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Characterization of Tan Spot Races in Kazakhstan

Akerke Maulenbay, Kunsulu Zakarya, Raushan Moldazhanova and Aralbek Rsaliyev *

Research Institute for Biological Safety Problems, Gvardeisky 080409, Kazakhstan
* Correspondence: aralbek@mail.ru; Tel.: +7-(701)-606-18-25

Abstract: Tan spot disease, which is caused by *Pyrenophora tritici-repentis* (*Ptr*), is one of the most significant wheat diseases in Kazakhstan, an important wheat-growing region in Central Asia. In this study, we aimed to investigate the race composition of *Ptr* responsible for tan spot in Kazakhstan through the phenotypic and genotypic characterization. During 2019–2020, samples of *Ptr* isolates were collected for analysis in six regions of the Republic of Kazakhstan from commercial and experimental fields of bread and durum wheat affected by tan spot disease. Race classification was based on inoculation bioassay of 167 isolates to four corresponding differential wheat genotypes, with a PCR assay used to identify the effector genes. The characterization of these isolates showed they belonged to four different known races (Races 1–4) in addition to one potential atypical race that does not fit into the current race system. We identified two races, Races 1 and 2, as critical for wheat production in Kazakhstan, as they occur throughout the study area. Most isolates exhibited amplification of the *ToxA* gene, a necrosis-causing effector, which is consistent with the inoculation results; only Races 3 and 4 did not show amplification of the *ToxA* gene. Inoculating wheat with the sixteen isolates of *Ptr* resulted in disease, the classification of which was similar to that caused by Race 8, according to the current wheat differential set. However, according to the genetic characterization, these isolates did not possess the associated effector gene expected for Race 8 assignment; therefore, we designated them as isolates of an atypical race. According to our results, race classification should be based on both phenotypic and genotypic analyses, where possible, to adequately capture the breadth of physiological variation among *Ptr* isolates, in addition to the possible expansion of the differential set.

Keywords: tan spot; *Pyrenophora tritici-repentis*; wheat; races; necrotrophic effectors

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1. Introduction

Tan spot of wheat is an important pathogen associated with foliar disease complexes and it is an economically important disease in worldwide wheat-growing regions [1]. The causal agent of this disease is the homothallic ascomycete *Pyrenophora tritici-repentis* (Died.) Drechsler (anamorph *Drechslera tritici-repentis* (Died.) Shoemaker). Tan spot causes serious yield losses due to a reduction in the photosynthetic area of leaves, which leads to reduced grain fill, a lower test weight, kernel shriveling, and a reduction in the number of kernels per head [2,3]. On average, tan spot causes from 5 to 15% yield losses; however, it can cause up to 50% yield losses under conditions that are favorable for disease development [2,3]. This pathogen can attack durum (*Triticum turgidum* L. var *durum* Desf.) and bread wheat (*Triticum aestivum* L. em Thell) as well as many other crops, such as barley, oats, rye, and various noncereal grass species [4–8]. Tan spot can appear over season on diseased seed, infested crop residue, and overwintered grass hosts [3]. The first report of tan spot dates back to 1823. Researchers isolated the first asexual stage of the pathogen and identified it on grasses in Germany [1]; next, in the United States, researchers described the sexual stage [9]. Later, the pathogen was identified on wheat in Japan by researchers [10]. Tan spot has since been reported worldwide and is considered a saprophyte, occasionally causing minor spotting to severe localized outbreaks and yield losses in wheat crops. Specifically, the disease reported by researchers in India in 1934, in Canada in 1937, in the United States

in 1940, in Australia in 1952, and in Kenya in 1954 [1]. The disease became economically important for wheat production in the mid-1970s, affecting major wheat-growing countries worldwide [1,11,12]. The first serious tan spot outbreak was observed in Canada in 1974 [13]. Disease with high incidence and severity has also been observed throughout the southern region of South America, in Argentina, Brazil, Chile, Paraguay, and Uruguay [11]. The first reports of tan spot in Kazakhstan and other countries of Central Asia (Uzbekistan, Kyrgyzstan, and Tajikistan) appeared in the 1980s, with leaf lesions observed on spring and winter wheat in six regions of Kazakhstan, two regions of Uzbekistan, and one region of Tajikistan [14,15]. Although the disease was observed during the 1980s in Kazakhstan, it was not considered a serious threat to wheat production until the early 2000s [16–18].

Ptr follows an inverse gene-for-gene interaction with its wheat host and is associated with the secretion of three identified necrotrophic effectors (NEs): ToxA, ToxB, and ToxC. ToxA is a proteinaceous necrosis-inducing effector, encoded by a single-copy of *ToxA* gene. ToxB is another proteinaceous but chlorosis-inducing effector encoded by multiple copies of the *ToxB* gene [19], while ToxC causes chlorosis in a genotype-specific manner but has not been as thoroughly characterized, and tools for its genotypic analysis are not yet available [20]. Each toxin interacts with a specific host-sensitivity locus: *Tsn1*, *Tsc2*, and *Tsc1*, respectively [21,22]. *Ptr* can germinate in both resistant and susceptible wheat genotypes, form appressoria, invade epidermal cells, and grow into the intercellular space of the mesophyll, stopping further growth in the mesophyll in the incompatible reaction while continuing in the compatible reaction [23]. In ToxA-containing isolates, where a single copy of the gene is sufficient to cause symptoms on susceptible crops, in contrast, ToxB-containing isolates appear to require more than one copy to cause significant symptoms [23,24]. Furthermore, a higher level of *ToxB* transcript correlates with the faster development of appressoria [25]. The classification of *Ptr* into races based on its pathogenicity and virulence began with isolates collected from wheat plants in Manitoba, Canada [26,27]. These studies led to the development of a wheat differential set for tan spot, a lesion type-based disease rating scale, and a basic race classification system. Up to now, researchers have identified eight races of this pathogen, which can induce necrosis, chlorosis, or both, on four effective host differentials of wheat [24]. Races 2, 3, and 5 produce a single toxin each (ToxA, ToxC, and ToxB, respectively). Races 1, 6, and 7 produce a combination of two NEs each (ToxA + ToxC, ToxB + ToxC, and ToxA + ToxB, respectively). Race 8 produces all three NEs, whereas Race 4 isolates do not produce any known NEs and are non-pathogenic [19,24,28]. Researchers use a visual assessment of the disease phenotype in the race designation of *Ptr*. However, race characterization based only on phenotypic features could result in incorrect classification [29]. Andrie et al. [20] proposed a combination of phenotypic and genotypic characterization for *Ptr* race identification for efficacy. Nowadays, the genotypic characterization of the *Ptr* population is widely conducted by researchers due to the incorporation of molecular tools and the intense use of PCR.

In several independent studies on the physiological variation in *Ptr* populations conducted from 2001 to 2017, diversity in the race structure of isolates from Kazakhstan has been observed. The predominant races in Kazakhstan were Races 1 and 2, though Races 2, 3, and 4 have also been detected [16,30,31]. Recently, the disease has expanded across Kazakhstan, and epidemics are often observed in regions in the south, southeast, north, and east [17,18]. Most of the cultivated commercial varieties of wheat lack sufficient resistance to *Ptr* and thus tan spot is of economic importance, particularly in locations where conditions are conducive to disease development. Changes in disease severity could be due to changes in pathogen virulence, the wide adoption of no-till and conservation tillage practices, and/or the introduction of susceptible cultivars [17]. Outbreaks of this disease in Kazakhstan are predicted to likely increase in severity and frequency due to an increase in the mean annual temperatures and altered precipitation patterns [32].

Despite the increasing importance of tan spot disease in Kazakhstan, the population structure of *Ptr* in Kazakhstan has not yet been characterized using molecular techniques

to identify NEs produced by the fungus [33,34] to support the results of a phenotypic method [20]. Considering the characterization of *Ptr* isolates as an imperative factor for the development of resistant wheat cultivars [35] this work aimed to identify races of *Ptr* in Kazakhstan and to test isolates from this country for the presence of the toxin-encoding genes *ToxA* and *ToxB*. In this way, knowledge about this pathogen in different wheat-growing regions of Kazakhstan can be generated and accurate information about the race structure in this country can be provided for the first time through phenotypic and genotypic characterization of *Ptr* isolates.

2. Materials and Methods

2.1. Fungal Isolates

Route surveys of tan spot disease were conducted in 2019–2020 on commercial wheat fields and experimental plots of bread and durum wheat cultivars in the Almaty, Turkestan, Zhambyl, East Kazakhstan, Akmola, and Kostanay regions of the Kazakhstan Republic. The surveys were carried out in 17 districts. Detailed information on the sites is presented in Supplementary Table S1. The samples were air-dried and stored in paper envelopes to avoid saprophyte growth [16]. The overall workflow is shown in Figure 1, which was created using Biorender.com (accessed on 14 August 2022). Leaves with visible lesions were cut into 1–2 cm segments, which were then surface-sterilized in 1% sodium hypochlorite solution for 2 min and rinsed twice in sterile distilled water for 30 s. The surface-sterilized leaf segments were placed in Petri dishes containing moistened Whatman® No. 1 filter paper (Whatman International Ltd., Maidstone, UK) [36]. These dishes were incubated at 20–25 °C under fluorescent light for 24 h to promote the production of conidiophores, followed by incubation for 18–24 h in the dark at 15 °C h to promote the production of conidia. Single conidia were transferred to V8-potato dextrose agar (V8-PDA) under a stereoscope, which was followed by incubation at 20 °C in the dark to reach a 3–4 cm diameter. Single-spore cultures were stored at 4 °C and used within three weeks to prepare the inoculum [26].

2.2. Inoculum Preparation and Bioassays

V8-PDA was used for inoculum production: small plugs (0.5 cm) of the 4–8-day cultures were transferred to individual Petri dishes, which was followed by incubation in the dark until colonies reached 4 cm in diameter. Cultures were flooded in sterile distilled water and then flattened using the bottom of a flame-sterilized glass tube, which was followed by incubation under fluorescent light for 18–24 h at room temperature to induce conidiophores. They were subsequently incubated at 15 °C in the dark for 18–24 h for conidia formation. For conidia harvesting, the cultures were flooded with sterile distilled water and gently dislodged using a sterilized wire loop; the concentration was 3000 conidia mL^{-1} with sterilized distilled water in a Goryaev Counting Chamber (IBSS, Sevastopol, Russia). The surface tension of the conidia suspension was reduced by adding a single drop of Tween 20 (polyoxyethylene sorbitan monolaurate) (Sigma-Aldrich, St. Louis, MI, USA) per 100 mL [26].

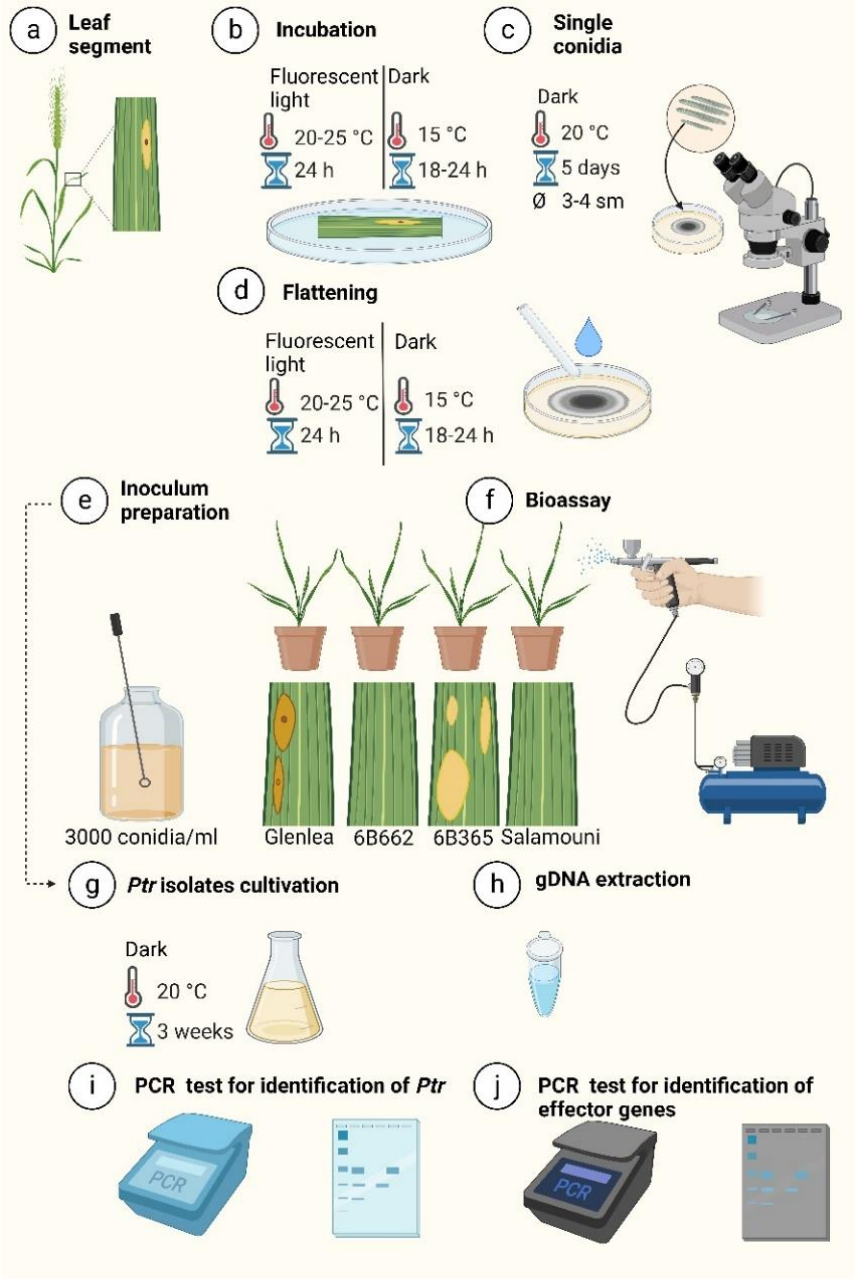

Figure 1. Step-by-step workflow of the characterization of tan spot races and identification of effector genes: (**a**) leaf segment affected by tan spot; (**b**) induction of conidia on leaf segment; (**c**) the cultivation of individual conidia on selective medium (V8-PDA); (**d**) flattening followed by incubation for conidia formation; (**e**) adjusting the concentration of inoculum; (**f**) inoculation with conidia suspension (Race 1 shown in the figure as an example of symptom appearance); (**g**) cultivation of *Ptr* isolates for gDNA extraction; (**h**) gDNA extraction; (**i**) PCR assay for identification of *Ptr*; (**j**) PCR assay for identification of effector genes.

2.3. Wheat Differential and Symptom Rating

The race structure of *Ptr* was determined by using a standard differential set consisting of the four bread wheat genotypes: "Glenlea", 6B365, 6B662, and "Salamouni". This set is used for classification according to the eight known races of the pathogen [24]. Seeds of each genotype were individually sown in 200 mL plastic pots ("Kvadro", Nur-Sultan, Kazakhstan) containing a mixture of soil, sand, and compost in a ratio of 2:1:1 ($v/v/v$), with 5–6 seeds per pot. The experiment was repeated twice. Wheat seedlings were grown to the two-leaf stage at 20/18 °C (day/night), with a 16 h photoperiod (180 mmol m^{-2} s^{-1}) [37].

Wheat seedlings were inoculated with the conidial suspension until runoff using a sprayer connected to an airline [26]. After inoculation, the seedlings were placed in the dark for 24 h at room temperature in a misting chamber (relative humidity: 95%), using an ultrasonic humidifier (Electrolux EHU-3510D, Stockholm, Sweden) to provide continuous humidification [37]. After incubation, the seedlings were maintained at 20/18 °C (day/night) with a 16 h photoperiod (180 mmol m^{-2} s^{-1}) and 60% relative humidity [26,37].

Symptom development was assessed 6–8 days after inoculation with *Ptr* races, using a 5-point scale, as follows: (1) small dark-brown to black spots without any surrounding chlorosis or tan necrosis (resistant); (2) small dark-brown to black spots with very little chlorosis or tan necrosis (moderately resistant); (3) small dark-brown to black spots completely surrounded by a distinct chlorotic or tan necrotic ring, with the lesions generally not coalescing (moderately resistant to moderately susceptible); (4) small dark-brown or black spots completely surrounded by chlorotic or tan necrotic zones, with some of the lesions coalescing (moderately susceptible); and (5) dark-brown or black centers distinguishable or indistinguishable, with most lesions consisting of coalescing chlorotic or tan necrotic zones (susceptible) [26]. Analysis of variance of the data for wheat differentials and *Ptr* isolates was performed on GraphPadPrism 8.0.1 (GraphPad Software, Inc., LaJolla, CA, USA).

2.4. Genomic DNA Extraction and PCR Assay for Identification of Ptr and Amplification of Effector Genes

Ptr isolates were grown at 20 °C in the dark for three weeks in Fries liquid medium [37,38]. Genomic DNA (gDNA) was extracted from 40 mg of lyophilized mycelia [36,37] using the Genomic DNA via Plant/Fungi DNA Isolation Kit (Norgen Biotek Co., Thorold, ON, Canada), following the manufacturer's instructions. DNA was quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

To confirm the identities of the *Ptr* isolates, PCR testing was conducted using a specific primer set: PtrUnique_F2 (5′-GGACTTGGCTTTCTATTGTGC-3′) and PtrUnique_R2 (5′-CTTGGTGAATGGTGAAGATGG-3′). The thermal cycling parameters were 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 1 min, followed by elongation at 72 °C for 10 min [39]. Multiplex PCR assay was conducted using specific primers for the detection of the effector genes *ToxA* (TA51F and TA52R) and *ToxB* (TB71F and TB60R), with specific primers for chitin synthase 1 (*CHS-1*), a conserved gene in fungi, as a control to confirm the presence of fungal DNA (Table 1) [20].

Table 1. The sequences of the oligonucleotide primers used for multiplex PCR for amplification of *ToxA* and *ToxB* genes in *Pyrenophora tritici-repentis* isolates.

Genes	Primer for Multiplex PCR	Sequence	Estimated Band Size	Reference
ToxA	TA51F	5′-GCGTTCTATCCTCGTACTTC-3′	573 bp	[20]
	TA52R	5′-GCATTCTCCAATTTTCACG-3		
ToxB	TB71F	5′-GCTACTTGCTGTGGCTATC-3	232 bp	[20]
	TB60R	5′-ACTAACAACGTCCTCCACTTTG-3′		[40]
CHS-1	CHS-79F	5′-TGGGGCAAGGATGCTTGGAAGAAG-3′	275 bp	[41]
	CHS-354R	5′-TGGAAGAACCATCTGTGAGAGTTG-3′		

PCR amplification included an initial denaturation step at 94 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, 58 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min [20]. The amplification products were analyzed by electrophoresis on a 1.5% agarose gel (Sigma-Aldrich, St. Louis, MI, USA) stained with ethidium bromide in 1× TBE buffer (Invitrogen, Waltham, MA, USA). The lengths of the amplified fragments were estimated through comparison with a 100 bp DNA ladder (Thermo Fisher Scientific, Waltham, MA, USA).

3. Results

3.1. Isolate Collection

We conducted a survey of wheat fields in 2019 and 2020 to collect samples of leaves that were affected by tan spot disease from bread and durum wheat cultivars. The surveyed sites include six regions and seventeen districts of the main wheat-growing areas of Kazakhstan. A total of 167 single-spore isolates were recovered from the collected samples (Supplementary Table S1). Among the six regions, most of the isolates were collected from the Zhambyl region (47), followed by Kostanay (35). The lowest number of isolates was recovered in the Akmola region (12). In 2019, we studied 75 isolates: 3 were collected from durum wheat cultivars and 72 from bread wheat cultivars. In 2020, 92 isolates were recovered and characterized, made up of 12 and 80 isolates from durum and bread wheat cultivars, respectively (Table 2). We confirmed the identity of all isolates as *Ptr* based on the morphological characteristics of conidia and PCR assays (Supplementary Table S1).

Table 2. The number of *Pyrenophora tritici-repentis* isolates recovered and characterized in 2019 and 2020 in six regions of Kazakhstan.

Region	Host	2019	2020	Total
Almaty	BW [1]	14	12	26
	DW [2]	1	4	5
Turkestan	BW	9	16	25
Zhambyl	BW	20	27	47
East Kazakhstan	DW	0	2	2
	BW	8	7	15
Akmola	DW	0	3	3
	BW	5	4	9
Kostanay	DW	2	3	5
	BW	16	14	30
Total	DW	3	12	15
	BW	72	80	152

[1] Bread wheat. [2] Durum wheat.

3.2. Race Characterization

We tested all recovered isolates for their ability to induce disease on the differential wheat lines in a host genotype-specific manner. Our results show that the 167 isolates collected from 2019–2020 belonged to four known races and an atypical race. Of these, two races (Races 1 and 2) were found in all six regions in both years, two races (Races 3 and 4) were found in four regions in 2020, and one atypical race was observed in five regions in 2020 (Table 3 and Supplementary Table S1).

According to the bioassay, we assigned isolates that caused necrosis on Glenlea and chlorosis on 6B365 but that were resistant to the host differential 6B662 to Race 1. Race 1 comprised 48 (64.0%) and 51 (55.4%) isolates in 2019 and 2020, respectively. We assigned the subsequent isolates that caused necrosis on Glenlea but that were non-pathogenic on Glenlea and 6B662 to Race 2. Race 2 was represented by 20 (26.7%) and 19 (20.7%) isolates in 2019 and 2020, respectively. The frequency of Race 1 was higher in the Zhambyl region in 2020, and it was lowest in the Akmola region in the same year. We observed Race 2 twice less frequently than Race 1 in all studied regions and years. The isolates assigned to Races 1 and 2 were collected from bread wheat.

In 2019 and 2020, we assigned seven isolates that caused chlorosis on 6B365 but that were avirulent on Glenlea and 6B662 to Race 3. Six isolates were avirulent on all wheat genotypes and were therefore designated as non-pathogenic Race 4. Races 3 and 4 were found exclusively on durum wheat from experimental agricultural plots in the Almaty (Karasay district, Almalybak), Kostanay (Karabalyk district, Nauchniy), East Kazakhstan

(Glubokov district, Solnechnoe), and Akmola (Shortandy district, Nauchnoe) regions (Supplementary Table S1). No representatives of Races 3 and 4 were found in the Turkestan and Zhambyl regions.

Table 3. Year, geographic origin, host, and race classification of *Pyrenophora tritici-repentis* isolates collected in Kazakhstan during 2019 and 2020.

Year	Race	Number of Isolates per Region (%)						Total
		Almaty	Turkestan	Zhambyl	East Kazakhstan	Akmola	Kostanay	
2019	Race 1	10 (13.3) [2]	6 (8.0) [2]	13 (17.3) [2]	6 (8.0) [2]	4 (5.3) [2]	9 (12.0) [3]	48 (64.0)
	Race 2	4 (5.3) [2]	3 (4.0) [2]	6 (8.0) [2]	2 (2.7) [2]	1 (1.3) [2]	4 (5.3) [2]	20 (26.7)
	Race 3	1 (1.3) [3]	-	-	-	-	1 (1.3) [3]	2 (2.7)
	Race 4	-	-	-	-	-	1 (1.3) [3]	1 (1.3)
	Atypical [1]	-	-	1 (1.3) [2]	-	-	3 (4.0) [2]	4 (5.3)
2020	Race 1	8 (8.7) [2]	9 (9.8) [2]	20 (21.7) [2]	6 (6.5) [2]	2 (2.2) [2]	6 (6.5) [2]	51 (55.4)
	Race 2	4 (4.3) [2]	4 (4.3) [2]	4 (4.3) [2]	1 (1.1) [2]	1 (1.1) [2]	5 (5.4) [2]	19 (20.7)
	Race 3	1 (1.1) [3]	-	-	1 (1.1) [3]	1 (1.1) [3]	2 (2.2) [3]	5 (5.4)
	Race 4	1 (1.1) [3]	-	-	1 (1.1) [3]	2 (2.2) [3]	1 (1.1) [3]	5 (5.4)
	Atypical [1]	2 (2.2) [3]	3 (3.3) [2]	3 (3.3) [2]	-	1 (1.1) [2]	3 (3.3) [2]	12 (13.0)

[1] Race lacks in *ToxB* gene, but it behaves similar to Race 8; that is, the molecular characterization contradicted the phenotypic characterization. [2] Isolates from bread wheat. [3] Isolates from durum wheat.

Sixteen isolates (four and twelve in 2019 and 2020, respectively) caused necrosis on Glenlea and chlorotic symptoms on 6B662 and 6B365. These isolates exhibited all three known toxin phenotypes, which initially led us to designate them as Race 8, as they were characterized by the production of ToxA, ToxB, and ToxC. However, the molecular and phenotypic characterization results are conflicting. According to the genotypic analysis, although these isolates caused chlorosis of 6B662, the lack of the ToxB gene rules out their classification as Race 8 in this study (molecular characterization of *Ptr* isolates is shown in detail in Section 3.3; Supplementary Table S1). These sixteen isolates did not fit into the current evaluation system and we therefore designated them as an atypical race (Table 3). In 2019, a single isolate assigned as an atypical race was found in the Zhambyl region and three isolates in Kostanay. However, in 2020, they were found more frequently, except in East Kazakhstan. Two isolates were collected from durum wheat and fourteen from bread wheat (Table 3 and Supplementary Table S1).

The frequencies of the races differ between the regional populations of *Ptr* in Kazakhstan during 2019 and 2020. In 2019, we observed the highest diversity in the Kostanay region, while in 2020, we found all detected races in the Almaty, Akmola, and Kostanay regions. The race pattern in East Kazakhstan differs from that of the other regions, with no atypical race isolates observed (Table 3).

ANOVA showed significant effects ($p < 0.0001$) of isolates, differentials, and interaction (isolates × differentials) based on *Ptr* symptom rating on a 5-point scale (1–5) (Table 4).

Table 4. Analysis of variance of the interaction between *Pyrenophora tritici-repentis* wheat differentials and isolates under greenhouse condition.

ANOVA Table	df	SS	MS	F
Isolates	166	521.47	3.1414	26.903 ****
Differentials	3	2789.1	929.69	7111.1 ****
Isolates × differentials	498	1171.9	2.3532	18.000 ****
Error	501	65.500	0.13074	

**** $p < 0.0001$. df, degree of freedom; SS, sum of squares; MS, mean squares.

To categorize the pathogenic groups of isolates, differentials with 1–2 points were classified as resistant and 3–5 as susceptible. The resistance and susceptibility pattern of four wheat genotypes is shown in Supplementary Table S1.

3.3. Presence of Effector Genes in Ptr Isolates

To support the results of the inoculation bioassay, all isolates were subjected to multiplex PCR to determine the presence or absence of the effector genes *ToxA* and *ToxB*. *CHS*-1 was also amplified as an internal control for the presence of fungal DNA in all tested isolates. The multiplex PCR amplification patterns for the *Ptr* isolates with an internal control for the presence of fungal DNA (*CHS-1*) are shown in Figure 2.

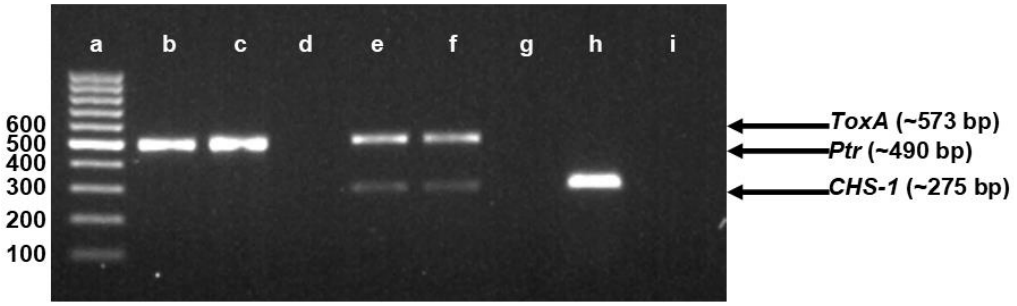

Figure 2. Example PCR amplification with primer sets specific for identification of *Ptr* isolates and *ToxA* and *ToxB* effector genes. Detection of effector genes in the *Ptr* population in Kazakhstan using multiplex PCR with specific primers *ToxA* (TA51F/TA52R) and *ToxB* (TB71F/TB60R) and the chitin synthase 1 gene (*CHS-1*) as an internal control for the presence of fungal DNA. The reaction products are visualized on a 1.5% agarose gel stained with ethidium bromide. An isolate collected in 2018 was used as a positive control, representing Race 1. (**a**) DNA ladder (100 bp); (**b**) isolate KZ/Kt-19-10 (*Ptr*); (**c**) positive control (*Ptr*); (**d**) negative control (water); (**e**) isolate KZ/Kt-19-10 (*ToxA* + *CHS-1*); (**f**) positive control (*ToxA* + *CHS-1*); (**g**) negative control (water); (**h**) isolate KZ/Kt-19-10 (*CHS-1*); (**i**) negative control (water).

The *ToxA* gene was amplified in 154 *Ptr* isolates in which necrotic symptoms on Glenlea were observed during the inoculation test (Supplementary Table S1). As expected, no amplicon corresponding to *ToxA* was found in the isolates previously assigned to Races 3 and 4. In contrast, the *ToxB* gene was not detected in any the isolates assigned to Race 8 according to the inoculation results. This represents an atypical result, in which the chlorosis development on 6B662 cannot be attributed to the presence of ToxB and, therefore, we refer to these isolates as atypical.

4. Discussion

Fungal foliar diseases, such as rusts (causal agents: *Puccinia triticina* Erikss, *Puccinia graminis* f. *tritici* Erikss. & Henning., and *Puccinia striiformis* Westend.), tan spot (*Ptr*), glum blotch (*Parastagonospora nodorum* (Berk.) Quaedvl. = *Septoria nodorum* (Berk.) Berk.), spot blotch (*Cochliobolus sativus* (S. Ito & Kurib.) Drechsler ex Dastur = *Bipolaris sorokiniana* (Sacc.) Shoemaker), and powdery mildew (*Blumeria graminis* (DC.) Speer), are major biological constraints of bread and durum wheat production in Kazakhstan [31,42–47].

In the current study, we aimed to identify the races of *Ptr* in Kazakhstan and test isolates from this country for the presence of the toxin-encoding genes *ToxA* and *ToxB*. Most of the characterized isolates of *Ptr* were from bread wheat varieties collected mainly from commercial wheat fields (Table 2). In Kazakhstan, bread wheat varieties are grown in more than 80% of the wheat area. Up to 20–25 million tons of bread wheat are grown annually and up to 5–7 million tons of the grain are exported [43,48]. In contrast, we collected durum wheat samples from the agricultural stations of Almalybak in the Karasay district, Karabalyk in the Karabalyk district, and the research nurseries in Glubokov and Shortandy (Supplementary Table S1). The small sample size for durum wheat in our study is due to

the narrow range of durum wheat in Kazakhstan, the unstable market demand, and large variations in the yield and quality. Furthermore, the production is under the strong pressure of abiotic factors during a crucial period of plant maturation, such as drought, heat, and heavy rains as well as periodic invasions of devastating durum wheat pathogens [45,49].

Two races, 1 (ToxA, ToxC) and 2 (ToxA), were identified as critical for wheat production in Kazakhstan because they were found throughout the study area. Researchers have observed a similar trend in a global collection of *Ptr* populations reviewed by Kamel et al., 2019 [36]. In our study, Race 2 was about 2.5 times less common than Race 1, which could be due to the lack of selection pressure from the widespread cultivation of wheat varieties carrying the *Tsn1* gene, which has led to a sharp increase in the incidence and severity of the disease worldwide [50]; the combination of NEs produced by Race 1 rather than Race 2 together with the homothallic nature of the pathogen reduce the chances of recombination [4]. Furthermore, during the evaluation, most Kazakh wheat varieties demonstrated susceptibility to *Ptr* Race 1, which suggests that the search for resistance to Race 1 is more challenging than for other races [51]. Isolates designated as Races 1 and 2 were recovered from bread wheat. Likewise, in a study of a Silk Road survey, isolates of Races 1 and 2 were predominantly collected from hexaploid wheat rather than tetraploid hosts [16].

The *ToxA* gene was amplified in 154 isolates that thus belong to the races producing the ToxA necrotrophic effector, which we confirmed using both inoculation bioassays and PCR tests. The worldwide collection of *Ptr* isolates mainly consists of isolates that possess the coding region of the *ToxA* gene [52–55]. Recently, researchers have linked the increased incidence of tan spot disease over other wheat diseases (North America) to the horizontal gene transfer of the *ToxA* gene from *P. nodorum*, which appears to play a critical role in the interaction between bread and durum wheat and *P. nodorum* [56]. However, *ToxA–Tsn1* does not play a significant role in the interaction between *Ptr* and durum wheat [57]. SSR markers spanning the *Ptr* genome and its various chromosomes showed significant differences between ToxA-producing and ToxA-non-producing isolates. While researchers have obtained most ToxA-non-producing isolates from tetraploid (durum) wheat, they have mainly collected ToxA-producing isolates from hexaploid wheat [58].

In the present study, we identified only a few isolates of *Ptr* as belonging to Races 3 and 4, which we exclusively collected from durum wheat (Table 3 and Supplementary Table S1). Races 3 and 4 have been reported in previous studies of Kazakh *Ptr* populations but with low frequencies [30,31,59]. The first report of Race 3 (ToxC) from the Central Kazakhstan region (KZ-44) was from the bread wheat varieties Somoni and Dangara (TJ-5) and Saratovskaya-42 [30]. In the study of the Silk Road *Ptr* population, most isolates of Race 3 were collected from durum wheat varieties, with a single isolate from the bread wheat genotype [16]. In contrast, researchers collected Canadian Race 3 isolates exclusively from durum wheat varieties [52]. The first Kazakhstan report of Race 4, a non-toxin-producing race, was in Mailyoshak, from the bread wheat Krasnodopadskaya-250 [30]. Researchers from other countries have reported a similar trend of low abundance for Race 4 on wheat (reviewed by Kamel et al., 2019) [36]. The low frequency of Race 4 is likely because this race does not contain any of the three known host-selective NEs and is avirulent on wheat, which makes it less competitive with other races in terms of establishment [60]. Although Race 4 cannot induce symptoms on differential wheat lines, several isolates from North Dakota induced necrosis on durum wheat but not on a bread wheat variety, which suggests the production of additional effectors [61]. Moreover, researchers reported Race 4 isolates on durum wheat in Tunisia [36,62]. The host itself may be responsible for the variability in the race structure of *Ptr*. Researchers have characterized ToxB- and ToxC-producing races by durum wheat and Race 4 is mainly from wild grasses [37,52].

Sixteen isolates in our study caused the same symptoms as Race 8, causing necrosis on Glenlea and chlorosis on 6B662 and 6B365. However, a comparison of the phenotypic and genotypic race characterization did not match the disease profiles. The mentioned isolates amplified the *ToxA* gene but they did not amplify the *ToxB* gene. The use of multiplex PCR

and the inclusion of a reference-positive fungal gene (*CHS-1*) amplified in all tested isolates, help to ensure the accuracy of such results [36]. The absence of the *ToxB* gene in the isolates rules out the possibility of Races 5, 6, 7, and 8 in this region [24]. Therefore, we classified these sixteen isolates as an atypical race. This report is the first of an atypical race discovered in Kazakhstan. Similarly, isolates of *Ptr* from Argentina [33], the United States [20], Nepal, and Georgia [63] were assigned to Race 8 in the phenotypic race characterization; however, according to the PCR amplification with the gene-specific primers, they carried *ToxA* but *ToxB* was not present. The identification of previously unknown phenotype–genotype combinations suggests the discovery of new races of *Ptr* that potentially exert new toxic activities on wheat, and the official race designation of these isolates is still pending until they are thoroughly characterized [20]. The role of the *ToxB* gene in *Ptr* isolates that lack *ToxB* activity has not been fully elucidated but some researchers have suggested that it plays other roles in the basic pathogenic capabilities of the fungus in addition to its role in inducing chlorosis on toxin-susceptible wheat lines [25,37].

Undoubtedly, the *Ptr* race system has greatly influenced the understanding of the interaction between necrotrophic effectors and hosts, and it has enabled researchers to distinguish between the eight known races of *Ptr*. There are also several reports of isolates that do not fit the known eight races; these isolates induce necrosis on Glenlea but did not harbor the *ToxA* gene according to PCR results [20,33,35,36,60,63,64]. These atypical responses could also indicate the presence of additional and unknown NEs in these races, or the use of different arsenals for pathogenicity on wheat [52]. The ability to identify all races of a pathogen is limited by the differential system used and, most likely, there are additional races and effectors in *Ptr* [24,36].

New races of the pathogen are circulating in nature and the emergence of isolates that do not fit into the current race classification system and are designated as atypical is of great concern. Therefore, our study represents the first comprehensive analysis of a collection of *Ptr* isolates from a large wheat-growing region using both virulence and molecular data, thus providing valuable information on the races and effector genes present in the Kazakh population. Breeding programs in Kazakhstan target high yield potential and superior grain quality with the focus on irrigated and rained areas and breeding for rusts and common bunt for the South Kazakhstan region [65], and early maturation to optimize grain yield and quality in the conditions of Northern Kazakhstan [66]. Breeding for resistant wheat varieties is the best option to control the disease sustainably and avoid excessive expenditure on fungicides, which are a cost-effective and environmentally friendly method of disease control. This discovery holds particular significance to breeders and pathologists interested in producing wheat lines with resistance to tan spot.

5. Conclusions

Differentiation of the Kazakhstani *Ptr* population resulted in the characterization of four known races and one atypical race. According to the phenotypic and genotypic examination, the collection of Kazakh isolates were predominantly Race 1 followed by Race 2, consistent with the fact that the corresponding *ToxA* effector gene was amplified in the majority of isolates. The frequencies of Races 3 and 4 were almost the same in both studied years. The corresponding effector gene *ToxB* was not amplified in any of the isolates, which rules out the presence of ToxB-producing races in the isolates of this study. Because there is a conflict between the genotypic and phenotypic race classification, we designated the isolates as an atypical race. According to these results, genotyping should be performed to support the assignments based on phenotypic tests as a whole to exclude erroneous assignments. Atypical races and their different arsenals for pathogenicity on wheat should be studied further. Breeding activities for resistance to tan spot diseases in wheat in Kazakhstan should be further detailed.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture12101564/s1, Supplementary Table S1. Isolate code, geographic origin, year, host, and phenotypic reaction on four differential wheat lines of *Pyrenophora tritici-repentis*; PCR reaction and race designation of *Ptr* isolates in Kazakhstan. Refs. [20,26,39–41] have been cited in the supplementary materials.

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