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Pathogenic and molecular variability among isolates of *Pyrenophora tritici-repentis*, causal agent of tan spot of wheat in Argentina

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Abstract Tan spot, caused by *Pyrenophora tritici-repentis*, is a common disease of wheat (*Triticum aestivum*) responsible for economic losses in some wheat growing areas worldwide. In this study the pathogenic and genetic diversity of 51 *P. tritici-repentis* isolates collected from different ecological regions of Argentina were analyzed. Virulence tests were conducted on 10 selected wheat cultivars: Buck Halcón, Chris, Gabo, Glenlea, Klein Dragón, Klein Sendero, Max, ND 495, ProInta Guazú and ProInta Imperial. Data revealed significant differences between all main factors evaluated and the interactions for 19 of the isolates analyzed. Based on the reaction type of each isolate/cultivar combination, 48 different pathogenic patterns were detected. The molecular analysis using Inter-Simple Sequence Repeats (ISSR) revealed the existence of 36 different haplotypes among 37 isolates of *P. tritici-repentis* originally selected for this study. These results indicate that *P. tritici-repentis* on wheat in Argentina is a heterogeneous fungus, implying that screening wheat germoplasm for resistance for tan spot disease requires a wide range of pathogen isolates.

Keywords Tan spot · *Triticum aestivum* · *Pyrenophora tritici-repentis* · ISSR · pathogenic diversity

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Introduction

Tan spot caused by *Pyrenophora tritici-repentis*, anamorph *Drechslera tritici-repentis*, is a major disease of wheat (*Triticum aestivum*) worldwide (Wiese 1987). The shift in farming practices toward stubble retention has resulted in a considerable increase in disease incidence in major wheat growing areas (Hosford 1982). Yield losses have ranged from 3% to 53%, depending on cultivar susceptibility,

environmental conditions, and virulence of the pathogen population (Hosford 1971; Rees and Platz 1983). The disease is very destructive on durum, winter and spring wheat (Lamari and Bernier 1989a, b; Misra and Singh 1972). Tan spot reduces total yield, kernel weight (Schilder and Bergstrom 1990; Shabeer and Bockus 1988), number of grains per head (Schilder and Bergstrom 1990), total biomass (Kremer and Hoffmann 1992), and/or grain quality because of red-smudge symptoms (Fernandez et al. 1994).

Tan spot is the fastest growing disease problem in the Southern Cone region of South America including Argentina, Brazil, Chile, Paraguay and Uruguay (Kohli et al. 1992). In the early 1980s, *P. tritici-repentis* was found for the first time affecting wheat crops in the north-central region of Buenos Aires, Argentina (Annone 1985). Subsequently, tan spot has gained predominance among foliar wheat diseases in most of the wheat growing areas in Argentina (Carmona et al. 1999; Kohli 1995; Kohli et al. 1992; Perelló et al. 2003). Increase in the severity of this disease has been linked with the change in the wheat varietal composition and the expansion of the area under conservation tillage practices, especially zero tillage, which allow the build-up of inoculum on the wheat stubble over time (Klein 2001). Most commercially accepted cultivars of wheat are susceptible to moderately susceptible to tan spot in Argentina (Klein 2001). This pathogen has received considerable attention in the past three decades, following the description of the etiology of tan spot of wheat by Hosford (1971). *Pyrenophora tritici-repentis* has been reported as a difficult organism to study because of the high level of variation in disease symptoms and significant interactions among isolates, genotype and environment (Krupinsky 1992).

Knowledge of pathogen variation in virulence and/or aggressiveness is an important component for developing durable resistant cultivars to combat plant diseases (Araya 2003). Physiological variation in virulence and/or aggressiveness in the *P. tritici-repentis* population have been reported using both quantitative and qualitative scales (Ali and Francl 2002; Ali et al. 2002; de Wolf et al. 1998; Diaz de Ackermann 1987; Krupinsky 1987, 1992; Strelkov et al. 2002). The tan spot syndrome consists of two phenotypically distinct and independent symptoms: tan necrosis and extensive chlorosis (Lamari and Bernier 1989b). Lamari et al. (1995) proposed a race-based system to describe isolates of *P. tritici-repentis* and currently 11 races of *P. tritici-repentis* have been identified (Ali and Francl 2002, 2003; Ali et al. 2002; Lamari et al. 2003; Manning et al. 2002). Five races of the pathogen were reported to occur based on virulence patterns on a standard differential set of wheat cultivars in the United States and Canadá (Ali and Francl 2001, 2002; Lamari and Bernier 1989a, b; Lamari et al. 1991, 1995, 1998). According to Ali and Francl (2002) *P. tritici-repentis* has a diverse population in South America. However, little is known about the genetic variation of the pathogen population in Argentina.

It is recognized that *P. tritici-repentis* presents great variability in its genome and pathogenicity (de Wolf et al. 1998). Several molecular methods have been used to analyze diversity of plant pathogens at the genome level, such as random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), enterobacterial intergenic consensus (ERIC), repetitive extragenic palindromic (REP), simple sequence repeats (SSRs) and inter simple sequence repeats (ISSRs; Mehta 2001; Mehta et al. 2004; Pujol Vieira dos Santos et al. 2002; Stenglein and Balatti 2006). ISSR consists of the amplification of DNA sequences between SSR by means of anchored or non-anchored SSR homologous primers (Zietkiewicz et al. 1994). SSRs are tandem repeat motifs composed of one to six nucleotides, which are ubiquitous, abundant and highly polymorphic in most eukaryotic genomes (Tautz and Renz 1984). ISSR does not require a previous knowledge of the sequence and generates specific and reproducible patterns due to the high stringent conditions of annealing (Bornet and Branchard 2001). A preliminary study with Brazilian isolates of *P. tritici-repentis* demonstrated high levels of polymorphisms, but did not find correlation between RAPD, geographic origin and/or pathogenic data (Pujol Vieira dos Santos et al. 2002). To date, the extent of genetic variability among and within isolates of *P. tritici-repentis* in Argentina is lacking.

The objective of this study was to assess the variability among *P. tritici-repentis* isolates collected from different wheat ecological growing areas in Argentina by pathogenic and molecular markers, and describe the relationship between this variability and the geographic origin of the isolates.

Materials and methods

Terminology

According to Van der Plank (1978, 1984) specificity in host-pathogen relationships is often indicated by significant isolate × cultivar interaction in the analysis of variance of an experiment, where a number of pathogen isolates are tested in all possible combinations on a set of host genotypes. Non-specificity is identified by a lack of such interaction.

Fungal isolates

The leaf samples were collected from wheat leaves of different cultivars growing in different regions of Argentina, in years 2000, 2001, 2002 and 2003 (Fig. 1). The leaves of each collected sample were cut into 1.0–1.5 cm pieces so that a lesion was cut through the centre. Five disks of each leaf were selected at random, washed under tap water, surface-sterilized by dipping successively into 70% ethanol for 2 min, 5% sodium hypochlorite (commercial 55 g Cl/l) for 2 min and finally rinsed twice in fresh sterilized distilled water (SDW). Five leaf disks were placed in each Petri dish containing 2% PDA acidified to pH 5 with 250 mg chloramphenicoll^{-1} to suppress bacterial growth. Plates were incubated for 4 or 5 days at 21+2°C with alternating light (3,500 lx dark cycles of 12 h plus the addition of near-UV light 365 nm). At the fifth day of incubation the conidia were taken from the colony and placed on the Petri dishes with 2% V8 for 6 days at 21+2°C with alternating light (3,500 lx dark cycles of 12 h plus the addition of near-UV light 365 nm). The isolates were stored under sterile mineral oil at 4°C.

Fifty-one *P. tritici-repentis* monosporic isolates were obtained and were identified according to species concepts and descriptions, and taxonomic standards for species separation, as presented in Ellis and Waller (1976) (Table 1).

Inoculum preparation and inoculation procedure

Mycelial plugs were taken from the advancing margin of each *P. tritici-repentis* isolate for 6 days grown on 2% PDA. These plugs of 0.5 cm diam were placed in the centre of a Petri dish with V8-agar (200 ml of V8 juice®, 3 g $CaCO_3$, 20 g agar and 800 ml distilled water) and incubated for 7 days at 21+2°C with alternating light (3,500 lx dark cycles of 12 h plus the addition of near-UV light 365 nm). At the seventh day of incubation the dishes were then incubated in continuous light for 24 h. The light cycle stimulated conidiophore formation and reduced mycelial fragments while the dark cycle induced completion of the sporulation process (Raymond and Bockus 1982).

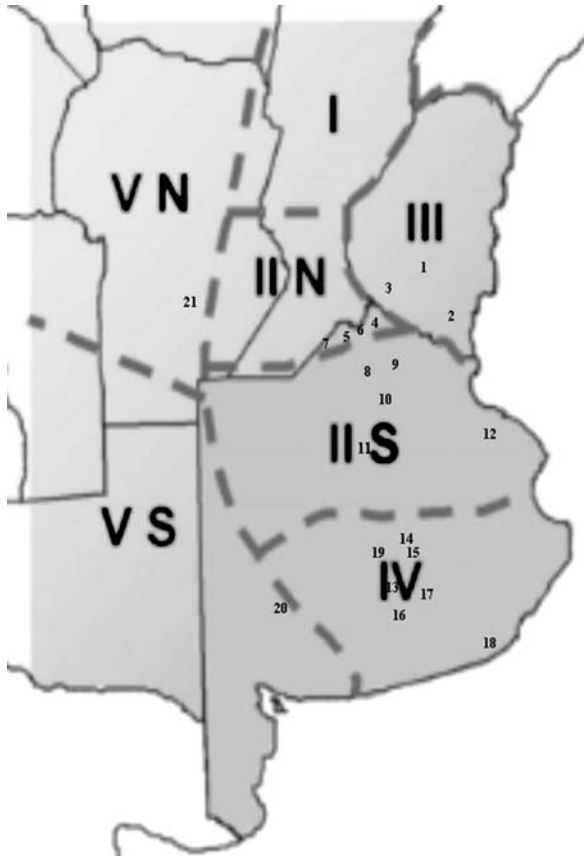

Fig. 1 Map of areas of wheat in Argentina showing the localities where *P. tritici-repentis* isolates were collected. Areas of wheat in Argentina: I, II North, II South, III, IV, V North and V South. Localities: *1* Rincón Nogoyá, *2* Gualeguaychú, *3* Victoria, *4* Salto, *5* Pergamino, *6* Arrecifes, *7* Comodoro Py, *8* Alberti, *9* Bragado, *10* 9 de Julio, *11* 25 de Mayo, *12* Los Hornos, *13* Chillar, *14* Tapalqué, *15* Azul, *16* Benito Juárez, *17* Tandil, *18* Orense, *19* Olavarria, *20* Coronel Suárez, *21* Marcos Juárez

Following the final incubation period, plates were examined with a stereomicroscope to verify conidial production. The conidial harvest was taken by flooding the plate with 5 ml of SDW and dislodging the conidia with a bent glass rod. The resulting suspension was filtered through cheesecloth. The number of conidia mm^{-2} was determined with a nematode-

Table 1 Isolate codes and origins of *Pyrenophora tritici-repentis* isolates used in this study

Code of isolate	Location	Wheat cultivar	Year of collection	Argentinian wheat region
A021	Alberti	Klein Escudo	2002	IISouth
A029	Alberti	Klein Escudo	2002	IISouth
A0210	Alberti	Klein Escudo	2002	IISouth
ARR031	Arrecifes	Buck Mataco	2003	II North
ARR035	Arrecifes	Buck Mataco	2003	II North
AZ021	Azul	Klein Don Enrique	2002	IV
AZ023	Azul	Klein Don Enrique	2002	IV
AZ024	Azul	Klein Don Enrique	2002	IV
BJ00	Benito Juárez	Klein Don Enrique	2000	IV
BJ0011	Benito Juárez	Klein Don Enrique	2000	IV
B021	Bragado	Klein Escorpión	2002	IISouth
B024	Bragado	Klein Escorpión	2002	IISouth
B028	Bragado	Klein Escorpión	2002	IISouth
CH002	Chillar	Klein Don Enrique	2000	IV
CH007	Chillar	Klein Don Enrique	2000	IV
CH0010	Chillar	Klein Don Enrique	2000	IV
CP021	Comodoro Py	Baguette 10	2002	II North
CS001	Coronel Suárez	Buck Sureño	2000	V South
CS003	Coronel Suárez	Buck Sureño	2000	V South
G037	Gualeguaychú	Buck Biguá	2003	III
G0310	Gualeguaychú	Buck Mataco	2003	III
H003	Los Hornos	Buck Brasil	2000	IISouth
H0011	Los Hornos	Buck Brasil	2000	IISouth
H0014	Los Hornos	Buck Brasil	2000	IISouth
H021	Los Hornos	Buck Brasil	2002	IISouth
9J031	9 de Julio	Klein Escorpión	2003	II South
9J032	9 de Julio	Klein Escorpión	2003	II South
MJ032	Marcos Juárez	Klein Escorpión	2003	V North
25M032	25 de Mayo	Buck Mataco	2003	II South
25M036	25 de mayo	Buck Mataco	2003	II South
OL022	Olavaria	Klein Don Enrique	2002	IV
O001	Orense	Buck Sureño	2000	IV
O008	Orense	Buck Sureño	2000	IV
O0018	Orense	Buck Sureño	2000	IV
P021	Pergamino	Klein Don Enrique	2002	II North
P027	Pergamino	Klein Don Enrique	2002	II North
P022	Pergamino	Klein Don Enrique	2002	II North
P023	Pergamino	Klein Don Enrique	2002	II North
N021	Rincón Nogoyá	Baguette 10	2002	III
N022	Rincón Nogoyá	Baguette 10	2002	III
SALTO1	Salto	Klein Escorpión	2003	II North
SALTO2	Salto	Klein Escorpión	2003	II North
TA021	Tandil	Baguette 10	2002	IV
TA022	Tandil	Baguette 10	2002	IV
TA024	Tandil	Baguette 10	2002	IV
T003	Tapalqué	Klein Don Enrique	2000	IV
T009	Tapalqué	Klein Don Enrique	2000	IV
T0017	Tapalqué	Klein Don Enrique	2000	IV
V024	Victoria	Baguette 10	2002	III
V025	Victoria	Baguette 10	2002	III
V0213	Victoria	Baguette 10	2002	III

counting dish (Neubauer haemacytometer) and a binocular stereoscope. Conidial suspension was adjusted to 3×10^3 conidia ml^{-1} and 0.05% Tween® 20 was added as a surfactant.

Pathogenicity test and statistical analysis

To evaluate virulence variability, a differential set of 10 wheat cultivars (Buck Halcón, Chris, Gabo, Glenlea, Klein Dragón, Klein Sendero, Max, ND495, ProInta Guazú and ProInta Imperial) was selected based on previous information and previous tests (Lamari and Bernier 1989a, b; Ali and Francl 2001; Krupinsky 1992). Three seeds of each differential were sown in plastic cones (15 cm diam and 12 cm in length) and grown in the greenhouse at 18+2°C with a 10 h photoperiod. The cones were arranged in a split-plot replicated three times in a completely randomized design (Sah and Fehrmann 1992; Schilder and Bergstrom 1990). Isolates were used as main-plots and cultivars as subplots. All differential wheat cultivars were inoculated individually using the 51 monosporic isolates.

Wheat seedlings at the three leaf stage were sprayed until run-off using a small manual atomizer. After inoculation the plants were placed in a plastic covered chamber for 48 h to ensure high humidity.

The plants were rated for symptom development and disease severity (percent leaf area infected) 9 days post-inoculation. Temperature and light conditions were maintained at 18±2°C with a 10 h photoperiod during the disease rating experiments.

The data obtained from the reaction type or symptoms generated by the 10 cultivars of wheat (necrosis; chlorosis and both necrosis+chlorosis) were transformed as reaction type present (1) or reaction type absent (0) and the data were assembled in a matrix for similarity analyses. Pathogenic similarities between all the isolates were computed using the Jaccard coefficient (Sneath and Sokal 1973), which does not consider the joint absence of a marker/type of reaction as an indication of similarity. The similarity index of Jaccard between i and j is given by $Sij=a/(a+b+c)$, where a is the number of characters present in both i and j, b is the number of characters present in i but not in j, and c the number of characters present in j but not in i. The data were used to construct a dendrogram using the unweighted pair group method with arithmetic average (UPGMA). The Cophenetic Correlation Coefficient (CCC) was chosen to indicate the level of distortion between the similarity matrix and cluster analysis. NTSySpc version 2.0 was used to perform these analyses.

Data of disease severity (percent leaf area infected) were arcsine square-root transformed and submitted to an ANOVA using the computer programme MSTAT-c (Russell). Means were separated by Tukey's test ($P=0.05$).

DNA extraction

Thirty-seven randomly selected isolates of *P. tritici-repentis* were grown on PDA media. For each isolate, 7 day-old cultures were carefully scraped from the surfaces of two Petri dishes using a scalpel, placed in a 1.5 ml Eppendorf tube, and mixed with 400 µl of CTAB (cetyltrimethylammonium bromide) extraction buffer (100 mM Tris–HCl pH 8.0+20 mM EDTA pH 8.0+1.4 M NaCl+0.2% (*w*/*v*) β mercaptoethanol)+ 100 µl CTAB 10%. DNA was extracted by heating the slurry at 60°C for 30 min. One volume of a chloroform/isoamyl alcohol (24:1 *v*/*v*) was then added, and the tubes were vortexed and centrifuged at 10,000×*g* for 5 min. The aqueous phase, containing the DNA, was transfered to a new tube and precipitated overnight by adding isopropanol. The DNA was pelleted by centrifugation at 10,000×*g* for 10 min, washed with 10 mM ammonium acetate–75% ethanol (10,000×*g*, 10 min) and then with 70% ethanol (10,000×*g*, 10 min). The pellet of DNA was then dried and dissolved in 80 µl of TE (10 mM Tris–HCl pH 8.0+1 mM EDTA pH 8.0). The DNA concentration was estimated by comparison against a 1 kb DNA ladder molecular marker of known concentration (Promega Biotech. Corp.) electrophoresed in 0.7% agarose.

ISSR analysis

Five of 28 ISSR primers tested, $(CAA)_5$, GAG $(CAA)_5$, $(AG)_8TA$, $(GA)_8ACC$, and $(GAG)_5CAG$ amplified polymorphic DNA fragments yielding consistent banding patterns and were selected to analyze diversity among *P. tritici-repentis* isolates. PCRs were performed in a 25 µl final volume containing 12 ng of genomic DNA, 10X reaction buffer (2 mM Tris–HCl pH 8.0+10 mM KCl+0.01

mM EDTA+1 mM DTT+50% glycerol+0.5% Tween®20+0.5% Nonidet® P40.), 0.7 μM of primer, 200 μM of each dNTP (Promega Biotech. Corporation), 2.5 mM $MgCl_2$, and 1.25 units of *Taq* DNA polymerase (Higway Molecular Biology-InBio-UNICEN-Tandil). Reaction mixtures were overlaid with a drop of mineral oil. DNA amplification was performed in a thermal cycler MJ Research (PTC-100) with an initial denaturing step at 94°C for 7 min, followed by 33 cycles at 94°C for 60 s, 48°C for 75 s, and 72°C for 4 min, and a final extension cycle at 72°C for 7 min. Each reaction was performed at least twice. PCR products from ISSR reactions were electrophoresed on 1.5% (*w*/*v*) agarose gels containing 0.2 μg μl^{-1} of ethidium bromide at 80 V in 5X Tris–borate–EDTA buffer for 4 h at room temperature. Fragments were visualized under UV light. The size of the DNA fragments was estimated by comparing the DNA bands with a 1 kb DNA ladder marker (Promega Biotech. Corp). Gel images were photographed with a Polaroid Camera DS34 and film type 667.

Analysis of genetic similarity

The amplified bands were scored as present (1) or absent (0). Fragments of the same size were considered homologous and only clear and reproducible amplified fragments were scored. The resulting data were assembled in a matrix. Genetic similarities between all the pairs of isolates were computed using the Jaccard coefficient (Sneath and Sokal 1973). The data were used to construct a dendrogram using the UPGMA and the CCC was chosen. Correlation between pathogenicity ratings and ISSR lineages of the 37 isolates selected for molecular analysis was determined using the Mantel test. NTSySpc version 2.0 was used to perform these analyses.

Results

Pathogenicity test

The reaction types observed were characterized by necrosis (Nec), chlorosis (Cl) and necrosis with chlorosis halo (NecCl; Fig. 2). After 72 h inoculation, wheat cv. ND495 showed symptoms in response to inoculation with isolates CS001, AZ024, CP021, OL022, N021, H0014, A0210, P022, AZ021, T009, N022, P027, and cv. Max reacted to inoculation with isolates CS001, AZ024, OL022, H0014, A0210, BJ0011, O0018, B021, T003, A029, N021. After 96 h from inoculation, the following wheat cultivars showed symptoms in response to inoculation with isolates CS001, CP021, A0210 (Glenlea); AZ024, A0210 (K. Dragón); A0210 (ProInta I.); N022 (Gabo) and N021 (B. Halcón).

All isolates of *P. tritici-repentis* were pathogenic to all wheat cultivars used in the experiment, except isolate P021. The most frequent reaction type was NecCl, represented by 175 (34%) isolate x cultivar interactions. The majority of these interactions were with the wheat cvs Max, ND495 and K. Sendero. Five isolates (A0210, BJ00, B024, 25 M032 and P022) produced NecCl on the wheat cv. ProInta Imperial. The reaction type Cl was represented by 97 (19%) of the isolate x cultivar interactions, and the majority of these interactions were with the wheat cvs Glenlea and ND495. The cv. Max showed low frequency of this reaction type with only four isolates (CH007, CP021, H003 and T0017). However, both reaction types (NecCl and Cl) were observed in all wheat cultivars tested. The reaction type Nec was observed only in six cultivars (Chris, Max, Glenlea, Gabo, B. Halcon and K. Dragon) and the frequency was low 2% (Table 2).

The isolate A0210 was the only isolate that caused symptoms on all the wheat cultivars tested. The isolates A0210, AZ021, BJ0011, B024, H003, H021, N022 and 9J031 caused all three reaction types. However, other isolates (A021, ARR035, B028, CH002, 9J032, 25 M032, 25 M036, MJ032, O008, P022, S031, S032, TA022 and V0213) produced only NecCl, and showed no specific interaction with any of the 10 wheat cultivars inoculated. Moreover, CH007 was the only isolate that caused only Cl on Max, Glenlea, K. Sendero and ND495 (Table 2).

The cluster analysis based on reaction types defined 48 groups among the 51 isolates evaluated according to the Jaccard coefficient (CCC=077) (Fig. 3). The isolates were grouped in two clusters (I and II) with an average similarity between groups of 20%. Cluster I included 13 isolates that produced Cl on the wheat cv. ND495, and the other cluster II, included the remaining 38 isolates that produced NecCl on the same cultivar. Within cluster I, the

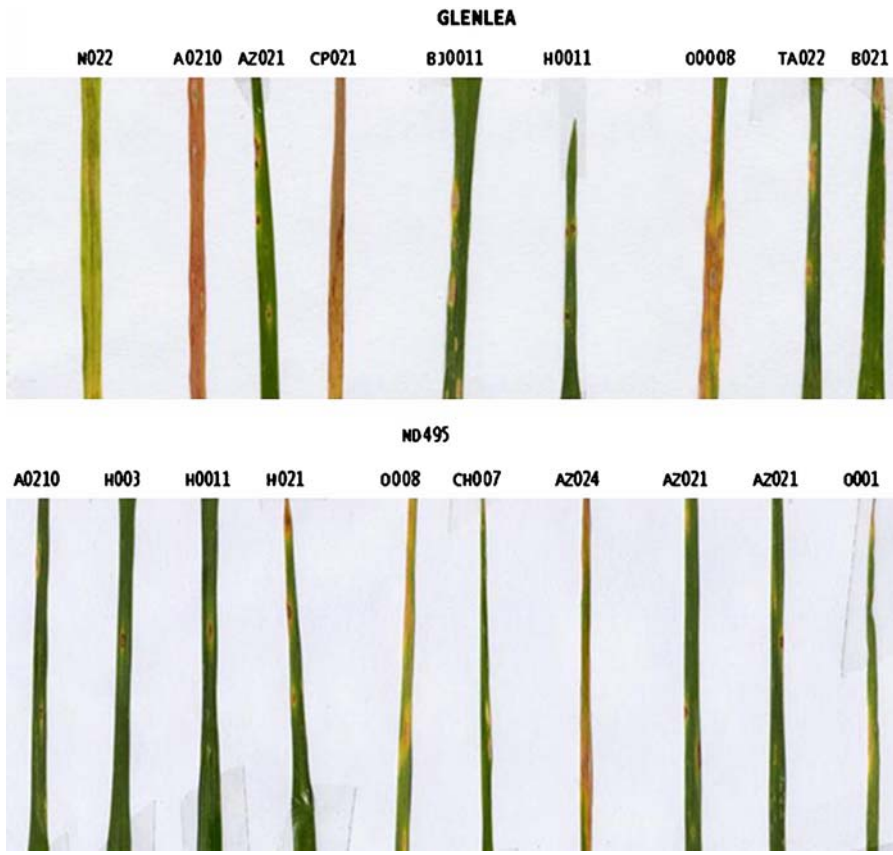

Fig. 2 Reaction types revealed by wheat cvs Glenlea and ND495 inoculated with some *P. tritici-repentis* isolates

isolates of *P. tritici-repentis* were grouped in two subclusters IA and IB with 25% reaction type similarity; the subcluster IA included those isolates of the group that produced symptoms on wheat cultivar B. Halcon. In subcluster IA, the isolate N021 uniquely produced NecCl in cultivar K. Dragon and in the subcluster IB the isolate BJ00 was unique in producing NecCl in ProInta Guazu and Nec in Max. Furthermore, within cluster II we observed two subclusters IIA and IIB with an average 27% similarity between groups. These subclusters were differentiated because the 11 isolates included in subcluster IIB produced symptoms on the wheat cv. Chris. The subcluster IIA included the remaining 27 isolates. The isolates of *P. tritici-repentis* were grouped by reaction type on the 10 wheat cultivars tested, but no relationship could be established between the reaction type, geographic origin and/or wheat cultivar.

Analysis of variance (ANOVA) of percent severity indicated significant differences among isolates, cultivars and isolate x cultivar interactions (Table 3). Means of severity comparisons with Tukey's test revealed significant differences for the isolate x cultivar interactions for 19 isolates of *P. tritici-repentis* tested (Table 4). Moreover, these isolates presented distinct patterns of interactions with the 10 wheat cultivars. The percent severity caused by the *P. tritici-repentis* isolates on the wheat cultivars was a variable trait. On wheat cv. ND495 we observed that isolates V025 and BJ0011 caused the highest and lowest severities, respectively. These differences suggested that a wheat cultivar could appear resistant or susceptible, depending on which isolate is used. However, the aim of this study was not to detect resistant and/or susceptible wheat cultivars, but to describe differential severity reactions.

Analysis of molecular markers

Diversity of isolates of *P. tritici-repentis* was analyzed by ISSR markers. All reactions were performed at least twice and five primers [$(CAA)_5$, $GAG(CAA)_5$, $(AG)_8TA$, $(GA)_8ACC$, $(GAG)_5CAG$] yielding poly-

Table 2 Different reaction types revealed by 10 wheat cultivars inoculated with 51 *P. tritici-repentis* isolates under greenhouse conditions

Wheat cultivar										
Isolates	Chris	ProInta imperial	Max	Glenlea	ProInta Guazú	Gabo	B. Halcón	K. Sendero	ND 495	K. Dragón
A021	–	–	NecCl	–	–	–	–	NecCl	NecCl	–
A029	Cl	Cl	NecCl	Cl	NecCl	NecCl	Cl	Cl	Cl	–
A0210	Nec	NecCl	NecCl	NecCl	Cl	Cl	NecCl	NecCl	NecCl	NecCl
ARR031	–	–	–	Nec	–	–	–	NecCl	NecCl	–
ARR035	–	–	–	–	–	NecCl	NecCl	NecCl	NecCl	–
AZ021	Nec	–	NecCl	NecCl	–	Cl	Cl	NecCl	NecCl	Cl
AZ023	NecCl	–	NecCl	Cl	Cl	–	–	NecCl	NecCl	–
AZ024	Cl	–	NecCl	–	–	–	–	NecCl	NecCl	–
BJ00	NecCl	NecCl	Nec	Cl	NecCl	–	–	NecCl	Cl	–
BJ0011	Cl	–	NecCl	Nec	–	–	–	–	NecCl	–
B021	Cl	–	NecCl	NecCl	NecCl	NecCl	NecCl	Cl	Cl	–
B024	Nec	NecCl	–	NecCl	Cl	NecCl	–	NecCl	NecCl	–
B028	–	–	NecCl	–	–	–	–	–	NecCl	–
CH002	–	–	NecCl	NecCl	NecCl	–	–	–	NecCl	NecCl
CH007	–	–	Cl	Cl	–	–	–	Cl	Cl	–
CH0010	–	–	NecCl	–	Cl	Cl	–	–	NecCl	–
CP021	–	–	Cl	Cl	–	–	–	Cl	Cl	NecCl
CS001	Cl	–	NecCl	Cl	Cl	Cl	–	NecCl	NecCl	–
CS003	NecCl	–	NecCl	NecCl	Cl	Cl	–	NecCl	NecCl	NecCl
G037	–	–	Nec	–	–	–	–	NecCl	NecCl	–
G0310	–	–	–	–	–	–	Nec	NecCl	–	–
H003	Nec	–	Cl	Cl	Cl	Cl	Cl	Cl	NecCl	NecCl
H0011	Cl	–	NecCl	NecCl	NecCl	NecCl	NecCl	NecCl	NecCl	NecCl
H0014	NecCl	–	NecCl	Cl	–	NecCl	–	Cl	Cl	–
H021	Nec	–	NecCl	–	Cl	NecCl	Cl	NecCl	NecCl	NecCl
9J031	–	Cl	NecCl	–	–	NecCl	Cl	NecCl	NecCl	Nec
9J032	–	–	NecCl	NecCl	–	NecCl	–	NecCl	–	–
MJ032	–	Cl	–	–	–	–	NecCl	NecCl	NecCl	–
25M032	–	NecCl	NecCl	–	–	–	NecCl	NecCl	NecCl	NecCl
25M036	–	–	NecCl	–	–	–	–	NecCl	NecCl	–
OL022	NecCl	–	NecCl	Cl	Cl	Cl	–	Cl	NecCl	Cl
O001	Nec	–	NecCl	NecCl	–	–	–	NecCl	NecCl	–
O008	NecCl	–	NecCl	NecCl	–	–	–	–	NecCl	–
O0018	NecCl	–	NecCl	–	–	–	–	–	Cl	NecCl
P021	–	–	–	–	–	–	–	–	–	–
P027	NecCl	–	NecCl	Cl	–	Cl	Cl	NecCl	NecCl	Cl
P022	–	NecCl	NecCl	–	–	–	–	NecCl	NecCl	–
P023	Cl	–	NecCl	Cl	NecCl	NecCl	Cl	Cl	Cl	–
N021	NecCl	Cl	NecCl	Cl	NecCl	Cl	Cl	–	Cl	NecCl
N022	NecCl	Cl	NecCl	Cl	–	Nec	–	Cl	NecCl	–
S031	–	–	NecCl	–	–	–	–	NecCl	NecCl	–
S032	–	–	NecCl	–	–	NecCl	NecCl	NecCl	NecCl	NecCl
TA021	–	–	NecCl	–	Cl	–	NecCl	–	NecCl	–
TA022	–	–	–	NecCl	–	–	NecCl	NecCl	NecCl	–
TA024	Cl	–	NecCl	NecCl	NecCl	NecCl	NecCl	Cl	Cl	–
T003	–	–	NecCl	–	NecCl	NecCl	NecCl	Cl	Cl	–
T009	Cl	–	NecCl	Cl	Cl	NecCl	–	NecCl	NecCl	Cl
T0017	NecCl	Cl	Cl	Cl	–	Cl	–	Cl	–	Cl
V024	–	–	NecCl	–	Cl	Cl	–	–	Cl	–
V025	NecCl	–	NecCl	Cl	–	Cl	NecCl	–	Cl	–
V0213	NecCl	–	NecCl	–	–	–	–	–	NecCl	–

Reaction types: *Cl* chlorosis; *Nec* necrosis; *NecCl* necrosis with chlorosis

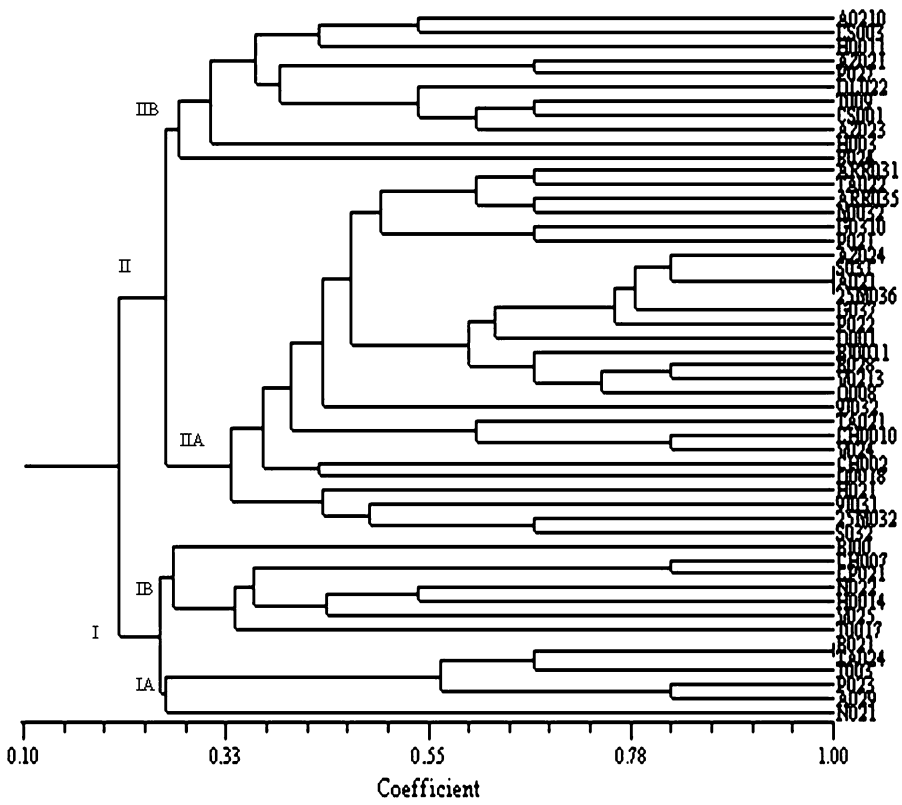

Fig. 3 Cluster analysis dendrogram based on reaction type produced by 10 wheat cultivars inoculated with 51 isolates of *P. tritici-repentis*

morphic banding patterns were selected to analyze diversity of the fungus. A total of 62 fragments was generated using the five ISSR primers and among them 43 were polymorphic (69%). A representative amplification reaction with primer AA3 is presented in Fig. 4. The genetic similarity between isolates was calculated based on the banding pattern generated by ISSR primers and a dendrogram was built based on the Jaccard coefficient (CCC=0.85). Cluster analysis of ISSR data defined 36 haplotypes among the 37 isolates analyzed, showing that all, except isolates CH007 and T003, had unique banding patterns (Fig. 5). The isolates were grouped in two groups (I, II) with an average similarity between groups of 62%. Cluster I included only three isolates V0213, CH007 and T003, isolated from Victoria, Chillar and Tapalqué, respectively, and obtained from wheat cvs Baguette 10 (V0213), and Baguette 10 and K. Don Enrique, respectively. Cluster II, included the remaining 34 isolates. Cluster II isolates were grouped in two subclusters, IIA and IIB, with 74% genetic similarity. Subcluster IIA included a total of 25 isolates and subcluster IIB included the remaining isolates MJ032, 9 J032, G037, T009, ARR031, S0321, S032 P023 and CH002.

In addition, the variability of reaction types was unrelated to variability at the DNA level (r=0.05) revealing the lack of congruency between ISSR markers and reaction type data. These groupings did

Table 3 ANOVA describing effects of isolate, wheat cultivar and interaction after inoculation with 51 *P. tritici-repentis* isolates

Source of variation	*df*	Mean square
Replication	2	76.996 ns
Isolates	50	223.483***
Error	100	62.727
Cultivars	9	598.397***
Isolates × cultivars	450	17.448***
Error	918	13.499
Total	1,529	

CV=45.45%.

ns nonsignificant

***F value significant at P<0.05

Table 4 Means of differences for the isolate x cultivar interaction according Tukey's test for disease severity caused by 19 *P. tritici-repentis* isolates

Wheat Cultivar	Isolates																		
	N021	AZ024	O0018	AZ021	A029	T009	A0210	V025	CH002	V024	H021	CS001	B021	T0017	H0011	AZ023	B024	BJ0011	H003
Chris	9.274 abc	7.619 ab	4.055 b	10.71 abc	8.717 b	7.182 bc	11.41 abc	12.30 bc	9.965 ab	11.28 ab	16.01 abc	8.873 ab	11.57 abc	9.639 ab	11.59 cd	8.192 b	5.215 b	5.938 b	11.10 ab
Prointa imperial	4.055 c	6.383 ab	4.055 b	5.155 c	4.836 b	8.639 abc	6.629 c	10.94 bc	5.598 b	17.48 a	12.61 bcd	6.736 b	4.849 c	4.055 b	8.121 d	5.155 b	5.167 b	5.215 b	6.313 b
Max	11.21 abc	14.82 a	21.86 a	17.01 ab	11.93 ab	17.79 a	20.06 a	16.62 ab	17.39 a	16.39 ab	14.72 abcd	13.80 ab	11.50 abc	15.50 a	20.22 abc	19.13 a	7.937 ab	15.77 a	11.98 ab
Glenlea	15.81 ab	12.85 ab	12.29 ab	18.85 a	7.387 b	16.63 ab	14.35 abc	15.54 abc	9.368 ab	14.22 ab	5.931 d	8.656 ab	17.08 a	6.772 ab	25.70 a	13.57 ab	9.955 ab	8.152 ab	19.12 a
Prointa guazu	9.178 abc	4.326 b	10.96 b	10.39 abc	8.633 b	8.206 abc	9.197 bc	8.110 bc	9.553 ab	11.29 ab	9.626 bcd	6.211 b	8.391 abc	5.598 b	6.814 d	7.390 b	7.287 ab	4.055 b	7.750 b
Gabo	9.178 abc	9.516 ab	11.16 b	14.84 abc	11.12 ab	9.926 abc	12.10 abc	11.76 bc	10.88 ab	11.68 ab	23.11 a	8.338 ab	8.892 abc	7.864 ab	11.10 cd	7.844 b	12.34 ab	8.532 ab	12.70 ab
Buck Halcon	12.95 abc	4.326 b	6.828 b	12.53 abc	4.326 b	5.297 c	8.514 bc	5.918 c	8.159 ab	7.656 b	7.070 cd	4.480 b	7.072 bc	5.867 ab	8.007 d	7.744 b	4.764 b	5.867 b	6.520 b
Klein Sendero	6.288 bc	8.231 ab	9.505 b	13.51 abc	12.66 ab	8.334 abc	10.67 abc	9.134 bc	8.680 ab	14.61 ab	18.99 ab	9.423 ab	5.686 c	7.685 ab	13.88 bcd	8.849 b	8.742 ab	4.055 b	15.08 ab
Nd495	18.34 a	15.31 a	8.732 b	17.90 ab	18.81 a	14.36 abc	16.52 ab	23.89 a	11.04 ab	15.61 ab	16.40 abc	16.60 a	15.66 ab	12.96 ab	22.02 ab	11.69 ab	14.97 a	6.197 ab	14.48 ab
Klein dragon	10.14 abc	8.891 ab	7.372 b	8.989 bc	6.323 b	8.804 abc	11.36 abc	7.256 bc	5.791 b	7.513 b	11.30 bcd	6.511 b	8.570 abc	6.735 ab	9.891 d	6.452 b	9.693 ab	4.055 b	8.075 b

Means of 19 observations (isolates × cultivars). Numbers followed by same letter are not significantly different at $P=0.05$ (Tukey's test).

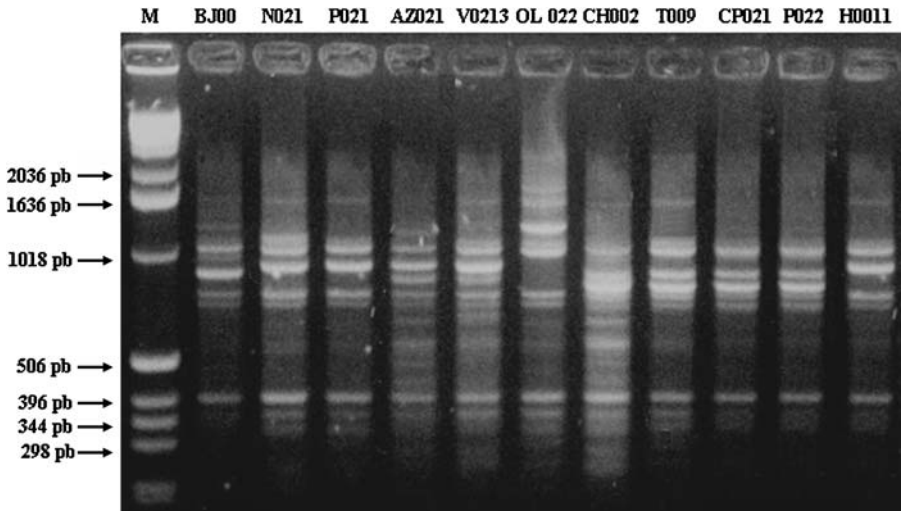

Fig. 4 Sample gel of ISSR (primer AA3) patterns produced by 11 isolates of *P. tritici-repentis*

not reflect pathogenic patterns, geographic origin, wheat cultivar origin of the *P. tritici-repentis* isolates.

Discussion

Using pathogenic and ISSR data we detected genetic diversity among the *P. tritici-repentis* isolates recovered from wheat leaves collected in different locations from Argentina.

In the pathogenicity test, the first disease symptoms were noticeable after 72 h of inoculation on the wheat cvs ND495 and Max, indicating thereby the relative level of susceptibility of these cultivars. On the rest of the cultivars the disease symptoms were noticed 96 h after inoculation.

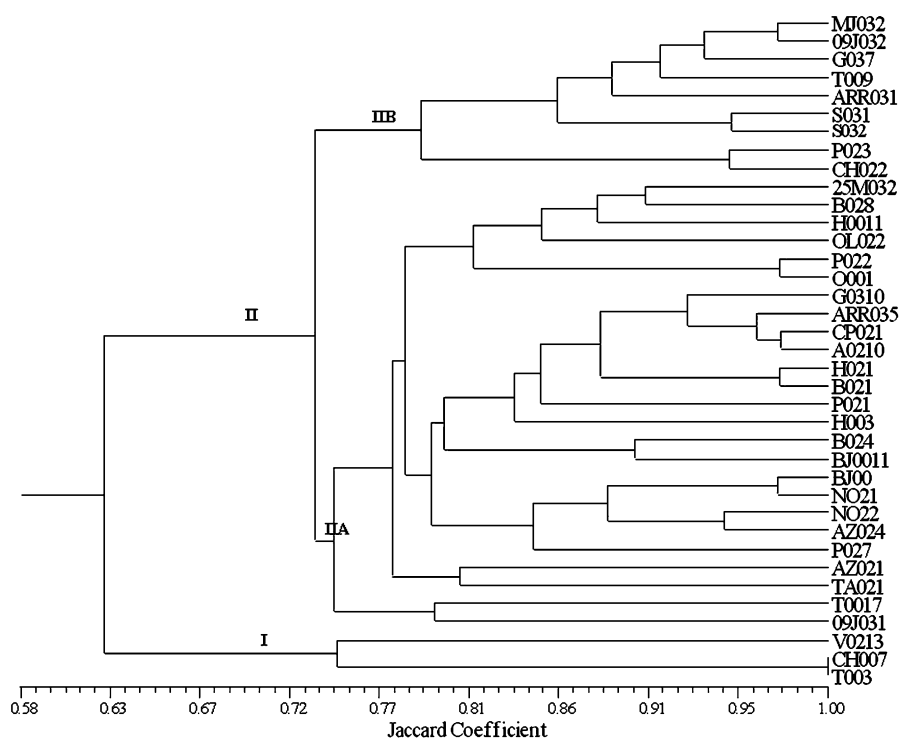

Fig. 5 Cluster analysis dendrogram based on ISSR markers

Various isolates caused different reaction types and different levels of severity on the same wheat cultivar. These results showed a considerable degree of variation in the isolates tested. According to the definition of Van der Plank (1978, 1984) we observed physiological specialization for 19 of the 51 isolates of *P. tritici-repentis* tested. Similar results were obtained in other studies, where several authors reported physiological variation in the fungal population (Hosford 1971; Lamari and Bernier 1989a, b; Schilder and Bergstrom 1990; Krupinsky 1992; de Wolf et al. 1998). In agreement with other authors (Krupinsky 1992; Schilder and Bergstrom 1990) the wheat cvs Max, ND495 and Glenlea revealed symptoms with the majority of *P. tritici-repentis* isolates tested. In race characterization of this pathogen, several authors observed only isolates causing necrosis on wheat cv. Glenlea (Ali and Francl 2002, 2003; Lamari and Bernier 1989a, b; Strelkov et al. 2002). However, in this study we report not only isolates causing Nec alone, but also 16 isolates producing Cl and 12 causing NecCl on the wheat cv. Glenlea. This result suggested that the Argentinean population of *P. tritici-repentis* is complex in reaction types and this characteristic should be considered in future race studies. The wheat cultivars used for this study did not show an evident susceptibility pathogenic pattern. These isolates should therefore be tested on the wheat set proposed by Lamari and Bernier (1989a, b).

This study confirmed a high degree of genetic variability among Argentinean *P. tritici-repentis* isolates. Di Zinno et al. (1998) observed similar results with Brazilian isolates of *P. tritici-repentis*. Other authors made similar observations for *Pyrenophora* species (Crous et al. 1995; Ganesahn 1997; Peltonen et al. 1996; Singh and Hughes 2006). Moreover, Pujol Vieira dos Santos et al. (2002) described a high degree of variability among *P. tritici-repentis* isolates from Brazil with RAPD markers.

The pathogenic and molecular analyses showed intraspecific variability within *P. tritici-repentis* isolates and it was not possible to establish a relationship between this variability and the geographical regions and/ or wheat cultivars from which *P. tritici-repentis* isolates were obtained. In all analyses based on pathogenic and ISSR data, it was shown that *P. tritici-repentis* isolates from the same geographic region appeared in different groups. Conversely, those that showed the highest similarity coefficients were collected from different geographic regions. In agreement with our study, Friesen et al. (2005) using AFLP markers determined a high level of variability among isolates of *P. tritici-repentis* and these results show no genetic grouping of pathotypes or grouping for geographic location.

Genetic variability at the DNA level among isolates of *P. tritici-repentis* based on ISSR markers was unrelated to pathogenic variability. This result is consistent with other findings suggesting that isolates with the same pathogenicity patterns are not necessarily closely related based on DNA analysis (Sicard et al. 1997; Stenglein and Balatti 2006).

The high degree of pathogenic and genetic variability among isolates compared in this study characterizes a diverse population of *P. tritici-repentis* in the region. According to Pujol Vieira dos Santos et al. (2002), there are many factors that could have been affecting polymorphism analysis, e. g. the intraspecific variants of a pathogen, the number of samples selected for analysis, genetic flow between populations, environmental adaptation and adaptation to a new host and selective pressure and migration. On the other hand, *P. tritici-repentis* is a homothallic fungus that readily produces the sexual stage on field stubble, giving this fungal population the opportunity for adaptation by sexual recombination. In Argentina, the sexual stage of *P. tritici-repentis* occurs on wheat stubble between crops, whereas the asexual stage *D. tritici-repentis* occurs during crop growth. The occurrence of sexual recombination in nature is likely to be the reason for the high level of genetic variability among isolates of *P. tritici-repentis* (Singh and Hughes 2006). Under favourable conditions conidia can travel 10–200 km (de Wolf et al. 1998). The tan spot fungus is also seed-borne, and thus long distance travel of fungal inoculum can occur by seed transmission (Singh and Hughes 2006). Hence, we can assume that the occurrence of the sexual state and long distance dispersal of inoculum could have contributed to pathogenic and genetic variability independent of geographic origin.

Consideration of variation in virulence in the *P. tritici-repentis* population is essential to understand the pathogen genetics of tan spot. Knowledge of the pathogen population helps in the development of a successful disease management programme, particularly resistant cultivars, effective fungicides and biological control agents. In particular, all wheat lines

should be screened with all races prevalent in the region prior to their commercialization.

The work presented here has laid the foundation for future studies by providing valuable information on the previously unknown pathogenic and genetic diversity of the Argentinean population of *P. tritici-repentis*. A greater number of isolates is currently under investigation to obtain a more comprehensive picture of the fungal race structure in the region.

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