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Genetic Variability among Presumed Clonal Pathotypes of *Puccinia graminis* f. sp. *tritici* in Australia

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Abstract

Evolution of aggressiveness *Puccinia graminis* f. sp. *tritici* pathotypes with high virulence against important resistance genes constitutes a major threat to the global wheat production, and consequently food security. Changes in pathogenicity and environmental adaptation of these fungi have resulted in serious epidemics and total crop loss. In order to develop appropriate rust control measures, there is a need to understand the current pathogen-environment interactions; mechanisms of pathotype evolution, and pathogenic dynamics against the existing resistance genes. This research examined the genetic variability among 157 Australian *Puccinia graminis* f. sp. *tritici* isolates collected over 39 years. The isolates represented two putative clonal lineages derived from founding pathotypes 326-1,2,3,5,6 (Lineage 3) and 194-1,2,3,5,6 (Lineage 4). Pathogenicity assessments found general consistency with previous determinations, indicating that pathotypes in Lineage 3 and Lineage 4 generally displayed similar avirulence and virulence patterns. Cluster analysis of 111 isolates based on phenotypic data collected into one group comprising multiple subgroups. The level of variation in pathogenicity observed among the subgroups of the 99 derivatives of 326-1,2,3,5,6 and 194-1,2,3,5,6 resulted from the pathotypes with uncommon or additional virulence. Six SSR markers showed 100% genetic similarity among Lineages 2, 3 and 4, and their distinctiveness from Lineage 1, consistent with independent origins. Seven highly polymorphic SSRs or microsatellites revealed 34 genotypes among 142 isolates with low genetic variation F-statistics (F_{ST} = 0.042) indicating high genetic similarity confirming their clonality arising *via* mutation. However, the existing pathotype genetic variations originate from the different forms and rates of mutation which vary per locus.

Keywords: Stem rust; Pathotype; Genetic variability; Microsatellites; Clonal population

Introduction

Although stem (black) rust caused by *Puccinia graminis* f. sp. *tritici* (*Pgt*) is a very important disease of wheat [1], it has not been a major threat to wheat production in Australia since 1974 because of the development and utilisation of cultivars with combinations of effective resistance genes [2-4]. However, the genetic plasticity, constant local pathotype evolution and periodic incursions of exotic pathotypes mean that this pathogen continues to pose a serious threat to wheat production in Australia. This is particularly so because Africa, the presumed origin of three founding pathotypes of *Pgt* that appeared in Australia in 1954 (21-0) and 1969 (326-1,2,3,5,6 and 194-1,2,3,5,6) [5-7], is currently a hotspot for stem rust with the appearance of highly virulent pathotypes viz. TTKTK, TTKTT (Ug99 variants) and the newly identified pathotype TKTTF that overcame *SrTmp* causing up to 100% yield losses in Africa [8,9].

Based on long-term pathogenicity studies of *Pgt* in Australia, pathotypes develop mainly *via* single-step mutation, but examples of somatic hybridisation [10,11] and occasional exotic introductions of new pathotypes exist [5,7]. Three instances of incursions of exotic *Pgt* pathotypes into Australia have been documented since annual pathogenicity surveys began in 1921. In each case, the three new pathotypes are presumed to have acted as the founding ancestors of many pathotypes that are thought to have emerged *via* single-step mutation, leading to clonal lineages within the pathogen population [3,7]: Lineage 1 derived from standard race 126 predominated during 1925-1950; Lineage 2 derived from standard race 21 first detected in 1954 was predominant during the 1970s; Lineages 3 and 4 derived from pathotypes 326-1,2,3,5,6 and 194-1,2,3,5,6, respectively, were first detected on wheat cultivars carrying *Sr6* in South Australia (Clinton) and NSW (Tichborne-Bowie) in 1969 [7]. Studies of spore size, pathogenicity and growth in axenic culture were consistent with the hypothesis that pathotypes 194-1,2,3,5,6 and 326-1,2,3,5,6 were introduced into Australia as wind-borne urediniospores from Africa (Angola and Mozambique). In Australia 194-1,2,3,5,6 and 326-1,2,3,5,6 were detected nine months apart, differing only in pathogenicity for resistance gene *Sr7b*, with 194-1,2,3,5,6 being virulent while 326-1,2,3,5,6 was avirulent [7,12]. The isozyme phenotypic similarity of standard race 21 with standard races 194 and 326, suggested that race 21 also originated from Africa [5-7].

Studies of isozymic variation in *Pgt* in Australia found that standard race 21 together with its postulated derivatives possessed similar isozyme phenotypes [5]. These studies further demonstrated that pathotypes 34-1,2,3,7 and 34-2,3,7,11 were indistinguishable from standard race 21 for the enzyme glutamate oxalate transaminase (GOT), and that they also resembled variants of standard race 126. Burdon *et al.* (1982) found that the isozyme phenotypes of isolates of standard race 326 plus its derivative standard races 98 and 343, and standard race 194 were very similar to race 21. These studies also determined that Australian isolates belonging to standard races 21, 194 and 326 as well as isolates of 194 and 222 collected from Africa had similar isozyme profiles. Although standard races 21 and 194 were introduced into Australia 15 years apart (Watson, 1981), they possessed the same isozyme phenotypes. In terms of pathogenicity, pathotype (pt.) 194-1,2,3,5,6 was avirulent to *Sr9g* and *Sr15*, but was virulent to *Sr11*, *Sr9b*, *Sr17, Sr8a*; while 21-0 was avirulent to these genes. According to Watson (1981), 10 years after the detection of pt. 326-1,2,3,5,6, its derivative 343-1,2,3,5,6 (virulent for *Sr5*) became the most prevalent pathotype (pt.) of *Pgt* because of its virulence on major cultivars Oxley,

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Condor and Egret in Australia during 1970-1990 [7,11,13]. Further mutational derivatives of pt. 343-1,2,3,5,6 have overcome more race-specific resistance genes including *Sr6, Sr7b, Sr8a, Sr8b, Sr9b, Sr9e, Sr9g, Sr11, Sr15, Sr17, Sr21, Sr30, SrAgi* and *Sr36* [2,3,14].

Pathotypes within a clonal lineage have a common genetic background [3,5,7], and their pathogenicity is phenotypically reflected through their avirulence and virulence patterns on various host genotypes carrying known resistance genes. Pathogenic variability studies in Australia have shown that virulence to many known resistance genes exist among derivatives of pts. 326-1,2,3,5,6 and 194-1,2,3,5,6. Genetic diversity within *Pgt* arises by various mechanisms that include crossing over of chromosomes during meiosis or somatic hybridisation [11], sexual recombination [14] and spontaneous process such as strand slippage during DNA replication [15]. Environmental factors also induce genetic variation, leading to DNA changes that include point or large scale mutation in the form of insertions, deletions, duplications, inversions, translocations and substitutions [15,16]. However, it is commonly known that variability of *Pgt* pathotypes is generated mostly through mutations [9,17]. In Australia where *Pgt* does not undergo sexual recombination, variation within endemic pathotypes is thought to arise *via* mutation and somatic hybridisation [3,11,12,18,19].

The genetic diversity of rust pathogen populations has been studied worldwide using various genetic markers including the protein based isozymes [5], SSR or microsatellites [16,20-26], RAPD, AFLP [27], and SNPs in genome sequencing [28,29]. The development and application of molecular markers has facilitated the identification of genetic variability between and within species of rust pathogens [20,25,30,31], and continues to increase the understanding of genetic diversity that exists in rust pathogen populations worldwide [26,32-35]. Microsatellite markers are highly informative based on the number and frequency of alleles, and provide efficient and accurate means of detecting genetic variations in organisms. Their multi-allelic, codominant nature allows individuals to be easily and uniquely genotyped, and their ability to distinguish between closely related individuals has increased their application in clonal pathogen population studies and plant species that have narrow genetic bases [36]. Because of their high polymorphism, abundance, informativeness and specificity, the co-dominant SSRs have proved highly efficient and useful in DNA fingerprinting for detecting population genetic diversity, identifying individuals and phylogenetic analyses [26,37-39].

However, concerns about the high mutation rate of SSRs that may lead to error in DNA during replication, limited knowledge about their evolution and polymorphism, difficulty in automation, primer sequencing, high cost of development and need to improve their statistical analysis are currently being resolved through various technologies [20,38,40]. Attempts to further improve the performance of molecular markers in population genetic studies, pathogen specific SSR markers for *Pgt* [20] and *Puccinia striiformis* f. sp. *tritici* (*Pst*) [30] were designed and successfully applied in Australia and other countries [25]. Berlin *et al.* (2012) detected high levels of genetic variation among formae speciales of *Puccinia graminis* f. sp. *avenae* and *Puccinia graminis* f. sp. *secalis* using SSR markers originally designed for *Pgt*. SSR markers were successfully used to investigate genetic diversity in *Pt* in South Africa [41], *Pgt* variants of Ug99 in South Africa, and Ethiopia [25,35,42], genetic diversity studies of *Pt* [31], population genetics of *Pgt* in Ethiopia [33] and world *Pst* population [32,43,44].

Whereas annual pathogenicity studies have contributed significantly to the understanding of the evolution and migration of *Pgt* pathotypes in Australia, knowledge of the mechanisms underlying genetic variability is limited. As part of a comprehensive study of genetic variability in *Pgt* in Australia based on SSR markers, 148 isolates from Lineages 3 (founding pt. 326-1,2,3,5,6) and 4 (founding pt. 194-1,2,3,5,6) were purified and validated based on their avirulence and virulence on the 35 standard differential genotypes. To test the hypothesis that these pathotypes were derived *via* mutation from a single ancestor, all were genotyped using *Pgt* specific polymorphic SSR markers, and analysed for genetic variability.

Materials and Methods

Pathogenicity validation of *Pgt* pathotypes

One hundred forty-eight isolates (Table S1) considered to be derived from founding pathotypes 326-1,2,3,5,6 and 194-1,2,3,5,6 were selected from the *Pgt* pathotype collection stored under liquid nitrogen at the Plant Breeding Institute (PBI), University of Sydney, NSW, Australia. These isolates were collected in Australia over a period of 39 years (1969-2008), and were characterised for pathogenicity on differential genotypes in annual surveys. Nine pathotypes belonging to standard races 126 (Lineage 1), 21 (Lineage 2), and 34 (Lineage 5, regarded as originating from a somatic hybridization event between isolates from Lineages 1 and 2) were included as controls for comparison.

Isolation, purification and multiplication of pathotype urediniospores

Pathotype (pt.) urediniospores were purified and increased *via* single pustules on a universal susceptible wheat cultivar (Morocco) in the greenhouse. Seedlings were raised in 9 cm diameter pots filled with composted pine bark plus coarse sand mixed in a 4:1 ratio. The mixture was fertilized with water soluble Aquasol (25 g Aquasol® Hortico Pty. Ltd., Revesby, NSW, Australia) dissolved in 9 litres of water. The fertilizer was uniformly applied at the rate of 25-30 g per 100 pots prior to sowing. Pots sown with clumps of 10-15 seeds were transferred to growth rooms where seedlings were grown under 17-22°C and daily irrigation. A week after sowing, a nitrogenous fertilizer (Urea) was applied at a rate of 25 g per 9 litres of water to the seedlings for proper plant vegetative growth and disease development.

Urediniospores or infected dried leaves (DL) were obtained from the liquid nitrogen storage, and heat shocked at 40°C for 4 minutes to break spore dormancy. Initial inoculation of one week old seedlings with each of the 10 isolates was done on 10-15 Morocco seedlings per pots. For each accession, subcultures using four isolated single pustules were established by transferring each to healthy Morocco seedlings in four separate pots. The inoculated seedlings were incubated for 48 hours on a water filled tray inside a naturally lit greenhouse maintained at 20°C. After incubation, the infected seedlings were transferred to temperature and irrigation controlled microclimate rooms maintained at 25-27°C for further disease development. To prevent cross-contamination each pot was covered with a transparent hollow plastic cylinder during the entire period of plant growth and infection. Four to six days after the first inoculations, the second sets of Morocco seedlings were grown in a clean room as described above. At 8 days after inoculating the first Morocco sets, four well separated single pustules were identified and all other pustules removed by cutting, after which the plastic cylinder was replaced. Prior to inoculating the second sets of 40 Morocco pots per cycle, all isolated single pustules were incubated inside tightly sealed plastic bottles placed on a water filled stainless steel tray for 24 hours to induce potentially extraneous spores to germinate and to encourage the development of a new crop of spores. The second Morocco seedlings were inoculated by lightly

rubbing the single sporulating pustule on the upper surfaces of three or four clean leaves per pot. The final pure single pustules selected in the third purification cycles on Morocco were increased on five to eight leaves of the fourth sets of clean Morocco seedlings following the above procedures.

At 7-8 days after inoculating the fourth sets, each flecking Morocco leaf was suspended inside a plastic straw fitted with a sterile Eppendorf tube to collect urediniospores. After full development of pustules, the infected leaves were tapped daily to allow the spores drop into the dry sterile Eppendorf tubes. At 6-7 days later, all tubes together with the straws carrying the infected leaves were removed from the plants, carefully attached together, sealed inside special cellophane bags that allowed moisture exchange, and dried over silica gel inside a dehumidified storage cold room. The spores of each pustule were carefully collected into one well labeled tube and tightly covered. The spores were further dried over the silica gel inside a tightly covered plastic container. Completely dry spores were weighed and then stored under liquid nitrogen until required.

Verification of *Pgt* pathotypes and disease assessment

The 35 standard differential genotypes with known stem rust resistance genes [45,46] were used to verify isolate purity and identity based on avirulence/virulence patterns. Eight to 10 seedlings of each differential genotype were raised in clumps as described in the above section, and inoculated. Inoculated seedlings were incubated for 48 hours, and then transferred to naturally lit microclimate rooms for disease development under controlled temperature (25-27°C) and with automated irrigation. At 10-12 days after inoculation, rust infection types (ITs) (Tables 1 and S2) were assessed using the modified Stakman *et al.* (1962) '0'-'4' scale [45,47] as follows: '0' = no visible uredinia (immune), ';' = hypersensitive flecks (very resistant), '1' = small uredinia with chlorosis or necrosis (resistant), '2' = small to medium sized uredinia with green islands surrounded by necrosis or chlorosis (resistant to moderately resistant), '3' = medium sized uredinia with or without chlorosis (moderately resistant-moderately susceptible), '3+' = large uredinia without chlorosis (susceptible), 'X' = a mesothetic reaction where all ITs were uniformly distributed over one leaf (resistant), 'Y' = variable uredinia sizes with larger ones towards the tip (resistant), 'Z' = variable uredinia sizes with larger ones towards the leaf base (resistant). Symbols 'C' and 'N' denoted more than the typical chlorosis and necrosis, whereas '+' and '-' indicated slight variation from the normal reactions. Intermediate ITs were recorded as 'X' or 'X+'. Low infection types (LITs) ranged from '0;' to '3', while HITs ranged from '33+' to '3+'. When the differentials expressed heterogeneous ITs, the most frequent was recorded first plus a coma then the least frequent recorded next.

Genomic DNA extraction

Genomic DNA was extracted from 30-50 mg of pure urediniospores per isolate treated with ethanol (70%) using cetyltrimethyl ammonium bromide (2xCTAB) [48] method modified by Karaoglu *et al.* (2013) for rust DNA extraction at the Molecular Laboratory, Plant Breeding Institute (PBI), Sydney University. CTAB was prepared using 0.5 M EDTA pH 8, 5 M NaCl, Polyvinylpyrrolidone 40000 MW, 1 M Tris-HCL pH 8 and water. DNA was quantified as concentration of Nucleic acid per 2 µl of DNA stock using the spectrophotometer *via* a computer-based Nanodrop (ND-1000 V3.3.0) programme. DNA qualities and quantities were further verified using 2% agarose gel dissolved in 100 ml of 1x TBE and stained with 2 µl of gel red by the electrophoresis system run at 90 volts for 1.5 hours. The DNA bands were analysed against the standard Lambda DNA, and gel images read on the UV radiation illuminator or Gel Doc. The stock DNA was stored at -20°C freezer for further use.

DNA amplification *via* Polymerase chain reactions based SSR markers

DNA Polymerase chain reactions (PCR) were performed using a 30 µl volume comprising 3 µl of 10x buffer, 3 µl of 2 mM deoxyribonucleotide triphosphates (dNTPs), Roche Diagnostics, Dee Why, NSW, Australia; 1.8 µl of 2 mM $MgCl_2$; 1.8 µl of forward and 1.8 µl of reverse labeled (6 FAM) SSR primer pairs indicated in Table 2; 0.3 µl of *Taq* polymerase (Applied Biosystems, Mulgrave, VIC, Australia); 3 µl of diluted genomic DNA and 15.3 µl of double distilled (dd) H_2O. DNA amplification was performed in the Eppendorf MasterCycler (Germany) set to initial denaturation of 95°C for 5 mins, denaturation at 95°C for 45 seconds, annealing temperatures (Tm) of 56-59°C for 30 seconds (depending on the primer size), and extension at 72°C for 30 seconds. Apart from initial denaturation, the other steps were repeated by the PCR for 35 cycles, followed by final extension at 72°C for 7 mins. The PCR finished the cycle by storing the products at 4°C. The PCR products were centrifuged for 1 min at 1000 rpm using the 5810R Eppendorf centrifuge. The 3 µl of each PCR product mixed with 3.5 µl of loading blue dye were loaded onto 3% agarose gel stained with gel red (Biotium, Jomar, Diagnostics Stepney, SA, Australia). Additionally 2 µl of a standard HyperLadder IV 100 Lanes (Lot No. H4-106F Invitrogen, Australia) were loaded in the first wells against which the separated SSR alleles were analysed by electrophoresis run at 100-110V for 1-4 hours depending on the primer sizes. SSR alleles were visualized under ultra violet light (GelDoc IT imaging System, Model M-26, Bio Imaging Systems, CA 917 86, USA).

Pathotype DNA fingerprinting and data analysis

The isolate DNA (21-25 µl of fluorescent labeled PCR products of more than 50 ng/µl concentration) amplified fragment length polymorphism analysis or genotyping was done by Macrogen Inc., using the GenScan services (AB13730XL capillary analyzer). The Macrogen generated 3730X1 RawData were analysed using the Applied Biosystems Peak Scanner software v1.0. The analysis outputs of specific primer band sizes were read *via* plot view. One to two main strong Alleles with band sizes not less than 100 bp were recorded as the actual primer sizes based on the published ranges [20]. Isolates with the same alleles or band sizes on the 3% agarose were considered to be genetically similar.

The polymorphic information content (PIC) was calculated using microsatellite allele frequencies using the following formula:

$$PIC = 1 - \sum_{i=1}^{1} \left(P_i^2 \right)$$

where P*i* was the frequency for the *i*th allele among a total of *l* alleles [49]. A marker with PIC value greater than 0.5 was highly informative [50]. The sizes of microsatellite marker fragments were scored manually on agarose gels as either "1" or "0", indicating presence or absence of a specific allele, respectively. The "1" or "0" data were converted to 'A' or 'T' in MS Excel as the formatting for Molecular Evolutionary Genetics Analysis (MEGA v6) [51]. In MEGA, the isolate cluster analysis was done through the UPGMA (unweighted pair-group method with arithmetic means) dendrogram. The MEGA-UPGMA dendrogram analysis was verified *via* NTSYS-pc V2 software using '0' or '1' data [52]. The bootstrap method was used to test the phylogeny analysis with 1000 iterations [53]. The pairwise *Nei* genetic distance analysis was done within the Genetic Analysis in Excel (GenALEx). Statistical genetic variation among isolates was determined using the analysis of molecular variance (AMOVA) performed with 9999 permutations [38].

This analysis yielded an estimate of Wright's F-statistics (F_{ST}), which is a measure of population genetic differentiation at significance level of 0.05. F_{ST} values greater than 0.25 indicated high genetic variation [54]. The principal coordinate analysis (PCoA) of a genetic similarity matrix was generated from SSR data to visualise the genetic pattern among isolates. The genetic similarity between pathotypes/isolates was analysed based on genetic distance using GenAlEx [38].

Results

Pathogenicity validation of *Pgt* pathotypes

One hundred twenty three isolates examined in current study belonged to Lineage 3 partly because the most common pathotypes during the 39 year period of collection belonged to standard races 321, 326, 343, 98 and 11. A total of 23 isolates (pathotypes belonging to standard races 194 and 222) were from Lineage 4. A 10 year interval assessment of the pathotypes showed that derivatives (343-1,2,3,5,6 and 98-1,2,3,5,6) of the founding pathotype of Lineage 3 (viz. pt. 326-1,2,3,5,6) were the most common. Pathotype 194-1,2,3,5,6 and its derivatives predominated mostly during the 1960s and 1970s. Previous studies reported that derivatives of pt. 326-1,2,3,5,6 were more frequent than derivatives of pt. 194-1,2,3,5,6 because the former were more adapted to Australia wheat cultivars and growing environments [3,55] especially during the 1980s and 1990s. Derivatives of pt. 326-1,2,3,5,6 within standard race 98 were most common during 2000-2008 (Table S1).

The stem rust differential genotypes used to verify the avirulence and virulence of 113 *Pgt* isolates (representing Lineages 1, 2, 3, 4 and 5) included six genotypes that represent the original international series (Table 1), which carry the resistance genes *Sr5; Sr7b; Sr9g; Sr9e; Sr21 and Sr13, Sr17*; Australian series comprising 13 cultivars with *Sr6; Sr11; Sr9b; Sr36; Sr17; Sr8a; Sr15; Sr30; SrAgi; SrEm; Sr8b; Sr27 and SrSatu,* and 16 additional genotypes with *SrNin; Sr2, Sr5, Sr6, Sr8a, Sr12; Sr5b, Sr7b, Sr9b; Sr26; Sr24; Sr42; Sr5, Sr6, Sr8a, Sr36; Sr5, Sr8a, Sr9b, Sr12; Sr11, Sr17, Sr36; Sr31; Sr9b; Sr22; Sr32; Sr35 and Sr38* [3,45].

Since the first detection of pts 326-1,2,3,5,6 and 194-1,2,3,5,6, in Australia in 1969 [56], the derivative clonal Lineage 3 and Lineage 4, respectively, have progressively generated numerous pathotypes *via* single-step mutation. These pathotypes have gained virulence to many known resistance genes including *Sr5* that was overcome by pt. 343-1,2,3,5,6 the first mutant of pt. 326-1,2,3,5,6 detected in 1973 [7] as shown in Figure 1. The 25 variants of pt. 326-1,2,3,5,6 have evolved

Differential	R-gene	690822	691042	334	540129	730033	750389	791111	780106	840837	82-L-1	820036	790225
		326-1,2,3,5,6	194-1,2,3,5,6	126-5,6,7,11	21-0	34-2,4,5,7	194-1,2,3,5,6,7	222-1,2,3,5,6	343-1,2,(3),5,6,11	343-1,2,3,4,5,6 Cook Rust	98-1,2,3,5,6 +Emmer +9e	11-1,2,3,5,6	21-1,2,3,5,6
Reliance	*Sr5*	0;	0;	3+	0;	3+	;0	33+	33+C	33+C	33+	3+	0;
Marquis	*Sr7b*	2+	3+	3+	33+	3+	3+	33+	2+	3+C	33+	33+	3+
Acme	*Sr9g, X*	2=	2=	X	3+	33+	2=	2=C	1=	2=C	3+	2=	3+
Emmer	*Sr9e*	;1-	;+	0;	;+	;2=	;+	;1-	;1=	;1=C	33+	;	;
Einkorn	*Sr21*	;1	;-	;1	;+	;	;1+	;1	;1	;1	;1	33+	;1-
Line S	*Sr13, Sr17*	2+	2=	2=	;	;2=	2=	2=	2=	2C	2=	1	2=
McMurachy	*Sr6*	3+	3+	;+	X	X	3+	3+	3+	33+	3+	3+	3+
Yalta	*Sr11*	3+	3+	0;	2=	3+	3+	33+C	3+	3+	3+C	33+	33+
W2402	*Sr7b, Sr9b*	33+	3+	;1	2C	2-	33+	33+	3+	3+	33+C	3+	3+
TD	*Sr36*	;1	0;	;	1+	3+	;	;1-	0;	3+	;	;1=(1P)	;1++
Renown	*Sr7b, Sr17*	33+	3+	33+Y	X	3+	33+	3+	3+	33+	3+	3+	33+
Mentana	*Sr8a*	3+	3+	3+	2=	22-	3+	3+	3+	3+	3+	3+	3+
Norka	*Sr15*	X	X	3+	X	3+	3+	2+	X	X	X	X	X
Festiguay	*Sr30*	2	2-	2=	;2=	2-	2=	2=	2=	2+C	2-	2-	;N2-
TAF 2	*SrAgi*	2=	1+	;N	;N	;CN	;CN1=	;N,33+	;+1	2-C	;+N,3+	;+N1	;+N
51209	*SrEm*	22+	X	0;	2-	;22-C	22+	;2+C	22+C	22+	;1+	;1	;2=
Barleta	*Sr8b*	X-	;12	3+	X-N	3+	X-	X	3+	X	2++	X-	X
Coorong	*Sr27*	;1	;	;N	;	;	;1-	;	;1=	;1=	;1-	;	;2=
Satu	*SrSatu*	;	;	;N	;	;	;	;CN	;	;	;	; (mottling)	;N
Sr Nin	*SrNin*	;1-	;	0;	;	;	;	;1-	;	;1=C	;+	;	;1
Gatcher	*Sr2,5,6,8a,12*	0;	0;C	X	;2=	;2-	0;C	2-C	;1=C	0;	3+C	1+ (mottling)	0;(mottling)
Comb X	*Sr5b,7b,9b*	0;	0;	1+	;	2-2C	0;C	2=3+	2+	2+	2+	2+	;2+
Kite	*Sr26*	2-C	1	;N	;	;2=	1+	2=C	1+	1-C	2=C	;N1-	2=
Agent	*Sr24*	2-C	2=	0;	2=	;2=C	2-C	2=	2	2-C	2=C	2-	2=
Norin 40	*SrNorin40*	2-C	2=	3+	2=	;2=C	2-C	2=C	1C	2=C	2=C	1+2-	2-
Cook	*Sr5,6,8a,36*	0;	0;	;	;	;	0;	;	0;,1=(1P)	33+	0;	;N1=	0;
Banks	*Sr5,8a,9b,12*	0;	0;	1+	;2=N	;CN	2++3	2++3	;1++	2C	2++3	2+	2+
Egret	*Sr5,8a,9b,12*	0;	0;	2=	0;	;	0;	3+	3+	3+	3+	3+	0;
Mendos	*Sr11,17,36*	;	0;	0;	0;	33+	;	0;	;	3+	0;	0;	;
Mildress	*Sr31*	2-	1+	2=	2=	;2=	1=	1+C	;	2=	;1=	2=	2=
Mokoan	*Sr9b*	3+	3+	;1	2C	;2=	33+C	3+	3+	3+	3+	3+	3+
W3534	*Sr22*	2+C	2=	22-	2+	2-2+C	22+	2=	2=	2+	2-	2	2+
Sr32	*Sr32*	22+C	22+	22+	2+	22+	2+	22+	22+	22+C	2+	2+	2+
Sr35	*Sr35*	0;	0;	0;	;	;	0;	0; (1 mottling)	0;	0;	0;	0;(mottling)	0;
Trident	*Sr38*	X	X	0;	X-	X	X	X	X-	X	X	X	X

Table 1: Infection types (ITs) of selected *Pgt* isolates on stem rust standard differential genotypes. P: Number of plants assessed. The first row lists accessions of the different pathotypes indicated in the second row.

via three standard races (326, 343 and 98), while the nine derivatives of pt. 194-1,2,3,5,6 evolved through standard races 194 and 222 (Figure 2). Previous studies have shown that since 1973, mutational derivatives of 343-1,2,3,5,6 [7] have overcome many single stem rust resistance genes including: *Sr6, Sr7b, Sr8b, Sr9e, Sr9g, Sr15, Sr21, SrAgi, Sr36* and *Sr30* [2,3,6,55,57]. Virulence for resistance genes *Sr8b, Sr9e, Sr30, Sr36, SrAgi* and *SrEm* developed in pathotypes 343-1,2,(3),5,6,11; 98-1,2,3,5,6 +*Sr9e* and 343-1,2,3,4,5,6, In this study, the differential genotype Barleta Benvenuto carrying *Sr8b* reacted with LITs to most pathotypes apart from 343-1,2,(3),(5),6,11 and 34-2,4,5,7 that caused HITs. *Sr9e* was highly effective against most isolates in Lineages 3 and 4, but virulence to this gene was found in accessions 82-L-1 and 77-L-3 of pts. 98-1,2,3,5,6 +Emmer +9e and 321-1,2,3,5,6, respectively. Although Einkorn differential genotype (*Sr21*) was highly resistant to a majority of the pathotypes derived from standard races 326, 343, 222, 194, 98, 321 and 11 (Table S2), virulence for *Sr21* was found in accessions 810843; 820036; 780128 of pts. 222-1,2,3,5,6,7 +Einkorn; 11-1,2,3,5,6 and 343-1,2,(3),(5),6 +Einkorn, respectively.

Festiguay (*Sr30*), TAF 2 (*SrAgi*) and 51209 (*SrEm*) were resistant to most pathotypes apart from 194-1,2,3,5,6,10 that was virulent to *SrEm*. In addition to the virulence to *Sr30* detected in pt. 343-1,2,3,5,6,8,9 (Park & Wellings, 1992), further virulence to *Sr30* and *SrAgi* was found in pathotypes 194-1,2,3,7,8,9 and 194-2,3,7,8,9 (Table S2). Assessment of 99 isolates out of the 148 derivatives of pts. 326-1,2,3,5,6 and 194-1,2,3,5,6 determined that all were avirulent to *Sr13; Sr22; Sr24; Sr26; Sr27; Sr31; Sr35; Sr38; Sr42; SrSatu* and *SrNin*. Apart from McMurachy (*Sr6*), Renown (*Sr7b, Sr17b*) and Mentana (*Sr8a*) that reacted with LITs to either 194-1,2,3,7,8,9 and/or 194-2,3,7,8,9; all derivative pathotypes of standard races 326 and 194 were virulent to *Sr6; Sr7b; Sr9b; Sr11* and *Sr17*. These results concurred with previous pathogenicity studies [3,13,55,57]. However, the differential genotype Marquis that carries *Sr7b* reacted with HITs to virulent derivatives of 194-1,2,3,5,6 but LITs ('2') were observed on plants inoculated with pts. 326-1,2,3,5,6; 343-1,2,(3),(5),6 +Einkorn; 343-1,2,(3),(5),6,11; 343-1,2,(3),(5),6; 343-1,2,3,4,5,6,10 Yellow; 343-1,2,3,5,6; 343-1,2,3,5,6 +*Sr13* higher; 343-1,2,3,5,6,8,9; 343-1,2,3,5,6,9; 343-1,2,3,5,6,9 +*Sr7B*. low; 98-1,2,(3),(5),6; 343-1,2,3,5,6 +*Sr7b*; 98-1,2,3,5,6 +*Sr7b*; 343-1,2,3,5,6,7 +*Sr7b*; 98-1,2,3,5,6,7 +*Sr7b* and 343-1,2,(3),5,6,11 that included mostly derivatives of standard races 343 and 98. Moreover, W2402,

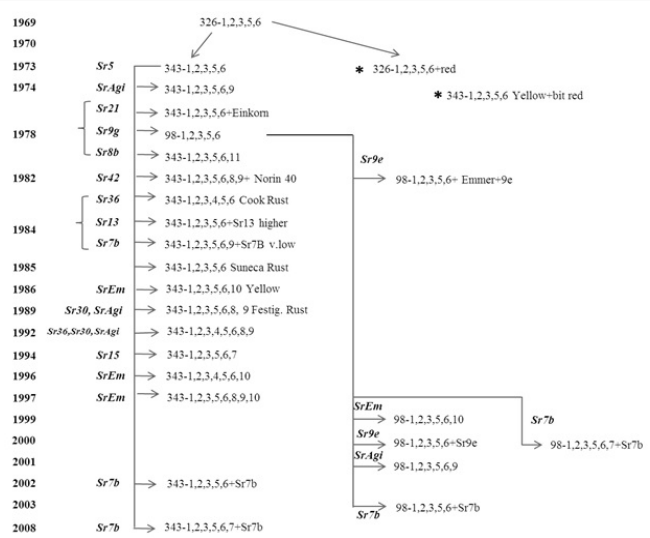


Figure 1: Evolution flow diagram of a set of pathotypes in clonal Lineage 3 from the founding pt. 326-1,2,3,5,6 since 1969 ˚Spontaneous greenhouse mutants with altered urediniospore colour.

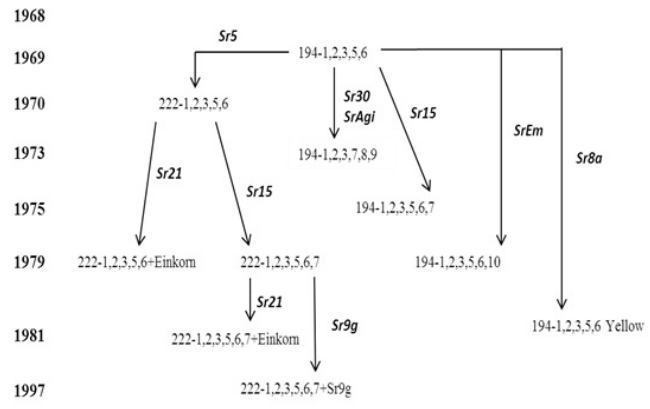
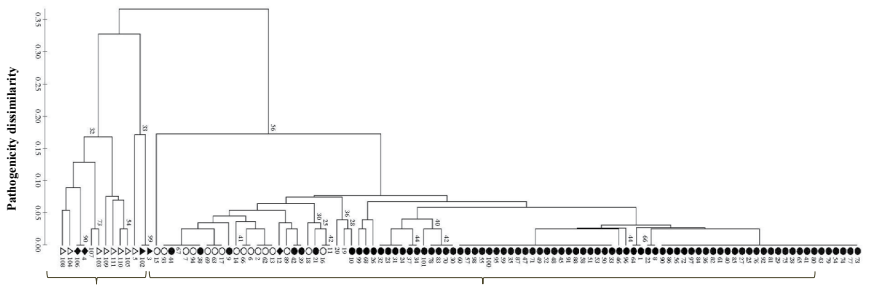


Figure 2: Evolution flow diagram of 10 pathotypes in clonal Lineage 4 from the founding pt. 194-1,2,3,5,6 since 1969.



1	326-1,2,3,5,6	16	222-1,2,3,5,6 + Eink	31	343-1,2,3,5,6,9	46	98-1,2,3,5,6,7 + Sr 7b	61	343-1,2,3,5,6	76	343-1,2,3,5,6	91	98-1,2,3,5,6	106	21-0
2	194-1,2,3,5,6	17	222-1,2,3,5,6,7	32	343-1,2,3,5,6,9 + Sr 7B v.low	47	98-1,2,3,5,6	62	194-1,2,3,5,6	77	343-1,2,3,5,6	92	343-1,2,3,5,6	107	11-2,12,13
3	126-5,6,7,11	18	222-1,2,3,5,6,7 + Eink	33	98-1,2,(3),(5),6	48	98-1,2,3,5,6	63	222-1,2,3,5,6	78	343-1,2,3,4,5,8,9	93	222-1,2,3,5,6,7	108	34-2
4	21-0	19	321-1,2,3,5,6	34	98-1,2,3,5,6,9	49	98-1,2,3,5,6	64	326-1,2,3,5,6 + red	P9	343-1,2,3,5,6	94	222-1,2,3,5,6	109	34-1,2,7,10+Sr38
5	34-2,3,5,7 ex. A. scab. A.	20	321-1,2,3,5,6	35	98-1,2,3,5,6	50	98-1,2,3,5,6	65	343-1,2,3,5,6	80	343-1,2,3,5,6	95	98-1,2,3,5,6	110	34-1,2,7+Sr38+Sr21
6	194-1,2,3,5,6,7	21	343-1,2,(3),(5),6 + Einkorn	36	343-1,2,3,5,6	51	98-1,2,3,5,6	66	194-1,2,3,5,6,7	81	343-1,2,3,5,6	96	98-1,2,3,5,6,10	111	34-1,2,7+Sr38+SrN40
7	222-1,2,3,5,6 + Eink	22	343-1,2,(3),(5),6,11	37	98-1,2,3,5,6,9	52	98-1,2,3,5,6	67	11-1,2,3,5,6	82	343-1,2,3,5,6	97	343-1,2,3,5,6		
8	343-1,2,(3),5,6,11	23	343-1,2,(3),(5),6,9	38	343-1,2,3,5,6 + Sr 7b	53	98-1,2,3,5,6	68	343-1,2,3,4,5,6	83	343-1,2,3,5,6,8,9	98	98-1,2,3,5,6		
9	343-1,2,3,4,5,6 Cook Rust	24	343-1,2,(3),(5),6,9	39	98-1,2,3,5,6+Sr 7b	54	343-1,2,3,5,6	69	222-1,2,3,5,6,7	84	343-1,2,3,5,6	99	343-1,2,3,4,5,6 Yellow		
10	98-1,2,3,5,6 + Emmer + 9e	25	343-1,2,(3),5,6	40	343-1,2,3,5,6	55	98-1,2,3,5,6	70	343-1,2,3,5,6,(8),9	85	343-1,2,3,5,6	100	98-1,2,3,5,6		
11	11-1,2,3,5,6	26	343-1,2,3,4,5,6,10 Yellow	41	343-1,2,3,5,6	56	343-1,2,3,5,6	71	98-1,2,3,5,6	86	343-1,2,3,5,6	101	343-1,2,3,5,6,(8),9,10		
12	21-1,2,3,5,6,7	27	343-1,2,3,5,6	42	98-1,2,3,5,6 +Sr7b	57	98-1,2,3,5,6	72	343-1,2,3,5,6	87	98-1,2,3,5,6	102	126-5,6,7,11		
13	194-1,2,3,5,6 Yellow	28	343-1,2,3,5,6 Suneca Rust	43	343-1,2,3,5,6	58	98-1,2,3,5,6	73	343-1,2,3,5,6	88	98-1,2,3,5,6	103	34-2,12,13		
14	194-1,2,3,5,6,10	29	343-1,2,3,5,6 + Sr13 higher	44	343-1,2,3,5,6,7 +Sr 7b	59	98-1,2,3,5,6	74	343-1,2,3,5,6	89	222-1,2,3,5,6,7+Sr9g	104	34-2,7,10		
15	194-1,2,3,7,8,9	30	343-1,2,3,5,6,8,9 Festig. Rust	45	98-1,2,3,5,6	60	98-1,2,3,5,6	75	343-1,2,3,5,6	90	343-1,2,3,5,6	105	34-1,2,7+Sr38+Sr21		

Figure 3: A dendrogram of the avirulence and virulence patterns of 111 *Pgt* isolates on stem rust differential genotype.

Mokoan and Renown that carry *Sr7b* and/or *Sr9b* were susceptible to these particular pathotypes. This result indicated that probably Marquis carries other unknown gene(s) interacting with *Sr7b* to confer enhanced resistance to the avirulent pathotypes. All accessions of race 194 were avirulent to *Sr5* but all derivatives of standard race 222 were virulent to *Sr5*. Acme carrying *Sr9g* was generally resistant to most accessions of standard races 326, 343, 222 and 194 but was susceptible to all derivatives of standard race 98 as earlier reported (Park, 1996). Although Norka (*Sr15*) reacted with varying ITs to all pathotypes, it responded with a range of mesothetic ('X') to HITs to most pathotypes.

Cluster analysis of avirulence and virulence

Many pathotypes within the same clonal lineage displayed similar avirulence/virulence patterns on common hosts thus clustering together (Figure 3). Cluster analysis grouped 111 isolates into two main groups of which the first comprised the control pathotypes within standard races 21, 34 and 126, while the second comprised all derivatives of standard races 194 and 326 plus pathotypes within standard races 21, 11 and 321. Group one was further sub-divided into two major subgroups: (i) pathotype derivatives of standard races 34 and 21, (ii) pathotype derivatives of standard race 126 and 34. Group two was comprised of (iii) pathotype 194-1,2,3,7,8,9 a derivative of standard race 194, and (iv) pathotype derivatives of standard races 326,194, 321 and 11.

The 93 derivatives of founding pts. 326-1,2,3,5,6 and 194-1,2,3,5,6 formed one main group comprising of multiple subgroups, each characterised with similarity in avirulence and virulent patterns (Figure 3). Interestingly, pt. 194-1,2,3,7,8,9 detected in 1973 that evolved from founding pt. 194-1,2,3,5,6 by gaining virulence to *Sr30* and *SrAgi* separated from the main group at 83% similarity, forming a genotype of its own. The second sub-cluster including pts. 326-1,2,3,5,6 and 194-1,2,3,5,6 derivatives further divided in two groups that subdivided in groups of varying sizes. Apart from few cases where isolates of different pathotypes grouped together, the final clustering was generally based on genotype implying that isolates of the same pathotype formed a cluster reflecting uniformity in avirulence and virulence patterns on common hosts. For example race 326 isolates within the following genotypes 326-1,2,3,5,6; 343-1,2,3,5,6; 343-1,2,3,5,6,9; 343-1,2,3,5,6,8,9; 343-1,2,3,5,6,11; 98-1,2,3,5,6 and 98-1,2,3,5,6,9; 98-1,2,3,5,6 +*Sr7b* grouped together. Similarly, isolates that constituted Lineage 4 sub-sub-cluster grouped according to their genotypes like 194-1,2,3,5,6; 194-1,2,3,5,6,7 and 222-1,2,3,5,6,7. This study determined that a loss or gain in virulence of a pathotype simply *via* single-step mutation of e.g., pt. 194-1,2,3,5,6 to 194-1,2,3,7,8,9 resulted in significant genetic variation of a pathotype becoming virulence to stem rust resistance genes *Sr30* and *SrAgi* three years after detecting the founding pathotypes (Table S2). Apart from mutating to pt. 222-1,2,3,5,6 detected in 1970; the founding pathotype 194-1,2,3,5,6 subsequently mutated to four pathotypes (194-1,2,3,7,8,9; 194-1,2,3,5,6,7; 194-1,2,3,5,6,10 and 194-1,2,3,5,6 Yellow) during a period of eight years (1973-1981) as shown in Figure 2. Around the same period, Pt. 222-1,2,3,5,6 also evolved into two pathotypes (222-1,2,3,5,6+Einkorn and 222-1,2,3,5,6,7) detected during 1979. Pt. 222-1,2,3,5,6,7 further evolved in two pathotypes (222-1,2,3,5,6,7+Einkorn and 222-1,2,3,5,6,7 +*Sr9g*) detected during 1981 and 1997, respectively. Similarly, the evolution of pt. 326-1,2,3,5,6 to 343-1,2,3,5,6 then to 343-1,2,3,5,6,9 detected in 1973 and 1974 (Figure 1) took four to five years resulting in new genotypes every time a mutation occurred. The variations in the avirulence and virulence of pathotypes within the same lineage was an implication of variation in their genetic composition, which was examined in greater detail using *Pgt*-specific SSR markers.

Genetic diversity among *Pgt* isolates and analysis of polymorphic SSR markers

Thirteen polymorphic pairs of *Pgt*-specific SSR markers amplified all genomic DNA under PCR at the annealing temperature (Tm) range of 56-59°C, indicating that they were suitable for genotyping the isolates (Table 2). The primers amplified a total of 37 alleles among 157 isolates with an overall average of 2.8 alleles per primer. Individually, primers *PgSUN47*, *PgSUN29* (TCTTT)6, *PgSUN29* (TTTTC)6, *PgSUN36* and *PgSUN55* amplified two alleles, while *PgSUN7*, *PgSUN51* and *PgSUN53* amplified three alleles (Table 2). Primers *PgSUN46*, *PgSUN43*, *PgSUN42* and *PgSUN44* amplified three alleles while the most polymorphic primer (*PgSUN40*) amplified six different alleles in the 148 isolates (numbers 10-157).

Genetic variability among microsatellite loci

The PIC and genetic diversity (heterozygosity) of the 13 microsatellite loci were calculated based on the number of alleles per locus and the corresponding relative frequencies. The overall, average PIC and H values for the 13 markers were 0.372 (ranging from 0.169 to 0.586) and 0.443 (ranging from 0.186 to 0.660), respectively, which indicated that these markers were suitable for determining the genetic variability among *Pgt* isolates. Based on the Botstein's (1980) principle [50], markers *PgSUN7* and *PgSUN51* were considered highly informative because their PIC values were >0.5, but markers *PgSUN53*, *PgSUN29* (TCTTT)6, *PgSUN29* (TTTTC)6, *PgSUN36*, *PgSUN46*, *PgSUN40*, *PgSUN42* and *PgSUN44* were moderately informative because their PIC ranged between 0.25 and 0.5. Primers *PgSUN55* and *PgSUN43* with PIC of 0.169 and 0.238, respectively, were slightly informative but useful because their PIC values were above zero (Table 2).

The statistical analysis of genetic variability based on the observed heterozygosity (*Ho*) showed that loci *PgSUN7*, *PgSUN29* (TCTTT)6, *PgSUN29* (TTTTC)6, *PgSUN51* and *PgSUN53* were heterozygous in all isolates, while locus *PgSUN47* was not (Table 3). The number of alleles per locus ranged from 1.0 (*PgSUN47*) to 2.0 (*PgSUN7*) with the mean of 1.6 (Table 3). The level of *Ho* per locus ranged from '0' for locus *PgSUN47* to '1' for locus *PgSUN7*, and the average was 0.600. The expected heterozygosity (*He*) ranged from '0' (*PgSUN47*) to 0.5 (*PgSUN7*) with an average of 0.3. Based on the six SSR markers of low polymorphism, the PIC ranged from 0.37 (*PgSUN47*) to 0.56 (*PgSUN7*) with an average of 0.45. Apart from loci *PgSUN47* and *PgSUN53*, which had F_{ST} (proportion of the total genetic variance within the subgroups relative to the total genetic variance) higher than the expected upper limit (0.5 in clonal populations), the F_{ST} of the remaining loci ranged from 0.17 (*PgSUN29*) to 0.39 (*PgSUN51*), implying moderate to high levels of genetic variation per locus across the 157 isolates. Based on the seven SSR markers with higher polymorphism, the number of alleles per locus ranged from 1.5 (*PgSUN55*) to 4.5 (*PgSUN40*) with a mean of 2.6. Apart from *PgSUN40* (*Ho* = 0.4) all other loci had *Ho* values of '0' (Table 3) indicating absence of heterozygosity. The *He* ranged from 0.008 (*PgSUN55*) to 0.412 (*PgSUN40*) with a mean of 0.319. The PIC values of these seven markers were lower ranging from 0.169 (*PgSUN55*) to 0.402 (*PgSUN40*) with a mean of 0.303. However, the F_{ST} values ranged from 0.001 (*PgSUN36*) to 0.057 (*PgSUN44*) with a mean of 0.027, indicating higher genetic similarity among the 148 isolates.

Genetic diversity among *Pgt* isolates within the putative clonal Lineages 3 and 4

Analysis of genetic variation across the lineages using loci *PgSUN7*, *PgSUN47*, *PgSUN29* (TCTTT)6, *PgSUN29* (TTTTC)6, *PgSUN51* and *PgSUN53* showed that the percentage of polymorphic

Primer No.	Locus	Repeat motifs	Primer sequences (5´-3´)	Annealing Tm (°C)	Allele size range (bp)	*Na*	PIC	*H*
F6-8	*PgSUN7*	$(ACA)_{18}(CAA)_{11}$	F: CTCAAGCACCCTCAACATCC R: CGTCGTCCCTCCATAGTCTT	59	204-233	3	0.563	0.640
F9-18	*PgSUN47*	$(CTCTT)_{14}$	F: ACCGCTATCAACCCTAATCA R: GGAGGAAAAGCCGAGAATC	59	226-289	2	0.365	0.480
F10-28	*PgSUN29*	$(TCTTT)_{6}$	F: CACGCTTTTCTCTGCTGATT R: CAGGCTAACTCTCCAGTGTG	59	198-237	2	0.365	0.480
F10-30	*PgSUN29*	$(TTTTC)_{6}$	F:CACGCTTTTCTCTGCTGATT R: CAGGCTAACTCTCCAGTGTG	59	198-237	2	0.365	0.480
F9-41	*PgSUN51*	$(GAGAAA)_{7}$	F: CCCAATCCCATCTCGTCTCA R: CTGCGCGTCTATCGATTCGT	59	137-157	3	0.586	0.660
F9-45	*PgSUN53*	$(TTTCAT)_{8}$	F: GCTGGGAATGGTTTATCTAA R: CACCAGAAGGGACTGATTAT	59	233-525	3	0.466	0.540
F2-21	*PgSUN46*	$(TGTTT)_{13}$	F:GGGGTGACTGAAAGATAAGA R:CGCTGGTAAAGGTGAAAAAC	58	275-296	3	0.333	0.406
F4-23	*PgSUN43*	$(CAAAA)_{12}$	F: GTTTGCCTTATTCGTCTCA R: GGGGTATAAAGTGTATGAAG	58	426-445	3	0.238	0.265
F6-31	*PgSUN36*	$(TTTGT)_{9}$	F: CGGGGTGACTGAAGATAGAG R: CCCTTTCTACCCTCCTTACTT	59	299-315	2	0.325	0.408
F6-33	*PgSUN40*	$(GAAAA)_{10}$	F: GTGGTGGTGGATTAGACATT R: CCTCCATCCTTCATACCTTA	57	163-230	6	0.402	0.421
F6-36	*PgSUN42*	$(TTTGG)_{12}$	F: GGGGGGTGACTGAAAAATA R: CCCTTTTCTACCTCCTTACTT	56	303-324	3	0.319	0.393
F10-40	*PgSUN44*	$(AAACA)_{13}$	F: ATCCGCTGGTAAAGGTGAAA R: GGGGGGTGACTGAAAGATAA	59	277-296	3	0.335	0.405
F9-44	*PgSUN55*	$(TGAAGA)_{8}$	F: AGAGGGGACATCAAGACTAT R: GGCGGGTTGGGGGGTTATTC	59	330-372	2	0.169	0.186

Table 2: Profile of 13 polymorphic simple sequence repeat (SSR) or microsatellite markers used to genotype *Puccinia graminis* f. sp. *tritici* isolates. Polymorphic information content (PIC); Heterozygosity/gene diversity (H) of SSR markers based on frequencies of 37 alleles. Repeat motifs of SSR markers. Tm °C: Annealing temperature; Alleles sizes were measured in bp (base pairs); *Na*: Number of alleles per locus; F: Forward primer sequences; R: Reverse primer sequences. Six markers of low polymorphism (*PgSUN7, PgSUN47, PgSUN29* (TCTTT)6, *PgSUN29* (TTTTC)6, *PgSUN51* and *PgSUN53*) amplified 15 alleles in 157 isolates. Seven highly polymorphic markers (*PgSUN46, PgSUN43, PgSUN36, PgSUN40, PgSUN42, PgSUN44* and *PgSUN55*) amplified 22 alleles in 148 isolates.

Number of pathotypes 157	Primer No.	Locus	*Na*	*Ho*	*He*	F_{ST}	*I*
	F6-8	PgSUN7	2.000	1.000	0.500	0.219	0.693
	F9-18	*PgSUN47*	1.000	0.000	0.000	1.000	0.000
	F10-28	*PgSUN29*	1.800	0.800	0.400	0.167	0.555
	F10-30	*PgSUN29*	1.800	0.800	0.400	0.167	0.555
	F9-41	*PgSUN51*	1.800	0.800	0.400	0.394	0.555
	F9-45	*PgSUN53*	1.200	0.200	0.100	0.815	0.139
SE			0.091	0.091	0.045	0.147	0.063
148	F2-21	*PgSUN46*	2.500	0.000	0.386	0.050	0.603
	F4-23	*PgSUN43*	2.500	0.000	0.256	0.030	0.458
	F6-31	*PgSUN36*	2.000	0.000	0.407	0.001	0.597
	F6-33	*PgSUN40*	4.500	0.004	0.412	0.019	0.831
	F6-36	*PgSUN42*	2.500	0.000	0.383	0.025	0.584
	F10-40	*PgSUN44*	2.500	0.000	0.381	0.057	0.603
	F9-44	*PgSUN55*	1.500	0.000	0.008	0.004	0.024
SE			0.272	0.001	0.042	0.008	0.024

Table 3: Genetic diversity among isolates at 13 SSR loci based on Heterozygosity, F-statistics and polymorphism by locus for co-dominant data. *Na*: Alleles per locus; *Ho*: Observed heterozygosity; *He*: Expected heterozygosity; F_{ST}: Wright' F-Statistics; *I*: Information index.

loci was low in Lineage 1 (33.0%), and high (66.7%) in the other four lineages (Table 4). Similarly, the Shannon diversity indices (I) and heterozygosity were higher among the four lineages, indicating diversity within each lineage, but lower in Lineage 1 isolates. However, based on the loci *PgSUN46*, *PgSUN43*, *PgSUN36*, *PgSUN40*, *PgSUN42*, *PgSUN44* and *PgSUN55*, higher polymorphism were observed in Lineages 3 and 4 whereas observed heterozygosity was low (Table 4). Based on the standard classification of the pairwise F-statistics, which range from 0 (genetically related) to ≥ 5 (population not sharing any genetic diversity), the significant (P = 0.001) pairwise F_{ST} values between the lineages ranged from 0 to 0.684, but for Lineage 3 and Lineage 4 the F_{ST} value were low (Table 5). However, higher levels of genetic variation were observed in pairs of lineages, *viz*. 1:2; 1:3; 1:4; 1:5 and 2:5. Similarly, comparisons of the genetic distances between Lineages 1:2; 1:3; 1:4; 1:5; 2:5; 3:5 and 4:5 indicated that all were high. The *Nei* genetic distances (*Nei D*) between the lineages ranged from 0 (Lineages 3:4) to 1.275 (Lineages 1:2), but the lineages with the lowest genetic differences or *Nei* distance were 3:4; 2:4 and 2:3, which was further supported by *Nei* genetic identity (*Nei I*) showing that Lineage 3 and Lineage 4 had the highest probability of having similar alleles at each locus. These results were further confirmed in the analysis involving loci *PgSUN46*, *PgSUN43*, *PgSUN36*, *PgSUN40*, *PgSUN42*, *PgSUN44* and *PgSUN55* (Table 5), where the F_{ST} (0.042) and *Nei D* (0.028) values were very low but not in significant levels of genetic variations between Lineage 3 and Lineage 4.

Two UPGMA-based phylogenetic trees/dendrograms were constructed using MEGA software for clustering and similarity analysis across the *Pgt* isolates using the SSR allele data based on the two categories of markers. To check the validity of the MEGA analysis, the same data were used to develop similar dendrograms using the NTSYS software, but due to the absence the bootstrap analysis in NTSYS the MEGA results were retained. The first phylogenetic tree (Figure 4) was based on the six markers that were monomorphic among isolates of Lineage 3 and Lineage 4, and included all 157 isolates to assess the relationship between these two Lineages and representative control isolates from Lineage 1 (isolates 1, 2 and 3), Lineage 2 (isolates 7, 8 and 9), and Lineage 5 (isolates 4, 5 and 6) (Table S1). The second phylogenetic tree (Figure 5) was based on the seven markers that were polymorphic among the Lineage 3 and 4 isolates, and included only the 142 isolates from these two lineages.

The first dendrogram (Figure 4) categorized the isolates into two major groups that separated Lineages 1 and 5 from Lineages 2, 3, and 4 at 35% similarity. Group 1 comprised three isolates of pt. 126-5,6,7,11; one isolate of pt. 34-2,4,5,6,7,11 and two isolates of pt. 34-2,3,5,7 with 52% genetic similarity. Pathotypes 34-2,4,5,6,7,11 and 34-2,3,5,7 formed the first cluster within group 1, which had 100% similarity at 100% bootstrap, which was as expected because all three isolates were established from single pustules of standard cultures of these pathotypes. This clearly illustrated the reproducibility of the SSR screening. The second cluster within group 1 comprised the three isolates of pathotype 126-5,6,7,11 with 100% similarity, which again was as expected given that all three were established from a single culture of this pathotype. The second major group comprised 151 isolates from Lineages 2, 3 and 4, which separated into two clusters at 98% similarity of which the first had only one isolate (pt. 194-1,2,3,5,6,7), and the rest formed the second sub cluster with 100% similarity. Overall, this analysis determined that derivatives of standard race 126 (Lineage 1) and standard race 34 (Lineage 5) were 52% genetically similar, but shared 35% genetic similarity with derivatives of standard races 326 (Lineage 3) and 194 (Lineage 4) that were 100% genetically similar. These results further agree with the previous isozyme-based studies that reported that pathotypes within standard races 194 and 326 not only share a common genetic background, but are also genetically related to pathotypes within standard race 21 that is also believed to have migrated from Africa to Australia. The 100% similarity among 151 isolates representing 43 genotypes and numerous accessions is evidence that they evolved clonally from the genetically related *Pgt* races (21, 194 and 326) over the 39 years forming Lineages 2, 3 and 4, each with a strong common genetic background.

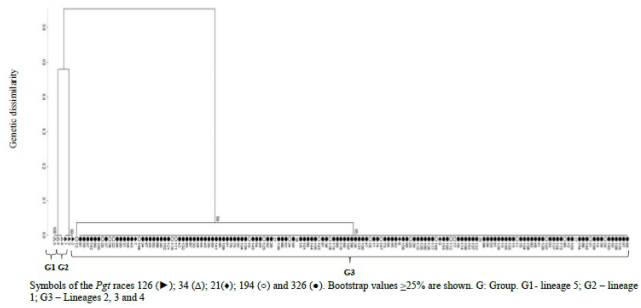
Further UPGMA analysis of the 142 isolates from Lineage 3 and Lineage 4 based on the seven highly polymorphic markers (Table 5) produced a complex dendrogram with two main groups, with representatives of Lineages 3 and 4 in both. Group 1 included isolates regarded as being representatives of the founders of Lineage 3 (isolate number 15, pt. 326-1,2,3,5,6) and Lineage 4 (isolate number 10, pt. 194-1,2,3,5,6) (Table S1). In total, the seven primers identified 34 SSR genotypes among the 142 isolates of lineages 3 and 4, of which 13 were represented by single isolates only. The founding isolates of pathotypes 194-1,2,3,5,6 and 326-1,2,3,5,6 differed in their SSR genotype at one locus (*PgSUN43*: pt. 194-1,2,3,5,6 allele size 436 bp; pt. 326-1,2,3,5,6 allele size 441 bp), which supports the original suggestion by Luig (1977) that although both were detected at about the same time, they represented different *Pgt* genotypes. The overall variation in SSR genotype seen among the 142 isolates presumed to be derived from these two founding pathotypes further implies significant evolution of variation at the SSR marker loci over the 39 year period that these isolates were collected.

Markers	Population	N	%P	*Na*	*I*	*Ho*	*He*
Six	Lineage 1	3	33.0	1.333	0.231	0.333	0.167
	Lineage 2	3	66.7	1.667	0.462	0.667	0.333
	Lineage 3	121	66.7	1.667	0.462	0.667	0.333
	Lineage 4	27	66.7	1.667	0.462	0.667	0.333
	Lineage 5	3	66.7	1.667	0.462	0.667	0.333
	SE	9	6.67	0.091	0.063	0.091	0.045
Seven	Lineage 3	121	100.0	2.857	0.493	0.001	0.277
	Lineage 4	27	85.7	2.286	0.564	0.000	0.361
	SE	14	7.1	0.272	0.068	0.001	0.042

Table 4: Genetic variation within lineages based on heterozygosity, F-statistics and polymorphism by lineage for co-dominant data using 13 markers. Six markers: Low polymorphism (*PgSUN*, *PgSUN47*, *PgSUN29*, *PgSUN29*, *PgSUN51* and *PgSUN53N*). Seven markers: High polymorphism (*PgSUN46, PgSUN43, PgSUN36, PgSUN40, PgSUN42, PgSUN44* and *PgSUN55*). N: Number of isolates; P: Percentage polymorphism; *Na*: Number of different alleles; *I*: Shannon's information index; *Ho*: Observed heterozygosity; *He*: Expected heterozygosity.

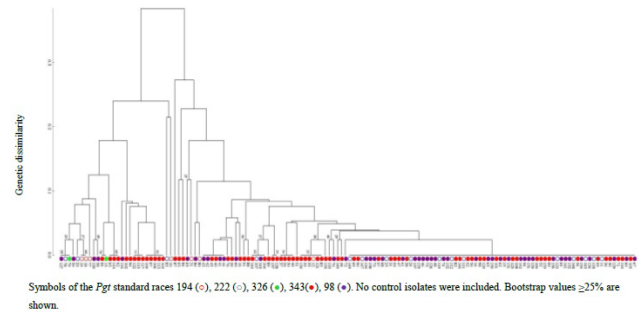
Markers	1st lineage	2nd lineage	F_{ST}	*LinFst*	*Nei D*	*Nei I*
Six	Lineage 1	Lineage 2	0.684	2.167	1.275	0.280
	Lineage 1	Lineage 3	0.622	1.645	1.275	0.280
	Lineage 2	Lineage 3	0.000	0.000	0.000	1.000
	Lineage 1	Lineage 4	0.631	1.711	1.275	0.280
	Lineage 2	Lineage 4	0.000	0.000	0.000	1.000
	Lineage 3	Lineage 4	0.000	0.000	0.000	1.000
	Lineage 1	Lineage 5	0.455	0.833	0.319	0.727
	Lineage 2	Lineage 5	0.556	1.250	0.981	0.375
	Lineage 3	Lineage 5	0.556	1.250	0.981	0.375
	Lineage 4	Lineage 5	0.556	1.250	0.981	0.375
Seven	Lineage 3	Lineage 4	0.042	0.044	0.028	0.973

Table 5: Pairwise lineage F_{ST}, pairwise *Nei* genetic distance genetic and *Nei* genetic identity values based on 13 markers of low polymorphism. F_{ST}: Wright's F-Statistics (a measure of genetic differentiation among populations); *LinFst*: Linearised F-Statistics; *Nei D*: *Nei* genetic distance; *Nei I*: *Nei* genetic identity.



Symbols of the *Pgt* races 126 (▶); 34 (Δ); 21(♦); 194 (○) and 326 (●). Bootstrap values ≥25% are shown. G: Group. G1- lineage 5; G2 – lineage 1; G3 – Lineages 2, 3 and 4

Figure 4: UPGMA dendrogram/phylogenetic tree based on six SSR markers of low polymorphism.



Symbols of the *Pgt* standard races 194 (○), 222 (○), 326 (♦), 343(●), 98 (●). No control isolates were included. Bootstrap values ≥25% are shown.

Figure 5: UPGMA dendrogram/phylogenetic tree based on seven highly polymorphic SSR markers.

The oldest isolates in Group 2 were collected in 1973 [isolates 20, 21 (pt. 194-1,2,3,5,6,8,9) and 32 (pt. 343-1,2,3,5,6); suggesting an initial divergence of a second SSR genotype around 1973, with further independent evolution of both variation in SSRs and in pathogenicity occurring among the two groups over the following years. Interestingly, 71 isolates representing 18 pathotypes were identical in SSR genotype to isolate 32 (pt. 343-1,2,3,5,6) (Figure 5). These isolates were collected over a 35 year period (1973 to 2008), consistent with clonality and single step acquisition of virulence within the Australian *Pgt* population. Isolates within this SSR genotype were found in all parts of Eastern Australia following its first detection in 1973, and it was not found in WA until 2000 when pt. 98-1,2,3,5,6 was isolated (Table S1 and Figure 5). This suggests that this SSR genotype arose in Eastern Australia, evolved and became widespread, and sometime around 2000, spread to WA.

The principal coordinate analysis (PCoA) of genetic similarity separated the 157 isolates into three distinct groups that included: group 1 comprising of Lineage 1 isolates; group 2 comprising Lineage 5 isolates and group 3 that included all isolates of clonal Lineages 2, 3 and 4 (Figure 6). The first axis explained 89.3% while the second axis explained 10.7% of allelic variation among the isolates in the five lineages. The clustering in the PCoA bi-plot indicated that clonal Lineages 2, 3, and 4 consisted of genetically related isolates that differed from Lineages 1 and 5. The PCoA of Lineages 3 and 4 grouped all isolates together but further sub-clustering occurred within the composite group (Figure 7). The first axis explained 54.3% while the second axis explained 12.1% of the genetic variation, and the accumulated proportion of the variation explained by both axes was 66.4%. Although Lineages 3 and 4 clustered together, the PCoA illustrated that genetic diversity exists among the isolates within these two lineages, and the closer the distances between the isolates the more genetically related they were.

Analysis of molecular variance (AMOVA) was used to statistically partition the genetic variation among the lineages and to estimate the significance of the F-statistics. The first AMOVA showed that 88% variation existed among 157 isolates within the five lineages; while the variation among the Lineages was 12% (Table 6).

The measure of genetic variation or F-Statistics value resulting from 9999 permutations was 0.222 (P=0.001), implying that based on the six SSR markers with low polymorphism a significantly moderate level of genetic variation existed among these isolates in the five lineages. The second AMOVA of Lineages 3 and 4 showed 4% genetic variation between Lineages while there was 0% genetic variation among the 148 individuals (Table 7). The F_{ST} value (0.042) was highly significant (P ≤ 0.005), and indicated a very low level of genetic variation among all isolates. These results confirmed that the *Pgt* pathotypes in Lineage 3 and Lineage 4 were clonal because the F_{ST} value was less than 0.5, but also the existing genetic variability among these pathotypes was close to zero or very low (F_{ST}=0.042) implying that the 34 genotypes (24 genotypes in Lineage 3 and 10 genotypes in lineage 4) were genetically related.

Discussion

The emergence of highly virulent stem rust pathotypes in East Africa [9], the development and spread of aggressive and high temperature tolerant stripe rust pathotypes in Europe, America, Africa, Asia and Australia [32,44,58], and the recent emergence of two new virulent leaf rust pathotypes in Australia (Park *et al.*, 2014 *unpublished*) constitute a major threat to global wheat production and consequently

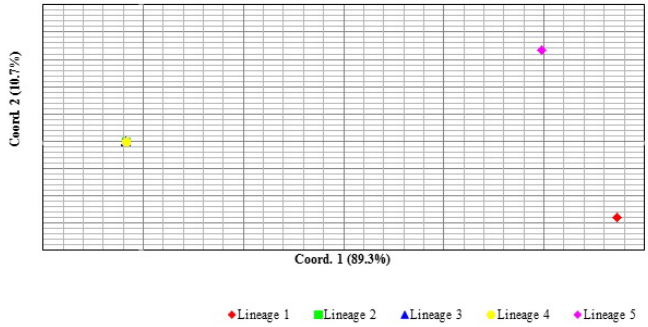


Figure 6: PCoA of genetic similarity matrix generated from six SSR alleles among the 5 lineages.

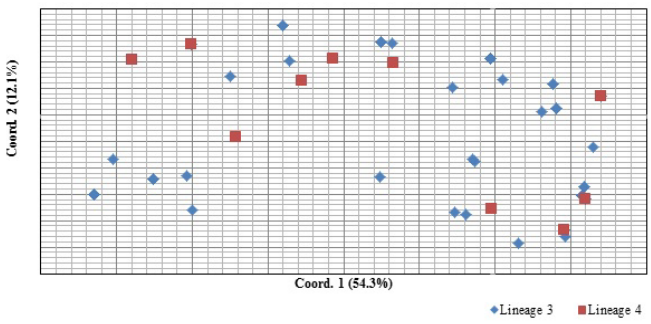


Figure 7: PCoA of genetic similarity matrix generated from seven SSR alleles within Lineage 3 and Lineage 4.

Source	df	ss	ms	est. var.	variation
Among Lineages	4	33.325	8.331	0.283	12%
Among Individuals	152	0.000	0.000	0.000	0%
Within Individuals	157	311.000	1.981	1.981	88%
Total	313	344.325		2.264	100%
F-Statistics	value	*P*-value			
Fst	0.222	0.001			
Fis	-1.000	1.000			
Fit	-0.556	1.000			

Table 6: Analysis of molecular variation among isolates in five lineages using six SSR-based allelic distance matrix for F-Statistics. df: degree of freedom; ss: sum of squares; ms: mean squares; est. variation: estimated variance and probability (P) values for F_{ST}, *FIS* and *FIT*. The F-statistics were based on standard permutation across the full data set.

Source	df	ss	ms	est. var.	variation
Among Lineages	1	6.128	6.128	0.046	4%
Among Individuals	146	302.024	2.069	1.033	95%
Within Individuals	148	0.500	0.003	0.003	0%
Total	295	308.652		1.082	100%
F-Statistics	value	*P*-value			
Fst	0.042	0.005			
Fis	0.997	0.000			
Fit	0.997	0.000			

Table 7: Analysis of molecular variation among *Pgt* isolates in Lineages 3 and 4 using seven SSR based allelic distance matrix for F-Statistics. df: degree of freedom; ss: sum of squares; ms: mean squares; est. variation: estimated variance and probability (P) values for F_{ST}, *FIS* and *FIT*. The F-statistics were based on standard permutation across the full data set.

food security. The changes in the pathogenicity and environmental adaptation of these fungi have resulted in serious epidemics and in some cases total crop loss [8,44]. Detailed studies have shown that many of the world's wheat cultivars are vulnerable to the 'Ug99' group of *Pgt* pathotypes [59-62] implying that if its variants were introduced into the major wheat growing regions, they would cause severe epidemics resulting in massive yield losses.

Ongoing surveys of pathogenic variability in *Pgt* in Australia since 1921 have provided strong evidence of periodic incursions of exotic isolates, simple mutational acquisition of virulence for resistance genes deployed in commercial wheat cultivars, and somatic hybridisation between *Pgt* pathotypes [6,7]. 'Step-wise' mutation is considered to be the major mechanism generating new pathotypes of *Pgt*, and is believed to have given rise to the five clonal lineages, each derived from a founding isolate [3,5-7,10,17]. Lineage 1, derived from standard race 126, predominated in Australia from 1925 when pt. 126-5,6,7,11 was first detected, until the 1950s. Lineage 2, derived from standard race 21, was first detected in 1954. Pathotypes within Lineage 2 developed following its initial detection, predominating during the 1960s and 1970s [56] and are still isolated in wheat growing regions. Lineage 3, derived from standard race 326, was first detected in 1969 and like Lineage 4 (standard race 194), also first detected in 1969, are thought to have originated from Africa and been transported to Australia by high altitude winds [3,5,7]. Derivative pathotypes from these two lineages are also still detected in Australia. Lineage 5 was derived from a pathotype within standard race 34, presumed to have developed *via* somatic hybridisation between isolates from Lineages 1 and 2 [6].

Pathogenicity assessments of the selected and purified *Pgt* isolates found general consistency with previous determinations, indicating that pathotypes in Lineage 3 and Lineage 4 generally displayed similar avirulence and virulence patterns on known stem rust resistance genes carried by differential genotypes. Cluster analysis based on phenotypic data grouped the 99 derivatives of founding pts. 326-1,2,3,5,6 and 194-1,2,3,5,6 into one big cluster comprising multiple subgroups, each similar in avirulence and virulent patterns. Interestingly, pt. 194-1,2,3,7,8,9, which is believed to have evolved from founding pt. 194-1,2,3,5,6 by gaining virulence to *Sr30* and *SrAgi* separated from the main group at 83%, forming a clade of its own.

Luig (1978), reported that mutant pathotypes with virulence for *Sr5*, *Sr15*, *Sr9e* and *Sr21* were common, whereas mutation from avirulence to virulence for *Sr6* and *Sr30* was low, and mutation for virulence to *Sr13*, *Sr24*, *Sr26* and *Sr27* was not detected [17]. Further studies have shown that subjection of a pathogen to intense selection pressure may lead to divergence of specific pathogen genetic compositions that affect the host-pathogen interaction [61,63]. Moreover, host driven pathogen evolution is known to be major in influencing the population structure of fungal pathogens including *Puccinia graminis* [16].

The high number of *Pgt* pathotypes confirmed in this study was probably a result of frequent replacement of cultivars carrying major resistance genes. A similar scenario occurred in Australia, when progressive accumulation of resistance genes to *Pgt* aligned with the evolution of pt. 21-2,3,4,5,6,7 from pt. 21-0 [56]. In situations where the host does not change, pathotypes may remain constant as in the case of *Puccinia triticina* pathotypes 26-3 and 26-1,3 that remained stable in New Zealand for >50 years because of extensive growing of the same susceptible cultivars [64]. Likewise, the widespread growing of wheat cultivars carrying specific leaf rust resistance genes have selected for specific pathotypes in certain regions [65] such as USA where soft red winter wheat cultivars grown in the Southern and Eastern states carrying *Lr9, Lr11, Lr18* and *Lr26* selected for pathotypes with matching virulence [66].

The varying levels of heterozygosity, F-statistics and polymorphism of the six SSR marker analysis across the 157 isolates revealed significant variation. These results were further confirmed *via* PCoA; pairwise population *Nei* genetic distance and identity, UPGMA and AMOVA derived F_{ST} analyses that detected moderate to high levels of genetic variation among all isolates of Lineage 1, Lineage 2, Lineage 3, Lineage 4 and Lineage 5. However, based on the same SSRs the PCoA, pairwise *Nei* genetic distances, UPGMA and F_{ST} analyses determined no genetic variation among isolates in Lineage 3 and Lineage 4, but a further analysis of 142 isolates from these two lineages using seven highly polymorphic SSRs detected 34 genotypes with very low level of genetic variation (F_{ST} = 0.042). The phenotypic and genotypic results confirmed that although the Lineage 3 and Lineage 4 founding pathotypes were originally detected in Australia as two pathotypes differing in their avirulence and virulence to *Sr7b*, they actually had a strong common genetic background. Although pathogenicity is only a small proportion of the total genetic variation in *Pgt* pathogen population, it can be subject to intense selection by resistance genes in common hosts, leading to rapid changes at the specific pathogenicity loci and the development of new pathotypes.

The 34 SSR genotypes detected among the isolates in Lineage 3 and Lineage 4, which are believed to have arisen *via* asexual means only, suggests significant evolution at these loci and could indicate that genetic changes have occurred within these pathogen populations for traits other than pathogenicity that favour survival, for example, aggressiveness. In this study, the number of alleles detected ranged from two to six per locus, while the heterozygosity, *Nei* genetic distance and genetic variation (F_{ST}) per locus were significantly low implying a high

level of genetic relatedness or similarity among isolates of the above genotypes. Rapid mutations over time have been found to facilitate the adaptation of the pathogen to host carrying specific resistance genes resulting in significant allelic variation within loci and varying levels of heterozygosity. Previous studies have determined that high levels of heterozygosity in clonally reproducing pathogen populations result from high rates of reproduction that lead to divergence of alleles [67]. A similar study of variability for aggressiveness of *Puccinia triticina* using microsatellite markers detected differences in aggressiveness among isolates of the same pathotypes and the same clonal lineage [68].

Another study carried out to understand the worldwide population structure of the stripe rust pathogen of wheat *Puccinia striiformis* f. sp. *tritici* using microsatellite genotyping of past and present populations detected low levels of genetic differentiation, and based on F_{ST} values clonal populations were found in samples from NW Europe (F_{ST}=0.044), Middle East (F_{ST}=0.084) and East Africa (F_{ST}=0.052) [43]. Additionally, highly recombinant populations with high genetic diversity were detected in isolates collected from China and South Asia. Based on this study, progressive deviation was observed among all populations apart from the one from NW Europe. This finding also applies to *Pgt* clonal populations in Australia that have evolved from the original six races to numerous genetically related pathotypes with virulence to many resistance genes.

Similarly, a study carried to determine the genetic relationship in *Puccinia triticina* among isolates on a diverse international panel of durum using 11 SSRs *via* AMOVA detected significant close relatedness among isolates from South America, North America and Europe, whereas isolates from either Ethiopia or Israel had distinct SSR genotypes [69]. It was concluded that the significant genetic similarity across different regions probably indicated recent common ancestry, while the genetic differentiation in isolates from Ethiopia indicated different origins. Based on virulence phenotypic analysis and SSR genotypes, a study found high level of genetic diversity within the *Puccinia triticina* populations on tetraploid and hexaploid wheats in Ethiopia [16]. This study established that the level of genetic diversity varied across the five virulence phenotypes or SSR groups, and from 48 isolates 17 SSR genotypes were detected. The uniqueness of the virulence phenotypes and the varying levels of genetic diversity within and across genotypes arise from the continuous existence of a diverse host population ranging from landraces to improved cultivars (diploid, tetraploid and hexaploid) that allow survival and adaptation of early/old *Pt* populations through selection [16]. Whole genome sequencing and phylogenetic studies of *Puccinia triticina* determined that genetic variations among the pathotypes within the same clonal lineage arise during evolution processes when individuals acquire virulence for different resistance genes. Interestingly, it was found out that the most recent evolved *Pt* pathotypes used in this study were more variable and virulent than the old races implying that the high level of virulence was probably responsible for the high degree of *Pt* adaptability [70]. The detection of 34 genotypes in 142 *Pgt* isolates in clonal Lineage 3 and Lineage 4 clearly illustrated that while the isolates within both appear to be genetically very similar, significant variation beyond pathogenicity has developed in each over a 39 year period. These results agree with previous studies that have concluded that some level of genetic diversity resulting from mutation exists in highly clonal rust pathogen populations [16,33,55,67].

In conclusion, by integrating pathogenicity and molecular analyses *via* SSR marker genotyping, the current study confirmed that *Pgt* clonal Lineages 3 and 4, which are derived from the founding pathotypes 326-1,2,3,5,6 and 194-1,2,3,5,6, respectively, are genetically related. The allelic variations existing among the individuals or groups of isolates result mainly from single-step mutations in form of deletions, insertions, substitutions and inversions, giving rise to new isolates varying in avirulence or virulence mechanisms. The distances between the clusters indicate the closeness of the genetic relatedness. Even with evolution of the 34 SSR genotypes that were detected, the level of genetic similarity has remained high supporting the belief that step-wise mutation is the major mechanism for new *Pgt* pathotypes evolution contributing to *Pgt* genetic diversity in Australia. Although mutation is believed to be the main mechanism *via* which these pathotypes evolve, further analysis of the DNA to understand the types and rate of mutation as well as whole genome sequencing to determine the actual nucleotide changes would provide more detailed knowledge about the pathogen.

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