FULL PAPER

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Three Phaeosphaeria species and Paraphaeosphaeria michotii isolated from Phragmites leaves in Osaka, Japan

Received: February 6, 2002 / Accepted: June 27, 2002

Abstract Four graminicolous fissitunicate ascomycetes, Phaeosphaeria (Pha.) oryzae, Pha. nigrans, Pha. donacina, and Paraphaeosphaeria michotii, are redescribed with reference to the anatomy of ascomata, asci, and pseudoparaphyses and the external morphology of ascospores. Phaeosphaeria donacina is new to Japan. Phaeosphaeria oryzae is the second record from Japan. These ascomycetes were isolated from the healthy prime leaves of two reed species, Phragmites (Phr) australis and Phr. karka, collected in Osaka Prefecture, central Japan, from 1995 to 1998.

Key words Fissitunicate ascomycetes · Graminicolous fungi · Morphology · Phaeosphaeria donacina · Phragmites

Introduction

During a survey of microfungi on living leaves of two reed species, Phragmites (Phr.) australis (Cav.) Trin. ex Steud. and Phr. karka (Retz.) Trin., I have isolated four fissitunicate ascomycetes, Phaeosphaeria (Pha.) oryzae I. Miyake, Pha. nigrans (Roberge in Desm.) L. Holm, Pha. donacina (Sacc.) Shoemaker & Babcock, and Paraphaeosphaeria michotii (Westendorp) O. Eriksson. These fungi occur on a wide variety of monocotyledons (Shoemaker and Eriksson 1967; Shoemaker and Babcock 1989). There have been only a few reports of the two genera from Japan until now.

In this article, I report the aforementioned fungi with detailed morphological descriptions including the ascomal

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anatomy, arrangement of asci and pseudoparaphyses, and ejaculation and external morphology of ascospores. Phaeosphaeria donacina is new to Japan, and Pha. oryzae is reported for the second time from Japan.

Materials and methods

Collection of plant materials

Living leaves of two Phragmites species, Phr. australis and Phr. karka, were collected on the banks of the River Yodo, Osaka Prefecture (34°42′10″ N, 135°28′50″ E), from 1995 to 1998. Leaf samples without disease symptoms were collected in sterile glass cylinders (40 mm in diameter, 190 mm deep) sheathed with filter sheets (Sun-Sheet 12-12; Asahi Techno Glass, Tokyo, Japan) and brought back to the laboratory.

Isolation of fungi

The washing technique of Harley and Waid (1955) as modified by Tokumasu (1978) was used for isolating fungi. Leaf samples were cut into 1-cm² pieces from the middle part of each leaf blade. Leaf pieces were put into sterile test tubes capped with aluminum foil, and 10 ml sterile 0.005% Aerosol OT (di-iso-octyl sodium sulfosuccinate) solution was added to the tubes as a washing detergent. Washing with the detergent was repeated twice with a vortical type mixer, each time for 1 min. Then, the leaf pieces were rinsed with 10 ml sterilized distilled water six times in the same manner, transferred onto sterile filter paper in 9-cm petri dishes, and dried for 1 day to suppress vigorous bacterial growth after plating. Dried pieces were cut into small segments (\sim 1 \times 1mm) and placed onto agar plates (LcA: 1g glucose, 1g KH_2PO_4 , 0.2 g $MgSO_4 \cdot 7H_2O$, 0.2 g KCl, 2 g $NaNO_3$, 0.2 g yeast extract, 13 g agar, 11 distilled water). The plates were incubated at room temperature. Fungi growing out of the segments were transferred to fresh culture media (MYP:

3.5g malt extract, 0.5g soytone, 0.25g yeast extract, 15g agar, 11 distilled water) for later identification.

Culture method

Isolates were induced to form reproductive structures by growing them on autoclaved *Phr. australis* leaves placed on LCA agar plates at room temperature.

Morphological observations

Internal morphology of reproductive structures was observed under a Nomarski differential interference contrast microscope and an optical microscope after squashing and mounting them in a drop of distilled water on glass slides. To examine details of the centrum of reproductive structures, the material was fixed with 4% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2, embedded in Spurr's low-viscosity epoxy resin (Spurr 1969), and sectioned at 0.8 µm with an ultramicrotome (Ultracut N; Reichert-Jung Optische Werke, Vienna, Austria). The sections were stained with warmed toluidine blue (0.5% aq.). For the scanning electron microscope (SEM) study, a part of the fixed materials was postfixed with osmium tetroxide, then dehydrated in a graded acetone series and critical-point dried. After being sputter-coated with gold, the materials were studied under SEM (Hitachi S-2150).

Deposition of fungal materials

All specimens are deposited in the Herbarium of the Osaka Museum of Natural History, Osaka (OSA). All living cultures are deposited at the Institute for Fermentation, Osaka, Japan (IFO). All specimens were also indicated by Fukuhara's sampling number in the following text.

Taxonomy

Phaeosphaeria oryzae I. Miyake, Bot. Mag. (Tokyo) 23:94, 1909; I. Miyake, J. Coll. Agric. Imp. Univ. Tokyo 2:246–247, 1910; O. Eriksson, Ark. Bot. 6:407–409, 1967; R.A. Shoemaker, and C.E. Babcock, Can. J. Bot. 67:1534–1535, 1989. Figs. 1–5, Table 1

Synonym: *Leptosphaeria oryzae* (Miyake) Hara, A monograph of rice diseases, p. 53, 1950. *teste* Shoemaker et Babcock (1989).

Trematosphaerella oryzae (Miyake) Padwick, A manual of rice diseases. p. 153, 1959. teste Shoemaker et Babcock (1989).

Leptosphaeria oryzicola K. Hara, A monograph of rice disease, 3rd edn., p. 113, 1959, as oryzaecola. teste Shoemaker et Babcock (1989).

Leptosphaeria oryzina Sacc., Accd. Veneto-Trent., p. 67, 1917. teste Shoemaker et Babcock (1989).

Ascoma scattered, immersed-subepidermal, becoming erumpent, globose, papillate, without clypeus, glabrous, in section 100-150 um diameter, 120-150 um high. Papilla central, short-conical, intraepidermal to slightly erumpent, 30–50 µm high, 30–50 µm diameter, composed of 3–5 layers of polygonal cells around ostiole, without periphyses. Ascomal wall of textura angularis in surface view, uniformly 5–7 µm thick in longitudinal section, of 2–4 layers of pseudoparenchymatic cells, all cells brown-colored. Asci numerous, broadly rounded above, cylindrical, shortstalked, bitunicate, $75.0-85.0 \times 8.5-9.5 \mu m$, containing 8 overlapping, linearly biseriate ascospores. Ascospores narrowly fusiform, straight or slightly curved, $19.0-19.5 \times$ 5.0 µm, 3-septate, rounded end cells, second cells enlarged, dark brown, with small guttules, finely echinulate, without sheath. Pseudoparaphyses present in abundance, filiform, septate, about 1.5–3 µm in diameter, hyaline.

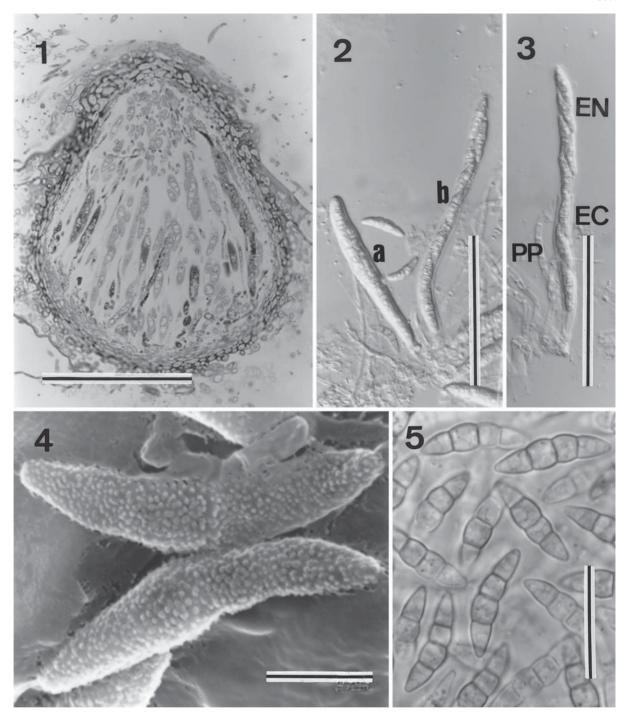
Specimens examined: Dried ascomata on autoclaved *Phr. australis* leaves produced by isolates from leaf samples were collected by M. Fukuhara in Kinki District, Honshu, Japan: Osaka Prefecture, Osaka-shi, Oyodo-kita, Yodo River, 34°42′10″ N, 135°28′50″ E, Oct. 30, 1995, 95680104 (OSA-MY50016, living culture IFO-33076).

Notes. The delimitation between *Phaeosphaeria* and *Leptosphaeria* has been obscure because they share many morphological features, i.e., glabrous ascomata, cylindrical to cylindric-clavate asci, fusiform ascospores that are 3- or more septate, and cellular pseudoparaphyses. Based on morphological data and ITS2 and partial 28S rDNA sequences, Khashnobish and Shearer (1996) supported the monophyly of *Phaeosphaeria* and suggested that the genus is delimited by the relatively thin peridium composed of thin-walled pseudoparenchyma with 2–4 cell layers, as was observed in the author's collections (Fig. 1).

Table 1. Morphological comparsion among three Phaeosphaeria (Pha.) species and Paraphaeosphaeria michotii

Character	Pha. oryzae	Pha. nigrans	Pha. donacina	Paraphaeosphaeria michotii
Ascomal size (µm) Asci size (µm) Ascospore size (µm) Number of septa Ascospore color Ascospore surface Conidia size (µm)	$100-150 \times 120-150$ $75.0-85.0 \times 8.5-9.5$ $19.0-19.5 \times 5.0$ 3 Dark brown Echinulate	$100-170 \times 100-170$ $60.0-90.0 \times 7.0-12.0$ $24.0-29.0 \times 5.0$ 5 Hyaline to yellow Smooth	$100-170 \times 100-170$ $72.0-75.0 \times 10.0-15.0$ $23.0-25.0 \times 5.0$ 3 Yellowish-brown Smooth	$70-170 \times 70-170$ $65.0-80.0 \times 5.0-10.5$ $19.0-19.5 \times 5.0$ 2 Reddish-brown Echinulate $1.5-3.0 \times 1.5-3.0$

a-, conidium production not known

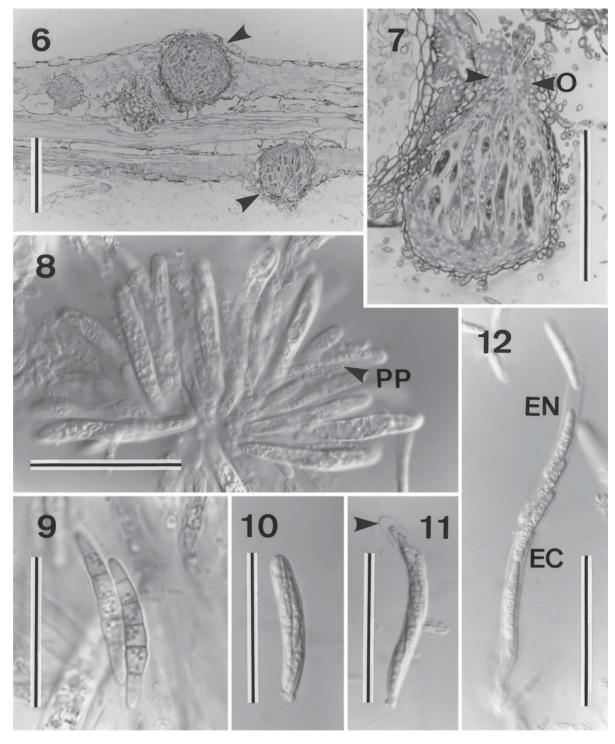


Figs. 1–5. *Phaeosphaeria oryzae*, 95680104. **1** Longitudinal section of ascoma. **2** Ascus with ascopores (*a*) and ascus releasing ascospores (*b*). **3** Extended endotunica (*EN*) with trapped ascospores and remains of

the base of ectotunica (EC) and pseudoparaphyses (PP). 4 Ascospores (SEM). 5 Ascospores. Bars 1 110 μ m; 2, 3 80 μ m; 4, 5 μ m; 5, 20 μ m

Phaeosphaeria oryzae is the type species of the genus Phaeosphaeria. Miyake (1909, 1910) provided Japanese and German descriptions for the species but cited no specimen. Eriksson (1967) referred to the specimens of Pha. oryzae collected by Miyake in Japan (F9572, F9573, Swedish Museum of Natural Histories) and designated them as the lectotypes.

The ascoma characters of the author's collections coincide well with the original descriptions of *Pha. oryzae* (Miyake 1909, 1910). The ascospores are regular in echinulation (Fig. 1), which is the most distinctive feature of this species (Shoemaker and Babcock 1989). *Phaeosphaeria oryzae* is morphologically similar to *Pha. tofieldiae* (Müller) Leuchtmann. However, *Pha. oryzae* is distinguished from



Figs. 6–12. *Phaeosphaeria nigrans*, 97090301. **6** Longitudinal section of ascomata in host tissue (*arrowheads*). **7** Longitudinal section of ascoma with ostiole (*O*). **8** Asci and pseudoparaphyses (*PP*). **9** Ascospores. **10** Ascus with ascospores. **11** Dehisced apical cap (*arrowhead*) of

ectotunica before spore discharge. 12 Extended endotunica (EN) with trapped ascospores and remains of base of ectotunica (EC). Bars 6 120 μ m; 7 110 μ m; 8, 10–12 80μ m; 9 20μ m

Pha. tofieldiae by having shorter and dark brown ascospores (Shoemaker and Babcock 1989).

Phaeosphaeria nigrans (Roberge in Desm.) L. Holm, Symb. Bot. Ups. 14:112, 1957; O. Eriksson, Ark. Bot. 6:428–

429, 1967; K. Katumoto, Jpn. Bot. 43:212–214, 1968; R.A. Shoemaker and C.E. Babcock, Can. J. Bot. 67:1551–1552, 1989. Figs. 6–12, Table 1 Synonym: *Sphaeria nigrans* Roberge in Desm., Ann. Sci.

Nat. Bot., Ser. 3, 6:79, 1846. *teste* Shoemaker et Babcock (1989).

Leptosphaeria nigrans (Roberge in Desm.) Ces. & De Not., Comment. Soc. Critt. Ital. 1:235, 1863. *teste* Shoemaker et Babcock (1989).

Leptosphaeria eustomella Sacc., Michelia, 2:251, 1881. teste Shoemaker et Babcock (1989).

Ascoma scattered, immersed-subepidermal, becoming erumpent, globose, papillate, without clypeus, glabrous, in section 100–170 µm diameter, 100–170 µm high. Papilla central, short-conical, intraepidermal to slightly erumpent, 35– 60μm high, 40–80μm diameter, composed of 3–5 layers of polygonal cells around a ostiole, without periphyses. Ascomal wall of textura angularis in surface view, laterally uniformly 8–10 µm thick in longitudinal section, composed of 2-4 layers of pseudoparenchymatic cells, all cells browncolored. Asci numerous, in a broad peripheral hymenium, cylindrical, $60-90 \times 7-12 \mu m$, short-stalked, with 8 overlapping, linearly tetraseriate to biseriate ascospores above. uniseriate below. Ascospores narrowly fusiform, straight or slightly curved, $24.0-29.0 \times 5.0 \mu m$, 5-septate, rounded end cells, second cells enlarged, hyaline to yellow with small guttules, smooth, without sheath. Pseudoparaphyses filiform, septate, about 1.5–3 µm diameter, hyaline.

Specimens examined: Dried ascomata on autoclaved *Phr. australis* leaves produced by isolates from leaf samples were collected by M. Fukuhara in Kinki District, Honshu, Japan: Osaka Prefecture, Osaka-shi, Oyodo-kita, Yodo River, 34°42′10″ N, 135°28′50″ E, June 29, 1996, 97090301 (from *Phr. australis*; OSA-MY50034); Aug. 1, 1997, 97151001 (from *Phr. australis*; OSA-MY50035); July 28, 1998, 98150702 (from *Phr. australis*; OSA-MY50049); June 30, 1996, 9612A0603 (from *Phr. karka*; OSA-MY50019, living culture IFO-33095); July 4, 1998, 98110304 (from *Phr. karka*; OSA-MY50046).

Notes. *Phaeosphaeria nigrans* is morphologically similar to *Pha. rousseliana* (Desm.) L. Holm. However, *Pha. nigrans* is distinguished from *Pha. rousseliana* by the ascospore characters (Shoemaker and Babcock 1989). The author's collections are characterized by 5-septate yellowish ascospores with an enlarged cell (Fig. 9). The ascomata and ascospores characters of these fungi agree with the previous descriptions of *Pha. nigrans* (Holm 1957; Katumoto 1968; Shoemaker and Babcock 1989), although the ascospores are slightly longer.

Katumoto (1968) reported this fungus on the dead leaves of *Triticum aestivum* L. in Yamaguchi Prefecture, Japan. He suggested that a fungus on *Oryza sativa* L., which Hara (1954) called *Leptosphaeria culmicola* (Fr.) Awd., also belongs to this species.

Phaeosphaeria donacina (Sacc.) Shoemaker & Babcock,
Can. J. Bot. 67:1524, 1989. Figs. 13–17, Table 1
Synonym: Leptosphaeria donacina Sacc., Atti Soc. Veneto-Trentina Sci. Nat., Padova 2:155, 1873. teste Shoemaker et Babcock (1989).

Ascoma scattered, immersed-subepidermal, becoming erumpent, globose, papillate, without clypeus, glabrous, in section $100-170\,\mu m$ diameter, $100-170\,\mu m$ high. Papilla central, short-conical, erumpent, $30\,\mu m$ high, $30\,\mu m$ diameter.

eter, composed of 5–7 layers of polygonal cells around ostiole, without periphyses. Ascomal wall of textura angularis in surface view; in longitudinal section laterally uniformly 8–10 μm thick, composed of 2–4 layers of pseudoparenchymatic cells, all cells brown-colored. Asci numerous, in a broad hymenium, cylindrical, 72.0–75.0 \times 10.0–15.0 μm , short-stalked, with 8 overlapping, linearly biseriate ascospores. Ascospores narrowly fusiform, straight or slightly curved, 23.0–25.0 \times 5.0 μm , 3-septate, rounded end cells, second cells slightly enlarged, yellowish-brown, with small guttules, smooth, sheath absent. Pseudoparaphyses filiform, septate, about 1.5–3 μm diameter, hyaline.

Specimens examined: Dried ascomata on autoclaved *Phr. australis* leaves produced by isolates from leaf samples were collected by M. Fukuhara in Kinki District, Honshu, Japan: Osaka Prefecture, Osaka-shi, Oyodo-kita, Yodo River, 34°42′10″ N, 135°28′50″ E: Sept. 4, 1995, 95410901 (from OSA-MY50011, living culture IFO-33074); Sept. 4, 1995, 95430601 (from OSA-MY50012); Oct. 2, 1996, 96350404 (OSA-MY50018); Oct. 2, 1996, 96450503 (from OSA-MY50032, living culture IFO-33075).

Notes. New to Japan. The author's collections are very similar to *Pha. donacina*, described by Shoemaker and Babcock (1989) in morphological characters, including the color of ascospores that distinguish *Pha. donacina* from closely related *Pha. sacchari* (van Breda de Haan) Shoemaker & Babcock (Shoemaker and Babcock 1989).

The Latin *donacinus* is derived from Greek *donax* (reed). This species was described based on a material on *Arundo donax* L. (Japanese name: Dan-tiku) (Shoemaker and Babcock 1989).

Paraphaeosphaeria michotii (Westendorp) O. Eriksson,
Ark. Bot., 6:406, 1967; R.A. Shoemaker and O. Eriksson,
Can. J. Bot. 45:1605–1608, 1967; Y. Otani, Bull. Natl. Sci.
Mus., Ser. B (Bot.) 2:95–97, 1976; K. Katumoto, Trans.
Mycol. Soc. Jpn 21:10–11, 1980; R.A. Shoemaker, and C.E.
Babcock, Can. J. Bot. 67:1592, 1989; M.P.S. Câmara et al.,
Mycol. Res. 105:49–50, 2001. Figs. 18–22, Table 1
Synonym: Sphaeria michotii Westendorp, Bull. Acad. R.
Sci. Belg., Sér. 2, 7:87, 1859. teste Shoemaker et Eriksson (1967).

Sphaerella michotii (Westendorp) Auersw. in Gonnermann and Rabenhorst., Mycol. Eur. 6:18, 1869. *teste* Shoemaker et Eriksson (1967).

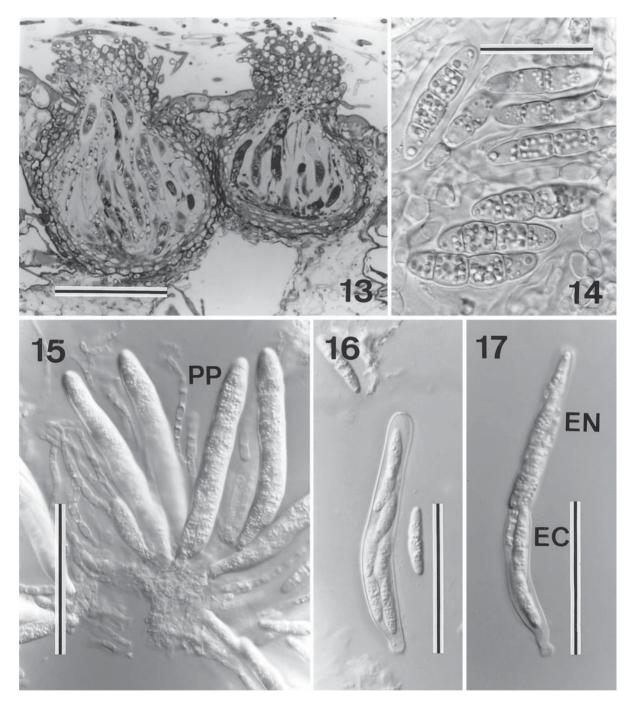
Leptosphaeria michotii (Westendorp) Sacc., Fung. Ital. 279, 1878 and Syll. Fung. 2:58, 1883. *teste* Shoemaker et Eriksson (1967).

Scleropleella michotii (Westendorp) Höhn., Ann. Mycol. 18:76, 1920. teste Shoemaker et Eriksson (1967).

Leptospaheria iwamotoi Miyake, J. Coll. Agric. Imp. Univ. Tokyo 2:249, 1910. teste Otani (1976).

Leptosphaeria folliculata Ell. & Ev., Proc. Phila. Acad. 237, 1890. teste Shoemaker et Eriksson (1967).

Pleospora monilispora (Fuckel) Fuckel, Jahrb. Nassau. Ver. Naturkd. 23, 24:138, 1869 (1870). teste Shoemaker et Babcock (1989).



Figs. 13–17. *Phaeosphaeria donacina*, 94450503. **13** Longitudinal section of ascomata. **14** Ascospores. **15** Asci and pseudoparaphyses (*PP*). **16** Ascus with ascospores and released ascospores. **17** Extended

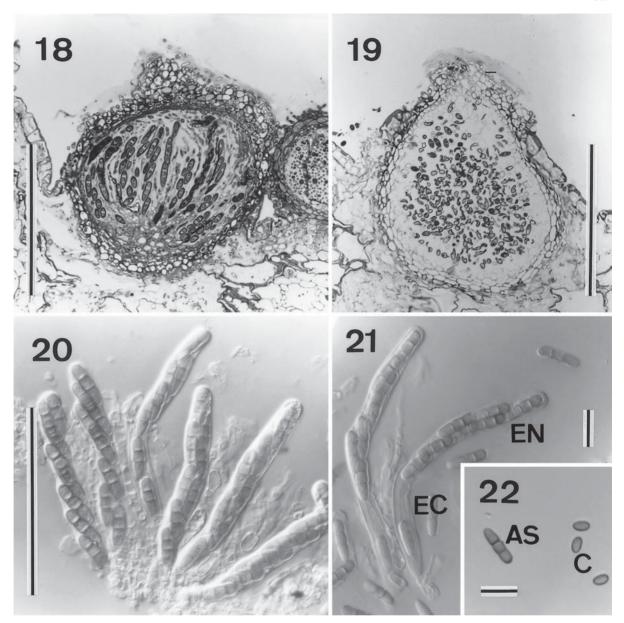
endotunica (EN) with trapped ascospores and remains of base of ectotunica (EC). Bars 13 60 μ m; 14 20 μ m; 15–17 50 μ m

Leptosphaeria zeae Stout, Mycologia 22:277, 1930. teste Shoemaker et Babcock (1989).

Anamorph: Coniothyrium sensu lato.

Ascomata scattered, immersed-subepidermal, becoming erumpent, globose, in section $70\text{--}170\,\mu\text{m}$ diameter, $70\text{--}170\,\mu\text{m}$ high. Papilla short, erumpent, $30\text{--}70\,\mu\text{m}$. Ascomal wall of textura angularis in surface view, in longitudinal section $5\text{--}8\,\mu\text{m}$ thick, composed of 3--6 layers of

pseudoparenchymatal cells, all cells brown-colored. Asci numerous, in a broad hymenium, cylindrical, short-stalked, broadly rounded above, bitunicate, 65.0– 80.0×10.5 – $15.0 \mu m$, containing 8 overlapping, linearly biseriate ascospores. Ascospores narrowly fusiform with upper part cylindrical and lower part tapered, 15.0– $19.0 \times 5.0 \mu m$, 2-septate, rounded end cells, central cells slightly enlarged, reddish brown. Pseudoparaphyses filiform, septate, about 1.5– $3 \mu m$ diameter, hyaline. Pycnidia globose, in section 70–



Figs. 18–22. *Paraphaeosphaeria michotii*, 97160104. **18** Longitudinal section of ascoma. **19** Longitudinal section of pycnidium. **20** Asci with ascospores. **21** Extended endotunica (*EN*) with trapped ascospores and

remains of base of ectotunica (EC). 22 Ascospore (AS) and pycnoconidia (C). Bars 18, 19 120 μm; 20 80 μm; 21, 22 20 μm

170 μm diameter, 70–170 μm high with a papillate ostiole, often oozing pycnospores, 1.5–3 μm, reddish-brown.

Specimens examined: Dried ascomata on autoclaved *Phr. australis* leaves produced by isolates from leaf samples were collected by M. Fukuhara in Kinki District, Honshu, Japan: Osaka Prefecture, Osaka-shi, Oyodo-kita, Yodo River, 34°42′10″ N, 135°28′50″ E: Sept. 1, 1996, 96250903 (from *Phr. australis*; OSA-MY50029, living culture IFO-33072); Sept. 1, 1996, 96250201 (from *Phr. australis*; OSA-MY50024); Sept. 1, 1996, 96250203 (from *Phr. australis*; OSA-MY50025); Aug. 1, 1997, 97160104 (from *Phr. karka*; OSA-MY50036, living culture IFO-33073).

Notes. The genus *Paraphaeosphaeria* was described as a segregate of *Phaeosphaeria* for species producing brown,

usually punctate ascospores with rounded ends, a submedian primary septum, an inflated cell above the primary septum, and a *Coniothyrium sensu lato* anamorph (Webster 1955). Twenty-two species have been described in this genus (Câmara et al. 2001). However, Câmara et al. (2001) pointed out that the traditional *Paraphaeosphaeria* is separated into three major clusters by a molecular approach and that the concept of the genus should be narrowed. They suggested that the ascospore septation, ascomal morphology, and anamorph characters are good indicators of phylogenetic affinity.

Paraphaeosphaeria (Pa.). michotii is distinguished from the other members of the genus by the ascospore septation and anamorph character (Câmara et al. 2001). The author's collections are identified as *Pa. michotii* by having the 2-septate, echinulate ascospores with the widest point below the middle (Fig. 22), and the *Coniothyrium* conidial state.

Two species of *Paraphaeosphaeria* have been reported in Japan. Hara (1918) reported *Pa. michotii* on *Morus alba* (Japanese name: Ma-guwa) in Gifu Prefecture, Japan, as *Leptospaheria michotii*, and pointed out the similarity to *Leptospaheria iwamotoi* Miyake. Katumoto (1980) reported the presence of *Pa. rusui* (Wallr.) O. Eriksson on *Ruscus aculeatus* L. (Japanese name: Nagi-ikada) in Yamaguchi Prefecture, Japan. *Paraphaeosphaeria rusui* is regarded as a synonym of *Pa. glauco-punctata* (Greville) Shoemaker & Babcock (Shoemaker and Babcock 1985). *Paraphaeosphaeria glauco-punctata* would be excluded from the *Paraphaeosphaeria* in the narrow sense (Câmara et al. 2001).

Acknowledgments I am grateful to Drs. Ken Katumoto and Naohiko Sagara for numerous comments during the course of this study; to Dr. Tatsundo Fukuhara for reading an earlier version of the manuscript; and to an anonymous reviewer for numerous comments that have improved the manuscript. This study was supported in part by the Sasagawa Scientific Research Grant of the Japan Science Society.

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