sakamoto & Miyamoto, 2005; Sato, Shoji, & Ohta, 1960). Racodium snow blight has been reported from northern Japan in regions such as Hokkaido and Tohoku, which experience heavy snowfall in winter. *Racodium therryanum* is an asexual fungus that was originally isolated from *Picea abies* and identified by De Thumen (1880); it was recently isolated from seeds of Korean fir (*Abies koreana*) in Korea (Cho, Miyamoto, Takahashi, Hong, & Kim, 2007). *Racodium therryanum* (as '*Rhacodium*' *therryanum* in Saccardo, 1899) is characterized by felty, dark brown mycelium and hyphae that are 6–7 µm in width (De Thumen, 1880; Saccardo, 1899). It produces oval or globose chlamydospores on mature cultures (Sakamoto & Miyamoto, 2005). In Japan, Sato et al. (1960) identified a snow mold fungus as *R. therryanum* based on morphological characteristics described by Saccardo (1899). The sexual stage of this fungus has not been found.

The taxonomic placement of *R. therryanum* is not clear. Recently, Abdollahzadeh, Groenewald, Coetzee, Wingfield, and Crous (2020) newly introduced the order Racodiales closely related to Capnodiales including the type species of the genus *Racodium* (*Racodium rupestre*), but they did not discuss the placement of *R. therryanum*. On the other hand, some researchers have pointed out the taxonomic relationship between *R. therryanum* and *Herpotrich-*

ia juniperi. Berlese (1892) suggested that *R. therryanum* and *H. juniperi* appear to be the same species, but made no definitive conclusion. Saccardo (1899) also indicated that *R. therryanum* is probably the subiculum of *H. juniperi*. In addition, Bose (1961) referred *R. therryanum* as a synonym of *H. juniperi*. However, their accurate taxonomic relationship has remained unclear. *Herpotrichia juniperi* is a member of the family Melanommataceae within the order Pleosporales (Dothideomycetes, Ascomycota); it has brown hyphae that are 4–6 µm in width, and produces ascospores within globose to subglobose pseudothecia that are 200–450 µm in diam (Sivanesan, 1971). It is also known that *H. juniperi* has pycnidia which develop on the old culture (Bose, 1961), but their role in its life cycle is unclear. *Herpotrichia juniperi* is a causal agent of brown felt blight in conifers of midwestern Eurasia and North America (Kuz'michev, Sokolova, & Kulikova, 2001; Sinclair & Lyon, 2005; Oskay, Lehtijärvi, Dogmuş-Lehtijärvi, & Halmschlager, 2011). Sakamoto and Miyamoto (2005) indicated the similarity of snow blight symptoms caused by *R. therryanum* and *H. juniperi*, and recommended that their relationship be investigated through molecular analyses. In this study, we conducted phylogenetic analysis of *R. therryanum* and *H. juniperi* using four major gene regions: 28S large subunit (LSU) rDNA, 18S small subunit (SSU) rDNA, translation elongation factor 1-alpha (*tef1*), and RNA polymerase II second largest subunit (*rpb2*).

We used four isolates of *R. therryanum*; one was identified by Sato et al. (1960) (MAFF 410406, deposited at the National Agriculture and Food Research Organization; Table 1) and the other three were collected from a nursery or natural forest at the University of Tokyo Hokkaido Forest (Furano, Hokkaido, Japan; Iwakiri, Sakaue,

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Table 1 Sequences of *Racodium therryanum* isolates used for the phylogenetic analysis

Isolates	Source	Locality	Latitude	Longitude	Collection date	LSU	GenBank accession numbers		
							SSU	<i>tef1</i>	<i>rpb2</i>
HFRN18-240	<i>Picea jezoensis</i> seedling	Furano, Hokkaido	43°17'03"N	142°26'28"E	May, 2018	LC618842	LC618848	LC618854	LC618860
HTSO20-756	<i>P. jezoensis</i> seed	Teshio, Hokkaido	45°02'34"N	142°01'18"E	Apr, 2020	LC618843	LC618849	LC618855	LC618861
MAFF 410406	<i>P. jezoensis</i> seedling	Tomakomai, Hokkaido	N/A	N/A	Apr, 1952	LC618844	LC618850	LC618856	LC618862
Rac09Pj11-1	<i>P. jezoensis</i> seedling	Furano, Hokkaido	43°13'11"N	142°22'51"E	May, 2009	LC618845	LC618851	LC618857	LC618863

Table 2 Sequences of *Herpotrichia juniperi* and related Melanommataceae species for phylogenetic analysis

Species	Strain	GenBank accession numbers ^a			
		LSU	SSU	<i>tef1</i>	<i>rpb2</i>
<i>Alpinaria rhododendri</i>	CBS 141994	KY189973	KY190004	KY190009	KY189989
<i>Aposphaeria corallinolutea</i>	CBS 131287	JF740330	–	–	–
<i>Bertiella macrospora</i>	IL 5005	GU385150	–	–	–
<i>Gemmamyces piceae</i>	CBS 141555	KY189976	KY190006	KY190011	KY189992
<i>Herpotrichia juniperi</i>	CBS 200.31	DQ678080	DQ678029	DQ677925	LC618864
	CBS 467.64	LC618846	LC618852	LC618858	LC618865
<i>Herpotrichia macrotricha</i>	GKM 196N	GU385176	–	GU327755	–
	JCM 14419	LC618847	LC618853	LC618859	LC618866
<i>Herpotrichia vaginatipora</i>	MFLUCC 13-0865	KT934252	KT934256	KT934260	–
<i>Melanocucurbitaria uzbekistanica</i>	MFLUCC 17-0829	MG829022	MG829129	MG829209	–
<i>Melanodiplodia tianschanica</i>	MFLUCC 17-0805	MG829023	MG829130	MG829210	MG829256
<i>Melanomma pulvis-pyrius</i>	CBS 124080	GU456323	GU456302	GU456265	GU456350
<i>Monotosporella tuberculata</i>	CBS 256.84	GU301851	–	GU349006	–
<i>Muriformistrickeria rosae</i>	MFLU 16-0227	MG829028	MG829135	MG829215	–
<i>Muriformistrickeria rubi</i>	MFLUCC 15-0681	KT934253	KT934257	KT934261	–
<i>Phragmocephala atra</i>	MFLUCC 15-0021	KP698725	KP698729	–	–
<i>Praetumpfia obducens</i>	CBS 141474	KY189984	KY190008	KY190019	KY190000
<i>Pseudotrickeria muriformis</i>	MFLUCC 13-0764	KT934254	KT934258	KT934262	–
<i>Pseudotrickeria mutabilis</i>	PM 1	KY189988	–	KY190022	KY190003
<i>Sarimanas pseudofluviatile</i>	MAFF 239465	LC001714	LC001711	–	–
<i>Sarimanas shirkamiense</i>	MAFF 244768	LC001715	LC001712	–	–
<i>Seifertia azaleae</i>	DAOM 239136	EU030276	–	–	–
<i>Seifertia shangrialaensis</i>	MFLUCC 16-0238	KU954100	KU954101	KU954102	–

^a Sequences newly obtained in this study are indicated in bold

Matsushita, & Fukuda, 2021) or the Hokkaido University Teshio Experimental Forest (Teshio, Hokkaido, Japan). The isolates were obtained either from seedlings of two conifer species (*Picea jezoensis* and *Picea glehnii*) showing snow blight symptoms or from decayed seeds in Apr or May in 2009, 2018, and 2020 (Table 1). All four isolates were identified as *R. therryanum* according to morphological characteristics described by Sato et al. (1960), namely, felty, dark green to dark gray mycelia.

DNA was extracted from mycelia grown on potato dextrose agar plates at 5 °C for 1 mo using the PrepMan Ultra sample preparation reagent (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The primers LR0R/LR5 (Rehner & Samuels, 1994), NS1/NS4 (White, Bruns, Lee, & Taylor, 1990), EF983F/EF2218R (Rehner & Buckley, 2005), and fRPB2-7cR (Liu, Whelen, & Hall, 1999) were used for polymerase chain reaction (PCR) amplification of LSU, SSU, *tef1*, and *rpb2*, respectively, under the following thermal conditions: 95 °C for 2 min, followed by 35–40 cycles at 95 °C for 10 s, 55 °C for 10 s, 72 °C for 45 s, with a final extension at 72 °C for 10 min. The annealing temperatures for *tef1* and *rpb2* were 60 °C and 52 °C, respectively. The PCR products were purified and sequenced at Macrogen Japan (Kyoto, Japan). In addition to the four *R. therryanum* isolates, sequences of the strains *H. juniperi* CBS 467.64 and CBS 200.31 (for *rpb2* only) and *Herpotrichia macrotricha* JCM 14419 were newly

generated. The sequence data were edited using BioEdit 7.2.5 (Hall, 1999) and aligned using MUSCLE (Edgar, 2004) in MEGA7 (Kumar, Stecher, & Tamura, 2016). Concatenated sequences were aligned with 21 Melanommataceae species (Table 2). *Melanomma pulvis-pyrius* was selected as the outgroup. All aligned sequences were deposited in TreeBASE (ID: S27997). A phylogenetic tree was constructed using the maximum likelihood (ML) and Bayesian inference (BI) methods. ML estimation was conducted using IQ-TREE ver. 1.6.12 (Nguyen, Schmidt, von Haeseler, & Minh, 2015) with 1000 ultrafast bootstrap replicates. K2P+R3 was selected as the best evolution model for LSU via ModelFinder (Kalyaanamoorthy, Minh, Wong, von Haeseler, & Jermin, 2017) in IQ-TREE. Similarly, F81+F+I, TIM2+F+G4, and TNe+G4 were selected as the best models for SSU, *tef1*, and *rpb2*, respectively. BI analysis was conducted using MrBayes 3.2.7 (Ronquist et al., 2012). The best model for each region was determined using MrModeltest v. 2.3 (Nylander, 2004) based on the hierarchical likelihood ratio test (hLRT). The best models were GTR+I+G for LSU, F81 for SSU, GTR+G for *tef1*, and SYM+I+G for *rpb2*. Four Markov chains were run for 1,000,000 generations and trees were sampled every 100 generations. The first 25% of generations were discarded as burn-in. The phylogenetic tree was visualized using FigTree 1.4.3 (<http://tree.bio.ed.ac.uk/software/figtree/>).

The sequences of the four DNA regions were 902 bp (LSU), 1094

bp (SSU), 1038 bp (*tef1*), and 1144 bp (*rpb2*) in length. The sequences of the four *R. therryanum* isolates showed high similarities with those of *H. juniperi* isolates CBS 200.31 (LSU, 99.7–99.9%; SSU 99.9–100%; *tef1*, 95.9–98.9%; *rpb2*, 95.9–97.6%) and CBS 467.64 (LSU, 99.3–99.6%; SSU, 99.9–100%; *tef1*, 95.4–98.4%; *rpb2*, 96.3–98.3%). Also, the combined LSU–SSU–*tef1*–*rpb2* phylogenetic tree showed that the four *R. therryanum* isolates were grouped with the *H. juniperi* isolates, which were distinctively branched from other Melanommataceae species with high ML bootstrap (MB: 99%) and

Bayesian posterior probability (BP: 0.99) values (Fig. 1). Therefore, all isolates tested in this study were identified as *H. juniperi*, and the snow mold fungus in Japan, which has been identified as *R. therryanum*, was revealed to phylogenetically be *H. juniperi*. Based on this result, we refer to the Japanese snow mold *R. therryanum* as *H. juniperi* in the following sections. Since *H. juniperi* was validly published as ‘*Sphaeria*’ *juniperi* in 1854 (Rabenhorst 1854), before the description of *R. therryanum* in 1880, Bose’s description (1961) which synonymized *R. therryanum* with *H. juniperi* seems to be

Fig. 1 – Maximum likelihood (ML) phylogenetic tree for *Racodium therryanum*, *Herpotrichia juniperi*, and related species in the family Melanommataceae based on concatenated LSU, SSU, *tef1*, and *rpb2* regions. ML bootstrap (MB; > 75%) and Bayesian posterior probability (BP; > 0.95) values are shown. The four *R. therryanum* isolates are indicated in bold.

authentic. Type specimens and European isolates of *R. therryanum* should be re-examined to confirm the synonymization.

Although *H. juniperi* specimens isolated in Japan, Eurasia, and North America are phylogenetically the same species, their life cycles exhibit significant differences. In Japan, *H. juniperi* mycelia on infected twigs usually disappear soon after snowmelt (annual growth), whereas in Eurasia and North America, they grow perennially on the plant surface (Sinclair & Lyon, 2005). In midwestern Eurasia and North America, *H. juniperi* produces ascospores in summer or autumn, whereas in Japan it is always asexual and its sexual stage has not yet been found. Population genetic analyses have indicated that *H. juniperi* in Japan (discussed as *R. therryanum*) may undergo sexual reproduction (Iwakiri et al., 2021). Further studies of the life cycle of *H. juniperi*, especially in seasons when ascospores can be produced, are needed to elucidate differences among *H. juniperi* in Japan and in other regions.

The phylogenetic tree indicated that four Japanese *H. juniperi* isolates were divided into two well-supported subclades (MB > 90, BP > 0.95, Fig. 1). Iwakiri et al. (2021) found that *H. juniperi* in Japan (as *R. therryanum*) included several genetically differentiated groups. These results suggest the genetic diversity of *H. juniperi* in Japan. Schneider, Grünig, Holdenrieder, and Sieber (2009) reported that *H. juniperi* isolates from a mountainous region in Switzerland were divided into five genetically differentiated groups that may represent cryptic species. Further phylogenetic analyses of many samples from various regions are necessary to discuss the genetic diversity and species delimitations of *H. juniperi*.

Disclosure

The authors declare no conflicts of interest.

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