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FUNGAL DISEASES

Genetic analysis and PCR-based identification of major *Fusarium* species causing head blight on wheat in Japan

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Abstract Identifying the *Fusarium* species cause *Fusarium* head blight (FHB) and produces mycotoxins in wheat and other cereal is difficult and time consuming because of confusing phenotypic classification systems. In Japan, the *F. graminearum* complex, *F. culmorum*, *F. avenaceum*, and *Microdochium nivale* predominantly cause FHB. The internal transcribed spacer (ITS) and 5.8S of rDNA, a partial sequence of β-tubulin and mitochondrial cytochrome *b* (*cytb*) genes of the four species were PCR-amplified and analyzed. On the basis of the ITS, β-tubulin and *cytb* sequences, *F. avenaceum* and *M. nivale* are distinct from the *F. graminearum* complex and *F. culmorum*, whereas the *F. graminearum* complex is closely related to *F. culmorum*. Moreover, thiophanate–methyl-resistant isolates of the *F. graminearum* complex and *F. culmorum* did not have an amino acid substitution at amino acid codon 198 or 200 of β-tubulin. In contrast, very highly or highly thiophanate–methyl-resistant isolates of *M. nivale* had Glu (GAG) substituted with Ala (GCG) or Lys (AAG) at codon 198, respectively. The allele-specific PCR assay was used to identify the *F. graminearum* complex and *F. culmorum*, and these *Fusarium* species could be distinguished rapidly.

Keywords *Fusarium* head blight · rDNA-ITS · β-tubulin · Cytochrome *b* · Allele-specific PCR

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Introduction

Fusarium species are important pathogens that cause *Fusarium* head blight (FHB) in wheat and other cereals and can reduce yield by 30–70% (Bai and Shaner 1994). More than ten *Fusarium* species, including *Microdochium nivale* (Fr.) Samuels and Hallett (=*F. nivale* Cesati ex Sacc.), can cause head blight throughout the world (Liddell 2003). Several of these species can produce two major groups of mycotoxins, trichothecenes (Mirocha et al. 2003), and zearalenone (Hesseltine et al. 1978). These *Fusarium* species can cause serious problems for the agricultural industry and are potential threat to the food supply (McMullen et al. 1997; Clear et al. 2000).

In Japan, 12 *Fusarium* species cause head blight on wheat, barley and rye (Aoki 2003). Of these 12, *F. graminearum* Schw. (=*Gibberella zeae* Schw. and Petch), *F. culmorum* (W. G. Smith) Sacc., *F. avenaceum* (Corda: Fr.) Sacc., and *M. nivale* predominate and are most important (Aoki 2003; Aoki et al. 2005). Although *F. graminearum* is distributed all over Japan, *F. culmorum*, *F. avenaceum*, and *M. nivale* are found mainly in the northern part of Honshu and Hokkaido. *Fusarium graminearum*, *F. culmorum*, and *F. avenaceum* produce trichothecenes such as nivalenol (NIV) and deoxynivalenol

(DON), which contaminate the grain (Aoki 2003; Souma 2003).

Recently, molecular technique has been developed to monitor and quantify the *Fusarium* species causing FHB and producing mycotoxins (Bluhm et al. 2002; Edel et al. 1997; Jurado et al. 2005; Konstantinova and Yli-Mattila 2004; Kulik et al. 2004; Láday et al. 2004; Mishra et al. 2003; Nicholson et al. 1998, 2003, 2004; Parry and Nicholson 1996; Suga et al. 2004; Waalwijk et al. 2004; Wilson et al. 2004; Yli-Mattila et al. 2002, 2004a, b; Yoder and Christianson 1998). These specific molecular markers were mostly designed from internal transcribed spacer (ITS) (Bluhm et al. 2002; Edel et al. 1997; Mishra et al. 2003; Wilson et al. 2004; Yli-Mattila et al. 2002, 2004a, b) or intergenic spacer (IGS) regions of rDNA (Jurado et al. 2005; Konstantinova and Yli-Mattila 2004; Yli-Mattila et al. 2002, 2004a, b), β-tubulin gene (Yli-Mattila et al. 2002, 2004b) or mitochondrial DNA (mtDNA) (Láday et al. 2004), and are highly specific for identifying *Fusarium* species. However, some of these species-specific markers have a differential sensitivity for isolates of same species of *Fusarium* from different areas (Yli-Mattila et al. 2004b). Thus, developing species-specific molecular makers to detect local isolates is important for reliable species identification.

Among the *Fusarium* species producing mycotoxins, the *F. graminearum* complex is the most widely distributed in the world and has been separated into seven lineage groups (O'Donnell et al. 2000a). Recently, two more species, *F. vorosii* Tóth et al. and *F. gerlachii* Aoki et al. were described within the *F. graminearum* complex (Starkey et al. 2007). However, only three species of the *F. graminearum* complex, *F. graminearum* Schwabe s. str. (lineage 7 of *F. graminearum*), *F. asiaticum* O'Donnell et al. (lineage 6 of *F. graminearum*), and *F. vorosii* have been found in Japan (Aoki 2003; O'Donnell et al. 2004; Starkey et al. 2007). The *F. graminearum* s. str. and *F. asiaticum* are regarded as genetically distinct according to PCR-based assay (Aoki et al. 2005; Suga et al. 2004). Thus, genetic analysis is very useful for designing new PCR (polymerase chain reaction) primers to rapidly and successfully identify different *Fusarium* species causing FHB.

The ITS and 5.8S regions of rDNA and β-tubulin gene are useful for identifying *Fusarium* species and studying their phylogeny (O'Donnell and Cigelnik 1997; O'Donnell et al. 2000b; Yli-Mattila et al. 2002). Recently, the cytochrome *b* (*cytb*) gene was also used to study the phylogenetic relationship in basidiomycetous yeasts (Biswas et al. 2001) and fungi (Sierotzki et al. 2003). The aim of the present study was to compare the diversity of nucleotide sequences in ITS and 5.8S rDNA regions, β-tubulin gene and *cytb* gene of *F. graminearum*-complex, *F. culmorum*, *F. avenaceum*, and *M. nivale* collected in Japan. The divergence of nucleotide sequences was used to design species-specific primers to identify these fungi. Thus, developing a one-step, allele-specific PCR is important to rapidly identify *F. graminearum*-complex and *F. culmorum*, in particular, which are most widely distributing pathogens causing FHB in Japan.

Thiophanate–methyl, a benzimidazole fungicide, has been used to control wheat FHB for a long period in Japan. Very recently, however, *Fusarium* isolates resistant to this fungicide have been detected occasionally in Aomori and Oita prefectures (Iwama et al. 2007; Yoshimatsu et al. 2006). Benzimidazole resistance was reported much earlier for *M. nivale* (Tsuboki 1984). Therefore, we included molecular characterization of benzimidazole resistance in these fungi in this study. In addition, partial *cytb* gene fragments, including codons 129 and 143 known to be responsible for resistance to QoI fungicides in fungi (Gisi et al. 2002), from the *F. graminearum*-complex and *F. culmorum* were amplified.

Materials and methods

Fungal isolates and DNA extraction

Fungal isolates used in this study are listed in Table 1. All *Fusarium* and *Microdochium* isolates were maintained on potato dextrose agar (PDA). The DNA extraction protocol followed that of Chung and Tsukiboshi (2005).

Polymerase chain reaction (PCR) amplification and sequencing of ITS and 5.8S rDNA regions, β-tubulin gene and *cytb* gene of *Fusarium* isolates

For ITS and 5.8S rDNA region amplification, primers of ITS1 and ITS4 (White et al. 1990) were used and PCR conditions were those of Chung and Tsukiboshi (2005). Partial β-tubulin gene fragment was amplified by primers FU-tubulin3 (5′-CGAGCCCGGTACCATGGACG-3′ and FU-tubulin2 (5′-GGTCGCCGTAAGAGGGGTTGG-3′), designed from sequences registered in GenBank, and PCR conditions were as follows: initial denaturation at 94°C for 3 min, 35 cycles of 94°C for 1 min, 60°C for 30 s, 72°C for 2 min; and final extension at 72°C for 5 min. Similarly, partial *cytb* gene fragment was amplified by primers RSCBF1 and RSCBR2 (Ishii et al. 2001), and PCR conditions were as follows: initial denaturation at 94°C for 2.5 min, 40 cycles of 94°C for 1 min, 50°C for 30 s, 72°C for 1.5 min; and final extension at 72°C for 8.5 min. Amplifications were done using 20 µl PCR reaction mixtures, containing 0.2 µM of each primer, 1 unit of Ex *Taq* DNA polymerase (Takara Bio, Otsu, Japan), and a dNTP mixture (containing 250 µM of each of dNTP) and

Table 1 Fungal isolates used in this study, origin, and DDBJ/EMBL/GenBank accession numbers

Species	Isolate number	Host	Origin	Accession
Fusarium graminearum complex	MAFF 101032[a]	Wheat	Mie, Japan	AB272111
	MAFF 101053[a]	Wheat	Hokkaido, Japan	AB272114
	MAFF 236484[a]	Wheat	Ibaraki, Japan	AB272112
	MAFF 305135[a]	Wheat	Tokushima, Japan	AB272113
	MAFF 235980	Wheat	Hokkaido, Japan	
	H3[a]	Wheat	Ibaraki, Japan	AB272110
	NIV7[a]	Wheat	Okinawa, Japan	AB272109
	ZF4	Wheat	Nanjing, China	
	ZF17	Wheat	Nanjing, China	
	ZF22	Wheat	Nanjing, China	
	ZF43[a]	Wheat	Nanjing, China	AB272107
	ZF43-6	Wheat	Nanjing, China	
	ZF43-17	Wheat	Nanjing, China	
	ZF52[a]	Wheat	Nanjing, China	AB272108
	ZF52-7	Wheat	Nanjing, China	
F. culmorum	MAFF 101144[a]	Wheat	Hokkaido, Japan	AB272118
	MAFF 101145[a]	Wheat	Iwate, Japan	AB272119
	MAFF 236454[a]	Wheat	Ibaraki, Japan	AB272115
	MAFF 236455[a]	Wheat	Ibaraki, Japan	AB272116
	MAFF 236456[a]	Wheat	Ibaraki, Japan	AB272117
F. avenaceum	MAFF 101041[a]	Wheat	Hokkaido, Japan	AB272120
	MAFF 101042[a]	Wheat	Hokkaido, Japan	AB272121
	MAFF 235547	Wheat	Ibaraki, Japan	
	MAFF 235548	Wheat	Ibaraki, Japan	
	MAFF 235734[a]	Wheat	Ibaraki, Japan	AB272122
Microdochium nivale	MAFF 101046[a]	Wheat	Hokkaido, Japan	AB272126
	MAFF 305033[a]	Rye	Ibaraki, Japan	AB272127
	Mn2-2	Wheat	Aomori, Japan	
	Mn4-2[a]	Wheat	Aomori, Japan	AB272124
	Mn4-3	Wheat	Aomori, Japan	
	Mn4-5	Wheat	Aomori, Japan	
	Mn4-6	Wheat	Aomori, Japan	
	Mn4-15[a]	Wheat	Aomori, Japan	AB272125
	Mn4-16	Wheat	Aomori, Japan	
	Mn7-3	Wheat	Aomori, Japan	
Fusarium sp.	19-1	Wheat	Aomori, Japan	
	20-1	Wheat	Aomori, Japan	
	20-2	Wheat	Aomori, Japan	
	20-3	Wheat	Aomori, Japan	
	1	Wheat	Oita, Japan	
	155	Wheat	Oita, Japan	
	156	Wheat	Oita, Japan	
	157	Wheat	Oita, Japan	
	329	Wheat	Oita, Japan	
	OB02	Wheat	Shimane, Japan	
	OB03	Wheat	Shimane, Japan	
	OB29	Wheat	Shimane, Japan	
	OB72	Barley	Shimane, Japan	
	OB73	Wheat	Shimane, Japan	
Total	49			

[a] Used for molecular phylogenetic study of ITS and 5.8S rDNA regions

Ex *Taq* reaction buffer (containing 2 mM $MgCl_2$). PCR products were first purified with a Wizard PCR Prep DNA Purification System (Promega, Madison, WI, USA), and then the products were reacted with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) using the same primers for PCR under the following conditions: 25 cycles of 96°C for 10 s, 50°C for 5 s, 60°C for 4 min. Cycle sequencing reaction products were purified by ethanol precipitation, then analyzed with an ABI PRISM 3100 automated sequencer (Applied Biosystems). The sequences obtained in this study were deposited in the DDBJ/EMBL/GenBank databases.

Sequence alignment, molecular phylogenetic analyses and species-specific primer design

DNA sequences were aligned using Clustal X v1.8 (Thompson et al. 1997). Further visual alignments were done with the program Sequence Alignment (Se-Al) Editor v.2.0 (Rambaut 2000). Phylogenetic analysis of the aligned sequences was done by distance parsimony methods. The distance matrix for the aligned sequences was calculated using Kimura's two-parameter method (Kimura 1980) and analyzed with the neighbor-joining (NJ) method (Saitou and Nei 1987), including positions with gaps as a fifth character because 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 with the program PAUP v4.0b (Swofford 2000) using the heuristic search option with 100 random stepwise-addition sequences to search for the most parsimonious tree. Bootstrap (Felsenstein 1985) values were generated with 1,000 replicate heuristic searches to estimate support for clade stability of the consensus tree using the same program. *Fusarium oxysporum* (AY928420) and *F. solani* (AY677295) from GenBank were used as outgroups in phylogenetic analyses. For increased reliability of the analyses, 17 ITS sequences from GenBank, including *F. graminearum*, *F. culmorum*, *F. avenaceum* and *M. nivale* (Table 2), were used to align our *Fusarium* species obtained from Japan. In addition, the consensus variation of nucleotides in ITS and 5.8S rDNA, β-tubulin gene and the *cytb* gene among *Fusarium* species was aligned by Clustal X v1.8 (Thompson et al. 1997) and used to design species-specific primers. The PCR condition were tested by gradient PCR using the TaKaRa PCR Thermal Cycler Dice (Otsu, Japan), and PCR products were run in 1.5% agarose gels containing 0.5 µg/ml ethidium bromide in Tris–acetate–EDTA (TAE) buffers.

Table 2 Additional *Fusarium* and *Microdochium* species selected for ITS sequence alignment and analysis, origin, and DDBJ/EMBL/GenBank accession numbers

Species	Host	Origin	Accession
F. graminearum	Sugarcane	Japan	AB250414
	Banana	Honduras	AF006344
	–[a]	France	AY188924
	–	Brazil	AY753988
	–	Poland	DQ453701
	Corn	USA	U34578
F. culmorum	–	UK	AF484956
	–	UK	AY147320
	–	UK	AY147341
	–	UK	AY147335
	–	UK	AY147362
	–	Poland	DQ453702
	–	Poland	DQ453703
	–	USA	U85535
F. avenaceum	–	France	AY188925
	–	Sweden	AY618233
M. nivale	–	USA	AF067642
F. equiseti	Wheat	Japan	AB425996

[a] – unknown

Evaluation of thiophanate–methyl resistance and determination of β-tubulin gene nucleotide sequence

Sensitivities of *Fusarium* and *Microdochium* isolates to thiophanate–methyl (Nippon/Soda, Japan) were tested using mycelial growth assay. Each isolate was cultured on PDA plates at 25°C for 5 days. Mycelial disks, 4 mm in diameter, were cut from the margins of colonies and transferred onto PDA plates amended with 0, 1, 10, 100, and 1,000 mg/l active ingredient (a.i.) of thiophanate–methyl. 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 measured and the minimum inhibitory concentration (MIC) of the fungicide was determined. The MIC value for the sensitive phenotype was $<$10 mg/l, between 10 mg/l and 100 mg/l for the less sensitive phenotype, and between 100 mg/l and 1,000 mg/l for the high resistance phenotype, and $>$1,000 mg/l for the very high resistance phenotype. The β-tubulin gene fragment, including amino acid codons 198 and 200, was amplified from each isolate by FU-tubulin 3 and FU-tubulin 2 primers, and its nucleotide sequence was determined. The PCR conditions and nucleotide sequencing followed as those described.

Results

Molecular phylogenetic analyses of *Fusarium* isolates based on ITS and 5.8S rDNA

The amplified sequences for ITS and 5.8S rDNA regions of *F. graminearum* complex and *F. culmorum* were 545 bp long. Those of *F. avenaceum* and *M. nivale* were 561 and 556 bp, respectively. The three *Fusarium* species are genetically closely related, in particular, only two nucleotide subtitutions were observed between the *F. graminearum* complex and *F. culmorum*. The three *Fusarium* species were distinct from *M. nivale* based on the differences in the ITS and 5.8S rDNA sequences (Table 3). Consequently, the aligned ITS sequences consisted of 624 characters.

Two methods were used to study the phylogenetic relationship of these four *Fusarium* species. In the MP analysis, 418 characters were constant, 66 parsimony uninformative, and 140 parsimony informative. The ITS and 5.8S rDNA phylogram has a consistency index (CI) of 0.804, a retention index (RI) of 0.911, retention consistency (RC) of 0.733 and a tree length of 327. The result indicated that the *F. graminearum*-complex and *F. culmorum* were distinct from *F. avenaceum* and *M. nivale*, and this result was supported by a high bootstrap value (Fig. 1). Similar to the MP analysis, the NJ analysis also showed that the *F. graminearum* complex was closely related to *F. culmorum* based on the sequence of ITS and 5.8S rDNA regions and supported by a high bootstrap value (100%) (Fig. 2).

Table 3 Comparison of nucleotide and deduced amino acid divergence of three regions in three species with *Fusarium graminearum* complex

Species	Divergence (%)		
	ITS-5.8S rDNA	Partial β-tubulin gene (amino acid)	Partial *cytb* gene (amino acid)
F. culmorum	0.4–1.6	0.6–1.1 (0.0)	0.0–0.7 (0.0)
F. avenaceum	10.6–10.9	11.9–12.1 (1.3–1.9)	3.8–10.5 (1.1–4.2)
Microdochium nivale	22.8–23.5	12.3–13.2 (2.6)	14.7–15.0 (7.4)

Divergence of β-tubulin and *cytb* genes

The partial β-tubulin gene fragments, 470 bp long, were amplified from these *Fusarium* species and *M. nivale*. Japanese isolates of the *F. graminearum* complex were highly homologous with *F. culmorum* isolates and only 0.6–1.1% nucleotide divergence was observed between the two species, and the homology of deduced amino acid sequences was 100%. On the other hand, the partial β-tubulin gene nucleotide sequences of *F. avenaceum* and *M. nivale* had higher divergence of 11.9–12.1 and 12.3–13.2%, respectively, than did the *F. graminearum* complex and *F. culmorum* (Table 3).

The partially amplified *cytb* gene fragments from the four species were 286 bp long, corresponding to 95 amino acids. *F. graminearum* complex isolates differed by 0.0–0.7% in their nucleotides from *F. culmorum* isolates,

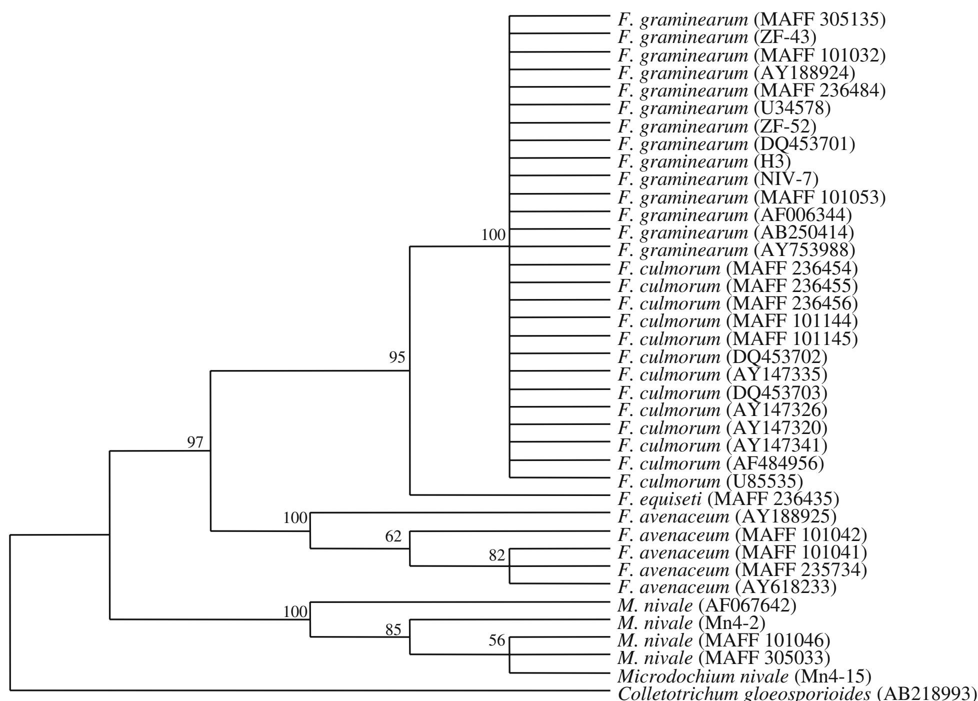

Fig. 1 The ITS and 5.8S rDNA sequence-based tree generated using most parsimony analysis. The numbers at branch node indicate the confidence values from bootstrap analysis using 1,000 replications. Outgroup: *Colletotrichum gloeosporioides*

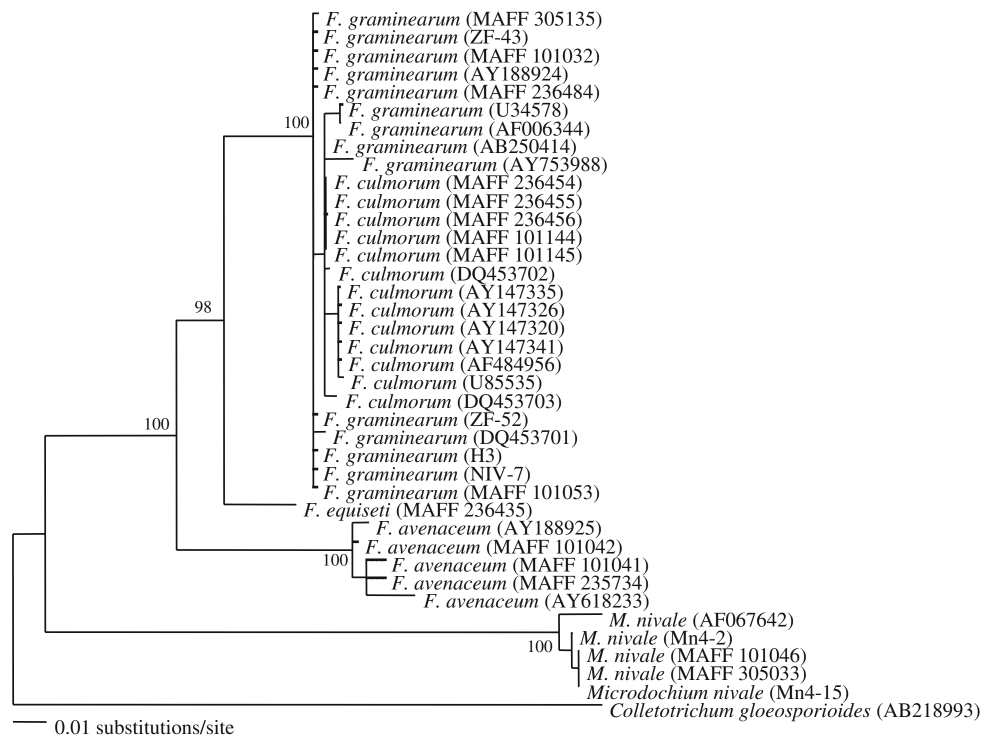

Fig. 2 The ITS- and 5.8S rDNA- sequence-based tree generated using neighbor-joining analysis. The numbers at branch node indicate the confidence values from bootstrap analysis using 1,000 replications. Bootstrap values above 52% are presented. Outgroup: *Colletotrichum gloeosporioides*

but the deduced amino acid sequences were the same as *F. culmorum* isolates. *F. avenaceum* isolates had 3.8–10.5% nucleotide and 1.1–4.2% amino acid divergence from *F. graminearum*-complex isolates, while isolates of *M. nivale* had 14.7–15.0% nucleotide and 7.4% amino acid divergence from those of *F. graminearum* complex (Table 3).

Relationship of genetic variations in β-tubulin gene with thiophanate–methyl sensitivity

In the present study, the isolates of the *F. graminearum* complex and *F. culmorum* sensitive to thiophanate–methyl had the sequence GAA (Glu) at codon 198, while *F. avenaceum* and *M. nivale* isolates had GAG (Glu) (Table 4). Similarly, less thiophanate–methyl sensitive Chinese isolates of *F. graminearum* (kindly supplied by Prof. Zhou Minggou, Nanjing Agric. Univ., China) also revealed GAA (Glu) at codon 198. Sequences at codon 200 were TTT (Phe) in *F. graminearum* complex isolates, whereas those of *F. culmorum*, *F. avenaceum*, and *M. nivale* isolates were TTC (Phe). Thus, the mutation leading to the amino acid substitution at codon 198 or 200 of β-tubulin was not observed in the *F. graminearum* complex, *F. culmorum* and *F. avenaceum* isolates. In contrast, isolates very highly resistant to thiophanate–methyl, MAFF101046 and Mn4-16, and one highly resistant isolate of *M. nivale*, Mn5-11, had mutation at codon 198 (Table 4). The sequences in very highly resistant and highly resistant isolates of *M. nivale* were GCG (Ala) and AAG (Lys), respectively, both had substitution at GAG (Glu) at codon 198.

Design of specific PCR primers for distinguishing *F. graminearum* complex from *F. culmorum*

The ITS and 5.8S rDNA regions of the isolates of *F. graminearum*-complex and *F. culmorum* analyzed in this study had high sequence homology. However, two consensus nucleotide substitutions were found in the ITS1 and ITS2 regions between *F. graminearum* complex and *F. culmorum* isolates (Fig. 3). Consequently, we designed the following species-specific primers to differentiate them: forward primer: ITS1-Fg1 (5′-GGACGGCCCGCCGCAGGAACCCT-3′) and reverse primer: ITS2-Fg (5′-CGCGACGATTACCAGTAACGATG-3′) for *F. graminearum*-complex, forward primer: ITS1-Fc1 (5′-GGACGGCCCGCCGCAGGAACCTT-3′) and reverse primer: ITS2-Fc1 (5′-CGCGACGATTACCAGTAACGATA-3′) for *F. culmorum*. PCR conditions were as follows: initial denaturation at 95°C for 2 min, 30 cycles of 95°C for 30 s, 68°C for 30 s, 72°C for 2 min; and final extension at 72°C for 5 min. Amplifications were done using 20 µl PCR reaction mixtures, containing 0.2 µM of each primer, 1 unit of Ex *Taq* DNA polymerase, and dNTP mixture (containing 250 µM of each of dNTP) and Ex Taq reaction buffer (containing 2 mM $MgCl_2$). About 350 bp were amplified, and the results from PCR indicated that the two primer

Table 4 Mutation and deduced amino acid substitutions in the partial sequence of the β-tubulin gene in isolates of the *Fusarium graminearum*-complex, *F. culmorum*, *F. avenaceum*, and *Microdochium nivale*, and different phenotypic responses to thiophanate–methyl

Species	Isolate	Response[a]	Sequence in codon (amino acid)	
			198	200
F. graminearum-complex	MAFF 101032	S	GAA (Glu)	TTT (Phe)
F. graminearum-complex	MAFF 101053	S	GAA (Glu)	TTT (Phe)
F. graminearum-complex	MAFF 236484	S	GAA (Glu)	TTT (Phe)
F. graminearum-complex	MAFF 305135	S	GAA (Glu)	TTT (Phe)
F. graminearum-complex	H3	S	GAA (Glu)	TTT (Phe)
F. graminearum-complex	ZF4	LS	GAA (Glu)	TTT (Phe)
F. graminearum-complex	ZF22	S	GAA (Glu)	TTT (Phe)
F. graminearum-complex	ZF43	S	GAA (Glu)	TTT (Phe)
F. graminearum-complex	ZF43-6	S	GAA (Glu)	TTT (Phe)
F. graminearum-complex	ZF43-17	LS	GAA (Glu)	TTT (Phe)
F. graminearum-complex	ZF52	LS	GAA (Glu)	TTT (Phe)
F. graminearum-complex	ZF52-7	LS	GAA (Glu)	TTT (Phe)
F. culmorum	MAFF 101144	S	GAA (Glu)	TTC (Phe)
F. culmorum	MAFF 101145	S	GAA (Glu)	TTC (Phe)
F. culmorum	MAFF 236454	S	GAA (Glu)	TTC (Phe)
F. culmorum	MAFF 236455	S	GAA (Glu)	TTC (Phe)
F. culmorum	MAFF 236456	S	GAA (Glu)	TTC (Phe)
F. avenaceum	MAFF 101041	S	GAG (Glu)	TTC (Phe)
F. avenaceum	MAFF 101042	S	GAG (Glu)	TTC (Phe)
F. avenaceum	MAFF 235547	S	GAG (Glu)	TTC (Phe)
F. avenaceum	MAFF 235548	S	GAG (Glu)	TTC (Phe)
F. avenaceum	MAFF 235734	S	GAG (Glu)	TTC (Phe)
M. nivale	MAFF 101046	VHR	GCG (Ala)	TTC (Phe)
M. nivale	MAFF 305033	S	GAG (Glu)	TTC (Phe)
M. nivale	Mn4-3	S	GAG (Glu)	TTC (Phe)
M. nivale	Mn4-4	S	GAG (Glu)	TTC (Phe)
M. nivale	Mn4-5	S	GAG (Glu)	TTC (Phe)
M. nivale	Mn4-8	S	GAG (Glu)	TTC (Phe)
M. nivale	Mn4-13	S	GAG (Glu)	TTC (Phe)
M. nivale	Mn4-16	VHR	GCG (Ala)	TTC (Phe)
M. nivale	Mn5-11	HR	AAG (Lys)	TTC (Phe)

[a] *S* sensitivity (MIC $\leq$ 10 ppm), *LS* less sensitivity (10 ppm < MIC $\leq$ 100 ppm, *HR* high resistance (100 ppm < MIC $\leq$ 1,000 ppm), *VHR* very high resistance (1,000 ppm < MIC)

pairs were successfully used for distinguishing the *F. graminearum* complex from *F. culmorum* (Fig. 4). Then the *Fusarium* isolates obtained from Aomori, Oita, and Shimane Prefectures in Japan were examined using these primers. The results indicated that two thiophanate–methyl-resistant isolates, 19-1 and 20-2, from Aomori were *F. culmorum* and that the other two isolates, 20-1 and 20-3, were part of the *F. graminearum* complex. Moreover, two isolates, 1 and 329, from Oita and isolates OB02, OB03, 0B29, OB72, and OB73 from Shimane were *F. graminearum* complex (Fig. 5). The PCR results were further confirmed by sequencing the ITS-5.8S rDNA regions of these isolates and subjected to a Blast search of GenBank. In addition, we also used these species-specific primer pairs to test other *Fusarium* species, including *F. tricinctum*, *F. equiseti*, *F. sporotrichioides*, and *M. nivale*, but the result indicated that these primer pairs did not amplify the ITS and 5.8S rDNA regions in these species (data not shown).

Discussion

The molecular sequence of ITS-5.8S rDNA and the partial sequence of β-tubulin and of cytochrome *b* (*cytb*) genes indicated that *F. graminearum* complex were closely related

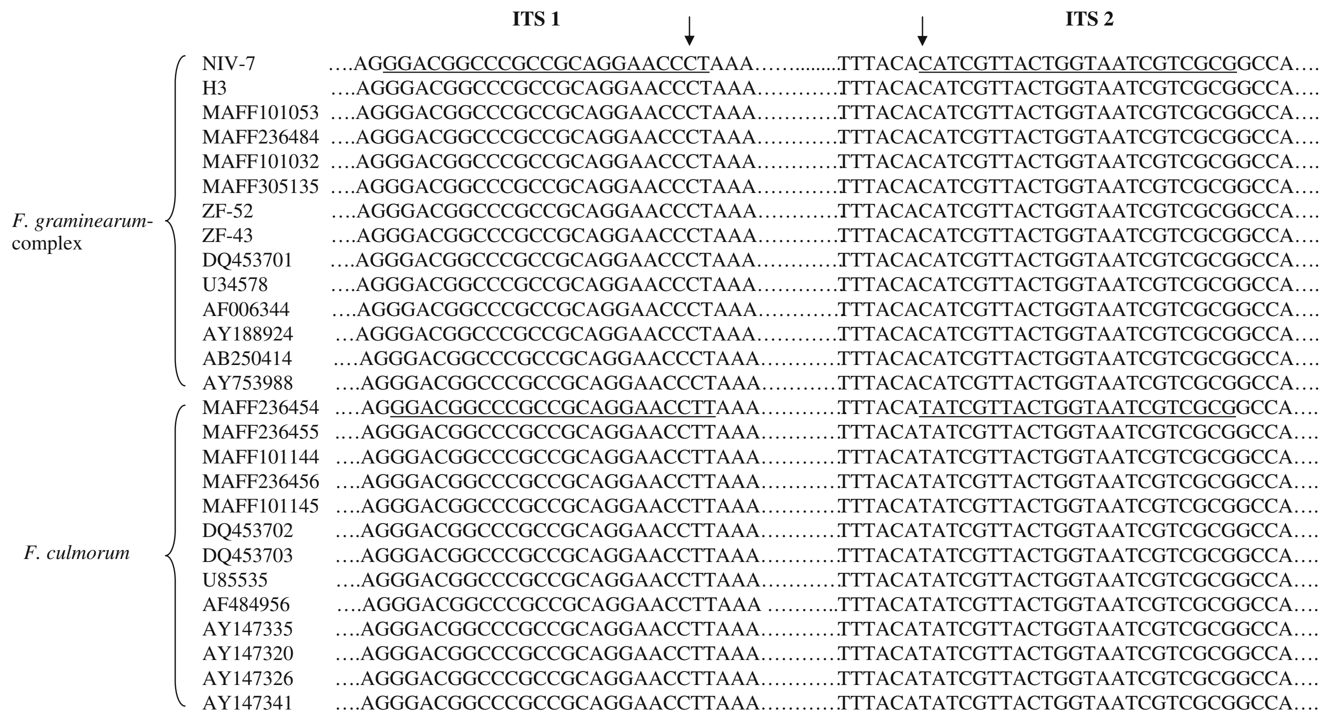

Fig. 3 The nucleotide sequence variation at ITS1 and ITS2 regions in *Fusarium graminearum*-complex and *F. culmorum*. The arrows indicate the change of bases. Nucleotides with underline are used to design the species-specific primers for identifying the *F. graminearum*-complex and *F. culmorum*

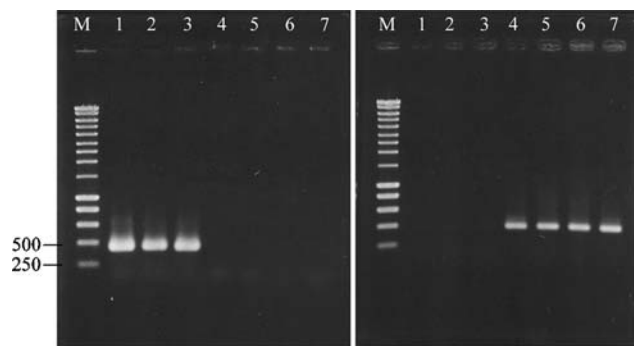

Fig. 4 The polymerase chain reaction (PCR) primer pairs ITS1-Fg1 + ITS2-Fg (*left*) and ITS1-Fc1 + ITS2-Fc1 (*right*) for identifying *Fusarium graminearum* (*line 1–3*) and *F. culmorum* (*line 4–7*). *M* DNA ladder marker, *Lane 1* NIV7, *Lane 2* H3, *Lane 3* MAFF101032, *Lane 4* MAFF236454, *Lane 5* MAFF236455, *Lane 6* MAFF236456, and *Lane 7* MAFF101144

with *F. culmorum* genetically, but distinct from *F. avenaceum* and *M. nivale* isolates. Molecular phylogenetic analysis of ITS and 5.8S rDNA regions showed that *F. graminearum* complex and *F. culmorum* were placed in the same clade, and *F. avenaceum* was phylogenetically closer to the *F. graminearum* complex and *F. culmorum* than to *M. nivale*.

In the present study, the sequence variations of ITS and 5.8S rDNA regions between the *F. graminearum* complex and *F. culmorum* were low. Thus, *F. graminearum* complex and *F. culmorum* could not be separated in the

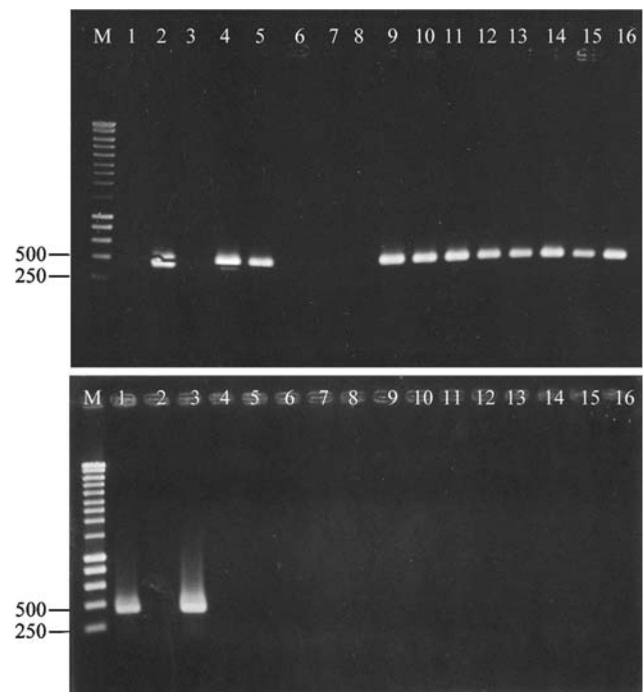

Fig. 5 The polymerase chain reaction (PCR) primer pairs ITS1-Fg1 + ITS2-Fg (*up*) and ITS1-Fc1 + ITS2-Fc1 (*down*) for identifying *Fusarium* isolates from Aomori Prefecture (*Lane 1–4*), Oita Prefecture (*Lane 5–9*) and Shimane Prefecture (*Lane 10–14*). *Lane 15* (ZF52) and *16* (ZF4) are *F. graminearum* isolates from China. *M* DNA ladder marker

phylogenetic tree based on the sequence of this region. Similarly, several researchers indicated that the ITS and 5.8S rDNA regions were not suitable to identify the *F. graminearum* complex and *F. culmorum* because of their low polymorphic nucleotide sequences (Bateman et al. 1996; Edel et al. 1997; Schilling et al. 1996; Yli-Mattila et al. 2004a). Although Schilling et al. (1996) mentioned that insertion/deletions of a few bases existed the between *F. graminearum* complex and *F. culmorum*, the sequence variations were not polymorphic enough to identify the two species. However, two fixed nucleotide substitutions were found in the ITS1 and ITS2 regions between *F. graminearum* complex and *F. culmorum*. We aligned the ITS sequences in Japanese isolates of *F. graminearum* complex and *F. culmorum* and compared with isolates of the *F. graminearum* complex and *F. culmorum* from different countries. The alignment indicated that the two fixed nucleotide substitutions also existed between the *F. graminearum*-complex and *F. culmorum* isolates obtained in different countries.

In the present study, the primers designed for the two fixed nucleotide differences were effective in specifically identifying Japanese isolates of the *F. graminearum* complex and *F. culmorum*. The results revealed that a single nucleotide polymorphism (SNP) is useful for the differential detection of genetically close isolates. However, the SNP assay has been generally used to diagnose fungal species not to quantitate them. Recently, new methods were developed to quantitatively analyze a population of *Fusarium* species (Bluhm et al. 2002; Nicholson et al. 1998, 2003; Waalwijk et al. 2004). Thus, our species-specific primers may be useful for quantifying species populations. Bushnell et al. (2003) indicated that *Fusarium* species causing FHB could invade different plant tissues; however, we did not try to isolate *Fusarium* species from various plant tissues. We will do such isolations in the future. As we did with the ITS and 5.8S rDNA regions, we aligned the nucleotide sequences of partial β-tubulin gene and only a few nucleotide difference existed between the *F. graminearum* complex and *F. culmorum*. O'Donnell et al. (2004) indicated that the fixed nucleotide characters of β-tubulin gene existed in the *F. graminearum* complex; however, the partial β-tubulin gene sequences that we amplified (400 bp) had the same nucleotides as *F. graminearum* s. str., and we had difficulty in further identifying our *F. graminearum* complex isolates in this study. While *F. avenaceum* and *M. nivale* were distinct from the *F. graminearum* complex and *F. culmorum*, molecular phylogenetic analysis of the β-tubulin gene showed that the *F. graminearum* complex and *F. culmorum* located in the same clade as found in the analysis of ITS regions (data not shown). The results revealed high consensus between the *F. graminearum* complex and *F. culmorum* in the ITS region and the β-tubulin gene.

According to O'Donnell et al. (2004) and Starkey et al. (2007), the Japanese *F. graminearum* complex isolates belong to *F. graminearum* s. str., *F. asiaticum* or *F. vorosii*; however, we did not check whether the *F. graminearum*-complex isolates belonged to *F. graminearum* s. str. or to *F. asiaticum*. It is important to establish a way to distinguish *F. graminearum* s. str. and *F. asiaticum* from *F. vorosii* and to monitor the population in the field in future. Similarly, Nicholson et al. (1996) indicated that *M. nivale* causing wheat head blight included two varieties, *M. nivale* var. *nivale* and *M. nivale* var. *majus*. These two varieties were recently elevated to species status based on variations in the EF-1 alpha gene sequences (Glynn et al. 2005). *M. nivale* var. *nivale* is mainly found in Hokkaido, Japan, but we did not check these sequences for our Japanese *M. nivale* isolates. Doing so will be the focus of future research.

In the present study, we also examined the relationship of thiophanate–methyl resistance with the sequence variations in the β-tubulin gene. However, no mutations were found at amino acid codons 198 and 200 in the thiophanate–methyl-resistant isolates of the *F. graminearum* complex we have tested so far. This phenomenon was also reported for benzimidazole-resistant isolates of *F. sambucinum* Fuckel (=*Gibberella pulicaris* (Fr.) Sacc.) (Kawchuk et al. 2002) and *F. graminearum* (Li et al. 2003). In contrast to *Fusarium* species, thiophanate–methyl-resistant isolates of *M. nivale* had a mutation at codon 198 in the β-tubulin gene. The result revealed that in highly resistant and very highly resistant isolates of *M. nivale*, Glu (GAG) was substituted with Lys (AAG) and Ala (GCG), respectively. However, there was no nucleotide change at codon 200. This result indicated that the mutation at codon 198 was responsible for high resistance and very high resistance to thiophanate–methyl in *M. nivale* isolates.

The *cytb* gene sequence has been used to study the phylogeny of fungi (Biswas et al. 2001) due to their high sequence diversity. However, the partial *cytb* gene sequence that we analyzed indicated that the *F. graminearum* complex was closely related to *F. culmorum* as did the ITS-5.8 S rDNA regions and the β-tubulin gene. In the present study, only a few isolates of the *F. graminearum* complex and *F. culmorum* were analyzed. It is, therefore, necessary to analyze more isolates in the future to check the stability of the species identification system developed in this study.

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