

FULL PAPER

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Taxonomic characterization of *Shiraia*-like fungi isolated from bamboos in Japan

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Abstract The molecular phylogeny of nuclear LSU rDNA sequences (D1/D2 domain), ITS regions, and beta-tubulin gene (*tub2*) showed that the seven strains of *Shiraia*-like fungi obtained from fresh bamboo tissues as endophytes were closely related to *Shiraia bambusicola* and had three distinctive lineages (groups A–C). The closest group (group A) to *S. bambusicola* produced distinctive prawn-shaped conidioma-like structures that differed from conidiomata in the anamorph of *S. bambusicola*. Currently, none of the morphological structures and molecular database records were compatible with our *Shiraia*-like fungi. These results reveal that *Shiraia*-like fungi group A is supposed to be a new species that should be assigned into a novel genus/species related to *S. bambusicola*.

Key words Anamorph · Bamboo · Nuclear rDNA · *Shiraia bambusicola* · *Shiraia*-like fungi

Introduction

Since the genus *Shiraia* was established by Hennings (1900) with *S. bambusicola* Henn. as type species, only the type species has been recorded and studied. The morphological features of its teleomorph were given precisely by Amano (1980, 1983). In the past few decades, the genus was reclassified into Pleosporales on the basis of its morphological characters (Luttrell 1973), and nuclear rDNA sequences

were analyzed by Cheng et al. (2004). In addition, Tsuda and Ueyama (1987) described the presence of the synamorph with macro- and microconidia.

Shiraia bambusicola is known as a parasitic fungus on twigs of several genera of bamboos. The stroma of this species has been used as a traditional medicine in China (Cheng et al. 2004). To date, extensive studies have been conducted on compounds of *S. bambusicola*, which have been found to be involved in antitumor activity and antiangiogenesis (Mazzini et al. 2001; Tong et al. 2004; Chen et al. 2005). The products, which were characterized by the name hypocrellins, form perylenequinone pigments. The compounds are photosensitizers that can possibly be used for photodynamic treatment (Wu et al. 1989). This fungus is distributed in several parts of Japan (Hino 1961; Kishi et al. 1991).

In our previous study of isolation and phylogenetic analysis (Morakotkarn et al. 2007), the diversity of endophytic fungi in bamboo tissues was investigated. Seven isolates of the 257 isolates from fresh bamboo tissues were found to be closely related to *S. bambusicola* based on rDNA sequence data. In this research, we focused on the characterization of the 7 bamboo isolates, tentatively called *Shiraia*-like fungi, using molecular analysis and their morphological features.

Materials and methods

Fungus collection and isolates

Seven strains (JP7, JP93, JP119, JP151, JP185, JP232, and JP256) were examined in this study, and were isolated from bamboo as mentioned in Morakotkarn et al. (2007). Reference strains were obtained from the NBRC culture collection (NBRC 30147, 30312, 30737–30739, 30752–30755, 30771, 30772, and 100220). All isolates were cultured on potato dextrose agar (PDA) at 25°C for 10–14 days to observe the morphology and provided for DNA examination. Information on the strains is shown in Table 1.

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Table 1. List of strains and accession numbers of DNA sequences

Strain	Scientific name	Source of isolation	Status of isolates	Accession numbers ^a		
				28S rDNA	ITS regions	β -Tubulin (<i>tub2</i>)
<i>Bamboo isolates</i> ^c						
JP7	<i>Shiraia</i> -like fungus	<i>Phyllostachys</i> sp. ^b	Endophytes	AB354974	AB255241	AB355008
JP93	<i>Shiraia</i> -like fungus	<i>Sasa</i> sp. ^b	Endophytes	AB354975	AB255277	AB355009
JP119	<i>Shiraia</i> -like fungus	<i>Phyllostachys</i> sp. ^b	Endophytes	AB354976	AB354993	AB355010
JP151	<i>Shiraia</i> -like fungus	<i>Sasa</i> sp. ^b	Endophytes	AB354977	AB255289	–
JP185	<i>Shiraia</i> -like fungus	<i>Sasa</i> sp. ^b	Endophytes	AB354978	AB354994	AB355011
JP232	<i>Shiraia</i> -like fungus	<i>Phyllostachys</i> sp. ^b	Endophytes	AB354979	AB255303	AB355012
JP256	<i>Shiraia</i> -like fungus	<i>Phyllostachys</i> sp. ^b	Endophytes	AB354980	AB354995	AB355013
<i>References</i>						
NBRC 30147	<i>Shiraia bambusicola</i> P. Hennings	<i>Phyllostachys bambusoides</i>	–	AB354962	AB354981	AB354996
NBRC 30312	<i>Shiraia bambusicola</i> P. Hennings	<i>Phyllostachys bambusoides</i>	–	AB354963	AB354982	AB354997
NBRC 30737	<i>Shiraia bambusicola</i> P. Hennings	<i>Phyllostachys bambusoides</i>	Monoconidia	AB354964	AB354983	AB354998
NBRC 30738	<i>Shiraia bambusicola</i> P. Hennings	<i>Phyllostachys bambusoides</i>	Monoconidia	AB354965	AB354984	AB354999
NBRC 30739	<i>Shiraia bambusicola</i> P. Hennings	<i>Phyllostachys bambusoides</i>	Immature ascus	AB354966	AB354985	AB355000
NBRC 30752	<i>Shiraia bambusicola</i> P. Hennings	<i>Phyllostachys bambusoides</i>	–	AB354967	AB354986	AB355001
NBRC 30753	<i>Shiraia bambusicola</i> P. Hennings	<i>Phyllostachys</i> sp. ^b	–	AB354968	AB354987	AB355002
NBRC 30754	<i>Shiraia bambusicola</i> P. Hennings	<i>Phyllostachys</i> sp. ^b	–	AB354969	AB354988	AB355003
NBRC 30755	<i>Shiraia bambusicola</i> P. Hennings	<i>Phyllostachys bambusoides</i>	–	AB354970	AB354989	AB355004
NBRC 30771	<i>Shiraia bambusicola</i> P. Hennings	<i>Phyllostachys</i> sp. ^b	–	AB354971	AB354990	AB355005
NBRC 30772	<i>Shiraia bambusicola</i> P. Hennings	<i>Phyllostachys</i> sp. ^b	Monoconidia	AB354972	AB354991	AB355006
NBRC 100220	<i>Shiraia bambusicola</i> P. Hennings	<i>Phyllostachys bambusoides</i>	Microconidia	AB354973	AB354992	AB355007

ITS, internal transcribed spacer region; –, not determined

^aThe accession numbers will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases

^bWe could not identify the species name of host plant

^cSeven isolates were deposited at NITE Biochemical Resource Center (NBRC) as NBRC 103846–103852

Morphological observation

The microscopic observation was conducted according to the method of Tanaka and Harada (2003). Sexual and asexual reproductions were induced by inoculating mycelium of each strain on different media: i.e., cornmeal agar (CMA), rice straw agar (RSA), and bamboo twig pieces onto agar.

Genomic DNA extraction and polymerase chain reaction (PCR) amplification

For each strain, the endophytes and references were cultured on PDA and incubated at 25°C for 10–14 days. The total genomic DNA of each strain was extracted by harvesting a mycelium from the PDA plates. The DNA was extracted using a fungal miniprep kit (E.Z.N.A.; Omega Bio-tek, Doraville, GA, USA). DNA amplification was performed using Ex Taq polymerase (Takara Bio, Shiga, Japan) in a Gene Amp PCR system 9700 (Applied Biosystems Japan, Tokyo, Japan) under each condition of the D1/D2 domain of large subunit (LSU) rDNA, internal transcribed spacer (ITS) regions, and beta(β)-tubulin subunit 2 gene (*tub2*). The following primer pairs were used: NL1 (5' GCATATCAATAAGCGGAGGAAAAG 3') and NL4 (5' GGTCCGTGTTTCAAGACGG 3') for the LSU rDNA gene described by O'Donnell (1993), ITS1 (5' TCCGTAG GTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCT TATTGATATGC 3') for ITS regions described by White et al. (1990), and Bt2a (5' GGTAACCAATCGGTGCT GCTTTC 3') and Bt2b (5' ACCCTCAGTGTAGTGAC

CCTTGGC 3') for *tub2* described by Glass and Donaldson (1995).

PCR reactions were performed in a 50- μ l PCR mixture under the following thermal conditions: 5 min at 94°C, 35 cycles of 30 s at 94°C, 50 s at 52°C for LSU rDNA (D1/D2 domain), and 55°C for ITS regions, 30 s at 72°C, and a final extension step of 7 min at 72°C. For the *tub2* gene, the conditions were 3 min at 95°C, 30 cycles of 1 min at 94°C, 50 s at 52°C, 1 min at 72°C, and a final extension step of 10 min at 72°C. The PCR products were purified using the QIAEX II Gel extraction kit (Qiagen, Tokyo, Japan) and a PCR-M cleanup system (Viogene, Sunnyvale, CA, USA).

DNA sequencing and phylogenetic analysis

The purified PCR products were directly sequenced on both strands with the same primers that were used for amplification. Reaction products were performed using the ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems). The DNA sequences were aligned with additional sequences obtained from Genbank using BioEdit (Hall 1999) and Clustal X 1.83 (Thompson et al. 1997) (see TreeBASE no. S2084). To generate the phylogenetic tree, MEGA software v. 3.1 was applied (Kumar et al. 2004). Ambiguous positions that may not be homologous were eliminated, and gap positions were completely deleted. Phylogenetic analyses were inferred by the neighbor-joining method (Kimura two-parameter distance calculation) and by maximum parsimony using the heuristic search (CNI level = 1) option (Nei and Kumar 2000). The bootstrap

values were simulated at 1000 replications for reliability of the tree topologies.

Results and discussion

Isolation information

In our previous study (Morakotkarn et al. 2007), we reported 257 fungal strains isolated from fresh bamboo tissues since 2006, of which 7 isolates were selected for this study. Six strains of these were isolated from nodes and internodes of bamboo tissues, except for JP151, which was recovered only from leaf tissue. No symptoms were observed in the bamboo plants used for isolation (*Phyllostachys* sp. and *Sasa* sp.). On the other hand, ascomata of *S. bambusicola* were found on *Phyllostachys*, mainly on *P. bambusoides*, and 12 refer-

ence strains of *S. bambusicola* derived from the NBRC culture collection were isolated from conidia and immature asci. The strains and accession numbers of the DNA sequences are shown in Table 1.

Phylogenetic analysis

The D1/D2 domain of LSU rDNA, the ITS regions (ITS1 + 5.8S + ITS2), and the beta(β)-tubulin subunit 2 (*tub2*) genes were completely sequenced in both directions. In the phylogenetic relationships, all the reference strains of *S. bambusicola* were clustered in a single clade. However, seven isolates of the *Shiraia*-like fungi were categorized into three different groups as group A–C (Figs. 1–3). In the LSU rDNA (D1/D2 domain) parsimony tree, four isolates of group A (JP7, JP93, JP232 and JP256) were clustered into the nearest clade to the *S. bambusicola* references (Fig. 1).

Fig. 1. Parsimony tree of bamboo isolates based on 562 bp of large subunit (LSU) rDNA gene (D1/D2 domain) sequences. Confidence values above 50% obtained from a 1000-replicate bootstrap analysis are indicated at the *branch nodes*. Bootstrap values from maximum-parsimony and neighbor-joining methods were determined (MP/NJ). The *scale bar* indicates the number of steps. The *bold line* emphasizes the clade of *Shiraia*-like fungi in group A

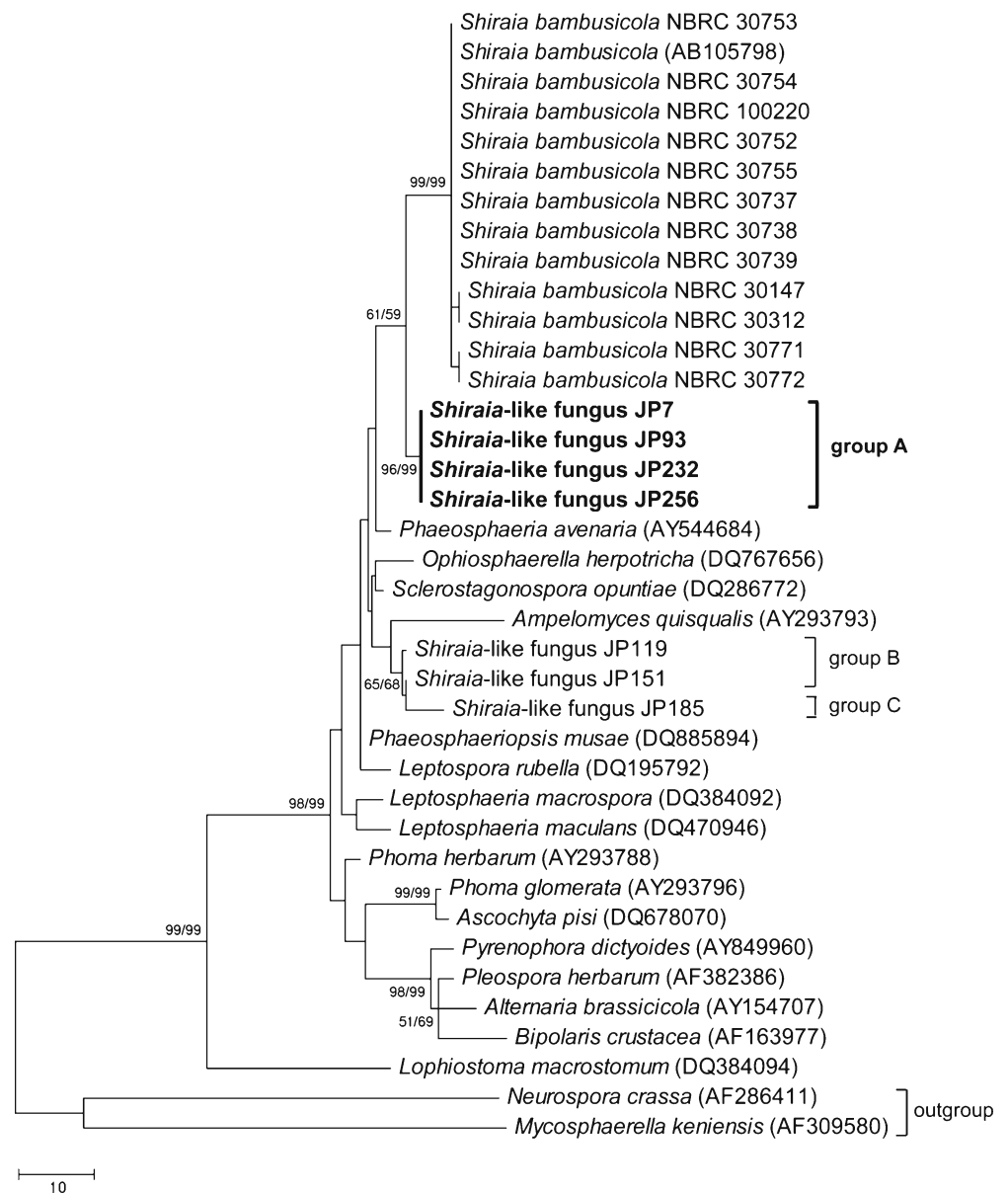
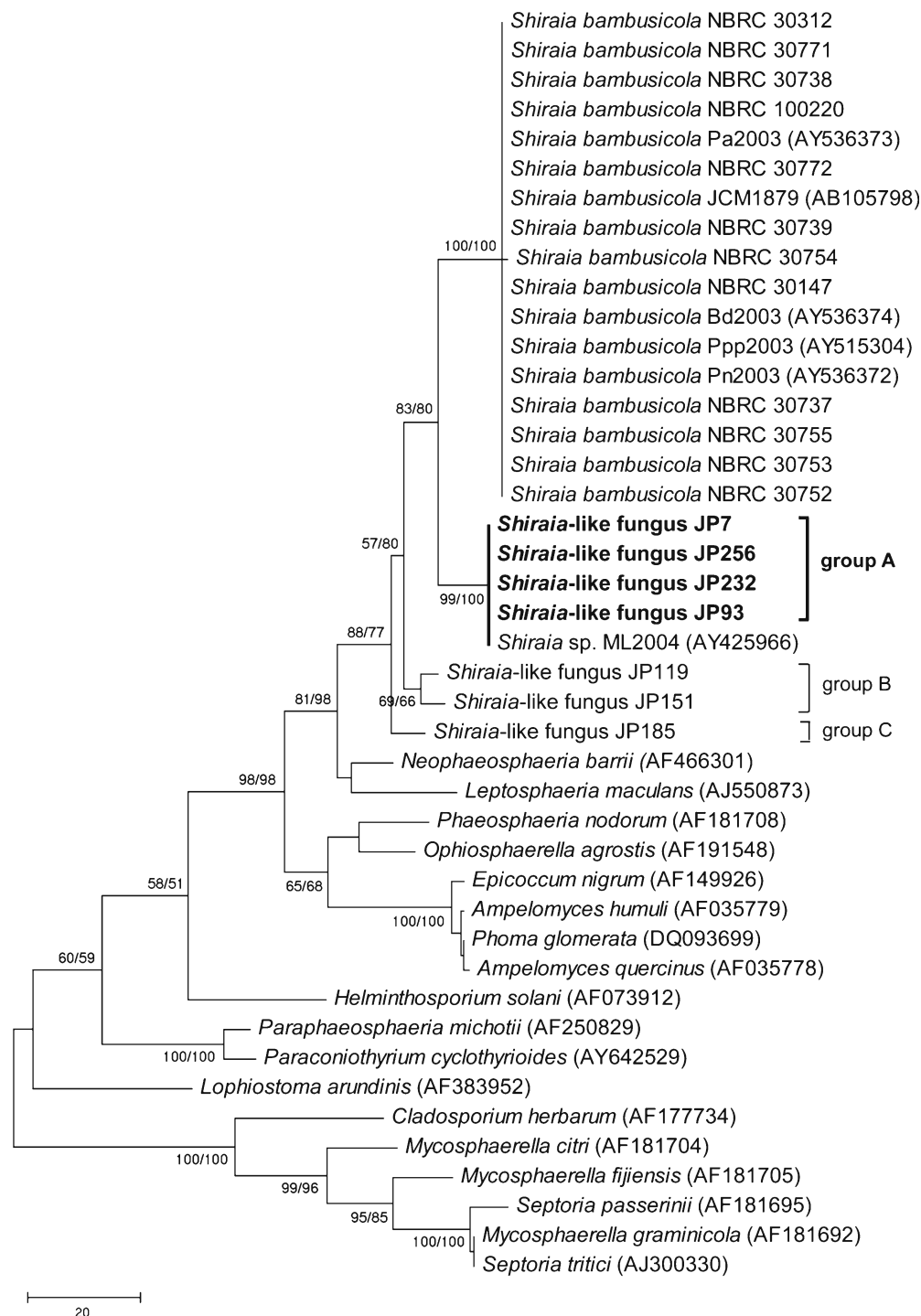


Fig. 2. Parsimony tree of bamboo isolates based on 385 bp of internal transcribed space (ITS)1–5.8S-ITS2 rDNA gene sequences. Confidence values above 50% obtained from a 1000-replicate bootstrap analysis are indicated at the *branch nodes*. Bootstrap values from the maximum-parsimony and neighbor-joining methods were determined (MP/NJ). The *scale bar* indicates the number of steps. The *bold line* emphasizes the clade of *Shiraia*-like fungi in group A



Other three isolates had low similarities with *S. bambusicola* and clustered in another clade.

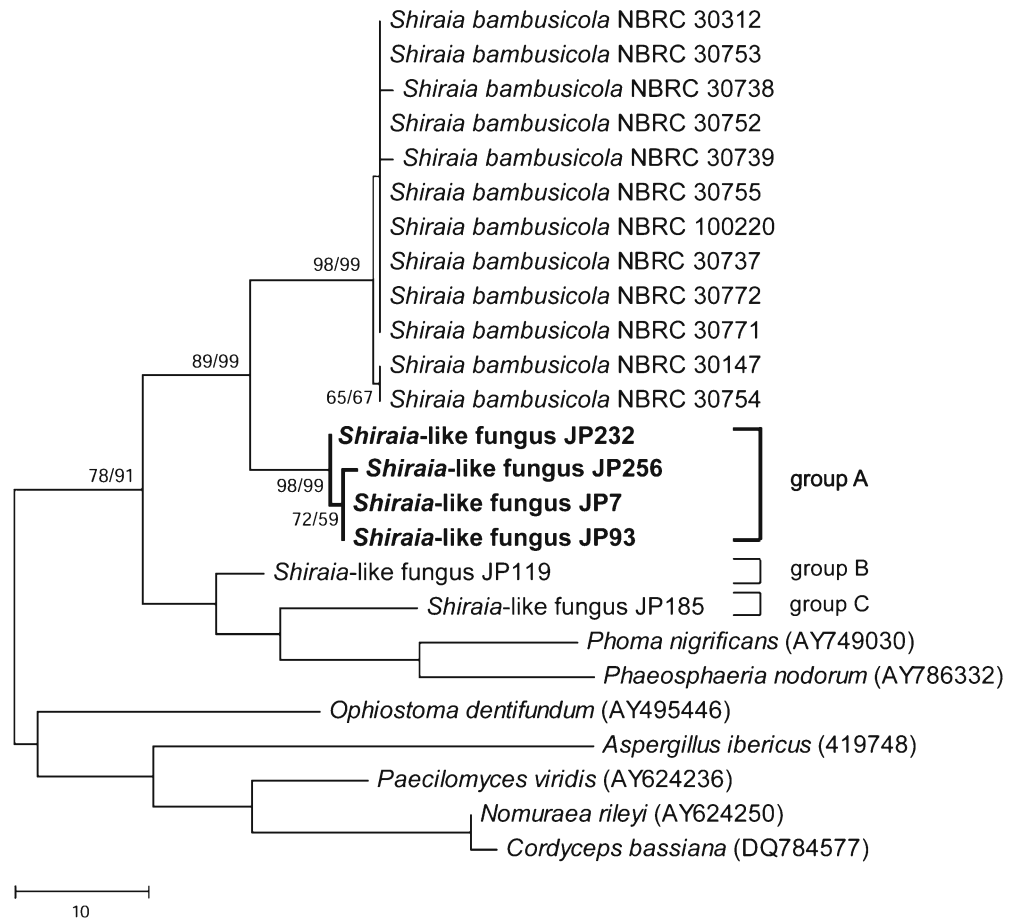
The ITS parsimony tree showed that group A was most similar to *Shiraia* sp. ML-2004 AY425966 (98% similarities) and clustered to the *S. bambusicola* clade (90%–93% similarities) with high bootstrap values. Although group B (JP119 and JP151) and group C were related to *Ampelomyces quisqualis* (AY293793) and its allies in the D1/D2 phylogram, they were clearly separated from group A, and no reference strain from the database was clustered within

these two groups. Moreover, the *tub2* parsimony tree showed a similar cluster pattern with ITS regions, categorizing our *Shiraia*-like fungi into three different groups.

Morphological characteristics

Seven isolates were cultured and observed in morphological features on three kinds of media following Tanaka and Harada (2003). As a result, three different morphological

Fig. 3. Parsimony tree of bamboo isolates based on 300 bp of beta-tubulin (*tub2*) gene sequences. Confidence values above 50% obtained from a 1000-replicate bootstrap analysis are indicated at the *branch nodes*. Bootstrap values from the maximum-parsimony and neighbor-joining methods were determined (MP/NJ). The *scale bar* indicates the number of steps. The *bold line* emphasizes the clade of *Shiraia*-like fungi in group A



types were recognized among them. The isolates JP119 and JP151 in group B produced numerous conidia (Fig. 5a) and one isolate, JP185 in group C, produced filiform conidia (Fig. 5b–d). In contrast, a peculiar structure was found in four other isolates of group A, JP7, JP93, JP232, and JP256: they produced prawn-shaped conidiomata (Fig. 4a–e) on RSA and CMA. The prawn-like structure is supposed to be a spermatogonium as the germination of conidia has not been observed. Their cultures on PDA for 7 days at 25°C produced colonies faintly tinted red. A pinkish to reddish pigment was produced on PDA after several more days. Honeydew exudates were detected after 1 month.

The microscopic photographs of isolate JP7, which was cultured on RSA medium, are shown in Fig. 4. The prawn-shaped conidioma-like structures were solitary, superficial, pycnidial, elongated, 80–230 × 22–40 μm, unilocular, with muriform, rough surface, dark brown at the base, pale brown to hyaline from the middle to tip, and produced on and merging into a basal sterile mycelium. The walls were about 2–8 μm thick and composed of thick-walled angular cells. The shapes of immature conidioma-like structures were doliform to ampulliform. When overmatured, they were aggregated, often in an irregular branching from one stalk. The ostioles were central and circular. The conidiogenous cells were ampulliform, enteroblastic, phialidic, discrete, about 4.5–5 × 2.5–3 μm, hyaline, and smooth. The

conidia were cylindrical to ellipsoid, aseptate, 2–3 × 1.5–2 μm, hyaline, thin-walled, and smooth.

In our molecular phylogenetic analysis using several genes, it is strongly suggested that *Shiraia* and our seven *Shiraia*-like fungi are members of Pleosporales. Especially in phylogenetic analysis using high-resolution molecules such as ITS regions and the beta-tubulin gene, *Shiraia*-like fungi are dispersed into three groups, groups A–C (see Figs. 2, 3). The phylogenetic relationships of groups B and C were rather far from other known genera, and we could not find any specific characters except molecular evidence in these two groups. More examination of these fungi is needed to clarify the taxonomic status of groups B and C.

The morphology of both the macroconidia and microconidia in the synanamorph of *S. bambusicola* (Tsuda and Ueyama 1987; Tsuda et al. 1989) were distinctly different from the conidia of group A, which were produced in a prawn-like conidioma. Moreover, the anamorphs of the neighboring genera (e.g., *Ampelomyces*, *Conidioxyphium*, *Capitorostrum*, and *Phaeosphaeria*) do not have the same morphology as in group A (see Sutton 1980). We suspect that the group A fungi may represent a novel genus related to *Shiraia* based on both molecular phylogeny and morphology. Induction of germination of the conidia of group A fungi was tried (see Fig. 4g); however, they did not germinate. Therefore, more investigation, especially on the

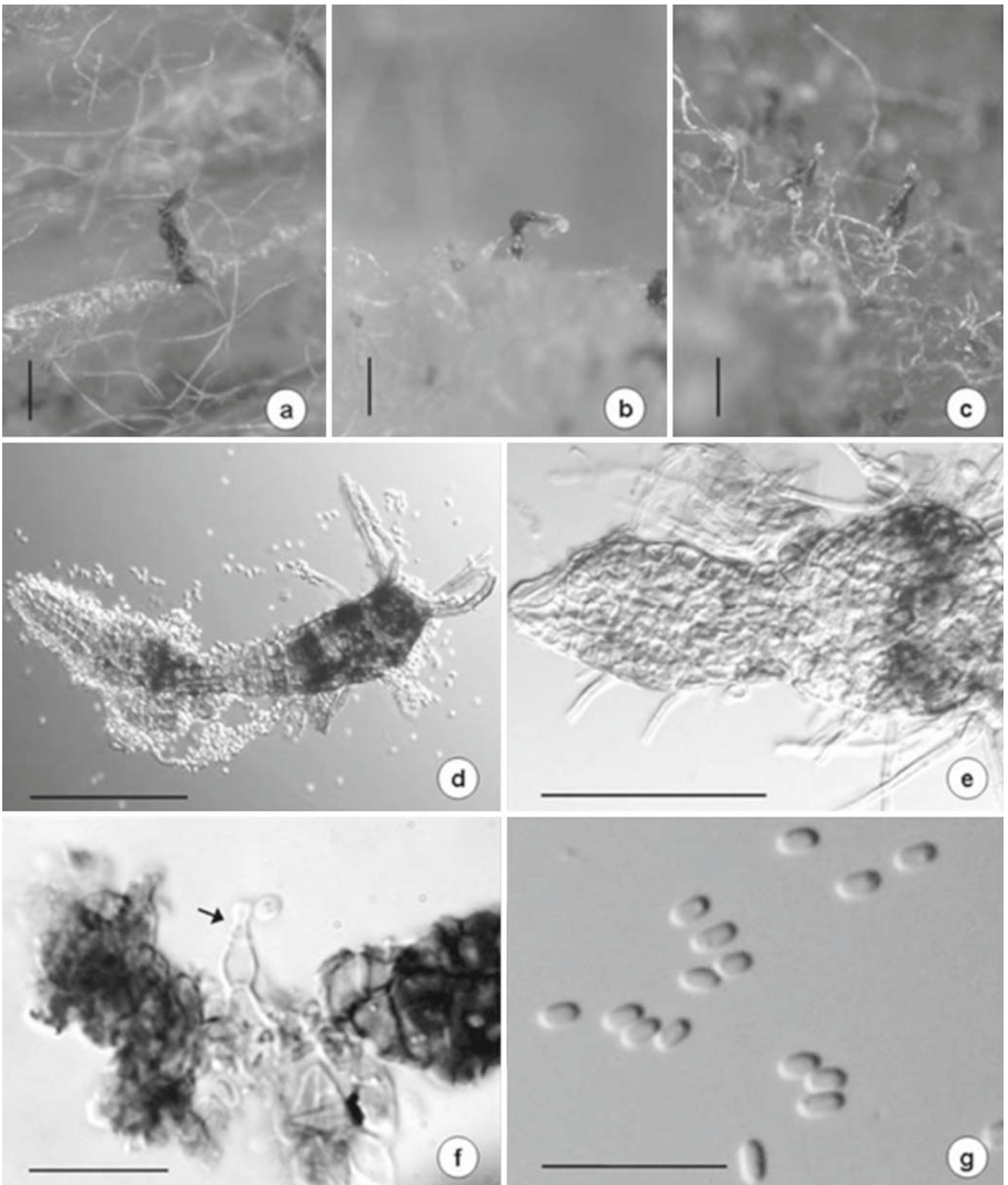


Fig. 4. Light microscopy of *Shiraia*-like fungus isolate JP7 on RSA. **a–c** Conidiomata on agar. **d** Mature conidioma. **e** Immature conidioma. **f** Conidiogenous cell (arrow). **g** Conidia. Bars **a–c** 100 μm ; **d, e** 50 μm ; **f, g** 10 μm

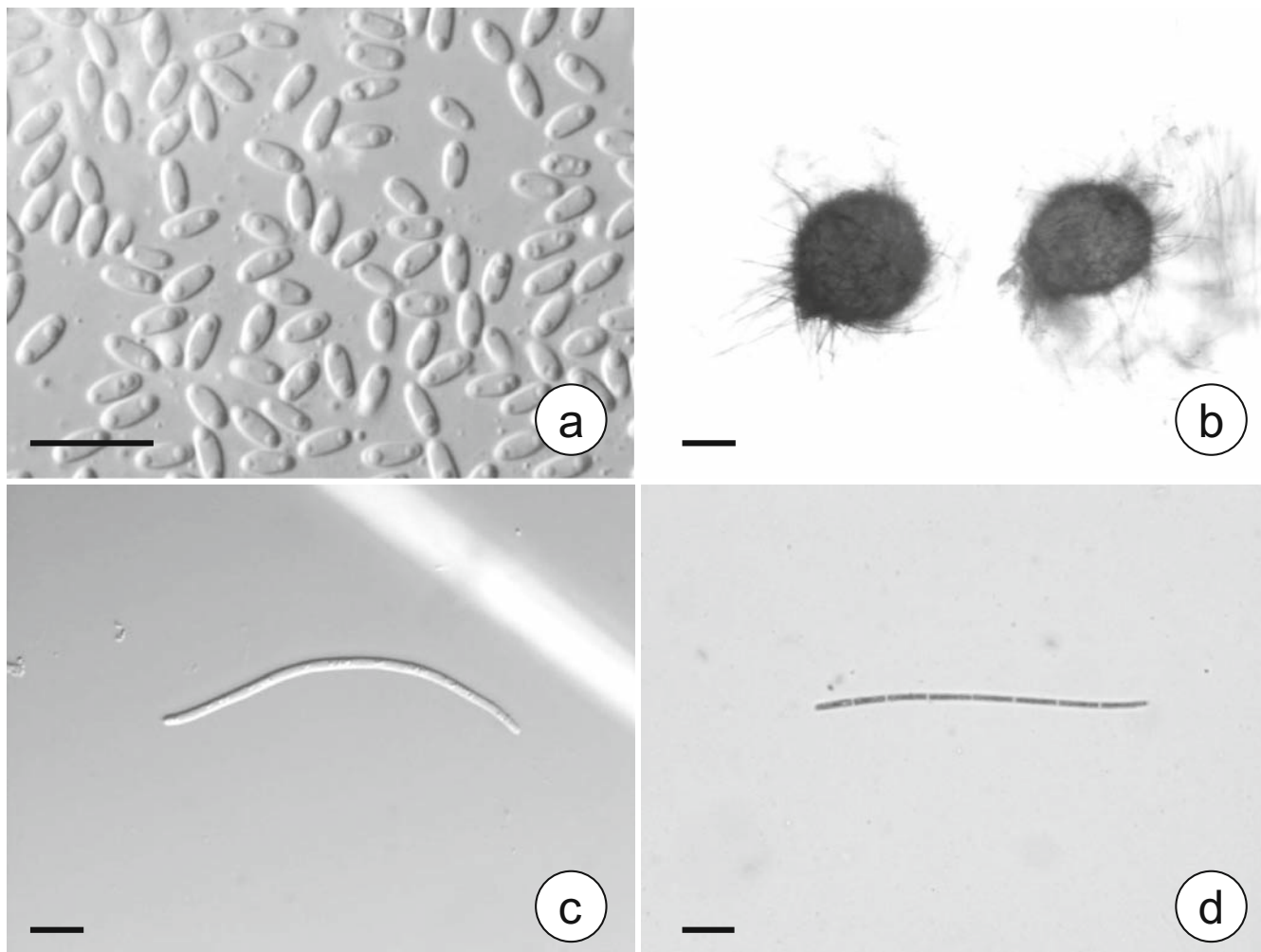


Fig. 5. Light microscopy of *Shiraia*-like fungi, isolates JP119 (group B) and JP185 (group C) on RSA. **a** Conidia, JP119. **b** Conidiomata, JP185. **c, d** Filiform conidia (**d** trypan blue stain), JP185. Bars **a, c, d** 10 μ m; **b** 100 μ m

function of the conidia, should be carried out to confirm the hypothesis.

The stomata of *S. bambusicola* have been observed on the twigs of several species of bamboos, e.g., *Phyllostachys nigra* Munro., *P. bambusoides* Siebold. et Zucc., and *P. nidularia* Munro. (Hino 1961). Our *Shiraia*-like isolates were discovered from fresh tissues of *Phyllostachys* sp. and *Sasa* sp. Interestingly, three isolates in group A were found from *Phyllostachys* sp. in two distinct places (Suita campus, Osaka University, and Hakunoshima, Minoh, Osaka Prefecture, Japan). In addition, the sources of the *S. bambusicola* reference strains were mainly isolated from *Phyllostachys* sp. (see Table 1). These facts suggest that *S. bambusicola* and our *Shiraia*-like fungi may have host preferences to *Phyllostachys* spp. *S. bambusicola* and our isolates appear to be endemic fungi inhabiting bamboos of East Asia, because the genus *Shiraia* and *Shiraia*-like fungi have been found only from Japan and China.

It has been reported that *S. bambusicola* could produce biologically active compounds. New perylenequinones were

isolated from the stomata tissues, and the structures of the compounds were determined (Kishi et al. 1991). Wu et al. (1989) revealed that several phytopathogenic fungi that produce deeply red pigmented mycelium expressed the presence of secondary metabolites containing a perylenequinone nucleus. These fungal metabolites exert photodynamic activity on bacteria and fungi. The deep red pigmentation on the culture media of *S. bambusicola* was reported by Wu et al. (1989). Remarkably, all the *Shiraia*-like fungi in group A produced a deep red pigment and expressed bioactivity against the plant pathogenic fungi (data not shown). This finding suggests that our *Shiraia*-like fungi might produce a valuable compound that represents a new bioresource.

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