Genetic Diversity in Australian Populations of *Puccinia graminis* f. sp. *avenae*

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ABSTRACT

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Sequence-tagged microsatellite profiling was used to develop 110 microsatellites for *Puccinia graminis* f. sp. *tritici* (causal agent of wheat stem rust). Low microsatellite polymorphism was exhibited among 10 pathogenically diverse *P. graminis* f. sp. *tritici* isolates collected from Australian cereal growing regions over a period of at least 70 years, with two polymorphic loci detected, each revealing two alleles. Limited cross-species amplification was observed for the wheat rust pathogens, *P. triticina* (leaf rust) and *P. striiformis* f. sp. *tritici* (stripe rust). However, very

high transferability was revealed with *P. graminis* f. sp. *avenae* (causal agent of oat stem rust) isolates. A genetic diversity study of 47 *P. graminis* f. sp. *avenae* isolates collected from an Australia-wide survey in 1999, and a historical group of 16 isolates collected from Australian cereal growing regions from 1971 to 1996, revealed six polymorphic microsatellite loci with a total of 15 alleles. Genetic analysis revealed the presence of several clonal lineages and subpopulations in the pathogen population, and wide dispersal of identical races and genotypes throughout Australian cereal-growing regions. These findings demonstrated the dynamic population structure of this pathogen in Australia and concur with the patterns of diversity observed in pathogenicity studies.

Rust diseases caused by *Puccinia* spp. have a significant impact on cereal production worldwide due to their ability to rapidly evolve new virulent pathotypes on previously resistant cultivars. In Australia, pathogenic variability in populations of the wheat rust pathogens *Puccinia graminis* f. sp. *tritici* (causal agent of stem or black rust) and *P. triticina* (causal agent of leaf or brown rust) has been monitored continuously since the 1920s, and *P. striiformis* f. sp. *tritici* (causal agent of yellow or stripe rust) has been monitored since it was first detected in Australia in 1979. Pathogenic variability in *P. graminis* f. sp. *avenae*, the stem rust pathogen of oats, also has been monitored in annual surveys since 1976. These surveys have demonstrated that, despite the absence of sexual recombination in these pathogens in Australia, populations are diverse in pathogenicity traits. This diversity is believed to be the result of periodic introductions of pathotypes, mutation, and somatic hybridization (27,28,35).

Molecular markers have been applied widely in fungal plant pathogen populations to characterize genetic diversity and phylogenetic relationships, and in the discrimination of isolates and pathotypes (2,20). The genetic diversity of Australian and international pathogen populations causing stem rust, leaf rust, and stripe rust diseases of wheat have been investigated previously using several types of multilocus DNA fingerprinting methods, including random amplified polymorphic DNAs (RAPDs) (21, 27), amplified fragment length polymorphisms (AFLPs) (18,30, 37), selectively amplified microsatellites (SAMs), and S-SAPs (18). In general, low levels of diversity were revealed among iso-

lates of the same species or formae speciales. The microsatellite-based SAM assay revealed significantly higher levels of genetic diversity between isolates of *P. graminis* f. sp. *avenae* and *P. graminis* f. sp. *tritici* compared with other methods tested previously, indicating microsatellite evolution within Australian populations (18). This finding prompted a search for single-locus microsatellite markers in these pathogens.

Microsatellites, or simple sequence repeats (SSRs), consist of tandem repeats of short (1- to 6-bp) nucleotide sequences. SSRs are widely used molecular markers because they are codominant, highly abundant, distributed randomly throughout most eukaryotic genomes (33), and tend to be highly polymorphic due to variation in the number of repeat units (10). SSR amplification requires only a small amount of genomic DNA using a pair of polymerase chain reaction (PCR) primers that flank the microsatellite repeat. More recently, PCR assays have been devised to amplify SSR loci using a single primer specific to DNA sequence flanking only one side of the microsatellite and a primer that anchors to the repeat motif itself (15,16). Markers amplified using these PCR assays are referred to as sequence-tagged microsatellites (STMs), and generally are developed using SAM analysis (16) and STM profiling (STMP) (15). These techniques offer a cost-efficient and rapid approach to developing SSRs compared with traditional hybridization-based methods (15).

In the present work, the STMP technique was used to develop single-locus microsatellites for *P. graminis* f. sp. *tritici*. These markers were assessed for their ability to differentiate pathogenically diverse isolates of *P. graminis* f. sp. *tritici*, and their utility for cross-species amplification in *P. triticina*, *P. striiformis* f. sp. *tritici*, and *P. graminis* f. sp. *avenae* was investigated. Due to a high level of STM amplification in *P. graminis* f. sp. *avenae*, a larger genetic diversity study was undertaken using a collection of isolates representing current and historical populations of this pathogen in Australian cereal growing regions.

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MATERIALS AND METHODS

Microsatellite library construction. DNA was extracted from 50 mg of *P. graminis* f. sp. *tritici* urediniospores (3). Two STM libraries were constructed using *P. graminis* f. sp. *tritici* DNA (accession 730879) (Table 1) according to the STMP procedure as described (15). Briefly, STMP employs PCR to directly amplify SSR-containing fragments from a DNA sample, thereby eliminating the need to construct and screen a DNA library to identify microsatellite-containing clones. This is achieved because STMP employs the principles of serial analysis of gene expression (SAGE) (32) to generate a short (21-bp) but characteristic nucleotide sequence tag for each microsatellite present within a DNA sample. These nucleotide tags then are ligated together to form long concatemers, which are cloned into plasmids and sequenced. Each concatemer typically contains 25 to 30 nucleotide tags; therefore, a large number of microsatellites can be characterized in a single sequencing reaction. Thus, the sequencing throughput of STMP vastly exceeds that of traditional library screening methods that characterize only one microsatellite locus per plasmid clone. STMP nucleotide tags contain sufficient flanking DNA sequence to uniquely characterize each microsatellite locus and for the design of PCR primers to amplify corresponding loci directly from

genomic DNA. This is achieved by PCR amplification of genomic DNA using a primer derived from a nucleotide sequence tag and a primer that anchors to the microsatellite repeat motif itself (15). The first STM library constructed for *P. graminis* f. sp. *tritici* was based on (AC)_n dinucleotide repeats and the second on (CT)_n repeats. Fifty-five STM primers were designed for each library and tested using a set of 10 pathogenically diverse isolates of *P. graminis* f. sp. *tritici* collected from Australian cereal-growing regions over a period of at least 70 years (Table 1).

STM analysis. STM amplification was performed in a 20-μl reaction mixture containing 0.2 mM dNTP, 1× PCR buffer, 1.5 mM MgCl₂, 1 unit of *Taq* DNA polymerase, 5 pmol STM primer, 10 pmol microsatellite anchoring primer labeled at the 5' end with hexachloro-6-carboxylfluorescein (HEX), and 20 ng of genomic DNA. Primer sequences are given in Table 2. For the STM primer set targeting (AC)_n repeats, PCR was performed for 39 cycles with the following profile: 60 s of denaturation at 92°C, 60 s of annealing (described below), and 30 s of extension at 72°C. The annealing temperature for the first cycle was 64°C, reducing by 1°C/cycle for nine consecutive cycles, followed by 30 cycles at 55°C, with the denaturation and annealing steps reduced to 30 s. The same PCR profile was used for the STM primer set targeting (CT)_n repeats, except that the annealing temperature range was 59

TABLE 1. List of *Puccinia graminis* f. sp. *tritici*, *P. striiformis* f. sp. *tritici*, *P. triticina*, and *P. graminis* f. sp. *avenae* isolates used for testing sequence-tagged microsatellites

Species, accession	Race ^a	Site of collection ^b	Year
<i>P. graminis</i> f. sp. <i>tritici</i>			
1. 840552	34-2, 12, 13	Kingaroy, QLD	1984
2. 540129	21-0	Southern NSW	1954
3. 690924	34-2	Walbundrie, NSW	1969
4. 691042	194-1, 2, 3, 5, 6	Tichbourne, NSW	1969
5. 334	126-5, 6, 7, 11	?	?
6. 781219	98-1, 2, 3, 5, 6	Southern NSW	1978
7. 730879	343-1, 2, 3, 5, 6	Kerang, VIC	1973
8. 751188	21-1, 2, 10	Ceduna, SA	1975
9. 690822	326-1, 2, 3, 5, 6	Clinton, SA	1969
10. 730560	34-2, 11	Cobar, NSW	1973
<i>P. striiformis</i> f. sp. <i>tritici</i>			
1. 861725	110 E143 A+	Richmond, TAS	1986
2. 821559	104 E137 A-	Grafton, NSW	1982
3. 821552	104 E137 A+	Forbes, NSW	1982
4. 881510	104 E9 A-	Trundle, NSW	1988
5. 821589	106 E139 A-	Springston, NZ	1982
6. 861773	109 E141 A-	Christchurch, NZ	1986
7. 831917	108 E141 A+	Rutherglen, VIC	1983
8. 881584	104 E137 A-, Yr17+	Cobbitty, NSW	1988
9. 911582	106 E139 A-, YrSk+	Lincoln, NZ	1991
<i>P. triticina</i>			
1. 710208	64-1, 3	Castle Hill, NSW	1971
2. 810043	53-1, (6), (7), 10, 11	Palmerston North, NZ	1981
3. 720469	10-1, 2, 3, 4	Narrabri, NSW	1972
4. 890163	76-1, 3, 10, 12	Manawatu, NZ	1989
5. 670028	26-1, 3	Takapara, NZ	1967
6. 760583	76-2, 3, 6, (7)	Narrabri, NSW	1976
7. 840045	104-2, 3, (6), (7), 11	Mt. Derimut, VIC	1984
8. 200347	104-1, 2, 3, (6), (7), 11, 13	Urania, SA	2000
9. 630550	135-2	Ravensworth, NSW	1963
10. 900053	64-(6), (7), (10), 11	Narrabri, NSW	1990
<i>P. graminis</i> f. sp. <i>avenae</i>			
1. 992579	94 + Pg13	Condobolin, NSW	1999
2. 992678	94 + PgSa	Mendooran, NSW	1999
3. 992655	94 + Pg13, a	Young, NSW	1999
4. 992664	94	Yeoval, NSW	1999
5. 992629	41 + PgSa	Stawell, VIC	1999
6. 992630	30 + Pga	Winchelsea, VIC	1999
7. 992584	94	Moona, WA	1999
8. 992573	94	Mindarabin, WA	1999
9. 992585	94	Moona, WA	1999
10. 992728	94	Roseworthy, SA	1999

^a Race nomenclatures were as described for *P. graminis* f. sp. *tritici*, *P. striiformis* f. sp. *tritici*, *P. triticina* (22), and for *P. graminis* f. sp. *avenae* (31).

^b Australian states: QLD = Queensland, NSW = New South Wales, VIC = Victoria, SA = South Australia, TAS = Tasmania, and WA = Western Australia.

^c W. L. Waterhouse, unpublished data (1926).

to 50°C. Following PCR, an equal volume of loading buffer (98% formamide, 1 mM EDTA, 0.2% carbonyl fuschin) was added to each reaction. The PCR products were denatured at 95°C for 3 min, chilled on ice, and separated using a 5% sequencing gel on a GelScan 2000 DNA fragment analyzer (Corbett Research, Mortlake, Australia). All STM amplifications were duplicated in independent reaction preparations.

Cross-species amplification. STMs were tested for cross-species amplification using isolates of the wheat rust pathogens *P. tritici* and *P. striiformis* f. sp. *tritici*, collected from Australia and New Zealand, and Australian isolates of the oat stem rust pathogen, *P. graminis* f. sp. *avenae* (Table 1). The 10 *P. tritici* and nine *P. striiformis* f. sp. *tritici* isolates were selected to represent the maximum pathogenic variation of current and historical pathogen populations. For *P. graminis* f. sp. *avenae*, the 10 isolates represent maximum geographic variation in the 1999 annual pathogenicity survey conducted at the Plant Breeding Institute Cobbitty, University of Sydney. All isolates were obtained from collections maintained at the Plant Breeding Institute

Cobbitty, University of Sydney, and pathotypes were determined for the wheat pathogens by assessing virulence patterns on sets of differential cultivars (22). For all isolates, DNA was extracted from 50 mg of urediniospores (3).

Based on positive results obtained with *P. graminis* f. sp. *avenae* during cross-species amplification testing, the isolate set was expanded to undertake a larger investigation of genetic diversity. DNA was obtained for a set of 63 *P. graminis* f. sp. *avenae* isolates, including 47 collected from the 1999 annual pathogenicity survey (Table 3). Standard race designations were assigned to each isolate as described (31) using the differential genotypes White Tartar (*Pg1*), Richland (*Pg2*), Joannette Strain (*Pg3*), Rodney (*Pg4*), Eagle² × CI 4023 (*Pg8*), Santa Fe selection (*Pg9*), and Saia (*PgSa*), plus the supplementary differentials Rodney M (*Pg13*) and Omega (*Pga*). Sixteen isolates were included to represent Australian *P. graminis* f. sp. *avenae* populations between 1971 and 1996. These were obtained from collections maintained at the Plant Breeding Institute Cobbitty, University of Sydney, and were selected to represent the range of pathogenicities identified during that period.

TABLE 2. Sequences of primers used to reveal 43 sequence-tagged microsatellite (STM) loci in *Puccinia graminis* f. sp. *tritici* (Pgt), *P. striiformis* f. sp. *tritici* (Pst), *P. tritici* (Pt), and *P. graminis* f. sp. *avenae* (Pga)

Primer	Primer sequence (5'→3') ^a	Pathogens revealing STM loci
5' anchored		
PAC6	KKR YRY YAC ACA CAC ACA C KKY RYR YCA CAC ACA CAC A	...
PCT6	KKV RVR VCT CTC TCT CTC T KKR VRV RTC TCT CTC TCT C	...
STM		
pMS5ca	GAT CGG TTT TAT CAC GCT TTA	Pga, Pgt, Pst
pMS6ca	GAT CCC TGT AGG ACC CAG	Pga
pMS8ca	GGC GAT GAA TCT AAC CTG	Pgt
pMS11ca	CCT CAG ATT CCA ATA ACC AAC	Pga
pMS12ca	GGT TTA GTG GGT AGG TTG A	Pga
pMS15ca	CAT CGT CCA CCA GGA TTT CAA	Pga, Pgt, Pst, Pt
pMS19ca	CGA CGA GGT GGC GGA CT	Pgt
pMS21ca	GCT TTG TGA GTG AGA AGT GTC	Pga, Pgt, Pt
pMS22ca	GAT CCT AAG GCA TGA TGA GA	Pga
pMS23ca	CGA AAC AAA AGG AAA GAT AA	Pgt
pMS24ca	CGG CAC GCT GCT GAT G	Pga
pMS31ca	GAT CAG GCT TGC AGC TAG ACG	Pgt
pMS33ca	CAC TTG TGG AAC TGC TCA G	Pga, Pgt, Pst, Pt
pMS37ca	CTT AAC ACA AGC CCC GAT G	Pga, Pgt
pMS39ca	GCT GTC TAA TAT CCT CAT AC	Pga, Pgt
pMS40ca	CTG GTC GGT CTC TTC TTC A	Pga, Pgt, Pst, Pt
pMS41ca	CGT TCA ACG AGA GGG AAA	Pga, Pgt, Pst, Pt
pMS43ca	GAT CTG TCT ACC TTC CTT CA	Pga
pMS46ca	TGC TAC TCT TAG TCG TCA TC	Pgt
pMS50ca	GCA GAT GCC CAG TGT CGT GA	Pga, Pgt, Pst, Pt
pMS54ca	CTT GCG GAA TCG GCT CA	Pgt
pMS55ca	GAT CGA CTT GAC AAA CGG TG	Pga, Pgt, Pst, Pt
pMS57ca	GTA AAA CTG CTC ACT TGC TG	Pgt
pMS58ca	GTG CCA ATA AAG GCT TGT GAG	Pga, Pgt, Pst, Pt
pMS61ca	GGC TTT TCC TCT TCG GCT T	Pga
pMS87ca	GAT CGG TGG CAA GTT GGT TG	Pga, Pgt
pMS1ct	GAT CAG CCA TCC GAC CCA	Pgt
pMS3ct	AGG ATC ACT CAC ACA CAT GCT	Pga
pMS14ct	CAG GAT CTA TAT CAC GAT GTT	Pga
pMS20ct	CTA CAG GAT CAG GGA AAA CTT	Pga
pMS21ct	CAG GAT CGT TTC AAG AGA GAG	Pga, Pgt, Pst, Pt
pMS23ct	GAT CGA GGA CGA GGA AAC AG	Pga
pMS34ct	GAT CCC AGC ACT AGG GCT TG	Pga
pMS42ct	GAT CCG CTC AAC TCT CCC A	Pga
pMS43ct	CAG GAT CAA AAA TAA GAC ACA	Pga
pMS52ct	CAG GAT CGT ACA CTC AAA TTC	Pgt
pMS62ct	GAT CTG CAG GCA CGG ACC CA	Pga
pMS68ct	GAT CCT TCG TGA CCG AGC	Pga
pMS78ct	GAT CGC TTT TGG CCC TGC TG	Pga, Pgt
pMS83ct	GAT CAC TCA CAC ACA TGC TG	Pga
pMS84ct	GAT CAG CAT CTC GGA GGC	Pga
pMS85ct	CAG GAT CAT GGA GAA TCA AAC	Pga
pMS110ct	GAT CTT TTG CGT TCC TCT CA	Pga

^a K = G or T; R = A or G; V = A, C or G; Y = C or T. Each primer consisted of equimolar amounts of the two listed oligonucleotides.

Statistical analysis. Microsatellite fragments were scored as present (=1) or absent (=0) across the 63 *P. graminis* f. sp. *avenae* isolates. The resulting binary matrix was used to construct a similarity matrix using the Jaccard coefficient, $GS(ij) = a/(a + b + c)$ (17), where *a* is the number of fragments shared by *i* and *j*, *b* is the number of fragments present in *i* and absent in *j*, and *c* is the number of bands present in *j* and absent in *i*. Cluster analysis was

performed using the unweighted pair group with arithmetic means method (UPGMA) (29) and support for the clusters was evaluated by bootstrap analysis with 1,000 iterations (13) using the program WinBoot (36). Allele frequencies and heterozygosity were determined for each SSR locus according to Nei (25).

Analysis of molecular variance (AMOVA) (11) was used to statistically partition the distribution of genetic variation revealed

TABLE 3. List of *Puccinia graminis* f. sp. *avenae* isolates used for genetic analysis using single-locus sequence-tagged microsatellites

Isolate no.	Accession	Race ^a	Virulence/avirulence phenotype	Site of collection ^b	Year
1	992549	94 + Pg13	Pg1, 2, 3, 4, 8, 9, 13/Sa, a	Northern NSW	1999
2	992579	94 + Pg13	Pg1, 2, 3, 4, 8, 9, 13/Sa, a	Condobolin, Northern NSW	1999
3	992593	94 + Pg13	Pg1, 2, 3, 4, 8, 9, 13/Sa, a	Badgingarra, WA	1999
4	992609	94 + Pg13	Pg1, 2, 3, 4, 8, 9, 13/Sa, a	Baan Baa, Northern NSW	1999
5	992612	94 + Pg13	Pg1, 2, 3, 4, 8, 9, 13/Sa, a	Northern NSW	1999
6	992629	41 + PgSa	Pg1, 2, 3, 4, 8, Sa/9, 13, a	Stawell, VIC	1999
7	992630	30 + Pga	Pg1, 2, 4, 8, Sa/3, 9, 13, a	Winchelsea, VIC	1999
8	992633	30 + Pga	Pg1, 2, 4, 8, Sa/3, 9, 13, a	Cressy, VIC	1999
9	992655	94 + Pg13, a	Pg1, 2, 3, 4, 8, 9, 13, a/Sa	Young, Southern NSW	1999
10	992658	94 + Pg13	Pg1, 2, 3, 4, 8, 9, 13/Sa, a	Cowra, Southern NSW	1999
11	992662	94 + Pg13	Pg1, 2, 3, 4, 8, 9, 13/Sa, a	Molong, Southern NSW	1999
12	992676	94 + Pg13, Sa	Pg1, 2, 3, 4, 8, 9, 13, Sa/a	Leadville, Northern NSW	1999
13	992678	94 + PgSa	Pg1, 2, 3, 4, 8, 9, Sa/13, a	Mendooran, Northern NSW	1999
14	992701	94 + PgSa	Pg1, 2, 3, 4, 8, 9, Sa/13, a	Boorowa, Southern NSW	1999
15	992708	94 + Pg13	Pg1, 2, 3, 4, 8, 9, 13/Sa, a	Ongerup, WA	1999
16	992712	94 + Pg13	Pg1, 2, 3, 4, 8, 9, 13/Sa, a	Newdegate, WA	1999
17	992714	94 + Pg13	Pg1, 2, 3, 4, 8, 9, 13/Sa, a	Coomalbidup, WA	1999
18	992715	94 + Pg13	Pg1, 2, 3, 4, 8, 9, 13/Sa, a	Newdegate, WA	1999
19	992724	94 + Pg13, Sa, a	Pg1, 2, 3, 4, 8, 9, 13, Sa, a/	Tottenham, Northern NSW	1999
20	992725	94 + Pg13	Pg1, 2, 3, 4, 8, 9, 13/Sa, a	Tottenham, Northern NSW	1999
21	992732	94 + Pg13, Sa	Pg1, 2, 3, 4, 8, 9, 13, Sa/a	Lake Bolac, VIC	1999
22	992733	94 + Pg13, Sa, a	Pg1, 2, 3, 4, 8, 9, 13, Sa, a/	Castle Hill, NSW	1999
23	992537	94	Pg1, 2, 3, 4, 8, 9/13, Sa, a	Rutherglen, VIC	1999
24	992584	94	Pg1, 2, 3, 4, 8, 9/13, Sa, a	Moona, WA	1999
25	992594	94 + Pg13, Sa, a	Pg1, 2, 3, 4, 8, 9, 13, Sa, a/	Leeton, Southern NSW	1999
26	992608	94 + PgSa	Pg1, 2, 3, 4, 8, 9, Sa/13, a	Gunnedah, Northern NSW	1999
27	992641	94 + Pg13	Pg1, 2, 3, 4, 8, 9, 13/Sa, a	Oaklands, Southern NSW	1999
28	992646	94 + Pg13	Pg1, 2, 3, 4, 8, 9, 13/Sa, a	Moama, Southern NSW	1999
29	992652	94 + Pg13	Pg1, 2, 3, 4, 8, 9, 13/Sa, a	Mitiamo, VIC	1999
30	992683	94 + Pg13, Sa	Pg1, 2, 3, 4, 8, 9, 13, Sa/a	Northern NSW	1999
31	992723	94 + Pg13, Sa	Pg1, 2, 3, 4, 8, 9, 13, Sa/a	Manildra, Southern NSW	1999
32	992727	94 + Pg13	Pg1, 2, 3, 4, 8, 9, 13/Sa, a	Gilgandra, Northern NSW	1999
33	992546	94 + Pg13	Pg1, 2, 3, 4, 8, 9, 13/Sa, a	Temora, Southern NSW	1999
34	992559	94 + Pg13, a	Pg1, 2, 3, 4, 8, 9, 13, a/Sa	Narrabri, Northern NSW	1999
35	992585	94	Pg1, 2, 3, 4, 8, 9/13, Sa, a	Moona, WA	1999
36	992611	94 + PgSa	Pg1, 2, 3, 4, 8, 9, Sa/13, a	Breeza, Northern NSW	1999
37	992647	94 + Pg13	Pg1, 2, 3, 4, 8, 9, 13/Sa, a	Southern NSW	1999
38	992656	94 + Pg13, Sa	Pg1, 2, 3, 4, 8, 9, 13, Sa/a	Yeoval, Southern NSW	1999
39	992687	94 + Pg13	Pg1, 2, 3, 4, 8, 9, 13/Sa, a	Northern NSW	1999
40	992690	94 + Pg13, Sa	Pg1, 2, 3, 4, 8, 9, 13, Sa/a	Caroona, Northern NSW	1999
41	992692	94 + Pg13, Sa, a	Pg1, 2, 3, 4, 8, 9, 13, Sa, a/	Mudgee, Northern NSW	1999
42	992697	94 + Pg13, Sa	Pg1, 2, 3, 4, 8, 9, 13, Sa/a	Tamworth, Northern NSW	1999
43	992707	94 + Pg13	Pg1, 2, 3, 4, 8, 9, 13/Sa, a	Yillamining, WA	1999
44	992716	94 + PgSa	Pg1, 2, 3, 4, 8, 9, Sa/13, a	Googong, WA	1999
45	992717	94 + PgSa	Pg1, 2, 3, 4, 8, 9, Sa/13, a	Ganmain, Southern NSW	1999
46	992718	94	Pg1, 2, 3, 4, 8, 9/13, Sa, a	Young, Southern NSW	1999
47	992722	94 + Pg13, Sa	Pg1, 2, 3, 4, 8, 9, 13, Sa/a	Tottenham, Northern NSW	1999
48	710112	8	Pg2, 3/1, 4, 8, 9, Sa, 13, a	University of Sydney, NSW	1971
49	710116	42	Pg1, Sa/2, 3, 4, 8, 9, 13, a	University of Sydney, NSW	1971
50	720091	22	Pg2, 3, 4/1, 8, 9, 13, Sa, a	Deniliquin, Southern NSW	1972
51	730147	77	Pg3, 9/1, 2, 4, 8, Sa, 13, a	Merriwagga, Southern NSW	1973
52	770098	4	Pg1, 2/3, 4, 8, 9, 13, Sa, a	Spring Creek, QLD	1977
53	770247	7	Pg1, 3/2, 4, 8, 9, 13, Sa, a	Forbes, Southern NSW	1977
54	770258	59	Pg1, 3, Sa/2, 4, 8, 9, 13, a	Bega, Southern NSW	1977
55	780026	1 or 2	Pg3/1, 2, 4, 8, 9, 13, Sa, a	Northfield, SA	1978
56	780933	20	Pg1, 2, 3, 4/8, 9, 13, Sa, a	Lawes, QLD	1978
57	781415	59 + Pg13	Pg1, 3, Sa, 13/2, 4, 8, 9, a	Tullamore, Northern NSW	1978
58	800459	22	Pg2, 3, 4/1, 8, 9, 13, Sa, a	Manjimup, WA	1980
59	810608	5	Pg3/1, 2, 4, 8, 9, 13, Sa, a	Eugowra, Southern NSW	1981
60	820917	18	Pg1, 2, 4/3, 8, 9, 13, Sa, a	Werribee, VIC	1982
61	820625	20	Pg1, 2, 3, 4/8, 9, 13, Sa, a	Mt. Barker, WA	1982
62	842589	2 + Pg13	Pg3, 13/1, 2, 4, 8, 9, Sa, a	Meandarra, QLD	1984
63	962508	41 + Pg13, a	Pg1, 2, 3, 4, 8, 13, Sa, a/9	Toowoomba, QLD	1996

^a Race nomenclature as described (31).

^b Australian state abbreviations: NSW = New South Wales, QLD = Queensland, SA = South Australia, VIC = Victoria, WA = Western Australia.

among *P. graminis* f. sp. *avenae* isolates between two levels: among isolates within the subpopulations of the 1999 survey and historical collection, and among the two subpopulations. The binary data matrix was used to construct a pairwise Euclidean distance matrix with the program AMOVA-PREP (24) in preparation for WINAMOVA. The significance of the AMOVA variance components and fixation indices were tested using nonparametric permutation procedures (12). The fixation index, F_{ST} , was calculated by the AMOVA analysis and provided a measure of the genetic differentiation of the two groups assessed. Values of F_{ST} greater than 0.25 indicate significant genetic differentiation (14).

RESULTS

Evaluation of STMs. For the set of 10 *P. graminis* f. sp. *tritici* isolates, 23 primers (21%) reproducibly amplified a distinct SSR fragment (Table 2). Of these, 19 STMs were amplified using the primer set targeting (AC)_n repeats and four were amplified based on (CT)_n repeats. Two STMs (amplified by pMS21ca and pMS46ca) exhibited codominant polymorphism among the 10 *P. graminis* f. sp. *tritici* isolates, with each locus revealing two alleles.

The STMs exhibited high transferability across the stem rust pathogens *P. graminis* f. sp. *avenae* and *P. graminis* f. sp. *tritici* (Table 2). Surprisingly, more STMs amplified in *P. graminis* f. sp. *avenae* than any of the three wheat rust pathogens tested, with a total of 34 (31%) loci reproducibly amplified. Only nine STMs reproducibly amplified in each of *P. striiformis* f. sp. *tritici* and *P. triticina*, and eight STMs across all four taxa. Furthermore, 20 STMs were amplified in *P. graminis* f. sp. *avenae* only, compared with nine unique STMs for *P. graminis* f. sp. *tritici*. All STMs amplified in *P. striiformis* f. sp. *tritici* and *P. triticina* were monomorphic.

Of the 34 functional STMs in *P. graminis* f. sp. *avenae*, six (18%) revealed polymorphism among the entire set of 63 isolates, with a total of 15 alleles observed (Table 4). Five of the polymorphic STMs were amplified using the STM primer set targeting (AC)_n repeats, with two to four alleles revealed. The remaining polymorphic STM was based on (CT)_n repeats and revealed two alleles.

Genetic diversity in *P. graminis* f. sp. *avenae*. Estimates of heterozygosity and allele frequencies for the six polymorphic SSR loci are given in Table 4. In this analysis, the *P. graminis* f. sp. *avenae* isolate set was divided into two groups, those that were sampled in 1999 and those sampled of the historical collec-

tion (collected from 1971 to 1996). In general, the entire isolate set and the isolates collected in 1999 exhibited similar heterozygosities, ranging from 0.37 to 0.67, with averages of 0.47 and 0.49, respectively. The historical isolates generally exhibited much lower heterozygosities at each locus, ranging from 0.06 to 0.48, with an average of 0.30. Similarly, the average number of alleles per locus was 2.50 for the entire isolate set and isolates collected in 1999, and 2.17 for the historical isolate collection.

The estimates of allele frequencies at each locus further illustrated genetic differences between isolates collected in 1999 and the historical collection (Table 4). For most of the SSR loci, alleles occurred at a much higher or lower frequency in the historical isolates compared with the 1999 isolates. Those alleles that occurred at a much higher frequency in the historical isolate set generally were the most predominant alleles at those loci, consistent with the lower heterozygosity estimates for this isolate set. In addition, two alleles that occurred at a low frequency in the 1999 isolates for loci amplified by pMS11ca and pMS39ca did not occur at all in the historical isolate collection.

Cluster analysis revealed an almost even division of the 63 isolates into two primary clusters separated at 56% genetic similarity (Fig. 1). The first of the two distinct clusters (Fig. 1, uppermost) consisted of 30 isolates divided into two main groups of 78% genetic similarity. One of these groups consisted primarily of isolates collected from northern and southern regions of New South Wales (NSW) in 1999 (14 of 16 isolates), whereas the second group comprised most of the isolates collected from 1971 to 1984 and six isolates from 1999. The cluster containing the historical isolates formed two subgroups, separated by 8% genetic dissimilarity. One of these subgroups comprised isolates collected from 1971 to 1978, and the other consisted of isolates collected from 1977 to 1984, with most of the isolates (except for one) of the 1971-to-1978 group collected from NSW. Three isolates collected in 1999 clustered more closely to the historical isolates than the other isolates collected in 1999.

The bottommost group of the two distinct clusters consisted of 33 isolates, of which 31 were collected in 1999, with an overall genetic similarity of 72%. Within this cluster, one group comprised 24 isolates with 88% genetic similarity and contained the isolate collected in 1996 (isolate 63) (Table 3). The remaining nine isolates grouped loosely, with an overall genetic similarity of 72%, and contained an isolate collected in 1971 (isolate 48) (Table 3). The isolates of this cluster were collected throughout the growing regions sampled in 1999. Within many of the subgroups revealed by cluster analysis, the majority of the isolates exhibited identical SSR genotypes.

TABLE 4. Polymorphic sequence-tagged microsatellites (STMs) detected in 63 Australian isolates of *Puccinia graminis* f. sp. *avenae*

Locus	Microsatellite primers		Allele size (bp)	Heterozygosity ^a			Allele frequencies ^a		
	5' anchored	STM		All	1999	Historical	All	1999	Historical
1	PAC6	pMS11ca	158	0.67	0.64	0.23	36.5	19.2	87.5
	...	...	163	...	...	...	35.7	46.8	3.1
	...	...	165	...	...	...	0.8	1.1	0.0
	...	...	168	...	...	...	26.9	32.9	9.4
2	...	pMS12ca	150	0.48	0.46	0.52	38.9	35.1	50.0
	...	...	155	...	...	...	61.1	64.9	50.0
3	...	pMS22ca	150	0.37	0.43	0.06	75.4	67.8	96.9
	...	...	155	...	...	...	24.6	32.2	3.1
4	...	pMS39ca	205	0.54	0.56	0.43	52.5	45.5	71.9
	...	...	220	...	...	...	4.2	5.7	0.0
	...	...	228	...	...	...	43.3	48.9	28.1
	...	pMS61ca	192	0.41	0.51	0.48	66.9	72.8	50.0
5	...	...	206	...	...	...	33.1	27.2	50.0
	...	pMS85ct	175	0.37	0.44	0.06	76.3	68.6	96.9
	...	...	185	...	...	...	23.7	31.4	3.1
Mean	...	...	...	0.47	0.49	0.30	...	...	...

^a All = all 63 isolates, 1999 = 47 isolates collected in the Australian 1999 annual pathogenicity survey, and Historical = 16 isolates collected from Australian cereal growing regions from 1971 to 1996.

Fig. 1. Dendrogram depicting patterns of genetic similarity among 63 isolates of *Puccinia graminis* f. sp. *avenae* detected by six microsatellite loci. Shown is the isolate number, race, the state where the isolate was collected, and year of collection. Values given on dendrogram branches are percent of bootstrap iterations supporting the cluster. Australian state abbreviations: NSW = New South Wales, Qld = Queensland, SA = South Australia, Vic = Victoria, WA = Western Australia.

Analysis of molecular variance statistically partitioned 65.2% of the genetic variation among the entire set of 63 *P. graminis* f. sp. *avenae* isolates and 34.8% among the two subpopulations of the 1999 survey isolates and historical isolate collection (Table 5). The moderately high F_{ST} value of 0.348 indicated significant genetic differentiation between the two subpopulations.

DISCUSSION

SSR polymorphism. The STMs developed for *P. graminis* f. sp. *tritici* revealed very little polymorphism among *P. graminis* f. sp. *tritici* isolates collected from Australian populations over a 70-year period. This finding was consistent with the low genetic diversity revealed in previous research using three multilocus DNA fingerprinting methods (AFLP, SAM, and S-SAP) on the same isolate set (18). However, the microsatellite-based SAM analysis revealed significantly higher levels of genetic variation among isolates than the other assays tested, indicating SSR evolution in Australian cereal rust populations. The higher levels of polymorphism revealed by SAM analysis relative to other multilocus fingerprinting techniques and single-locus SSRs can be explained. This assay reveals polymorphism both as presence or absence of the fragment or SSR repeat length variation. Presence or absence polymorphism arises due to sequence variation in regions flanking SSRs, regions that have been reported to be sites of point mutations (7). Higher levels of SSR polymorphism with the SAM assay compared with single-locus SSRs in the present work could arise due to the SAM assay simultaneously revealing a large number of loci; in the case of the previous study, 953 loci were scored (18). Therefore, the chances of detecting SSR polymorphism with the SAM assay were greater compared with investigating 110 SSR loci in the present work.

The sample sizes for the wheat rust pathogens tested in the present work were small, and it is highly probable that STMs found to be monomorphic would be more informative when assessed with isolates from regions outside of Australia. Of the two polymorphic codominant loci detected in *P. graminis* f. sp. *tritici*, one appeared double banded (pMS46ca), indicating two microsatellite loci or heterozygosity of the dikaryotic urediniospores as reported previously for SSR loci investigated in *P. triticina* (8) and *P. striiformis* f. sp. *tritici* (9).

Many of the STM primers developed for *P. graminis* f. sp. *tritici* were transferable across the rust pathogens tested. The conservation of SSR flanking sequences among species has been reported and thus provides a useful molecular tool for phylogenetics studies (7). More STMs amplified in or were specific to *P. graminis* f. sp. *avenae* than any of the wheat rust pathogens. Furthermore, the genetic diversity detected among *P. graminis* f. sp. *avenae* isolates representing the Australian pathogen population in 1999 ($n = 47$) was greater than that detected among 10 *P. graminis* f. sp. *tritici* isolates representing 70 years of pathogenic variation in Australia. This finding is consistent with previous research with three multilocus DNA fingerprinting methods in which the greatest genetic diversity among isolates of five Australian *Puccinia* pathogens (*P. graminis* f. sp. *avenae*, *P. graminis* f. sp. *tritici*, *P. striiformis* f. sp. *tritici*, *P. triticina*, and a variant of *P. striiformis* f. sp. *tritici* referred to as Barley Grass

Stripe Rust [34]) was revealed in *P. graminis* f. sp. *avenae* (18). The low polymorphism revealed among isolates of *P. triticina* and *P. striiformis* f. sp. *tritici* was consistent with the lack of SSR polymorphism detected in the present work, as well as previous reports where single-locus SSRs developed for *P. triticina* (8) and *P. striiformis* f. sp. *tritici* (9) revealed low polymorphism.

The genetic diversity revealed in *P. graminis* f. sp. *avenae* in this work complements the findings of annual surveys of pathogenic diversity which have reported high levels of variability in Australian *P. graminis* f. sp. *avenae* populations (1; unpublished data). The higher genetic diversity of this pathogen compared with *P. graminis* f. sp. *tritici* and other cereal rusts that have been examined in Australia is likely to be a consequence of the susceptibility of most Australian oat cultivars to *P. graminis* f. sp. *avenae*, and the large populations of *P. graminis* f. sp. *avenae* that persist in extensive wild oat communities (and other related grasses) throughout Australia. These populations enable the persistence of the disease throughout the year and provide a large inoculum pool.

An important finding of this work is that the level of polymorphism detected within one species of *Puccinia* with a particular set of SSRs does not predict or preclude their usefulness as genetic markers within another. To our knowledge, this is the first report of the successful development and application of single-locus SSRs for genetic diversity studies in a local population of a cereal rust pathogen. Also, all polymorphic codominant loci detected in *P. graminis* f. sp. *avenae* displayed the same double-banded pattern observed for *P. graminis* f. sp. *tritici* (pMS46ca), and reported previously for *P. triticina* (8) and *P. striiformis* f. sp. *tritici* (9), indicating high heterozygosity of the dikaryotic urediniospores.

Relationships between races. Cluster analysis of the STM polymorphism detected among the 63 *P. graminis* f. sp. *avenae* isolates revealed two distinct groupings, or subpopulations, of only 56% genetic similarity (Fig. 1). Within each of these primary clusters, two further subgroups formed of 72 to 78% genetic similarity. One of these subgroups contained 14 (of 16) isolates collected prior to 1999 (Table 3). These isolates were separated into two clusters of low (8%) genetic dissimilarity consisting of isolates collected from 1971 to 1978 and from 1977 to 1984. Therefore, each of these clusters represents a 7-year period, with an overlap of 2 years, and most of the isolates (except for one) composing the 1971-to-1978 cluster were collected in NSW.

The isolates collected in 1999 consisted of eight races, none of which occurred in the isolates collected prior to 1999 (Table 3). Isolates collected from 1971 to 1996 consisted of 13 races, of which six occurred in each of the 1971-to-1978 and 1977-to-1984 clusters, and races 20 and 22 occurred in both groups. Race 94+Pg13 was the most prevalent (21 of the 47 isolates collected in 1999) of the 21 races, occurring in all of the geographical regions surveyed, but it occurred in the greatest frequency in samples from NSW and Western Australia. This race also was distributed evenly throughout the groups revealed by cluster analysis (Fig. 1).

Although there is no clear difference in race composition between the two primary clusters and subgroups revealed by cluster analysis for the 63 *P. graminis* f. sp. *avenae* isolates, some of the

TABLE 5. Analysis of molecular variance for six microsatellite loci revealed in 63 isolates of *Puccinia graminis* f. sp. *avenae* representing two subpopulations: the Australian 1999 annual pathogenicity survey and a historical collection from Australian cereal-growing regions from 1971 to 1996^a

Source of variation	df	SSD	MSD	Variance component	Percent of total	P	F_{ST}
Among populations	1	27.07	27.07	1.05	34.80	<0.001	0.348
Within populations	61	120.20	1.97	1.97	62.20	...	...
Total	62	147.27	...	...	...	...	...

^a Statistics include degrees of freedom (df), sums of squared deviations (SSDs), mean squared deviations (MSDs), variance component estimates, the percentages of total variance contributed by each component, the probability (P) of obtaining a more extreme component estimate by chance alone (estimated from 1,000 sampling realizations), and the fixation index (F_{ST}) value.

distinctions may be explained, in part, by geographical origin, with many of the grouped isolates originating from NSW. The high frequency of isolates of identical race phenotype and SSR genotype collected from distant geographical regions was evidence for long-distance migration of this pathogen throughout Australia. Furthermore, AMOVA significantly partitioned 65.2% of the genetic variation among the entire isolate set (Table 5). The phenomenon of long-distance dispersal in the cereal rust pathogens, on both a continental and global scale, is well documented (4). Interestingly, the cluster analysis revealed that isolates of the same race can be genetically identical or up to 44% genetically dissimilar. Similarly, isolates of different races also showed a high incidence of clustering at 100% genetic similarity.

Overall, the clustering of isolates did not follow expectations based on virulence, indicating a poor correlation between pathogenicity and molecular variation. This finding has been reported previously for cereal rusts with various types of markers, including isozymes (26), double-stranded RNA (6), RAPDs (5), DAF (3), and AFLPs (18,27), as well as for other fungal phytopathogens (2). Examination of the SSR data generated in this work revealed that many of the groups formed by the cluster analysis (Fig. 1) were based on an allele occurring in one group but not the other (F. J. Keiper, *unpublished data*). For example, the SSR locus amplified by pMS11ca revealed four alleles of 158, 163, 165, and 168 bp across the 63 isolates. The isolates grouped in the uppermost primary cluster in Figure 1 revealed the 158- and 163-bp alleles, with the 165-bp allele occurring at a low frequency, whereas the isolates of the bottommost cluster revealed 163- and 168-bp alleles. Similarly, the discrimination of the isolates collected from 1971 to 1984 into the 1971-to-1978 and 1977-to-1984 clusters was based on the occurrence of a unique allele for one SSR locus in the 1977-to-1984 group. These findings show genome evolution; however, it has been proposed that the genome as a whole evolves at a much faster rate than genes governing virulence (5,19).

Race evolution. The evolution toward greater virulence in the *P. graminis* f. sp. *avenae* population is evident in this collection of races, with a shift in the virulence phenotype over time (Table 3). In the isolates sampled from 1971 to 1984, virulence for resistance genes *Pg1*, *Pg2*, *Pg3*, *Pg4*, *PgSa*, *Pg9*, and *Pg13* was present in various combinations; however, the number of genes for which individual races were avirulent far outnumbered the number of genes for which each was virulent. Also, no race sampled prior to 1996 exhibited virulence on resistance genes *Pg8*, *Pg13*, or *Pga*. By 1996, virulence phenotypes more closely resembled those observed for the isolates collected in 1999, with virulence on the majority of resistance genes assayed (five to nine genes), and avirulence on few (one to three) genes. These observations were complemented by the heterozygosities obtained in this work (Table 4). The more virulent isolates (those collected in 1999) exhibited higher heterozygosities at each SSR locus compared with the less virulent isolates collected prior to 1996.

The predominant races sampled in this study were virulent on the greatest number (at least seven) of resistance genes. Race 94 and its derivatives comprised the majority of the isolates collected in 1999. The 16 historical isolates exhibited 14 races, with race 22 occurring in both 1972 and 1980 and race 20 occurring in both 1978 and 1982. These races generally were more virulent (*Pg2*, *Pg3*, and *Pg4*) compared with the other races collected prior to 1984 (Table 3), enabling them to persist in the pathogen population over time. Genetic variation between the two isolates collected in different years for each race was evident with separate clustering (1971-to-1978 and 1977-to-1984 groups) in Figure 1. The identities of resistance genes in some Australian oat cultivars are known; however, the information available is not comprehensive. Cultivars deployed over the past 20 to 30 years have carried *Pg2* (e.g., Kalgan), *Pg4* (e.g., Bettong), *Pga* (e.g., Culgoa II), or combinations such as *Pg1* and *Pg2* (e.g., Glider) and *Pg2*,

Pg3, and *Pg4* (e.g., Quamby). Virulence for all of these genes or gene combinations, except gene *Pga*, existed in at least some races isolated between 1971 and 1984. It is possible that the cultivars carrying these genes may have influenced the frequencies of corresponding virulences in the pathogen population. Virulence for the resistance gene *Pga* first was detected in 1996 in Queensland (23). This gene first was deployed in Australia in Queensland in 1991, and an additional five cultivars carrying *Pga* were released in that state from 1991 to 1996. The initial detection of virulence for *Pga* in 1996, followed by an increase in the frequency of this virulence, is no doubt related to the deployment of cultivars carrying this gene. The five isolates with virulence for *Pga* that were included in the present study were found to have three different SSR genotypes, suggesting independent mutations to virulence for this resistance gene. One of these races, 94+*Pg13*, *PgSa*, *Pga*, (isolates 22, 25, and 41, Table 3) is virulent on all of the differential genotypes used to classify races and also is virulent on all current Australian oat cultivars.

Population evolution. The evolution of subpopulations and clonal lineages in cereal rust pathogens in Australia is a well-documented phenomenon. For the wheat rust pathogens, where sexual recombination is absent, ongoing surveys of pathogenic variation have provided strong evidence for the evolution of subpopulations through the introduction of exotic, genetically distinct isolates, followed by mutational events. For pathogens like the rusts, where host-pathogen coevolution is strong due to cultivar replacement, population evolution is associated with random mutation from avirulence to virulence, believed to occur via a single-step mutation, followed by host selection (28,35). Distinct subpopulations within the Australian oat crown rust pathogen (*P. coronata* f. sp. *avenae*) population also were revealed through analysis of pathogenicity and DNA amplification fingerprinting (3).

The findings of the present work indicated the existence of up to five lineages of *P. graminis* f. sp. *avenae* during the time period investigated. The two groups revealed in the cluster analysis indicated two distinct lineages. Further clustering patterns within these two primary groups suggested that several genetically distinct founding isolates have been sampled in this work. For example, within the period from 1971 to 1996, cluster analysis demonstrated low similarity between race 8, collected in 1971, and race 42, also collected in 1971. Race 8 also exhibited low genetic similarity to the other isolates collected prior to 1996, and most of the isolates collected in 1999. This indicated that the two isolates collected in 1971 were (or originated from) separate introductions and belonged to separate lineages in the pathogen population. The two groups formed by the cluster analysis of isolates collected from 1971 to 1978 (including race 42) and 1977 to 1984 suggested a third lineage. However, the genetic dissimilarity between the two groups was low at $\approx 8\%$. Therefore, these subgroupings may reflect lineages arising from two separate introductions of genetically similar isolates, or provide evidence for the evolution of a second lineage (1977-to-1984 group) from the first (1971-to-1978 group). The evolution of lineages with similar genetic backgrounds is believed to be the result of mutational events (3), and evidence for this process in the present work was the occurrence of a single unique allele in the 1977-to-1984 group. Such events are believed to occur at a high frequency in a large pathogen population (3). Race 41+*Pg13*, collected in 1996, exhibited high genetic similarity to races collected in 1999, and may represent a fourth lineage giving rise to races observed in the 1999 collection, or a separate introduction of a genetically similar isolate.

In conclusion, the results of the present work illustrate the dynamic population structure of *P. graminis* f. sp. *avenae* in Australia over a 30-year period. We demonstrated for the first time that it is possible to develop informative SSRs to examine the relationships between isolates and races of local cereal rust

populations. The STM markers were highly reproducible and highly transferable between two formae speciales of *P. graminis*, indicating that these markers do not need to be developed for each pathogen under investigation. Genetic analysis revealed the presence of several subpopulations and lineages in the *P. graminis* f. sp. *avenae* population. However, correlations between molecular variation and diversity in race or changes in virulence were not evident. It is possible that these correlations may improve with larger numbers of informative markers.

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