Simple Sequence Repeat Diversity of a Worldwide Collection of *Puccinia triticina* from Durum Wheat

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ABSTRACT

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Isolates of *Puccinia triticina* collected from durum wheat from Argentina, Chile, Ethiopia, France, Mexico, Spain, and the United States were analyzed with 11 simple sequence repeat (SSR) markers in order to determine the genetic relationship among isolates. These isolates also were compared with *P. triticina* isolates from common wheat from North America, and an isolate collected from *Aegilops speltoides* from Israel, to determine genetic relationships among groups of *P. triticina* found on different telial hosts. The large majority of isolates from durum wheat were identical for SSR markers or had <8% genetic dissimilarity, except for isolates from Ethiopia, which had 55% dissimilarity with respect to the other durum isolates. Isolates from common wheat had >70% genetic dissimilarity from isolates from durum wheat, and the isolate from *A. speltoides* was >90% dissimilar from all isolates tested. Analysis of molecular variance tests showed significant levels ($P = 0.001$) of genetic differentiation among regions and among isolates within countries. Isolates of *P. triticina* from durum wheat from South America, North America, and Europe were closely related based on SSR genotypes, suggesting a recent common ancestor, whereas *P. triticina* from Ethiopia, common wheat, and *A. speltoides* each had distinct SSR genotypes, which suggested different origins.

Additional keywords: population genetics, *Triticum turgidum*.

Rust fungi are highly successful obligate parasites of plants and important pathogens of wheat (2). Wheat leaf rust caused by *Puccinia triticina* is a major disease of common wheat (*Triticum aestivum* L.) and durum wheat (*Triticum turgidum* L. var. *durum*) that occurs regularly where the crop is grown (44). Wheat is the most widely cultivated crop in the world, and world trade for wheat is greater than for all other crops combined (11). Durum wheat is grown widely in southern Europe, the Middle East, and northern Africa (15). Smaller areas of durum wheat are grown in Argentina, Canada, Chile, Mexico, and the United States (15). Wheat leaf rust can cause millions of dollars in yield losses annually, and the use of resistant cultivars is the most economical and effective measure for control of the disease; however, durable host resistance against *P. triticina* has been difficult to achieve. Virulent races of leaf rust can overcome the resistance that has been bred into commercial hexaploid (AABBDD) common wheat cultivars relatively easily; on average, only 3 years after release (47). In the case of tetraploid (AABB) durum wheat, however, resistance to *P. triticina* has proven to be longer lasting. For example, certain durum wheat cultivars released in northwestern Mexico maintained their resistance to leaf rust for 25 years (47). The genetics of leaf rust resistance in durum wheat is relatively poorly understood in comparison with common wheat (28). Genetic studies have indicated that leaf rust resistance in durum wheat is inherited in a Mendelian fashion, with both dominant and recessive resistance genes (43,50,51,55,56). Resistance genes *Lr14a* and *Lr23* originated from tetraploid wheat, but it generally is not known whether durum and common wheats share the same leaf rust resistance genes on the A and B genomes, or if the genes in the two types of wheat are distinct. Studies that examined leaf rust resistance in durum wheat often have used isolates of *P. triticina* that were virulent to common wheat and avirulent to durum wheat.

In recent years, leaf rust on durum wheat has become more prevalent in several parts of the world. In Mexico during 2001 to 2003, a new race of *P. triticina* virulent on durum wheat accounted for losses of >US$32 million to wheat growers (47). Recently, yield losses of durum wheat in Spain, caused by leaf rust, ranged from 10 to 37%, which forced extensive fungicide applications (10). Similarly, in France, *P. triticina* on durum wheat has become widespread (19).

Rust uredioniospores can move over vast distances carried by wind, traveling across continents and oceans (30). Movement of rust urediniospores is an important factor in management of the disease, as is the genetic composition of the rust populations. Changes in populations of rust fungi have been detected using virulence to specific resistance genes (7,8,31,38,40,47) and molecular markers, such as amplified fragment length polymorphism (AFLP) (29,40), random amplified polymorphic DNA (RAPD) (13,31,32,38,40), restriction fragment length polymorphism (RFLP) (25), and simple sequence repeat (SSR) (35). These methods have allowed researchers to discriminate between local, introduced, and new emerging populations of rust fungi. The recent development of microsatellites or SSR markers for *P. triticina* (12,52) has made possible the genetic analysis of leaf rust pathogen populations with codominant genetic markers, which were not previously available for this pathogen.

Understanding the genetic variability of *P. triticina* populations occurring over large geographic areas is crucial for the development of effective control strategies for this disease. The objective of our present work was to characterize collections of *P. triticina* currently infecting durum wheat in Europe, Eastern Africa, and North and South America for genetic variation using microsatellite markers. We wanted to determine whether isolates

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of *P. triticina* that are virulent to durum wheat shared a common origin, as would be indicated if these collections were genetically similar, or if they arose from genetically different populations. We also wanted to determine the genetic relationship among isolates from durum wheat from different countries and a collection of *P. triticina* isolates from common wheat in North America.

MATERIALS AND METHODS

***P. triticina* samples.** A worldwide collection of 46 samples of *P. triticina* from durum wheat was used in this study. Part of the collection was obtained from the following countries and cooperators: Argentina and Chile, S. German; France, H. Goyeau and J. P. Hardouin; Mexico, R. Singh; Spain, A. del Olmo; and the United States, L. Jackson. In all, 4 samples from Argentina, 1 from Chile, 8 from Ethiopia, 10 from France, 13 from Mexico, 8 from Spain, and 2 from the United States were used. From each collection, one to three single-uredinial isolates were derived (Table 1). Single-uredinial isolation was carried out as previously described (26) using the susceptible durum wheat cv. Local Red. The isolates were increased on 7-day-old seedlings of Local Red, which had been watered with a 0.33% solution of maleic hydrazide, in order to enhance spore production (33). Also included in the study were a set of 11 *P. triticina* isolates of representative races from common wheat from the United States and 1 isolate from common wheat from Ethiopia. The isolates from durum wheat and common wheat correspond to group I of *P. triticina* according to the classification of Anikster et al. (1). The common wheat isolates from the United States were selected for their diversity in virulence to near-isogenic lines of cv. Thatcher. The virulence of these races had been determined earlier according to the nomenclature described by Long and Kolmer (36) (Table 1) and had diverse molecular polymorphism based on previous RAPD and AFLP analyses (29,32). Additionally, a sample with virulence specific to *Aegilops speltoides*, collected from Israel, was included in this study. *A. speltoides* has the S genome, which is thought to be the donor of the B genome in wheat. This isolate also is considered to be in group I for *P. triticina* (1). Isolates from common wheat were increased on seedlings of the susceptible cv. Thatcher, and the Israeli isolate was increased on *A. speltoides* seedlings. All inoculated plants were grown in a greenhouse with temperatures between 18 and 25ºC and 16 h of supplemental light.

Urediniospores were collected from infected seedlings that were kept in plexiglass isolation chambers in order to avoid cross contamination and grown under the same greenhouse conditions as described above. Urediniospores were germinated in a solution of 0.6 mg of gramicidin D (MP Biomedicals, Inc., Solon, OH), dissolved in 125 µl of absolute ethanol, per 100 ml of double-distilled (dd)H_2O. The gramicidin solution (20 ml) plus 100 µl of a nonanol solution (1.5 µl of nonanol [Sigma-Aldrich, St. Louis] in 1 ml of acetone) were placed in the bottom of a 10-cm glass petri dish and used to suspend 25 mg of rust spores. In addition, a filter paper moistened in the nonanol solution was placed inside the top of the petri dish and the plates were kept in darkness overnight, at room temperature. Germinated spore mats were collected and placed in 1.5-ml centrifuge tubes. The mats were snap frozen in liquid nitrogen, immediately lyophilized, and finally stored at –80ºC.

SSR genotypes. In total, 72 single-uredinial isolates from durum wheat were characterized for SSR genotypes (Table 1). In order to extract DNA, the lyophilized fungal material first was ground by shaking each sample with 25 mg of sterilized glass beads for 20 s in a Savant FastPrep shaker (FP120; Holbrook, New York). The OmniPrep genomic DNA extraction kit (GenoTech, St. Louis) was used according to the manufacturer's instructions. DNA concentration was determined using a DyNA Quant 200 Fluorometer (Hoefer, San Francisco), previously standardized with calf thymus DNA (Sigma-Aldrich). A final concentration of *P. triticina* DNA at 1 ng/µl was used for DNA amplification.

Eleven SSR primer pairs were used in this study (Table 2). Previously, SSR-enriched libraries were developed (BC Research Inc., Vancouver, BC, Canada) from genomic DNA of *P. triticina* isolate CDL97NE406, and clones that showed di-nucleotide and tri-nucleotide motifs were selected. Approximately 200 clones were sequenced from the library, and 54 primer pairs were designed and screened for reproducible amplification products on a set of 21 representative *P. triticina* isolates from common wheat and durum wheat (52). Eleven primer pairs that amplified a number of polymorphic bands were used for this study.

For SSR amplification, the final concentrations of the master mix consisted of 0.5 mM each primer, 0.2 mM dNTPs, 0.05% casein, 1× Phusion HF buffer (New England Biolabs, Ipswich, MA), 0.1 U of Phusion high-fidelity DNA polymerase (New England Biolabs), and 1 ng of DNA in a final reaction volume of 10 µl. The forward primer was fluorescently labeled with IRDye700 (LI-COR, Lincoln, NE). Amplification of DNA was done with a Peltier Thermal Cycler-200 (MJ Research, Waltham, MA) with a temperature profile of 98ºC for 30 s; 30 cycles of 98ºC for 30 s, 62ºC for 30 s, and 72ºC for 30 s; and 10 min of final extension at 72ºC. Prior to electrophoresis, amplification products were diluted 1:100 in ddH_2O, 5 µl of Blue stop solution (LI-COR) was added, and samples were denatured for 5 min at 95ºC. Amplification products were separated on a 7% polyacrylamide gel, with electrophoresis conditions of 1,500 V, 35 mA, 40 W, and 48ºC, for 3.5 h, and visualized through a DNA analyzer Gene Readir 4200 system (LI-COR). An IRDye700 fluorescent DNA size standard ranging from 50 to 350 bp (LI-COR) was

TABLE 1. Single-uredinial isolates of *Puccinia triticina* collected from durum wheat, common wheat, and *Aegilops speltoides* used for simple sequence repeat genotyping

Sample origin	Number of isolates	Virulence nomenclature[a]
Durum wheat		
Argentina	5	BBB-*10*, BBB-*10,14a*
Chile	3	BBB-*10*, BBB-*14a*
Ethiopia	11	BBB, BBB-*10,14a*
France	14	BBB-*10*, BBB-*10,14a*
Mexico	25	BBB-*10*
Spain	8	BBB-*10*, MGDS
United States	6	BBB-*10*
Common wheat		
Ethiopia	1	CBDM
United States	11	BBBD, FBMT, KFBJ, MBRJ, MCDS, MJBJ, NBGS, PBDQ, PBLR, SBDG, THBJ
Aegilops speltoides		
Israel	1	…
Total	85	…

[a] Virulences were determined according to the nomenclature described by Long and Kolmer (36) on near-isogenic lines of cv. Thatcher. The isolate from Israel was avirulent to Thatcher (6).

included in each gel run. Individual isolates were scored visually for dikaryotic genotypes for each SSR locus.

Data analysis. Data were recorded by assigning a letter code to each allele per locus, resulting in a two-letter designation for all isolates for each locus. Dissimilarity values were calculated for each pairwise comparison between isolates using the Kosman and Leonard (KL) (34) dissimilarity coefficient for codominant markers for diploid organisms. Genetic dissimilarity (*as*) between individuals i_1 and i_2 is calculated by averaging within locus genetic dissimilarity [$as(t_{i1}, t_{i2})$] across all n loci:

$$as(i_1, i_2) = \frac{1}{n} \cdot \sum_{j=1}^{n} as(t_{j1}, t_{j2})$$

where t_{i1} and t_{i2} designate the genetic states of the individuals within each n loci. For one locus, a value of 1 corresponds to two isolates that do not share any common alleles, a value of 0.5 corresponds to two individuals sharing one allele in common, and a value of 0 corresponds to two individuals sharing two common alleles. For example, a pairwise comparison between three isolates having the following genetic states (AA, AB, and BC for the same locus) would give the following values: $as(AA,AA) = 0$, $as(AA,AB) = 0.5$, and $as(AA,BC) = 1$. The dissimilarity matrix of the KL dissimilarity coefficient then was used to construct a dendrogram using the unweighted pair group method with arithmetic means (UPGMA) clustering method in NTSYS-pc (version 2.1; Exeter Software, Setauket, NY). Binary data matrices also were generated for all loci based on the presence (= 1) or absence (= 0) of amplification products per locus. Genetic distances were calculated based on the Dice coefficient of similarity, and clustering was done using UPGMA in NTSYS-pc.

In order to determine how well the UPGMA dendrograms represented each of the original matrices of similarity (Dice coefficient) and dissimilarity (KL coefficient), a matrix of cophenetic values was generated for each of the matrices using the COPH module in NTSYS-pc, and then the MXCOMP program was used to calculate the level of correlation between the cophenetic matrices with the original corresponding dissimilarity and similarity matrices. The level of correlation between the Dice similarity and KL dissimilarity matrices was determined with the MXCOMP module.

To visualize relationships between SSR genotypes of the isolates in two-dimensional principal coordinate (PC) plots, eigenvectors were derived using NTSYS-pc. The symmetric KL dissimilarity matrix was transformed with DCENTER, and the resulting double-centered matrix then was used with EIGEN to calculate principal coordinates. To test the goodness of fit, a distance matrix was constructed from the eigenvectors using the SIMINT module in NTSYS-pc and then compared with the corresponding double-center dissimilarity matrix using the MXCOMP module. The resulting correlation measured the extent to which the principal coordinate analysis showed the pattern of relative distances among the isolates.

Collections of isolates were defined based on country of origin of the collections and whether they came from durum wheat, common wheat, or *A. speltoides*. Durum wheat isolates originated from seven countries (Argentina, Chile, the United States, Mexico, France, Spain, and Ethiopia); common wheat isolates came from the United States, with one isolate from Ethiopia; and one isolate from *A. speltoides* came from Israel. Five regions were defined for analysis of molecular variance (AMOVA): (i) South America, with isolates from Argentina and Chile; (ii) North America, with isolates from Mexico and the United States; (iii) Europe, with isolates from France and Spain; (iv) Ethiopia; and (v) isolates from common wheat in the USA,

Ethiopia, plus three isolates collected from durum wheat from Chile, Ethiopia, and Spain but with common wheat type virulence characteristics. The isolate from *A. speltoides* was not included in the AMOVA analysis. Percentage of polymorphic loci and number of private alleles by country of origin were obtained with GenAlEx6 (41) software, and χ^2 tests of allele frequencies over regions by locus were done with POPGENE version 1.31 (54). Nei's genetic distance (39) based on allele frequencies by region was calculated using GenAlEx6. An unrooted tree was constructed using PHYLIP version 3.6 (17) from allele frequency data by regions, with the NEIGHBOR clustering option.

AMOVA, as described by Excoffier et al. (14), was carried out using GenAlEx6 to test differences within and between the regional isolate groups of *P. triticina*. Estimation of genetic differentiation was based on F_{ST} and R_{ST} statistics from AMOVA, as described in GenAlEx6. The AMOVA option for codominant data with regional data structure was used to calculate F_{ST} as the proportion of the variance among populations and regions, relative to the total variance, which assumes an infinite-allele model (42):

$$F_{ST} = \frac{V_{AP} + V_{AR}}{V_{WP} + V_{AP} + V_{AR}}$$

where V_{AP} is the variance among populations within regions, V_{AR} is the variance among regions, and V_{WP} is the variance within populations. F_{RT} is calculated as the proportion of the variance among regions, relative to the total. F_{SP} is calculated as the proportion of the variance among populations within regions, relative to the variance both within and among populations. R_{ST} calculations in GenAlEx6 parallel those for F_{ST}; however, a stepwise mutation model is assumed for genetic differentiation based on codominant microsatellite loci, as proposed by Slatkin (48). Genotypic variation based on number of alleles, and heterozygosity statistics, were calculated with POPGENE.

TABLE 2. Simple sequence repeat primers used in this study, number of alleles per locus, number of genotypes, and size of alleles generated from the analysis of 85 *Puccinia triticina* isolates collected from durum wheat, common wheat, and *Aegilops speltoides*

Primer pair[a]	Number of alleles	Number of genotypes	Size of alleles (bp)
PtSSR3	4	6	284, 297, 300, 325
PtSSR61	4	6	295, 297, 299, 301
PtSSR62	2	3	299, 301
PtSSR68	10	16	294, 304, 310, 312, 314, 316, 317, 319, 321, 332
PtSSR92	7	11	233, 242, 244, 247, 249, 251, 253
tSSR152	3	3	388, 392, 394
PtSSR154	9	15	240, 243, 251, 254, 257, 262, 265, 268, 274
PtSSR158	6	10	223, 226, 229, 232, 254, 257
PtSSR164	5	7	203, 215, 217, 220, 222
PtSSR173	6	6	209, 215, 217, 219, 228, 233
PtSSR187	6	8	259, 261, 264, 267, 273, 277
Total	62	…	…

[a] Primer sequences described in Szabo and Kolmer (52).

Smouse's (49) multilocus analysis of population subdivision also was calculated with POPGENE.

RESULTS

From the 85 isolates used in this study, 62 alleles were amplified by 11 SSR primer pairs (Table 2). The number of alleles per locus ranged from 2 to 10, with an average of 5.63 alleles/locus. Amplification product sizes varied between 203 and 394 bp. In all, 3 to 16 SSR genotypes were found per locus. The number of SSR genotypes found for isolates from each country is presented in Table 3. The percentage of polymorphic loci ranged from 18% for the isolate from *A. speltoides* to 100% for the isolates from common wheat, with an overall mean of 71% (Table 3). Isolates from Spain, Ethiopia, common wheat, and *A. speltoides* had private alleles (Table 3).

The genetic dissimilarity dendrogram using the KL coefficient based on UPGMA analysis is shown in Figure 1. Fifty-nine isolates, corresponding to the majority of durum wheat isolates from Argentina, Chile, Mexico, France, and the United States, were either identical or had <8% dissimilarity (Fig. 1, cluster 2). The *P. triticina* isolates from durum wheat from Ethiopia (Fig. 1, cluster 3) had 55% dissimilarity with respect to the other durum wheat isolates in cluster 2. All common wheat leaf rust isolates in cluster 4 had >70% dissimilarity from the durum wheat isolates in clusters 2 and 3. Three isolates from durum wheat, from Chile (CH14.32), Ethiopia (E17.4), and Spain (S28), were in the same cluster with the common wheat leaf rust isolates. These isolates had virulence characteristics on isogenic lines of 'Thatcher' (races BBBD and MGDS) similar to common wheat isolates. The isolate from *A. speltoides* from Israel (cluster 1) had an average of >90% dissimilarity to all other isolates in clusters 2, 3, and 4. The cophenetic correlation of the dendrogram with the original dissimilarity matrix was very high, with $r = 0.98$. The correlation between the KL dissimilarity matrix and the Dice similarity matrix was very high, with $r = 0.99$; therefore, only the KL dissimilarity data was used for further analysis.

The representation of the SSR genotypes in two-dimensional PC plots is shown in Figure 2. The first two dimensions accounted for 78.9% of the variation. The double-centered matrix and the matrix constructed from the eigenvectors had a correlation coefficient of $r = -0.67$. Three general groups can be distinguished on the PC plot. Group A included durum isolates from all countries tested except for Ethiopia. Isolates from Ethiopia formed a second separate group B, and group C was composed mainly of common wheat isolates from the United States plus the three isolates from durum wheat from Chile, Ethiopia, and Spain that clustered with the common wheat types in the UPGMA analysis (Fig. 1). The coordinates for these three samples were Chile, 0.575, –0.158; Ethiopia, 0.582, –0.168; and Spain, 0.4, –0.111. The common wheat isolate from Ethiopia with coordinates 0.199, –0.006 was closest to isolates in group A. The isolate virulent to *A. speltoides* from Israel with coordinates 0.25, 0.12 and the isolate from common wheat from the United States with virulence designation SBDG and coordinates 0.69, 0.17 were distinct from all other isolates.

An unrooted tree (Fig. 3) was generated based on Nei's genetic distance (Table 4) among *P. triticina* populations. The tree showed that the *P. triticina* isolates from durum wheat from South America, North America, and Europe clustered very close together, *P. triticina* from durum wheat from Ethiopia was separate from the other durum isolates, and *P. triticina* from common wheat was separate from all the durum isolates.

Based on the results from the UPGMA and PCA analyses, the three isolates originally collected from durum wheat but that clustered with the common wheat types (Figs. 1 and 2) were included in the common wheat population for AMOVA analysis. Results from AMOVA (Table 5) showed that significant ($P \leq 0.008$) genetic differentiation was found among regions ($R_{RT} = 0.1364$, $F_{RT} = 0.3339$) and within countries ($R_{ST} = 0.0887$, $F_{ST} = 0.3268$). Calculation of R_{ST} showed that 13% of the variation occurred among regions and 87% was between isolates within countries. The AMOVA with F_{ST} calculations showed that 33% of the variation was among regions and 67% of the variation occurred between isolates within countries.

The R_{ST} and F_{ST} values from pairwise comparisons between the *P. triticina* populations are shown in Table 4. Little genetic differentiation was found between durum isolates from South America, North America, and Europe, as shown by low F_{ST} and R_{ST} values ($P > 0.05$), with the exception of a significant F_{ST} value ($P = 0.008$, $F_{ST} = 0.073$) between durum isolates from Europe and North America. Significant genetic differentiation ($P = 0.001$) was found between pairwise comparisons with isolates from Ethiopia and common wheat for F_{ST} and R_{ST}, except for a nonsignificant R_{ST} value ($P = 0.093$, $R_{ST} = 0.048$) between durum isolates from South America and common wheat. Isolates from Ethiopia and common wheat were mostly different from the isolates of *P. triticina* from durum wheat from South America, North America, and Europe.

The correlation between the matrices of R_{ST} and F_{ST} values as calculated in NTSYS was 0.68. Additionally, the correlation between Nei's genetic distance and F_{ST} was $r = 0.90$, and between Nei's genetic distance and R_{ST} was $r = 0.35$. The χ^2 tests on the allele frequencies for isolates based on country of origin and telial host by locus showed significant differences in frequencies ($P < 0.001$) for all loci across isolate groups.

The genotypic variation and heterozygosity statistics are shown in Table 6. The results show the mean value of the 11 loci for each

TABLE 3. Number of simple sequence repeat (SSR) genotypes, percentage of polymorphic loci, and summary of private alleles from *Puccinia triticina* isolates collected from durum wheat, common wheat, and *Aegilops speltoides*, analyzed with 11 SSR markers

				Private alleles		
P. triticina collections	No. of isolates	No. of SSR genotypes[a]	Polymorphic loci (%)	No. of loci	No. of alleles	No. of individuals (%)[b]
Durum wheat						
Argentina	5	2	55	0	0	0
Chile	2	1	55	0	0	0
United States	6	3	64	0	0	0
Mexico	25	5	82	0	0	0
France	14	5	91	0	0	0
Spain	7	5	91	1	1	1 (14.3)
Ethiopia	10	4	82	3	4	10 (100)
Common wheat	15	14	100	5	8	15 (100)
A. speltoides	1	1	18	7	9	1 (100)
Mean	…	…	71	…	…	…

[a] Total number of different SSR genotypes within a *P. triticina* collection.

[b] Number of individuals from the collections possessing private alleles; percentage of individuals within the collections having a private allele is shown in parenthesis.

collection. The durum isolates from Argentina, Chile, the United States, Mexico, France, Spain, and Ethiopia had mean values of Shannon's information index *I* ranging from 0.3781 to 0.6461. The common wheat isolates had the highest gene diversity (*I* = 0.9509), whereas the isolates from Chile had the lowest (*I* = 0.3781). The mean observed heterozygosity (H_o) for all groups of durum isolates was very similar, ranging from 0.5325 to 0.5649. The common wheat isolates had H_o of 0.7212. For Nei's expected heterozygosity (H_e), the durum wheat isolates had similar intermediate values (0.3924 to 0.2727) and the common wheat isolates had the highest H_e of 0.5451. The mean H_e was lower than the mean H_o for all collections.

Smouse's multilocus analysis of population subdivision showed the average correlation between alleles at different loci within countries and telial host to be 0.5982 (χ^2 = 3304.23, df = 76, *P* = 0.000) and among collections to be 1.0504 (χ^2 = 1069.59, df = 88, *P* = 0.000). The results indicated multilocus linkage disequilibrium between alleles at SSR loci in the isolates under study, as expected for asexually reproducing rust pathogen isolates.

DISCUSSION

Isolates of *P. triticina* from durum wheat from North America, South America, and Europe were closely related for SSR geno-

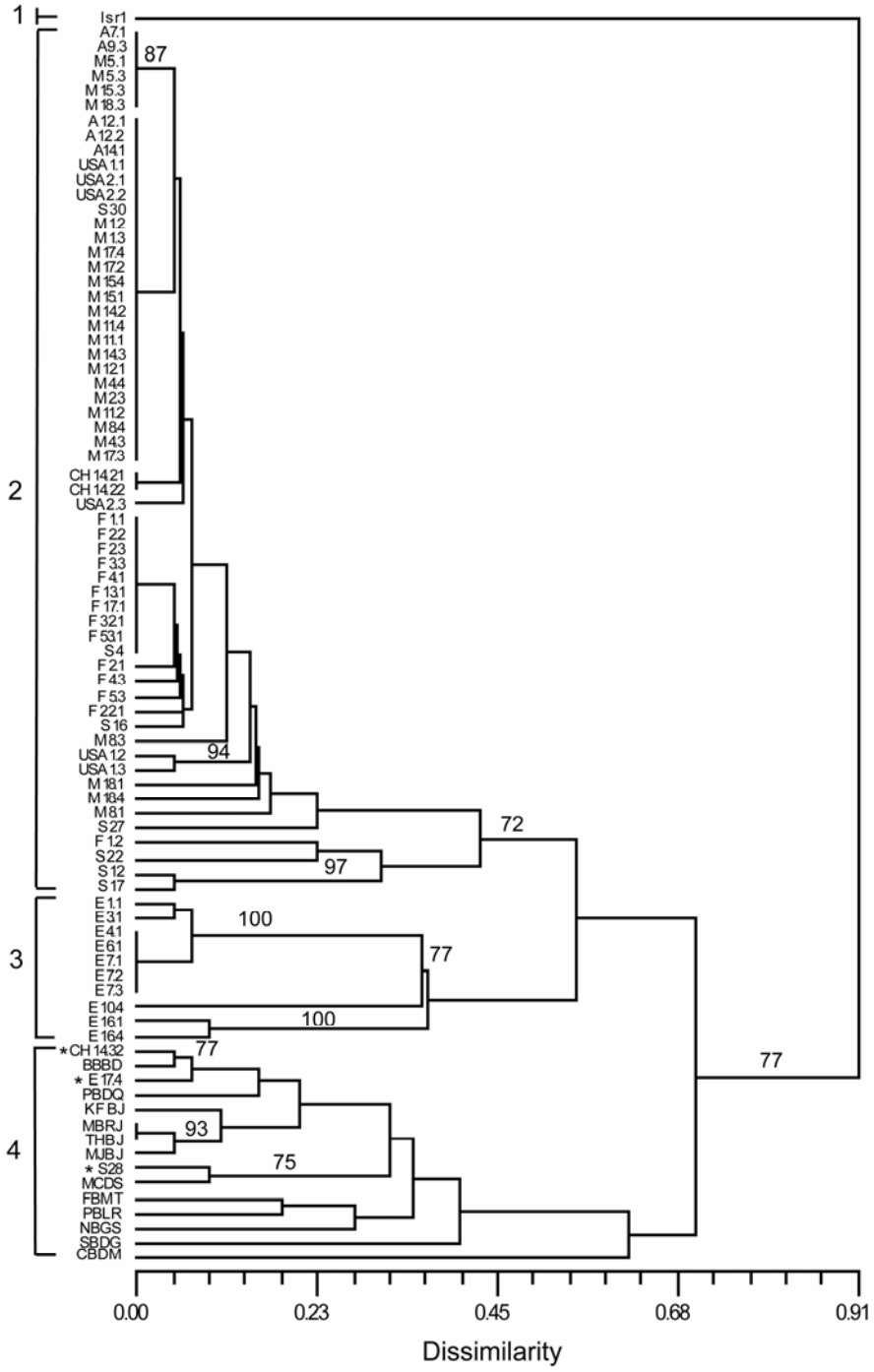

Fig. 1. Genetic dissimilarity dendrogram of *Puccinia triticina* isolates collected from durum wheat, common wheat, and *Aegilops speltoides* based on unweighted pair group method with arithmetic means clustering method, using the Kosman-Leonard (34) genetic distance coefficient calculated from 11 simple sequence repeat loci. Vertical lines define the four main clusters. Isolates from durum wheat in cluster 4 are marked with an asterisk. Numbers along the nodes are bootstrap values >70% in 1,000 replicas. A = Argentina, CH = Chile, E = Ethiopia, F = France, Isr = Israel, M = Mexico, USA = Unites States, S = Spain, and four-letter code = common wheat isolates.

types, which suggests a recent common ancestor for these isolates. The durum isolates from Ethiopia are genetically distinct from the durum isolates in Europe, North America, and South America, which suggests a different origin for these two major groups. The results also showed that isolates of *P. triticina* from durum wheat, common wheat, and *A. speltoides* have distinct SSR genotypes, which indicates a divergent selection of *P. triticina* genotypes based on host selection.

Cluster analyses indicated a high degree of genetic similarity among durum isolates from Argentina, Chile, the United States, Mexico, France, and Spain. Migration could explain how a population of *P. triticina*, after becoming established in one area, could move large distances between continents. Urediniospores of *P. triticina* are capable of movement within and between continents (30). Races of *P. triticina* virulent on wheat cultivars with the resistance gene *Lr17a* are thought to have been introduced into the United States and Canada in the mid 1990s, possibly from Mexico, based on analysis of virulence data and AFLP phenotypes (29). Phenotypes with virulence to *Lr17a* have spread quickly across the different wheat-growing regions of North America, and are now a common group of leaf rust isolates in the United States (33). In Europe, migration of a single wheat stripe rust pathogen (*P. striiformis*) population occurred between the United Kingdom, France, Germany, and Denmark as evidenced by virulence patterns and AFLP data (23). Park et al. (40) found *P. triticina* isolates from widely separated locations in Europe to have the same RAPD phenotype. Kolmer and Liu (31) found that *P. triticina* isolates from South America, western Canada, and central Europe had very similar RAPD phenotypes.

AMOVA analysis with R_{ST} and F_{ST} statistics showed significant levels of genetic differentiation among regions and isolates from different countries. Most of the variation was present within isolates from individual countries. Our data showed that, when the isolate from *A. speltoides* was not considered, isolates from France, Spain, Ethiopia, and common wheat had several private SSR alleles each. The presence of private alleles in isolates from individual countries could explain why most of the variation was found within countries. F_{ST} values from pairwise comparisons between isolates from different regions showed the same relationship as did pairwise analyses of Nei's genetic distances, evidenced by a high correlation value between F_{ST} and Nei (r = 0.91). A low correlation was found between Nei's genetic distance and R_{ST} (r = 0.35), however, which was not surprising because R_{ST} is calculated based on allele sizes, whereas F_{ST} and Nei consider the presence or absence of an allele regardless of molecular weight. Nonsignificant genetic differentiation in comparisons between South America and common wheat, North America and Europe, and Ethiopia and common wheat with R_{ST}, but not with F_{ST}, could be due to length homoplasies at one or

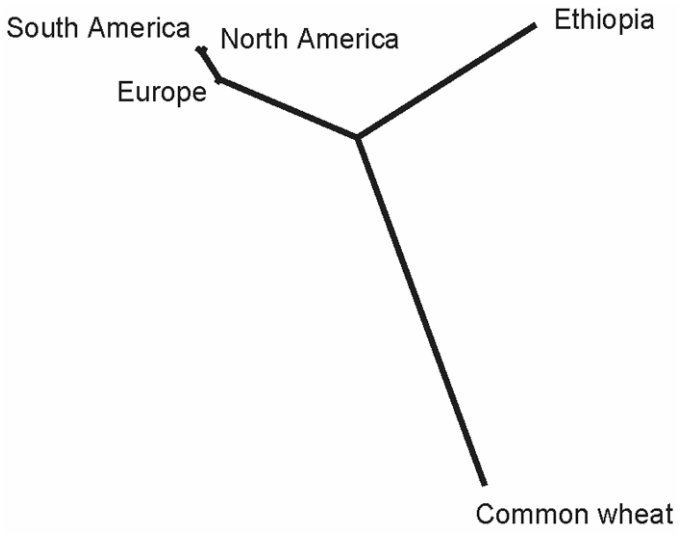

Fig. 3. Unrooted tree based on Nei's genetic distance calculated from 11 simple sequence repeat loci of *Puccinia triticina* isolates collected from durum wheat and common wheat. For *P. triticina* from durum wheat, South America includes isolates from Argentina and Chile, North America includes isolates from the United States and Mexico, and Europe includes isolates from France and Spain.

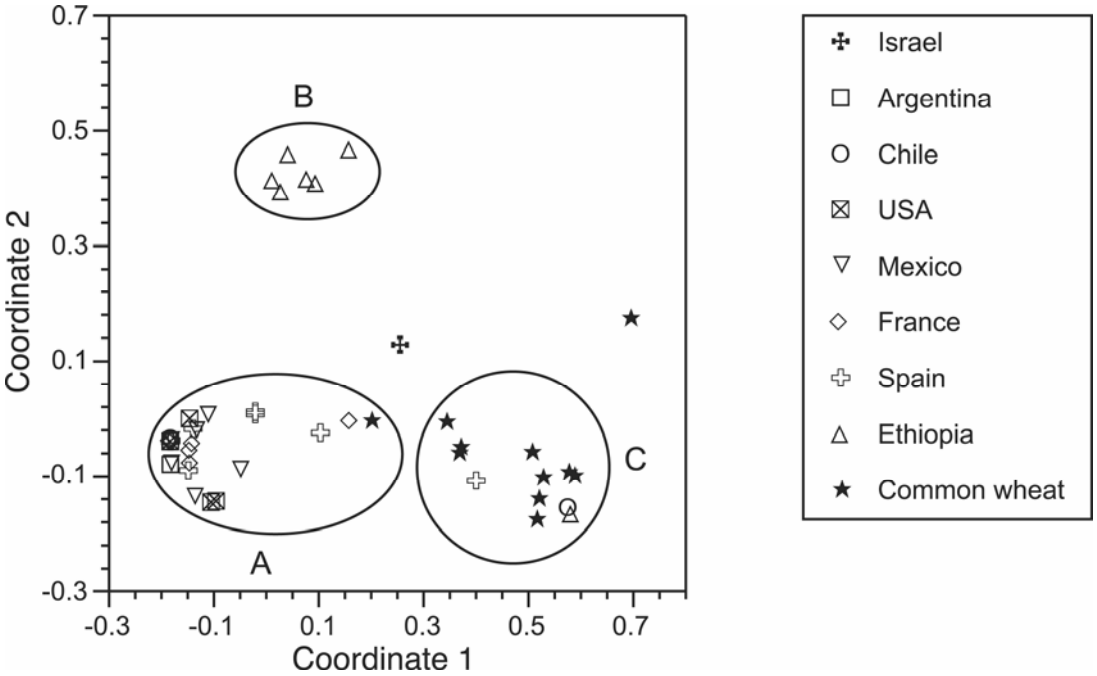

Fig. 2. Two-dimensional principal coordinate plot of 85 *Puccinia triticina* isolates collected from durum wheat, common wheat, and *Aegilops speltoides*. The analysis was based on Kosman-Leonard (34) genetic dissimilarity values from 11 simple sequence repeat loci. Three groups are delimited by circles: **A** = durum wheat isolates from Argentina, Chile, the United States, Mexico, France, and Spain plus one common wheat isolate from Ethiopia; **B** = durum wheat isolates from Ethiopia; and **C** = common wheat isolates from the United States plus three durum wheat isolates from Chile, Ethiopia, and Spain.

more loci (18) in those collections. The results presented here regarding genetic differentiation of *P. triticina* collections calculated from R_{ST} and F_{ST} violate assumptions of random mating under which inbreeding coefficients originally were derived (53). However, Excoffier et al. (14) derived their AMOVA methodology using mitochondrial DNA haplotype data, which is comparable to asexual cereal rust fungi, in that neither genome undergoes sexual recombination. The calculations of R_{ST} and F_{ST} for our pairwise comparisons were done through AMOVA using a resampling with replacement procedure that generated 999 population permutations, therefore generating reliable statistics for population differentiation. Results indicate that *P. triticina* populations from durum wheat from Europe and North and South America are very similar, whereas the populations from durum wheat from Ethiopia and common wheat are genetically distinct.

Shannon's information index showed that, on a locus basis, the durum leaf rust isolates from different countries had similar levels of genetic diversity, whereas the isolates from common wheat had the highest genetic diversity and the highest mean number of observed SSR alleles. The isolates from common wheat were purposely chosen for this study based on their genetic and phenoltypic diversity from previous RAPD and AFLP analyses (29,32). The observed heterozygosity of SSR alleles for all populations was higher than expected, based on expectations under Hardy-Weinberg equilibrium. High levels of heterozygosity at microsatellite loci have been maintained in these asexually reproducing populations. In 15 *P. triticina* isolates from Europe, Duan et al. (12) found higher levels of heterozygosity than expected at 8 of 12 polymorphic SSR loci. High levels of heterozygosity also have been found for virulence or avirulence alleles in populations of *P. triticina* (27). Burdon and Roelfs (9) found an excess of heterozygotes at four isozyme loci in an asexual population of the wheat stem rust pathogen *P. graminis* f. sp. *tritici*. Groth et al. (20) found that asexual collections of the bean rust fungus *Uromyces appendiculatus* had a higher frequency of putative heterozygotes for isozyme loci and avirulence or virulence alleles than in sexual collections. A selective advantage that maintains heterozygosity may be present in rust fungi, although mechanisms for such selection are unknown.

The *P. triticina* isolates from common wheat varied in molecular and virulence phenotypes, but all common wheat isolates tested were distinct from the durum population for SSR genotypes. Two isolates originally collected from durum wheat (CH14.32 and E17.4) that clustered with the common wheat isolates had virulences similar to the common wheat isolate BBBD, which is an older race of *P. triticina* rarely found on modern common wheat cultivars, because it is avirulent to many *Lr* genes. It is not likely, however, that the recent population of *P. triticina* on durum wheat found worldwide originated from any of the common wheat races tested in this study, as evidenced by the high genetic dissimilarity between these two groups. The Spanish isolate S28, which had virulence similar to the common wheat types (race MGDS) and also clustered with these isolates, most likely was a result of cross-contamination of isolates increased in the greenhouse, based on results from virulence tests on Thatcher differential lines for this isolate (data not shown). Martinez et al. (37) examined the virulence specificities of *P. triticina* populations from common wheat and durum wheat in Spain from 1998 to 2000 and did not report collections with race designation on durum wheat similar to the one found for isolate S28. Goyeau et al. (19) also found *P. triticina* populations from common wheat and durum wheat in France to be distinct, based on virulence phenotypes. Therefore, isolates S28, CH14.32, and E17.4 were included in the common wheat leaf rust population for analysis of genetic differentiation, because these isolates had virulence characteristics of common wheat leaf rust isolates.

The *P. triticina* isolate from *A. speltoides* was very distinct from the other *P. triticina* isolates studied here. Previous work done by Ben-Yehuda et al. (5,6) showed that, based on teliospore and basidiospore dimensions, nuclear DNA content, and aecial host preference, this rust resembles *P. triticina* found on wheat. Ben-Yehuda et al. (5) crossed the leaf rust from *A. speltoides* with

TABLE 4. Nei's genetic distance, R_{ST}, and F_{ST} between *Puccinia triticina* isolates from durum wheat, grouped by regions, and *P. triticina* isolates from common wheat, based on 11 simple sequence repeat loci[a]

Regions	Nei's genetic distance	R_{ST}[b]	F_{ST}[b]
South America–North America	0.003	0.000 (0.320)	0.000 (0.376)
South America–Europe	0.054	0.000 (0.361)	0.051 (0.089)
South America–Ethiopia	0.588	0.418 (0.001)	0.430 (0.001)
South America–Common wheat	0.847	0.048 (0.093)	0.389 (0.001)
North America–Europe	0.049	0.002 (0.266)	0.073 (0.008)
North America–Ethiopia	0.576	0.301 (0.001)	0.461 (0.001)
North America–Common wheat	0.822	0.141 (0.001)	0.450 (0.001)
Europe–Ethiopia	0.552	0.339 (0.001)	0.402 (0.001)
Europe–Common wheat	0.767	0.095 (0.001)	0.385 (0.001)
Ethiopia–Common wheat	0.866	0.069 (0.139)	0.369 (0.001)

[a] Regions were as defined in the text. South America: Argentina and Chile; North America: Mexico and the United States; Europe: France and Spain; and Ethiopia. For R_{ST} and F_{ST}, the common wheat collection was composed of isolates from common wheat from the United States and Ethiopia, plus common wheat type isolates from durum wheat from Chile (CH14.32), Spain (S28), and Ethiopia (E17.4).
[b] Probability values based on 999 permutations are shown in parenthesis. Negative pairwise R_{ST} and F_{ST} were converted to zero.

TABLE 5. Analysis of molecular variance with calculation of R_{ST} and F_{ST} within and between collections of *Puccinia triticina* isolates from different countries collected from durum wheat and common wheat, based on 11 simple sequence repeat loci

Source of variation[a]	df	Sum of squares	Mean squares	Estimated variance	Variation (%)	Statistic	Value	*P* value[b]
Among regions	4	19,619.52	4,919.88	164.76	13	R_{RT}	0.1364	0.001
Among countries within regions	3	779.42	259.81	0.00	0	R_{SR}	0	0.924
Within countries	160	176,053.09	1,100.33	1,100.33	87	R_{ST}	0.0887	0.008
Among regions	4	136.73	34.18	1.04	33	F_{RT}	0.333	0.001
Among countries within regions	3	5.32	1.77	0.00	0	F_{SR}	0	0.654
Within countries	160	334.64	2.09	2.09	67	F_{ST}	0.3268	0.001

[a] Regions were as defined in the text: South America, North America, Europe, Ethiopia, and common wheat. Isolates were defined based on country of origin and host preference. The common wheat collection was composed of isolates from common wheat from the United States and Ethiopia, plus common wheat type isolates collected from durum wheat from Chile (CH14.32), Spain (S28), and Ethiopia (E17.4).
[b] Probability of a larger value than observed with 999 permutations.

P. triticina and obtained viable F1 progeny. However, the two rust pathogens were very distinct for telial host range. The *A. speloides* rust fungus was avirulent to cultivated wheat but could infect hundreds of *A. speltoides* accessions that were immune to *P. triticina* from wheat. The authors proposed to designate this collection of leaf rust fungi as a forma specialis *speltoides* within *P. triticina*. Our results indicate that, based on SSR loci, *P. triticina* with virulence to common wheat and durum wheat and *P. triticina* virulent to *A. speltoides* are genetically very distinct. The isolate from *A. speltoides* had private alleles in seven of the nine SSR loci examined. Additionally, cluster analysis showed >90% dissimilarity between the *A. speltoides* isolate and the other *P. triticina* isolates tested.

Previous work on *P. triticina* revealed relatively little molecular polymorphism using RAPD markers when compared with the high virulence polymorphism present in the populations studied (31,40). Isolates with different virulence phenotypes often had the same RAPD phenotypes or differed by only one or two RAPD bands (32). The use of AFLPs later proved more successful in differentiating between leaf rust isolates with identical or similar virulence and RAPD phenotypes (29). The use of RAPD and AFLP markers has the advantage that multiple loci can be analyzed in a single experiment, although there is some concern regarding reproducibility of the results with RAPDs and the visualization of multiple bands with AFLPs to accurately determine the molecular phenotypes of the isolates. A disadvantage in the use of dominant molecular markers on *P. triticina*, however, is that these generate only phenotypic data, because cereal rust urediniospores are dikaryotic. The development and use of codominant SSR markers on *P. triticina* populations has the advantage of providing genotypic data from which more accurate genetic and evolutionary relationships can be developed between individuals and populations. The use of SSR markers for *P. triticina* in this study has proven very effective in distinguishing among collections of the pathogen on durum wheat, common wheat, and *A. spletoides* from worldwide locations, as well as detecting variation within collections, particularly in the common wheat rust pathogen collections. Keiper et al. (24) found that selectively amplified microsatellites revealed the greatest polymorphism within species of *P. triticina*, *P. graminis* f. sp. *tritici*, and *P. striiformis* f. sp. *tritici* from Australia compared with AFLP and sequence-specific amplification polymorphisms. Duan et al. (12) also identified 12 polymorphic microsatellite loci in a collection of *P. triticina* isolates from Europe. As more adequate statistical methods are developed for clonal dikaryotic organisms, the use of SSR markers will be of greater value to better understand the population genetics of *P. triticina* and other cereal rust pathogens. Kosman and Leonard's (34) new index to assess genetic dissimilarity between individuals in homozygous and heterozygous states offers a valuable alternative to the use of other matching coefficients such as Dice or Jaccard that present some difficulties for diploid genotypes, as discussed by those authors. In addition, there are drawbacks in the use of either R_{ST} or F_{ST} for analysis of genetic differentiation based on microsatellites, as discussed by Balloux and Lugon-Moulin (3). R_{ST} can have high variance, whereas F_{ST} follows the infinite allele model of mutation, which might not be suitable for microsatellites. Fisher et al. (18) compared sequence information of the flanking regions of microsatellite loci for determining the phylogeny of *Coccidioides immitis*. They found that genetic distances using microsatellite alleles based on the stepwise mutation model, similar to R_{ST}, did not perform as well as simple allele frequency data when compared with the sequence data obtained from the microsatellite flanking regions. They attributed this to the effects of insertion and deletions in the flanking sequences of the microsatellite repeat units.

This study determined that a population of *P. triticina* with enhanced virulence on durum wheat has spread rapidly throughout Europe, South America, and Mexico. It is possible that this new population arose from mutation and selection from a previous *P. triticina* population already present on durum wheat. The new *P. triticina* race found in Mexico in 2001 (47) has similar virulence on the *Lr* genes in the Thatcher differentials to one of the most common durum leaf rust pathogen races present in Mexico in the 1980s (46). Although the origin of the new race of *P. triticina* on durum wheat cannot be determined here, it is possible that it had a single origin and spread to cultivated durum in North America, South America, and Europe. Because there is relatively little genetic variation within the *P. triticina* population on durum wheat in North America, South America, and Europe, durum cultivars with resistance to these durum isolates in one location also should be resistant to the durum isolates in the other locations. The isolates of *P. triticina* found on durum wheat in Ethiopia, however, are genetically dissimilar from the other durum isolates tested in this study, suggesting a different origin and evolutionary history than the *P. triticina* isolates from durum wheat in North America, South America, and Europe. Therefore, it is likely that resistance to *P. triticina* on durum wheat in Ethiopia might not be effective against populations of the pathogen in North America, South America, and Europe and vice versa. Ethiopia is considered a center of diversity of the cultivated tetraploid wheat (21,45). It is estimated that emmer wheat (*T. dicoccum*) arrived in the highlands of Ethiopia from the Near East some 5,000 years ago (16,22). Durum wheat presumably originated later by accumulation of mutations to obtain a free-threshing wheat around 1,000 B.C.E. (16). Approximately 60% of the wheat cultivated in Ethiopia is tetraploid, and it is grown by small-scale peasant farmers who utilize mostly landrace varieties (4). The *P. triticina* isolates found in Ethiopia would have evolved on a very different type of durum host compared with the North American, South American, or European isolates.

TABLE 6. Genotypic variation and heterozygosity statistics from 11 simple sequence repeat loci of nine groups of *Puccinia triticina* isolates collected from durum wheat and common wheat

Isolates	No. of isolates	Mean *na*[a]	Mean *ne*[b]	Mean *I*[c]	Mean H_o[d]	Nei's H_e[e]
Durum wheat						
Argentina	5	1.6364	1.6029	0.4087	0.5455	0.2836
Chile	2	1.5455	1.5455	0.3781	0.5455	0.2727
United States	6	1.8182	1.6608	0.4608	0.5455	0.3056
Mexico	25	2.2727	1.6215	0.4658	0.5564	0.3029
France	14	2.3636	1.6428	0.5128	0.5649	0.3268
Spain	7	2.4545	1.8145	0.6461	0.5325	0.3924
Ethiopia	10	2.5455	1.9062	0.6360	0.5455	0.3773
Common wheat	15	3.7273	2.3325	0.9509	0.7212	0.5451

[a] Mean number of observed alleles.
[b] Mean number of effective alleles.
[c] Mean Shannon's information index.
[d] Mean observed heterozygosity.
[e] Mean Nei's expected heterozygosity.

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