Amplified fragment length polymorphism and virulence polymorphism in *Puccinia hordei*

Y. Sun, S. Zhong, B.J. Steffenson, T.L. Friesen, and S.M. Neate

Abstract: *Puccinia hordei* is the causal agent of barley leaf rust. To study the genetic diversity in *P. hordei*, 45 isolates with diverse virulence patterns and geographical origins were analyzed using amplified fragment length polymorphism markers. Two pathotypes of *Puccinia graminis* f. sp. *tritici* and one isolate of *P. graminis* f. sp. *secalis* were included in the analysis for comparison. Six primer-pair combinations of amplified fragment length polymorphism were used and a total of 782 polymorphic markers were generated. Cluster analysis showed that *P. graminis* f. sp. *tritici* and *P. graminis* f. sp. *secalis* were distinctly different from *P. hordei*. The *P. hordei* isolates were clustered into five groups: group I contained a single, rare isolate that was virulent on all resistance genes except *Rph13* and *Rph15*; group II contained a single isolate found to be virulent on the resistance gene *Rph15*; group III contained 2 isolates; group IV contained 24 isolates, 11 from the United States and 13 from diverse locations around the world; and group V contained 17 isolates, 7 from California, 7 from other states of United States, and 3 from central Europe. The study revealed that molecular diversity in *P. hordei* can be associated with virulence, but not well with geographic origin.

Key words: Hordeum vulgare, DNA markers, *Puccinia hordei*.

Résumé : Le *Puccinia hordei* est l'agent responsable de la rouille des feuilles de l'orge. Dans le but d'étudier la diversité génétique du *P. hordei*, 45 isolats avec divers types de virulence et d'origines géographiques variées ont été analysés avec des marqueurs de polymorphisme de la longueur des fragments amplifiés. Deux pathotypes du *Puccinia graminis* f. sp. *tritici* et un isolat du *P. graminis* f. sp. *secalis* ont été incorporés à l'analyse pour fin de comparaison. Six combinaisons de paires d'amorces du polymorphisme de la longueur des fragments amplifiés ont été employées et un total de 782 marqueurs polymorphes ont été obtenus. L'analyse typologique a révélé que le *P. graminis* f. sp. *tritici* et le *P. graminis* f. sp. *secalis* étaient nettement distincts du *P. hordei*. Les isolats du *P. hordei* étaient rassemblés en cinq groupes : le groupe I contenait un seul et rare isolat virulent envers tous les gènes de résistance sauf *Rph13* et *Rph15*; le groupe II contenait un seul isolat virulent envers le gène de résistance *Rph15*; le groupe III contenait 2 isolats; le groupe IV contenait 24 isolats, 11 des États-Unis et 13 de divers endroits autour du monde; et le groupe V contenait 17 isolats, 7 de la Californie, 7 d'autres États américains et 3 d'Europe centrale. L'étude a montré que la diversité moléculaire du *P. hordei* pouvait être associée à la virulence, mais qu'il était difficile de la relier à l'origine géographique.

Mots-clés : Hordeum vulgare, marqueurs d'ADN, *Puccinia hordei*.

Introduction

Leaf rust, caused by the fungal pathogen *Puccinia hordei* G. Otth, is an important disease of barley in many production areas of the world. Yield losses over 30% have been recorded in Australia (Cotterill et al. 1992), Europe (King and Polley 1976) and the United States (Griffey et al. 1994) under epidemic conditions. Plant resistance is the most effective and economical means of controlling the disease. However, *P. hordei* isolates can be highly variable in their virulence spectrum (Jin and Steffenson 1991), and race-specific type of resistance is often lost with the rapid emergence of new virulent pathotypes of the pathogen (Clifford 1984). Information about the virulence diversity of leaf rust is important for resistance breeding. Breeding lines are typically screened for resistance to rust by inoculation with either specific pathotypes or mixtures of pathotypes and (or) evaluation in nurseries where natural rust infection occurs (Jin et al. 1995).

The virulence of *P. hordei* isolates can be characterized by their unique virulence–avirulence patterns on barley differential lines with single genes for resistance to leaf rust

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(Steffenson and Jin 1992). The virulence patterns provide useful information regarding the effectiveness of host resistance genes, which is essential for breeding programs. However, virulence assessments may detect only a limited number of virulence loci because the number of genetically characterized differentials used is limited. Furthermore, virulence types are subject to strong selection by the host (Kolmer 1993); this selection thus limits their application in genetic-diversity studies (McDonald and McDermott 1993). Biochemical and molecular markers such as isozymes, restriction fragment length polymorphism, randomly amplified polymorphic DNA (RAPD), and amplified fragment length polymorphism (AFLP) can be used in plant pathogens to evaluate genetic diversity independent of environmental factors and effects on gene expression levels (McDermott and McDonald 1993). In addition, DNA techniques allow for the assessment of a very large number of neutral polymorphic marker loci. A previous study found no significant differences in eight Middle East isolates from three *Hordeum* spp., using sequence analysis of internal transcribed spacer of ribosomal DNA (Szabo et al. 1996). No extensive genetic studies on *P. hordei* with molecular techniques have been reported since. AFLP analysis has proved suitable to study populations with a low genetic diversity (Powell et al. 1996). It has previously been used to detect genetic diversity in other rust pathogens such as *Puccinia graminis* Pers.:Pers. (Zambino et al. 2000), *Puccinia triticina* Eriks. (Kolmer 2001), and *Puccinia striiformis* Westend. f. sp. *tritici* Eriks. (Markell et al. 2004). However, the genetic diversity among *P. hordei* isolates remains unexplored.

The combined information on virulence variation and DNA polymorphisms should improve our understanding of *P. hordei* and its genetic structure. Knowledge about genetic relationships among the different pathotypes is vital for developing strategies for breeding new resistant cultivars, as well as for managing the disease. The objectives of the present study were to analyze the virulence and genetic diversity of selected *P. hordei* isolates from different barley-producing regions and to explore the possible associations between virulence patterns and molecular phenotypes. AFLP was used to assess the genetic diversity. The virulence phenotypes of the isolates were evaluated on 15 differential lines of barley.

Materials and methods

Isolates

Forty-five *P. hordei* isolates originating from the barley-growing regions of the United States and also from other parts of the world were studied (Table 1). Isolates were chosen to represent both geographical and virulence diversity. Isolates from outside of the United States were chosen to represent different virulence patterns, and isolates from within the United States, to represent the diversity found on barley in annual leaf-rust surveys. Two *Puccinia graminis* f. sp. *tritici* Eriks & E. Henn. pathotypes (QCC and MCC) and one *Puccinia graminis* f. sp. *secalis* Eriks & E. Henn. isolate (92-MN-90) were also included in the study as controls. Representative samples and cultures used in this work are permanently accessioned in the Cereal Disease Laboratory, at the US Department of Agriculture, St. Paul, Minnesota.

Single-spore isolation

To obtain single-spore isolates, urediniospores from each of the 45 *P. hordei* isolates were placed on a glass microscope slide. Individual urediniospores were transferred from the slide to detached leaves of the susceptible *Hordeum vulgare* L. 'Moore', using a dissecting microscope and a fine glass needle. The inoculated leaves were placed on distilled-water agar media containing 0.5% of benomyl, and were incubated at room temperature (20–25 °C). About 12–14 days after inoculation, spores were collected from a pustule derived from a single spore. Spores of each isolate were then propagated on 'Moore' barley in an isolated greenhouse to ensure the purity of the cultures. Urediniospores were collected and stored at –80 °C until used.

Virulence analysis

The virulence phenotypes of the *P. hordei* isolates were evaluated on a set of 15 differential lines of barley, each thought to contain a single gene for resistance to *P. hordei*. These differential lines, and their corresponding resistance genes provided within parentheses, included: Sudan (*Rph1*), Peruvian (*Rph2*), Aim (*Rph3*), Estate (*Rph4*), 'Gold' (*Rph5*), Bolivia (New) (*Rph6*), 'Cebada Capa' (*Rph7*), Egypt 4 (*Rph8*), Hor 2596 (*Rph9*), 'Triumph' (*Rph10*), 'Clipper' BC8 (*Rph11*), 'Clipper' BC67 (*Rph12*), PI 531849 (*Rph13*), PI 531901-1 (*Rph14*), and I-95-282-2 (*Rph15*). Seven days after emergence, plants were inoculated with rust on the first fully expanded leaves. A concentration of 5.4 mg of urediniospores per millilitre of mineral oil (Soltrol 170; Phillips Petroleum, Bartlesville, Okla.) was applied at a rate of approximately 0.035 mg per plant with an atomizer pressured at 25–30 kPa. After inoculation, plants were kept in mist chambers at 20 °C for 16 h in the dark. During this time, the plants were kept moist by periodic misting (32 s of mist every 16 min) with ultrasonic humidifiers. Plants were transferred to a growth chamber at 24–25 °C with a 14 h photoperiod provided by 115 W cool white bulbs (photosynthetic photon flux density, 150–350 μmol m^{-2} s^{-1}). After 12–14 days of incubation, the infection types (ITs) were assessed on each differential line, primarily on the basis of pustule size, using a 0–4 scale as described by Levine and Cherewick (1952). In this study, ITs of 2 or less (i.e., 0, 0;, 1, 2, or combinations thereof: 00;, 0;1, 12, 21, and 23–) were considered indicative of low virulence (incompatibility), whereas ITs of 3– or more (e.g., 3–2, 3–, 3–3, 33–, or 3) were considered indicative of high virulence (compatibility). Virulence assays were repeated at least twice for each isolate.

DNA extraction

Approximately 150 mg of spores of each isolate were used for DNA extraction by the method described by Weiland (1997). Briefly, dry urediniospores were placed in a mortar to which 1 g of white quartz sand (–50 + 70 mesh, product No. S-9887; Sigma Co., Oakville, Ont.) was added. Sufficient (0.8 mL) extraction buffer (Tris–HCl, 100 mmol/L; ethylenediaminetetraacetic acid, disodium salt (Na_2EDTA), 20 mmol/L; NaCl, 0.5 mol/L; sodium dodecylsulfate, 1%; pH 8.0) was added to the mortar so that the urediniospores–sand mixture became saturated. Then, a 0.25 mL mixture of buffer-saturated phenol – chloroform – isoamyl alcohol

Table 1. *Puccinia hordei* isolates used for analysis of molecular diversity, and their virulence patterns on 15 differential lines of barley with single resistance genes to leaf rust.

Isolate	Octal code[a]	Virulence to *Rph* genes[b]	Virulence group (VG)	Molecular group (MG) and (or) subgroup	Geographical origin	Year collected[c]
93-9	31321	*Rph1*, *Rph2*, *Rph4*, *Rph7*, *Rph8*, *Rph11*, *Rph13*	6	V	N.C., United States	1993
90-23	31321	*Rph1*, *Rph2*, *Rph4*, *Rph7*, *Rph8*, *Rph11*, *Rph13*	6	V-1	Calif., United States	1990
93-20	31321	*Rph1*, *Rph2*, *Rph4*, *Rph7*, *Rph8*, *Rph11*, *Rph13*	6	V-1	Va., United States	1993
93-33	31321	*Rph1*, *Rph2*, *Rph4*, *Rph7*, *Rph8*, *Rph11*, *Rph13*	6	V-1	Va., United States	1993
95-19	31321	*Rph1*, *Rph2*, *Rph4*, *Rph7*, *Rph8*, *Rph11*, *Rph13*	6	V-1	N.C., United States	1995
93-18	31221	*Rph1*, *Rph2*, *Rph4*, *Rph8*, *Rph11*, *Rph13*	6	V-2	Calif., United States	1993
94-2	31221	*Rph1*, *Rph2*, *Rph4*, *Rph8*, *Rph11*, *Rph13*	6	VI-1	Tex., United States	1994
94-14	31221	*Rph1*, *Rph2*, *Rph4*, *Rph8*, *Rph11*, *Rph13*	6	VI-2	Calif., United States	1994
93-27	31331	*Rph1*, *Rph2*, *Rph4*, *Rph7*, *Rph8*, *Rph10*, *Rph11*, *Rph13*	6	VI	Va., United States	1993
95-15	31201	*Rph1*, *Rph2*, *Rph4*, *Rph8*, *Rph13*	6	V-2	Calif., United States	1995
94-4	31201	*Rph1*, *Rph2*, *Rph4*, *Rph8*, *Rph13*	6	V-2	Tex., United States	1994
93-5	31201	*Rph1*, *Rph2*, *Rph4*, *Rph8*, *Rph13*	6	V-2	Calif., United States	1993
94-12	31201	*Rph1*, *Rph2*, *Rph4*, *Rph8*, *Rph13*	6	VI-2	Calif., United States	1994
94-9	31201	*Rph1*, *Rph2*, *Rph4*, *Rph8*, *Rph13*	6	VI-2	Calif., United States	1994
95-13	31201	*Rph1*, *Rph2*, *Rph4*, *Rph8*, *Rph13*	6	V-2	Calif., United States	1995
93-55	11221	*Rph1*, *Rph4*, *Rph8*, *Rph11*, *Rph13*	6	V-1	Wisc., United States	1993
95-14	31021	*Rph1*, *Rph2*, *Rph4*, *Rph11*, *Rph13*	6	V-2	Calif., United States	1995
93-3	71221	*Rph1*, *Rph2*, *Rph3*, *Rph4*, *Rph8*, *Rph11*, *Rph13*	6	V-2	Ariz., United States	1993
95-10	31000	*Rph1*, *Rph2*, *Rph4*	6	V-2	Calif., United States	1995
95-31	35020	*Rph1*, *Rph2*, *Rph4*, *Rph6*, *Rph11*	6	VI-3	N.D., United States	1995
95-30	11210	*Rph1*, *Rph4*, *Rph8*, *Rph10*	5	VI-1	N.D., United States	1995
92-74	11230	*Rph1*, *Rph4*, *Rph8*, *Rph10*, *Rph11*	5	VI-2	Wash., United States	1992
Tel Aviv	11230	*Rph1*, *Rph4*, *Rph8*, *Rph10*, *Rph11*	5	VI-1	Israel	—
95-40	11230	*Rph1*, *Rph4*, *Rph8*, *Rph10*, *Rph11*	5	VI-1	N.D., United States	1995
93-39	11230	*Rph1*, *Rph4*, *Rph8*, *Rph10*, *Rph11*	5	VI-1	Neb., United States	1993
Tomome	11230	*Rph1*, *Rph4*, *Rph8*, *Rph10*, *Rph11*	5	VI-1	Morocco	—
Race 8	11230	*Rph1*, *Rph4*, *Rph8*, *Rph10*, *Rph11*	5	VI-2	N.D., United States	—
Ger5	51230	*Rph1*, *Rph3*, *Rph4*, *Rph8*, *Rph10*, *Rph11*	5	V-1	Germany	1991
China	10230	*Rph1*, *Rph8*, *Rph10*, *Rph11*	5	III	China	1995
92-2	31230	*Rph1*, *Rph2*, *Rph4*, *Rph8*, *Rph10*, *Rph11*	5	VI-3	Mexico	1992
92-3	31030	*Rph1*, *Rph2*, *Rph4*, *Rph10*, *Rph11*	5	VI-2	Mexico	1992
57-19	00232	*Rph8*, *Rph10*, *Rph11*, *Rph14*	5	III	Va., United States	1957
Czech	71661	*Rph1*, *Rph2*, *Rph3*, *Rph4*, *Rph8*, *Rph9*, *Rph11*, *Rph12*, *Rph13*	4	V-1	Czech Republic	1992
Octal1567	71671	*Rph1*, *Rph2*, *Rph3*, *Rph4*, *Rph8*, *Rph9*, *Rph10*, *Rph11*, *Rph12*, *Rph13*	4	V-1	Denmark	1993
Izmir	37630	*Rph1*, *Rph2*, *Rph4*, *Rph5*, *Rph6*, *Rph8*, *Rph9*, *Rph10*, *Rph11*	3	VI-4	Turkey	—
Arg231	37230	*Rph1*, *Rph2*, *Rph4*, *Rph5*, *Rph6*, *Rph8*, *Rph10*, *Rph11*	3	VI-4	Argentina	—
Rabat	37230	*Rph1*, *Rph2*, *Rph4*, *Rph5*, *Rph6*, *Rph8*, *Rph10*, *Rph11*	3	VI-4	Morocco	1991
76-12	77230	*Rph1*, *Rph2*, *Rph3*, *Rph4*, *Rph5*, *Rph6*, *Rph8*, *Rph10*, *Rph11*	3	VI-4	UK	1976
Neth202	67232	*Rph2*, *Rph3*, *Rph4*, *Rph5*, *Rph6*, *Rph8*, *Rph10*, *Rph11*, *Rph14*	3	VI-4	Netherlands	—
HOMS	27232	*Rph2*, *Rph4*, *Rph5*, *Rph6*, *Rph8*, *Rph10*, *Rph11*, *Rph14*	3	VI-4	Syria	—
Aust220	06220	*Rph5*, *Rph6*, *Rph8*, *Rph11*	2	VI	Australia	1991

Table 1 (*concluded*).

Isolate	Octal code[a]	Virulence to *Rph* genes[b]	Virulence group (VG)	Molecular group (MG) and (or) subgroup	Geographical origin	Year collected[c]
Ger12	17220	*Rph1*, *Rph4*, *Rph5*, *Rph6*, *Rph8*, *Rph11*	2	VI	Germany	1988
Neth28	36372	*Rph1*, *Rph2*, *Rph5*, *Rph6*, *Rph7*, *Rph8*, *Rph10*, *Rph11*, *Rph12*, *Rph14*	1	VI-2	Netherlands	—
90-3	37676	*Rph1*, *Rph2*, *Rph4*, *Rph5*, *Rph6*, *Rph8*, *Rph9*, *Rph10*, *Rph11*, *Rph12*, *Rph14*, *Rph15*	1	II	Israel	1990
92-7	77772	*Rph1*, *Rph2*, *Rph3*, *Rph4*, *Rph5*, *Rph6*, *Rph7*, *Rph8*, *Rph9*, *Rph10*, *Rph11*, *Rph12*, *Rph14*	1	I	Morocco	1992

[a]0 indicates an incompatible reaction, and 1 indicates a compatible reaction on the differentials, following inoculation. Octal digits were assigned as follows: 000 = 0, 100 = 1, 010 = 2, 001 = 4, 110 = 3, 101 = 5, 011 = 6, and 111 = 7. The octal digits in this system are sorted according to the number of virulences per triplet (Herrmann et al. 1999).
[b]Compatible (virulence) reactions of *P. Hordei* on barley differential lines carrying single resistance genes *Rph1* to *Rph15*.
[c]Dashes indicate that the collection date is not available.

(25:24:1, *v*/*v*/*v*) was added. The mixture was ground vigorously for 1 min with a pestle to form a thick paste. A further 1 mL of extraction buffer and 0.5 mL of buffered phenol – chloroform – isoamyl alcohol were added, and the solution was mixed thoroughly before transfer into microfuge tubes. The microfuge tubes were centrifuged at 16 000 *g* for 5 min at room temperature. The aqueous phase was transferred to a new tube and was mixed with isopropanol in a ratio of 1:0.6 (*v*/*v*). Samples were incubated at room temperature for 10 min followed by 15 min at 13 000 *g* to recover the precipitate. The pellets were washed with 95% ethanol and allowed to air dry briefly before resuspension in 340 µL of TE (Tris–HCl, 10 mmol/L; Na_2EDTA, 1 mmol/L; pH 8.0) containing ribonuclease A at 20 µg/mL. Samples were incubated at 37 °C for 30 min and then extracted with 0.3 mL of phenol – chloroform – isoamyl alcohol. The aqueous phase (~300 µL) was transferred to a new tube and mixed with ammonium acetate at a concentration of 7.5 mol/L and ethanol in a ratio of 1:0.5:2.5 (*v*/*v*/*v*). The samples were then incubated for 30 min or longer at 20 °C before centrifugation for 15 min at 13 000 *g*. The DNA pellets were rinsed with 95% ethanol, air dried and resuspended in 100 µL of TE. DNA quality and quantity were determined by agarose gel electrophoresis and UV spectrophotometry. DNA was kept at –20 °C for later use. A working solution was made by dilution of the stock DNA solution to a final concentration of 50 ng/µL.

AFLP analysis

AFLP analysis was conducted according to Vos et al. (1995). The restriction reaction was carried out with 1 µg of genomic DNA, using 5 U (1 U ≈ 16,67 nkat) of *Eco*RI and 1 U of *Mse*I enzymes in 1× T4 buffer (Promega Co., Madison, Wisc.). Restriction fragments were ligated to the *Mse*I and *Eco*RI adapter in a single step at 37 °C for 4 h. The ligation product (2.5 µL) was preamplified in 30 µL of PCR reaction mixture, using a Dyad thermal cycler (Bio-Rad, Hercules, Calif.), *Mse*I and *Eco*RI primers without any additional selective nucleotide at the 3′ end, and the following cycling parameters: 20 cycles of 94 °C for 30 s, 56 °C for 60 s, and 72 °C for 60 s. After preamplification, the template was diluted in water 1:40 for selective amplification. Randomly selected primer pairs were employed for selective amplification, which was conducted with two selective bases at the 3′ end of both primers. Cycling parameters were as follows: 1 cycle of 94 °C for 30 s, 65 °C for 60 s, and 72 °C for 30 s; 12 cycles in which the annealing temperature was reduced by 0.7 °C each cycle from 65 to 56 °C; 23 cycles of 94 °C for 30 s, and 56 °C for 30 s. As a result of the inclusion of a second labeled *Eco*RI primer in the final amplification, two *Eco*RI primers amplified two differentially labeled sets (with IRD800 and IRD700, respectively heptamethine cyanine dye and pentamethine carbocyanine dye) of AFLP fragments in parallel, with the same *Mse*I primer being used to double the AFLP data. Primer pairs used for the study were E12 + M16, E13 + M17, E16 + M16, E16 + M17, E17 + M13, and E21 + M13 (Table 2). The products of the multiplexed AFLP reactions were separated on a DNA analyzer NEN Global IR^2 System (model 4200, infrared two-dye system; LI-COR, Inc., Lincoln, Neb.). One microlitre of the AFLP reaction mixture was loaded

Table 2. Primer combinations and the number of polymorphic AFLP markers generated in molecular diversity analysis of *Puccinia hordei*.

	Number of AFLP markers in *Puccinia hordei*[a]		Number of AFLP markers in *Puccinia* spp.[b]	
Primer combination	Polymorphic	Monomorphic	Polymorphic	Monomorphic
E12 + M16	65	38	164	3
E13 + M17	30	24	120	2
E16 + M16	41	44	159	2
E16 + M17	32	24	102	0
E17 + M13	51	26	130	5
E21 + M13	23	25	107	6
Total	242	181	782	18

Note: AFLP, amplified fragment length polymorphism.
[a]From 45 isolates of *P. hordei.*
[b]From 45 isolates of *P. hordei,* two pathotypes of *Puccinia graminis* f. sp. *tritici*, and one isolate of *P. graminis* f. sp. *secalis*.

into each well in a 6% polyacrylamide gel made with 1× TBE buffer (Tris base, 10.8 g; boric acid, 5,5 g; Na_4EDTA, 9.3 g; H_2O, 1 L). Electrophoresis was done in 1× TBE running buffer for 2.5 h with voltage set at 2000 V, current at 40 mA, power at 50 W, and temperature at 45 °C.

Data analysis

The AFLP polymorphic bands between 50 and 700 base pairs (bp) were scored manually for each of six primer pairs. Only unambiguous bands were scored. DNA bands larger than 700 bp were sometimes not repeatable and were therefore not scored. AFLP bands that were present in some isolates but absent in other isolates were considered polymorphic markers. Each isolate was scored for each polymorphic band: 1 for the presence and 0 for the absence. Likewise, for virulence analysis, each isolate was scored on each of the differential host lines: 1 for a compatible reaction and 0 for an incompatible one. Allelic diversity was assessed using Nei's allelic diversity index (Nei 1973). The similarity matrix was constructed for the qualitative data (Dice 1945) with the NTSYSpc 2.1 software package (version 2.1; Exeter Software, Setauket, N.Y.). A similarity matrix was subjected to cluster analysis by unweighted pair-group method with arithmetic averages (UPGMA) in the sequential agglomerative hierarchical and nested clustering method (SAHN) program in NTSYSpc 2.1. Dendrograms of both virulence and AFLP diversity were constructed for *P. hordei* isolates. A bootstrap analysis (500 replications) was conducted with the Winboot program (Nelson et al. 1994) to estimate the strength of AFLP molecular grouping. Bootstrap analysis was not conducted for the virulence data because of the small size of the data set. Correlation between the AFLP and virulence phenotypic data was determined by matrix comparison of the symmetric similarity matrices, using MX-COMP in NTSYSpc 2.1.

Results

Virulence variation

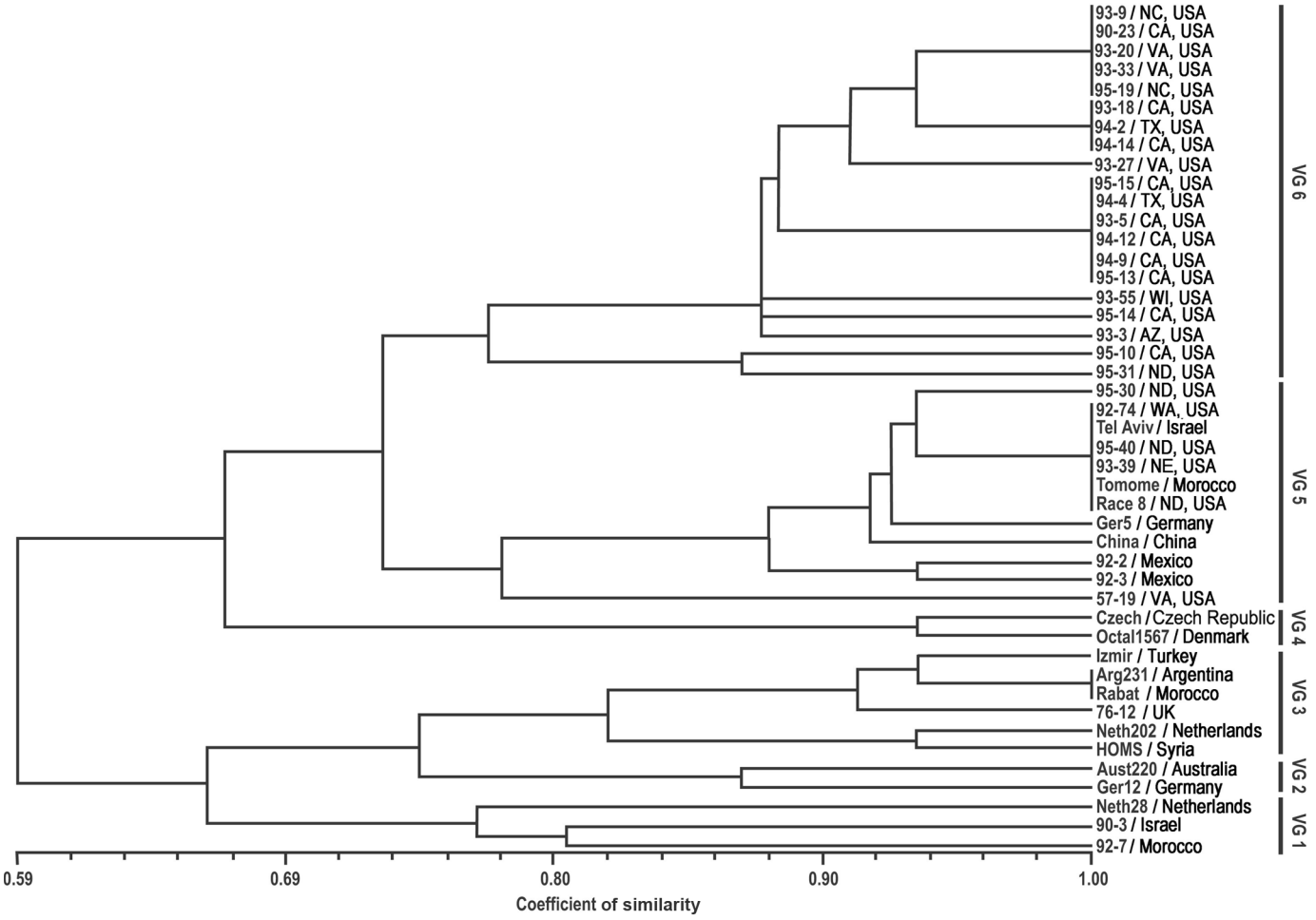
Virulence phenotypes for the 45 *P. hordei* isolates are shown in Table 1. Twenty-eight virulence patterns were identified on the set of 15 differential host lines. Most isolates had unique virulence patterns on the 15 resistance genes to leaf rust. However, some isolates exhibited the same virulence pattern. Isolates 92-74, Tel Aviv, 93-39, Tomome, Race 8, and 95-40 exhibited the same virulence phenotype and were compatible on the differential lines with *Rph1*, *Rph4*, *Rph8*, *Rph10*, and *Rph11*. Similarly, other groups of isolates exhibiting the same virulence patterns were found: 95-15, 94-4, 93-5, 94-12, 94-9, and 95-13 were all virulent for *Rph1*, *Rph2*, *Rph4*, *Rph8*, and *Rph13*; 93-18, 94-2, and 94-14 were all virulent for *Rph1*, *Rph2*, *Rph4*, *Rph8*, *Rph11*, and *Rph13*; 93-9, 90-23, 93-20, 93-33, and 95-19 were all virulent for *Rph1*, *Rph2*, *Rph4*, *Rph7*, *Rph8*, *Rph11*, and *Rph13*; and Arg231 and Rabat were all virulent for *Rph1*, *Rph2*, *Rph4*, *Rph5*, *Rph6*, *Rph8*, *Rph10*, and *Rph11*. Virulence frequencies for individual resistance genes varied considerably from 2% for *Rph15* to 91% for *Rph1* and *Rph8*.

Seven virulence dendrograms were obtained with the FIND option in the SAHN program. The virulence dendrograms revealed minor differences among a few isolates within the clusters, but these differences did not influence any of the major groupings. A 76% similarity cutoff allowed classification of the 45 isolates into six virulence groups (Fig 1). Virulence group (VG) 1 included isolates Neth28, 90-3, and 92-7. This group had the widest virulence spectrum. Specifically, isolate 92-7 had virulence for all resistance genes, except *Rph13* and *Rph15*. Isolate 90-3 was the only isolate that exhibited a compatible reaction for *Rph15*. VG 2 included Aust220 and Ger12, both of which had virulence for *Rph5*, *Rph6*, *Rph8*, and *Rph11*. VG 3 comprised Izmir, Arg231, Rabat, 76-12, Neth202, and HOMS and were virulent for *Rph2*, *Rph4*, *Rph5*, *Rph6*, *Rph8*, *Rph10*, and *Rph11*. VG 4 contained isolates Czech and Octal1567, which were virulent for *Rph1*, *Rph2*, *Rph3*, *Rph4*, *Rph8*, *Rph9*, *Rph11*, *Rph12*, and *Rph13*. VG 5 included 95-30, 92-74, Tel Aviv, 95-40, 93-39, Tomome, Race 8, Ger5, China, 92-2, 92-3, and 57-19, which were virulent for *Rph10*. VG 6 consisted of the rest of isolates, and these were virulent on differential lines with *Rph1* and *Rph4*. Notably, no single resistance gene was effective against all of the tested isolates.

AFLP variation

The six primer-pair combinations generated 782 AFLP markers among all tested *Puccinia* spp. isolates. Among the

Fig. 1. A dendrogram of virulence diversity of 45 isolates of *Puccinia hordei* on 15 resistance genes to leaf rust revealed by cluster analysis with unweighted pair-group method with arithmetic averages (UPGMA). The bootstrap analysis was not performed because of the small virulence database.



423 markers identified in the *P. hordei* isolates, 242 (57.2%) were polymorphic (Table 2). Cluster analysis showed that the three *P. graminis* isolates (QCC, MCC, and 92-MN-90) were distinctively different from the *P. hordei* isolates. Two formae speciales, *Puccinia graminis* f. sp. *tritici* (races QCC and MCC) and *Puccinia graminis* f. sp. *secalis* (92-MN-90), were also clearly different from each other, with a similarity level of about 70%. The similarity between races QCC and MCC of *Puccinia graminis* f. sp. *tritici* was 85%, which is lower than that among the *P. hordei* isolates in which five clusters were resolved at the 91% similarity level to form the following five AFLP molecular groups (MGs). Isolate 90-3 from Israel and 92-7 from Morocco fell into their own individual groups, designated MG I and MG II, respectively; MG III comprised two isolates, including China, an isolate collected from China, and 57-19, an isolate collected in 1957 from Virginia, United States; MG IV and V consisted of 24 and 17 isolates, respectively. The grouping was supported by bootstrap analysis. In MG IV, four major subgroups were identified: MG IV-1 (95-30, Tel Aviv, Tomome, 95-40, 93-39, and 94-2), MG IV-2 (92-74, Neth28, 94-12, 94-9, Race 8, 94-14, and 92-3), MG IV-3 (92-2 and 95-31), and MG IV-4 (Izmir, Arg231, HOMS, 76-12, Rabat, and Neth202). The bootstrap values of these four subgroups were 98.1, 100, 94.5, and 100, respectively. The rest of the isolates in MG IV were Ger12, 93-27, and Aust220. In MG V, two major subgroups, MG V-1 and MG V-2, were recognized. MG V-1 had bootstrap values of 100 and included isolates Czech, 93-20, 90-23, 93-33, 95-19, 93-55, Octal1567, and Ger5. MG V-2 contained isolates 93-18, 95-15, 93-5, 95-13, 95-10, 93-3, 95-14, and 94-4 with a bootstrap value of 89.0 (Fig. 2). 93-9 was the only isolate in MG V that did not fall into one of the two subgroups.

Relationship between virulence and AFLP variation

To reveal the relationships between virulence and AFLP patterns, AFLP and virulence similarity matrices were compared by correlation analysis, using MXCOMP analysis in NTSYSpc 2.1. The correlation coefficient (r) was 0.45. The low coefficient implies that the correlation between virulence and AFLP patterns was low in general. However, when specific groups of isolates were compared in the two dendrograms generated from virulence and AFLP data, a close relationship between virulence phenotype and AFLP pattern was identified. For example, isolates in VG 3 (Izmir, Arg231, Rabat, 76-12, Neth202, and HOMS) all fell into MG IV-4. Both VG 4 isolates Czech and Octal1567 were placed within MG V-1. Isolates 95-30, Tel Aviv, 95-40, 93-

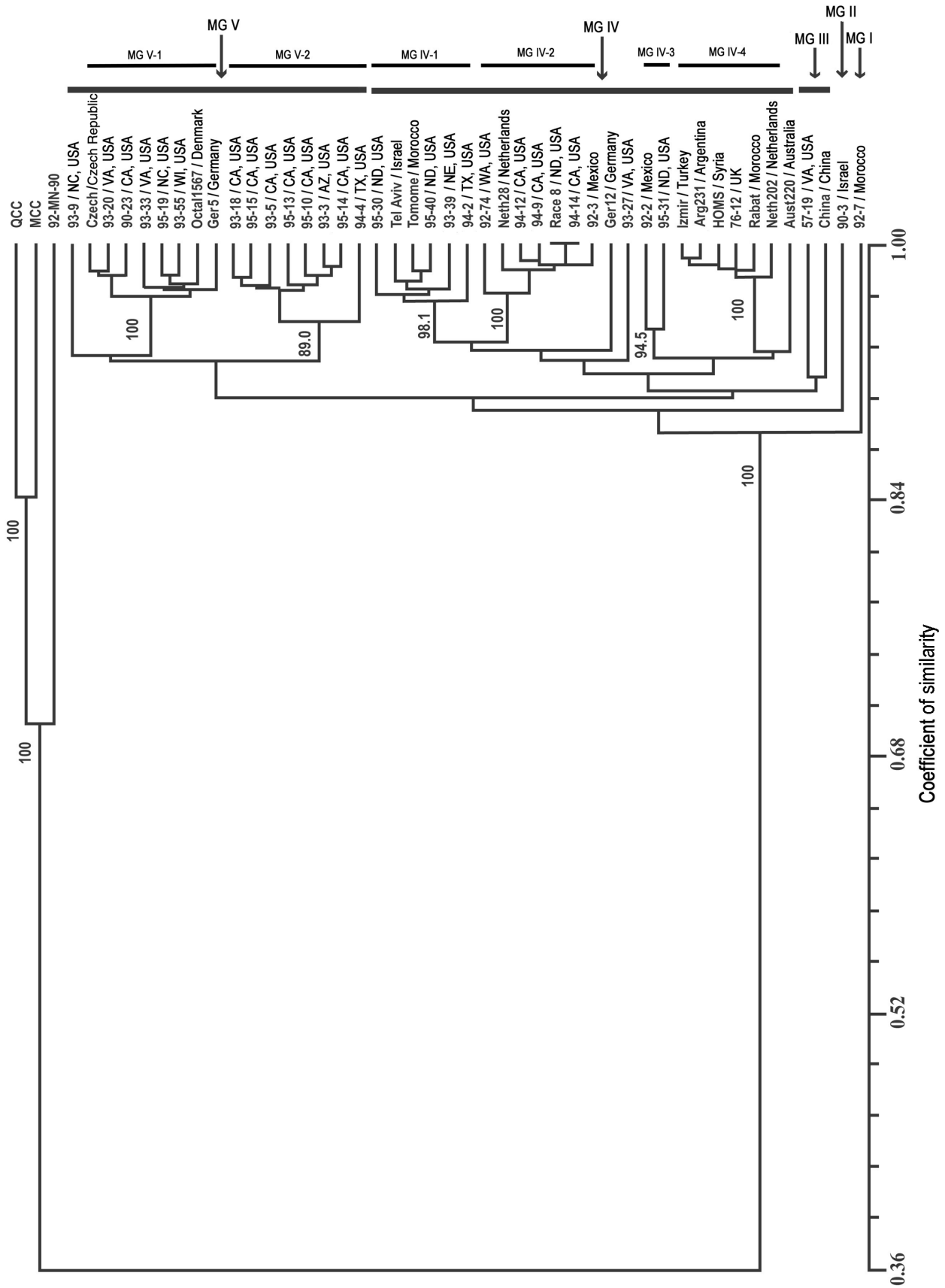


Fig. 2. A dendrogram of 45 isolates of *Puccinia hordei* and 3 isolates of *Puccinia graminis* constructed by cluster analysis with unweighted pair-group method with arithmetic averages (UPGMA). Five *P. hordei* clusters were resolved at the 91% similarity level to form five amplified fragment length polymorphism (AFLP) molecular groups (MGs): MG I, MG II, MG III, MG IV, and MG V. The bootstrap values greater than 89.0 are labeled at the corresponding nodes. The bootstrap values were generated by 500 replications, using the Winboot program (Nelson et al. 1994).

39, and Tomome in VG 5 were also classified within MG IV-1. 93-18, 95-15, 94-4, 93-5, 95-13, 95-14, 93-3, and 95-10 were all VG 6 isolates and were also in the same MG subgroup V-2. Isolates 90-23, 93-20, 93-33, and 95-19 had an identical virulence pattern and were all classified into MG V-1. Nevertheless, some pathotypes or virulence patterns were independent of their AFLP molecular data. For example, isolates 93-18, 94-2, and 93-20 were classified into different AFLP groups even though they had identical virulence phenotypes. Isolates in the same AFLP group sometimes had different virulence patterns, such as those in MG IV-2: Neth28 belongs to VG 1; 92-74, Race 8, and 92-3 were clustered in VG 5; and 94-14, 94-9, and 94-12 were grouped in VG 6.

Discussion

Twenty-eight pathotypes were identified among the *P. hordei* isolates tested in this study. These pathotypes represent the majority of the virulence patterns detected worldwide (Fetch et al. 1998). The virulence spectra of tested isolates ranged from narrow (i.e., isolate 95-10 with virulence for only 3 *Rph* genes) to very wide (isolate 92-7 with virulence for 13 *Rph* genes). The natural variation in virulence of *P. hordei* is undoubtedly greater than the variation that we observed, because identification of a pathotype depends on the differential lines used, i.e., a pathotype could be separated further if more host genotypes (resistance genes) were included. In addition, while we collected isolates that represent both geographic and virulence diversity, a larger collection would most likely have revealed further variation in virulence.

Cluster analysis indicated that there were five virulence groups. Some relationships were detected between VGs and the origin of the isolates. All the isolates in VG 1, 2, 3, and 4 were from regions outside of the United States. VG 1 had the widest virulence spectrum with the isolates 92-7 and 90-3. Isolate 92-7 was collected from Morocco and had virulence for most of the *Rph* genes. 90-3, a highly virulent isolate collected from Israel, was the only isolate compatible on *Rph15*. *Rph15* is a highly effective resistance gene identified from a wild barley (*Hordeum vulgare* subsp. *spontaneum* C. Koch.) accession collected in Israel. In nature, the alternate hosts of *P. hordei*, *Ornithogalum* L. spp., exists in the same habitats as wild barley (Anikster 1982; Wahl et al. 1984) and are required for *P. hordei* to complete its life cycle. The coevolution between the host and pathogen may have contributed to the selection and emergence of highly virulent isolates such as 90-3 from this region (Anikster and Wahl 1979). VG 2 and VG 3 isolates originated from every continent, except Asia and North America. VG 4 isolates Czech and Octal1567 originated from Europe (Czech Republic and Denmark). VG 5 comprises isolates from various parts of the world. The geographical diversity of this group indicates that isolates with common virulence for *Rph10* have worldwide distribution. Finally, in VG 6, all 20 isolates were from the United States. These isolates lack virulence for *Rph5*, *Rph9*, *Rph12*, *Rph14*, and *Rph15*. *Rph1* was identified in a very old cultivar (*H. vulgare* 'Manchuria') and presumably has been used widely. This may be why virulence for *Rph1* was frequently found. In commercial cultivars, a few other *Rph* genes have been deployed. *Rph2*, *Rph3*, *Rph4*, *Rph7*, and *Rph12* have been used in Europe (Dreiseitl and Steffenson 2000), and virulence to these genes was discovered in isolates from that region. *Rph2* and *Rph7* have regularly been integrated in cultivars planted in the United States (Steffenson et al. 1993). Virulence for *Rph7* and *Rph2* were found respectively in 23% and 69% of the United States isolates. In North America, *Rph5*, *Rph9*, *Rph12*, *Rph14*, and *Rph15* are especially important for resistance breeding programs because isolates tested to date from this region are avirulent for these genes. At this time, the resistance gene *Rph15*, which is effective against a wide range of races of the pathogen, is being incorporated into two-rowed barley breeding lines being developed in North Dakota (J.D. Franckowiak, personal communication).

The AFLP information generated from the two pathotypes of *P. graminis* f. sp. *tritici* (QCC and MCC) shows a similarity of 85% (Fig. 2). The diversity level is higher than what we showed in our much larger worldwide collection of *P. hordei*. Previously, Szabo et al. (1996) analyzed leaf rust isolates from different *Hordeum* spp. (*H. vulgare*, *H. murinum* L., *H. vulgare* subsp. *spontaneum*, and *H. bulbosum* L.), using sequence analysis of internal transcribed spacer of ribosomal DNA, and found that they were phylogenetically very close in the parsimony analysis. In the present study, molecular variation was identified not only in isolates with different virulence patterns, but also in isolates with the same virulence pattern. Although AFLP polymorphisms detected among isolates clearly demonstrated that *P. hordei* is highly variable, the diversity level is not as high as found in other pathogens. In *P. striiformis*, five RAPD groups were detected among 115 isolates at 80% similarity level (Chen et al. 1993). In *P. triticina*, 69 isolates were divided into five AFLP groups and had approximately 71% similarity (Kolmer 2001). In *Cochliobolus sativus* (Ito & Kuribayashi) Drechs. ex Dastur, 58 isolates were classified into five AFLP groups at 64% similarity level (Zhong and Steffenson 2001). In *Magnaporthe grisea* (T.T. Hebert) Yaegashi & Udagawa, six discretely distinct genetic lineages were classified at 85% similarity level (Levy et al. 1993). In barley-producing regions such as North America, where *P. hordei* can overwinter as uredinia in the south and asexual urediniospores are the means of reproduction, the role of the alternate hosts, *Ornithogalum* spp., is considered unimportant for generating genetic variation. In these systems, mutations are likely the common source of variation for *P. hordei*. This may explain the low diversity found in *P. hordei* isolates from these regions. In barley-producing regions where the alternate hosts *Ornithogalum* spp. are involved in sexual reproduction, such as in Israel where the only *Rph15*-virulent isolate (90-3) was found, recombination may play an important role in diversity, but a larger collection from this region should be analyzed to test this hypothesis.

Among the five *P. hordei* AFLP groups identified in the present study, some had unique relationships with virulence. For example, the Moroccan isolate 92-7, which had the widest virulence spectrum, overcoming most of the *Rph* genes, was clearly separated by the AFLP analysis from all the other tested isolates to form the AFLP group MG I. Similarly, 90-3 was the single isolate in AFLP group MG

II; it has a unique virulence: it is the only isolate with virulence for resistance gene *Rph15*. The overall correlation between virulence and AFLP was relatively low, as indicated by comparison of similarity matrices, where the matrix *r* was 0.45. A low correlation between virulence and molecular markers is common in sexually reproducing populations (Burdon and Roelfs 1985a), but sexual production of *P. hordei* is expected only in the regions where *Ornithogalum* spp. exist. The reason for the low correlation between virulence and molecular markers in *P. hordei* is unknown. Although not statistically significant, some degree of correlation is present between molecular and virulence variations, because many isolates in the same virulence group were also classified into the same MG subgroup. Each of these MG subgroups, IV-1, IV-4, V-1, and V-2, contains a number of isolates, if not all, from a same VG cluster. These correlations may be the result of *P. hordei* reproducing asexually in nature, especially in North America where most of the isolates originated. In *P. striiformis*, the low correlation between virulence and molecular markers may be a result of frequent parasexual recombination within the natural population (Chen et al. 1993; Little and Manners 1969). In other rusts pathogens that reproduce asexually, such as *P. graminis* f. sp. *tritici* (Burdon and Roelfs 1985b) and *P. triticina* (Kolmer et al. 1995), there is a close association between virulence and molecular pattern, and the reason why this is not so with *P. hordei* is unclear.

Often, isolates had the same virulence pattern but showed different molecular phenotypes. In those cases, a difference in virulence pattern might become detectable if differential lines with additional resistance genes were available. Isolates Race 8 and 94-14 had the same AFLP phenotypes but had different virulence phenotypes, indicating that the two isolates may have diverged from a common ancestral genotype via host selection.

AFLP is a powerful technique, which allowed for discrimination within *P. hordei*, a pathogen with a low diversity level. This study revealed the diversity of the selected *P. hordei* isolates in both virulence and AFLP molecular profiles. This information is useful for deploying resistance genes to leaf rust in barley breeding programs.

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