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MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF *Fusarium verticillioides* IN MAIZE

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ABSTRACT

Seed health is one of the important aspects in preventing the transmission of pathogenic seed-borne microorganisms and in identifying and minimizing possible mycotoxin producers in food. This study was conducted to investigate the occurrence of fungi in four accessions of rice and maize obtained from the seed bank of the National Institute of Agrobiological Sciences, Japan (NIAS Gene bank). Fungal isolation and morphological identification were conducted according to the published taxonomic procedures. Molecular analysis based on DNA sequencing of specific gene regions (Histone H3 gene region, ITS region of ribosomal DNA) were also performed on 14 species out of 24 isolated species. The nucleotide - nucleotide BLAST search and subsequent phylogenetic analysis were performed for further precise identification. Based on the DNA sequencing data, four species of *Fusarium*, two species of *Penicillium* and three species of *Aspergillus* were identified. The *Fusarium* (F2) isolate from the maize showed 99% DNA homology with the BLAST search and it was nested within a clade together with the Gene bank accession of two strains of *Fusarium verticillioides* (AF 150858, AF 150860) supported with a high (100%) bootstrap value. This is the first report of this species from Japan.

KEYWORDS: DNA sequencing, Ear rot, Fumonisin, *Fusarium verticillioides*, Maize, Seed health.

INTRODUCTION

Seeds are an internationally important commodity in world agriculture and approximately 90% of the crops are propagated by seeds. Use of clean and healthy seed is an important prerequisite for better crop establishment and production. Seed health concerns the quality status of the seed which may be used either as farmer's planting material or as germplasm for crop improvement. Seed health testing is a tool for managing and minimizing seed borne diseases and it is a part of the entire disease control.

Seeds are the passive carriers for many forms of fungi, bacteria, viruses and insects. Systemic transmission of fungi from seed to seedling is well documented (Manandhar *et al.*, 1998, Shrestha *et al.*, 2000). Hutchins and Reeves (1997) have reported that the *Curvularia* spp., *Alternaria padwickii*, *Fusarium moniliform* and *bipolaris oryzae* are major pathogens in rice. Rust, southern corn leaf blight and stalk rot or ear and kernel rots are the most prominent diseases among maize fields (Li and Wu, 1986). In addition, mycotoxin production in food is apparent. *Fusarium* spp. *Penicillium* spp. and *Aspergillus* spp. are the three most important mycotoxin producing fungal genera with respect to animal and human health (Moss, 1996).

Moreover, it is important to test the seed health together with better knowledge of the specific species of fungi for the increased production of food for the densely populated world. Correct identification of species is an important factor in seed health analysis. Morphological characters are not sufficient for the precise identification of the species. Molecular analysis of strains has made it possible to assess their genetic differences at the DNA level. This study was performed at the NIAS, Gene Bank, National Institute of Agrobiological Sciences, Tsukuba. Japan to identify the associated fungal species in rice and maize seed samples by morphological and molecular analysis.

METHODOLOGY

Fungal isolation

Two (2) accessions of rice variety Hatsutokachi (L 9440. L 9441) and Two (2) accessions of maize seed variety "Kawaginu" (L 1802. L 208067) obtained from the NIAS Gene bank were analyzed. Ten seeds were selected from each accession, and were placed separately on the SNA (Synthetic low Nutrient Agar) plates in 9 cm petri dishes for the detection and isolation of microorganisms without washing or sterilization procedures. These cultures were examined microscopically for the fungal occurrence at one week interval up to one month. Isolated fungal species were cultured on SNA and the pure cultures were maintained at room temperature.

Morphological identification

For morphological identification. four different types of culture media were used. Synthetic Nutrient Agar (SNA) medium to induce conidiogenesis and to examine conidial structures, and Potato Dextrose Agar (PDA), Czapek Yeast Agar (CYA) and Malt Extract Agar (MEA) to study the colony characteristics. Cultures were examined microscopically under low magnification (x 150) of a Nikon optical microscope to study morphological features of the aerial mycelia. When sporulation was observed in the cultures. agar blocks containing conidial structures were mounted on a microscopic slide with a drop of sterile distilled water and examined at a high magnification (x 600). Diagnostic morphological features were recorded for each strain and morphological identification was conducted according to the published taxonomic procedures.

Molecular identification

Fourteen fungal isolates were cultured on SNA plates in 5 mm diameter paper disks and incubated for 5 to 7 days at the room temperature. Mycelia covering and penetrating paper disks were harvested and DNA templates for PCR (Polymerase Chain Reaction) were prepared using chloroform-isoamyl-alcohol (CIA) method (T.Aoki, personal communication). Amplification of DNA fragments by PCR was conducted for *Penicillium* spp. and *Aspergillus* spp. using ITS (Internal Transcribed Spacer) primers (White *et al.*, 1990) and for *Fusarium* spp. using H3-la, H3-lb primer pair (Steenkamp *et al.*, 1999) and H3F1, H3R1 primer pair (O'Donnell *et al.*, 2004) with the following program: initial denaturation at 95°C for 15 min, primer annealing at 94°C for 30 sec, and 54°C for 1 min 40 cycles and 72°C for 10 min for extension. Amplified products were examined using 2% Agarose gel electrophoresis in 0.5x TBE buffer. Gels were observed using a Gel Imager.

PCR products were purified using QIA Quick PCR purification Kit (QIAGEN) and DNA concentration was checked using Nano Drop^{tm} (Nano Drop Technologies Inc.). A set of eight different primers for ITS region (ITS5, ITS2, NL1, NL3, NL4, NL3C, ITS4 and ITS2), and two sets of primers for Histone H3 regeion (H3-1a + H3-1b or H3F1 + H3R1) were used for sequencing reaction. Big Dye Terminator version 1.1 (Applied Biosystems Inc.), 5x PCR buffer, and distilled water were used for the sequence reaction, under the following program, 96°C for 1.5 min., 96°C 10 sec, 50°C 5 sec, 60°C 4 min. (25cycles) and holding at/4°C. After the sequencing reaction, products were purified by using DyeEx Spin Columns (QIAGEN) and were run on an Applied Biosystem 377 DNA sequencer. Data was analyzed using GENEDOC package. The sequencing matrix of *Fusarium* species were obtained by multiple alignment, using Clustal X, version 1.81 (Thompson *et al.*, 1997) and phylogenetic analysis was performed. The nucleotide-nucleotide BLAST (Basic Local Alignment Search Tool) equipped in the internet site of NCBI (National Center for Biotechnology Information, USA; http://www.ncbi.nlm.nih.gov/BLAST/) was used to determine the DNA homology of these isolates.

RESULTS AND DISCUSSION

Fungal identification based on morphological and molecular characters

Out of twenty four isolates obtained from the seed samples examined, seven isolates of *Fusarium*, four isolates of *Penicillium* and three isolates of *Aspergillus* were further subjected to precise identification at species level. Morphological characters were examined in detail according to the published taxonomic literature. The fourteen fungal isolates identified on the morphological characters are shown in Table 1.

Table 1. Morphological identification of representative fungal isolates.

Isolates	*Origin*	*Morphological I.D.*
F1	Rice	*Fusarium fujikuroi*
F2	Maize	*Fusarium* cf. *verticillioides*
F3	Rice	*Fusarium sporotrichioides*
F4	Rice	*Fusarium avenaceum*
F5	Rice	*Fusarium incarnatum*
F6	Rice	*Fusarium equiseti*
F7	Rice	*Fusarium asiaticum*
P1	Rice	*Penicillium* sp.
P2	Maize	*Penicillium* sp.
P3	Rice	*Penicillium* sp.
P4	Maize	*Penicillium* sp.
A1	Rice	*Aspergillus* cf. *japonicus*
A2	Maize	*Aspergillus* cf. *flavus*
A3	Rice	*Aspergillus* sp.

Based on morphology, especially on those of conidial structures, seven isolates (species) of *Fusarium* were identified to species level. However, four isolates of *Penicillium* and one isolate of *Aspergillus* were unable to be identified further due to the difficulty which arise at the morphological (phenotypic) characterization. Two isolates of *Aspergillus* were considered to be closely related morphologically to two known species of the genus, *A. japonicus* and *A. flavus*.

Results of identification of the 14 fungal isolates based on the DNA sequence data are given in Table 2.

The amplification of DNA fragments of Histone H3 gene regions of the isolates F1 and F7 was unsuccessful; no DNA sequence data were obtained for these isolates. The procedures followed for obtaining DNA sequence data on Histone H3 gene region were successful on the isolates F6. However, no comparable DNA sequence data were found for the identification of the strain at the species level with the BLAST or T. Aoki's data. Based on the DNA sequence data, four isolates (species) of *Fusarium*, two isolates (species) of *Penicillium* and three isolates (species) of *Aspergillus* were identified at species level. However, two isolates of *Penicillium* could not be well identified at species level, because the Genebank accessions of the DNA that were closely related to the sequence data on these isolates were for an unidentified species of *Penicillium*, i.e. NRRL 28143. In total 9 species were then identified from 14 isolates examined.

Table 2. Molecular identification of representative fungal isolates.

Isolates	*Molecular I.D.*	*Identification Criteria*
F1	(-)*	
F2	*Fusarium verticillioides*	Histone H3 / BLAST SEARCH (99%I.D.)**+ phylogenetic analyses***
F3	*Fusarium sporotrichioides*	Histone H3 / phylogenetic analyses
F4	*Fusarium avenaceum*	Histone H3 / phylogenetic analyses
F5	*Fusarium incarnatum*	Histone H3 / phylogenetic analyses
F6	(-)****	
F7	(-)*	
P1	*Penicillium* sp. (most related to NRRL 28143)	ITS / BLAST SEARCH (99% I.D.)
P2	*Penicillium verruculosum*	ITS / BLAST SEARCH (98% I.D.)
P3	*Penicillium* sp. (most related to NRRL 28143)	ITS / BLAST SEARCH (99% I.D.)
P4	*Penicillium citrinum*	ITS / BLAST SEARCH (99% I.D.)
A1	*Aspergillus japonicus*	ITS / BLAST SEARCH (99% I.D.)
A2	*Aspergillus flavus*	ITS / BLAST SEARCH (99% I.D.)
A3	*Aspergillus versicolor*	ITS / BLAST SEARCH (99% I.D.)

*	Amplification of DNA fragments was unsuccessful
**	% DNA homology
***	Phylogenetic analyses together with the data-set on Histone H3 region for *Fusarium* species compiled at the NIAS Genebank
****	Sequence data on Histone H3 region obtained, but no comparable data found

Molecular phylogeny of *Fusarium* isolates

Neibour-joining (NJ) analysis based on the DNA sequence data of the Histone H3 gene regions was conducted on the seven *Fusarium* isolates together with the data downloaded from the Genbank site http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide) and T. Aoki's personal DNA sequence data-set on Japanese species of *Fusarium* compiled at the NIAS Genebank (T. Aoki, Personal Communication). A NJ-phylogenetic tree created by the analysis is presented in Figure 3.

Bootstrapping values more than 50 % are also indicated on the node of each clade of the tree. The F2 isolate from maize was nested to a clade together with the accessions of two strains of *Fusarium verticillioides* (AF 150858, AF 150860) supported with a high (100%) bootstrap value. However, it deviated from the *Fusarium verticillioides* position for the isolate from the imported banana from Mexico to Japan, although the whole *F. verticillioides*-clade was supported with a high (100%) bootstrap value. Similarly, the isolates F3, F4 and F5 were nested to the *Fusarium sporotrichioides*-clade, the *Fusarium avenaceum*-clade and the *Fusarium incarnatum*-clade, respectively, based on DNA sequences of the Histone H3 gene region. The F1, F6 and F7 isolates from rice were not identified at the rank of species based on DNA sequences, however, these isolates were identified as *Fusarium fujikuroi*, *F. equiseti*, and *F. asiaticum* based on morphological characters.

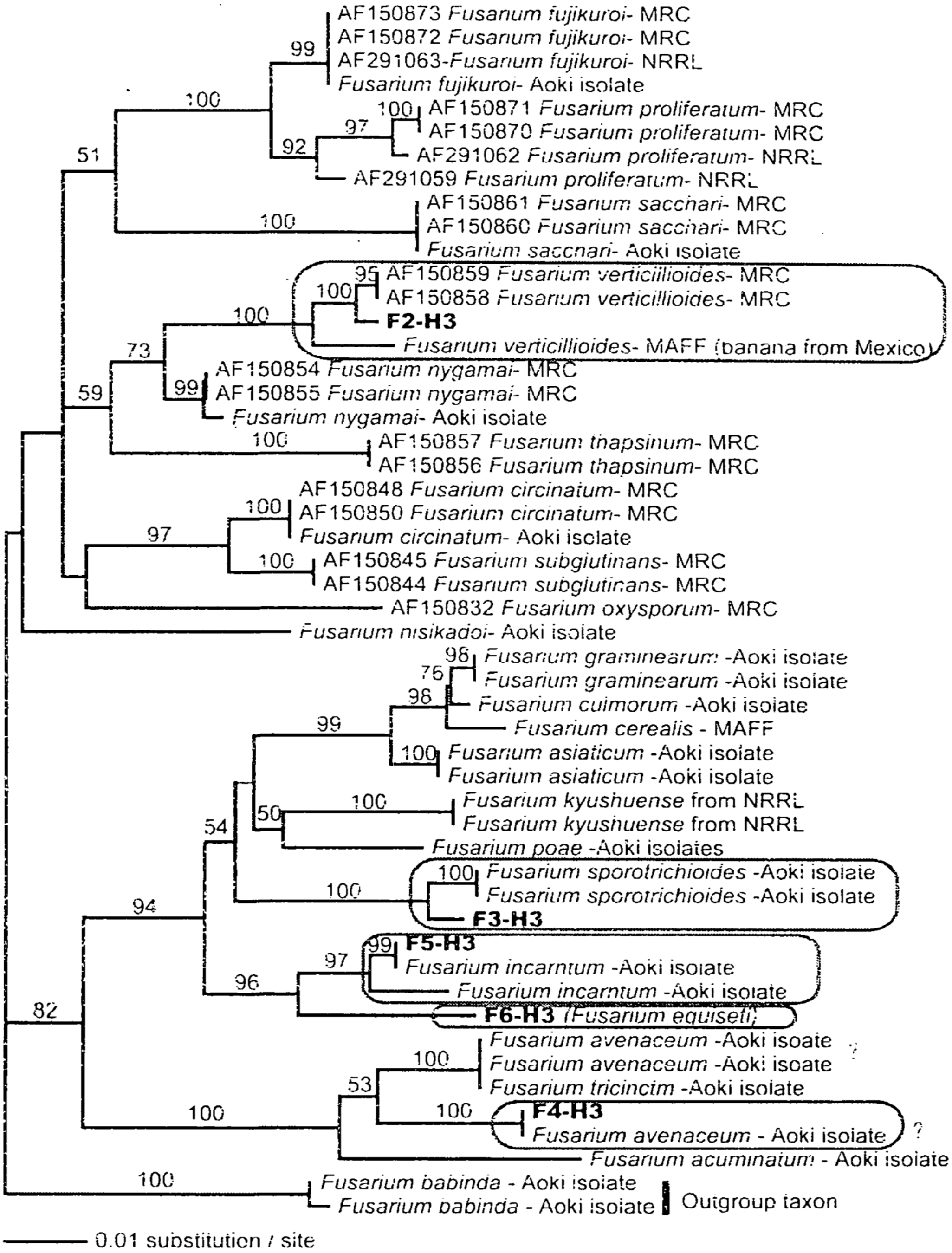

Figure 3. A NJ-phylogenetic tree on *Fusarium* species examined based on the DNA sequence data of the Histone H3 gene regions.
NJ Tree, Histone H3, 458 bps, #. % bootstrap value

In this study, based on the morphological and molecular analysis, the maize isolate (F2; Fig. 1)) was identified as *F. verticillioides* and was deposited at the Microorganisms Section of the NIAS Genebank under the accession no. MAFF 240085. This is the first report of this species isolated from Japan. However, this species has been reported from Japan only on the trapped materials during the plant quarantine inspection process of imported banana from Mexico (Hirata *et al.*, 2001).

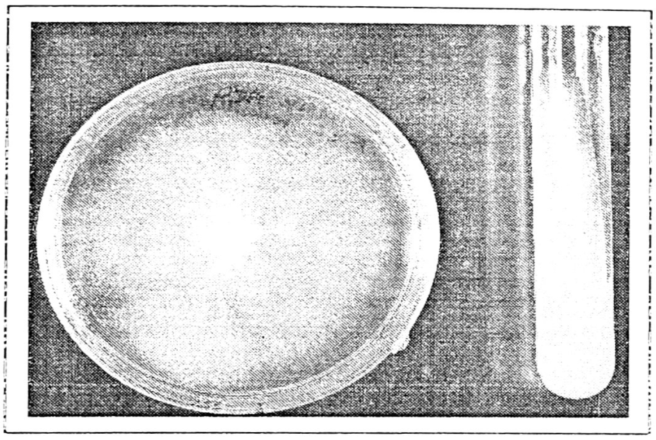

Figure 1. Mycelium of *Fusarium verticillioides* grown in a culture plate.

Fusarium verticillioides is a widely distributed pathogen which causes corn seedling blight, root rot, stalk rot or ear rot (Kommedahl and Windels, 1981). The main feature of this species is low level of symptoms. When the plant is at stressed condition, fungus can cause disease. The most important infection method is through seed. An important feature of the species is the production of fumonisins (B1) that has a potential toxicity for human which cause esophageal cancer in human (Marass, 1995). It also has some effect on brain, liver and lungs, kidneys, gastrointestinal track and blood cells.

CONCLUSIONS

The two accessions of maize seed samples, Japanese variety "Kawaginu" which had been stored in gene bank L 1802 (28/01/1999) and L 208067 (27/05/2002) were infected with *Fusarium verticillioides* which has not yet been identified in Japan. Molecular analysis based on DNA sequencing is the highly sensitive and accurate method for characterizing fungi present in maize seeds.

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