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Mycosphaerella buna sp. nov. with a *Pseudocercospora* anamorph isolated from the leaves of Japanese beech*

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A species of *Mycosphaerella* with a *Pseudocercospora* anamorph was collected on overwintered fallen leaves of Japanese beech, *Fagus crenata*. Based on comparison of morphology with *Mycosphaerella* species on Fagaceae, the fungus was newly described as *Mycosphaerella buna*. The *Pseudocercospora* anamorph derived from a single ascospore of the fungus was morphologically identical to an endophytic anamorph isolated from asymptomatic living leaves of Japanese beech.

Key Words——endophytic fungus; *Fagus crenata*; *Mycosphaerella buna*; *Pseudocercospora*.

Pseudothecia of a *Mycosphaerella* species were frequently found on the lower surface of overwintered fallen leaves of Japanese beech, *Fagus crenata* Blume collected at Ogawa Reserved Forest, Kitaibaraki, Ibaraki Pref., Japan. A *Pseudocercospora* anamorph was obtained from single ascospore cultures isolated from the pseudothecia. The genus *Mycosphaerella* Johanson includes more than 1800 species and is one of the largest ascomycete genera (Corlett, 1991). Many *Mycosphaerella* species are known to be pathogens of various plants (Crous, 1998; Ito, 1973; Katsuki, 1965; Katumoto, 1983), and the pseudothecia usually occur on fallen leaves of deciduous trees (Tomilin, 1979; Sivanesan, 1983; Vasilyeva, 1998). Among them, 21 species of *Mycosphaerella* were reported on the plants of Fagaceae (Tomilin, 1979). Morphological comparison with these species was carried out to identify the *Mycosphaerella* species collected on *F. crenata*.

From asymptomatic living leaves of *F. crenata* collected at the same site, a species of *Pseudocercospora* was isolated. This anamorph was morphologically similar to the *Pseudocercospora* anamorph obtained from the ascospores of the above *Mycosphaerella* species. Therefore, we carried out inoculation experiments to clarify the relationship between these anamorphs.

We report here the morphological and cultural characteristics of the fungus on *F. crenata*, and discuss its taxonomy and its endophytic nature.

Materials and Methods

Single ascospore isolation from pseudothecia on fallen leaves Overwintered fallen leaves of *F. crenata* with pseudothecia of *Mycosphaerella* species were collected several times in spring of the years 1997 to 1999 at Ogawa Reserved Forest, Kitaibaraki, Ibaraki Pref., Japan. A fascicle of asci was obtained from a pseudothecium on the fallen leaves with a sterilized needle and was placed in a drop of sterilized water on sterilized glass slide. The fascicle of asci was separated into ascospores under a light microscope, and the ascospores were transferred onto plates of water agar (WA: 10 g of Wako agar, 1000 ml of deionized water), which were kept at 20°C in the darkness. After the ascospores had germinated (24–48 h), single ascospores were transferred to plates of malt extract agar (MA: 10 g of Difco malt extract, 20 g of Wako agar, 1000 ml of deionized water). Single ascospores were also inoculated onto autoclaved leaves of *F. crenata*.

Isolation of the fungus from asymptomatic leaves Asymptomatic living leaves of *F. crenata* were collected in May to July, 1997 to 1999 at the same site where fallen leaves were collected. They were cut into small fragments (ca. 7 mm in diam), and four fragments per leaf were sterilized in 70% ethanol solution for 1 min, followed by NaClO (1% available chlorine) for 2 min, 70% ethanol solution for 1 min again, then immersed in sterilized distilled water two times for 1–2 min, and dried for 2–3 h on sterilized filter paper. The fragments were incubated on plates at 20°C in the darkness. Conidial caespituli were produced on them in 7–10 d. For single conidium isolation, a conidial mass was suspended in sterilized water, and 50 μl of the suspension was spread out on water agar plates. After the conidia had germinated at 20°C in the darkness (24–48 h), single conidia were transferred to MA plates. Conidia were also inoculated onto autoclaved or living leaves of *F. crenata*.

Culture of the fungus Isolates obtained from a single ascospore or a single conidium were cultured on three ar-

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tificial media at 20°C in the darkness. The media were MA, potato dextrose agar (PDA: 39 g of Nissui potato-dextrose-agar, 1000 ml of deionized water) and Miura's medium (LcA; 1 g of glucose, 1 g of KH_2PO_4, 0.2 g of $MgSO_4$, 0.2 g of KCl, 2 g of $NaNO_3$, 0.2 g of Difco Yeast Extract, 13 g of Wako agar, 1000 ml of deionized water).

Inoculation of the fungus Inoculation was performed onto the leaves of 2-y-old potted seedlings of *F. crenata* grown from seeds, which were kept in a growth cabinet maintained at 20°C, about 90% relative humidity, with illumination for 15 h (max. 12,600 lux, daylight strip lamps). Six seedlings were used for inoculation. Germinated single conidia on small pieces of WA were inoculated on the lower side of leaves of four seedlings on 8 June 1998. For control, small pieces of WA were inoculated on the lower side of leaves of two seedlings. The leaves were kept at 100% relative humidity in darkness for 3 d, then transferred in the growth cabinet. After 5 mo, they were covered with vinyl net and moved outdoors. All the fallen leaves of seedlings were collected and kept moist in petri dishes at 4°C. When spermatia were produced on them, they were mated once with a sterile needle to produce the teleomorph.

Morphological observations Specimens producing pseudothecia on fallen leaves of *F. crenata* were used for morphological observation. Pseudothecia from the holotype specimens of *M. aquatica* (Cooke) J. H. Mill. (BPI) were also observed for comparison of morphology. Vertical sections were prepared with a microtome equipped with a freezing unit for light microscopic observation. For morphological observations of the anamorph, the caespituli which were produced on the lower surface of the inoculated autoclaved leaves or asymptomatic leaves of *F. crenata* in MA plates were used. Vertical sections of them were also prepared with the microtome. Dimensions of 30 spores were measured under the light microscope. For scanning electron microscopy (SEM), caespituli on the leaves were fixed with 2.5% glutaraldehyde for 24 h at 4°C, dehydrated in an alcohol series, dried in a critical point dryer, then coated with platinum-palladium using a Hitachi E-1030 Ion Sputter. These preparation was observed with a Hitachi S-4200 SEM operating at 15 Kv.

Morphology and Taxonomy

Mycosphaerella buna R. Kaneko et Kakishima, sp. nov. Fig. 1A–J, Fig. 2A–G

Pseudothecia hypophylla, solitaria, subepidermalia, globosa vel subglobosa, ostiolata, nigra, 70–90 μm diam, 70–90 μm alta. Asci fasciculati, clavati vel subcylindracei, bitunicati, subsessiles, 8-sporis, imbricato-biseriati, 45–55 × 7–11 μm. Ascosporae fusiformes vel elliptico-fusiformes, ad apices plus minusve acutae, rectae vel parum curvatae, medio uniseptatae, ad septum non constrictae, hyalinae, guttulatae, laeves, 14–23 × 2.5–4.5 μm. Spermogonia subepidermalia, ostiolata, nigra, 45–80 μm diam, 50–85 μm alta. Spermatia hyalina, bacilliformia, 2.5–4.0 × 0.5–1.0 μm.

Anamorphosis: *Pseudocercospora* sp. Mycelium internum et externum, hyalinum vel olivaceum, ex hyphis ramosis laevibus 1.5–4.0 mm diam constans. Caespituli olivaceo-brunnei vel atro-brunnei, praecipue hypophylli, usque ad 120 μm diam. et 100 μm alti. Conidiophora ex mycelio superficiali singulatim exorientia vel in fasciculis in cellulis extermis stromatis dense aggregata, subhyalina vel pallide brunnea, 0–3 (–4) septata, oblonga vel cylindracea, non ramosa, 4–20 × 2.5–4.0 μm. Cellulae conidiogenae terminales, conicae vel cylindraceae, rectae vel geniculatae, hyalinae, sympodiales, raro percurrentes. Conidia solitaria, laevia, hyalina, recta vel curvata, guttulata, anguste obclavata, apice subacuta, basi obconico-truncata, 1–7 septata, (11–) 15–60 × 1.0–3.0 μm, pariete tenui; hila 1.0–2.0 μm lata, inconspicua, hyalina.

Pseudothecia hypophyllous, solitary, subepidermal, globose to subglobose, ostiolate, black, 70–90 μm in diam, 70–90 μm in height; asci fasciculate, clavate to almost cylindrical, bitunicate, subsessile, eight-spored overlapping biseriate, 45–55 × 7–11 μm; ascospores fusiform or elliptic fusiform with more or less pointed ends, straight or slightly bending, 1-septate at the center, widest at septum, tapering toward both apices, two cells almost same shape, not constricted at septum, hyaline, guttulate, smooth, 14–23 × 2.5–4.5 μm. Spermogonia subepidermal, ostiolate, black, 45–80 μm in diam, 50–85 μm in height; spermatia hyaline, rod-shaped, 2.5–4.0 × 0.5–1.0 μm.

Anamorph: *Pseudocercospora* sp. Mycelium internal and external, hyaline to olivaceous, branched, smooth hyphae, 1.5–4.0 μm in diam. Caespituli olive-brown to dark brown, mainly hypophyllous, up to 120 μm in diam and 100 μm in height, often covered with subhyaline slimy mass of conidia; conidiophores arising singly from superficial mycelium, or aggregated in dense fascicles from the upper cells of stroma, subhyaline to pale-brown, 0–3 (–4) septate, oblong to cylindrical, unbranched, 4–20 × 2.5–4.0 μm; conidiogenous cells often integrated in conidiophores, conical or cylindrical, straight to geniculate, hyaline, sympodial, occasionally percurrent, scars unthickened; conidia solitary, smooth, hyaline, straight to curved, thin-walled, guttulate, narrow-obclavate with subacute apex, base obconic-truncate, 1–7-septate, (11–)15–60 × 1.0–3.0 μm; hila 1.0–2.0 μm wide, inconspicuous, hyaline.

Holotype: On fallen leaves of *Fagus crenata* Blume, TSH-A0001 (=TNS-F-107964, isotype, culture MSP-9901=MAFF410884), Ogawa, Kitaibaraki, Ibaraki Pref., Japan. 18 May 1999, R. Kaneko (R. K.)

Etymology: buna=Japanese name of *Fagus crenata*

Additional specimens examined: Teleomorph: On fallen leaves of *F. crenata*, TSH-A0006, A0007, Ogawa, Kitaibaraki, Ibaraki Pref., 5 May 1997, R. K.; TSH-A0008, 4 May 1998, R. K.; TSH-A0002, A0003, A0004, 30 Mar. 1999, R. K.; TSH-A0012, Juniko-Lake, Aomori Pref., 25 May 1999, R. K.; TSH-A0013, Hachimantai, Akita Pref., 25 May 1999, R. K.; On inoculated fallen leaves of a seedling of *F. crenata*, TSH-A0005, Tsukuba, Ibaraki Pref., 5 July 1999, R. K., Anamorph: TSH-0009, dried culture of autoclaved leaves inoculated with single ascospores isolated from TSH-

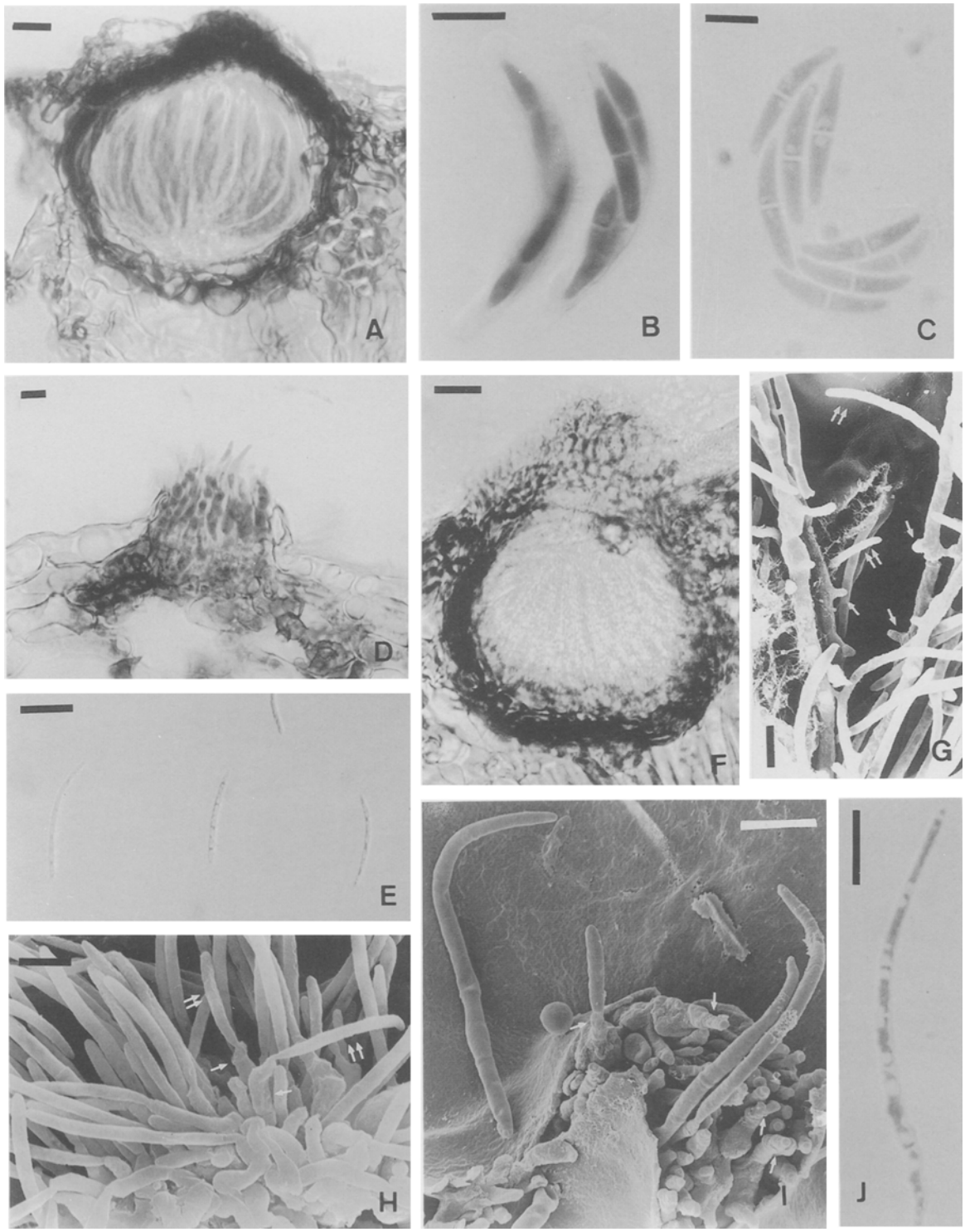

Fig. 1. *Mycosphaerella buna* (THS–A0001) and its *Pseudocercospora* anamorph (TSH–A0009).
A. A vertical section of a pseudothecium. B. Asci. C. Ascospores. D. A vertical section of a young stroma. E. Conidia. F. A vertical section of a spermogonium. G. Conidiogenous cells (arrow) and conidia (double arrows) growing from external hyphae (SEM). H. Conidia (double arrows) and conidiophores (arrow) in a stroma (SEM). I. Tortuous conidia and percurrent conidiogenous cells (arrow) (SEM). J. A conidium with guttulae. Scale bars: 10 μm.

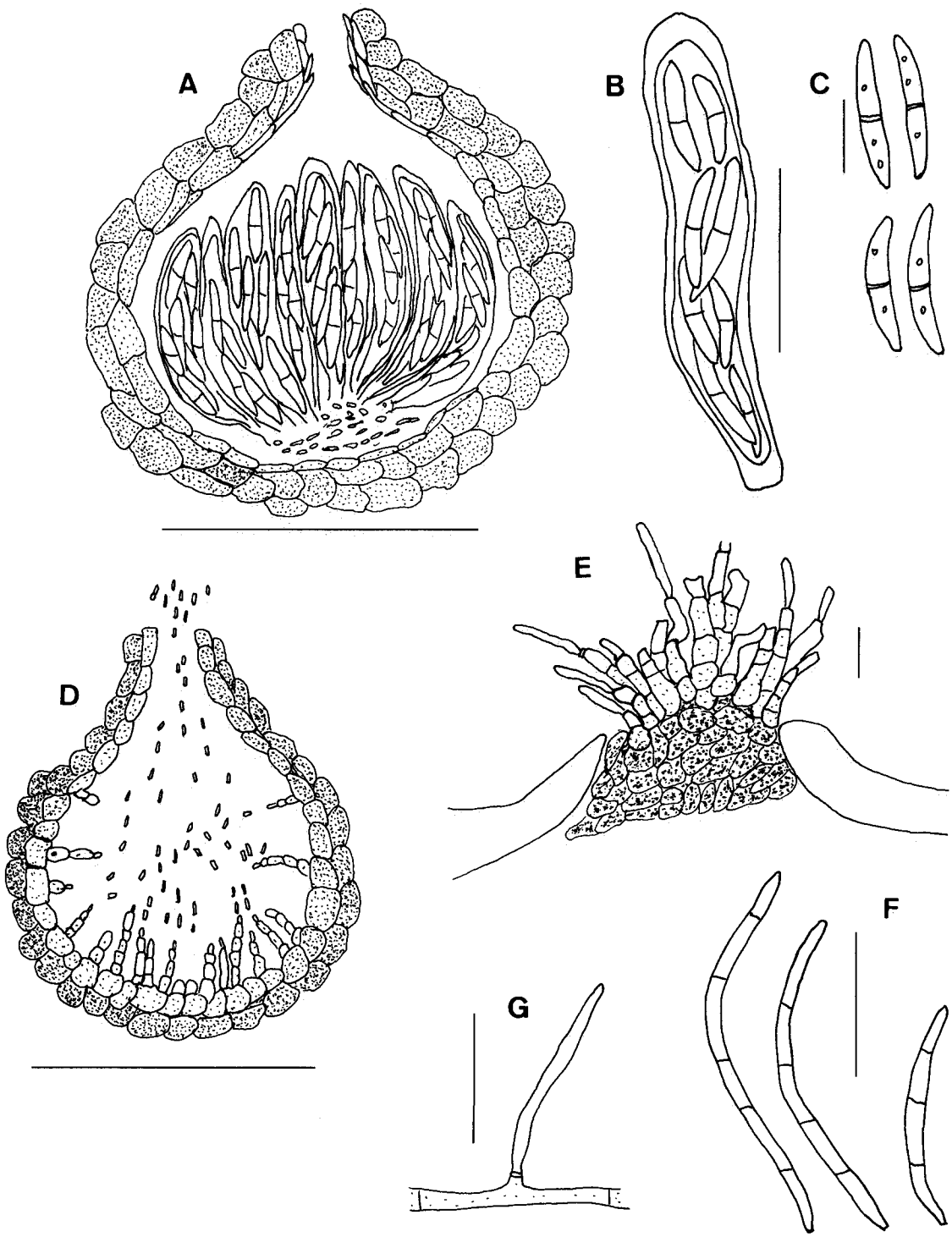

Fig. 2. *Mycosphaerella buna* and its *Pseudocercospora* anamorph.
A. A pseudothecium. B. An ascus. C. Ascospores with guttulae. D. A spermogonium. E. A stroma. F. Conidia. G. A conidium and a conidiogenous cell from an external hypha. Scale bars: A, D=50 μm. B, E–G=20 μm. C=10 μm.

A0001, Tsukuba, Ibaraki Pref., 25 May 1999, R. K.; A0010, dried culture isolated from asymptomatic living leaves of *F. crenata*, Ogawa, Kitaibaraki, Ibaraki Pref., 13 June 1999, R. K.; TSH-A0011, 18 July 1999, R. K.

All specimens and dried cultures examined are deposited in the Mycological Herbarium, Institute of Agriculre & Forestry, University of Tsukuba (TSH) or Department of Botany, National Science Museum, Tokyo (TNS).

Cultural characteristics: Single ascospore cultures at

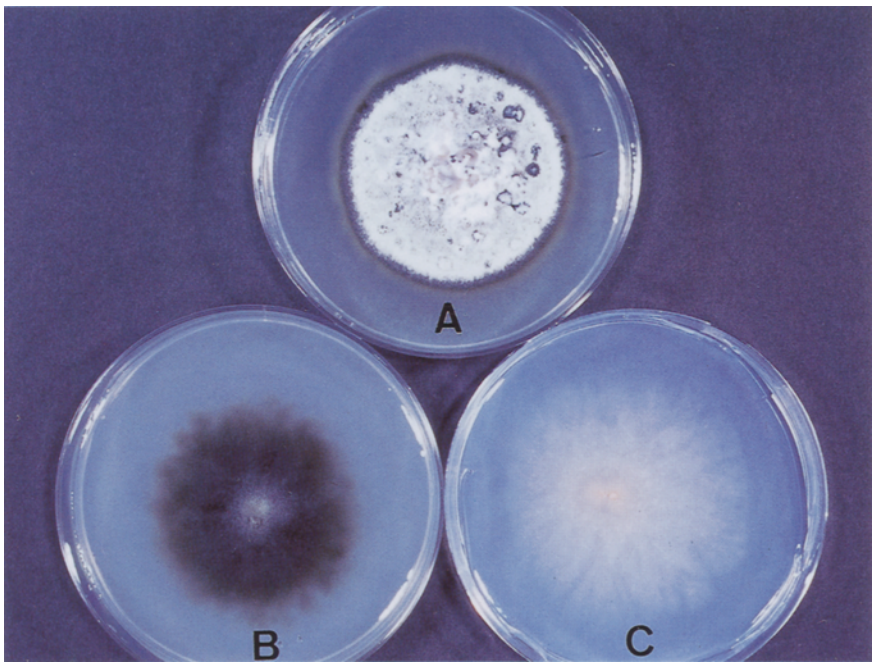

Fig. 3. Colonies of *Mycosphaerella buna* (MSP9901) on three media in 35 d at 20°C in the dark. A. PDA medium. B. MA medium. C. LcA medium.

20°C for 30 d on three different artificial media (PDA, MA, LcA) were clearly different from each other (Fig. 3). Colonies on PDA were dark green and lamellate, and covered with white aerial mycelium. The margins were sometimes pale pink to orange. Colonies on MA were not raised, grayish dark-green and hygrophanous. Colonies on LcA were not raised, pale pink and submerged. The fungus did not produce caespituli on the artificial media, but produced conidia directly from every single submerged hypha. Sporulation from the aerial hyphae was not observed. Colonies on PDA and LcA produced a number of conidia, but those on MA produced few. There was no difference in the growth rate of colonies among the three media. They attained a diameter of 30–36 mm in 30 d at 20°C in the dark.

Note: Twenty-one *Mycosphaerella* species have been reported on fagaceous trees (Saccardo, 1882, 1891, 1899, 1926; Dennis, 1968; Tomilin, 1979; Doi, 1983). Among them, four *Mycosphaerella* species were morphologically similar to *M. buna* in their hosts or the dimensions of asci and ascospores (Table 1).

Mycosphaerella maculiformis (Pers.:Fr.) J. Schrot. has been reported from several countries including Japan as a pathogen causing a leaf-spot disease of various broad-leaved trees (Ito, 1973; Saccardo, 1891). *Mycosphaerella maculiformis* is similar in ascal dimensions, but differs in the distinctly shorter dimensions of its ascospores and in having ascospores constricted at the septum.

Mycosphaerella fagi (Auersw.) Lindau on *Fagus silvatica* L. has been reported from Europe (Tomilin, 1979). This species has also been reported on *F. crenata* from Japan (Doi, 1983) and was found with *M. buna* on its fallen leaves. However, *M. fagi* differs in the much smaller dimensions of its asci and ascospores and in having ascospores constricted at the septum. Doi (1983) reported an unidentified *Mycosphaerella* species on fallen leaves of *F. crenata*. The ascospores of this species are not constricted at the septum, like those of *M. buna*, but the fungus differs in the shorter dimensions of its ascospores.

Mycosphaerella flageoletiana (Sacc. et Traverso) Tomilin on *F. silvatica* has been reported from Europe (Tomilin, 1979). *Mycosphaerella flageoletiana* is mainly epiphyllous, which differs from *M. buna*. *Mycosphaerella flageoletiana* is similar in ascal dimensions, but differs in its longer ascospores.

Mycosphaerella aquatica (Cooke) J.H. Mill. is morphologically similar to *M. buna*. We examined the holotype (BPI690) of *M. aquatica* on *Quercus aquatica* L. collected in U. S. A. *Mycosphaerella aquatica* (38–52 × 5–8 μm) and *M. buna* (45–55 × 7–11 μm) are similar to each other in the dimensions of asci. However, the ascospores of *M. aquatica* (11–16 × 3–5 μm) are shorter and wider than those of *M. buna* (14–23 × 2.5–4.5 μm). Moreover, ascospores of *M. buna* are widest at the septum, whereas those of *M. aquatica* are widest in the middle of the apical cell.

Any species of *Pseudocercospora* has not been reported on Fagaceae (Deighton, 1976, 1987; Guo and Hsieh , 1995; Katsuki, 1965; Sivanesan, 1983). Two species of *Cercospora* have been reported on Fagaceae, which might be assigned to *Pseudocercospora.* They are *Cercospora macrochaeta* on *Quercus chrysolepis* Liebm. (Saccardo, 1896) and *C. polytricha* on *Q. virens* Aiton (Saccardo, 1886), but these fungi differ from the

Table 1. Morphological characteristics of *Mycosphaerella buna* and related species.

Character	*M. buna*	*M. aquatica* (Holotype, BPI690)	*M. aquatica* (Tomilin, 1979)	*M. maculiformis* (Tomilin, 1979)	*M. fagi* (Doi, 1983)	*M. flageoletiana* (Tomilin, 1979)
Pseudothecium						
position	hypophyllous	hypophyllous	hypophyllous	hypophyllous	amphigenous mainly hypophyllous	epiphyllous
dimension (μm)	70–90	90–100	90–100	90–120	70–100	65–80
Ascus						
dimension (μm)	45–55 × 7–11	38–56 × 5–8	52–56 × 7–8	42–46 × 8–9	22–27 × 4–5.5	45–50 × 7
shape	clavate or almost cylindrical	clavate or cylindrical	clavate or almost cylindrical	cylindrical or clavate	cylindrical or clavate	narrow clavate or narrow cylindrical
Ascospore						
dimension (μm)	14–23 × 2.5–4.5	11–16 × 3–5.5	17–20 × 3.5–4	12–14 × 3–4	6–8 × 2–2.5	22–26 × 3–3.5
shape	fusiform or elliptic fusiform	narrow obovoid or clavate	narrow obovoid or clavate	obovoid or oblong	obovoid-oblong to ellipsoid	narrow clavate or almost cylindrical
constriction at septum	not constricted	not constricted	not constricted	constricted	constricted	not constricted
Anamorph	*Pseudocercospora* sp.	?	?	*Phyllosticta ludoviciana*	?	?
Host	*Fagus crenata*	*Quercus aquatica*	*Q. aquatica*	Deciduous trees	*F. crenata*	*F. silvatica*

Pseudocercospora anamorph of *M. buna* in the dimensions of their conidia.

Mycosphaerella buna was frequently found on overwintered fallen leaves of Japanese beech at Ogawa, Ibaraki Pref. This fungus was also collected on these leaves at Lake Juniko, Aomori Pref. and Hachimantai, Akita Pref. We suspect that the fungus is widely distributed in Japan.

Relationship between *Mycosphaerella buna* on the fallen leaves and an endophytic *Pseudocercospora* anamorph in asymptomatic leaves

Living leaves of seedlings inoculated with conidia of an endophytic *Pseudocercospora* anamorph isolated from asymptomatic living leaves developed no symptoms during the growing season. When leaves began to yellow, a number of spermatia were observed on the lower side of inoculated leaves attached to twigs. Asci and ascospores were produced on a fallen leaf 5 mo after artificial mating with spermatia. Neither asci nor ascospores were produced on the fallen leaves of non-inoculated seedlings. The dimensions of these asci and ascospores were 50–55 × 8.0–9.0 μm and 21–24 × 2.5–4.0 μm, respectively, and were morphologically identical to those of *M. buna*.

Caespituli were produced on sterilized leaves inoculated with ascospores 7 d after incubation at 20°C in the dark (Figs. 1D, 2E). Spermogonia were also produced 3 wk after inoculation. Caespituli and spermatia were also produced on sterilized leaves inoculated with conidia, which were obtained from cultures isolated from asymptomatic leaves. Both caespituli were morphologically similar to each other. The dimensions of the former conidia produced in caespituli from ascospores were 15–60 × 1.0–2.5 μm, those from conidia were 36–64 × 1.0–3.0 μm.

Colony characteristics including growth rate of both cultures derived from ascospores obtained from asci on fallen leaves and conidia isolated from asymptomatic leaves were similar. The dimensions of conidia on these cultures were morphologically identical: the former ones were 10.5–48.0 × 1.0–3.0 μm and the latter ones were 10.0–47.5 × 1.5–3.0 μm.

These results demonstrate that *M. buna* on the fallen leaves is identical to an endophytic *Pseudocercospora* anamorph in the asymptomatic living leaves.

Endophytic fungi are characterized by having a phase of interrupted growth following infection, or at least in having a cryptic phase where asymptomatic colonization of living host tissue occurs. Their presence in host tissue ordinarily becomes apparent only after the onset of natural senescence or induced necrosis (Stone and Petrini, 1997). We conclude that *M. buna* is present in living leaves of Japanese beech without symptoms as an endophytic fungus.

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