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FULL PAPER

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Three new *Ophiostoma* species isolated from Japanese red pine

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Abstract Three new species of *Ophiostoma* found on Japanese red pine are described as *Ophiostoma pusillum* sp. nov., *O. botuliforme* sp. nov., and *O. nigrogranum* sp. nov. *Ophiostoma pusillum* is characterized by oblong ascospores and a *Hyalorhinocladiella* anamorph. *Ophiostoma botuliforme* has ostioles covered with a hyaline gelatinous cap, allantoid ascospores, and a *Pesotum* anamorph with hyaline to pale brown stipes. *Ophiostoma nigrogranum* has hyaline ostiolar hyphae with rounded tips, allantoid ascospores, and sclerotium-like structures.

Key words *Ophiostoma botuliforme* · *Ophiostoma nigrogranum* · *Ophiostoma pusillum* · *Pinus densiflora*

Introduction

Ophiostoma Syd. & P. Syd., previously considered to be a member of the *Ceratocystis sensu lato* complex, is a genus characterized by perithecia with a long neck, evanescent asci, and often ascospores with mucilaginous sheaths. Historically, its position in the complex has been controversial (Upadhyay 1993; Samuels 1993), but recently it has been shown by molecular data to be phylogenetically unrelated to *Ceratocystis sensu stricto* (Hausner et al. 1992, 1993a; Spatafora and Blackwell 1994).

Most *Ophiostoma* species depend on insects, particularly bark beetles, for their dissemination (Lagerberg et al. 1927; Mathiesen-Käärik 1960; Mathre 1964; Dowing 1984; Malloch and Blackwell 1993). Although many species of *Ophiostoma* are continually isolated from particular bark beetle species, others are isolated from unspecific bark beetles at a low frequency. These associations with beetles are thought to be the result of complicated interactions between bark beetles and fungi. Various factors (e.g., nutritional and moisture requirement of the beetles and fungi, the presence of mycangia on the beetles, and competition between fungi and beetles) are thought to affect the interactions (Mathiesen-Käärik 1960; Francke-Grosmann 1963; Whitney 1982).

The genus *Ophiostoma* includes economically important species that cause wilt diseases in trees and sap stain in timber (Lagarberg et al. 1927; Seifert 1993). Therefore, they have been extensively studied (Grosmann 1931; Siemaszko 1939; Mathiesen-Käärik 1953; Solheim 1986), and about 140 species of *Ophiostoma* have been described. However, most of them have been reported from Europe or North America with relatively few reports from other areas.

In Japan, the first study of *Ophiostoma* was conducted by Kasai (1917), who investigated *O. piliferum* (Fr.: Fr.) Syd. & P. Syd. and its ability to cause blue stain in timber. Subsequently, Nisikado and Yamauti (1933, 1934, 1935) recorded the distribution of *Ophiostoma*, *O. ips* (Rumbold) Nannf., *O. minus* (Hedgc.) Syd. & P. Syd., and *O. piceae* (Münch) Syd. & P. Syd. in Japan. Aoshima (1965) reported several additional species of *Ophiostoma*. Subsequently, Yamaoka et al. (1997, 1998) recorded 11 *Ophiostoma* species associated with the bark beetles *Ips typographus* L. f. *japonicus* Niijima and *I. cembrae* (Heer). However, many species remain to be described and given the appropriate names (Masuya et al. 1998, 1999; Yamaoka et al. 1998). Therefore, our study focused on the elucidation of the fungal flora of *Ophiostoma* in Japan. During the survey of *Ophiostoma* species in Japan, we have isolated three species of *Ophiostoma* from Japanese red pine and bark beetles

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infesting this pine. They are presented here as newly described species.

Materials and methods

Sampling and isolation of fungi

Fallen branches or cut logs of *Pinus densiflora* Siebold & Zucc. infested with bark beetles (*Tomicus piniperda* L., *Cryphalus fulvus* Niijima, *Cryphalus* sp., and an unidentified bark beetle) were brought to the laboratory. Bark was peeled with a flame-sterilized scalpel and hatchet to expose beetle broods and adjacent tissues. Two small pieces (about 2 mm^3) were cut from bark or sapwood near the galleries with a sterilized scalpel and placed directly onto 1% malt agar plates (1% MA; 10 g malt extract, 15 g agar, 1 l distilled water) without surface sterilization. In addition, putatively infested adult beetles were also placed on 1% MA plates. Inoculated plates were kept at 15°C in the dark. After 2 months, pure cultures were obtained by picking hyphae or conidia from the plates and putting these on the plates with 2% malt agar (2% MA; 20 g malt extract, 15 g agar, 1 l distilled water). These plates were incubated at 15°C in the dark. Cultures obtained were incubated for 2 weeks, and small pieces of autoclaved pine twigs or bark were added to the plates to stimulate sporulation. The plates were additionally incubated until perithecia were produced.

Living cultures were deposited at MAFF (Genetic Resource Center, Culture Collection of National Institute of Agrobiological Resources, Japan) and JCM (Japan Collection of Microorganisms, The Institute of Physical and Chemical Research). Dried specimens were deposited at TFM: FPH (Forest Pathology Herbarium of Forestry and Forest Products Research Institute, Japan).

Morphological observations

Perithecia, conidiophores, and conidia were mounted on glass slides in 1% lacto-fucsin for observation, measurement, and photography using a light microscope. Perithecia were also mounted after bleaching with sodium hypochlorite (1% available chlorine). Measurements were made of 50 random perithecia, ascospores, conidiophores, and conidia.

For scanning electron microscopy (SEM) observations, 8-mm-diameter agar disks were cut from the colonies with a sterilized scalpel and fixed in 3% glutaraldehyde overnight. The disks were then dehydrated in graded ethanol series, passed through ethanol isoamylacetate, and dried with a Hitachi Critical Point Drier. The materials were subsequently coated with platinum-palladium and examined using a Hitachi S-4200 scanning electron microscope.

Growth experiments

The growth rates of each of two isolates of three *Ophiostoma* species were determined at 20°C in the dark. In addition, cycloheximide tolerance of isolates was examined at a 1.0 g/l concentration. Three replicate plates of 2% MA containing 1.0 g/l cycloheximide and three not containing cycloheximide were prepared for each isolate. Agar disks 5 mm in diameter were cut from the margins of actively growing colonies of each isolate and placed at the center of the plates. Colony diameter on each plate was measured after 4 and 9 days incubation at 20°C, and growth rates were calculated as millimeters per day (mm/day).

Taxonomy

Ophiostoma pusillum Masuya, sp. nov. Figs. 1–10.

Anamorph: *Hyalorhinocladiella* sp.

Perithecia superficialia vel partim immersa, basi nigra, globosa vel subglobosa, 75–95 μm diam, appendicibus hyphoideis externis brunneolis septatis ornata, 10–100 × 0.5–2.2 μm; peridium ectostratum ex cellulis in forma inaequilateralibus vel irregularibus compositum. Collum cylindraceum, curvatum vel rectum, basi nigrum, pallidiore ad apicem, 55–100 μm longum, ad basim 12–20 μm latum, ad apicem 5.5–10 μm latum, apice obtusum vel truncatum et pileo hyalino tectum, hyphis ostioli non praeditum. Asci juveniles clavati vel subglobosi, usque 6.5 × 3.5 μm; asci maturi non visi. Ascosporae hyalinae, aseptatae, aspectu laterali vel frontali oblongae, aspectu extremo quadrangulatae, vagina hyalina circumdantes, 2.5–4 × 0.7–1.5 μm cum vagina, ad apicem colli in guttula conglobatae. Conidiophora hyalina, septata, micronematosa, mononematosa. Cellulae conidiogenae sympodiales, hyalinae, 15–17 × 0.9–1.8 μm. Conidia hyalina, aseptata, oblonga vel ellipsoidea, 2–4.5 × 0.5–1.5 μm, solitaria, dein ad apicem conidiophori in muco aggregata.

Holotype: TFM:FPH 7586, dried culture of MAFF410945 (JCM11704) from Takasu, Gifu Pref., Japan, on *Pinus densiflora*, isolated by H. Masuya on August 26, 1996, from conidial mass.

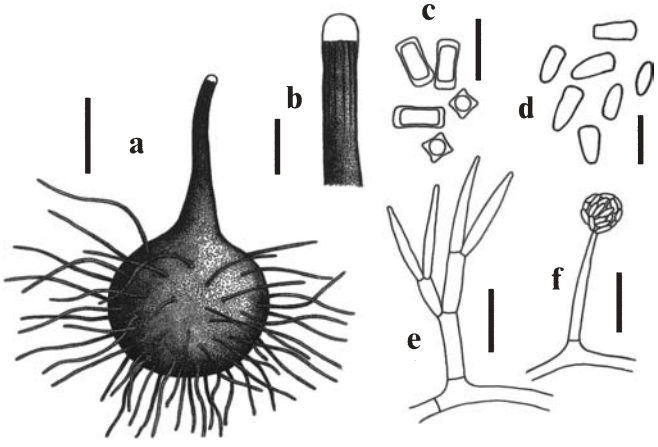
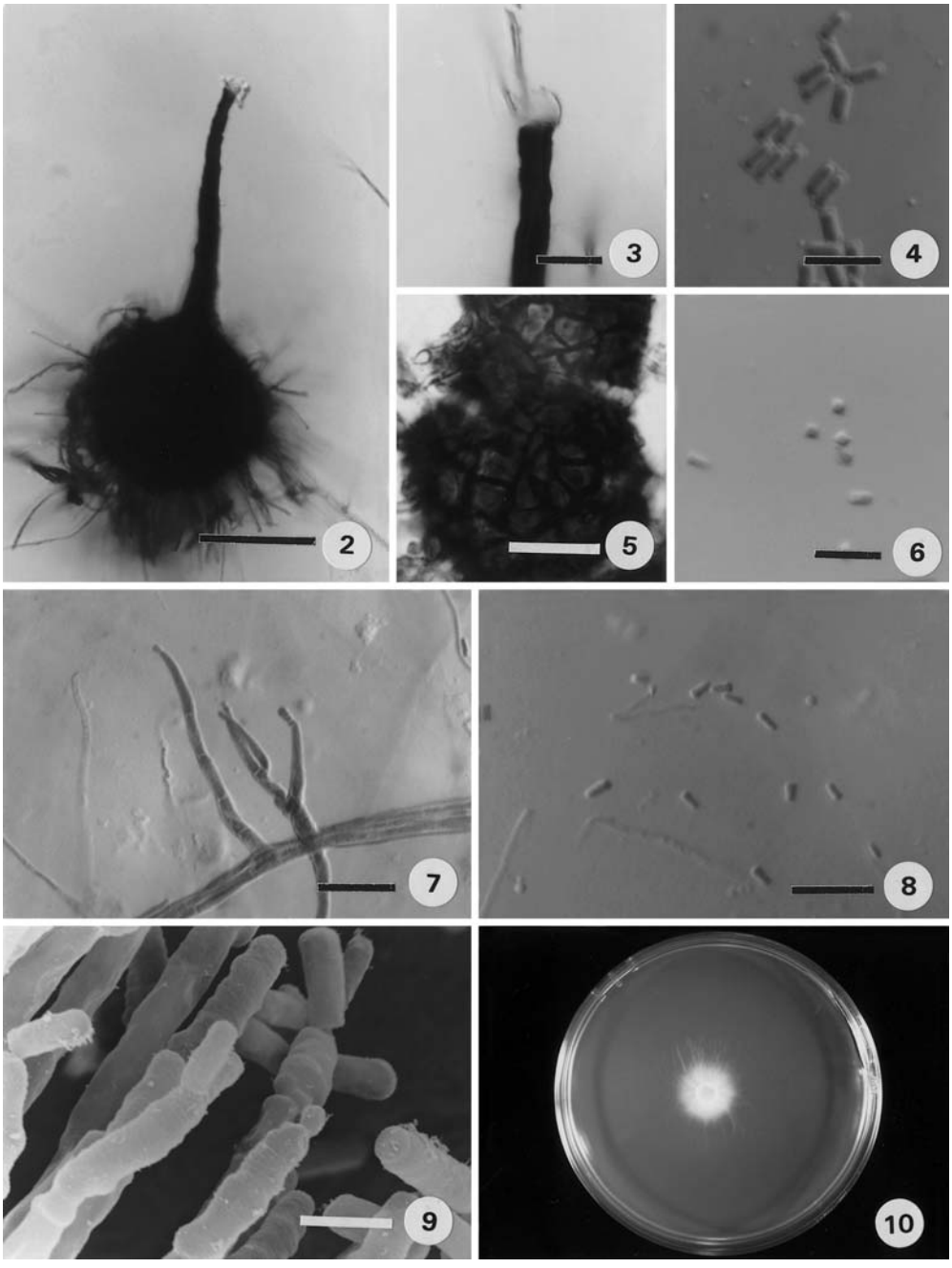


Fig. 1. *Ophiostoma pusillum* (MAFF410945). **a** Perithecium. **b** Tip of a perithecial neck. **c** Ascospores. **d** Conidia. **e** Hyaline *Leptographium*-like conidiophore. **f** Simple conidiophore. *Bars* **a** 50 μm; **b**,**e**,**f** 10 μm; **c**,**d** 5 μm



Figs. 2–10. Morphological characteristics of *Ophiostoma pusillum*. **2** Perithecium. **3** Tip of perithecial neck with a gelatinous cap. **4** Ascospores in side view. **5** Outer layer of peridium. **6** Ascospores in end view. **7** Conidiophores. **8** Conidia. **9** Scanning electron micrographs of conidiogenous cells and conidia. **10** Colony grown on 2% malt agar (MA) for 2 weeks at 20°C. *Bars* **2** 50 μm; **3**,**5**,**7**,**8** 10 μm; **4**,**6** 5 μm; **9** 3 μm

Paratype: TFM:FPH 7587, dried culture of MAFF410946 (JCM11705) from Tateyama, Toyama Pref., Japan, on *Pinus densiflora*, isolated by H. Masuya on August 27, 1996, from conidial mass.

Etymology: *pusillus* = tiny in Latin; referring to small perithecia of the species.

Colony: Colonies hyaline, sometimes becoming pale brown, oppressed to occasionally floccose on 2% MA. Margins irregular (Fig. 10). Hyphae straight or curved, spreading radially on the medium and immersed in the medium, hyaline to pale brown and 1–5.5 μm diameter, 2.5 μm on average. Growth of colonies on 2% MA, 36–48 mm, 42 mm on average, in 4 days at 20°C. Resistance to cycloheximide with approximately 86% reduction in growth on 2% MA with 1.0 g/l cycloheximide at 20°C. Protoperithecia sometimes formed.

Teleomorph: Perithecia superficial on, or partly embedded in, the substratum. Basal part black, globose to subglobose, 70–95 μm diameter, 85 μm on average, ornamented with brown, unbranched, thin-walled, irregular or straight, septate hyphal appendages, 10–100 × 0.5–2.2 μm, 38 × 1.3 μm on average (Figs. 1a, 2), outer layer of the peridium composed of thick-walled, inequilaterally or irregularly shaped cells, 5–12.5 × 4–9 μm, 8 × 6 μm on average (Fig. 5). Neck black at base, becoming lighter colored (brown) at the apex, either nearly cylindrical or tapered,

Table 1. Morphological characteristics of *Ophiostoma pusillum*, *O. nigrum*, and *C. tubicollis*

Characters	*O. pusillum*	*O. nigrum*[b]	*C. tubicollis*[b]
Perithecia base width (μm)	48–80	60–140	85–150
Perithecial character	Globose to subglobose	Globose to subglobose	Globose to subglobose, ornamented with spine
Perithecial neck length (μm)	54–95	120–350	53–115 (315)
Tip of perithecial neck	Ostiolar hyphae absent, with gelatinous cap	Ostiolar hyphae occasionally present	Truncate, ostiolar hyphae absent
Ascospore size[a] (μm)	2.5–4 × 0.5–1.5	3–5 × 1.5–2.5	3–4.5 × 1.5–2
Ascospore shape	Oblong, with sheath	Oblong, with sheath	Oblong, with sheath
Conidiophore character	Unbranched, or penicillately branched, without distinct denticles	Unbranched, penicillately branched, or synnematous, without distinct denticles	Loosely branched, without distinct denticles
Conidial size (μm)	1.3–4.5 × 0.5–1.5	1.5–5 × 1–3.5	2–6.5 × 0.7–1.5
Conidial shape	Oblong to clavate	Globose to subglobose	Clavate

[a] Including sheath
[b] Data from Olchowecki and Reid (1974) and Upadhyay (1981)

curved or straight, 55–100 μm long, 85 μm on average, 12–20 μm wide at base, 18 μm on average, 5.5–10 μm wide at tip, 7 μm on average, composed of dark, laterally fused, thick-walled, septate, hyphal elements, 2–2.5 μm wide, 2.2 μm on average, terminating in an obtuse to truncate apex, and covered by a hyaline gelatinous cap (Figs. 1b, 3). Ostiolar hyphae absent. Immature asci, clavate to subglobose when young, maximum size 6.5 × 3.5 μm, then evanescent. Ascospores hyaline, one celled, oblong in side and top view, quadrangular in end view, enclosed in a thin, distinct hyaline sheath slightly thickened at either end of spores (Figs. 1c, 4, 6), 2.5–4 × 0.7–1.5 μm, 3.8 × 1.4 μm on average (including sheath), accumulating in a white drop at the tip of neck.

Anamorph: Conidiophores arising directly from mycelium, hyaline, septate, mononematous, sometimes developed to a *Leptographium*-like form with a hyaline short stipe and up to three series of branches and conidiogenous cells (Figs. 1e, 7). Conidiogenous cells annellidic, cylindrical, 15–17 × 0.9–1.8 μm, 16 × 1.5 μm on average (Fig. 9). Conidia hyaline, one celled, oblong to ellipsoidal, sometimes slightly curved, 2–4.5 × 0.5–1.5 μm, 4 × 1.4 μm on average (Figs. 1d, 8), becoming aggregated in slimy masses at the tip of conidiophores (Fig. 1f).

Ophiostoma pusillum, which is characterized by small perithecia with a short neck and oblong ascospores, exhibits a combination of teleomorph characteristics also found in *O. nigrum* (R.W. Davidson) de Hoog & R.J. Scheffer (Davidson 1958; Upadhyay 1981) and *C. tubicollis* Olchow. & J. Reid (Olchowecki and Reid 1974). However, the width of the ascospores of this fungus is narrower than those of *O. nigrum* and *C. tubicollis* (Table 1). Perithecia of *O. nigrum* have comparatively long necks with occasionally a few ostiolar hyphae, but lacking a gelatinous cap at the apex, whereas those of *O. pusillum* have short necks without ostiolar hyphae and with a gelatinous cap (Figs. 1, 3). Therefore, *O. pusillum* and *O. nigrum* can be distinguished by perithecial neck and ascospores. Comparison of *O. pusillum* with the type specimen of *O. nigrum* (BPI595711) also supported these differences. *Ophiostoma pusillum* and *C. tubicollis* differ in perithecial size and ornamentation of perithecia (Table 1). The ornaments of the perithecia of *C. tubicollis* are conical spines, but those of *O. pusillum* are hyphal. In addition, the perithecial neck of *C. tubicollis* lacks a gelatinous cap at the tip.

Conidiogenesis in *O. pusillum* is annellidic with the anamorph being referable to *Hyalorhinocladiella* H.P. Upadhyay & W.B. Kendri (Upadhyay and Kendrick 1975). *O. nigrum* and *C. tubicollis* both have *Hyalorhinocladiella* anamorphs (Olchowecki and Reid 1974), but *O. nigrum* also produces *Acremonium*-like conidiophores. The hyphal width of *C. tubicollis* is very narrow (up to 2 μm) (Olchowecki and Reid 1974), but up to 5.5 μm in *O. pusillum* (Table 1). Thus the anamorphic state of *O. pusillum* also differentiates this species from *C. tubicollis* and *O. nigrum*.

Ophiostoma pusillum was isolated from the bark beetles *Tomicus piniperda* L. and *Cryphalus fulvus* Murayama, and from the gallery walls of an unidentified bark beetle species that had infested an already dead red pine. The frequency of isolation from *T. piniperda* and *C. fulvus* was very low.

Ophiostoma botuliforme Masuya, sp. nov. Figs. 11–21.

Anamorph: *Pesotum* sp.

Perithecia superficialia vel immersa, basi nigra, globosa vel subglobosa, 70–100 μm diam, appendicibus hyphoideis externis brunneolis septatis ornata, 14–156 × 1.8–4 μm; peridium ectostratum ex cellulis in forma inaequilateralibus vel irregularibus compositum. Collum cylindraceum, curvatum vel rectum, basi nigrum, pallidiore ad apicem, 80–190 μm longum, ad basim 14–23 μm latum, ad apicem 9–12 μm latum, apice obtusum vel truncatum et pileo hyalino tectum, hyphis ostioli non praeditum. Asci juveniles subglobosi vel clavati, ad 6.5 × 5 μm cum vagina; asci maturi non visi. Ascosporae hyalinae, aseptatae, botuliformes, vagina hyalina circumdantes, 3–5 × 1–2 μm, ad apicem colli in guttula conglobatae. Conidiophora mononematosa hyalina, septata. Cellulae conidiogenae annelides, hyalinae, 6.5–43.5 × 1.2–3 μm. Conidia hyalina, aseptata, oblonga vel ellipsoidea, 4–8.5 (–14) × 0.8–2.7 μm. Conidiophora synnematosa hyalina vel pallide brunnea, septata, 55–130 × 9.5–38 μm. Cellulae conidiogenae annelides, hyalinae,

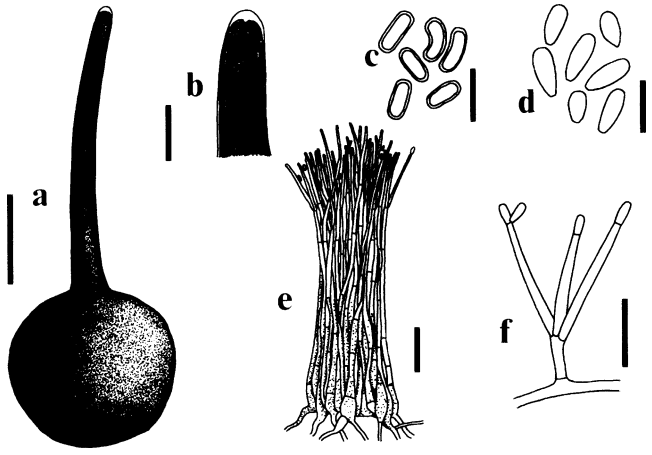


Fig. 11. *Ophiostoma botuliforme* (MAFF410947). **a** Perithecium. **b** Tip of perithecial neck. **c** Ascospores. **d** Conidia produced by synnematous conidiophore. **e** A hyaline *Pesotum*-like conidiophore. **f** Simple conidiophore. *Bars* **a** 50 μm; **b**,**e**,**f** 10 μm; **c**,**d** 5 μm

cylindricae, 8.7–26 × 0.7–1.5 μm. Conidia hyalina, aseptata, oblonga vel ellipsoidea, 2.5–6 × 0.8–2.5 μm, solitaria, dein ad apicem conidiophori in muco aggregata.

Holotype: TFM:FPH 7588, dried culture of MAFF410947 (JCM11706) from Takizawa, Iwate Pref., Japan, on *Pinus densiflora*, isolated by H. Masuya on November 28, 1996, from conidial mass.

Paratype: TFM:FPH 7589, dried culture of MAFF410948 (JCM11707) from Takizawa, Iwate Pref., Japan, on *Pinus densiflora*, isolated by H. Masuya on November 28, 1996, from conidial mass.

Etymology: *botuliforme* = sausage-shaped in Latin, refers to the allantoid shape of ascospores.

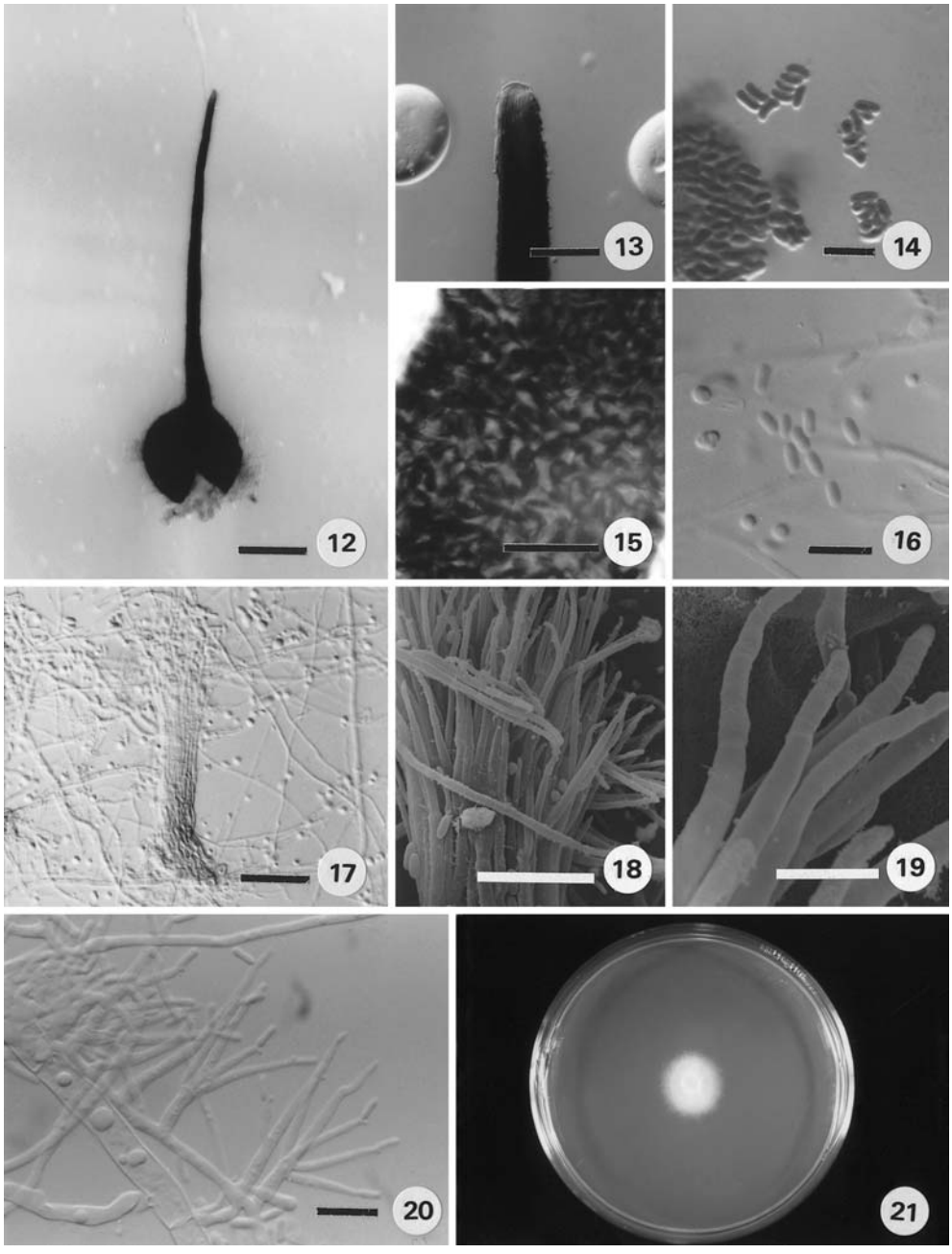
Colony: Colonies hyaline, oppressed to occasionally floccose on 2% MA. Margins slightly irregular (Fig. 21). Hyphal elements straight or curved, spreading radially on, as well as immersed in the medium, hyaline, 0.5–5.7 μm diameter. Growth of colonies on 2% MA 12.8–19.2 mm, 16.4 μm on average, in 4 days at 20°C. Resistance to cycloheximide with approximately 81% reduction in growth on 2% MA with 1.0 g/l cycloheximide at 20°C. Perithecia rarely produced.

Teleomorph: Perithecia either superficial or immersed in the substratum. Bases part black, globose to subglobose, 70–100 μm diameter, 85 μm on average, with or without sparse hyphal appendages, 14–156 × 1.8–4 μm, 90 × 3 μm on average (Figs. 11a, 12), outer layer of the peridium composed of thick-walled, inequilaterally or irregularly shaped cells, 2–17.5 × 2–12.5 μm, 9.5 × 6.5 μm on average (Fig. 15). Necks black at their base, becoming light colored (brown) at the apex, nearly cylindrical or tapered, curved or straight, 80–190 μm long, 150 μm on average, 14–23 μm wide at base, 20 μm on average, 9–12 μm wide at tip, 10 μm on average, composed of dark, laterally fused, thick-walled, septate, hyphal elements, 2–2.5 μm wide, 2.2 μm on average, terminating in an obtuse apex, that is covered by a hyaline gelatinous cap (Figs. 11b, 13). Ostiolar hyphae absent. Asci evanescent, clavate to subglobose when young, up to 6.5 × 5 μm, mature asci not seen. Ascospores hyaline, one celled, somewhat allantoid, enclosed in a distinct uniform hyaline sheath (Figs. 11c, 14), 3–5 × 1–2 μm, 4.5 × 1.8 μm on average (including sheath).

Anamorph: Conidiophores mononematous to synnematous. Mononematous conidiophores arising directly from hyphal elements, hyaline, septate sometimes developed to *Leptographium*-like forms with a short stipe and up to three series of branches and conidiogenous cells. Conidiogenous cells annellidic, cylindrical, tapered (Figs. 11f, 19, 20), 6.5–43.5 × 1.2–3 μm, 20 × 2 μm on average. Conidia hyaline, one celled, oblong to ellipsoidal, rounded at both ends, or with a slightly truncated base, 4–8.5 (–14) × 0.8–2.7 μm, 6 × 2 μm on average (Fig. 16). Stipes of the synnematous conidiophores hyaline to pale brown (Figs. 11e, 17, 18). Synnemata with loosely fused outer stipe cells, with gradations to mononematous conidiophores, 55–130 × 9.5–38 μm excluding conidial masses, 90 × 25 μm on average. Conidiogenous cells of synnemata conspicuously divergent (Fig. 18). Conidiogenous cells annellidic, cylindrical, 8.7–26 × 0.7–1.5 μm, 20 × 1.3 μm on average. Conidia hyaline, one celled, oblong to ellipsoidal, rounded at both ends of conidia, or with a slightly truncated base, 2.5–6 × 0.8–2.5 μm, 5 × 2.2 μm on average (Fig. 11d), becoming aggregated in slimy white masses at the tip of conidiophores.

Ophiostoma botuliforme is characterized by having perithecial necks with the tips covered by a hyaline gelatinous cap, reniform ascospores, and a *Pesotum* anamorph with hyaline to pale brown stipes. The teleomorph of *O. botuliforme* resembles that of both *C. acericola* H.D. Griffin and *C. allantospora* H.D. Griffin (Griffin 1968; Upadhyay 1981), and by examining the type specimens of *C. acericola* (DAOM110143) and *C. allantospora* (DAOM110145) and their original descriptions, we found that (1) *C. acericola* lacks gelatinous caps at the neck tips, *O. botuliforme* has them; and (2) the ascospore size and form of *O. botuliforme* are different from that of *C. acericola*. Ascospores of *O. botuliforme* are reniform, whereas those of *C. acericola* are orange-section shaped and smaller in size than those of *O. botuliforme*. Conversely, ascospores of *O. botuliforme* and *C. allantospora* are indistinguishable, but the perithecial necks of *C. allantospora* are longer than those of *O. botuliforme* (Table 2). Perithecial size is significantly larger in *C. allantospora* than in *O. botuliforme* (Table 2).

The anamorph of *O. botuliforme* is synnematal with the synnemata having hyaline to pale brown colored stipes. In the past, anamorphs with a similar characteristic were referred to as *Hyalopesotum* H.P. Upadhyay & W.B. Kendri., which was established as a hyaline counterpart of *Pesotum* J.L. Crane & Schokn. (Upadhyay and Kendrick 1975). However, Okada et al. (1998) argued that synnematous anamorphs of *Ophiostoma* should be treated under *Pesotum* with a narrower concept, including *Hyalopesotum*. The anamorph of *O. botuliforme* is therefore referable to *Pesotum sensu* Okada et al. On the other hand, Harrington et al. (2001) accepted the definition of Crane and Schoknecht (1973) that restricts *Pesotum* to anamorphs with affinities to the *O. piceae* complex that also have a *Sporothrix* synanamorph. *O. botuliforme* cannot be treated as *Pesotum sensu* Crane & Schoknecht, because the



Figs. 12–21. Morphological characteristics of *Ophiostoma botuliforme*. **12** Perithecium. **13** Tip of a perithecial neck with gelationous cap. **14** Ascospores. **15** Outer layer of peridium. **16** Conidia. **17** Hyaline, synnematous conidiophore (*Pesotum* type). **18** Scanning electron micrographs of a synnematous conidiophore. **19** Scanning electron micrographs of conidiogenous cells. **20** Simple conidiophores. **21** Colony grown on 2% MA for 2 weeks at 20°C. *Bars* **12**,**17** 50 μm; **15**,**18** 20 μm; **13** 10 μm; **14**,**16**,**20** 5 μm; **19** 3 μm

anamorph of *O. botuliforme* does not have a *Sporothrix* synanamorph. Also, other *Pesotum*-like anamorphs of *Ophiostoma*, including *Hyalopesotum*, are not yet fully defined. We will now accept the definition of Okada et al. (1998) and treat the anamorph of *O. botuliforme* as a *Pesotum* species.

The anamorph of *O. botuliforme* cannot be compared, because *C. acericola* has never been obtained in culture and its anamorph has never been reported (Griffin 1968). The anamorph of *C. allantospora* is a *Hyalorhinocladiella* and different from that of *O. botuliforme*.

This species was only isolated from an unidentified *Cryphalus* species.

Ophiostoma nigrogranum Masuya, sp. nov. Figs. 22–32.

Anamorph: *Sporothrix* sp.

Perithecia in medio superficialia vel partim immersa, basi nigra, globosa vel subglobosa, 80–130 μm diam, appendicibus hyphoideis externis simplicibus brunneolis septatis ornata, 7–60 (–100) × 1.3–2.7 μm; peridium ectostratum ex cellulis in forma inaequilateralibus vel irregularibus. Collum cylindraceum, curvatum vel rectum, basi nigrum, pallidiore ad apicem, 270–505 μm longum, ad basim 24–27 μm latum, ad apicem 6–15 μm latum, hyphis ostioli hyalinis septatis divergentibus cylindricis apice rotundatis 9–19 μm longuis ad basim 1–2 μm latis terminatum. Asci juveniles anguste fusiformes, clavati vel

Table 2. Morphological characteristics of *Ophiostoma botuliforme*, *C. acericola*, and *C. allantospora*

Characters	*O. botuliforme*	*C. acericola*[b]	*C. allantospora*[b]
Perithecia base width (μm)	70–100	75–100	110–300
Perithecial character	Globose to subglobose	Globose to subglobose	Globose to subglobose
Perithecial neck length (μm)	80–190	100–400	Up to 1500 in culture, up to 4000 on wood
Tip of perithecial neck	Ostiolar hyphae absent, with gelatinous cap	Ostiolar hyphae absent	Ostiolar hyphae occasionally present in culture
Ascospore size[a] (μm)	3–5 × 1–2	2.5–4 × 1.5–2.5	3–5.5 × 1.5–2.5
Ascospore shape	Reniform, with sheath	Orange-section shape, with sheath	Allantoid, with sheath
Conidiophore character	Unbranched, penicillately branched, or synnematous with hyaline stipes	No data	Unbranched, or penicillately branched, without distinct denticles
Conidial size (μm)	2.5–6 × 0.8–2.5	No data	2–8 × 1–3
Conidial shape	Oblong to ellipsoidal	No data	Cylindrical to elliptical, mostly ovobate

[a] Including sheath
[b] Data from Griffin (1968) and Upadhyay (1981)

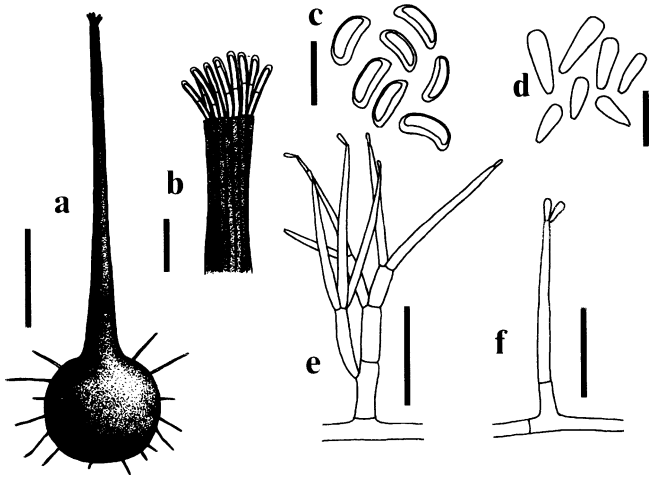


Fig. 22. *Ophiostoma nigrogranum* (MAFF410943). **a** Perithecium. **b** Tip of perithecial neck. **c** Ascospores. **d** Conidia. **e** Hyaline branched conidiophore. **f** Simple conidiophore. *Bars* **a** 50 μm; **b**,**e**,**f** 10 μm; **c**,**d**, 5 μm

subglobosi, ad 10 × 4 μm; asci maturi non visi. Ascosporae hyalinae, aseptatae, allantoideae, vagina hyalina circumdantes, 2.5–4 × 0.7–1.5 μm cum vagina, ad apicem colli in guttula conglobatae. Conidiophora hyalina, septata, micronematosa, mononematosa. Cellulae conidiogenae sympodiales, hyalinae, 7.5–30 × 0.9–2.3 μm. Conidia hyalina, aseptata, oblonga vel clavata, 2–6 × 0.5–2 μm, solitaria, dein ad apicem conidiophori in muco aggregata.

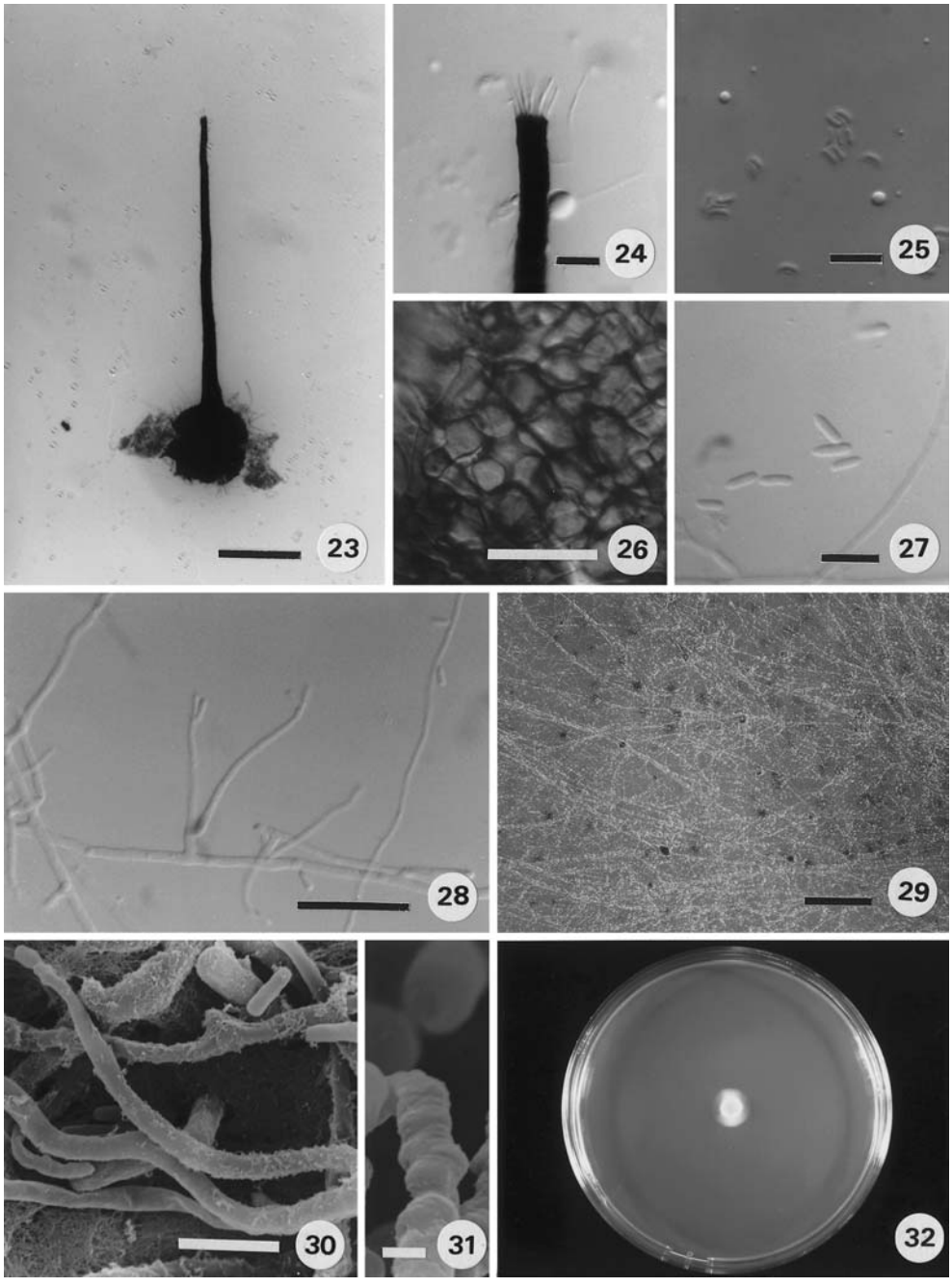
Holotype: TFM:FPH 7590, dried culture of MAFF410943 (JCM11702) from Himeji, Hyougo Pref., Japan, on *Pinus densiflora*, isolated by H. Masuya on September 24, 1996, from ascospore mass.

Paratype: TFM:FPH 7591, dried culture of MAFF410944 (JCM11703) from Himeji, Hyougo Pref., Japan, on *Pinus densiflora*, isolated by H. Masuya on September 24, 1996, from ascospore mass.

Etymology: *nigrogranum* = black grain in Latin, refers to the appearance of sclerotium-like structures in culture.

Colony: Colonies hyaline, oppressed, aerial hyphae rarely produced on 2% MA. Margins smooth (Fig. 32). Hyphal elements straight or curved, spreading radially and densely on the medium and immersed in the medium, hyaline, 0.5–6.5 (–8.5) μm diameter, 2.3 μm on average. Aerial hyphae absent. Growth of colonies in 2% MA, 8.4–14 mm, 11.2 mm on average, in 4 days at 20°C. Resistance to cycloheximide with approximately 81% reduction in growth on 2% MA with 1.0 g/l cycloheximide at 20°C. Protoperithecia and sclerotium-like structures often produced on and in the medium. Sclerotium-like structures, black, globose or irregularly shaped, up to 200 μm diameter (Fig. 29). Perithecia produced on the substratum and medium after about 1.5 months of incubation.

Teleomorph: Perithecia superficial or partly embedded in the medium. Bases part black, globose to subglobose, 80–130 μm diameter, 110 μm on average, densely ornamented with brown, unbranched, thin-walled, curved or straight, septate hyphal appendages, 7–60 (–100) × 1.3–2.7 μm, 38 × 2 μm on average (Figs. 22a, 23), outer layer of the peridium composed of thick-walled, inequilaterally or irregularly shaped, sometimes globosed cells, 2.5–23 × 2.5–12.5 μm, 9 × 6.5 μm on average (Fig. 26). Necks black at the base, becoming light colored (brown) at the apices, nearly cylindrical or tapered, curved or straight, 270–505 μm long, 350 μm on average, 24–27 μm wide at base, 26 μm on average, 6–15 μm at tip, 10 μm on average, composed of dark, laterally fused, thick-walled, septate, hyphal elements, 1.7–3.5 μm wide, 2 μm on average, terminated in a corona of divergently arranged ostiolar hyphae (Figs. 22b, 24). Ostiolar hyphae, cylindrical, 9–19 μm long, 14 μm on average, and 1–2 μm wide at base, 1.4 μm on average, rounded at the apices (Figs. 22b, 24). Asci evanescent, narrowly fusiform or clavate to subglobose, maximum 10 × 4 μm when young, mature asci not seen. Ascospores hyaline, one celled, allantoid, enclosed in an uniform, distinct hyaline sheath (Figs. 22c, 25), 2.5–4.5 × 0.9–2 μm (including sheath), 4 × 1.8 μm on average, accumulating in a white drop at the tip of necks.



Figs. 23–32. Morphological characteristics of *Ophiostoma nigrogranum*. **23** Perithecium. **24** Tip of a perithecial neck with ostiolar hyphae. **25** Ascospores. **26** Outer layer of peridium. **27** Conidia. **28** Simple conidiophores. **29** Sclerotium-like structures produced in a culture grown for 1 month at 20°C. **30** Scanning electron micrographs of conidiophores. **31** Scanning electron micrographs of conidiogenous cells. **32** Colony grown on 2% MA for 2 weeks at 20°C. *Bars* **23** 100 μm; **24** 10 μm; **26**,**28** 20 μm; **25**,**27**,**30** 5 μm; **29** 1 mm; **31** 0.5 μm

Anamorph: Conidiophores arising directly from the hyphal elements, hyaline, septate, mononematous, rarely developed to a *Leptographium*-like form with a short stipe and up to two series of branches and conidiogenous cells (Figs. 22e,f, 28). Conidiogenous cells annellidic or sympodial, cylindrical, tapered, without distinct denticules (Figs. 30, 31), 7.5–30 × 0.9–2.3 μm, 20 × 1.5 μm on average. Conidia hyaline, one celled, oblong to clavate, rounded at the apices and slightly truncated at the bases (Figs. 22d, 27), 2–6 × 0.5–2 μm, 5 × 1.8 μm on average, becoming aggregated in slimy masses at the tip of conidiophores.

Ophiostoma nigrogranum has hyaline, ostiolar hyphae with rounded apices, allantoid ascospores with a distinct sheath and sclerotium-like structures. The species is similar to *O. stenoceras* (Robak) Melin & Nannf., *C. tenella* R.W. Davidson (1958), and *C. coronata* Olchow. & J. Reid (1974) in perithecial characteristics.

Ophiostoma nigrogranum is distinguished from *O. stenoceras* by the shape of the ascospore (Table 3). The anamorphic characteristics of *O. nigrogranum* are also different from those of *O. stenoceras*. The former has conidiogenous cells without distinct denticles, but the latter has denticles. Also, globose conidia are often found in *O. stenoceras*, but never in *O. nigrogranum*. Sclerotium-like structures have never been reported in cultures of *Ophiostoma stenoceras*.

Table 3. Morphological characteristics of *Ophiostoma nigrogranum*, *O. stenoceras*, *C. tenella*, and *C. coronata*

Characters	*O. nigrogranum*	*O. stenoceras*[b]	*C. tenella*[c]	*C. coronata*[d]
Perithecia base width (μm)	80–130	80–180	120–140	100–175
Perithecial neck length (μm)	270–505	400–1400	350–500	355–1930
Ostiolar hyphae size (μm)	9–19 × 1–2	18–35(55) × 1.5–2.5	30–45 × 2–2.5	16–65 × 1.5–2.1
Ostiolar hyphae character	Cylindrical, with septa	Tapered, with septa	Cylindrical, with septa	Tapered, with septa
Ascospore size[a] (μm)	2.5–4.5 × 0.9–2.0	2.5–4.5 × 1–1.5	3.5–5 × 1.3–1.7	3.0–5.2 × 0.8–1.3
Ascospore shape	Allantoid, with sheath	Orange-section shape, without sheath	Crescent shape, without sheath	Crescent shape, rounded at both ends, without sheath
Conidiophore character	Branched, without distinct denticle	Branched, with denticles (0.5–1 μm length)	Denticle absent	Denticles present
Conidial size (μm)	2–6 × 0.5–2	2–7 × 1–2.5	2.5–6 × 1.2–2.5	4.0–10 × 0.8–1.5
Conidial shape	Ellipsoidal to clavate	Ovoid to clavate	Oblong to ellipsoidal, sometimes curved at base	Clavate, sometimes curved, with ramoconidia

[a] Including sheath
[b] Data from Upadhyay (1981)
[c] Combined data from Davidson (1958) and Olchowecki and Reid (1974)
[d] Data from Hutchison and Reid (1988)

Ceratocystis tenella and *C. coronata*, both of which should be transferred to *Ophiostoma* because of their anamorphic characteristics, have often been confused. Although described by Davidson in 1958, *C. tenella* was not fully characterized by him, and therefore it was redescribed by Griffin (1968). However, according to Olchowecki and Reid (1974), Griffin (1968) confused *C. tenella* with *C. coronata*, which Olchowecki and Reid (1974) described later. In contrast, Upadhyay (1981) reduced *C. coronata* to synonymy with *C. tenella* based on his broad concept of species delimitation among species of *Ophiostoma*. However, his decision was rejected by Hutchison and Reid (1988) on the basis of the distinct ascospore morphology of the two species and differences in the anamorphs. Although these fungi need to be the subject of further study, at this time we have elected the taxonomy of Olchowecki and Reid (1974).

Ascospores of *O. nigrogranum* are more rounded at either end than those of *C. tenella* and *C. coronata*. *O. nigrogranum* has ostiolar hyphae that are cylindrical and relatively short, whereas those of *C. tenella* are tapered ostiolar hyphae and those of *C. coronata* are longer than those of *O. nigrogranum* (see Table 3). We recognized these differences from comparison with the type specimen of *C. tenella* (BPI70809) and a well-documented description and photograph of *C. coronata* (Hutchison and Reid 1988).

The occurrence of sclerotium-like structures, well known in *O. minus*, is a noteworthy characteristic of *O. nigrogranum*. However, *O. nigrogranum* is easily distinguished from *O. minus* by the shape of ascospores, morphology of the prithecia, and colony appearance.

Ophiostoma nigrogranum was isolated frequently from galleries of an unidentified bark beetle, as well as less frequently from *T. piniperda*.

Discussion

Many species of *Ophiostoma* have been described but not always fully characterized. Upadhyay's monograph (1981) reduced many, until then accepted, species of *Ophiostoma* to synonyms or deemed them nonaccepted species. Therefore, some species may still require taxonomic revision. Indeed, recent studies employing molecular data have rejected some of his synonymy, and some species [i.e., *O. floccosum* Math.-Käärik, *O. adjuncti* (R.W. Davidson) T.C. Harr.] are again accepted (Hausner et al. 1993b; Harrington et al. 2001). The reappraisal of other *Ophiostoma* species is hampered by a lack of type specimens, ex-type cultures, or full descriptions. Therefore, identification of *Ophiostoma* species is difficult even when using molecular data or mating experiments. Type specimens, ex-type cultures, and full descriptions are essential if we are to understand the phylogeny and taxonomy of *Ophiostoma*.

From the results of morphological comparisons with other *Ophiostoma* species in this study, we recognized three new species particularly by features of the perithecial neck tip. The perithecial necks of *O. pusillum* and *O. botsuliforme* have a rounded tip with a gelatinous cap, but those of *O. nigrogranum* have a truncate neck with ostiolar hyphae surrounded by a gelatinous sheath. These characteristics were consistent in all isolates and supported by the uniqueness of species when combined with other characteristics, e.g., those of ascospores and anamorphs. Additional critical studies on the structure and development of the perithecial neck tip may well enhance our ability to characterize all *Ophiostoma* species.

Only five other species of *Ophiostoma*, all recognized as blue-stain fungi, from Japanese red pine have been recorded previously (Kasai 1917; Nisikado and Yamauti 1933, 1934, 1935; Masuya et al. 1999). However, the species newly described here form white colonies, two species in particular, *O. botuliforme* and *O. nigrogranum*, did not stain the wood substrates in the cultures. Nonstaining *Ophiostoma*

species are hard to detect from substrates because of a lack of staining ability and their coexistence with other blue-stain fungi that may mask their presence (Whitney and Cobb 1972). Thus, these species have probably gone undetected. Therefore, many other nonstaining *Ophiostoma* may be potentially distributed on *P. densiflorae* in Japan.

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