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Characterization of *Puccinia graminis* f. sp. *tritici* isolates derived from an unusual wheat stem rust outbreak in Germany in 2013

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An unusual stem rust infestation occurred in German wheat fields in summer 2013. This study analysed 48 isolates derived from 17 *Puccinia graminis* f. sp. *tritici* (Pgt) samples and six races were identified: TKTTF, TKKTF, TKPTF, TKKTP, PKPTF and MMMTF. Infection type and genotypic data confirmed that none of these races belonged to the TTKS (Ug99) race group. German isolates of race TKTTF are phenotypically different to the ones responsible for the stem rust epidemic in Ethiopia in 2013–2014. Forty isolates were genotyped using a custom SNP array. Phylogenetic analysis showed that these 40 isolates represented two distinct lineages (clade IV and clade V). Thirty-eight isolates clustered into clade IV, which previously was defined by Ethiopian isolates of race TKTTF. Race TKKTP is of special concern due to its combined virulence to stem rust resistance genes *Sr24*, *SrTmp* and *Sr1RS^{Amigo}*. The vulnerability to race TKKTP in US and international winter wheat was confirmed as 55% of North American and international cultivars and breeding lines resistant to race TTKSK (Ug99) became susceptible to TKKTP. Races identified in Germany in 2013 confirmed the presence of virulence to important resistance genes that are effective against race TTKSK. This information should be useful for breeders to select diverse and effective resistance genes in order to provide more durable stem rust resistance and reduce the use of fungicides.

Keywords: genotyping, race typing, surveillance, wheat stem rust

Introduction

Wheat stem rust, caused by *Puccinia graminis* f. sp. *tritici* (Pgt), is one of the most devastating diseases of wheat (*Triticum aestivum*). Stem rust is favoured by warm temperatures up to 35 °C (Roelfs *et al.*, 1992) and can develop quickly, causing severe yield losses when conditions for its development are favourable. Severe stem rust epidemics occurred in Europe in the 19th and early 20th centuries (Zadoks, 1963), mostly associated with the presence of common barberry (*Berberis vulgaris*; Zadoks & Bouwman, 1985). The barberry eradication programmes in western Europe in the early 1900s (Hermansen, 1968), coupled with the widespread use of systemic broad-spectrum fungicides, have had a significant effect in reducing the frequency and severity of stem rust. In Germany, stem rust has not been observed for decades, but occurred in five Federal States in 2013 (Fig. 1). In mid-June the disease was first observed in winter wheat, then 2 weeks later in spring wheat nurseries of breeding companies located in central parts of Germany. Julius Kuehn-Institut received samples from breeding companies mainly from Lower Saxony and Saxony-Anhalt. Infected field trials were also found in Thuringia, Brandenburg and Saxony. The samples mainly came from the widespread winter wheat cultivars Patras, Julius, JB Asano and Akteur and the spring wheat cultivars KWS Chamsin, KWS Kadrilj, KWS Scirocco and Sonett.

Routine surveillance and monitoring of stem rust occurrence are essential for managing wheat stem rust disease, especially when host resistance is a key component of the strategy. Characterization of Pgt isolates using standard wheat differentials provides critical information on effectiveness of specific host resistance genes and pathogen variation (Newcomb *et al.*, 2016). Recently, the development of single nucleotide polymorphism (SNP) technologies has added new tools for understanding Pgt diversity (Olivera *et al.*, 2015; Newcomb *et al.*, 2016). Given the long-range aerial dispersal of Pgt urediniospores, knowing not only the local but also the regional pathogen populations is essential in understanding effectiveness of host resistance in current cultivars and helping inform breeding programmes. The objectives of this study were to: (i) identify and characterize the Pgt

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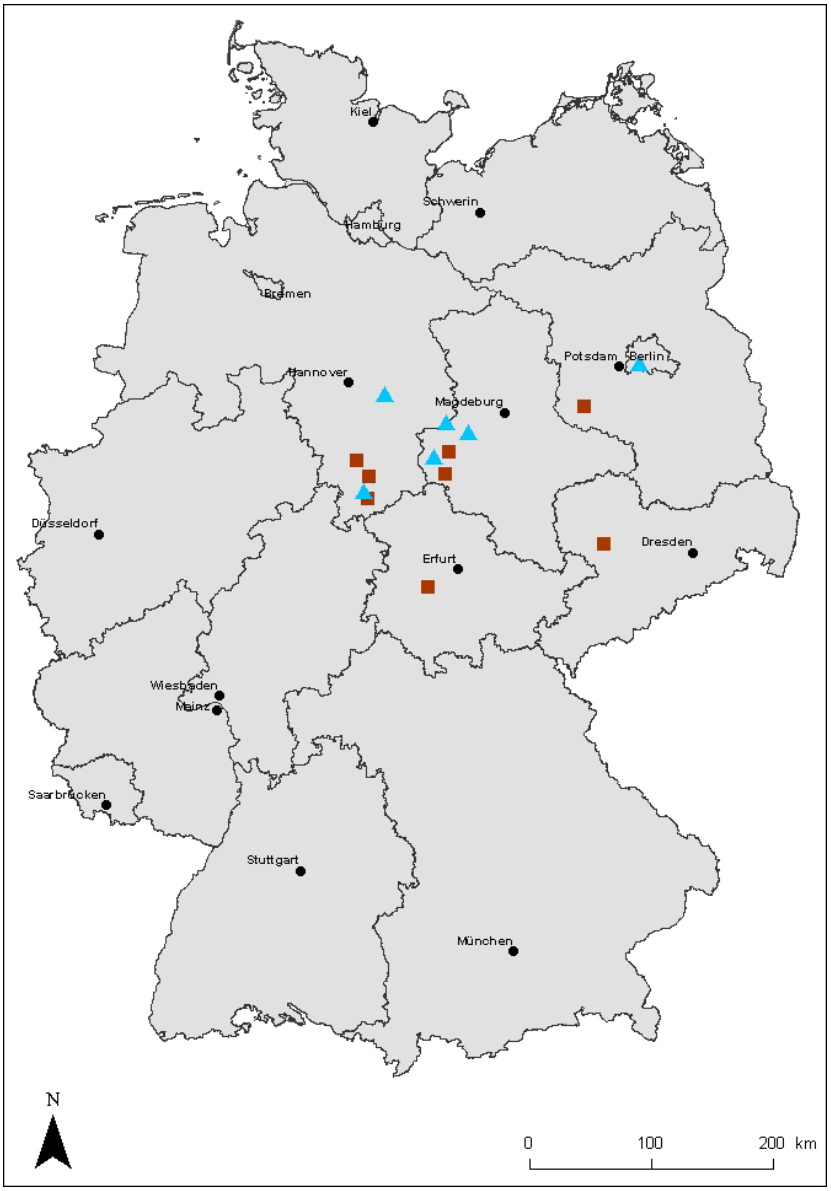

Figure 1 Map of Germany showing the geographic distribution of the stem rust outbreak that occurred in 2013. The blue triangles indicate the collection sites of the samples processed in this study. The red squares indicate the location of additional sites where stem rust infection was observed. The black circles correspond to the capitals of the Federal States of Germany.

races that occurred in Germany in 2013, (ii) genotype representative isolates, and (iii) determine the level of vulnerability of North American and international winter wheat cultivars and breeding materials to these potentially new virulence combinations.

Materials and methods

Sample collection and storage

Seventeen samples of dried Pgt urediniospores collected in Germany in 2013 were sent to the Biosafety Level 3 (BSL-3) Plant Pathogen Containment Facility at the USDA-ARS Foreign Disease – Weed Science (FD-WS) Research Unit, Ft Detrick, MD, USA in September 2013. Details of sample location and cultivars are given in Figure 1 and Table 1. Shipping and receiving protocols were followed according to USDA APHIS permit conditions for handling international cultures of Pgt. Upon arrival, samples were increased on the susceptible wheat McNair 701 (CItr 15288) and collected into gelatin capsules (size 00). After increase, all samples were sent to USDA-ARS Cereal Disease Laboratory (CDL), St Paul, MN, USA, and stored in a −80 °C freezer until processing after 1 December 2013.

Race identification and characterization

The North American stem rust differential set (Roelfs & Martens, 1988; Roelfs *et al*., 1992) that was modified to further delineate Pgt races in the TTKS race group (Jin *et al*., 2008) was used for race identification. All samples were further characterized on additional monogenic lines carrying the following resistance genes: *Sr13*, *Sr22*, *Sr25*, *Sr26*, *Sr27*, *Sr32*, *Sr33*, *Sr35*, *Sr37*, *Sr39*, *Sr40*, *Sr44*, *Sr47*, *Sr50*, *SrSatu* and the 1AL.1RS translocation in winter wheat (*Sr1RS*^{Amigo}). Durum cultivars

Table 1 Origin of 17 samples of *Puccinia graminis* f. sp. *tritici* collected in 2013 in Germany

Sample no.	Federal state	Location	Host	Cultivar/breeding line
13GER01	Saxony-Anhalt	R.A.G.T. Saaten Deutschland GmbH, Silstedt	Winter wheat	Not known
13GER02	Brandenburg	Julius Kuehn-Institut, Berlin-Dahlem	Winter wheat	Breeding line
13GER03	Brandenburg	Julius Kuehn-Institut, Berlin-Dahlem	Winter wheat	Capone
13GER04	Brandenburg	Julius Kuehn-Institut, Berlin-Dahlem	Winter wheat	Ritmo
13GER05	Brandenburg	Julius Kuehn-Institut, Berlin-Dahlem	Winter wheat	Divimar
13GER06	Lower Saxony	Limagrain GmbH, Peine/Rosenthal	Winter wheat	Tobak
13GER07	Saxony-Anhalt	Lantmännen SW Seed Hadmersleben GmbH, Oschersleben	Winter wheat	Breeding line
13GER08	Saxony-Anhalt	Lantmännen SW Seed Hadmersleben GmbH, Oschersleben	Spring wheat	KWS Chamsin
13GER09	Lower Saxony	Saatzucht Josef Breun GmbH & Co. KG, Lenglern	Winter wheat	Breeding line
13GER10	Lower Saxony	Saatzucht Josef Breun GmbH & Co. KG, Lenglern	Winter wheat	Breeding line
13GER11	Lower Saxony	Strube Research GmbH & Co KG, Söllingen	Spring wheat	Breeding line
13GER12	Lower Saxony	Strube Research GmbH & Co KG, Söllingen	Spring wheat	Breeding line
13GER13	Saxony-Anhalt	R.A.G.T. Saaten Deutschland GmbH, Silstedt	Winter wheat	Breeding line
13GER14	Saxony-Anhalt	R.A.G.T. Saaten Deutschland GmbH, Silstedt	Winter wheat	Colonia
13GER15	Saxony-Anhalt	R.A.G.T. Saaten Deutschland GmbH, Silstedt	Winter wheat	Breeding line
13GER16	Saxony-Anhalt	R.A.G.T. Saaten Deutschland GmbH, Silstedt	Winter wheat	Breeding line
13GER17	Lower Saxony	Strube Research GmbH & Co KG, Söllingen	Barley	Not known

Iumillo (*Sr9g*, *Sr12*+) and Leeds (*Sr9e*, *Sr13*+) were also included in the evaluation. After the evaluation, one to five single-pustule isolates were derived from each original sample from either the differential plants or additional resistant lines. Experimental procedures for storage retrieving, inoculation, incubation and disease assessment were performed as described by Jin *et al.* (2007) and Olivera *et al.* (2015). Single-pustule derived cultures (pure cultures) were evaluated two to four times on differential lines before a race phenotype was designated. Race designation was based on the letter code proposed by Roelfs & Martens (1988). Urediniospores from the original samples and the pure cultures were increased on the susceptible wheat McNair 701 and Line E in pots enclosed in cellophane bags (Zellglas Boden-Beutel), and stored at −80 °C. A phenotypic comparison among two German, two Ethiopian (from 2013 (Olivera *et al.*, 2015)), and one Turkish (from 2012 (Newcomb *et al.*, 2013)) representative isolates of race TKTTF was conducted on a series of 148 wheat lines carrying single or combinations of 75 stem rust resistance (*Sr*) genes.

Genotyping of Pgt isolates

DNA was extracted from either Pgt-infected wheat leaf tissue or urediniospores (Olivera *et al.*, 2015). Genotyping was performed using a custom Illumina Infinium SNP array (PgtSNP 3.0k chip) as described by Newcomb *et al.* (2016) with the exception that a cut-off for missing data was set at 2%. Phylogenetic analysis of this dataset was performed using R (v. 3.2.1; R Core Development Team, 2015), with the package POPPR v. 2.0.2 (Kamvar *et al.*, 2015). Neighbour-joining analysis was used with 5000 bootstrap replicates. A set of 15 Pgt reference isolates was included in the analysis: Clade I – Ug99 race group (04KEN156-04, race TTKSK; 06KEN19V-3, race TTKST; 07KEN24-4, race TTTSK), Clade II – JRCQC (14YEM115-1, race JRCQC; 14YEM123-1, race JRCQC; 14YEM149-5, race JRCQC), Clade III – TRTTF race group (06YEM34-1, race TRTTF; 14ETH123-1, race RRTTF; 14ETH136-3, race RRTTF), Clade IV-A – TKTTF (13ETH18-1, race TKTTF; 14ETH02-2, race TKTTF; 14ETH128-1, race TKTTF), and Clade IV-B – TKTTF (13ETH20-1, race TKTTF; 14ETH126-1, race TKTTF; 14ETH132-2, race TKTTF).

Seedling evaluation of winter wheat germplasm

Isolate 13GER16-1 was increased on susceptible wheat McNair 701 for use in seedling screening assays conducted inside a University of Minnesota BSL-3 facility. Seedling screening entries include 448 cultivars and breeding lines of the 2014/2015 North American winter wheat regional nurseries, 56 lines from the 18th and 19th Facultative and Winter Wheat Observation Nurseries (FAWWON), and 23 from the winter wheat stem rust regional nursery (C4WWSRRN) from the International Maize and Wheat Improvement Centre (CIMMYT). All these entries were previously evaluated and selected for resistance to race TTKSK as a part of regional nursery evaluation for stem rust resistance (Y. Jin, unpublished data). Methods for planting, seedling maintenance, and inoculations were the same as described above for race analysis on differential sets. Seedling infection types (ITs) were determined at 14 days after inoculation following the 0–4 scale developed by Stakman *et al.* (1962). Infection types greater than or equal to 2^+ were categorized as susceptible reactions, while those less than 2^+ were categorized as resistant reactions.

Results

Phenotyping of Pgt isolates

Forty-eight single pustule isolates were derived from 16 viable samples collected in Germany in 2013. From these isolates, six races were identified: TKTTF, TKKTF, TKPTF, TKKTP, PKPTF and MMMTF (Table S1). The most frequently observed races were TKKTF (19 isolates) and TKTTF (16 isolates), whereas races TKPTF, TKKTP, PKPTF and MMMTF occurred in two, five, three and three isolates, respectively.

Race TKTTF produced high ITs on differentials carrying *Sr5*, *Sr6*, *Sr7b*, *Sr8a*, *Sr9a*, *Sr9b*, *Sr9d*, *Sr9e*, *Sr9g*, *Sr10*, *Sr17*, *Sr21*, *Sr30*, *Sr36*, *Sr38*, *SrTmp* and *SrMcN* (Table 2). Races TKKTF and TKPTF have a similar virulence profile to race TKTTF, but exhibiting a low IT on

Table 2 Infection types produced on stem rust differentials using isolates of races TKTTF, TKKTF, TKPTF, TKKTP, PKPTF and MMMTF of *Puccinia graminis* f. sp. *tritici*

Line	Gene	TKTTF (13GER15-1)	TKKTF (13GER01-1)	TKPTF (13GER02-2)	TKKTP (13GER16-1)	PKPTF (13GER06-1)	MMMTF (13GER05-1)
ISr5-Ra	*Sr5*	4	4	3^+	4	3^+	3^+
CnS_T_mono_deriv	*Sr21*	3^+	3^+	3^+	4	22^+	2^-
Vernstein	*Sr9e*	4	3^+	3^+	3^+	3^+	2^-
ISr7b-Ra	*Sr7b*	3^+	3^+	3^+	3^+	3^+	3^+
ISr11-Ra	*Sr11*	2^-;	2^-	2^-	2^-;	;2^-	3^+
ISr6-Ra	*Sr6*	4	4	3^+	4	4	1;
ISr8a-Ra	*Sr8a*	3^+	4	3^+	4	4	22^-
CnSr9g	*Sr9g*	4	4	3^+	4	4	3^+
W2691SrTt-1	*Sr36*	4	0	4	0;	3	3^+
W2691Sr9b	*Sr9b*	33^+	33^+	22^+	3	2	2^+
BtSr30Wst	*Sr30*	4	4	3^+	3^+	4	2^-
Combination VII[a]	*Sr17* (+*Sr13*)	2^+	2	2	2	2^-;	22^-
ISr9a-Ra	*Sr9a*	4	3^+	3^+	3^+	3^+	4
ISr9d-Ra	*Sr9d*	4	3^+	3^+	3^+	3^+	4
W2691Sr10	*Sr10*	4	4	3^+	3^+	3^+	4
CnsSrTmp	*SrTmp*	4	3^+	3^+	3^+	3^+	3^+
LcSr24Ag	*Sr24*	2^-	2	2^-	32^+	22^-	2
Sr31/6*LMPG	*Sr31*	2^-;	2^-	1;	2^-;	22^-	1^-;
VPM1	*Sr38*	3^+	3^+	3^+	3^+	3	33^+
McNair 701	*SrMcN*	4	4	4	4	4	4

Infection types (ITs) observed on seedlings at 14 days post-inoculation using a 0–4 scale according to Stakman *et al.* (1962), where ITs of 0, ;, 1, 2, or combinations thereof are considered as a low IT and ITs of 3 or higher are considered as a high IT.
[a]The low IT produced by avirulent isolates on *Sr17* is 0; to ;. IT 2^- or higher on this line indicates virulent to *Sr17* but avirulent to *Sr13*.

Sr36 (IT = 0) and *Sr9b* (IT = 22^+), respectively (Table 2). Race PKPTF produced high ITs on differentials carrying *Sr5*, *Sr6*, *Sr7b*, *Sr8a*, *Sr9a*, *Sr9d*, *Sr9e*, *Sr9g*, *Sr10*, *Sr17*, *Sr30*, *Sr36*, *Sr38*, *SrTmp* and *SrMcN* (Table 2). Isolates of races TKTTF, TKKTF, TKPTF and PKPTF were avirulent to all the additional stem rust resistance genes tested in this study (Table 3). Race TKKTP produced high ITs on differentials carrying *Sr5*, *Sr6*, *Sr7b*, *Sr8a*, *Sr9a*, *Sr9b*, *Sr9d*, *Sr9e*, *Sr9g*, *Sr10*, *Sr17*, *Sr21*, *Sr24*, *Sr30*, *Sr38*, *SrTmp* and *SrMcN* (Table 2). In addition, race TKKTP exhibited a high IT (IT = 3) to stem rust resistance conferred by the 1AL.1RS translocation (*Sr1RS^{Amigo}*; Table 3). Race MMMTF has the narrowest virulence spectrum of all races, exhibiting virulence to differentials carrying *Sr5*, *Sr7b*, *Sr9a*, *Sr9d*, *Sr9g*, *Sr10*, *Sr11*, *Sr17*, *Sr36*, *SrTmp* and *SrMcN* (Table 2). High ITs were not observed in the additional resistance genes to race MMMTF (Table 3).

Two representative isolates (13GER10-4 and 13GER11-3) of race TKTTF from Germany were phenotypically compared with two Ethiopian isolates (13ETH18-1 and 13ETH22-2) and one Turkish isolate (12TUR3M2-3) of race TKTTF on a number of wheat lines carrying single or combinations of different *Sr* genes. Isolates of TKTTF from Ethiopia and Turkey produced similar ITs on these lines, but isolates of TKTTF from Germany reacted differently on wheat lines carrying *Sr7a*, *Sr33*, *Sr45* and *SrTt-3* (Table 4). The two German isolates were virulent on *Sr7a*, *Sr45* and *SrTt-3* while the Ethiopian and Turkish isolates of TKTTF were avirulent. Higher ITs were also observed on *Sr33*.

Genotyping

A selected set of 40 Pgt isolates representing the six different race phenotypes characterized from the 2013 German samples and 15 reference isolates were genotyped using a custom PgtSNP 3.0k chip. After filtering, 2188 SNP loci were used for analysis. Thirty-eight (95%) isolates from Germany grouped into clade IV, while the remaining two isolates (13GER04-1 and 13GER05-1) formed a new clade (V) (Fig. 2). Clade IV was divided into five subclades (IV-A, IV-B, IV-C, IV-D and IV-E). The majority of the isolates belonged to either subclade IV-A (32.5%) or IV-E (47.5%). Subclade IV-A was further divided into two subgroups (IV-A.1 and IV-A.2). Three reference isolates (13ETH18-1, 14ETH02-2 and 14ETH128-1) and five German isolates formed clade IV-A.1. The remaining eight German isolates made up clade IV-A.2. None of the 40 German isolates clustered with the three reference isolates representing subclade IV-B (13ETH20-1, 14ETH126-1 and 14ETH132-2). Subclade IV-C consisted of four isolates (13GER06-1, 13GER06-2, 13GER06-3 and 13GER16-3) and subclade IV-D consisted of two isolates (13GER01-1 and 13GER01-2). The largest subclade (IV-E) consisted of 19 German isolates further divided into two subgroups subclade IV-E.1 (10.5%) and subclade IV-E.2 (89.5%).

Table 3 Infection types observed on lines carrying additional resistance genes to isolates of *Puccinia graminis* f. sp. *tritici* representing races TKTTF, TKKTF, TKPTF, TKKTP, PKPTF and MMMTF

Line	Gene	Type	TKTTF (13GER15-1)	TKKTF (13GER01-1)	TKPTF (13GER02-2)	TKKTP (13GER16-1)	PKPTF (13GER06-1)	MMMTF (13GER05-1)
ST464	*Sr13*	Durum wheat	2^{+}	2+	2^{+}	2	2	2^{-};
SwSr22T.B	*Sr22*	Bread wheat	2^{-}	2^{-}	2^{-}	2^{-}	22^{-}	22^{-};
Agatha/9*LMPG	*Sr25*	Bread wheat	2^{+}	2	2^{+}	2^{+}	22^{+}	2^{-};N
Eagle	*Sr26*	Bread wheat	2^{-}	22^{-}	2^{-}	2^{-}	2^{-}	;2^{-}
73,214,3-1/9*LMPG	*Sr27*	Bread wheat	;1^{-}	;	1^{-}	;1^{-}	;	;
Federation*4/Kavkaz	*Sr31*	Bread wheat	2^{-}	2^{-}	;2^{-}	2^{-};	2^{-}	;2^{-}
ER5155	*Sr32*	Bread wheat	2	22^{+}	2^{-}	2	2	22^{-}
Tetra Canthatch/*Ae. squarrosa*	*Sr33*	Bread wheat	2^{-}	2	;	2^{-}	2^{-}	;N
Mq(2)5XG2919	*Sr35*	Bread wheat	0;	0;	0;	;	0;	;
W3563	*Sr37*	Bread wheat	;13^{-}	$11^{+}3^{-}$	;1	$2^{+}1$;	;11^{+}	;1
RL6082	*Sr39*	Bread wheat	2^{-}	2	2^{-};	2^{-}	1^{-};	2^{-}
RL6088	*Sr40*	Bread wheat	2	22^{+}	2^{-}	2	22^{-}	2^{-};
TAF 2	*Sr44*	Bread wheat	$11^{+}3^{-}$	13	;13^{+}	;	;	1;
DAS15	*Sr47+*, *SrAes7t*	Durum wheat	;1^{-}	;1^{-}	;1^{-}	;	;N	;1^{-}
Fed*3/Gabo*51BL.1RS-1-1	*Sr50*	Bread wheat	2^{-};	2^{-}	2^{-}	;	2^{-}	;1
Satu	*SrSatu*	Triticale	0;	;	;	;	;	;
TAM 107-1	*Sr1RS^{Amigo}*	Bread wheat	2^{-};	22^{-}	2^{-};	3	2^{-};	1;
Leeds	*Sr9e*, *Sr13+*	Durum wheat	;	;	;	;	;	0;
Iumillo	*Sr9g*, *Sr12+*	Durum wheat	0;	;	0;	;	;	;

Infection types (ITs) observed on seedlings at 14 days post-inoculation using a 0–4 scale according to Stakman *et al.* (1962), where ITs of 0, ;, 1, 2, or combinations thereof are considered as a low IT and ITs of 3 or higher are considered as a high IT.

Table 4 Wheat genotypes that differentiated isolates of race TKTTF of *Puccinia graminis* f. sp. *tritici* from Germany, Ethiopia and Turkey

Gene	Line	13GER10-4 Germany	13GER11-3 Germany	13ETH18-1[a] Ethiopia	13ETH22-2[a] Ethiopia	12TUR3M2-3[b] Turkey
Sr7a	Na101/MqSr7a	3^{+}	3^{+}	13^{-}	13^{-}	13^{-}
Sr33	Tetra Canthatch/*Ae. squarrosa*	2	2	;1^{-}	;1^{-}	;1^{-}
Sr45	CSID 5406	3^{+}	3^{+}	;1^{-}	;1^{-}1	;1^{-}
SrTt-3	Federation*2/SrTt-3	3^{+}	3^{+}	3^{-}1;	3^{-}1;	3^{-}1;

Infection types (ITs) observed on seedlings at 14 days post-inoculation using a 0–4 scale according to Stakman *et al.* (1962), where ITs of 3 or higher are considered as a high IT.

[a]Data were presented in Olivera *et al.* (2015).

[b]Data were presented in Newcomb *et al.* (2013).

All of the isolates from the 2013 German collections with TKTTF race phenotype grouped into subclade IV-A and these were split between the two subgroups (IV-A.1 and IV-A.2). The other main race phenotype in this collection (TKKTF) comprised the majority (94%) of the isolates in subclade IV-E.2. The remaining two isolates of race TKKTF (13GER01-1 and 13GER01-2) were genetically distinct and formed subclade IV-D. The three isolates of race TKKTP analysed were split between subclade IV-E.1 (13GER16-4 and 13GER16-5) and IV-E.2 (13GER10-6). Isolates of race PKPTF (13GER06-1, 13GER06-3 and 13GER16-3) grouped together in subclade IV-C. The two isolates of race TKPTF were split between subclades IV-A.1 (13GER02-2) and IV-C (13GER06-2). The two isolates that were phenotypically most distinct (13GER04-1 and 13GER05-1, race MMMTF) were also the most genetically different (clade V).

Germplasm evaluation

Due to the unique virulence combination of Pgt race TKKTP on *Sr24* and *Sr1RS^{Amigo}*, two important stem rust resistance genes common in US winter wheat, the reactions of the US and international elite winter wheat breeding germplasm that were selected for resistance to TTKSK at the seedling stage were examined (Table 5). Of the 448 TTKSK-resistant North American breeding lines tested against isolate 13GER18-1 of race TKKTP, 246 (55%) lines were susceptible to race TKKTP (Table 5). Of the 79 TTKSK-resistant lines from the international stem rust nurseries (18th and 19th

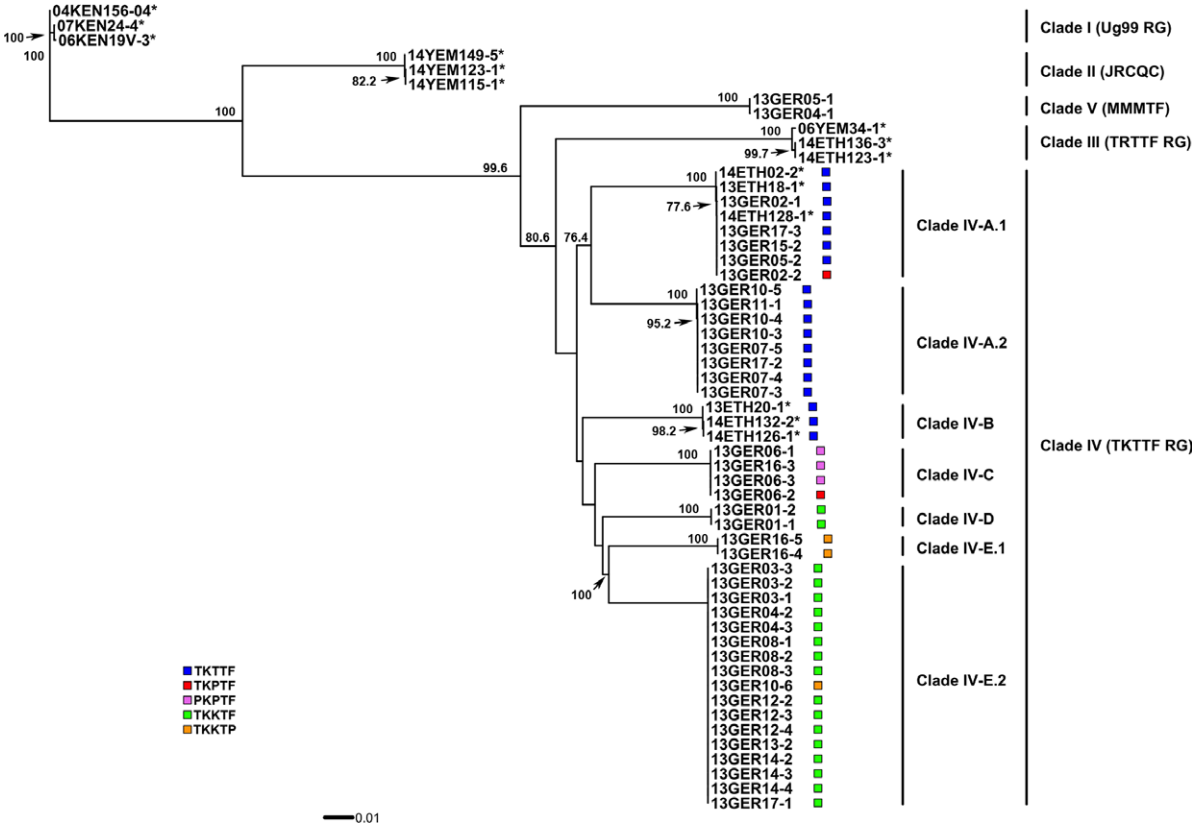

Figure 2 Neighbour-joining phylogenetic tree of 40 German *Puccinia graminis* f. sp. *tritici* isolates from 2013 based on 2188 single nucleotide polymorphism loci. Fifteen reference isolates were included in the analysis (*). Clades, subclades, and race or race group are indicated. Race phenotypes for isolates in clade IV are indicated by colours. The tree is unrooted. Bootstrap values for 5000 replicates are shown (>75%). Branch lengths are measured in the number of substitutions per site.

FAWWON and C4WWSRRN), 44 (56%) lines were susceptible to race TKKTP.

Discussion

Wheat stem rust is a re-emerging disease, posing a new threat to wheat production worldwide, which is highlighted by the occurrence and spread of *Sr31*-virulent races in the Ug99 race group (Singh *et al.*, 2015). Since first reported in Uganda (Pretorius *et al.*, 2000), the TTKS (or Ug99) race group has been rapidly evolving and expanding its geographical range. To date, 11 races of the TTKS race group have been identified in eastern Africa and the Middle East (Singh *et al.*, 2015; Newcomb *et al.*, 2016), and it is projected to spread further, threatening critical wheat-growing regions in the world (Park *et al.*, 2011). Race TTKSK and its variants have combined virulence to widely deployed and important stem rust resistance genes including *Sr31*, *Sr24*, *Sr36*, *SrTmp* and *Sr9h* (Pretorius *et al.*, 2012; Singh *et al.*, 2015; Newcomb *et al.*, 2016). In addition to the Ug99 race group, other unrelated races with important virulence combinations have emerged and caused severe localized epidemics. In Ethiopia, race TKTTF (virulence on *SrTmp*) was responsible for severe yield losses (up to 100%) in the 2013–2014 crop seasons (Olivera *et al.*, 2015). Race TKTTF has been found in East Africa, the Caucasus and the Middle East (Mert *et al.*, 2012; Olivera *et al.*, 2015; Singh *et al.*, 2015). The wide geographic distribution of this race and the large inoculum load produced after these localized epidemics pose a threat if it reaches the neighbouring wheat-producing regions.

Wheat stem rust has not been a major concern in western Europe since the epidemics in late 19th and early 20th centuries due largely to the eradication of the alternate host, common barberry (Zadoks, 1963). The disease has not been observed in Germany for decades prior to 2013 (Flath *et al.*, 2014). Thus, the occurrence of stem rust infections was unusual, and one of the first concerns was to determine if Ug99 had reached Europe. Through virulence and genotypic analyses, it was concluded that none of the Pgt isolates derived from the 17 samples collected belonged to the Ug99 race group. Delayed wheat crop development due to a cold, wet spring followed by a hot summer might be a contributing factor for the occurrence of wheat stem rust in 2013 in Germany (Flath *et al.*, 2014). In 2016 wheat stem rust was observed again in spring wheat nurseries of two breeding

Table 5 Susceptibility of elite winter wheat TTKSK-resistant breeding germplasm from US and international nurseries to race TKKTP of *Puccinia graminis* f. sp. *tritici*

TTKSK-resistant germplasm[a]	No. of lines tested	No. of lines susceptible to TKKTP
Selections from 2014 US winter wheat regional nurseries	448	246 (55%)
Selections from the 18th and 19th FAWWON[b]	56	28 (50%)
C4WWSRRN[c]	23	16 (69%)
Total	527	290 (55%)

[a]Lines were selected for TTKSK resistance in a germplasm evaluation programme that was not a part of this study (Y. Jin, unpublished data).
[b]Facultative and Winter Wheat Observation Nursery, with participation worldwide.
[c]The fourth winter wheat stem rust resistant nursery from the CIMMYT/Turkey winter wheat breeding programme.

companies situated in the middle of Germany (Lower Saxony). In the last decade, stem rust has been observed in neighbouring countries, such as Austria (Oberforster *et al*., 2010) and Italy (Nocente *et al*., 2011). Stem rust is also frequently observed late season on spring-planted cereals in the mountainous regions of Switzerland (F. Mascher, Agroscope, Changins/Cadenazzo Switzerland, personal communication). These occurrences appeared to be sporadic and epidemics on regional scales have not been reported. Where information is available, the races reported in Italy were different from that found in Germany.

Six Pgt races were identified and characterized from 48 single-pustule isolates. Four of these races are similar to each other (TKKTF, TKTTF, TKPTF, TKKTP), as they differ in virulence to one or two stem rust resistance gene(s) included in the standard differential set (*Sr36*, *Sr9b* or *Sr24*). A race designated as TKTTF was responsible for the wheat stem rust epidemic that occurred in southern Ethiopia in 2013 (Olivera *et al*., 2015). Isolates of this race have also been observed in Turkey (Mert *et al*., 2012; Newcomb *et al*., 2013), Lebanon and Iran (Singh *et al*., 2015), and Yemen (P. D. Olivera, unpublished data). However, the results of the present study indicate that the isolates of race TKTTF (clade IV-A.2) from Germany are different to the ones observed in Ethiopia and Turkey, based on reactions on several stem rust resistance genes that were not represented in the standard stem rust differentials, suggesting that the composition of these isolates are more complex. In particular, German isolates of race TKTTF (clade IV-A.2) were virulent to *Sr* genes (*Sr7a*, *Sr45*, *SrTt-3*) that were effective against the TKTTF isolates from Ethiopia and Turkey. Representative isolates of TKTTF (clade IV-A.1) are in the process of being analysed on these additional differential wheat lines to determine if they show the same virulence differences as the TKTTF clade IV-A.2 German isolates.

Forty isolates derived from the German 2013 Pgt collections were genotyped using a custom SNP array (PgtSNP 3.0k chip). Phylogenetic analysis showed that these 40 isolates represented two distinct lineages (clade IV and clade V). The majority of the isolates clustered into clade IV, which was previously defined by Ethiopian isolates of race TKTTF and is composed of two subclades (IV-A and IV-B) (Olivera *et al*., 2015). The 38 German isolates belonging to clade IV clustered into four subclades, three of them being new (IV-C, IV-D and IV-E). All of the isolates with TKTTF race phenotype grouped into subclade IV-A and were divided into two subgroups (IV-A.1 and IV-A.2). The two German isolates (13GER10-4 and 13GER11-3) that showed phenotypic differences with an extended wheat stem rust resistance gene set belonged to subclade IV-A.2, while the Ethiopian and Turkish reference isolates used in this analysis belonged to subclade IV-A.1. Preliminary genotyping of additional Pgt isolates from Turkey has identified a type IV-A.2 isolate, suggesting that subclade IV-A.2 has a broad geographical distribution similar to type IV-A.1 (L. J. Szabo, unpublished data). Additional analysis of isolates in subclade IV-A.1 and IV-B.2 will need to be done to confirm the correlation between phenotypic variation and these genetic subgroups.

Over half of the 2013 German Pgt isolates belonged to the three new subclades within clade IV, and represent phenotypic variants of race TKTTF (collectively called TKTTF race group). Given that each of these subclades represent distinct phylogenetic groups, based on genetic distance and bootstrap support, it is likely that subclades IV-C, IV-D and IV-E are not recently derived or specific to Germany. In support of this hypothesis, preliminary genotyping of Pgt samples from Israel identified an isolate that is a member of subclade IV-E.2 (L. J. Szabo, USDA-ARS, Cereal Disease Laboratory, St Paul, USA & H. Sela, Tel Aviv University, Israel, unpublished data). It is interesting to note that Pgt isolates of subclade IV-B have only been observed in Ethiopia (Olivera *et al*., 2015).

The variation in virulence shows a somewhat different pattern from that of the SNP genotyping. Although there is a general clustering of races in different subclades (for example, isolates of race TKTTF are found only in subclades IV-A and IV-B and isolates of race TKKTF are only observed in subclades IV-D and IV-E), three of the four subclades containing isolates from Germany consist of two different race phenotypes. In addition, the same race phenotype is often found in more than one genotype subclade. This supports the idea that changes in virulence occur frequently, resulting in isolates with common race phenotypes but different genetic backgrounds. This makes it difficult to predict evolution of lineages based solely on race phenotypes. It is tempting to postulate that isolates representing races with lower frequencies within a genetic group were derived from the dominant race type. For example, the derivation of race phenotype TKKTP (subclade IV-E.2) from race TKKTF is consistent with the hypothesis of mutations from avirulence to

virulence. However, in the subclade IV-A.1 the opposite would be true with the derivation of race type TKPTF from race type TKTTF resulting in a gain in avirulence to *Sr9b*. A similar pattern was observed in the Ug99 race group with the recent identification of race TTHST, a variant of the common race TTKST (Newcomb *et al*., 2016). This highlights the need for expanded population genetics studies that include more extensive samples across broader geographical areas and years.

Three isolates derived from the same sample (13GER06-1 [PKPTF], 13GER06-2 [TKPTF] and 13GER06-3 [PKPTF]) were all assigned, as expected, to clade IV-C based on SNP data. However, one isolate differs in the virulence observed on *Sr21* (race TKPTF exhibits a high IT on *Sr21*). Resistance conferred by *Sr21* has been shown to be variable, influenced by temperature, environmental conditions and genetic backgrounds (Chen *et al*., 2015). Even though these isolates were evaluated three times before the final race assignment was made, this race designation has to be considered as tentative. This uncertainty underlines the importance of having environment-controlled conditions for accurate race phenotyping experiments.

Even though the sample size was small and from a single year, a relatively high degree of diversity in virulences and genotypes was found. Some of these virulence combinations have not been found anywhere else and appeared to be unique. The alternate host, common barberry, appeared to have re-emerged and become widespread in Germany (Anonymous, 2016). This species has probably contributed to the high diversity observed in the *P. graminis* f. sp. *secalis* population in Germany (Miedaner *et al*., 2016). Barberry is also persistent and widespread in mountainous regions in Italy, Spain and Switzerland. Although the contemporary role of the alternate host in wheat stem rust pathogen variation and disease epidemiology has not been established for western Europe, it is probable that some of these new virulence types may have resulted from sexual recombination on the alternate host. A similar situation has occurred in Sweden, where the re-emergence of common barberry has led to widespread stem rust epidemics in oat fields (Berlin *et al*., 2013).

Race TKKTP is of special concern due to its combined virulence to *Sr24*, *SrTmp* and *Sr1RS^{Amigo}*. This is the first known Pgt race with a virulence combination to these TTKSK-effective genes that are widely present in North American winter wheat singly or in combinations (Jin & Singh, 2006; Olson *et al*., 2010; Yu *et al*., 2010; Zhang *et al*., 2014). TKKTP is the second known Pgt race with virulence to stem rust resistance conferred by *Sr1RS^{Amigo}* after the identification of race TRTTF from Yemen and Ethiopia (Olivera *et al*., 2012). Evaluation of elite breeding germplasm of winter wheat revealed the vulnerability as expected due to the unique virulence combination.

Since the re-emergence of wheat stem rust in East Africa, increased pathogen monitoring activities allowed the detection of new races and virulence combinations that result in breakdown of resistance in currently grown cultivars. Races of Pgt identified in Germany in 2013 confirmed the presence of virulence to critical stem rust resistance genes that are effective against race TTKSK such as *Sr36*, *Sr24*, *SrTmp* and *Sr1RS^{Amigo}*. This information could be useful for breeders to select diverse and effective resistance genes in order to provide more durable stem rust resistance and reduce the use of fungicides.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Table S1. Race phenotypes and SNP genotypic clades of 48 isolates of *Puccinia graminis* f. sp. *tritici* and infection types on selected stem rust resistance genes.