Fungicide Sensitivity and Phylogenetic Relationship of Anthracnose Fungi Isolated from Various Fruit Crops in Japan

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ABSTRACT

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Anthracnose diseases of fruit crops are mainly caused by *Colletotrichum gloeosporioides* and *C. acutatum*. In these *Colletotrichum* species, intra- and interspecific variation in fungicide sensitivity has been reported; however, the relationship between fungicide sensitivity and molecular phylogeny has not been analyzed. Fifty-one isolates from 10 fruit crops, acacia, and tea were tested for their sensitivities to thiophanate-methyl, diethofencarb, and iminoctadine-triacetate, and their internal transcribed spacer (ITS) and 5.8S regions of rDNA were analyzed. *C. gloeosporioides* isolates were divided into sensitive, less sensitive, intermediate resistant, or resistant to the three fungicides. In contrast, *C. acutatum* isolates were all less sensitive. In molecular phylogenetic analyses, *C. gloeosporioides* isolates fell into the same genetic group, whereas *C. acutatum* isolates were placed into two genetic groups. Although phylogenetic relationship was not closely related to fungicide sensitivity, the isolates of *C. gloeosporioides* most resistant to iminoctadine-triacetate were found in the same phylogenetic subgroup.

Additional keywords: β -tubulin gene, benzimidazole fungicides

Colletotrichum gloeosporioides (Penz.) Penz. & Sacc. and *C. acutatum* J.H. Simmonds cause important anthracnose diseases of fruit crops worldwide (2,12,38,42). In Japan, *C. gloeosporioides* is one of the most common fungi and causes anthracnose on 86 crops, whereas *C. acutatum* causes anthracnose on 18 crops (41). Several fungicides, such as the benzimidazoles, diethofencarb in a mixture with thiophanate-methyl, and iminoctadine-triacetate, have been used effectively to control anthracnose diseases of various fruit crops (13,18,19,30). However, *C. gloeosporioides* isolates that are less sensitive or resistant to these fungicides have been detected (14,15,18,28,44). Similarly, *C. acutatum* isolates obtained from grapevine, apple, and Japanese pear were less sensitive to benomyl and diethofencarb (13,15,18,28,33). A single-point mutation in the gene encoding the fungicide-targeted protein β -tubulin has been associated with moderate or high levels of resistance to benzimidazole fungicides in

several fungal pathogens (1,17,18,22,28,44,45).

Molecular analysis is a useful tool for studying phylogenetic relationships of filamentous fungi and for relating it to host-specificity, geographical variation, and gene-diversity among intra- and inter-species groups (26). For instance, Freeman (10) used arbitrarily primed polymerase chain reaction (ap-PCR) to analyze *Colletotrichum* spp. obtained from various fruit crops and found that DNA polymorphisms corresponded to the host and geographic distribution. Similarly, based on the nucleotide sequence of the internal transcribed spacer (ITS) regions of rDNA, species-specific primers were designed and used successfully for identifying *C. gloeosporioides* and *C. acutatum* (23,24,35,36). Further, Freeman and Shabi (12) compared the polymorphism of genomic DNA of *Colletotrichum* spp. from various hosts and reported that molecular divergence existed among *C. gloeosporioides* and *C. acutatum*. Moreover, the sequence of D2 domain of large-subunit rDNA showed that intra- and interspecies variation existed in *Colletotrichum* spp. (9,11,20).

To effectively control anthracnose diseases, it is necessary to determine the sensitivity of isolates of the *Colletotrichum* species causing the disease to fungicides. The objectives of this study were (i) to examine sensitivity of *C. gloeosporioides*

and *C. acutatum* isolates obtained from various fruit crops in Japan to the three fungicides, thiophanate-methyl, diethofencarb, and iminoctadine-triacetate using in vitro methods, (ii) to analyze ITS and 5.8S regions to understand the relationships between fungicide sensitivity and molecular phylogeny of the isolates examined, and (iii) to sequence the partial β -tubulin gene, including the amino acid codons 198 and 200, which have been reported to be responsible for resistance or lower sensitivity to benzimidazoles of *C. gloeosporioides* and *C. acutatum*, respectively. Brief results from this work have been reported previously (14,15,17,44).

MATERIALS AND METHODS

Fungal isolates and fungicides. Fifty-one *Colletotrichum* isolates obtained from fruit trees (Japanese pear (*Pyrus pyrifolia* var. *culta*), grapevine (*Vitis* spp.), apple (*Malus pumila* var. *domestica*), peach (*Prunus persica*), loquat (*Eriobotrya japonica*), prune (*P. domestica*), Japanese plum (*P. salicina*), Japanese chestnut (*Castanea crenata*), blueberry (*Vaccinium* spp.) and akebia (*Akebia* spp.)), soft fruit (strawberry (*Fragaria* $\times$ *ananassa*)), and woody plants (tea (*Camellia sinensis*), acacia (*Acacia* spp.)) were used (Table 1). The benzimidazole fungicide thiophanate-methyl (70% WP), iminoctadine-triacetate (25% WP), and the *N*-phenylcarbamate fungicide diethofencarb (25% WP) were used in the study.

Determination of sensitivity of *Colletotrichum* isolates to fungicides. Sensitivities of *Colletotrichum* isolates to thiophanate-methyl, diethofencarb, and iminoctadine-triacetate were tested using mycelial growth assays. Each isolate was cultured on potato dextrose agar (PDA) dishes at 25°C for 5 days. Mycelial disks, 4 mm diameter, were cut from the margins of colonies and transferred onto PDA dishes amended with 0, 1, 10, and 100 mg/liter active ingredient (a.i.) of thiophanate-methyl or diethofencarb, and 0, 1, 10, 100, and 1,000 mg a.i./liter of iminoctadine-triacetate. Diethofencarb and iminoctadine-triacetate were added to PDA after autoclaving, and thiophanate-methyl was added to PDA before autoclaving. After incubation at 25°C in the dark for 3 days, the diameter of each colony was

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measured and the EC₅₀ for each fungicide was calculated using log-linear model software kindly supplied by ZEN-NOH (Tokyo).

DNA extraction and PCR amplification. DNA extraction followed the method of Goodwin and Lee (16). From this crude DNA extract, 1 to 2 µl was used directly for each PCR amplification.

Identification and assignment of isolates into *C. gloeosporioides* and *C. acutatum* were performed using the species-specific primers CgInt (24) and CaInt2 (37). For molecular phylogenetic analysis, the ITS1,

5.8S rDNA, and ITS2 regions were amplified using a primer pair of ITS1 and ITS4 (43). Amplifications were done using 20 µl of PCR reaction solutions. The PCR reaction mixture contained total DNA, 1.5 mM MgCl₂, 200 µM each dNTP, and 2.5 units of *Taq* DNA polymerase (Nippon Gene, Toyama, Japan). For an initial amplification, the PCR reaction program followed the procedure of Chung and Tsukiboshi (4). PCR products were run on 1.5% agarose gels in TAE (Tris-acetate, EDTA) buffers then stained with 0.5 µg/ml ethidium bromide.

Analysis of β-tubulin gene. The β-tubulin gene, including the amino acid codons 198 and 200, was amplified by C' and F primers (1). The PCR reaction mixture and reaction program followed were those reported by Albertini et al. (1). PCR products were first purified by Wizard PCR Preps DNA Purification System (Promega, Madison, WI). Purified PCR products were reacted with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) under the following conditions: 25 cycles of 96°C for 10 s, 50°C for 5 s, 60°C for 4 min.

Table 1. Source of *Colletotrichum* isolates used in this study and sensitivity to thiophanate-methyl, diethofencarb, and iminoctadine-triacetate

Isolate	Host	Location	Species ^b	Phenotype ^a		
				Thiophanate-methyl	Diethofencarb	Iminoctadine-triacetate
GC1-1 ^c	Japanese pear	Akita	<i>C. acutatum</i>	LS	LS	LS
GC1-2 ^c	Japanese pear	Akita	<i>C. acutatum</i>	LS	LS	LS
I5-1 ^d	Japanese pear	Kochi	<i>C. gloeosporioides</i>	S	LS	S
I6-1 ^d	Japanese pear	Kochi	<i>C. gloeosporioides</i>	IR	LS	S
I9-1 ^d	Japanese pear	Kochi	<i>C. gloeosporioides</i>	S	LS	S
M14-2 ^d	Japanese pear	Kochi	<i>C. gloeosporioides</i>	IR	LS	S
M19-1 ^d	Japanese pear	Kochi	<i>C. gloeosporioides</i>	IR	LS	S
SA02-1-1	Japanese pear	Saga	<i>C. gloeosporioides</i>	HR	S	S
SA02-3-2	Japanese pear	Saga	<i>C. gloeosporioides</i>	S	LS	HR
SA02-5-1	Japanese pear	Saga	<i>C. gloeosporioides</i>	HR	S	S
SA02-6-1	Japanese pear	Saga	<i>C. gloeosporioides</i>	S	LS	S
SA02-8-1	Japanese pear	Saga	<i>C. gloeosporioides</i>	HR	S	S
Nashi-2	Japanese pear	Aichi	<i>C. gloeosporioides</i>	S	LS	HR
Nashi-10	Japanese pear	Aichi	<i>C. gloeosporioides</i>	S	LS	HR
B-60	Grapevine	Akita	<i>C. gloeosporioides</i>	S	LS	S
C1-5	Grapevine	Akita	<i>C. gloeosporioides</i>	S	LS	S
C1-8	Grapevine	Akita	<i>C. gloeosporioides</i>	S	LS	S
GC-2	Grapevine	Akita	<i>C. gloeosporioides</i>	S	LS	S
B-23	Grapevine	Akita	<i>C. acutatum</i>	LS	LS	LS
C1-19	Grapevine	Akita	<i>C. acutatum</i>	LS	LS	LS
C1-40	Grapevine	Akita	<i>C. acutatum</i>	LS	LS	LS
G5-1-7	Grapevine	Akita	<i>C. acutatum</i>	LS	LS	LS
YH1-9	Grapevine	Akita	<i>C. acutatum</i>	LS	LS	LS
FGGC01011	Grapevine	Fukuoka	<i>C. gloeosporioides</i>	S	LS	S
FGGC01084	Grapevine	Fukuoka	<i>C. gloeosporioides</i>	S	LS	S
FGGC01088	Grapevine	Fukuoka	<i>C. gloeosporioides</i>	S	LS	S
FGGC01100	Grapevine	Fukuoka	<i>C. gloeosporioides</i>	HR	S	S
FGGC01101	Grapevine	Fukuoka	<i>C. gloeosporioides</i>	HR	S	HR
S-7	Apple	Akita	<i>C. gloeosporioides</i>	S	LS	HR
S-12	Apple	Akita	<i>C. gloeosporioides</i>	S	LS	HR
S-20	Apple	Akita	<i>C. gloeosporioides</i>	S	LS	HR
H-44	Apple	Akita	<i>C. acutatum</i>	LS	LS	LS
M-82	Apple	Akita	<i>C. acutatum</i>	LS	LS	LS
T-22	Apple	Akita	<i>C. acutatum</i>	LS	LS	LS
FPeCG-9301	Peach	Fukuoka	<i>C. acutatum</i>	LS	LS	LS
MAFF305138	Peach	Unknown	<i>C. acutatum</i>	LS	LS	LS
MAFF305596	Loquat	Chiba	<i>C. acutatum</i>	LS	LS	LS
GC-P-38	Prune	Nagano	<i>C. acutatum</i>	LS	LS	LS
MAFF410044	Japanese plum	Nagano	<i>C. acutatum</i>	LS	LS	LS
Chestnut-1	Japanese chestnut	Ibaraki	<i>C. gloeosporioides</i>	S	LS	IR
Chestnut-2	Japanese chestnut	Ibaraki	<i>C. gloeosporioides</i>	S	LS	IR
GC-M-1	Japanese chestnut	Nagano	<i>C. acutatum</i>	S	LS	LS
GC-B-1	Blueberry	Nagano	<i>C. acutatum</i>	LS	LS	LS
NM-1	Strawberry	Tochigi	<i>C. gloeosporioides</i>	S	LS	IR
Na91-016	Strawberry	Tochigi	<i>C. acutatum</i>	LS	LS	LS
AL-5	Strawberry	Nagasaki	<i>C. acutatum</i>	LS	LS	LS
AL-9	Strawberry	Nagasaki	<i>C. acutatum</i>	LS	LS	LS
Cf: 5-0	Strawberry	Nagasaki	<i>C. gloeosporioides</i>	S	LS	S
GC-AK-1	Akebia	Nagano	<i>C. acutatum</i>	LS	LS	LS
GC-RP-8	Acacia	Nagano	<i>C. acutatum</i>	LS	LS	LS
1073R	Tea	Shizuoka	<i>C. gloeosporioides</i>	S	LS	S

^a S = sensitive; LS = less sensitive; IR = intermediately resistant; HR = highly resistant; R = resistant.

^b Identified by polymerase chain reaction (PCR) using species-specific primers of CgInt and CaInt2.

^c Published by Fukaya (15).

^d Published by Yano et al. (44).

Cycle sequencing reaction products were purified by ethanol precipitation, and then sequenced using ABI PRISM 3100 automated sequencer (Applied Biosystems).

Molecular phylogenetic analysis of ITS and 5.8S regions of rDNA. The ITS and 5.8S regions of rDNA were amplified. Both forward and reverse nucleotides were sequenced and assembled; once completed the nucleotide bases were aligned using Clustal X v1.8 (40). Further visual alignments were done in Sequence Alignment (Se-Al) Editor v2.0 (31). The aligned sequences were analyzed together with the out-group sequence of *C. crassipes* (accession no. AJ536230) obtained from GenBank.

Phylogenetic analysis of the aligned sequences was done by distanced parsimony methods. The distance matrix for the aligned sequences was calculated using Kimura's two-parameter method (21) and was analyzed with the neighbor-joining (NJ) method (32). The maximum-parsimony analysis was also done. Included in the analysis were positions with gaps as a fifth character since they may be informative for inferring phylogenies. Reliability of the inferred trees was estimated by 1,000 bootstrap resampling using the same program. Parsimony analysis was done by PAUP v4.0b (39) using the heuristic search option with 100 random stepwise-addition sequences to search for the most parsimonious tree. Bootstrap (8) values were generated with 1,000 replicate heuristic searches to estimate support for clade stability of the consensus tree using the same program.

RESULTS

Response to fungicides. The phenotypic difference of sensitivities to thiophanate-methyl, diethofencarb, and iminotadine-triacetate are shown in Table 1. Five isolates of *C. gloeosporioides* had EC₅₀ values of >100 mg/liter of thiophan-

ate-methyl and were grouped as highly resistant (HR), whereas three isolates had EC₅₀ values between 10 and 100 mg/liter and were classified as intermediately resistant (IR), and 21 isolates with EC₅₀ values <1 mg/liter were sensitive (S). With respect to diethofencarb, five isolates of *C. gloeosporioides* with an EC₅₀ value of <1 mg/liter were determined to be sensitive (S), and 24 isolates with EC₅₀ values of >100 mg/liter were considered less sensitive (LS). With iminotadine-triacetate, seven isolates with EC₅₀ values between 100 and 1,000 mg/liter were highly resistant (HR), three isolates with EC₅₀ values between 10 and 100 mg/liter were intermediately resistant (IR), and 19 isolates with EC₅₀ values of <1 mg/liter were sensitive (S). On the other hand, *C. acutatum* isolates were inherently less sensitive to the three fungicides, and the EC₅₀ values for all isolates excluding GC-M-1 were between 1 and 10 mg/liter (thiophanate-methyl), >100 mg/liter (diethofencarb), and between 100 and 1,000 mg/liter (iminotadine-triacetate).

Species identification by PCR. Using the species-specific primers CgInt and Calnt2, 29 isolates were identified as *C. gloeosporioides* and 22 isolates as *C. acutatum*. The isolates from apple, Japanese pear, grapevine, Japanese chestnut, and strawberry were included in these two species. The isolates from peach, prune, loquat, Japanese plum, akebia, acacia, and blueberry were exclusively *C. acutatum*, whereas the only isolate from tea was *C. gloeosporioides* (Table 1).

Mutation of β -tubulin gene. Forward and reverse nucleotides of the β -tubulin gene were sequenced and assembled. These sequences were confirmed using two replicates to ensure accuracy. The mutation at the deduced amino acid codons 198 and 200 in partially amplified β -tubulin gene obtained from 11 isolates of *C. gloeosporioides* and 4 isolates of *C.*

acutatum are shown in Table 2. Three isolates of *C. gloeosporioides*, SA02-1-1, SA02-5-1, and SA02-8-1, highly resistant to thiophanate-methyl, showed a point mutation from GAG to GCG at codon 198, resulting in substitution of Glu with Ala. However, the sequences of codon 198 were GAG (Glu) in intermediately thiophanate-methyl resistant and sensitive isolates. The sequence at codon 200 for intermediately resistant isolates of *C. gloeosporioides*, M14-2 and M19-1 derived from Japanese pear, possessed the change from TTC (Phe) to TAC (Tyr). In *C. acutatum*, two isolates, G5-1-7 and Na91-016, showed less sensitivity to thiophanate-methyl and had the sequences of AAG (Lys) at codon 198. On the other hand, two other isolates, GC1-1 and GC1-2, had the wild type sequences of GAG (Glu) and TTC (Phe) at codons 198 and 200, respectively.

Phylogenetic analysis inferred from ITS and 5.8S rDNA sequence. For molecular phylogenetic analysis, 572 to 585 base pairs of the ITS and 5.8S rDNA regions were analyzed. Of the 615 total characters including gaps, 543 characters were constant and 15 variable characters were parsimony-uninformative. Moreover, 57 characters were parsimony-informative and gaps were treated as missing. In a neighbor-joining tree constructed from the ITS and 5.8S rDNA sequences, *C. gloeosporioides* isolates fell into one major clade and *C. acutatum* isolates fell into two major clades with 100, 97, and 83% bootstrap support, respectively (Fig. 1). The ITS and 5.8S rDNA phylogram had a consistency index (CI) of 0.927, a retention index (RI) of 0.994, retention consistency (RC) of 0.922, and a tree length of 82. In a maximum-parsimony tree constructed from the ITS and 5.8S rDNA sequences, results were similar to those obtained from neighbor-joining analysis: *C. gloeosporioides* isolates fell into one major clade, and *C. acutatum* isolates fell into two major clades with 100, 90, and 77% bootstrap support, respectively (Fig. 2). The ITS and 5.8S rDNA phylogram had a CI of 0.767, a RI of 0.977, RC of 0.749, and a tree length of 103. The *C. acutatum* isolates showed higher sequence divergence on ITS2 region (4.4%) than the ITS1 region (2.7%), whereas no sequence divergence on 5.8S rDNA region was observed. Similarly, *C. gloeosporioides* isolates showed higher sequence divergence in the ITS2 region (3.2%) than in the ITS1 regions (2.9%), and no sequence divergence in the 5.8S rDNA region was recognized.

DISCUSSION

C. gloeosporioides isolates from various fruit trees and other hosts in Japan showed different sensitivity to thiophanate-methyl, diethofencarb, and iminotadine-triacetate, whereas all *C. acutatum* isolates were inherently less sensitive to the three fungicides. Benzimidazole resis-

Table 2. Mutation and deduced amino acid substitutions in the partial sequence of the β -tubulin gene in isolates of *Colletotrichum* spp. with different phenotypic responses to thiophanate-methyl

Isolate	Species	Response ^a	Sequence in codon (amino acid)	
			198	200
I5-1 ^b	<i>C. gloeosporioides</i>	S	GAG (Glu)	TTC (Phe)
I9-1 ^b	<i>C. gloeosporioides</i>	S	GAG (Glu)	TTC (Phe)
M14-2 ^b	<i>C. gloeosporioides</i>	IR	GAG (Glu)	TAC (Tyr)
M19-1 ^b	<i>C. gloeosporioides</i>	IR	GAG (Glu)	TAC (Tyr)
SA02-1-1	<i>C. gloeosporioides</i>	HR	GCG (Ala)	TTC (Phe)
SA02-3-2	<i>C. gloeosporioides</i>	S	GAG (Glu)	TTC (Phe)
SA02-5-1	<i>C. gloeosporioides</i>	HR	GCG (Ala)	TTC (Phe)
SA02-6-1	<i>C. gloeosporioides</i>	S	GAG (Glu)	TTC (Phe)
SA02-8-1	<i>C. gloeosporioides</i>	HR	GCG (Ala)	TTC (Phe)
GC-2 ^c	<i>C. gloeosporioides</i>	S	GAG (Glu)	TTC (Phe)
NM-1 ^c	<i>C. gloeosporioides</i>	S	GAG (Glu)	TTC (Phe)
GC1-1	<i>C. acutatum</i>	LS	GAG (Glu)	TTC (Phe)
GC1-2	<i>C. acutatum</i>	LS	GAG (Glu)	TTC (Phe)
G5-1-7 ^c	<i>C. acutatum</i>	LS	AAG (Lys)	TTC (Phe)
Na91-016 ^c	<i>C. acutatum</i>	LS	AAG (Lys)	TTC (Phe)

^a S = sensitive; IR = intermediately resistant; HR = highly resistant; LS = less sensitive.

^b Published by Yano et al. (44).

^c Published by Ishii (17).

tance in *C. gloeosporioides* and lower sensitivity to both benzimidazoles and diethofencarb in *C. acutatum* have been reported by several researchers in Japan (13,15,17,18,33,44).

In this study, most of the *C. gloeosporioides* isolates tested were still sensitive to thiophanate-methyl and iminooctadine-triacetate. Thiophanate-methyl-resistant isolates were obtained from Japanese pear and grapevine. However, thiophanate-methyl-resistant isolates were also

detected from apple before (18,33). According to our results, isolates of *C. gloeosporioides* were classified into three groups: sensitive, intermediately resistant, and highly resistant to thiophanate-methyl. Highly thiophanate-methyl-resistant isolates of *C. gloeosporioides* showed negative cross-resistance to diethofencarb as previously reported (18,33,44). On the other hand, isolates of *C. gloeosporioides* with intermediate resistance to thiophanate-methyl were found only in Japanese

pear orchards in Kochi Prefecture, Japan, and they did not show negative cross-resistance to diethofencarb as reported by Yano et al. (44). Sato et al. (33) reported that *C. gloeosporioides* could be distinguished from *C. acutatum* based on sensitivity to benomyl and diethofencarb. However, the difference in sensitivity to benzimidazole fungicides and diethofencarb cannot always be used to distinguish the two *Colletotrichum* species because we found some isolates of *C. gloeosporioides*

Fig. 1. Internal transcribed spacer (ITS) and 5.8S sequence-based tree generated using neighbor-joining analysis. Numbers at the branch node indicate the confidence values from bootstrap analysis using 1,000 replications. Bootstrap values above 60% are presented. *Colletotrichum gloeosporioides* isolates in bold type were resistant to iminooctadine-triacetate. Number in parentheses is accession number in GenBank.

with fungicide sensitivity similar to that of *C. acutatum*.

Although Fukaya (13) found that the dormant spray of iminoctadine-triacetate was effective in controlling grapevine ripe rot caused by *C. gloeosporioides* and *C. acutatum* in vineyards, isolates of the two species less sensitive to this fungicide have been detected from grapevine and apple (14). Moreover, iminoctadine-triacetate-resistant isolates of *C. gloeosporioides* were also found from Japanese pear, strawberry, and Japanese chestnut in this study. This suggests that iminoctadine-triacetate-resistant *C. gloeosporioides* isolates may be distributed widely in various fruit crops in Japan.

Several researchers have reported that high resistance or intermediate resistance to benzimidazole fungicides was caused by a single point mutation at codon 198 or 200 of β -tubulin in filamentous fungi (1,17,22,28,44). In the present study, the partial sequence of the β -tubulin gene of *C. gloeosporioides* isolates highly resistant to thiophanate-methyl also had the amino acid substitution of Glu (GAG) with Ala (GCG) at codon 198. In intermediately resistant isolates of *C. gloeosporioides*, Phe (TTC) was substituted with Tyr (TAC) at codon 200. On the other hand, the sequence in *C. acutatum* isolates was GAG or AAG at codon 198. The isolates of *C.*

acutatum used for this study showed the same sequence at codon 200 as *C. gloeosporioides*. Also in our study, the amino acid substitution of glutamic acid (wild type) with lysine was confirmed to codon 198 in *C. acutatum*. Albertini et al. (1) reported that codon 198, which encodes glutamic acid in benzimidazole-sensitive strains, could convert to a codon for alanine, glycine, lysine, or glutamine in benzimidazole-resistant strains. However, glutamic acid was replaced by alanine, glycine, lysine, or glutamine in two isolates of *C. acutatum*, GC1-1 and GC1-2, obtained from Japanese pear. Peres et al. (29) mentioned that isolates of *C. acu-*

Fig. 2. Internal transcribed spacer (ITS) and 5.8S sequence-based tree generated using maximum-parsimony analysis. Numbers at the branch node indicate the confidence values from bootstrap analysis using 1,000 replications. Bootstrap values above 60% are presented. *Colletotrichum gloeosporioides* isolates in bold type were resistant to iminoctadine-triacetate. Number in parentheses is accession number in GenBank.

tatum were neither highly sensitive nor highly tolerant to benomyl. This suggests that less sensitivity to thiophanate-methyl found in two isolates may be governed by other factors. De Waard (6) and Del Sorbo et al. (7) reported that ATP binding cassette (ABC) transporter systems can play a role in multidrug resistance in filamentous fungi. Thus, factors other than the mutation of the β -tubulin gene, such as transporter systems, might play a role in less sensitivity to benzimidazoles in cells of *C. acutatum*.

The molecular phylogenetic analysis showed that the *C. gloeosporioides* and *C. acutatum* are distinct species based on the nucleotide sequence of ITS and 5.8S rDNA regions, and the result corresponds with the species-specific PCR primer reaction. According to Moriwaki et al. (25), *C. gloeosporioides* isolates obtained from various host plants could be separated into three groups based on the sequence of the ITS1 region, and isolates from fruit crops were in the same ITS group. Our results also showed that the ITS1 sequence divergence of *C. gloeosporioides* isolates from various fruit crops was low. Freeman (10) and Correll et al. (5) used random amplified polymorphic DNA and restriction fragment length polymorphism analyses to group the *Colletotrichum* species from different geographic regions and host plants; however, the sequence divergence of ITS and 5.8S rDNA regions did not correspond with geographical differences, host specificity, and fungicide sensitivity in our study. Although nine of 10 *C. gloeosporioides* isolates showing resistance to iminoctadine-triacetate fell into the same subgroup, there was no significant relationship between the ITS and 5.8S rDNA sequence and sensitivity to iminoctadine-triacetate. Additional isolates resistant to this fungicide should be analyzed in the future.

C. acutatum isolates separated into two distinct ITS groups, and the differentiation was supported by a highly reliable bootstrap value. Moriwaki et al. (25) reported that *C. acutatum* isolates obtained from various host plants did not show significant divergence in the ITS1 sequence and belonged to one ITS group. Similarly, our sequence data of *C. acutatum* isolates obtained from various fruit crops also showed low divergence in the ITS1 region. However, the sequence data of the ITS2 region within *C. acutatum* isolates showed higher divergence than the ITS1 region, and sequence variation of the ITS2 region might relate to intraspecies differences in *C. acutatum*. Sreenivasaprasad et al. (36) suggested that ITS1 sequence variation was useful in explaining the phylogenetic relationship within *Colletotrichum*. However, Sherriff et al. (34) and Freeman et al. (11) considered the ITS2 sequence too highly variable to be useful in intraspecies differentiation. Similarly, O'Donnell and

Cigelnik (27) reported that divergence of the ITS2 sequence of *Fusarium* species was discordant with the species phylogeny inferred from three unlinked loci. In our opinion, divergence of ITS2 sequence in *C. acutatum* isolates obtained from various fruit crops in Japan might not correspond to species difference but might correspond with intraspecies differences. Recently, Peres et al. (29) mentioned that *C. acutatum* is a group species and the relationships among populations correspond to different host plants and infection strategies. Intraspecies were recognized to exist in *Colletotrichum* species, especially in isolates that possess differences in pathogenicity (3,5,10,34). Although ITS and 5.8S rDNA sequences did not show any phylogenetic relationship to geography, host plant, and fungicide sensitivity, the results suggest that a subspecies might exist in *C. acutatum* obtained from various fruit crops in Japan.

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