



HOKKAIDO UNIVERSITY

Title	Genotyping <i>Cephalosporium gramineum</i> and development of a marker for molecular diagnosis
Author(s)	Baaj, D. Wafai; Kondo, N.
Citation	Plant Pathology, 60(4), 730-738 https://doi.org/10.1111/j.1365-3059.2011.02429.x
Issue Date	2011-08
Doc URL	https://hdl.handle.net/2115/49679
Rights	The definitive version is available at wileyonlinelibrary.com
Type	journal article
File Information	PP60-4_730-738.pdf



Genotypes of Cephalosporium gramineum and a DNA marker

Genotyping *Cephalosporium gramineum* and development of a marker for molecular diagnosis

Authors: D. Wafai Baaj and N. Kondo*

*Corresponding author: Norio Kondo, Professor

E-mail: norikon@res.agr.hokudai.ac.jp

Fax: +81 11 706 4938

Address: Laboratory of Plant Pathology, Research Faculty of Agriculture, Hokkaido University. Kita 9 Nishi 9, Kita-ku, Sapporo, Hokkaido, 060-8589, Japan.

Cephalosporium gramineum is the causal fungus of Cephalosporium stripe disease of wheat. The disease has been known since the 1930s, mostly from Japan, the United Kingdom and the northern winter wheat belt of North America. However, the population genetic structure of the causal fungus is not clear. We investigated the genetic variation of 40 isolates of *C. gramineum*, based on variations in internal transcribed spacers (ITS) and intergenic spacers (IGS) of rDNA. Of the isolates, 29 were from Japan and the rest from the United States and Europe. The ITS region was about 600 bp and almost identical among these isolates. In the IGS region (~5 kbp), restriction fragment length polymorphism analysis detected four genotypes among the 40 isolates. One representative isolate was selected from each of the four genotypes, and the IGS region was sequenced. Attempts to design a genotype-specific marker based on the size of PCR products amplified with selected primers failed to differentiate among the four genotypes. Alternatively, we developed a species-specific primer set (CGIGS1 and CGIGS2) that annealed within the conserved region, producing a DNA fragment of about 1.8 kbp. Our tests of this primer set on a wide range of other fungi from 11 genera confirmed that it was specific to *C. gramineum*. This primer set could serve as an effective tool in the molecular diagnosis of *C. gramineum* and has the potential to assist in a better understanding the host–pathogen interaction.

Keywords: *Cephalosporium gramineum*, Cephalosporium stripe, genotyping, IGS-RFLP, molecular marker, species-specific primers.

Introduction

Cephalosporium stripe, caused by the soilborne fungus *Cephalosporium gramineum* Nisikado & Ikata (*Hymenula cerealis* Ell. & Ev.), is an important vascular disease of winter wheat. Spring grains are susceptible but either escape infection or do not permit infections to build to a damaging level. Symptoms appear during jointing and heading as chlorotic, longitudinal stripes on leaves. The continuity of the stripes and one or more darkened veins within them throughout the culm, leaf sheath and blade are diagnostic. Near harvest, the culm of infected plants may darken at and below nodes. Such plants typically are or will be stunted, prematurely ripe and white-headed (Wiese, 1987). Yield loss of up to 80% can occur when conditions are favourable (Johnston & Mathre, 1972).

The disease was initially described from Okayama Prefecture in Japan in 1930. Within a few years, the disease had spread to other prefectures (Hiroshima, Hyougo, Ehime, Kagawa, Aichi, Nagano and Iwate), severely affecting wheat production (Nishikado *et al.*, 1934; Ikata & Kawai, 1937; Yunoki & Sakurai, 1965).

In 1955, Bruehl (1957) discovered the disease on winter wheat in the United States, but previously, Orton (1931) had recorded *Cephalosporium* sp., isolated from wheat in Minnesota in 1924, without referring to the stripe disease itself. In Europe, Ciferri (1959) first recorded *C. gramineum* in meadow foxtail (*Alopecurus pratensis*) in Italy followed by Gray and Noble (1960), who reported the disease in Scotland.

In 1982, Kobayashi *et al.* (1982) first reported the disease in Hokkaido (the northernmost Japanese island), the main wheat production area of Japan, where it has seriously affected wheat production (Ozaki *et al.*, 1988). Although *Cephalosporium* stripe is not currently the major disease affecting wheat production in Japan, it still occurs almost every year in Hokkaido, especially in fields where winter wheat is continuously cultivated. In the United States, *Cephalosporium* stripe is still a chronic yield-reducing disease that occurs every year in the Pacific Northwest region (Murray, 2006).

The genetic structure of *C. gramineum* has yet to be determined. Knowledge of the amount and distribution of genetic variation is of great importance in managing pathogenic fungi (McDonald, 1997). In addition, the identification of *C. gramineum* is still limited to culture morphology, even though in the last decade, molecular-based techniques have made a significant impact on the speed and reliability of fungal species identification.

Both the internal transcribed spacer (ITS) and the intergenic spacer (IGS) of rDNA are highly polymorphic and provide useful tools for taxonomic studies at the species and

subspecies level (Chen *et al.*, 1992; Henrion *et al.*, 1994). Also, many studies have used the IGS region to develop species- and subspecies-specific markers (Chen *et al.*, 2000; Konstantinova & Mattila, 2004).

The objectives of this study were to detect genetic variation within *C. gramineum* based on differences in the ITS and IGS regions of rDNA and to develop a molecular marker for the specific identification of *C. gramineum*.

Materials and methods

Fungal isolates

Table 1 lists the fungal isolates used in this study. *C. gramineum* was isolated from winter wheat and orchard grass plants with typical *Cephalosporium* stripe symptoms. Leaves with diagnosed stripes were cut into small fragments, surface-sterilised with 70% EtOH, air-dried and placed on cornmeal agar (Sigma) acidified with lactic acid to pH 4.8. After incubation at 20°C for about 1 week, colonies growing out from the fragments were identified as *C. gramineum* by cultural characteristics (slow-growing, wet and white-grey or yellow colonies, morphology of conidia forming false heads on conidiophores; Kobayashi *et al.*, 1982). Isolates were further purified by the isolation of a single conidium. Reference isolates were obtained from the American Type Culture Collection (ATCC), the culture collection of the

Centraalbureau voor Schimmelcultures (CBS), Department of Plant Pathology, Washington State University, Pullman, USA and the National Institute of Agrobiological Science (NIAS) Gene Bank, Japan. For long-term preservation, isolates were maintained in green-pea agar slants (200 g frozen green peas boiled 20 min and filtered through four layers of cheesecloth, with 15 g agar L⁻¹). Isolates were also kept as plugs of potato dextrose agar (PDA; 24 g Difco dextrose broth, 15 g agar L⁻¹) containing mycelia and conidia, in 15% glycerol at -80°C.

The pathogenicity of new isolates collected from Hokkaido was verified by inoculation onto susceptible spring wheat (*Triticum aestivum* cv. Haruyutaka). A conidial inoculum was prepared by culturing in 100 mL potato dextrose broth (Difco) in 500-mL flasks on a shaker for 5–6 days at 24°C. Fungal mycelia were removed by filtering through four layers of cheesecloth, and conidia were washed twice via centrifugation (5000 rpm). Conidia were resuspended in distilled water, with the concentrations of final suspensions adjusted to 1.2×10^7 using a haemocytometer. Two-week-old seedlings of spring wheat cv. Haruyutaka grown in vermiculite were used for the inoculation test. We gently removed seedlings from their containers, washed the roots in tap water and then trimmed off one-third of the roots with sterilised scissors. The roots of eight plants were dipped into each conidial suspension for 6 h and then transplanted to 12-cm pots. Plants dipped in water were used as negative controls. The isolate MAFF305546 was included in the test as

a positive control. Two weeks after flowering, plants were observed for symptoms. Isolates were considered pathogenic when stripes or necrotic vascular bundles were observed in any of the uppermost four leaves.

DNA extraction

For DNA extraction, isolates were grown in potato dextrose broth for 2 weeks, and the mycelia were harvested via vacuum filtration. The harvested mycelia either were used immediately for DNA extraction or frozen at -80°C until use. The mycelia were ground to a fine powder in liquid nitrogen, and DNA was extracted using the CTAB method of Watanabe & Sugiura (1989), modified from Murray & Thompson (1980). The DNA concentration was determined using a spectrophotometer (DU-640, Beckman-Coulter), and DNA extracts were stored at -20°C until use.

PCR amplification and ITS sequencing

For all *C. gramineum* isolates, the ITS region was amplified using the universal primer pair ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3'; White *et al.*, 1990). PCR consisted of 0.1 μM of each primer, 200 μM dNTPs, 1 unit *Taq* DNA polymerase, 1 $\times$ PCR buffer (Labo Pass) and 0.5–1.0 ng DNA template in a total volume of 20 μL . The thermal cycler (PCR Thermal

Cycler MP, Takara) programme was as follows: initial denaturing step at 94°C for 3 min, followed by 35 cycles at 94°C for 1 min, 58°C for 30 s and 72°C for 30 s, with a final extension at 72°C for 5 min. The temperature was then maintained at 4°C. Negative controls (no DNA) were included to test for contamination of reagents and reaction mixtures. PCR products were electrophoresed through 1.0% agarose gel in 1× TBE buffer, stained with EtBr and visualised on a UV transilluminator. The amplified ITS region of 32 isolates was sequenced (Table 1). Following visualisation, target bands were excised, and the DNA from the excised bands was purified using QIAquick gel extraction kit (Qiagen), according to the manufacturer's instructions. Purified products were sequenced using the dye terminator protocol (Hokkaido System Science).

Blast searches were conducted using new sequences as queries. Sequences of two *C. gramineum* isolates, CG1 and CG5 (GenBank Accession Nos. AY428791.1 and AY428792, respectively), were downloaded to facilitate comparison. New sequences were trimmed to the region corresponding to the beginning and end of isolates CG1 and CG5. Clustal W (Thompson *et al.*, 1994) was used to align new sequences with downloaded sequences.

C. gramineum isolates with unsequenced ITS regions (Table 1) were subjected to ITS-restriction fragment length polymorphism (RFLP) using *Hinf*I (Takara) according to the manufacturer's instructions. Restriction fragments were separated via 2% NuSieve gel electrophoresis, stained with EtBr and photographed under UV light.

PCR amplification of IGS and IGS-RFLP analysis

The primers 28SF (5'-CTGAACGCCTCTAAGTCAGAA-3') and CNS1R (5'-GAGACAAGCATATGACTAC-3'; Chen *et. al.*, 2000) were used to amplify the IGS region. PCR reactions consisted of 0.3 μ M of each primer, 200 μ M dNTPs, 1.5 mM MgSO₄, 1 unit Platinum *Taq* DNA Polymerase High Fidelity, 1 $\times$ PCR buffer and 0.5–1.0 ng DNA template. Except for the primers, all reagents and buffers were purchased from Invitrogen Japan. Thermal cyclor conditions were one cycle at 94°C for 90 s, 58°C for 30 s and 70°C for 4 min, followed by 40 cycles at 94°C for 30 s, 58°C for 30 s and 70°C for 4 min. Other procedures related to PCR and electrophoresis were the same as above.

To detect variations in the PCR-amplified IGS region, we conducted restriction enzyme digestions using the following enzymes: *EcoRI*, *HincII*, *HindIII*, *HinfI*, *PvuII*, *SacI*, *Sau3AI*, *SspI* and *XhoI* (Takara) according to the manufacturer's instructions. Restriction fragments were separated via 2% agarose gel electrophoresis, stained with EtBr and photographed under UV light. Only those enzymes that resulted in different restriction patterns among isolates were used for further analysis.

Sequencing of IGS region and design of specific primers for *C. gramineum*

One representative isolate was selected from each of the four genotypes of *C. gramineum*, as

shown in Table 3: MK08201 (genotype A), ATCC36969 (genotype B), MK08301 (genotype C) and CBS132.34 (genotype D). The IGS region was amplified using the universal primer pair 28 SF and CNS1R, and then the PCR products were purified using a Qiagen DNA purification kit. Purified products were sequenced using the primer-walking strategy (Hokkaido System Science).

Clustal W was used to align the sequences and to determine the conserved DNA region among the four isolates. Two specific primers were designed to flank the conserved region using Primer3 (Rozen & Skaletsky, 2000): CGIGS1 (5'-GGGTAGATGGGATGTGGATG-3') and CGIGS2 (5'-GAAGGAGGCAGAGTTGTTGC-3').

PCR reactions consisted of 0.3 μ M of each primer, 200 μ M dNTPs, 0.5–1.0 ng DNA and 1 unit *G-Taq* in 1 $\times$ PCR buffer in a total volume of 20 μ L. Temperatures cycles were 94°C for 1 min for the first cycle, followed by 29 cycles at 94°C for 1 min, 65°C for 30 s and 72°C for 30 s, with a final extension of 5 min at 72°C.

The specificity of the primer pair to *C. gramineum* was tested against fungi from 11 genera (Table 2). The DNeasy Plant DNA Extraction Mini-Kit (Qiagen) was used for DNA extraction. DNA concentration was determined using a spectrophotometer (DU-640, Beckman-Coulter), and DNA extracts were stored at –20°C until use.

Primer pair was assessed for its ability to detect the fungus in infected wheat plants

under same conditions. For this purpose, the susceptible wheat cultivar Haruyutaka was artificially inoculated same as mentioned above. Negative control plants were mock inoculated by the same method using distilled water. After flowering when diagnosed stripes became clear, wheat leaves were collected, thoroughly washed to remove residual dirt, surface-sterilised with 70% EtOH and air-dried. Leaves were cut into small pieces, ground in liquid nitrogen, and DNA was extracted with the DNeasy Plant DNA Extraction Mini-Kit (Qiagen).

To determine sensitivity, we used genomic DNA of *C. gramineum* (isolate UR0901) to prepare a 10-fold serial dilution from 0.7 ng/μL to 0.7 fg/μL. These seven DNA concentrations were then used for PCR templates under the conditions described above.

Results

Pathogenicity test

All isolates collected from Hokkaido were highly pathogenic to the spring wheat cultivar Haruyutaka (Table 1).

Amplification of ITS region and DNA sequencing

The size of the amplified ITS was approximately 600 bp in all isolates (Fig. 1a). Blast

searches identified the new sequences as *C. gramineum*. The nucleotide sequence of the ITS region was almost identical among all isolates and was identical to sequences found in GenBank. However, a point mutation in one nucleotide at position 173 bp (Fig. 2; performed using Geneious Pro, Drummond *et al.*, 2010) added a restriction site for the enzyme *HinfI* in 7 of the 40 isolates (Table 3). ITS-RFLP analysis using *HinfI* identified isolates with this mutation (Table 3). However, the difference among isolates of one nucleotide in the ITS region was not sufficient to identify distinctive genotypes or to reveal the level of genetic variation within *C. gramineum*. Sequences were deposited in GenBank database under Accession Nos. HQ322339 to HQ322369 and HQ322374.

Amplification of IGS region and IGS-RFLP analysis

Amplification with primers 28SF and CNS1 resulted in an approximately 5-kb fragment from all *C. gramineum* isolates (Fig. 1b). Of the nine restriction enzymes used for digestion, only two, *EcoRI* and *HinfI*, generated different banding patterns among isolates (Fig. 3). *PvuII*, *SacI* and *SspI* did not have restriction sites in this region. The other enzymes, *HincII*, *HindIII*, *Sau3AI* and *XhoI*, had the same restriction patterns among isolates (data not shown).

Digestion with *EcoRI* identified two groups among the 40 isolates, one without and the other with the *EcoRI* restriction site, whereas digestion with *HinfI* identified four groups with different restriction patterns (Fig. 3). By combining the results of the *EcoRI* and *HinfI*

digestions, we divided the *C. gramineum* isolates into four IGS genotypes: A, B, C and D (Table 3). Sequences of IGS region in the isolates representing the four genotypes were deposited in GenBank database under Accession Nos. HQ322370 to HQ322373.

Primer design and specificity

Figure 4 shows the annealing sites of primers (performed using Geneious Pro, Drummond *et al.*, 2010). PCR amplification of DNA from all *C. gramineum* isolates used in this study or from artificially inoculated wheat plants using the CGIGS1/CGIGS2 primer pair yielded a single PCR product of the expected size of ~1880 bp (Figs 5 and 7), whereas no PCR products were detected in other fungi (Fig. 6) or in mock inoculated wheat plants (Fig. 7).

To determine the sensitivity of the PCR assay using the CGIGS1/CGIGS2 primer pair, we tested a dilution series of *C. gramineum* genomic DNA. A single PCR band was reliably produced from as little as 0.7 pg fungal DNA (Fig. 8).

Discussion

This is the first study on genetic variability at the molecular level in *C. gramineum*. Understanding the genetic variability of plant pathogens is important to determine the most effective strategy of breeding resistant varieties. This not only helps in understanding the

population structure of the pathogen, but also improves screening procedures, since the number of isolates used depends on the variation among them (Quincke, 2009). In addition, better predictions of the durability of host resistance are possible, since higher levels of variation in pathogens offer a greater evolutionary potential to overcome the resistance gene (Keller *et al.*, 2000; Stuthman *et al.*, 2007).

In this study, we investigated genetic variation within *C. gramineum* by exploring the ITS and IGS regions of nuclear rDNA. The ITS region was almost totally conserved within the 40 isolates of *C. gramineum* used in this study, whereas the IGS region revealed some genetic variation.

RFLP analysis of the IGS region with two restriction enzymes distinguished four genotypes within the 40 isolates (Fig. 3, Table 3). The majority of isolates from the United States and those from Europe were in the same genotypes (genotypes C and A, respectively), although due to the limited number of isolates, especially from Europe, an association between genotypes and the geographical origins of *C. gramineum* isolates is speculative.

Isolates from Hokkaido were assigned to each of the four genotypes. Notably, the three isolates in genotype D included the oldest registered isolate of *C. gramineum*, CBS 132.32, isolated in 1932 from Okayama Prefecture, where the diseases was first discovered; the other two, IKD0905 and BH0909, were isolated in 2009 from Hokkaido (Ikeda and Bihoro, respectively). This might indicate that genotype D isolates have been transferred from

Okayama Prefecture in the southern part of Japan to Hokkaido. The existence of four distinct genotypes of the IGS region suggests the existence of at least four evolving populations of *C. gramineum*. Although the IGS region of nuclear rDNA is unlikely to be involved in the infection process, genotypic differences in *P. gregata* f. sp. *sojae* between the two identified IGS genotypes correspond to the severity of symptoms among *P. gregata* f. sp. *sojae* isolates (Hughes *et al.*, 2002; Harrington *et al.*, 2003). Also, the two genotypes displayed cultivar preference (Chen *et al.*, 2000; Meng *et al.*, 2005; Malvick & Impullitti, 2007).

The existence of *C. gramineum* races has not yet been confirmed. Mathre *et al.* (1977) and Van Wert *et al.* (1984) suggested that *C. gramineum* races might exist, but after assessing isolates from different regions, Cowger & Mundt (1998) found no significant cultivar $\times$ inoculum-source interaction, and thus no evidence of substantial pathogenic variability in this fungus.

Further work is necessary with a larger number of isolates to detect any correlation between the genetic variation found in this study and other phenotypic traits, such as sporulation and pathogenicity, and to reveal the evolutionary history of this pathogen.

Sequencing of the entire IGS region provided more information about differences among genotypes. Alignment of the IGS region of four isolates representative of each genotype identified the variable region between bp 780 and 1763 (Fig. 4). Sequence variability was due to both nucleotide substitutions and insertion/deletion events. However, all

trials to develop a genotype-specific primer flanking the variable region failed to differentiate among the four genotype isolates based on PCR product sizes amplified with the primers. Only genotype A could be distinguished from the others, owing to a size difference of about 100 bp. Alternatively, we sought to develop a *C. gramineum*-specific primer pair that was not genotype-based. Using this primer pair, we could detect the pathogen in artificially infected wheat plants under relatively high annealing temperature (65°C). Primers destined to detect a specific pathogen from plant materials must be able to distinguish between target and non-target nucleic acids. However, specific primers that perform well at relatively low annealing temperatures might also hybridize to non target annealing sequences, because of low stringency conditions leading to costly positive results. Therefore, in addition to sequence specificity, the ability of a primer set to perform at a high annealing temperature is an important advantage for diagnosis (Bahar *et al*, 2008). Under these temperature conditions, the primers CGIGS1/CGIGS2 could amplify as little as 0.7 pg of *C. gramineum* DNA (Fig. 8). Further research is under way to confirm the efficiency of this primer pair in the specific detection of *C. gramineum* in naturally infected hosts and soils. The development of such a sensitive detection method is essential to understand the host–pathogen interaction and illuminate unresolved questions related to pathogen ecology.

Until recently, quantification of *C. gramineum* is determined by dilution plating onto a semiselective medium, which is labor intensive and time-consuming method. While many

workers have succeeded to develop a molecular assays based on sequence-specific probes labeled with fluorescent reporters and quencher, and combined with flanking primers to identify and quantify specific fungi in plant and soil (Hughes *et al.*, 2009; Shishido *et al.*, 2010). These real-time quantitative PCR assays give higher lever of specificity and sensitivity reduce time, labor, and overall cost. Our objective now is to develop a quantitative assay to quantify *C. gramineum* in infested field soils for use in studying pathogen survival and crop rotation efficiency in disease suppression.

Acknowledgements

We would like to thank Dr. Timothy. D. Murray from the Department of Plant Pathology, Washington State University for providing *Cephalosporium gramineum* isolates. We also thank Dr. Naoyuki Matsumoto from the National Agriculture Research Center for Hokkaido Region for providing *Typhulla incarnata* and *T. ishikariensis* isolates and Dr. Osamu Fujine from the Hokkaido Central Agriculture Experimental Station for providing *Gaeumannomyces graminis* isolates.

References

- Bahar O, Efrat M, Hadar E, Dutta B, Walcott RR, Burdman S, 2008. New subspecies-specific polymerase chain reaction-based assay for the detection of *Acidovorax avenae* subsp.citrulli. *Plant Pathology* **57**, 754-63.
- Bruehl GW, 1957. Cephalosporium disease of wheat. *Phytopathology* **47**, 641-49.
- Chen W, Hoy JW, Schneider RW, 1992. Species-specific polymorphisms in transcribed ribosomal DNA of five *Pythium* species. *Experimental Mycology* **16**, 22-34.
- Chen W, Grau CR, Adey EA, Meng X, 2000. A molecular marker identifying subspecific populations of the soybean brown stem rot pathogen, *Phialophora gregata*. *Phytopathology* **90**, 875-83.
- Ciferri R, 1959. Cephalosporium stripe disease on meadow foxtail. *FAO Plant Protection Bulletin* **7**, 54-5.
- Cowger C, Mundt CC, 1998. A hydroponic seedling assay for resistance to Cephalosporium stripe of wheat. *Plant Disease* **82**, 1126-31.
- Drummond AJ, Ashton B, Buxton S, Cheung M, Cooper A, Heled J, Kearse M, Moir R, Stones-Havas S, Sturrock S, Thierer T, Wilson A (2010) Geneious v5.1, Available from <http://www.geneious.com>.
- Gray EG, Noble M, 1960. Cephalosporium stripe in cereals in Scotland. *FAO Plant*

Protection Bulletin **8**, 46.

Harrington TC, Steimel J, Workneh F, Yang XB, 2003. Characterization and distribution of two races of *Phialophora gregata* in the North-Central United States. *Phytopathology* **93**, 901-12.

Henrion B, Chevalier G, Martin F, 1994. Typing truffle species by PCR amplification of the ribosomal DNA spacers. *Mycological Research* **98**, 37-43.

Hughes TJ, Atallah ZK, Grau CR, 2009. Real time PCR assays for the quantification of *Phialophora gregata* f.sp. *sojae* IGS genotypes A and B. *Phytopathology* **99**, 1008-14.

Hughes TJ, Chen W, Grau CR, 2002. Pathogenic characterization of genotype A and B of *Phialophora gregata* f. sp. *sojae*. *Plant Disease* **86**, 729-35.

Ikata S, Kawai I, 1937. Studies on the stripe disease of wheat. *Bulletin of Okayama Agricultural Experiment Station* **41**, 1-111.

Johnston RH, Mathre DE, 1972. Effect of infection by *Cephalosporium gramineum* on winter wheat. *Crop Science* **12**, 817-19.

Keller B, Feuillet C, Messmer M, 2000. Genetics of disease resistance. In: Slusarenko AJ, *et al.* eds. *Mechanisms of Resistance to Plant Diseases*. Kluwer Academic Publisher, Dordrech, The Netherlands, 101-60.

Kobayashi K, Ui T, Saito I, 1982. Cephalosporium stripe of winter wheat in Hokkaido. *Annals of the Phytopathological Society of Japan* **48**, 542-43.

- Konstantinova P, Mattila TY, 2004. IGS–RFLP analysis and development of molecular markers for identification of *Fusarium poae*, *Fusarium langsethiae*, *Fusarium sporotrichioides* and *Fusarium kyushuense*. *International journal of food microbiology* **95**, 321-31.
- Malvick DK, Impullitti AE, 2007. Detection and quantification of *Phialophora gregata* in soybean and soil samples with a quantitative, real- time PCR assay. *Plant Disease* **91**, 736-42.
- Mathre DE, Johnston RH, McGuire CF, 1977. Cephalosporium stripe of winter wheat: pathogen virulence, sources of resistance, and effect on grain quality. *Phytopathology* **67**, 1142-48.
- McDonald BA, 1997. The population genetics of fungi: tools and techniques. *Phytopathology* **87**, 448-53.
- Meng X, Grau CR, Chen W, 2005. Cultivar preference exhibited by two sympatric and genetically distinct populations of the soybean fungal pathogen *Phialophora gregata* f. sp. *sojae*. *Plant Pathology* **54**, 180-88.
- Murray MG, Thompson WF, 1980. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research* **8**, 4321-26.
- Murray TD, 2006. Seed transmission of *Cephalosporium gramineum* in winter wheat. *Plant Disease* **90**, 803-6.

- Nisikado Y, Matsumoto H, Yamauti K, 1934. Studies on a new *Cephalosporium*, which causes the stripe disease of wheat. *Berichte des Ohara Instituts für Landwirtschaftliche Forschungen*, Kurashiki, Japan. **6**, 275-306.
- Orton CR, 1931. Seed-borne parasites, a bibliography. *West Virginia Agricultural Experiment Station Bulletin* **245**, 1- 47.
- Ozaki M, Kondo N, Akai J, Kodama F, 1988. Effect of residue management on incidence of *Cephalosporium* stripe of winter wheat. *Bulletin of Hokkaido Prefectural Agricultural Experiment Station* **58**, 121-26.
- Quincke MC, 2009. *Phenotypic response and quantitative trait loci for resistance to Cephalosporium gramineum*. Oregon, USA: Oregon State University, PhD thesis.
- Rozen S, Skaletsky HJ, 2000. Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S, eds. *Bioinformatics Methods and Protocols: Methods in Molecular Biology*. Humana Press, Totowa, NJ, 365-86.
- Stuthman DD, Leonard KJ, Miller-Garvin J, 2007. Breeding crops for durable resistance to disease. *Advances in Agronomy* **95**, 319-67.
- Shishido M, Sato K, Yoshida N, Tsukui R, Usami T, 2010. PCR - based assays to detect and quantify *Phomopsis sclerotioides* in plants and soil. *Journal of General Plant Pathology* **76**, 21–30.
- Thompson JD, Higgins DG, Gibson TJ, 1994. CLUSTAL W: improving the sensitivity of

progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**, 4673-80.

Van Wert SL, Ravenscroft AV, Fulbright DW, 1984. Screening wheat lines as seedlings for resistance to *Cephalosporium-gramineum*. *Plant Disease* **68**, 1036-38.

Watanabe I, Sugiura M, 1989. Method for isolation plant DNA. In: Watanabe I, Sugiura M, eds. *Cloning and Sequencing, Plant Biotechnology Manual* (Japanese). Tokyo, Japan: Nouseon Bunkasha, 253-58.

White TJ, Bruns SL, Taylor JW, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. *PCR Protocols: A Guide to Methods and Applications*. New York, USA: Academic Press, Inc., 315–22.

Wiese MV, 1987. *Compendium of Wheat Disease*, 2nd ed. St Paul, MN, USA: APS Press, 26-8.

Yunoki T, Sakurai Y, 1965. Control of the *Cephalosporium* stripe disease of wheat. *Bulletin of Chugoku Agricultural Experiment Station* **A-11**, 113- 44.

Figure 1 Agarose gel (1%) showing (a) ITS-PCR amplification products and (b) IGS-PCR amplification products of four isolates of *Cephalosporium gramineum*. Lane M, DNA size marker, (a) Ø 174 *Hae* III digest, (b) 500 bp DNA ladder; lanes 1-4: 1, OKC83; 2, MK08302; 3, CBS132.34; 4, ATCC60208; lane 5, negative control (no DNA).

Figure 2 Alignment of a part of ITS sequence for four *Cephalosporium gramineum* isolates showing the point mutation.

Figure 3 Agarose gel (2%) showing restriction patterns of PCR amplified IGS region after digestion with *Hinf*I (upper gel) and *Eco*RI (lower gel), and different banding patterns of isolates within each genotype. Lane M, DNA size marker (upper gel, 100 bp DNA ladder; lower gel, 500bp DNA ladder); lanes1-12: 1, CBS 502.81; 2, KR08106; 3, KT0801; 4, ATCC15017; 5, KR09108; 6, MAFF 305546; 7, ATCC60205; 8, Cg94-1; 9, MK09215; 10, BH0909; 11, CBS132.34; 12, IKD0905.

Figure 4 Alignment of the sequences of PCR amplified IGS using universal primers 28SF and CNS1R from *Cephalosporium gramineum*. ATCC36968 (genotype B), CBS132.34 (genotype D), MK08201 (genotype A), MK08302 (genotype C). Arrows show specific primers annealing sites.

Figure 5 Agarose gel (1%) showing amplification of *Cephalosporium gramineum* isolates used in this study using the specific primers, CGIGS1 and CGIGS2. Lane M: 500bp DNA ladder; lane N: negative control (no DNA); lanes 1-40: 1, MK09101; 2, MK09215; 3, NAG0807; 4, OKC83; 5, URH0901; 6, ATCC15017; 7, ATCC46044; 8, Cg84-30; 9, Cg94-1; 10, Cg95-1; 11, Cg95-4; 12, CBS 759.69; 13, KR0605; 14, KR08309; 15, KR08601; 16, KR9101; 17, KT0801; 18, KR9104; 19, CBS502.81; 20, ATCC32622; 21, ATCC36969; 22, KR08507; 23, KR08202; 24, MK08101; 25, MK08201; 26, MK08302; 27, TY0802; 28, NAN0803; 29, OTF0809; 30, ATCC60205; 31, ATCC60208; 32, MAFF305546; 33, KR08106; 34, BH0909; 35, KR0601; 36, KR0603; 37, IKD0905; 38, KR9108; 39, CBS132.34; 40, KR08402.

Figure 6 Agarose gel (1%) showing PCR amplification using the specific primers, CGIGS1 and CGIGS2. Lane M, 500bp DNA ladder; lanes 1–2, *Cephalosporium gramineum*: 1, isolate ATCC32622; 2, isolate MAFF305546; lanes 3-22: 3, *Cadophora gregata* isolate T96-1; 4, *Cadophora gregata* isolate T96-5; 5, *Fusarium graminearum*; 6: *Fusarium oxysporum*, 7, *Microdochium nivale*; 8, *Pythium graminicola*; 9, *Pythium rostratum*; 10, *Pythium paddicum*: 11, *Gaeumannomyces graminis* isolate GGS2; 12, *Gaeumannomyces graminis* isolate GGS5; 13, binucleate *Rhizoctonia* AG-D II; 14, unknown fungus No.5; 15, unknown fungus No.6; 16, *Helgardia herpotrichoides*; 17: *Sclerotinia sclerotiorum*; 18, *Typhula incarnata*; 19, *Typhula ishikariensis* ; 20, *Verticillium dahliae* isolate 86101; 21, *Verticillium dahliae* isolate 84011; 23, *Rhynchosporium secalis*; lane N, negative control (no DNA).

Figure 7 Detection of *Cephalosporium gramineum* in infected wheat plants using the specific primers, CGIGS1 and CGIGS2. Lane M, 500 bp ladder; lanes 1-2, genomic DNA of *Cephalosporium gramineum*: 1, isolates OTF0809; 2, isolate KR08106; lanes 3-4, DNA from infected wheat plants (leaves); lane 5, DNA from mock inoculated wheat plants (leaves); lane 6, negative control (no DNA).

Figure 8 Agarose gel (1%) showing sensitivity of *Cephalosporium gramineum*-specific primers, CGIGS1 and CGIGS2, using a dilution series of genomic DNA of *Cephalosporium gramineum* isolate UR090. Lane M: 500bp DNA ladder; lanes 1-7: 0.7ng to 0.7fg, respectively; lane 8: negative control (no DNA).

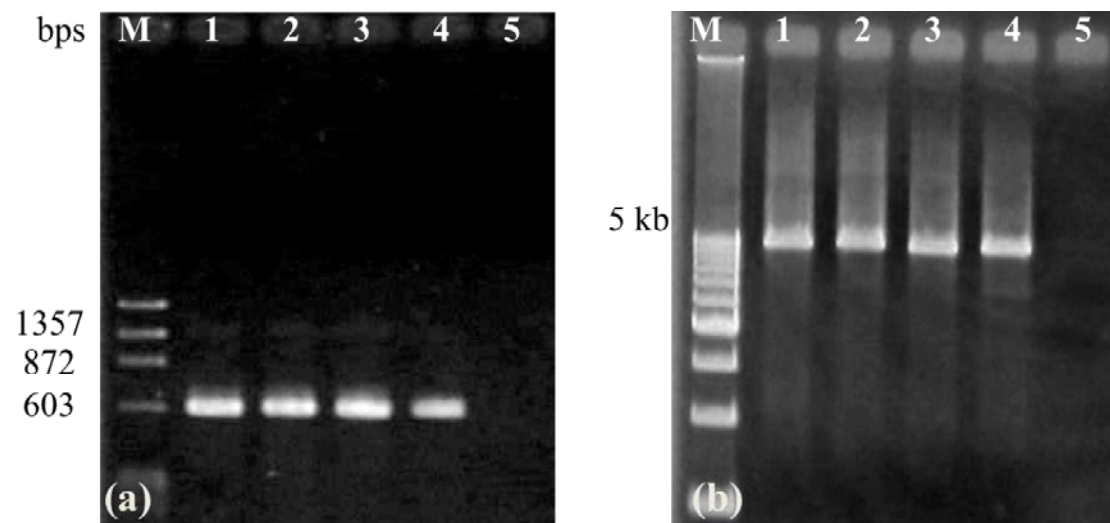


Figure 1

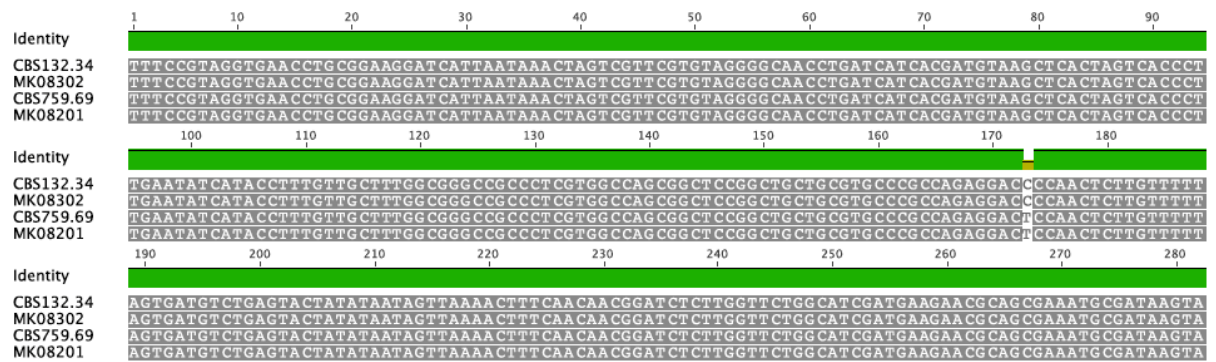


Figure 2

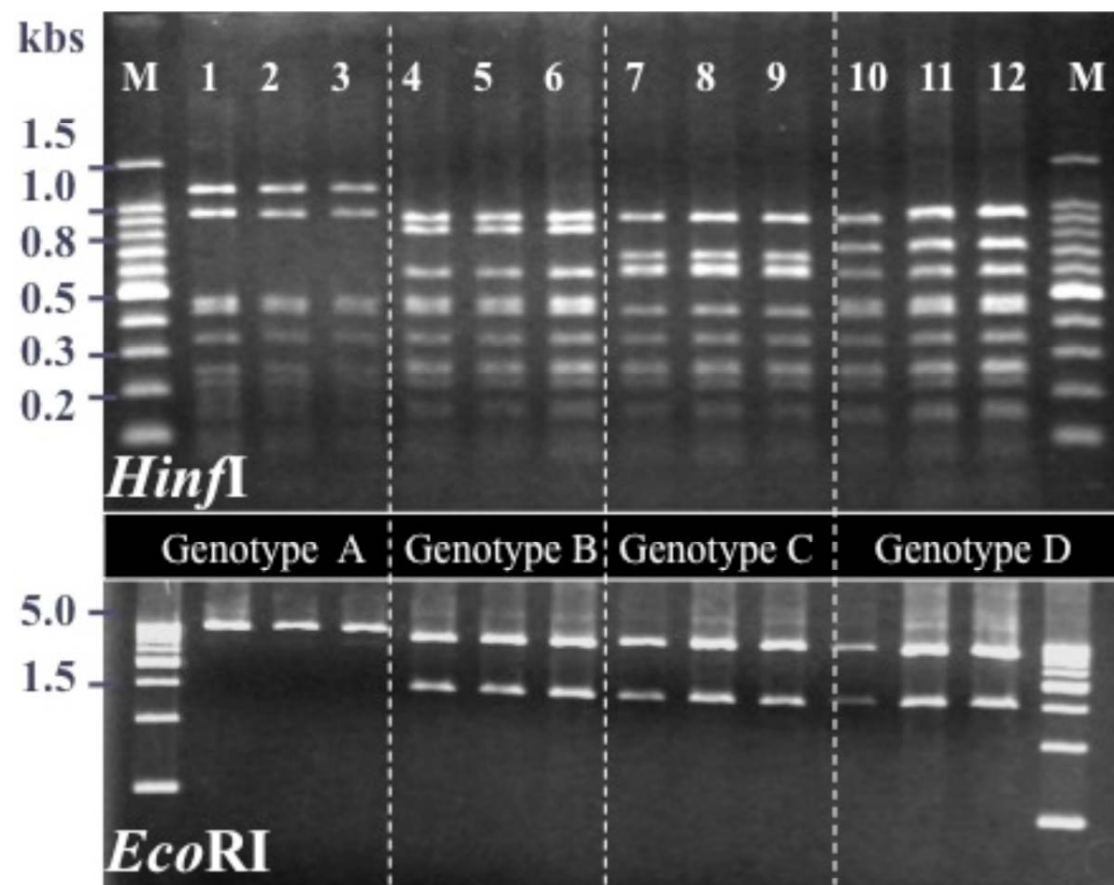


Figure 3

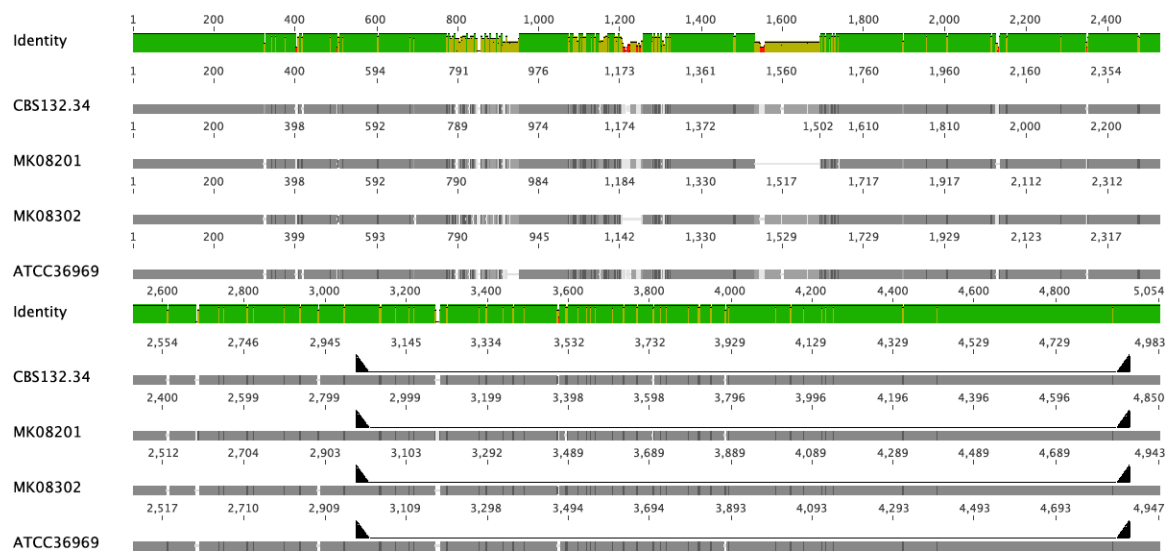


Figure 4

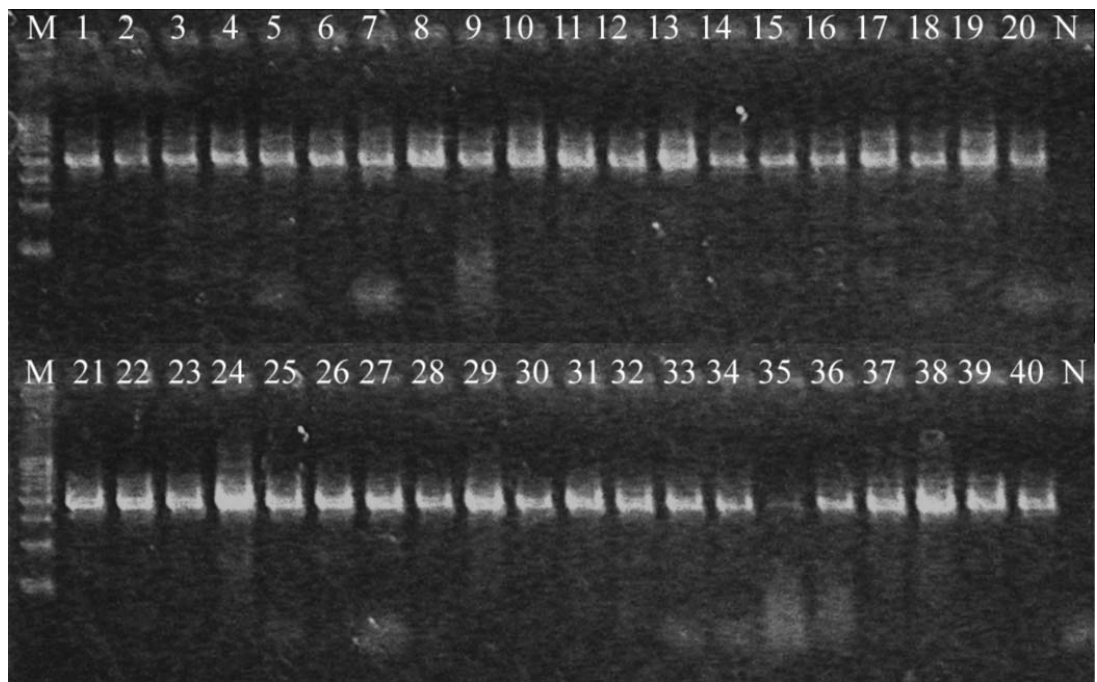


Figure 5



Figure 6

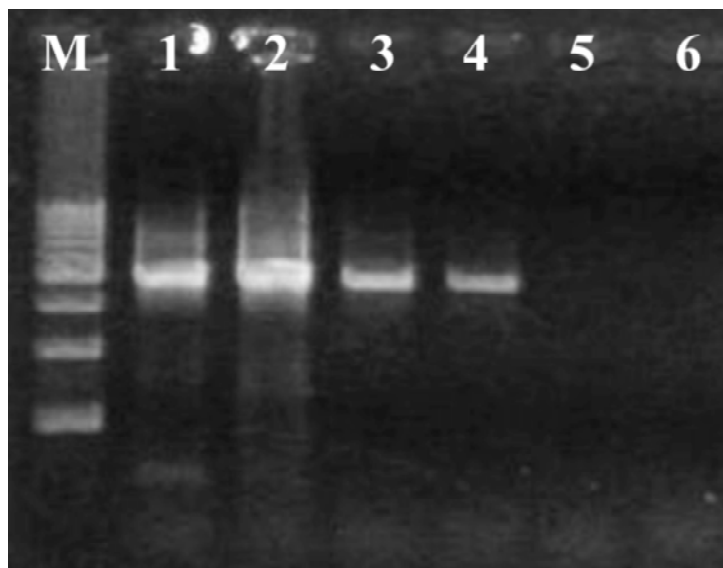


Figure 7

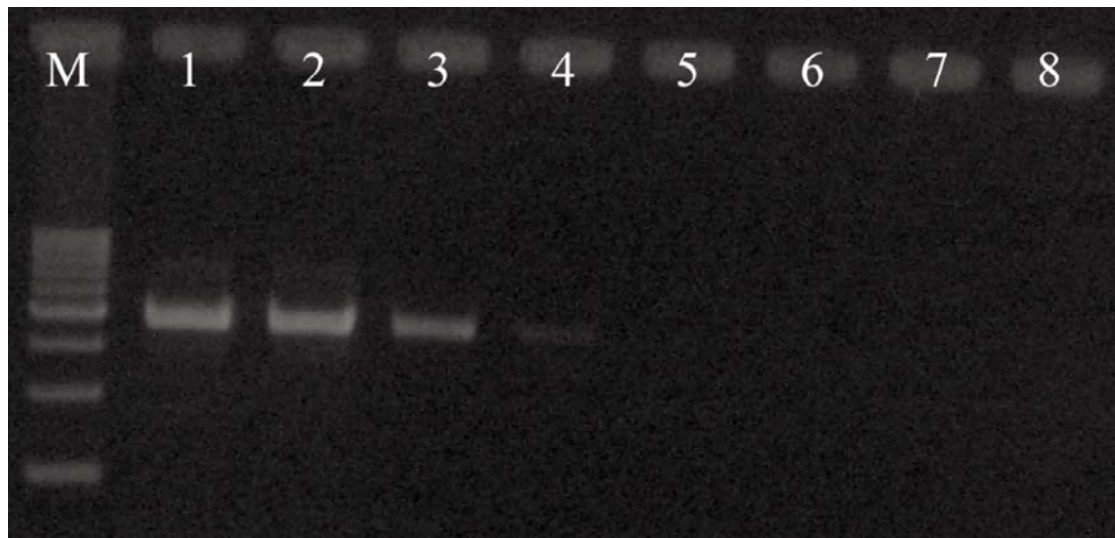


Figure 8

Table 1 *Cephalosporium gramineum* and reference isolates used in this study

Isolate name	Origin/ Source	Host species	Pathogenicity to wheat	Isolation year/ Reference
BH0909	Ikeda, Hokkaido, Japan	<i>Triticum aestivum</i> , cv.Hokushin, leaves	+	2009
IKD0905	Ikeda, Hokkaido, Japan	<i>T. aestivum</i> , cv.Hokushin, leaves	+	2009
KR0601 [*]	Kuriyama, Hokkaido, Japan	<i>T. aestivum</i> , cv.Hokushin, leaves	+	2006
KR0603 [*]	Kuriyama, Hokkaido, Japan	<i>T. aestivum</i> , cv.Hokushin, leaves	+	2006
KR0605 [*]	Kuriyama, Hokkaido, Japan	<i>T. aestivum</i> , cv.Hokushin, leaves	+	2006
KR08106	Kuriyama, Hokkaido, Japan	<i>T. aestivum</i> , cv.Hokushin, leaves	+	2008
KR08202 [*]	Kuriyama, Hokkaido, Japan	<i>T. aestivum</i> , cv.Hokushin, leaves	+	2008
KR08309 [*]	Kuriyama, Hokkaido, Japan	<i>T. aestivum</i> , cv.Hokushin, leaves	+	2008
KR08402 [*]	Kuriyama, Hokkaido, Japan	<i>T. aestivum</i> , cv.Hokushin, leaves	+	2008
KR08507 [*]	Kuriyama, Hokkaido, Japan	<i>T. aestivum</i> , cv.Hokushin, leaves	+	2008
KR08601 [*]	Kuriyama, Hokkaido, Japan	<i>T. aestivum</i> , cv.Hokushin, leaves	+	2008
KR9101 [*]	Kuriyama, Hokkaido, Japan	<i>T. aestivum</i> , cv.Hokushin, leaves	+	1991
KR9104 [*]	Kuriyama, Hokkaido, Japan	<i>T. aestivum</i> , cv.Hokushin, leaves	+	1991
KR9108 [*]	Kuriyama, Hokkaido, Japan	<i>T. aestivum</i> , cv.Hokushin, leaves	+	1991

KT0801 [*]	Kitami, Hokkaido, Japan	<i>Dactylis glomerata</i> , leaves	+	2008
MK08101 [*]	Makubetsu, Hokkaido, Japan	<i>T. aestivum</i> , cv.Hokushin, leaves	+	2008
MK08201 [*]	Makubetsu, Hokkaido, Japan	<i>T. aestivum</i> , cv.Hokushin, leaves	+	2008
MK08302 [*]	Makubetsu, Hokkaido, Japan	<i>T. aestivum</i> , cv.Hokushin, leaves	+	2008
MK09101	Makubetsu, Hokkaido, Japan	<i>T. aestivum</i> , cv.Hokushin, leaves	+	2009
MK09215	Makubetsu, Hokkaido, Japan	<i>T. aestivum</i> , cv.Hokushin, leaves	+	2009
NAG0807 [*]	Naganuma, Hokkaido, Japan	<i>T. aestivum</i> , cv.Hokushin, leaves	+	2008
NAN0803 [*]	Nanporo, Hokkaido, Japan	<i>T. aestivum</i> , cv.Hokushin, leaves	+	2008
OKC83 [*]	Oiwake, Hokkaido, Japan	<i>T. aestivum</i> , cv.Hokushin, leaves	+	1983
OTF0809 [*]	Otofuke, Hokkaido, Japan	<i>T. aestivum</i> , cv.Hokushin, leaves	+	2008
TY0802 [*]	Toyoura, Hokkaido, Japan	<i>T. aestivum</i> , cv.Hokushin, leaves	+	2008
URH0901	Urahoru, Hokkaido, Japan	<i>T. aestivum</i> , cv.Hokushin, leaves	+	2009

Reference Isolates

ATCC15017 ^a	USA	<i>T. aestivum</i>	ND ^d	Phytopathology, 1963
ATCC32622 [*]	Montana, USA	Winter wheat, <i>T. aestivum</i>	ND	Phytopathology, 1975

ATTC36969 [*]	Kitami, Japan	Winter wheat, <i>T. aestivum</i>	ND	Physio. Plant Pathology, 1977
ATTC46044 [*]	Montana, USA	Winter wheat, <i>T. aestivum</i>	ND	Phytopathology, 1977
ATCC60205	Michigan,USA	<i>T. aestivum</i>	ND	Plant Disease, 1984
ATCC60208 [*]	Michigan,USA	<i>T. aestivum</i>	ND	Plant Disease, 1984
CBS132.34 ^{b,*}	Kurashiki, Japan	<i>T. aestivum</i> , culm	ND	1932
CBS502.81 [*]	Sweden	<i>T. aestivum</i>	ND	-
CBS759.69 [*]	Scotland; Edinburgh,UK	<i>Hordeum vulgare</i> , stem	ND	1966
Cg84-30 [*]	WSU, Prof. T.D.Murray	<i>T. aestivum</i>	ND	Plant Disease, 1988
Cg94-1 [*]	WSU, Prof. T.D.Murray	-	ND	-
Cg95-1 [*]	WSU, Prof. T.D.Murray	-	ND	-
Cg95-4 [*]	WSU, Prof. T.D.Murray	-	ND	-
MAFF305546 ^{c,*}	Hiroshima, Japan	<i>T. aestivum</i>	+	1960

^a ATCC: American Type Culture Collection

^b CBS: Centraalbureau voor Schimmelcultures

^c MAFF: Ministry of Agriculture, Forestry, and Fisheries, Japan

^d ND: Not done

* Isolates in which ITS region was sequenced

Table 2 Fungal species used to test the specificity of *Cephalosporium gramineum* PCR primer pair

Fungal species	Isolate Code	Host	Source
<i>Cadophora gregata</i>	T96-1	<i>Vigna angularis</i>	LPPHU
	T96-5	<i>V. angularis</i>	LPPHU
<i>Fusarium graminearum</i>		<i>Triticum aestivum</i> , cv. Haruyutaka	LPPHU
<i>Fusarium oxysporum</i>	OTO2	<i>T. aestivum</i> , cv. Haruyutaka	LPPHU
<i>Gaeumannomyces graminis</i>	GG52	<i>T. aestivum</i>	HCAES, Dr. O. Fujine
	GG55	<i>T. aestivum</i>	HCAES, Dr. O. Fujine
<i>Microdochium nivale</i>	MN546	<i>T. aestivum</i>	LPPHU
<i>Helgardia herpotrichoides</i> *	235896	<i>T. aestivum</i> , stem	NIAS
<i>Pythium graminicola</i>	305578	Corn field soil	NIAS
<i>Pythium paddicum</i>	241108	<i>T. aestivum</i>	NIAS
<i>Pythium rostratum</i>	24111	<i>T. aestivum</i>	NIAS
Binucleate <i>Rhizoctonia</i> AG-D II	MW-EF1	<i>Zoysia</i> sp.	LPPHU
<i>Rhynchosporium secalis</i>	239446	<i>Hordeum vulgare</i>	NIAS

<i>Sclerotinia sclerotiorum</i>	Ch-1	<i>Brassica juncea</i>	LPPHU
<i>Typhula incarnata</i>		<i>Spergula arvensis</i>	NARC, Dr. N. Matsumoto
<i>Typhula ishikariensis</i>		<i>Lolium perenne</i>	NARC, Dr. N. Matsumoto
<i>Verticillium dahliae</i>	86101	<i>Glycine max</i>	LPPHU
	84011	<i>Solanum esculentum</i>	LPPHU
Unknown fungus	No.5	<i>T. aestivum</i> , cv. Haruyutaka	LPPHU
Unknown fungus	No.6	<i>T. aestivum</i> , cv. Haruyutaka	LPPHU

HCAES, Hokkaido Central Agriculture Experimental Station, Japan

NIAS, National Institute of Agrobiological Sciences, Japan

NARC, National Agriculture Research Center for Hokkaido Region, Japan

LPPHU, Laboratory of Plant Pathology, Hokkaido University, Japan

* *Helgardia herpotrichoides* is the old nomenclature. The new one should be either *H. yallundae* or *H. acuformis*, however, such information is not available on the website of the NIAS, from which we purchased the *Helgardia* isolate. Thus we used the old nomenclature.

Table 3 Genotyping of isolates used in this study based on IGS-RFLP analysis

Genotype A	Genotype B	Genotype C	Genotype D
CBS502.81	ATCC15017	ATCC32622	BH0909
CBS759.81 [*]	ATCC36969	ATCC60205	CBS132.34
KR08106 [*]	Cg95-1	ATCC60208	IKD0905
KR08309 [*]	KR08202	ATCC46044	
KT0801 [*]	KR08507	Cg84-30	
MK08201 [*]	KR08601	Cg94-1	
MK09101 [*]	MAFF305546	Cg95-4	
OKC83 [*]	KR9101	KR0601	
TY0802	KR9104	KR0603	
	KR9108	KR0605	
		KR08402	
		MK08101	
		MK08302	
		MK09215	
		NAG0807	
		NAN0803	
		OTF0809	
		URH0901	

^{*} Isolates in which point mutation in ITS region added a restriction site for the enzyme *Hinf*I