

Incidence, Molecular Characteristics and Pathogenicity of *Gibberella fujikuroi* Species Complex Associated with Rice Seeds from Asian Countries

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Abstract *Gibberella fujikuroi* species complex (GFSC) was isolated from rice (*Oryza sativa* L.) seed samples from ten Asian countries and investigated for incidence of GFSC, molecular characteristics, and pathogenicity. Regardless of geographic origin, GFSC was detected with incidences ranging from 3% to 80%. Four species, *Fusarium fujikuroi*, *F. concentricum*, *F. proliferatum*, and *F. verticillioides*, were found to show an association with rice seeds, with *F. fujikuroi* being the predominant species. In phylogenetic analyses of DNA sequences, no relationship was found between species, isolates, and geographic sources of samples. Unidentified fragments of the β -tubulin gene were observed in ten isolates of *F. fujikuroi* and *F. verticillioides*. With the exception of three isolates of *F. fujikuroi*, *F. fujikuroi*, *F. proliferatum*, and *F. verticillioides* were found to have *FUM1* (the fumonisin biosynthetic gene); however, *FUM1* was not found in isolates of *F. concentricum*. Results of pathogenicity testing showed that all isolates caused reduced germination of rice seed. In addition, *F. fujikuroi* and *F. concentricum* caused typical symptoms of bakanae, leaf elongation and chlorosis, whereas *F. proliferatum* and *F. verticillioides* only caused stunting of seedlings. These findings provide insight into the characteristics of GFSC associated with rice seeds and might be helpful in development of strategies for management of bakanae.

Keywords *Gibberella fujikuroi* species complex, Incidence, Molecular characteristics, Pathogenicity, Rice seed

Rice (*Oryza sativa* L.), a staple crop for many people worldwide, is subject to a number of fungal diseases. Among the major diseases affecting rice, 'bakanae' is widely distributed in all rice-growing countries and causes significant yield losses of up to 50% [1]. Occurrence of bakanae has shown a recent increase as environmentally-friendly cultivation of rice increases. Despite continuous

development of new chemical and sterilization methods, control of this disease remains difficult. The causal organism of bakanae was first identified as *Fusarium heterosporum* Nees by Hori in 1898, and its teleomorph was described as *Lisea fujikuroi* by Sawada [2]. This organism was later transferred to *Gibberella* as *G. fujikuroi* (Sawada) Ito [3]. *G. fujikuroi* (Sawada) Ito is a polytypic species complex with anamorphs in *Fusarium* species from section *Liseola* [4, 5]. Some members of *G. fujikuroi* species complex (GFSC) (*F. fujikuroi* Nirenberg [6], *F. proliferatum* (Mats.) Nirenberg [6, 7] and *F. verticillioides* (Sacc.) Nirenberg [6, 7]), have been found to show an association with bakanae [6, 7]. However, it is unclear whether all three species are associated with symptoms of bakanae or if *F. proliferatum* and *F. verticillioides* are present only as saprophytes [8].

Studies of the incidence, genetic diversity, and pathogenicity of GFSC in rice plants and seed materials have been conducted in several countries. The incidence and severity of bakanae were investigated in Asian countries, including Malaysia [9], Indonesia [9], Pakistan [10], and India [11]. Genetic diversity, pathogenicity, and toxigenicity of GFSC strains isolated from rice seeds from nine Asian and African countries have been reported [8]. A recent study also

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investigated the species composition and genetic diversity of putative fumonisin-producing GFSC associated with rice seeds collected in Korea [12].

Species identification of *Fusarium* based on morphological characteristics is complicated and time-consuming [4]. In addition, species of GFSC have similar morphologies and differentiation of *F. fujikuroi* from *F. proliferatum* morphologically is almost impossible. Therefore, molecular identification of fungal species by DNA sequence has been used to support morphological identification of *Fusarium* species. In addition, phylogenetic analysis of DNA sequences has been used for differentiation and evaluation of the genetic relationship among closely related *Fusarium* species. The internal transcribed spacer (ITS) regions of nuclear ribosomal RNA gene repeats have commonly been applied; however, many fusaria within the *Gibberella* clade possess non-orthologous copies of ITS2, which can lead to incorrect phylogenetic inference [13, 14]. The translation elongation factor-1 α gene (TEF) is regarded as a good tool for use in species identification for *Fusarium* because it is highly informative at the species level and non-orthologous copies of the gene have not been detected in the genus [15]. The histone H3 (H3) and β -tubulin (BT) genes were also used for species identification of *Fusarium*.

The most evident symptom of bakanae is chlorotic and abnormally elongated leaves induced by gibberellic acid (GA) produced by the pathogens [16]. The angle of the leaf with the stem was found to be wider in diseased seedlings than in healthy seedlings [17]. However, not all infected seedlings have symptoms of bakanae, and they are sometimes only stunted or normal in appearance. Currently, it is still not clear which species are associated with the various symptoms. Each species of GFSC has a specific profile of mycotoxins, including fumonisin. A gene cluster of fumonisin biosynthesis (*FUM*) was identified and characterized in *F. verticillioides* [18], revealing that fumonisin non-producing species lack the *FUM* genes [19]. Nevertheless, little information is available regarding the incidence, pathogenicity to rice, and mycotoxin profiles of *F. concentricum*, a member of GFSC.

The current study was conducted using rice seed samples from Asian countries in order to: 1) determine infection status with GFSC, 2) identify species of GFSC isolates, 3) characterize molecular traits of the isolates, 4) determine the presence of the *FUM1* gene in the isolates, and 5) evaluate the pathogenicity of the isolates on rice.

MATERIALS AND METHODS

Seed samples and isolation of GFSC. A total of 110 seed samples of rice (*Oryza sativa* L.) from ten Asian countries, including 45 samples from Korea were used for isolation of the GFSC. Seed samples had been stored at 4°C for 3~28 years. A total of 100 seeds of each sample were placed on two layers of filter paper (Advantec No. 2; Advantec, Dublin, CA, USA) moistened with sterile distilled water in a 9-cm Petri dish and then incubated at 25°C

under near ultra violet light for seven days. White and fluffy mycelia were removed from the seed surface under a stereomicroscope and transferred to potato dextrose agar (PDA) medium supplemented with 100 mg/L of streptomycin sulfate. The samples were then incubated at 25°C under the same light conditions for seven days; single spores were then isolated from suspected colonies and incubated at 25°C in the dark for seven days. Isolates grown on slant PDA media were stored at 4°C for further investigation.

DNA extraction, PCR amplification, and sequencing.

Mycelia grown in potato dextrose broth medium were removed and transferred to a 1.5-mL Eppendorf tube. After lyophilization, the dry mycelia were ground to fine powder using TissueLyser II (Qiagen, Hilden, Germany) with 7-mm stainless beads. Genomic DNA was extracted from the mycelia powder using a DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. The DNA concentration was adjusted to 20 ng/ μ L and 1 μ L of DNA template was used for PCR amplification. The incidence of GFSC was determined by PCR using the Gib2-F (5'-GAGGCGCGGTGTCGGTGTGCTTG-3') and Fgc-R (5'-CTCTCATATACCCTCC G-3') primer set [20], which specifically detects strains of GFSC. Amplification reactions of three genes, TEF, H3, and BT, were performed for subsequent sequence analyses. Briefly, reactions were performed in 20 μ L of *AccuPower* PCR premix (Bioneer, Daejeon, Korea), composed of 250 μ M dNTP, 10 mM Tris-HCl (pH 9.0), 30 mM KCl, 1.5 mM MgCl₂, and 1 unit of *Top* DNA polymerase. Each gene was partially amplified using the specific primer sets as described previously: EF-1 (5'-ATGGGTAAGGAGGACGACAAGAC-3') and EF-2 (5'-GGAAGTACCAGTGATCATGTT-3') for TEF [12], H3-1a (5'-ACTAAGCAGACCGCCCGCAGG-3') and H3-1b (5'-GCGGGCGAGCTGGATGTCTT-3') for H3 [21], and Bt2a (5'-GGTAACCAAATCGGTGCTGCTTTC-3') and Bt2b (5'-ACCCTCAGTGTAGTGACCCTTGGC-3') for BT [21]. The PCR products were then purified using a *HiGene* PCR Purification Kit (Solgent, Daejeon, Korea) according to the manufacturer's instructions; then, they were directly sequenced in both directions using the same primers. DNA sequences were edited using SeqMan Pro (DNASTAR Inc., Madison, WI, USA) and the consensus sequences were used for species identification. The *FUM1* gene was amplified using the rp32 (5'-ACAAGTGTCTTGGGGTCCAGG-3') and rp33 (5'-GATGCTCTTGAAGTGG CCTACG-3') primers as described by Procter *et al.* [18].

Molecular characterization and phylogenetic analysis.

The DNA sequences of the TEF, H3, and BT genes were analyzed for species identification of GFSC isolates. Sequences were then compared with other sequences found in NCBI database (<http://blast.ncbi.nih.gov>) and Fusarium-ID (<http://isolate.fusariumdb.org>) [15] using Clustal W with a gap opening penalty of 3.0 and a gap extension penalty of 1.8. The neighbor-joining (NJ) method was used for phylogenetic

analysis. For tree construction, the combined sequences were analyzed using the Tamura-Nei model as a nucleotide substitution model, including transitions and transversions with MEGA 5.1. The reliability of the NJ tree was estimated using the bootstrap method with 1,000 replications. Sequences of 12 strains of Asian and African clades of GFSC established by O'Donnell *et al.* [14] were used as references: NRRL13566 and KACC44004 (*F. fujikuroi*), NRRL25181 and NRRL26794 (*F. concentricum*), NRRL22944 and NRRL53578 (*F. proliferatum*), NRRL25226 (*F. mangiferae* Britz, M. J. Wingf. & Marasas), NRRL13999 [*F. sacchari* (E. J. Butler & Hafiz Khan) W. Gams], NRRL22172 (*F. verticillioides*), NRRL13308 (*F. acutatum* Nirenberg & O'Donnell), NRRL22045 (*F. thapsinum* Klittich, J. F. Leslie, P. E. Nelson & Marasas), and NRRL25446 (*F. brevicatenulatum* Nirenberg, O'Donnell, Kroschel & Andrianaivo). The tree was rooted with NRRL34079 (*F. graminearum* Schwabe). The partial BT sequence of *Ophiocordyceps sinensis* (Berk.) G. H. Sung, J. M. Sung, Hywel-Jones & Spatafora (accession No. JX968023) from the NCBI GenBank was used for analysis of the 360-bp fragments of BT.

Pathogenicity tests. Pathogenicity tests were performed using seed inoculation assays. Seeds of the Chucheongbyeon rice cultivar were tested for the presence of any seed-borne *Fusarium* spp. by incubation on Komada's medium. Briefly, seeds were sterilized by soaking in 62°C water for 10 min and then air-drying on sterile filter paper in a flow cabinet; then, seeds found to be *Fusarium*-free were used for inoculation of strains. Fungal inoculum was prepared for each strain from single spore cultures. The concentration of the inoculum suspension was determined using a hemocytometer and adjusted to 1×10^6 spores/mL with sterile water. Thirty seeds were soaked in 10 mL of the inoculum suspension in a 50-mL conical tube with gentle shaking for 20 hr at room temperature. Control seeds were soaked in sterile water instead of the inoculum suspension. Inoculated and control seeds were then placed on solidified MS basal medium in a glass test tube with a diameter of 30-mm and height of 200-mm (four tubes per strain/five seeds per tube) and incubated at 28°C under alternating 12-hr fluorescent light/12-hr darkness for 14 days. Seven days after incubation, the number of germinated seeds was counted. At seven and 14 days after incubation, the seedlings were observed for symptoms of bakanae, slender and chlorotic leaves, elongated seedlings, leaves with wide angles between stems, and withered seedlings. Analysis of variance and Duncan's multiple range tests ($p < 0.05$) in each species were performed using R version 2.15.2 [22].

RESULTS

Infection of rice seeds with GFSC and molecular identification of isolates. The infection status of rice seeds with GFSC was examined in 110 seed samples from ten Asian countries using the blotter method (Table 1). At

Table 1. Geographic sources of rice seed samples, number of samples tested, occurrence and incidence of *Gibberella fujikuroi* species complex (GFSC), and storage period of seed samples

Geographical origin	No. of samples tested	Occurrence ^a (%)	Incidence ^b (%)	Year of sample storage ^c
Bangladesh	5	40	5~24	28
China	8	63	3~48	8~12
India	9	44	4~13	28
Japan	14	64	5~26	7~11
Korea	45	64	4~80	6~21
Myanmar	5	100	12~45	3
Philippines	10	50	22~45	28
Russia	2	50	12	17
Taiwan	6	33	4~5	28
Uzbekistan	6	100	6~48	6

^a% of samples infected with GFSC.

^bLowest and highest % of seed infected with GFSC in a sample.

^cLongest and shortest period of the infected sample storage at 4°C and with 45% relative humidity.

least 33% of samples from each country were found to be infected with GFSC, regardless of the geographic sources of the seed samples. All samples from Myanmar and Uzbekistan and more than 50% of samples from China, Japan, the Philippines, Russia, and Korea were found to be infected with GFSC, and the incidence (% infected seeds in a sample) of GFSC varied from 3% to 80%. After molecular identification with DNA sequences of TEF and histone H3 (H3), 48 isolates of four species were finally selected (39 *F. fujikuroi*, 2 *F. concentricum* Nirenberg & O'Donnell, 4 *F. proliferatum* and 3 *F. verticillioides*) (Table 2). *F. fujikuroi* was the species found most frequently, regardless of geographic sources. However, seed samples from Myanmar were only infected with *F. proliferatum*.

Phylogenetic analysis. The phylogenetic tree constructed from combined DNA sequences of TEF, H3, and BT genes (1,283 sites with gaps) consisted of four main clades (Fig. 1). Clade I was composed of 39 *F. fujikuroi* isolates from samples from nine countries with 97% of the bootstrap value, clade II consisted of four isolates of *F. proliferatum* from samples from Myanmar and Korea with 99% of the bootstrap value, and clade III consisted of two isolates of *F. concentricum* from samples from China and Japan with 99% unity. Isolates of Clades I, II, III, NRRL25226 (*F. mangiferae*), and NRRL13999 (*F. sacchari*) were grouped into a large clade with bootstrap support of 98%. Three isolates of *F. verticillioides* were grouped into clade IV with 99% of the bootstrap values and this clade was included in a single clade with NRRL13308 (*F. acutatum*), NRRL22045 (*F. thapsinum*), and NRRL25446 (*F. brevicatenulatum*) with 48% of the bootstrap value. No relationship was observed between geographic sources of seed samples and phylogenetic position of the isolates. Of particular interest, PCR for

Table 2. Isolates of *Gibberella fujikuroi* species complex recovered from rice seed samples

Identification	Isolate	Seed sample	Geographic source	<i>FUM1</i> ^a	BT ₃₆₀ ^b	
<i>F. fujikuroi</i>	RGF-1	JPNR-1	Japan	+		
	RGF-7	JPNR-3	Japan	+		
	RGF-11	JPNR-4	Japan	nd		
	RGF-29	PHLR-3	Philippines	+		
	RGF-45	PHLR-4	Philippines	+		
	RGF-47	PHLR-11	Philippines	+	+	
	RGF-48	PHLR-12	Philippines	+		
	RGF-54	KORR-2	Korea (Suwon)	+	+	
	RGF-55	KORR-5	Korea (Miryang)	+		
	RGF-56	CHNR-9	China	+	+	
	RGF-61	BGDR-4	Bangladesh	nd		
	RGF-62	TWNR-4	Taiwan	+		
	RGF-63	JPNR-2	Japan	+		
	RGF-64	CHNR-5	China	+		
	RGF-65	RUSR-1	Russia	+		
	RGF-67	INDR-1	India	+		
	RGF-69	BGDR-1	Bangladesh	+		
	RGF-77	THAR-3	Thailand	+		
	RGF-78	THAR-4	Thailand	+		
	W1	KORR-21	Korea (Dalseong)	+		
	W4	KORR-37	Korea (Gangreung)	+		
	W10	KORR-8	Korea (Goheung)	+		
	W11	KORR-13	Korea (Gongju)	+		
	W17	KORR-10	Korea (Namhae)	+		
	W18	KORR-28	Korea (Danyang)	+		
	W19	KORR-23	Korea (Dalseong)	+		
	W24	KORR-3	Korea (Bonghwa)	+		
	W25	KORR-40	Korea (Sinan)	+	+	
	W26	KORR-30	Korea (Samcheok)	+		
	W28	KORR-16	Korea (Seocheon)	+		
	W29	KORR-11	Korea (Sunchang)	+	+	
	W30	KORR-39	Korea (Wando)	+		
	W31	KORR-41	Korea (Sinan)	+		
	W34-1	KORR-31	Korea (Yeongweol)	+		
	W34-2	KORR-31	Korea (Yeongweol)	+	+	
	W36	KORR-19	Korea (Jangseong)	+		
	W37	KORR-24	Korea (Daegu)	nd	+	
	W44	KORR-36	Korea (Hoengseong)	+		
	W45	KORR-38	Korea (Hoengseong)	+		
	<i>F. concentricum</i>	RGF-33	JPNR-5	Japan	nd	
		RGF-36	CHNR-6	China	nd	
	<i>F. proliferatum</i>	RGF-16	MMRR-1	Myanmar	+	
		RGF-18	MMRR-2	Myanmar	+	
		RGF-22	MMRR-3	Myanmar	+	
		RGF-31	KORR-4	Korea (Miryang)	+	
<i>F. verticillioides</i>	RGF-37	JPNR-1	Japan	+	+	
	RGF-39	UZBR-5	Uzbekistan	+	+	
	RGF-53	KORR-1	Korea (Eumseong)	+	+	

Species identification was based on DNA sequences of translation elongation factor-1 α , histone H3, and β -tubulin genes.

^a '+' indicates that *FUM1* was detected, 'nd' indicates that the gene was not detected.

^b '+' indicates that 360-bp fragments were detected.

amplification of the BT gene revealed that seven isolates of *F. fujikuroi* and three isolates of *F. verticillioides* had an additional fragment with a size of 360 bp. Sequence analysis of the fragments (302 sites with gaps) showed that those of *F. verticillioides* and *F. fujikuroi* were highly homologous

(Fig. 2). The sequence identities of the additional fragments to the original BT fragments (291 bp) were 74~76% in *F. fujikuroi* and 68~69% in *F. verticillioides*. The greatest similarity was observed in the partial BT gene of *Ophicordyceps sinensis*, with identity of 83%.

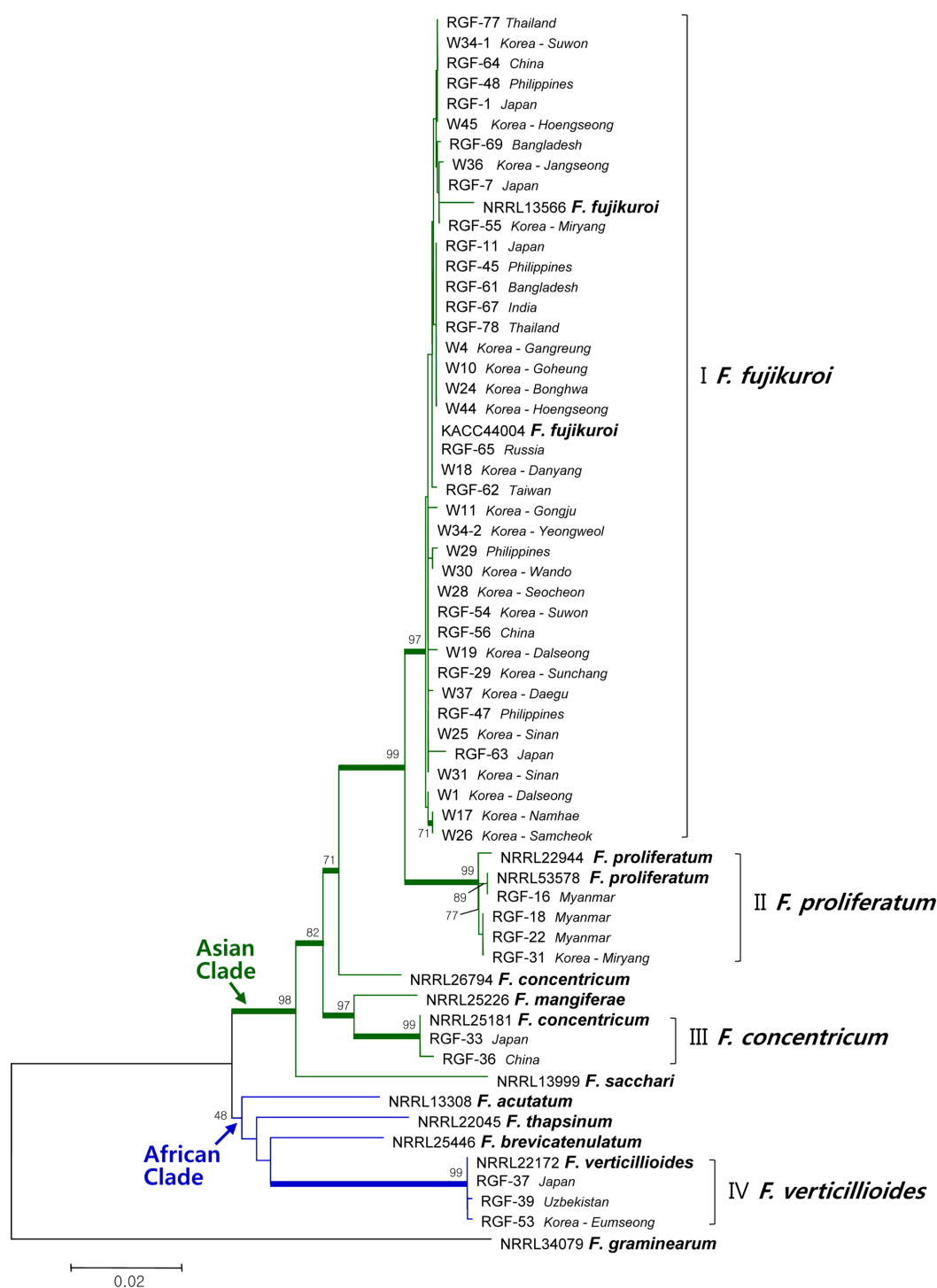


Fig. 1. Neighbor-Joining tree based on combined DNA sequences of translation elongation factor-1 α , histone H3, and β -tubulin of *Gibberella fujikuroi* species complex isolated from rice seed samples. The bootstrap values based on 1,000 replications are indicated as percentages in the internodes. Thick branches are supported by bootstrap values > 70%. The tree is rooted with *Fusarium graminearum* (NRRL 34079).

Distribution of the *FUM1* gene. Most isolates were found to contain the *FUM1* gene (Table 1), and the size of the amplified PCR fragments was approximately 680 bp. All isolates of *F. proliferatum* and *F. verticillioides* were found to contain the gene. In addition, 36 isolates of *F.*

fujikuroi had specific fragments that encompassed all isolates of this organism except RGF-61, RGF-11, and W37. The size of isolates of *F. concentricum* (RGF-33 and RGF-36) was approximately 430-bp fragments instead of 680-bp fragments.

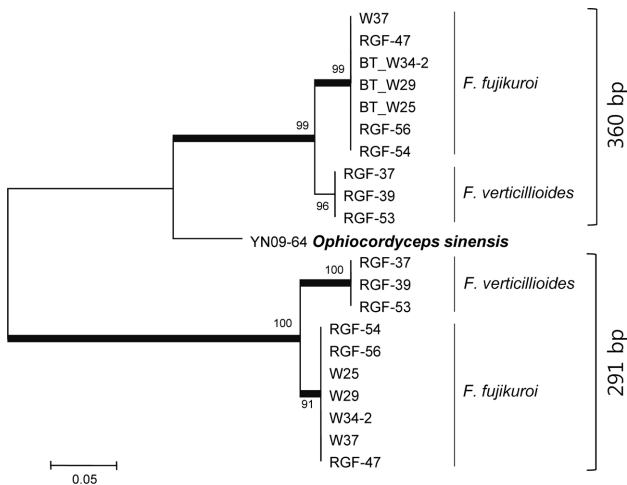


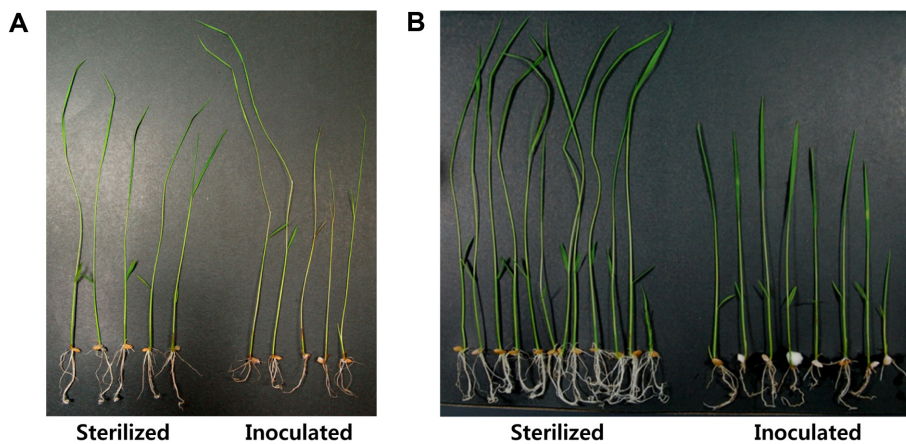
Fig. 2. Neighbor-Joining tree based on DNA sequences of β -tubulin in ten isolates of *Fusarium fujikuroi* and *F. verticillioides*. β -Tubulin sequences with sizes of 360 bp and 291 bp from the same isolates were compared. The bootstrap values based on 1,000 replications are indicated as percentages in the internodes. Thick branches are supported by bootstrap values > 70%. YN09-64 had the β -tubulin sequence with maximum homology to the 360-bp fragment.

Pathogenicity. Compared with the control treatment, all isolates examined inhibited seed germination (Fig. 3),

with germination rates of 67.0% for *F. fujikuroi*, 52.5% for *F. concentricum*, 81.3% for *F. proliferatum*, and 73.8% for *F. verticillioides*. However, results of ANOVA and Duncan's multiple range tests showed no significant differences. Isolates of *F. fujikuroi* and *F. concentricum* induced leaf chlorosis, slender leaves and/or elongation of seedlings (Fig. 3A and 3C). Overall, a wide angle between leaves and stems was observed in 2.6% of rice seedlings inoculated with isolates of *F. fujikuroi* (Fig. 3C), while isolates of *F. proliferatum* and *F. verticillioides* showed no symptoms of leaf chlorosis, slender leaves, or elongation, however, they did show stunted seedlings with a mean of 70.0% and 48.8%, respectively (Fig. 3B and 3C).

DISCUSSION

The results of this study demonstrate the importance of GFSC associated with rice seeds in Asian countries, including Korea. Bakanae is primarily seed-borne, and high levels of its incidence in rice seeds have been reported in recent studies. In the current study, the occurrence and incidence of GFSC in rice seeds was found to vary independently of sample sources and storage period of seeds. Seed samples dried to 15% seed moisture content were stored at 4°C in air-tight containers; therefore, they had little chance of being contaminated during storage. It is notable that GFSC survived in dry seeds for up to 28



Species	Mean of germination (%)	Means of number of				
		Chlorotic and slender leaves (%)	Elongated seedling (%)	Leaves with wide angle (%)	Withered seedling (%)	Stunted seedling (%)
<i>F. fujikuroi</i>	67.0 ab	24.7 ab	15.4 a	2.6 a	26.7 a	0.6 c
<i>F. concentricum</i>	52.5 b	45.0 a	30.0 a	0 a	0 a	0 b
<i>F. proliferatum</i>	81.3 a	0 b	0 a	0 a	1 a	70.0 a
<i>F. verticillioides</i>	73.8 ab	0 b	0 a	0 a	0 a	48.8 a

Means were statistically compared using ANOVA and Duncan's new multiple range test for value ($\alpha=0.05$). Means with the same letters were not significantly different.

Fig. 3. Pathogenicity of *Gibberella fujikuroi* species complex isolates to rice. Leaf elongation, chlorosis, and wide angle between leaf and stem in rice seedlings inoculated with isolates of *Fusarium fujikuroi* or *F. concentricum* (A), stunted seedlings inoculated with isolates of *F. proliferatum* or *F. verticillioides* (B), pathogenicity of each species to rice (C).

years because *F. moniliforme* (synonym of *G. fujikuroi*) was reported to be viable in seeds of *Zea mays* for eight years [23].

Three species of GFSC, *F. fujikuroi*, *F. proliferatum*, and *F. verticillioides*, which are known to be the predominant *Fusarium* species [6], were recovered from rice seed samples. In addition, *F. concentricum*, which was first described in banana (*Musa sapientum*) from Guatemala and Costa Rica and Asian brown leaf hopper (*Nilaparvata lugens*) from Korea [5], was also identified from rice seed samples from China and Japan. Among GFSC isolates, *F. fujikuroi* was the predominant species, and 92% of samples collected from Korea were infected with these species. The most abundant contamination of rice seeds with *F. fujikuroi* was previously reported in another study [12]. In addition, in the current study, seed samples from Myanmar were found to be infected with *F. proliferatum*, making this the first report of their occurrence in rice seeds from Myanmar.

Through multigene phylogenetic analysis, O'Donnell *et al.* [14] established three large clades in species of GFSC, which were designated as the "African", "American", and "Asian" based on the geographical origins of the plant hosts. The "Asian" clade is the smallest of the three GFSC clades. All isolates of *F. fujikuroi*, *F. proliferatum*, and *F. concentricum* recovered in the current study were grouped into this clade together with strains of *F. mangiferae* and *F. sacchari*. The "African" clade, the largest of the three clades, includes the agriculturally important pathogens, *F. verticillioides*, *F. thapsinum*, *F. acutatum*, and *F. brevicatenuatum*. Three isolates of *F. verticillioides* found in the current study were grouped into this clade, although the bootstrap value was low. Phylogenetic analysis with DNA sequences of isolates from rice seed samples found no relationship between species, isolates, and geographic sources of seed samples. Phylogenetic species recognition appeared to be the most effective method for differentiation of *F. fujikuroi* and *F. proliferatum* because they were closely related in terms of morphology and biology. Topologies of the trees from the TEF and H3 sequences were the same as that from combined sequences, whereas the tree from the original BT sequences had slightly different topology (data not shown); *F. fujikuroi*, *F. concentricum*, and *F. verticillioides* formed a large clade, however, *F. proliferatum* was separated into a distinct group in the BT tree. Grouping of *F. fujikuroi* and *F. verticillioides* was supported by 93% of bootstrap value. Additional unidentified fragments (approximately 360 bp) of the BT gene observed in ten isolates of *F. fujikuroi* and *F. verticillioides* showed sequence identities to the original BT below 76%. These findings are interesting because the BT gene is known to be non-orthologous and there is no possibility of contamination by other fungal species at any experimental stage. More detailed investigations are needed in order to determine the identity of these additional BT fragments and why *F. fujikuroi* (Asian clade) and *F. verticillioides* (African clade) are closely located in the tree of BT, different from those from TEF and H3.

Investigation of any relationship between these unidentified BT and resistance to chemicals such as benomyl is worthwhile because the BT gene was shown to confer cold-sensitive benomyl resistance to *F. moniliforme* [24]. The intraspecific variability of *F. fujikuroi* isolates was found to be very low, which was confirmed by Universally Primed-PCR analysis in the current study (data not shown).

Inhibition of seed germination is a primary symptom induced by spore inoculation of GFSC isolates in rice. Most isolates inhibited seed germination compared to the control treatment independent of species. Typical symptoms of bakanae, such as chlorotic/slender leaves and elongated seedlings are caused by most *F. fujikuroi* and *F. concentricum* isolates. In addition, *F. fujikuroi* was found to cause an increase in the angles between the rice leaf and stem, as reported by Imura [17], as well as withering of shoots. However, the pathogenicity of *F. fujikuroi* and *F. concentricum* to rice varied among isolates. These differences do not originate from the environment and nutritional status of the rice seedlings because the pathogenicity tests were performed under well-controlled conditions with MS agar media in a growth chamber. Although *F. concentricum* was determined not to present a threat to agricultural crops [25], in the current study, it induced disease symptoms in rice. Abnormal leaf elongation is known to be induced by production of GA by the pathogen. Although there have been no reports of GA production in *F. concentricum* to date, reevaluation of the effects and role of *F. concentricum* on agricultural crops, including rice, is needed. The widespread occurrence of *F. proliferatum* suggests that its role in the complex symptoms of bakanae of rice may be significant [6]. In a recent study reported by Wulff *et al.* [8], *F. verticillioides* and *F. proliferatum* isolates from rice seeds showed pathogenicity to rice; therefore, they were considered potential pathogens of rice and not merely saprophytes. However, in the current study, rice seedlings inoculated with *F. proliferatum* or *F. verticillioides* isolates only showed stunted growth and did not show typical bakanae symptoms. Conduct of a more detailed study using a large number of isolates will provide a better understanding of the relationship between symptoms of bakanae and *F. proliferatum* and *F. verticillioides*.

The *FUM1* gene is a member of the fumonisin biosynthetic gene cluster (*FUM*), which has been identified and characterized in *F. verticillioides* [18]. Fumonisin, non-producing species of *Fusarium*, lack this gene [19]. *FUM1* was detected in all isolates of *F. proliferatum* and *F. verticillioides* examined in the current study, which is reasonable because these are well known as fumonisin-producers in maize. Approximately 90% of isolates of *F. fujikuroi* examined contained *FUM1*; however, production of fumonisin in each isolate should be investigated further because *FUM1* was also detected in isolates that did not produce fumonisins [19], and most *F. fujikuroi* isolates obtained from rice seeds in Korea produced only a small amount of fumonisins [12]. Two isolates of *F. concentricum*

were found to lack *FUM1*, which was consistent with the results of a previous study showing that *F. concentricum* was a non-producer [23]. Although fumonisin production does not appear to be directly related to disease occurrence in rice, production of fumonisins might facilitate colonization of plant tissue and competition against other fungal species during infection [26]. Glenn *et al.* [27] reported that complementation of a *FUM* gene cluster deletion in *F. verticillioides* resulted in restoration of both fumonisin production and pathogenicity in maize seedlings; however, in the current study, the pathogenicity of the isolates examined was not related to *FUM1*, indicating that fumonisin production does not play a role in bakanae occurrence in rice and might depend on the host plant.

The current study provides insight into understanding the incidence, molecular characteristics, and pathogenicity of GFSC in rice seeds from Asian countries. A more detailed analysis of DNA sequences, pathogenicity, toxin production, and longevity of the pathogens in rice seeds might be helpful in development of more accurate tools for diagnosis of the disease and strategies for management of bakanae.

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