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Genetic variation among Australasian pathotypes of P. hordei using a PCR-fingerprinting technique

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Two PCR-fingerprinting primers, $(GACA)_4$ and M13, were tested across 22 pathotypes of *Puccinia hordei* Otth collected from Australasia over a 30 year period, to assess their usefulness in revealing genetic variability in this pathogen. Both primers revealed polymorphisms among the pathotypes, with $(GACA)_4$ generating a higher level of polymorphism. Molecular analyses revealed evidence of clonality among the *P. hordei* pathotypes, supporting the hypothesis that some arose from mutational changes in the pathogenicity of a founding pathogen genotype. Evidence was also obtained of sexual recombination within *P. hordei* in Australia on the alternate host *Ornithogalum umbellatum*. This is the first study of genetic variation among Australasian pathotypes of *P. hordei* using a PCR-fingerprinting technique.

Key words: *Puccinia hordei*, genetic diversity, fingerprinting, $(GACA)_4$, M13.

INTRODUCTION

The fungus *Puccinia hordei* (*Ph*) belongs to the genus *Puccinia*, the largest genus of the order Pucciniales with 3,000 to 4,000 species (Littlefield, 1981). *Ph* is the casual agent of barley leaf rust, an economically important disease which affects barley production in many parts of the world (Clifford, 1985). The pathogen is present in all barley growing regions of Australia (Park et al., 2003), reaching epidemic levels in Queensland during 1978, 1983, 1984 and 1988 (Cotterill et al., 1995). A severe epidemic of leaf rust can reduce the yield of a susceptible cultivar by up to 62% (Cotterill et al., 1992), and significant yield losses have been experienced in Australia (Cotterill et al. 1995; Cotterill et al., 1992; Waterhouse, 1927), New Zealand (Arnst et al., 1979), Europe and the USA (Griffey et al., 1994; Melville et al., 1976). *Ph* is a macrocyclic and heteroecious rust pathogen that forms its aecial stage on various species of *Ornithogalum*, *Leopoldia* and *Dipcadi* in the family Liliaceae (Clifford, 1985).

Different barley genotypes with resistance genes, known collectively as a differential set, were used by Levine and Cherewick (1952) and Clifford (1977) to characterise pathotypes (pts) among different isolates of *Ph*. The differential set used to characterise pts of *Ph* at the University of Sydney, Plant Breeding Institute (PBI) comprises 30 different barley genotypes with one or more resistance (*Rph*) genes (Park, 2003). The first assessment of pathogenic variability in *Ph* in Australia

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was made in 1920 by Waterhouse (1927), who detected two pts, one similar to a European pt and another that differed in virulence on some genotypes compared to a pt found in North America (Waterhouse, 1952; Watson and Butler, 1947). In a later Australian study, Cotterill et al. (1995) found substantial pathogenic variation among *Ph* isolates collected between 1966 and 1990. This study identified 11 different pts among 154 isolates, of which pt 210P$^+$ was the most common. Up to 1995, virulence was detected for the leaf rust resistance genes *Rph1*, *Rph2, Rph4, Rph5*, *Rph6*, *Rph8*, *Rph9* and *Rph12*, and the genes *Rph3* and *Rph7* remained effective (Cotterill et al., 1995). Pathotype 4610P$^+$ virulent on *Rph12* was first detected in 1991 from Tasmania, after which (1996 to 2002), more pathogenic variation was detected in *Ph* including the identification of two new *Rph12* virulent pathotypes (pts) with added virulence for the resistance gene *Rph10* (viz. pts 5610P$^+$ and 5453P$^-$) (Park, 2003). While no virulence was detected in these studies for genes *Rph3*, *Rph7*, *Rph11*, *Rph14*, *Rph15* and *Rph18* (Park, 2003), virulence for *Rph3* was detected in 2009 (pt 5457P$^+$) in northern New South Wales (NSW) (Park, 2010). This pathotype is believed to have arisen from pt 5453P$^-$, first detected in Western Australia in 2001 (Park, 2006), via sequential single step mutations for virulence to *Rph19* (pt 5453P$^+$) and then *Rph3* (pt 5457P$^+$) (Park et al., 2015; Park, 2010).

While annual surveys of pathogenic variability in rust pathogens that infect cereal crops in Australia have provided evidence that variation arises via either the introduction of exotic genotypes, simple mutation, and asexual hybridisation (Wellings and McIntosh, 1990), sexual recombination is also thought to contribute to variability in the case of *P*. *hordei* (Park, 2008; Park et al., 1995). The alternate host *Ornithogalum umbellatum* occurs in Australia, where it is present on the Yorke Peninsula of South Australia (SA) (Wallwork et al., 1992) and in the Murrumbidgee catchment areas including Henty and Junee in NSW. While six pts of *Ph* were identified among uredinial isolates derived from aeciospores collected from infected plants of *O. umbellatum* from the Yorke Peninsula (Wallwork et al., 1992), the contribution of sexual recombination to overall genetic variability in *Ph* in Australia is largely unknown.

Although information on variability obtained from pathogenicity on differential genotypes is important in the genetic control of rusts, it is of limited use in assessing genetic variation in these pathogens. Both biochemical and molecular markers have been applied to evaluate genetic diversity among various plant pathogens (McDermott and McDonald, 1993). Amplified fragment length polymorphism (AFLP) analyses were used to study genetic diversity among isolates of *Ph* in relation to their virulence (Sun et al., 2007). This study revealed an association between molecular diversity and virulence patterns in *Ph* isolates collected from different geographical regions of the world. Keiper et al. (2003) studied the genetic structure of several cereal rust pathogens using various polymerase chain reaction (PCR) based tools like AFLP, selectively amplified microsatellites (SAM) and sequence-specific amplification polymorphisms (S-SAP). This study was able to discriminate fungal pathogens from five rust taxa [*P. triticina* (*Pt*), *P. graminis* f. sp*. tritici* (*Pgt*), *P. striiformis* f. sp*. tritici* (*Pst*), barley grass stripe rust caused by *P. striiformis* f. sp*. pseudohordei* (*Psph*) and *P. graminis* f. sp*. avenae* (*Pga*)], although the level of polymorphism observed within individual taxa was low. In a separate study that used AFLPs and random amplified polymorphic DNA (RAPDs), Steele et al. (2001) found no polymorphism among Australian and New Zealand isolates of *Pst*. However, the same AFLP primers showed five to 15% polymorphic fragments among isolates of *Pst* from the UK, Denmark and Colombia. These results were consistent with clonality in Australian populations of *Pst*. Microsatellites, or simple sequence repeats (SSRs) have also been developed and applied to study polymorphisim among different rust pathogens (Dambroski and Carson, 2008; Kolmer et al., 2011; Ordoñez et al., 2010; Mantovani et al., 2010; Keiper et al. 2006; Visser et al., 2011; Karaoglu and Park, 2014).

Another useful tool for assessing genetic diversity is “PCR-fingerprinting”. This technique uses microsatellites $(GACA)_4$ and $(GTG)_5$ and the minisatellite M13 derived from the core sequence of the wild type phase M13 bacterium, as single primers in PCR to amplify hypervariable DNA sequences (Meyer et al., 2001). The PCR-fingerprinting technique has been used successfully to reveal polymorphism among various fungal and bacterial pathogens. For example, Vuyst et al. (2008) used $(GTG)_5$ to identify acetic acid bacteria in cocoa beans and the primers GTG, GACA and M13 were used to study population dynamics in several human pathogens (Cogliati et al., 2007; Delhaes et al., 2008; Meyer et al., 2001; Roque et al., 2006; Trilles et al., 2008). Selective amplification of the microsatellite polymorphic loci (SAMPL) markers $(GACA)_4$ + H-G and R1 + H-G were used to study polymorphism among 44 (25 Australasian and 19 European) isolates of *Phragmidium violaceum* (causal agent of blackberry rust), revealing more diversity in European isolates than in Australasian isolates, with 37 and 22% polymorphic loci, respectively (Gomez et al., 2006). In all of these studies, the primers GACA and M13 generated the most discriminating and informative DNA profiles. Efforts have been made for the first time to study genetic variation in Australasian populations of *Ph* using PCR-fingerprinting profiles with primers $(GACA)_4$ and M13.

MATERIALS AND METHODS

Isolates of pathogens and DNA extraction

A total of 22 pts of *Ph*, comprising 20 from Australia and two from

Table 1. Details of *Puccinia hordei* pathotypes and control pathotypes of *P. triticina*, *P. graminis* f. sp. *tritici*, *P. striiformis* f. sp. *tritici*, *P. graminis* f. sp. *avenae* and *P. striiformis* f. sp. *pseudohordei* analysed using PCR-fingerprinting markers $(GACA)_4$ and M13.

Isolate ID	Pathogen	Pathotype	Culture No.	Origin	Host/Cultivar	Year
1-*Ph*	*P. hordei*	$211P^+$	484	Coonamble, NSW	Barley/O'Connor	1992
2-*Ph*	*P. hordei*	$220P^+$	485	Yanco, NSW	Barley/Nigrinudum	1992
3-*Ph*	*P. hordei*	$253P^-$	490	Grafton, NSW	Barley/?	1992
4-*Ph*	*P. hordei*	$243P^+$	537	Grafton, NSW	Barley/?	1999
5-*Ph*	*P. hordei*	$200P^+$	570	Yanco, NSW	Barley/Gus	2002
6-*Ph*	*P. hordei*	$232P^+$	506	Balaclava, SA	Barley/Galleon	1994
7-*Ph*	*P. hordei*	$201P^-$	480	St Leonards, VIC	Barley/?	1992
8-*Ph*	*P. hordei*	$201P^+$	481	Rochester, VIC	Barley/?	1992
9-*Ph*	*P. hordei*	$242P^+$	531	Borung, VIC	Barley/?	1998
10-*Ph*	*P. hordei*	$5653P^-$	569	Byaduk, VIC	Barley/Franklin	2002
11-*Ph*	*P. hordei*	$243P^+$	489	Monto, QLD	Barley/?	1992
12-*Ph*	*P. hordei*	$243P^-$	507	Toowoomba, QLD	Barley/Dampier	1994
13-*Ph*	*P. hordei*	$5453P^-$	560	Esperance, WA	Barley/Schooner	2002
14-*Ph*	*P. hordei*	$5653P^+$	584	Wongan Hills, WA	Barley/?	2004
15-*Ph*	*P. hordei*	$4610P^+$	491	Cressy, TAS	Barley/Franklin	1992
16-*Ph*	*P. hordei*	$5653P^+$	542	Glen Esk, TAS	Barley/Gairdner	2000
17-*Ph*	*P. hordei*	$211P^-$	483	Aorangi, NZ	Barley/?	1992
18-*Ph*	*P. hordei*	$231P^+$	486	Aorangi, NZ	Barley/?	1992
19-*Ph*	*P. hordei*	$5610P^+$	520	Ravensthorpe, WA	Barley/?	1997
20-*Ph*	*P. hordei*	$220P^+$	577	SA	*O. umbellatum*	2003
21-*Ph*	*P. hordei*	$200P^-$	518	SA	Barley/?	1995
22-*Ph*	*P. hordei*	$5457P^+$	612	Legume, QLD	Barley/?	2009
23-*Pt*	*P. triticina*	104-2,3,(6),(7),11	423	Mt Derimut, VIC	Wheat/Nebraska	1984
24-*Pgt*	*P. graminis* f. sp. *tritici*	194-2,3,7,8,9	344	Hermitage, QLD	Wheat/?	1980
25-*Pst*	*P. striiformis* f. sp. *tritici*	110 E143 A^+	444	Richmond, TAS	Wheat/Hartog	1987
26-*Psph*	*P. striiformis* f. sp. *pseudohordei*	981549	589	Turretfield, SA	Barley/?	1998
27-*Pga*	*P. graminis* f. sp. *avenae*	41+*Pg9*	496	Rutherglen, VIC	Oat/?	1993

Source: Cereal Rust Collection, University of Sydney, PBI, Cobbitty.

New Zealand, along with isolates of five control pathogens (*Pt*, *Pgt*, *Pst*, *Psph* and *Pga*) were included in this study (Table 1). All the pts used in the study were sourced from the rust collection maintained in liquid nitrogen at PBI, University of Sydney. The *Ph* pts used were selected to represent those identified in different regions within Australia and New Zealand in annual pathogenicity surveys conducted from 1980 to 2009.

Freshly collected urediniospores were desiccated over silica for 12 h. A sample of 25 to 30 mg of urediniospores of each rust isolate was put in labelled Lysing Matrix C tubes (Impact resistant tubes with 1.0 mm silica spheres, Mp Biomedical, Ohio, USA). One milliliter of 2x Cetyl-trimethylammonium bromide (CTAB) extraction buffer [(CTAB 2% (w v^{-1}), 20 mM EDTA (pH 8.0), 1.4 M NaCl, Polyvinylpyrrolidone (PVP; 40000 MW) 1% (w v^{-1}), 100 mM Tris-HCl (pH 8.0) and ddH_2O (double distilled autoclaved water)] was added to each sample, mixed well by inversion and tubes were submerged in ice for 2 min. Tubes were then shaken for 15 s on a FastPrep® Cell Distrupter (M.P. Biomedicals, Irvine, CA, USA) at speed 6, returned to ice for 3 min and shaken again for 20 s at the same speed. Tubes were kept in a pre-warmed water bath at 65˚C for 30 min and inverted every 10 min, after which they were removed, mixed well by inversion and the solution in each tube/sample was divided (~500 µl in each tube) into two new 1.5 ml Eppendorf tubes to generate duplicate extractions. DNA extraction was carried in a fume hood by adding ~ 250 µl of cold phenol, followed by ~ 250 µl of cold chloroform: isoamyl alcohol (24:1 v v^{-1}), to each tube. Samples were mixed gently by inverting (~ 100 times) the tubes until a thick emulsion formed. Tubes were centrifuged at 13,000 rpm for 15 min and the supernatant was transferred into sterile 1.5 ml Eppendorf tubes. The process of phenol and chloroform: isoamyl alcohol extraction was repeated. About 50 µl of 3 M NaOAc and ~ 500 µl of cold isopropanol were added to each tube and tubes were then stored at -20˚C. The following day, the tubes were centrifuged at 13,000 rpm for 30 min and the DNA pellet thus formed was drained carefully. The pellets were washed with 500 µl of ethanol (70% v v^{-1}), centrifuged at 13,000 rpm for 15 min, drained carefully and allowed to air dry. The dried pellet was re-suspended in 100 µl ddH_2O and stored overnight at 4˚C. The following day, 5 µl of Rnase-A (10 µg $µl^{-1}$) was added to each tube and incubated at 37˚C for 2 h. All DNA samples were quantified using a Nanodrop ND-1000 spectrophotometer (Nanodrop® Technologies) and diluted to working dilution of 10 ng $µl^{-1}$ using ddH_2O.

PCR-fingerprinting

Two oligonucleotide primers were used in fingerprinting the isolates

of *Ph* and control pts: The microsatellite-specific [$(GACA)_4$ (5'GACAGACAGACAGACA3')] (Ali et al., 1986; Meyer et al., 2001) and [M13 (5'GAGGGTGGCGGTTCT3') minisatellite specific core sequence derived from the wild-type phage M13 vector] (Vassart et al., 1987; Meyer et al., 2001).

PCR reactions were performed in a final volume of 50 µl which contained 3.0 µl of genomic DNA (10 ng μl^{-1}), 5.0 µl of dNTPs (0.2 mM), 5.0 µl of 10x PCR buffer (NH4 Reaction buffer, Bioline), 3.0 µl of 50 mM $MgCl_2$ (Bioline), 5.0 µl of primer (2 mM), 0.5 µl (5 u μl^{-1}) of *Taq* DNA (Immolase DNA polymerase from Bioline) and 28.5 µl of ddH_2O. PCR amplification profile comprised of an initial denaturation step at 94°C for 5 min, followed by 35 cycles of 30 s denaturation at 94°C, 60 s annealing at 47°C if M13 or at 40°C if $(GACA)_4$ primer was used, 30 s extension at 72°C and a final extension of 7 min at 72°C. Reactions were performed in a 96-well DNA theromocycler (Eppendorf Mastercycler, Germany). PCR products were concentrated to 30 µl by placing in a fan forced oven for 45 min at 65°C and resolved on 2% high resolution agarose (MetaPhor® Agarose, Lonza, Rockland Inc.USA) gels at 80 V electrophoresis for 6 h. Five kilobite DNA marker HyperLadder™ III (Bioline) was used as reference. The separated fragments were visualised under an ultra violet light unit fitted with a GelDoc-IT UVP Camera (Bio-rad, Australia Pty. Ltd. Gladesville NSW).

Data analyses

Gel images were scored and analysed using the software GelCompar II (6^{th} edition, Applied Maths, Belgium). Fragment position optimisation and tolerance was set to 1 and 1.5%, respectively. Fragments were selected automatically by the GelCompar and unclear fragments were deselected manually. Based on the standard DNA ladder used, molecular weights of selected fragments were assigned automatically. Fragment scoring for the both primers ranged from 500 to 2500 bp. Genetic diversity among the *Ph* pts examined was evaluated using Unweighted pair group method for arithmetic averages (UPGMA) cluster analyses based on a distance matrix calculated using the Dice coefficient of similarity. The quality of similarity clusters was tested using the cluster validity index Cophenetic correlation coefficient (CPCC) using software GelCompar II. The CPCC was used to test the efficiency of the similarity clusters that resulted from the individual analyses of markers M13 and $(GACA)_4$. The CPCC is a simple correlation coefficient between the original dissimilarity matrix and the final dissimilarity matrix (Cophenetic matrix) produced after the clustering algorithm recalculates the dissimilarities (Lessig, 1972). Dendrograms were constructed and based on similarity clusters of both primers $(GACA)_4$ and M13, the *Ph* pts were clustered accordingly.

RESULTS

Both oligonucleotides $(GACA)_4$ and M13 amplified all pts, producing fragments in the range of 500 to 2500 bp. After deselecting unclear fragments manually, a total 27 and 28 fragments were scored automatically for markers $(GACA)_4$ and M13, respectively (Table 3). The UPGMA similarity dendrograms produced from the cluster analyses based on markers $(GACA)_4$ and M13 data grouped all 22 *Ph* pts and control pathogens (Figures 1 and 2). Both primers $(GACA)_4$ (Figure 1) and M13 (Figure 2) out-grouped representative control isolates of *Pt*, *Pgt*, *Pst*, *Psph* and *Pga* from the *Ph* pts examined. Both fingerprinting primers produced distinct clades for *Pst* and *Psph*, *Pgt* and *Pga*, while *Pt* was in a standalone group (Figures 1 and 2).

Cluster analysis based on marker M13 produced seven groups among the *Ph* pts with 75.9% to 100% similarities (Figure 2), while marker $(GACA)_4$ revealed higher variability among the *Ph* pts and produced 10 different groups with 70.5 to 100% similarities (Figure 1). Markers clustered pts $211P^-$ and $231P^+$ together (Figures 1 and 2), both of which originated from New Zealand.

Marker $(GACA)_4$ resolved the greatest genetic variation among the *Ph* pts and different "GACA" and "M13" groups were defined (Table 2). GACA group one (^{GGP1}) contained pts $211P^+$, $220P^+$, $253P^-$, $243P^+$, $200P^+$, $232P^+$, $201P^-$, $201P^+$, $242P^+$ and $243P^-$. All 10 pts have virulence for *Rph8* in common (Park 2003). Marker M13 also grouped these pts in one group (^{MGP2}), except pts $201P^+$ and $243P^-$, which were grouped in ^{MGP1} (Table 2). Both markers out-grouped pts $200P^-$ (^{GGP8} and ^{MGP3}), $5653P^+$ (^{GGP3} and ^{MGP4}) and $5653P^-$ (^{GGP6} and ^{MGP5}) from all others (Table 2). Pathotype $200P^-$ is virulent to *Rph8* only whereas pt $5653P^-$ carries aditional virulence for genes *Rph1*, *Rph2*, *Rph4*, *Rph6*, *Rph9*, *Rph10* and *Rph12*. Pathotype $5653P^+$ posses additional virulence for *Rph19* compared to pt $5653P^-$ (Park, 2003). In both cases, pts $211P^-$ and $231P^+$ were grouped together in distinct groups of GACA (^{GGP7}) and M13 (^{MGP7}) as detailed in Table 2.

Marker $(GACA)_4$ produced distinct clusters for pts $5453P^-$ (Isolate 13-*Ph*) and $5457P^+$ (Isolate 22-*Ph*) but with 90.9% similarity (Figure 1), whereas these two pts were shown to be 100% similar (Figure 2) when genotyped using the fingerprinting marker M13. Pathotype $5457P^+$ carries additional virulence for *Rph3* and *Rph19* as compared to the pt $5453P^-$ though both pts share virulence for genes *Rph1*, *Rph2*, *Rph4*, *Rph6*, *Rph9*, *Rph10*, *Rph12* (Park et al., 2015). Marker $(GACA)_4$ grouped pts $243P^+$, $4610P^+$ and $5653P^+$ together in a single group (^{GGP4}) and discriminated pt $220P^+$ in a distinct group ^{GGP5} (Table 2), but genotyping based on M13 marker grouped these four pts in a group (^{MGP2}) with other pts (Table 2).

DISCUSSION

The evolution of new virulent pts of *Ph* is a significant constraint in the economical production of barley in Australia and worldwide. Understanding genetic diversity in *Ph* is fundamental in the efforts to develop cultivars of barley with resistance to this pathogen. For example, genetically diverse fungal pathogens may have a greater potential to evolve new pts with the ability to overcome resistance. In earlier work, six pts of *Ph* were identified from aeciospores collected from infected plants of *O. umbellatum* in SA (Wallwork et al., 1992). Furthermore, high diversities of *Ph* pts have been reported in SA in pathogenicity surveys, suggesting that sexual recombination is contributing to pathogen diversity (Park, 2010).

Table 2. Groups of *P. hordei* pathotypes based on the cluster analyses using PCR-fingerprinting markers $(GACA)_4$ and M13.

Isolate ^{MGP}	Pathotype	Isolate ^{GGP}	Pathotype	Virulence to *Rph* genes*
8 ^{MGP1}	201P$^+$	1^{GGP1}	211P$^+$	*Rph1*, *Rph4*, *Rph8*, *Rph19*
12 ^{MGP1}	243P$^-$	2 ^{GGP1}	220P$^+$	*Rph5*, *Rph8*, *Rph19*
16 ^{MGP1}	5653P$^+$	3 ^{GGP1}	253P$^-$	*Rph1*, *Rph2*, *Rph4*, *Rph6*, *Rph8*
1 ^{MGP2}	211P$^+$	4 ^{GGP1}	243P$^+$	*Rph1*, *Rph2*, *Rph6*, *Rph8*, *Rph19*
2 ^{MGP2}	220P$^+$	5 ^{GGP1}	200P$^+$	*Rph8, Rph19*
3 ^{MGP2}	253P$^-$	6 ^{GGP1}	232P$^+$	*Rph2*, *Rph4*, *Rph5*, *Rph8*, *Rph19*
4 ^{MGP2}	243P$^+$	7 ^{GGP1}	201P$^-$	*Rph1*, *Rph8*
5 ^{MGP2}	200P$^+$	8 ^{GGP1}	201P$^+$	*Rph1*, *Rph8*, *Rph19*
6 ^{MGP2}	232P$^+$	9 ^{GGP1}	242P$^+$	*Rph2*, *Rph6*, *Rph8*, *Rph19*
7 ^{MGP2}	201P$^-$	12 ^{GGP1}	243P$^-$	*Rph1*, *Rph2*, *Rph6*, *Rph8*
9 ^{MGP2}	242P$^+$	19 ^{GGP2}	5610P$^+$	*Rph4*, *Rph8*, *Rph9*, *Rph10*, *Rph12*, *Rph19*
11 ^{MGP2}	243P$^+$	14 ^{GGP3}	5653P$^+$	*Rph1*, *Rph2*, *Rph4*, *Rph6*, *Rph8*, *Rph9*, *Rph10*, *Rph12*, *Rph19*
15 ^{MGP2}	4610P$^+$	11 ^{GGP4}	243P$^+$	*Rph1*, *Rph2*, *Rph6*, *Rph8*, *Rph19*
19 ^{MGP2}	5610P$^+$	16 ^{GGP4}	5653P$^+$	*Rph1*, *Rph2*, *Rph4*, *Rph6*, *Rph8*, *Rph9*, *Rph10*, *Rph12, Rph19*
20 ^{MGP2}	220P$^+$	15 ^{GGP4}	4610P$^+$	*Rph4*, *Rph8*, *Rph9*, *Rph12*, *Rph19*
21 ^{MGP3}	200P$^-$	20 ^{GGP5}	220P$^+$	*Rph5*, *Rph8*, *Rph13*, *Rph19*
14 ^{MGP4}	5653P$^+$	10 ^{GGP6}	5653P$^-$	*Rph1*, *Rph2*, *Rph4*, *Rph6*, *Rph8*, *Rph9*, *Rph10 Rph12*
10 ^{MGP5}	5653P$^-$	17 ^{GGP7}	211P$^-$	*Rph1*, *Rph4*, *Rph8*
13 ^{MGP6}	5453P$^-$	18 ^{GGP7}	231P$^+$	*Rph1*, *Rph2*, *Rph4*, *Rph5*, *Rph8*, *Rph19*
22 ^{MGP6}	5457P$^+$	21 ^{GGP8}	200P$^-$	*Rph8*
17 ^{MGP7}	211P$^-$	13 ^{GGP9}	5453P$^-$	*Rph1*, *Rph2*, *Rph4*, *Rph6*, *Rph9*, *Rph10*, *Rph12*
18 ^{MGP7}	231P$^+$	22 ^{GGP10}	5457P$^+$	*Rph1*, *Rph2*, *Rph3*, *Rph4*, *Rph6*, *Rph9*, *Rph10*, *Rph12*, *Rph19*

Isolate: Isolate ID as given in Table 1; ^{MGP}Groups of *P. hordei* pathotypes based on M13 analysis; ^{GGP}Groups of *P. hordei* pathotypes based on GACA analysis; *with respect to the resistance genes listed in Park (2003), virulence to *Rph* genes shown in last column is corresponding to the pathotypes shown in the previous column.

Prior to the current study, no attempt had been made to study the genetic diversity of *Ph* in Australia, using PCR-fingerprinting. The usefulness of the PCR-fingerprinting primers M13 and GACA in discriminating fugal pathogens has been shown in several studies (Cogliati et al., 2007; Delhaes et al., 2008; Meyer et al., 2001; Roque et al., 2006; Trilles et al., 2008). In view of this, PCR-fingerprinting primers M13 and $(GACA)_4$, were assessed for their utility in *Ph*.

Cluster analyses of marker data revealed seven to 10 clusters among the 22 *Ph* pts and both markers out-grouped the control pathogens. As expected, a high percentage of similarity was observed among the *Ph* clusters, whereas the control pathogens were more diverse. Both PCR-fingerprinting primers $(GACA)_4$ and M13 clearly differentiated *Pt*, *Pgt*, *Pst*, *Psph*, *Pga* from each other and from the pts of *Ph*. Markers M13 and $(GACA)_4$ revealed only 26.4 and 33.3% genetic similarities between *Ph* and the control rust pts. These findings are in accordance with earlier studies in which isolates of *Pgt* were clearly differentiated from isolates of *Ph* using AFLP markers (Sun et al., 2007).

Both markers distinguished *Pst* and *Psph* with 57.1 to 83.3% genetic similarities, which is in accordance with an earlier study of these rust pathogens by Keiper et al. (2003) in which *Pst* and *Psph* were distinct but more similar compared to other rust pathogen species. Both markers M13 and $(GACA)_4$ formed distinct clades of *Pga* and *Pgt* and differentiated these two from the wheat rust pathogens *Pst* and *Pt*, also consistent with earlier results of an AFLP study on these rust pathogens (Keiper et al., 2003). The current results support the informative value and usefulness of the PCR-fingerprinting markers in differentiating species of rust pathogens.

The PCR-fingerprinting primer M13 clustered the 22 *Ph* pts into seven groups, while the marker $(GACA)_4$ resolved 10 groups among the *Ph* pts (Table 2) and detected more polymorphism. Interestingly, both markers grouped *Ph* pts 211P- and 231P$^+$ with 100% similarity (^{GGP7} and ^{MGP7}, Table 2) and differentiated them from all other *Ph* pts. Both pts originated from New Zealand and differ only in virulence on *Rph2*, *Rph5* and *Rph19*. It is therefore possible that these two pts are simply related and their distinctiveness from the Australian pts indicates that *Ph* populations in the two countries are distinct. This contrasts with results from long-term surveys of pathogenic variability in wheat rust pathogens across Australia and New Zealand, which have provided

Table 3. GelCompar selected fragments accross the amplifications produced by PCR-fingerprinting markers $(GACA)_4$ and M13 where unclear fragments were deselected manually.

S/N	$(GACA)_4$ fragments (bp)	M13 fragments (bp)
1	510	543
2	550	557
3	572	571
4	584	668
5	675	768
6	707	811
7	741	853
8	765	895
9	790	914
10	839	950
11	956	999
12	961	1023
13	1061	1084
14	1089	1122
15	1126	1195
16	1173	1255
17	1278	1327
18	1276	1410
19	1380	1542
20	1502	1574
21	1568	1629
22	1639	1699
23	1852	1787
24	2000	1875
25	2078	2014
26	2250	2140
27	2480	2268
28		2485

substantial evidence of rust migration between the two land masses (Luig, 1985). These studies have also provided evidence that wheat rust movement is predominantly from west to east (Luig, 1985; Wellings et al., 2003). In view of this, the distinctiveness of the two pts of *Ph* from New Zealand from those in Australia suggests that they may have originated from a region outside Australasia and that they have remained localized to New Zealand.

Based on pathogenicity, Cotterill et al. (1995) suggested that the appearance of a group of pts distinct from pt $243P^-$ and typified by pt $200P^-$ and its subsequent single-step mutations in the form of pts $201P^-$, $210P^-$ and $220P^-$in the 1980s, may have resulted from an exotic incursion. The present results support this hypothesis.

Studies of pathogenic variability in all three wheat rust pathogens in Australia have provided strong evidence of clonality, with presumed clonal lineages comprising closely related pts derived by sequential single-step mutations from a common ancestor (Keiper et al., 2006). In contrast, pts of *Ph* detected in Australia between 1992 and 2001 did not appear to be so simply related based on pathogenicity (Park, 2003). Of the pts examined in the present study, pt $5457P^+$ is believed to have originated from pt $5453P^-$ via step-wise mutation for virulence for *Rph19* and then for *Rph3* (Park, *unpublished*). Surprisingly, while markers $(GACA)_4$ and M13 grouped these two pts and separated them from all other pts, they were not identical (Figures 1 and 2, respectively). These results show that the relationship between these two pts is not as simple as thought.

The molecular analyses in the present study did, however, provide some evidence of clonal lineages in *Ph* in Australasia. Marker $(GACA)_4$ revealed pts $201P^+$ and $201P^-$ to be 100% genetically similar (Figure 1) and given that pt $201P^+$ differs from $201P^-$ only in being virulent for *Rph19*, together these results are consistent with pt $201P^+$ arising via a single step mutation in pt $201P^-$ with

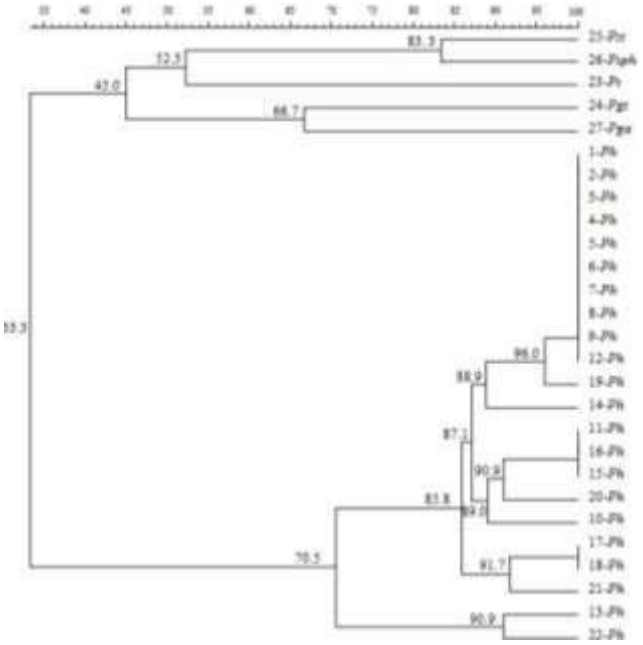

Figure 1. Genetic similarity dendrogram of 22 *P. hordei* pathotypes and five control pathotypes (*Pt*, *Pgt*, *Pst*, *Psph* and *Pga*) based on PCR-fingerprinting marker $(GACA)_4$ data. UPGMA cluster analyses conducted using Dice coefficient of similarity. Similarity percentage values are shown on the left hand side of the group nodes. Pathotypes detail is provided in Table 1.

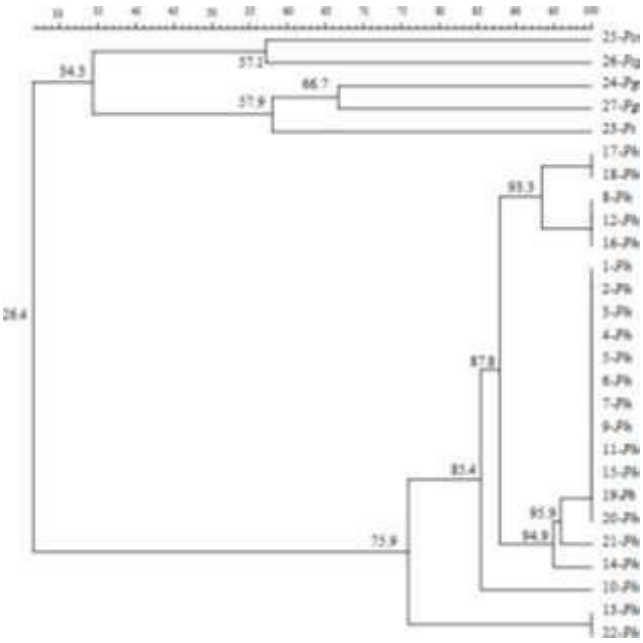

Figure 2. Genetic similarity dendrogram of 22 *P. hordei* pathotypes and five control pathotypes (*Pt*, *Pgt*, *Pst*, *Psph* and *Pga*) based on PCR-fingerprinting marker M13 data. UPGMA cluster analyses conducted using Dice coefficient of similarity. Percent similarity values are shown on the left hand side of the group nodes. Pathotypes detail is provided in Table 1.

added virulence for *Rph19*. The lack of molecular variation among some of the pts studied support the hypothesis of single-step mutation being an important source of pathogenic variation in *Ph*, which is consistent with the results published by Steele et al. (2001) who found a similar situation among Australian isolates of *Pst*. Marker $(GACA)_4$ revealed more informative fragments compared to the M13. So PCR-fingerprinting technique using marker $(GACA)_4$ can be a very efficient and an effective tool to find genetic variations in *Ph* and other rust pts.

Conflict of Interests

The authors have not declared any conflict of interests.

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