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Isolation, Morphology, and Molecular Characteristics of *Ustilago maydis* from the Philippines

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ABSTRACT

The basidiomycete *Ustilago maydis* causes smut in corn (*Zea mays*). The pathogen was first reported in the Philippines in 1919. However, characterization data and knowledge of its current isolate for use as reference are scarce. This study provides knowledge of the morphocultural characteristics of a Laguna isolate of *Ustilago maydis* and, for the first time, confirms the species and identity through DNA sequencing of the partial sequence of internal transcribed spacer (ITS) in the ribosomal DNA gene region. The teliospore size (n = 30) ranged from 8.09 to 11.36 μM (averaged at 9.21 μM). Washed teliospores were successfully isolated in a potato dextrose agar (PDA) medium supplemented with $CuSO_4$. The teliospore and the haplotype DNA (from PDA) were extracted and used in a polymerase chain reaction assay. A ~800 base pair product was amplified, and sequences of the ITS rDNA revealed teliospore and haplotype of the Philippine isolate were 100% similar to known *U. maydis*. Phylogenetic analysis also revealed a close resemblance of the Philippine isolate to *U. maydis* isolated from corn in Mexico. Description and DNA sequence information of the *U. maydis* Philippine isolate MBZM001 provided in this study can be used as references for future studies.

Keywords: *Zea mays*, maize, fungal plant disease, phylogenetic analysis

INTRODUCTION

Corn (*Zea mays*) is a major cereal crop grown in the tropics. It is one of the staple crops in the Philippines, second to rice. The volume of corn production in the country is generally stable in the past decades, but there have been some episodes of yield reductions due to biotic and abiotic disturbances. Pests and diseases account to significant yield loss in corn production. For instance, the incursion of fall armyworm has brought severe destruction in corn fields over the last few years. Diseases caused by fungi (Balendres, 2023), bacteria, viruses, and nematodes (Tangonan, 1999) are also major concerns. Among the major diseases are stalk rots, ear rots, and leaf blights.

One of the not-so-well-studied but still popular disease infecting corn is smut. This disease is caused by *Ustilago maydis* (DC.) Corda, a semi-obligate plant pathogenic fungus under the phylum Basidiomycota (Alexopoulos et al., 1996). The disease can be easily distinguished due to the tumor-like galls formed in infected corn ears. Severe infection by *U. maydis* results in black masses. The pathogen was first reported in the Philippines in 1919. It has long been established as a model system for studying biotrophic pathogens (Brefort et al., 2009). Studies have shown that its life cycle and dissemination mechanisms (Shutleff, 1980) generally resulted in mild grain yield losses only under field conditions. This basidiomycete fungus infects all aerial parts of the corn in the form of prominent tumors. It can be cultured in its haploid yeast phase on standard laboratory media (Pataky & Snetselaar, 2006).

This study aims to isolate and characterize the corn smut pathogen *U. maydis* in the Philippines and to provide new information on the molecular characteristics of this pathogen. The information will be useful as a reference for future research on *U. maydis* in the Philippines and in Southeast Asia.

MATERIALS AND METHODS

Sample and Spore Processing

Infected samples showing tumor-like galls in corn cobs were collected from a corn field in Los Baños, Laguna, Philippines. The diseased samples were provided by a grower who observed the disease in the field. Samples were brought to the Plant Pathology Laboratory of the Institute of Plant Breeding, College of Agriculture and Food Science, University of the Philippines Los Baños. Isolation was performed following the method described by Bosch and Djamei (2017). Infected grain with teliospores was carefully ground using a micro pestle in a 1.5-mL microcentrifuge tube. Then, 500 µL of sterile distilled water was added. Ground spores were incubated for 1 hour at room temperature before adding 3% CuSO4 and mixed using a vortex to disinfect the contaminants in the solution. The spore/CuSO4 mixture was incubated for another 15 min at room temperature and then centrifuged for 5 min at 1,200 × g for the spores to pellet. The supernatant was decanted, and the pellet was resuspended in 1 mL of sterile distilled water. Washing of the pellet was done three times, and spores were resuspended in 300 µL of sterile distilled water. Dilution series (100-104) was made and plated into potato dextrose agar (PDA) for incubation for two days at room temperature. Single colonies were then picked out and purified into new PDA plates for further characterization.

DNA Extraction

DNA was extracted from the teliospores of the infected corn and pure culture of the pathogen in PDA using the procedure described by Chen and Kuo (1993). The infected sample collected black masses of teliospores and was ground in a 1.5-mL microcentrifuge tube with 300 µL of lysis buffer (40 mM Tris-Acetate pH 7.8, 20 mM sodium acetate, 1 mM ethylenediaminetetraacetic acid [EDTA], 1% sodium dodecyl sulfate). On the other hand, a loopful of the yeast-like colonies of the pathogen in PDA was also suspended in the same buffer. After careful grinding, 66 µL of 5 M NaCl solution was added and centrifuged at 12,000 rpm for 10 min. Next, the supernatant was transferred, and equal amounts of chloroform isoamyl alcohol (24:1) were added, mixed, and spun for 3 min at 12,000 rpm. The aqueous sample was then transferred into a new microcentrifuge tube containing two volumes of absolute ethanol, mixed gently, and incubated for 1 hr in the freezer. Then, the samples were spun for 5 min at 12,000 rpm to collect DNA pellets. Next, the supernatant was decanted. The pellet was washed twice with 70% ethanol, air dried, and resuspended in 50-µL Tris-EDTA (TE) buffer (1 M Tris buffer, pH 8.0, 0.5 M EDTA). DNA quality was checked by electrophoresis in 1.2% agarose gel in 0.5× TAE buffer.

Molecular Assay

The genomic DNA was used as a template for the polymerase chain reaction (PCR) assay to amplify the partial sequence of the internal transcribed spacer (ITS) gene region using primer pairs ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990). The PCR reaction mix contained 1× PCR buffer (Invitrogen), 2 mM MgCl2 (Invitrogen), 0.2 mM dNTP mix (Invitrogen), 0.2 µM of each primer, 1 U of Taq polymerase (Invitrogen), 1-µL DNA template, and volume up to 25 µL with diethyl pyrocarbonate-treated water (Invitrogen). PCR amplification was carried out in MyCycler™ Thermal Cycler System (Bio-Rad Laboratories) with an initial denaturation step of 94°C for 5 min, followed by 24 cycles of denaturation at 94°C for 45 sec, annealing at 55°C for 45 sec and extension of 72°C for 1 min. A final extension step at 72°C for 7 min was then employed. The amplified bands were subjected to gel electrophoresis with 1.5% agarose (Vivantis) dissolved in 0.5X TAE buffer containing 2-µL GelRed solution (Biotium) and run at 100 V for 30 min. Gel bands were visualized using the Molecular Imager Gel Doc™ XR+ with Image Lab software (Bio-Rad Laboratories). PCR products were sent to Apical Scientific Sdn. Bhd. (Malaysia) for DNA sequencing. A consensus sequence of the forward and reverse DNA sequences of the genes was made using the Geneious software. Following this, a sequence similarity check was performed using the NCBI BLASTn software. A phylogenetic tree was generated through the MEGA X software, and bootstrap analysis using 1,000 replications was used to assess the stability of the branches.

RESULTS AND DISCUSSION

Tumor-like galls with varying sizes were observed in harvested corn (Figure 1A) collected from Los Baños, Laguna, Philippines. Galls are white, firm, and semi-glossy. Severe infection was also observed where galls ruptured, releasing black masses of teliospores. Corn smut pathogen *Ustilago maydis* is a well-

studied pathogen making it a model organism for fungal genetics. However, little to no research in the Philippines dealt with this pathogen. In this study, we reported corn smut and characterization of the pathogen up to DNA sequencing for accurate identification.

Several different colonies were observed growing in PDA after two days of incubation. First, a yeast-like colony was picked out and purified. Then, colonies were cream or pale yellow with rough surfaces (Figure 2). Finally, microscopically, a bud-like manner of growth was seen where cells were cigar shaped. From the teliospores, cream-colored yeast-like colonies, the haploid sporidia of the pathogen were isolated in PDA. It is considered basidiospores, the fungus saprophytic stage (Pataky & Snetselaar, 2006). *U. maydis* cells observed corroborated with the work of Nadal et al. (2016), where cells measured between 2 and 4 µM in width and 20 and 30 µM in length, which are cigar shaped. Under appropriate environmental conditions, genetically compatible sporidia mate and form dikaryotic infection hyphae and are considered the pathogenic stage of the fungus. This is yet to be investigated. Under the microscope, spherical to ellipsoid echinulate teliospores were olive brown (Figure 3).

Figure 1. Infected corn showing tumor-like galls. Severe infection showed black masses of teliospores ruptured around the infected sample.

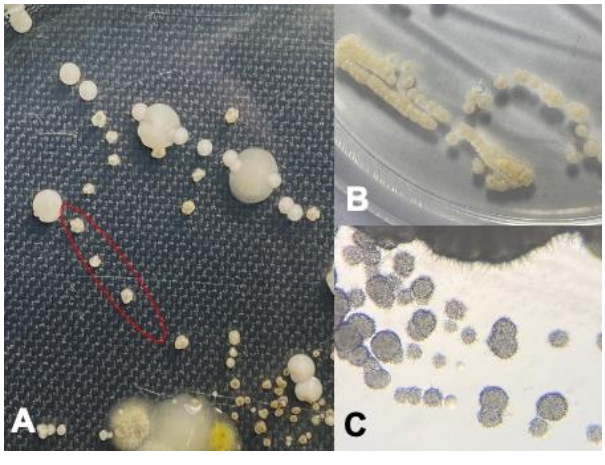

Figure 2. Yeast-like colony that is cream or pale yellow with rough surfaces. Microscopically, a bud-like manner of growth was seen where cells are cigar shaped.

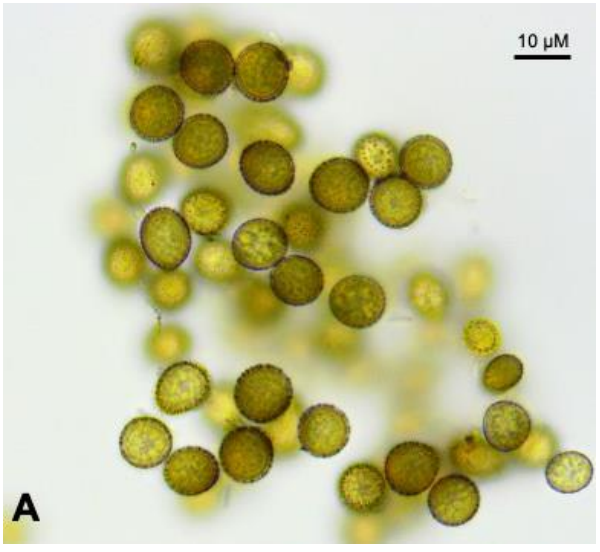

Figure 3. Teliospores that are spherical to ellipsoid and olive brown in color (magnification = 1000×).

PCR analysis of the isolate's partial sequence ITS gene region yielded ~800 base pair amplicons in gel electrophoresis. Initial analysis of the DNA sequence using the BLASTN software showed a 100% similarity to *Ustilago maydis* and uncultured *Pseudozyma* accessions submitted to GenBank and 98%–99% similarities to other *U. maydis* accessions as shown in the constructed phylogenetic tree (Figure 4). The sequences from the GenBank, like the isolates, were from fungal community-related studies and had no details on the specific pathogen itself.

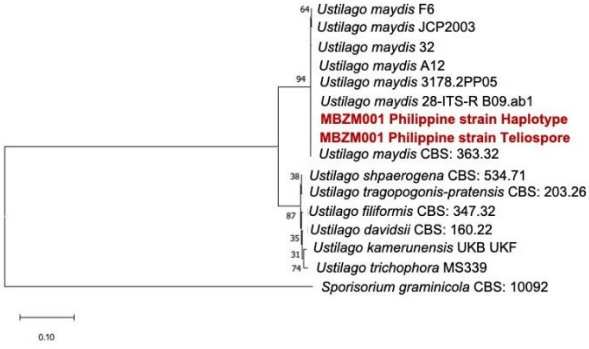

Figure 4. Phylogenetic tree using the partial ITS gene region showing the relatedness of the Philippine isolates of *U. maydis* (haplotype and teliospore) to other world collections.

CONCLUSION

This study isolated and characterized *Ustilago maydis* causing corn smut in the Philippines. The pathogen was successfully isolated in a culture media. The DNA sequencing of the partial ITS region further validated the identity of the pathogen. Analysis of the ITS gene region of the isolates revealed 100% sequence similarity to *Ustilago maydis*. To our knowledge, this is the first DNA-based analysis of a Philippine isolate of *U. maydis*. The resulting sequence could be used as reference for future phylogeny analysis. The affinity of the Philippine collection with other world collections was also elucidated in this study.

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