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FULL PAPER

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Morphological and molecular characterization of *Colletotrichum boninense* sp. nov. from Japan

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Abstract Eleven isolates of a species of *Colletotrichum* were collected from eight plant species (*Crinum asiaticum* var. *sinicum*, *Passiflora edulis*, *Cucumis melo*, *Cymbidium* sp., *Clivia miniata*, *Cattleya* sp., *Prunus mume*, and *Dendrobium kingianum*) at six locations on the Pacific Coast of Japan. Although the fungus had been once identified as *Colletotrichum gloeosporioides sensu lato*, it was clearly different from *C. gloeosporioides sensu stricto* in its wide conidia [l/b ratio: (1.8–) 2–3 (–3.3)], having a hilum-like conidial base and cream- to orange-colored colonies on PDA. The intraspecific DNA homologies of the ITS1 sequence were 96.9%–100%, but interspecifically 80.2%–82.3% with *C. gloeosporioides*. Based on the morphological and molecular characterization, the fungus is proposed as a new species, *Colletotrichum boninense*.

Key words *Colletotrichum boninense* · New species · Taxonomy

Introduction

Eleven isolates of a species of *Colletotrichum* Corda, which had hitherto been morphologically included in *C. gloeosporioides* (Penz.) Penz. & Sacc. *sensu lato*, were isolated from eight plant species collected at six locations in Japan. Some features of them, however, were different from those of *C. gloeosporioides sensu stricto*. As a result of taxonomic revision of the species belonging to *Colletotrichum* by von Arx (1957), *C. gloeosporioides* had more than 600 synonyms and contained many morphological and physiological variations. Although some species were separated from *C. gloeosporioides* (van der Aa 1978; Holliday 1980; Sutton 1980; von Arx 1981), Sutton (1992) described *C. gloeosporioides* with seven formae speciales and recognized it as a heterogeneous group with variations in morphological characteristics. In addition, some new species with differences in morphology and/or pathogenicity have hitherto been reported and segregated from *C. gloeosporioides* (Waller et al. 1993; Shivas et al. 1998).

The purpose of this study was to compare these *Colletotrichum* isolates with *C. gloeosporioides* and the other similar *Colletotrichum* species morphologically based on the description by von Arx (1957) and Sutton (1992) and to investigate the molecular characteristics to reveal the taxonomic distinctiveness of the *Colletotrichum* isolates.

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Materials and methods

Fungal isolates

The 11 isolates of a *Colletotrichum* species collected in Japan are shown in Table 1. These isolates were isolated from eight plant species, *Crinum asiaticum* var. *sinicum* L., *Passiflora edulis* Sims, *Cucumis melo* L., *Cymbidium* sp., *Clivia miniata* Regel, *Cattleya* sp., *Prunus mume* Siebold & Zucc., and *Dendrobium kingianum* (Bidwill) Lindl., collected in Tokyo (mainly the Bonin Islands), Kagawa, Kochi, Kagoshima, or Ibaraki Prefecture from 1988 to 1996 (Horie et al. 1990; Sato 1991) and deposited in MAFF (Ministry of Agriculture, Forestry and Fisheries, Japan) Genebank.

Taxonomic characters

Cultural characters had been taken from the culture on potato dextrose agar (PDA; Eiken, Tokyo, Japan) grown under the alternating lighting condition of 12 h black light/12 h dark at 25°C for 7 days. Conidia were produced on PDA plate or hydrangea leaves by the agar-leaf disk method (Kishi 1994). Appressoria were observed in slide

Table 1. Isolates of *Colletotrichum* spp. examined in the present study

Isolates	Host plants	Geographic origin	Year	Refference	DDBJ accession no.
C. boninense					
MAFF 305972	*Crinum asiaticum* var. *sinicum*	Bonin islands	1988	Horie et al. 1990	AB051400
MAFF 305973	*Passiflora edulis*	Bonin islands	1989	Horie et al. 1990	AB051401
MAFF 305998	*Cucumis melo*	Bonin islands	1988	Horie et al. 1990	AB051402
MAFF 306094	*Crinum asiaticum* var. *sincium*	Bonin islands	1990	Sato 1991	AB051403
MAFF 306100	*Cymbidium* sp.	Ibaraki Pref.	1989		AB042313
MAFF 306162	*Crinum asiaticum* var. *sinicum*	Kagoshima Pref.	1991	Sato 1991	AB051406
MAFF 306204	*Clivia miniata*	Tokyo Met.	1991		AB051404
MAFF 306205	*Clivia miniata*	Tokyo Met.	1991		AB051405
MAFF 238641	*Cattleya* sp.	Kagawa Pref.	1995		AB087213
MAFF 238656	*Prunus mume*	Tokyo Met.	1996		AB087214
MAFF 238642	*Dendrobium kingianum*	Kochi Pref.	1996		AB087215
C. gloeosporioides					
MAFF 305752	*Passiflora edulis*	Bonin islands	1988	Horie et al. 1990	AB087216
MAFF 305913	*Fragaria* × *ananassa*	Tochigi Pref.	1987	Ishikawa et al. 1989	AB042315
MAFF 306173	*Carica papaya*	Okinawa Pref.	1991		AB087217
MAFF 306439	*Diospyros kaki*	Fukuoka Pref.	1993		AB087218
MAFF 306553	*Fagopyrum esculentum*	Ibaraki Pref.	1998	Moriwaki and Tsukiboshi 1999	AB087219
C. musae					
MAFF 305595	*Musa sapientum*	Bonin islands	1987		AB087220
C. fragariae					
MAFF 744017	*Fragaria* × *ananassa*	Fukuoka Pref.	1981		AB087221
C. lindemuthianum					
MAFF 305390	*Phaseolus vulgaris*	Tochigi Pref.	1974		AB087222
C. orbiculare					
MAFF 306518	*Cucumis melo*	Miyagi Pref.	1997	Kanno and Moriwaki 2000	AB042308
C. trifolii					
MAFF 510487	*Medicago sativa*	Tochigi Pref.	1972		AB087223

MAFF, Genebank, Ministry of Agriculture, Forestry and Fisheries, Japan

cultures on potato carrot agar (PCA) under the same conditions (Sutton 1980). Length and width of each 50 conidia and appressoria were measured for every isolate. Colony diameters on PDA were measured for calculating mycelial growth rate after culturing for 4–7 days at 10°, 17°, 22°, 25°, 28°, 33°, and 36°C. An isolate of *C. gloeosporioides* (MAFF 306553 isolated from *Fagopyrum*) was also examined for comparison.

PCR amplification and sequencing

The regions of ribosomal DNA (rDNA) were amplified with polymerase chain reaction (PCR) conditions using ITS5 and ITS4 primers (White et al. 1990). DNA sequencing of the PCR products were obtained for both strands using direct sequencing in an ABI PRISM 377 sequencer (Applied Biosystems, Foster City, CA, USA). The sequence reactions were conducted using the BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems) following the manufacturer's protocol. Two primers, ITS1 and ITS2 (White et al. 1990), were used for the sequencing in both directions.

Phylogenetic analysis

For phylogenetic analysis, the ITS1 region was sequenced (see Table 1). *Colletotrichum orbiculare* (Berk. & Mont.) von Arx, *C. trifolii* Bain & Essary and *C. lindemuthianum* (Sacc. & Magnus) Briosi & Cav. were included as an outgroup for comparison. Multiple sequence alignment of the data was initially carried out using the alignment subroutines on CLUSTAL X (Thompson et al. 1997). The alignment of all sequences was checked visually. Phylogenetic trees were obtained from the data by distance and parsimony methods. A tree showing the phylogenetic relatedness between the isolates was constructed from distance matrix values by the neighbor-joining (NJ) method (Saitou and Nei 1987), using CLUSTAL X. The distances in the ITS1 region were determined by Kimura's two-parameter model (Kimura 1980). Sites where gaps existed in any of the sequences were excluded. A bootstrap analysis using 10000 resamples of the sequence data was carried out (Felsenstein 1985). For parsimony analysis, the PAUP program version 4b10 (Swofford 2001) was used, and heuristic search was performed with 100 repeats of random addition sequences in Stepwise-Addition Options and TBR swapping algorithm in Branch-Swapping Options. Confidence limits for the branches based on parsimony criteria were estimated by bootstrap analysis of 1000 replicates.

Results and discussion

Taxonomic description

Colletotrichum boninense J. Moriwaki, Toy. Sato & T. Tsukiboshi, sp. nov. Figs. 1–7

Coloniae in PDA, cremeae vel aurantiacae, pannosae cum mycelio aerio, raro setosae; reversum cremeum vel persicinum. Sclerotia absentia. Appressoria sepiacea vel fusco-brunneo, margine irregulari, solitaria vel in catenulis efformata, (6–) 8–12.5 (–17) × (4–) 5.5–9 (–15) μm. Conidia in massa aurantiaca, recta, cylindrica, utrinque obtusa, basi protuberatione hilo simili praedita, (11.5–) 13–15.5 (–17) × (4–) 5–6 (–7) μm.

Habitat: in foliis *Crini asiatici* var. *sinici*, *Passiflorae edulis*, *Cucumeris melo*, *Cymbidii* sp., *Cliviae miniatae*, *Cattleyae* sp., *Pruni mume* et *Dendrobii kingiani*.

Holotypus: NIAES 20520, cultura sicca (MAFF 305972), isolata e foliis morbo affectis *Crini asiatici* var. *sinici*, Bonin insulae in Japonia, 1988, T. Sato, in Herbario Instituti Nationalis Agro-Environmentalis Scientiae, Tsukuba, Japonia. Paratypus: NIAES 20521, cultura sicca (MAFF 306094), isolata e foliis morbo affectis *Crini asiatici* var. *sinici*, Bonin insulae in Japonia, 1990, T. Sato, in Herbario NIAES, Tsukuba, Japonia.

Colonies on PDA cream to orange, felted with aerial mycelium, reverse cream to pink. Setae rare. Sclerotia absent. Appressoria sepia to dark brown, edge very irregular, formed solitaly or catenate, (6–) 8–12.5 (–17) × (4–) 5.5–9 (–15) μm. Conidia formed in orange masses, straight, cylindrical, obtuse at both ends, with a hilum-like low protuberance at the base, (11.5–) 13–15.5 (–17) × (4–) 5–6 (–7) μm in size.

On and from *Crinum asiaticum* var. *sinicum*, *Passiflora edulis*, *Cucumis melo*, *Cymbidium* sp., *Clivia miniata*, *Cattleya* sp., *Prunus mume*, and *Dendrobium kingianum*.

Etymology: boninense, referring the geographic origin, the Bonin Islands, where the fungus was first found.

Holotype: NIAES 20520, a dried culture (MAFF 305972), isolated from a diseased leaf of *Crinum asiaticum* var. *sinicum*, Bonin Islands, Japan, 1988, T. Sato, deposited in the herbarium of NIAES (National Institute for Agro-Environmental Sciences, Tsukuba, Ibaraki), Japan.

Paratype: NIAES 20521, a dried culture (MAFF 306094), isolated from a diseased leaf of *Crinum asiaticum* var. *sinicum*, Bonin Islands, Japan, 1990, T. Sato, deposited in the herbarium of NIAES, Japan.

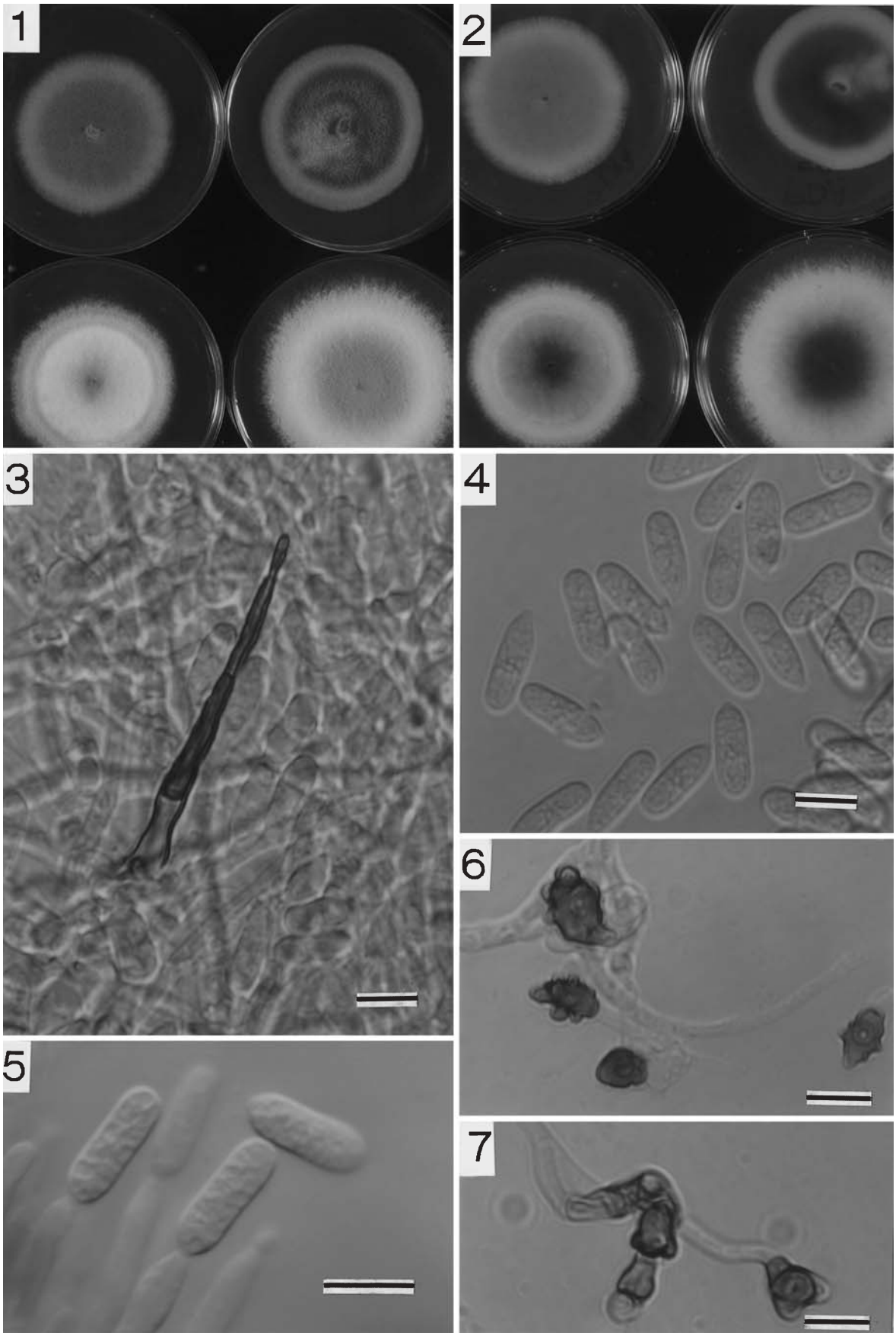
Isolates examined: MAFF 305972 from *Crinum asiaticum* var. *sinicum*, Bonin Islands, Japan, 1988, T. Sato (holotype, NIAES 20520); MAFF 306094 from *Crinum asiaticum* var. *sinicum*, Bonin Islands, Japan, 1990, T. Sato (paratype, NIAES 20521); MAFF 306162 from *Crinum asiaticum* var. *sinicum*, Kagoshima Pref., Japan, 1991, T. Sato; MAFF 305998 from *Cucumis melo*, Bonin Islands, Japan, 1988, T. Sato; MAFF 305973 from *Passiflora edulis*, Bonin Islands, Japan, 1988, T. Sato; MAFF 306100 from *Cymbidium* sp. Ibaraki Pref., Japan, 1989, T. Sato; MAFF 306204 from *Clivia miniata*, Tokyo Met., Japan, 1991, T. Sato; MAFF 306205 from *Clivia miniata*, Tokyo Met., Japan, 1991, T. Sato.

Colletotrichum boninense is characterized by its cylindrical and rather broad conidia with an obtuse apex and protruding base (Figs. 4, 5). Conidial length : breadth (l/b) ratio was (1.8–) 2–3 (–3.3) (Table 2). Appressoria produced on

Table 2. Comparison of morphological and cultural characteristics of *Colletotrichum boninense* with *C. gloeosporioides*

Species and reference	Conidium (size, μm)	Length : breadth ratio	Appressorium (size, μm)	Colony characteristic	Mycerial growth rate (optimum temperature)
C. boninense					
This study	Cylindrical, base with a scarlike hilum, (11.5–) 13–15.5 (–17) × (4–) 5–6 (–7)	(1.8–) 2–3 (–3.3) mean, 2.5	Irregular in shape, sepia to dark brown, (6–) 8–12.5 (–17) × (4–) 5.5–9 (–15)	White aerial mycelium, reverse cream to orange	5.2–6.1 mm/days (25°C)
C. gloeosporioides					
Sutton 1992	Cylindrical, base truncate, 12–17 × 3.5–6	–	Clavate, ovate, obovate, sometimes lobed, sepia brown, 6–20 × 4–12	Grayish white to dark gray, aerial mycelium, reverse unevenly white to gray or darker with age variable	–
Arx 1970	Cylindrical, ellipsoid, base truncate, 12–21 × 3.5–6	–	–		
MAFF 306553	(11–) 12.5–16 (–17.5) × 3–4.5 (–5.5)	(2.4–) 2.8–4.2 (–5) mean, 3.5			6–9 mm/days (28°C)

–, Not described


1
2
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6
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7

PCA slide culture are irregular in shape, sepia to dark brown, formed solitary or catenate, and smaller size (Figs. 6, 7; Table 2) and are quite different from those of *C. gloeosporioides*. Setae are rarely produced in acervuli and only on the isolate, MAFF 305973, produced setae of 2–3 (–5) cells and 38.4–76.7 × 3.3–6.0 μm in size on PDA in the dark (Fig. 3). Colonies on PDA are cream to orange with orange conidial masses, felty with white aerial hyphae, and reverse is cream to pink (Fig. 1). They lack sclerotia on PDA under the alternating lighting condition. *Colletotrichum boninense* (MAFF 305972, 305973, 305998, 306094, 306100, and 306205) grows at 10°–36°C with an optimum at 25°C (Fig. 8, Table 2), and the mycelial growth rate is 5.2–6.1 mm/day at 25°C. On the other hand, *C. gloeosporioides* (MAFF 306553) grows at an optimum of 28°C with a mycelial growth rate of 10.3 mm/day.

Colletotrichum boninense preliminarily fell within the broad species concept of *C. gloeosporioides sensu lato* (von Arx 1957; Sutton 1980), but differed from *C. gloeosporioides sensu stricto* in morphological features. It can be distinguished from *C. gloeosporioides* by its colony morphology, shape, and l/b ratio of conidia. Colonies of *C. boninense* were cream to orange, covered with coalescing orange conidial masses, in comparison with those of *C. gloeosporioides*, which were grayish-white to dark gray. Although the size of conidia of *C. boninense* was in the range of (11.5–) 13–15.5 (–17) × (4–) 5–6 (–7) μm and overlapped the size range of *C. gloeosporioides* (12–17 × 3.5–6 μm; Sutton 1992), the conidia had a protuberance at the base and were generally shorter and wider than those of *C. gloeosporioides*, resulting in a l/b ratio of (1.8–) 2–3 (–3.3). Sato (1997) reported that three isolates of *C. boninense* [MAFF 238656 (Apr1-1), 305972, and 305998] were slightly more tolerant to benomyl than *C. gloeosporioides*, but more susceptible to it than *C. acutatum*, and they were tolerant to diethofencarb as well as both species. *Colletotrichum boninense* was different in morphology from *C. kahawae* (Waller et al. 1993) and *C. xanthorrhoeae* (Shivas et al. 1998), distinguished from other *Colletotrichum* species with straight spores. The breadth of conidia in *C. boninense* was (4–) 5–6 (–7) μm, broader than those of *C. kahawae* (4 μm) or of *C. xanthorrohoeae* (3–5 μm).

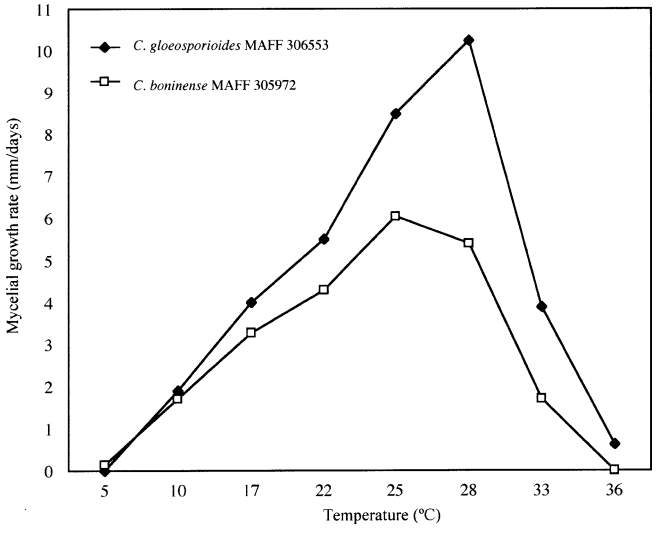

Fig. 8. Mycelial growth rates of *C. boninense* (MAFF 305972), and *C. gloeosporioides* (MAFF 306553) at different temperatures on PDA

Molecular characterization and phylogeny

The ITS1 sequences of *C. boninense* were analyzed phylogenetically with those of other *Colletotrichum* species (Table 1). The ITS1 region of *C. boninense* had 190 bp with 0%–3.1% divergences among 11 isolates, whereas that of *C. gloeosporioides* had 171 bp with 0%–3.5% divergences among 5 isolates. On the other hand, the internal divergences between *C. boninense* and *C. gloeosporioides* were 17.7%–19.8% (data not shown). Cannon et al. (2000) pointed out that ITS1 sequence divergence between individual pairs of *Colletotrichum* strains showed a bimodal frequency distribution, with one peak between 0% and 5% and the other between 7% and 23% divergence, and that the former peak appeared to correspond to infraspecific variation and the latter to interspecific divergence. Consequently, the differences between *C. boninense* and *C. gloeosporioides* should reflect interspecific ones. The sequence data of *C. boninense* were deposited in DDBJ with accession numbers AB042313, AB051400–AB051406, and AB087213–AB087215 (see Table 1).

Neighbor-joining (NJ) and most parsimonious (MP) trees were constructed by using the ITS1 sequences, and almost the same topologies were obtained in both methods. MP analysis generated 18 MP trees with 53 steps, and 50% majority-rule consensus tree showed the isolates of *C. boninense* as a monophyletic lineage with a bootstrap value of 95% (group A in Fig. 9). On the other hand the isolates of *C. gloeosporioides*, *C. musae*, and *C. fragariae* made another clade with high bootstrap supports (97%, group B in Fig. 9). The tree made by the NJ method also indicated the monophyly of groups A and B. *Colletotrichum orbiculare*, *C. trifolii*, and *C. lindemuthianum* made a different clade far from group A and B in both analyzing methods. Molecular phylogenetic analyses on ITS1 sequences clearly distinguished *C. boninense* from *C. gloeosporioides* as well as

Figs. 1, 2. Colonies formed on potato dextrosc agar (PDA) plates at 25°C under black light for a week. Colony surface (**1**) and reverse (**2**) of *Colleotrichum boninense* MAFF 305973 and 306100 (*upper left to right*) and *C. gloeosporioides* MAFF 305913 and 306009 (*lower left to right*)

Fig. 3. A seta of *C. boninense* (MAFF 305973) formed on PDA slant under the dark. *Bar* 10 μm

Fig. 4. Conidia of *C. boninense* (MAFF 305972) formed on potato carrot agar (PCA) slide culture at 25°C under black light for a week. *Bar* 10 μm

Fig. 5. Conidiogenesis of *C. boninense* (MAFF 306100) on a hydrangea leaf at 25°C under black light for a week. *Bar* 10 μm

Fig. 6, 7. Appressoria of *C. boninense*, MAFF 305972 (**6**) and MAFF 306100 (**7**), formed on PCA slide culture at 25°C under black light for a week. *Bar* 10 μm

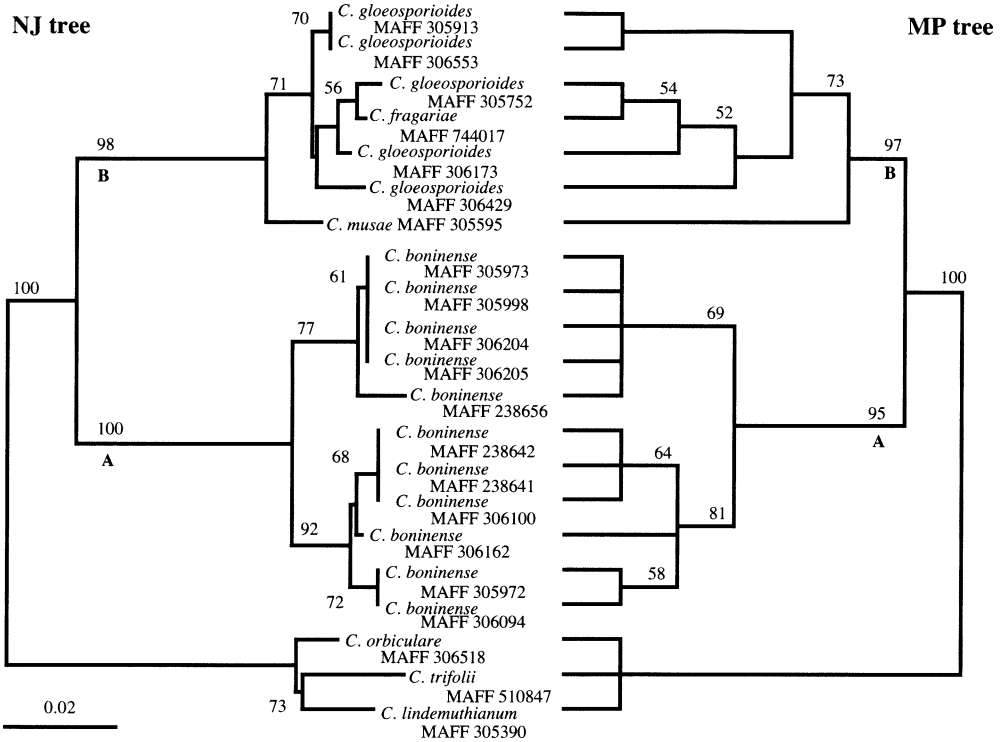

Fig. 9. Tree illustrating relatedness of *C. boninense* and *C. gloeopsporioides*, based on neighbor-joining (*NJ*) and most parsimonius (*MP*) analysis of the ITS1 regions. For NJ tree, distances were determined by Kimura's two-parameter method. *Bar* indicates a distance of 0.02. In MP analysis, 18 trees had a tree length of 53 steps, and the 50% majority-rule consensus tree was reconstructed. In both trees, *numbers* beside the branches are the bootstrap values indicating the frequencies with which a given branch appeared in 10 000 (NJ) or 1000 (MP) replications. Bootstrap values greater than 50% are shown. *Colletotrichum orbiculare*, *C. trifolii*, and *C. lindemuthianum* were used as outgroups

other similar *Colletotrichum* species such as *C. musae* (Berk. & M.A. Curtis) von Arx and *C. fragariae* Brooks.

Morphological distinctiveness and monophyly based on molecular phylogenetic analyses of ITS1 sequences showed the taxonomic individuality of *C. boninense*. *Colletotrichum boninense* was hitherto found to inhabit a wide range of host plants such as *Crinum*, *Clivia*, and *Cymbidium* and to be distributed on the Pacific Coast of Japan. Further studies on the host range and geographical distribution are necessary to clarify the ecological niche of the fungus.

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