Original peer-reviewed article（原著論文；査読有）

First report of a subhypogeous basidiomycete, *Scleroderma yunnanense* (Boletales) from Japan

半地下生担子菌 *Scleroderma yunnanense*（シラガニセショウロ，イグチ目）の日本初記録

Taiga Kasuya[1*], Kazumasa Imai[2], Kentaro Hosaka[3]

糟谷 大河 [1*], 今井 一雅 [2], 保坂 健太郎 [3]

[1] Department of Biology, Keio University, 4-1-1 Hiyoshi, Kohoku-ku, Yokohama-shi, Kanagawa 223-8521, Japan
慶應義塾大学生物学教室，〒 223-8521 神奈川県横浜市港北区日吉 4-1-1

[2] Shin-ike-cho, Chikusa-ku, Nagoya-shi, Aichi 464-0027, Japan
〒 464-0027 愛知県名古屋市千種区新池町

[3] Department of Botany, National Museum of Nature and Science, 4-1-1 Amakubo, Tsukuba-shi, Ibaraki 305-0005, Japan
国立科学博物館植物研究部，〒 305-0005 茨城県つくば市天久保 4-1-1

* Corresponding author（主著者）
E-mail: tkasuya@keio.jp

Abstract

A subhypogeous sclerodermataceous fungus was collected in Nagoya, Aichi Prefecture, central Japan. Based on morphological observations and phylogenetic analyses using nuclear ribosomal DNA sequences, the present fungus was identified as *Scleroderma yunnanense*, belonging to Sclerodermataceae (Boletales). *Scleroderma yunnanense* is new to Japan and characterized by not stellately dehiscent basidiomata when mature, thick (2–7 mm) peridium covered by cream to pale yellowish scales, basidiomata covered with whitish mycelial strands, clamped hyphae, and globose to subglobose basidiospores densely covered with pyramidal warts.

要旨

愛知県名古屋市において、ニセショウロ型の半地下生菌の一種が採集された。本菌について、形態的特徴の観察および子実体より得られた核リボソーム DNA の塩基配列 を用いた系統解析を行った。その結果、本菌はイグチ目ニセショウロ科に属する *Scleroderma yunnanense* と同定された。本種は日本新産であり、子実体が成熟しても星形に裂開しない点、殻皮が厚く（2–7 mm）、クリーム色～淡黄色を帯びる鱗片に覆われる点、子実体の表面に白色の菌糸束が存在する点、菌糸にクランプ結合を有する点、そして担子胞子が球形～類球形で角錐状のいぼ状突起により密に覆われる点で特徴づけられる。本菌の和名をシラガニセショウロとする。

Article Info: Submitted: 29 December 2022 Accepted: 17 March 2023 Published: 31 July 2023

Introduction

Among ca. 40 known species of the genus *Scleroderma* Pers. (Boletales, Sclerodermataceae), at least five subhypogeous or hypogeous species, i.e., *S. hypogaeum* Zeller, *S. michiganense* (Guzmán) Guzmán, *S. paradoxum* G.W. Beaton, *S. patagonicum* Nouhra & Hern. Caff. and *S. yunnanense* Y. Wang have been recognized in the world (Beaton & Weste, 1982; Guzmán, 1970; Nouhra et al., 2012; Zhang et al., 2013). Although sixteen taxa of the genus were hitherto recorded in Japan, only one species, *S. capeverdeanum* M.P. Martín, M. Dueñas & Tellería has been known as a subhypogeous taxon (Kasuya et al., 2022). During our continuous studies of the species diversity of *Scleroderma* in Japan (Kasuya & Guzmán, 2007; Kasuya et al., 2002, 2022), several subhypogeous basidiomata of sclerodermataceous fungus were collected from Nagoya, Aichi Prefecture. Based on morphological observations and phylogenetic analyses using nuclear ribosomal DNA sequences, the present fungus was identified as *S. yunnanense*. This species was originally described from China and it is known as a subhypogeous species (Zhang et al., 2013). Our morphological observations and phylogenetic analyses of the Japanese specimen revealed that it is the first distributional record of *S. yunnanense* in Japan.

Materials and methods

Sample collecting and morphological observations

Fresh subhypogeous basidiomata were collected from fallen leaves of broad-leaved trees and rich soil in Heiwagaoka Park, Meito-ku, Nagoya, Aichi Prefecture in July 2021. Basidiomata were photographed and observed macroscopically. Fresh basidiomata were air-dried using a food dehydrator (Snackmaster Express FD-60; Nesco/American Harvest, Milwaukee, WI, USA) under 46°C for 46 hours. For light microscopy, hand-cut sections of dried basidiomata were mounted in 3% (w/v) KOH or 70% (v/v) ethanol reagent. Dimensions of basidiospores were measured from KOH-mounted sections. Fifty randomly selected basidiospores were measured under a light microscope at 1000× magnification. All measurements were performed with Photoruler 1.1.3 (http://inocybe.info/_userdata/ruler/PhotoRuler.html). In addition, the surface features of basidiospores were observed by scanning electron microscopy (SEM). For SEM, a small portion from glebal tissue was put onto double-sided adhesive tape on a specimen holder and coated with platinum-palladium using a JFC-1600 Ion Sputter Coater (JEOL, Tokyo, Japan). Specimen was examined with a JSM-6480LV SEM (JEOL, Tokyo, Japan) operating at 10 kV. Specimen examined in this study was deposited at the mycological herbarium of National Museum of Nature and Science (TNS) in Japan.

DNA preparation, PCR and sequencing

DNA extraction, PCR and DNA sequencing of the specimen examined in the present study were carried out according to the methods introduced by Kasuya et al. (2012, 2022). DNA sequence data were obtained from the nuclear ribosomal internal spacer region (ITS) and a part of the large subunit gene (LSU). For amplifying the ITS region, the primer combination of ITS5 and ITS4 (White et al., 1990) was used. For amplifying the LSU, the combination of LR0R and LR5 (Vilgalys & Hester, 1990) was used. PCR and sequencing were carried out following the same methods previously described in Kasuya et al. (2012, 2022). A total of two newly generated sequences from this study were deposited in GenBank (Table 1).

Phylogenetic analyses

Newly generated ITS and LSU sequences from the Japanese specimen were used for the phylogenetic analyses. Additionally, 30 ITS and eight LSU sequences of *Scleroderma* were retrieved from the NCBI GenBank databases (https://www.ncbi.nlm.nih.gov/) and included in the analyses (Table 1). DNA sequences were initially aligned using Muscle v.3.6 (Edgar, 2004), followed by manual alignment in the data editor of BioEdit ver. 7.0.1 (Hall, 1999). A total of 66 ITS and 98 LSU nucleotide positions were respectively excluded from the analyses because of the presence of ambiguously aligned regions. Phylogenetic analyses were performed for the combined dataset of ITS and LSU sequences under maximum parsimony (MP) and maximum likelihood (ML) criteria. MP analysis was conducted by PAUP version 4.0b10 (Swofford, 2002) following the same method reported by Kasuya et al. (2022). ML analysis of the combined dataset of ITS and LSU was performed using MEGA X (Kumar et al. 2018) after testing the best models according to the methods previously introduced by Kasuya et al. (2022). Tamura 3-parameter (Tamura, 1992) with gamma-distributed rate heterogenetic and a proportion of invariant sites (T92+G+I) was chosen as the optimal substitution model for the ML analysis of the combined ITS and LSU dataset. Three sequences of *S. areolatum* Ehrenb. (Table 1) were selected for outgroups, which were strongly supported as the sister of the major clade containing *S. yunnanense* (Ortiz-Rivero et al., 2021).

Results

Morphological observations

The specimen examined was morphologically identical to *S. yunnanense* (Zhang et al., 2013) in its following features: (1) irregularly dehiscent basidiomata when mature, (2) thick (2–7 mm)

Table 1. Specimen identification, voucher specimen and/or isolate numbers, origin and GenBank accession numbers for ITS and LSU sequences used for the present phylogenetic analyses

Species names	Herbarium voucher; isolate	Origin	GenBank accession numbers	
			ITS	LSU
Scleroderma anomalosporum	INPA 271001; n/a*	Brazil	KX792084	n/a*
S. areolatum	K (M) 125392; n/a	UK: England	EU784407	n/a
S. areolatum	TNS F-82295; n/a	Japan: Chiba, Choshi	OQ025272	OQ025269
S. areolatum	INM 2-213463; n/a	Japan: Ibaraki, Kamisu	OQ025273	OQ025270
S. australe	MEL 269386; n/a	Australia	MT270609	n/a
S. bermudense	BZ3961; n/a	Belize	EU718118	DQ644137
S. camassuense	INPA 271114; n/a	Brazil	KX792085	n/a
S. dictyosporum	n/a; IR215	Burkina Faso	FJ840443	n/a
S. dictyosporum	SD-4901; n/a	Burkina Faso	FJ840449	n/a
S. duckei	INPA 272127; n/a	Brazil	KX792086	n/a
S. duckei	UFRN Fungos-2795; n/a	Brazil	KX792087	n/a
S. geaster	MA-Fungi 34025; n/a	Spain	MT270638	n/a
S. geaster	MA-Fungi 68558; n/a	Spain	MT270642	n/a
S. geaster	E 157436; n/a	Greece	MT270644	n/a
S. guzmanii	IBUG J.A. Garcia Valle 24; n/a	Mexico	MT270645	n/a
S. guzmanii	OSC 41254; n/a	USA	MT270648	n/a
S. polyrhizum	MA-Fungi 32214; n/a	Spain	MT270660	n/a
S. polyrhizum	ILLS 56824; n/a	USA	MT270661	n/a
S. polyrhizum	MA-Fungi 39352; n/a	Spain	MT270662	n/a
S. sinnamariense	n/a; n/a	India: Southwestern Ghats	AB908177**	AB908177**
S. texense	AWW216; n/a	USA	EU718123	EU718153
S. texense	F C0296202F; n/a	USA	MT270649	n/a
S. texense	VPI F-0004156; n/a	USA	MT270650	n/a
S. xanthochroum	AWW311; n/a	Malaysia	EU718126	EU718154
S. yunnanense	KUN-HKAS 79633A; Ji001A	China: Yunnan	JQ639040	n/a
S. yunnanense	KUN-HKAS 79633B; Ji001B	China: Yunnan	JQ639041	n/a
S. yunnanense	KUN-HKAS 79633C; Ji001C	China: Yunnan	JQ639402	n/a
S. yunnanense	KUN-HKAS 79633D; Ji001D	China: Yunnan	JQ639403	n/a
S. yunnanense	KUN-HKAS 80386; n/a	China	MW493647	MW493703
S. yunnanense	**TNS F-82294; n/a**	**Japan: Aichi, Nagoya**	**OQ025271*****	**OQ025268*****
Scleroderma sp.	G509; n/a	Guyana	KJ786660	KJ786558

*"n/a" means information not available.
**Identical accession number for ITS and LSU indicates a single DNA sequence containing both regions.
***Sequences newly generated in the present study.

peridium covered by cream to pale yellowish scales (Fig. 1A–B), (3) well-developed whitish mycelial strands on the surface of basidiomata (Fig. 1C), (4) clamped hyphae (Fig. 1D–E), and (5) globose to subglobose basidiospores densely covered with pyramidal warts (Fig. 1F–G). A detailed description and illustrations of the salient features of Japanese specimens are given below.

Phylogenetic analyses

The The combined dataset of ITS and LSU consisted of 28 ingroups and three outgroups. It had an aligned in length of 1,808 characters inclusive of gaps, of which 164 characters were constant, 1,552 variable and phylogenetically uninformative, and 256 phylogenetically informative. The MP analysis of the combined ITS and LSU dataset yielded 10,000 most parsimonious trees, of which 3,384 trees were found in the first step of the heuristic search. The consistency index, retention index, and rescaled consistency index of the most parsimonious trees are 0.6349, 0.8268, and 0.5249, respectively. The highest log-likelihood of the resulting ML tree of the combined ITS and LSU dataset is -5316.67. The MP and ML analyses resulted in trees that were almost identical in topology. Hence, only the MP tree topology of the combined ITS and LSU dataset is shown in Fig. 2.

The combined ITS and LSU sequences generated from a Japanese specimen and Chinese samples of *S. yunnanense* were placed within a strongly supported clade in both MP and ML phylogenies [MP BS (%) /ML BS (%) =100/100; Fig. 2], and were distinct from those of the other *Scleroderma* species. Present phylogenetic analyses support the identity of Japanese and Chinese *S. yunnanense* based on morphology.

Taxonomy

***Scleroderma yunnanense* Y. Wang**, Mycotaxon 125: 195 (2013).

Fig. 1.

Description: Basidiomata (Fig. 1A–B) subhypogeous, globose, depressed globose to subglobose, 3–8 cm broad, dirty white, cream to pale yellowish brown, sessile or rarely with a short pseudostipe up to 1 cm long, often covered with well-developed whitish mycelial strands (Fig. 1C), arising from whitish mycelial tuft attached to soil. Mycelial

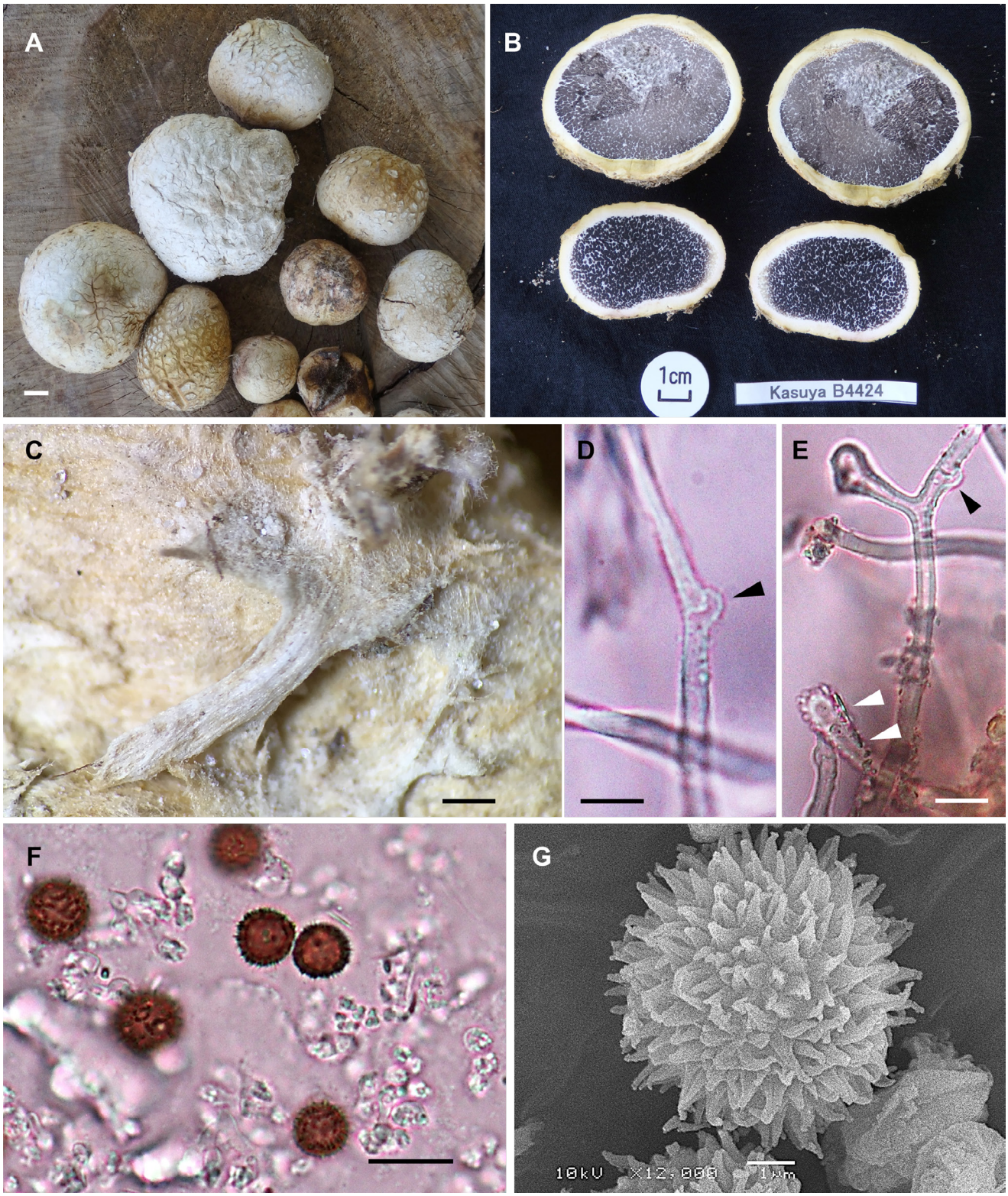

Fig. 1. Morphological features of *Scleroderma yunnanense* (TNS-F-82294). A: Surface of basidiomata. B: Vertical sections of mature basidiomata showing peridium and gleba. C: Whitish mycelial strands on the surface of basidiomata. D: Hypha with a clamp-connection (black arrow) of mycelial strands on the surface of a basidioma. E: Mycelial strands on the surface of a basidioma composed of clamped (black arrow) hyphae sparsely covered with amorphous, hyaline crystalline materials (white arrows). F: Basidiospores. G: Scanning electron microscopy image of a basidiospore. Bars: A = 1 cm; C: 1 mm; D, E = 5 μm; F = 10 μm; G = 1 μm.

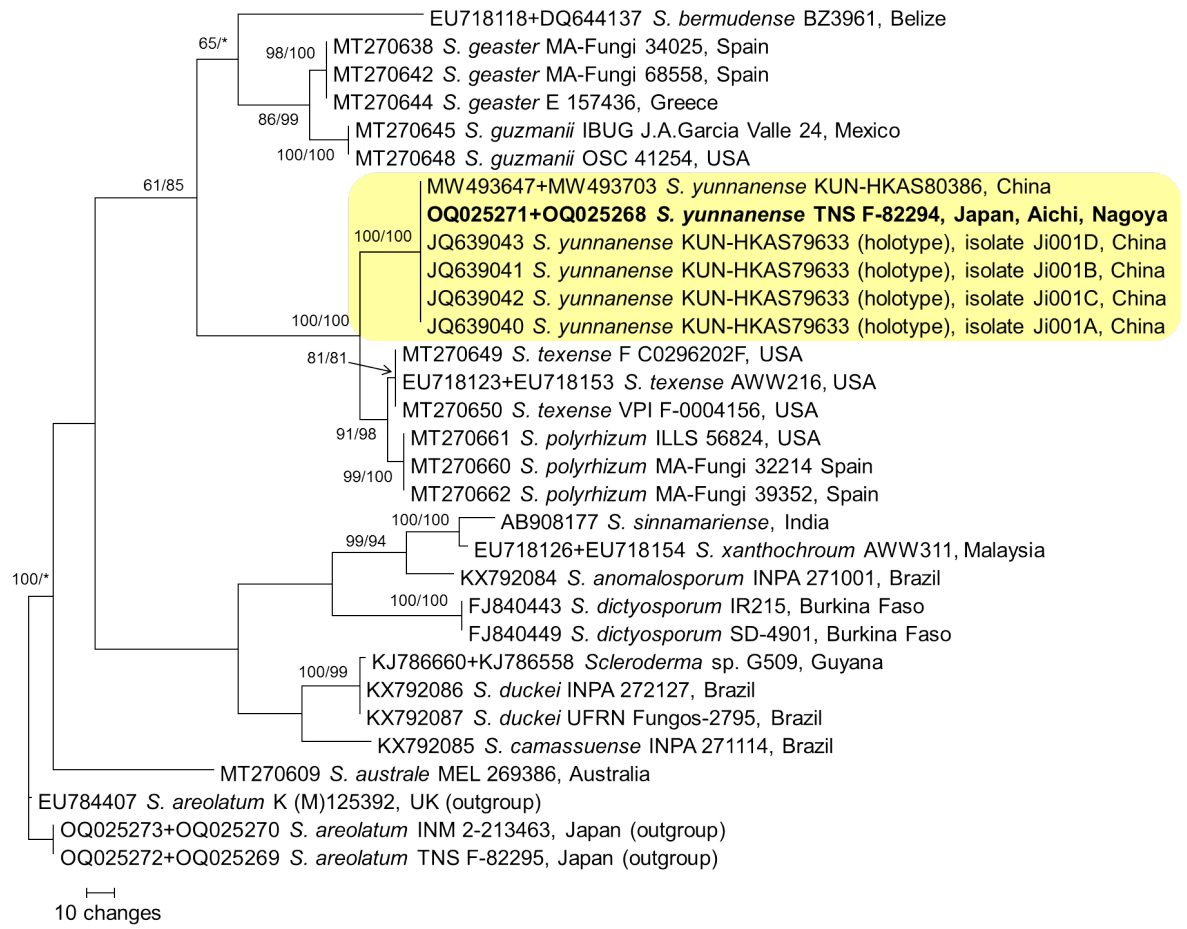

Fig. 2. A phylogenetic tree generated from maximum parsimony (MP) analysis based on the combined dataset of the nuclear rDNA ITS region and LSU sequences of selected members of Sclerodermataceae. Taxon names indicated by bold are newly generated sequences from the present study. Bootstrap support values (BS) of MP and maximum likelihood (ML) greater than 60% are shown for each node (MP/ML), and BS less than 60% are indicated by an asterisk (*).

strands on surface of basidiomata (Fig. 1D–E) interwoven, septate, thin-walled, 2–5 µm broad, hyaline in 3% KOH and 70% ethanol reagent, with numerous clamp-connetctions; surface sometimes sparsely covered with amorphous, hyaline crystalline materials (Fig. 1E). Peridium (Fig. 1A–B) thick, ca. 2–7 mm thick, two-layered. Exoperidium thin (up to ca. 0.5 mm thick), pale yellowish brown to pale brown, covered with cream to pale yellow scales. Endoperidium thick, firm, pale yellowish brown to cream near exoperidium, white near gleba. Apical part of peridium irregularly dehiscent but not stellate when mature. Gleba (Fig. 1B) firm, white to cream when young, then becoming purplish gray, grayish brown to blackish brown with whitish capillitial threads, finally powdery when mature. Rudimentary hyphae of gleba interwoven, septate, thin-walled, 1–2.5 µm broad, hyaline in 3% KOH and 70% ethanol reagent; surface sparsely covered with amorphous, hyaline gelatinous materials. Basidia not observed. Basidiospores (Fig. 1F–G) globose to subglobose, 6.5–8 µm in diam. (mean = 7.6 µm, n = 50) including surface ornamentation, dark brown in 3% KOH (Fig. 1F), dark reddish brown in 70% ethanol reagent; surface densely echinulate composed of pyramidal warts up to 1.5 µm long (Fig. 1G).

Habitat: Subhypogeous, small groups or gregarious among fallen leaves, mosses and rich soil under *Quercus glauca* Thunb. trees. Fruiting in the Japanese locality occurs in summer (July).

Specimens examined: JAPAN, Aichi Prefecture, Nagoya, Meito-ku, Heiwagaoka (approx. 35°10'39"82N, 136°58'54"20E, alt. approx. 64 m asl.), July 16, 2021, coll. K. Imai, TNS-F-82294, GenBank accession no.: OQ025271 (ITS), OQ025268 (LSU).

Known distribution: China (Zhang et al., 2013) and Japan (new record).

Japanese name: *Shiraga-nise-shoro* (newly proposed here; "*Shiraga*" = white hair in Japanese, referring to the whitish mycelial strands on the surface of basidiomata; "*nise-shoro*" = the Japanese name of *Scleroderma*).

Discussion

As outlined above, the Japanese specimen was morphologically almost identical to the original description of *S. yunnanense* (Zhang et al., 2013). Moreover, our phylogenetic analyses (Fig. 2) show the monophyly of the present species, as indicated by Zhang et al. (2013) and Ortiz-Rivero et al. (2021). The present species is morphologically similar to *S. cepa* Pers. in its echinulate basidiospores and thick peridium (Guzmán, 1970; Coccia et al., 1990), but is clearly distinguishable by its clampless hyphae. The present phylogenetic analyses demonstrate that *S. polyrhizum* (J.F. Gmel.) Pers. and *S. texense* Berk. are closely related to *S. yunnanense* (Fig. 2). Morphologically, these two species differ from *S. yunnanense* by subreticulate to reticulate basidiospores (Ortiz-Rivero et al., 2021).

Zhang et al. (2013) reported that specimens of *S. yunnanense* were collected under *Pinus kesiya* var. *langbianensis* (A. Chev.) Gaussen ex Bui and *Betula alnoides* Buch.-Ham. ex D.Don in China. The Japanese specimen was collected under *Quercus glauca* trees. While it is unclear whether the mycorrhizal hosts of the present species are varied among geographically distant regions, *S. yunnanense* presumably forms ectomycorrhizae with pinaceous, betulaceous, or fagaceous trees.

Scleroderma yunnanense is known as an edible fungus and is widely consumed by the local people in the Yunnan area of China (Zhang et al., 2013). Further investigations are required to clarify whether the Japanese population of the present species is also edible or not.

Acknowledgements

We are very much obliged to Ms. Kyung-Ok Nam and Ms. Megumi Otsuka of the National Museum of Nature and Science for help with assisting molecular experiments. We also thank Ms. Nako Hirata for facilitating the fieldwork. We are grateful to Dr. Tamao Hatta of Chiba Institute of Science for his skillful technical assistance and for helpful suggestions to SEM observation. This work was supported in part by JSPS KAKENHI Grant Number JP20K06805.

References

Beaton G., Weste G. (1982) A new species of *Scleroderma* from Victoria, Australia. Transactions of the British Mycological Society 79: 41–43.

Coccia M., Migliozzi V., Lavorato C. (1990) Studio sul genere *Scleroderma* Persoon. Bollettino dell' Associazione Micologia ed Ecologica Romana 20–21: 3–59.

Edgar R.C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research 32: 1792–1797.

Guzmán G. (1970) Monografia del género *Scleroderma* Pers. emend. Fr. (Fungi-Basidiomycetes). Darwiniana 16: 233–407.

Hall T.A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41: 95–98.

Kasuya T., Guzmán G. (2007) *Veligaster nitidum*, a pantropical sclerodermataceous fungus new to Japan and Thailand. Mycoscience 48: 259–262.

Kasuya T., Guzmán G., Ramírez-Guillén F., Kato T. (2002) *Scleroderma laeve* (Gasteromycetes, Sclerodermatales), new to Japan. Mycoscience 43: 475–476.

Kasuya T., Hosaka K., Uno K., Kakishima M. (2012) Phylogenetic placement of *Geastrum melanocephalum* and polyphyly of *Geastrum triplex*. Mycoscience 53: 411–426.

Kasuya T., Uzawa M., Hosaka K. (2022) *Scleroderma capeverdeanum*, a subhypogeous fungus new to Japan, collected from the *Eucalyptus* plantation in Ibaraki Prefecture. Truffology 5: 23–32.

Kumar S., Stecher G., Li M., Knyaz C., Tamura K. (2018) MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Molecular Biology and Evolution 35: 1547–1549.

Nouhra E.R., Caffot M.L.H., Pastor N., Crespo E.M. (2012) The species of *Scleroderma* from Argentina, including a new species from the *Nothofagus* forest. Mycologia 104: 488–495.

Ortiz-Rivero J., Watling R., Guzmán-Dávalos L., Martín M.P. (2021) The many-rooted earthball—*Scleroderma geaster* and *S. polyrhizum* revisited, with the description of a new species. Phytotaxa 510: 1–17.

Swofford D.L. (2002) PAUP*. Phylogenetic analysis using parsimony (*and other methods). MA Sinauer Associates, Sunderland.

Tamura K. (1992) Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G+C-content biases. Molecular Biology and Evolution 9: 678–687.

Vilgalys R., Hester M. (1990) Rapid genetic identification and mapping of enzymatically amplified DNA from several *Cryptococcus* species. Journal of Bacteriology 172: 4238–4246.

White T.J., Bruns T., Lee S., Taylor J.W. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M.A., Gelfand D.H., Sninsky J.J., White T.J. (eds.) PCR protocols. Academic Press, New York, pp. 315–322.

Zhang C., Xu X.-E., Liu J., He M., Wang W., Wang Y., Ji K. (2013) *Scleroderma yunnanense*, a new species from South China. Mycotaxon 125: 193–200.