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# Molecular and Morphological Identification of *Ustilaginoidea Virens*, the Causal Agent of Rice False Smut in Sri Lanka

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**Abstract**

Seed-borne fungal pathogens significantly impact on both the quality and quantity of rice production of rice. Among these, *Ustilaginoidea virens*, the causal agent of rice false smut, directly impacts grain yield and quality by partially or completely replacing kernels with spore masses. Historically considered a minor issue, false smut has recently emerged as a major rice disease of concern. Accurate identification and confirmation of *U. virens* are thus critical for developing effective disease management strategies. In this study, rice panicles with characteristic false smut symptoms were collected from the wet zone of Sri Lanka. The associated fungal pathogen was isolated on potato sucrose agar (PSA) and designated as isolate WC2.04. Colony and micro-morphological features of the isolate obtained were documented, followed by DNA extraction, Polymerase chain reaction and bidirectional Sanger sequencing of the internal transcribed spacer (ITS) region. Sequence data were analyzed using the Basic Local Alignment Search Tool (BLAST) against the NCBI database, and phylogenetic analyses were performed. The results confirmed the identity of WC2.04 as *U. virens*. This study presents the first molecular confirmation of the occurrence of *U. virens* in Sri Lanka, highlighting the importance of integrating morphological and molecular approaches for the accurate identification of rice seed-borne fungi. Accurate identification is crucial for developing and implementing effective disease management strategies to mitigate the impact of rice false smut in the region.

***Keywords:*** Cereal rust fungi, false smut, seed-borne pathogens, *Ustilaginoidea virens*

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## Introduction

Rice (*Oryza sativa*) is a vital and staple food for over half of the global population, particularly in Asia, Africa, and Latin America, where demand continues to increase (Zeigler and Barclay, 2008). In Sri Lanka, paddy seeds for sowing are typically grown by farmers and exchanged among communities, emphasising the need for rigorous seed quality control. However, rice seeds harbour a diverse array of microorganisms, with 56 fungal pathogens identified on rice, 41 of which are seed-borne (Ackaah et al., 2023). Among these, *Ustilaginoidea virens* (Cooke) Takahashi, the causal agent of false smut disease, was first documented in 1878 in Tamil Nadu, India (Cooke, 1878). In folk belief, farmers in South Asian countries consider that the golden-green false smut spore balls occurring on rice as a symbol of prosperity, since they appear most often in lush, well-irrigated, high-yielding fields., However, false smut has now transitioned from a minor issue to a significant disease threat. This shift has been attributed to the widespread use of nitrogenous fertilisers, extensive cultivation of high-yielding varieties, and conducive environmental conditions, temperatures of 22–28 °C, high humidity, and rainy weather during rice booting stages (Ladhalakshmi et al., 2012).

The pathogen's life cycle includes both sexual (sclerotia) and asexual (chlamydospore) stages. Sclerotia act as primary inoculum sources, germinating to produce ascocarps with infective ascospores, while airborne chlamydospores enable secondary infections (Ashizawa et al., 2010). Disease symptoms manifest as false smut balls, transitioning from yellow to yellowish-orange, green, and eventually greenish black, becoming apparent after flowering when spikelets are covered by the fungus. Recent studies reveal that genetic variation of *U. virens* is more closely associated with geographic distribution than host cultivar (Wang et al., 2008). Functional genomics analyses indicate the presence of genes suppressing host hypersensitive responses (Sun et al., 2020) and high expression of genes encoding secreted proteins related to toxins and secondary metabolism (Wang et al., 2008).

In Sri Lanka, research on false smut has predominantly relied on morphological assessments, with no prior DNA sequence-based characterization of *U. virens* isolates. This study addresses this gap, providing the first molecular confirmation of false smut pathogens in Sri Lanka, thereby contributing essential data for disease diagnostics and management strategies.

## Materials and Methods

### *Sample collection, isolation, and morphological characterization*

Symptomatic rice panicles were collected from the 6°47'55.0"N 79°56'30.8" E (Piliyandala, Colombo, wet zone, Sri Lanka). Infected rice seeds were surface sterilized for 2 min in 1% (v/v) sodium hypochlorite solution and subsequently rinsed three times with sterile distilled water and dried using sterile filter papers. Seeds were cut into two parts by using a sterile scalpel, and a sterilized needle was used to dust the spores from the inner surface of the false smut balls onto the Petri plates containing the potato sucrose agar (PSA) medium. The plates were incubated at room temperature (Bashyal et al., 2021). Herbarium samples were prepared for the deposition of reference material in the USJCC herbarium.

Colony morphology of the false smut isolate was observed on 14-day-old cultures on PSA medium amended with 0.01 gL-1 of streptomycin sulfate antibiotic. Colony characteristics, including elevation, margin, shape, and form, were recorded using standard terminology to determine the colony characteristics. The surface and reverse colors of fungal colonies were recorded for the isolation on PSA media using standard color chart. For micromorphological characterization, spores of false smuts were directly taken from the false smut balls and wet mount was prepared using sterile distilled water. Prepared slides were observed under a calibrated compound light microscope (Carl Zeiss Primo Star, Germany). Conidial length and width measurements were taken for at least 30 conidia whenever possible. Conidial width measurements were taken from the widest part of each conidium. Magnification and all the values were recorded. Conidial length and width are presented as ranges as mean ± standard deviation. Extreme measurements are given in parentheses with mean and standard deviation.

For molecular identification, false smut balls of *Ustilaginoidea virens* were carefully surface sterilized with 70% ethanol, washed with sterile distilled water, and then dissected to remove rice husks and leaf parts. Grounded samples were subjected to DNA extraction (Manamgoda et al.,2012). The extracted genomic DNA was dissolved in PCR-grade water and stored at -20 °C for downstream applications. The internal transcribed spacer (ITS) region, the formal fungal barcode, was PCR amplified using ITS1 and ITS4 primers (White et al., 1990) (Table 1), along with smITS-F and smITS-R1 primers (Kruse et al., 2017).

**Table 1:** Primer *sequences used in PCR amplifications.*

| Primer | Sequence | References |
|---|---|---|
| Forward primer ITS1 | 5′-TCC GTA GGT GAA CCT GCG G-3′ | White et al., 1990 |
| Reverse primer ITS4 | 5′-TCC TCC GCT TAT TGA TAT GC-3′ | White et al., 1990 |
| Forward primer smITS-F | 5′-CAAACTYGGTCATTTAGAGGAAGTAA-3′ | Kruse et al., 2017 |
| Reverse primer smITS -R1 | 5′- AGATGGCATTTACCACCCATTTTGM-3′ | Kruse et al., 2017 |

PCR amplification was performed in a BIORAD® T100™ thermal cycler with conditions following Manamgoda et al. (2012) and Kruse et al. (2017), using positive (*Bipolaris maydis* culture code USJCC-0131) and negative (PCR-grade water) controls for the ITS protocol. Amplicons were visualized by electrophoresis on a 2% agarose gel stained with ethidium bromide, and the successful amplicons were sequenced bidirectionally by Macrogen, Korea. Consensus sequences from forward and reverse reads were assembled using BioEdit v7.7.1 and initially identified via NCBI BLAST search. Sequences were aligned using ClustalW and MAFFT v7, and phylogenetic analyses were conducted with maximum likelihood (ML) using the RAxML-HPC BlackBox tool in the CIPRES online resource. Phylogenetic trees were visualized in FigTree v1.4.(Rambaut and Drummond 2008).

**Results**

In the initial stage of the infection, false smut balls appeared yellowish-orange in color and gradually turned olive green and finally into a greenish-black color. On PSA media colony, milky white to yellowish white, fluffy, and compact, raised, circular colonies with entire margin. The upper surface and reverse are white, approximately 4.4 cm diam. after 21 days.
Conidia (5–)6–8(–7) × (4–)5–7(–7) μm ($\bar{x}$ = 7±1× 6±1μm, n = 30), single-celled, yellowish, globose, and smooth. Sexual morph not observed.

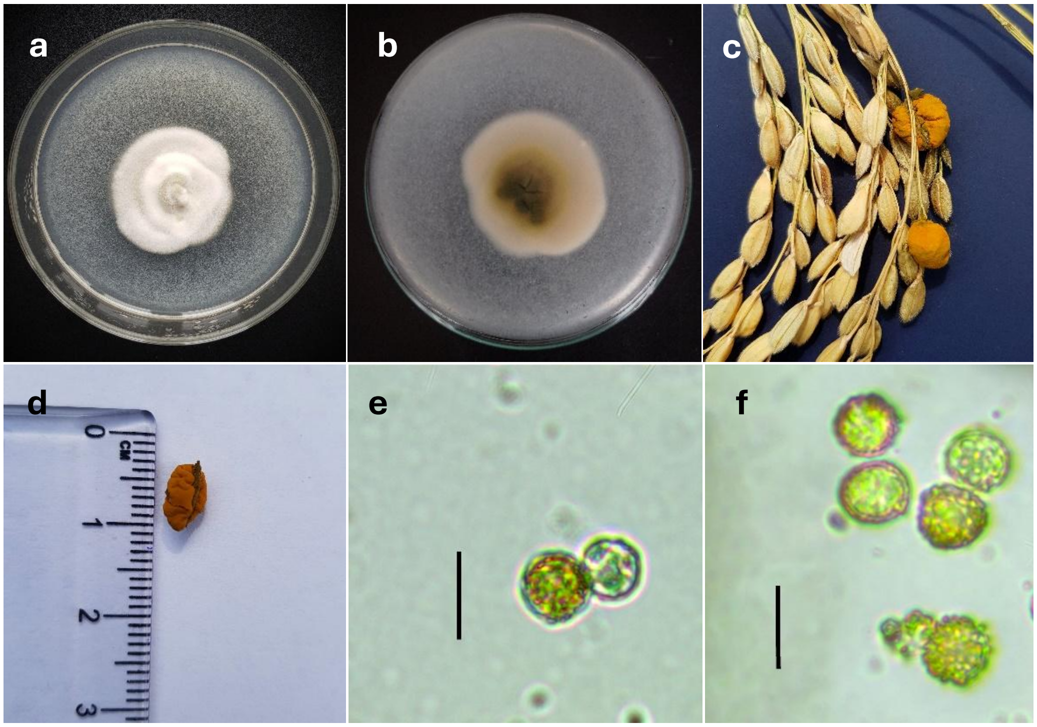


**Figure 1**: *21-day-old colony of WC2.04 on PSA, a) upper surface, b) the reverse surface of the colony. c ) Infected rice panicle, d) Infected rice seeds. e,f ) chlamydospores of false smut balls. Bar in e and f=10 µm.*

Isolate WC2.04, recovered from rice seeds in Sri Lanka, was identified as *Ustilaginoidea virens* through phylogenetic analysis. The phylogram constructed showed no bootstrap values above 50% the phylogenetic tree (Figure 2) revealed slight population-level variation among the isolates.

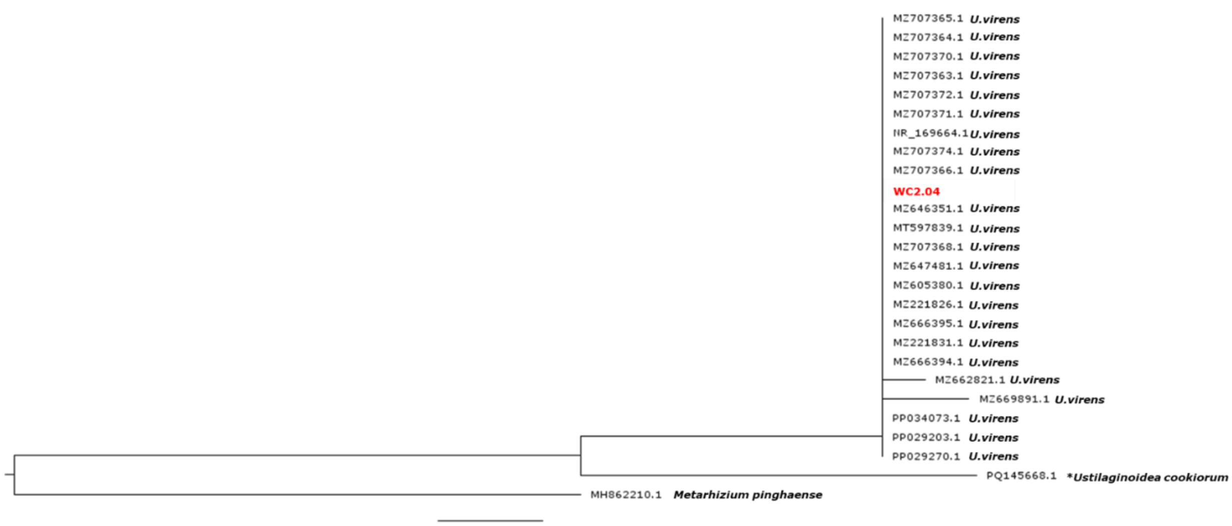


**Figure 2:** *Phylogram of Ustilaginoidea spp. generated from maximum likelihood analysis based on ITS sequence data. The tree is rooted with MH862210.1 Metarhizium pinghaense. Bootstrap values of more than 50 are shown in the tree. The isolate from this study is highlighted in red colour; ex-type cultures were marked (*).*

## Discussion

*Ustilaginoidea virens* (Cooke) was first described by Cooke in 1878 and has since emerged as a significant rice pathogen in rice-growing regions worldwide, including the United States, Egypt, India, China, and Japan (Ashizawa et al., 2010). From 2008 to 2016, the annual average occurrence of false smut in rice-growing areas in China was estimated at 3.062 million hectares, resulting in an annual yield loss of 158.6 million kg. Some studies have reported that during epidemic years, false smut can cause up to a 40% yield loss (Han et al., 2015). Emerging fungal diseases like false smut pose a major threat to future rice production, particularly under changing climatic conditions and evolving agricultural practices.

Accurate identification of seed-borne fungi such as *U. virens* is challenging when relying solely on morphological characters. It is therefore recommended to integrate morphological characters with molecular and phylogenetic analyses, employing ex-type and other reference strains for precise species identification. The isolation of *U. virens* is difficult due to its slow-growth rate (Bashyal et al., 2021), and its growing colonies are often overgrown by fast-growing saprophytes during isolation and incubation. Various methods for isolating false smut pathogens have been explored in previous studies, including pathogen isolation from sterilised, teased false smut balls on PDA (Baite and Sharma, 2015), chlamydospore streaking (Ladhalakshmi et al., 2012), and single spore isolation using optimized media (Bashyal et al., 2021).

In this study, the dust spore method was employed, whereby spores from the inner layer of false smut balls were used. This approach is advantageous because surface sterilisation can compromise the viability of outer-layer spores (Bashyal et al., 2021). According to the literature, PSA is considered the most suitable medium for isolating the false smut pathogen (Bashyal et al., 2021). Growth on PSA medium typically becomes apparent after 14 days of incubation, and by 30 days, white fluffy mycelia transform into dense, velvety, leathery colonies. Similar growth patterns have been reported by (Bashyal et al.,2021) and Lin et al. (2018). Unlike true smuts, which typically cannot be cultured, false smut replaces all or part of a rice kernel with yellowish-golden spores and, although challenging, has some culturable potential (Brooks et al., 2009).

For DNA extraction of *U. virens*, both direct samples and cultures were used. For direct samples, sterilised false smut balls and spores from the inner layer were separately collected. Careful execution of surface sterilization is essential to minimize contamination with other fungi (Bashyal et al., 2021). In this study, the fungal barcode ITS region was amplified using the primers ITS1 and ITS4 (White et al., 1990), as well as smITS-F and smITS-R1 (Kruse et al., 2017). The smITS-F and smITS-R1 primers were specifically designed to reduce amplification of host plant DNA and other contaminating fungi, while maximizing coverage for various smut genera (Kruse et al., 2017). A phylogenetic tree for *U. virens* was constructed using ITS sequences, as they represent the most widely available data in GenBank. However, the limited availability of ITS sequences for *Ustilaginoidea* species in the NCBI GenBank makes constructing a robust backbone phylogeny challenging compared to some other fungal genera. This study represents the first molecular identification and confirmation of *Ustilaginoidea virens* in Sri Lanka.

## Conclusion

This study presents the first molecular confirmation of *Ustilaginoidea virens*, the causal agent of rice false smut, in Sri Lanka. By integrating detailed morphological observations with robust molecular and phylogenetic analyses, we accurately identified the pathogen from infected rice seeds. These findings fill a critical gap in local pathogen diagnostics and lay the foundation for improved disease surveillance and management strategies against false smut in Sri Lankan rice cultivation.

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