Genetic comparison of Ug99 with selected South African races of *Puccinia graminis* f.sp. *tritici*

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SUMMARY

Using simple sequence repeat (SSR) and amplified fragment length polymorphism (AFLP) marker analyses, the genetic structure of selected South African wheat stem rust races was compared with Ug99. SSR analysis divided the population into two distinct groups with 24.5% similarity between them. A local race, UVPgt55 (North American race notation TTKSF), grouped with Ug99 (TTKSK) with a 100% similarity. When AFLP data were included, the same groups were found, but with an increased similarity of 66.7%. Although the SSR data were unable to distinguish between all individual isolates, the AFLP data alone and in combination with the SSR data discriminated between the isolates. The grouping of individual isolates resembled the pathogenicity profile of the different races. On the basis of its similarity with Ug99, it was concluded that UVPgt55 was most probably an exotic introduction into South Africa, whereas the other races specialized locally through mutational adaptation.

INTRODUCTION

Stem rust of wheat is caused by the obligate biotrophic pathogen *Puccinia graminis* f.sp. *tritici*. The historical importance and damage potential of stem rust are well known (Leonard and Szabo, 2005), requiring ongoing breeding for resistance in many parts of the world (Knott, 1989). However, the success of resistance breeding is often short lived as a result of pathogenic variability, manifested by the appearance of new races or pathotypes within *P. graminis* f.sp. *tritici*. The probability of a new race is particularly high when a particular resistance gene is used extensively in breeding (Keiper *et al.*, 2003).

The specialization of new rust races occurs primarily as a result of sexual and somatic recombination and mutation (Knott, 1989). McIntosh (1992) mentioned migration and chance as additional mechanisms in cereal rust population dynamics. Although sexual recombination has been important in the development of

P. graminis f.sp. *tritici* races, especially in North America, the sexual cycle is less important in other areas. In Australia, the cereal rust population is mainly determined by exotic introductions, mutation and somatic hybridization (Park *et al.*, 1995, 1999; Wellings and McIntosh, 1990). Likewise, sexual recombination has not been reported in wheat stem rust in South Africa where standard races 21 and 34, first identified during the 1920s (De Jager, 1980), adapted into several variants over time (Boshoff *et al.*, 2000; Le Roux, 1985, 1989; Le Roux and Rijkenberg, 1987; Lombard, 1986; Pretorius *et al.*, 2007).

A new stem rust race UVPgt55 (2SA88, Agricultural Research Council race notation; TTKSF, North American race notation) was detected in South Africa in 2000 (Boshoff *et al.*, 2002). It was the first documented South African race to show virulence towards *Sr8b* and *Sr38*. In addition, it exhibited a similar avirulence/virulence profile to Ug99 (North American race notation TTKSK) (Pretorius *et al.*, 2000), with the exception of avirulence towards *Sr31* (Pretorius *et al.*, 2007). The potential threat of Ug99 to global wheat production has been emphasized in several reports (Jin *et al.*, 2007; Singh *et al.*, 2006). More recently, the matter has been intensified by the movement of Ug99 to Yemen (Mackenzie, 2007) and Iran (FAO Newsroom, 2008) with an anticipated further spread to the Middle East and Asia. A further adaptation of Ug99 (TTKST), in which it acquired virulence to *Sr24* (Jin *et al.*, 2008), has reduced the number of effective genes needed to combat this race.

In view of the similar virulence profiles of UVPgt55 and Ug99, the question arose as to how closely related were these two races. If they are closely related, it is possible that both races originated in eastern Africa, proving that these stem rust races are spreading in a southerly direction to other African countries. However, if UVPgt55 has a South African origin, it is possible that races similar to Ug99 could develop independently in other areas.

Several molecular techniques have been developed to determine the genetic diversity and structure of fungal plant pathogen populations, such as rusts. These include random amplification of polymorphic DNA (RAPD; McCallum *et al.*, 1999), simple sequence repeats (SSRs; Weber and May, 1989), amplified fragment length polymorphism (AFLP; Hovmöller *et al.*, 2008; Keiper *et al.*, 2003), selectively amplified microsatellites (SAMs; Keiper *et al.*, 2003) and sequence-tagged microsatellites (STMs; Keiper *et al.*, 2006).

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AFLP fingerprinting has been increasingly used to characterize the genetics of fungal plant pathogen populations and to identify races or pathotypes. It is a powerful diagnostic tool that is sensitive and repetitive (Hurtado and Ramstedt, 2002). It has been used successfully to discriminate and identify *Fusarium* species (Abdel-Satar *et al.*, 2003), to identify inter-isolate variation and population structure in *Alternaria* species (Bock *et al.*, 2002) and to distinguish closely related organisms at the species to strain level (Schmidt *et al.*, 2004), even down to clonal level (Chulze *et al.*, 2000). With regard to *P. graminis*, Szabo (2007) developed 24 polymorphic and highly specific dinucleotide SSR markers.

The aim of this study was to evaluate whether SSR and AFLP markers could determine the genetic structure of selected South African wheat stem rust races, including the eastern African race Ug99.

RESULTS

Stem rust phenotypes

The seedling infection types of one isolate from each race are presented in Table 1. All infection types were as expected.

Marker polymorphism

SSR analysis

Twenty of the 24 published dinucleotide SSR primers of Szabo (2007), amplifying the largest number of alleles, were selected for SSR analysis of 25 South African and four Ug99 stem rust isolates (Table 2). These 20 primer combinations amplified a total of 42 alleles across the 29 pathogen isolates, 29 (69%) of which were polymorphic. The 13 monomorphic alleles were amplified by nine primer combinations. Four of the 15 polymorphic primer combinations amplified null alleles. Most primer combinations amplified two alleles (average of 2.1 alleles per primer combination). PgtSSR68 and PgtSSR164 amplified the largest number of alleles (four). The 15 polymorphic primer combinations amplified only one type of polymorphism, namely polymorphic fragments that were present in both UVPgt55 and Ug99 and absent in all other races, or vice versa. The only additional polymorphism was a unique fragment that was amplified from isolate 2 of UVPgt56.

Pairwise genetic similarity coefficients ranged from 0.2432 (between all isolates of races UVPgt55 and Ug99 and the rest of the isolates) to 1.000 (between isolates of races UVPgt50,

Table 1 Infection types* produced by isolates of South African races of *Puccinia graminis* f.sp. *tritici* on selected stem rust differentiating lines.

Sr gene†	Stem rust race‡						
	UVPgt50/1	UVPgt52/1	UVPgt53/1	UVPgt55/3§	UVPgt56/1	UVPgt57/1	UVPgt58¶
<i>Sr5</i>	3+	3	0	3+	0	0	0
<i>Sr8b</i>	;1	;1	;1	4	;1	;1	;1
<i>Sr9e</i>	3	;c	;c	2+	;c	;c	;c
<i>Sr24</i>	1	2 + 3	1	1	1	1	1
<i>Sr27</i>	;	;	3++	;c	3	3++	3++
<i>Sr31</i>	1	;1	1	2	1	1	1
<i>Sr38</i>	X–	;1	;	3	;	;	;
<i>SrKiewiet</i>	;c	;1	1	;c	3+	3	1
<i>SrSatu</i>	;	;	;	;	;	3	;

*Infection types were rated according to a 0–4 scale (McIntosh *et al.*, 1995) on primary leaves.

†The avirulence/virulence phenotypes of the selected races are as follows:

UVPgt50: *Sr8b,9g,21,24,27,31,36, Kiewiet, Satu/Sr5,6,7b,8a,9b,9e,17,30*

UVPgt55: *Sr21,24,27,31,36, Kiewiet, Satu/Sr5,6,7b,8a,8b,9b,9e,9g,17,30*

UVPgt52: *Sr8b,9e,9g,21,27,30,31,36, Kiewiet, Satu/Sr5,6,7b,8a,9b,17,24*

UVPgt53: *Sr5,6,7b,8b,9b,9e,17,21,24,30,31,36, Kiewiet, Satu/Sr8a,9g,27*

UVPgt58: *Sr5,6,7b,8b,9b,9e,9g,17,21,24,30,31,36, Kiewiet, Satu/Sr8a,27*

UVPgt56: *Sr5,6,7b,8b,9b,9e,17,21,24,30,31,36, Satu/Sr8a,9g,27, Kiewiet*

UVPgt57: *Sr5,6,7b,8b,9b,9e,17,21,24,30,31,36/Sr8a,9g,27, Kiewiet, Satu*

‡Host lines were Reliance (*Sr5*), Barleta Benvenuto (*Sr8b*), SST66 (*Sr9e*), LC/Sr24Ag (*Sr24*), Coorong (triticales, *Sr27*), Sr31/6*LMPG (*Sr31*), Trident (*Sr38*), Kiewiet (triticales, *SrKiewiet*) and Tobie (triticales, *SrSatu*).

§Based on these differentiating genes, Ug99 is similar to UVPgt55, except for *Sr31*, on which it produces infection type 4 (Ug99 not phenotyped in this experiment).

¶UVPgt58 is similar to UVPgt53, except for avirulence to *Sr9g*.

'X' and 'c' indicate a mixed host response and extensive chlorosis, respectively. Plus or minus signs indicate variation in pustule size within an established infection type class. Flecking is indicated by 'f'.

Table 2 Genetic information generated by 20 simple sequence repeat (SSR) primer combinations. Monomorphic allele sizes are indicated in normal type, and polymorphic allele sizes are indicated in bold type.

Locus*	N_a	N_p	Allele size (bp)
PgtSSR1	1 (null allele)	1	259
PgtSSR4	2	2	337 333
PgtSSR6	1 (null allele)	1	165
PgtSSR11	2	0	173 168
PgtSSR12	2	0	167 157
PgtSSR13	2	2	232 205
PgtSSR14	3	2	213 201 191
PgtSSR20	1	0	165
PgtSSR21	2	0	165 160
PgtSSR47	2	2	197 188
PgtSSR68	4	3	275 271 262 259
PgtSSR90	2 (1 null allele)	1	278 274
PgtSSR119	2	2	312 309
PgtSSR134	2	2	345 340
PgtSSR140	1 (null allele)	1	260
PgtSSR147	2	2	212 209
PgtSSR149	2	2	248 232
PgtSSR151	2	0	255 248
PgtSSR164	4	4	123 118 114 110
PgtSSR180	3	2	215 212 207
Total	42	29	

*SSR loci as given in Szabo (2007).

N_a , number of alleles.

N_p , number of polymorphic alleles.

Table 3 *EcoRI* and *MseI* adapter and primer sequences used in amplified fragment length polymorphism (AFLP) analysis.

Enzyme	Type	Sequence (5'–3')
<i>EcoRI</i>	Adapter-F	CTCGTAGACTGCGTACC
	Adapter-R	AATTGGTACGCAGTCTAC
<i>MseI</i>	Adapter-F	GACGATGAGTCCTGAG
	Adapter-R	TACTCAGGACTCAT
<i>EcoRI</i>	Primer +0	GACTGCGTACCAATTC
	Primer +1	GACTGCGTACCAATTCA
	Primer +2	GACTGCGTACCAATTCNN
		NN = AA, CC, TG
<i>MseI</i>	Primer +0	GATGAGTCCTGAGTAA
	Primer +2	GATGAGTCCTGAGTAANN
		NN = AT, AG
	Primer +3	GATGAGTCCTGAGTAACAT

UVPgt52, UVPgt53, UVPgt56, UVPgt57 and UVPgt58), with an average of 0.6836.

AFLP analysis

Twenty-nine rust isolates were screened with four AFLP primer combinations (E-AA/M-AT; E-CC/M-AG; E-TG/M-AG; E-A/M-CAT; Table 3). Repeatability of the AFLP results was confirmed by a high correlation between the subsets of AFLP reactions. The

Table 4 Genetic information generated by four amplified fragment length polymorphism (AFLP) primer combinations.

AFLP primer combination	Total number of fragments	Polymorphic fragments	Polymorphism (%)	Isolated unique fragments
E-AA/M-AT	84	19	22.6	1
E-CC/M-AG	112	65	58.0	12
E-TG/M-AG	83	36	43.4	6
E-A/M-CAT	85	34	40.0	6
Total	364	154		25
Average	91	39	41.0	6

results indicated that only nine of the amplified fragments (2.4%) were inconsistent. A total of 364 reliably amplified fragments was amplified, 154 (42.3%) of which were polymorphic (Table 4). The number of polymorphic fragments per primer combination ranged from 19 (E-AA/M-AT) to 65 (E-CC/M-AG), with an average of 39 polymorphic fragments per primer combination. AFLP primer combinations with a high A + T content amplified the smallest number of polymorphic fragments (E-AA/M-AT and E-A/M-CAT), whereas the primer with the highest C + G content (E-CC/M-AG) amplified the largest number of polymorphic fragments (65), as well as the largest total number of fragments (112). Primer combination E-AA/M-AT amplified a race-specific fragment for all four isolates of UVPgt52. A total of 25 isolate-specific fragments was amplified. Six uniquely amplified fragments were detected in isolate 2 of UVPgt53.

Pairwise genetic similarity coefficients ranged from 0.6790 to 0.9961, with an average of 0.8384. The highest pairwise genetic similarity coefficient was recorded between isolates 1 and 4 of Ug99, and the lowest was observed between isolate 2 of race UVPgt53 and isolate 4 of UVPgt55.

Genetic diversity

SSR analysis

A dendrogram for the 29 isolates was constructed on the basis of the 42 amplified SSR loci (Fig. 1) using Jaccard's coefficient of similarity and the unweighted pair group method with arithmetic averages (UPGMA) for clustering. Cluster analysis classified isolates into two main groups. Isolates from UVPgt55 and Ug99 clustered into one main group, and the rest of the isolates into a second. Isolate 2 from UVPgt56 clustered separately from the other isolates in the second main group, with a similarity of 91.7% to the other isolates in the group. SSR analysis could not uniquely distinguish between isolates within a specific race or between most of the isolates from different races. SSR analysis revealed races UVPgt55 and Ug99 (group I) to be genetically diverse from UVPgt50, UVPgt52, UVPgt53, UVPgt56, UVPgt57 and UVPgt58 (group II), with a similarity of 24.5% between the two main groups (Fig. 1). The cophenetic correlation coefficient

Fig. 1 Dendrogram of 29 tested stem rust pathogen isolates based on UPGMA (unweighted pair group method with arithmetic averages) cluster analysis and the Jaccard similarity coefficients calculated from 38 simple sequence repeat (SSR) alleles.

Fig. 2 Dendrogram of 29 tested stem rust pathogen isolates based on UPGMA (unweighted pair group method with arithmetic averages) cluster analysis and the Jaccard similarity coefficients calculated from 364 amplified fragment length polymorphism (AFLP) fragments.

of 0.999 indicated a good fit between the Jaccard coefficient matrix and the symmetrical matrix produced from the UPGMA-based dendrogram (cophenetic correlation coefficient $r > 0.9$ indicates a very good fit; $r = 0.9-0.8$ indicates a good fit and $r < 0.8$ indicates a poor fit).

AFLP analysis

Cluster analysis using AFLP data (both monomorphic and polymorphic fragments) revealed the same two major groups as found for the SSR data (Fig. 2). All 29 isolates could be distinguished from each other using the four tested AFLP primer

combinations. The two main groups revealed a genetic similarity of 71.9%. The four isolates of Ug99 showed the lowest levels of genetic variation, with a similarity of 99.3% between isolates which clustered within UVPgt55 in the first main group. The highest level of genetic variation between isolates of the same race was recorded for UVPgt53. Isolates 1 and 4 of Ug99 had a genetic similarity of 99.6%.

Isolates of the same race generally clustered together, except for UVPgt53, where isolates 2 and 4 were genetically diverse and clustered separately from all isolates in the second main group. Furthermore, the single isolate of UVPgt58 clustered with two

Fig. 3 Dendrogram of 29 tested stem rust pathogen isolates based on UPGMA (unweighted pair group method with arithmetic averages) cluster analysis and the Jaccard similarity coefficients calculated from combined simple sequence repeat (SSR) and amplified fragment length polymorphism (AFLP) data.

isolates of UVPgt53. The genetic variation was 93.5% between isolates of UVPgt50, 94.5% for UVPgt52, 95.4% for UVPgt56 and 94.5% for UVPgt57. The maximum genetic similarity between isolates within the second main group was 87.6%. The cophenetic correlation coefficient was 0.990, suggesting a good fit between the dendrogram and the genetic similarity matrices.

Combined SSR and AFLP analysis

Cluster analysis using combined SSR and AFLP data (Fig. 3) classified isolates identical to the dendrogram based on AFLP data alone (Fig. 2). The level of genetic similarity between isolates using combined AFLP and SSR data was 66.7%, compared with 71.9% using AFLP data alone. The cophenetic correlation coefficient was 0.995, suggesting the presence of a very good fit between the dendrogram and the genetic similarity matrices.

To assess the genetic variation among races and among isolates within races, analysis of molecular variance (AMOVA) was performed. Two different types of analysis were performed. During the first analysis, the distribution of genetic variation between isolates within and among races was tested. AMOVA revealed that most of the molecular variability (78.4%, $P < 0.001$) could be attributed to differences between the different rust races, with 21.6% of the variation distributed among isolates within each race. During the second analysis, the distribution of genetic variation between isolates within and among races was tested based on the clustering results using combined SSR and AFLP data. The hypothesized relationship between races UVPgt55 and Ug99 was included in the AMOVA structure. AMOVA revealed that more than 80% ($P < 0.001$) of the detected genetic variation was attributed to variation among the two groups (group I, Ug99 and UVPgt55; group II, rest of the races), with

6.7% distributed among races within the two groups and 12% among isolates within each race. High F_{ST} values for both analyses (> 0.75) indicated high genetic differentiation among races.

The minimum-spanning network constructed from the 29 tested rust isolates (Fig. 4) showed a similar grouping to the dendrogram analysis (Fig. 3). The minimum-spanning network analysis indicated that 94 mutational events separated the Ug99 and UVPgt55 genotypes from the rest of the genotypes. The number of mutational events between isolates within the Ug99/UVPgt55 group varied between 1 and 13, whereas it varied between 1 and 22 for the isolates in the other group. This result confirmed that the high level (80%) of genetic variation contributed to variation among the two main groups seen in the second AMOVA results.

DISCUSSION

The continuous monitoring of the genetic diversity of fungal pathogens is crucial to the understanding of genetic variation and host–pathogen co-evolution (Aradhya *et al.*, 2001). By appreciating the origin and potential of variation in cereal rust fungi, future breeding programmes for genetic resistance in the host crop can be managed proactively.

Since the beginning of wheat cultivation in South Africa, stem rust has evolved to form various races (Pretorius *et al.*, 2007). From the first two described standard races (34 and 21 in 1922 and 1929, respectively; De Jager, 1980), several pathways of specialization have been proposed. One such proposed pathway led to the putative specialization of race UVPgt55 from 2SA43 (Pretorius *et al.*, 2007). Race UVPgt55, which is similar to Ug99 except for avirulence on *Sr31*, has become the

Fig. 4 Minimum-spanning network of 29 tested stem rust isolates based on simple sequence repeat (SSR) and amplified fragment length polymorphism (AFLP) data. Each node represents a unique genotype. Genotypes connected by thin lines differ by one to nine markers, whereas those connected by thicker lines differ by 10 or more markers. A total of 94 markers separated the Ug99 and UVPgt55 genotypes from the rest of the genotypes.

most prevalent stem rust race in South Africa since its detection in 2000 (Komen, 2007).

SSR analysis (Table 2) indicated that the genetic diversity of the 29 tested South African isolates was much lower than that of the 25 isolates analysed by Szabo (2007). In the present study, a total of 42 alleles were found at the 20 tested loci, at an average of 2.1 per locus, compared with the 105 alleles, at an average of 4.8, in the study by Szabo (2007). Likewise, the number of alleles per locus and the number of null alleles were lower. As it was not stated by Szabo (2007) whether the isolates used came from North America only, or from different parts of the world, it is difficult to explain the different levels of variation detected between the two studies.

Considering the AFLP results (Table 4), it is clear that the second primer combination (E-CC/M-AG) yielded the most informative results by giving the largest number of amplified fragments, the highest percentage of polymorphisms and the largest number of fragments unique to a particular isolate. The latter presents an opportunity to develop sequence characterized amplified regions (SCARs) to allow for the rapid identification of particular isolates.

Both SSR (Fig. 1) and AFLP (Fig. 2) data divided the stem rust population into two different groups, with the percentage similarity much higher for the AFLP data (71.9%) than the SSR data (24.5%). A similarity of 66.7% was obtained between the two groups for the two combined datasets (Fig. 3), suggesting a split from a common ancestor leading to two quite diverse groups. AMOVA results further indicated that the highest percentage variation was caused by the variation between, not within, races. This was expected, as all isolates within a race were tested to be phenotypically similar on the differentiating testers used. When the cluster results were used as a basis for AMOVA, and races

were placed into two main groups (Ug99 and UVPgt55 in one group and the rest of the isolates in another group), the highest percentage variation was caused by differences between the two groups, thereby confirming the validity of the generated data. The percentage of variation explained by differences within isolates of the same races was higher (12.2%) than the percentage variation explained among races within each group (6.7%). This confirmed that races within each main group are closely related.

In the present study, AFLP analysis was able to uniquely distinguish all 29 tested stem rust isolates, even the four isolates from any given race. Bock *et al.* (2002) indicated that the source of variation in many apparently asexual fungi is unknown and somewhat difficult to explain, although the level of recombination can be typical of a sexual system. According to Burdon and Silk (1997), plant pathogenic fungi most commonly rely on mutation and recombination as the main sources of genetically based variation. Genetic recombination occurs during meiosis, when reassortment and crossing over produce gametes or progeny with different combinations of alleles from those in the parental genomes. In fungi, non-meiotic recombination, or parasexuality, is also capable of producing progeny with new combinations of genes (Taylor *et al.*, 1999).

Based on the AFLP results, the first group consisted of UVPgt50, UVPgt52, UVPgt53, UVPgt56, UVPgt57 and UVPgt58, with a combined similarity of 88%, and the second group consisted of UVPgt55 and Ug99, with a 92% similarity. Within Ug99, the different isolates were 99.5% similar, whereas UVPgt55 isolates showed a 92% similarity. This difference in similarity between isolates of the same race could be a result of the fact that UVPgt55 (as well as the other isolates of the different races) was multiplied several times, allowing for the generation of variance, whereas Ug99 was multiplied only once.

Within the first group of the AFLP dendrogram, two subgroups were found. The first consisted of UVPgt50 and UVPgt52, and the second consisted of UVPgt53, UVPgt56, UVPgt57 and UVPgt58. The grouping of UVPgt58 with UVPgt53 was expected, as the latter is a single-step variant with additional virulence against *Sr9g*. This was confirmed by the minimal-spanning network (Fig. 4). The same applies to UVPgt56 and UVPgt57 that grouped together with a 93% similarity. The two races differ only in virulence for *SrSatu*. The genetic data therefore clearly supported the groupings of the different races previously based on infection type data.

The grouping of UVPgt55 and Ug99 supported the similar virulence/avirulence profiles of the two races (Pretorius *et al.*, 2007). The most likely explanation is that UVPgt55 appeared in South Africa as an exotic introduction, most probably from eastern Africa and not through local adaptation as proposed previously (Pretorius *et al.*, 2007). Assuming that virulence is more often gained than lost, it is possible that UVPgt55 may have been the progenitor of Ug99.

This was confirmed by the minimum-spanning network analysis of the data (Fig. 4). The same two groups as shown in Fig. 3 were found, with a total of 94 proposed mutational events separating them. Within each of the two groups, the number of mutational events between the different isolates was much lower (maximum of 22), indicating that each group probably developed from a separate ancestor. The analysis indicated that, within group 2, Ug99 most probably developed from UVPgt55.1, needing only four mutational events. As Ug99 was isolated in eastern Africa, it is suggested that UVPgt55 may also have originated in eastern Africa, where it gave rise to the development of Ug99. UVPgt55, however, spread to South Africa, where it has become the most prevalent pathotype since 2000 (Komen, 2007).

Exotic introductions of new pathogen races in wheat production regions have often been reported. A RAPD study performed on the genetic structure of *P. graminis* f.sp. *tritici* in South America revealed that the current stem rust population shared a common ancestor with both European and North American populations (McCallum *et al.*, 1999). The level of similarity between isolates from each continent (South America, 0.72; Europe, 0.73) was similar to that between isolates from the different continents (0.7), indicative of a common ancestor. This high level of similarity clearly indicated that the pathogen was introduced into South America, most probably partly through North America.

In Australia, some of the changes in rust populations have been attributed to exotic introductions (Keiper *et al.*, 2003; Park *et al.*, 1999). Using three different molecular techniques, Keiper *et al.* (2003) determined the genetic structure of different Australian isolates of five cereal rust species. Although the genetic similarity of eight of the *P. graminis* f.sp. *tritici* isolates was high (> 85%), two isolates were different. One was race 126, which showed a pathogenic and molecular distinctness from the other described races, leading to the conclusion that it was an

exotic introduction to Australia (Keiper *et al.*, 2003; Luig, 1977). The second isolate, assumed to be a somatic hybrid between races 126 and 21 (Luig, 1977), could also be differentiated by molecular data. Together with these results, the current study confirms the establishment of exotic introductions in other areas.

Low correlations were found between the pathogenicity of the Australian cereal rust species and their clustering within the dendrogram (Keiper *et al.*, 2003). Similar results from other studies led to the conclusion that DNA polymorphisms are independent of pathogenicity, indicating that the genome evolves faster than the genes that cause the pathogenic phenotype (Chen *et al.*, 1993). In the current study, however, a good correlation was found between the pathogenicity and molecular fingerprints of the isolates, confirming the use of molecular techniques as a supplement to pathogenicity tests.

To conclude, although all known South African *P. graminis* f.sp. *tritici* races were not included, this study clearly indicates that little genetic variation exists between South African races. This could be a result of the small geographical area in which wheat is cultivated, as well as the genetic uniformity of the cultivars used and the absence of the barberry alternative host that could promote sexual recombination. Population evolution would thus be driven by single-step mutations, leading to the formation of new virulent races (Park *et al.*, 1995; Wellings and McIntosh, 1990). However, the close relationship between UVPgt55 (2SA88) and Ug99 indicates that exotic introductions also occur in South Africa, highlighting the migration potential of wheat stem rust. It thus confirms the potential threat posed by TTKS and its variants (Mackenzie, 2007).

EXPERIMENTAL PROCEDURES

Pathogen materials

The South African races UVPgt50, UVPgt52, UVPgt53, UVPgt55, UVPgt56, UVPgt57 and UVPgt58 of *P. graminis* f.sp. *tritici* were used in this study. The UVPgt notations reflect the wheat stem rust cultures held at the University of the Free State. UVPgt50, UVPgt52, UVPgt53, UVPgt55, UVPgt56 and UVPgt58 are isolates of, and similar to, races 2SA4, 2SA100, 2SA102, 2SA88, 2SA102K and 2SA103 (Agricultural Research Council notation), respectively, described by Pretorius *et al.* (2007). UVPgt57 appears to be a single-gene mutant of UVPgt56, differing only in virulence for *SrSatu*. Likewise, UVPgt58 is similar to UVPgt53, except for avirulence to *Sr9g*. Ug99 (TTKSK; Jin *et al.*, 2008), from the original Ugandan collection in 1999 (Pretorius *et al.*, 2000), was also included. Four single-pustule isolates of the South African races were established, except for UVPgt58 which was represented by one isolate. To minimize contamination, cultures were increased in isolation cubicles on selective hosts, namely SST66 (*Sr9e*, UVPgt50), Gamka (*Sr24*, UVPgt52), Coorong (*Sr27*, UVPgt53),

Trident (*Sr38*, UVPgt55), Kiewiet (triticale, UVPgt56) and Tobie (triticale, *SrSatu*, UVPgt57). UVPgt58 was collected from the triticale cultivar Pan 299.

The purity of all isolates was confirmed by inoculating them individually onto an abbreviated differential set. The set included *Sr5*, *Sr8b*, *Sr9e*, *Sr24*, *Sr27*, *Sr31*, *Sr38* and the triticales Kiewiet and Tobie (*SrSatu*). UVPgt58 was also tested on *Sr9g*. Infection types (0–4 scale; McIntosh *et al.*, 1995) on the primary leaves of plants maintained in a glasshouse at 18–24 °C were determined 14 days after inoculation.

Four single-pustule isolates of Ug99, established during the initial characterization of this race (Pretorius *et al.*, 2000), were retrieved from a –70 °C freezer and germinated under controlled conditions in the dark at 25 °C. Urediniospores were seeded onto sterile discs of dialysis tubing placed onto 0.6% (w/v) agar plates in Petri dishes. To enhance germination, spores were heat shocked at 45 °C for 6 min before placement on dialysis tubing. In addition, filter paper saturated with 0.01% (v/v) nonanol was placed within the Petri dish lid. Spores and germ tubes were harvested from the cellophane discs after 24 h.

SSR analysis of stem rust races

Analysis was performed using 20 (Table 2) of the 24 SSR primer pair combinations described by Szabo (2007). Primers were synthesized by Integrated DNA Technologies Inc. (Coralville, IA, USA). After grinding infected wheat leaf tissue in liquid nitrogen, total genomic DNA was extracted using the GenElute Plant Genomic DNA Miniprep Kit (Sigma, St. Louis, MO, USA). The quality and quantity of extracted genomic DNA were confirmed on a 1.0% (w/v) agarose gel using 0.5 × TAE (20 mM Tris-acetate pH 8, 0.5 mM EDTA) as running buffer (Sambrook *et al.*, 1989). Each 15 µL polymerase chain reaction (PCR) contained 10 ng total genomic DNA, 10 pmol of each primer and a 1 × concentration of KapaTaq ReadyMix (KapaBiosystems, Cape Town, South Africa). The amplification regime consisted of 94 °C for 1 min, followed by 31 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s. A final elongation step of 10 min at 72 °C was included. To confirm the success of the amplification reactions, 5 µL of each PCR was analysed on a 1.5% (w/v) agarose gel (Sambrook *et al.*, 1989).

After mixing the remainder of each PCR with 5 µL of loading buffer [98% (v/v) formamide, 10 mM ethylenediaminetetraacetic acid (EDTA) pH 8, 1 mg/mL bromophenol blue, 1 mg/mL xylene cyanol], amplified DNA was denatured for 5 min at 95 °C. A total of 5 µL of each reaction was separated for 1 h at 80 W on a 5% (w/v) denaturing polyacrylamide gel [19 : 1 acrylamide : bis-acrylamide, 7 M urea, 1 × TBE buffer (89 mM Tris-borate pH 8, 2.0 mM EDTA)] using 1 × TBE as running buffer (Sambrook *et al.*, 1989). After separation, the amplified fragments were visualized by silver staining using the Silver Sequence™ DNA Sequencing System (Promega, Madison, WI, USA). The gels were air dried and

photographed by exposing photographic paper (Ilford Multi-grade IV RC) directly under the gel to dim light for approximately 20 s. SSR fragment lengths were determined by comparison with a 25-bp DNA ladder (Promega).

AFLP analysis of stem rust races

Genomic DNA extraction for AFLP analysis

Single-spore isolates of all *P. graminis* races were germinated on agar plates as described. Germinated mycelia were collected and total genomic DNA was isolated using cetyltrimethylammonium bromide (CTAB) according to Saghai-Marooof *et al.* (1984). Freeze-dried mycelia were disrupted in CTAB extraction buffer [100 mM Tris-Cl pH 8.0, 20 mM EDTA, 1.4 M NaCl, 2% (w/v) CTAB, 0.2% (v/v) β-mercaptoethanol] using a TissueLyser (Qiagen, Hilden, Germany). After incubation for 1 h at 65 °C, the suspension was extracted once with chloroform–isoamyl alcohol (24 : 1, v/v) and precipitated with isopropanol (Sambrook *et al.*, 1989). After washing the DNA pellet with 70% (v/v) ethanol, it was dissolved overnight at 4 °C in TE buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA). Contaminating RNA was removed by the addition of DNase-free RNase to a final concentration of 100 µg/mL and incubation for 2 h at 37 °C. The DNA was again extracted with chloroform–isoamyl alcohol (24 : 1, v/v) in the presence of 0.75 M ammonium acetate, and precipitated for 2 h at –20 °C with ice-cold 100% (v/v) ethanol. After washing the precipitated DNA twice with ice-cold 70% (v/v) ethanol, the dried DNA was dissolved in TE buffer for 2 h at 37 °C and the concentration was determined (Sambrook *et al.*, 1989). The DNA was diluted to a final concentration of 200 ng/µL.

AFLP analysis

AFLP analysis was performed using *Mse*I primers in combination with *Eco*RI primers (Table 3). Primers and adapters were synthesized by Integrated DNA Technologies Inc. The prepared oligonucleotides used for adapters were polyacrylamide gel electrophoresis (PAGE) purified. Adapters were prepared by mixing equimolar amounts of both forward and reverse strands, heating them for 10 min at 65 °C and then leaving them at room temperature to cool. AFLP analyses were performed according to Herselman (2003). The reproducibility of AFLP reactions was tested on a subset of nine randomly selected isolates from the original set of 29 isolates.

Data analysis

A binary matrix recording specific SSR and AFLP fragments as present (1) or absent (0) was generated for each genotype. For the AFLP analysis, only reliable (between 150 and 700 bp) bands were considered. Pairwise genetic distances were expressed as the complement of Jaccard's similarity coefficient (Jaccard, 1908). Cluster analyses were performed using UPGMA (Sokal

and Michener, 1958). Statistical analyses were performed using NTSYS-pc version 2.02i (Rohlf, 1998; Exeter Software, New York, NY, USA). Dendrograms were created using the SAHN program of NTSYS. The robustness of the dendrogram was tested by estimating the cophenetic correlation values for each dendrogram and comparing them with the original genetic similarity matrix using Mantel's matrix correspondence test (Mantel, 1967). Values were calculated using the COPH and MXCOMP programs. In order to assess the genetic variation among races and among isolates within races, AMOVA (Excoffier *et al.*, 1992) was performed using the statistical program Arlequin version 3.1 (Excoffier *et al.*, 2005). AMOVA was performed to test the structure: (i) among races with no previous knowledge with regard to genetic clustering, and (ii) among races and groups based on UPGMA clustering results. The significance level for all AMOVA tests was set at 0.05. The fixation index F_{ST} was calculated and provided a measure of the genetic differentiation of the groups. Values of F_{ST} greater than 0.25 indicate significant genetic differentiation (Hartl and Clark, 1997).

A minimum-spanning network was constructed based on the minimum number of markers between different genotypes. The network was constructed using NETWORK 4.5.0.0 software (www.fluxus-engineering.com), employing the median-joining approach (Bandelt *et al.*, 1999).

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