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FULL PAPER

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Strobilomyces verruculosus sp. nov. from Japan

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Abstract A new species, *Strobilomyces verruculosus*, is described and illustrated. It is morphologically distinct from other species of *Strobilomyces* by having a verruculose pileus with small subpyramidal scales, a long and thick stipe with small warty to appressed scales, subdecurrent tubes, and incompletely reticulate basidiospores. Molecular phylogenetic analyses using the mitochondrial cytochrome oxidase subunit 3 (*cox3*) gene support that it belongs to the genus *Strobilomyces* and is highly differentiated from the other members of this genus found in Japan.

Key words *cox3* · Taxonomy

Introduction

The genus *Strobilomyces* (Boletaceae, Boletales) is defined as having blackish-brown globose to subglobose basidiospores with reticulate or verrucose to echinate ornamentation, a dry pileus densely covered with woolly or rigid scales, and flesh that discolors to reddish, changing to blackish, when bruised (Singer 1986). A previous molecular phylogenetic study elucidated that *Strobilomyces* is closely related to *Boletus*, *Leccinum*, and *Xerocomus* (Bruns et al. 1998). Ecological observations indicate that *Strobilomyces* is an ectomycorrhizal genus that forms mutualistic relationships with host plant families such as Fagaceae and Pinaceae (Matsuda and Hijii 1999; Sato et al. 2007).

Species of *Strobilomyces* are mostly reported from the subtropical and tropical areas of Asia (Chiu 1948; Corner 1972; Ying and Ma 1985; Zang 1985) and Africa (Heinemann 1954; Pegler 1977), but they are also observed in temperate areas of Asia (Kawamura 1954; Hongo 1979, 1982; Nagasawa 1987, 1997), North America (Singer 1945; Bessette et al. 2000), Europe (Breitenbach and Kränzlin 1991), and Australia (May and Wood 1997). So far approximately 20 species of *Strobilomyces* have been described in the world (Kirk et al. 2001). In Japan, 4 species of *Strobilomyces* are hitherto reported: *S. strobilaceus* (Scop.: Fr.) Berk., *S. confusus* Singer, *S. seminudus* Hongo, and *S. mirandus* Corner (Kawamura 1954; Hongo 1982; Nagasawa 1987, 1997; Sato et al. 2005). However, based on morphological characters, the current taxonomy of *Strobilomyces* is confusing. Using DNA sequence data of nuclear and mitochondrial DNA, Sato et al. (2007) exhibited evidence that *Strobilomyces* might contain a number of cryptic species.

In recent field surveys of fungi, we collected a putative *Strobilomyces* that possessed morphological characters distinct from any known Japanese members of this genus. Thus, we compared the specimens to non-Japanese members of the genus as described in the literature to identify it. In addition, we performed molecular phylogenetic analyses comparing the nucleotide sequences of the mitochondrial cytochrome oxidase subunit 3 gene (*cox3*) from our *Strobilomyces* with several other representative species of the genus. We chose to use the *cox3* gene for these analyses, according to the report by Sato and Murakami (2008) that the nucleotide sequences of the *cox3* gene are informative for detecting interspecific genetic differentiation in *Strobilomyces*. As a result, we propose that our specimens represent a new species of *Strobilomyces*, and we describe it herein as *S. verruculosus*.

Materials and methods

Fungal specimens studied

In 2004, 2005, and 2007, ten specimens of *Strobilomyces* were collected from evergreen *Castanopsis-Quercus* forests in Kyoto, Kumamoto, and Miyazaki Prefectures. Specimens examined (Table 1), including the holotype, are deposited in the Makino Herbarium of Tokyo Metropolitan University (MAK, Tokyo).

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Table 1. Taxa and voucher sample information of the fungal samples, and GenBank accession numbers for sequence data obtained in this study

Taxa	Specimen no.	Locality of collection	GenBank acc. no.
Strobilomyces verruculosus Hirot. Sato	MAK, s007	Mt. Tatsuta, Kurokami-cho, Kumamoto-shi, Kumamoto Pref., Japan	AB275176
	MAK, s010	Nango-cho, Higashiusuki-gun, Miyazaki Pref., Japan	AB426522
	MAK, s245	Mito-shrine, Joyo-shi, Kyoto Pref., Japan	AB275176
	MAK, s246	Mito-shrine, Joyo-shi, Kyoto Pref., Japan	AB275176
	MAK, s247	Mito-shrine, Joyo-shi, Kyoto Pref., Japan	AB275176
	MAK, s302	Mito-shrine, Joyo-shi, Kyoto Pref., Japan	AB275176
	MAK, s359	Mito-shrine, Joyo-shi, Kyoto Prefecture, Japan	AB275176
	MAK, s376	Fukakusa-yabunouchi-cho, Kyoto-shi, Kyoto Pref., Japan	AB275176
	MAK, s660	Mito-shrine, Joyo-shi, Kyoto Pref., Japan	AB275176
	MAK, s693	Mito-shrine, Joyo-shi, Kyoto Pref., Japan	AB275176
Strobilomyces strobilaceus (Scop.:Fr.) Berk.	MAK, s174	Mino-shi, Osaka Pref., Japan	AB426523
	MAK, s192	Minenohara-Hill, Susaka-shi, Nagano Pref., Japan	AB426524
	MAK, s290	Ohmi-Shrine, Jingu-cho, Otsu-shi, Shiga Pref., Japan	AB426525
	MAK, s310	Fu-shan Research Station, Taipei, Taiwan	AB275188
	MAK, s380	Mt. Yoshida, Sakyo-ku, Kyoto-shi, Kyoto Pref., Japan	AB353787
	MAK, s404	Mt. Tanakami, Otsu-shi, Shiga Pref., Japan	AB368445
Strobilomyces confusus Sing.	MAK, s386	Mt. Yoshida, Sakyo-ku, Kyoto-shi, Kyoto Pref., Japan	AB353784
	MAK, s409	Miidera-cho, Otsu-shi, Shiga Pref., Japan	AB353790
Strobilomyces seminudus Hongo	MAK, s346	Mt. Yoshida, Sakyo-ku, Kyoto-shi, Kyoto Pref., Japan	AB353783
	MAK, s381	Mt. Yoshida, Sakyo-ku, Kyoto-shi, Kyoto Pref., Japan	AB353788
Strobilomyces sp.	MAK, s309	Fu-shan Research Station, Taipei, Taiwan	AB275189
Boletus pseudocalopus Hongo	MAK, bl002	Mt. Yoshida, Sakyo-ku, Kyoto-shi, Kyoto Prefecture, Japan	AB426526
Tylopilus ferrugineus (Frost) Sing.	MAK, t003	Mt. Daimonji, Sakyo-ku, Kyoto-shi, Kyoto Prefecture, Japan	AB426527
Austroboletus subvirens (Hongo) Wolfe	MAK, A002	Mt. Kiyomizu, Higashiyama-ku, Kyoto-shi, Kyoto Prefecture, Japan	AB426528
Boletellus emodensis (Berk.) Sing.	MAK, b033	Mt. Kiyomizu, Higashiyama-ku, Kyoto-shi, Kyoto Prefecture, Japan	AB426529
Aureoboletus thibetanus (Pat.) Hongo & Nagasawa	MAK, ar001	Mt. Kiyomizu, Higashiyama-ku, Kyoto-shi, Kyoto Prefecture, Japan	AB426530
Heimiella japonica Hongo	MAK, h003	Mt. Tanakami, Otsu-shi, Shiga Prefecture, Japan	AB426531

Voucher specimens are deposited in the Makino Herbarium of Tokyo Metropolitan University (MAK)

Observation of morphological characters

Microscopic observation was made under a VK-9700 digital microscope (Keyence, Osaka, Japan) with material (sections or fragments of the basidiocarp tissues) mounted in 5% ammonia solution. Basidiospores were also observed under a scanning electron microscope (Philips XL-series; Eindhoven). Fifty basidiospores obtained from the spore prints of two specimens (MAK, s660; MAK, s693) were measured at 1000× under the VK-9700 digital microscope.

DNA extraction, PCR amplification, and sequencing

Total DNA was extracted from the fruit body tissue of all samples by the same methods as described in Sato et al. (2007). The region of *cox3* gene was amplified with polymerase chain reaction (PCR) primers COX3F1 and COX3R1 (Sato and Murakami 2008). PCR amplification was done using 0.5 µl total DNA in a 20-µl reaction mixture containing 1× PCR buffer, 2 nmol dNTPs, 10 pmol of both forward and reverse primers, and 0.5 units Ex-Taq polymerase (Takara Bio, Otsu, Japan). Cycling parameters for PCR were as follows: denaturation at 95°C for 3 min, followed by 35 cycles of 30 s at 95°C, 30 s at 43°C, and 72°C for 45 s, followed by a final extension of 7 min at 72°C. Before nucleotide sequencing, PCR products were purified using ExoSAP-IT (GE Healthcare, NJ, USA) according to the manufacturer's instructions. The purified PCR products were sequenced using the same primers as used for amplification. Nucleotide sequencing was performed using an ABI 3100 automated sequencer (Applied Biosystems, Foster City, CA, USA) with Big Dye-Terminator ver. 3.1 (Applied Biosystems), following the manufacturer's instructions.

Molecular phylogenetic analyses

Maximum-parsimony (MP) analysis and Bayesian analysis were conducted based on the partial nucleotide sequences of *cox3* gene for the 27 specimens listed in Table 1 as well as four nucleotide sequences (AF002160, AF002149 AF002155, AF002153) obtained from DDBJ GenBank nucleotide sequence databases. Sequence data were aligned using the CLUSTAL W program (Thompson et al. 1994). The alignment was deposited in TreeBASE (http://www.treebase.org/) under the accession number SN4210.

Maximum-parsimony (MP) analysis was conducted using PAUP 4.0b10 (PAUP*) (Swofford 2002). The MP trees were obtained using the heuristic search option, with 100 replications of random sequence addition, and tree bisection and reconnection (TBR) branch swapping, holding one tree at each step during stepwise addition, and with the "MulTrees" option on. MaxTrees was set to 50000 trees. For nonparametric bootstrapping, 10000 bootstrap repli-

cates were performed for each maximum-parsimony analysis.

For Bayesian analysis, we adopted the general time-reversible model with site-specific rates (GTR + SSR). Bayesian inference analysis was performed using MrBayes ver. 3.1.2 (Ronquist and Huelsenbeck 2003). Separate and combined analysis consisted of four simultaneous runs each with four simultaneous Markov Chain Monte Carlo (MCMC) chains initially run for 1 000 000 generations, saving the current tree to a file every 100 generations. Default cold and heated chain parameters were used. At the end of each run, we considered the sampling of the posterior distribution to be adequate if the average standard deviation of split frequencies was <0.01. MCMC runs were summarized and further investigated for convergence of all parameters, using the "sump" and "sumt" commands in MrBayes. Trees before log-likelihood stabilization and convergence (burn in = 2500) were discarded before a majority rule consensus tree was generated.

Results

Molecular phylogenetic analyses

Jukes–Cantor genetic distances were calculated based on the nucleotide sequences of *cox3* gene obtained from our specimens. Two different nucleotide sequences were found in our samples of *S. verruculosus*. The average similarities between the lineages of *S. verruculosus* and those of "*S. confusus* and *S. seminudus* complex," "*S. strobilaceus*," or "*S. mirandus*" were 96.66% (SD = 0.38, 96.20%–97.31%), 95.08% (SD = 0.34, 94.47%–95.62 %), and 94.59 (SD = 0.13, 94.49%–94.68 %), respectively.

The mitochondrial *cox3* sequence data contained 23 taxa and 571 total characters, of which 150 were parsimony informative. No indels were found in these data, and thus all the data of nucleotide sequences were included in Bayesian and MP analyses. The topology of the consensus MP tree (23 MP trees: TL = 455, CI = 0.651, RI = 0.706) was almost the same as that of the Bayesian trees, and the Bayesian inference topology is depicted in Fig. 1. The lineage names shown in Fig. 1 correspond to those presented in Sato et al. (2007). It was implied by Sato et al. (2007) that these lineages might indicate discrete biological species.

The monophyly of *S. verruculosus* was supported by high posterior probability (1.00) and high bootstrap value (100%) (Fig. 1). High Bayesian posterior probability (1.00) and high MP bootstrap value (81.2%) also supported the monophyly of the group composed of lineages 1, 2, 3, 4, and *S. verruculosus*.

Taxonomy

Strobilomyces verruculosus Hirot. Sato, sp. nov. Figs. 2–9

Pileus 3–12 cm diametro, primo covexus demum planus, pagina siccus dense squamulosus, squamulis fibrosis nigricantibus vel pullatis verruculosis vel subconoideis 0.25–1 mm latis. Stipes 6–12 cm longus, cylindricus vel basin versus decrescens, apice 1–3.5 cm prope basin 0.8–2.5 cm crassus, solidus, pileo concolor, apice reticulatus, prope apicem saepe cum zona lata annulari tomentosa, basin versus dense squamulosus squamulis verruculosis vel appressis, prope basin saepe squamuloso-tomentosus. Tubuli ad 15 mm longi, subdecurrentes, albi dein fuliginosi ad maturitatem; pori ad 1 mm diametro, albi dein fuliginosi,

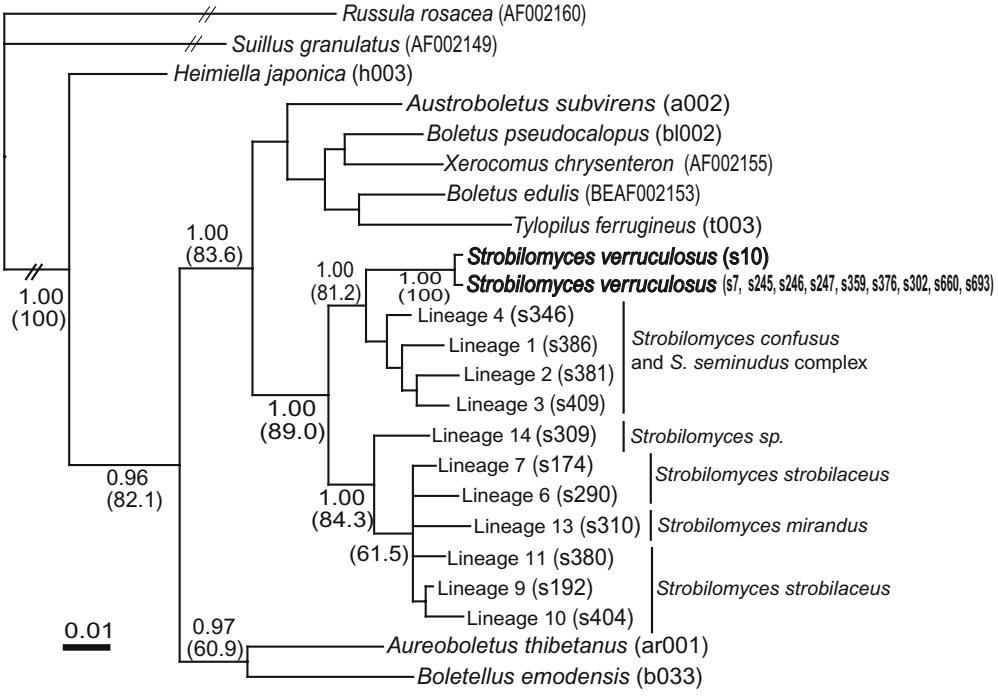
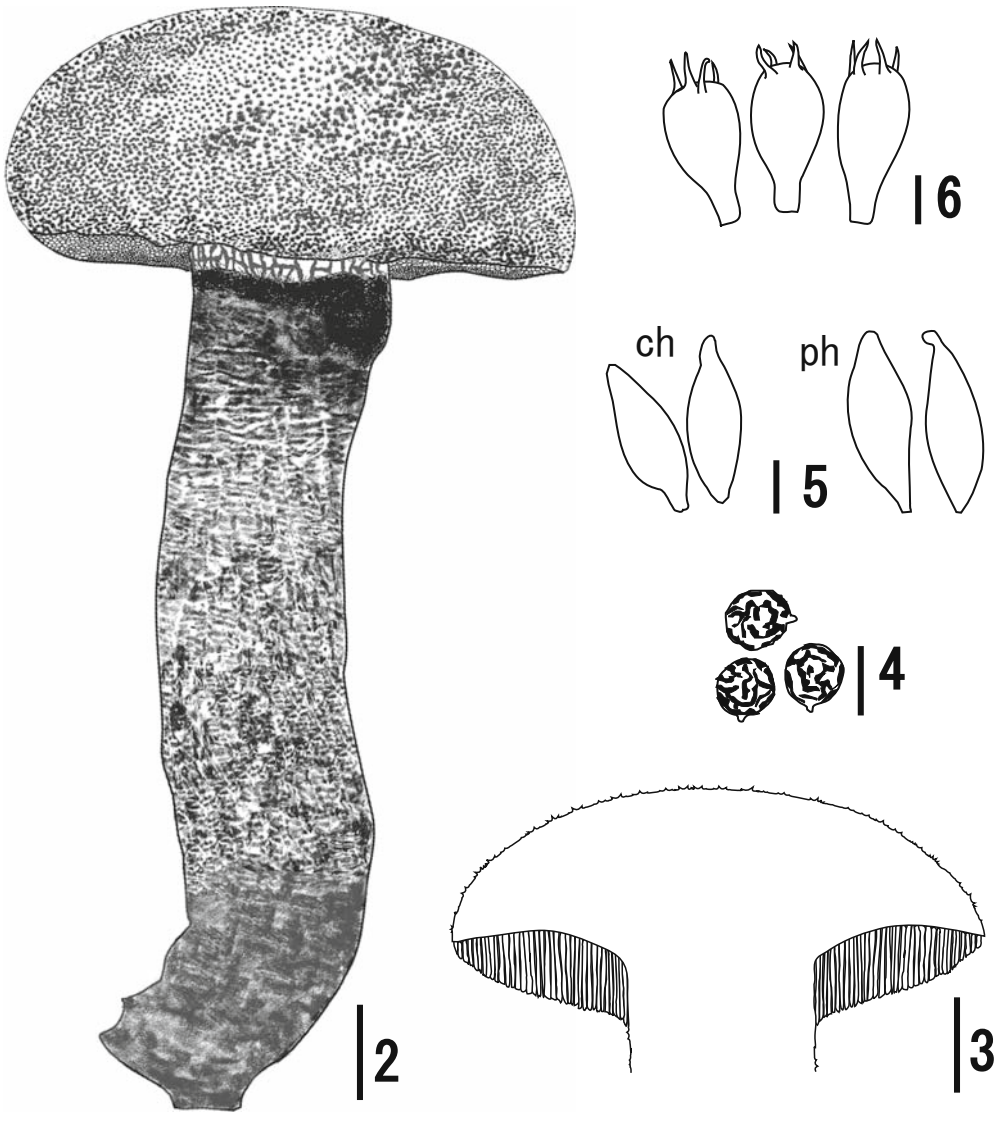


Fig. 1. Bayesian 50% majority-rule consensus tree for *Strobilomyces* as inferred from the nucleotide sequence of the *cox3* gene. *Numbers on branches* are posterior probabilities (>0.5); bootstrap frequencies by maximum-parsimony analysis (>50%) are shown in the *parentheses below*. The lineage names shown in Fig. 1 correspond to those presented in Sato et al. (2007). *Bar* 0.01 expected changes/site



Figs. 2–6. *Strobilomyces verruculosus* (holotype). **2** Basidioma. **3** Vertical section view of basidioma. **4** Basidiospores. **5** Cheilocystidia (*ch*) and pleurocystidia (*ph*). **6** Basidia. *Bars* **2, 3** 1 cm; **4–6** 10 μm

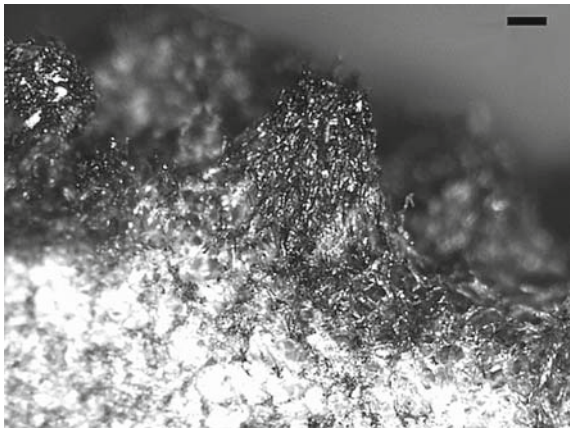


Fig. 7. A scale of the pileus surface of *Strobilomyces verruculosus* (holotype) at 200×, under digital microscope. *Bar* 100 μm

contusis distincte rubescentes demum nigricantes. Basidiosporae 9.1–11.0 × 9.0–10.9 μm ornamento incluso, 7.8–9.5 × 7.4–8.6 μm eo excluso, globosae vel subglobosae, fusco-brunneae in aqua ammoniae, incomplete reticulatae.

Holotype: Mito-shrine, Joyo-shi, Kyoto Prefecture, Japan, in an evergreen forest occupied by *Castanopsis cuspidata* (Thunb) Schottky, July 25, 2007, Hirot. Sato (MAK, s693).

Pileus 3–12 cm in diameter, at first convex then plane, surface dry, blackish, or purple black when young, becoming dark brown or almost black when matured, densely verruculose, with minute scales that are fibrous, warty, or subpyramidal, blackish, 0.3–0.6 mm high, 0.25–1 mm wide at the base. Stipe 6–12 cm long, equal or narrowing to the base, straight or curved, 1–3.5 cm thick at apex, 0.8–2.5 thick near the base, concolorous with the pileus; surface reticulate with shallow, elongate meshes above, often with a broad annular zone near the apex as a thin cottony-membranous belt, minutely warty or appressed scaly, often squamulose-tomentose near the base. Tubes up to 15 mm long, subdecurrent, whitish then fuliginous at maturity; pores up to 1 mm wide, whitish to grayish. Tubes and pores distinctly turning reddish then black on bruising. Flesh of the pileus up to 20 mm thick in the center of the pileus, up to 10 mm thick halfway to the margin, whitish, the color changing

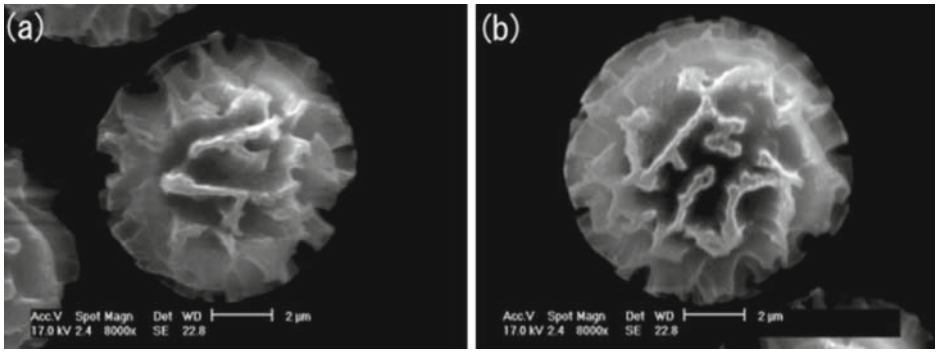


Fig. 8. a, **b** Basidiospores of *Strobilomyces verruculosus* (MAK, s007) at 8000×, under sc anning electron microscopy (SEM). *Bars* 2 μm

Fig. 9. Basidiomata of *Strobilomyces verruculosus* (MAK, s302) in its habitat. *Bar* 5 cm

similar to the tubes and pores when injured. All parts of the basidiocarp more or less changing into reddish then black on bruising. Color of the spore prints fuscous brown.

Basidiospores 9.1–11.0 × 9.0–10.9 μm (Q = 1.00–1.10, 1.04 ± 0.03; range, mean ± SD) including ornamentation, spore body 7.8–9.5 × 7.4–8.6 μm (Q = 1.00–1.14, 1.06 ± 0.03), globose to subglobose, dark brown in ammonia water, incompletely reticulate. Basidia 14–19 × 30–48 μm, clavate, 4-spored; sterigmata 5–11 μm long. Cheilocystidia 38–54 × 8–17 μm, numerous, clavate to fusiform or subfusoid, hyaline or with brownish to pale fuscous contents, thin walled. Pleurocystidia 37–65 × 13–19 μm, abundant, similar to cheilocystidia but somewhat larger. Hymenophoral trama strongly bilateral divergent, composed of hyphae 6–16 μm wide, scarcely branched. Pileipellis composed of subradially arranged cylindrical whitish hyphae, 6–22 μm wide. Warty scales of the pileus composed of blackish hyphae, ascending in bundles, the end cells slightly attenuated toward the apex, 24–72 μm in length, 10–22 μm wide. Pileus trama composed of interwoven hyphae, 2–12 μm wide. Hyphae of scales on stipitipellis slightly ascending in bundles, blackish, 6–14 μm wide. Stipe trama consisting of cylindrical hyphae, whitish to brownish, 6–20 μm wide. All hyphae without clamp connections.

Japanese name: Tsubukasa-oni-iguchi.

Etymology: verruculosus = verruculose in Latin, referring to the morphology in the pileus surface of the species.

Specimens examined: Mt. Tatsuta, Kurokami, Kumamoto-shi, Kumamoto Prefecture, Japan, coll. by Mr. T. Shiotsu, July 23, 2002 (MAK, s007); Nango-cho, Higashiusuki-gun, Miyazaki Prefecture, Japan, coll. by Mr. T. Shiotsu, July 19, 2002 (MAK, s010); Mito-shrine, Joyo-shi, Kyoto Prefecture, Japan, coll. by Mr. K. Maruyama, July 4, 2004 (MAK, s245; MAK, s246; MAK, s247); September 5, 2004 (MAK, s302–holotype); July 22, 2005 (MAK, s359); July 13, 2007 (MAK, s660); July 25, 2007 (MAK, s693); Fushimi-inari-shrine, Fukakusa-yabunouchi-cho, Kyoto-shi, Kyoto Prefecture, Japan, September 16, 2005 (MAK, s376).

Habitat: Gregarious on the ground in mixed forests of *Castanopsis cuspidata* and *Quercus* spp. dominated by *C. cuspidata*.

Distribution: Honshu (Kyoto Prefecture) and Kyushu (Miyazaki Prefecture and Kumamoto Prefecture) in Japan.

Discussion

The fungus newly described in this work, *S. verruculosus*, is distinguished from other known members of *Strobilomyces* in having several conspicuous macroscopic morphological characteristics, such as a pileus densely covered with minutely warty (up to 1 mm) scales, and a long and thick stipe with an annular zone near the apex. Besides these macroscopic features, the micromorphology of the basidiospores (ornamented with incomplete reticula) is also useful for distinguishing *S. verruculosus* from other members of *Strobilomyces*. Among all these, the morphology of the pileus is the most peculiar morphological character unique to *S. verruculosus*.

Among the members of *Strobilomyces*, *S. annulatus* Corner and *S. giganteus* Zang somewhat resemble *S. verruculosus* in having a large basidiocarp and incompletely reticulate basidiospores. However, *S. annulatus* is distinct

from *S. verruculosus* in having a pileus with larger (2–3 mm) scales and having a stipe with an ample ring (Corner 1972). In addition, *S. giganteus* differs from *S. verruculosus* in having a pileus with rimose-areolate patches and having smaller basidiospores (Zang 1985).

The molecular phylogenetic trees based on the nucleotide sequences of mitochondrial *cox3* gene support the results of these morphological observations. The molecular phylogenetic analyses suggest that *S. verruculosus* forms a clear single clade and that it is genetically differentiated from any other lineage of currently recognized *Strobilomyces*. Sato and Murakami (2008) showed that the average similarity of *cox3* gene sequences among lineages 1, 3, and 4 was 97.77% (SD = 0.95), and that these three lineages were reproductively isolated. The average similarity between *S. verruculosus* and other lineages is lower than that value. In addition, Sato et al. (2007) reported high genetic differentiation between *S. verruculosus* (as "lineage 5" in Sato et al. 2007) and the other members of *Strobilomyces* in nuclear *rpb1*, internal transcribed spacer (ITS)-2, and mitochondrial *atp6* regions. These molecular data suggest that *S. verruculosus* cannot be attributed to any known species of *Strobilomyces* in Japan.

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