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Pathogenic and genetic diversity in *Puccinia hordei* Otth in Australasia

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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 5453P) 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., 2010). While annual surveys of pathogenic variation 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 uredinal 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. psuedocondar (Paps) and P. graminis f. sp. avenae (Pg),], 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 polymorphism among different rust pathogens (Dambrosi 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)4 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)3 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; Dehaes 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
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), 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. After centrifuging at 13,000 rpm for 15 min, the DNA pellet was re-suspended in 500 µl of ethanol (70% v:v) and allowed to air dry. The dried pellet was re-suspended in cold ddH₂O and stored overnight at 4°C. The following day, the tubes were centrifuged at 13,000 rpm for 30 min and the DNA pellet thus formed was re-suspended in 100 µl ddH₂O 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 ddH₂O.

### PCR-fingerprinting

Two oligonucleotide primers were used in fingerprinting the isolates.

### 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), 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         | Balaklava, 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-Pl      | P. triticina | 104-2,3,(6),(7),11 | 423 | Mt Derimit, 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-Psp     | P. striiformis f. sp. pseudohordei | 981549 | 589 | Turrettfield, 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.
of Ph and control pts: The microsatellite-specific [(GACA)₄ (5′GACACGACAGACGACA3′)] (Ali et al., 1986; Meyer et al., 2001) and [M13 (5′GAGGGTGGCCGGTTC3′)] 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 MgCl₂ (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 ddH₂O. 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 thermocycler (Eppendorf Mastercycler, Germany). PCR products were concentrated to 30 μl by placing in a fan forced oven (MetaPhor® for 45 min at 65°C and resolved on 2% high resolution agarose (Agarose, Lonza, Rockland Inc.USA) gels at 80 V electrohoresis for 6 h. Five kilobite DNA marker HyperLadder™ II (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 (6th 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 stability 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, 241P, 242P and 243P. All 10 pts have virulence for Rph8 in common (Park 2003). Marker M13 also grouped these pts in one group (MGP1, except pts 201P and 243P, which were grouped in the other GGP1 group (Table 2). Both markers out-grouped pts 200P (GGP4 and MGP4), 5653P (GGP6 and MGP6) and 5635P (GGP7 and MGP7) from all others (Table 2). Pathotype 200P is virulent to Rph8 only whereas pt 5635P carries additional virulence for genes Rph1, Rph2, Rph4, Rph6, Rph9, Rph10 and Rph12. Pathotype 5653P possesses additional virulence for Rph19 compared to pt 5635P (Park, 2003). In both cases, pts 211P and 231P were grouped together in distinct groups of GACA (GGP1) and M13 (MGP1) 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 possesses 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 and 4610P and 5635P together in a single group (GGP1) and discriminated pt 220P in a distinct group (GGP6) (Table 2), but genotyping based on M13 marker grouped these four pts in a group (MGP6) 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).
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 *Psph* was 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 (GACA$_4$ and M13, Table 2) and differentiated them from all other *Ph* pts. Both pts originated from New Zealand and differ only in virulence on *Rph* genes shown in last column is corresponding to the pathotypes shown in the previous column.

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       | MGP | 201P*     | 1       | GGP | 211P*     | Rph1, Rph4, Rph8, Rph19 |
| 12      | MGP | 243P      | 2       | GGP | 220P*     | Rph5, Rph8, Rph19      |
| 16      | MGP | 5653P*    | 3       | GGP | 253P*     | Rph1, Rph2, Rph4, Rph6, Rph8 |
| 1       | MGP | 211P*     | 4       | GGP | 243P*     | Rph1, Rph2, Rph6, Rph8, Rph19 |
| 2       | MGP | 220P*     | 5       | GGP | 200P*     | Rph8, Rph19            |
| 3       | MGP | 253P*     | 6       | GGP | 232P*     | Rph2, Rph4, Rph5, Rph8, Rph19 |
| 4       | MGP | 243P*     | 7       | GGP | 201P      | Rph1, Rph8              |
| 5       | MGP | 200P*     | 8       | GGP | 201P*     | Rph1, Rph8, Rph19      |
| 6       | MGP | 232P*     | 9       | GGP | 242P*     | Rph2, Rph6, Rph8, Rph19 |
| 7       | MGP | 201P*     | 10      | GGP | 243P      | Rph1, Rph2, Rph6, Rph8 |
| 9       | MGP | 242P*     | 11      | GGP | 5610P*    | Rph4, Rph8, Rph9, Rph10, Rph12, Rph19 |
| 11      | MGP | 243P*     | 12      | GGP | 5653P*    | Rph1, Rph2, Rph4, Rph6, Rph8, Rph9, Rph10, Rph12, Rph19 |
| 15      | MGP | 4610P*    | 13      | GGP | 243P*     | Rph1, Rph2, Rph6, Rph8, Rph19 |
| 19      | MGP | 5610P*    | 14      | GGP | 5653P*    | Rph1, Rph2, Rph4, Rph6, Rph8, Rph9, Rph10, Rph12, Rph19 |
| 20      | MGP | 220P*     | 15      | GGP | 4610P*    | Rph4, Rph8, Rph9, Rph12, Rph19 |
| 21      | MGP | 200P*     | 16      | GGP | 220P*     | Rph5, Rph8, Rph13, Rph19 |
| 14      | MGP | 5653P*    | 17      | GGP | 5653P*    | Rph1, Rph2, Rph4, Rph6, Rph8, Rph9, Rph10, Rph12, Rph19 |
| 10      | MGP | 5653P*    | 18      | GGP | 211P*     | Rph1, Rph4, Rph8        |
| 13      | MGP | 5453P     | 19      | GGP | 231P*     | Rph1, Rph2, Rph4, Rph5, Rph8, Rph19 |
| 22      | MGP | 5457P*    | 20      | GGP | 200P      | Rph8                   |
| 17      | MGP | 211P      | 21      | GGP | 5453P*    | Rph1, Rph2, Rph4, Rph6, Rph9, Rph10, Rph12, Rph19 |
| 18      | MGP | 231P*     | 22      | GGP | 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.
Table 3. GelCompar selected fragments across 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)\textsubscript{4} revealed more informative fragments compared to the M13. So PCR-fingerprinting technique using marker (GACA)\textsubscript{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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