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FULL PAPER

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Typhula maritima, a new species of *Typhula* collected from coastal dunes in Hokkaido, northern Japan

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Abstract *Typhula maritima* on coastal dunes in Ishikari and Yoichi, Hokkaido, northern Japan, is illustrated as a new species on the basis of morphology and molecular evidence. The sclerotia in the sand had mycelial strands that extended upward to support basidiocarps. Mycelial strands also connected sclerotia with plant tissues of the dunegrass *Eylmus mollis* Trin. *Typhula maritima* is psychrotrophic and halotrophic, and sclerotia floated on seawater for 1 week. These physiological characteristics are important factors for adaptation to the coastal dune environment. Inoculation tests indicated that *T. maritima* could grow on living *E. mollis*, but it was not pathogenic. The results suggested that this fungus was, at least, not an active pathogen in the coastal dune ecosystem. Ex-holotype is TH-AIST-Tm-1 (= NBRC 104266).

Key words Coastal dune ecosystem · Taxonomy · Typhulaceae

Introduction

The genus *Typhula* (Pres.) Fr., a member of Typhulaceae, Agaricales (Dentinger and McLaughlin 2006), has slender, fertile clavulae and distinct, filiform stipes arising from sclerotia or directly from mycelia (Remsberg 1940); this genus includes more than 50 species mainly in cool temperate and frigid regions (Corner 1950; Berthier 1976). Most species are saprophytic and psychrotrophic or psychrophilic, and several species [*Typhula incarnata* Lasch ex Fr., *T. ishikariensis* S. Imai, *T. japonica* Terui, *T. phacorrhiza* (Reichardt) Fr., *T. trifoli* Rostr., and *T. variabilis* Riess] are phytopathogens in grasslands from cool temperate regions to the Arctic (Hsiang et al. 1999; Matsumoto et al. 2001; Hoshino 2005).

We have been surveying the biodiversity of macrofungi in a coastal dune area, Ishikarihama, Ishikari-shi, Ishikari Province, Hokkaido, northern Japan, since 2004 (Kasuya et al. 2007; Takehashi et al. 2007). Several taxa of macrofungi belonging to ascomycetes and basidiomycetes have been found in coastal sandy dunes in cool temperate to frigid regions in Europe, including Nordic countries (Andersson 1950) and northern Scotland (Watling and Rotheroe 1989). However, no species with spathulate or clavate basidiocarps resembling *Typhula* spp. have been listed in these checklists or in monographs on *Typhula* and related genera (Remsberg 1940; Corner 1950; Parmasto 1965; Berthier 1976). In this article, we describe the taxonomic characteristics as well as physiological and ecological traits of *Typhula* sp., indicating adaptations to the coastal dune environment.

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Materials and methods

Collection

The specimens of *Typhula* sp. were collected from coastal dunes in Ishikarihama, Kotan, and Muenhama, Ishikari-shi, Ishikari Province, and Yoichihama, Yoichi-cho, Shiribeshi Province, Hokkaido, Japan (Fig. 1). Dates of collection are shown below. These specimens were kept in the mycological herbaria of National Museum of Nature and Science, Tokyo (TNS) and The Hokkaido University Museum, Sapporo (SAPA).

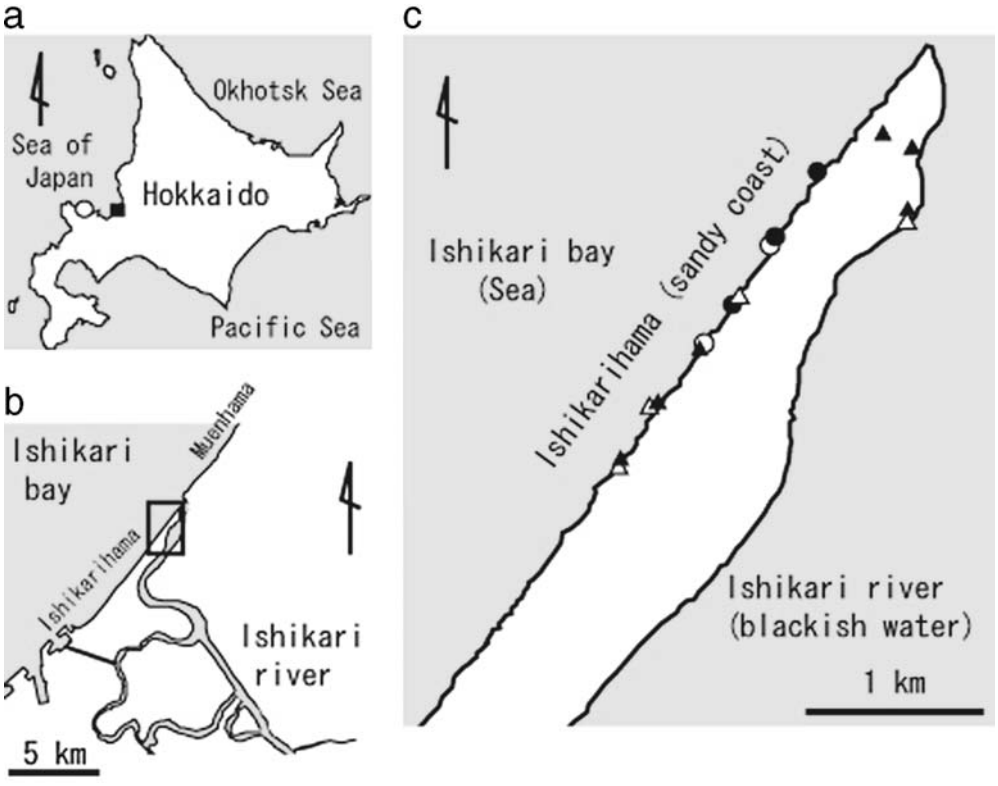


Fig. 1. Localities of *Typhula maritima*. **a** *Closed square and open circle* show the locations of Ishikarihama (Ishikari, Ishikari Province) and Yoichihama (Yoichi, Shiribeshi Province), respectively. **b** *Rectangular area* indicates the research site in Ishikarihama. **c** *Open and closed circles* and *open and closed triangles* show where basidiocarps were found in 2004 (*open circles*), 2005 (*closed circles*), 2006 (*open triangles*), and 2007 (*closed triangles*). All symbols indicate the spots where we found more than 25 basidiocarps in late September to late November

Morphological observations

Colors of basidiocarps and sclerotia were described according to the color identification chart of the Royal Botanic Garden Edinburgh (*Flora of British Fungi*) (Anonymous 1969). Basidiospores from fresh specimens were mounted in water, 3% or 5% (w/v) KOH, and phloxine B solution for light microscopic examination. About 30 spores were randomly chosen for determination of length and width excluding the apiculus.

Surface features of basidiospores, mycelial strands, sclerotia, and stipes of basidiocarps were observed by scanning electron microscopy (SEM). For SEM, basidiocarps and sclerotia were cut on a piece of double-sided adhesive tape attached to a specimen holder and then coated with platinum-palladium using a JFC-1100 Ion Sputter (JEOL, Tokyo, Japan). They were examined using a JSM-T330A SEM (JEOL) operating at 10 kV.

Phylogenetic analyses

DNA was extracted from the specimens by the protocol of DNeasy Plant Mini (Qiagen, Hilden, Germany). The internal transcribed spacer (ITS) region of genomic rDNA was amplified with the primer pairs ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), as described by Hsiang and Wu (2000). The polymerase chain reaction (PCR) product was purified using a QIAquick PCR Purification Kit (Qiagen) and sequenced on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using the primer ITS1. Multiple alignments of the ITS sequences were performed, and the nucleotide substitution rate was calculated. The alignments were deposited in TreeBASE (http://www.treebase.org/) under the accession number SN4375. A phylogenetic tree was constructed by the neighbor-joining method (Kimura 1980; Saitou and Nei 1987) using the program CLUSTAL W (Thompson et al. 1994) with bootstrap values based on 1000 replications (Felsenstein 1985).

Isolation and culture

Collected sclerotia were put in paper envelopes and dried at room temperature. Sclerotia were surface-sterilized in 70% (v/v) ethanol and 0.5% (as active chlorine) sodium hypochlorite solution and thoroughly washed with sterilized distilled water. They were then cut with sterilized razor blades, placed on potato dextrose agar (PDA; Difco, Becton Dickinson Microbiology Systems, Sparks, MD, USA) so that cut surfaces were in contact with the agar, and incubated at 4°C.

Collected basidiocarps were put in plastic cases with wet cotton balls and kept in a refrigerator. Stipes and mycelial strands were removed from basidiocarps. Cut basidiocarps were hung on the inside of covers of Petri dishes containing PDA with double-sided adhesive tape and incubated at 4°C for 1 or 2 days. Basidiospores that fell on PDA germinated, mated with each other, and formed sclerotia. These sclerotia were inoculated on new PDA at 4°C for 2 weeks. Isolates were maintained on PDA slant cultures at –1°C.

Growth temperature relationships and salt tolerance of mycelia

Mycelial disks 5 mm in diameter were cut from the margin of an actively growing colony, transferred to the centers of PDA plates with various concentrations of NaCl (0%–10%, v/v), and inoculated at five different temperatures from –1° to 25°C, in duplicate. After 1, 2, and 3 weeks of inoculation, the colony diameters were determined. The linear mycelial growth rate per week was calculated after the initial lag period. An isolate of *T. incarnata* was also used as a reference.

Floating of sclerotia in seawater

Sclerotia were prepared from PDA cultures of *Typhula* sp. from Ishikarihama, *T. incarnata*, *T. ishikariensis* biological species I (Matsumoto 1997), *T. phacorrhiza*, and *T. variabilis* in our laboratory collection. Seawater was collected from Zenibako beach in Sapporo in May 2006. Fifty sclerotia of each species were put in 100 ml sterilized seawater in 500-ml Erlenmeyer flasks at 4°C with vigorous shaking for 1 week. Floating sclerotia in sterilized seawater were counted every day. Sclerotia that were floating after 1 week were inoculated on PDA plates at 10°C for 2 weeks.

Pathogenicity

Ten mature dunegrass plants, *Eylmus mollis* Trin., that were more than 20 cm tall were collected in Ishikarihama, Hokkaido, Japan, in October 2006. Yellowish leaves were removed, and plants were transplanted in plastic pots. Each plant received 5-mm-diameter mycelial disks on leaf sheaths and were incubated in a moist container at –1°C for 3 months in triplicate. Then inoculated plants were regrown in a phytotron (MLR-350H; Sanyo, Tokyo, Japan) at 25°C under 12 h light condition for 3 weeks. The estimate of aggressiveness was based on dry weight of plants after regrowth. Control plants received PDA disks without fungal mycelia.

Results and discussion

Field observations

Spathulate or clavate basidiocarps were found on the sands between the seashore and foredunes in Ishikarihama during the period from late September to late November before persistent snow cover began (Figs. 2–4a). Sclerotia were found on the sands just after snowmelt (Fig. 5a) and connected to *E. mollis* leaves with mycelial strands (Fig. 5b). *Eylmus mollis* and Japanese sedge, *Carex kobomugi* Ohwi, are major plant species in foredunes in Ishikarihama (Sasaki et al. 2002). However, we did not find any sclerotia on *C. kobomugi*.

Basidiocarps of this fungus fruited in the research site every year between 2004 and 2007 but were found in different spots from one year to the next (Fig. 1c). During the research period from summer to autumn, typhoons battered the coast, disturbing its habitat. After a typhoon had passed through in Yoichihama in October 2006, although *E. mollis* were almost covered by sand, we could collect sclerotia on the sands. These observations suggest that sclerotia of this fungus can float in seawater and survive the natural disturbance by autumn storms.

Taxonomy

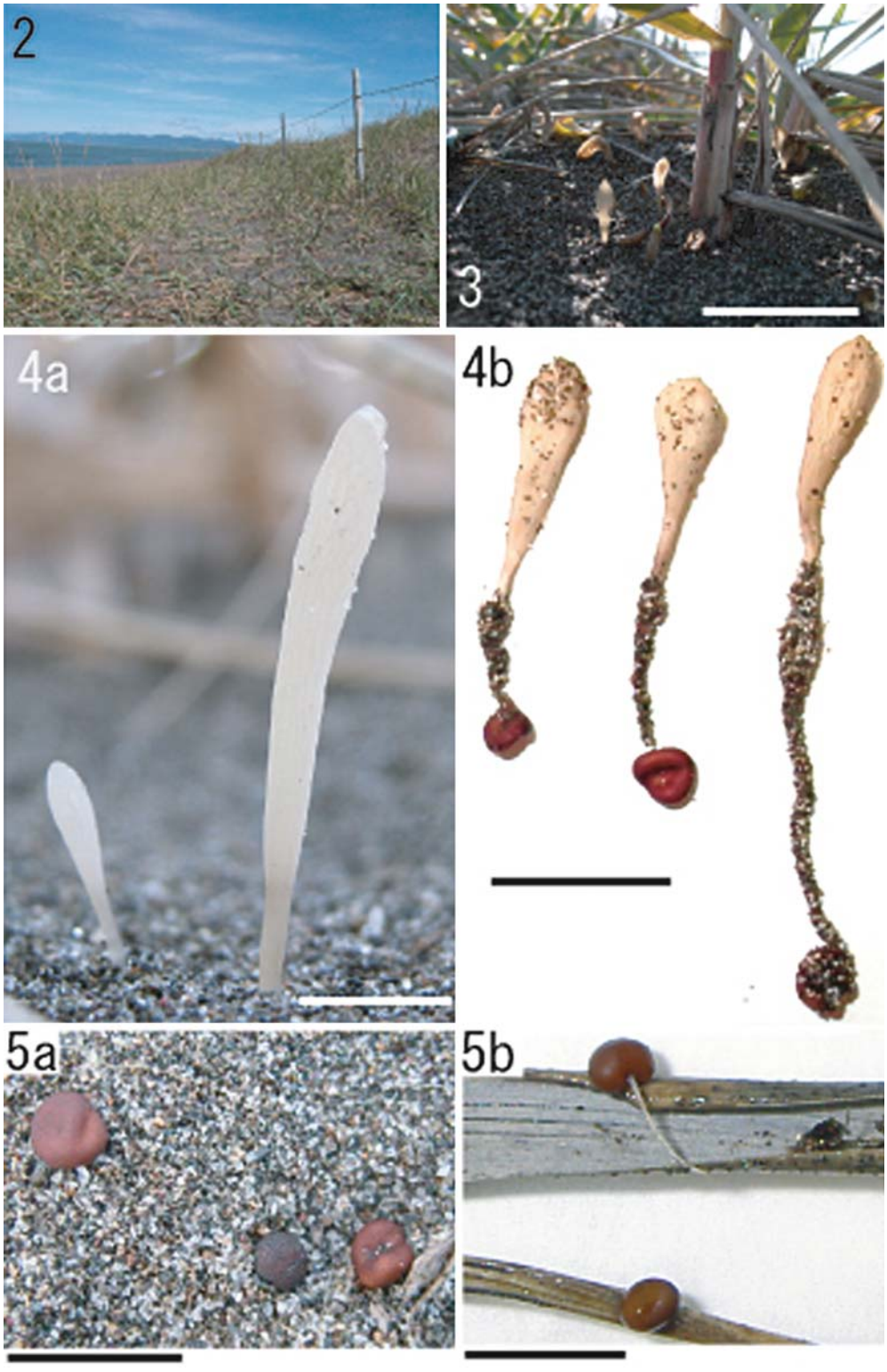
Typhula maritima T. Hoshino, Takehashi & T. Kasuya, sp. nov. Figs. 4–13

MycoBank no.: MB515232

Sclerotia brunnea, castanea vel nigra, globosa vel subglobosa, 1–4 × 1–5 mm, superficie glabra, ex plectenchymate 3–8 mm; sporophora clavata vel spathulata, erecta, recta vel curva, simpliciter raro ramosa, glaba, ad apicem crassa, rotunda vel acuta, unum didyma ex sclerotio submaritimo, 10–45 mm alta, cineracea dein eburnea; clavula recta, spathulata vel fistulosa, 3–30 mm longa, 3–8 mm lata; stipes indistinctus, rectus, glaber vel leviter pubescens ad basim, 4–6 mm longus, 1–2 mm diametro; rhizomorphae ex sclerotiis arenosis, 8–27 mm; basidia cylindrica, crassa ad apicem, cellulae confibula conjunctae ad basem, quadrispora, 35–50 µm longa, 7–11 µm lata; basidiosporae ellipsoideae, hyalinae, tenuitunicatae, guttulatae, apiculatae, 8–14 × 4–8 µm.

Sclerotia reddish brown to purplish brown [20 (dark brick) or 21 (purplish chestnut) or occasionally 38 (violaceous black)], minute, 1–4 × 1–5 mm, globose to distorted subglobose (similar to beans of beach pea, *Lathyrus japonicus* Willd.) (Fig. 5a), surface glabrous smooth, thinly covered with hyphae that are loosely bundled into mycelial strands (Figs. 5b, 7a,b). Mycelial strands are approximately 3–8 mm long and connected to the host substrate. Rind cells of sclerotium versiform, often irregularly polyhedral to elliptical, and interlocking with each other (Fig. 6).

Basidiocarps (Fig. 4) one to two per sclerotium, 10–45 mm high, attached at the base to comparatively long (8–27 mm) mycelial strands that are connected to the sclerotium, narrowly to broadly clavate, or spheropedunculate in small ones, obscurely stipitate, simple or rarely dichotomously branched, apex obtuse or rounded, watery white to whitish (1A or 7 white) at first, buff to cinnamon (2B to 6F) when mature; stipe 4–6 mm long, 1–2 mm wide, darker than the fertile head in color, but often difficult to distinguish from it; head 3–30 mm long and 3–8 mm wide, stuffed at first then hollow. Context hyphae of basidiocarp clamped, made up of cylindrical-fusiform to swollen cells (up to 25 µm across) that are thin- to thick walled with yellowish-brown to yellow pigments and granular, refractive contents (Figs. 10, 13). Basidia clamped at the base, cylindrical, 35–50 (avg. 42) × 7–11 (avg. 9.2) µm, four-spored (Figs. 9, 12). Basidiospores cylindrical-ellipsoid to oblong, 8–14 (avg. 10.5) × 4–8 (avg. 5.7) µm, hyaline, smooth, thin walled, with a prominent obtuse apicule (Figs. 8, 11).



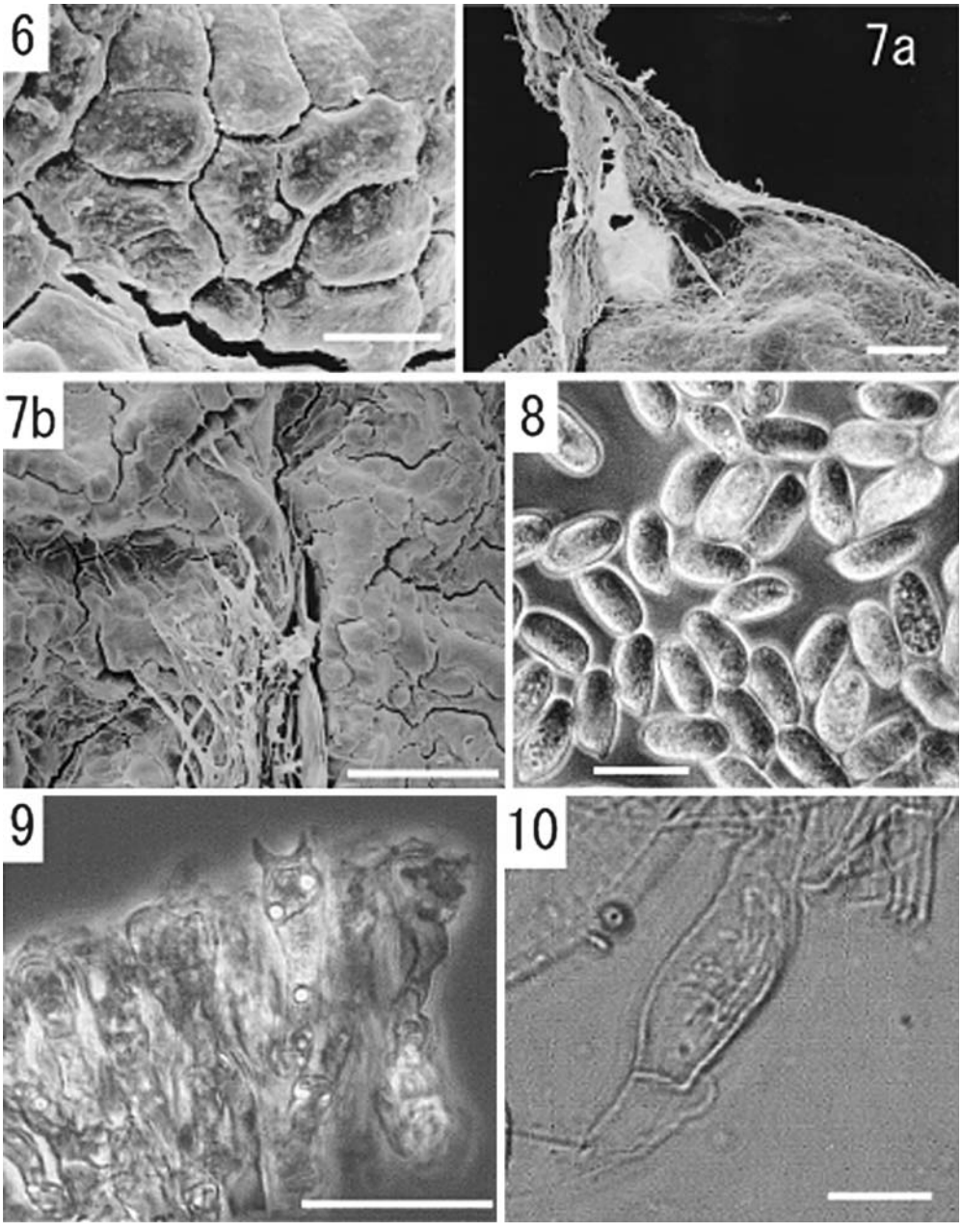
Figs. 2–5. *Typhula maritima* in nature. **2** Foredunes in Ishikarihama. **3**, **4** Basidiocarps (TNS-F-17093). **5** Sclerotia (TNS-F-17094). *Bars* **3** 5 cm; **4**, **5** 1 cm

Holotypus: Japan. Hokkaido: Ishikarihama, Hamacho, Ishikari-shi, on the sands, Nov. 3, 2007, coll. T. Hoshino, kept in the mycological herbarium of National Museum of Nature and Science, Tokyo (TNS-F-17093)

Etymology: Latin, *maritima* = maritime, referring to the habitat of the fungus.

Japanese name: Sunahama-gamanoho-take (sandy beach-bulrush-mushroom, newly named). Ainu name: Pis-un-sikina-karus (sandy beach-present-bulrush-mushroom, newly named).

Habitat: On the sands and sclerotia on *Eylmus mollis* Trin. in foredunes.



Figs. 6–10. Microscopic characteristics of *Typhula maritima*. **6**, **7** Sclerotium and mycelial strand (TNS-F-17094). **8** Basidiospores (TNS-F-17093). **9** Basidia (TNS-F-17093). **10** Medullary hyphae (TNS-F-17093). *Bars* **6** 5 µm; **7a**, **9** 50 µm, **7b** 25 µm; **8** 10 µm; **10** 10 µm

This species is unique in the genus in its habitat. Basidiocarps are produced on the end of relatively long (8–27 mm) mycelial strands emergent from sclerotia. Similar structures to support sporocarps have been reported from various fungal taxa fruiting in coastal dunes (Andersson 1950; Bon 1970; Watling and Rotheroe 1989). Mycelial strands are easily detached from sclerotia by the wind when dry, and sclerotia are dispersed on the sands. Such a feature has not been reported in other species of *Typhula* (Remsberg 1940; Corner 1950; Parmasto 1965; Berthier 1976).

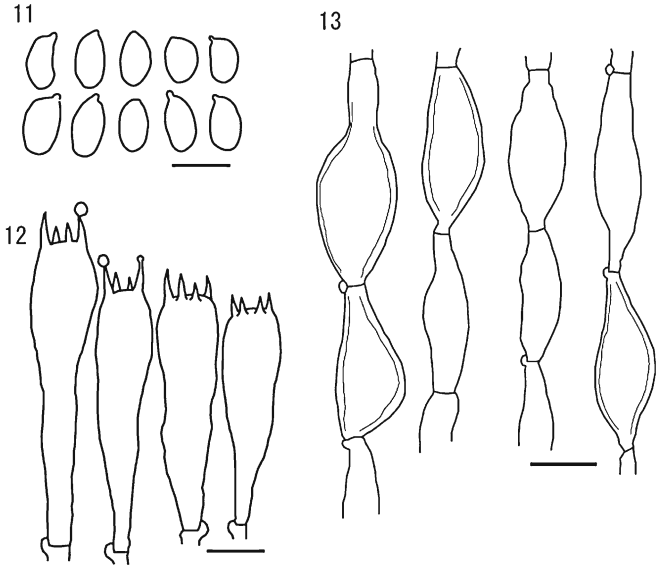
Specimens examined: TNS-F-17093 (holotype) and SAPA 1102 (isotype), basidiocarps from Ishikarihama (Hamacho, Ishikari-shi), Hokkaido, Nov. 3, 2007, coll. T. Hoshino; SAPA 1103, sclerotia from Kotan (Atsuta-ku, Ishikari-shi), Hokkaido, Sept. 27, 2006, coll. M. Ishioka; SAPA 1104, sclerotia from Yoichihama (Sakae-machi, Yoishi-cho), Hokkaido, Oct. 9, 2006, coll. T. Hoshino; TNS-F-17094, sclerotia from Ishikarihama, Apr. 10, 2006, coll. T. Hoshino; TNS-F-18189, basidiocarps from the same locality, Sept. 27, 2004, coll. S. Takehashi; TNS-F-18190, sclerotia from the same locality, Apr. 30, 2006, coll. T. Kasuya; TNS-F-18191, basidiocarps from Muenhama (Atsuta-ku, Ishikari-shi), Hokkaido, Sept. 23, 2006, coll. T. Kasuya; TNS-F-18192, sclerotia from Muenhama, Oct. 3, 2006, coll. S. Takehashi.

Ex-holotype culture: The strain TH-AIST-Tm-1 originated from a sclerotium produced on a mass sporous PDA culture from the holotype. This strain was deposited in Biological Resource Center, National Institute of Technology

and Evaluation (NBRC, NITE), Kazusa, Japan as NBRC 104266.

Phylogenetic analysis

ITS sequences were obtained from *T. maritima* from Ishikarihama (NBRC 104266; AB375430), Kotan (SAPA1103; AB430447), and Yoichihama (SAPA1104; AB430448). Phylogenetic comparisons of our isolates with previous data on ITS sequences of *Typhula* spp. and related genera are shown in Fig. 14. The type strain of *T. maritima* was highly homologous (more than 99%) with other isolates from the same species and was separated from other *Typhula* spp. in the dendrogram based on ITS sequences. DNA sequence data also indicated that this fungus is a new species in the genus *Typhula*.



Figs. 11–13. *Typhula maritima*. **11** Basidiospores (TNS-F-17093). **12** Basidia (TNS-F-17093). **13** Medullary hyphae (TNS-F-17093). *Bars* **11**, **12** 10 μm; **13** 20 μm

Cultural and physiological characteristics

Colonies of *T. maritima* on PDA were white, appressed, and had few aerial mycelia with sclerotia produced in concentric rings near the inoculum. Hyphae had clamp connections. This fungus grew on various media such as PDA, cornmeal agar, lima bean agar, malt extract agar, oatmeal agar, Sabouraud dextrose agar, and tomato juice agar. The ex-holotype, strain NBRC 104266, grew on PDA plates at temperatures between –1°C and 20°C but did not grow at 25°C. The optimal temperature for mycelial growth was 10°C, and mycelial growth rate at 10°C was ~1 mm/day. Stipe-like pletenchymata were observed in cultures that had been kept for more than 3 months at 10°C in a cold room. We did not find basidiocarps from sclerotia on sand, suggesting that germination of sclerotia required moist conditions for a given period.

Typhula maritima had higher halotrophic characteristics than those of the typical snow mold *T. incarnata*, which was distributed in backdunes and grassland in Ishikarihama

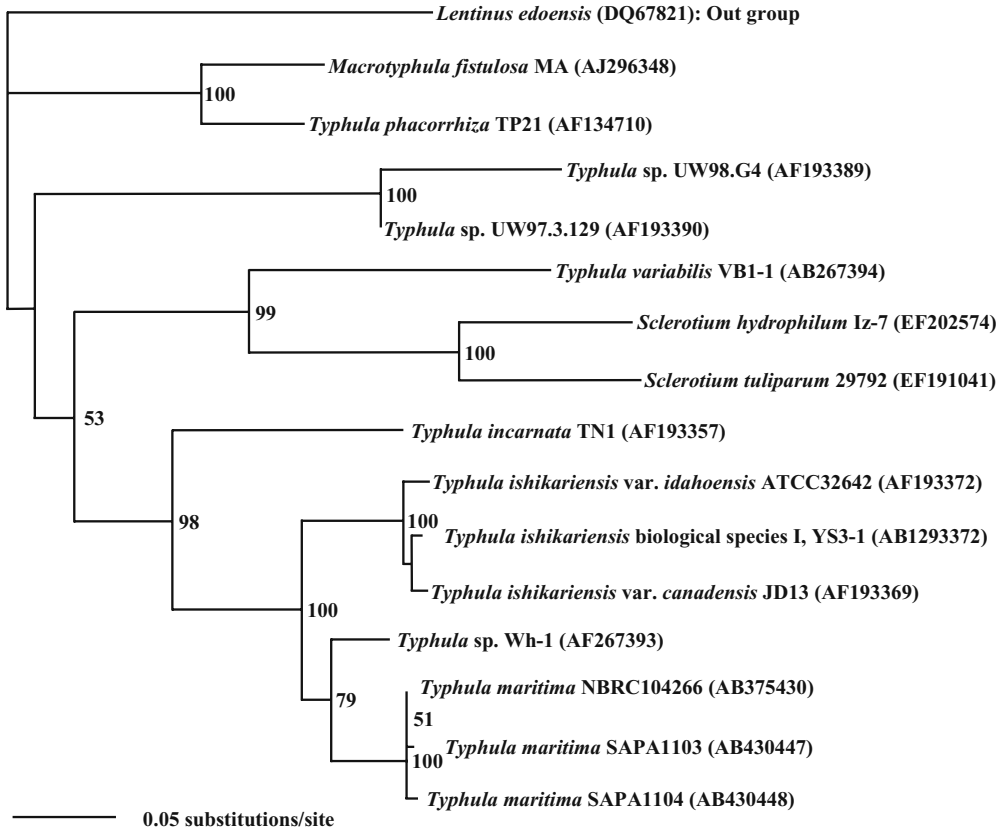


Fig. 14. Neighbor-joining (NJ) tree based on sequences of the internal transcribed spacer 1 (ITS1)-5.8S-ITS2 region for phylogenetically related species of the genus of *Typhula*. Bootstrap percentages (from 1000 replications) greater than 50% are shown at *branch points*. Accession numbers are given in *parentheses*

Table 1. Inoculation test of *Typhula maritima* on dunegrass, *Eylmus mollis*

	Inoculated on:		
	Uninoculated control	Living plants	Dead leaves
Regrowth after inoculation (g dry weight/plant)	28.8	25.3	–
Number of sclerotia produced per plant	0	19.0	12
Reisolation of *T. maritima* from leaves (%)	0	100.0	–

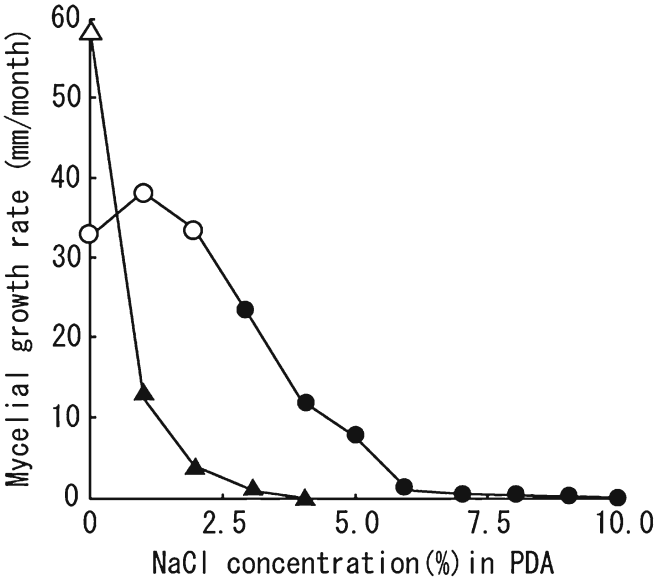


Fig. 15. Effects of NaCl concentration on mycelial growth and sclerotium production of *Typhula maritima* and *T. incarnata*. *Circles and triangles* represent *T. maritima* and *T. incarnata*, respectively. *Open symbols* indicate that sclerotium formation was observed on potato dextrose agar (*PDA*) plates; *closed symbols* indicate that sclerotia were not found in culture plates. Cultures were incubated at 10°C

(Fig. 15). Mycelia of *T. maritima* could survive and grow on PDA plates containing NaCl up to 9%, and optimal mycelial growth was obtained at 1% NaCl. However, production of the sclerotium was inhibited on PDA plates containing NaCl of more than 3%. The mycelial growth rate of *T. maritima* was slower than that of *T. incarnata*, which has phytopathogenic and saprophytic activities. Smaller numbers of basidiocarps and sclerotia of *T. maritima* were found in backdunes including areas adjacent to foredunes. Other species such as *T. incarnata*, *T. ishikariensis*, and *T. phacorrhiza* were also present from backdunes to grassland. The observations indicate a cline in halotolerance among species of *Typhula*.

Numerous sclerotia of *T. maritima* floated in seawater and remained viable, implying that *T. maritima* migrated in the form of sclerotia by storms in the period from summer to autumn and, consequently, its habitat continuously shifted along the wind; the location in Ishikarihama changed each year (see Fig. 1c). A similar mechanism seems to operate in *T. ishikariensis* group II from Oppland, Norway, whose sclerotia are covered by abundant aerial mycelia (Matsumoto et al. 1996). *Typhula maritima* was also probably distributed by marine currents. Other species in *Typhula* did not have halotrophic and floating sclerotia in seawater, and these characteristics represent an adaptation of *T. maritima* to the coastal dune environment.

Pathogenicity

Typhula maritima could grow on living leaves of *E. mollis* under the same conditions as those under snow cover (Table 1). However, regrowth of host plants after inoculation was similar to that of uninoculated, control plants. This fungus also grew on dead leaves of *E. mollis*. Therefore, we concluded that this fungus was not a phytopathogen in natural vegetation but a saprophyte exploiting specific substrates of *E. mollis*.

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