Genotyping-by-Sequencing for the Study of Genetic Diversity in *Puccinia triticina*

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Abstract

Leaf rust, caused by *Puccinia triticina* Erikss., is globally the most widespread rust of wheat. Populations of *P. triticina* are highly diverse for virulence, with many different races found annually. The genetic diversity of *P. triticina* populations has been previously assessed using different types of DNA markers. Genotyping technologies that provide a higher density of markers distributed across the genome will be more powerful for analysis of genetic and phylogenetic relationships in *P. triticina* populations. In this study, we utilized restriction-associated DNA (RAD) genotyping-by-sequencing (GBS) adapted for the Ion Torrent sequencing platform for the study of population diversity in *P. triticina*. A collection of 102 isolates, collected mainly from tetraploid and hexaploid wheat, was used. The virulence phenotypes of the isolates were determined on 20 lines of Thatcher wheat near isogenic for leaf rust resistance genes. Seven races were found among 57 isolates collected from tetraploid wheat, and 21 races were observed among 40 hexaploid wheat type isolates. This is the first

study to report durum wheat virulent races to *Lr3bg* in Tunisia, *Lr14a* in Morocco, and *Lr3bg* and *Lr28* in Mexico. Ethiopian isolates with high virulence to durum wheat but avirulent on Thatcher (hexaploid wheat) were tested for virulence on a set of durum (tetraploid) differentials. A subset of 30 isolates representing most of the virulence phenotypes in the 102 isolates were genotyped using RAD-GBS. Phylogenetic analysis of 30 isolates using 2,125 single nucleotide polymorphism (SNP) markers showed nine distinct clusters. There was a general correlation between virulence phenotypes and SNP genotypes. The high bootstrap values between clusters of isolates in the phylogenetic tree indicated that RAD-GBS can be used as a new genotyping tool that is fast, simple, high throughput, cost effective, and provides a sufficient number of markers for the study of genetic diversity in *P. triticina*.

Keywords: pathogen diversity, fungi, field crops, cereals and grains

Leaf rust is a common disease in all wheat (*Triticum* spp.) growing regions and is the most commonly found of the three wheat rusts (Chester 1946), reducing the quantity and quality of kernels. The severity of leaf rust is usually the highest in areas where wheat grows under warm to hot climates such as the Great Plains of North America and the steppes of Central Asia. Similarly, high leaf rust severity has also been reported in regions with warm and humid climate such as the Mediterranean basin, South America, and the coastal regions of North America (Liu et al. 2014). Leaf rust is found on hexaploid common wheat (*Triticum aestivum* L.) globally, whereas leaf rust occurs on tetraploid durum wheat (*Triticum turgidum* L. var. *durum*) mainly in the Mediterranean basin, Middle East, Ethiopia, Mexico, and South America (Ordoñez and Kolmer 2007a).

Puccinia triticina is a macrocyclic-heteroecious rust, producing five spore stages. Wheat and wild relatives are the primary hosts on which urediniospores, teliospores, and basidiospores are produced. The pycniospores and aeciospores are produced on the alternate hosts, mainly *Thalictrum speciosissimum* L. (Mains and Jackson 1921), which is native to Spain and Portugal, where the sexual cycle has been observed. In addition, sexual reproduction on *Thalictrum*

spp. was observed in northeast Kazakhstan (J. A. Kolmer, unpublished data) and in Siberia on *Isopyrum fumarioides* (Chester 1946). Because susceptible alternate hosts are absent in many parts of the world, *P. triticina* reproduces mainly by clonal production of dikaryotic urediniospores.

Despite the rare contribution of sexual recombination, *P. triticina* population continues to evolve asexually and shows high diversity for virulence to leaf rust resistance (*Lr*) genes in wheat (Kolmer 2013; McCallum et al. 2007; Roelfs et al. 1992). Over 50 virulence phenotypes are detected annually in North America (Kolmer 2013; Kolmer and Hughes 2016; McCallum et al. 2007). Genetic drift, migration, and host selection contribute to genetic diversity in *P. triticina* populations, and parasexual recombination in *P. triticina* has also been reported in Australia (Park et al. 1999). Mutation is the primary source of *P. triticina* diversity, resulting in the rapid appearance of virulent races in response to the use of wheat cultivars with effective leaf rust resistance genes (Ordoñez and Kolmer 2007a).

In recent years, *P. triticina* races highly virulent on durum wheat (Goyeau et al. 2006; Martinez et al. 2005; Ordoñez and Kolmer 2007a; Singh et al. 2004) have spread to most of the durum producing regions of the world. As a result, many durum wheat cultivars are now susceptible to leaf rust. Isolates of *P. triticina* virulent on durum wheat are avirulent to most of the *Lr* genes identified in common (hexaploid) wheat (Goyeau et al. 2006; Ordoñez and Kolmer 2007b). In addition, *P. triticina* isolates collected from durum wheat are less diverse for virulence phenotypes and simple sequence repeat (SSR) genotypes compared with those collected from common wheat (Goyeau et al. 2012; Kolmer and Acevedo 2016; Martinez et al. 2005; Ordoñez and Kolmer 2007a, 2007b). Isolates of *P. triticina* virulent on durum wheat in Mexico (Singh et al. 2004) were very similar for virulence phenotypes and SSR genotypes to isolates collected on durum wheat in Spain, France, Mexico, Argentina, and the United States, suggesting a common ancestor (Ordoñez and Kolmer

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2007a, 2007b). However, some *P. tritricina* isolates collected in Ethiopia on tetraploid wheat had a virulence phenotype and SSR genotype distinct from all other worldwide collections of isolates from both durum and common wheat (Kolmer and Acevedo 2016). These Ethiopian durum-specific isolates are avirulent to leaf rust susceptible common wheat cultivars such as Thatcher and Little Club.

Over the last 20 years, molecular markers have been used to assess genetic diversity in *P. tritricina* populations. Random amplified polymorphism of DNA markers were the first used to genotype *P. tritricina* populations (Kolmer et al. 1995; Kolmer and Liu 2000; Park et al. 2000), followed by amplified fragment-length polymorphisms (AFLPs) (Kolmer 2001) and then by SSRs (Duan et al. 2003). A set of 23 SSRs (Kolmer et al. 2019; Ordoñez et al. 2010; Szabo and Kolmer 2007) have been used in studies to assess the genetic diversity of clonal populations of *P. tritricina*. Moderate correlation between SSR genotypes and virulence phenotypes has been observed (Kolmer 2015; Kolmer and Acevedo 2016; Kolmer et al. 2011, 2013; Ordoñez and Kolmer 2007b, 2009; Ordoñez et al. 2010).

Population genetic studies require high numbers of markers in order to draw reliable inferences regarding the genetic relatedness of different population groups. A large number of single nucleotide polymorphic (SNP) markers can be obtained with the genotyping-by-sequencing (GBS) method. GBS is a low-cost approach used to achieve a reduced-complexity sequence of the whole genome by targeting subsets of genomic regions (Elshire et al. 2011; Poland et al. 2012a). This technique has been used in several studies involving genomic selection, genetic mapping, dissecting the genetic basis of disease resistance traits, and genetic diversity studies in several organisms (Adhikari and Missaoui 2019; Elshire et al. 2011; Li et al. 2015; Lu et al. 2013; Poland et al. 2012a, 2012b). Restriction-associated DNA GBS (RAD-GBS) uses restriction enzymes to capture targeted regions of the genome. Adaptors are ligated to the flanking regions of the restriction sites to create barcoded libraries, which allows the sequencing of several individuals at a time (Mascher et al. 2013). The RAD-GBS method has mainly been based on Illumina GAI and HiSeq platforms (Poland and Rife 2012). However, recently an optimized RAD-GBS protocol, adapted for the Ion Torrent platform, has become available (Rothberg et al. 2011) and has been used to generate sufficient genotyping density for a number of plant pathogens (Carlsen et al. 2017; Gao et al. 2016; Leboldus et al. 2015). The reference genome of *P. tritricina* (https://www.broadinstitute.org/annotation/genome/puccinia_group/Downloads.html) allowed mapping of the RAD-GBS generated SNPs to specific locations on contigs. We hypothesized that the use of high-density SNPs across the *P. tritricina* genome will provide better precision in the study of genetic diversity of this pathogen. The objectives of this study were to (i) assess the application of RAD-GBS using the Ion Torrent sequencing platform as a new genotyping tool for the study of genetic diversity in *P. tritricina* and (ii) develop durum differential lines to distinguish between virulence phenotypes of Ethiopian isolates that are avirulent to Thatcher wheat.

Materials and Methods

***Puccinia tritricina* isolates.** Forty-five samples of wheat leaves infected with *P. tritricina* were received from Morocco, Tunisia, Spain, and Ethiopia. Each sample corresponds to multiple leaves with leaf rust infections, collected from a single plant. Sampling was done randomly in the field. A set of 12 samples was collected in 2014 from research plots of durum wheat, common wheat, and cultivated emmer at the Debre Zeit Agricultural Research Center (DZARC), Ethiopia. A second set of eight samples were collected in 2014 in Spain from common wheat, durum wheat, and triticale fields at Peralta, Learza (Navarra), Huesca, Coril (Cadiz), and Jerez de la Frontera locations. Six samples were also collected in 2014 in Tunisia from research plots of common wheat, durum wheat, and triticale at the National Institute of Agronomic Research of Tunisia (INRAT), Beja experiment station, Grombalia, and Mateur. An additional 19 samples were collected in Morocco in 2014 and 2015 from durum wheat and common wheat fields at Zemamra, Jemaa-Shaam, Rabat, Marchouch, and Tahrir. The samples (infected leaves) were placed in paper

envelopes and air dried at room temperature. The samples were then shipped to North Dakota State University, where they were stored at -80°C until processed.

The samples were processed during winter months (December to February) at the North Dakota Agricultural Experiment Station Greenhouse Complex (ND-AESGC), Fargo, ND. Urediniospores from each sample were collected using a cotton swab immersed in Soltrol-170 mineral oil (Phillips Petroleum). The swab containing the collected spores was used to inoculate 7-day-old seedlings of the leaf-rust-susceptible durum wheat line RL6089 for spore increase. The seedlings were treated with a 0.3% solution of maleic hydrazide to increase sporulation and prevent secondary leaves from forming (Goyeau et al. 2006). Seven days after inoculation, spores from a single uredinium were collected with a cotton swab, which was in turn used to inoculate seedlings of RL6089 and Thatcher for increase of the purified single-uredinium isolates. From each sample, one to four single-uredinial isolations were derived. The single-uredinial isolates were increased on seedlings and covered with an isolation box to avoid cross-contamination. Incubation and greenhouse conditions were as described by Aoun et al. (2016).

A total of 86 single-uredinial isolates were obtained from the samples collected in Ethiopia, Morocco, Spain, and Tunisia. Seven more single-uredinial isolates from Mexico (collected in 2001 to 2014) were acquired from CIMMYT-Mexico. Five isolates from Chile (collected in 2013) and five isolates from Pakistan (collected in 2013 and 2014) were acquired from the USDA-ARS Cereal Disease Laboratory (USDA-CDL) in St. Paul, MN. In addition, two isolates from the United States, race 1 and CA1.2, were included in the analysis for comparison. Race 1 is a North American common wheat type race and was used in this study as a control because its whole genome sequence is available; isolate CA1.2 was collected on durum wheat in California in 2009. In total, 102 single-pustule isolates were used for this study, with 51 collected from durum wheat, 40 from common wheat, five from triticale, and six from cultivated emmer wheat (*Triticum dicoccum*) (Table 1).

Virulence phenotypes of *P. tritricina* isolates. Urediniospores of each of the 102 single-uredinial isolates were increased to generate enough inoculum for virulence phenotyping and DNA extraction. Eighty-one isolates were phenotyped at the USDA-CDL, and the remaining 21 isolates were phenotyped at ND-AESGC, Fargo, ND. Five sets of four Thatcher wheat near-isogenic lines (NILs) carrying different *Lr* genes were used for the isolate phenotyping. The first set included lines with genes *Lr1* (isogenic line RL6003), *Lr2a* (RL6000), *Lr2c* (RL6047), and *Lr3a* (RL6002); the second set included lines with genes *Lr9* (RL6010), *Lr16* (RL6005), *Lr24* (RL6064), and *Lr26* (6078); the third set comprised lines with genes *Lr3ka* (RL6007), *Lr11* (RL6053), *Lr17* (RL6008), and *Lr30* (RL6049); the fourth set included lines with genes *LrB* (RL6047), *Lr10* (RL6004), *Lr14a* (RL6013), and *Lr18* (RL6009); and the fifth set had lines carrying genes *Lr3bg* (RL6042), *Lr14b* (RL6006), *Lr20* (RL6092), and *Lr28* (RL6079). Thatcher was included as a check. The plants were inoculated at the first-leaf stage (7 to 8 days after planting) with fresh urediniospores suspended in Soltrol-170 mineral oil (Phillips Petroleum) using an inoculator pressurized by an air pump. The inoculated plants were then placed in a mist chamber overnight and later transferred and kept in the greenhouse at 18 to 25°C and a 16-h photoperiod. The seedlings were evaluated for infection types (ITs) 10 to 12 days after inoculation. At ND-AESGC, Fargo, ND, the virulence phenotype of these isolates was based on two replicates per isolate. The virulence phenotypes of the isolates, at the USDA-CDL, MN, was based on single tests. However, in the case in which disease development or plant development was suboptimal and ITs were difficult to determine, a second test was performed. Seedlings showing ITs of 0 to 2⁺ and mesothetic reaction (or “X” reaction) were classified as resistant, whereas those showing ITs of 3 to 4 were classified as susceptible. Based on the ITs on the isogenic lines, a five-letter designation was assigned to each isolate following the nomenclature of Long and Kolmer (1989).

Some of the Ethiopian isolates with high virulence to durum wheat that were avirulent on Thatcher were given the race code EEEEE,

because these isolates could not be phenotyped for virulence with the Thatcher NILs. To be able to determine potential virulence differences across these isolates, seven Ethiopian EEEEE isolates were tested on a set of 23 durum wheat accessions using Thatcher as a resistant control. Twelve of these accessions were selected from the USDA National Small Grain Collection (NSGC) of durum wheat, because these showed resistance to several *P. triticina* isolates in previous studies (Aoun et al. 2016, 2017, 2019). The 12 accessions were PI 45442, PI 192051, PI 195693, PI 209274, PI 244061, PI 278379, PI 313096, PI 324928, PI 342647, PI 387263, PI 519832, and PI 534304. The remaining 11 genotypes were durum cultivars ‘Divide’, ‘Rusty’, ‘Capelli’, ‘Mindum’, ‘Russello S.G.7’, ‘Alred’, ‘Creso’ with *Lr14c*, ‘Llaretta INIA’ (*Lr14a*), ‘Camayo’ (*LrCamayo*), ‘Jupare C2001’ (*Lr27+Lr31*), and ‘Mexicali 75’.

DNA extraction, library preparation, and sequencing. A total of 44 isolates, selected from the 102 isolates, representing diverse virulence phenotypes and collected from different hosts and countries, were chosen for genotyping. These isolates included two from the United States, four from Mexico, five from Chile, five from Pakistan, four from Ethiopia, six from Tunisia, eight from Morocco, and 10 from Spain. Twenty-one isolates from common wheat, 17 from durum wheat, four from triticale, and two from emmer wheat were genotyped (Table 2).

For the 12 isolates obtained from the USDA-CDL, ~25 mg of urediniospores from each isolate were germinated, and the fungal material was lyophilized and ground as described by Ordoñez and Kolmer (2007b). The DNA was extracted using an OmniPrep (G-BioSciences) extraction kit according to the manufacturer’s instructions. For the remaining 32 isolates, 20 to 30 mg of urediniospores from each isolate were germinated at room temperature in a glass Petri dish containing 500 ml of a germination solution (499 ml of Milli Q water and 1 ml of 500× stock solution). The stock solution was made from 72 µl of nonyl alcohol, 0.5 µl of Tween 20, 10 ml of ethanol, and 10 ml of distilled water (Barnstead International, Dubuque, IA) (Nirmala et al. 2011). The urediniospores were left to germinate overnight, and the DNA was extracted from the germinated spore mats using a cetyltrimethylammonium bromide protocol (Riede and Anderson 1996). The DNA of the 44 isolates was quantified using a Qubit2.0 fluorometer (Invitrogen, Life Technologies). In total, 250 ng of DNA was used per isolate for genotyping.

For library preparation, the DNA from each isolate was digested with *HhaI* enzyme (NEB, Ipswich, MA) for 2 h 30 min at 37°C. The samples were then digested with a second enzyme, *ApeKI* (NEB), for 2 h 30 min at 75°C. The digested DNA was cleaned up using magnetic particles (Agencourt AMPure XP, manufactured by Serafiny). The DNA pellet was then washed twice with 75% ethanol and air dried, before resuspension in H₂O. Ligation reactions containing 3 µl of 10× ligase buffer (Promega, Madison, WI), 3 units of T4 DNA ligase (Promega), 100 µM universal P1-HhaI adaptor, 4 µl of H₂O, and 100 µM *ApeKI* specific barcoded adaptor were added to 20 µl of DNA of each isolate. The sequences of adaptors are as described by Leboldus et al. (2015). The ligation reaction was left for 16 to 20 h at 4°C and thereafter inactivated for 20 min

at 65°C. The uniformity of DNA concentration of all the reactions was checked on 1% agarose gel before the reactions were combined. Each 22 reactions (barcoded isolates) were combined into one sample. Unligated adaptors from the two pooled samples were removed using a PureLink Quick Gel Extraction and PCR Purification Combo kit (Invitrogen, Thermo Fisher Scientific). The two pooled samples each containing 22 isolates were then size selected for 275-bp fragments on 1.5% agarose cassettes using the Pippin Prep (Sage Science, Beverly, MA) size selection system. The size-selected samples were then amplified (GBS library) as described by Leboldus et al. (2015). The quality of PCR reactions was verified on 1% agarose gel before the two samples were normalized to a working concentration of 70 pM. The library sequencing was performed on an Ion Torrent PGM Sequencer (Life Technologies, Grand Island, NY) using two Ion 318 Microprocessor Chips for the two created samples (one chip per sample pool). The sequencing was performed following the Ion Torrent PGM sequencing protocol at the barley pathology laboratory at North Dakota State University, Fargo, ND.

Sequencing data analysis. The sequencing reads of each isolate were individually aligned to the *P. triticina* reference genome (race1-BBBD) available on the Broad Institute website (https://www.broadinstitute.org/annotation/genome/puccinia_group/Downloads.html). This alignment was done using the Burrows-Wheeler Aligner “mem” algorithm with default settings (Li and Durbin 2009). The SNP calling was conducted using the Genome Analysis Toolkit Unified Genotyper and subsequently filtered using “vcftools” for a minimum genotype quality of 10 and a minimum read depth per SNP per individual of three (Danecek et al. 2011; DePristo et al. 2011). The variant call format file was then imported into Golden Helix SNP & Variation Suite software. SNPs and isolates with missing data > 10% were discarded. In addition, markers with minor allele frequency < 5% were eliminated from further analysis.

Neighbor-joining phylogenetic analysis. Data analysis was performed using R 3.4.1 (R Core Team 2017). The generated SNPs were used to create a neighbor-joining phylogenetic tree using the package “Poppr” (Kamvar et al. 2014), including Ape version 3.1-4 (Paradis et al. 2004), Adegnet version 1.4-2 (Jombart 2008), and Pegas version 0.6 (Paradis 2010). The neighbor-joining tree was generated using the following parameters: Nei’s distance (Nei 1972, 1978), neighbor joining (Saitou and Nei 1987), and 5,000 bootstrap replicates with a cutoff of 75%.

Results

Virulence phenotypes. Virulence phenotypes on Thatcher differential lines showed that races of *P. triticina* collected from tetraploid wheat (durum and emmer wheat) differ from those collected from hexaploid common wheat. Isolates with virulence on tetraploid wheat had fewer races compared with isolates found on common wheat (Table 2). In 57 isolates collected from tetraploid wheat, only seven races were observed, whereas in 40 isolates collected from common wheat, 21 races were identified. Therefore, one race per 1.9 isolates was found among *P. triticina* common wheat type isolates compared with one race per 8.14 isolates among *P. triticina*

Table 1. Number of single-uredinial isolates collected from different countries on durum wheat, common wheat, emmer wheat, and triticale

Country	Number of collections	Number of isolates	Number of isolates per host species			
			Common wheat	Durum wheat	Emmer wheat	Triticale
Ethiopia	12	17	1	10	6	0
Mexico	— ^a	7	2	5	0	0
Morocco	19	32	14	18	0	0
Chile	— ^a	5	3	2	0	0
Pakistan	— ^a	5	5	0	0	0
Spain	8	21	12	5	0	4
Tunisia	6	13	2	10	0	1
U.S.A.	— ^a	2	1	1	0	0
Total	45	102	40	51	6	5

^a Single-uredinial isolates were obtained from collaborators.

tetraploid wheat type isolates. In addition, tetraploid wheat type races showed virulence to a lower number of *Lr* genes (zero to five *Lr* genes). In contrast, the common wheat type races were virulent to many *Lr* genes.

Race EEEEE, which is avirulent on Thatcher wheat, was only collected from Ethiopia on durum wheat (10 isolates) and emmer wheat (six isolates). In Morocco and Chile, races BBBQJ and BBBSJ were among the isolates collected on durum wheat, indicating that virulence to *LrB*, *Lr10*, *Lr14a*, *Lr14b*, and *Lr20* exists in these two countries. In Tunisia, the virulence phenotypes BBBQJ, BBBSJ, and BBBSQ were found among the isolates collected from durum wheat, showing that virulence to *LrB*, *Lr3bg*, *Lr10*, *Lr14a*, *Lr14b*, and *Lr20* is present in Tunisia. In Spain, only BBBSJ was identified on durum wheat, with virulence to *LrB*, *Lr10*, *Lr14a*, *Lr14b*, and *Lr20*. Four races (BBBQJ, BBBQK, BCBQJ, and CBBQS) were observed on durum wheat in Mexico. Three of these races (BBBQK, BCBQJ, and CBBQS) were not present in other countries. The Mexican races were virulent to *LrB*, *Lr3a*, *Lr3bg*, *Lr10*, *Lr14b*, *Lr20*, *Lr26*, and *Lr28*. In the United States, the only described race on durum wheat was BBBQJ, with virulence to *LrB*, *Lr10*, *Lr14b*, and *Lr20*. Twenty-one races were observed in the isolates collected from common wheat across experimental sites. These common wheat type races were virulent to many of the Thatcher differential lines. Triticale isolates collected from Spain had virulence phenotypes CGBNQ, FBBPQ, and FBBNQ, and race FCBPQ was collected from triticale in Tunisia.

Durum differential lines for the Ethiopia durum wheat isolates. Unlike all other isolates collected for this study, race designations were not determined for the Ethiopian EEEEE isolates, because these unique isolates were avirulent on Thatcher. Further phenotyping of seven EEEEE isolates, collected from durum and emmer wheat, on a set of 23 durum accessions showed that there were virulence differences between the EEEEE isolates. Twelve durum accessions were resistant to all the seven EEEEE isolates, whereas Alred and Rusty were susceptible to all isolates. The remaining nine durum accessions (PI 209274, PI 244061, PI 278379, PI 387263, Mindum, Llaeta INIA, Camayo, Mexicali 75, and Russello G.S.7) displayed differential reactions to the tested EEEEE isolates and were retained as differential lines (Table 3). Previous mapping studies showed that Llaeta INIA, Camayo, PI 209274, PI 244061, and PI 387263 carry *Lr14a* on chromosome arm 7BL (Herrera-Foessel et al. 2008b), *LrCamayo* on chromosome 6BL (Herrera-Foessel et al. 2007), *LrPI 209274* on chromosome 6BS (Aoun et al. 2017), *LrPI 244061* on chromosome 2BS (Aoun et al. 2017), and *LrPI 387263* on chromosome 6BL (Aoun et al. 2017), respectively. The *Lr* genes in the remaining four differential lines are unknown. Based on the virulence phenotypes on the nine durum differentials, each of the four isolates 50-4, 49-1, 57-2, and 65-2 had a unique virulence/avirulence profile. The remaining three isolates (63-1, 63-3, and 64-1) shared the same virulence/avirulence profile on the durum differentials (Table 3). Therefore, based on the durum differential set, five virulence phenotypes were found among the seven EEEEE isolates. This revealed that the EEEEE isolates are diverse for virulence on durum wheat genotypes.

Neighbor-joining phylogenetic analysis. Of the genotyped isolates, 30 were kept for further analysis after filtering missing data points, and 2,125 SNPs were retained. Of these 30 isolates, 18 were collected from common wheat, six from durum wheat, four from triticale, and two from emmer wheat. These isolates were of diverse origin, with eight from Spain, five each from Pakistan and Chile, three each from Mexico and Morocco, and two each from Ethiopia, Tunisia, and the United States.

Phylogenetic analysis using a neighbor-joining tree based on Nei's genetic distance grouped the 30 isolates into 10 clades with high bootstrap support values. The Nei's genetic distances between isolates ranged from 0.02 to 0.63. The lowest Nei's genetic distance was observed between isolates CHL_03-D2_MCDSS and CHL_04-D2_MBDSS and between isolates PAK_1-2_BBBDH and PAK_1-3_BBBDH. The highest Nei's genetic distance was found between the isolates TUN_18_BBBSQ and MEX_12_TBDKT.

The phylogeny showed clustering based on the host from which the isolates were collected. Durum and emmer wheat isolates were clustered in two well-supported clades with 100% bootstrap values. The durum type phenotypes BBBQJ, BBBSJ, BBBSQ, and CBBQS were clustered in clade I, despite coming from Tunisia, Mexico, and Morocco. This suggests that these phenotypes may share a common origin. The two Ethiopian isolates with phenotype EEEEE were in clade II with a Nei's genetic distance of 0.34. The isolates collected from triticale with virulence phenotypes FBBNQ, FBBPQ, CGBNQ, and FCBPQ from Tunisia and Spain were grouped in clade III. The isolate MEX_14_BBBQK (clade IV) was not clustered with the other durum type isolates in clade I. However, the bootstrap value associated with the MEX_14_BBBQK was lower than the bootstrap cutoff of 75%. The isolates USA_CA1.2_BBBQJ and MOR_34-2_MBDSS were grouped together in clade V. The dissociation of USA_CA1.2_BBBQJ from the other durum isolates may have resulted from possible spore contamination or DNA sample contamination. The Pakistani isolates with virulence phenotypes CCPSL and FHPSQ were grouped together in clade VI. The common wheat Race1 from the United States (BBBDJ), its variants from Pakistan (BBBDH and CBBDK), and the Mexican race TBDKT were clustered in clade VII. Clades VIII, IX, and X included common wheat M-races. Isolates from Chile with virulence phenotypes MFPNQ, MCDSS, and MBDSS clustered in clade VIII, whereas races MCPSS and MCPSSQ from Spain were grouped in clade IX. Clade X contained Spanish races MCTNQ and MHTNQ (Fig. 1).

The isolates within the same clade have similar virulence phenotypes. The number of virulence differences among isolates in each clade based on the 20 *Lr* genes in Thatcher NILs ranged from zero in clade II to 10 differences in clade VII. On average, only four virulence differences were observed between isolates within the same clade (Fig. 1). The average correlation coefficients between the isolate virulence/avirulence phenotypes were 0.8, 1.0, 0.8, 0.5, 0.7, 0.6, 0.7, 0.8, and 0.9 in clade I, clade II, clade III, clade V, clade VI, clade VII, clade VIII, clade IX, and clade X, respectively. This suggests high correlation between the SNP genotypes and virulence phenotypes (Fig. 1).

Discussion

In the current study, we used the RAD-GBS based on the Ion Torrent platform to genotype *P. tritricina* isolates collected from different hosts in several countries and representing several virulence phenotypes. This genotyping method was fast, low-cost, and provided a high density of SNPs compared with previous genotyping methods. There was a relationship between the SNP genotypes and virulence phenotypes, which is expected in clonal populations. This finding is in agreement with several studies, showing moderate correlation between *P. tritricina* virulence phenotypes and SSR genotypes (Kolmer 2015; Kolmer et al. 2011, 2013; Ordoñez and Kolmer 2007b, 2009; Ordoñez et al. 2010; Wang et al. 2010a, 2010b). The present study also revealed clear phylogenetic separation between isolates collected from different hosts. In addition, there were more phylogenetic clades observed for the common wheat isolates compared with those observed for tetraploid wheat isolates. This agrees with previous studies that showed limited molecular diversity among isolates from durum wheat and high molecular variation among isolates from common wheat (Kolmer et al. 2019). In contrast to previous phylogenetic trees based on SSRs or AFLPs, a phylogenetic tree based on SNPs was supported by high bootstrap values. This indicates that RAD-GBS using Ion Torrent is a reliable tool providing sufficient and robust SNPs for accurate estimation of genetic distance between *P. tritricina* isolates. Applying this genotyping method on a larger number of *P. tritricina* isolates collected globally will provide a better understanding of the population dynamics and genotypic diversity of *P. tritricina*. SNP markers developed from this low-cost and fast genotyping method could also be used for genome-wide association studies to map genes involved with virulence in *P. tritricina*.

The virulence phenotypes of 102 *P. tritricina* isolates showed distinct differences between durum wheat isolates and common wheat

isolates. Durum wheat races were avirulent to many *Lr* genes found in Thatcher differentials, indicating that the selection and spread of these races occur only on durum wheat cultivars. The phylogenetic analysis in this study showed that apart from the Ethiopian EEEEE isolates, most of the tetraploid wheat type isolates were related, suggesting a common ancestor. This limited genetic variation of durum wheat specific isolates indicates that most of the *Lr* genes deployed in one region could also be used in other regions worldwide (Ordoñez and Kolmer 2007b). For example, the leaf rust resistance gene *Lr.ace-4A* in the USDA-NSGC accession PI 192051 conferred resistance against all isolates collected from durum wheat in Tunisia, California, Morocco, and Ethiopia (Aoun et al. 2019).

Six races named BBBQJ, BBBSJ, BBBQK, BBBSQ, BCBQJ, and CBBQS were identified on durum wheat, indicating virulence to nine known resistance genes (*LrB*, *Lr3a*, *Lr3bg*, *Lr10*, *Lr14a*, *Lr14b*, *Lr20*, *Lr26*, and *Lr28*) in the Thatcher differential lines. Virulence to some of these *Lr* genes was previously reported among durum wheat isolates. For instance, virulence to *LrB*, *Lr3a*, *Lr10*, *Lr14a*, *Lr14b*, *Lr20*, and *Lr28* was identified in previous studies (Gharbi et al. 2013; Goyeau et al. 2012; Herrera-Foessel et al. 2014b; Huerta-Espino et al. 2009; Kolmer and Acevedo 2016; Ordoñez and Kolmer 2007a; Soleiman et al. 2016). This is the first study to report virulent races to *Lr3bg* in Tunisia, *Lr14a* in Morocco, and *Lr3bg* and *Lr28* in Mexico. The high virulence diversity observed

Table 2. Races of *Puccinia triticina* identified from samples collected from different countries and hosts

Isolate ID	Country	Location	Host	Variety/line	Race	Virulence to <i>Lr</i> genes	GBS ^a
CHL03-D2	Chile		<i>T. aestivum</i>		MCDSS	<i>Lr1, 3a, 3bg, 10, 14a, 14b, 17, 20, 26, B</i>	X
CHL04-D2	Chile		<i>T. aestivum</i>		MBDSS	<i>Lr1, 3a, 3bg, 10, 14a, 14b, 17, 20, B</i>	X
CHL33-D2	Chile		<i>T. aestivum</i>		MFPNQ	<i>Lr1, 3a, 3ka, 3bg, 14a, 14b, 17, 24, 26, 30, B</i>	X
CHL02-D1	Chile		<i>T. turgidum</i>		BBBSJ	<i>Lr10, 14a, 14b, 20, B</i>	X
CHL14-1	Chile		<i>T. turgidum</i>		BBBQJ	<i>Lr10, 14b, 20, B</i>	X
52-1 ^b	Ethiopia	DZARC- Bishoftu	<i>T. aestivum</i>	B21	MBJS-	<i>Lr1, 3a, 10, 11, 14a, 17, B</i>	
45-6 ^b	Ethiopia	DZARC- Bishoftu	<i>T. dicoccum</i>		EEEE	–	
47-2 ^b	Ethiopia	DZARC- Bishoftu	<i>T. dicoccum</i>		EEEE	–	
48-3 ^b	Ethiopia	DZARC- Bishoftu	<i>T. dicoccum</i>		EEEE	–	X
49-1 ^b	Ethiopia	DZARC- Bishoftu	<i>T. dicoccum</i>		EEEE	–	
49-2	Ethiopia	DZARC- Bishoftu	<i>T. dicoccum</i>		EEEE	–	
50-1 ^b	Ethiopia	DZARC- Bishoftu	<i>T. dicoccum</i>		EEEE	–	
50-4 ^b	Ethiopia	DZARC- Bishoftu	<i>T. dicoccum</i>		EEEE	–	X
57-2	Ethiopia	DZARC- Bishoftu	<i>T. turgidum</i>	182/Sr30	EEEE	–	
58-1 ^b	Ethiopia	DZARC- Bishoftu	<i>T. turgidum</i>	Rusty	EEEE	–	
62-2 ^b	Ethiopia	DZARC- Bishoftu	<i>T. turgidum</i>	PI 272553 (NSGC)	EEEE	–	X ^c
63-1	Ethiopia	DZARC- Bishoftu	<i>T. turgidum</i>	PI 387346 (NSGC)	EEEE	–	
63-2	Ethiopia	DZARC- Bishoftu	<i>T. turgidum</i>	PI 387346 (NSGC)	EEEE	–	
63-3	Ethiopia	DZARC- Bishoftu	<i>T. turgidum</i>	PI 387346 (NSGC)	EEEE	–	X ^c
64-1 ^b	Ethiopia	DZARC- Bishoftu	<i>T. turgidum</i>	PI 478427 (NSGC)	EEEE	–	
64-3 ^b	Ethiopia	DZARC- Bishoftu	<i>T. turgidum</i>	PI 478427 (NSGC)	EEEE	–	
65-2	Ethiopia	DZARC- Bishoftu	<i>T. turgidum</i>	PI 298547 (NSGC)	EEEE	–	
12	Mexico		<i>T. aestivum</i>		TBDKT	<i>Lr1, 2a, 2c, 3a, 3bg, 10, 14a, 14b, 17, 18, 20, 28</i>	X
17	Mexico		<i>T. aestivum</i>		CBDSS	<i>Lr3a, 3bg, 10, 14a, 14b, 17, 18, 20, 28, B</i>	X ^c
13	Mexico		<i>T. turgidum</i>		BBBQJ	<i>Lr10, 14b, 20, B</i>	
10	Mexico		<i>T. turgidum</i>		BCBQJ	<i>Lr10, 14b, 20, 26, B</i>	
11	Mexico		<i>T. turgidum</i>		CBBQS	<i>Lr3a, 10, 3bg, 14b, 20, B</i>	X
14	Mexico		<i>T. turgidum</i>		BBBQK	<i>Lr10, 14b, 20, 28, B</i>	X
15	Mexico		<i>T. turgidum</i>		BBBQJ	<i>Lr10, 14b, 20, B</i>	
28-1	Morocco	Zemamra	<i>T. aestivum</i>		MCDSS	<i>Lr1, 3a, 3bg, 10, 14a, 14b, 17, 20, 26, B</i>	
28-2	Morocco	Zemamra	<i>T. aestivum</i>		MCDSS	<i>Lr1, 3a, 3bg, 10, 14a, 14b, 17, 20, 26, B</i>	
29-1	Morocco	Zemamra	<i>T. aestivum</i>		MCDSS	<i>Lr1, 3a, 3bg, 10, 14a, 14b, 17, 20, 26, B</i>	
29-2	Morocco	Zemamra	<i>T. aestivum</i>		MCDSS	<i>Lr1, 3a, 3bg, 10, 14a, 14b, 17, 20, 26, B</i>	
30-1 ^b	Morocco	Zemamra	<i>T. aestivum</i>		BBBQJ	<i>Lr10, 14b, 20, B</i>	
30-2	Morocco	Zemamra	<i>T. aestivum</i>		BBBQJ	<i>Lr10, 14b, 20, B</i>	
30-3	Morocco	Zemamra	<i>T. aestivum</i>		BBBQJ	<i>Lr10, 14b, 20, B</i>	X
34-2	Morocco	Jemaa-Shaam	<i>T. aestivum</i>		MBDSS	<i>Lr1, 3a, 3bg, 10, 14a, 14b, 17, 20, B</i>	X
35-1	Morocco	Jemaa-Shaam	<i>T. aestivum</i>		FBBPQ	<i>Lr2c, 3a, 3bg, 14a, 14b, B</i>	
41-1	Morocco		<i>T. aestivum</i>	Taza (seisii)	MCDST	<i>Lr1, 3a, 3bg, 10, 14a, 14b, 17, 20, 26, 28, B</i>	X ^c
43-1 ^b	Morocco		<i>T. aestivum</i>	Meknes	MCDSS	<i>Lr1, 3a, 3bg, 10, 14a, 14b, 17, 20, 26, B</i>	
43-3	Morocco		<i>T. aestivum</i>	Meknes	MCDSS	<i>Lr1, 3a, 3bg, 10, 14a, 14b, 17, 20, 26, B</i>	X ^c
44-1 ^b	Morocco		<i>T. aestivum</i>	Seiz	MCDSS	<i>Lr1, 3a, 3bg, 10, 14a, 14b, 17, 20, 26, B</i>	
25-1	Morocco	Zemamra	<i>T. turgidum</i>	MERZAV	BBBQJ	<i>Lr10, 14b, 20, B</i>	
25-2 ^b	Morocco	Zemamra	<i>T. turgidum</i>	MERZAV	BBBQJ	<i>Lr10, 14b, 20, B</i>	
25-6 ^b	Morocco	Zemamra	<i>T. turgidum</i>	MERZAV	BBBQJ	<i>Lr10, 14b, 20, B</i>	
26-1	Morocco	Zemamra	<i>T. turgidum</i>		BBBQJ	<i>Lr10, 14b, 20, B</i>	X ^c
26-2	Morocco	Zemamra	<i>T. turgidum</i>		BBBQJ	<i>Lr10, 14b, 20, B</i>	
26-3	Morocco	Zemamra	<i>T. turgidum</i>		BBBSJ	<i>Lr10, 14a, 14b, 20, B</i>	

(Continued on next page)

^a The samples with symbol “X” were genotyped using restriction-associated DNA genotyping-by-sequencing (GBS).

^b Isolates phenotyped at the North Dakota Agricultural Experiment Station Greenhouse Complex, Fargo, ND; the rest of the isolates were phenotyped at the USDA-ARS Cereal Disease Laboratory, St. Paul, MN.

^c Isolates removed from analysis because of missing genotypic data points > 10%.

in fields in Mexico is probably due to the extensive planting of durum wheat in the country and selection pressure caused by the sequential utilization of a number of *Lr* genes in the CIMMYT durum breeding lines and cultivars. Recent leaf rust outbreaks on durum wheat have been recorded in Mexico over the past decades (Herrera-Foessel et al. 2005, 2007, 2008a, 2008b, 2014a), due the appearance of races with virulence to the newly deployed leaf rust resistance genes in the durum cultivars. Utilization of more complex leaf rust resistance such as stacking of multiple genes and use of non-race-specific genes in the newly released cultivars can reduce the chance of future outbreaks and potentially slow down the selection process for new virulences (Ayliffe et al. 2008).

Isolates with virulence to tetraploid wheat designated as race EEEEE types were only observed in Ethiopia. The phylogenetic analysis based on SNP genotypes distinguished the EEEEE isolates from all other isolates. Similar observations were made in previous studies based on virulence phenotypes and SSR genotypes (Huerta-Espino and Roelfs 1992; Kolmer and Acevedo 2016; Ordoñez and Kolmer 2007a, 2007b). These isolates have most likely evolved and been maintained in Ethiopia due to the high diversity of tetraploid wheat population grown in the country (Kolmer and Acevedo 2016). In this study, we observed small genotypic variation between EEEEE isolates, which was similar to results obtained by whole genome sequencing of a number of EEEEE isolates (J. A. Kolmer,

Table 2. (Continued from previous page)

Isolate ID	Country	Location	Host	Variety/line	Race	Virulence to <i>Lr</i> genes	GBS ^a
27-1	Morocco	Zemamra	<i>T. turgidum</i>	CARIOCA	BBBQJ	<i>Lr10, 14b, 20, B</i>	
27-2 ^b	Morocco	Zemamra	<i>T. turgidum</i>	CARIOCA	BBBQJ	<i>Lr10, 14b, 20, B</i>	X ^c
32-2	Morocco	Jemaa-Shaam	<i>T. turgidum</i>		BBBSJ	<i>Lr10, 14a, 14b, 20, B</i>	
33-1 ^b	Morocco	Jemaa-Shaam	<i>T. turgidum</i>		BBBSJ	<i>Lr10, 14a, 14b, 20, B</i>	X ^c
36-1 ^b	Morocco	Jemaa-Shaam	<i>T. turgidum</i>		BBBSJ	<i>Lr10, 14a, 14b, 20, B</i>	
36-3	Morocco	Jemaa-Shaam	<i>T. turgidum</i>		BBBQJ	<i>Lr10, 14b, 20, B</i>	
37-2 ^b	Morocco	Rabat	<i>T. turgidum</i>	RL7075	BBBQJ	<i>Lr10, 14b, 20, B</i>	
38-1	Morocco	Rabat	<i>T. turgidum</i>	Cali (A33File1)	BBBQJ	<i>Lr10, 14b, 20, B</i>	
38-2	Morocco	Rabat	<i>T. turgidum</i>	Cali (A33File1)	BBBQJ	<i>Lr10, 14b, 20, B</i>	X
38-3	Morocco	Rabat	<i>T. turgidum</i>	Cali (A33File1)	BBBQJ	<i>Lr10, 14b, 20, B</i>	
39-9	Morocco	Marchouch	<i>T. turgidum</i>	Kristal/Sebatel #7	BBBQJ	<i>Lr10, 14b, 20, B</i>	
40-1	Morocco	Marchouch	<i>T. turgidum</i>	Kristal	BBBQJ	<i>Lr10, 14b, 20, B</i>	
13PAK15-1	Pakistan		<i>T. aestivum</i>		FHPSQ	<i>Lr2c, 3a, 3ka, 3bg, 10, 14a, 14b, 16, 17, 26, 30, B</i>	X
13PAK17-1	Pakistan		<i>T. aestivum</i>		CCPSL	<i>Lr3a, 3ka, 3bg, 10, 14a, 17, 26, 30, B</i>	X
14PAK1-2	Pakistan		<i>T. aestivum</i>		BBBDH	<i>Lr14a, 14b, 28</i>	X
14PAK1-3	Pakistan		<i>T. aestivum</i>		BBBDH	<i>Lr14a, 14b, 28</i>	X
14PAK1-4	Pakistan		<i>T. aestivum</i>		CBBDK	<i>Lr3a, 14a, 14b, 20, 28</i>	X
6-1	Spain	Jerez de la Frontera	<i>Triticale</i>		FBBNQ	<i>Lr2c, 3a, 3bg, 14a, 14b, B</i>	X
6-2	Spain	Jerez de la Frontera	<i>Triticale</i>		FBBPQ	<i>Lr2c, 3a, 3bg, 14a, 14b, 18, B</i>	X
6-3	Spain	Jerez de la Frontera	<i>Triticale</i>		CBGNQ	<i>Lr3a, 3bg, 11, 14a, 14b, B</i>	X
6-4	Spain	Jerez de la Frontera	<i>Triticale</i>		FBBPQ	<i>Lr2c, 3a, 3bg, 14a, 14b, B</i>	
2-1	Spain	Learza (Navarra)	<i>T. aestivum</i>	NSA11-8606	MCPSS	<i>Lr1, 3a, 14a, 18, 26, B</i>	X
2-2	Spain	Learza (Navarra)	<i>T. aestivum</i>	NSA11-8606	MCPSS	<i>Lr1, 3a, 14a, 18, 26, B</i>	
2-3	Spain	Learza (Navarra)	<i>T. aestivum</i>	NSA11-8606	MCPSS	<i>Lr1, 3a, 14a, 18, 26, B</i>	
3-1	Spain	Huesca	<i>T. aestivum</i>	L6W14-8082	MCDSQ	<i>Lr1, 3a, 3bg, 10, 14a, 14b, 17, 26, B</i>	
3-2	Spain	Huesca	<i>T. aestivum</i>	L6W14-8082	MCDSQ	<i>Lr1, 3a, 3bg, 10, 14a, 14b, 17, 26, B</i>	
7-1	Spain	Huesca	<i>T. aestivum</i>	12-15-1515	MCTNQ	<i>Lr1, 3a, 3ka, 3bg, 11, 14a, 14b, 17, 26, 30, B</i>	X
7-2	Spain	Huesca	<i>T. aestivum</i>	12-15-1515	MHTNQ	<i>Lr1, 3a, 3ka, 3bg, 11, 14a, 14b, 16, 17, 26, 30, B</i>	X
7-3	Spain	Huesca	<i>T. aestivum</i>	12-15-1515	MCTNQ	<i>Lr1, 3a, 3ka, 3bg, 11, 14a, 14b, 17, 26, 30, B</i>	X
8-1	Spain	Peralta/Navarra	<i>T. aestivum</i>	125B0254-B	MCPSS	<i>Lr1, 3a, 3ka, 3bg, 10, 14a, 14b, 17, 20, 26, 30, B</i>	
8-2	Spain	Peralta/Navarra	<i>T. aestivum</i>	125B0254-B	MCPSS	<i>Lr1, 3a, 3ka, 3bg, 10, 14a, 14b, 17, 20, 26, 30, B</i>	
9-1	Spain		<i>T. aestivum</i>	Garcia	MHPSQ	<i>Lr1, 3a, 3ka, 3bg, 10, 14a, 14b, 16, 17, 26, B</i>	
9-2	Spain		<i>T. aestivum</i>	Garcia	MCPSQ	<i>Lr1, 3a, 3ka, 3bg, 10, 14a, 14b, 17, 26, B</i>	X
4-1	Spain	Coril (Cadiz)	<i>T. turgidum</i>	Don Jaime <i>Lr14a</i>	BBBSJ	<i>Lr10, 14a, 14b, 20, B</i>	X ^c
4-2	Spain	Coril (Cadiz)	<i>T. turgidum</i>	Don Jaime <i>Lr14a</i>	BBBSJ	<i>Lr10, 14a, 14b, 20, B</i>	
4-3	Spain	Coril (Cadiz)	<i>T. turgidum</i>	Don Jaime <i>Lr14a</i>	BBBSJ	<i>Lr10, 14a, 14b, 20, B</i>	
5-1	Spain	Coril (Cadiz)	<i>T. turgidum</i>	Gallareta <i>Lr72</i>	BBBSJ	<i>Lr10, 14a, 14b, 20, B</i>	
5-2	Spain	Coril (Cadiz)	<i>T. turgidum</i>	Gallareta <i>Lr72</i>	BBBSJ	<i>Lr10, 14a, 14b, 20, B</i>	X ^c
19-1	Tunisia		<i>Triticale</i>		FCBPQ	<i>Lr2c, 3a, 3bg, 14a, 14b, 26, B</i>	X
18-1	Tunisia	Beja exp. station	<i>T. aestivum</i>	UNK	BBBSQ	<i>LrB, 3bg, 10, 14a, 14b</i>	X
18-2	Tunisia	Beja exp. station	<i>T. aestivum</i>	UNK	BBBSJ	<i>Lr10, 14a, 14b, 20, B</i>	
20-1	Tunisia	Grombalia	<i>T. turgidum</i>	Maali	BBBSJ	<i>Lr10, 14a, 14b, 20, B</i>	
20-2	Tunisia	Grombalia	<i>T. turgidum</i>	Maali	BBBSJ	<i>Lr10, 14a, 14b, 20, B</i>	
20-4	Tunisia	Grombalia	<i>T. turgidum</i>	Maali	BBBSJ	<i>Lr10, 14a, 14b, 20, B</i>	X ^c
22-2	Tunisia	Jendouba	<i>T. turgidum</i>	Maali	BBBSJ	<i>Lr10, 14a, 14b, 20, B</i>	X ^c
22-3	Tunisia	Beja exp. station	<i>T. turgidum</i>	Maali	BBBSJ	<i>Lr10, 14a, 14b, 20, B</i>	
23-1	Tunisia	Mateur	<i>T. turgidum</i>	Nasr	BBBSJ	<i>Lr10, 14a, 14b, 20, B</i>	
23-2	Tunisia	Mateur	<i>T. turgidum</i>	Nasr	BBBSJ	<i>Lr10, 14a, 14b, 20, B</i>	
23-3	Tunisia	Mateur	<i>T. turgidum</i>	Nasr	BBBSJ	<i>Lr10, 14a, 14b, 20, B</i>	
24-1	Tunisia	INRAT	<i>T. turgidum</i>		BBBQJ	<i>Lr10, 14b, 20, B</i>	X ^c
24-2	Tunisia	INRAT	<i>T. turgidum</i>		BBBSQ	<i>LrB, 3bg, 10, 14a, 14b</i>	X ^c
Race1	U.S.A.		<i>T. aestivum</i>		BBBDJ	<i>Lr14a, 14b, 20</i>	X
CA1.2	U.S.A.	California	<i>T. turgidum</i>		BBBQJ	<i>Lr10, 14b, 20, B</i>	X

unpublished data). Coalescence and phylogenetic analysis indicated that the EEEEE isolates were the first form of *P. triticina* to have virulence to either tetraploid or hexaploid wheat (Liu et al. 2014). The unique wheat population in Ethiopia that includes

landrace durum and emmer wheat cultivars may have selected this particular phenotype (Kolmer and Acevedo 2016). We identified five virulence phenotypes among seven tested EEEEE isolates based on durum differentials. This differential set can be useful to further study

Table 3. Virulence/avirulence profile of seven Ethiopian EEEEE isolates tested on durum wheat accessions^a

Line	Type	Country	49-1: ^b EEEE	50-4: EEEE	57-2: EEEE	63-1: EEEE	63-3: EEEE	64-1: EEEE	65-2: EEEE
PI 45442	Cultivated	South Africa	0;	;13	0;	;	;1	;1-	;1
PI 192051	Landrace	Portugal	0;	;1	;1-	0;	0;	0;	0;
PI 195693	Landrace	Ethiopia	;1	1+3	;1-	;1	;1	;1-	;1
PI 209274 ^c	Breeding line	Australia	3	3	;1+	3	3	3	3
PI 244061 ^c	Landrace	Yemen	.	31	0;	;	;	;	0;
PI 278379 ^c	Landrace	Malta	1	3	2-	3	3	.	;1
PI 313096	Landrace	Cyprus	0	;1	;1	0	0	1-	0
PI 324928	Breeding line	Argentina	0;	;13	1-	;1	;	;13-	;1-
PI 342647	Cultivated	Lebanon	0;	;1+	;1-	;	0;	;	0
PI 387263 ^c	Landrace	Ethiopia	1+	3	;1	3	3	3-	1+
PI 519832	Cultivar	Lebanon	0	;1	;1-	1	;1	0;	;1
PI 534304	Landrace	Ethiopia	0;	;13	0	;	0;	0;	0;
Divide	Cultivar	U.S.A.	2+3-	13	1	;1	1+	2+3	;1
Rusty	Line	U.S.A.	3	3	3-	3	.	3	3
Capelli	Cultivar	Italy	;1-	;1+	;1	;1	1	;1-	1
Mindum ^c	Cultivar	U.S.A.	3	;1	1	;1	;1	1	;1-
Creso	Cultivar	Italy	0;	;1-	;1-	;	0;	;1-	0
Llaret INIA ^c	Cultivar	Mexico	3	3+	1	;13	;13	1+	;13
Camayo ^c	Cultivar	Mexico	;1-	3	0;	1	;1	;1-	;1
Jupare C2001	Cultivar	Mexico	;1-	;	1	;1	;1-	;1	;1-
Mexicali 75 ^c	Cultivar	Mexico	3	3	;1	3	3-	3-	2
Alred	Line	Mexico	3	4	3	3	32+	3	32+
Russello S.G.7 ^c	Cultivar	Italy	3	3	2	3	3	3	3
Thatcher	Cultivar	U.S.A.	;1	;1-	2+	;1-	1-	;1	2

^a All the accessions are *Triticum turgidum* spp. *durum*, except Thatcher is *T. aestivum* (resistant check).

^b Isolate number.

^c Accessions that differentiated between the Ethiopian EEEEE isolates.

Fig. 1. Neighbor-joining phylogenetic tree of 30 *Puccinia triticina* isolates collected on durum wheat, emmer wheat, common wheat, and triticale, based on Nei's genetic distances, calculated using 2,125 single nucleotide polymorphism markers. Isolate name was based on country (USA = United States, CHL = Chile, MEX = Mexico, PAK = Pakistan, MOR = Morocco, TUN = Tunisia, and ETH = Ethiopia), collection number, and race-based virulence phenotypes on Thatcher differentials. Numbers along the nodes are bootstrap values >75% in 5,000 replicates.

virulence diversity in durum wheat type isolates. Previously, a differential set adapted for *P. trititica* isolates collected on durum wheat was implemented by Goyeau et al. (2012). With the increased susceptibility of durum to leaf rust worldwide, there is a need to develop a standardized differential set adequate to assess virulence diversity in the durum type *P. trititica* population.

In summary, the current study is the first of its kind in using RAD-GBS to study the genetic variation in *P. trititica*. The abundant and robust SNPs that are distributed across the genome provided higher levels of support for estimating the genetic distances between isolates. There was a clear separation between isolates from different hosts, with higher genotypic diversity in isolates collected from common wheat compared with that observed in isolates collected from tetraploid wheat. In contrast to common wheat races, virulence to *Lr* genes *LrB*, *Lr3a*, *Lr3bg*, *Lr10*, *Lr14a*, *Lr14b*, *Lr20*, *Lr26*, and *Lr28* was observed among the durum wheat races. The EEEEE isolates collected from tetraploid wheat in Ethiopia showed a unique virulence phenotype and SNP genotype. Durum differentials were developed to identify the virulence diversity between the Ethiopian EEEEE isolates.

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