Full paper

Taxonomic reappraisal of *Typhula variabilis*, *Typhula laschii*, *Typhula intermedia*, and *Typhula japonica*

Sachiko Ikeda[a,b,*], Tamotsu Hoshino[c], Naoyuki Matsumoto[d], Norio Kondo[e]

[a] Department of Phytopathology and Entomology, Hokkaido Research Organization, Kitami Agricultural Experiment Station, Yayoi 52, Kunneppu, Hokkaido 099-1496 Japan

[b] Graduate School of Agriculture, Hokkaido University, Kitaku Kita 9, Nishi 9, Sapporo 060-8589, Japan

[c] National Institute of Advanced Industrial Science and Technology (AIST) Hokkaido, 2-17-2-1, Tsukisamu Higashi, Toyohira, Sapporo 062-8517, Japan

[d] Graduate School of Agriculture, Hokkaido University, Kitaku Kita 9, Nishi 9, Sapporo 060-8589, Japan

[e] Research Faculty of Agriculture, Hokkaido University, Kitaku Kita 9, Nishi 9, Sapporo, 060-8589, Japan

*Corresponding author:

S. Ikeda

Tel.: +81 157-47-2184

Fax: +81 157-47-2774

E-mail: ikeda-sachiko@hro.or.jp

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Abstract

We have redefined *Typhula variabilis*, *T. laschii*, *T. intermedia*, and *T. japonica* on the basis of morphological and molecular evidence. *Typhula variabilis*, *T. laschii*, and *T. intermedia*, hitherto regarded as synonymous, were compared by critical observations of sclerotial rind cells. Rind cells of *T. variabilis* were thick and plateaued in the center, whereas of *T. laschii* had a ridge in the center. An isolate from winter wheat that we had previously identified as *T. variabilis* was reidentified as *T. intermedia* because it failed to mate with *T. variabilis*, even though rind cells of *T. intermedia* were digitate and occasionally had double-line contours, as in the case of *T. variabilis*. Sequencing of the internal transcribed spacer regions, including 5.8S, supported these differences, indicating that *T. variabilis*, *T. laschii*, and *T. intermedia* are separate species. *Typhula japonica* was characterized by two-spored basidia and basidiospores that often remained agglutinated with each other and germinated on basidiocarps. Its single basidiospores normally developed into dikaryotic mycelia and rarely into monokaryotic mycelia.

Keywords

Morphology, Taxonomy, Typhulaceae

1. Introduction

The genus *Typhula* (Pers.: Fr.) Fr. has clavarioid basidiocarps that arise from sclerotia or directly from mycelia, and includes more than 50 species. Several species are phytopathogenic and have been studied in detail in terms of their systematics and genetics, but most have been characterized only on the basis of their morphology, with little or no comparison of specimens. We found two fungi on overwintered leaves of dicots (carrot, canola, and sugar beet) that resembled *T. variabilis* Riess and *T. japonica* Terui. To date, characterization of *T. variabilis* has been ambiguous, often resulting in confusion with *T. laschii* Rabenhorst and *T. intermedia* Appel & Laubert. *Typhula variabilis* has not so far been reported in Japan, and we compared our isolates with previous descriptions. *Typhula japonica* was first described by Terui (1941) on rapeseed (*Brassica rapa* L. var. *nippo-oleifera* (Makino) Kitam.). However, the description was incomplete and specimens were not available. Hence, we characterized both fungi on the basis of morphological and molecular studies and of mating reactions in the present study, and found that they represent separate species. We present more detailed descriptions of *T. variabilis* and *T. japonica*, and show that *T. laschii* and *T. intermedia* are distinct from *T. variabilis*.

2. Materials and methods

2.1. Fungal materials

Isolates of *T. variabilis* and *T. japonica* were obtained from sclerotia that formed on carrot leaves and crowns and on canola leaves in Memuro, Utoro, and Takikawa, Hokkaido, Japan, shortly after snowmelt in 2010. Representative isolates were selected with stratified random sampling for detailed study (Table 1). *Typhula intermedia* was isolated from sclerotia attached to a wheat leaf in Sapporo, Hokkaido, in May 2009. All isolates were maintained on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA) plates at 0 °C until use.

A specimen labeled as *T. laschii* (Herb. Univ. Upsaliensis F-608676) was provided by the herbarium of Uppsala University, Sweden.

2.2. Growth temperature relations

Minimum, maximum, and optimal growth temperatures of all isolates on PDA plates were determined in the dark at 0 to 25 °C. The diameter of each colony was measured every 7 d after inoculation. The experiments were conducted three times with three replicates.

2.3. Morphological observations

Sclerotia of isolates S1, S3, RMM1112, TK1118, and UT1114 that formed on oat grain medium, which was prepared in a similar way of barley grain medium (Pierson and Gaskill 1961), were incubated outdoors in pots planted with canola (*Brassica napus* cv. Kizakinonatane) in Memuro for 2 mo from Sept in 2011 and 2012 to develop basidiocarps. Colors of basidiocarps and sclerotia follow the *Flora of British Fungi Colour Identification Chart* (RBG Edinburgh 1969).

Basidia and basidiospores were mounted on glass slides for light microscopic examination. Spore dimensions, excluding the apiculus, were determined using 50–100 basidiospores of each isolate by eye against a graduated scale. Basidia and basidiospores were observed by scanning electron microscopy (SEM; JSM-5310LV, JEOL, Tokyo Japan) as described in Woo et al. (2012). The surface and fractures of the sclerotia were also observed by SEM.

For transmission electron microscopy (TEM), sclerotia were first soaked in sterilized distilled water for 1 h, cut in dual-partitioning with a razor, and prefixed and postfixed as described by Xing and Guo (2008). They were then dehydrated through a 50% to 100% ethanol series and embedded in Luft's low-viscosity resin (Glauert and Lewis 1998). Ultrathin sections were prepared for TEM observations (JEM-2100, JEOL), using an ultramicrotome (Leica EM UC7, REICHERT-NISSEI URTRACUT Ñ, Leica, Wetzlar, Germany) and stained with 2% uranyl acetate solution for 15 min and lead citrate for 2 min.

2.4. Mating tests

2.4.1. Isolation of monokaryons

One mature basidiocarp was selected from each of isolates S1 and S3 and secured to Petri dish lids with double-sided adhesive tape to collect spores in sterile water in the dish at 4 °C for 3 d. The spore suspension was spread over PDA plates and incubated at 4 °C for 4–7 d. Colonies with smooth hyphae were selected to obtain monokaryons. Ten monokaryons were randomly selected from each isolate for the monokaryon-monokaryon (mon-mon) mating tests described in section 2.4.2.

Since basidiospores of isolates RMM1112 and TK1118 were stuck together, one basidiocarp of each isolate was soaked in 2 mL of sterile water with or without 0.1% Tween 20 in a 2-mL microtube to collect the spore masses: spore suspensions were vortexed 10 times for 10 s each time and then left still for 10 min. The proportion of single spores that were separated from the clusters was then determined using light microscope. Aliquots of the spore suspension were spread on PDA plates. After incubation at 4 °C for 7–10 d, single hyphae from single colonies were transferred onto fresh PDA plates. They were then incubated at 10 °C for 2 wk and examined under a microscope for the presence or absence of clamp connections.

2.4.2. Monokaryon-monokaryon (mon-mon) mating tests

Two monokaryons were inoculated 2 cm apart at the periphery of PDA plates and incubated at 10 °C for 2 wk. Agar blocks with mycelia were taken from the colony junction and transferred to an unoccupied area of the plate. Seven d after incubation, the presence of clamp connections in mycelia grown from the blocks was examined under a microscope. The presence of clamp connections was the criterion for mating compatibility.

2.4.3. Dikaryon-monokaryon (di-mon) mating tests

Monokaryons of *T. variabilis* isolate S3 were used for di-mon mating tests with isolates S1, S2, and S4. Four monokaryons of S3 were also tested with *T. intermedia* VB1-1 (AB267394). Monokaryons of isolates TK1118 and UT1114 were paired with their respective parent isolates and RMM1112. Four monokaryons of *T. ishikariensis* Imai provided by AIST Hokkaido were paired with isolates S1, S2, and S4. Pairing was performed as in mon-mon mating tests. Agar blocks with mycelia were taken from colonies of tester monokaryons and transferred to an unoccupied area of the plate. Mycelia grown for 7 d after incubation were

examined for the presence or absence of clamp connections under a microscope.

2.5. Sequencing of internal transcribed spacer regions

DNA was extracted from sclerotia and the herbarium specimen using a DNeasy Plant Mini Kit according to the manufacture's protocol (Qiagen, Hilden, Germany). The internal transcribed spacer (ITS) region of rRNA genes, including the 5.8S rRNA gene, was amplified by polymerase chain reaction (PCR) with the primer pair ITS1 (5′-TCCGTAGGTGAACCT-GCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), as described by Hsiang and Wu (2000). The products were purified using a QIAquick PCR Purification kit (Qiagen) and sequenced on an ABI PRISM 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) with primer ITS1. Multiple alignments of the ITS sequences were performed, and the nucleotide substitution rates were calculated, using MEGA5 (Tamura et al. 2011). The alignments were deposited in TreeBASE (http://www.treebase.org/) under accession number 15606. A phylogenetic tree was constructed by maximum-likelihood analysis (Fisher 1936) using the CLUSTALW program (Thompson et al. 1994) with bootstrap values based on 1000 replicates (Felsenstein 1985). Sequence data were deposited in GenBank (Table 1).

3. Results

3.1. Culture characteristics

Colonies of *T. variabilis* on PDA were white (1A, 7 white, 78 white, or 84 white) to beige (4D, 32 clay buff, or 52 buff) or light brown (27 hazel, 28 milky coffee, or 29 fawn) with thin (thick in some isolates) aerial mycelia. Pale spines, which resembled to be basidiocarp stipes, ejected directly from the surface. Cultures sometimes became crustaceous when incubated for more than 4 mo. Sclerotia were produced on the surface in rings or irregularly and were variable in color. *Typhula variabilis* grew at temperatures from 0 to 25 °C on PDA, with an optimum temperature between 5 and 15 °C. The maximal mycelial growth rate ranged from 1.0 to 2.0 mm per d.

Colonies of *T. japonica* on PDA were white (1A, 7 white, 78 white, or 84 white) to beige (4D, 32 clay buff or 52 buff), turned brown (17 snuff brown or 24 date brown) with time, and

had thin aerial mycelia. Pale, thread-like basidiocarp stipes emerged from sclerotia or mycelia. Sclerotia were scattered over the surface or produced in rings and were variable in color. Isolate UT1114 required more than 5 mo to produce sclerotia on PDA plates but 2 mo in oat grain culture. The fungus grew at temperatures from 0 to 20 °C on PDA, with an optimum temperature of around 10 °C, and failed to grow at 25 °C. The maximal mycelial growth rate ranged from 2.0 to 4.0 mm per d.

3.2. Isolating monokaryons and mating tests

3.2.1. Features of single basidiospore isolates of T. japonica

Unlike *T. variabilis* basidiospores, those of *T. japonica* were agglutinated (Fig. 1B). Only a few spore clusters were separated after treatment with Tween 20 (Table 2). Most colonies had clamped hyphae, and a few colonies from basidiocarps of isolate TK1118 had smooth hyphae. These monokaryons were dikaryotized by isolates RMM1112, TK1118, and UT1114 (Table 3).

3.2.2. Mon-mon mating test

Ten monokaryons of isolate S3 were paired in all possible combinations to obtain the bifactorial mating pattern. Four monokaryons—S3e1, S3e2, S3e5, and S3e7—were selected as a set that differed in mating incompatibility alleles (Raper 1966).

3.2.3. Di-mon mating test

The four monokaryons from *T. variabilis* isolate S3 were dikaryotized by isolates S1, S2, and S4 (Table 3). They were not dikaryotized by *T. intermedia* VB1-1. The four monokaryons of *T. ishikariensis* also failed to mate with any of the isolates.

3.3. Taxonomy

Typhula variabilis Riess, Hedwigia 5: 21, 1853. Figs. 2–5.

MycoBank no.: MB152949.

One or more basidiocarps emerging from a sclerotium, clavate or cylindrical, simple or sometimes branched, 8.0–28.4 mm long under natural conditions (Fig. 2). Head often tapered, grayish white (1A, 7 white, 78 white, or 84 white) or pale beige (2B or 4D), 4.2–20.1 mm long. Head often paler than stem, which is 1.2–8.3 mm long, with 5–20-μm-long lateral hairs. Basidiospores 3.8–5.0 × (6.3–)8.8–12.5 μm; X_m (interval of mean values per collection) = 4.0–4.5 × 9.5–10.5 μm; *Q* (basidiospore length/width ratio, given as an interval of mean value) = 1.8–3.0; white (1A, 7 white, 78 white, or 84 white), cylindrical ellipsoid, fusiform to subfusiform, slightly apiculate (Figs. 3A, 4B). Basidia (22.5–)24.0–30.0 × 5.0–7.0 μm, cylindrical, base-clamped, and 4-spored (Figs. 3B, 4A). Sterigmata 3–4 μm long.

Sclerotia globose to subglobose, light (13 rust, 14 rusty tawny, or 15 brick) to dark brown (19 bay, 22 purplish date, or 24 date brown) on PDA. Sclerotia on diseased plants dark (36 fuscous black) when dry, and light or dark red-brown (19 bay, 20 dark brick, 41 blood red) when wet; (0.8–)1.2–1.8(–2.5) × (0.8–)1.0–1.6 mm when dry, (0.8–)1.0–1.8(–2.1) × (0.8–)1.0–1.7(–2.1) mm when wet. Rind rugged, thick (Fig. 5A), often cracked (Fig. 5B). Rind cells coalesced at the base but separate on the surface (Fig. 5A). Rind cell contour unclear, typically in double lines (Fig. 3C–E). The features of the rind and rind cells of *T. variabilis* (Fig. 5A, B) were not as evident as in *T. japonica* (Fig. 6A–C) and differed from those of *T. laschii*: each rind cell of *T. variabilis* had a plateau (Fig. 5A, B). The rind in oat grain culture was 7–9 μm thick. That on carrot leaves was as thick as that in oat grain culture, but the surface was not rugged. That on PDA plates was flat and thin (4–5 μm).

Materials examined: Stems, living and dead leaves of *Daucus carota*, Memuro, Hokkaido, Japan, coll. S. Ikeda, April 2010 (specimen: SAPA 100032, 100033; living cultures: MAFF 244291, 244292, 244293).

Lectotype (designated here): Hedwigia 5: TAF III, Fig. 2. 1853.

Typhula laschii Rabenhorst, Botanische Zeitung 3: 293, 1849. Fig. 7.

MycoBank no.: MB155574.

One or more basidiocarps emerging from a sclerotium, clavate or cylindrical, simple or sometimes branched, 10.0–50.0mm long on Herb. Univ. Upsaliensis F-608676. Head often

tapered, grayish white (1A, 7 white, 78 white, or 84 white) or pale beige (2B or 4D), and usually paler than stem. Basidia, cylindrical and 4-spored.

Sclerotia globose, subglobose to flattened disk, dark (36 fuscous black), 1.0–1.8 mm × 0.5–1.8 mm. Rind rugged and 7–11 μm thick; rind cell has a ridge in its center (Fig. 7A–D).

Neotype (designated here): Herb. Univ. Upsaliensis F-608676.

Typhula intermedia Appel & Laubert, Arbeiten aus der Kaiserlichen Biologischen Anstalt für Land- und Forstwirtschaft 5(3): 153, 1905. Fig. 8.

MycoBank no.: MB155939.

One or more basidiocarps emerging from a sclerotium, clavate or filiform, simple or sometimes branched, 18–40 mm long. Basidiospores 4.3–7.8 × 11.7–16.0 μm, X_m = 6.2 × 13.9 μm, Q = 1.8–2.7, elongate, cylindrical. Basidia 25–30 × 9–10 μm, 4-spored.

Sclerotia globose to subglobose, dark red-brown (19 bay, 22 purplish date, or 24 date brown) on PDA, always single, never coalesced. Sclerotia on dead plants dark (36 fuscous black) when dry, and light or dark red-brown (19 bay, 20 dark brick, 41 blood red) when wet; 0.5–1.0 × 0.5–1.0 mm when dry, 0.6–1.3 × 0.6–1.5 mm when wet. Rind flat and thin (3–5 μm) on PDA. Rind cell contour in double lines and digitate (Fig. 8A, B); rind of sclerotia on PDA somewhat rugged, flat and thin (3–5 μm). The first description of *T. intermedia* did not show the holotype or any illustrations (Appel and Laubert 1905).

Materials examined: Living leaves of *Triticum aestivum*, Sapporo, Hokkaido, Japan, coll. T. Hoshino, May 2009 (specimen: SAPA 100038; living culture: MAFF 244400).

Typhula japonica Terui, Transaction of the Sapporo Natural History Society 17: 40, 1941. Figs. 1, 6, 9, 10.

MycoBank no.: MB291705.

One or more basidiocarps emerging from a sclerotium, clavate or cylindrical, simple or sometimes branched, 10–60 mm long under natural conditions (Fig. 9). Head often tapered, grayish white (1A, 7 white, 78 white, or 84 white), gray (34 smoke gray), or pale beige (2B or 4D), 5.0–40.0 mm long. Head often paler than stem, which is 5.0–30.0 mm long, with 20–100-μm-long lateral hairs and longer mycelia tangled at the base. Basidiospores (9.0–)9.5–

10.5(–11.3) × 5.0–7.0 μm, X_m = 10.0–10.5 × 5.5–5.7 μm, Q = 1.6–2.0, elongate, white, fusiform to subfusiform, conspicuously apiculate (Figs. 1A–D, 10B). Basidia (20.0–)23.0–26.0 × 5.0–6.0 μm, unclamped, 2-spored (Figs. 1D, 10A), not cylindrical. Sterigmata 4.0–6.0 μm long.

Sclerotia on carrot leaves and PDA light (4D or 85 buff) to brown (10 cinnamon or 11 sienna); (0.8–)1.2–3.3 × (0.7–)1.2–2.5 mm when dry, (0.9–)1.5–3.5 (–4.6)× (0.9–)1.5–3.3(–4.1) mm when wet; hemispherical, subglobose, flattened, convex on the top, flat or concave with a pit below. Rind cells variable in shape (Fig. 6A–C).

Materials examined: Both living and dead leaves of *Brassica napus*, Memuro, Hokkaido, Japan, coll. S. Ikeda, Apr. 2010 (specimen: SAPA 100036; living culture: MAFF 244279).

Lectotype (designated here): *Trans. Sapporo Nat. Hist. Soc.* 17: 40, 1941. Fig. 1.

Epitype (designated here): SAPA 100036 (RMM1112).

Ex-epitype: MAFF244279 (RMM1112).

3.4. Sequencing of ITS regions

ITS sequences were obtained from three *T. variabilis* isolates (S1 [AB889545], S3 [AB889546], and S4 [AB889547]), two *T. japonica* isolates (RMM1112 [AB889549] and UT1114 [AB889550]), a *T. intermedia* isolate (VB1-1 [AB267394]), and a *T. laschii* specimen from Uppsala University (AB889548), and from other accessions. Phylogenetic analysis revealed the separation of *T. variabilis*, *T. laschii*, and *T. intermedia* (Fig. 11); *T. japonica* was positioned in the clade of *T. laschii* despite their differences in basidia (4 spores in *T. laschii*, 2 in *T. japonica*) and sclerotia (dark and 1 mm globose to subglobose in *T. laschii*, light to brawn and 1‑4 mm hemispherical to subglobose in *T. japonica*).

4. Discussion

Most species in the genus *Typhula* (Pers.: Fr.) Fr. are ambiguously defined, and few comparative studies have been performed. *Typhula variabilis*, *T. laschii*, and *T. intermedia* are similar (Corner 1950), and Berthier (1976) regarded *T. intermedia* as a synonym of *T. variabilis*. The original papers describing each were written by Riess in 1853 (*T. variabilis*),

by Rabenhorst in 1849 (*T. laschii*), and by Appel and Laubert in 1905 (*T. intermedia*), and their descriptions were not rigorously compared to validate the separate species identities, unlike current taxonomic classification. *Typhula variabilis* was described only by drawings of basidiocarps and basidiospores, with little information on their size and none on rind cells. In addition, the paper on *T. laschii* described the form and color of basidiocarps and sclerotia in only 26 words, with no mention of size or other features. Owing to this scarcity of information, Berthier (1976) questioned whether *T. variabilis* and *T. laschii* were identical, and I. Olariaga (Universidad del País Vasco, personal communication, 2013) supported the suggestion to rename a specimen labeled *T. variabilis* at Uppsala University (Herb. Univ. Upsaliensis F-608676) as *T. laschii* in 2006 owing to the priority of the latter.

Typhula variabilis has featureless basidiocarps and basidiospores, and characteristic thick rind cells with precipitous margins whose contours were drawn in double lines by Berthier (1976) and Dynowska (1986). However, the rind cells of specimen F-608676 have ridges in the center, which we recognized as lines in the center of cells (Fig. 7A–D). Thus, F-608676 is not *T. variabilis*. On the other hand, rind cells of our isolates S1, S3, and S4 were thick and flat on top with precipitous margins, giving them a plateau-like shape (Figs. 3C–E, 5A, B). These features are consistent with those illustrated by Berthier (1976) and Dynowska (1986) and described by Remsberg (1940). These characteristics of rind cells indicate that isolates S1, S2, S3, and S4 are *T. variabilis*. In addition, rind cells were not completely coalesced in *T. variabilis* (Fig. 5A, B), making microscopic observation difficult. The rugged surface of *T. intermedia* sclerotia (Fig. 8B) complicated observations of rind cells intended to distinguish the fungus from *T. variabilis*. However, they are separate species because monokaryons of *T. variabilis* were not dikaryotized by *T. intermedia* (Table 3). In addition, *T. intermedia* is pathogenic on wheat (T. Hoshino, unpublished), whereas *T. variabilis* is not, but is pathogenic on members of the Apiaceae and *Beta vulgaris* (data not shown). Repeated attempts to develop basidiocarps of *T. intermedia* were unsuccessful, so we have based our description of the taxonomic features of basidiocarps, basidia, and basidiospores on the original article (Appel and Laubert 1905) and Remsberg's monograph (1940). The original descriptions of *T. intermedia* and *T. laschii* did not designate the holotype or show any illustrations; typification is needed. Remsberg (1940) described the characteristics of *T. intermedia* in detail, but no other descriptions of the fungus were available. We think Remsberg's specimen is worthy of typification as a neotype, but we were unable to examine it. The typification of *T. intermedia* requires future surveys.

No species described by Remsberg (1940), Corner (1950), or Berthier (1976) conformed

to the Herb. Univ. Upsaliensis F-608676 specimen. The original report of *T. laschii* (Rabenhorst 1849) was poor, and identification of existing species was difficult. In addition, we were concerned that frequent changes in the name of a specimen would result in further confusion. Hence, we propose to refer to F-608676 as *T. laschii* and set it as the neotype, because the original article did not indicate the holotype and had practically no taxonomic criteria (McNeill et al. 2012: Art. 9.7, Melbourne Code). In the Taxonomy section (3.3), we described the sclerotium and features of basidiocarps and basidia based on the specimen. It also needs further research, especially on living materials.

Riess (1853) drew only basidiocarps and basidiospores of *T. variabilis* (lectotype, Art. 9.2, Melbourne Code), but the descriptions by Remsberg (1940) and Berthier (1976) have additional information, especially on sclerotia. The identification of *T. variabilis* requires morphological observation of rind cells as described above. The species needs an epitype to identify unambiguously. Remsberg's drawings, photographs, and specimen are adequate, but they were collected in North America. A specimen from Europe that exactly matches the descriptions by Remsberg (1940) and Berthier (1976) should be designated as the epitype, because an epitype should ideally be collected from the same location and host as the original protolog (Hyde and Zhang 2008).

Typhula japonica was first described in Japan (Terui 1941). Only basidiocarps and basidia of the lectotype of Terui (1941) were illustrated (and their bases were not clearly indicated), and basidiospore measurements were not made. However, it has not been reported since, and no specimen was available. Our isolates RMM1112, TK1118, and UT1114 have two-spored basidia, are pathogenic on canola (data not shown), and originate from Hokkaido, conforming to the original description. We identified these three isolates as *T. japonica*, and applied isolate RMM1112 (SAPA100036) as the epitype (Art. 9.8, Melbourne Code), because the original description by Terui (1941) did not indicate details such as spore size.

Typhula japonica has two-spored basidia, as have *Typhula pulgensis* (Khurana 1980) and *T. ochraceosclerotiata* (Olariaga and Salcedo 2009). These three species are probably closely related but require critical comparisons. The sclerotial rind cell pattern of *T. japonica* (Fig. 6) resembles that of *T. ochraceosclerotiata* (Olariaga and Salcedo 2009). Basidiospores of *T. japonica* are sticky and coherent with each other on basidiocarps (Fig. 1B). Most single basidiospores developed into dikaryotic mycelia, and only a few were monokaryotic. In addition, *T. japonica* basidiospores often germinate on basidia (Fig. 1C). They may not be ejected into the air but are likely to remain on basidiocarps: this species requires further investigations of epidemiology and comparisons with other two-spored species. Several

monokaryons from isolates TK1118 and UT1114 were dikaryotized with the isolate RMM1112, which confirmed that these three isolates are all *T. japonica* (Table 3).

In this study, morphological investigations, mating tests, and analysis of ITS sequences revealed that *T. variabilis*, *T. laschii*, and *T. intermedia* are separate species with different genetic backgrounds (Fig. 11). Diagnostic features of the three species are summarized in Table 4. A phylogenic tree of all species of the genus *Typhula* whose ITS sequences have been registered in GenBank divided the species roughly into three groups—A, including pathogenic species such as *T. ishikariensis* and *T. incarnata* Lasch; B, including *T. phacorrhiza* (Reichardt) Fr. and *Macrotyphula juncea* (Alb. & Schwein.) Berthier; and C, including *T. laschii* and *T. japonica*—and *T. intermedia* as a standoff (Fig. 11). In addition, the results suggest that the genus *Typhula* is genealogically polyphyletic; thus, detailed investigations are needed.

Disclosure

The authors declare no conflicts of interest. All the experiments undertaken in this study comply with the current laws of Japan.

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444 **Figure legends**

445

446 Fig. 1 – *Typhula japonica*. A: Basidiospores (isolate UT1114). B: Basidiospore mass (isolate
447 RMM1112, SEM). C: Basidiospores germinating from the apex (isolate RMM1112, SEM).
448 D: Surface of basidiocarp head (isolate RMM1112, SEM). *Bars*: 10 µm.

449

450 Fig. 2 – Basidiocarps of *Typhula variabilis* (isolate S3), developed under leaves of canola in a
451 pot in the open air.

452

453 Fig. 3 – *Typhula variabilis*. A: Basidiospores (isolate S4). B: Basidia (isolate S3). C–E: Rind
454 cell pattern (isolate S3). C, D, on carrot leaves; E, in oat grain culture. *Bars*: 10 µm.

455

456 Fig. 4 – *Typhula variabilis*. A: Basidia (isolate S3). B: Basidiospores (isolate S3). *Bars*: 10
457 µm.

458

459 Fig. 5 – Rind of *Typhula variabilis* (isolate S3, in oat grain culture). A: Cross-section of rind
460 cells by TEM. B: Surface of a sclerotium by SEM. *Bars*: 10 µm.

461

462 Fig. 6 – Rind cell pattern of *Typhula japonica* (isolate UT1114). A, B, on carrot leaves; C, in
463 oat grain culture. *Bars*: 10 µm.

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465 Fig. 7 – Rind cells of *Typhula laschii* (specimen from Univ. of Uppsala). A–C: Rind cell
466 pattern. D: Side view of rind; the center of the rind cells is ridged (arrows). *Bars*: 10 µm.

467

468 Fig. 8 – Rind cells of *Typhula intermedia* VB1-1. A: Digitate cells. B: Contour in double lines.
469 *Bars*: 10 µm.

470

471 Fig. 9 – Basidiocarps of *Typhula japonica* (isolate RMM1112), developed under leaves of
472 canola in a pot in the open air.

473

474 Fig. 10 – *Typhula japonica*. A: Basidia (isolate RMM1112). B: Basidiospores and a germing
475 basidiospore (isolate RMM1112). *Bars*: 10 µm.

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Fig. 11 – Maximum-likelihood analysis tree of *Typhula* species based on sequences of the ITS1–5.8S–ITS2 region. Bootstrap percentages (from 1000 replicates) are shown at branch points. The tree is drawn to scale, with branch length measured in the number of substitutins per site. The outgroups are *Cantharellus cibarius* and *Arrhenia auriscalpium*.

Table 1 – Inventory of fungal materials used in this study.

Species	Isolate	Isolation source	Locality	GenBank acc. no. (ITS)	MAFF no.[a]	SAPA no.[b]
Typhula	S1	Carrot	Memuro, Japan	AB889545	244291	100032
variabilis	S2	Carrot	Memuro, Japan			
	S3	Carrot	Memuro, Japan	AB889546	244292	100033
	S4	Carrot	Memuro, Japan	AB889547	244293	
T. laschii[c]			Gästribland, Sweden	AB889548		
T. intermedia	VB1-1	Wheat	Sapporo, Japan	AB267394	244400	100038
T. japonica	RMM1112	Canola	Memuro, Japan	AB889549	244279	100036
	TK1118	Carrot	Takikawa, Japan		244280	100035
	UT1114	Carrot	Utoro, Japan	AB889550	244278	100037

[a] Cultures deposited in Genebank of the National Institute of Agrobiological Sciences, Japan (MAFF).

[b] Specimens deposited in the Hokkaido Univ. Museum, Sapporo, Japan (SAPA).

[c] Specimen from Herb. Univ. Upsalienesis F-608676.

Table 2 – Rate of single basidiospores of *Typhula japonica* and their karyotic conditions[a].

Isolate	Suspended in	No. basidio-spores/mL	% separate spores[b]	No. monokaryons / no. single-spore colonies examined
T. japonica	Sterilized water	9.3×10^3	67.1	0/39
RMM1112	0.1% Tween 20	5.6×10^3	37.9	0/50
T. japonica	Sterilized water	6.0×10^4	26.0	0/39
TK1118	Sterilized water	7.9×10^3	49.4	1/38
	0.1% Tween 20	5.0×10^2	60.0	2/25
	0.1% Tween 20	9.0×10^2	55.6	3/40
T. variabilis S3[c]	Sterilized water	2.2×10^4	94.5	32/34

[a] One basidiocarp was examined for each isolate.

[b] No. separate spores / (No. separate spores + No. clustering spores) × 100

[c] Determined as a reference.

Table 3 – Di-mon mating tests between dikaryons of *Typhula variabilis, T. japonica, T. intermedia* and monokaryons of *T. variabilis, T. japonica, T. ishikariensis.*

Monokaryon		Dikaryon							
		T. variabilis				*T. japonica*			*T. intermedia*
		S1	S2	S3	S4	RMM 1112	TK 1118	UT 1114	VB1-1
T. variabilis	S3e1	+[a]	+	+	+	-[b]	-	-	-
	S3e2	+	+	+	+	-	-	-	-
	S3e5	+	+	+	+	-	-	-	-
	S3e7	-	+	+	+	-	-	-	-
T. japonica	TK1118Am1	-	-	-	-	+	+	+	-
	TK1118Am2	-	-	-	-	+	+	+	-
	TK1118Bm1	-	-	-	-	+	+	+	-
	TK1118Bm2	-	-	-	-	-	+	-	-
	UT1114Am1	-	-	-	-	+	+	+	-
	UT1114Am2	-	-	-	-	+	+	+	-
T. ishikariensis[c]	9-4-3(A)[d]	-	-	-	-	-	-	-	-
	7-6-7(A)	-	-	-	-	-	-	-	-
	8-2(B)[e]	-	-	-	-	-	-	-	-
	35-8(B)	-	-	-	-	-	-	-	-

[a] +: A monokaryon was dikaryotized.

[b] -: A monokaryon was not dikaryotized.

[c] Provided by AIST.

[d] (A): Biotype A.

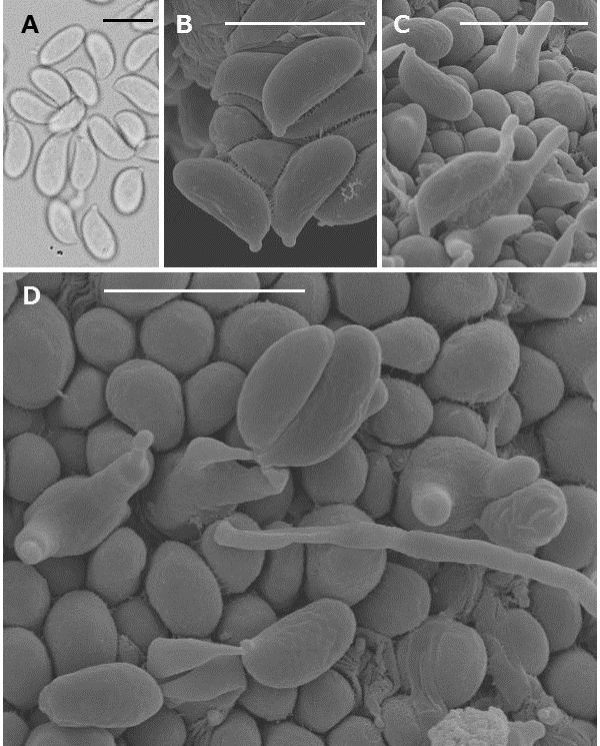
[e] (B): Biotype B.

19 Table 4 – Morphological comparisons of sclerotia of *Typhula variabilis*, *T. laschii*, and *T.*

20 *intermedia.*

Sclerotium	*T. variabilis*	*T. laschii*	*T. intermedia*
Size	1–2 mm[d,e]	1–3 mm[e]	0.5–1 mm[e]
Shape	Hemispherical[e], subglobose[c,e], resemble large brassica seeds[a,b,e]	Hemispherical[e], sub-globose[e], flattened[e]	Hemispherical[e], subglobose [a,b,e], always single[a,e]
Color	Black[a,b,d,e], dark brown[a,b,d,e] dark red-brown[b,e]	Black [b,e], dark brown[e]	Black[a,e], dark brown[a,b,e], dark red-brown[a,b,e]
Surface	Very bumpy[a,d,e], rugged[a,d,e], raised [d,e], but not incisive[e]	Incised[e], wrinkled[b]	Bumpy[a,b,e], raised[e], wrinkled [a,b]
Rind cell	Plateaued[c,d,e], thick in the center and thin at the margin[c,d,e]	Steep ridge in the center[e]	

21 Descriptions assembled from: [a] Remsberg (1940); [b] Corner (1950); [c] Berthier (1976); [d] Dynowska (1986); [e] Present study.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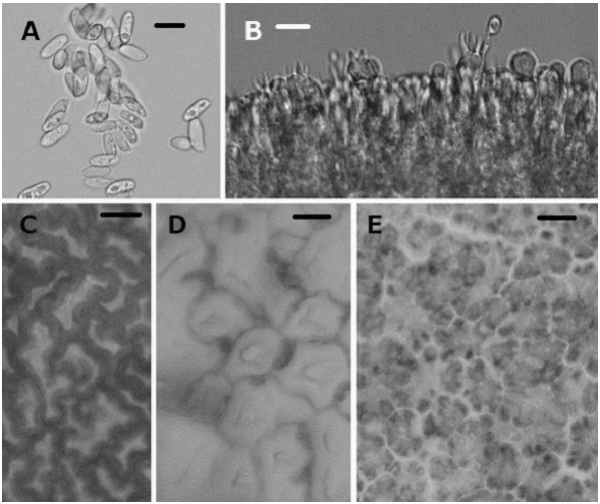
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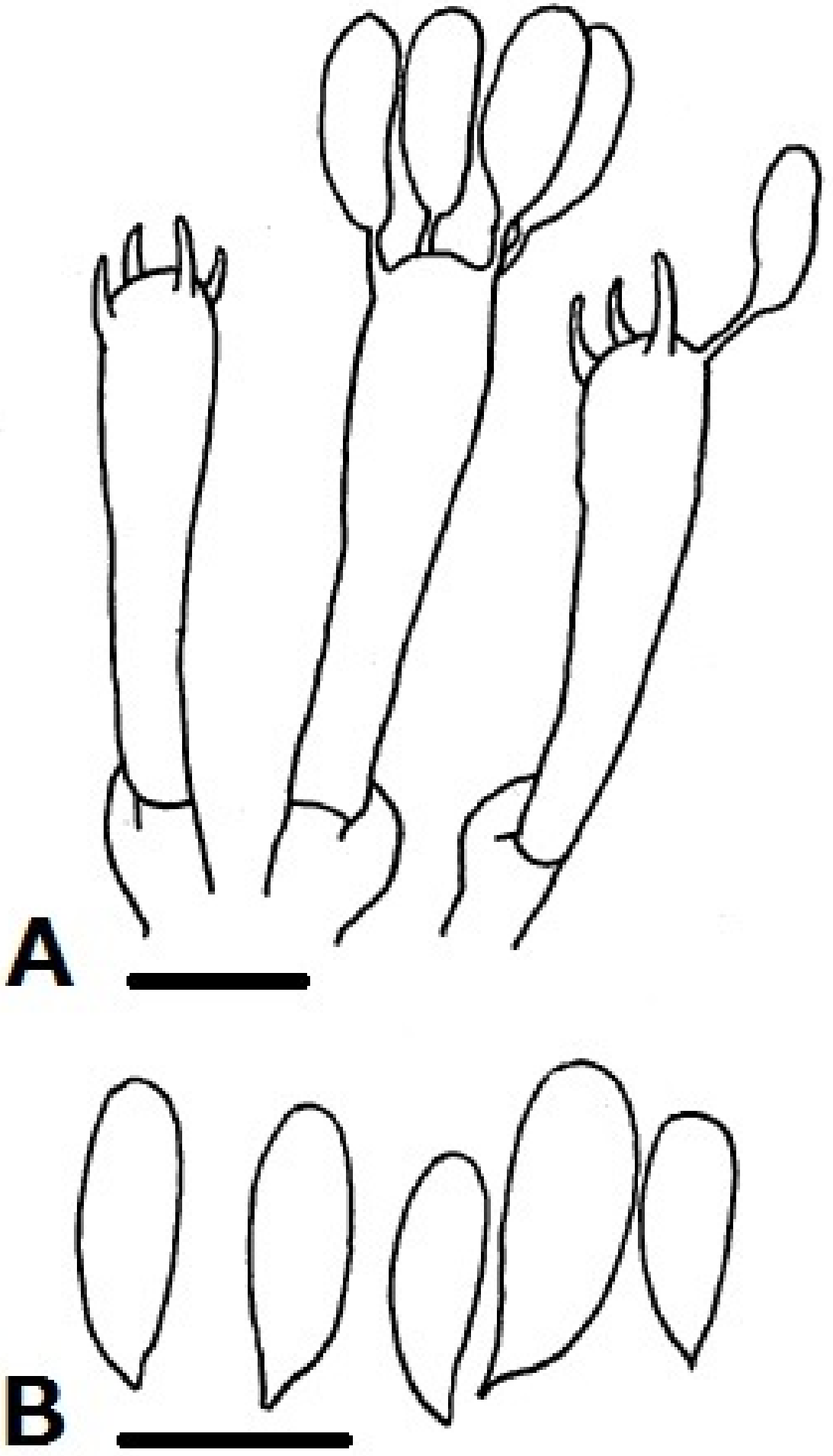
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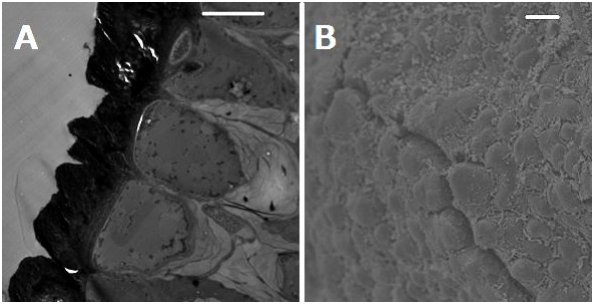
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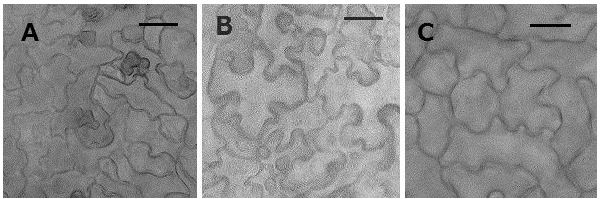
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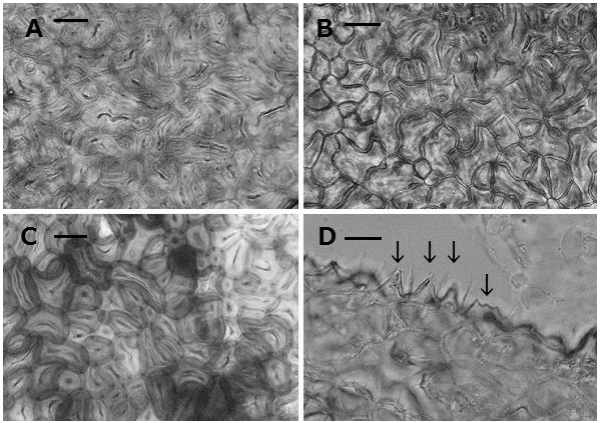
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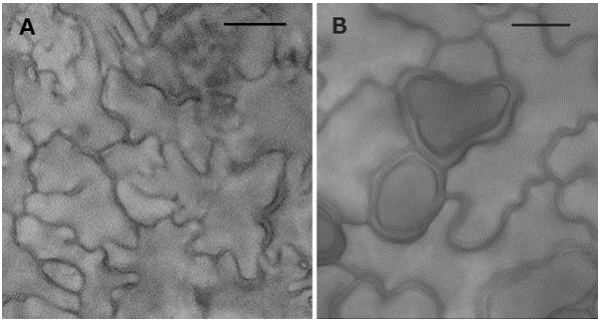
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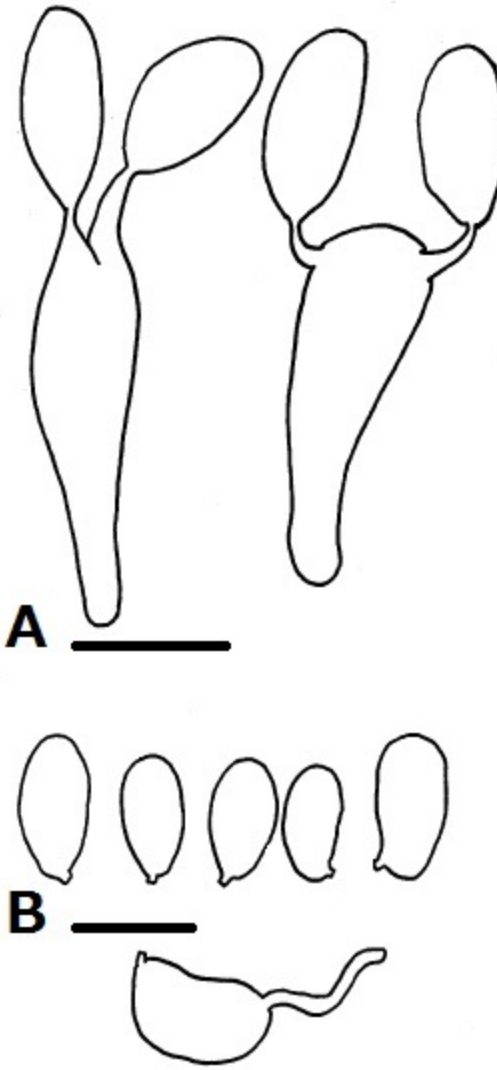
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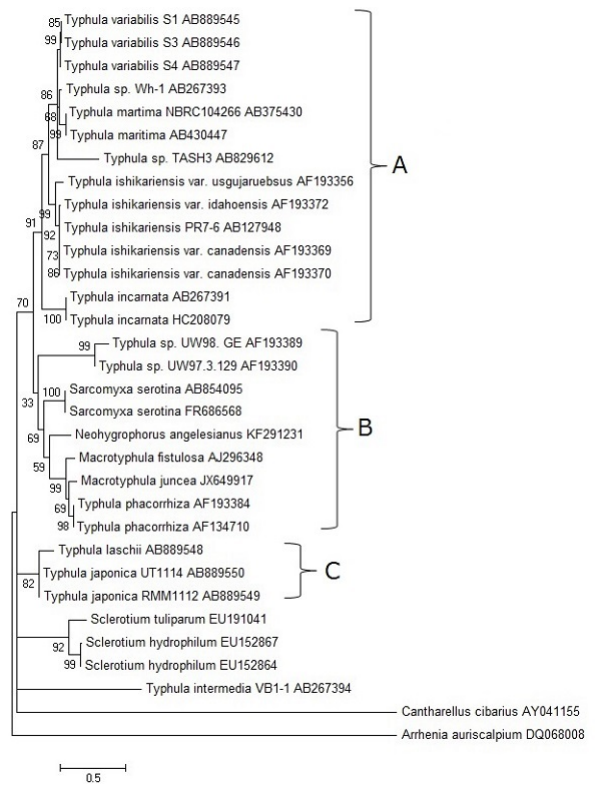
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Typhula variabilis S3 AB889546
Typhula variabilis S4 AB889547
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Typhula martima NBRC104266 AB375430
Typhula maritima AB430447
Typhula sp. TASH3 AB829612
Typhula ishikariensis var. usgujaruebsus AF193356
Typhula ishikariensis var. idahoensis AF193372
Typhula ishikariensis PR7-6 AB127948
Typhula ishikariensis var. canadensis AF193369
Typhula ishikariensis var. canadensis AF193370
Typhula incarnata AB267391
Typhula incarnata HC208079
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Typhula sp. UW98. GE AF193389
Typhula sp. UW97.3.129 AF193390
Sarcomyxa serotina AB854095
Sarcomyxa serotina FR686568
Neohygrophorus angelesianus KF291231
Macrotyphula fistulosa AJ296348
Macrotyphula juncea JX649917
Typhula phacorrhiza AF193384
Typhula phacorrhiza AF134710
B
Typhula laschii AB889548
Typhula japonica UT1114 AB889550
Typhula japonica RMM1112 AB889549
C
Sclerotium tuliparum EU191041
Sclerotium hydrophilum EU152867
Sclerotium hydrophilum EU152864
Typhula intermedia VB1-1 AB267394
Cantharellus cibarius AY041155
Arrhenia auriscalpium DQ068008
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