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A new species of *Plagiostoma* (Ascomycota-Diaporthales) with a *Phomopsis* anamorph isolated from *Phragmites* leaves in Japan

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A new ascomycete species, *Plagiostoma phragmiticola* inhabiting two reed plants, *Phragmites australis* and *P. karka*, is described and illustrated. This new species is characterized by having non-stromatic, solitary perithecia, laterally and obliquely protruding perithecial beaks, and relatively small asci and ascospores. Black zone-lines in colonized leaf tissues were not observed. Its anamorph belongs to *Phomopsis*.

Key Words——Ascomycetes; *Phomopsis*; *Phragmites*; *Plagiostoma*; systematics.

An undescribed valsaceous fungus was isolated from living, symptomless leaves of two reed species collected on the Lake Biwa and River Yodo system, Kinki District, central Japan. Six isolates of the fungus were obtained from 1995 to 1998, and three of them produced pycnidial anamorphs on agar media.

After detailed observations, we have come to the conclusion that the teleomorph of the fungus is a new species to be accomodated in the genus *Plagiostoma* Fuckel of the family Valsaceae. *Plagiostoma* was introduced for valsaceous fungi having horizontal ascomata with an eccentric ostiolate beak and 2-celled hyaline ascospores (von Arx, 1951; Barr, 1978; Monod, 1983). In Japan, only one species of this genus, *P. polyascum* (Tochinai et Yamagiwa) Y. Otani et Igarashi, was known (Otani, 1995).

The obtained anamorph of our fungus belongs to *Phomopsis* (Sacc.) Bubák, which is known as the anamorph of *Diaporthe* Nitschke (Uecker, 1988). On the other hand, the anamorph of *Plagiostoma* has not been confirmed (Barr, 1978), although Monod (1983) reported the formation of unicellular conidia on agar media in *P. tormentillae* (Lind) Bolay and *P. bavaricum* (Rehm) M. E. Barr. Since the exact congenetic relation of the teleomorph and anamorph of our fungus has been confirmed by cultural and inoculation experiments, we propose the existence of a *Phomopsis* anamorph for the genus *Plagiostoma*.

Materials and Methods

Collection Living leaves of two *Phragmites* species, *P. australis* (Cav.) Trin. ex Steud. and *P. karka* (Petz.) Trin., were collected on the banks of Lake Biwa, Shiga Pref., and of River Yodo, Osaka Pref., from 1994 to 1998. Leaf samples without disease symptoms were collected into sterile glass cylinders (40 mm across, 190 mm deep) sheathed with filter sheets (SUN-SHEET 12–12; Asahi Techno Glass Corp.) and carried to the laboratory for isolation.

Isolation The washing technique of Harley and Waid (1955) as modified by Tokumasu (1978) was used for isolating fungi. Leaf samples were cut into 1-cm-square pieces at the middle part of each leaf blade. Leaf pieces were put into sterile test-tubes capped with aluminum foil, and 10 ml of sterile 0.005% Aerosol OT (di-*iso*-octyl sodium sulfosuccinate) solution was added to the tubes as the washing detergent. Washing with the detergent was repeated twice by agitation with a vortical type mixer, each time for one minute. Then the leaf pieces were rinsed with 10 ml of sterilized distilled water six times in the same manner. Rinsed leaf pieces were transferred onto sterile filter paper in 9-cm petri dishes and dried for one day to suppress vigorous bacterial growth after plating. Dried pieces were cut into small segments (ca. 1 × 1 mm) and placed onto agar plates (LCA: 1 g glucose, 1 g 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, 1 L distilled water). The plates were incubated at room temperature. Fungi growing out of the segments were transferred to fresh culture media (MYP: 3.5 g malt extract, 0.5 g soytone, 0.25 g yeast extract, 15 g agar, 1 L distilled water) for later identification.

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

Single ascospore and conidial mass isolation To obtain isolates from single ascospores and conidial masses, subcultures were made as follows. Stroma-like structures taken from a culture were squashed in 10 ml of sterilized

water. This suspension was then poured into petri dishes containing a thin layer of water-agar. After confirming the germination of ascospores and conidia under × 10 objective lenses, single ascospore and conidial mass isolates were cut out from the agar and transferred to LcA slants in test tubes.

Obtaining reproductive structures To obtain teleomorph and/or anamorph specimens on leaves from these isolates, 1-mm agar cubes containing the growing mycelia on the LcA slants were cut out and inoculated onto autoclaved *Phragmites* leaves placed on LcA agar plates. These plates were incubated at room temperature for 30 d.

Observation External morphology of reproductive structures was examined under a dissecting microscope and a scanning electron microscope (SEM). Their internal morphology was observed with squashed materials under a phase contrast microscope and an optical microscope, after mounting them in one drop of distilled water on glass slides. To examine details of the centrum of the 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 mm with an ultramicrotome (ULTRACUT N, Reichert-Jung Optische Werke). The sections were stained with warmed Toluidine Blue (0.5% aq.). For observation by SEM (Hitachi S-2150), a part of the fixed materials was postfixed with osmium tetroxide, dehydrated in a graded acetone series, critical-point dried, and sputter-coated with gold.

Results

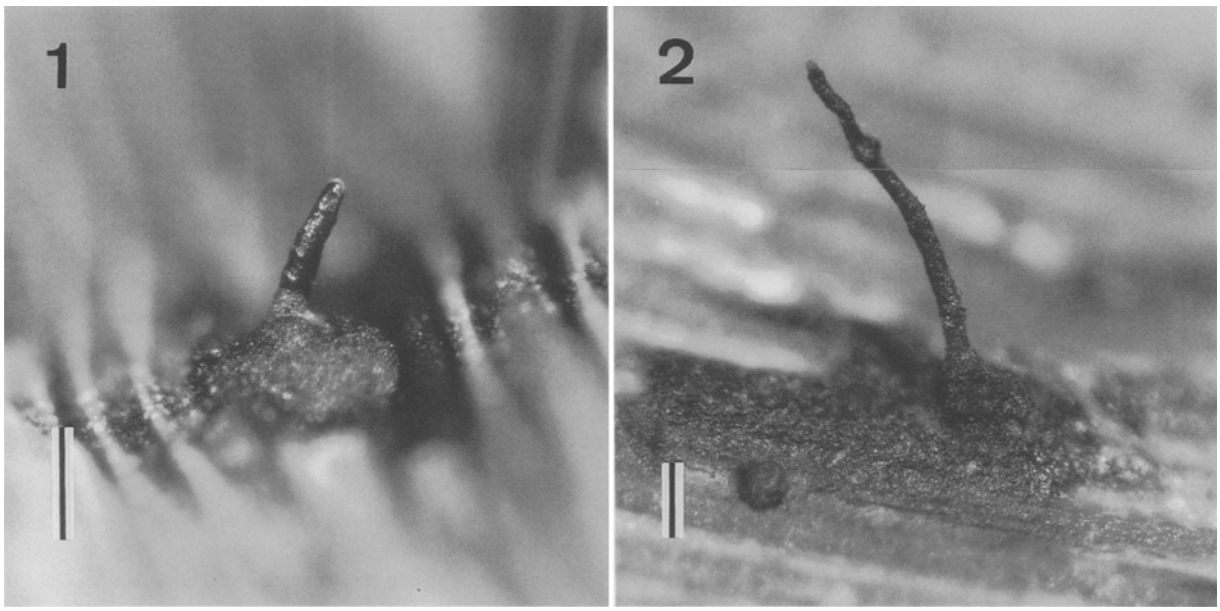
Cultural characteristics Six isolates were obtained from 1995 to 1998 (Table 1). Colonies on 2% malt extract agar spreading broadly, attaining a diam of 85 mm in 10 d at 20°C, smooth, whitish, aerial mycelium scanty, without zonations; reverse whitish. Stromatic development absent. All the cultures produced black perithecia scattered on autoclaved *Phragmites* leaves on LcA, and three of them produced pycnidia with pycnidiospores (Table 1).

Table 1. Isolates of *Plagiostoma phragmiticola* and their pycnidium production on agar medium (LcA).

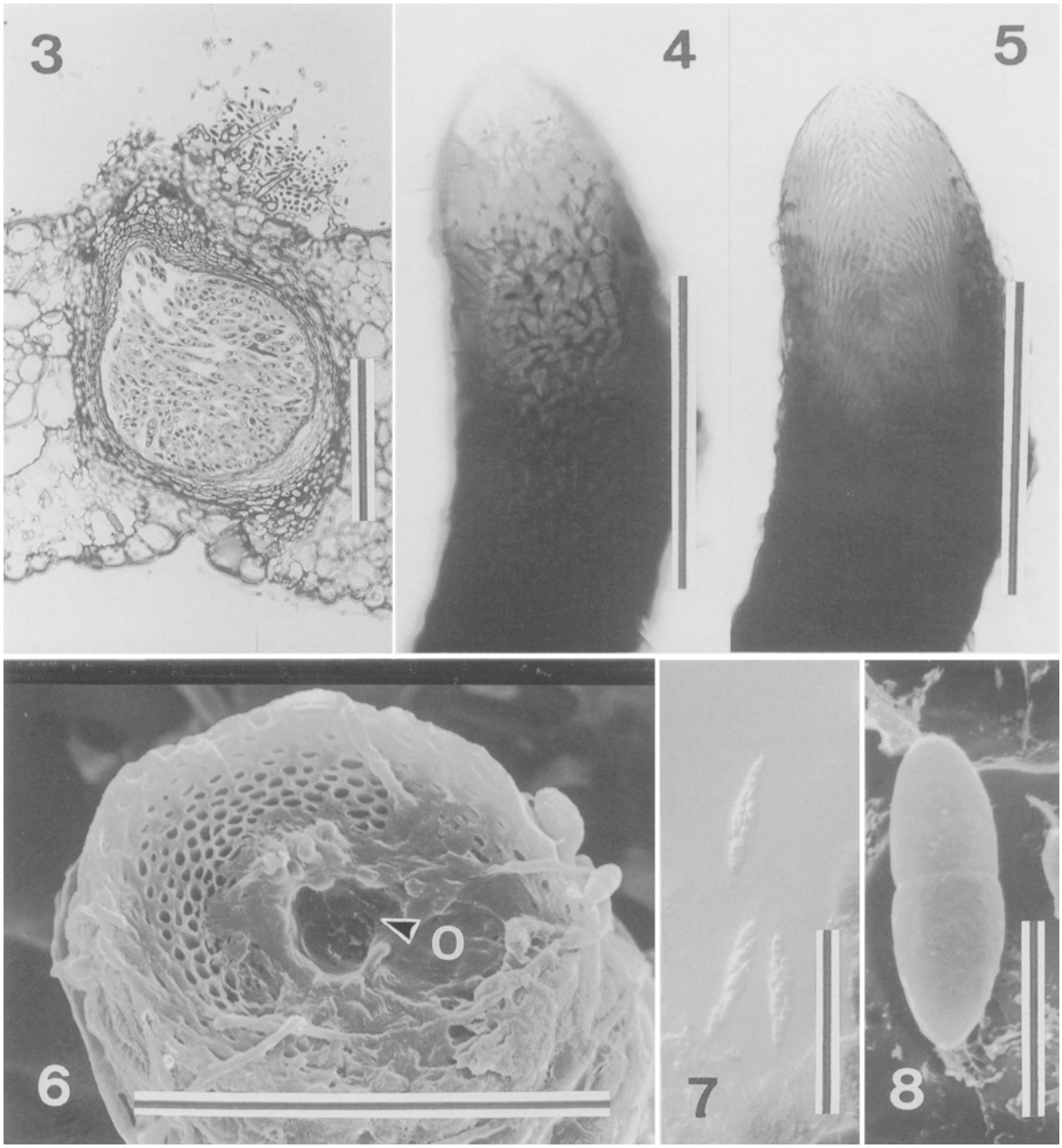
Isolate No.	Year of collection and isolation	Host	Pycnidium production[a)]
95223a02	1995	*P. karka*	+
95431002	1995	*P. australis*	−
96290303	1996	*P. karka*	−
97380202	1997	*P. australis*	+
98100701	1998	*P. australis*	−
98150701	1998	*P. australis*	+

a) +: Occurrence of pycnidia, −: no occurrence.

Morphology on the leaf tissue on LcA The perithecia solitary, gregarious, obliquely immersed, then erumpent through leaf periderm, without stromata and black zonelines, flask-shaped to globose, 150–280 μm diam, protruding a long eccentric to lateral beak (Figs. 1, 2); peridia black, membranous, pseudoparenchymatous, consisting of 5–7 layers of polygonal cells (Fig. 3). Perithecial beaks straight to slightly curved, almost isodiametrical, 250–1000 μm long, 40–80 μm wide, black, apex amber according to color nomenclature by Rayner (1970), with a periphysate ostiolar canal (Figs. 1, 2, 4–6). Asci with an evanescent short stipe, unitunicate, clavate and slightly truncate, and somewhat thickened at the apex with a refractive and non-amyloid apical ring, 35–45 × 5–



Figs. 1, 2. *Plagiostoma phragmiticola* (95431002) perithecia produced 35 d after inoculation on autoclaved *Phragmites australis* leaves. Scale bars: 250 μm.

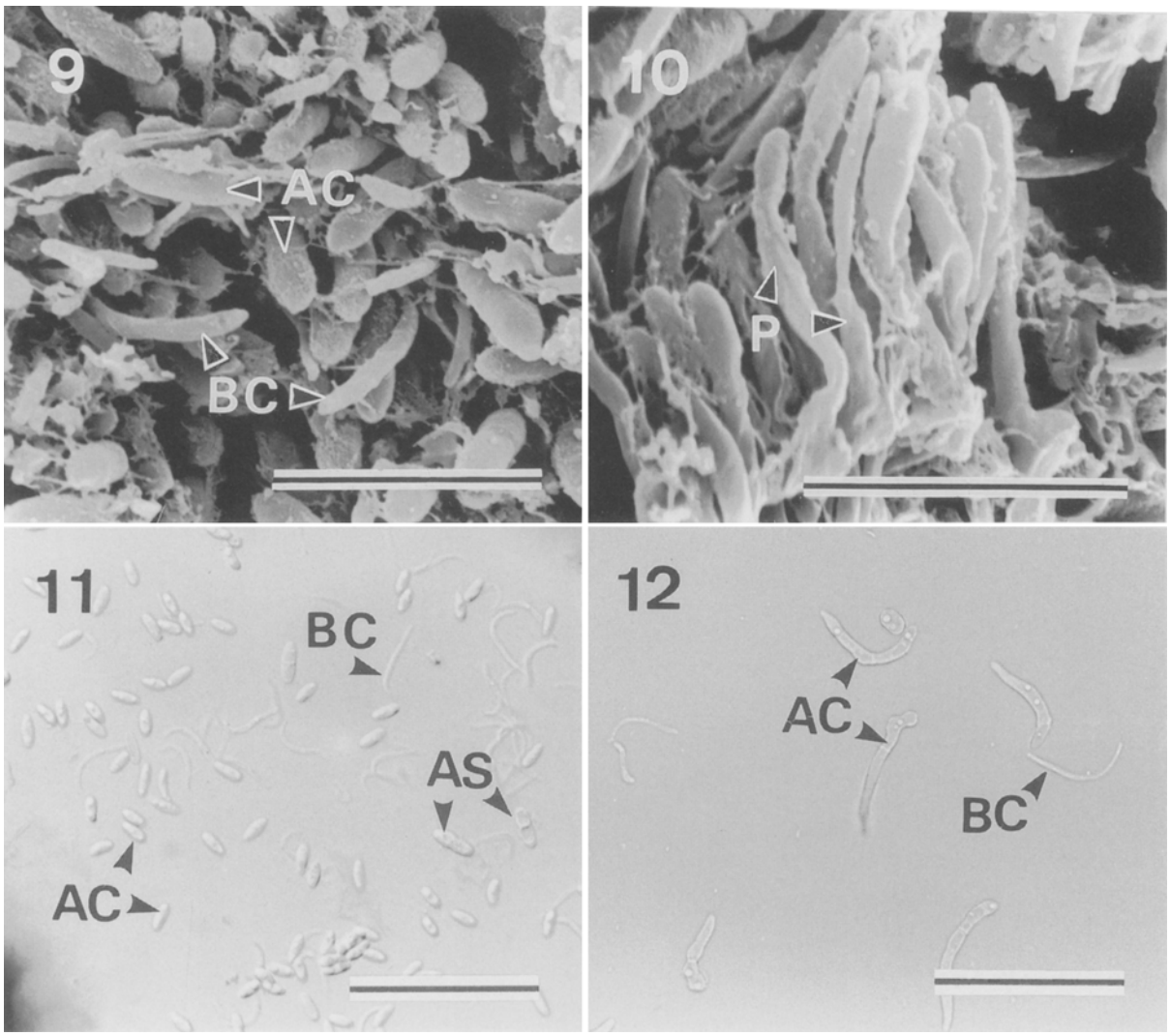


Figs. 3–8. *Plagiostoma phragmiticola* (95431002) 35 d after inoculation on autoclaved *P. australis* leaves. 3. Longitudinal section of a perithecium, with asci floating. 4, 5. Tip of perithecial beak focused at different phases. 6. A perithecial beak with a ostiolar canal (O) (viewed from above after the beak tip was cut off; SEM). 7. Asci with ascospores (phase contrast microscopy). 8. Ascospore (SEM). Scale bars: Figs. 3–7, 50 μm; Fig. 8, 5 μm.

7.5 μm, 8-spored, aparaphysate (Fig. 7). At maturity, the perithecial cavity full of free and floating asci (Fig. 3). Ascospores biseriate in asci, spindle-shaped, rounded to obtuse at both ends, 10 × 2.5–3 μm, with a median septum, slightly constricted at the septum, smooth (Fig. 8), hyaline, rarely hazel on age according to color nomenclature by Rayner (1970).

Isolates 95223a02, 97380202 and 98150701 produced pycnidia (Table 1). The pycnidia contained elongate, cylindric phialides that produced two types of non-septate hyaline conidia (Figs. 9–10). One (alpha conidia) was elliptic to oblong, 5–7 × 2.5–3 μm, and the other (beta conidia) was filiform and curved, 15–25 × 0.8–1 μm (Fig. 11). Conidial germination occurred in the alpha conidia, but not in the beta conidia (Fig. 12).

Subcultures originating from conidial masses developed perithecia/pycnidia complex on autoclaved *Phragmites* leaves (Figs. 13–16), confirming the anamorph-tel-



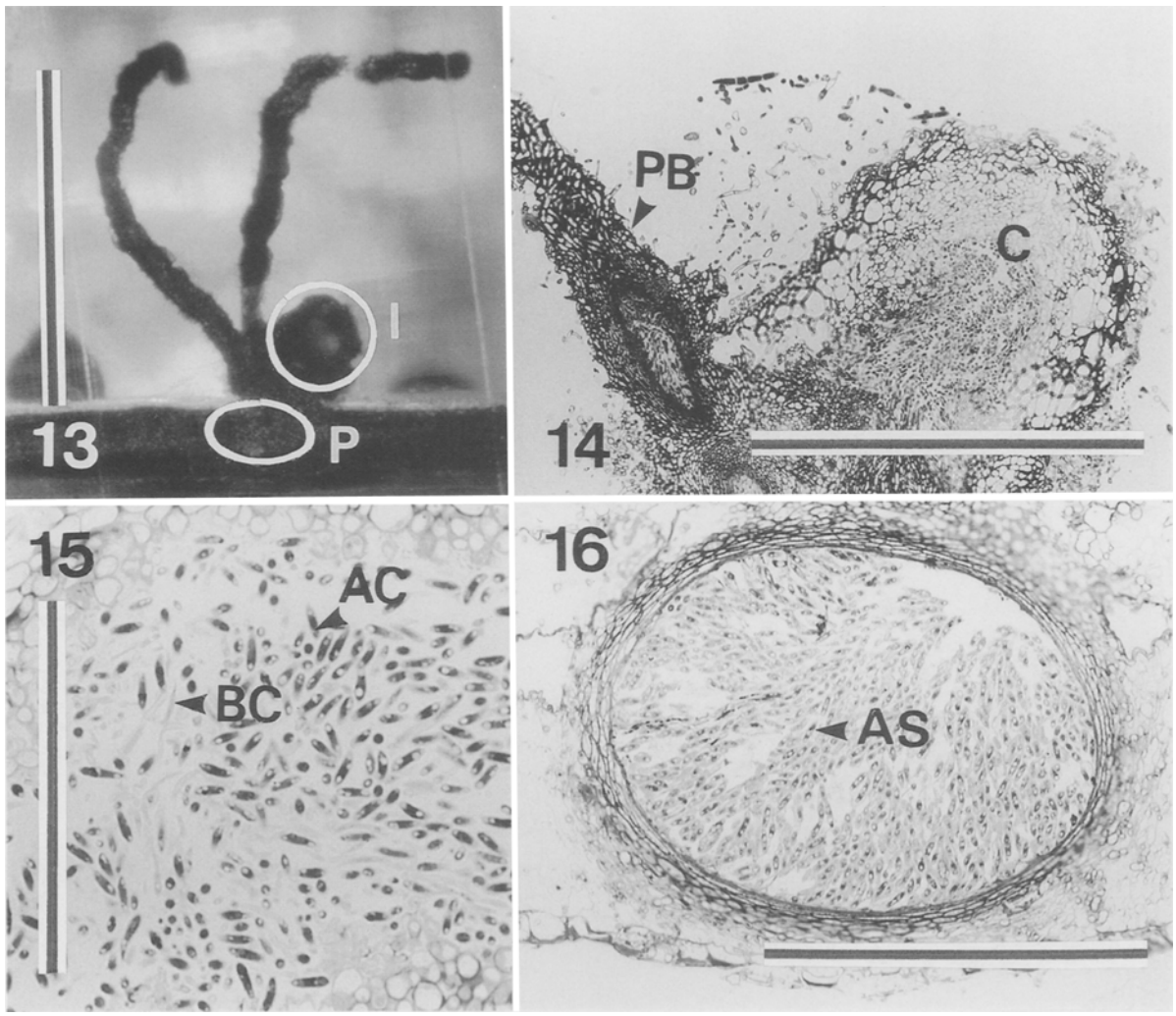
Figs. 9–12. *Plagiostoma phragmiticola* (95223a02) anamorph formed 28 d after inoculation on agar medium (LcA). 9. Alpha conidia (AC) and beta conidia (BC) (SEM). 10. Cylindric phialides (P) (SEM). 11. Alpha conidia (AC), beta conidia (BC) and ascospores (AS) (phase contrast microscopy). 12. Germinating alpha conidia (AC) and beta conidia (BC). Scale bars: Figs. 9, 10, 10 μm; Fig. 11, 50 μm, Fig. 12, 25 μm.

eomorph connection of this fungus. Subcultures derived from single ascospores also produced perithecia/pycnidia complex on autoclaved reed leaves in culture. This fungus should be homothallic because perithecia developed in single ascospore isolates.

Characteristics of the stroma-like structure on agar media Stroma-like structures developed on agar media, i.e., LcA (Figs. 17, 18) and MYP, but not on the inoculated *Phragmites* leaves (Fig. 16). They were carbonaceous, partially embedded in the agar, and usually produced pycnidia and perithecia (Figs. 19, 20). The perithecia developed singly or in irregular, non-valsoid clusters and produced long beaks.

Discussion

Assignation to the genus *Plagiostoma* In the classification of genera within the order Diaporthales, the presence or absence and the type of stromatic tissue, the position of perithecium and beak in relation to the substrate, and the shape and septation of ascospores have been used as the main taxonomic criteria (Barr, 1978). The genus *Plagiostoma* comprises the non-stromatic species that have oblique or horizontal perithecia, eccentric or lateral beaks, and ellipsoid ascospores with a median, supramedian or submedian septum characteristic of the Diaporthales (von Arx, 1951; Barr, 1978; Monod, 1983). The fungus reported here showed the same characters as *Plagiostoma* in the perithecia and ascospores on autoclaved reed leaves. However, it produced stroma-like structures on agar media. Furthermore, the anamorph produced in the stroma-like structure has a diagnosis for *Phomopsis* (Sacc.) Bubák as it produces cylindric phialides that bear two types of hyaline nonseptate



Figs. 13–16. *Plagiostoma phragmiticola* (95223a02) teleomorph/anamorph complex formed 30 d after inoculation on autoclaved *P. australis* leaves. 13. Lateral view of a teleomorph (P)/anamorph (I) complex on an autoclaved leaf (dissecting microscopy). 14. Microtome section of a perithecial beak (PB) and pycnidial conidioma (C) (from circle I in Fig. 13). 15. Alpha conidia (AC) and beta conidia (BC) in a conidioma (enlarged from Fig. 14C). 16. Asci (AS) floating in a perithecium (from circle P in Fig. 13). Scale bars: Fig. 13, 1 mm; Fig. 14, 300 μm.; Fig. 15, 50 μm.; Fig. 16, 100 μm.

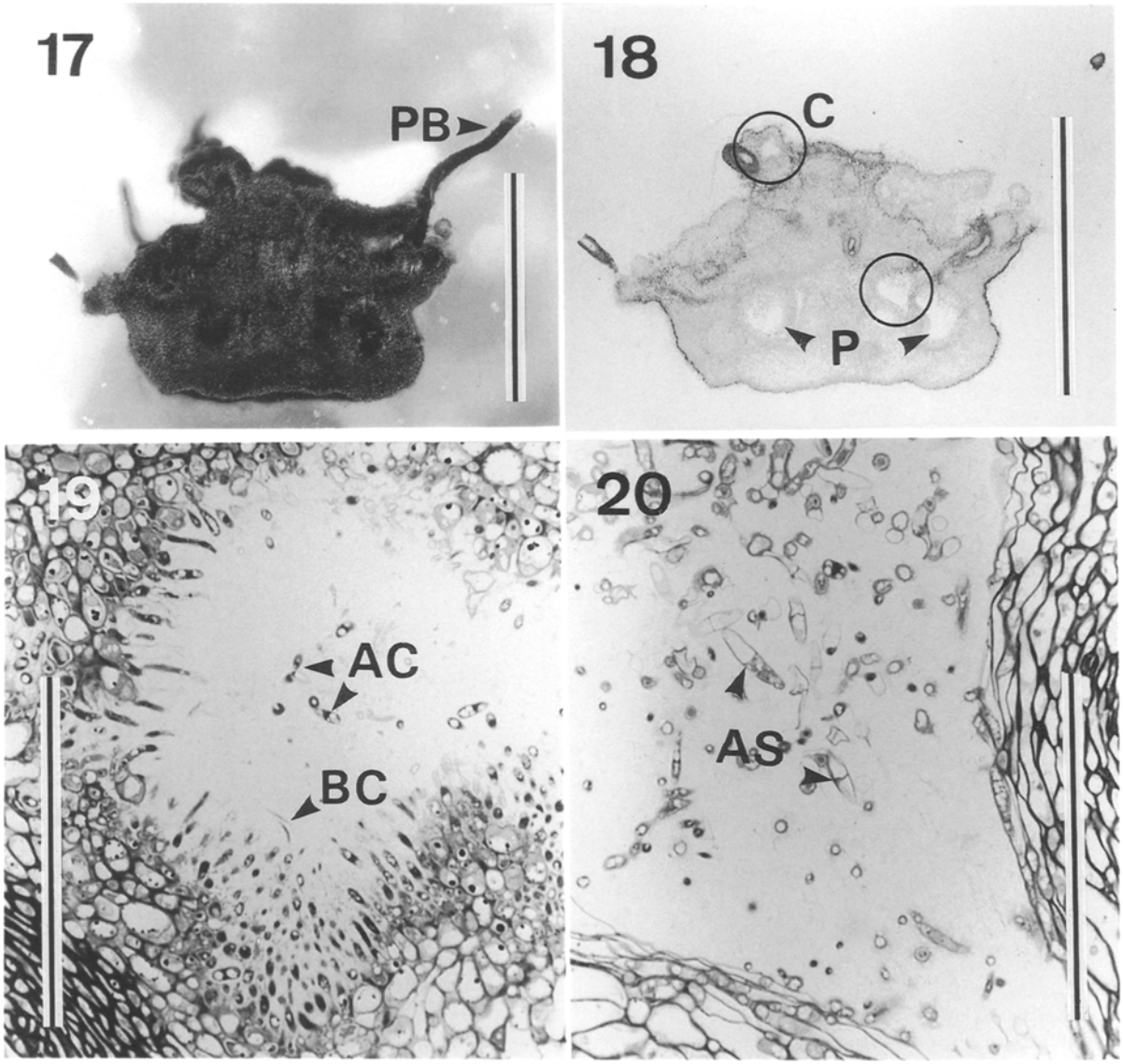
conidia, alpha and beta. *Phomopsis* is the only known anamorph of *Diaporthe*, a stromatic genus of the Diaporthales. This apparently suggests the possibility that our fungus belongs to the genus *Diaporthe*.

Perithecia of *Diaporthe* form in valsoid clusters in a stromatal tissue, whereas those of *Plagiostoma* form solitarily without stromata (Barr, 1978). However, in Diaportales, stromatal morphology and perithecial arrangement in culture often differ from those on the host substratum (Whemeyer, 1927; Fernández and Hanlin, 1996). Furthermore, conidiomatal structure of *Phomopsis* on agar media is extremely plastic (Brayford, 1990a). Hence we think that characteristics on host substratum rather than in culture should be taken as diagnostic characters for Diaporthales.

We consider that the characteristics on *Phragmites* leaves, although they were on agar, are more important as the diagnostic characters than those on LCA and MYP. Our species has a diagnosis for the genus *Plagiostoma* as it produces perithecia solitarily embedded within leaf tissue without stromata. Added to this, perithecia of *Diaporthe* are produced in colonized plant tissues with narrow black lines often termed 'zone-lines' (Brayford, 1990b), but our species was without zone-lines. Therefore, we assign this species to the genus *Plagiostoma*.

The present finding of the teleomorph-anamorph connection in this species may suggest an affinity between *Plagiostoma* and *Diaporthe*, and this new species may be a key to understanding the phylogeny of these two genera.

Differences from other *Plagiostoma* species Our species is characterized by relatively small asci (35.0–40.0 × 5.0–7.5 μm) and ascospores (10.0 × 2.5–3.0 μm). These characteristics are similar to those of two other species,

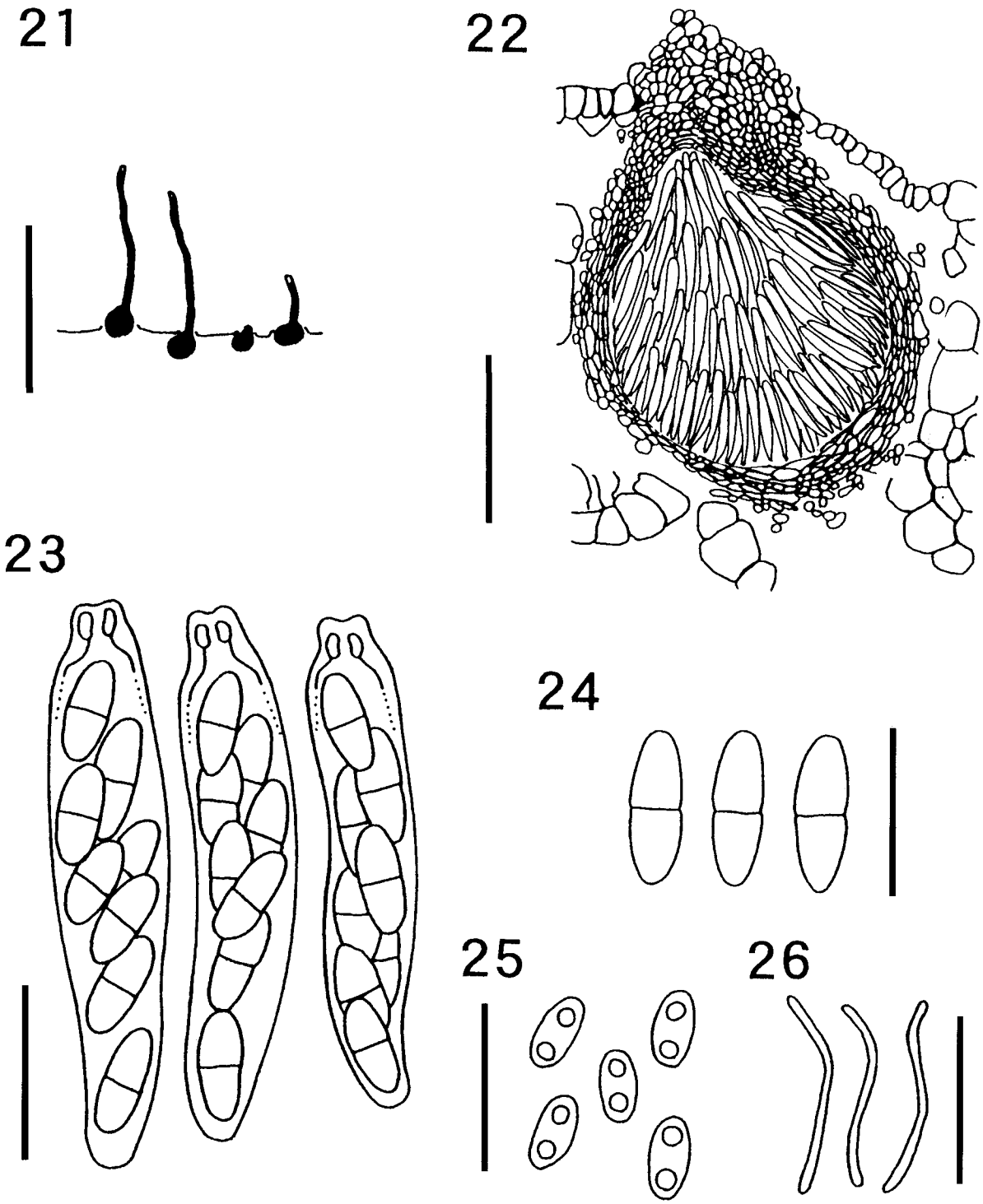


Figs. 17–20. *Plagiostoma phragmiticola* (95223a02) stromatal tissue formed 30 d after inoculation on agar medium (LcA). 17. Lateral view of stromatal tissue with a perithecial beak (PB). 18. Microtome section of a stromatal tissue containing pycnidial conidioma (C) and perithecia (P: a circle and arrowheads). 19. Alpha conidia (AC) and beta conidia (BC) in a pycnidial conidioma (from C in Fig. 18). 20. Ascospores (AS) in a perithecium (from P in Fig. 18). Scale bars: Figs. 17, 18, 1 mm; Figs. 19, 20, 50 μm.

P. conradii (Ellis) M. E. Barr and *P. devexum* (Desm.) Fuckel, which were reported from Cistaceae (*Hudsonia* spp.) and Polygonaceae (*Polygonum* spp. and *Rumex* spp.), respectively (Barr, 1978). Our species is distinguishable from *P. conradii* by its slender asci and ascospores. Our species most closely resembles *P. devexum*, having asci and ascospores of the same form and the same range of size. However, our species is distinguishable by its smooth ascospores, in contrast to the setose ascospores of *P. devexum* (Barr, 1978).

Another salient character of our species is the long perithecial beak. However, the length of the perithecial beak is not considered a significant enough characteristic to diagnose the new species, since a large variation in the length of perithecial beaks, apparently due to humidity and light conditions, has been observed in Diaporthales (Brayford, 1990a; Hodges, 1980; Huang and Luttrell, 1982).

Further, our species is distinguished from the other *Plagiostoma* by inhabiting reed plants. *Plagiostoma* species have hitherto been reported only from dicotyledons (Barr, 1978, Monod, 1983). Even within the order Diaporthales, there are few records from monocotyledons: three species of *Clypeoporthe* Hörnel (Barr, 1978) and *Gnomonia oryzae* I. Miyake (1910) from Gramineae, and *Maculatipalma frondicola* J. Fröhlich et K. D. Hyde (1995) from Palmae. Although many *Plagiostoma* species exhibit host specificity and are found exclusively on one plant genus or closely related plant genera (Barr, 1978), we have not yet examined whether the associa-



Figs. 21–26. *Plagiostoma phragmiticola*. 21. Perithecia. 22. Longitudinal section of a perithecium. 23. Asci with ascospores. 24. Ascospores. 25. Alpha conidia. 26. Beta conidia. Scale bars: Fig. 21, 1 mm; Figs. 22, 50 μm; Fig. 23, 15 μm; Figs. 24, 10 μm; Figs. 25, 20 μm.

tion of the new species with the two reed species is specific.

A formal description of the species is presented below.

Taxonomy

Plagiostoma phragmiticola Mi. Okada & Katumoto, sp. nov. Figs. 21–26

Storomata absentia. Perithecia solitaria, gregaria,

primo immersa, dein erumpentia, lageniformia, 150–280 μm diam, apice rostrata; paries ater, pseudoparenchymaticus, 10–15 μm crassus, ex cellulis 5–7 stratosis polygonalibus compositus; rostrum excentricum vel interdum fere laterale, longe cylindricum, oblique protrudens, 250–1000 μm longum, 40–80 μm crassum, atrum, ad apicem succineum. Asci unitunicati, apice leviter truncati et paulo incrassati, annulo apicali jodo non cyanescenti praediti, non vel brevissime stipitati, octospori, 35–45 × 5–7.5 μm. Ascosporae fusoideae, medio 1-septatae, apice utrinque obtusatae, hyalinae vel rarius avellaneae, laeves, 10 × 2.5–3 μm.

Status anamorphus: *Phomopsis*.

Habitus: In foliis vivis *Phragmitis australis* (Cav.) Trin. ex Steud. et *P. karkae* (Petz.) Trin.

Holotypus: Mi. Okada 95223a02 (TNS-F-843), exsiccatum folii *Phragmitis australis* inoculati cum ascospora singula in cultura ex foliis vivis *P. karkae*, Flumen Yodo, Yawata, Kyoto Pref., Japonia, M. Okada leg. et id Herb. "the National Science Museum, Tsukuba" (TNS) conservatum.

Etymology: From generic name of the host plants and from the Latin *cola* which means 'dweller', referring to the habitat of this species on reeds.

Living cultures: Deposited at the Institute for Fermentation, Osaka, Japan, M. Okada 96223a02 (IFO-33170).

Known distribution: Japan: Honshu (Osaka Pref., Kyoto Pref. and Shiga Pref.)

Additional materials examined: Dried specimens on autoclaved *P. australis* leaves that have been inoculated with single-ascospore isolates from *P. australis* or *P. karka* in Kinki District, Honshu, Japan;—Osaka Pref., Osaka-shi, Oyodo-kita, Yodo River, 34°42′10″N, 135°28′50″, 4 Sep. 1995, 95431002 (from *P. australis*; TNS-F-844, living culture IFO-33171); 2 Oct. 1997, 97380202 (from *P. australis*; TNS-F-846, living culture IFO-33173; 28 Jul. 1998, 98150701 (from *P. australis*; TNS-F-848, living culture IFO-33175);—Kyoto Pref., Yawata-shi, Miyuki-basi, Yodo River, 34°53′10″N, 135°42′10″E, 9 Sep. 1996, 96290303 (from *P. karka*; TNS-F-845, living culture IFO-33172);—Shiga Pref., Takashima-cho, Hagi-nohama, Lake Biwa, 35°21′40″N, 136°3′30″E, 8 Apr. 1998, 98100701 (from *P. australis*; TNS-F-847, living culture IFO-33174); all by M. Okada.

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