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Ophiostoma ssiori sp. nov. (Ophiostomatales, Ascomycetes) isolated from a bark beetle in *Prunus* species

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Abstract An *Ophiostoma* species frequently isolated from *Polygraphus ssiori* infesting *Prunus* species was described as *Ophiostoma ssiori* sp. nov. This fungus is characterized by allantoid to crescent-shaped ascospores and synnematous anamorph, similar to *O. subalpinum*, *O. torticiliata* and many species of the *O. piceae* complex, but can be distinguished by morphological characteristics of ostiolar hyphae, conidia, and synnema. Molecular data also show that these fungi differ from each other.

Key words: new species, ophiostomatoid fungi, *Ophiostoma ssiori*, *Polygraphus ssiori*, *Prunus* sp.

Introduction

The genus *Ophiostoma* H. & P. Sydow is characterized by long-necked perithecia, evanescent asci, and hyaline one-celled ascospores accumulating in mucilaginous masses at the tip of the perithecial neck. These characteristics are adapted for dispersal by insects. Indeed, many species of *Ophiostoma* have been isolated from insects, especially bark beetles (Coleoptera; Scolytidae), which primarily infest trees. Some of these species are exclusively associated with specific beetles, and in this case, the bark beetles have mycangia, cuticular structures that carry fungal spores and mycelia (Francke-Grosmann, 1967), and the fungi function as food for the larvae. In addition, vectored fungi may influence the distribution and abundance of bark beetles by altering the host-plant quality and availability (Whitney, 1982). Thus, the fungi have multiple interactions with the bark beetles and are thought to have important impacts on the beetles and host trees in the forest ecosystem. However, basic information regarding fungal flora of this group is mainly restricted to North America and Europe. In particular, there have been only a few reports regarding fungal species isolated from beetle species distributed in Asia, including Japan. We therefore have attempted to address this information deficit by examining the *Ophiostoma* species, which has been isolated from bark beetles in Japan.

In our survey of the *Ophiostoma* species in Japan, we conducted isolation tests from *Polygraphus ssiori* Niijima (Masuya *et al.*, 2001). *Polygraphus ssiori* is a bark beetle species infesting *Prunus* spp. and distributed in Asia (Wood and Bright, 1992). This beetle species is common in Northern Japan and is frequently found on *Prunus* spp. Based on the results of isolation tests, the *Ophiostoma* species is frequently isolated from these beetles (65–95%, Masuya *et al.*, 2001). This species is morphologically similar to *Ophiostoma subalpinum* Ohtaka & Masuya, *O. piceae* complex (Harrington *et al.*, 2000), and *O. torticiliata* (Olchow. & J. Reid) Seifert & Okada. We did, however, find some morphological differences, and the fungi also appear to be quite different from each other ecologically. We therefore describe herein the fungus isolated from *P. ssiori* as *Ophiostoma ssiori* sp. nov.

Materials and Methods

Fungi isolation

Unidentified *Prunus* sp. fungus was isolated from the bark beetle, *P. ssiori*, and from the gallery walls of the insects in the bark of dead tree. The fungus was collected on the same species of tree at two different localities in northern Japan (Higashidohri Village, Aomori Prefecture and Morioka City, Iwate Prefecture) during May and June 2000. A total of 162 beetles and 100 pieces of bark bearing galleries of the insects were used for the isolations.

Adult beetles were placed directly on the surface of 1% malt agar (malt extract, 10 g; agar, 15 g; distilled water, 1000 mL) without surface-sterilization, and the plates were incubated at 15°C in the dark. After 2 months, fungi that had grown on the plates were isolated by transferring hyphal tips or by lifting conidial masses using a sterilized tungsten needle to Petri dishes containing 2% malt agar (malt extract, 20 g; agar, 15 g; distilled water, 1000 mL). These dishes were incubated at 15°C in the dark for an additional 2 weeks.

Morphology

The isolates were incubated at 15°C in the dark, and after 2 weeks, small pieces of sterilized twigs of *Prunus jamasakura* Sieb. were added to the plates to stimulate sporulation. Fungal structures (e. g. perithecia or conidiophores) that were produced in cultures were mounted on glass slides in 1% lacto-fuchsin and observed and measured using a light microscope. Fifty measurements were made of each structure, and the ranges and averages were computed.

The growth rates of isolates were determined at 4, 10, 15, 20, 25, 30, and 35°C. Agar disks 5 mm in diameter were cut from actively growing margins of colonies of each isolate to be tested and placed at the center of plates containing 2% malt extract agar. Three replicate plates were prepared for each isolate. In addition, the cycloheximide tolerance of isolates was tested at 1.0 g/L concentration using the method of Harrington (1981). The colony diameter on each plate was measured after 4 and 9 days of incubation at 20°C, and growth rates were calculated as mm/day.

DNA sequence comparisons

Five isolates of the fungus obtained in this study and two *O. subalpinum* isolates (MAFF 410923 (ex-type) and 410924) were used for DNA analysis. Cultures were incubated on 2% MA plates within 4 weeks. DNA was directly amplified using the polymerase chain reaction (PCR) in a GeneAmp 9600 thermal cycler (Perkin-Elmer). The method used was that of Suyama *et al.* (1996) with slight modification. A small amount of mycelium or conidia was removed from cultures and crushed in a 100 μm PCR tube with a pipette tip under a dissection microscope. Fifty microliters of reaction mixture containing 5 μL 10×buffer, 6 μL of 25 mM $MgCl_2$, 10 mM of dNTPs, 20 pmol of each primer, ITS5 and ITS4 for the ITS1-5.8s-ITS2 region (White *et al.*, 1990), 10 μL 5×CG-RICH solution (with FastStart Taq DNA polymerase, Roche Molecular Biochemicals), and 2.5 units of Fast Start Taq DNA polymerase (Roche Molecular Biochemicals) were added to the template. The PCR conditions were as follows: 4 min at 95°C, annealing at 94°C for 20 s, and at 56°C for 1 min. This cycle was repeated 40 times, and the final elongation reaction was carried out at 72°C for 10 min. The PCR products were purified with Microcon-30 Microconcentrators (Amicon, Inc., USA) and used for sequencing using the Big Dye Terminator Cycle Sequencing FS Ready Reaction kit and the ABI PRISM 310 genetic analyzer (Perkin Elmer Applied Biosystems). Obtained sequences have been deposited in GenBank (AB096205–AB096209 for *O. ssiori*, AB096210 and AB096211 for *O. subalpinum*).

The obtained sequences were analyzed together with several sequences previously reported by Harrington *et al.* (2001) for the ITS1-5.8S-ITS2 regions of the ribosomal DNA operon. Overall, the data set for ITS1-5.8srDNA-ITS2 regions included 28 sequences, including our obtained se-

quences.

Sequences were aligned using Clustal X version 1.81 (Thompson *et al.,* 1997). Alignments were manually adjusted using the program BioEdit version 5.0.9 (Hall, 1999). The aligned data set was analyzed using the programs PAUP*4.0 beta10 (Swofford, 1999). A parsimony analysis was carried out using a heuristic search with random stepwise addition and the tree-bisection reconnection (TBR) option of the program. Gaps were treated as missing data. All characters were equally weighted. The MAXTREE option set to auto-increase. Bootstrap values (500 replicates) were also calculated with the MAXTREE option set to 1000.

Results and Discussions

Ophiostoma ssiori is characterized by allantoid to crescent-shaped ascospores and synnematous anamorph with a *Sporothrix*–like synanamorph. These characteristics are shared by many other *Ophiostoma* species, particularly, *O. piceae* complex (*O. piceae* (Münch) H. & P. Syd., *O. setosum* Uzunovic *et al*., *O. floccosum* Math.-Käärik, O. querci (Georgév.) Nannf., *O. ulmi* (Buisman) Nannf., *O. novo-ulmi* Brasier, *O. himal-ulmi* Brasier & Mehrotra, *O. canum* (Münch) H. & P. Syd., and *O. catonianum* (Goid.)Goid.). However, species of this complex are easily distinguished from *O. ssiori* based on their more divergent ostiolar hyphae and the long stipe of the synnema (Table 1). Also, the species in the *O. piceae* complex frequently produce synnema and distinct *Sporothrix*–like synanamorphs with ramoconidia on their colonies, but *O. ssiori* less frequently or does not produce them on the colony and its synanamorph does not have distinct denticles on the conidiogenous cells (Figs. 8–10). *O. torticiliata* (Olchow. & J. Reid) Seifert & Okada also has crescent-shaped ascospores and a synnematous anamorph, but is distinguished from *O. ssiori* based on the length of ostiolar hyphae, the stipe length of the synnemata and the morphology of the ascospores. *O. torticiliata* has longer ostiolar hyphae and a longer synnema stipe and shorter ascospores than *O. ssiori* (Table 1). *Ophiostoma subalpinum* is particularly similar to *O. ssiori* with respect to the relatively short stipes of their synnema. However, ostiolar hyphae of *O. subalpinum* are shorter than those of *O. ssiori*. These two species can also be distinguished from one another by the difference in their ascospore morphology, with *O. ssiori* having more slender ascospores than *O. subalpinum* (Table 1). The conidial shapes of the synnemata are also distinguishable. Conidia of *O. ssiori* are oblong, while those of *O. subalpinum* are ellipsoidal to ovoid. In addition, the habitats and vectors of the two species differ significantly. *O. subalpinum* is frequently isolated from *Abies veitchii* and its infesting bark beetles, *Cryphalus piceae* Ratzeburg, *C. montatus* Nobuchi, and *Polygraphus proximus* Blandford (Ohtaka *et al*., 2002). In contrast, *O. ssiori* is isolated from *P. ssiori*, which infests *Prunus* sp. with high frequency. *Ophiostoma ssiori* can therefore be distinguished from previously known species of *Ophiostoma* with regard to both morphology and ecology (Table 1).

Our molecular data from the present study show that *O. ssiori* is not related to the *O. subalpinum* and the species of the *O. piceae* complex (Fig.1). *Ophiostoma subalpinum* is clearly included in the clade of the *O. piceae* complex. In contrast, the clade of *O. ssiori* is clearly distinguished from the others based on its high bootstrap value (100%). Also, other than the species discussed above, there appear to be no species with a sequence highly similar to that of *O. ssiori* listed in the BLAST and FASTA database (not published). Therefore, based on molecular data, it is appropriate that *O. ssiori* be treated as separate from these other species.

Ophiostoma ssiori has frequently been isolated from *P. ssiori*, suggesting that this fungus is closely associated with *P. ssiori*. Some *Ophiostoma* species are known to have close relationships with bark beetles, and these fungi are thought to function nutritionally or as tools for overcoming the host defense (Paine *et al*., 1997). However the role of *O. ssiori* in relation to *P.*

Table 1. Morphological and ecological characteristics of *Ophiostoma ssiori*, *O. subalpinum*, *O. piceae* complex and *O. torticiliata*.

	Characters	*Ophiostoma ssiori*	*O. subalpinum**	*O. piceae***	*O. torticiliata****
Teleomorph	Perithecial base diam. (μm)	75–150	85–195	80–225(–280)	150–250
	Neck lengh (μm)	400–1000	355–650	500–2000	(400–)700–1500
	Ostiolar hyphae length	10–46	5–16	10–25(–40)	Up to 135
	Ostiolar hyphae type	Parallel, rarely divergent	Divergent	Divergent	Divergent
	Ascospore size (μm)	(4–)4.5–5.5(–7)×1–2	3–4×1.5–2	2–5×1.5–2	2.5–3.5×1–1.5
	Ascospore shape	Crescent-shaped, without sheath	Oblong or allantoid, sometimes with sheath	Oblong, lunate, or allantoid, without sheath	Lunate or orange-section shaped, with sheath
Anamorph	Synnemata length	80–360	185–340	470–1200(–1500)	Up to 1200
	Conidia size (μm)	1.5–7(–10)×0.5–2	2.7–4.2×1.7–2.5	2.5–6×1–2.5	2.5–4×1–2
	Conidia shape	Oblong to ellipsoidal	Ellipsoidal or ovoid	Ellipsoidal, oblong, or allntoid	Clavate, ovoid, or oblong
Synanamorph	Conidiophore character	Unbranched, or branched, without distinct denticles	No data	Unbranched, branched, with ramoconidia	No data
	Conidia size (μm)	2.5–7.5×0.5–2	No data	(3–)5–15×1–3.5	No data
	Conidia shape	Oblong, ellipsoidal, or fusiform	No data	Cylindrical, elliptical, or fusiform	No data
Beetle associates		*Polygraphus ssiori*	*Cryphalus piceae*, *C. montatus* *Polygraphus proximus*	Various	Nodata
Host		*Prunus* sp.	*Abies veitchii*	Various	*Populus balsamifera*

* Data from Ohtaka *et al.* (2002), ** Combined data from Hunt (1956), Upadhyay (1981), and Ohtaka *et al.* (2002), *** Combined data from Olchowecki & Reid (1974), and Uadhyay (1981).

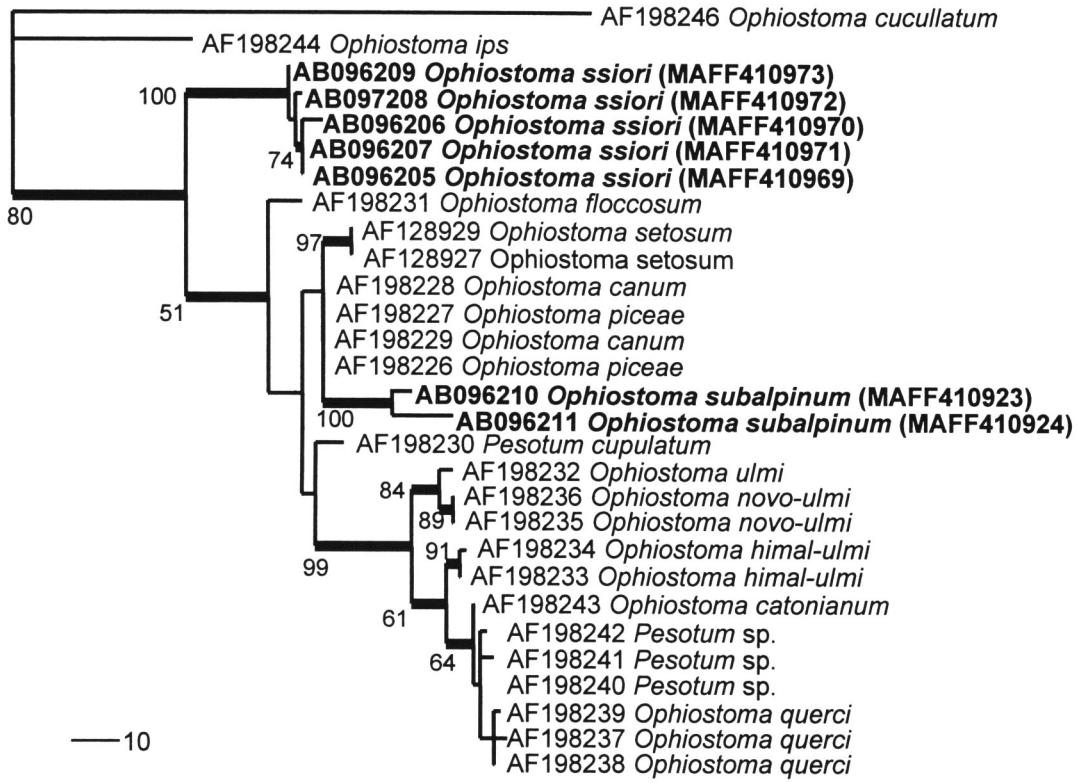


Fig. 1. One of the 604 most parsimonious trees of *Ophiostoma piceae* complex, other *Ophiostoma* species and its related anamorph based on 644 characters, including gaps, of ITS1-5.8S-ITS2 regions of rDNA operon. From a total of 644 characters, 459 characters are constant, 113 variable characters are parsimony-uninformative and 72 are informative. The tree is unrooted. 500 replicates of bootstrap values>50% indicated below the branches. Tree length=256, Consistency index (CI)=0.8633, Homoplasy index (HI)=0.1367, Retention index (RI)=0.9072.

ssiori remains uncertain, and additional studies are required.

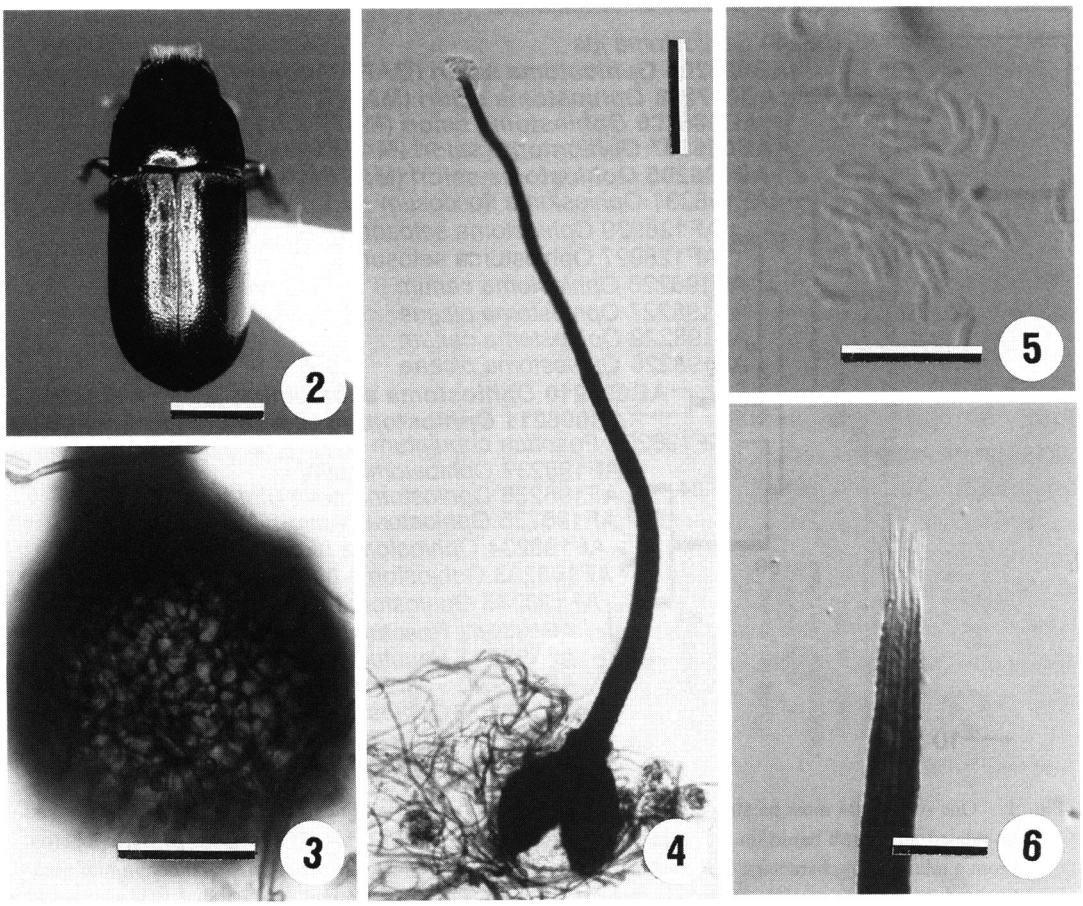
Many *Ophiostoma* species have been obtained from coniferous trees, although little information regarding species obtained from angiosperm is available. In addition, there are few reports regarding the *Prunus* species. However *O. ssiori* was easily isolated from *Prunus* species and a bark beetle in the present study, suggesting that there may be many opportunities to collect *Ophiostoma* species from non coniferous trees. Such collections would be very important for improving our knowledge of this group of fungi.

Taxonomy

Ophiostoma ssiori Masuya, Kubono et Ichihara sp. nov. (Figs. 3–13)

Anamorph: *Pesotum* sp.

Perithecia basi nigra, globosa vel subglobosa, 75–150 μm diam, peridium ectostratum ex cellulis forma inaequilateralis vel irregularis, 5–15 μm diam. Collum cylindraceum, curvatum vel rectum, basi nigrum, pallidiore ad apicem, 400–1000 μm longum, ad basim 20–45 μm latum, ad apicem 8–17 μm latum, apice obtusum vel truncatum et pileo hyalino tectum, hyphis ostioli, hyalina, septata, parallea, cylindrica, apice rotundata, 10–46 μm longum, ad basim 1.5–2.5 μm latum. Asci juveniles clavati, ad 9×3.5 μm cum



Figs. 2–6. *Polygraphus ssiori* Niijima, and morphological characteristics of teleomorph of *Ophiostoma ssiori*. 2. *Polygraphus ssiori* Niijima female. 3. Outer layer of peridium. 4. Perithecium. 5. Ascospores. 6. Tip of a perithecial neck with ostiolar hyphae. Scale bars=1 mm in Fig. 2, 50 μm in Fig. 4, 20 μm in Fig. 3, 10 μm in Figs. 5 and 6.

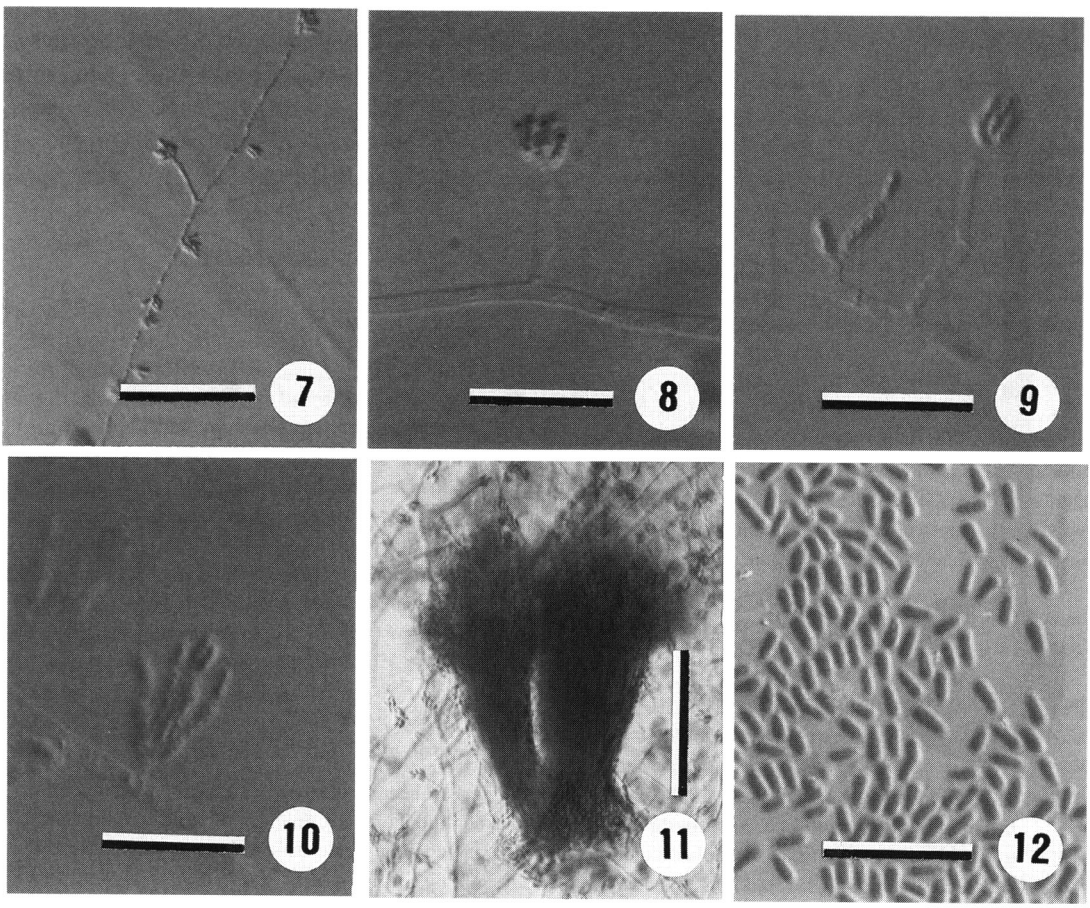
vagina, asci maturi non visi. Ascosporae hyalinae, aseptatae, lunatae, vagina nulla, 4.5–5.5× 1–2 μm, ad apicem colli in guttula conglobatae. Conidiophora mononematosa hyalina, septata. Cellulae conidiogenae annelides, hyalinae, 8–56×1.4–3.6 μm. Conidia hyalina, aseptata, oblonga, ellipsoidea vel clavata, interdum curvata, 2.5–7.5×0.5–2 μm. Conidiophora synnematosa pallido—brunnea vel nigra, septata, 80–360× 16–152 μm. Cellulae conidiogenae annelides, hyalinae, cylindricae, 8.7–26×0.7–1.5 μm. Conidia hyalina, aseptata, oblonga vel ellipsoidea, 1.5–7(−10)×0.5–2 μm, solitaria, dein ad apicem conidiophori in muco aggregata.

HOLOTYPE: TNS-F-6023, dried culture of MAFF410973 from Morioka, Iwate Pref., Japan, on *Prunus* sp., isolated by H. Masuya on 24 July 2000, from mycelium.

PARATYPE: TNS-F-6024, dried culture of MAFF410970 from Higashidohri, Aomori Pref., Japan, on *Prunus* sp., isolated by H. Masuya on 14 June 2000, from mycelium.

ETYMOLOGY: *ssiori*, refered to the name of isolation source, *Polygraphus ssiori* Niijima.

Colonies on 2% malt extract agar reaching 60–70 (mean 65) mm diam. in 9 days at 20°C, no growth at 4 and 35°C, their mean growth rate 52 mm/day at 20°C, at first hyaline to pale brown, after 8 weeks becoming brownish olive (Rayner, 19"m). Hyphae hyaline to brown, ver-



Figs. 7–12. Morphological characteristics of anamorph of *Ophiostoma ssiori*. 7. Simple conidiophores. 8. Simple conidiophore. 9. Simple conidiophores and a secondary conidium. 10. catenulate conidia and secondary conidia. 11. Synnematous conidiophores. 12. Conidia. Scale bars=50 μm in Fig. 11, 20 μm in Fig. 7, 10 μm in Figs. 8, 9, 10, and 12.

rucose, 1.2–7.5 μm wide. aerial hyphae oppressed or erected, hyaline to brown, smooth-walled, 1–7 μm wide, often aggregated in strands of hyphae and somewhat rod-shaped, moist conidial masses rarely appearing as cream-colored droplets on the colony surface. The fungus is tolerant of cycloheximide, with growth at 20°C reduced by approximately 20% on 2% malt extract agar containing 1.0 μg/mL.

Teleomorph: Perithecia superficial or partly embedded on the substratum and medium. Basal part black, globose to subglobose, 75–150 (mean 125) μm diam. without hyphal ornamentation, outer layer of the peridium composed of a thick wall, isodiametric to irregularly shaped cells, 5–15 (mean 9) μm diameter. Necks dark brown to black, broad at the base, becoming cylindrical to slightly tapered, sometimes flaring slightly at the tip, straight or curved, 400–1000 (mean 740) μm long, 20–45 (mean 34) μm wide at base, 8–17 (mean 11) μm wide near the tip, composed of dark, laterally fused, thick-walled, septate, hyphal elements, 1.7–3 (mean 2.5) μm wide. Ostiolar hyphae, when present, hyaline, straight, tapered, paralleled, up to 12 in number, 10–46 μm long, 1.5–2.5 μm wide at base, develop as extensions of the hyphal elements composing the outer layer of the neck. Asci evanescent, clavate when young, up to 9×3.5 μm, mature asci not seen. Ascospores, hyaline, one-celled, crescent-shaped,

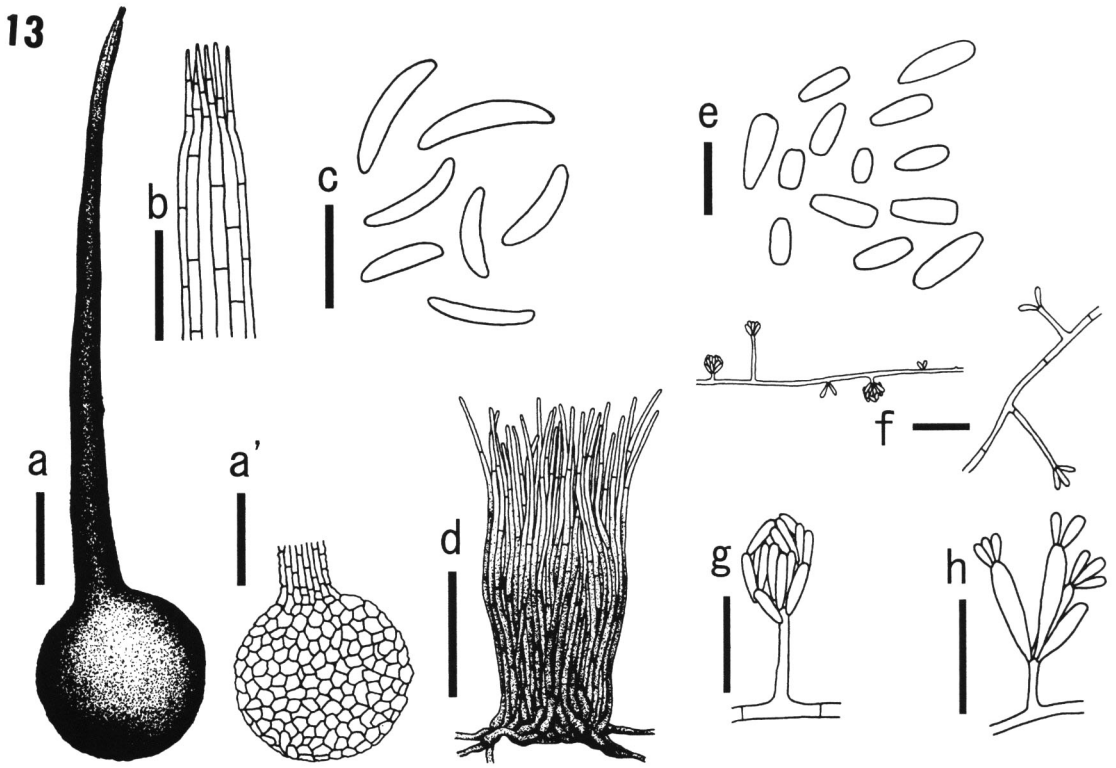


Fig. 13. *Ophiostoma ssiori* (MAFF910970). a. Perithecium. a'. Perithecium bleached with sodium hypochlorite. b. Tip of perithecial neck. c. Ascospores. d. Synnematous conidiophore. e. Conidia. f. Simple conidiophores. g. Simple conidiophore. h. catenulate conidia and secondary conidia. Scale bars=50 μm in Figs. 13a, 13a' and 13d, 10 μm in Figs. 13b, 13f, 13g, and 13f, and 5 μm in Figs. 13c and 13e.

(4–)4.5–5.5(–7)×1–2 (mean 5×1.4) μm, without sheath, accumulated in a cream-colored mass at the tip of neck.

Anamorph: Conidiophores macronematous to micronematous, mononematous to synnematous, sometimes directly produce conidia on hyphal elements. Mononematous conidiophores arising directly from hyphal elements, hyaline, septata, *Sporothrix*–like forms with a short stipe and conidiogenous cells. Conidiogenous cells sympodial, cylindrical, tapered, 2.5–56×1.4–3.6 (mean 21×2) μm. Conidia hyaline, 1–celled, oblong, ellipsoidal or clavate, sometimes curved, rounded at both ends, or with a slightly truncated base, 2.5–7.5(–12)×0.5–2 (mean 5×1.2) μm, sometimes produce secondary conidia, become intercalary. Secondary conidia, hyaline, one-celled, subglobe to ellipsoidal, 2–4×0.5–1.5 (mean 3.5×1.2) μm. Stipes of the synnematous conidiophores pale brown to black. Synnemata with loosely fused outer stipe cells, 80–360×16–152 (mean 170×51) μm excluding conidial masses. Conidiogenous cells of synnemata conspicuously divergent. Conidiogenous cells annellidic, cylindrical, 8.7–26×0.7–1.5 (mean 20×1.3) μm. Conidia hyaline, 1–celled, oblong to ellipsoidal, rounded at the both end of conidia, or with truncated bases, 1.5–7(–10)×0.5–2 (mean 4×1.5) μm, becoming aggregated in slimy creamed masses at the tip of the conidiophores.

Materials examined: from *Polygraphus ssiori* in *Prunus* sp., Morioka, Iwate, 2000-V-9, H. Masuya, MAFF410969; from *Polygraphus ssiori* in *Prunus* sp., Higashidohri, Aomori, 2000-VI-14, H. Masuya, MAFF410970; from *Polygraphus ssiori* in *Prunus* sp., Higashidohri, Aomori, 2000-VI-14, H. Masuya, MAFF410971; from

Polygraphus ssiori in *Prunus* sp., Morioka, Iwate, 2000-VI-12, H. Masuya, MAFF410972; from *Polygraphus ssiori* in *Prunus* sp., Morioka, Iwate, 2002-XII-24, H. Masuya, MAFF410973; from *Polygraphus ssiori* in *Prunus* sp., Morioka, Iwate, 2000-V-12, H. Masuya, MAFF410974.

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