Cautionary Notes on Use of the MoT3 Diagnostic Assay for *Magnaporthe oryzae* Wheat and Rice Blast Isolates

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ABSTRACT

The blast fungus *Magnaporthe oryzae* is comprised of lineages that exhibit varying degrees of specificity on about 50 grass hosts, including rice, wheat, and barley. Reliable diagnostic tools are essential given that the pathogen has a propensity to jump to new hosts and spread to new geographic regions. Of particular concern is wheat blast, which has suddenly appeared in Bangladesh in 2016 before spreading to neighboring India. In these Asian countries, wheat blast strains are now co-occurring with the destructive rice blast pathogen raising the possibility of genetic exchange between these destructive pathogens. We assessed the recently described MoT3 diagnostic assay and found that it did not distinguish between wheat and rice blast isolates from Bangladesh. The assay is based on primers matching the WB12 sequence

corresponding to a fragment of the *M. oryzae* MGG_02337 gene annotated as a short chain dehydrogenase. These primers could not reliably distinguish between wheat and rice blast isolates from Bangladesh based on DNA amplification experiments performed in separate laboratories in Bangladesh and in the United Kingdom. Specifically, all eight rice blast isolates tested in this study produced the WB12 amplicon. In addition, comparative genomics of the WB12 nucleotide sequence revealed a complex underlying genetic structure with related sequences across *M. oryzae* strains and in both rice and wheat blast isolates. We, therefore, caution against the indiscriminate use of this assay to identify wheat blast and encourage further development of the assay to ensure its value in diagnosis.

Outbreaks caused by fungal diseases have increased in frequency and are a chronic threat to global food security (Fisher et al. 2012). A prime case is blast, a disease caused by the ascomycete fungus *Magnaporthe oryzae* (syn. *Pyricularia oryzae*), which is best known as the most destructive disease of rice. However, in addition to rice, *M. oryzae* can also infect other cereal crops such as wheat, barley, oat, and millet destroying food supply that could feed hundreds of millions of people (Fisher et al. 2012; Liu et al. 2014; Pennisi 2010). Increased global trade, climate change, and the propensity of this pathogen to occasionally jump from one grass host to another, have resulted in increased incidence of blast diseases. For example, only a few decades ago blast was not known to affect wheat, a main staple crop critical to ensuring global food security. But in 1985, blast disease on wheat was first reported in Paraná State, Brazil (Igarashi et al. 1986). It has since spread throughout Brazil and subsequently moved into neighboring South American countries where it is now a major threat to wheat production (Goulart et al. 1992, 2007; Kohli et al. 2011). Currently, wheat blast affects as many as 3 million hectares of cultivated wheat, seriously limiting the potential for wheat production in the vast grasslands region of South America.

More recently, in February 2016, wheat blast was detected for the first time in Asia, following reports of a severe outbreak in Bangladesh (Islam et al. 2016). The outbreak was particularly destructive, affecting ~16% of the cultivated wheat area in Bangladesh and with yield losses reaching up to 100% (Islam et al. 2016). In 2017, the pathogen spread to the neighboring West Bengal region of India and hundreds of hectares were cleared by burning to limit the accumulation and spread of the pathogen according to local press reports. An open source population genomics project led by Islam et al. (2016) revealed that the Bangladeshi wheat blast outbreak was caused by a South American genotype of *M. oryzae*, and therefore most likely introduced from South America (OpenWheatBlast <http://s620715531.websitehome.co.uk/owb/>). The 2016 Bangladeshi epidemic vividly illustrates the clear and present danger caused by blast diseases in an era of global trade. It is especially worrisome because blast could spread further to other wheat-producing areas in South Asia, such as India and Pakistan, thus threatening food security across South Asia.

Until recently, the genetic structure of *M. oryzae* remained somewhat unclear. Whole-genome sequence analyses of 76 isolates from 12 different grass hosts confirmed the status of *M. oryzae* as a single species and revealed genetic exchanges among the different host-specific lineages (Gladieux et al. 2018). Overall, *M. oryzae* lineages exhibit low levels of nucleotide polymorphisms within single-copy genes, with π ranging from 7.75×10^{-4} to 1.24×10^{-3} in the various host-specific lineages of this species. This is one order of magnitude lower than divergence between *M. oryzae* and its closest relatives *Magnaporthe grisea* and *Magnaporthe pennisetigena* (Gladieux et al. 2018). Nonetheless, the different lineages of *M. oryzae* exhibit significant genome plasticity probably due to the activity of transposable elements and selection for optimal disease effector repertoires (Yoshida et al. 2016). Notably, effector genes often exhibit presence/absence polymorphisms, and particular

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effector genes can be associated with a given lineage probably contributing to the specialization of individual genotypes to certain hosts (Inoue et al. 2017).

Given the propensity of *M. oryzae* to jump hosts and spread to new geographic regions, reliable and cost-effective diagnostic tools are needed to monitor this pandemic pathogen at the genotype level. This is not necessarily a straightforward problem considering the relatively low genetic diversity and potential for gene flow among lineages of *M. oryzae*. Pieck et al. (2017) recently reported a polymerase chain reaction (PCR) assay diagnostic for wheat blast based on a single molecular marker named MoT3. The assay is based on primers matching the WB12 sequence corresponding to a fragment of the *M. oryzae* MGG_02337 gene annotated as a short chain dehydrogenase. When evaluated with 285 *M. oryzae* isolates from various hosts, the assay produced the expected amplicon for 113 of 115 wheat blast isolates from North and South America, whereas it did not yield amplicons for the four rice blast isolates tested (Pieck et al. 2017). Here, we report that these primers could not reliably distinguish between wheat and rice blast isolates from Bangladesh based on DNA amplification experiments performed in separate laboratories in Bangladesh and in the United Kingdom. In addition, comparative genomics of the WB12 nucleotide sequence revealed a complex underlying genetic structure with WB12-related sequences occurring across *M. oryzae* strains and in both rice and wheat blast isolates. We, therefore, caution against the indiscriminate use of this assay to identify wheat blast. Moving forward, we recommend the design of new primers that unambiguously distinguish between the WB12 sequence from related sequences, and to take into account the distribution of the WB12 sequence across the various lineages of *M. oryzae*.

MATERIALS AND METHODS

MoT3 assay. To confirm that MoT3 primers amplify sequences from wheat blast isolates, we (in Sophien Kamoun's UK Lab) used genomic DNA extracted from a *M. oryzae* wheat blast isolate from Bangladesh (BTJP4-1) and one from Brazilian wheat blast (BR32). To test the specificity of MoT3 primers, we also included genomic DNA from three *M. oryzae* rice blast isolates from Bangladesh (RB9c, RB11a, and RB13-1c) and one from Japan (INA168). In M. Tofazzal Islam's Lab in Bangladesh, genomic DNA was independently extracted from four Bangladeshi wheat blast isolates (BTMP 13-1, BTMP 13-2, BTJP 4-5, and BTJP 4-6) and four rice blast isolates (RB-3b, RB-9d, RB-11b, and RB-11d) and one isolate of *Colletotrichum* sp. All remaining procedures for PCR and gel electrophoresis were similar in both labs and are described below. In these experiments, we used PoT2 transposon primers as a positive control for the presence of *M. oryzae* genomic DNA.

PCR assays with MoT3 primers (MoT3F 5'-GTCGTCATCAACGTGACCAG-3' and MoT3R 5'-ACTTGACCCAAGCCTCGAAT-3') (Pieck et al. 2017), elongation factor-1 α primers (forward 5'-CTYGGTGTAGGCAGCTCA-3' and reverse 5'-GAAMTTGCAGGCRATGTGGG-3'); and in the Bangladesh lab, PoT2 (forward 5'-CGTCACACGTTCTTCAACC-3' and reverse 5'-CGTTTCACGCTTCTCCG-3') (Castroagudin et al. 2016; Harmon et al. 2003) were performed following the methods described in Pieck et al. (2017). Briefly, 50 μ l PCR reactions containing 1 $\times$ DreamTaq Green buffer (Thermo-Fisher Scientific, Waltham, MA), 0.2 mM dNTPs, 200 nM each primer, 100 ng of template genomic DNA, and one unit of DreamTaq polymerase (Thermo-Fisher) were set up and amplification was performed using a C1000 Thermal Cycler (Bio-Rad, Hercules, CA) with the following program: initial denaturation at 94°C for 60 s; 30 cycles of 94°C for 30 s, 62°C for 30 s, 72°C for 90 s; and extension at 72°C for 120 s.

PCR products (10 μ l each) were run on 1.5% agarose gel and stained with ethidium bromide. The rest of the PCR products were purified with a PCR purification Kit (Qiagen, Hilden, Germany) and used for sequencing from both strands by GATC Biotech (Konstanz,

Germany). DNA sequence contigs were assembled from sequencing chromatograms using Sequencer (Genecodes, Ann Arbor, MI) for each amplicon and exported in fasta format for further analysis.

Sequences from each amplicon contig were aligned together with genomic regions from several *M. oryzae* isolates corresponding to the target sequence WB12 described in Pieck et al. (2017). These regions were identified by BLASTN in the genomic sequences of *M. oryzae* isolates from rice (FR13, GUY11, PH14-rn, and TH16), *Eleusine indica* (CD156), *Setaria italica* (US71), and wheat (BR32), as well as one *M. grisea* isolate (BR29) from *Digitalis sanguinalis* (downloaded from <http://genome.jouy.inra.fr/gemo/> [Chiapello et al. 2015]). Primer sequences were removed from the alignment and a gene tree was constructed using ClustalW2 neighbor joining algorithm and bootstrapped 1,000 times.

WB12 sequence similarity analyses. We used BLASTN from BLAST suite version 2.2.26 (Altschul et al. 1997) to search for the WB12 sequence (Pieck et al. 2017) in the genome assemblies of

Fig. 1. Amplification of the MoT3 regions from rice and wheat infecting *Magnaporthe oryzae* isolates using MoT3 primers. **A**, Amplification performed in the Bangladesh laboratory using MoT3 primers and using PoT2 primers as a positive control for amplification of PoT2 region. **B**, Amplification performed in the U.K. laboratory using MoT3 primers and elongation factor (EF) primers. EF serves as a positive control for amplification.

their identity, we sequenced the amplicons (Supplementary Fig. S2). We then constructed a multiple sequence alignment using the amplicon nucleotide sequences (excluding the primer sequences) along with genomic sequences from WB12-like regions of several *M. oryzae* isolates identified by BLASTN search of genome sequences available from the *Magnaporthe* GEMO Database website (<http://genome.jouy.inra.fr/gemo/>) (Chiappello et al. 2015) (Fig. 2A). Amplicon sequences from *M. oryzae* from Bangladeshi rice blast isolates (RB9c, RB11a, and RB13-1c) showed that they bear different WB12 sequences despite the fact that MoT3 primers were able to produce amplicons with expected size from these isolates (Fig. 2A). The Bangladesh wheat blast isolate shows high sequence identity to the WB12 sequence itself, whereas the Bangladeshi rice isolates have a high degree of similarity with genome sequences of reference rice isolates (FR13, GUY11, PH14-m, and TH16) and the one isolate from *Eleusine* (CD156). The WB12-like sequence of *M. oryzae* wheat isolate BR32 from Brazil was more similar to those from rice isolates than to the wheat isolates indicating that the distribution of the different WB12 sequences is not strictly correlated with host or origin (Fig. 2B). We compared the sequence chromatograms corresponding to Figure 3 and found that MoT3 reverse primer sequences were recovered in amplicons from various *M. oryzae* samples including Bangladeshi wheat and rice blast isolates. We conclude that the reverse primer showed mis-priming despite the single mismatch at the 3' extension end (based on FR13 sequence, described below), allowing WB12-like sequences to be amplified.

MoT3 PCR amplifications yield diverse sequences. To further explore the PCR assays shown in Figure 1 and determine

Comparative genomics analyses reveal that WB12-paralogous sequences can serve as templates for MoT3 primers. To confirm the amplicon sequencing analyses and assess

Fig. 2. MoT3 amplicon sequences of *Magnaporthe oryzae*. **A**, Multiple sequence alignment of WB12 sequence (boxed), MoT3 amplicon sequences (indicated by light gray/red dots), and WB12-like genomic regions of several isolates. Sequence conservation for each site is indicated by box-shading the nucleotide with either black (>50%) or gray (conservative mutation). **B**, A gene tree based on sequences of WB12 region in a selection of *M. oryzae* isolates. Light gray (red) dots = Amplicon sequences; Dark gray (blue) dots = Bangladeshi isolates; the rest of the sequences were extracted from the genomic sequences.

Alignment between WB12 sequence and BR32_scaffold00014

Query= WB12
Subject= BR32_scaffold00014

Score = 370 bits (410)
Expect = 1e-103
Identities = 391/513 (76%)
Gaps = 25/513 (5%)
Strand=Plus/Plus

Query	5	CAGTTCGACGTCAATTTTTGGGGCCCCATGGAGCTGACCAGACATGCCGTCAGGATTATG	64
Sbjct	247737	CAGTTCGACGTCAACTTCTGGGGCCCCATGGAGCTGACCAGGCATGCCGTCAGGGTCATG	247796
Query	65	CGCGAGGAGAACCCAAAGAACGGGGGTGCTCCCGGCGGC	124
Sbjct	247797	CGCGAGGAGAACCCAAAGAACGG---TCCCATCGGCGGCGTCATCATCAACGTCACCAGC	247853
Query	125	GCGGTTGGCTTTCTGGCCATACCCGGCAGCCCCTTTTATTGCGCATCCAAGTTTGCTATG	184
Sbjct	247854	TTCGTTGGCTTCGTGTCGATACCGGGCTGCCCTTTTACTGCGCATCCAAGTTTGCTCTG	247913
Query	185	GAGGGATTTACAGAGACCTTTGCCAAGGATGTATGCCCTGACTGGAATAGTAAGTTTGTT	244
Sbjct	247914	GAGGGTTTCACCGAGACATTTGCTAAAGAGGTGCACCCGGACTGGAACAGTAGGTTTAC	247973
Query	245	GTTACAACATC-----ACGAATCGAATGG-----AGACAAAACTACAAGAAA	287
Sbjct	247974	TCTAGCACTTCCTTTGCCGTGATGATTCGAATGGGAGCGAAAGAAAAAAGGAAAAAAA	248033
Query	288	ATTGAAGTACAGCTTTCTAACGGTTTGCAATTGCACAAAACAACCCAGTTCACTTTGC	347
Sbjct	248034	A--GGATTACAGCTTTCTAACGGTATG-ACTTTTACAAAAC--CACAGTTCACTTTGGC	248088
Query	348	TCGCCGAGCCCGGCGGTGTCGCTACGGAATTCATAAACAACGTCTCATTTGGACCCACC	407
Sbjct	248089	TCGCCGAGCCCGGCGGTGTCGCTACGGCATTCGCGGACAACAGAAAATTTGGGGTCCCTC	248148
Query	408	ACCCGGCATAACGAAGCCCCCAACACGCCGGCGCGGGC	467
Sbjct	248149	ATCCGGCATAACAGGCCCGGCACGCCGACGCGAGAGTTCGAGGCTTGGGCCCAAGATC	248208
Query	468	CATCCACCACGAGTTTCTCGTCGGCCGAAGGGA	500
Sbjct	248209	TATCCGACATGGGCTTCTCGACGGCAGAGGGGA	248241

MoT3 Primers (positive strands):
Forward: 5' –GTCGTCATCAACGTGACCAG – 3'
Reverse: 3' – ATTCGAGGCTTGGGTCAAGT – 5'

Fig. 3. Alignment of second WB12 region in BR32 against WB12 isolate of *Magnaporthe oryzae* with MoT3 primers highlighted: forward primer in light gray/blue; reverse primer in dark gray/pink.

the diversity and distribution of the WB12 sequence within populations of *M. oryzae*, we searched the genome sequences of 67 *M. oryzae* isolates from various host plants available in GenBank using BLAST v.2.2.26. We found significant hits with >98% identity across at least 400 bp in the majority of isolates from the wheat-infecting lineage, including *Bromus* isolate P29, which was previously grouped as a member of a wheat-infecting lineage (Supplementary Table S1). However, two wheat isolates, notably BR32, and a *Bromus* isolate P28 did not yield a BLAST match to WB12. We conclude that the WB12 sequence is not present in all wheat-infecting isolates.

BLASTN searches also produced sequences related to WB12 (we refer to as WB12-like) in several contigs of the assembled genomes of the 67 *M. oryzae* isolates. The first set of WB12-like sequence had 70 to 98% identity to the WB12 sequence over a 400 bp matched region, and was present in 65 isolates except in US71 and GrF52 from *Setaria* spp. The second set of WB12-like sequences had 60 to 98% identity over 100 to 400 bp of the matching region and was only found in US71 and GrF52. We conclude that the WB12 region is specific to a subset of wheat-infecting isolates of *M. oryzae* and missing in other lineages. In contrast, the WB12-like sequences are present in almost all wheat and non-wheat isolates.

We checked the degree to which the MoT3 primers could match the WB12-like sequences. MoT3 primer sequences can be aligned with high identity to the WB12-like sequence. For example, MoT3 primer sequences can be aligned to WB12-like sequence of *M. oryzae* wheat isolate BR32 (Fig. 3).

Although there were a few mismatches between primers and BR32 genome sequence, these sequence differences apparently do not interfere with PCR amplification under the conditions we described in the methods. Our findings lead us to caution against the indiscriminate use of the MoT3 assay to identify wheat blast. The MoT3 primers can under certain amplification conditions yield positive amplicons with the great majority of *M. oryzae* isolates independent of their host of origin. Moving forward, we recommend the design of new primers, ideally nested within the current MoT3 primers, that unambiguously distinguish between the WB12 sequences from different isolates. We also advise to take into account the uneven distribution of the WB12 sequence across the various lineages of *M. oryzae*. Identifying additional diagnostic loci for wheat blast that could be used in conjunction with the MoT3 primers would also provide improved reliability and robustness to the assay.

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