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

A new distributional record of *Elaphomyces fuscus* from Japan: as a new host of *Tolypocladium japonicum*

ゴマタマツチダンゴの日本における新たな分布記録：タンポタケモドキの新宿主として

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Abstract

An *Elaphomyces* species parasitized by *Tolypocladium* sp. was observed in Itako, Ibaraki Prefecture, central Japan. Based on morphological observations and molecular identification using nuclear ribosomal DNA sequences of both *Elaphomyces* and *Tolypocladium* spp., the present fungus and its parasite were identified as *Elaphomyces fuscus* and *Tolypocladium japonicum*, respectively. We report *E. fuscus* as a first record outside the type locality and as a new host of *T. japonicum*.

要旨

茨城県潮来市において、トリポクラディウム属菌が寄生したツチダンゴ属菌の一種が観察された。本菌ならびにトリポクラディウム属菌のそれぞれについて、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分子同定を行った。その結果、本菌はゴマタマツチダンゴ *Elaphomyces fuscus*、寄生していたトリポクラディウム属菌はタンポタケモドキ *Tolypocladium japonicum* とそれぞれ同定された。ゴマタマツチダンゴを基準標本産地以外から初めて記録するとともに、タンポタケモドキの新宿主として報告する。

Article Info: Submitted: 18 May 2023 Accepted: 10 January 2024 Published: 31 May 2024

Introduction

Among ca. 20 known species of the genus *Elaphomyces* T. Nees (Eurotiales, Elaphomycetaceae) in Japan, *E. fuscus* M. Shirakawa and *E. marmoratus* M. Shirakawa have been described recently as new taxa (Hatakeyama & Orihara, 2022; Shirakawa & Tanaka, 2020). These two species were described based on specimens collected in secondary mixed forests of Ome, Tokyo, and known only from the type locality (Shirakawa & Tanaka, 2020). In 2022, a mushroom foray organized by Ibaraki Nature Museum was held in Suigo Prefectural Forest Park, Itako, Ibaraki Prefecture, central Japan. During the foray, several collections of *Tolypocladium* sp. (Hypocreales, Ophiocordycipitaceae) parasitized to fruiting bodies of *Elaphomyces* sp. were obtained by its participants (Fig. 1A, B). Simultaneously ascomata of *Elaphomyces* sp. without stromata of *Tolypocladium* sp. were also collected at the same locality (Fig. 1C). Based on morphological observations and molecular identification

Fig. 1. Fruiting bodies of *Tolypocladium japonicum* and *Elaphomyces fuscus*. A: Stromata of *T. japonicum* in the natural habitat (INM-2-229315). B: Stromata of *T. japonicum* arising from fruiting bodies of *E. fuscus*. (INM-2-229315). C: Immature ascomata of *E. fuscus* (INM-2-229316).

using nuclear ribosomal DNA sequences of both *Elaphomyces* and *Tolypocladium* spp. specimens, they were identified as *E. fuscus* and *Tolypocladium japonicum* (Lloyd) C.A. Quandt, Kepler & Spatafora, respectively. Our morphological observations and molecular identification of both specimens revealed that it is the first distributional record of *E. fuscus* outside the type locality along with a new host fungus of *T. japonicum*.

Materials and methods

Sample collecting and morphological observations

Fresh samples were collected in secondary mixed forests dominated by *Quercus serrata* Murray and *Pinus densiflora* Siebold & Zucc. at Suigo Prefectural Forest Park, Itako, Ibaraki Prefecture in July 2022. Fresh samples were photographed and observed macroscopically and then they were air-dried using a food dehydrator (Snackmaster Express FD-60; Nesco/American Harvest, Milwaukee, WI, USA) under 46 °C for 46 hours. Dried specimens examined in this study were deposited at the mycological herbaria of Ibaraki Nature Museum (INM) and National Museum of Nature and Science (TNS) in Japan.

For light microscopy, dried fungal tissues were revived with water and fixed with neutral buffered 10% formalin. The fixed tissues were dehydrated, replaced with xylene, and then embedded with paraffin. Deparaffinized thin sections (6 µm thick) were stained with hematoxylin and eosin (Feldman & Wolfe, 2014), a routine staining method for formalin-fixed paraffin-embedded (FFPE) tissue sections (Bass et al., 2014; Van den Tweel et al., 2010). Measurements of various lengths were done on photomicrographs of the FFPE fungal sections and analyzed with a free image analyzing application, Image J (Abramoff et al., 2004; Schneider et al., 2012). Measurements of the sizes of ascospores of *Elaphomyces* specimens, which were much larger than the width of fungal tissue sections, were done on photomicrographs of ascospores smeared on a glass slide mounted with 3% KOH. In addition, the surface features of the ascospores were observed by scanning electron microscopy (SEM). For SEM, a small portion from spore mass 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.

DNA preparation, PCR and sequencing

DNA extraction, PCR and DNA sequencing of specimens examined in the present study were carried out according to the methods introduced by Kasuya et al. (2012, 2022). DNA was

extracted from an inner peridium and/or a gleba of *Elaphomyces* sp., and fertile portions of *Tolypocladium* specimens. DNA sequence data were obtained from the internal trascribed spacer region (ITS) and a part of the large subunit (LSU) of the nuclear rDNA. 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.

Molecular identification

Newly generated ITS and LSU sequences from Japanese specimens were used for DNA barcoding for species identification. The obtained raw sequences were assembled using ATGC version 6.0.1 (GENETYX Corporation, Tokyo, Japan). Assembled sequences were analyzed using GenBank (https://www.ncbi.nlm.nih.gov/genbank/) Basic Local Alignment Search Tool (BLAST) search (blastn) to confirm their phylogenetic affinities. Default settings of blastn option were used for the analyses. A total of four newly generated and assembled sequences from this study were deposited to the International Nucleotide Sequence Databases (INSD; accession nos.: OQ991235–OQ991238).

Results

Morphological observations

One to four stromata of *Tolypocladium* sp. grown directly from underground ascomata of *Elaphomyces* sp. (Fig. 1A, B), they were clavate, unbranched, with a slightly compressed apex, 5–20 mm long, 5–7 mm broad at fertile parts which were tan-colored to blackish brown, on a cylindrical stipe, surface roughened by ostioles, solid in the center but holes possibly eaten by fly larvae were observed (Figs 1A and 2A, B). Stipes were 25–65 mm long, 2–5 mm broad, cylindrical, sometimes bifurcate, white, cream or pale yellow to pale gray, lacking the rhizomorphous structure on the basal part (Figs. 1B and 2A). Perithecia were wholly or partially immersed, ovoid to ellipsoid, 400–450 ×130–180 µm, with papillate ostioles. Ascospores were hyaline, long cylindrical, disarticulated into part-spores within asci; part spores were cylindrical, 10–20 × 3.0–3.4 µm (Fig. 2C). In addition to the stipe of *Tolypocladium* sp., another two primordia of possible this fungus were visible in the FFPE thin sections (Fig. 2D).

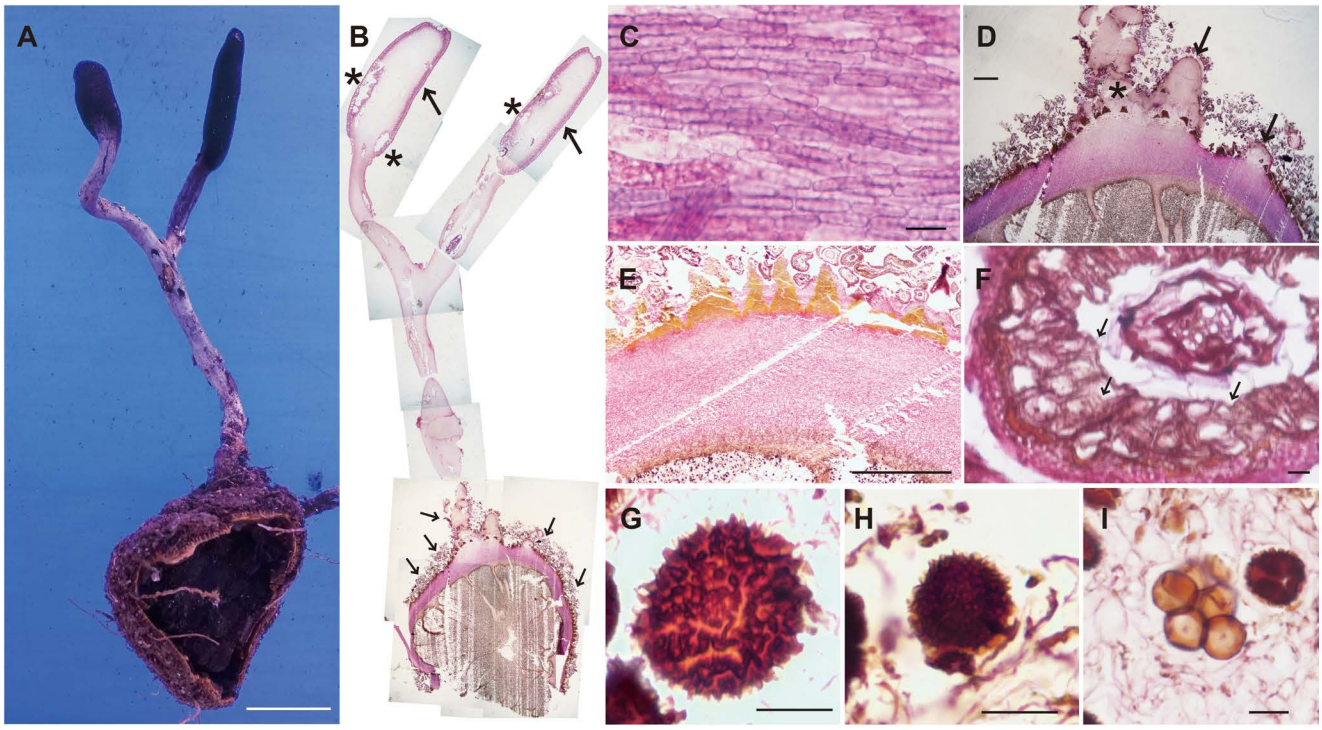

Fig. 2. Morphological features of *Elaphomyces fuscus* with mature stromata of *Tolypocladium japonicum* (INM-2-229315). A: The ascoma of *E. fuscus* with stromata of *T. japonicum* after formalin-fixation. B–I: Whole structures of ascomata of *E. fuscus* with stromata of *T. japonicum* showing by the formalin-fixed and paraffin-embedded (FFPE) thin (6 µm thick) sections stained with hematoxylin and eosin. B: Layers of perithecia just beneath the surface of the fertile parts of *T. japonicum* (large arrows), ectomycorrhizal root tips (small arrows) and the inner structures of *E. fuscus* are clearly visible. Asterisks show holes possible eaten by fly larvae in the stroma of *T. japonicum*. C: Segmentalized and articulated ascospores of *T. japonicum*. D: A stipe of mature stroma of *T. japonicum* growing out of the surface of *E. fuscus* (asterisk), with another two primordia (arrows) of possible *T. japonicum* in its vicinity. E: Peridium and surface warts of the ascoma of *E. fuscus*. F: The vertical section of ectomycorrhizal roots covering the ascoma of *E. fuscus*. Formation of the Hartig net beyond the epidermis to enclose several layers of cortical cells are frequently observed (arrows). G: A large-sized ascospore of *E. fuscus*. H: A small-sized ascospore of *E. fuscus*. I: An ascus of *E. fuscus* containing five ascospores or more. Spores do not show ornamentation. Bars: A = 1 cm; C, F–I: 10 µm; D, E = 1 mm.

Underground ascomata of *Elaphomyces* sp. parasitized by *Tolypocladium* sp. were 20–25 × 22–25 mm, globose, subglobose to oval, rigid and covered with soil, earthy crust and ectomycorrhizal roots (Figs. 1B and 2A, B). Warts of ascomata surfaces were conical, dark yellow, partly with pale purplish tinge, the peridium was white in the outer layer and brown in the inner layer, not marbled, and the inside of the peridium was packed with powdery, dark brown to blackish gleba (Figs. 1B and 2A, B). The warts were dark yellow under microscopy, 334 ± 81.0 µm high, and were covered with thin (80–150 µm) layer of hyaline hyphae on the peridium (Fig. 2E). The peridium was composed of compactly woven hyaline hyphae, outer layer was composed of hyaline hyphae, and inner layer was of brown hyphae. The membranous septae radiated from the center and connected to the peridium (Fig. 2B, D). An inner gleba was packed with numerous dark brown to blackish ascospores and sparsely distributed hyalinous hyphae which presumably originated from parasitized *Tolypocladium* (Fig. 2B, D and E). Ascospores were globose to subglobose, and the mean of the quotient was 1.08 ± 0.06 µm. The dispersion of spore lengths (mean = 24 µm, median = 26 µm) was high, (15–) 20–28 (–33) µm (Fig. 3, box-whisker plots of INM-2-229315) with ornamentation, and the distribution pattern did not show Gaussian but they were biphasic (Fig. 3, scatter plots of INM-2-229315) with larger and smaller ones. Surfaces of large-sized ascospores were reticulate, 1.5–3.0 µm high, dark reddish brown under the microscope, and apices of each reticulation were conical, capitulate or obtuse head and irregularly shaped, broadly base to costate with labyrinthine pattern (Fig. 2G). Surfaces of small-sized ascospores were also dark reddish brown under microscopy, and reticulate with conical, capitulate or obtuse tips and with polygonal to labyrinthine bases, 1.5–2.5 µm high (Fig. 2H). A few, 12–15 µm in lengths of asci containing smooth ascospores were observed (Fig. 2I).

There were numerous ectomycorrhizal roots (Figs. 1B and 2B, F) both on the surface of the *Elaphomyces* ascomata and at the stipe base of *Tolypocladium* sp., even around the primordia of possible this fungus (Fig. 2B, D). Ectomycorrhizal root tips were directly connected to the hyphae both on the *Elaphomyces* and *Tolypocladium* fruiting bodies. The vertical section of a mycorrhizal tip covering the ascoma of *E. fuscus* (Fig. 2F) clearly shows formation of the Hartig net beyond the epidermis to enclose several layers of cortical cells and it suggests the typical ectomycorrhizal structure of gymnosperm trees (Smith & Read, 2008).

Underground *Elaphomyces* ascomata without stromata of *Tolypocladium* sp. were 8–23 × 8–18 mm, globose, subglobose to depressed-globose, rigid and covered with soil, earthy crust and ectomycorrhizal roots (Figs. 1C and 4A). Warts of ascomata surfaces were conical, dark yellow, partially with pale purplish tinge; the outer part of the peridium was white, partially with purplish tinge, and the inner part of the peridium was brown, not marbled; the inside of the peridium was packed with gleba which were compact and white to brown when immature, but becoming powdery and black-brown at maturity (Figs. 1C and 4A). Ascomata have short protrusions on the top, which turned out to be the possible primordia of *Tolypocladium* stromata by the thin FFPE sections, although we have not sequenced this possible primordia. (Fig. 4B). Ascospores were globose to subglobose, and the mean of the quotient was 1.12 ± 0.14 µm. The dispersion of spore lengths (mean = 20 µm, median = 18 µm) was (11–) 17–23 (–31) µm (Fig. 3, box-whisker plots of INM-2-229316) with ornamentation, and similar to the case of the infected ascomata, the distribution pattern showed biphasic (Fig. 3, scatter plots of INM-2-229316 and Fig. 4C). Both the mean and the median of ascospores of *Elaphomyces* sp. without mature *Tolypocladium* stromata were significantly smaller than those with mature stromata of *Tolypocladium* sp. Further, numbers

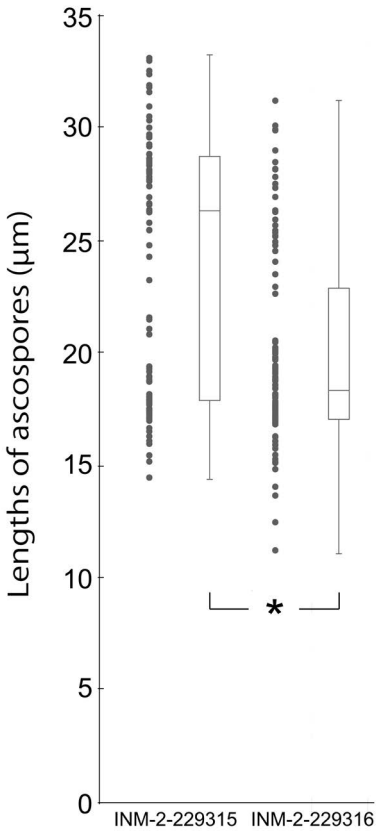

Fig. 3. Scattered plots and box-whisker plots showing the distribution or dispersion of spore lengths of *E. fuscus* with (INM-2-229315, n = 87) and without (INM-2-229316, n = 107) mature stromata of *T. japonicum*. Scatter plots show biphasic patterns of the distribution. Box and whisker plots show the high dispersion and the difference of medians. An asterisk (*) indicates the significant difference ($p = 0.0000039$) using Mann-Whitney U test.

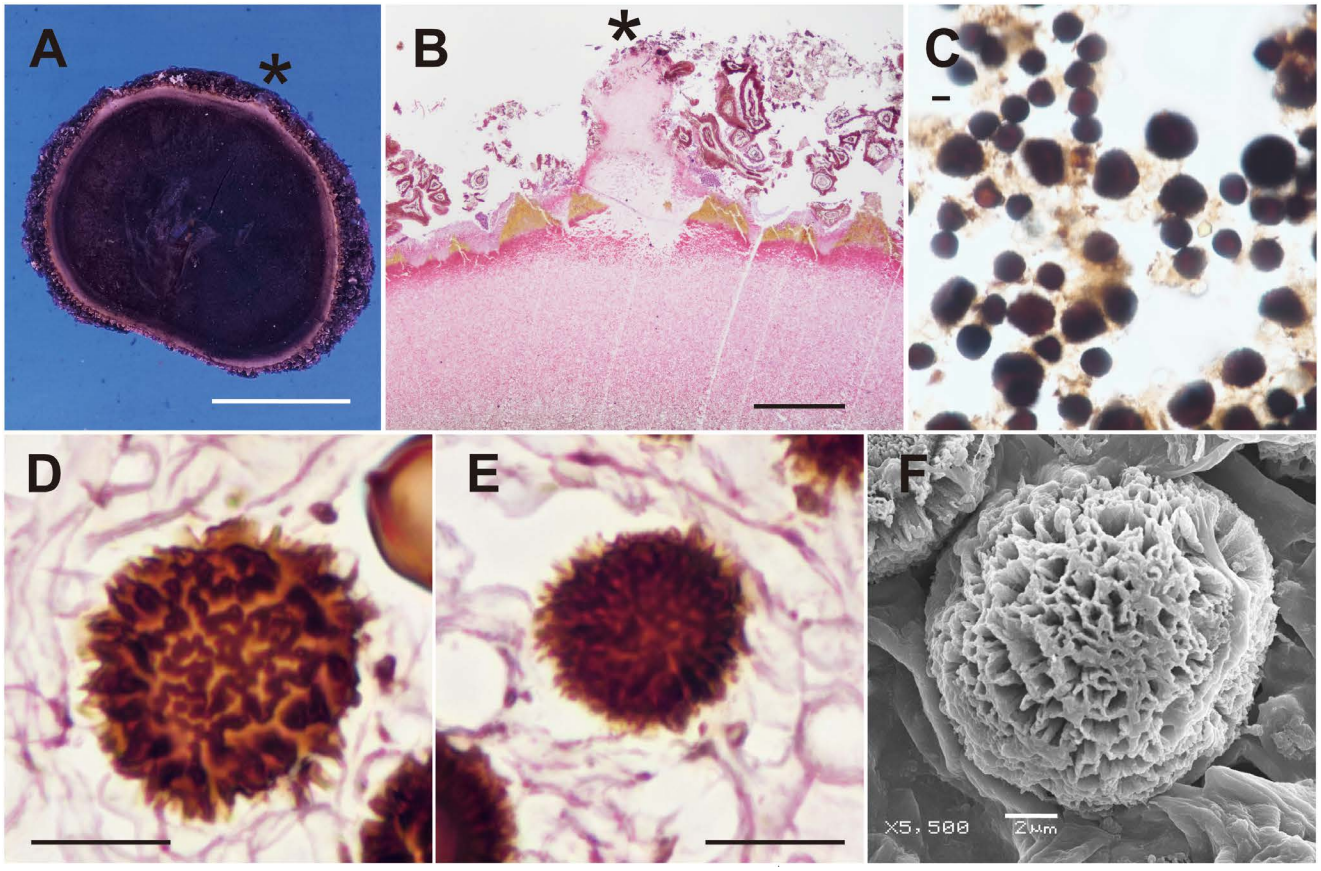

Fig. 4. Morphological features of *Elaphomyces fuscus* without mature stromata of *Tolypocladium japonicum* (INM-2-229316). A: The ascoma of *E. fuscus* after formalin fixation. B–D: Microscopic features of *E. fuscus* showing by the FFPE thin section stained with hematoxylin and eosin. B: The primordium of possibly *T. japonicum* (asterisk) growing out of *E. fuscus* and breaking through the warts. The structure is also visible macroscopically as a short protrusion on the top of *E. fuscus* (asterisk in A). C: Unstained spores directly smeared on the glass slide showing the biphasic pattern of the spore sizes. D: A large-sized ascospore. E: A small-sized ascospore. F: Scanning electron microscopy image of a small-sized ascospore. Bars: A = 1 cm; B = 500 μm; C–E: 10 μm; F = 2 μm.

of large-sized ascospores were much larger and numbers of small-sized spores were much smaller than those in *Elaphomyces* sp. with mature *Tolypocladium* stromata. Surfaces of large-sized ascospores were reticulate, dark reddish brown under the microscope, and apices of each reticulation were conical, capitulate or obtuse and irregularly shaped, broad base to costate with labyrinthine pattern (Fig. 4D). Surfaces of small-sized ascospores were also dark reddish brown under the microscope, and reticulate with conical, capitulate or obtuse tips and with polygonal to labyrinthine bases (Fig. 4E, F). Only a few asci were observed with non-reticulate ascospores.

Molecular identification

The BLAST search indicated that sequences of the present *Tolypocladium* specimen (ITS: OQ991237, 565 bp; LSU: OQ991235, 895 bp) share highest similarities with *T. japonicum* (Tables 1 and 2). For ITS, the top two hits of BLAST search were both *T. japonicum* (98.56 to 99.29% similarities), and next hits were *T. guangdongense* (T.H. Li, Q.Y. Lin & B. Song) V. Papp (INSD accession nos. MT386337–MT386340) from China, showing a similarity of 93.18% (Table 1). For LSU, the top two hits of BLAST search (99.77 to 100.00%) were also *T. japonicum* (AB027366 and DQ518761; Table 2).

The BLAST search indicated that the ITS sequence of the present *Elaphomyces* specimen (OQ991238, 680 bp) shares the highest similarities with *E. fuscus* (Table 3). For ITS, the top two hits of BLAST search were all *E. fuscus* (99.63 to 99.84% similarities) including the sequence from the holotype (Table 3), and the next hit was "Uncultured *Elaphomyces* YM56" (LC175083) from Iwate, Japan at 97.40% similarity. No LSU sequences from *E. fuscus* are currently available from INSD. The top six hits of BLAST search (all of 99.78%) of LSU sequence (OQ991236, 911 bp) of the present *Elaphomyces* specimen were *E. asperulus* Vittad. (KR029753–KR029755 and KR064762) from Denmark, Italy and Sweden (Molia et al., 2020), and *E. roseoviolaceus* A. Molia & E. Larss. (KR029750 and KR029751) from Norway (Molia et al., 2020).

Table 1. Similarities of the ITS sequence (OQ991237, 565 bp) of the present *Tolypocladium* specimen (INM-2-229315) resulted by BLAST search

INSD accession no.	Taxon names	Origin	Sequence similarities	Sequence lengths (bp)
AB027366	*Tolypocladium japonicum*	Japan	99.29%	1690
KT873533	*T. japonicum*	Taiwan	98.56%	601
MT386340	*T. guangdongense*	China	93.18%	597
MT386339	*T. guangdongense*	China	93.18%	572
MT386338	*T. guangdongense*	China	93.18%	614
MT386337	*T. guangdongense*	China	93.18%	597

Table 2. Similarities of the LSU sequence (OQ991235, 895 bp) of the present *Tolypocladium* specimen (INM-2-229315) resulted by BLAST search

INSD accession no.	Taxon names	Origin	Sequence similarities	Sequence lengths (bp)
AB027366	*T. japonicum*	Japan	100.00%	1690
DQ518761	*T. japonicum*	unknown	99.77%	886
OP207731	*T. capitatum*	China	97.43%	901
AB027364	*T. capitatum*	Japan	97.43%	1653
OP207735	*T. paradoxum*	Japan	97.43%	900

Table 3. Similarities of the ITS sequence (OQ991238, 680 bp) of the present *Elaphomyces* specimen (INM-2-229316) resulted by BLAST search

INSD accession no.	Taxon names	Origin	Sequence similarities	Sequence lengths (bp)
LC500967*	*Elaphomyces fuscus*	Japan	99.84%	615
LC500968	"uncultured *Elaphomyces* " (mycorrhizal root tips of *E. fuscus*)	Japan	99.82%	568
LC523911	*E. fuscus*	Japan	99.63%	546
LC175083	"uncultured *Elaphomyces* YM56"	Japan	97.40%	583
KX238833	*E. asperulus*	Spain	95.90%	698
KR029750	*E. roseoviolaceus*	Norway	95.59%	2067
KR029751	*E. roseoviolaceus*	Norway	95.59%	2070
KX165346	*E. asperulus*	Norway	95.26%	602

*Sequence from the holotype of *E. fuscus* .

Discussion

Morphological features of *Tolypocladium* specimens examined in the present study such as clavate stromata on a cylindrical stipe, and lacking of the rhizomorphous structure on the basal part of stromata were well agreement in the previous descriptions of *T. japonicum* (Imai, 1935; Ke & Ju, 2015; Kobayasi & Shimizu, 1960). Microscopic features of perithecia and ascospores were also almost identical to descriptions of *T. japonicum* (Ke & Ju, 2015; Kobayasi & Shimizu, 1960). Moreover, ITS and LSU sequences newly generated in the present study showed high similarities with those of *T. japonicum* from Japan and Taiwan (Tables 1 and 2). We therefore identified the *Tolypocladium* specimens examined as *T. japonicum* as below.

***Tolypocladium japonicum* (Lloyd) C.A. Quandt, Kepler & Spatafora**, IMA Fungus 5: 126 (2014).

[MycoBank ID: 808705]

Figs. 1 and 2.

Specimens examined: JAPAN, Ibaraki Prefecture, Itako, Shimasu, Suigo Prefectural Forest Park, (approx. 35°58'52"48N, 140°32'22"32E, alt. approx. 24 m asl.), July 3, 2022, coll. Y. Otaki, INM-2-229315 [INSD accession no.: OQ991237 (ITS), OQ991235 (LSU)]; same locality, July 3, 2022, coll. T. Kasuya, TNS-F-83036.

Habitat: On ascomata of *Elaphomyces* spp.

Known distribution: Japan (Imai, 1935; Kobayasi & Shimizu, 1960), China (Liang et al., 2003), Taiwan (Ke & Ju, 2015) and Austria (Kobayasi & Shimizu, 1960; Mains, 1957).

Japanese name: Tanpotake-modoki (Imai, 1935).

Morphologically, ascomata of *Elaphomyces* with mature stromata of *T. japonicum* and without them were very similar. These *Elaphomyces* specimens shared morphological similarities with *E. fuscus* in the following features: (1) dark yellow warts of ascomata, (2) whitish outer layer of the peridium, (3) dark brown to blackish powdery gleba, and (4) reticulate, dark reddish brown ascospores under the microscope. Aforementioned characteristics were main diagnostic features of *E. fuscus* and the present *Elaphomyces* specimens were morphologically almost identical to the original description of *E. fuscus* (Shirakawa & Tanaka, 2020). In addition, the ITS sequence newly generated in the present study showed high similarity (99.84%) with those of from the holotype of *E. fuscus* (Table 3). We therefore identified those specimens as *E. fuscus* as below.

***Elaphomyces fuscus* M. Shirakawa**, Mycoscience 61: 319 (2020).

[MycoBank ID: 833108]

Figs. 1, 2 and 4.

Specimens examined: JAPAN, Ibaraki Prefecture, Itako,

Shimasu, Suigo Prefectural Forest Park, (approx. 35°58'52"48N, 140°32'22"32E, alt. approx. 24 m asl.), July 3, 2022, coll. T. Kasuya, INM-2-229316 [INSD accession no.: OQ991238 (ITS), OQ991236 (LSU)]; same locality, July 3, 2022, coll. Y. Otaki, TNS-F-83037.

Habitat: Hypogeous, growing in clusters 3–10 cm underground under *Quercus serrata* and *Pinus densiflora* trees.

Known distribution: Japan (Tokyo: Shirakawa & Tanaka, 2020; Ibaraki: new record).

Japanese name: Gomatama-tsuchidango (Shirakawa & Tanaka, 2020).

Notes: Reticulate ascospores of *E. fuscus* are main distinguishable features from *E. granulatus* Fr., and also the present species differs from *E. japonicus* Lloyd in its reticulate ascospores and yellowish warts of ascomata (Shirakawa & Tanaka, 2020). Similarities of ITS and LSU sequences between *E. fuscus* and *E. asperulus* are high (ITS: 95.26 to 95.90%; LSU: 99.78%), and also, both species are morphologically similar, but the color of the peridium of the former is not homogeneously purplish hue such as the latter species (Paz et al., 2017; Shirakawa & Tanaka, 2020). Ascospores of *E. asperulus* show confluent warty ornamentation while those of *E. fuscus* are labyrinthine, irregular meshes (Paz et al., 2017; Shirakawa & Tanaka, 2020). Although ITS and LSU sequences of *E. roseoviolaceus* also shows high similarity (ITS: 95.59%; LSU: 99.78%) to those of *E. fuscus*, morphological features of both species are quite different. *Elaphomyces roseoviolaceus* has spiny to verrucose ascospores and dark pink to violaceous peridium (Molia et al., 2020).

Elaphomyces fuscus was previously known only from the type locality. Thus, this study is the first distributional record of *E. fuscus* outside the type locality. As above mentioned, the vertical section of a mycorrhizal tip covering the ascoma of *E. fuscus* (Fig. 2F) shows the typical ectomycorrhizal structure of gymnosperms. Because the present *Elaphomyces* specimens were collected underneath *Q. serrata* and *P. densiflora* trees, we treat this is a portion of *P. densiflora* roots. Previously, ectomycorrhizal association of *Q. serrata* with *E. fuscus* has only been reported (Shirakawa & Tanaka, 2020), therefore we here add *P. densiflora* as a novel host plant of *E. fuscus*. Additionally, we report *E. fuscus* as a new host fungus of *T. japonicum*. Although ascomata of *E. fuscus* with mature stromata of *T. japonicum* and without them are morphologically very similar each other, sizes of ascospores are different (Fig. 3). Further studies are required to clarify the mechanism of formation of different sizes of ascospores.

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 deeply thank Ms. Yua Otaki, Ms. Mihoko Uzawa, Mr. Yoshinori Inaba and volunteer stuffs of Ibaraki Nature Museum 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 Numbers JP20K06805 and JP23K05895.

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