Characterization of two new wheat stem rust races within the Ug99 lineage in South Africa

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Abstract Two new races of the wheat (*Triticum aestivum* L.) stem rust pathogen, representing the fifth and sixth variants described within the Ug99 lineage, were detected in South Africa. Races TTKSP and PTKST (North American notation) were detected in 2007 and 2009, respectively. Except for *Sr24* virulence, race TTKSP is phenotypically identical to TTKSF, a commonly detected race of *Puccinia graminis* f. sp. *tritici* (*Pgt*) in South Africa. PTKST is similar to TTKSP except that it produces a lower infection type on the *Sr21* differential and has virulence for *Sr31*. Simple sequence repeat (SSR) analysis confirmed the genetic relationship amongst TTKSF, TTKSP, PTKST and TTKSK (Ug99). TTKSK, PTKST and TTKSF grouped together with 99% similarity, while sharing 88% genetic resemblance with TTKSP. These four races in turn shared only 31% similarity with other South African races. It is proposed that TTKSP arose locally as a single step mutation from race TTKSF, whereas PTKST probably represents an exotic introduction of *Pgt* to South Africa.

Key words Pathotype · *Puccinia graminis* f. sp. *tritici* · *Triticum aestivum* · Ug99

Introduction

Puccinia graminis f. sp. *tritici* (*Pgt*) is the causal agent of stem rust, a historically important (Saari and Prescott 1985) and recurring (Singh et al. 2006; 2008) disease of wheat. It is highly adapted to long distance migration through wind dispersal and rain deposition of urediniospores (Rowell and Romig 1966; Singh et al. 2006). In addition to natural dispersal mechanisms, accidental transport by means of contaminated clothing or goods may also contribute to the spread of spores (Singh et al. 2006).

Currently more than 60 numbered or temporarily designated *Sr* genes for resistance to stem rust have been listed in the Komugi Wheat Genetics Resource Database (www.shigen.nig.ac.jp, accessed 16 April 2010). Utilization of many of these genes in breeding programs has resulted in the effective control of stem rust in most countries (Singh and McIntosh 1987; McIntosh et al. 1995; Singh et al. 2008). However, the detection of Ug99 (syn. TTKSK, North American [NA] race notation, Jin et al. 2008b) in East Africa, a *Pgt* race with broad virulence (Pretorius et al. 2000; Wanyera et al. 2006; Jin et al. 2007) and adaptive capacity (Jin et al. 2008a; 2008b; 2009), indicated that continued efforts are necessary to control this disease.

The rapid adaptation of Ug99 for *Sr24* virulence (Jin et al. 2008b) was of particular concern as this gene occurs in Kenyan commercial varieties (MacKenzie 2008). *Sr24* continues to be widely used in countries such as Australia (Park and Bariana 2008) and South Africa (McIntosh et al. 1995) and was initially identified as a source of resistance to Ug99 (Jin et al. 2007).

It was recently shown that, in addition to step-wise mutations, an exotic introduction contributed to the genetic diversity of the South African *Pgt* population (Visser et al. 2009). TTKSF, the most prevalent race in South Africa since its first detection in 2000, shares an identical virulence profile with Ug99, except for avirulence towards *Sr31* (Pretorius et al. 2007). This resemblance was confirmed at molecular level using SSR and AFLP analyses (Visser et al. 2009). Ug99 and TTKSF were distinctly different from the other South African races, suggesting that TTKSF was an exotic introduction into South Africa, most probably from Ug99 ancestry in East Africa.

Two races with *Sr24* virulence, namely 2SA100 and 2SA101 (Agriculture Research Council [ARC] notation), were detected in South Africa during the mid 1980s (Le Roux and Rijkenberg 1987). These closely related races are avirulent and virulent to *Sr9g*, respectively, and were distinctly different from Ug99 (Jin et al. 2008b; Visser et al. 2009). Considering the data of Le Roux and Rijkenberg (1987) on comparable entries in the current NA differential set, 2SA100 and 2SA101 code to LSH and LTH, respectively. In 2007, TTKSP (2SA106) with *Sr24* virulence, was detected in the Western Cape, South Africa (Terefe et al. in press). Phenotypically, this race is similar to TTKST except for avirulence to *Sr31*, and similar to TTKSF except for virulence to *Sr24*. A second new race, PTKST, was detected at two locations in KwaZulu-Natal, South Africa, at the end of 2009 (Pretorius et al. 2010). PTKST is virulent to both *Sr24* and *Sr31*.

The objective of this study was to determine the relationship between TTKSF, TTKSP, PTKST, TTKSK and other *Pgt* stem rust races using SSR markers.

Materials and methods

Stem rust isolates

In this study, the South African *Pgt* races were represented by four single pustule isolates each of UVPgt50, 52, 53, 55, 56, 57, 59 and 60. UVPgt58 was represented by a single isolate. All single pustule isolates were sub-samples from the type culture of each race and do not reflect different field collections. The UVPgt notations reflect the wheat stem rust cultures held at the University of the Free State. UVPgt50, 52, 53, 55, 56, 58 and 59 are similar to races 2SA4, 2SA100, 2SA102, 2SA88, 2SA104, 2SA103 and 2SA106 named by the ARC-Small Grain Institute, Bethlehem. 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* and UVPgt59 is similar to UVPgt55 except for *Sr24* virulence in the former. Ug99 (TTKSK, Jin et al. 2008b), from the original Ugandan collection in 1999 (Pretorius et al. 2000), was also included.

UVPgt59 (TTKSP) was received as stem rust field collection Pg-KGI-49 from the ARC-Small Grain Institute in 2007, sampled from an unknown wheat line at the Tygerhoek experimental farm, Western Cape, on 26 September, 2007. UVPgt60 (PTKST) was collected on 17 November, 2009 from a wheat cultivar suspected of carrying *Sr31* in a disease nursery near Greytown, KwaZulu-Natal. Virulence was confirmed on several wheat cultivars known to possess the *Sr31* resistance gene (Pretorius et al. in press). Both races were phenotypically characterized on a differential set (Table 1) using standard procedures for inoculation of seedlings and recording of infection types (Jin et al. 2008b). Phenotyping was repeated in at least three independent experiments. Single-pustule isolates of UVPgt59 and UVPgt60 were increased on wheat lines LCSr24Ag (*Sr24*) and Federation*4/Kavkaz (*Sr31*), respectively. Urediniospores were harvested and germinated as previously described (Visser et al. 2009).

Genomic DNA extraction for SSR analysis

Total genomic DNA was isolated from fungal tissue of the UVPgt59 and UVPgt60 isolates using CTAB according to Saghai-Maroof et al. (1984) and as described in Visser et al. (2009). For the other races, previously extracted genomic DNA (Visser et al. 2009) was used in the SSR analyses.

SSR analysis of stem rust races

SSR analysis of all isolates was done using 24 primer combinations that were developed at the Plant Breeding Institute Cobbitty, University of Sydney, Australia (H. Karaoglu and R.F. Park unpublished data). The isolates of UVPgt59 and UVPgt60 were also fingerprinted with SSR primer combinations described by Szabo (2007) and used by Visser et al. (2009). Selected isolates previously used by

Visser et al. (2009) were again fingerprinted using both SSR primer sets to facilitate correlation between the two data sets.

Each 15 µl PCR reaction contained 10 ng total genomic DNA, 10 pmol of each primer and a 1x concentration of KapaTaq ReadyMix (KapaBiosystems, Cape Town, South Africa). The amplification regime was 94°C for 1 min, followed by 31 cycles of 94°C for 30 s, 53 or 55°C for 30 s (depending on the primer pair used) and 72°C for 30 s. A final elongation step of 10 min at 72°C was included. To confirm success of the amplifications, 5 µl of each PCR reaction was analysed on a 1.5% (w/v) agarose gel (Sambrook et al. 1989). Polyacrylamide gel electrophoresis was performed as described in Visser et al. (2009).

Data analysis

A binary matrix recording specific SSR fragments as present (1) or absent (0) was generated for each isolate. Pairwise genetic distances were expressed as the complement of Jaccard's similarity coefficient (Jaccard 1908). Cluster analyses were performed using UPGMA (unweighted pairgroup method using arithmetic averages; Sokal and Michener 1958). Statistical analyses were computed using NTSYS-pc version 2.02i (Rohlf 1998; Exeter Software, NY, USA) and dendrograms were created using the SAHN programme of NTSYS-pc. The robustness of the dendrogram was tested by estimating the co-phenetic 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. To assess the genetic variation among races and among isolates within races, analysis of molecular variance (AMOVA) (Excoffier et al. 1992) was performed using the statistical programme Arlequin version 3.1 (Excoffier et al. 2005). AMOVA was performed to test the structure among races and groups based on UPGMA clustering results (Visser et al. 2009). The significance levels for all AMOVA tests were set at 0.05. The fixation index F_{ST} was calculated and provided a measure of genetic differentiation of 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).

Results and discussion

Virulence profiles of TTKSP and PTKST

The infection type (IT) data in Table 1 show that UVPgt59 differed from UVPgt55 (TTKSF) only by its virulence to *Sr24* and it was thus coded TTKSP (Table 1) according to the NA nomenclature system (Jin et al. 2008b). In addition to *Sr31* virulence, isolate UVPgt60 differed from TTKSF and TTKSP only in regard to the IT on the *Sr21* differentials. All eight single-pustule isolates, sub-cultured from UVPgt60, produced an intermediate IT (2 to 2++) on the *Sr21* differential line CS_T mono_deriv (Table 1) and coded to PTKST. The UVPgt60 isolates also produced a clear low IT (;1=) on *T. monococcum* cv. Einkorn, the original Stakman et al. (1962) differential for *Sr21* (Pretorius et al. 2010). TTKSF and TTKSP produced IT 3 on CS_T mono_deriv and IT 2 on Einkorn. Based on stem rust phenotype, TTKSP appeared to be a single step mutant from TTKSF. Given the existence of TTKSP in South Africa, the initial conclusion was that it added virulence for *Sr31*. However, the low reaction conferred by *Sr21* suggested that PTKST may be an introduction rather than a local adaptation from TTKSP. IT data recorded during the original description of Ug99 (TTKSK) (Pretorius et al. 2000) are also included in Table 1.

TABLE 1 HERE

Marker polymorphism

Among the 24 SSR markers developed at the University of Sydney and tested on genomic DNA extracted from 33 South African and four Ug99 isolates of *Pgt*, six primer pairs failed to amplify fragments. Another two each amplified a single faint fragment. The latter amplifications were not repeatable and these eight primer pairs were therefore excluded from the analysis. The remaining 16 SSR primer combinations amplified a total of 69 alleles of which 54 (78%) were polymorphic (Table 2). The number of alleles ranged from one for primer pair A19 to 17 for primer pair A2 with a mean of 4.3 per primer pair. Fifteen monomorphic alleles were amplified by nine primer pairs. In contrast to the SSR study by Visser et al. (2009), the amplified polymorphic alleles were not only present in TTKSK and TTKSF and absent in the other races or vice versa, but polymorphisms were evident between different races that originally grouped together.

TABLE 2 HERE

Genetic diversity

Previously, SSR analysis of UVPgt50, UVPgt52, UVPgt53, UVPgt55, UVPgt56, UVPgt57, UVPgt58 and Ug99 using primer combinations developed by Szabo (2007) divided the races into two groups with one consisting of Ug99 and UVPgt55 and the second group containing the rest (Visser et al. 2009). It was not possible to distinguish between races within each group, as well as between isolates of each race, except for one isolate (56.2) of UVPgt56 which had a unique banding pattern. After combining the SSR data of UVPgt59 and UVPgt60 with that generated for the other races using the Szabo (2007) primers, a dendrogram was constructed using Jaccard's coefficient of similarity and UPGMA for clustering. Based on the available data, the 37 isolates were again divided into two groups (Fig. 1). There was a good fit between the Jaccard's coefficient matrix and symmetrical matrix produced by the UPGMA-based dendrogram with the cophenetic correlation coefficient (r) being 0.9987.

The first group (Fig. 1) consisted of UVPgt55, UVPgt59, UVPgt60 and Ug99, whereas the second group contained the remaining races. The genetic similarity between the two groups increased from 24.5% previously (without UVPgt59 and UVPgt60) to 36%. Within the first group, UVPgt55, UVPgt60 and Ug99 clustered separately from UVPgt59 with a similarity of 97%, but it was again impossible to distinguish between the other races.

FIGURE 1 HERE

To improve the resolution between the individual isolates of the different races, a dendrogram (r = 0.9958) was constructed using the 69 SSR alleles generated using SSR primer pairs developed at the University of Sydney (Fig. 2). Again, two major groups were found with the genetic similarity between the two groups decreasing to 27%. Within the first group, UVPgt55, UVPgt60 and Ug99 clustered separately from UVPgt59 with an 81% similarity between the two subgroups. Isolates Ug99.1, Ug99.2 and Ug99.3, clustering with UVPgt60, showed a genetic similarity of 97% with isolate Ug99.4 that clustered with UVPgt55.

FIGURE 2

Within the second group, two subgroups were evident (genetic similarity of 73%) with isolates from UVPgt50, UVPgt52, UVPgt53 and UVPgt57 clustering together with a similarity of 93%. Isolates of UVPgt53 fell into two smaller groups indicating genetic heterogeneity between the four isolates. The second subgroup consisted of isolates from UVPgt56 and UVPgt58 with a genetic similarity of 92%.

When the two SSR data sets were used to construct a combined dendrogram (Fig. 3), a similar pattern was observed (r = 0.9997) except that the genetic similarity of the different associations was lower than that using the Szabo (2007) primers, but higher than when the University of Sydney

primers were used. It was still impossible to distinguish between UVPgt50, UVPgt52 and UVPgt57, whereas there was some differentiation between different isolates from UVPgt53 and UVPgt56.

FIGURE 3 HERE

Analysis of molecular variance (AMOVA) was used to determine the main source of genetic variation. When isolates of all races were divided into two groups (UVPgt55, UVPgt59, UVPgt60 and Ug99 in group 1 and the other races in group 2 based on dendrogram results) 91.3% (P<0.001) of the molecular variability could be attributed to variation between the two groups, with 7.9% attributed to variation among races within groups and 0.71% among isolates within each race. The F_{ST} value of 0.993 was indicative of the high genetic differentiation among the races.

A minimum-spanning network of the combined data (Fig. 4) produced a pattern similar to Fig. 3. A total of 74 mutational events separated the two main groups. Within one group, all UVPgt59 isolates were separated by nine mutational events from three Ug99 isolates and the UVPgt60 isolates and by one further mutational step from the four isolates of UVPgt55 and one isolate of Ug99. In the other group, the cluster consisting of all four isolates of each of UVPgt50, UVPgt52 and UVPgt57 were separated by 10 events from a hypothetical intermediate isolate linked to UVPgt56 and UVPgt58. Results indicated the possibility of recombination between isolates of UVPgt56 and UVPgt58.

FIGURE 4 HERE

Conclusions

Based on infection type and SSR results, the close genetic relationship between TTKSP (UVPgt59), TTKSF (UVPgt55), PTKST (UVPgt60) and TTKSK (Ug99) was confirmed. With TTKSF being a likely exotic introduction into South Africa and TTKSP a putative mutational derivative, it can be concluded that the latter evolved locally. TTKSP is the fifth variant within the Ug99 lineage (Jin et al. 2008a). PTKST, the sixth described race in this group, most likely represents an exotic *Pgt* introduction to South Africa and highlights the vulnerability of the wheat industry to foreign pathogenic races. This emphasizes the need for regular monitoring of the stem rust pathogen, in particular isolates in the variable Ug99 lineage, as well as continued resistance breeding.

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Table 1 Infection types produced by *Pgt* races TTKSK (Ug99), TTKSP (UVPgt59) and PTKST (UVPgt60) on differentiating lines and scored according to the 0 to 4 scale (Stakman et al. 1962).

Entry	*Sr* gene	Infection type		
		TTKSK[a]	TTKSP	PTKST
ISr5-Ra	*5*	3+[b]	4	4
Cns_T_mono_deriv	***21***	**Not tested**	**3**	**22++**
Einkorn	***21***	**1**	**2**	**;1=**
Vernstein	*9e*	3+	4	4
ISr7b-Ra	*7b*	4	4	4
ISr11-Ra	*11*	3+	4	4
ISr6-Ra	*6*	4	4	4
ISr8a-Ra	*8a*	3+	4	4
Acme	*9g*	3+	4	4
W2691SrTt-1	*36*	;1=c	0;	0;
W2691Sr9b	*9d*	3+	4	4
Festiguay	*30*	3+	3++	3++
Renown	*17*	3+	3++	3++
ISr9a-Ra	*9a*	Not tested	4	4
ISr9d-Ra	*9d*	Not tested	4	4
W2691Sr10	*10*	Not tested	4	4
CnsSrTmp	*Tmp*	Not tested	2	2
LCSr24Ag	***24***	**1**	**3**	**3**
Sr31/6*LMPG	***31***	**4**	**1**	**4**
Trident	*38*	3+	3+	3++
McNair 701	*McN*	4	4	4
Barleta Benvenuto	*8b*	4	4	4
Coorong (triticale)	*27*	;	;	;
Kiewiet (triticale)	*Unknown*	;	;	;
Satu (triticale)	*Satu*	Not tested	;	;

[a]Data from the original description of Ug99 (Pretorius et al. 2000). Tester lines different from the current set were Reliance (*Sr5*), Vernal (*Sr9e*), Marquis (*Sr7b*), Yalta (*Sr11*), W2402 (*Sr9b*), Gamka (*Sr24*) and Federation4*/Kavkaz (*Sr31*)

Table 2 Number of alleles and allele sizes generated by 16 SSR primer pairs developed at the University of Sydney. Monomorphic allele sizes are underlined while the polymorphic allele sizes are in normal script.

Locus	N_a [a]	N_p [b]	Allele size (bp)
A1	2	2	405 402
A2	17	15	336 333 331 329 327 326 324 323 321 316 313 310 308 303 297 295 269
A4	3	2	241 238 230
A6	2	0	320 305
A7	10	9	311 306 299 297 294 291 285 279 276 273
A8	3	3	286 283 277
A10	3	3	594 389 358
A11	3	2	364 330 327
A12	5	2	241 235 230 222 216
A13	2	2	464 457
A15	3	2	247 243 231
A17	5	2	243 236 231 221 216
A19	1	0	244
A20	2	2	318 312
A21	5	5	233 229 225 219 134
A23	3	3	322 320 316
Total number of alleles	69	54	
% polymorphic alleles		78	
Average number of alleles per primer set	4.3	3.4	

[a] N_a, number of alleles
[b] N_p, number of polymorphic alleles

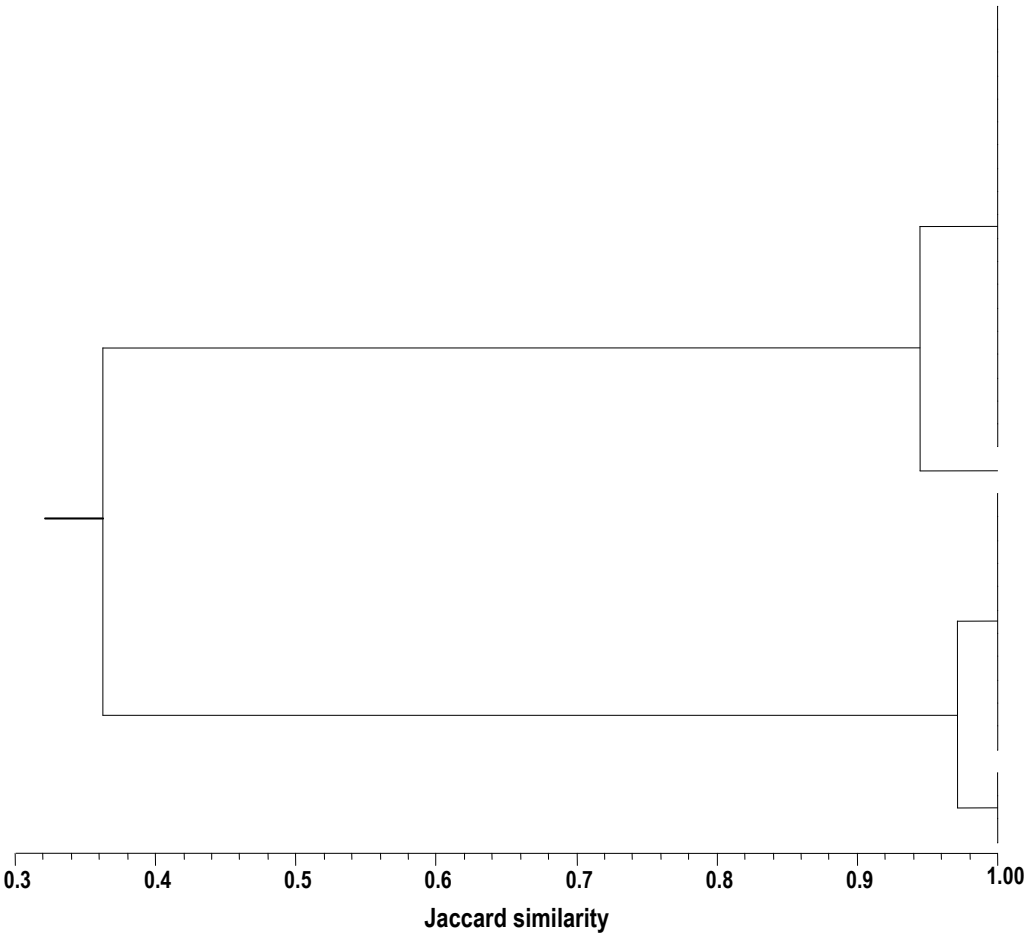

Fig. 1 Dendrogram of 37 *Pgt* isolates based on UPGMA cluster analysis and the Jaccard similarity coefficients calculated from 38 SSR alleles (Visser et al. 2009) generated using SSR primer pairs developed by Szabo (2007)

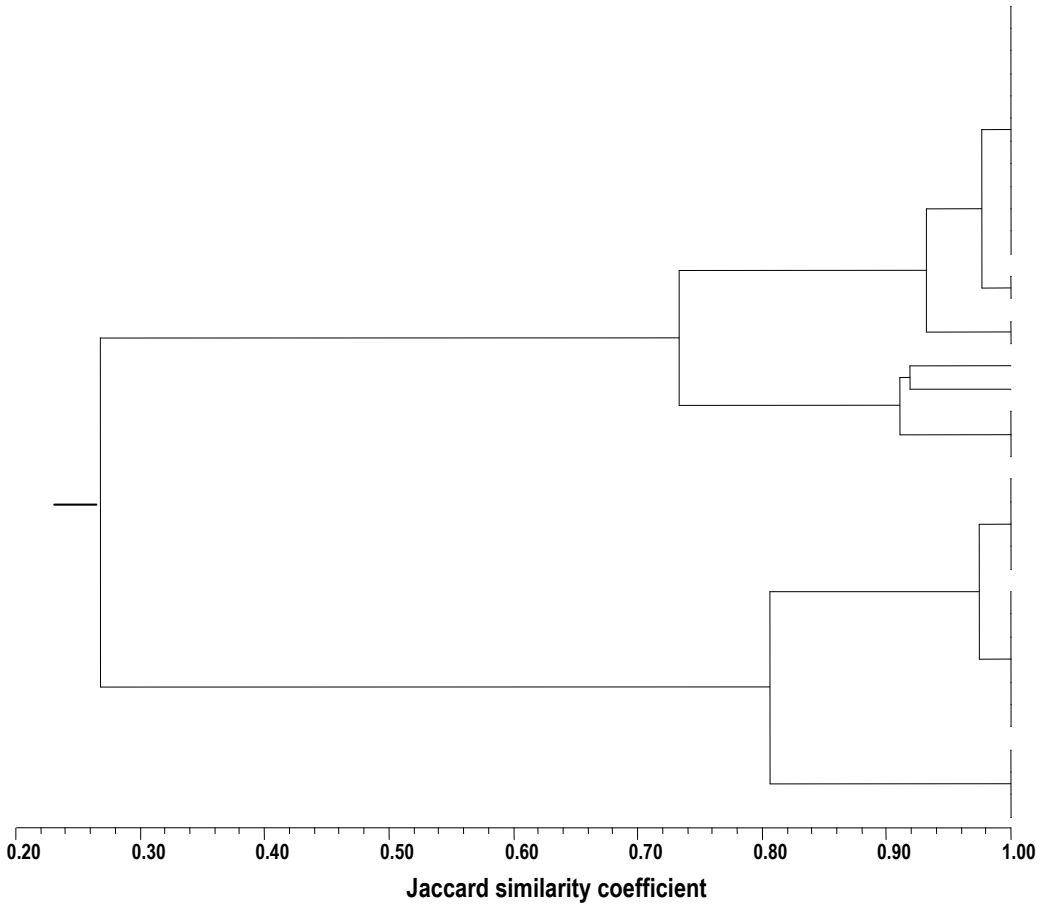

Fig. 2 Dendrogram of 37 *Pgt* isolates based on UPGMA cluster analysis and the Jaccard similarity coefficients calculated from 69 SSR alleles generated using SSR primer pairs developed at the University of Sydney

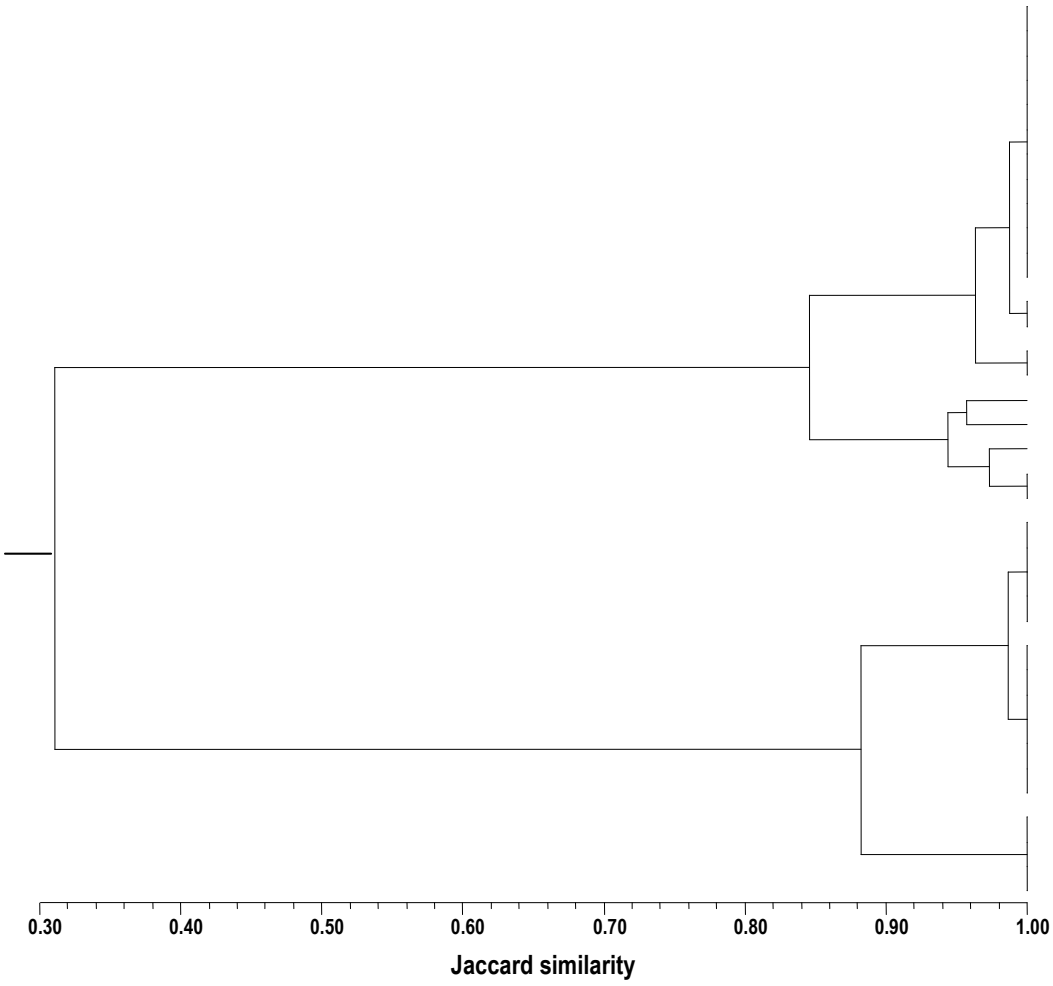

Fig. 3 Dendrogram of 37 *Pgt* isolates based on UPGMA cluster analysis and the Jaccard similarity coefficients calculated from 107 SSR alleles generated using SSR primer pairs developed by Szabo (2007) and the University of Sydney

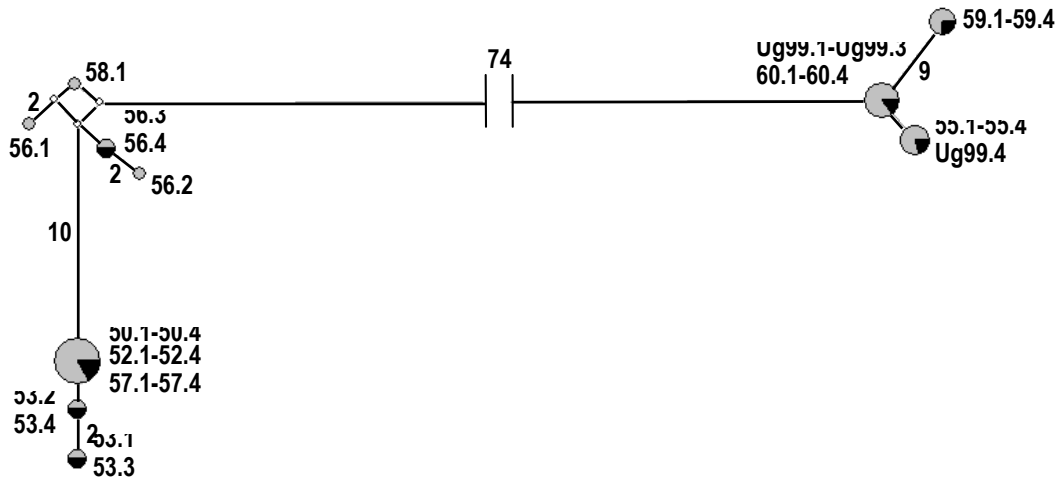

Fig. 4 Minimum-spanning network of 37 tested *Pgt* isolates based on the combined SSR data set generated by primers developed by Szabo (2007) and the University of Sydney. The diameter of each filled node is proportional to the number of rust isolates and the dark coloured sections represent a single isolate with that specific genotype. Open nodes indicate hypothetical intermediate genotypes. The number indicated next to the connecting lines represents the number of mutational events separating each genotype. No number represents a single event.