



Molecular diagnostics for *Tilletia barclayana* causing kernel smut of rice

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ABSTRACT

Kernel smut of rice, caused by fungal pathogen *Tilletia barclayana* has emerged as a serious challenge in rice (*Oryza sativa* L.) growing regions worldwide. The disease leads to considerable yield losses ranging from 5–20% during rice production and also adversely affects seed quality. In view of the importance of this pathogen of the most traded crop and the lack of a quick and reliable molecular detection protocol, the present study aimed to develop species-specific molecular markers for the direct detection and identification of *T. barclayana* from seeds in 2024. Two sets of specific primers, TbarF2&R2 and TbarF2&R3 were developed from partial sequences of internal transcribed spacer (ITS) DNA region. Species-specific fragments of 308 bp was produced using TbarF2&R2 and 328 bp was amplified with the primer, TbarF2&R3 in *T. barclayana*. No amplification was obtained from the DNA of other *Tilletia* spp. and several other unrelated fungal plant pathogens tested in the present work indicating the specificity of the primers for the detection of the target pathogen. The specific primers detected the presence of *T. barclayana* in infected rice samples received for quarantine clearance from different countries. This qPCR based diagnostic assay developed in the present study can be applied for the direct and rapid detection and identification of *T. barclayana* during quarantine processing and seed health testing to facilitate rice seed certification as well as quick quarantine clearance for international exchange of rice germplasm.

Keywords: Diagnostics, Paddy bunt, qPCR, Quarantine, Rice

Rice (*Oryza sativa* L.) is a major staple food crop with global annual production reaching 503 million metric tonnes approximately (FAOSTAT 2022). To meet the growing food demand, high-yielding and hybrid rice varieties have been developed in which male sterile lines have been crucial for hybrid seed production. Among various biotic stresses, kernel smut caused by *Tilletia barclayana* (Bref., Sacc. and P. Syd.), is widely distributed in rice-growing regions worldwide (Whitney 1989, Rush *et al.* 2000, Webster 2005, Elshafey 2018). In recent years, kernel smut has gained increasing importance as an economically significant disease of rice due to its rising incidence and severity.

Rice kernel smut was first reported in Japan in 1896 and later reported in the United States in 1899 (Khanal *et al.* 2023a). Initially reported as a minor disease, but over the past decade, it has emerged as a significant challenge in United States (Allen *et al.* 2020, Wang *et al.* 2021). The kernel smut is characterised by the partial or full replacement of rice grain with black, sooty mass of teliospores (*T. barclayana*) resulting into significant yield losses ranging from 5–20% during the production of rice (Whitney and

Cartwright 2018; Wang *et al.* 2018, 2020, 2022; Khanal *et al.* 2023b). Most often panicles infected with a smut have only one or a few infected grains. However, up to 20% of smutted kernels have been reported in heavily infected fields (Cui 2021, Zhou *et al.* 2021). The disease is reported to cause declines in both yield and quality between 20–50% in the parent lines of hybrid rice (Chen *et al.* 2016) and has become a major constraint in the production of hybrid rice seeds in various rice-producing countries worldwide.

In India also, kernel smut of rice has also emerged as a serious concern for seed production, particularly under favourable environmental conditions, with frequent occurrence in the North-western regions. To date, no commercial rice cultivars in India have shown complete resistance against to this pathogen (Sharma *et al.* 2021). This disease is classified as an objectionable disease under the Indian Minimum Seed Certification Standards (Sharma *et al.* 2023). Internationally, regulatory limits are also in place; for instance, milled rice in the United States has a restriction that permits up to 3% of smutted grains (USDA 2020). Similarly, in India, certified seed standards allow a maximum of 0.5% smutted rice seeds (Chahal 2001).

As per the information available, there is lack of molecular detection tools to detect the pathogen directly from the seeds. Hence, the present study was aimed to develop species-specific primers for detection of this pathogen to

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accelerate the seed health testing for bunt-free rice seeds for import and export quarantine, seed certification as well as conservation of bunt-free rice seeds.

MATERIALS AND METHODS

Collection and morphological identification of teliospores: Bunted rice seeds were collected from rice samples received for quarantine clearance and conservation in the Genebank. Rice seed samples showing the symptoms of kernel smut were brought to the Plant Pathology Lab at Division of Plant Quarantine, ICAR-National Bureau of Plant Genetic Resources, New Delhi in 2024. For isolation of *T. barclayana*, the suspected seeds carrying typical symptoms were taken and black powdery mass of teliospores were collected on butter paper. The identification of teliospores was identified as *T. barclayana*, by conventional morphological methods using teliospore characters (Fig. 1). Black powdery mass of the teliospores was used for DNA extraction.

DNA extraction: Genomic DNA was extracted from teliospores of *T. barclayana* using DNeasy Plant mini kit. The purity and concentration of the DNA was further checked using the nanodrop spectrophotometer by NanoPhotometer[®] and DNA concentration was adjusted by dilution to 25 ng/μL using PCR-grade water and was used as template for PCR amplification.

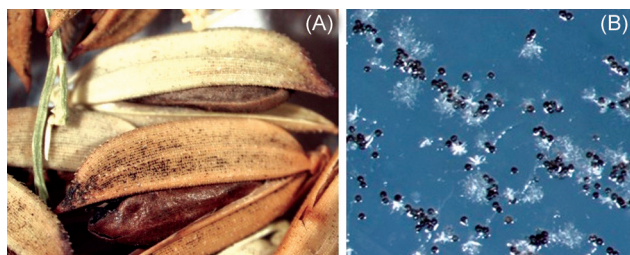


Fig. 1 (A) Smutted rice seed showing teliospores on seed surface; and (B) Germinating teliospores as infective propagule.

ITS amplification and DNA sequencing for molecular confirmation: Genomic DNA of the *T. barclayana* was subjected to PCR amplification targeting the Internal Transcribed Spacer (ITS) region using primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). The amplification was performed in a total reaction volume of 25 μL, comprising 1× Taq polymerase buffer, ~25 ng genomic DNA, 1 U Taq DNA polymerase, 0.6 mM of each dNTP, 1.5 mM MgCl₂, and 5 pmol of each primer. The thermal cycling conditions for ITS amplification followed the protocol described by Tripathi *et al.* (2023). PCR was carried out using a Biorad C1000 Touch[™] Thermal Cycler. The amplified products were run on 1.5% agarose gel stained with ethidium bromide and visualised under a UV gel documentation system (G:Box, Syngene). The desired bands were then excised and purified using a gel extraction and purification kit.

Designing of species-specific primers: ITS region

nucleotide sequences of different *Tilletia* spp. namely, *T. indica* (MT498030.1), *T. caries* (MH781423.1), *T. horrida* (DQ827714.1), *T. controversa* (MH781412.1), *T. walkeri* (AF310185.1), *T. laevis* (OL636487.1), *T. ehrhartae* (AY770433.1) along with other fungal pathogens involved in infecting rice namely, *Alternaria padwickii*, *Bipolaris oryzae*, *Pyricularia oryzae*, *Rhizoctonia solani* were retrieved from National Center for Biotechnology Information Genebank database. Sequences alignment was performed in Bioedit using Clustal W including ITS sequences of *T. barclayana* (Accession no. AF310168.1) and also from the ITS sequence generated from the present study.

Two forward and four reverse primers were manually designed from ITS region based on observed nucleotide variations. Primer design was carried out by considering several parameters, including melting temperature, primer length, GC content, presence of a GC clamp, self-dimer and cross dimer formation, hairpin structures, secondary structure formation, repeats and runs, stability at 3' end, avoidance of cross-homology and template secondary structures, product length, melting temperature, optimum annealing temperature and compatibility between primer pairs in terms of melting temperature differences (Kumar and Chordia 2015). Multiple Primer Analyser (MPA) was also used to analyse melting temperature and to avoid any inter- and intra-primer interactions. Primers were synthesised by IDT (Integrated DNA Technologies Inc.). The designed primers were subjected to BLASTn analysis at NCBI to confirm their specificity.

Specificity of designed primers: All possible forward and reverse primer combinations were evaluated to obtain specific amplification of the target pathogen. To optimise the annealing temperature of each primer pair, gradient PCR gradient PCR was conducted using a thermal cycler (C1000 Touch, BioRad) with temperature range of 55–65°C. Primer specificity was assessed by performing PCR reaction in a 25 μL reaction mixture containing 1× Taq polymerase buffer in mix containing 25 ng of genomic DNA extracted from teliospores, 1U Taq DNA polymerase, 0.6 mM of each dNTPs, 1.5 mM of MgCl₂ and 5 pmol of each primer. PCR amplification was carried out under the following thermal cycler conditions: Initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 65°C for 30 sec for both primers and extension at 72°C for 30 sec, with a final extension at 72°C for 5 min. The amplified products were initially separated on 1.5% agarose gel prepared in 1× TAE buffer, stained with ethidium bromide, and visualised using a UV-gel documentation system. A 100 bp ladder was used as a molecular size marker. For better resolution the PCR products were further resolved on 2.5% agarose gel in 1× TAE buffer stained with ethidium bromide, and visualised using a UV-gel documentation system with a 100 bp ladder as a reference. The desired amplicons were excised, eluted, and purified using a gel purification kit and given for sequencing to confirm the identity of the amplified fragment.

Sensitivity test using conventional and qPCR: The

sensitivity of two marker sets developed for *T. barclayana* was evaluated using a series of genomic DNA concentrations ranging from 100 to 0.0001 ng. Conventional PCR conditions were carried out in a total volume of 25 µL containing 1× *Taq* polymerase buffer, 1 U *Taq* DNA polymerase, 0.6 mM of each dNTPs, 1.5 mM of MgCl₂, 5 pmol of each primer and template DNA at the specified concentrations. Amplification was performed under previously described thermal cycling conditions. The PCR products were separated on 2.5% agarose gel prepared in 1× TAE buffer, stained with ethidium bromide and visualised using a UV-gel documentation system. In addition, primer sensitivity was assessed through qPCR using the same range of DNA concentrations. The qPCR assay was conducted with an initial denaturation at 98°C for 4 min, followed by 40 cycles of denaturation at 98°C for 15 sec and combined annealing and extension at 65°C for 60 sec. Each 20 µL reaction mixture consisted of Sso Advanced universal SYBR green supermix (BioRad), 10 pmole of each of forward and reverse primers and 1 µL template DNA at different concentrations.

Validation: For validation, infected seeds from the samples received for long term conservation or for quarantine clearance were collected. Total genomic DNA was isolated from both infected and healthy rice seeds using the DNeasy Plant mini kit according to the manufacturer's instructions. Both conventional and qPCR assays were carried out using the same reaction components and thermal cycler conditions described previously for each primer set, with 2.5 µL of template DNA extracted directly from seed samples. To access the marker specificity, the primers were also tested against wheat seeds infected with *T. caries* and *T. indica* under same PCR conditions. The amplification product obtained from conventional PCR was separated on 2.5% agarose gel and subsequently visualised using a gel documentation system.

RESULTS AND DISCUSSION

The identity of the fungal pathogen was confirmed with morphological characterisation. Teliospores globose to subglobose, light brown to dark brown enclosed in a hyaline to pale yellow sheath with or without short apicules 17–25 µm in diameter. The identity was further confirmed with the nucleotide sequences of ITS region. A PCR product of approximately 650 bp (gel not shown) was obtained using ITS primers. The amplified fragments were sequenced, assembled, and edited using BioEdit software, and the resulting sequences were analysed through NCBI BLASTn. The pathogen was identified based on 99% nucleotide sequence similarity with the target organism. The ITS sequence was subsequently submitted to the NCBI Genebank database under the assigned accession no. PQ345543.

Evaluation of the specificity of the species-specific primers: The specificity of the designed species-specific primers was assessed through uniplex PCR. Comprehensive details of the primer pairs, including their sequences are presented in Table 1.

Table 1 Species-specific primers for *T. barclayana* developed from ITS region

Pathogen	Target Gene	Primer name	Sequence (5'-3')
<i>Tilletia barclayana</i>	ITS	TbarF2	CGCCCTCTTTAACTCAT-ACTTA
		TbarR2	CGAGCCAGACTTATTCAG-CATGGC
		TbarR3	AGCTACTGATGTCTTTAAGG

ITS, Internal transcribed spacer.

From the two forward and four reverse primers designed from the ITS region for *T. barclayana*, a total of eight primer pair combinations were generated. The optimal annealing temperature was determined for each primer set.

Out of eight combinations tested, two combinations of primers i.e. TbarF2& R2 and TbarF2&R3 produced specific amplicon in target pathogen. The primer sets, TbarF2&R2 amplified 308 bp and TbarF2&R3 amplified 328 bp size of specific amplicon in target pathogen whereas, no amplification was obtained in other spp. namely *T. caries* and *T. indica* and other common fungal and bacterial plant pathogens infecting rice crop namely, *Alternaria padwickii*, *Bipolaris oryzae*, *Pyricularia oryzae*, *Fusarium verticillioides*, *Rhizoctonia solani*, *B. hawaiiensis*, *B. tetramera*, *Curvularia lunata*, *C. geniculata*, *Xanthomonas oryzae* pv. *oryzae*, *X. campestris* pv. *compestris* and *Burkholderia glumae*. The nucleotide sequences of amplified fragment from both the primers sequenced and confirmed that the primers amplified the same ITS region from where it is designed (Fig. 2).

Sensitivity assessment: A sensitivity of each specific primer sets PCR was evaluated using both conventional

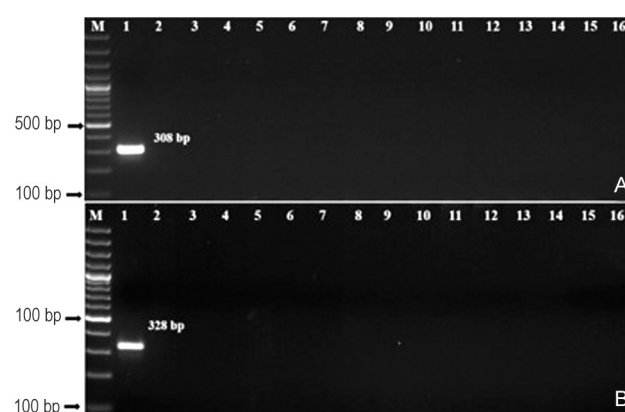


Fig. 2 Amplification achieved using the primer set TbarF2&R2 (A) and TbarF2&R3 (B).

Lane M-100bp plus ladder; 1, *T. barclayana*; 2, *T. indica*; 3, *T. caries*; 4, *A. padwickii*; 5, *B. oryzae*; 6, *P. oryzae*; 7, *F. verticillioides*; 8, *R. solani*; 9, *B. hawaiiensis*; 10, *B. tetramera*; 11, *C. lunata*; 12, *C. geniculata*; 13, *X. oryzae* pv. *Oryzae*; 14, *X. campestris* pv. *compestris* pv. *oryzae*; 15, *B. glumae*; and 16, NTC (non-template control).

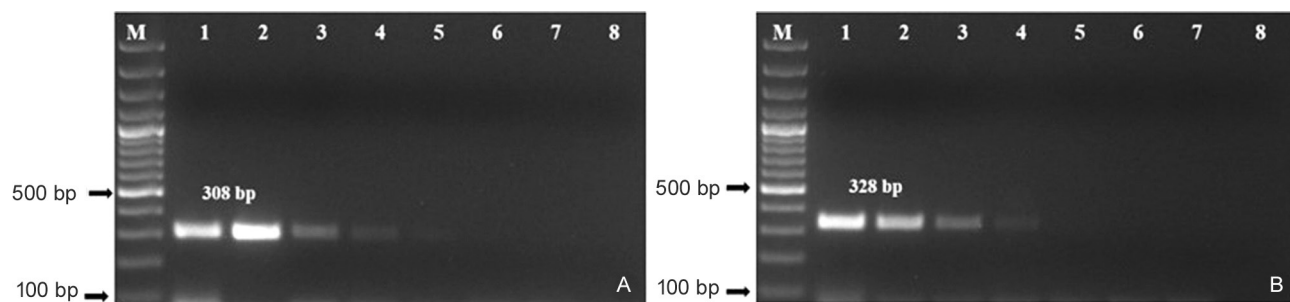


Fig. 3 Sensitivity of *T. barclayana* specific primers TbarF2&R2 (A) and TbarF2&R3 (B) using different concentrations of fungal genomic DNA using conventional PCR.

Lane M, 100 bp ladder; 1, 100 ng; 2, 10 ng; 3, 1 ng; 4, 0.1 ng; 5, 0.01 ng; 6, 0.001 ng; 7, 0.0001 ng and 8, NTC (non-template control).

and qPCR. The genomic DNA was quantified at an initial concentration of 100 ng and subsequently subjected to serial dilution. Among the markers, TbarF2&R2 demonstrated the higher specificity, detecting the pathogen down to 0.01 ng of DNA, whereas TbarF2&R3 showed detection up to 0.1 ng under conventional PCR conditions (Fig. 3).

Both primer sets demonstrated a detection limit of 0.01 ng in the qPCR assay, indicating high sensitivity for detecting the fungal pathogen from genomic DNA samples (Fig. 4). The standard curve exhibited strong and significant correlation ($R^2=0.901$ and 0.980), with C_t values showing slopes of -2.006 and -2.440 for the TbarF2&R2 and TbarF2&R3 primer sets, respectively. Primer specificity was further confirmed by the presence of a single distinct melting peak at 83°C .

Validation: The species-specific markers were validated using DNA extracted from both infected and healthy rice seeds. The primers successfully detected the target pathogen through conventional PCR and qPCR, producing specific amplification only in infected samples (Fig. 5 and 6). These primers also proved effective in identifying the pathogen in the rice samples received for quarantine screening as well as seed health testing. The markers consistently generated pathogen-specific amplicons of expected size in infected seed, while no specific amplification was observed in healthy

samples. The markers were also checked with wheat grains infected with *T. caries* and *T. indica* and they did not produce any amplification in both the samples using these primers (gel picture not shown).

Kernel smut, caused by *T. barclayana*, commonly referred paddy bunt, has evolved from a paddy bunt to a sporadic and relatively minor disease into a major constraint in hybrid rice production worldwide. Previously considered an isolated issue, it has now reached epidemic proportions in rice-growing regions such as United States, Brazil, Vietnam,

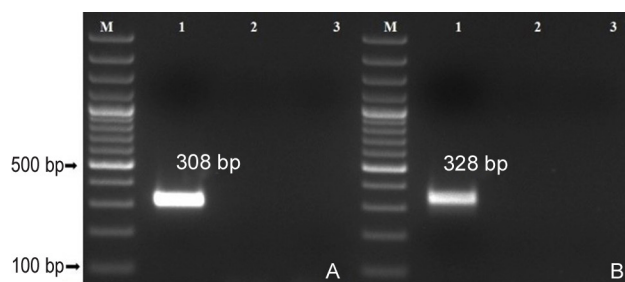


Fig. 5 Validation of markers TbarF2&R2 (A) and Tbar F2&R3 (B) with *T. barclayana* infected and healthy rice seeds using conventional PCR.

Lane M, 100 bp DNA ladder; 1, Infected seeds; 2, Healthy seeds; and 3, NTC (non-template control).

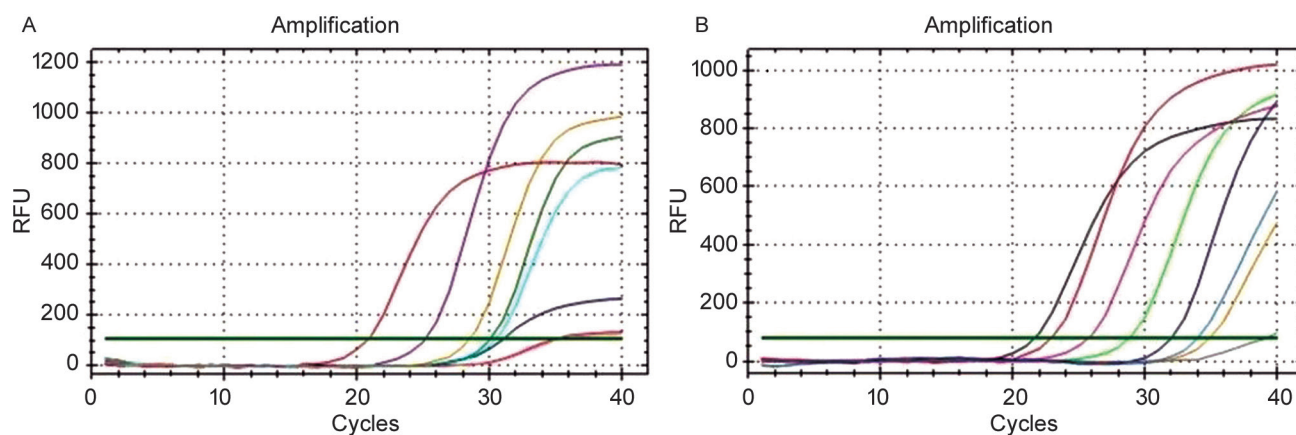


Fig. 4 Sensitivity test of *T. barclayana* specific markers TbarF2&R2 (A) and TbarF2&R3 (B) at different concentrations of fungal genomic DNA using qPCR.

RFU, Relative fluorescence unit.

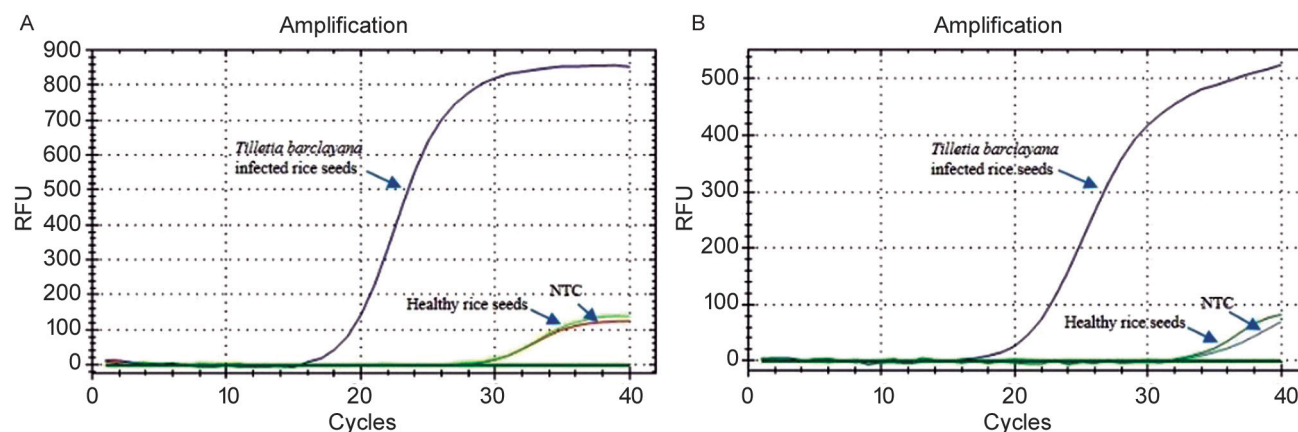


Fig. 6 Validation of markers TbarF2&R2 (A) and Tbar F2&R3 (B) with *T. barclayana* infected and healthy rice seeds using qPCR. RFU, Relative fluorescence unit.

and several South-east Asian countries (Carris *et al.* 2006, Brooks *et al.* 2009, Wang *et al.* 2022). The primary risk associated with kernel smut is not just yield loss, but, more crucially, the decline in grain quality, which significantly impacts global rice trade, seed certification standards, and the exchange of germplasm.

The International Rice Research Institute, Philippines has identified *T. barclayana* as a seed-borne pathogen of quarantine significance, noting that the primary economic losses from kernel smut arise from degraded seed quality. Consequently, strict regulations limit the allowable smutted grains in international rice trade. In the United States, the rising incidence and severity of kernel smut over the past decade have caused widespread concern among rice growers (Allen *et al.* 2020). For example, 2021 outbreaks in Texas and Louisiana impacted up to 50% of panicles, with kernel infection levels reaching 20% (Zhou *et al.* 2021). Such epidemics severely reduce yield and milling quality, creating substantial financial burdens on rice farmers through lost market value and necessitates additional quarantine measures.

Kernel smut poses a major barrier to quality seed production, seed certification, and international rice germplasm exchange due to stringent international phytosanitary rules (Pannu *et al.* 2002, Akhtar *et al.* 2022). Countries are progressively tightening quarantine regulations; Guinea designates it as a regulated non-quarantine pest, while the European Union includes it in EPPO's A2 list (EPPO 2024). Documented occurrences in Colombia, Taiwan, and Australia demonstrate its expanding distribution and acknowledging its potential threat to regional agriculture, which climate change may accelerate. Despite its impact, research on physiological races and variation in pathogenicity remains limited. However, notable pathogenic variability has been observed among strains in China (Wang *et al.* 2018) and the USA (Khanal *et al.* 2022). This virulence diversity complicates disease management and raises the urgent need for reliable and robust diagnostic tools.

India imports global rice genetic resources exclusively

through ICAR-NBPGR, New Delhi. Quarantine testing routinely intercept *T. barclayana* from imported germplasm originating in Brazil, China, Nepal, the Philippines, USA, and Vietnam (Singh *et al.* 2018). Conventional seed health testing methods such as visual inspection, stereomicroscopy, and KOH soaking are routinely used (Dev *et al.* 2012). However, these methods often fail to detect symptomless infections, leading to false results that may inadvertently allow smutted seeds to bypass quarantine, transport to new regions and establish in new regions and creating further quarantine challenges.

Given the increasing global spread of *T. barclayana* and the diagnostic limitations, the development of a robust molecular diagnostic protocol is essential. In our study, we developed conventional and qPCR-based assays targeting the ITS region to rapidly and accurately identify *T. barclayana* directly from rice seeds. Building upon earlier diagnostics (Chen *et al.* 2016), our study significantly advances this work by providing two novel species-specific molecular markers that facilitate the precise identification of *T. barclayana*. These markers enhance the accuracy of seed health testing and reduce the time required for quarantine clearance, ensuring safe transboundary movement of rice germplasm while maintaining stringent phytosanitary standards.

The pathogen's ability to persist in soil and on seed surfaces for years (Webster and Gunnell 1992, Carris *et al.* 2006), coupled with an asymptomatic early infection stage (Zhu *et al.* 1998), makes early detection difficult and underscores the need for effective quarantine measures. By the time symptoms appear, grain filling is compromised and kernels are replaced by dark spore masses, drastically reducing yield and quality (Liu 2008). Asymptomatic periods make molecular detection tools indispensable for early identification and prevention of pathogen spread.

Germplasm is conserved for both current use and future security; however, its true value can only be realised when the seeds intended for preservation possess high initial quality. Hence, for safe international exchange and long-term conservation of rice germplasm, testing all seed samples for their health status is essential. In this endeavour,

our protocols offer a substantial upgrade over traditional detection methods, equipping regulatory agencies with a more reliable tool to safeguard against introducing exotic strains of *T. barclayana* into unaffected areas, especially those with no prior history of the pathogen. As rice remains a heavily traded global commodity (USDA 2020), swift molecular testing is vital for protecting agricultural trade, plant genetic resource (PGR) exchange, and crop biosecurity. Because *T. barclayana* is a designated objectionable disease in India, high initial seed health is mandatory for long-term conservation. Our findings are intended to strengthen existing knowledge, raise awareness of the potential threat, enhance disease surveillance, and enable early detection, thereby helping to minimise future losses in this important food crop.

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