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Genetic differentiation of the wheat leaf rust fungus *Puccinia triticina* in Europe

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The objective of this study was to determine whether genetically differentiated groups of *Puccinia triticina* are present in Europe. In total, 133 isolates of *P. triticina* collected from western Europe, central Europe and Turkey were tested for virulence on 20 lines of wheat with single leaf rust resistance genes, and for molecular genotypes with 23 simple sequence repeat (SSR) markers. After removal of isolates with identical virulence and SSR genotype within countries, 121 isolates were retained for further analysis. Isolates were grouped based on SSR genotypes using a Bayesian approach and a genetic distance method. Both methods optimally placed the isolates into eight European (EU) groups of *P. triticina* SSR genotypes. Seven of the groups had virulence characteristics of isolates collected from common hexaploid wheat, and one of the groups had virulence characteristics of isolates from tetraploid durum wheat. There was a significant correlation between the SSR genotypes and virulence phenotypes of the isolates. All EU groups had observed values of heterozygosity greater than expected and significant fixation values, which indicated the clonal reproduction of urediniospores in the overall population. Linkage disequilibria for SSR genotypes were high across the entire population and within countries. The overall values of R_{ST} and F_{ST} were lower when isolates were grouped by country, which indicated the migration of isolates within Europe. The European population of *P. triticina* had higher levels of genetic differentiation compared to other continental populations.

Keywords: brown rust, *Lr* resistance genes, simple sequence repeat, specific virulence, *Triticum aestivum*, *Triticum turgidum*

Introduction

Leaf rust, caused by *Puccinia triticina*, is a common disease of wheat (*Triticum aestivum*) in Europe. Leaf rust is considered to be important in northwest, southern and southeast Europe (Zadoks & Bouwmann, 1985), and in eastern Europe (Mesterhazy *et al.*, 2000). Breeding of wheat cultivars with leaf rust resistance has been conducted in some European countries, but fungicides are regularly applied to control the disease.

Virulence surveys of *P. triticina* have been conducted in the former Czechoslovakia (Hanzalova *et al.*, 2008), France (Goyeau *et al.*, 2006), Spain (Martinez *et al.*, 2005), Hungary (Manninger, 1994), Germany (Lind & Gultyaeva, 2007) and the UK (Bayles & Borrows, 2011). These studies used different wheat lines as host differentials and also used various types of nomenclature to describe the races or virulence phenotypes of *P. triticina*. As urediniospores of *P. triticina* are wind-dispersed it would be expected that the major virulence phenotypes would be found in more than one country across Europe, as was the case for race 77 and its derivatives from 1960 to 1980 (Zadoks & Bouwmann, 1985). An extensive study of *P. triticina* virulence in western Europe was conducted in 1995 (Park & Felsenstein, 1998) and was notable in that a large number of collections were obtained from a number of different countries and were characterized for virulence using the same differential set and description nomenclature. The identities of leaf rust resistance genes present in European winter wheat cultivars have also been postulated (Singh *et al.*, 2001; Pathan & Park, 2006; Goyeau & Lannou, 2011) by comparing infection types produced on the cultivars with infection types produced on near-isogenic lines of wheat with single resistance genes when tested with specific *P. triticina* virulence phenotypes. In general, the most common virulence phenotypes of *P. triticina*, as determined from the surveys, were virulent to the most common leaf rust resistance genes in the wheat cultivars, indicating selection for virulence in the *P. triticina* populations.

Molecular markers have also been used to describe variation in *P. triticina* in Europe. Randomly amplified polymorphic DNA (RAPD) markers (Park *et al.*, 2000) were used to characterize isolates from the 1995 Europe-wide

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virulence study. There was little evidence of distinct groupings of isolates based on RAPD phenotype, and little relationship between molecular polymorphism and virulence variation. *Puccinia triticina* isolates collected from different cultivars and locations in France were characterized for virulence and variation at microsatellite or simple sequence repeat (SSR) loci (Goyeau *et al.*, 2007). The *P. triticina* population in France was highly structured with a significant relationship between molecular variation and virulence. Goyeau *et al.* (2007) also determined that the *P. triticina* population in France reproduced by the clonal production of urediniospores, with no evidence of sexual recombination. However, pycnial infections and aeciospores of *P. triticina* were found on the alternate host *Thalictrum* sp. in Portugal (Palyart & Freitas, 1954; d'Oliveira & Samborski, 1966) and Italy (Sibilia, 1960; Casulli, 1988), raising the possibility of some sexual recombination events in parts of Europe. Also, *Thalictrum speciosissimum* is present in Turkey (Tatlidil *et al.*, 2005), although pycnial infections of *P. triticina* have not been recorded. Host selection has also affected *P. triticina* in Europe as isolates collected from *Triticum turgidum* (durum wheat) have virulence (Goyeau *et al.*, 2006) and molecular genotypes (Mantovani *et al.*, 2010) distinct from those of isolates collected from *T. aestivum* (common wheat).

The objective of this study was to determine if there was significant genetic differentiation in *P. triticina* isolates collected from across Europe, specifically whether distinct groups of *P. triticina* based on SSR genotypes and virulence phenotypes were found in Europe, as opposed to a single group of *P. triticina* genotypes showing no genetic differentiation. In addition, as the alternate host is present in Europe, different parameters of genotypic variation were examined for indications of sexual recombination in the *P. triticina* population. A collection of *P. triticina* isolates from western Europe, central Europe and Turkey were tested for virulence with a common set of wheat differential lines under standardized greenhouse conditions. The European isolates were genotyped at the same SSR loci that were used to characterize *P. triticina* isolates from North America (Ordoñez & Kolmer, 2009), South America (Ordoñez *et al.*, 2010), the Middle East (Kolmer *et al.*, 2011) and Central Asia (Kolmer & Ordoñez, 2007).

Materials and methods

Puccinia triticina isolates

Isolates of *P. triticina* were obtained from the Czech Republic and Slovakia (21 isolates), Germany (two isolates), Spain (three isolates), France (eight isolates), the UK (35 isolates), Hungary (six isolates), Italy (15 isolates), Romania (two isolates), Turkey (39 isolates) and Ukraine (two isolates), giving a total of 133 isolates. Isolates from the Czech Republic and Slovakia were collected between 1995 and 2009; isolates from Germany and Spain in the mid-1990s; isolates from France in 2004; isolates from the UK in 1976–1994 and 2009; isolates from Hungary in 1994–1995; isolates from Italy in 1994–2006; and isolates from Romania, Turkey and Ukraine in 2009. All isolates originated from collections of dried leaves with uredinial infections from a single wheat cultivar or breeding line at a single location. One to two single-uredinial isolates were derived from each collection and increased. Urediniospores were dried in a desiccator for 2 days and stored at −80°C.

Virulence phenotypes

Urediniospores of each isolate were used to inoculate 7-day-old seedlings of wheat cv. Thatcher (CI 1003) as previously described (Kolmer *et al.*, 2009) in order to increase urediniospores for virulence testing and DNA extraction. To determine the virulence phenotypes of the *P. triticina* isolates, five sets of four Thatcher near-isogenic lines of wheat each carrying one leaf rust resistance gene were used: set 1, *Lr1* (isogenic line RL6003), *Lr2a* (RL6000), *Lr2c* (RL6047) and *Lr3* (RL6002); set 2, *Lr9* (RL6010), *Lr16* (RL6005), *Lr24* (RL6064) and *Lr26* (RL6078); set 3, *Lr3ka* (RL6007), *Lr11* (RL6053), *Lr17* (RL6008) and *Lr30* (RL6049); set 4, *LrB* (RL6047), *Lr10* (RL6004), *Lr14a* (RL6013) and *Lr18* (RL6009); and set 5, *Lr3bg* (RL6042), *Lr14b* (RL6006), *Lr20* (RL 6092) and *Lr28* (RL6079). Thatcher was included as a susceptible control. Urediniospores of each isolate were spray-inoculated to each set of 7- to 8-day-old differentials. Virulence phenotypes were determined 10–12 days after inoculation for each isolate on each Thatcher differential line using a 0–4 scale (Long & Kolmer, 1989). Infection types $0–2^+$ (immune response to moderate uredinia with necrosis and/or chlorosis) were classified as avirulent and infection types 3–4 (moderate to large uredinia without chlorosis or necrosis) were classified as virulent. Each isolate was given a five-letter code based on virulence/avirulence to each of the five sets of four differentials, adapted from the North American nomenclature for virulence in *P. triticina* (Long & Kolmer, 1989). For analysis, virulence phenotypes were described with a 20-digit binary number based on avirulence/virulence.

Molecular genotypes

DNA was extracted from 25 to 30 mg urediniospores of each isolate by first grinding the spores with 25 mg glass beads in a Savant FastPrep shaker (FP120, Holbrook) for 20 s, and then using an OmniPrep extraction kit (GenoTech) according to the instructions. Between 1 and 2 ng DNA was used for each PCR amplification.

Twenty-three SSR microsatellite primer pairs developed from genomic libraries of *P. triticina* were used to characterize the collection: PtSSR 3, PtSSR 13, PtSSR 50, PtSSR 55, PtSSR 61, PtSSR 68-1, PtSSR 76, PtSSR 91, PtSSR 92, PtSSR 151A, PtSSR 152, PtSSR 154, PtSSR 158, PtSSR 161, PtSSR 164, PtSSR 173, PtSSR 184, PtSSR 186 (Szabo & Kolmer, 2007), RB 1, RB 8, RB 11, RB 26 and RB 35 (Duan *et al.*, 2003). Amplification and

electrophoresis were carried out as previously described (Szabo & Kolmer, 2007). Allele sizes in base pairs were scored visually for each primer pair by using a LI-COR 4200 or 4300 DNA sequencer that was calibrated with IRDye 700 molecular weight size standards. DNA bands generated by each primer pair were standardized with the allele sizes in the initial characterization of the SSR primers (Szabo & Kolmer, 2007) and also with other *P. triticina* isolates previously characterized using the same set of SSR primers. Separate DNA samples of isolates included in both previous studies and in the current study as controls had the same SSR genotypes.

Data analysis

The molecular weights for alleles at each of the 23 SSR loci for all isolates were recorded. The number of SSR genotypes was determined with GENODIVE v. 2.021b (Meirmans & Van Tienderen, 2004), and isolates were assigned genotype numbers. Isolates from the same country that had identical virulence phenotypes and SSR genotypes were eliminated, leaving 121 isolates for further analysis. The SSR data was formatted for analysis with INSTRUCT (Gao *et al.*, 2007), which uses a Bayesian approach similar to STRUCTURE (Pritchard *et al.*, 2000) to assign genotypes into subpopulations. However, as INSTRUCT was developed for use with inbreeding plant species, the Hardy–Weinberg equilibrium is not assumed in assigning individuals to subpopulations. INSTRUCT was run in the mode to infer population structure with admixture, with 200 000 Markov chain Monte Carlo (MCMC) iterations, a burn in of 100 000, thinning of 10, and testing for k groups of 1–20 with five separate chains for each k grouping. The SSR genotypes were also grouped using the K means clustering in GENODIVE v. 2.021b (Meirmans & Van Tienderen, 2004). This method assigns individual genotypes to k number of groups such that the within-groups sum of squares distance of the individuals to the group centroid is minimized, and the among-groups sum of squares is maximized. The random start method and simulated annealing method that uses MCMC steps to assign individuals to subpopulations were both used. A matrix of SSR allele frequency differences of the 121 isolates was used with both methods. One thousand random starts were used, and 100 000 steps were used for the simulated annealing, with k set between 2 and 20 for both methods. The pseudo-F statistic (Calinski & Harabsz, 1974) was used to determine the optimal number of subpopulations. Neighbour-joining trees (1001 in total) of the SSR genotypes of the 121 isolates were generated with POWERMARKER v. 3.25 (Liu & Muse, 2005) using Nei's distance coefficient and bootstrap values for support of the SSR groups were obtained with the CONSENSE program in PHYLIP v. 3.6 (Felsenstein, 1989).

Averages of single-locus parameters for the isolates in the SSR groups: number of alleles, number of effective alleles (N_E), Shannon's information index (I), observed heterozygosity (H_O), expected heterozygosity (H_E) and fixation index (F) were calculated with GENALEX v. 6 (Peakall & Smouse, 2006). Genetic differentiation via the AMOVA (Excoffier *et al.*, 1992) with 999 permutations of the data set was calculated for the SSR genotypes with R_{ST} (Slatkin, 1995) that assumes a stepwise mutation model and by F_{ST} that assumes the infinite alleles model. An analogous measure developed for binary data, Φ_{PT}, was used to calculate differentiation of the virulence phenotypes in the SSR groups. Pairwise values of R_{ST}, F_{ST} and Φ_{PT} were calculated via AMOVA amongst SSR groups. A Mantel correlation coefficient was calculated between the SSR distance matrix and the virulence distance matrix with GENALEX v. 6. Linkage disequilibrium across all SSR loci was calculated with the index of association (I_A), and also with a measure corrected for the number of loci, $\bar{r}_D$, using MULTILOCUS v. 1.3 (Agapow & Burt, 2001). Tests of departure from random mating for both indices were done with 1000 randomizations of the data set. The significance in differences of frequency (%) of virulence to leaf rust resistance genes in different SSR groups of *P. triticina* isolates was determined with Fisher's exact test (Steel & Torrie, 1980).

Results

Population assignment

The 133 isolates were tested for SSR genotype at 23 loci and for virulence to 20 Thatcher near-isogenic lines. After removal of isolates with identical SSR genotypes and virulence phenotypes within each country, 121 isolates remained for further analysis. Based on INSTRUCT, the log-likelihood posterior mean for assignment of SSR genotypes was smallest (−2667) with k at eight subpopulations. There were no differences in isolate grouping between the five individual runs with k at eight populations. The large majority of isolates had a posterior probability of assignment to their respective group of >0·90 (Table 1). A few isolates had lower assignment probabilities between one or more European (EU) groups. Isolate #9 from the UK had an assignment probability of 0·529 to EU1 and a probability of 0·420 to EU8 (Table 1). Other isolates with relatively low probabilities for two EU groups are also listed in Table 1. Using k means clustering in GENODIVE, the results of the random start and simulated annealing procedures were identical, as both methods indicated that the optimal number of subpopulations was eight based on the pseudo-F statistic. The assignments of individuals to the eight subpopulations using INSTRUCT and k means clustering in GENODIVE were identical. EU2 was the largest group, with 29 isolates, and had 24 virulence phenotypes and 16 SSR genotypes (Table 2). EU6 was the smallest group, with six isolates, and had the smallest number of virulence phenotypes and SSR genotypes. The EU SSR groups were cosmopolitan, as all groups had isolates from more than one country. EU3 had isolates from only Italy and Spain. All other EU groups had isolates from three or more countries. EU7 was the most diverse geographically, with isolates from Italy, Turkey, Germany, UK, France and Spain. A total of

Table 1 Isolates of *Puccinia triticina* with virulence phenotype[a] and European (EU) group based on *k* means clustering[b] of simple sequence repeat (SSR) genotypes and posterior probability from INSTRUCT[c]

Isolate no.	Country	Designation	Virulence phenotype	SSR genotype	EU SSR group	Posterior probability	EU SSR group	Posterior probability
1	Czech/Slovakia	CS20-09	BBBQB	1	1	0·95	–	–
2	Czech/Slovakia	CS9-09	DBGQG	2	1	0·91	–	–
3	France	FR60	DGGQG	3	1	0·94	–	–
4	UK	GB1-1-09	DGGQG	4	1	0·94	–	–
5	UK	GB10-1-09	DGGQG	5	1	0·94	–	–
6	UK	GB15-3-09	BBBQG	1	1	0·95	–	–
7	UK	GB13-3-09	BBBSG	6	1	0·84	–	–
8	UK	GB14-1-09	DHJSG	1	1	0·95	–	–
9	UK	GB7-3-09	BHBQG	7	1	0·53	8	0·42
10	UK	GB76-1-2	BBBQJ	8	1	0·94	–	–
11	UK	GB80-1-1	DCJQG	9	1	0·89	–	–
12	UK	GB81-2-1	DBGGG	10	1	0·91	–	–
13	UK	GB81-5-1	DCLJG	9	1	0·93	–	–
14	UK	GB81-5-2	DCLJG	11	1	0·89	–	–
15	UK	GB85-31-2	DCJLG	12	1	0·91	–	–
16	UK	GB90-10-2	DHDNJ	13	1	0·94	–	–
17	UK	GB90-11-1	BBBQG	14	1	0·95	–	–
18	UK	GB90-12-2	DGJQL	15	1	0·94	–	–
19	UK	GB90-26-2	DCLJG	16	1	0·92	–	–
20	Czech/Slovakia	CS4-09	FHPNQ	17	2	0·97	–	–
21	Turkey	TK10-1-09	FHPTQ	18	2	0·96	–	–
22	Turkey	TK11-1-09	PGPSS	19	2	0·97	–	–
23	Turkey	TK11-3-09	PBFSS	20	2	0·95	–	–
24	Turkey	TK13-3-09	PCFSQ	21	2	0·95	–	–
25	Turkey	TK14-3-09	FHPTS	22	2	0·97	–	–
26	Turkey	TK17-3-09	PHPTQ	23	2	0·97	–	–
27	Turkey	TK18-1-09	PBPSQ	21	2	0·95	–	–
28	Turkey	TK18-3-09	PBFSL	21	2	0·95	–	–
29	Turkey	TK19-1-09	FCTSQ	24	2	0·94	–	–
30	Turkey	TK20-1-09	PCFSL	21	2	0·95	–	–
31	Turkey	TK20-2-09	FCPSQ	18	2	0·96	–	–
32	Turkey	TK20-3-09	FHPSQ	25	2	0·97	–	–
33	Turkey	TK22-2-09	FCMNQ	25	2	0·97	–	–
34	Turkey	TK23-1-09	FCPSQ	26	2	0·92	–	–
35	Turkey	TK23-2-09	FCPNQ	25	2	0·97	–	–
36	Turkey	TK23-3-09	FHPNQ	25	2	0·97	–	–
37	Turkey	TK24-1-09	PCPPQ	25	2	0·97	–	–
38	Turkey	TK24-2-09	PCPNQ	27	2	0·97	–	–
39	Turkey	TK25-2-09	FHPPQ	28	2	0·97	–	–
40	Turkey	TK25-3-09	PHPPQ	25	2	0·97	–	–
41	Turkey	TK27-1-09	PBPSN	21	2	0·95	–	–
42	Turkey	TK29-1-09	PBPSL	29	2	0·95	–	–
43	Turkey	TK30-1-09	PBPSL	21	2	0·96	–	–
44	Turkey	TK30-2-09	FCPSL	30	2	0·92	–	–
45	Turkey	TK6-3-09	CCTSL	31	2	0·89	–	–
46	Turkey	TK9-3-09	FCPTQ	25	2	0·97	–	–
47	Ukraine	UK33-1-09	PBFSL	21	2	0·95	–	–
48	Ukraine	UK33-2-09	PBFSL	32	2	0·96	–	–
49	Spain	ES1-1	DBBGJ	33	3	0·96	–	–
50	Spain	ES14-1	FGBNQ	34	3	0·48	2	0·14
51	Italy	PSB1-3	BBBGJ	35	3	0·98	–	–
52	Italy	PSB16-2	BBBQG	36	3	0·96	–	–
53	Italy	PSB7-3	FGBQQ	37	3	0·86	–	–
54	Italy	ITA1-1	BBBGG	38	3	0·98	–	–
55	Italy	ITA1-2	DBBGJ	38	3	0·98	–	–
56	Italy	ITA15-1	FGBQQ	38	3	0·98	–	–
57	Italy	ITA2-2	FGBQS	38	3	0·98	–	–
58	Italy	ITA7-1	BBBGK	39	3	0·96	–	–
59	Czech/Slovakia	CS95-2-2	FCPQQ	40	4	0·96	–	–

Table 1 (Continued)

Isolate no.	Country	Designation	Virulence phenotype	SSR genotype	EU SSR group	Posterior probability	EU SSR group	Posterior probability
60	Czech/Slovakia	CS95-4-2	FCPQS	40	4	0·96	–	–
61	Czech/Slovakia	CS95-7-1	FCPNQ	40	4	0·96	–	–
62	Czech/Slovakia	CS95-9-2	FCFDL	40	4	0·96	–	–
63	Czech/Slovakia	CS18-09	FCPNS	41	4	0·95	–	–
64	Czech/Slovakia	CS19-09	FCPNS	42	4	0·95	–	–
65	France	FR59	FCPSS	40	4	0·96	–	–
66	France	FR61	FCPSL	40	4	0·96	–	–
67	France	FR64	FCPNQ	40	4	0·96	–	–
68	UK	GB12-3-09	BBDQG	43	4	0·68	7	0·16
69	UK	GB5-2-09	FCPSQ	42	4	0·95	–	–
70	UK	GB8-2-09	FCPSQ	44	4	0·96	–	–
71	UK	GB94-1-1	FCPLL	45	4	0·94	–	–
72	UK	GB94-1-2	FCPLS	46	4	0·95	–	–
73	Turkey	TK3-1-09	CCPSL	42	4	0·95	–	–
74	Czech/Slovakia	CS10-09	FHMQQ	47	5	0·78	2	0·15
75	France	FR58	NBGQG	48	5	0·65	3	0·16
76	UK	GB11-2-09	DCBGG	49	5	0·95	–	–
77	UK	GB11-3-09	DBBQG	49	5	0·95	–	–
78	UK	GB4-2-09	DBBQH	50	5	0·95	–	–
79	UK	GB82-1-2	DBBQJ	51	5	0·93	–	–
80	UK	GB93-1-2	DBBQG	52	5	0·94	–	–
81	Italy	PSB13-2	FBBQQ	53	5	0·83	–	–
82	Italy	PSB2-2	FGBQS	53	5	0·83	–	–
83	Italy	PSB3-3	FGBQQ	53	5	0·83	–	–
84	Turkey	TK19-2-09	KCMQQ	54	5	0·78	2	0·15
85	Czech/Slovakia	CS95-6-1	TCPDL	55	6	0·89	–	–
86	Germany	DL62-1-1	SCJBJ	56	6	0·55	2	0·31
87	Hungary	HG95-10-1	TCPBN	57	6	0·95	–	–
88	Hungary	HG95-3-2	TCBBD	57	6	0·95	–	–
89	Hungary	HG95-9-1	TCTBN	57	6	0·95	–	–
90	Hungary	HG95-4-2	TCTLN	57	6	0·95	–	–
91	Germany	DL24-4-2	FCMLQ	58	7	0·95	–	–
92	Spain	ES9-1	FGBQQ	59	7	0·87	–	–
93	France	FR56	FBPSQ	58	7	0·95	–	–
94	UK	GB93-1-1	FBMSQ	60	7	0·94	–	–
95	Italy	IT12-2	FGPSQ	58	7	0·94	–	–
96	Italy	IT14-1	FGMNS	61	7	0·66	4	0·20
97	Italy	IT4-1	FCTSQ	62	7	0·77	4	0·16
98	Turkey	TK14-1-09	FBMSQ	63	7	0·95	–	–
99	Turkey	TK14-2-09	FHPTQ	63	7	0·95	–	–
100	Turkey	TK19-3-09	FCMTQ	64	7	0·70	4	0·20
101	Turkey	TK3-2-09	FCMSS	64	7	0·69	4	0·16
102	Turkey	TK4-1-09	FBMSQ	65	7	0·94	–	–
103	Turkey	TK6-2-09	CBMSQ	63	7	0·95	–	–
104	Turkey	TK8-2-09	CBMSQ	66	7	0·93	–	–
105	Czech/Slovakia	CS11-09	MHPSS	67	8	0·94	–	–
106	Czech/Slovakia	CS13-09	MCPSS	68	8	0·94	–	–
107	Czech/Slovakia	CS16-09	MCPSQ	68	8	0·94	–	–
108	Czech/Slovakia	CS2-09	LBDSQ	69	8	0·66	6	0·20
109	Czech/Slovakia	CS5-09	MHPSQ	70	8	0·91	–	–
110	Czech/Slovakia	CS6-09	MHPSQ	71	8	0·92	–	–
111	Czech/Slovakia	CS27-09	MNPSS	68	8	0·94	–	–
112	France	FR55	MCDSS	72	8	0·95	–	–
113	France	FR57	MBDSS	72	8	0·95	–	–
114	UK	GB14-3-09	MFPSS	73	8	0·95	–	–
115	UK	GB7-1-09	PCDSS	74	8	0·63	4	0·24
116	UK	GB7-2-09	PCDSJ	74	8	0·64	4	0·25
117	UK	GB9-1-09	MCPSQ	75	8	0·94	–	–
118	Hungary	HG94-4-1	TBDKT	76	8	0·91	–	–

Table 1 (Continued)

Isolate no.	Country	Designation	Virulence phenotype	SSR genotype	EU SSR group	Posterior probability	EU SSR group	Posterior probability
119	Turkey	TK1-1-09	MCDSS	73	8	0·95	–	–
120	Turkey	TK2-3-09	MCDSS	77	8	0·95	–	–
121	Turkey	TK9-1-09	MBPSL	78	8	0·66	2	0·26

[a]A five-letter code describes virulence to 20 Thatcher near-isogenic wheat lines as adapted from Long & Kolmer (1989)
[b]Meirmans & Van Tienderen (2004).
[c]Gao *et al.* (2007).

Table 2 Genotypic diversity in eight groups of *Puccinia triticina* from Europe as grouped by simple sequence repeat (SSR) genotypes for virulence to 20 Thatcher lines of wheat with different leaf rust resistance genes and for 23 SSR loci

	European (EU) group								
Parameter	EU1	EU2	EU3	EU4	EU5	EU6	EU7	EU8	Total
Number of isolates	19	29	10	15	11	6	14	18	121
Number of virulence phenotypes	13	24	9	12	9	6	11	13	88
Number of SSR genotypes	16	16	7	7	8	3	9	12	78

88 virulence phenotypes and 78 SSR genotypes were characterized amongst the 121 isolates.

Isolates from different countries with identical SSR genotypes and closely related virulence phenotypes were found. In EU1, isolate #1 CS20-09 from the Czech Republic/Slovakia with virulence phenotype BBBQB had SSR genotype #1, as did isolate #6 GB15-3-09 BBBQG from the UK. Phenotypes BBBQB and BBBQG differed only for virulence to *Lr14b*. In EU2, isolate #47 UK33-1-09 from the Ukraine had SSR genotype #21, as did six isolates from Turkey. In EU4, four isolates from the Czech Republic/Slovakia had identical SSR genotype #40 with three isolates from France. All seven isolates were highly related for virulence with a FCP– virulence phenotype. Also in EU4, single isolates from the Czech Republic/Slovakia, the UK and Turkey had SSR genotype #42. In EU7, single isolates from France, Germany and Italy had SSR genotype #58. In EU8, isolate #114 from the UK had SSR genotype #73, as did isolate #119 from Turkey.

Although the isolates were sampled from 1976 to 2009, this had little discernable effect on their grouping, as most of the EU groups included isolates that were sampled over a period of more than 15 years. Some isolates within EU groups sampled over a number of years also had highly similar virulence phenotypes. In EU1, isolate #10 BBBQJ collected from the UK in 1976 differed in virulence to only one Thatcher isoline compared with isolate #6 BBBQG, collected in 2009, also from the UK. Similar pairs of isolates that were highly related for virulence, yet were collected over a span of 15 or more years, were also present in the groups EU4, EU5 and EU7. Isolates in EU6 were an exception, as all were collected in the mid-1990s from Hungary and Germany.

Single-locus parameters

The eight EU groups varied for mean number of SSR alleles per locus from 3·13 in EU1 to 2·0 in EU6. EU1 and EU5 had the highest mean effective allele value of 2·23, whilst EU6 had the lowest value of 1·49 (Table 3). EU1 and EU5 had the highest values of average Shannon diversity for SSR loci of 0·835 and 0·78, respectively, whilst

Table 3 Average of single-locus parameters of *Puccinia triticina* isolates from Europe in groups of simple sequence repeat (SSR) genotypes

	European (EU) SSR group								
Parameter	EU1	EU2	EU3	EU4	EU5	EU6	EU7	EU8	Total
Number of alleles	3·13 (0·30)[a]	2·04 (0·21)	2·49 (0·21)	2·21 (0·18)	2·91 (0·34)	2·00 (0·19)	2·35 (0·27)	2·91 (0·28)	2·50 (0·09)
Number of effective alleles	2·23 (0·19)	1·59 (0·11)	1·55 (0·11)	1·81 (0·17)	2·23 (0·22)	1·49 (0·134)	2·04 (0·21)	1·91 (0·15)	1·85 (0·06)
Shannon *I*	0·835 (0·08)	0·443 (0·08)	0·50 (0·07)	0·58 (0·07)	0·78 (0·10)	0·40 (0·08)	0·64 (0·10)	0·68 (0·08)	0·61 (0·03)
Number of private alleles	10	5	5	2	6	2	4	5	39
H_O	0·735 (0·06)	0·472 (0·09)	0·43 (0·08)	0·70 (0·09)	0·71 (0·08)	0·30 (0·08)	0·68 (0·09)	0·64 (0·08)	0·58 (0·03)
H_E	0·491 (0·04)	0·29 (0·05)	0·29 (0·04)	0·38 (0·05)	0·46 (0·05)	0·23 (0·05)	0·40 (0·06)	0·41 (0·04)	0·37 (0·02)
F	−0·47 (0·07)	−0·55 (0·07)	−0·31 (0·09)	−0·67 (0·11)	−0·53 (0·06)	−0·18 (0·10)	−0·71 (0·06)	−0·48 (0·07)	−0·49 (0·30)

[a]Standard error.

EU6 had the lowest diversity of 0·40. Similarly, EU1 and EU5 had the highest number of private alleles, whilst EU6 had the lowest number. All EU groups had values of observed heterozygosity (H_O) that were higher than the values expected (H_E) under the Hardy–Weinberg equilibrium. All EU groups had significant negative fixation indices (F).

Linkage disequilibrium

Linkage disequilibrium was high amongst the 78 SSR genotypes with an I_A of 3·59 ($P < 0·001$) and an $\bar{r}_D$ of 0·165. Linkage diversity was also high amongst SSR genotypes within individual countries. The 20 SSR genotypes from Turkey had an I_A of 3·692 ($P < 0·001$) and an $\bar{r}_D$ of 0·191, and the 25 SSR genotypes from the UK had an I_A of 3·915 ($P < 0·001$) and an $\bar{r}_D$ of 0·1841.

Differentiation of SSR groups

The overall value of R_{ST} for the eight EU groups was 0·506, with 38% of the SSR variation between groups, 0% between individuals and 62% within individuals. All pairs of EU groups were differentiated for R_{ST} at the 0·001 confidence level, except for groups EU2 and EU8; EU2 and EU7; and EU7 and EU8 (Table 4). The overall F_{ST} value was 0·317, with 23% of the SSR variation between groups, 0% between individuals and 77% within individuals. All EU groups were differentiated for F_{ST} at the 0·001 confidence level. Nei's genetic distance between the EU groups is indicated in Figure 1.

Virulence phenotypes

The eight EU groups differed for frequency of virulence to 16 of the Thatcher near-isogenic lines that were tested (Table 5). The EU groups did not differ for virulence to lines with genes *Lr9*, *Lr16*, *Lr24* and *Lr28*. Isolates in EU3 had virulence frequencies of 0% or 10% to 13 of the Thatcher lines, and were nearly fixed for virulence to *Lr10*. This virulence profile is characteristic of isolates collected from durum wheat (Goyeau *et al.*, 2006). Isolates in the other EU groups had virulence characteristic

Table 4 R_{ST} values (above diagonal) and F_{ST} values (below diagonal) of genetic differentiation of simple sequence repeat (SSR) genotype groups of *Puccinia triticina* from Europe

Group	EU1	EU2	EU3	EU4	EU5	EU6	EU7	EU8
EU1	–	0·39*	0·59*	0·92*	0·30*	0·20*	0·51*	0·26*
EU2	0·34*	–	0·62*	0·38*	0·50*	0·41*	0·02	0·04
EU3	0·36*	0·60*	–	0·98*	0·44*	0·75*	0·69*	0·52*
EU4	0·26*	0·32*	0·47*	–	0·96*	0·97*	0·38*	0·47*
EU5	0·21*	0·31*	0·31*	0·30*	–	0·35*	0·59*	0·38*
EU6	0·31*	0·26*	0·63*	0·34*	0·32*	–	0·50*	0·28*
EU7	0·28*	0·30*	0·39*	0·20*	0·22*	0·35*	–	0·07
EU8	0·27*	0·29*	0·42*	0·25*	0·21*	0·31*	0·18*	–

*Significant at $P < 0·001$.

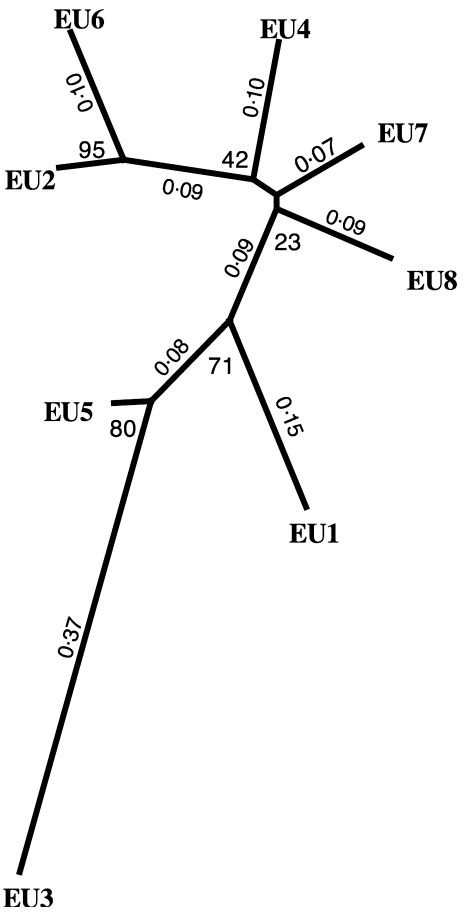

Figure 1 Neighbour-joining plot of Nei's genetic distance between groups of SSR genotypes of *Puccinia triticina* from Europe. Numbers on branch lengths indicate Nei's genetic distance. Numbers at branch junctions indicate bootstrap values.

of *P. triticina* collected from common wheat. The overall Φ_{PT} value of differentiation for the eight EU SSR groups was 0·464, with 46% of the variation between groups and 54% within groups. All pairs of EU groups were differentiated for Φ_{PT} at the 0·001 confidence level except for EU3 and EU5 (Table 6). The Mantel correlation of the virulence distance matrix with the SSR distance matrix for all 121 isolates was 0·549, with a significance level of 0·001.

Geographic differentiation

Isolates from the Czech Republic/Slovakia, France, the UK, Italy, Turkey, and Hungary were grouped on the basis of country of origin to determine if there was any geographic basis to the distribution of the SSR genotypes and virulence phenotypes. The overall R_{ST} was 0·188, with 18% of the variation between countries and 82% amongst isolates within countries. Isolates from Germany, Spain, Romania and Ukraine were not considered in pairwise comparisons because of sample sizes less than five. Based on R_{ST}, isolates from France and the Czech

Table 5 Frequencies of virulence to leaf rust resistance genes in isolates of *Puccinia triticina* from Europe in groups of simple sequence repeat (SSR) genotypes

Gene	EU1	EU2	EU3	EU4	EU5	EU6	EU7	EU8	Difference
Lr1	0·00	0·52	0·00	0·00	0·09	1·00	0·00	1·00	**
Lr2a	0·00	0·00	0·00	0·00	0·09	1·00	0·00	0·06	**
Lr2c	0·68	0·97	0·60	0·87	1·00	1·00	0·86	0·17	**
Lr3	0·00	1·00	0·40	0·93	0·46	0·83	1·00	0·94	**
Lr9	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·06	ns
Lr16	0·36	0·31	0·40	0·00	0·27	0·00	0·27	0·17	ns
Lr24	0·00	0·00	0·00	0·00	0·00	0·00	0·00	0·11	ns
Lr26	0·42	0·70	0·00	0·93	0·27	1·00	0·36	0·72	**
Lr3ka	0·15	0·79	0·00	0·87	0·18	0·67	0·93	0·56	**
Lr11	0·47	0·07	0·00	0·00	0·09	0·50	0·07	0·00	**
Lr17	0·26	0·97	0·00	1·00	0·00	0·83	0·29	1·00	**
Lr30	0·00	1·00	0·00	0·93	0·18	0·67	0·92	0·56	**
LrB	0·78	1·00	0·50	0·93	0·91	0·17	1·00	0·94	**
Lr10	0·89	0·73	0·90	0·53	1·00	0·00	0·85	1·00	**
Lr14a	0·31	1·00	0·10	0·67	0·00	0·17	0·85	1·00	**
Lr18	0·00	0·24	0·00	0·00	0·00	0·00	0·14	0·06	*
Lr3bg	0·05	1·00	0·40	0·93	0·46	0·67	1·00	0·89	**
Lr14b	0·89	0·69	1·00	0·73	1·00	0·16	1·00	0·94	**
Lr20	0·10	0·14	0·50	0·33	0·18	0·83	0·14	0·67	**
Lr28	0·00	0·00	0·10	0·00	0·09	0·00	0·00	0·06	ns

ns: no significant difference.
Significance: $^{**}P < 0{\cdot}001$; $^{*}P < 0{\cdot}05$.

Table 6 Φ_{PT} values of genetic differentiation of virulence phenotypes of *Puccinia triticina* isolates from Europe in groups of SSR genotypes

Group	EU1	EU2	EU3	EU4	EU5	EU6	EU7	EU8
EU1	–							
EU2	0·56*	–						
EU3	0·16*	0·60*	–					
EU4	0·54*	0·14*	0·59*	–				
EU5	0·13*	0·52*	0·07	0·51*	–			
EU6	0·61*	0·54*	0·63*	0·53*	0·60*	–		
EU7	0·50*	0·20*	0·50*	0·25*	0·41*	0·64*	–	
EU8	0·55*	0·29*	0·56*	0·38*	0·54*	0·55*	0·43*	–

*Significant at $P < 0{\cdot}001$.

Republic/Slovakia; the Czech Republic/Slovakia and Turkey; Hungary and Italy; and France and Turkey were not differentiated for SSR genotype. All other country pairs were differentiated for R_{ST} at the 0·01 confidence level (Table 7). The overall F_{ST} for grouping isolates by country was 0·146, with 12% of the SSR variation between isolates in different countries and 88% amongst isolates within countries. Isolates from France and the Czech Republic/Slovakia; and France and the UK were not significantly differentiated for F_{ST} at the 0·01 confidence level.

Discussion

The European collections of *P. triticina* isolates were characterized by highly differentiated groups of SSR genotypes. These groups also differed significantly for virulence to Thatcher lines with leaf rust resistance genes, with a significant association between molecular genotype and virulence phenotype. Isolates within the groups were highly related for SSR genotype, and also related for virulence phenotype. Recurrent mutations in the *P. triticina* isolates probably account for the molecular and virulence variation.

In Europe, seven groups of *P. triticina* with virulence and SSR genotypes characteristic of isolates from common wheat were found in comparison with five groups in North America (Ordoñez & Kolmer, 2009) and South America (Ordoñez *et al.*, 2010), two groups in the Middle East (Kolmer *et al.*, 2011) and four groups in Central Asia (Kolmer & Ordoñez, 2007). The high diversity of molecular genotypes in Europe may be related to the long-term cultivation of wheat; diverse wheat genotypes that differ for leaf rust resistance genes; migration of *P. triticina* from the Middle East and Central Asia, and occasional sexual or parasexual recombination in populations of *P. triticina*. Cultivated emmer wheat was brought to Europe from the Fertile Crescent region over 5000 years ago (Feldman, 2001). *Puccinia triticina* probably also came to Europe shortly afterwards from the same region. Aecial infections on *Thalictrum* sp. that are pathogenic to wheat have been found in southern (Casulli, 1988) and northern Italy (Sibilia, 1960; Tommasi *et al.*, 1980) and in Portugal (Palyart & Freitas, 1954; d'Oliveira & Samborski, 1966). It is feasible that aeciospores occasionally infected wheat that led to the establishment of new genotypes. Mutation in a long-established population combined with some recombination events may have resulted in highly diverse groups of *P. triticina* genotypes. Past migration events from the presumed centre of origin of *P. triticina* in Central Asia and the Middle East may also

Table 7 R_{ST} values (above diagonal) and F_{ST} values (below diagonal) of genetic differentiation of simple sequence repeat (SSR) genotypes of *Puccinia triticina* isolates from Europe

Number of isolates	Country	Country					
		Czech/Slovakia	France	UK	Italy	Turkey	Hungary
18	Czech/Slovakia	–	0·00	0·13**	0·40**	0·00	0·42**
8	France	0·01	–	0·09*	0·37**	0·00	0·42**
31	UK	0·05*	0·03	–	0·17**	0·15**	0·17**
14	Italy	0·22**	0·17**	0·15**	–	0·42**	0·00
38	Turkey	0·09**	0·12**	0·14**	0·34**	–	0·43**
5	Hungary	0·14*	0·19**	0·18**	0·40**	0·18**	–

Significance: $^{**}P < 0{\cdot}001$; $^{*}P < 0{\cdot}01$.

have contributed to high diversity. SSR genotype groups in the Middle East are closely related to groups in Europe (J. A. Kolmer, unpublished data) indicating the possibility of some migration or a common origin amongst the populations in these regions. Differences in genotypic diversity between the various continental regions may also be affected by the different collection methods used to obtain the *P. triticina* isolates in the different studies. Additional distinct SSR genotypes may have been detected if a greater number of isolates had been obtained from throughout Europe.

In this study the various population parameters indicated clonal reproduction in the European collections of *P. triticina*. The significant correlation of virulence phenotypes with SSR genotypes, the high levels of observed heterozygosity relative to the expected values (Halkett *et al.*, 2005) and the high levels of linkage disequilibrium amongst the SSR genotypes are characteristics of clonal reproduction by urediniospores. Goyeau *et al.* (2007) also found no evidence for sexual recombination in the *P. triticina* population in France. However, isolates with a sexual origin may occasionally arise and then be clonally dispersed across Europe. Isolates with posterior assignment probabilities to more than one EU group may have possibly originated from sexual recombination. Isolate #9 from the UK had nearly equal assignment probabilities for EU1 and EU8, and isolate #86 from Germany had values of 0·547 for EU6 and 0·312 for EU2. An isolate of *P. striiformis* from Pakistan with mixed ancestry between two SSR genotype groups was hypothesized to have originated from sexual recombination (Bahri *et al.*, 2011). In a simulation model, Balloux *et al.* (2003) found that populations with even a very small proportion of sexual reproduction had values of F_{IS} and intralocus allelic diversity similar to populations with high rates of sexual reproduction. If sexual reproduction does occur in the European populations of *P. triticina* it is probably very rare. The *P. triticina* populations in North America (Ordoñez & Kolmer, 2009) and South America (Ordoñez *et al.*, 2010), where suitable alternate hosts are not native, had similar high levels of H_O and linkage disequilibrium. If parasexual events were commonplace in *P. triticina* populations then some lower levels of H_O and linkage disequilibrium would be expected.

Puccinia triticina genotypes that are specialized to durum wheat with virulence and SSR genotypes similar to those in EU3 were previously characterized in France (Goyeau *et al.*, 2006) and Italy (Mantovani *et al.*, 2010). These isolates are characterized by avirulence to many *Lr* genes in common wheat and have SSR genotypes that are distinct from those of isolates from common wheat. Durum wheats have been grown in southern Europe for over 2000 years (Feldman, 2001). Selection caused by telial host genotype has probably driven the divergence of the *P. triticina* types adapted to common wheat and durum wheat.

The virulence phenotypes described in this study were similar to those found previously in Europe. Park & Felsenstein (1998), in a continent-wide survey in 1995, found that five races were widespread. These five races had similar virulences to the isolates in EU1, EU2, EU4, EU5, EU6 and EU7. Hanzalova *et al.* (2008) found the three most common *P. triticina* virulence types in Slovakia from 1994 to 2004 had virulence equivalent to the isolates in EU2, EU4 and EU8. In an extensive study of *P. triticina* virulence in France from 1999 to 2001, Goyeau *et al.* (2006) characterized races with virulence equivalent to isolates in EU1, EU2, EU4, EU5 and EU7.

Goyeau *et al.* (2006) identified in France in 2000 and 2001 a small number of isolates with virulence directly equivalent to phenotypes MBDS- and MCDS- in EU8. Isolates with these virulence phenotypes were first found in North America in 1996 (Kolmer, 1998) and in South America in 1999 (German *et al.*, 2007). The MCDSS and MBDSS isolates from North America and South America were highly related for SSR genotype (Ordoñez *et al.*, 2010). The similarity in SSR genotype and virulence phenotype suggests the possibility of *P. triticina* movement between the three continental regions.

The overall lower values of F_{ST} and R_{ST} when the SSR genotypes were grouped based on country of origin indicated the dispersal of similar SSR genotypes across Europe. Isolates from France and the UK were not differentiated based on F_{ST} and had a low level of R_{ST} differentiation. Park & Felsenstein (1998) also found that isolates from northern France and southern England had identical and similar virulence phenotypes. Isolates within countries were also diverse for SSR genotype. Isolates from Turkey had genotypes in EU1, EU4, EU5, EU7 and EU8; isolates from the Czech Republic/Slovakia had genotypes in EU2, EU4, EU5, EU6 and EU8; isolates from the UK had genotypes in EU1, EU4, EU5, EU7 and EU8.

The *P. triticina* virulence phenotypes in this study were probably affected by host selection. Winter wheat cultivars with seedling leaf rust resistance genes *Lr1*, *Lr3*, *Lr10*, *Lr14a*, *Lr20* and *Lr26* (Singh *et al.*, 2001; Goyeau *et al.*, 2006) are grown in Europe. Since 2006, leaf rust infection severity on Thatcher lines with genes *Lr1*, *Lr3*, *Lr10*, *Lr13*, *Lr14a*, *Lr16*, *Lr26* and *Lr37* has increased (Serfling *et al.*, 2011). Isolates with virulence to *Lr3*, *Lr14a*, *Lr20* and *Lr26* were widespread and were in most of the EU groups.

The two models of genetic differentiation, F_{ST} and R_{ST}, gave dissimilar results for differentiation of the EU SSR groups. Neither R_{ST} nor F_{ST} is completely satisfactory to describe *P. triticina* populations as mutation and genetic drift both probably contribute to genetic variation. The short-lived effectiveness of leaf rust resistance genes in wheat cultivars in the USA is testament to the rapid rate at which mutations from avirulence alleles to virulence alleles can occur in populations of *P. triticina* (Kolmer *et al.*, 2007), supporting the use of R_{ST}. However, SSR genotypes of *P. triticina* associated with different wheat market classes that are grown in different regions of the USA (Ordoñez & Kolmer, 2009) may have evolved in part as a result of genetic drift. In the absence of mutation, values of R_{ST} and F_{ST} should converge (Hardy *et al.*, 2003). The overall value of R_{ST} (0·506) was considerably

greater than the overall value of F_{ST} (0·317), suggesting the occurrence of mutation in these populations. The clonal nature of the European *P. triticina* population would also effectively eliminate gene flow between the different EU SSR groups, with new genotypes generated by mutation within each group. Isolates in EU3, the durum-adapted isolates from Italy and Spain, had an average R_{ST} value of 0·65 with the other EU groups and an average F_{ST} value of 0·45. The R_{ST} model probably better accounts for the evolutionary distance between the *P. triticina* isolates from common wheat and those adapted to durum wheat. Based on R_{ST}, EU2, EU7 and EU8 were not significantly differentiated, suggesting a common evolutionary history. If isolates in the three different groups were highly related for SSR genotype based on R_{ST}, then it might be expected that the same groups would be more closely related for virulence, given clonal reproduction. However, the relationship between virulence differentiation and R_{ST} was not consistent amongst the three groups. EU2 has relatively low values of Φ_{PT} with EU7 and EU8, but EU7 and EU8 had a Φ_{PT} differentiation value near to the overall Φ_{PT} value.

In conclusion, the *P. triticina* collections from Europe were highly differentiated for SSR genotype and virulence phenotype. Collections from common wheat were placed into seven different SSR genotype groups that were dispersed across Europe. The high diversity of SSR genotype groups could be a result of remnants of sexual reproduction because aecial infections on alternate hosts have been reported in southern Europe. In addition, the long-term cultivation of wheat in Europe would provide opportunity for the introduction of new genotypes from regions such as from the presumed centre of origin of *P. triticina* in the Fertile Crescent of southwest Asia. Isolates collected from durum wheat were highly distinct for SSR genotype compared to isolates from common wheat, indicating the effects of telial host selection in differentiating genotypes of *P. triticina*. Further comparative examination of worldwide *P. triticina* populations for SSR genotype and virulence similarity (Kolmer & Ordoñez, 2007; Ordoñez *et al.*, 2010) may provide some insight into the origin of the European genotype groups.

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