Genetic Diversity of the Mating Type and Toxin Production Genes in *Pyrenophora tritici-repentis*

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Accepted for publication 5 January 2010.

ABSTRACT

Lepoint, P., Renard, M.-E., Legrève, A., Duveiller, E., and Maraite, H. 2010. Genetic diversity of the mating type and toxin production genes in *Pyrenophora tritici-repentis*. *Phytopathology* 100:474-483.

Pyrenophora tritici-repentis, the causal agent of tan spot on wheat, is a homothallic loculoascomycete with a complex race structure. The objectives of this study were to confirm the homothallic nature of the pathogen, characterize mating type diversity and toxin production genes in a global collection of strains, and analyze how these traits are associated between each other and with existing races. The pseudothecia production capacity, race identification, mating type locus (*MAT*), internal transcribed spacer, and glyceraldehyde-3-phosphate dehydrogenase regions were analyzed in a selection of 88 strains originating from Europe, North and South America, North Africa, and Central and South Asia. Some (60%) strains produced pseudothecia containing ascospores, independent of their origin. Race identification obtained using the multiplex polymerase chain reaction targeting host-selective toxin (HST) genes was

consistent, overall, with the results based on the inoculation of a set of differential wheat cultivars and confirmed the predominance of race 1/2 strains (≈83%). However, discrepancies in race identification, differences from the reference tester strains, and atypical *ToxA* profiles suggest the presence of new races and HSTs. The *MAT1-1* and *MAT1-2* coding regions are consecutively arranged in a single individual, suggesting putative heterothallic origin of *P. tritici-repentis*. Upstream from the *MAT* is an open reading frame of unknown function (*ORF1*) containing a *MAT*-specific degenerate carboxy-terminus. The phylogenetic analysis of the *MAT* locus reveals two distinct groups, unlinked to geographical origin or *ToxA* profile. Group I, the best-represented group, is associated with typical tan spot lesions caused by races 1, 2, 3, and 5 on wheat. It is more homogenous than group II encompassing race 4 strains, as well as isolates associated primarily with small spot lesions on wheat leaves or other hosts. Group II could contain several distinct taxa.

Additional keywords: *Drechslera tritici-repentis*, Ptr ToxB, *tox*b.

Pyrenophora tritici-repentis (Died.) Drechsler (anamorph, *Drechslera tritici-repentis* (Died.) Shoemaker) is the causal agent of tan spot, a major foliar blight disease in temperate and warmer wheat-growing areas throughout the world (17,29,33,36,37,56). *P. tritici-repentis* nomenclature has undergone changes since its initial description in 1903 by Diedicke (14), having previously been referred to as *Helminthosporium gramineum* f. sp. *tritici-repentis*, *H. tritici-vulgaris*, and *H. tritici-repentis* for the anamorph, and *Pleospora trichostoma* and *Pyrenophora trichostoma* for the teleomorph (31).

P. tritici-repentis is isolated mainly from wheat but it has been reported on other cereals, such as rye and barley, in addition to numerous grasses (i.e., *Agropyron* spp.) (1,24). Symptoms observed on susceptible wheat cultivars are tan necrosis or fairly extensive chlorosis (25). The recent increase in the importance of this disease is due partly to reduced or zero-tillage practices that allow stubble retention on the soil, shorter crop rotations, or continuous wheat cultivation. These practices favor the pathogen overwintering on residues and the formation of the teleomorph, thus completing the full disease cycle (9,19). The extension of wheat cultivation into nontraditional, warmer environments also plays a major role in the increase in tan spot incidence. In these regions, the crop is grown in the cool season and disease control relies primarily on host genetic resistance.

P. tritici-repentis has been reported as a homothallic (self-fertile) loculoascomycete, implying that it does not require a

partner to mate. In nature, a single sexual cycle is completed in the growing season after overwintering on the stubble left in the field, whereas several cycles of asexual reproduction can occur during the same period (11,19). Conversely, large quantities of fertile pseudothecia can be produced in vitro on maize leaf water agar in as little as 4 weeks under specific light and temperature conditions (13,21).

The techniques used to characterize *P. tritici-repentis* isolates have evolved in conjunction with a better understanding of the pathogen and its interaction with its main host, wheat (25–27). Currently, a race designation system based on putative necrosis or chlorosis symptoms induced on a wheat differential set (27) distinguishes at least eight races (4,27,28,43,47,48). The number of races described is linked to the number of wheat cultivars included in the differential set, which could hamper the identification of new races. In practice, phenotypic race classification appears rather subjective and can depend on the observer, as well as being potentially restrictive as shown by Andrie et al. (4). The chlorosis symptom may evolve rapidly to necrosis, leading to confusion. Ideally, therefore, it should be accompanied by molecular confirmation of the presence of host-selective toxins (HSTs) associated with the necrosis or chlorosis symptoms observed. This can be carried out in view of the fact that the necrosis symptoms observed on certain wheat genotypes are induced by the HST Ptr ToxA (synonyms: Ptr necrosis toxin, Ptr toxin, ToxA) (6,51,52,58) and that chlorosis is induced on certain wheat differential genotypes by the production of either Ptr ToxB (synonym: Ptr chlorosis toxin) (10) or Ptr ToxC (18). ToxB and ToxC can be differentiated with selected wheat genotypes (48). Also, *ToxB*-like sequences (*ToxBv*, high sequence identity to *ToxB*) have been identified in race 3 strains (46,47) and *tox*b sequences (lower se-

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doi:10.1094/PHYTO-100-5-0474

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quence identity to *ToxB*) in race 4 strains (34,47), although neither produce Ptr ToxB. Representative strains of races 1 to 5, as well as two field isolates, were characterized by Andrieu et al. (4) using traditional phenotypic and a new genotypic multiplex polymerase chain reaction (PCR) method allowing the presence or absence of *ToxA*, *ToxB*, *ToxB*-like, and *tox*b sequences to be detected. However, there is room for improvement because the multiplex PCR is unable to distinguish race 1 from race 2 and race 3 from race 5. Also, for some strains, phenotypic and genotypic race identification does not always correspond, suggesting the existence of at least two supplementary races and possibly an additional HST Ptr ToxD (4).

Phylogenetic analysis based on combined data sets of internal transcribed spacer (ITS) regions, nuclear gene coding region glyceraldehyde-3-phosphate dehydrogenase (*gpd*), and mating type locus (*MAT*)-2 HMG box sequences of *Pyrenophora* spp. and other members of the family *Pleosporaceae* revealed *P. bromi* as *P. tritici-repentis*'s closest relative (5,8,57). Homologs of *ToxB* have been demonstrated in the genome of *P. bromi* and other members of the Ascomycota, suggesting that this gene originated at an early stage of fungal evolution and has been lost or retained by some lineages (5). In contrast, Friesen et al. (22) discovered a gene in the genome sequence of *Phaeosphaeria nodorum* with 99.7% similarity to *ToxA*, suggesting a lateral gene transfer that occurred just before 1941. This transfer appears to correspond to the sudden increase in tan spot and the reports of more severe symptoms. Intraspecific genetic diversity has been detected in *Pyrenophora tritici-repentis* by random amplified polymorphic DNA analysis (15,39,43) and on a minor scale in ITS sequences (57). However, no correlation has yet been described linking *P. tritici-repentis* genetic diversity to geographic origin or race.

Within the context of collaborative research programs on leaf blights on wheat between Université catholique de Louvain (UCL) and Centro Internacional de Mejoramiento de Maíz y Trigo (CIMMYT) (the Spanish acronym for the International Wheat and Maize Improvement Center), an extensive collection of *P. tritici-repentis* strains from Europe, North and South America, North Africa, and Central and South Asia has been collected over two decades. This collection provides the opportunity to study *P. tritici-repentis* diversity around the world.

The objectives of this study, based on the global collection of strains mentioned above, were to (i) verify the homothallic nature of *P. tritici-repentis* from the various origins by analyzing the teleomorph production capacity in vitro, (ii) analyze the organization and the putative diversity of the *MAT* gene within the pathogen population, (iii) compare the phenotypic and genotypic race identification of a large set of strains for the possible identification of individuals with unexpected toxin production profiles that could be good candidates for the description of new races, and (iv) explore the link between genetic diversity, geographic origin, and pathogenicity.

MATERIALS AND METHODS

***P. tritici-repentis* strains.** An extensive collection of *P. tritici-repentis* strains has been established from naturally infected leaves collected mainly through a series of field surveys conducted with CIMMYT since the late 1980s in wheat-growing areas worldwide. A selection of 82 monoconidial *P. tritici-repentis* strains was used for this study and deposited at the Mycothèque de l'Université catholique de Louvain (MUCL, Louvain-la-Neuve, Belgium), the agro-industrial fungi and yeast collection of the Belgian coordinated collections of microorganisms, Brussels. The collection was completed for comparison with the following *P. tritici-repentis* representative strains for races 1 to 5: Pt2 for race 1 (20), 86-124 for race 2 (4,15,20,26,27), 331-9 for race 3 (15,20), 90-2 for race 4 (27,43), and DW7 for race 5 (2,4). Strain

MUCL 18687 (= Centraalbureau voor Schimmelcultures [CBS] 259.59) (Utrecht, The Netherlands), previously identified as a syntype of *H. tritici-vulgaris* (Nisikado) (later reidentified as *P. tritici-repentis*), was also included in the study performed in 2007. Conidia production of *P. tritici-repentis* colonies on V8PDA (15% [vol/vol] V8 juice, 0.3% [wt/vol] CaCO₃, 1% [wt/vol] potato dextrose agar [Oxoid, Cambridge, UK], and 1% [wt/vol] agar) was induced using the conditions described by Lamari and Bernier (25,26). The confirmation that the strains belonged to *P. tritici-repentis* was based on the typical "snake-head" form of terminal conidial cells (16) and, in dubious cases, on ITS analysis. The race phenotype of 75 strains, including the reference strains, had been previously characterized at Unité de Phytopathologie (15,33) by inoculation on a set of four hexaploid wheat lines or cultivars (Glenlea, 6B-365, 6B-662, and Salamouni) with known reaction type to the eight races of *P. tritici-repentis* (28). Seedlings were produced on a 1:1 compost:peat substrate in an air-conditioned greenhouse (20 to 22 and 16 to 17°C day and night temperatures, respectively, with a 16-h photoperiod). At the three-leaf stage, they were inoculated with 2,000 conidia/ml until run-off. Inoculated seedlings were moved for 24 h to a mist chamber at 21°C with a 16-h photoperiod, before returning to the greenhouse or a growth room with similar conditions. They were rated for reaction type 7 days after inoculation using the scale described by Lamari and Bernier (25). Race identification was inferred from these ratings (28) (Table 1).

Teleomorph production. The strains were tested for their capacity to produce pseudothecia containing viable ascospores, using a modified version of the Friesen et al. (21) method. Autoclaved green maize leaf sections, ≈4 cm long, were placed on 2% water agar plates. Instead of inoculating the leaves with a calibrated conidial suspension, 4-mm plugs taken from an active colony margin of a young (≈5 days old) culture grown on V8PDA were used. Each plate was inoculated with three equidistant (≈1.5 cm apart) plugs of the same strain along the length of the leaf, mycelium side down. Parafilm-sealed plates were incubated for 3 days at 25°C in the dark before being placed under cool white fluorescent tubes (≈3,200 Lux, Sylvania Standard F58W/133-T8; Osram-Sylvania, München, Germany) at 15 ± 1.5°C and a 12-h photoperiod. Triplicates per strain were conducted on at least two consecutive occasions. If no fertile pseudothecia were produced, a third trial was carried out.

DNA extraction and amplification of ITS and *gpd* regions. DNA was extracted according to the supplier's recommendations using the FastDNA Kit (Qbiogene, Carlsbad, CA) and quantified with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). The ITS regions were PCR amplified using universal primers NS7 and ITS4 (54). The region coding for the nuclear gene *gpd* was amplified using primer pair *gpd1/gpd2* (8). The ITS PCR amplifications were performed with 50 ng of DNA template in a total volume of 50 µl containing 2.5 mM MgCl₂ (Fermentas GmbH, St. Leon-Rot, Germany), 300 µM each dNTP, 400 nM each primer, *Taq* DNA polymerase buffer, and 0.25 U of *Taq* DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA). The *gpd* PCR reaction mixtures (total volume of 50 µl) contained 20 ng of DNA in a 1× PCR buffer, 2.5 mM MgCl₂, 0.25 mM concentration of each dNTP, 100 nM concentrations of each primer, and 0.5 U of *Taq* DNA polymerase. All PCRs were performed in an MJ Mini Gradient Thermal Cycler (PTC-1148; Bio-Rad, CA) with the following ITS cycling conditions: initial denaturation at 94°C for 3 min followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min. A final extension was performed at 72°C for 7 min. The *gpd* cycling conditions were 95°C for 3 min; followed by 30 cycles of 94°C for 1 min, 60°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 10 min. The PCR products were purified with a MSB Spin PCRapace Kit 250 (Invitrogen GmbH, Berlin) following the suppliers' recommendations.

Amplification of MAT region. Nine *MAT* idiomorph primers spanning the entire hypothetical idiomorph were designed, based on the alignment of the *P. tritici-repentis* Pt-1C-BFP strain genome sequence obtained from the *P. tritici-repentis* sequencing project (Broad Institute of Harvard and MIT, <http://www.broad.mit.edu>) and available *MAT1-1* (AY950585) and *MAT1-2* (AY950586) sequences of *P. teres* strains. The PCR strategy used, and the primer locations and organization of coding regions within the *MAT* region, are shown in Figure 1. Primer sequences are given in Table 2. The PCR and amplification conditions used were identical to those used for the *gpd* gene.

Sequence diversity analysis. Sequencing reactions were performed using a CEQ DTCS Quick start Kit (Beckman Coulter Inc., Fullerton, CA), and nucleotide sequence chromatograms were obtained with a CEQ 2000 XL capillary automated sequencer (Beckman Coulter Inc.). Raw data were analyzed and edited using the sequence analysis software program Sequencher

v.4.6 (Gene Code Corporation, Ann Arbor, MI), before being aligned automatically with ClustalW 1.82 and manually corrected using BioEdit Sequence Alignment Editor v.5.0.9 (North Carolina State University). Searches were done against the GenBank/EMBL databases using BLASTN 2.2.9 (3).

The accession numbers for the *gpd*, *ITS*, and *MAT* sequences deposited in the EMBL database were AM884257 to AM884276, AM887493 to AM887511, and AM884582 to AM884620, respectively.

The phylogenetic relationships between *P. tritici-repentis* strains were inferred from the maximum parsimony analysis of *MAT* sequences using the PAUP* version 4.0b10 phylogenetics package (Swofford). Heuristic searches (1,000 replicates) were performed using random sequence addition by means of a tree-bisection-reconnection branch-swapping algorithm, with gaps considered to be a fifth character state. Confidence in the branching points was established by performing 1,000 bootstrap repli-

TABLE 1. *Pyrenophora tritici-repentis* strains analyzed in this investigation and synopsis of results^a

Strain ^b	Location	Host	Year	Sporulation			PCR ^c				Race	
				Ana. ^d	Tele. ^e	<i>MAT</i> ^f	<i>ToxA</i>	<i>ToxB</i>	<i>ToxBv</i>	<i>toxb</i>	PCR	Phenotype
Reference												
<u><i>Pti2</i>^{o*} 49351</u>	South Dakota	Wheat	1973	+	+	Ia	+	0	0	0	1/2	1
<u><i>86-124</i>^{o*} 49347</u>	Canada	Wheat	1980s	+	+	Ia	+	0	0	0	1/2	2
<u><i>331-9</i>^{o*} 49348</u>	Canada	Durum wheat	1980s	+	0	If	0	+	+	0	3/5	3
<u><i>90-2</i>^{o*} 49349</u>	Canada	Wheat	1990s	0	0	IId	0	+	(+)	+	4	4
<u><i>DW7</i>^{o*} 49350</u>	North Dakota	Durum wheat	1990s	+	+	Ig	0	+	+	0	3/5	5
Syntype												
<u><i>CBS259.59</i> 18687^{o*}</u>	Japan	Wheat	<1959	0	0	Q	(+)	0	0	0	D	–
Survey												
<u>42316^{o*}</u>	Hungary	<i>Agropyron repens</i>	1992	0	0	IIb	(+)	+	(+)	+	D	1–4
<u>42328^{o*}</u>	Mexico	Bread wheat	1993	±	0	Ie	+	0	0	0	1/2	2
<u>42329^{o*}</u>	Mexico	Bread wheat	1993	+	0	Ib	+	0	0	0	1/2	2
42330	Mexico	Bread wheat	1993	±	0	–	+	0	0	0	1/2	1
42335	Nepal	Bread wheat	1993	±	0	–	+	0	0	0	1/2	–
<u>42337</u>	India	Bread wheat	1993	±	0	Ia	+	0	0	0	1/2	2
42383	Morocco	Durum wheat	1995	+	+	–	+	0	0	0	1/2	1
<u>42384^{o*}</u>	Morocco	Bread wheat	1995	0	+	IIa	+	0	0	0	1/2	–
<u>42385</u>	Morocco	Bread wheat	1995	±	+	Ia	+	0	0	0	1/2	1
<u>42386^{o*}</u>	Morocco	Bread wheat	1995	±	+	IIa	+	0	0	0	1/2	–
<u>42387^{o*}</u>	Morocco	Barley	1995	0	0	IIa	0	0	0	0	D	4
42410	Argentina	Bread wheat	1994	±	+	–	+	0	0	0	1/2	1
42413	Argentina	Bread wheat	1994	+	0	–	+	0	0	0	1/2	1
42414	Argentina	Bread wheat	1994	+	+	–	+	0	0	0	1/2	–
<u>42416</u>	Argentina	Bread wheat	1994	±	0	Ia	+	0	0	0	1/2	1
42417	Argentina	Bread wheat	1994	+	0	–	+	0	0	0	1/2	–
<u>42418</u>	Mexico	Bread wheat	1994	±	+	Ie	+	0	0	0	1/2	1
42435	Bangladesh	Bread wheat	1994	+	0	–	+	0	0	0	1/2	2
<u>42436</u>	Bangladesh	Bread wheat	1994	±	0	Ia	+	0	0	0	1/2	2
42499	Argentina	Bread wheat	1995	+	+	–	+	0	0	0	1/2	1
<u>42500</u>	Argentina	Bread wheat	1995	+	+	Ic	+	0	0	0	1/2	2
42502	Argentina	Bread wheat	1995	±	0	–	+	0	0	0	1/2	–
42503	Argentina	Bread wheat	1995	+	+	–	+	0	0	0	1/2	–
42521	Belgium	Bread wheat	1996	±	0	–	0	0	0	0	D	1
<u>42522^{o*}</u>	Belgium	Bread wheat	1996	0	(+)	Ia	(+)	0	0	0	1/2	3–4
<u>42523^{o*}</u>	Mexico	Bread wheat	1996	0	0	Ia	0	0	0	0	D	–
42524	Mexico	Wheat	1996	±	0	–	+	0	0	0	1/2	–
42600	Argentina	Wheat	1990s	+	0	–	+	0	0	0	1/2	–
44678	Kazakhstan	Bread wheat	2003	+	+	–	+	0	0	0	1/2	1
44692	Kyrgyzstan	Bread wheat	2003	±	(+)	–	+	0	0	0	1/2	1

(continued on next page)

^a D indicates results different from reference strains and — indicates information not available.

^b Reference = race reference strains and Survey = Strains from own surveys. Reference strain number in italics, Mycotoxine de l'Université catholique de Louvain accession number in normal figures. Strains for which the glyceraldehyde-3-phosphate dehydrogenase (^c), internal transcribed spacer (^{*}), and mating type region (underlined accession number) were sequenced in this study and submitted to the EMBL database are indicated.

^c Multiplex polymerase chain reaction (PCR) (4) with primers TA51F/TA52R (*ToxA* in races 1 and 2), TB71F/TB60R (*ToxBv* for *ToxB*-like in races 3 and 5), and TB71F/TB58R (*toxb* in race 4); + = fragment of expected size, (+) = weak amplification of fragment of expected size, 0 = no amplicon observed, and * = fragment of unexpected size.

^d Anamorph production of strains was identified as: + conidia production average to very abundant, ± below average, 0 no conidia or conidiophores observed.

^e Teleomorph production on maize water agar was identified as: + pseudothecia producing ascospores, (+) pseudothecial initials, 0 no structures observed.

^f Mating type locus (*MAT*) haplotypes are groups of strains with identical mating-type sequences. The roman numerals indicate the phylogenetic group and lowercase letters indicate subgroups. Underlined entries indicate a unique haplotype for that strain and O indicates that all expected fragments were not amplified.

cates using maximum parsimony as the criterion and random sequence addition. Phylogenetic analysis was carried out in this way by using subsequently, as outgroups, available *P. teres* sequences and the *P. tritici-repentis* sequence MUCL 45128 (grouping with no other *P. tritici-repentis* strain). Given that *P. teres* is a heterothallic fungus, *MAT1-1* (AY950585) and *MAT1-2* (AY950586) sequences were combined to reflect the *MAT* structure of *P. tritici-repentis*.

Identification of races by PCR. In order to complement the visual assessment of disease phenotype for race designation, race genotype identification was carried out on *P. tritici-repentis* strains using the multiplex PCR designed by Andrieu et al. (4), targeting specific portions of the *ToxA* (primers TA51F/TA52R) and *ToxB/ToxB-like/toxB* (TB71F/TB6R, TB60R, or TB58R) open reading frames (ORFs). In theory, *ToxA* primers amplify an ~600-bp fragment only in race 1 and 2 strains, whereas races 3, 4, and 5 yield an ~250-bp amplicon specific to *ToxB* using primer

pair TB71F/TB6R. Reverse primer TB60R paired with TB71F is specific to *ToxB*-like sequences that are characteristic of races 3 and 5, whereas reverse primer TB58R is specific to *toxB* in race 4.

RESULTS

Morphological diversity and teleomorph development. All the sporulating strains presented the characteristic *P. tritici-repentis* conidial morphology despite the variations observed in the length of the conidiophores. Some of the strains had lost the capacity to produce conidia. Of the 88 strains studied, 53 (~60%) produced pseudothecia containing fertile ascospores and a further 12 (~14%) produced only pseudothecial initials (Table 1). There was no clear link between the capacity of production of pseudothecia or pseudothecia initials and the origin of the strains. Nevertheless, only 43% of the strains isolated before 1995 were still able to produce pseudothecia or pseudothecia initials when

TABLE 1. (continued from preceding page)

Strain ^b	Location	Host	Year	Sporulation		<i>MAT</i> ^f	PCR ^c				Race	
				Ana. ^d	Tele. ^e		<i>ToxA</i>	<i>ToxB</i>	<i>ToxBv</i>	<i>toxB</i>	PCR	Phenotype
44693	Kyrgyzstan	Bread wheat	2003	+	+	Ia	+	0	0	0	1/2	1
44710	Kyrgyzstan	Bread wheat	2003	±	+	—	+	0	0	0	1/2	1
45018	Kazakhstan	Bread wheat	2003	+	+	Ia	0	0	0	0	D	1
45038	Nepal	Bread wheat	2003	+	+	Ia	+	0	0	0	1/2	1–8
45040	India	Bread wheat	2003	+	+	—	+	0	0	0	1/2	2
45041	India	Bread wheat	2003	±	+	—	+	0	0	0	1/2	1–2
45125	Russia	Bread wheat	2003	+	0	Ia	+	0	0	0	D	—
45128	Russia	Bread wheat	2003	+	(+)	Ile	0	0	0	0	D	—
45176	Kazakhstan	Bread wheat	2003	+	+	Ia	0	0	0	0	D	3
45179	Russia	Bread wheat	2003	+	+	—	+	0	0	0	D	1
45269	Kazakhstan	Bread wheat	2003	+	+	—	+	0	0	0	D	1
45758	Tajikistan	Bread wheat	2004	+	+	Ia	+	0	0	0	1/2	2
45761	Tajikistan	Durum wheat	2004	+	+	—	+	0	0	0	1/2	1
45766	Tajikistan	Bread wheat	2004	+	+	Ia	0	0	0	0	D	3
45770	Tajikistan	Bread wheat	2004	+	+	—	+	0	0	0	1/2	1
45774 ^{o*}	Uzbekistan	Bread wheat	2004	+	+	Ia	+	0	0	0	1/2	2
45776	Uzbekistan	Bread wheat	2004	+	+	—	+	0	0	0	1/2	1
45777	Uzbekistan	Bread wheat	2004	+	+	—	+	0	0	0	1/2	1
45780 ^{o*}	Kazakhstan	Wheat	2004	±	(+)	Ile	0	+	(+)	+	4	4
45792	Kazakhstan	Wheat	2004	+	+	—	+	0	0	0	1/2	2
45953 ^{o*}	Belgium	Bread wheat	1982	±	0	Ia	+	0	0	0	1/2	1–4
45954	Belgium	Bread wheat	1985	+	+	—	+	0	0	0	1/2	1
45959	Kazakhstan	Wheat	2004	+	+	Ia	0	0	0	0	D	1–3
46242	Argentina	Bread wheat	2001	+	+	—	+	0	0	0	1/2	1
46243 ^{o*}	Argentina	Bread wheat	2001	+	+	Ie	+	0	0	0	1/2	1
46244	Luxembourg	Bread wheat	2000	+	(+)	—	+	0	0	0	1/2	1
46245	Luxembourg	Bread wheat	2000	+	(+)	Ia	0	0	0	0	D	3
46246	Nepal	Bread wheat	2001	±	(+)	—	+	0	0	0	1/2	2
46247 ^{o*}	Nepal	Bread wheat	2001	+	+	Ia	+	0	0	0	1/2	2
46248	Nepal	Bread wheat	2001	0	+	—	+	0	0	0	1/2	2
46249	Nepal	Bread wheat	2001	+	+	—	+	0	0	0	1/2	2
46250	Nepal	Bread wheat	2002	+	+	—	+	0	0	0	1/2	2
46251	Nepal	Bread wheat	2002	±	+	—	+	0	0	0	1/2	1
46825	Iran	Bread wheat	2005	±	(+)	Ia	+	0	0	0	1/2	1
46826	Armenia	Bread wheat	2005	±	+	—	+	0	0	0	1/2	2
46827	Turkey	Bread wheat	2005	0	(+)	Ia	+	0	0	0	1/2	1
46828	Turkey	Bread wheat	2005	0	(+)	—	+	0	0	0	1/2	1
46832	Georgia	Bread wheat	2005	+	+	—	+	0	0	0	1/2	1
46835	Georgia	Bread wheat	2005	+	+	Ia	+	0	0	0	1/2	1–8
46836	Georgia	Bread wheat	2005	+	+	—	+	0	0	0	1/2	1
46842	Azerbaijan	Wheat	2005	±	+	—	+	0	0	0	1/2	1
46843 ^{o*}	Azerbaijan	<i>Triticum manati</i>	2005	±	+	Id	+	0	0	0	1/2	1
46845	Azerbaijan	<i>T. turgilum</i>	2005	+	+	—	+	0	0	0	1/2	1
46860	Armenia	Bread wheat	2005	+	+	Ie	+	0	0	0	1/2	2
46861	Uzbekistan	Bread wheat	2005	+	(+)	—	+	0	0	0	1/2	1
47953	Mexico	Bread wheat	1994	+	+	—	+	0	0	0	1/2	1
47954	Mexico	Bread wheat	1994	+	+	—	+	0	0	0	1/2	1
47955	Mexico	Bread wheat	1993	+	+	—	+	0	0	0	1/2	1
47957	Mexico	Bread wheat	1993	+	+	—	+	0	0	0	1/2	1
47958	Mexico	Bread wheat	1993	+	(+)	—	+	0	0	0	1/2	1
47959	Mexico	Bread wheat	1993	+	+	—	+	0	0	0	1/2	1
47961	Mexico	Bread wheat	1993	±	0	—	+	0	0	0	1/2	1–4

tested in 2007, suggesting a loss of capacity of teleomorph production during storage. The morphology of the teleomorph was extremely variable in terms of the abundance, thickness and length of the setae produced, as well as the length of pseudo-theical necks.

Race identification. Validation of the Andrie et al. (4) multiplex PCR was initially confirmed with our reference strains for races 1 to 5, yielding identical race profiles to those described, except for strain 90-2. This race 4 reference strain from Canada (27,43) yielded a weak amplicon (≈250 bp) using primer pair TB71F/TB60R, whereas the race 4 strain SD20 used by Andrie et al. (4) but not available to us did not. Nevertheless, race 4 strain MUCL 45780 from Kazakhstan behaved similarly to strain 90-2. The *P. tritici-repentis* syntype strain MUCL 18687 yielded only a weak amplicon using primer pair TA51F/TA52R for *ToxA* detection. For the remaining 82 strains, the genotypic race profiles were compared with the profiles obtained for the reference race strains used in this study as well as with the race phenotype data available. Loss of conidia production capacity and pathogenicity affected race phenotyping of some strains (Table 1). Of the 82 strains, 68 showed a normal amplicon with the *ToxA*

primers. Race phenotype data were available for 59 of these strains and confirmed the concordance with race 1 (≈61%) or race 2 (≈29%). For ≈10% of the *ToxA*-positive strains, race phenotyping was uncertain because of intermediate or small lesion types on various differentials. The strains MUCL 45125, MUCL 45179, and MUCL 45269, originating from an area encompassing Western Siberia and Northern Kazakhstan, produced larger fragments than expected (>2,000 bp) when *ToxA* primers were used. MUCL 45179 and MUCL 45269 appeared as race 1 phenotype. For nine strains (MUCL 42387, MUCL 42521, MUCL 42523, MUCL 45018, MUCL 45128, MUCL 45176, MUCL 45766, MUCL 45959, and MUCL 46245), neither *ToxA* nor *ToxB* primer pairs were able to amplify the expected fragments, despite confirmation of the presence of the DNA template with *MAT* primers used as internal PCR control. The race phenotype of these strains appeared to be heterogeneous or uncertain, with strains MUCL 42521 showing a race 1 phenotype and MUCL 45176, MUCL 45766, and MUCL 46245 having a race 3 phenotype. Finally, MUCL 42316 isolated from *Agropyron repens* showed normal amplicons with the *ToxB* and *toxb* primers and weak amplicons with the *ToxA* and *ToxB*-like primers but, on Glenlea

Fig. 1. Organization and distribution of polymorphism types in the *Pyrenophora tritici-repentis* mating type locus (*MAT*), drawn to proportion and showing introns (black boxes); coding regions open reading frame (*ORF1*), *MAT1-1*, and *MAT1-2* (white boxes); intergenic regions (gray boxes); and flanking DNA (thin, boxed motif). Long arrows within coding regions indicate the transcription direction for each ORF and PCR primer locations are also represented (short black arrows). All the listed primers were designed in this study apart from PtMat_fw (41). A variable region (dark gray box, shown in Figure 2) observed between the *MAT1-1* and *MAT1-2* ORFs is indicated. Triangles indicate the location of synonymous mutations (white), nonsynonymous mutations (black), and indels (gray). Mutations and indels are indicated in bold, as well as their exact location (in base pairs) with regard to the consensus sequence Mycothèque de l'Université catholique de Louvain 42384 (*MAT* haplotype IIa). Associated *MAT* haplotypes are indicated for each haplotype by the accession number of one representative (shown on the right).

TABLE 2. List of primers used to amplify the mating type locus region in *Pyrenophora tritici-repentis* strains

Primer	Sequence 5'–3'	Origin
PtMat_fw	CAG GAC GTT CGC CAT CGC CAT	41
PtrPLP1	CAC TGC CCA CTC ACC TTG TGC	This study
PtrPLP2	CAG AAC AAA GGC AGG ACT GTG AGC	This study
PtrPLP3	CAG ACA AGG CGC TCT CGG TTG AAG	This study
PtrPLP4	ATG CGC TCA GCA AGG AAG GTC G	This study
PtrPLP5	AAC TCG GAA CGC TGA GCC TAA TGG	This study
PtrPLP6	TTG ACG CCA CAG ACG GAA CAG G	This study
PtrPLP7	GCT TTA CTA CAA CTT TCC TCT ACC	This study
PtrPLP8	CTG GAA GTG CTG GAC GAG AAG ATC	This study
PtrPLP10	GTA CGG GCC AGC ATG ACG TGC	This study

and 6B365, produced small, dark-brown to black spots sometimes surrounded with tan necrotic and chlorotic zones. None of the tested strains from the CIMMYT/UCL surveys showed the genotype profile of the race 3 and race 5 reference strains.

Amplification of *MAT* idiomorph. The *MAT* idiomorph was successfully amplified in 82 *P. tritici-repentis* strains as well as in the reference strains for races 1 to 5 and syntype strain MUCL 18687 (Table 1) via five overlapping PCRs. Four observations are worth mentioning.

First, regarding the organization of the *MAT* gene, the *MAT1-1* gene is followed by the *MAT1-2* gene, with both ORFs transcribed from left to right (Fig. 1). This gene is preceded (5' flanking region) by a conserved ORF previously described for *Phaeosphaeria nodorum* (7) and *Pyrenophora teres* (40) and

termed *ORF1*. As for *P. teres*, *P. graminea*, and *Phaeosphaeria nodorum*, the *Pyrenophora tritici-repentis* *ORF1* sequence is conserved only in its 5' region (beginning of the sequence). It is this change from the conservation to nonconservation of its sequence in the C-terminus (3' region) that marks the beginning of the *MAT* idiomorph.

Second, strains 90-2, MUCL 42316, MUCL 42384, MUCL 42386, MUCL 42387, and MUCL 45780 yielded fragments slightly larger than expected when primer pairs PtrPLP3/PtrPLP6 and PtrPLP5/PtrPLP8 were used. This corresponds to an ≈90-bp insertion in these strains located between primers PtrPLP5 and PtrPLP6 in a variable region. Careful analysis of sequences in this region (Fig. 2) showed that the *MAT1-1* ORF stop codon (TAA) is followed by a 13-bp sequence similar to the incomplete 3' termi-

Fig. 2. Alignment of the variable region (described in Figure 1) situated between the mating type locus (*MAT*)*1-1* and *MAT1-2* open reading frames (ORFs) for the 12 distinct *Pyrenophora tritici-repentis* *MAT* haplotypes identified, as well as *P. teres* *MAT1-2* sequence AY950586 (Pt0-1) and *P. graminea* *MAT1-2* sequence DQ823080 (CBS 100866). Stop codons are indicated by a short horizontal line, the transition from the *MAT1-1* to *MAT1-2* sequence is indicated by an asterisk, and the repetitive sequences are indicated by vertical dotted lines.

nus of the *P. teres/P. graminea MAT1-1* idiomorph sequence (positions 7 to 19). Adjacent to this sequence is the *MAT1-2* degenerate C-terminus of *ORF1* (beginning at the asterisks and ending with the TGA stop codon), followed by a sequence similar to the 5' *MAT1-2* idiomorph sequence in *P. teres/P. graminea*. It is within the latter region that repetitive sequences containing indels are observed. Similarly, a 9-bp sequence (GGGTTTCGA) situated further on in the *MAT1-2* coding region (Fig. 1) is repeated three times in all strains sequenced and a fourth time only in strains MUCL 42316, MUCL 42384, MUCL 42386, and MUCL 42387.

Third, *P. tritici-repentis* strains 86-124, MUCL 42328, MUCL 42329, MUCL 46243, and MUCL 46843 produced a nonspecific profile with primer pair PtMat_fw (41) and PtrPLP2 under standard PCR conditions. The expected unique amplicon was observed for these strains when the annealing temperature was increased from 60 to 69°C.

Finally, primer pairs targeting the *MAT1-2* region (PtrPLP3/PtrPLP6, PtrPLP5/PtrPLP8, and PtrPLP7/PtrPLP10) failed to amplify the expected fragments in syntype strain MUCL 18687.

Molecular diversity. In an exploratory genetic diversity study entailing amplification and sequencing of ITS, *gpd*, and *MAT* genes, 20 strains were selected on the basis of their diverse geographical and host origin as well as of their genotype and phenotype race profiles (Table 1). The results indicate that all the *gpd* sequences are identical to each other and to the *P. tritici-repentis* reference sequence AY004838 (DAOM 107224). All but two ITS sequences are identical, with sequences for strains 86-124 and 90-2 differing by a single base pair from one another and from the remaining strains. However, 51 single-nucleotide polymorphisms (of which 8 are nonsynonymous) and two indels were observed in the 12 *MAT* sequences haplotypes identified (Fig. 1). In a next step, the *MAT* gene was sequenced from an additional 20 strains. An exhaustive search resulted in three equally parsimonious trees resolving, independent of geographical origin, two major *P. tritici-repentis* sequence types (Fig. 3). The first group contains most of the strains (32 of 40) represented by seven distinct haplotypes with sequences differing, at most, by 3 bp. This group, referred to hereafter as group I, contains strains from all races apart from race 4. The second group (group II) includes seven strains representing five distinct haplotypes. Confirmed *P. tritici-repentis* race 4 strains such as 90-2 are included in this group but other races are also represented. BLAST results indicate that the most similar *MAT* sequences in databases are those for *P. teres/P. graminea*, with 86 and 92% identity for *MAT1-1* and *MAT1-2* sequences, respectively. The *gpd* and ITS sequences obtained for syntype strain MUCL 18687 are very distinct from the reference *P. tritici-repentis* sequences, with BLAST results inducing maximum similarity (100%) with the *D. catenaria* (Drechsler) Ito strain CBS 191.29.

DISCUSSION

P. tritici-repentis is known to have a very complex interaction with its host. To date, eight distinct races that differentially express three HSTs have been described (27,28,44,47,48). The existence of a fourth HST and additional races has also been suggested (4). Data from conventional phenotype race identification using a wheat differential set were confronted in this study with the results of the multiplex PCR designed by Andrieu et al. (4) that enables genotypic race identification. The multiplex PCR race identification method was validated on 88 strains and, overall, supported the results obtained using the conventional method. However, discrepancies between both methods and possible new toxin gene profiles were observed, suggesting that phenotypic race identification is rather subjective. Indeed, a continuum of symptom severity and type is reduced to a conclusion on the ability or not to induce extensive necrosis or chlorosis. In addition, the size of the differential set used limits the number of

possible races identified. Second, genotypic race identification still has room for improvement because new *Tox* genes are being discovered or suspected (44), *ToxC* primers await designing, and atypical profiles can be observed. Our results underline atypical *ToxA* profiles (amplification of a larger fragment) for strains MUCL 45125, MUCL 45179, and MUCL 45269 originating from Russia and Kazakhstan, whereas phenotype reactions clearly linked MUCL 45179 and MUCL 45269 to *P. tritici-repentis* race 1 (29,33). Interestingly, a similar host-specific toxin, *ToxA*, is produced by both wheat pathogens *P. tritici-repentis* (*PtrToxA*) and *Phaeosphaeria nodorum* (*SnToxA*), and it is thought that this gene was acquired by *Pyrenophora tritici-repentis* from *Phaeosphaeria nodorum* in a recent horizontal gene transfer (49). Mixed infections by *Pyrenophora tritici-repentis* and *Phaeosphaeria nodorum* occur frequently on wheat leaves in Western Siberia and Northern Kazakhstan (33). No further strains containing the *ToxB* and *ToxBv* of the race 3 and race 5 strains were detected among the worldwide collection, suggesting a rather restricted distribution of these races. Strains MUCL 42387 (Morocco), MUCL 42521 (Belgium), MUCL 42523 (Mexico), MUCL 45018 (Kazakhstan), MUCL 45128 (Russia), MUCL 45176 (Kazakhstan), MUCL 45766 (Tajikistan), MUCL 45959 (Kazakhstan), and MUCL 46245 (Luxembourg) do not possess the genes coding for the HSTs associated with the symptoms observed on the wheat differential set, highlighting the probability of new races and HSTs. The results support previous reports of a worldwide predominance of races 1 and 2 and that phenotypic race identification should ideally be accompanied by a genotypic confirmation so as to maximize the chances of revealing the existence of new races and improving our understanding of the host-pathogen interaction (4,11).

Pyrenophora tritici-repentis is clearly a homothallic loculo-ascomycete with a simple *MAT* locus organization containing both idiomorphs tandemly arranged in a single individual (Fig. 1). Upstream from the *MAT* locus is a region termed *ORF1* coding for an unknown function. The C-terminus region of this ORF is *MAT* degenerate in heterothallic species such as *P. teres* (40) and *Phaeosphaeria nodorum* (7), meaning that *MAT1-1* and *MAT1-2* strains each have a specific *ORF1* 3' end for this sequence that marks the beginning of the respective *MAT* idiomorph. In the case of homothallic *Pyrenophora tritici-repentis*, the *ORF1* initiating the *MAT* locus encloses the *MAT1-1* degenerate pattern. The *MAT1-2* degenerate *ORF1* pattern is situated after the *MAT1-1* coding region, and lacks its conserved 5' sequence. Yun et al. (55) suggested that the self-fertile reproductive lifestyles (homothallism) of some *Cochliobolus* spp. have evolved from self-sterile (heterothallic) ancestors via an unequal crossing-over event between islands of identity. Similarly, Inderbitzin et al. (23) reported a lateral transfer of the *MAT1-1* and *MAT1-2* gene fusion across lineages in the evolution of *Stemphylium* spp. but the mechanism and direction of the transfer is unknown. In our study, it is likely that *P. tritici-repentis* originated from a *MAT* recombination of heterothallic ancestors via the islands of homology located in the 5' *ORF1* sequence. However, we were unable to pinpoint the crossing-over site clearly. Similarly, the results suggest that subsequent recombination events in a handful of *P. tritici-repentis* strains might have occurred in this region where repetitive sequences are observed (Fig. 3). A repetitive 9-bp sequence within the *MAT1-2* coding region could also originate from a crossing-over.

The complete sequence for the *MAT* locus, including the 5' flanking region that contains *ORF1*, was sequenced from 40 *P. tritici-repentis* strains worldwide. Two distinct groups of strains were identified. The seven haplotypes in group I include most of the strains originating from around the world, differing at most by 3 bp ($\approx 0.06\%$) in their sequence. Group II is more heterogeneous, with five distinct haplotypes represented within the seven strains sequenced and sequences diverging $\leq 0.6\%$ (without counting

indels) between MUCL 45128 and MUCL 45780. The only clear-cut criterion distinguishing the two groups is that race 4 strains are found only in group II; however, the results suggest that races 1 or 2 are also present in this group.

The exact status of group II strains deserves more attention. Strains such as MUCL 42316 have been isolated from alternative hosts (*A. repens*) and MUCL 42387 from barley, whereas strains MUCL 45128 and MUCL 45780 were isolated from small necrotic spots on wheat leaves. Does group II reflect *P. tritici-repentis*'s pool of diversity on grasses with overall low pathogenicity on wheat, but from where some strains with toxin profiles matching the susceptible genes in widely grown wheat genotypes (44) emerged and diversified into group I? To answer this question, it is necessary to increase the number of strains studied in this group. It should be kept in mind that the "*tritici-repentis*" species name originated from the description in 1903 by

Diedicke (14) of a fungus collected in Germany on *Triticum repens*, an ancient name for *A. repens* (L.) Beauv. (31). Furthermore, it is interesting to note that *ToxA* was detected in some group II strains (e.g., MUCL 42384 and MUCL 42386 from wheat [Morocco] and MUCL 42316 from *A. repens* [Hungary]). This also raises the question of the moment and of the frequency of a putative horizontal gene transfer of *ToxA* from *Phaeosphaeria nodorum* to *Pyrenophora tritici-repentis* (49).

In view of a possible new distinction between the strains identified as *P. tritici-repentis*, one can already question the intra- and intergroup taxonomic status. It has previously been reported that infraspecific variation is low using *MAT* sequences (7,53). However, the importance of this gene has been validated for species-level phylogenies (7,30,38,40,53) and is reported to be more pertinent than other commonly used genes. Indeed, the ITS and *gpd* sequence analysis carried out in this study revealed

Fig. 3. One of the three most parsimonious trees generated with PAUP* v.4.0 b10 and *Pyrenophora tritici-repentis* strain Mycothèque de l'Université catholique de Louvain (MUCL) 45128 as an outgroup using the entire mating type locus (*MAT*) sequences and a portion of open reading frame (*ORF*)1 for *P. tritici-repentis* representatives from diverse geographical origins. Accession numbers, geographical origin, putative race, and telomorph production, as well as associated haplotypes, are indicated next to terminal clades. Bootstrap values based on 1,000 replications are indicated in percentages at internodes when replication frequencies exceed 50%. Telomorph production was identified as + = pseudothecia producing ascospores, (+) = pseudothecial initials, and 0 = no sexual structures observed. Strain MUCL 45128 was identified as basal to group II when *P. teres* was used as an outgroup.

almost no diversity at all between these two groups. The ITS and *gpd* data suggest that another species, *P. bromi*, for which the *MAT* region has not yet been sequenced, unfortunately, is one of *P. tritici-repentis*'s closest relatives. For the moment, heterothallic *P. teres* and closely related *P. graminea* are *P. tritici-repentis*'s closest phylogenetically related species for which the *MAT* gene has been completely sequenced. Two distinct formae speciales associated with distinct symptomologies (net form and spot form) have been described for *P. teres* (45). The taxonomic status of these formae speciales was recently proposed for consideration as a distinct species by Rau et al. (40), based on the divergence between the *P. teres* formae speciales *MAT* sequences being of similar magnitude to those between *P. teres* and *P. graminea* *MAT* sequences. In order to compare the *P. teres* case with the *P. tritici-repentis* one, it is necessary to quantify the magnitude of the divergence of the *P. teres*/*P. graminea* sequences. *P. teres* (net form) differs by 5 bp (*MAT1-1*) and 4 bp (*MAT1-2*) from *P. graminea*. It should be noted that the *P. teres* (spot form) is less divergent. In the case of *P. tritici-repentis*, this level of sequence diversity is easily reached between the two groups identified as well as within haplotypes belonging to group II, suggesting that there might also be several distinct taxa.

When morphological, geographical, sporulation, and teleomorph production data are combined with the *MAT* sequence analysis, the results suggest that there is no direct link between any of the traits. The *MAT* tree does not reflect the variability of the anamorph and teleomorph morphology, the capacity to produce the teleomorph, or the geographical origin of strains. In addition, for the same *MAT* haplotype (strains with identical sequences), teleomorph production is variable. This suggests that independent factors apart from the *MAT* gene intervene in the sexual cycle. Similarly, with the possible exception of race 4, the assignation of a given race is not linked to a *MAT* haplotype. In addition, the previously described *P. tritici-repentis* syntype strain MUCL 18687 is clearly not *P. tritici-repentis*. The heterothallic nature of this strain (*MAT1-1*), coupled with the ITS and *gpd* sequence analysis, suggest that it could belong to *D. catenaria* (Drechsler) Ito. Subcultures of this strain in other culture collections should be checked to clarify its status and redefine a *P. tritici-repentis* syntype.

It is very important to assess the genetic variation potential of a pathogen population, considering that this plays a major role in the evolution rate of the fungus to a changing environment and its potential to overcome host and fungicide resistance (35,50). It is also very important to keep track of the appearance of new haplotypes (races), which could potentially stem from lateral gene transfer or mutation and confer new, more aggressive properties on isolates. In this study, a new *MAT* PCR enabling the amplification of the entire *MAT* idiomorph was developed. Primer pairs PtrPLP3/PtrPLP6 and PtrPLP5/PtrPLP8 allow a visual distinction between two distinct *P. tritici-repentis* lineages, enabling a rapid screening. Also, apart from race analysis (toxin production), *MAT* is the first gene that has been able to distinguish distinct lineages within *P. tritici-repentis* that could constitute distinct taxa. *MAT* sequence analysis also revealed that *P. tritici-repentis* could have originated from a heterothallic ancestor. However, unlike the *ToxA* gene, *P. tritici-repentis* *MAT* shows great haplotype diversity, suggesting evolution over time of the *MAT* gene rather than a lateral transfer from another pathogen. The fact that the same *MAT* haplotype occurs worldwide raises the issue of the mode of dissemination of the pathogen. Although ascospores are actively discharged from pseudothecia for only short distances, meso-distance (10 to 200 km) dispersal of conidia can occur (12,32). Intercontinental transfer is possible through contaminated seed (42) and straw. Additional knowledge of the emergence, race structure, and speciation of *P. tritici-repentis* is essential for a better understanding of the possible molecular co-evolution of the pathogen and its hosts (44).

ACKNOWLEDGMENTS

This work was conducted within the framework of a CIMMYT-UCL collaboration program funded by the Belgian Directorate General for Development Cooperation. We thank L. Lamari (University of Manitoba, Winnipeg, Canada) for the *P. tritici-repentis* reference strains 86-214, 331-9, and 90-2; and S. Ali and the late L. Francé (Department of Plant Pathology, NDSU), who contributed reference strains Pti2 and DW7.

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