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FULL-PAPER

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Colletotrichum echinochloae, a new species on Japanese barnyard millet (*Echinochloa utilis*)

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Abstract Five isolates of a species of *Colletotrichum* were collected from Japanese barnyard millet (*Echinochloa utilis*) in Japan. Although the fungus had once been identified as *C. graminicola* sensu lato, it was clearly different from *C. graminicola* isolated from maize (*Zea mays*) in its falcate and short conidia, 18.0–22.2 μm in length, cultural characteristics, and specific pathogenicity to *E. utilis*. Moreover, molecular phylogenetic analyses using sequences of rDNA-ITS, HMG, and SOD2 indicated a monophyly of the isolates. A new species, *Colletotrichum echinochloae*, is then proposed based on the morphological, pathological, and molecular characteristics.

Key words *Colletotrichum echinochloae* · *Echinochloa utilis* · New species

Introduction

Colletotrichum graminicola (Ces.) G. W. Wilson was originally described as *Dicladium graminicola* Ces. from *Echinochloa crus-galli* (L.) Beauv. and *Zea mays* L. (Cesati 1852). von Arx (1957) included many species infecting gramineous plants, causing anthracnose disease, in *C. graminicola*. As a result of the taxonomic revision of the species described above, *C. graminicola* had 35 synonyms, as expanded in Wilson's opinion (Wilson 1914), and contained many morphological and physiological variations (von Arx 1957). Sutton (1980, 1992) recognized *C. graminicola* as a heterogeneous group with variations in morphological characteristics, and separated three graminicolous *Colletotrichum* species, namely, *C. caudatum* (Sacc.) Peck producing unique conidia, *C. falcatum* Went infecting *Saccharum*, and *C. sublineolum* Henn. infecting *Sorghum*, on the basis of reports by Mordue (1967), Nag Raj (1973), Holliday (1980, 1989), von Arx (1981), Baxter et al. (1983), and Baxter and van der Westhuizen (1984). In addition, *C. cereale* Manns from Pooideae grasses was also separated from *C. graminicola* based on its specific pathogenicity and nucleotide data of the ribosomal DNA and internal transcribed spacer region (rDNA-ITS), the high mobility group-box region of the *MAT1-2* mating-type locus (HMG), and the manganese-type superoxide dismutase gene (SOD2) (Crouch et al. 2006).

Concerning relationships of *Colletotrichum* species, there are many reports using phylogenetic analyses based on the rDNA-ITS region (Sherriff et al. 1994; Sreenivasaprasad et al. 1996; Johnston and Jones 1997; Moriwaki et al. 2002). These topologies reflect morphological classification but possess lower statistical reliability. Du et al. (2005) used HMG sequences, and the results achieved through analysis of HMG sequences correlated well with those obtained by analysis rDNA-ITS sequences but provided significantly better phylogenetic resolution.

Sutton (1966) reported the potential lectotype material of *C. graminicola* to be probably *Z. mays*. Sutton (1980) proposed that the name *C. graminicola* should be strictly applied to the fungus on *Z. mays*, and if other graminicolous collections were found to differ, it was necessary to apply different names to them. On the other hand, we tried to group Japanese *Colletotrichum* species based on rDNA-ITS1 sequences (Moriwaki et al. 2002). The isolate of *C. graminicola* from *Z. mays* was different from the isolate from *E. utilis* Ohwi & Yabuno. Moreover, some *Colletotrichum* isolates collected from *E. utilis* in Japan had features quite different from those of *C. graminicola* sensu Sutton (1980). The purpose of this study is to reveal the taxonomic status within the graminicolous *Colletotrichum* species containing *C. graminicola* from *Z. mays* from the aspects of morphology, pathogenicity, and molecular characteristics.

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Materials and methods

Fungal isolates

Three conidial isolates of *Colletotrichum* species were obtained from anthracnose-diseased Japanese barnyard millet (*Echinochloa utilis*) collected in a field in Nasushiobara-shi, Tochigi Prefecture, Japan, in September 2007 (Table 1). Symptomatic lesions on the plants, which were observed from August to October 2007, were yellowish brown to brown, spindle- or irregularly shaped with black acervuli inside that were produced in the midrib or edge of the leaves (Fig. 1A). Monoconidial isolation was done on agar-leaf piece media using the diseased leaves of Japanese barnyard millet (Furukawa and Kishi 2002; Sato and Moriwaki 2003). A germinated conidium was transferred onto modified Weitzman and Silva-Hunter (mWSH) agar (Weitzman and Silva-Hunter 1967; Hosoya and Otani 1997) for each isolate. The isolates were maintained on mWSH slants and deposited in National Institute of Agrobiological Sciences (NIAS) Genebank (MAFF) in Japan as MAFF 511471 to 511473. Two isolates of *C. graminicola* sensu lato established by N. Nishihara from Japanese barnyard millet were also used: MAFF 511152 from Kochi Prefecture in 1977 and 511328 from Tochigi Prefecture in 1980. Isolates of *C. graminicola* (ATCC 26416; from *Zea mays*), *C. sublineolum* (MAFF 511474; from *Sorghum bicolor* Moench), *C. falcatum* (MAFF 306170; from *Saccharum officinarum* L.), *C. caudatum* (MAFF 238574; from *Zoysia pacifica* (Goudsw.) M. Hotta & Kuroki), and *C. gloeosporioides* (Penz.) Penz. & Sacc. (MAFF 239930 and 239933; from *Litchi chinensis* Sonn.) were also examined for comparison.

Cultural characters and inoculation test

Cultural characters and conidial morphologies were observed in a culture grown on potato dextrose agar (PDA; Difco-BD Diagnostics, Sparks, MD, USA) under an alternating lighting conditions of 12 h black light/12 h dark at 25°C for 7–11 days. Appressoria were observed in slide cultures on potato carrot agar (PCA; Sato and Moriwaki 2003) following the same conditions as reported by Sutton (1980). The length and width of 100 conidia and appressoria were measured for every isolate. The *Colletotrichum* isolates, MAFF 511471 to 511473, were cultured on PDA at 5°, 10°, 15°, 20°, 23°, 25°, 28°, 30°, 35°, or 40°C in the dark for 6 days to estimate their mycelial growth.

Inoculation tests were performed to check pathogenicity. *Colletotrichum* isolates (MAFF 511471 to 511473) from *E. utilis*, *C. graminicola* ATCC 26416, and *C. sublineolum* MAFF 511474 were used. Conidial inocula were prepared by culturing the isolates on PDA under alternating lighting conditions of 12 h black light/12 h dark at 25°C for 7 days. Suspensions of the conidia at 10^5 conidia/ml were sprayed on whole plants of *E. utilis* (cv. Green millet), *Z. mays* (cv. Pioneer 115), and *S. bicolor* (cv. Hazuki) of the third- to fifth-leaf stages. Two pots of three plants each were inoculated with each fungal isolate. Three healthy plants were simultaneously sprayed with sterilized distilled water without conidia to serve as controls. The inoculated plants were kept under high humidity in the dark at 25°C for 2 days, then at 15°–25°C for 14–16 days in a greenhouse. Disease symptoms were recorded. The experiments were carried out twice. Reisolations were made from diseased tissue. The diseased leaves were incubated in moist plastic boxes under alternating lighting conditions of 12 h black light/12 h dark at 25°C for 7 days. The same fungus

Table 1. Isolates of *Colletotrichum* spp. examined in this study

Isolate	Host plant	Geographic origin	Year	DDBJ accession no.		
				ITS	HMG	SOD2
C. echinochloae						
MAFF 511152	*Echinochloa utilis*	Nankoku, Kochi Pref., Japan	1977	AB439807	AB439816	AB440149
MAFF 511328	*E. utilis*	Nasushiobara, Tochigi Pref., Japan	1980	AB439808	AB439817	AB440150
MAFF 511471	*E. utilis*	Nasushiobara, Tochigi Pref., Japan	2007	AB439809	AB439818	AB440151
MAFF 511472	*E. utilis*	Nasushiobara, Tochigi Pref., Japan	2007	AB439810	AB439819	AB440152
MAFF 511473[a]	*E. utilis*	Nasushiobara, Tochigi Pref., Japan	2007	AB439811	AB439820	AB440153
C. graminicola						
ATCC 26416	*Zea mays*	Kentucky, USA	ND	AB439812	AB439821	AB440154
C. sublineolum						
MAFF 511474	*Sorghum bicolor*	Nasushiobara, Tochigi Pref., Japan	2000	AB439813	AB439822	AB440155
C. falcatum						
MAFF 306170	*Saccharum officinarum*	Kagoshima, Kagoshima Pref., Japan	1991	AB462376	AB462378	AB462380
C. caudatum						
MAFF 238574	*Zoysia pacifica*	Miki, Hyogo Pref., Japan	1998	AB462377	AB462379	AB462381
C. gloeosporioides						
MAFF 239930	*Litchi chinensis*	Ishigaki, Okinawa Pref., Japan	2000	AB439814	AB439823	AB440156
MAFF 239933	*L. chinensis*	Okinawa Pref., Japan	2000	AB439815	AB439824	AB440157

ITS, internal transcribed spacer region; HMG, high mobility group-box region of the *MAT1-2* mating-type locus; SOD2, manganese-type superoxide dismutase gene (SOD2); ND, not determined

[a] Ex-holotype

was consistently reisolated from the inoculated, diseased plants.

Polymerase chain reaction (PCR) and sequencing of DNA

Total DNA was extracted following the protocol of Moriwaki et al. (2002). Regions of rDNA-ITS, HMG, and SOD2 were amplified by PCR using the following primer pairs: ITS5 and ITS4 (White et al. 1990), CgHMG-1 and -2 (Chen et al. 2002), and SOD 625-F and -R (Crouch et al. 2006). PCR conditions followed the methods reported by the respective sources for each primer set. For amplification of the SOD2 region of *C. gloeosporioides*, SODglo2-F (5′-CAG ATC ATG GAG CTG CAC CA-3′) and -R (5′-TAG TAC GCG TGC TCG GAC AT-3′) primers were newly designed in this study based on the sequence of *C. gloeosporioides* reported by Barhoom and Sharon (2007). The cycle parameters with these primers were an initial denaturation at 95°C for 4 min; followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 2 min, and a final extension for 5 min at 72°C. DNA sequences of the PCR products were analyzed for both strands using the same primers for the PCR reactions for direct sequencing in a CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA). The sequence reactions were conducted using the DTCS Quick Start Kit (Beckman Coulter) following the manufacturer's protocol. The sequence data were deposited in DDBJ with accession numbers AB439807 to AB439824, AB440149 to AB440157, and AB462376 to AB462381 (see Table 1).

Phylogenetic analysis

For phylogenetic analysis, the sequence data of the rDNA-ITS, HMG, and SOD2 regions of *C. graminicola* (MO-100178; epitype), *C. sublineolum* (S3.001; epitype), and *C. cereale* [NJ-4990 (epitype) and NJ-6795] were also included as references (Crouch et al. 2006). *Colletotrichum gloeosporioides* (MAFF 239930 and MAFF 239933) was used as an outgroup for comparison. A multiple sequence alignment of rDNA-ITS, HMG, and SOD2 was initially carried out using the alignment subroutines on CLUSTAL X (Thompson et al. 1997). All sequence alignments were checked visually, and the alignments were deposited in TreeBASE (SN4130). Phylogenetic trees were obtained by distance and parsimony methods. A phylogenetic tree for the isolates was constructed from distance matrix values by the neighbor-joining (NJ) method (Saitou and Nei 1987) using CLUSTAL X. The distances 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 1000 resamples of the sequence data was carried out (Felsenstein 1985). For maximum-parsimony (MP) analysis, the PAUP program version 4b10 (Swofford 2002) was used, and a heuristic search was performed with 100 repeats of random addition sequences in stepwise-addition options and the tree-bisection-reconnection (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

Colletotrichum echinochloae Moriwaki & Tsukib. sp. nov. Fig. 1B–G

Coloniae in agaro decocto tuberorum, hyalinae, acervulis nigris formantes, cum massis conidicis auranticis, interdum pannosae cum mycelio aerio. Setae abundantes in acervulo, piceae, 79.8–145.5(–186.3) µm. Sclerotia in cultura absentia. Appressoria sepiacea vel fusco-brunnea, clavata, ovata, globosa, lobulata, (8–)10.2–14.7(–20.3) × (6–)7.5–10.5(–12.7) µm. Conidia, hyalina, falcata, apices valde curvata, (14.0–)18.0–22.2(–24.0) × (3.5–)4.2–5.4(–6.7) µm.

Habitat: In foliis *Echinochloae utilis* Ohwi & Yabuno.

Holotypus: NIAES 20584 (ut cultura sicca), isolata e foliis morbidis *Echinochloae utilis*, Senbonmatsu, Nasushiobara-shi, Tochigi Pref., Japonia, 17 Sep. 2007, T. Tsukiboshi, in Herbario Instituti Nationalis Agro-Environmentalis Scientiae, Tsukuba, Japonia conservata.

Colonies on PDA hyaline, formed black acervuli with orange conidial masses, sometimes felted with aerial mycelium. Setae abundant in acervulus, black, 2–5 septated, 79.8–145.5(–186.3) µm. Sclerotia on culture absent. Appressoria sepia to dark brown, clavate, ovate, globose, slightly lobed, (8–)10.2–14.7(–20.3) × (6–)7.5–10.5(–12.7) µm. Conidia hyaline (orange in mass), falcate, apex strongly curved, (14.0–)18.0–22.2(–24.0) × (3.5–)4.2–5.4(–6.7) µm.

Found: On and from *Echinochloa utilis* Ohwi & Yabuno.

Etymology: Echinochloae, referring to the biological origin, *Echinochloa utilis*.

Isolates examined: MAFF 511152 isolated from *E. utilis*, Nankoku-shi, Kochi Pref., Japan, 1977, collected by N. Nishihara; MAFF511328, isolated from *E. utilis*, Senbonmatsu, Nasushiobara-shi, Tochigi Pref., Japan, 1980, collected by N. Nishihara; MAFF 511471, isolated from *E. utilis*, Senbonmatsu, Nasushiobara-shi, Tochigi Pref., Japan, 17 Sep. 2007, collected by T. Tsukiboshi; MAFF511472, isolated from *E. utilis*, Senbonmatsu, Nasushiobara-shi, Tochigi Pref., Japan, 17 Sep. 2007, collected by T. Tsukiboshi; MAFF 511473 (ex-holotype), isolated from a diseased leaf of *E. utilis*, Senbonmatsu, Nasushiobara-shi, Tochigi Pref., Japan, 17 Sep. 2007, collected by T. Tsukiboshi.

Colletotrichum echinochloae is characterized by its falcate and rather short conidia (18.0–22.2 × 4.2–5.4 µm) (Fig. 1D–G). As shown in Table 2, these conidial characters are quite different from those of *C. graminicola*, *C. sublineolum*, *C. falcatum*, *C. caudatum*, and *C. cereale*. The length : breadth (l/b) ratio of *C. echinochloae*, 3.5–5.0, is also different from that of *C. graminicola*. Appressoria on PCA slide culture are clavate, ovate, globose, and slightly

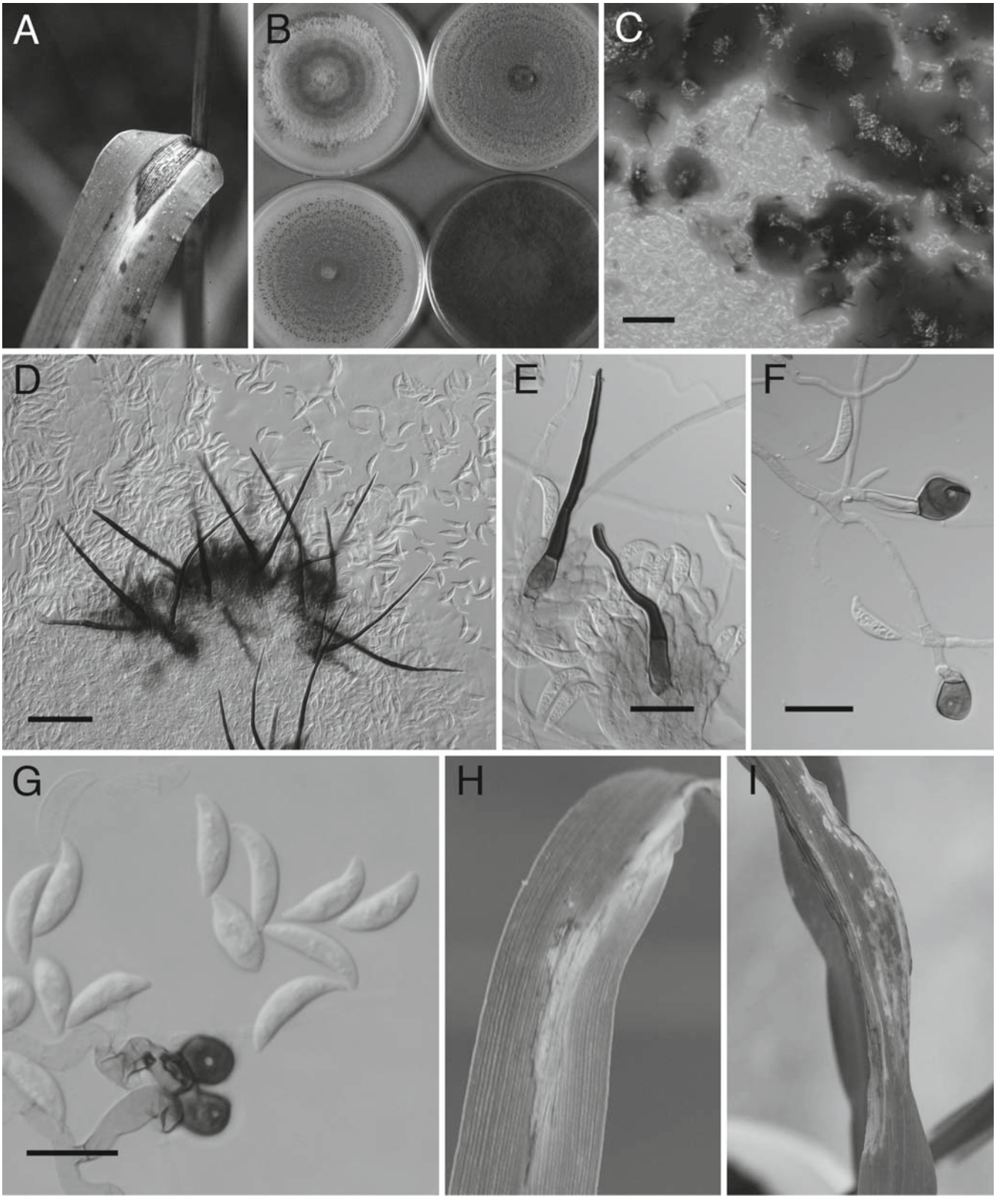

Fig. 1. Disease symptom on *Echinochloa utilis* and morphologies of *Colletotrichum echinochloae*. **A** Natural anthracnose symptom on a leaf of *E. utilis*. **B** Colony surface 11 days after incubation on potato dextrose agar (PDA) under black light with a 12-h photoperiod at 25°C for *C. echinochloae* MAFF 511471, 511472 (*upper, left to right*), 511473 (ex-holotype), and *C. graminicola* ATCC 26416 (*lower, left to right*). **C** Conidial masses on setose acervuli of MAFF 511473 on PDA under black light with a 12-h photoperiod at 25°C. **D, E** Acervuli with brown setae and falcate conidia of MAFF 511473 on PDA under black light with a 12-h photoperiod at 25°C (**D**) and 511152 on potato carrot agar (PCA) (**E**). **F, G** Appressoria and conidia of MAFF 511152 (**F**) and 511473 (**G**) formed on a PCA slide culture under black light with a 12-h photoperiod at 25°C. **H, I** Lesions artificially inoculated on a leaf of *E. utilis* with MAFF 511473 after 16 days (**H**) and on a leaf of *Zea mays* with MAFF 511471 after 9 days (**I**). *Bars* **C** 200 µm; **D** 100 µm; **E–G** 20 µm

Table 2. Comparison of morphological and cultural characteristics of *Colletotrichum echinochloae* with related species

Species and reference	Conidium		Appressorial shape and size (μm)	Colony characteristics on PDA	Mycelial growth rate (optimum temperature)
	Shape and size (μm)	length : breadth ratio			
C. echinochloae					
This study	Falcate, fusiform, apex strongly curved, (14.0–)18.0–22.2(–24.0) × (3.5–)4.2–5.4(–6.7)	(2.8–)3.5–5.0(–5.6) mean: 4.3	Clavate, ovate, globose, slightly lobed, sepia to dark brown, (8–)10.2–14.7(–20.3) × (6–)7.5–10.5(–12.7)	Hyaline, black acervuli with orange conidial masses	4.5 mm/day (25°–28°C)
C. graminicola					
Sutton 1992	Falcate, fusiform, gradually tapered toward each end, 23.5–29 × 3.5–5	ND	Edge very irregular, medium brown, 17.5–20(–30) × 12.5–14	Grey, margin diffuse and irregular, fluffily aerial mycelium, reverse vinaceous lilac	ND
This study (ATCC 26416)	(21.4–)23.5–28.2(–30.4) × (3.9–)4.4–5.0(–5.4)	(4.3–)4.8–6.3(–6.9) mean, 5.5	(16.0–)16.9–24.9(–26.7) × (11.5–)12.6–20(–25.5)	Dark brown, fluffily aerial mycelium	5.6 mm/day (28°C)
C. sublineolum					
Sutton 1992	Falcate, fusiform, gradually tapered toward each end, 18.5–27.5 × 3–4.5	ND	Edge irregular, medium brown, 11.5–15 × 8.5–9	Delimited edge, aerial mycelium grey, felty, occasionally woolly and undulate, reverse grey to greenish-grey	ND
C. cereale					
Crouch 2006	Falcate, fusiform, apices acute, 6.0–33.8 × 2.2–6.3 (avg. 23.3 × 3.4)	ND	Smooth, irregular or lobate, dark brown, black, 8.5–11.6 × 6.5–10.2	Variable; usually dark mat of setae, orange conidial masses, sometimes fluffy aerial mycelium, grey	ND

PDA, potato dextrose agar; ND, not determined

lobed (Fig. 1F, G) and are quite different from those of other *Colletotrichum* species. Setae of 3–5(–6) cells are produced in acervuli and 79.8–145.5(–186.3) μm in length on PDA (Fig. 1C, D). PDA colonies of *C. echinochloae* are hyaline and usually form black acervuli with orange conidial masses, whereas those of *C. graminicola* are grey to dark brown (Table 2, Fig. 1B). In addition, colonies of *C. echinochloae* lack sclerotia on PDA under alternating lighting conditions. *Colletotrichum echinochloae* preliminarily falls within the broad species concept of *C. graminicola* (von Arx 1957), but differs from *C. graminicola* sensu Sutton in its morphological features. It can be distinguished from *C. graminicola* and other graminicolous species by its colony morphology and the shape of conidia.

Colletotrichum echinochloae (MAFF 511471 to 511473) grows at 5°–30°C with an optimum at 25°–28°C (Fig. 2, Table 2), and the mycelial growth rate is 4.5 mm/day at 25°–28°C. In contrast, *C. graminicola* (ATCC 26416) grows at 15°–35°C with an optimum of 28°C with a mycelial growth rate of 5.6 mm/day.

Colletotrichum echinochloae is pathogenic to *E. utilis*, producing leaf blight and grayish-white lesions with brown surroundings in leaves (Fig. 1H). Although it causes small and necrotic lesions in *Z. mays* (Fig. 1I), the lesions do not expand. *Colletotrichum echinochloae* never infects *S. bicolor*. *Colletotrichum graminicola* and *C. sublineolum* also infect only *Z. mays* and *S. bicolor*, respectively, and they never infect *E. utilis*. These comparable results were reported previously (Le Beau 1950; Nishihara 1961; Jamil and Nicholoson 1987). Distinct host specificity was recognized in these plant inoculation tests.

The sequenced regions of rDNA-ITS were 499 to 533 bp. The aligned region included 489 characters and excluded 72 gaps. Of these, 420 were invariant, 51 were parsimony informative, and 18 were variable but parsimony uninformative. A heuristic search gave two MP trees differing only in the branching orders of clades *C. graminicola* and *C. cereale*; one of the trees is shown in Fig. 3 [tree length = 103 steps, consistency index (CI) = 0.806, and retention index (RI) =

Fig. 2. Mycelial growth of *Colletotrichum (C.) echinochloae* and *C. graminicola* (ATCC 26416) at different temperatures on PDA. [a]Ex-holotype

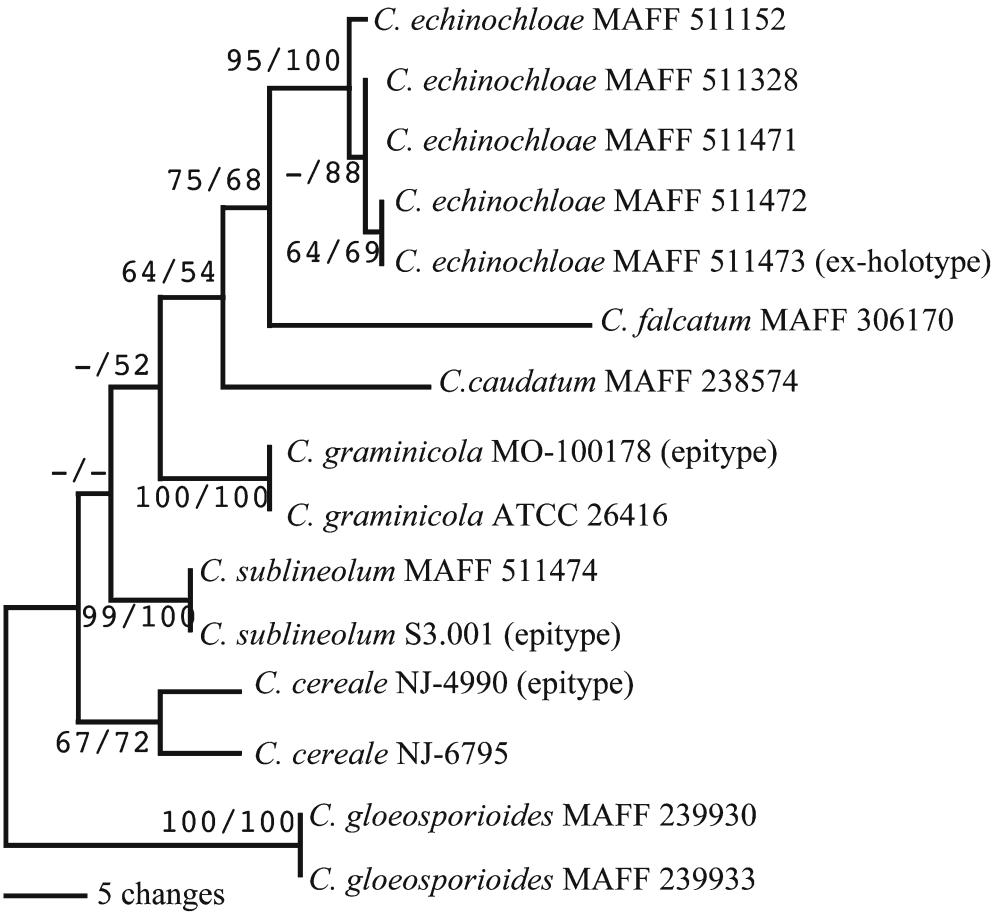

Fig. 3. One of two equally parsimonious trees of *Colletotrichum* species derived from rDNA-IT sequences. Numbers at each branch indicate percentage of occurrences of that branch in 1000 MP bootstrap replications (*before slash*) and in 1000 NJ bootstrap replications (*after slash*), where dashes (–) indicate less than 50% support. *Colletotrichum gloeosporioides* was used as an outgroup

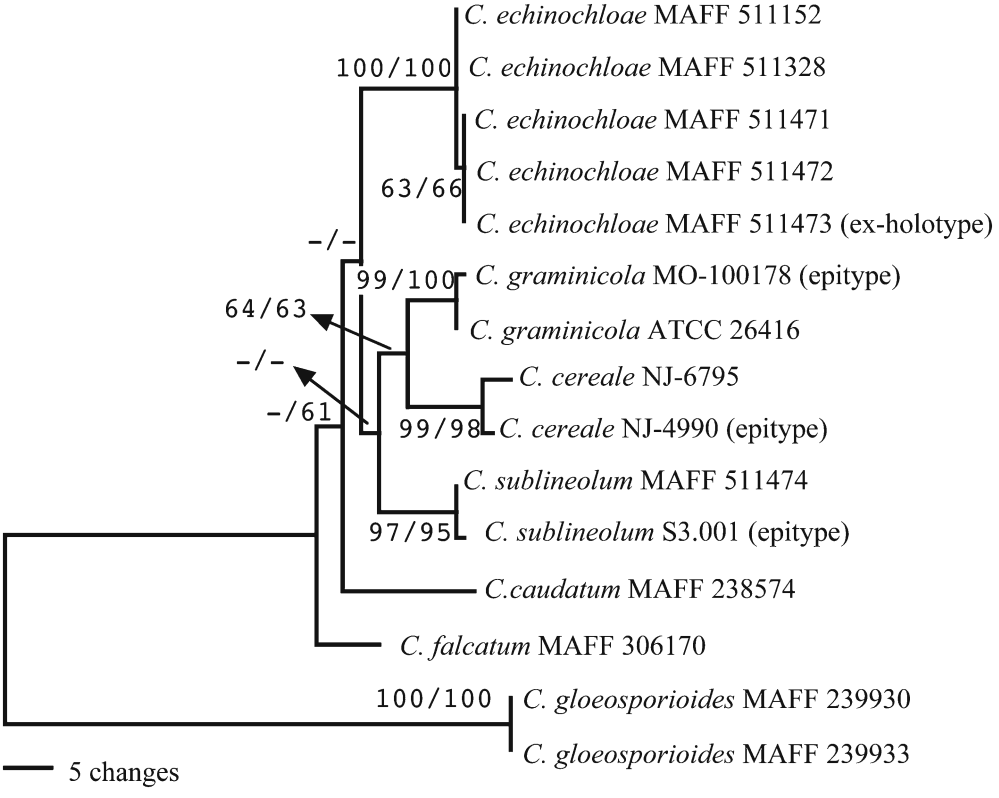

Fig. 4. One of four equally parsimonious trees of *Colletotrichum* species derived from HMG sequences. *Numbers at each branch* indicate percentage of occurrences of that branch in 1000 MP bootstrap replications (*before slash*) and in 1000 NJ bootstrap replications (*after slash*), where dashes (–) indicate less than 50% support. *Colletotrichum gloeosporioides* was used as an outgroup

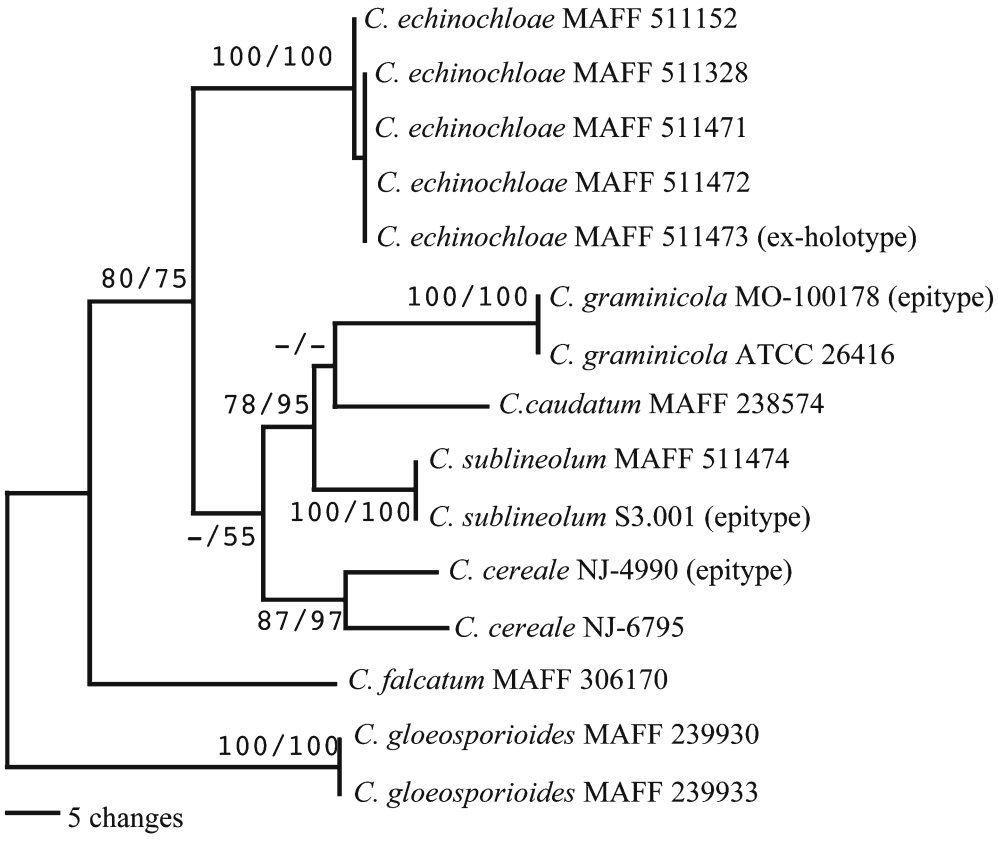

Fig. 5. Parsimonious tree of *Colletotrichum* species derived from SOD2 sequences. *Numbers at each branch* indicate percentage of occurrences of that branch in 1000 MP bootstrap replications (*before slash*) and in 1000 NJ bootstrap replications (*after slash*), where dashes (–) indicate less than 50% support. *Colletotrichum gloeosporioides* was used as an outgroup

0.846]. The MP trees were similar to the NJ tree (data not shown).

The sequenced regions of HMG were 219 to 226 bp. The aligned region included 219 characters and excluded 11 gaps. Of these, 103 were invariant, 110 were parsimony informative, and 6 were variable but parsimony uninformative. A heuristic search gave four MP trees differing only in the branching orders of clades; one of the trees is shown in Fig. 4 (tree length = 157 steps, CI = 0.847, and RI = 0.881). The MP trees were similar to the NJ tree (data not shown).

The sequenced regions of SOD2 were 395 bp. The aligned region included 395 characters. Of these, 270 were invariant, 100 were parsimony informative, and 25 were variable but parsimony uninformative. A heuristic search gave the MP tree shown in Fig. 5 (tree length = 178 steps, CI = 0.820, and RI = 0.873). The MP tree was similar to the NJ tree (data not shown).

All analyses using rDNA-ITS, HMG, and SOD2 sequences showed the isolates of *C. echinochloae* as a monophyletic lineage with a bootstrap value of 95%–100% (see Figs. 3–5). On the other hand, the isolates of *C. graminicola*, *C. sublineolum*, and *C. cereale* formed individual clades with 67%–100% bootstrap supports. *Colletotrichum gloeosporioides* formed a different clade far from the graminicolous *Colletotrichum* species in both MP and NJ analyses. Molecular phylogenetic analyses clearly distinguished *C. echinochloae* from *C. graminicola* as well as other similar *Colletotrichum* species such as *C. sublineolum*, *C. cereale*, *C. falcatum*, and *C. caudatum*.

In this study, morphological distinctiveness, pathogenicity, and monophyly based on molecular phylogenetic analyses showed the taxonomic individuality of *C. echinochloae*. *Colletotrichum graminicola* s. l., which has been recognized by Sutton (1992), is separated into six species, and the epithet of *C. graminicola* should be strictly applied to the fungus on *Z. mays*. Further studies on other grasses infected with *Colletotrichum* species may reveal other cryptic species similar to *C. echinochloae*.

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