Re-classification of previously identified toxigenic Fusarium and Gibberella strains based on PCR detection using species-specific primers

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Abstract
According to the current Fusarium taxonomy, F. asiaticum and F. graminearum sensu stricto are known to cause scabby disease in Japanese wheat and barley. In this study, the previously identified F. roseum KU-117 and eight Gibberella zeae strains were re-classified based on PCR detection using species-specific primers. As a result, the strain KU-117 was identified as F. asiaticum. Of the G. zeae strains, three strains isolated from Aomori were identified as F. graminearum sensu stricto, but the remaining five strains derived from Iwate, Tokyo, Kumamoto, and Nagasaki belonged to F. asiaticum. The RAPD patterns of the G. zeae strains tested showed low genetic diversity. The results obtained here reaffirmed that F. asiaticum and F. graminearum sensu stricto are distributed regionally in Japanese crop fields.

Fusarium graminearum is responsible for Fusarium head blight (scabby disease) in wheat and barley and is well-known to produce trichothecene mycotoxins such as nivalenol (NIV) and deoxynivalenol (DON). DON and its acetyl derivatives were found in the cultural broth of a Fusarium roseum isolate. This DON-producing isolate was preserved as the strain KU-117 derived from moldy barley grains harvested in Saita, Mitoyo-gun (presently Saita-cho, Mitoyo-shi), Kagawa Prefecture in 1970, and the strain was identified as F. roseum based on the taxonomic criterion of Snyder and Hansen. Later, the strain KU-117 was re-identified as F. graminearum based on the Fusarium taxonomy of Nelson, Toussoun, and Marasas. From a field survey of wheat grains contaminated with trichothecenes by Yoshizawa et al., different strains of F. graminearum distributed in southern Japan produced either DON or NIV and led to the co-occurrence of these two trichothecenes in cereals. Based on this evidence, the perithecia of Gibberella zeae, which is the teleomorph state of F. graminearum based on the taxonomic system of Booth, were widely collected from wheat and barley fields in Japan, and eight ascospores contained in an ascus were independently isolated and tested for their production of DON or NIV. As a summary of the results, G. zeae was divided into two groups: the DON-chemotype and NIV-chemotype. Later, the naming of the chemotype was expanded widely up to toxin-producing Fusarium species such as F. graminearum, F. culmorum, and F. crookwellense (presently F. cerealis).

Based on molecular phylogenetic studies, the F. graminearum species complex was first divided into nine groups (lineage species). Since then, the F. graminearum species complex has been expanded to 16 phylogenetically distinct species, listed as follows: F. acacia-mearnsii, F. aethiopicum, F. asiaticum, F. australasicum, F. boothii, F. brasiliicum, F. cortaderiae, F. gerlachi, F. grahamii, F. louisianense, F. meridionale, F. mesoamericanum, F. F. ussurianum, F. vorosii, and Fusarium sp. NRRL34461. These species are geographically dependent and are distributed separately in Asia, Central America, North America, South America, or the Southern Hemisphere.

According to the current Fusarium taxonomy, F. asiaticum, F. graminearum sensu stricto (= F. graminearum in a strict sense), and F. vorosii belonging to the F. graminearum species complex have been reported in Japan. Morphologically, F. asiaticum and F. graminearum s.s. are very similar and difficult to distinguish from each other. Also, the teleomorph state of these two species shows an identical morphology. Therefore, it is difficult to identify F. asiaticum or F. graminearum s.s. without molecular phylogenetic analysis. In this study, the historically well-known DON-pro-
ducing strain\(^7\) and eight \textit{G. zeae} strains identified by a previous classification system\(^7\) were re-examined for the modern classification of \textit{Fusarium} species based on PCR detection using species-specific primers. (Part of this study was presented at the 74th Meeting of the Japanese Society of Mycotoxicology held at Azabu University in Kanagawa in 2014).

The \textit{F. roseum} KU-117 and eight \textit{G. zeae} strains tested are listed in Table 1. As reference strains, four \textit{Fusarium} strains were selected (Table 1). These strains were cultured on PDA plate medium at 25ºC for 5 days. DNA was extracted with a FastDNA Kit (MP Biomedicals, LLC, Ohio, USA) according to the manufacturer’s instructions. As shown in Table 2, amplification was performed using three kinds of species-specific primer pairs: histone H3, mating-type (MAT), and reductase region genes. PCR was performed according to the method of Aoki\(^{13}\). Briefly, the PCR cycle for the histone H3 gene was as follows: keeping at 95ºC for 2 min, followed by 40 cycles of 95ºC for 30 sec, 55ºC for 30 sec, and 72ºC for 30 sec. Then, a final extension of 72ºC for 5 min, and kept at 4ºC. The annealing temperature and time for MAT and reductase genes were changed from 55ºC for 30 sec to 48ºC for 45 sec. All primer pairs were tested for the detection of \textit{F. asiaticum} or \textit{F. graminearum} s.s. (Table 2). As related species, \textit{F. cerealis} and \textit{F. culmorum} (Table 1) were used as negative controls.

All sets of primer pairs\(^{13}\), shown in Table 2, were examined for the tester and reference strains. However, of these primers tested, no band was observed with the reductase gene primers to detect \textit{F. asiaticum}. To solve this problem, the primer pairs of ASred10 and ASred20 were searched for on the BLAST site. Both primers hybridized the same forward side of the reductase gene, and so these primers did not amplify the target gene. Then, the primer ASred20 was rearranged to hybridize the reverse side on the opposite gene in the reductase region and designated as ASred20R (Table 2).

Table 1 Test strain, source, and re-classification

| Test strain     | Source | Re-classification |
|-----------------|--------|-------------------|
| \textit{Fusarium roseum} |        |                   |
| KU-117          | Saita  | Kagawa            | \textit{F. asiaticum} |
| TH-4-3          | Hirosaki | Aomori         | \textit{F. graminearum} s.s. |
| TH-4-7          | Hirosaki | Aomori         | \textit{F. graminearum} s.s. |
| TH-5-8          | Hirosaki | Aomori         | \textit{F. graminearum} s.s. |
| MM-1-6          | Mizusawa | Iwate         | \textit{F. asiaticum} |
| TT-1-4          | Tachikawa | Tokyo         | \textit{F. asiaticum} |
| KK-2-1          | Kikuchi | Kumamoto        | \textit{F. asiaticum} |
| KK-2-8          | Kikuchi | Kumamoto        | \textit{F. asiaticum} |
| NS-2-7          | Unzen  | Nagasaki        | \textit{F. asiaticum} |

Reference strain
\textit{F. cerealis} (= \textit{F. crookwellense} KH-2-1)\(^{18}\) (NBRC 3285 = JCM 9874 = ATCC 96539)
\textit{F. cerealis} (= \textit{F. crookwellense}) CL-3-2
\textit{F. culmorum} (KF-98)\(^{18}\)
\textit{F. graminearum} s.s. (= \textit{G. zeae} TH-5-1)\(^{9}\) (JCM 9873)

The primer pairs of ASred10 and ASred20R worked well, and a positive band for detecting \textit{F. asiaticum} was observed (Fig. 1). Recently, it was found that the sequence of the ASred20 primer was incorrect (T. Aoki, personal communication).

As with the primers in the reductase gene (Fig. 1), the other primer pairs for histone H3 and MAT genes functioned well and selectively detected \textit{F. asiaticum} or \textit{F. graminearum} s.s. (data not shown). No non–species-specific bands were observed in this study. Therefore, these species-specific primers were effective and useful tools for the identification of \textit{F. asiaticum} or \textit{F. graminearum} s.s. (Table 2). However, a false positive result was found with the AS1F15 and H3R1-18 primers for the histone H3 gene. These primers for detecting \textit{F. asiaticum} reacted positively with the reference strain of \textit{F. culmorum} KF 98 (Fig. 2). According to Aoki’s explanation\(^{13}\), with the use of primers designed to detect \textit{F. asiaticum} in the histone H3 gene, there is a potential risk of detecting similar genes in \textit{F. culmorum}, but no false-positive result was noted in his previous study (T. Aoki, personal communication).

Randomly amplified polymorphic DNA (RAPD) analysis is a useful technique for the simple and rapid differentiation of organisms\(^{19}\). This method can also be used to detect genetic diversity\(^{15}\). To compare \textit{G. zeae} strains located in different regions, along with the strain KU-117 and two reference strains, RAPD analysis was conducted. For this study, MightyAmp™ DNA polymerase (TaKaRa Bio, Shiga) and the primer\(^{18}\) 5’-GGACTCCACG-3’ were selected. A 50-μl reaction mixture for PCR was prepared according to the manufacturer’s instructions. The MightyAmp polymerase was able to generate a RAPD pattern only at an annealing temperature of 55±1ºC, and the amplification was programmed for 2 min of initial denaturation at 98ºC, followed by 40 cycles of denaturation for 1 min at 98ºC, annealing for 1 min at 55ºC, and an extension for 1 min at 68ºC. Then, after a final extension for 7 min at 68ºC,
Table 2  Species-specific primers for *F. asiaticum* and *Fusarium graminearum* s.s.\textsuperscript{12}

| Primer                  | Sequence                  |
|-------------------------|---------------------------|
| **Fusarium asiaticum**  |                           |
| Histone H3 gene         | forward: AS1F15            |
|                         | reverse: H3R1-18           |
| MAT gene                | forward: ASmat13           |
|                         | reverse: ASmat22           |
| Reductase gene          | forward: ASred10           |
|                         | reverse: ASred20           |
|                         | reverse: ASred20R          |
| **Fusarium graminearum**|                           |
| s.s. Histone H3 gene    | forward: GR05F             |
|                         | reverse: H3R1-18           |
| MAT gene                | forward: GRmat13           |
|                         | reverse: GRmat22           |
| Reductase gene          | forward: GRRed10           |
|                         | reverse: GRRed20           |

Fig. 1  Species-specific detection of *F. asiaticum* (A) and *F. graminearum* s.s. (B) using primers for the reductase region gene
Lane: M: 100 bp ladder, 1: *G. zeae* TH-5-8, 2: *G. zeae* TH-4-3, 3: *G. zeae* TH-4-7, 4: *F. graminearum* s.s., 5: *F. cerealis*, 6: *F. culmorum*, 7: *F. roseum* KU-117, 8: *G. zeae* TT-1-4, 9: *G. zeae* NS-2-7, 10: *G. zeae* KK-2-8, 11: *G. zeae* MM-1-6, 12: *F. cerealis* CL-3-2, 14: Negative control

Fig. 2  Species-specific detection of *F. asiaticum* using primers for the histone H3 region gene
Lane: M: 100 bp ladder, 1: *G. zeae* TH-5-8, 2: *G. zeae* TH-4-3, 3: *G. zeae* TH-4-7, 4: *F. graminearum* s.s., 5: *F. cerealis*, 6: *F. culmorum*, 7: *F. roseum* KU-117

Fig. 3  Amplified DNA polymorphisms of the tested *Fusarium* and *Gibberella* strains
Lane: M: 100 bp ladder, 1: *G. zeae* TH-5-8, 2: *G. zeae* TH-4-3, 3: *G. zeae* TH-4-7, 4: *F. graminearum* s.s., 5: *G. zeae* MM-1-6, 6: *G. zeae* KK-2-8, 7: *G. zeae* NS-2-7, 8: *F. cerealis*, 9: *F. culmorum*, 10: *F. graminearum* s.s., 11: *F. roseum* KU-117
it was kept at 4°C. As shown in Fig. 3, the RAPD patterns of G. zaeae TH-5-8, TH-4-3, and TH-4-7 and F. graminearum s.s. were identical and showed no genetic diversity, while those of G. zaeae MM-1-6, KK-2-8, and NS-2-7 and F. roseum KU-117 showed low genetic diversity due to a slightly different pattern of The strain KK-2-8. The patterns of F. graminearum s.s., F. roseum KU-117, and G. zaeae were different from F. cerealis and F. culmorum (Fig. 3). This limited study suggests that the RAPD patterns of G. zaeae strains tested showed low genetic diversity in their populations.

Table 1 summarizes the results of re-classification obtained from this study. The F. roseum KU-117 was re-classified as F. asiaticum KU-117 and deposited at the NITE Biological Resource Center (NBRC), Chiba Prefecture with the strain number NBRC 113358. Of the G. zaeae strains tested, the strains TH-4-3, TH-4-7, and TH-5-8 collected from the northern region were identified as F. graminearum s.s., but the other G. zaeae strains harvested from the central and southern regions belonged to F. asiaticum. Suga et al.\(^\text{17}\) reported that F. asiaticum was the predominant species in wheat and barley fields in the southern region of Japan, and that F. graminearum s.s. may be better adapted to cooler regions compared with F. asiaticum. The results obtained in this study were consistent with the report mentioned above. Among G. zaeae strains identified previously, re-classified F. asiaticum and F. graminearum s.s. strains are distributed regionally in the central to southern and northern fields of Japan, respectively.

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