A Genomic Approach to Develop a New qPCR Test Enabling Detection of the *Pyricularia oryzae* Lineage Causing Wheat Blast

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Abstract

Rapid detection is key to managing emerging diseases because it allows their spread around the world to be monitored and limited. The first major wheat blast epidemics were reported in 1985 in the Brazilian state of Paraná. Following this outbreak, the disease quickly spread to neighboring regions and countries and, in 2016, the first report of wheat blast disease outside South America was released. This Asian outbreak was due to the trade of infected South American seed, demonstrating the importance of detection tests in order to avoid importing contaminated biological material into regions free from the pathogen. Genomic analysis has revealed that one particular lineage within the fungal species *Pyricularia oryzae* is associated with this disease: the *Triticum* lineage. A comparison of 81 *Pyricularia* genomes highlighted polymorphisms specific to the *Triticum*

lineage, and this study developed a real-time PCR test targeting one of these polymorphisms. The test's performance was then evaluated in order to measure its analytical specificity, analytical sensitivity, and robustness. The C17 quantitative PCR test detected isolates belonging to the *Triticum* lineage with high sensitivity, down to 13 plasmid copies or 1 pg of genomic DNA per reaction tube. The blast-based approach developed here to study *P. oryzae* can be transposed to other emerging diseases.

Keywords: cereals and grains, emerging disease, field crops, fungi, genome comparison, infraspecific detection, pathogen detection, *Pyricularia oryzae*, wheat blast

Emerging infectious diseases (EIDs) are defined as diseases that have recently appeared on a new host or in a new area, or whose incidence has rapidly increased (Morse 2001). Various infectious disease agents and hosts may be involved in EIDs. For example, among the EIDs that have recently made the headlines, the *Zika virus* can cause fetal microcephaly in human populations (Garcez et al. 2016), the bacterium *Xylella fastidiosa* is responsible for Olive Quick Decline Syndrome (OQDS) in olive trees (Loconsole et al. 2014), and the fungus *Geomyces destructans* fulfills Koch's postulates for White-Nose Syndrome (WNS), which leads to mass bat mortalities (Fisher et al. 2012). Although these examples belong to different kingdoms and have highly contrasting biological characteristics, they have in common a sudden outbreak in a limited geographical area: the *Zika* outbreak occurred in 2007 in Micronesia (Ios et al. 2014), OQDS appeared suddenly in 2010 in Italy (Loconsole et al. 2014), and WNS was described for the first time in March 2007 in New York State (Fisher et al. 2012). Therefore, avoiding the spread of EIDs as soon as possible after their outbreak is essential. To reach this goal, accurate and early diagnosis of the causal agent is a key element in EID management. It allows close monitoring of the pathogen's spread through epidemiological surveillance, thereby enabling a rapid reaction if the pathogen is introduced into novel geographical areas or preventing the trade and exchange of contaminated biological materials whenever possible (detection at points of entry and quarantine measures, for example).

Pyricularia oryzae Cavara (synonym: *Magnaporthe oryzae*) is a phytopathogenic, hemibiotrophic ascomycete fungus responsible for

wheat blast in several species of the family Poaceae. Blast symptoms include necrotic lesions on an infected plant's leaves, panicle necks, stems, and grains (Asuyama 1963; Te Beest et al. 2007). *P. oryzae* encompasses multiple genetic lineages that, together, affect a broad range of Poaceae hosts. Blast has been recorded on more than 25 hosts (crops or wild species) such as rice (*Oryza sativa*), wheat (*Triticum aestivum* and *Triticum durum*), foxtail millet (*Setaria italica*), or perennial ryegrass (*Lolium perenne*) (Asuyama 1963; Klaubauf et al. 2014; Maciel 2011; Milazzo et al. 2019). However, spore inoculation experiments have shown that, individually, *P. oryzae* isolates are only pathogenic on a limited range of hosts and the species contains multiple host specificity groups (Couch et al. 2005; Kato et al. 2000; Tosa et al. 2004). Population genetics and phylogenomic analyses of *P. oryzae* isolates collected from different species of Poaceae have revealed that host specificity groups defined from pathogenicity data actually represent multiple divergent lineages within the species. Each lineage is associated with a limited range of specific hosts (Gladieux et al. 2018). For instance, almost all isolates sampled on wheat belong to the same host-specific lineage: the *Triticum* lineage. Genetic divergence between host-specific lineages is very low (less than 1% genome-wide pairwise distance, measured as the number of nucleotide differences per kilobase) and genetic exchanges have recently occurred between lineages (Gladieux et al. 2018). However, host specificity is not strict, with some isolates capable of infecting host genera that are different from their host of origin, although generally with less severe symptoms than on the original host (Asuyama 1963; Farman et al. 2017). Some of these opportunistic infections have been well documented, such as that of isolate WBKY11, which was sampled on a single infected wheat head in Kentucky, the United States, despite a previous total absence of wheat blast epidemics in the state. WBKY11 has been described as genetically similar to local isolates infecting ryegrass (*Lolium* spp.) and belonging to the *Lolium* lineage, which is the closest host-specific lineage to that of *Triticum* (Farman et al. 2017; Gladieux et al. 2018). At the genetic level, host specificity has been linked in some cases to effector-triggered immunity. For example, almost all isolates infecting rice had lost the effector *AVR1-CO39* that encodes a protein recognized by rice resistance proteins (Cesari et al. 2013; Couch et al. 2005; Kang et al. 1995), and expression of the

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PWL2 gene inhibits the infection of weeping lovegrass. Effector genes *PWT3* and *PWT4* have also been shown to prevent infection of wheat varieties carrying the corresponding resistance genes *Rwt3* and *Rwt4*, respectively (Inoue et al. 2017). Thus, a hypothesis for the appearance of wheat blast is the substantial use of *rwt3* wheat varieties (lacking the *Rwt3* resistance gene and, hence, susceptible to *P. oryzae* isolates carrying *PWT3* such as ryegrass isolates), followed by functional losses of the *PWT3* gene, allowing a massive outbreak among all *Rwt3*-carrying wheat varieties (Inoue et al. 2017).

Blast is an ancient fungal disease, first recorded on rice in 1637 in China and in 1704 in Japan (Ou 1985). The causal agent was isolated and named in 1891 but had certainly existed long before these dates, and the fungus is now found worldwide. However, before 1985, there were only sporadic reports of this disease among wheat crops (India in 1922, Pakistan in 1943, and Louisiana, the United States in 1973) (Cruz and Valent 2017; Malik and Khan 1944; Mcrae 1922; Rush and Carver 1973). The first wheat blast outbreak was reported in 1985 in the Brazilian state of Paraná, in which major symptoms were observed in wheat fields across six different municipalities. This first epidemic represented a major transition in wheat blast, evolving from causing episodic symptoms to major epidemics. Indeed, this outbreak was followed by a rapid spread to other regions in Brazil (northwestern São Paulo State and southern Mato Grosso do Sul) within a year (Anjos et al. 1996; Goulart 1992; Goulart et al. 2007; Igarashi et al. 1986; Picinini and Fernandes 1990). Soon afterward, the disease spread to three neighboring countries (1996 in Bolivia, 2002 in Paraguay, and 2007 in Argentina) (Cruz and Valent 2017). The first outbreak outside the center of origin was reported in Bangladesh in 2016 and attributed to the introduction of infected seed from South America (Islam et al. 2016; Malaker et al. 2016). Approximately 15,500 ha of wheat were infected by the wheat blast pathogen in Bangladesh in 2016 (Aman 2016; Islam et al. 2016). The *P. oryzae* isolates causing this recent EID are very aggressive. They greatly harm yield and grain quality, and up to 100% yield loss has been reported on susceptible wheat varieties under field conditions (Aman 2016; Cruz and Valent 2017; Islam et al. 2019, 2016).

It is of paramount importance to ensure early diagnosis of the *P. oryzae* isolates responsible for wheat blast epidemics (hereafter called “epidemic wheat blast isolates”) in order to protect wheat crops (Ceresini et al. 2018). The economic impact of misidentifying wheat blast disease may be substantial. Inaccurate identification of the causal agent can lead to drastic measures such as bans on exportation or the destruction of vast numbers of uncontaminated seed. Conversely, false-negative results (i.e., failure to detect epidemic wheat blast isolates in contaminated material) can start an epidemic in a new area. Given these considerable economic risks, the diagnostic tests used for control and surveillance must fulfill strict conditions: they must discriminate between *P. oryzae* isolates at an infraspecific level in order to distinguish isolates causing epidemic wheat blast from isolates capable of causing only opportunistic wheat infections, despite the isolates sharing identical morphological features in pure culture.

The *Triticum* lineage identified through phylogenomic analyses includes almost all of the isolates sampled on wheat (Gladieux et al. 2018). In this study, a single isolate had not been sampled on wheat and yet belonged to the *Triticum* lineage (isolate P29). P29 was collected from a *Bromus* plant in a wheat field severely affected by blast and was strongly pathogenic on wheat during pathotyping experiments (Pieck et al. 2017). On the other hand, only three isolates sampled from wheat were assigned to the *Lolium* lineage. Among these three are the previously mentioned U.S. isolate WBKY11 and isolate PY5010, described by Pieck et al. (2017) as mildly pathogenic on wheat under laboratory conditions while being highly pathogenic on *Lolium* spp. The third isolate is PY86.1, for which no data about its pathogenicity on wheat are available. These elements strongly suggest that the isolates belonging to the *Triticum* lineage are responsible for wheat blast epidemics and their regrouping within the same lineage implies the existence of genomic regions specific to these isolates. Hereafter, the term “*Triticum* lineage isolates” is used to denote isolates that were genetically assigned to

the *Triticum* lineage and capable of causing wheat blast epidemics (thus, synonymous with epidemic wheat blast isolates), and “wheat-borne isolates” is the term used to refer to those isolated from wheat regardless of their genetic assignment.

DNA-based detection methods such as PCR or real-time PCR are widely used to discriminate pathogens indistinguishable by morphology and have proved their effectiveness in detecting and identifying major plant pathogens, including fungi (Schena et al. 2004). At the species level, classical phylogenetic markers used in fungal taxonomy such as internal transcribed spacers (ITS), translation elongation factors, or β -tubulins are generally relevant targets for these species-specific PCR assays. However, in cases of taxa that have recently diverged such as *P. oryzae* lineages, it is necessary to identify genomic regions with higher variability than those used at the species level (Feau et al. 2018). Furthermore, given the very low level of divergence between *P. oryzae* lineages and the existence of recent genetic exchanges between them (Gladieux et al. 2018), it is unlikely that a single test targeting one genomic region would perfectly meet all of the requirements; for instance, in terms of specificity. A recent study showed that the presence of the MoT3 locus was a discriminant feature of most isolates belonging to the *Triticum* lineage (Pieck et al. 2017). Therefore, conventional PCR, quantitative PCR (qPCR), and loop-mediated isothermal amplification oligonucleotides have been designed to specifically amplify this genomic region (Pieck et al. 2017; Yasuhara-Bell et al. 2018). However, certain isolates assigned to the *Triticum* lineage do not carry the MoT3 sequence (for example, isolate BR0032) (Gladieux et al. 2018), suggesting that some *Triticum* lineage isolates would remain undetectable despite using some of these oligonucleotides (Gupta et al. 2019; Yasuhara-Bell et al. 2019). This pitfall indicates the need to develop a complementary test targeting a different genomic region.

In this study, we (i) searched for new genomic regions carrying DNA polymorphisms that are specific to the *Triticum* lineage isolates, focusing on alleles of genes known to be involved in pathogenicity to wheat or using a global genomic comparison of *P. oryzae* genomes; (ii) developed a real-time PCR diagnostic test that complements currently existing tools; and (iii) evaluated the specificity of this new test with *P. oryzae* isolates from a broad host range such as *P. oryzae* isolates from previously untested wild hosts such as *Eriochloa* or *Cenchrus* spp.

Materials and Methods

Fungal genome assembly. In all, 81 published *Pyricularia* genome assemblies (Gladieux et al. 2018) were compared: 4 *P. grisea* genomes, 1 *P. pennisetigena* genome, and 76 *P. oryzae* genomes. Of the latter, 20 belonged to the *Triticum* lineage, 19 had been collected from *Triticum* spp., and 1 was from *Bromus tectorum* (isolate P29) (Supplementary Table S1).

Fungal DNA extraction. DNA was extracted from a collection of 114 fungal isolates, including 110 *P. oryzae*. Of the latter, 30 had been isolated from wheat and 80 had been sampled from 21 different Poaceae genera. Some of these isolates were sequenced and genetically assigned to a host-specific lineage by Gladieux et al. (2018) (Supplementary Table S2). Upstream of this study, a population genetic analysis was conducted on 90 wheat-borne isolates collected in Brazil and Bolivia between 1989 and 2013. This analysis allowed us to maximize the genetic diversity of the wheat-borne isolates tested in this study, retaining one isolate for each multilocus genotype detected. The integrity of all 114 DNA extracts was verified by their successful amplification by PCR using ITS5-4, ITS1-4, or *RPB2* (5cR/7cR) primers (O'Donnell et al. 2007; White et al. 1990) prior to further testing.

Construction of plasmids for use as positive control. The genomic region targeted by the C17 primer set was amplified with forward and reverse primers located upstream and downstream: seqF_C17 (5'-TGC GTC CCA AGA TTT TCG TGA-3') and seqR_C17 (5'-CTA CGC ATG GTG GGG AGA GA-3'). The amplicon was inserted in a pCR4-TOPO plasmid (Invitrogen, Carlsbad, CA, U.S.A.), which was used to transform chemically competent TOP10 cells (Invitrogen), according to the manufacturer's instructions. The

bacterial cells were then subcultured overnight at 37°C, and the plasmids were purified using a NucleoSpin Plasmid kit (Macherey-Nagel, Düren, Germany). The plasmid solution was used as a positive control for C17 real-time PCR.

Seed samples. *P. oryzae*-free wheat seed (variety Filon) from a field in Montpellier, France were used for the negative control and artificial inoculation with the pathogen. The seed were provided by C. Rudelle (GEVES, Montpellier, France). A culture of *Triticum* lineage isolate BL0044 was grown on potato dextrose agar (PDA) medium at 26°C, in a level 3 biosafety room at ANSES, Malzéville, France. A plug of mycelium was taken from the edge of the actively growing culture and used to inoculate a sample of 400 wheat grains laid over 15 ml of potato dextrose broth (PDB) in a sterile Petri dish. After 48 h of incubation, an additional 15 ml of PDB was added and the mixture was ground in a 125-ml sterilized glass bowl using a Microtron MB 550 laboratory mixer (Kinematica, Lucerne, Switzerland). A negative control seed sample was also prepared, using a sterile PDA plug for inoculation. For each seed sample, one 500-µl sample was taken from the homogenate using a truncated 1-ml pipet tip and used for total DNA extraction with NucleoSpin Plant II DNA extraction kit (Macherey-Nagel), following the manufacturer's recommendations. *Triticum* lineage was detected by real-time PCR under optimized conditions, using these raw DNA extracts as templates.

Plant varieties for pathogenicity tests. *P. oryzae* isolates were tested for pathogenicity on wheat variety Thésée, which is susceptible to wheat blast, and on a commercial mixture of three ryegrass varieties (Pronto, Fandango, and Funk) used as susceptibility checks. Compatibility between *P. oryzae* isolates and wheat or ryegrass was evaluated under controlled conditions, by inoculating wheat leaves (5 plants/isolate) or ryegrass leaves (more than 10 plants/isolate) with a conidial suspension using two protocols. First, as described for rice by Gallet et al. (2016), 10 ml of conidial suspension (20,000 conidia/ml) with 1% gelatin was sprayed on 2-week-old seedlings for each isolate. This method of inoculation is softer and is closer to natural inoculation but was not adapted to inoculate epidemic wheat blast isolates as positive controls in our biosafety facilities. Second, a suspension of 50,000 conidia/ml was applied using a brush in a delimited wheat leaf region. This method may be deemed as more invasive because brush movements can injure leaf tissues but it enabled the introduction of positive controls (isolates BR0032 and BL0028) in the compatibility experiment. In both cases, the inoculated plants were kept overnight at 27°C and 100% humidity and then for a further 7 days with alternation of day and night (13 h at 27°C and 11 h at 21°C, respectively) before noting symptoms.

Search for PWT3 and PWT4 alleles in *P. oryzae* genomes. To determine whether the sequences of the avirulence genes *PWT3* and *PWT4* could be used as genomic hallmarks of the *Triticum* lineage, BLASTN (version 2.6.0) was used to search for alleles of these genes in the 81 *P. oryzae* genomes (options -task blastn and e-value 0.0001). The query sequences used were the nucleotide sequences of the avirulent alleles; that is, A0 for *PWT3* (accession number LC202650.1) and Br58 for *PWT4* (accession number LC202655.1). Some of the DNA sequences located upstream or downstream of these genes were repeated multiple times in the assembled genomes, such as positions 1 to 159 and 531 to 603 for the *PWT3* A0 allele, and positions 1 to 211 and 853 to 927 for the *PWT4* Br58 allele. Therefore, every hit that only mapped these repeated regions was removed. In addition, when two parts of the gene were identified on the same scaffold (indicating a large insertion in the gene sequence) or in different scaffolds on the assembly (due to either a genomic rearrangement or an assembly issue), the sequences were merged. All sequences were aligned with Muscle (version 3.8.31) and compared (data not shown).

Comparison of *P. oryzae* genomes to identify DNA regions unique to the *Triticum* lineage. To detect DNA regions that were specific to the *Triticum* lineage, we designed a bioinformatics approach using PYTHON scripts and based on comparing 81 previously published assembled genomes. We focused on polymorphisms that were fixed in the *Triticum* lineage. The genome of isolate BR0032 sampled from wheat and belonging to the *Triticum* lineage was chosen as a reference. This genome was fragmented into sequences of 500

nucleotides with 250 bp overlap. To select fragments of interest, we conducted a homology analysis with other genomes using BLASTN (version 2.6.0), specifying -task blastn. Fragments were then selected if two criteria were fulfilled: (i) a sequence strictly identical to the candidate fragment was found in each of the genomes belonging to the *Triticum* lineage and (ii) no sequence 100% identical to the candidate fragment was found in any of the genomes belonging to other host-specific lineages. A first screening of the fragments was performed using only three genomes (from *Triticum*, *Lolium*, and *Oryza* lineages), and every fragment retained was then screened using the other 78 genomes. Finally, for each selected fragment, the homologous sequence in each assembled genome was extracted and all the sequences were aligned using Muscle (version 3.8.31). This study's bioinformatics analysis is summarized and sketched in Figure 1.

Development of a qPCR diagnostic test. *Primer design.* Candidate primers and probes were designed to specifically amplify DNA from the *Triticum* lineage genotypes, targeting the *Triticum* lineage-specific positions identified by bioinformatics analyses. Forward and reverse primers were designed so as to amplify a DNA fragment of 90 to 120 bp using PCR. The polymorphisms specific to the *Triticum* lineage were preferentially positioned at the 3' end of the forward and reverse primers, in order to enhance PCR specificity. Primers and 5' 6-FAM/3' BHQ-1 hydrolysis probes were custom synthesized by Eurogentec (Seraing, Belgium).

Screening candidate primers for inclusivity and specificity. Inclusivity (i.e., ability to target all epidemic wheat blast isolates) and specificity (i.e., ability to avoid cross-reacting with nontarget DNA) were the main criteria for primer selection. Primers were first screened for specificity and inclusivity using conventional PCR. These two parameters were assessed using DNA from a small panel consisting of seven isolates sampled on different hosts (three on ryegrass: AG0064, PL2-1, and 365; one on finger millet: IN0113; one on rice: BR0019; and two wheat-borne isolates: AG0103 and BL0093). PCRs to enable screening used the qPCR core kit No ROX (Eurogentec) with the following reaction mixture: 2 µl of DNA template (0.5 ng/µl), 1× polymerase buffer, 5 mM MgCl₂, 0.3 µM forward and reverse primers, 4× 0.2 mM dNTPs, HotGoldStar DNA polymerase at 0.025 U/µl, and molecular-grade water to 20 µl. The PCR conditions were 10 min at 95°C, then 40 cycles of 15 s at 95°C and 55 s at 60°C. Amplification products were resolved by ethidium bromide staining after 50 min of electrophoresis on 2% agarose gel.

Specificity and inclusivity were then further assessed by combining the best candidate-specific primer pairs with a hydrolysis probe in real-time PCR. The hydrolysis probes were designed to target polymorphisms specific to the *Triticum* lineage, whenever possible. The specificity of primer and probe combinations was tested using DNA from a larger panel of 114 isolates, including 110 *P. oryzae* isolates sampled on 21 different host genera. Real-time PCRs were carried out in a 20-µl reaction volume containing 2 µl of DNA template (0.5 ng/µl), 1× qPCR core kit No ROX (Eurogentec) polymerase buffer, 5 mM MgCl₂, 0.3 µM forward and reverse primers, 0.1 µM probe, 4× 0.2 mM dNTPs, HotGoldStar DNA polymerase at 0.025 U/µl, and molecular-grade water to 20 µl. The following PCR profile was used: 10 min at 95°C, then 40 cycles of 15 s at 95°C and 55 s at 60°C. The specificity and inclusivity of the MoT3 test were assessed on the same 114-isolate panel, using the PCR conditions described above.

C17-F/R/P specific test performance values. For the C17 primer/probe combination, the master mix composition and cycling conditions were adjusted to optimize specificity and sensitivity (mean cycle threshold [Ct] values). Optimal real-time PCR conditions were as follows: 2 µl of DNA template, 1× polymerase buffer (qPCR core kit No ROX; Eurogentec), 5 mM MgCl₂, 0.3 µM forward C17-F (5'-CGA TAG AAA CTT GAG GAA GAT CAA GTA AG-3') and reverse C17-R (5'-TCA CCG AGA GAT GTG CCA C-3') primers, 0.05 µM C17-P probe (5'-FAM- TCG CTA ACA ATG TCC ACC CCG CC-BHQ1-3'), 4× 0.2 mM dNTPs, HotGoldStar DNA polymerase at 0.025 U/µl, and molecular-grade water to 20 µl. PCR cycles were identical to those used for primer screening, except that the

hybridization or polymerization temperature was increased to 65°C. The following performance criteria were assessed: analytical specificity, analytical sensitivity, transferability, and robustness. Analytical specificity, analytical sensitivity, and robustness measures were determined with data generated by a Stratagene Mx3005P real-time thermocycler (Agilent Technologies, La Jolla, CA, U.S.A.) and extracted using Mx3005P software (V4.10) with a threshold set at 400. Analytical specificity was assessed using duplicated reactions using genomic DNA (gDNA) from the 114 *P. oryzae* isolates

previously adjusted to 0.5 ng/μl. Analytical sensitivity was assessed using a 10-fold dilution series of the plasmid DNA-positive controls containing the C17-F/R amplicon diluted in ultrapure water and with a dilution of gDNA from isolates BR0032 and BL0028. The dilution series ranged from 13×10^3 to 1.3 plasmid copies/μl and gDNA at 0.5 ng/μl to 0.05 pg/μl. The limit of detection (LOD) was determined as the minimal quantity of target DNA that could be consistently amplified by qPCR (at least 10 replicates). A standard curve was constructed based on these data.

Fig. 1. Bioinformatics approach used to identify polymorphisms specific to the *Triticum* lineage of *Pyricularia oryzae*. Colors of the schematic genomes represent isolate lineages.

The test's transferability was assessed using three replicates with different brands of qPCR reagents: 2× Takyon for Probe Assay, No ROX blue (Eurogentec), LightCycler 480 Probes Master (Roche, Meylan, France), and Takyon Core Kit for Probe Assay, No ROX blue (Eurogentec), another type of thermocycler (Rotor-Gene 6500; Corbett Research, Mortlake, Australia), and a different laboratory (ANSES Plant Health Laboratory). To assess the robustness of the qPCR assay, several reaction parameters were deliberately modified and the assay was carried out using 10 replicates of different DNA templates. The qPCR assay's robustness was challenged by varying the reaction volume or DNA template volume by $\pm 10\%$, and slightly varying the hybridization temperature ($\pm 2^\circ\text{C}$) to verify whether the test could withstand experimental variations without compromising sensitivity and specificity. Transferability and robustness were determined using a panel of different DNA types as templates. The panel consisted of (i) target DNA close to the LOD (10× LOD and 100× LOD plasmid DNA) and *Triticum* lineage BL0044 gDNA at 0.5 ng/ μL , (ii) nontarget DNA (AG0064 ryegrass isolate at 0.5 ng/ μL and IN0113 finger millet isolate gDNA), and (iii) total DNA extracted from healthy wheat seed and wheat seed artificially inoculated with BL0044.

Results

PWT3 and PWT4 avirulence genes are not specific markers to the *Triticum* lineage. The *PWT3* and *PWT4* alleles (including presence or absence polymorphisms) were examined in 81 published and assembled genomes, including 76 *P. oryzae* genomes, in order to determine whether alleles of these effectors can be diagnostic of the *Triticum* lineage. The *PWT4* avirulence gene sequence was absent in 50 of the 81 genomes studied, including 14 isolates from the *Triticum* lineage and 36 isolates from other lineages. The complete and functional allele (i.e., the complete coding sequence and its upstream region) was only identified in the genome of isolate Br58, as already described by Inoue et al. (2017). Other genomes carried truncated alleles lacking various genic regions; one of these alleles was shared among isolates of *Lolium* lineage and certain isolates of the *Triticum* lineage (T25 and Br48). *PWT3* was found in all genomes except *P. oryzae* isolate EI9411 sampled on *Eleusine* spp. and *P. pennisetigena* isolate Pm1 sampled from *Pennisetum* spp. Various *PWT3* gene alleles were retrieved from these assemblies, including alleles displaying large insertions or gene fragmentation (i.e., fragments of the gene on different assembly scaffolds).

Sequence comparison, based on the regions conserved between different *PWT3* alleles, highlighted that *Triticum* lineage isolates shared alleles with non-*Triticum* lineage isolates such as *Lolium* or *Stenotaphrum* lineage isolates. For both *PWT3* and *PWT4*, some alleles were shared between isolates of the *Triticum* lineage and other lineages. Therefore, *PWT3* and *PWT4* avirulence genes were not suitable for designing markers specific to the *Triticum* lineage and were not further used in developing a diagnostic test.

Comparative genomics identifies candidate genetic markers unique to the *Triticum* lineage. The genome of isolate BR0032, used as a reference for the *Triticum* lineage, was fragmented in silico into 167,487 fragments. Our bioinformatics approach retained only fragments for which (i) a strictly identical (100% identity over 500 bp) sequence was found in all genomes from the *Triticum* lineage and (ii) hits in genomes from other lineages had <100% identity (Fig. 1). In total, 239 fragments were selected and their corresponding alignments were generated. At the end of this process, 323 polymorphisms specific to the *Triticum* lineage were identified. These positions were spread over 135 fragments belonging to four distinct scaffolds of the reference genome BR0032 (Supplementary Table S3).

Design of *Triticum* lineage-specific oligonucleotide candidates. In total, 34 primer pairs (forward and reverse primers) were designed to specifically amplify the DNA of *Triticum* lineage isolates using PCR. These oligonucleotide combinations target the different regions on the BR0032 genome that carry some of the *Triticum* lineage-specific positions identified by our bioinformatics scripts. As much as possible, the regions selected (i) were physically distant

from each other within the BR0032 genome and (ii) carried several polymorphisms in order to improve the oligonucleotides' specificity. Oligonucleotide sequences are given in Supplementary Table S4.

Prescreening of inclusive and specific primer sets. Among the 34 primer pairs tested, 14 (41%) yielded a stronger amplification signal with DNA from the two wheat-borne isolates included in the small panel, confirming by PCR the in silico results provided by the bioinformatics approach. However, of these 14 primer pairs, only 5 (C5, C7, C9, C17, and C19) generated no cross-reaction with DNA from the nontarget isolates in our small panel, and 4 of these (C5, C9, C17, and C19) were selected for further analyses. An associated hydrolysis probe was designed for use in combination with each selected primer pair in real-time PCR.

Final selection and evaluation of *Triticum* lineage-specific oligonucleotides. The four selected oligonucleotide combinations target sequences located in different regions of genome BR0032. Combinations C5, C9, and C17 target scaffold 15 of genome BR0032 (with an approximately 29,500-bp distance between C9 and C5 and an approximately 109,000-bp distance between C5 and C17), whereas combination C19 targets scaffold 17. The previously published primer/probe combination MoT3_1F, MoT3_1R, and MoT3_FAM2, which targets epidemic wheat blast isolates of *P. oryzae* (Pieck et al. 2017), was also included for comparison. The specificity and inclusivity of the combinations were assessed using real-time PCR, with a larger panel of 114 DNA extracts. The results showed that none of them were able to achieve full inclusivity and full specificity. In our PCR conditions, combinations MoT3 and C19 failed to yield positive results with DNA from 2 and 3 different wheat-borne isolates, respectively, out of the 30 in our panel, whereas they yielded cross-reactions with a single but different DNA extract from nontarget isolates (Fig. 2). When PCR used the three oligonucleotide combinations designed on scaffold 15 (C5, C9, and C17), it successfully amplified DNA from 100% of the 30 wheat-borne isolates tested. However, using these combinations, cross-amplifications were observed with DNA from isolates sampled on other host plants (Fig. 2). Based on these results, combination C17 was selected because, under our PCR conditions, it successfully amplified DNA from all 30 wheat-borne isolates in our panel yet yielded the lowest rate of cross-reactions (only four nontarget isolates: CR0023, CR0057, JP0033, and IN0113). After adjusting the primer mix composition and increasing the hybridization temperature to 65°C , all 30 DNA extracts from wheat-borne isolates were amplified (with Ct values ranging from 25.74 to 30.69, including isolate P29, sampled from *Bromus* spp. but belonging to the *Triticum* lineage), whereas late amplification was not observed with DNA from isolate IN0113 sampled from *Eleusine* spp. (Fig. 2).

C17-F/R/P test performance. Test sensitivity. The analytical sensitivity of test C17 was determined by analyzing three replicates of a serial 10-fold dilution in ultrapure water of gDNA of the isolates BR0032 and BL0028 or a dilution of the PCR target inserted into plasmid DNA. The test successfully amplified 100% of the replicates down to 1 pg of gDNA for both isolates and down to 13 plasmid copies/reaction tube (Fig. 3). This limit of detection was confirmed by a further test using 10 replicates. The regression coefficient (R^2) was 0.981, showing a good correlation between Ct values and the initial quantity of target DNA loaded (Fig. 3). As a proof of concept, DNA extracted from noninoculated seed and DNA extracted from seed artificially inoculated with isolate BL0044 were tested by real-time PCR using the C17-F/R/P set. The test consistently detected BL0044 DNA in the inoculated seed, whereas no signal was observed with the control seed sample.

C17 test robustness and transferability. When minor changes in volume and temperature were applied to the original PCR conditions, the mean Ct values obtained with the DNA templates tested were significantly modified (P value = 2.2×10^{-16}). However, Ct values measured under these different conditions are of the same order of magnitude without altering the qualitative results (+/−) and, thus, corroborate the specificity and sensitivity of the C17 test. An exception to this was when late amplification of the DNA from isolate IN0113 (Ct = 38.83) was observed for 1 of the 10 replicates when

the hybridization temperature was decreased to 63°C. The test's transferability in terms of other lab conditions was evaluated by changing the type of thermocycler, the real-time master mix brand, and the operator. In most cases, specificity and sensitivity of the C17 test were not qualitatively affected by these modifications. However, it is worth mentioning that changing the real-time PCR master mix type led to late amplifications of DNA from the IN0113 *Eleusine*

isolate. Quantitatively, Ct values were generally improved (i.e., determined earlier) using the Rotorgene thermocycler. Data are presented in Figure 4.

Characterizing cross-amplified taxa. Because DNA from isolates CR0023, CR0057, and JP0033 were consistently cross-amplified by the C17-F/-R/-P test, PCR primers were designed to flank the region amplified by primer set C17 in order to

	primer information	BR0032 scaffold	15	15	15	17	abs	15
		BR0032 position	373250	402500	511250	432500	abs	511250
		oligonucleotide combination	C9	C5	C17	C19	MoT3	C17
Isolates and hosts of origin	AG0103	<i>Triticum aestivum</i>						
	BL0017	<i>Triticum aestivum</i>						
	BL0018	<i>Triticum aestivum</i>						
	BL0020	<i>Triticum aestivum</i>						
	BL0023	<i>Triticum aestivum</i>						
	BL0028	<i>Triticum aestivum</i>						
	BL0037	<i>Triticum aestivum</i>						
	BL0044	<i>Triticum aestivum</i>						
	BL0046	<i>Triticum aestivum</i>						
	BL0063	<i>Triticum aestivum</i>						
	BL0092	<i>Triticum sp.</i>						
	BL0093	<i>Triticum sp.</i>						
	BR0031	<i>Triticum sp.</i>						
	BR0032	<i>Triticum sp.</i>						
	BR0034	<i>Triticum sp.</i>						
	BR0036	<i>Triticum sp.</i>						
	BR0039	<i>Triticum sp.</i>						
	BR0040	<i>Triticum sp.</i>						
	BR0041	<i>Triticum sp.</i>						
	BR0043	<i>Triticum sp.</i>						
	BR0045	<i>Triticum sp.</i>						
	BR0047	<i>Triticum sp.</i>						
	BR0080	<i>Triticum sp.</i>						
	BR0086	<i>Triticum sp.</i>						
	BR0087	<i>Triticum sp.</i>						
	BR0088	<i>Triticum sp.</i>						
	BR0123	<i>Triticum aestivum</i>						
	BTGP16	<i>Triticum sp.</i>						
	BTJP4-1	<i>Triticum sp.</i>						
	BTMP13-1	<i>Triticum sp.</i>						
	P29	<i>Bromus sp.</i>						
	AG0054	<i>Bromus sp.</i>						
	AG0055	<i>Bromus sp.</i>						
	AG0061	<i>Bromus unioloides</i>						
	AG0062	<i>Lolium sp.</i>						
	AG0063	<i>Lolium sp.</i>						
	AG0064	<i>Lolium sp.</i>						
	AG0065	<i>Stenotaphrum sp.</i>						
	AG0132	<i>Oryza sativa</i>						
	BF0017	<i>Pennisetum typhoides</i>						
	BR0019	<i>Oryza sativa</i>						
	BR0029	<i>Digitaria sanguinalis</i>						
	BR0030	<i>Cenchrus echinatus</i>						
	BR0062	<i>Eleusine indica</i>						
	BR0070	<i>Eragrostis sp.</i>						
	Br58	<i>Avena sp.</i>						
	CD0143	<i>Digitaria exilis</i>						
	Cd88215	<i>Cynodon dactylon</i>						
	CH0333	<i>Oryza sativa</i>						
	CH1120	<i>Oryza sativa</i>						
	CHRF	<i>Lolium sp.</i>						
	CHW	<i>Lolium sp.</i>						
	CR0021	<i>Panicum miliaceum</i>						
	CR0023	<i>Echinochloa crus-galli</i>						
	CR0026	<i>Lolium sp.</i>						
	CR0029	<i>Festuca elalior</i>						
	CR0030	<i>Setaria viridis</i>						
Isolates and hosts of origin	CR0031	<i>Setaria italica</i>						
	CR0057	<i>Lolium sp.</i>						
	EG0028	<i>Cyperus rotundus</i>						
	FH	<i>Lolium sp.</i>						
	FR0013	<i>Oryza sp.</i>						
	FR1069	<i>Lolium sp.</i>						
	GG11	<i>Lolium sp.</i>						
	GN0001	<i>Zea mays</i>						
	GR0001	<i>Ctenanthe oppenheimiana</i>						
	GY0011	<i>Oryza sativa</i>						
	HO	<i>Lolium sp.</i>						
	IN0003	<i>Panicum repens</i>						
	IN0005	<i>Panicum maximum</i>						
	IN0022	<i>Setaria sp.</i>						
	IN0023	<i>Setaria sp.</i>						
	IN0082	<i>Oryza sativa</i>						
	IN0108	<i>Setaria sp.</i>						
	IN0113	<i>Eleusine sp.</i>						
	IN0115	<i>Oryza sativa</i>						
	IR0013	<i>Zea mays</i>						
	IR0015	<i>Zea mays</i>						
	IR0095	<i>Zea mays</i>						
	IR0102	<i>Echinochloa sp.</i>						
	IS0001	<i>Cyperus rotundus</i>						
	JP0028	<i>Eragrostis curvula</i>						
	JP0030	<i>Panicum bisulcatum</i>						
	JP0031	<i>Panicum coloratum</i>						
	JP0033	<i>Eriochloa villosa</i>						
	JP0047	<i>Hordeum vulgare</i>						
	JP0048	<i>Hordeum vulgare</i>						
	KN0001	<i>Hordeum vulgare</i>						
	KN0006	<i>Hordeum vulgare</i>						
	Lc8401	<i>Leptochloa chimensis</i>						
	LpKY97	<i>Lolium sp.</i>						
	ML0031	<i>Pennisetum sp.</i>						
	Pd88413	<i>Paspalum distichum</i>						
	Pg1054	<i>Stenotaphrum secundatum</i>						
	Pg1213-22	<i>Festuca sp.</i>						
	PH0052	<i>Cyperus rotundus</i>						
	PH0053	<i>Cyperus rotundus</i>						
	PH0062	<i>Paspalum distichum</i>						
	PH0075	<i>Bracharia mutica</i>						
	PH0078	<i>Echinochloa sp.</i>						
	PH0097	<i>Paspalum paspaloides</i>						
	PL 2-1	<i>Lolium sp.</i>						
	PL 3-1	<i>Lolium sp.</i>						
	P28	<i>Bromus sp.</i>						
	PR0069	<i>Stenotaphrum secundatum</i>						
	Pr8202	<i>Panicum repens</i>						
	RW0043	<i>Eleusine coracana</i>						
	TF05-1	<i>Festuca sp.</i>						
	US0064	<i>Setaria sp.</i>						
	US0066	<i>Cenchrus ciliaris</i>						
	US0077	<i>Lolium perenne</i>						
	US0078	<i>Lolium perenne</i>						
	US0084	<i>Stenotaphrum secundatum</i>						
	VT0032	<i>Leersia hexandra</i>						

Fig. 2. List of *Pyricularia oryzae* isolates used to assess the specificity and inclusivity of the selected primer/probe combinations in real-time PCR. The color of the boxes indicates the amplification status of the 114 DNA extracts tested using primer sets C5, C9, C17, C19, and MoT3 (dark gray = amplification, light gray = late amplification, and white = no amplification). Last column (red) corresponds to the C17 primer set test using optimized quantitative PCR parameters (including higher hybridization temperature). Isolates in blue characters were genetically assigned to the *Triticum* lineage by Gladieux et al. (2018).

Sanger-sequence the entire locus. Sequencing confirmed that isolates CR0023, CR0057, and JP0033 shared 100% identity with *Triticum* lineage isolates at this locus. Within the C17-F/-R amplicon, a single-nucleotide polymorphism (SNP) was identified for isolate CR0023. Sequencing isolate IN0113 showed that the C17F primer's target region differed by two substitutions, located near the 3' end (Supplementary Fig. S1). These substitutions lead to the appearance of a sequence that is identical to the last five bases at the 3' end of C17's forward primer and could explain the late amplification of this DNA at a lower hybridization temperature. The pathogenicity of isolates CR0023, CR0057, and JP0033 was assessed by two methods. A brush inoculation confirmed the high leaf susceptibility of the wheat variety Thésée to wheat-borne isolates BR0032 and BL0028. Concerning non-wheat-borne isolates, except for CR0023, no reaction was observed. With isolate CR0023, limited necrosis could be observed at the margin or in the central vein of the leaves with this brush inoculation protocol for 5 of the 12 leaves inoculated. Spraying conidia on wheat cultivar leaves as well as on susceptible ryegrass varieties is a less invasive method that mimics the natural inoculation process. Using this inoculation protocol, none of the non-wheat-borne isolates tested induced any symptoms on wheat leaves but they created at least one lesion on susceptible checks of ryegrass, confirming their pathogenic potential in these

conditions. On ryegrass, the strongest symptoms were observed for CR0023 (sampled from *Echinochloa crus-galli*), intermediate symptoms for CR0057 (*Lolium* sp.) and only one lesion was observed for JP0033 (*Eriochloa villosa*) on ryegrass. Finally, because DNA from AG0055 isolated from *Bromus* spp. cross-reacted with the MoT3-based PCR test (Pieck et al. 2017) in our conditions, and because no genomic data are available concerning this isolate to validate the lineage it belongs to, this isolate was inoculated during the same experiment on wheat leaves (leaf brush) to validate its incompatibility with this host. This isolate was not able to induce any symptoms and, therefore, was deemed unable to infect wheat (Supplementary Fig. S2).

Discussion

Infecting a novel host is a major route of transmission in the emergence of infectious disease (Giraud et al. 2010; Gladieux et al. 2015). The management of new emerging diseases requires tools that can discriminate between emerging and established populations, in order to detect the pathogen at the earliest point and to monitor and limit its spread. Detection based on DNA amplification is a rapid and sensitive alternative to morphology-based identification and to pathogenicity testing and, today, is favored for infraspecific detection (Feau et al. 2018; Firth and Lipkin 2013). Indeed, morphology-based

Fig. 3. Sensitivity of the primer set C17 analyzed using a 10-fold serial dilution of plasmid DNA for positive control: gray = 1.3 copies/ μ l, green = 13 copies/ μ l, blue = 13E+01 copies/ μ l, yellow = 13E+02 copies/ μ l, and red = 13E+03 copies/ μ l). **A**, Amplification plot of a serial plasmidic 10-fold dilution and **B**, associated standard curve. Ct = cycle threshold.

identification is generally not diagnostic at the infraspecific scale in fungi, whereas pathogenicity tests are resource consuming and their outcome may be unclear (for instance, if the isolates surveyed represent opportunistic infections and do not constitute a self-sustainable population on the new host). The DNA-detection method can target either known host specificity determinants or other markers specific to the subgroup of isolates infecting a particular host (Bühlmann et al. 2013; Fernandez and Orth 2018; Franco Ortega et al. 2018; Singh and Kapoor 2018; Van Dam et al. 2018). In this study, an overall genome comparison of 81 assembled genomes enabled the identification of 323 polymorphisms specific to the *Triticum* lineage of *P. oryzae*, the causal agent of wheat blast. Some of these polymorphisms were successfully exploited to develop a qPCR-based detection test that specifically targets these positions, facilitating diagnosis of the causal agent in any particular case of wheat blast. We conducted an extensive validation process to clearly identify this detection test's limits, analyzing its inclusivity, specificity, sensitivity, robustness, and transferability.

Before starting an extensive search for new genetic targets to design a diagnostic test, we set out to assess the usability of previously described determinants of host specificity as markers specific to the *Triticum* lineage of *P. oryzae*. Inoue et al. (2017) showed that functional losses of the *PWT3* and *PWT4* effectors, which trigger an immune response in wheat varieties carrying cognate resistance genes, were key determinants in the spread of *P. oryzae* as a wheat pathogen, suggesting that *PWT3* and *PWT4* alleles would be ideal genetic markers for discriminating the *Triticum* lineage from other lineages. Our study clearly showed high sequence heterogeneity of *PWT3* within the *Triticum* lineage and allele sharing between the *Triticum* lineage and other lineages. This pattern can be explained by several evolutionary scenarios, including the emergence of the *Triticum* lineage through admixture with several other lineages, incomplete lineage sorting at these genes, or gene flow into the *Triticum* lineage subsequent to its emergence. Regardless of the cause of allelic polymorphism at *PWT3* and *PWT4*, no allele was specific to the *Triticum* lineage, thus precluding the use of these genes as targets for specific markers.

As an alternative means to identify further regions specific to the *Triticum* lineage, we developed a bioinformatics approach based on comparing the genomes from *P. oryzae* isolates of different lineages. We also included four genomes from *P. grisea* isolates and one

genome from *P. pennisetigena* isolates. Our approach was not designed to find all polymorphisms specific to the *Triticum* lineage but to select only those located in fixed regions of the *Triticum* lineage. In fact, the only SNPs selected were *Triticum*-specific and identified in fixed 500-bp sequences of *Triticum* lineage genomes. Interestingly, the 323 polymorphisms identified were not evenly scattered all over the genome but clustered in a limited number of regions, with 88% of polymorphisms found between positions 348,000 and 720,750 of scaffold 15 of the reference *Triticum*-lineage genome BR0032, and 11% of polymorphisms found between positions 216,750 and 595,750 of scaffold 17. In all, almost all of the positions found (99%) were concentrated in two regions accounting for less than 2% of the reference genome. These two regions may have experienced positive selection in the *Triticum* lineage, or may be linked to regions that have experienced positive selection. Therefore, the regions under positive selection might be functionally important in terms of pathogenicity to wheat. A similar strategy was used to detect the MoT3 marker (Pieck et al. 2017) but, though both studies have investigated the same pathogen species, this study used different genomic regions. In fact, several key parameters differed between the two studies, such as the reference genome (isolate B2 versus isolate BR0032), number of genomes studied, and threshold value used for selecting genome fragments. This example highlights that these parameters strongly affect the output when performing genome comparisons.

Based on the set of candidate-specific positions identified, 34 primer pairs were designed. These primers' specificity (ability to detect only isolates of *Triticum* lineage) and inclusivity (ability to detect any isolate of *Triticum* lineage regardless of origin) was checked using DNA samples that represented a set of isolates broader than that which was used in the bioinformatics detection process. The surveyed isolates' host of origin was known but, for most of them, genetic data were not available to confirm their lineage. However, all of the isolates in our study that were sampled on wheat (referred to here as wheat-borne isolates) were obtained from wheat blast epidemics. Of the 34 initially designed primer pairs, 14 showed stronger affinity to DNA from wheat-borne isolates. This high ratio confirmed the efficiency of our bioinformatics approach in identifying positions specific to a particular lineage within a species. However, in several cases, there were very few differences between targeted sequences of wheat-borne isolates and non-wheat-borne isolates, sometimes down to one SNP. The high sequence identity likely enabled a

		ROBUSTNESS						TRANSFERABILITY				
HYBRIDIZATION T*		63	67	65	65	65	65	65	65	65	65	
DNA VOLUME		2	2	1.8	2.2	2	2	2	2	2	2	
TOTAL VOLUME		20	20	20	20	18	22	20	20	20	20	
THERMOCYCLER		A	A	A	A	A	A	B	B	B	B	
KIT		1	1	1	1	1	1	1	2	3	4	
DNA	plasmid	100x T+LOD	29.6 +0.1	30 +0.3	29.4 +0.1	29.5 +0.1	29.1 +0.2	29.6 +0.2	22.2 +0.1	22.7 +0.2	22.5 +0.2	22.9 +0.1
		10x T+LOD	31.4 +0.3	32.36 +0.5	31.9 +0.3	32.2 +0.2	30.9 +0.2	31.4 +0.3	25.8 +0.4	26.3 +0.1	25.4 +0	26.4 +0.1
	genomic	(wheat) BL0044	27.1 +0.2	27.3 +0.2	27.1 +0.1	26.8 +0.2	27 +0.2	26.9 +0.1	24.4 +0.3	25.8 +0.2	25.7 +0.5	24.9 +0.3
		(eleusine) IN0113	38.83 (1/10)	-	-	-	-	-	-	-	-	-
		(lolium) AG0064	-	-	-	-	-	-	-	-	-	-
	seeds	inoculated seeds	31.8 +0.2	32.2 +0.3	32 +0.1	31.4 +0.2	31.6 +0.2	31.7 +0.2	25.2 +0.2	25.6 +0.2	25.8 +0.1	25.6 +0.4
		non-inoculated seeds	-	-	-	-	-	-	-	-	-	-

Fig. 4. Assessment of C17 test performance measuring amplification signals under different PCR conditions by varying hybridization temperature, DNA volume, total volume of PCR mix, thermocycler (A = Stratagene Mx3005P and B = Corbett rotor-gene 6005), or PCR kit brand (1 = Core kit NO ROX, Eurogentec; 2 = 2x Takyon for Probe Assay, No ROX blue; 3 = LightCycler 480 Probes Master; and 4 = Takyon Core Kit for Probe Assay, No ROX blue).

sufficient annealing of the primers to non-wheat-borne isolate DNA. This combination would have resulted in the weak nonspecific amplifications sometimes observed. Finally, five primer pairs successfully amplified only the two DNA extracts from wheat-borne isolates in the reduced panel, and four of them were considered for the development of a diagnostic test. *Triticum* lineage-specific hydrolysis probes were designed for each of these four primer pairs. The inclusivity and specificity of these primer/probe combinations were challenged by real-time PCR reactions with DNA from a wide range of 111 *P. oryzae*, including 30 wheat-borne isolates and 81 *P. oryzae* isolates sampled on other members of the Poaceae family. We also included the previously published MoT3 test (Pieck et al. 2017) for comparison. All primer/probe combinations tested (C5, C9, C17, C19, and MoT3) proved to be highly but not 100% specific. Because nondetection of *Triticum* lineage isolates could lead to the introduction of the pathogen in disease-free regions with potentially disastrous consequences, we ultimately selected the C17-F/R-P set that was able to amplify DNA from 100% of the wheat-borne isolates of our panel. Surprisingly, false-positive results were observed when C17 real-time PCR was carried out with DNA from four isolates that had not been sampled on wheat. DNA from isolates CR0023, CR0057, and JP0033 was amplified with a Ct value equivalent to *Triticum* lineage isolates while that of IN0113 led to a late amplification signal. These isolates had originally been sampled on wild Poaceae spp. in Asia: CR0023 had been sampled on *Echinochloa crus-galli* in Korea, CR0057 on *Lolium* spp. in Korea, JP0033 on *Eriochloa villosa* in Japan, and IN0113 on *Eleusine* spp. in India. Sequencing the genomic region targeted by the C17-F/R primers confirmed that CR0023, CR0057, and JP0033 carried a sequence 100% identical to that targeted for *Triticum* lineage isolate. Such patterns (i.e., isolates carrying genomic regions specific to another lineage) have previously been observed in *P. oryzae* (Gladioux et al. 2018; Inoue et al. 2017) and explained by gene flows between lineages. In our case, wheat blast epidemics have never occurred in Japan or Korea, which rules out the possibility that these isolates belong to the *Triticum* lineage or are due to recent genetic exchanges between these isolates and *Triticum* lineage isolates. Furthermore, these isolates were also positive in the C5 and C9 tests designed on the same scaffold of the BR0032 genome, suggesting that they actually share a significant genomic stretch with *Triticum* lineage isolates and making the hypothesis of a convergent evolution unlikely. Given these facts, the hypothesis of incomplete lineage sorting is favored to explain these “false positives”. Pathotyping tests confirmed that these three isolates were not able to infect wheat by spraying a suspension of 20,000 conidia/ml on wheat leaves and, thus, would unlikely occur on wheat showing blast symptoms. In addition, C17-F/R amplicon sequencing confirmed that the target sequence in IN0113 is comparable with non-*Triticum* lineage isolates but differs by two SNPs, thus allowing weak primer annealing and, therefore, resulting in a late amplification signal by PCR. This nonspecific amplification could be avoided by increasing the hybridization temperature during the amplification cycle.

It was essential to evaluate the performance values of the C17 test in order to validate the method and highlight potential drawbacks. In our conditions, the test proved highly repeatable and reproducible. The limit of detection was very low, with the test consistently able to detect as few as 13 plasmid copies of the target per PCR tube (or 1 pg of genomic wheat blast DNA), and the detection of *Triticum* lineage DNA in wheat seed was successful. Our experiments confirmed the test’s robustness by showing that small errors or drifts in temperature or volume would not qualitatively affect its sensitivity. However, we observed that using other brands or types of real-time PCR master mix than the ones originally used here for optimization may lead to weak cross-amplification of DNA from *Eleusine* isolate IN0113. This transferability assay confirmed that caution is required when a PCR protocol is implemented in conditions deviating from the original description (Ioos et al. 2019). This observation has recently been confirmed by additional data regarding the specificity of the MoT3 test (Gupta et al. 2019; Yasuhara-Bell et al. 2019).

The C17 diagnostic test developed here improves the detection of wheat blast to the extent of detecting *Triticum* lineage isolates that had, in previously available tests, been undetectable. On the one hand, the C17 test proved able to detect all epidemic wheat blast isolates in our possession. They consisted of 20 isolates belonging to the *Triticum* lineage tested by in silico analysis, and 30 isolates sampled on wheat with blast symptoms that were then tested by wet lab analysis. These 48 isolates had been sampled between 1988 and 2018 in five countries affected by the disease and, thus, cover a significant proportion of their genetic diversity. In our conditions, the MoT3 test (Pieck et al. 2017) failed to yield positive results for two of them (BR0032 and BR0043) by real-time PCR. On the other hand, the C17 test yielded false-positive results with DNA from three *P. oryzae* isolates not sampled on wheat; namely, CR0023, CR0057, and JP0033. We demonstrated that all three isolates were unable to induce any symptoms when inoculated by spray on a susceptible variety of wheat. Therefore, the presence of these genotypes on wheat blast showing symptoms is very unlikely and false-positive results are not expected when testing wheat tissue with the C17 test.

Prior to this study, it had been hypothesized that the isolates causing wheat blast epidemics belong to a species distinct from *P. oryzae*, and the concept of host specificity within *P. oryzae* had also been questioned (Castroagudín et al. 2016; Ceresini et al. 2019, 2018). However, further studies had refuted this hypothesis by explaining the biases that may have led to such a conclusion (Gladioux et al. 2018; Valent et al. 2019). In the present study, we chose to develop a test targeting isolates from the *Triticum* lineage of *P. oryzae* as described by Gladioux et al. (2018), assuming that only isolates belonging to the *Triticum* lineage are able to cause wheat blast epidemics. Under this assumption, the C17 test represents an efficient means to quickly assess whether any *P. oryzae* isolate sampled on wheat could lead to a wheat blast epidemic (with a positive C17 result) or, alternatively, belongs to a closely related lineage, only capable of causing opportunistic wheat infections (with a negative C17 result), such as the WBKY11 isolate in Kentucky (Farman et al. 2017). This rationale was validated because all of the isolates sampled on wheat affected by wheat blast epidemics yielded positive results with the C17 test. However, it is known that isolates belonging to other *P. oryzae* lineages can cause opportunistic infections on wheat and, if a new independent host jump were to occur with such isolates, it is very likely that these isolates would have a distinct genetic background and would remain undetectable by tests targeting markers specific to the *Triticum* lineage such as the one described here. Therefore, new wheat blast outbreaks should be thoroughly investigated.

The C17 test would be useful in studying the epidemiology and spread of wheat blast. In particular, it could be used to assess whether other members of the Poaceae family are a reservoir for *Triticum* lineage isolates. It is important to note, however, that some isolates sampled on hosts other than wheat generated false-positive results. Therefore, when testing isolates sampled on other species of Poaceae, all positive results should be confirmed using at least one other complementary test (DNA based or by pathotyping the isolate), especially in areas where wheat blast has not previously been reported. Finally, using an appropriate sampling strategy, the real-time PCR tool we developed may also be very useful for safeguarding wheat seed trade, and preventing the introduction of wheat blast disease in areas where it has been absent thus far. Wheat blast is seed borne and seed transmitted (Goulart 1988). Our preliminary experiments on artificially contaminated wheat seed suggest that the C17 test is able to detect *Triticum* lineage DNA in this matrix but, nonetheless, this result should be validated with naturally contaminated seed lots.

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