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FULL PAPER

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Cochliobolus heveicola sp. nov. (*Bipolaris heveae*) causes brown stripe of bermudagrass and Zoysia grass

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Abstract The pathogen of brown stripe in leaves of *Cynodon dactylon* (bermudagrass) and *Zoysia japonica* (Zoysia grass) in Japan is identical with *Bipolaris heveae*, a rubber tree pathogen, based on morphological and phylogenetic characteristics, following pathogenicity studies. Crossing isolates used in the study with each other, the obtained teleomorph is described as *Cochliobolus heveicola* sp. nov.

Key words Bermudagrass · *Bipolaris* · Brown stripe · *Cochliobolus* · Zoysia grass

Introduction

Cynodon dactylon (L.) Pers. (bermudagrass) and *Zoysia japonica* Steud. (Zoysia grass) are important turf grasses, mainly in the subtropical regions of the world but also in the southern part of Japan. They are widely used, especially as greens in parks and fairways in golf courses, because of their persistence and drought tolerance during warm to hot weather.

Some species of *Bipolaris*, a fungal genus that has a *Cochliobolus* or *Pseudocochliobolus* teleomorph (Loculoascomycetes), have been reported to cause diseases of turfgrasses all over the world (Smiley et al. 1992). *B. cynodontis* (Marignoni) Shoemaker, *B. hawaiiensis* (M.B. Ellis) Uchida & Aragaki, *B. sorokiniana* (Sacc.) Shoemaker, *B. spicifera* (Bainier) Subram., and *B. stenospila* (Drechsler) Shoemaker cause leaf spot or stem and crown rot of *Cynodon* or *Zoysia*. Leaf blight caused by *B. cynodontis* is the most severe foliar disease of bermudagrass in the world and has been reported to occur also in Japan (Tsuda and Ueyama 1981).

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In 1994, a new *Bipolaris* disease producing short brown stripes in the leaves of *C. dactylon* and *Z. japonica* was observed in Japan. The occurrence of the disease has since become more prevalent in Japan. The purpose of this article is to describe the symptom of the disease and to report the identity of the causal organism.

Materials and methods

Collection and isolation of the fungus

Diseased leaves of *Z. japonica* were collected from three sites in Tochigi and Yamanashi Pref., the central part of Japan, from 1994 to 2001. Infected leaves of *C. dactylon* were collected in 2002 at two sites in Okinawa Pref., the southern most part of Japan. The samples were stored dry in a refrigerator at 5°C until the pathogen was isolated. Single leaf lesions were excised and surface-sterilized for 30 s. in 70% ethanol, followed by immersion for 2–3 min in 1% sodium hypochlorite, then washed in distilled water. The specimens were incubated on water agar under an alternating 12 h darkness/12 h BLB FL20S·BLB (Toshiba, Japan) light cycle at 25°C for 5 days. A single conidium was transferred to V8 juice agar (V8) using a thin glass needle. Thirteen isolates were obtained; Zoy-1–11 from *Z. japonica*, and Cyn-1–2 from *C. dactylon*. The isolates, ATCC26447 of *Bipolaris heveae* (Petch) Arx emend Muchovej & R. Muchovej, and ATCC13447 and CBS156.36 of *B. stenospila* (Drechsler) Shoemaker [=*Cochliobolus stenospilus* T. Matsumoto & W. Yamam. nom. inval.], were used for comparison with the Japanese isolates. Morphological characteristics of the anamorph of the isolates were taken from the cultures on V8 juice agar under the alternate BLB light cycle described above.

Molecular phylogenetic analyses

Whole genomic DNA was extracted from the mycelium of each isolate grown on V8 by homogenizing them in a stan-

dard sodium dodecyl sulfate (SDS) detergent lysis buffer, followed by a phenol:chloroform extraction and precipitation in ethanol with sodium acetate (Sambrook et al. 1989). The internal transcribed spacer (ITS) regions and 5.8S rDNA were amplified with polymerase chain reaction (PCR) conditions using a primer pair of ITS1 and ITS4 (White et al. 1990). Purified PCR products were sequenced by ABI PRISM 3100 automated sequencers (Applied Biosystems, Foster City, CA, USA). For phylogenic comparison, the GenBank sequences of four species of *Bipolaris*, nine species of *Cochliobolus*, and *Alternaria alternata* (Fr.) Keissler as an outgroup taxon, were also included in the analysis (Berbee et al. 1999). The DNA sequences were aligned using Clustal X version 1.8 (Thompson et al. 1997). Further visual alignments were done in Sequence Alignment (Se-Al) Editor version 2.0 (Rambaut 2000). Phylogenetic analyses of the data were done by distance methods. The distance matrix for the aligned sequences was calculated using Kimura's two-parameter method (Kimura 1980) and was analyzed with the neighbor-joining (NJ) method (Saitou and Nei 1987) using the program PAUP* 4.0 beta 10 (Swofford 2002). Bootstrap values were generated with 1000 replicate heuristic searches to estimate support for clade stability of the consensus tree using the same program.

Inoculation

The isolates, Zoy-1, Cyn-1, and ATCC26447, were incubated on V8 in 9-cm Petri dishes for 3 days, following which the aerial hyphae were removed using a spatula. The isolates were then incubated under alternating BLB light for 3 days to produce conidia. The conidial suspension was made by pouring distilled water onto the conidiated colony and rubbing the surface with a glass rod. The conidial suspension was adjusted to approximately 10^5 spores/ml with water containing 0.1% wetting agent (Tween 20), and was sprayed on the leaves of *Z. japonica* (cv. unknown) and *C. dactylon* (cv. u2) plants that had been grown from seeds in a greenhouse for about 4 weeks. The inoculated plants were kept in a moist chamber at 25°C for 16h, then transferred to a greenhouse maintained at 25°C. The pathogenicity was checked 10 days after inoculation.

Crossing of isolates

The isolates were tested for production of the teleomorph by crossing them to each other. Two isolates on V8 medium were transferred to either side of a sterilized rice straw in the center of a plate containing Sach's medium (Tsuda and Ueyama 1981). All possible combinations were made among all the isolates. They were incubated at 25°C under an alternating fluorescent light for 4 weeks. The pseudothecia produced on rice straw were checked for the presence of asci and ascospores.

Results

Symptom

The new disease was observed from February in the leaves of *C. dactylon* and from May to June in those of *Z. japonica*, from sports turf and pastures. The lesions were initially reddish-brown, resembled pinholes, and were aligned parallel to the longitudinal leaf axis of leaves. As leaves aged the lesions became brown to dark brown stripes 2–10 × 0.5–2mm in size and were surrounded by a yellow halo (Fig. 1C). In severe infections, many lesions were present on each leaf, resulting in the leaf becoming yellow and later dead. The disease occurred most severely in infrequently mown turfs.

Pathogenicity

Typical brown stripes were reproduced in the leaves of the *Z. japonica* and *C. dactylon* plants 5–7 days after inoculation of the pathogen obtained from these grasses (Table 1, Fig. 1D,E). The disease occurred more severely in *C. dactylon* than in *Z. japonica*. The standard isolate of *B. heveae* also produced brown stripes on *C. dactylon*, although the pathogenicity was weak. This fungus was readily reisolated from infected leaves of inoculated plants.

Morphology of anamorph

The 13 isolates obtained from the diseased samples were similar in morphology and were identified as belonging to the genus *Bipolaris* based on the color and fusoid shape of the conidia, which gradually tapered to each end, with bipolar germination. Conidiophores of the isolates were straight or flexuous, pale to mid-brown, paler toward the apex, smooth, septate, 65–223.2μm long and 5.2–10μm thick (Table 2). Conidia were slightly curved, ellipsoidal, or broadly fusiform, pale to mid-golden-brown or reddish-brown, smooth, 77.5–131.3μm long, 11.3–23.2μm wide, with 6–13 distoseptatae and an inconspicuous scar (see Fig. 1A). The morphology of the isolates from turfs in Japan was similar to both *B. heveae* and *B. stenospila,* which based on their original descriptions are difficult to distinguish (Ellis 1971, 1976; Muchovej and Muchovej 1990). However, our isolates and the standard isolate of *B. heveae* (ATCC26447) were very similar in morphology (see Fig. 1B). The color of the conidia of our isolates and *B. heveae* was usually mid-brown whereas those of *B. stenospila* have been reported to be usually light golden-brown (Drechsler 1928; Edgerton 1955; Farris 1928). We failed to produce conidia of the *B. stenospila* strain (CBS156.36), probably because of the long period of time since it was deposited.

Molecular phylogenetic analyses and identification

Upon comparison with all ITS regions and 5.8S rDNA sequences included in this study and available in databases,

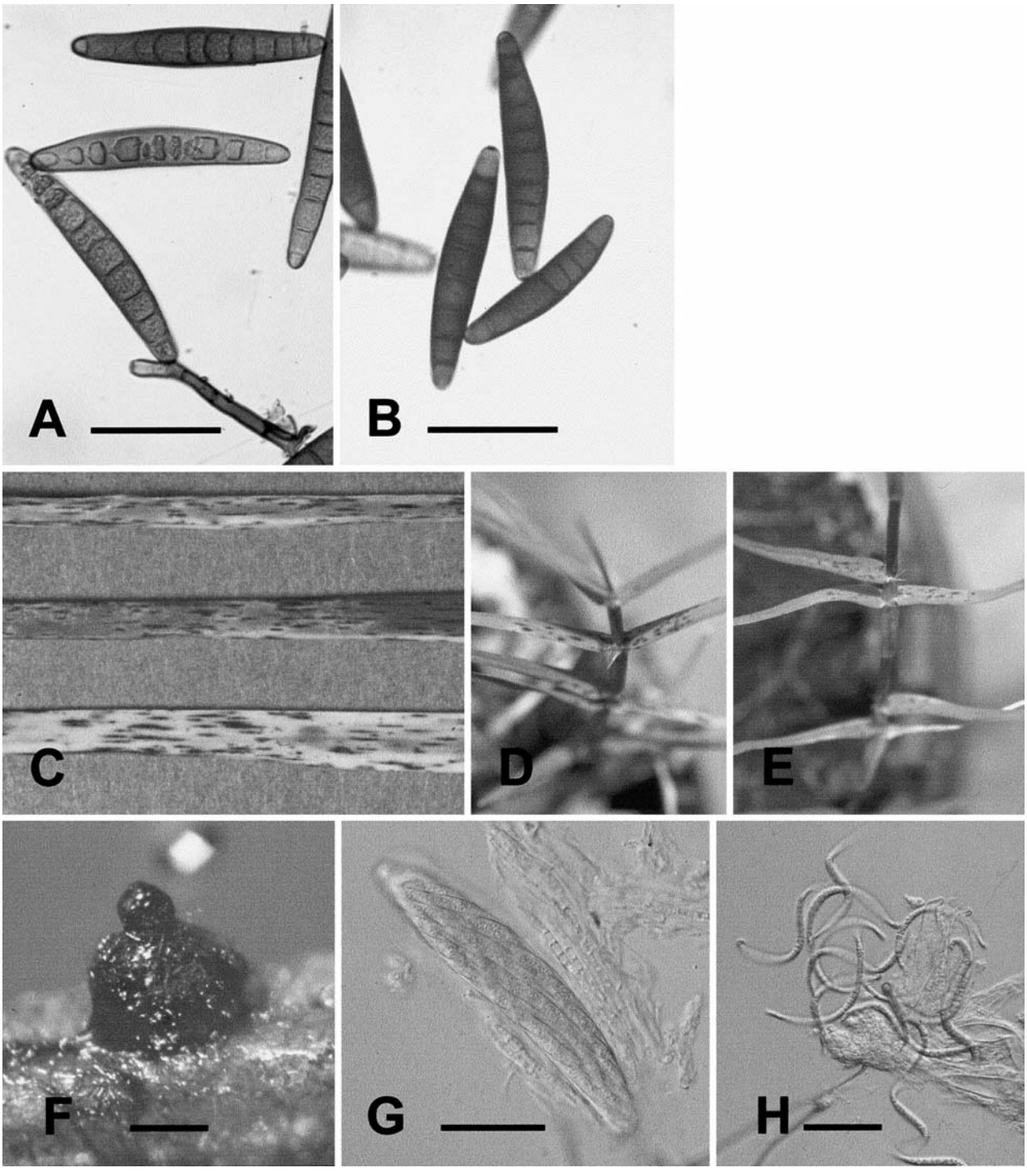

Fig. 1. *Bipolaris heveae*. **A** Conidia from Zoysia grass; **B** conidia of ATCC26447; **C** natural symptoms on Zoysia grass; **D** symptom on bermudagrass inoculated with Cyn-1; **E** symptom on bermudagrass inoculated with ATCC26447; **F** pseudothecium; **G** ascus; **H** Ascospores. *Bars* **A**,**B** 50 μm; **F–H** 100 μm

the Japanese isolates from turfs formed a group with *B. heveae* (Fig. 2). According to the NJ tree, they formed a monophyletic group supported with 100% bootstrap value. In contrast, *B. stenospila* (CBS156.36) was grouped with *B. cynodontis* and was distinctly different from our isolates. The *B. stenospila* isolate ATCC13447 formed a clade with *B. sacchari* and was concluded from the phylogenetic and morphological characteristics to have been misidentified. We thus have identified the Japanese isolates from *Z. japonica* and *C. dactylon* as *B. heveae* based on their morphology of the anamorph and the phylogenic analyses. The sequence data of the Japanese isolates of *B. heveae* and the isolates from ATCC and CBS were deposited in DDBJ with accession numbers AB179831–AB179837.

Table 1. Pathogenicity of the isolates of *Bipolaris heveae* to *Cynodon dactylon* (bermudagrass) and *Zoysia japonica* (Zoysia grass)

Inoculated plants	Isolates		
	Zoy-1	Cyn-1	ATCC26447
C. dactylon	++	++	+
Z. japonica	++	−	−

++, large lesions produced; +, small lesions produced; −, no pathogenicity

Teleomorph

Cochliobolus heveicola Tsukib. & W.H. Chung, sp. nov.

Pseudothecia atro-brunnea vel atra, globosa vel subglobosa, 223–245 μm diametro, 347 ad 415 μm alta, unilocularia, pseudoparaphysibus et ascis impleta. Asci hyalini, cylindrici vel clavati, 158–211 × 29–38 μm, 1–8 ascosporis includentes. Ascosporae hyalinae, filiformes, arcte torsivae, 155–208 × 8–10 μm, 5–9 septatae.

Table 2. Comparison of the morphology of anamorph among the turf isolates *Bipolaris heveae* and *B. stenospila*

	Conidiophore		Conidium			Host
	Color	Size (μm)	Color	Size (μm)	Septa	
Turf isolates	Pale to mid-brown	65–223.2 × 5.2–10	Pale to mid-golden or reddish-brown	77.5–131.3 × 11.3–23.2	6–13	*Cynodon Zoysia*
B. heveae (Ellis 1971)	Pale to mid-brown	Up to 200 × 6–8	Pale to mid-golden or reddish-brown	90–130 × 15–21	6–11	*Hevea*
B. stenospila (Ellis 1976)	Mid to mid-dark brown	Up to 200 × 5–9	Dark olivaceous or golden-brown	70–135 × 14–22	6–14	*Saccharum*

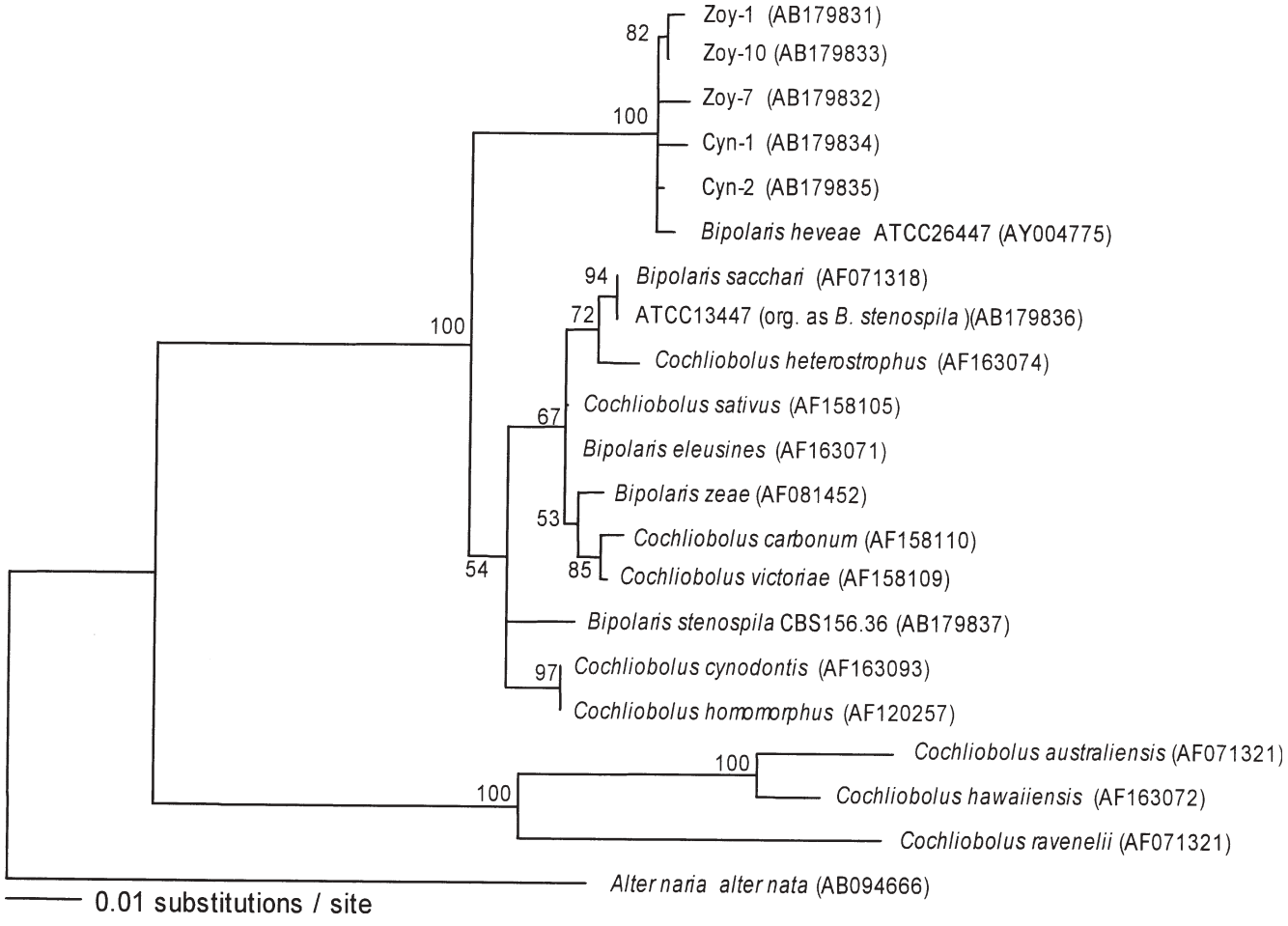

Fig. 2. A neighbor-joining tree inferred from the internal transcribed spacer (ITS) regions and 5.8S rDNA sequences from 15 taxa. The *number in front* of represented isolates shows the bootstrap value in 1000 bootstrap replicates. The accession numbers of rDNA-ITS data in GenBank are shown in *parentheses*. Length of branches is proportional to number of base changes, indicated by the *scale*

Anamorph: *Bipolaris heveae* (Petch) Arx emend. J.J. Muchovej & R.M.C. Muchovej.
Holotypus: Japonia, Tsukuba, cultura sicca, NIAES 20555, T. Tsukiboshi, in Herbario Instituti nationalis scientiae agroenvironmentalis.

Pseudothecia dark brown to black, globose to subglobose, 223–245 μm in diameter, 347–415 μm high, uniloculate and filled with pseudoparaphyses and asci. Asci were hyaline, cylindrical to clavate, 158–211 × 29–38 μm, producing 1–8 ascospores per ascus. Ascospores hyaline, filiform, coiled tightly, 155–208 × 8–10 μm with 5–9 septa.

The teleomorph of *B. heveae* was produced for the first time by crossing the isolates from the turfs. Pseudothecia were produced partly immersed in rice straw tissues with slightly or distinctly protruding ostioles and never on a developed columnar stroma (see Fig. 1F). Filamentous ascospores were coiled tightly in the ascus (Fig. 1G,H). These characteristics indicate it belongs to the genus *Cochliobolus*. It was produced in the crossing combination of the isolates, including Zoy-2 × Zoy-3, Cyn-1 × Zoy-4, and Cyn-2 × Zoy-8. The holotype, NIAES 20555, a dried culture specimen, was obtained from the crossing of Cyn-1 (collected in Okinawa Pref. on Feb. 19, 2002 by T. Tsukiboshi) and Zoy-7 (collected in Tochigi Pref. on June 5, 1995 by T. Tsukiboshi). However, some isolates such as Zoy-1, Zoy-9, and *B. heveae* (ATCC26447) never crossed in any combinations tested. From this crossing study, three isolates were assigned to one mating group and five to a second mating group.

Discussion

B. heveae was first reported as the pathogen of rubber trees (*Ficus elastica* Roxb.), causing minute, orbicular purple spots with a brown border on leaves. In this study, the isolates causing brown stripes on leaves of *Z. japonica* and *C. dactylon* were identified as *B. heveae* based on morphological characteristics and the molecular phylogenetic analysis. This is the first time that the fungus has been reported as a pathogen of gramineous plants. The pathogenicity of the standard isolate of *B. heveae* to these grasses also supported the conclusion, although the pathogenicity was weak. The standard isolate, ATCC26447, of *B. heveae* did not cross with the isolates from grasses the same as Zoy-1 and Zoy-9. It was assumed to be due to the low fertility of these isolates or some incompatible factors for the production of the teleomorph. The fungus is known to be distributed widely on rubber trees in the tropics, but this plant is not present in the areas of Japan where the disease occurred on grasses. The most likely source of the pathogen is seeds and plants of *Z. japonica* and *C. dactylon*, or other gramineous plants, imported into Japan.

Brown stripe of *C. dactylon* is recorded in the United States, and the pathogen has been identified as *B. stenospila* (Freeman 1957). Because the morphology of the causal pathogen is very similar to our isolates and it is difficult to distinguish *B. stenospila* from *B. heveae* based on morphological characteristics as described above, the causal pathogen and our isolates are possibly the same.

Two mating groups were present in the isolates used for the mating study. The mating type, MAT1-1 or MAT1-2 of each compatible group, should be determinable by detecting the specific genomic regions for mating, such as HMG-box (Turgeon et al. 1993).

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