Pathway-specific regulation of the botcinic acid biosynthetic gene cluster in the grey mould fungus Botrytis cinerea

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Population Biology

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Multilocus Genotypes of the Wheat Leaf Rust Fungus *Puccinia triticina* in Worldwide Regions Indicate Past and Current Long-Distance Migration

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ABSTRACT

Many plant pathogenic fungi have a global distribution across diverse ecological zones and agricultural production systems. *Puccinia triticina*, the wheat leaf rust fungus, is a major pathogen in many wheat production areas of the world. The objective of this research was to determine the genetic relatedness of *P. triticina* in different worldwide regions. A total of 831 single-uredinial isolates collected from 11 regions were characterized for multilocus genotype at 23 simple sequence repeat loci and for virulence to 20 lines of wheat with single genes for leaf rust resistance. A total of 424 multilocus genotypes and 497 virulence phenotypes were found. All populations had high heterozygosity and

significant correlation between virulence and molecular variation, which indicated clonal reproduction. The populations from North America and South America, Central Asia and Russia, and the Middle East and Europe were closely related for multilocus genotypes and many individual isolates from other continental regions were closely related. Twenty-seven multilocus genotypes were found in more than one continental region, and 13 of these had isolates with identical virulence phenotypes. The wide geographic distribution of identical and highly related multilocus genotypes of *P. triticina* indicated past and more recent migration events facilitated by the spread of clonally produced urediniospores.

Many important plant pathogens and pests are widespread throughout the major agricultural and ecological regions of the world. Plant pathogenic fungi in particular are distributed across different continents and are rarely endemic to only one country or continental region (Bebber et al. 2014). Although fungi have the narrowest host range of various pathogens and pests, they have the highest saturation rate (the number of countries where the pathogen or pest was found/the number of countries where the pathogen or pest could be present based on host population) compared with other pathogens and insect pests. Among crop pathogens and pests, fungal pathogens have the lowest rate of regional endemicity at 5.8% and are the leading category of global plant pathogen invaders (Bebber et al. 2014).

The wide distribution is evidence of the ability of fungal plant pathogens to adapt to natural host environments (Gladieux et al. 2015) and also to exploit the genetic uniformity of many host populations in human-guided agricultural production systems. Fungal pathogens have achieved this in various ways, depending on their means of dispersal. Pathogen distribution is affected by human activities, as in the case of *Cryphonectria parasitica*, the chestnut blight fungus, and *Ophiostoma ulmi*, the Dutch elm disease fungus, both of which were introduced to North America in the last century

by transport of infected plant material from Europe (Brown and Hovmøller 2002). Other types of fungal pathogens, such as rust fungi, have infective spores that are adapted for wind dissemination over thousands of kilometers (Kolmer 2005; Visser et al. 2019), in addition to being transported over long distances by human activities (Park et al. 1995; Steele et al. 2001).

Puccinia triticina Erikss., the wheat leaf rust fungus, is found throughout most of the nonarid wheat production areas of the world (Roelfs et al. 1992; Saari and Prescott 1985; Samborski 1985). The principal uredinial-telial hosts are hexaploid common wheat (*Triticum aestivum* L.), tetraploid durum wheat (*T. turgidum* L.), and wild emmer wheat (*T. dicoccoides* L.) (Bolton et al. 2008). Clonal reproduction of urediniospores occurs on the uredinial-telial hosts. Since *P. triticina* is a heteroecious rust, an alternate host is required for completion of the sexual cycle. The native habitat of the most compatible alternate host (pycnial-aecial spore stages), *Thalictrum speciosissimum* L., is limited to southern Europe (Casulli 1988; Casulli and Siniscalco 1987; Sibilia et al. 1963; Tommasi et al. 1980) and parts of West Asia (Tatlidil et al. 2005).

Individual populations of *P. triticina* have been examined for molecular variation and virulence in numerous studies. Annual virulence surveys of *P. triticina* are conducted in the United States (Kolmer and Hughes 2017) and Canada (McCallum and Seto-Goh 2006), which provide immediate information to help inform and support breeding programs in the selection of resistant wheat germplasm. Although there is considerable knowledge regarding genetic variation within different continental regions, no previous study has examined the genetic relationship and diversity of *P. triticina* populations between different continental regions. At the U.S. Department of Agriculture Agricultural Research Service (USDA-ARS) Cereal Disease Laboratory, *P. triticina* populations from North America (Ordoñez and Kolmer 2009), South America

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(Ordoñez et al. 2010), the Middle East (Kolmer et al. 2011), Central Asia (Kolmer and Ordoñez 2007), Europe (Kolmer et al. 2012), Russia (Kolmer et al. 2015), China (Kolmer 2015), East Africa (Kolmer and Acevedo 2016), and Pakistan (Kolmer et al. 2017) were examined for virulence to a standard set of wheat differential lines and for multilocus genotype (MLG) using a standard set of simple sequence repeat (SSR) markers. The objective of this study was to determine the genetic relatedness among these *P. triticina* populations. We also wished to determine whether MLGs and virulence phenotypes of *P. triticina* are closely associated with geographic regions or if individual isolates from different continents are highly related for both SSR variation and virulence, which would support the hypothesis of migration of clonal urediniospores between continental regions.

MATERIALS AND METHODS

Isolates of *P. triticina*. Isolates of *P. triticina* were obtained from field surveys and collections held in national laboratories in North America, South America, the Middle East, Central Asia, Europe, East Africa, Russia, China, and Pakistan (Supplementary Table S1). Collections were made from both common hexaploid wheat and from tetraploid durum wheat. Additional field collections were obtained from New Zealand in the mid-1990s, while isolates from South Africa were obtained in the mid-1990s and in 2012. All collections and isolates were purified by increasing urediniospores taken from a single uredinium on susceptible wheat seedlings in isolation chambers in a greenhouse (Kolmer and Hughes 2017). All single-uredinial isolates were assessed for seedling virulence to a set of 20 Thatcher wheat lines that are near isogenic for the following single leaf rust resistance genes: *Lr1*, *Lr2a*, *Lr2c*, *Lr3*, *Lr9*, *Lr16*, *Lr24*, *Lr26*, *Lr3ka*, *Lr11*, *Lr17*, *Lr30*, *LrB*, *Lr10*, *Lr14a*, *Lr18*, *Lr3bg*, *Lr14b*, *Lr20*, *Lr28*, and Thatcher as a susceptible control. The procedures and greenhouse conditions were as previously described (Kolmer and Hughes 2017; Ordoñez and Kolmer 2009). The isolates were rated for virulence to the individual Thatcher differential lines 10 to 12 days after inoculation. Isolates with infection types 3 and 4 (moderate to large uredinia lacking necrosis or chlorosis) were considered virulent, and infection types 0 to 2+ (immune response with no sign of infection to moderate size uredinia with prominent chlorosis) were considered avirulent to the particular Thatcher line. The virulence phenotypes were described using a five-letter code, in which each letter described virulence or avirulence to four Thatcher differential lines (Supplementary Table S2), adapted from the hexadecimal nomenclature used to describe virulence in *P. triticina* (Long and Kolmer 1989). Each isolate was also given a 20-digit binary number based on the avirulent/virulent response to the Thatcher differential lines.

Molecular genotyping. DNA was extracted using the Omniprep kit (G-Biosciences) from at least one isolate of every virulence phenotype (or race) from each country or region in each of the continental populations. If multiple isolates with the same virulence designation were found in a country or region, DNA was extracted from at least two isolates of the same phenotype. All isolates were genotyped for allelic variation with a set of 23 SSR primers developed from genomic libraries of *P. triticina*. Primers *Pt3*, *Pt13*, *Pt50*, *Pt55*, *Pt61*, *Pt68-1*, *Pt76-2*, *Pt91*, *Pt92*, *Pt138*, *Pt152*, *Pt154*, *Pt158*, *Pt161*, *Pt164*, *Pt173*, *Pt184*, and *Pt186* were developed by Szabo and Kolmer (2007), and primers *Fr1*, *Fr8*, *Fr11*, *Fr26*, and *Fr35* were developed by Duan et al. (2003). The PCR products were labeled with fluorescent primers and allele sizes in base pairs scored visually for each primer pair using a LI-COR 4200 or 4300 DNA sequencer with IRDye 700 molecular weight standards. Molecular weights for all alleles of each isolate were standardized with alleles from two isolates that were used as controls in all studies. All isolates were assigned an MLG number in GenAlex 6.502 (Peakall and Smouse 2012).

A total of 831 *P. triticina* isolates from 11 regions were analyzed for various measures of genetic diversity, genetic distance, and differentiation. All populations were clone corrected, with only a single isolate for each virulence phenotype-MLG combination within each country or region in each population retained. Since different phenotypes could have identical MLGs, multiple isolates could have the same MLG within the same population, while the same phenotypes could have different MLGs.

Analysis of regional populations. The R-based program Poppr 2.02 (Kamvar et al. 2014) was used to determine the number of MLGs and the genetic distance between regional populations. An unrooted neighbor-joining dendrogram of Nei's genetic distance between regional populations was generated in the R-based program APE (Paradis et al. 2004) with 1,000 bootstrap samples. Values of F_{ST} between the regional populations were determined with GenAlex. Allelic diversity (average number of alleles/locus, average number of effective alleles/locus), observed (H_o) and expected (H_e) heterozygosity, and the inbreeding coefficient F_{IS} averaged over all loci for each population were determined with GenAlex. Minimum spanning trees of MLGs in the regional populations were generated in Poppr.

Analysis of MLG groups. In previous studies of *P. triticina* variation in individual regions (Kolmer 2015; Kolmer and Acevedo 2016; Kolmer and Ordoñez 2007; Kolmer et al. 2011, 2012, 2015, 2017; Ordoñez and Kolmer 2009; Ordoñez et al. 2010), a total of 40 MLG groups within the regional populations were determined using principal coordinate plots in GenAlex, Bayesian methods in Structure (Pritchard et al. 2000), and *k* means clustering in Genodive version 2.0b27 (Meirmans and van Tienderen 2004). The number of MLGs, effective number of MLGs, and Simpson's unbiased (λ) diversity for each MLG group were determined with Genodive. An unrooted neighbor-joining tree of Nei's genetic distance between the MLG groups was generated with APE with 1,000 bootstrap samples. Analysis of molecular variation (AMOVA) and values of F_{ST} between the MLG groups were determined with GenAlex.

Values of P_{sex} (Stenberg et al. 2003) for each MLG that occurred in more than one regional population were calculated with GenAlex. The relationship between the virulence phenotype of all of the isolates and their MLGs was determined by comparing the distance matrix of the 20-digit binary numbers with the genetic distance matrix of the MLGs, using the Mantel correlation coefficient in GenAlex. The correlation between MLG and virulence was also determined for the 11 regional populations individually.

RESULTS

A total of 172 alleles at the 23 SSR loci were used to genotype the *P. triticina* populations from 11 regions. Of the 831 *P. triticina* isolates that were genotyped, there were a total of 424 MLGs. The 23 SSR loci were sufficient to detect variation in the *P. triticina* populations as the number of MLGs began to flatten out between 20 and 22 SSR loci (Supplementary Fig. S1) in the genotype accumulation curve. Fifty-eight MLGs were found in North America, 75 in South America, 81 in Europe, 47 in the Middle East, 21 in East Africa, 36 in Russia, 28 in Pakistan, 72 in Central Asia, 36 in China, 9 in New Zealand, and 11 in South Africa.

Regional MLG differentiation and genetic diversity. The isolates were initially grouped based on regions for measures of MLG genetic differentiation, diversity, and heterozygosity. All populations were significantly differentiated with F_{ST} at $P < 0.05$, and most pairs were significant at $P < 0.001$ (Table 1). The populations from Russia and East Africa and from Russia and North America had the highest F_{ST} values of 0.272 and 0.259, respectively. The populations from South America and North America and from the Middle East and East Africa were the most closely related, with the lowest F_{ST} values of 0.016 and 0.025, respectively. The population from New Zealand, when paired with populations from

South Africa, Pakistan, and the Middle East, was significantly differentiated from these populations at $P < 0.05$ but not at lower probability levels. This was likely attributable to the small number of isolates ($n = 10$) in the New Zealand population. A larger sample size in the New Zealand population would have likely resulted in higher levels of F_{ST} and lower probabilities as seen in the other population pairings.

The genetic relationship between the regional populations based on MLGs was also seen with Nei's genetic distance in an unrooted neighbor-joining tree (Fig. 1). The population pairs from North America and South America, the Middle East and East Africa, Pakistan and Europe, and Central Asia and Russia were closely related, with high bootstrap values. The populations from Russia and East Africa and from Russia and North America were the most distantly related.

In separate minimum spanning trees of the closely related population pairs, the MLGs were interspersed among each other, with little or no separation (Supplementary Fig. S2). Nine MLGs were in common in North America and South America, two in Europe and Pakistan, two in East Africa and the Middle East, and two in Russia and Central Asia.

The regional populations were also assessed for heterozygosity and allelic diversity in order to determine whether there were significant differences in the distribution of genetic diversity within the regions. Nine of the 11 populations had F_{IS} values significantly less than zero, which indicated that these populations had significantly greater observed heterozygosity (H_o) per locus compared with expected heterozygosity (H_e) under Hardy-Weinberg equilibrium (Fig. 2A). Only the populations from East Africa and South Africa had nonsignificant values of F_{IS}. Significant negative values of F_{IS} are considered to be indicators of clonal reproduction (Halkett et al. 2005), since they represent the deviation from random mating within populations in the absence of sexual recombination. The number of SSR alleles per locus in the regional populations ranged from 2.96 in South Africa to 4.96 in Europe (Fig. 2B). In all of the populations, the number of effective alleles per locus, which is the number of equally frequent alleles that would result in the same level of H_e, was significantly less than the actual number of alleles, which indicated an uneven distribution of alleles within each population. Taken together, the high levels of heterozygosity and the uneven distribution of alleles were indicators of deviations from random mating populations and of clonal reproduction in the regional populations.

Virulence phenotypes and MLG correlation. Among the isolates, a total of 497 virulence phenotypes were identified based on the avirulent/virulent responses to the 20 near-isogenic lines of Thatcher wheat. There were 89 virulence phenotypes in North America, 85 in South America, 106 in Europe, 77 in the Middle East, 22 in East Africa, 41 in Russia, 24 in Pakistan, 66 in Central Asia, 48 in China, eight in New Zealand, and 18 in South Africa. Nine Ethiopian isolates that were virulent to durum wheat had a low infection type of fleck to Thatcher wheat and could not be evaluated
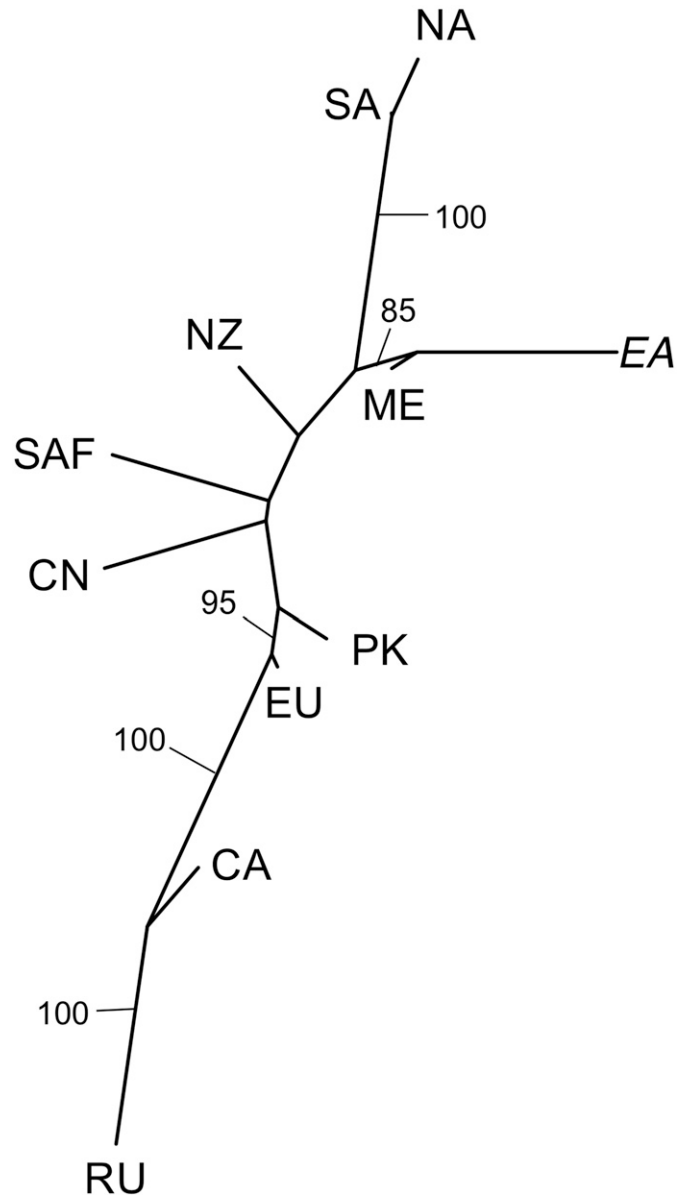
Fig. 1. Neighbor-joining plot of Nei's genetic distance of multilocus genotypes between *Puccinia triticina* isolates in 11 regional populations. The plot is based on 1,000 bootstrap samples with support values >50% indicated at nodes. NA = North America, SA = South America, EA = East Africa, ME = Middle East, NZ = New Zealand, SAF = South Africa, CN = China, PK = Pakistan, EU = Europe, CA = Central Asia, and RU = Russia.

TABLE 1. Estimates of genetic differentiation using F_{ST} in 11 regional populations of *Puccinia triticina* based on multilocus genotypes at 23 microsatellite loci[a]

North America	Central Asia	New Zealand	South Africa	Russia	China	Pakistan	Europe	Middle East	South America	East Africa	Population
0.000											North America
0.202*	0.000										Central Asia
0.088**	0.124*	0.000									New Zealand
0.144*	0.102*	0.035***	0.000								South Africa
0.259*	0.043*	0.210*	0.187*	0.000							Russia
0.135*	0.108*	0.056**	0.069*	0.191*	0.000						China
0.120*	0.060*	0.055***	0.069*	0.148*	0.057*	0.000					Pakistan
0.125*	0.051*	0.043**	0.055*	0.121*	0.056*	0.026*	0.000				Europe
0.072*	0.131*	0.034***	0.085*	0.220*	0.060*	0.037*	0.044*	0.000			Middle East
0.016**	0.193*	0.078*	0.133*	0.249*	0.131*	0.109*	0.113*	0.074*	0.000		South America
0.114*	0.179*	0.065**	0.114*	0.272*	0.093*	0.074*	0.067*	0.025**	0.105*	0.000	East Africa

[a] Significant at *$P < 0.001$, **$P < 0.01$, and ***$P < 0.05$.

for virulence to the Thatcher differential lines. These isolates were given an EEEEE race designation. All other isolates from durum wheat were phenotyped on the Thatcher differentials. There was signifi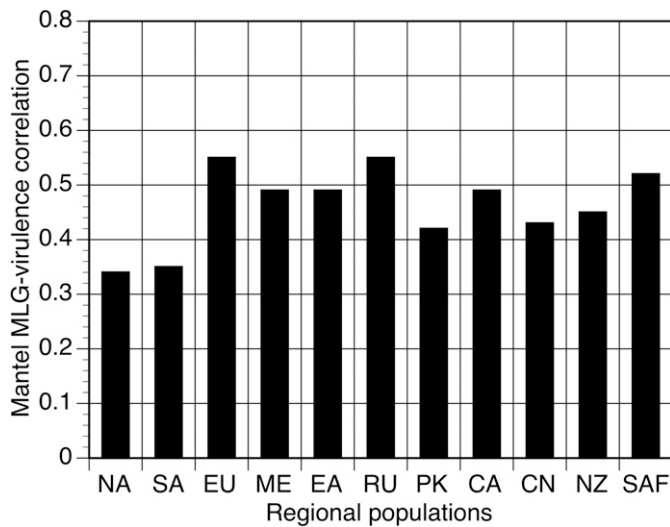cant correlation between the MLGs and virulence phenotypes at the level of the individual isolates with no geographic grouping, isolates within regions, and isolates in different regions. When all 831 isolates were considered without any grouping, the Mantel correlation between MLGs and virulence phenotypes was 0.32 ($P < 0.001$). All regional populations had significant ($P < 0.01$) within correlation for MLG and virulence phenotypes from 0.32 for North America to 0.56 for Russia (Fig. 3). When the isolates were grouped by region, the Mantel correlation of Nei's genetic distance between populations for MLG and virulence phenotypes was

0.64 ($P < 0.002$). The significant relationship between MLG and virulence was an additional indication of clonal reproduction in these populations.

MLG group diversity and differentiation. There were a total of 40 MLG groups from the previous studies of the individual regional populations, with six groups in North America (NA), five in South America (SA), eight in Europe (EU), three in the Middle East (ME), four in East Africa (EA), two in Russia (RU), three in Pakistan (PK), four in Central Asia (CA), and three groups in China (CN) (Table 2). Isolates from New Zealand (NZ) and South Africa (SAF) were each placed into one group each, as a result of the small number of isolates in these regions. MLG groups of isolates with virulence to durum wheat from North America, South America,
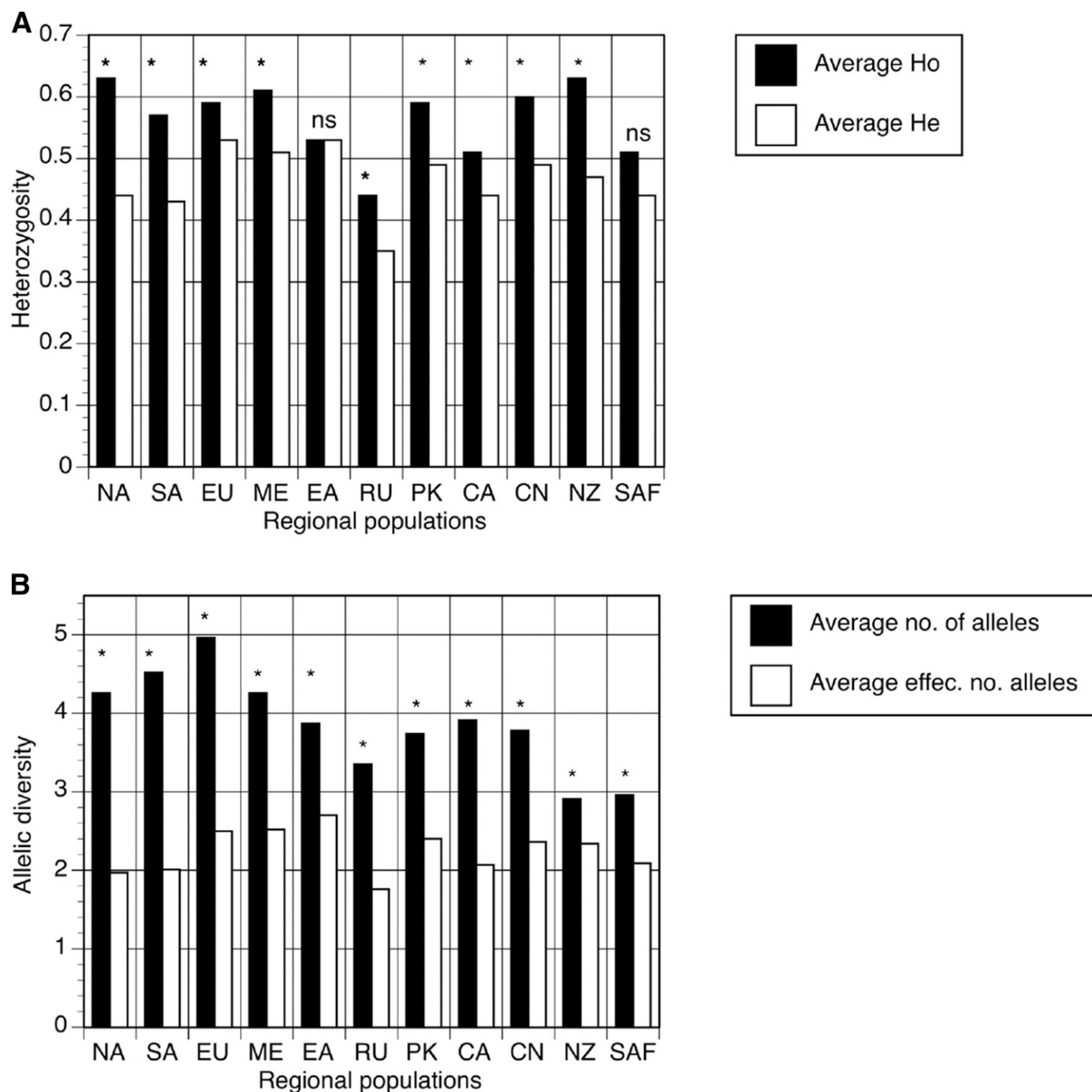
Fig. 2. Measures of genetic diversity in regional populations of *Puccinia triticina*. **A,** Average observed (H_o) and average expected (H_e) heterozygosity. **B,** Average number and average effective number of alleles per SSR locus. NA = North America, SA = South America, EU = Europe, ME = Middle East, EA = East Africa, RU = Russia, PK = Pakistan, CA = Central Asia, CN = China, NZ = New Zealand, SAF = South Africa, and ns = nonsignificant value of inbreeding coefficient F_{IS}.

Fig. 3. Mantel correlation coefficient between multilocus genotypes (MLGs) and virulence phenotypes of *Puccinia triticina* isolates in regional populations. All correlation coefficients were significant at $P < 0.01$. NA = North America, SA = South America, EU = Europe, ME = Middle East, EA = East Africa, RU = Russia, PK = Pakistan, CA = Central Asia, CN = China, NZ = New Zealand, and SAF = South Africa.

Europe, the Middle East, and East Africa were indicated with the suffix durum. Isolates from Ethiopia virulent to durum wheat and avirulent to Thatcher were in the EA durum E group. In the AMOVA, the overall F_{ST} was 0.30, with 78% of the variation within individuals (heterozygosity), 22% among the 40 groups, and 0% among individuals within groups, which indicated that the groups were useful for determining the genetic relationships of isolate groups from different regions.

Most of the MLG groups had isolates that were collected over a span of years, with only a few groups with isolates that were collected in a specific year. Historical reference isolates in NA1, NA4, and NA5 from the 1950s had similar or identical MLGs to isolates from the mid-2000s (Table 2). The groups SA1, SA2, EU1, and EU5 had isolates collected in the early 1980s up to the mid- to late 2000s. Isolates in EA1, EA2, and the EA durum E group had isolates that were collected in the 1980s up to 2012. Most of the other groups had isolates that were collected in the mid-1990s to the mid- to late 2000s or from the mid-2000s to 2013. There were some MLGs found in a specific year, as MLGs in EU2, EU6, and PK3 were found only in 2009, 1995, and 2013, respectively. Twenty-seven of the 29 isolates in EU2 were collected in Turkey in 2009, and all six isolates in EU6 were from Germany, Hungary, and Czechoslovakia in 1995. All isolates in PK3 were from Pakistan and Bhutan in 2013. Isolates were obtained from New Zealand in only 1997. Overall, however, there appeared to be relatively few MLG groups that were found in only 1 year.

TABLE 2. Isolates of *Puccinia triticina* in 11 regional populations: multilocus genotype (MLG) groups, number of isolates, diversity measures, number of virulence phenotypes, and years collected

| Continental population	MLG group	Isolates (n)	MLGs (n)	Simpson unbiased (λ) diversity	Effective MLGs (n)	Virulence phenotypes (n)	Year(s)
North America	NA1	12	11	0.903	10.3	9	1954 to 2005
	NA2	24	13	0.795	4.8	19	1984 to 2004
	NA3	21	11	0.780	4.5	10	1996 to 2005
	NA4	6	3	0.500	2.0	4	1959 to 2005
	NA5	62	16	0.720	3.6	46	1959 to 2005
	NA durum	7	4	0.694	3.3	1	2002 to 2009
South America	SA1	9	7	0.840	6.2	6	1982 to 2004
	SA2	50	25	0.928	13.9	41	1981 to 2006
	SA3	45	26	0.925	13.4	25	2004 to 2008
	SA4	7	7	0.857	7.0	7	1987 to 2008
	SA durum	11	10	0.893	9.3	6	1988 to 2008
Europe	EU1	19	14	0.903	10.3	14	1981 to 2006
	EU2	29	14	0.828	5.8	24	2009
	EU4	15	5	0.444	1.8	12	1995 to 2009
	EU5	16	12	0.898	9.8	15	1982 to 2009
	EU6	6	3	0.500	2.0	6	1995
	EU7	14	7	0.704	3.4	11	1993 to 2009
	EU8	23	14	0.896	9.6	14	1995 to 2009
	EU durum	15	12	0.880	8.3	10	1994 to 2015
Middle East	ME1	31	14	0.743	3.8	30	1998 to 2006
	ME2	39	20	0.810	5.3	35	1992 to 2006
	ME durum	15	13	0.916	11.8	12	1990 to 2015
East Africa	EA1	12	4	0.417	1.7	8	1984 to 2012
	EA2	12	6	0.694	3.3	12	1988 to 2012
	EA durum	7	2	0.245	1.3	1	2002 to 2012
	EA durum E	9	9	1.00	9.0	1	1987 to 2012
Russia	RU1	40	16	0.906	10.6	23	2006 to 2010
	RU2	26	20	0.923	13.0	18	2006 to 2010
Pakistan	PK1	17	9	0.789	4.7	11	2010 to 2013
	PK2	12	10	0.833	6.0	10	2011 to 2014
	PK3	9	9	0.889	9.0	3	2013
Central Asia	CA1	18	14	0.901	10.1	13	2002 to 2005
	CA2	46	39	0.971	34.1	33	2002 to 2005
	CA3	15	10	0.726	6.1	13	2002 to 2005
	CA4	20	9	0.620	2.6	17	2002 to 2004
China	CN1	36	23	0.924	13.2	25	2009 to 2010
	CN2	32	9	0.682	3.1	19	2009 to 2010
	CN3	13	4	0.568	2.3	4	2009 to 2010
New Zealand	NZ	10	9	0.880	8.3	8	1997
South Africa	SAF	21	11	0.807	5.2	18	1996 to 2010

The effect of clonality can be seen in the genotypic diversity measures of the MLG groups (Table 2). For example, isolates in the NA5 group had 16 MLGs and 46 virulence phenotypes among the 62 isolates and a Simpson diversity (λ) of 0.72. Many of these isolates had the same MLG but had different virulence phenotypes. The effective number of MLG was 3.6, which indicated the presence of many clones in this group, since this value is the number of evenly distributed MLGs needed to obtain the observed Simpson diversity. Other MLG groups with over 25 isolates (SA2, SA3, EU2, ME1, ME2, RU1, RU2, CN1, and CN2) also had large differences in the effective number of MLGs compared with the observed numbers. Most of these groups had an effective number of MLGs that were less than half the observed number. However, not all of the larger MLG groups had diversity indicators of clonality, since group CA2 was highly diverse with a Simpson index of 0.971 and an effective number of MLGs of 34.1, which was close to the observed 39 MLGs. This group was also highly diverse for virulence phenotypes with 33 among the 46 isolates. Groups SA4, EA durum E, and PK3 that had fewer than 10 isolates were also highly diverse with equal numbers of isolates and total numbers of observed MLGs.

The genetic distance between the MLG groups is visualized in Figure 4. Isolates that are virulent to durum wheat in groups ME durum, NA durum, SA durum, EU durum, and EA durum were highly related and were the most genetically distant groups. Isolates in EA durum E were also genetically distinct and were not closely related to any other MLG group. Isolates from common wheat in CN3 and EU1 were also not closely related to any other MLG groups. With the exception of EU5, the remaining MLG groups grouped in three branches. Each branch had MLG groups that were from different regions. Although isolates in NA2 and CN2 were in branches with other MLG groups, they were not closely related to other groups. Most pairwise combinations of MLG groups had significant levels of F_{ST} with $P < 0.001$ (*data not shown*); however, 44 pairs of MLG groups from different regions were more closely related and had F_{ST} values with significance levels of $P \geq 0.01$ (Supplementary Table S3). Most of the MLG group pairs with lower values of F_{ST} were also closely related for Nei's genetic distance (Fig. 4).

Occurrence of MLGs across regions. Among the 424 MLGs, 27 were found in more than one region (Table 3; Supplementary Table S4). The P_{sex} values for each genotype, which is the probability of sampling two or more identical MLGs in sexual populations (Stenberg et al. 2003), were exceedingly low ($P < 0.001$), which indicated that isolates with identical MLGs in different continents were clones. Isolates with identical MLGs were also more related for virulence phenotype, as these had a significantly smaller average virulence difference (0.00 to 4.95) compared with the average difference of 7.56 (0.004 standard error) in the total population. MLG 46 had the highest number of isolates at 47, as well as the highest number of virulence phenotypes at 35 (Table 3). Isolates with this MLG were very common in the United States, Canada, and Egypt and with a single isolate in Chile (Fig. 5). MLG 99 was the second most common, with 22 isolates and 19 virulence phenotypes (Table 3), which were found in Ethiopia, Kenya, Turkey, Egypt, and Israel (Fig. 5). Isolates in MLG 33 were found in Uruguay, the United States, the United Kingdom, Turkey, and Ethiopia, and isolates in MLG 36 were found in the United States, Chile, Ethiopia, Pakistan, Turkey, Kenya, and France (Fig. 5). These two MLGs are closely related and were dominated by virulence phenotypes that are virulent to *Lr17* and *Lr3bg* and avirulent to *Lr2a* and *Lr28*. Isolates in MLG 35 are highly related to MLG 33 and MLG 36, are also highly similar for virulence, and were found in Uruguay, Argentina, and the United States (Table 3). Isolates in MLG 6 were found in Uruguay, Egypt, France, the United Kingdom, the Czech Republic, Turkey, and Uzbekistan (Fig. 5). Isolates in MLG 25 were widespread and were found in Egypt, Ukraine, Russia, Turkey, Kazakhstan, Uzbekistan, and China. Isolates in MLG 72 and MLG 75 were highly related, with virulence to durum wheat cultivars (Table 3), and were found in Ethiopia, France, and Israel and in the United States, Mexico, and Ethiopia, respectively. Isolates in the other MLGs shown in Table 3 were lower in number but were still found in two to four regions.

Thirteen of the MLGs listed in Table 3 also had isolates with identical virulence phenotypes (Table 4). Isolates in MLG 36 with virulence phenotype MCDSS were the most widespread and were found in Kenya, Ethiopia, Chile, Turkey, France, and the United States from 2003 to 2011. Isolates in MLG 33 that also had virulence phenotype MCDSS were found in Uruguay, Turkey, and the United States from 2000 to 2009. Isolates with rare virulence
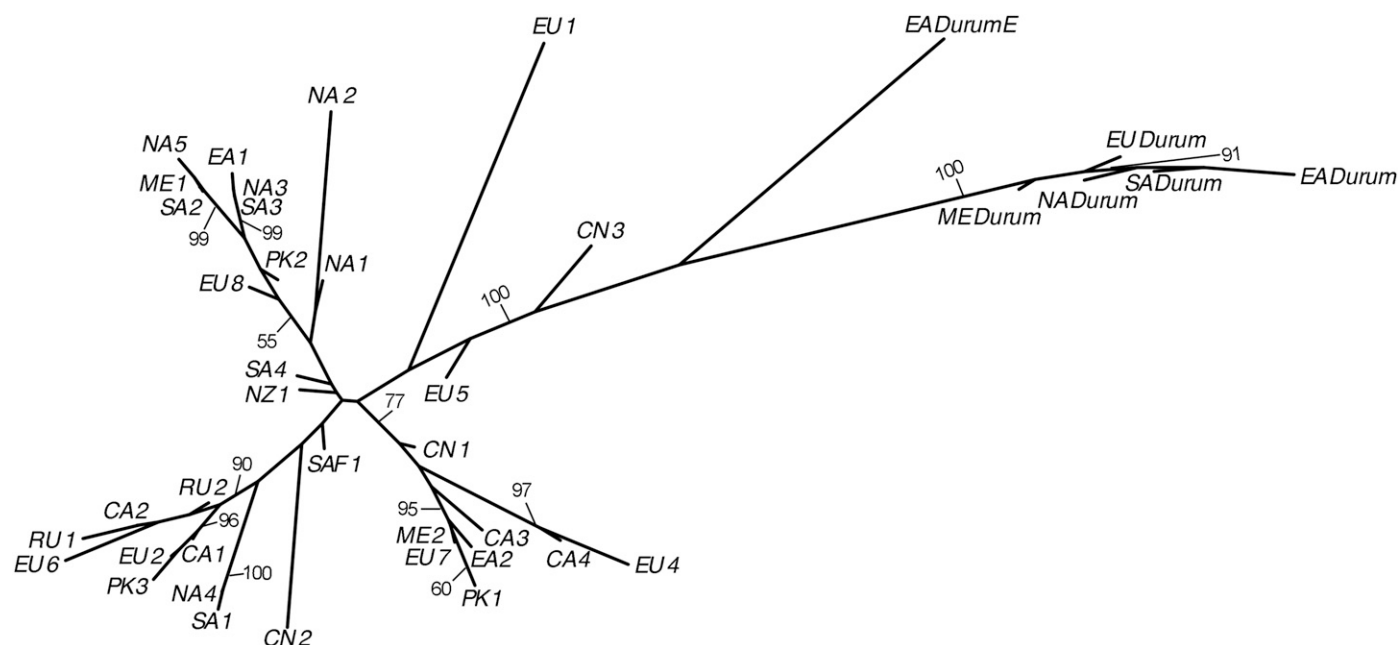
Fig. 4. Neighbor-joining plot of Nei's genetic distance of multilocus genotypes between *Puccinia triticina* isolates in 40 multilocus genotype groups within regional populations. The plot is based on 1,000 bootstrap samples with support values >50% indicated at nodes. NA = North America, SA = South America, EA = East Africa, ME = Middle East, NZ = New Zealand, SAF = South Africa, EU = Europe, PK = Pakistan, CN = China, CA = Central Asia, and RU = Russia.

phenotypes and identical MLGs were also found in widely separated regions. Isolates in MLG 39 with the rare virulence phenotype LCGHK (virulent to *Lr1*, *Lr26*, and *Lr11* and avirulent to *Lr2a*, *Lr2c*, and *Lr3*) were found in Brazil, Israel, and New Zealand.

Isolates with virulence phenotype SBDGK (avirulent to *Lr3* and virulent to *Lr17*, *Lr10*, *Lr14b*, *Lr20*, and *Lr28*) in MLG 79 were found in Uruguay and Canada in 2004 and 1959, respectively. Isolates in MLG 58 were unique since the five isolates had virulence

TABLE 3. Multilocus genotype (MLG) groups of *Puccinia triticina* sampled across more than one regional population and average virulence difference of isolates within and between MLG groups

MLG no.	Isolates (*n*)	MLG groups (isolates, *n*)[a]	P_{sex}	Virulence phenotypes (*n*)	Average virulence difference (standard error)
6	15	SA4 (1), ME2 (2), EU4 (11)[b], CA4 (1)[b]	0.00	13	2.64 (0.13)
7	3	ME2 (1), EU7 (1), CN1 (1)	9.23E-14	3	2.66 (1.52)
9	3	ME2 (2), PK1 (1)	2.77E-13	3	4.0 (0.99)
25	18	ME2 (1), EU2 (7),[b] CN1 (1), RU2 (5),[b] CA1 (4)	1.16E-11	14	2.75 (0.13)
33	19	EA1 (1), SA3 (7),[b] EU8 (2),[b] NA3 (9)[b]	7.94E-09	13	4.11 (0.18)
35	5	SA3 (4), NA3 (1)	6.75E-09	5	3.4 (0.32)
36	17	EA1 (9),[b] SA3 (3),[b] EU8 (3),[b] PK2 (1), NA3 (1)	3.15E-09	7	1.54 (0.12)
39	6	SA2 (4),[b] ME1 (1),[b] NZ (1)[b]	4.56E-10	4	2.20 (0.49)
41	7	EA (1), ME (1), NA5 (5)	1.05E-10	7	4.95 (1.01)
42	9	SA2 (1), ME1 (1), NA5 (7)	4.08E-11	8	3.61 (0.34)
46	47	SA2 (1), ME1 (15), NA5 (31)	3.11E-09	35	4.89 (1.44)
47	3	ME1 (2),[b] NA5 (1)[b]	3.24E-10	2	0.67 (0.58)
49	5	SA2 (1), ME1 (2), NZ (1), NA5 (1)	6.48E-10	5	4.6 (0.65)
52	4	EA1 (1),[b] EU8 (1),[b] NA5 (2)	1.08E-11	3	2.5 (0.98)
58	5	SA2 (2),[b] NZ (1),[b] NA1 (2)[b]	7.32E-10	1	0.0
59	3	ME2 (1), EU5 (2)	0.00	3	1.3 (0.33)
65	5	EU5 (3),[b] CN3 (2)[b]	0.00	3	1.00 (0.26)
67	3	PK2 (1), NA2 (2)	0.00	3	3.00 (0.58)
72	8	ME durum (1), EU durum (1), ETH durum (6)	0.00	2	0.72 (0.15)
75	4	NA durum (3), ETH durum (1)	0.00	1	0.0
77	5	ME2 (3), EU7 (2)	3.41E-12	5	3.40 (0.34)
79	5	SA (1),[b] NA4 (4)[b]	1.25E-09	3	0.60 (0.16)
82	2	SA1 (1), NA4 (1)	5.08E-09	2	1.0 (0.0)
83	3	EU2 (2), PK3 (1)	7.75E-12	3	1.33 (0.34)
84	11	EU2 (9), RU2 (1), CA1 (1)	1.52E-11	10	2.65 (1.27)
92	2	ME2 (1), EU7 (1)	9.23E-14	2	3.0 (0.0)
99	22	EA2 (6),[b] ME2 (16)[b]	1.61E-11	19	4.22 (0.15)
Total isolates	831			497	7.56 (0.004)

[a] SA = South America, ME = Middle East, EU = Europe, CA = Central Asia, CN = China, PK = Pakistan, RU = Russia, EA = East Africa, NA = North America, NZ = New Zealand, and ETH = Ethiopia.
[b] MLGs with identical virulence phenotype across continental regions.
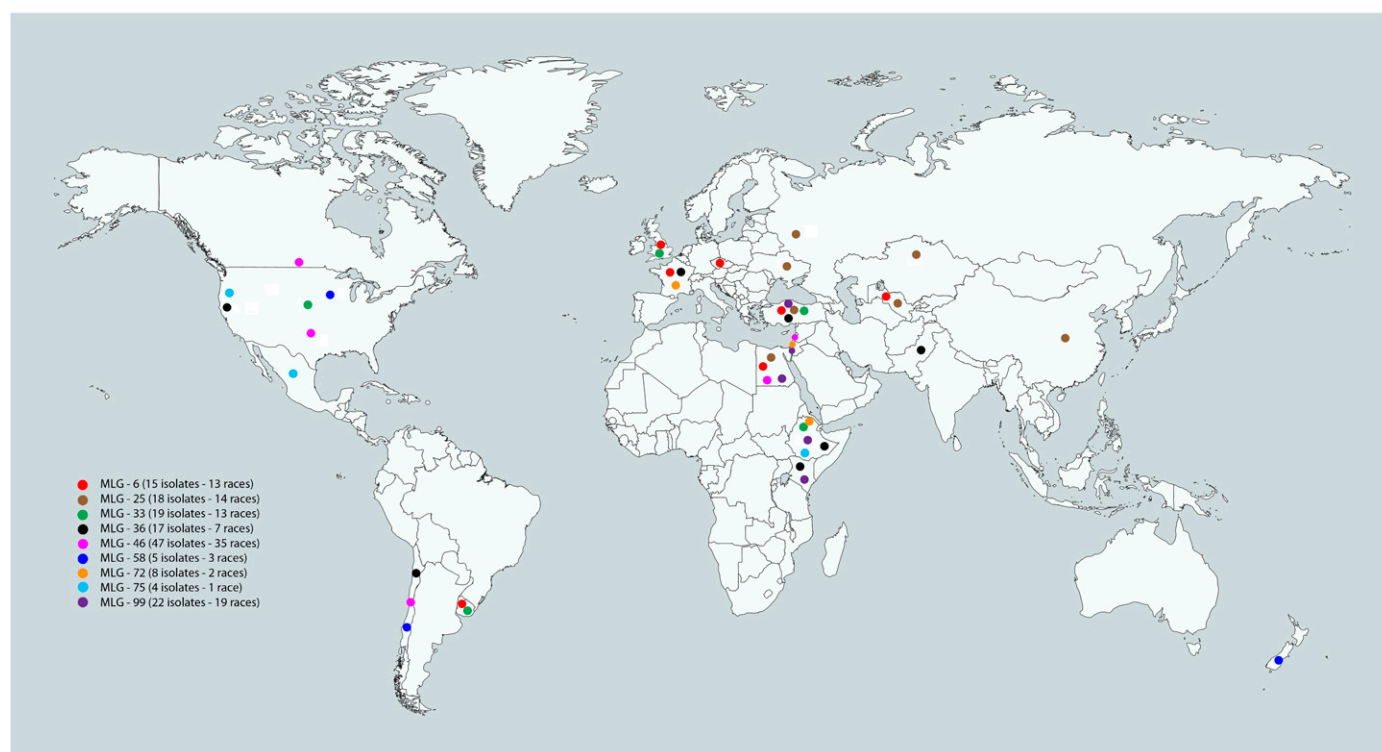

Fig. 5. Distribution of nine multilocus genotypes (MLGs) of *Puccinia triticina* across different continental regions.

phenotype BBBDJ, with virulence only to genes *Lr14a*, *Lr14b*, and *Lr20*. These relatively avirulent isolates were widely dispersed in time and geographically and were found in Chile, the United States, and New Zealand from 1954 to 2006 (Table 4). Isolates with virulence phenotype BBBQJ that are highly virulent to durum wheat in MLG 72 were found in Israel, France, and Ethiopia and isolates in MLG 75 with the same virulence were found in Mexico, the United States, and Ethiopia. Isolates in MLGs 6, 25, 47, 52, 65, and 99 also had isolates with identical virulence phenotypes that were found in at least two regions (Table 4).

DISCUSSION

This study showed that at a global level, populations of *P. triticina* are highly diverse for molecular genotype and also for virulence to leaf rust resistance genes. The widespread occurrence of MLGs across different regions and decades in time indicated that *P. triticina* has the potential for long-distance migration, which has likely occurred in the past and in more recent years. Host selection, asexual reproduction, and movement of genotypes–virulence phenotypes have influenced genetic variation in the regional *P. triticina* populations across different regions.

The regional populations all had estimates of genetic diversity that were characteristic of clonal populations. This is also observed with the consistent level of association between virulence phenotypes and MLGs across the regional populations and the small number of virulence differences between isolates with identical MLGs. Selection for virulence phenotypes occurs within clonal MLG groups, which maintains the correlation between molecular genotypes and virulence. Despite the lack of susceptible aecial hosts in most regions and dependence on clonal reproduction, *P. triticina* is a very successful pathogen of wheat, occurring regularly in many wheat production regions of the world, with many virulence phenotypes. It has the largest genome of the three wheat rust pathogens at 135 Mb (Cuomo et al. 2017), and mobile elements and repetitive DNA accounted for 51% of contig bases. The high proportion of mobile and repetitive elements in the genome may be a source of diversity for genes that are involved with virulence to host resistance genes (Ali et al. 2014b), resulting in the regular appearance of new races. In contrast, populations of *P. graminis* f. sp. *tritici* (wheat stem rust pathogen) have much less virulence diversity in areas such as North America, where the aecial host *Berberis vulgaris* is largely absent (Roelfs and Groth 1980).

Some MLG groups were found only in specific years. This could be attributable to sampling of isolates, as greater numbers in other years may have detected these MLGs, or the occurrence of MLGs in only one single year could be a result of their subsequent decline in later years. For example, in North America, isolates with a SBDGK virulence phenotype in the NA4 group, which is avirulent to *Lr3*, were predominant from the 1920s to the mid-1940s (Kolmer 1991). However, this virulence phenotype declined rapidly after the release of winter wheat cultivars in the mid-1940s with the *Lr3* resistance gene (Browder 1980; Samborski 1985). In current annual virulence surveys (Kolmer and Hughes 2017), isolates with the SBDGK phenotype are detected rarely and at low frequencies. Differentiation based on time of collection was also detected in populations of *P. striiformis* f. sp. *tritici* (wheat stripe rust) in China, Nepal, the Middle East, East Africa, and the Mediterranean region (Thach

TABLE 4. Multilocus genotypes (MLGs) of *Puccinia triticina* with identical virulence phenotypes found across different regional populations

MLG no.	MLG group[a]	Country	Year(s)	Virulence phenotype	Virulence
6	EU4	France	2004	FCPNQ	*2c, 3, 26, 3ka, 17, 30, B, 14a, 3bg, 14b*
	EU4	Czechoslovakia	1995		
	CA4	Uzbekistan	2003		
25	EU2	Turkey	2009	PBPSQ	*1, 2c, 3, 3ka, 17, 30, B, 10, 14a, 3bg, 14b*
	RU2	Russia	2008		
33	SA3	Uruguay	2004	MCDSS	*1, 3, 26, 17, B, 10, 14a, 3bg, 14b, 20*
	EU8	Turkey	2009		
	NA3	United States	2000		
36	EA1	Kenya	2011	MCDSS	*1, 3, 26, 17, B, 10, 14a, 3bg, 14b, 20*
	EA1	Ethiopia	2012		
	SA3	Chile	2008		
	EU8	Turkey	2009		
	EU8	France	2006		
	NA3	United States	2003		
39	SA2	Brazil	1996	LCGHK	*1, 26, 11, 10, 18, 14b, 20, 28*
	ME1	Israel	1998		
	NZ	New Zealand	1998		
47	ME1	Egypt	1999	TDBJK	*1, 2a, 2c, 3, 24, 10, 14a, 14b, 20, 28*
	NA5	United States	1982		
52	EA1	Kenya	1984	TBDKT	*1, 2a, 2c, 3, 17, 10, 14a, 18, 3bg, 14b, 20, 28*
	EU8	Hungary	1994		
58	SA2	Chile (2)	2006	BBBDJ	*14a, 14b, 20*
	NZ	New Zealand	1997		
	NA1	United States	2004		
	NA1	Canada	1954		
65	EU5	Italy	2006	FBBQQ	*2b, 3, B, 10, 3bg, 14b*
	CN3	China	2009		
72	ME_Durum	Israel	1990	BBBQJ	*B, 10, 14b, 20*
	EU_Durum	France	2006		
	ETH_Durum	Ethiopia	2011		
75	NA_Durum	Mexico	2002	BBBQJ	*B, 10, 14b, 20*
	NA_Durum	United States	2006		
	ETH_Durum	Ethiopia	2011		
79	SA1	Uruguay	2004	SBDGK	*1, 2a, 2b, 17, 10, 14b, 20, 28*
	NA4	Canada	1959		
99	EA2	Ethiopia	2007	TCJSQ	*1, 2a, 2c, 3, 26, 11, 17,B, 10, 14a, 3bg, 14b*
	ME2	Egypt	1999		

[a] EU = Europe, CA = Central Asia, RU = Russia, SA = South America, NA = North America, EA = East Africa, ME = Middle East, NZ = New Zealand, and CN = China.

et al. 2016). This was attributed to migration of new *P. striiformis* genotypes and displacement of older genotypes.

Sampling is a critical aspect in all population studies. Populations with small sample sizes that were collected in only one or a few years may not be representative of the overall populations. This can lead to questionable estimates of genetic diversity and also give misleading estimates of genetic relatedness between populations. A limitation of this study was the variation in size of the sampled collections across different years in the regional populations. Ideally all of the populations would have sampled in equal numbers within a uniform time period. The population from New Zealand had the smallest sample size and was collected in only 1 year. Additional collections over a period of years would have been a better representation of this population. Despite this issue, we were still able to detect isolates from New Zealand that had identical MLGs to other isolates across different regions over a period of years, indicating migration of *P. triticina* MLGs and virulence phenotypes. We were also able to detect isolates with identical MLGs that had other rare virulence phenotypes in more than one regional population across decades in time.

There was evidence of migration between adjacent regions, as the regions in closer proximity (North America–South America, Russia–Central Asia, and Middle East–East Africa) had a smaller genetic distance compared with more distant regions and also had MLGs in common. North America and South America may have received common introductions of *P. triticina* during European colonization and establishment of agricultural settlements. The similarity of the Russian and Central Asia and the East Africa and Middle East populations is likely attributable to the gradual stepwise expansion of MLGs/phenotypes by windblown uredinio-spores across these regions. Migration of wheat rust pathogens across large land areas is well documented. Phenotypes of *P. graminis* f. sp. *tritici* with virulence to *Sr31* and *Sr38* have gradually spread across East Africa (Jin et al. 2008, 2009; Wanyera et al. 2006) into parts of the Middle East and Southwest Asia. Similarly, phenotypes of *P. striiformis* f. sp. *tritici* with virulence to *Yr9* spread from East Africa to the Middle East and Asia (Ali et al. 2014a; Hovmøller et al. 2011). Phenotypes of *P. triticina* that are found in the winter wheat region of the southern Great Plains of the United States (Kolmer et al. 2006) are also detected later in the same year on spring wheat in the prairies of western Canada (McCallum and Seto-Goh 2006).

MLG groups from almost all regions were highly related to MLG groups from other regions, reducing the association between geographic origin and MLG. Long-distance migration of *P. triticina* between distant continents must be occurring. This can also be hypothesized because isolates with the same MLG and virulence phenotype were found in different continents. Migration events must have occurred in the past, as exemplified by MLG 58 found in New Zealand, Canada, the United States, and Chile from 1954 to 2007. More recent migration events have also occurred. MCDSS, MBDSS, and other phenotypes with virulence to *Lr17* and *Lr3bg* and avirulence to *Lr2a* and *Lr28* were first found in large numbers in the mid-1990s in the United States (Long et al. 2000) and Canada (Kolmer 1998), in South America in 1999 (Germán et al. 2007), and in France in 2000 and 2001 (Goyeau et al. 2006). These isolates are all highly related for MLGs and are in groups NA3, SA3, and EU8, respectively. Isolates with MCDSS phenotype in MLG 33 and MLG 36 were found in North America, South America, Europe, Southwest Asia, and East Africa. In addition, isolates in PK2 with MCDSS and MBDSS phenotypes were highly related for MLGs to isolates with the identical and similar phenotypes in EU8, NA3, SA3, and EA1. Similarly, the spread of highly related phenotypes and genotypes of *P. triticina* with virulence to durum wheat cultivars in many durum wheat regions also indicates migration between distant continents.

Examples of long-distance migration of wheat rust fungi are replete. The most well-known migration event is the introduction of

P. striiformis f. sp. *tritici* to Australia from northwest Europe in 1979 (Wellings and McIntosh 1990). Genotypes of *P. striiformis* with adaptation to higher temperatures have migrated to Australia, North America, and Europe since the early 2000s (Ali et al. 2014a; Hovmøller et al. 2008; Markell and Milus 2008). Stripe rust was also introduced to South Africa in 1996 (Pretorius et al. 1997), likely from the Mediterranean region. Virulence data and SSR genotyping support the hypothesis that wheat stem rust migrated from South Africa to Australia in the late 1960s (Visser et al. 2019).

Overall the *P. triticina* collections from hexaploid wheat in all regions were more diverse for molecular and virulence variation, compared with isolates from tetraploid durum wheat. While collections of isolates from durum wheat in geographically distant regions were closely related for molecular genotypes, MLG groups of isolates from common wheat from the same region were often highly genetically divergent. Isolates of *P. triticina* from durum wheat have closely related molecular genotypes and are not diverse for virulence phenotype, as found in France (Goyeau et al. 2006, 2011), Israel (Kosman et al. 2013), and Mexico (Singh et al. 2004). The reduced level of genetic and virulence diversity may be attributable to a smaller genetic basis of leaf rust resistance in the durum wheat cultivars. Most durum cultivars are susceptible to *P. triticina* phenotypes with virulence to *Lr72* (Herrera-Foessel et al. 2014) that have spread to South America (Ordoñez and Kolmer 2007a), Mexico (Singh et al. 2004), the Middle East (Ordoñez and Kolmer 2007b), and Europe (Goyeau et al. 2011) since the early 2000s. The preponderance of durum cultivars that are susceptible to these phenotypes (Singh et al. 2004) suggests that durum wheat germplasm as a whole is not very diverse for effective leaf rust resistance genes. In contrast, many leaf rust resistance genes have been documented in common wheat (McIntosh et al. 1995, 2017). Selection for virulence to these numerous leaf rust resistance genes has diversified *P. triticina* populations. For example, in the United States numerous common wheat cultivars in different market classes are grown that differ for leaf rust resistance genes. As a result, regional populations of *P. triticina* differ for predominant virulence phenotypes, and 50 to 70 virulence phenotypes are found annually (Kolmer and Hughes 2017). The diversity of deployed resistance genes in the cultivars has placed a continual selection pressure on *P. triticina* in North America, resulting in a population that is highly diverse for virulence phenotypes. In North America, six MLG groups were described (Ordoñez and Kolmer 2009), each with virulence phenotypes that have been selected by host resistance genes.

Isolates in the EA durum E MLG group were found only in Ethiopia. These isolates are highly virulent to durum wheat cultivars but unlike isolates in the other durum MLG groups, they are avirulent to many susceptible common wheat cultivars such as Thatcher. Ethiopia is a unique host environment because land races of emmer and durum wheat are still grown (Eticha et al. 2006) together with hexaploid wheat cultivars (International Maize and Wheat Improvement Center 2015). The durum adapted isolates that are avirulent to the susceptible common wheat cultivars have been collected from tetraploid landraces and modern durum wheat cultivars (Kolmer and Acevedo 2016). These isolates have been hypothesized to be the oldest form of *P. triticina* on tetraploid or common wheat (Liu et al. 2014).

The center of origin of *P. triticina* is likely the Fertile Crescent region of Mesopotamia, where alternate hosts (Tatlidil et al. 2005) and wheat progenitors are found (Feldman 2001). Coalescence analysis (Liu et al. 2014) of DNA sequence indicated that *P. triticina* types adapted to either tetraploid durum wheat or hexaploid common wheat diverged from a common ancestor found only on the diploid wheat progenitor *Aegilops speltoides* (Yehuda et al. 2004) in a process that paralleled the evolution of polyploid wheat in the Fertile Crescent (Salamini et al. 2002). As such, *P. triticina* is a long-established pathogen of wheat found in many wheat growing regions, as indicated by the occurrence of isolates with similar or

identical MLGs in distant continents. Leaf rust on wheat was first noted to be present in Australia in 1825 (McAlpine 1895) shortly after European settlement and in South Africa in 1911 (Pole Evans 1911). In contrast, *P. striiformis*, which was hypothesized to originate in the Himalayan region (Ali et al. 2014a), only arrived in Australia in 1979 and in South Africa in 1996. Regional populations of *P. striiformis* were also strongly associated with MLG groups (Ali et al. 2014a), compared with *P. triticina* that had highly related MLG groups from different continents. Although *P. striiformis* and *P. triticina* are both wind-dispersed rust pathogens of wheat with many different virulence phenotypes, they are very different with regard to distribution of genetic variation across regions and evolutionary history.

In conclusion, our study found that *P. triticina* is a highly diverse pathogen of wheat, with many highly related MLG groups widely distributed across wheat growing regions worldwide. The relative broad environmental adaptation (Roelfs 1989; Roelfs et al. 1992) and the ability of *P. triticina* to adapt to the continuously changing landscape of resistance genes in wheat cultivars, combined with the capability to produce millions of infective clonal urediniospores that can be wind dispersed over thousands of kilometers, has contributed to the persistence and widespread occurrence of this pathogen.

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