Research Article

Genetic Diversity of *Pyrenophera tritici-repentis* Isolates, the Causal Agent of Wheat Tan Spot Disease from Northern Iran

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Background: The tan spot disease of wheat caused by *Pyrenophera tritici-repentis* has become a major disease in most wheat growing areas worldwide.
Objectives: Here we used ISSR and RAPD markers to study the genetic diversity of 34 *P. tritici-repentis* isolates collected from North of Iran.
Materials and Methods: The leaves having the typical symptoms of tan spot disease were collected and after fungal isolation, purification and identification, DNAs were extracted. After PCR amplification using each primer, PCR products were run in agarose gels, and the resulting bands were scored. Cluster analysis was performed using Un-Wighted Nighbor Joining method.
Results: A total of 178 reproducible bands were scored. Out of which 115 (65%) were polymorphic corresponding to an average of 8 polymorphic bands per primer. The average PIC values for ISSR and RAPD markers were 0.38 and 0.43, respectively. A high degree of genetic variability among Iranian *P. tritici-repentis* isolates was identified. Cluster analysis based on un-weighted neighbor-joining method using the combined molecular data revealed five distinct clusters. The results from the cluster analysis indicated that the genetic similarity among the Iranian *P. tritici-repentis* isolates could be partly explained by geographic origins where the isolates were collected.
Conclusions: Genetic variability of *P. tritici-repentis* along with relatively high level of geographic diversity observed in this study may suggest longer evolutionary period for the isolates from the Middle East, wheat center of origin, as opposed to other places.

Keywords: Cluster analysis; Drechslera tritici-repentis; ISSR and RAPD markers; Genetic variability

1. Background

The fungus *Pyrenophera tritici-repentis* (Died.) Drechs. [anamorph: *Drechslera tritici-repentis* (Died.) Shoem] is the causal agent of wheat tan spot. It has become a major disease in most wheat growing areas worldwide (1). Under favorable environmental condition, the disease causes significant economic losses up to 50% of wheat production, particularly where susceptible cultivars are planted (2). The economic impact of tan spot disease is increasing due to the introduction of high yielding susceptible cultivars that are improved for resistance to other biotic and abiotic stresses. In addition, reduced or zero-tillage practices, shorter crop rotations and continuous wheat cultivation favor pathogen overwintering on plant residues. The pathogen overwintering allows the formation of the teleomorph stage. This stage not only provides primary inoculum of *P. tritici-repentis*, but also increases genetic recombination developing more races (3-4).

In Iran, *P. tritici-repentis* has intensified recently in some wheat-growing areas, mainly because of the introduction of susceptible wheat cultivars of International Maize and Wheat Improvement Center (CIMMYT) that have been planted in a monoculture fashion. As a result, severe epidemics have occurred in major wheat-growing provinces, including Mazandaran and Golestan (4). Accordingly, development of resistant wheat cultivars to replace the susceptible ones seems inevitable.

P. tritici-repentis is a homothallic fungus that read-

ily enters into generative phase, allowing the fungal population to recombine and generate new races (2). It was shown that sexual reproduction plays a significant role in genetic variability of fungal pathogens, resulting in increased biological fitness (5). This enables the fungal pathogens to rapidly evolve to overcome resistance genes as a response to selection pressure imposed by resistant cultivars, which has been frequently reported in many pathosystems (6). Therefore, study on the genetic variability of plant pathogen populations could likely lead to better management of disease in agricultural ecosystems and is required for proper decisions in breeding programs (7).

During the past two decades, molecular markers have been extensively used to study the population structure of many fungal pathogens (8, 9). However, to date, limited information is available on the molecular biodiversity and genetic structure of *P. tritici-repentis* populations. The pathogen is reported in many wheat growing regions around the world, but so far genetic diversity of limited populations have been studied. These populations consisted of the isolates from North America including Canada and the USA (2, 7, 10), South America including Argentina and Brazil (2, 11, 12), and Europe including Czech Republic, Germany, Slovak Republic and Russia (2, 7, 13). Although the Fertile Crescent is considered to be the center of origin of wheat and most of its fungal pathogens (14, 15), to date no information is available on the genetic structure of natural populations of the pathogen in Iran.

2. Objectives

Knowledge of the genetic structure of *P. tritici-repentis* in Iran is useful for improving wheat cultivars that are resistant to the pathogen populations. The relevant data helps to develope a breeding strategy to achieve durable disease resistance. Here, the genetic variability of *P. tritici-repentis* isolates originated from different wheat growing areas of Northern Iran was analyzed using Inter-Simple Sequence Repeat (ISSR) and Random Amplified Polymorphic DNA (RAPD) markers.

3. Materials and Methods

3.1. Fungal Isolates and DNA Extraction

During 2012-2013, the naturally infected wheat fields from different regions of Northern Iran were surveyed to collect the leaves having the typical symptoms of tan spot disease. The infected leaf samples were used to collect fungal isolates. Tan spot lesions were scraped using a sterile laboratory loop to collect *P. tritici-repentis* spores under stereomicroscope. The spores were scratched onto potato dextrose agar (PDA; potato 200 $g.L^{-1}$, dextrose 20 $g.L^{-1}$l, agar 15 $g.L^{-1}$) and allowed to germinate. After 10 h, the germinated spores were individually isolated and transferred on new PDA plates as single spore isolate that incubated at 20ºC. The mycelial biomass were collected, transferred onto Eppendorf tube and kept at -80ºC for long term storage. Totally, *P. tritici-repentis* isolates (n=34) were collected (Table 1; Figure 1).

For DNA isolation, a scrape of each isolate growing on PDA was cut and transferred to liquid YGM (yeast extract 10 $g.L^{-1}$, dextrose 20 $g.L^{-1}$) and incubated on a rotating shaker at 100 rpm, 20ºC for 5 days.

Table 1. List of *Pyrenophera tritici-repentis* isolates and their sampling geographical coordinates

Isolate code	Isolate name	Latitude	Longitude
P1	91M10B1S2	36.74000	53.85333
P2	91M10B2S1	36.74000	53.85333
P3	91M3B2S1	36.63056	53.27139
P4	91M28B6S1	36.98750	54.97361
P5	91M8B13S2	36.71417	53.69583
P6	91M9B1S1	36.72694	53.75972
P7	91M11B2S1	36.74361	53.88194
P8	91M7B1S1	36.70194	53.61889
P9	91M51B1S1	36.71417	53.69417
P10	91M28B7S1	36.98750	54.97361
P11	91M13B2S1	36.76889	54.00583
P12	91M44B1S3	36.80667	54.14028
P13	91M43B2S1	36.82056	54.23111
P14	91M52B4S1	36.70139	53.57611
P15	91M43B6S1	36.82056	54.23111
P16	91M52B5S2	36.70139	53.57611
P17	91M15B7S1	36.80222	54.12361
P18	91M49B2S3	36.72444	53.74472
P19	91M15B4S1	36.80222	54.12361
P20	91M32B1S2	37.03083	55.02806
P21	91M15B6S2	36.80222	54.12361
P22	91M3B5S1	36.63056	53.27139
P23	91M6B2S1	36.69694	53.50806
P24	91M32B4S1	37.03083	55.02806
P25	91M32B6S1	37.03083	55.02806
P26	91M32B7S1	37.03083	55.02806
P27	91M32B8S3	37.03083	55.02806
P28	91M32B5S1	37.03083	55.02806
P29	91M11B3S1	36.74361	53.88194
P30	91M11B6S1	36.74361	53.88194
P31	91M10B4S1	36.74000	53.85333
P32	91M3B4S1	36.63056	53.27139
P33	91M3B5S1	36.63056	53.27139
P34	91M7B1S1	36.70194	53.61889

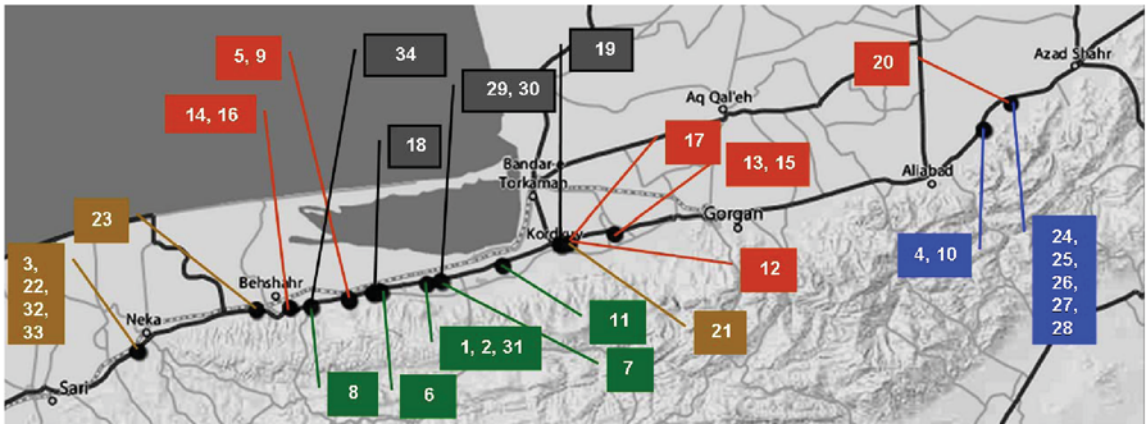

Figure 1. Sampling locations of *Pyrenophora tritici-repentis* isolates from Mazandaran and Golestan provinces based on GPS coordinates. The numbers showed in each box correspond to isolate codes as indicated in the Table 1

Table 2. Polymorphism and PIC values of ISSR and RAPD markers used for genetic diversity of 34 *Pyrenophera tritici-repentis* isolates

Marker	Primer names	No. of amplified bands	No. of polymorphic bands	polymorphism %	PIC value
ISSR	UBC807	10	9	90	0.49
	UBC815	15	10	67	0.43
	UBC816	16	9	56	0.39
	UBC818	14	7	50	0.35
	UBC828	10	9	90	0.48
	UBC880	17	9	53	0.36
	UBC874	16	8	50	0.31
	UBC822	14	6	42	0.27
RAPD	OPC20	10	7	70	0.46
	OPB7	11	10	91	0.48
	OPA2	13	8	61	0.39
	OPE10	12	9	75	0.44
	OPF2	9	6	66	0.41
	OPD6	11	8	72	0.43

Fungal biomass was harvested by filtration through Miracloth, rinsed with distilled water and was finely grinded in liquid nitrogen and was subjected to DNA extraction according to SDS method (4). DNA concentration was estimated by spectrophotometer at a wavelength of 260 nm.

3.2. ISSR and RAPD Analysis

For ISSR analysis, a set of 10 primers representing di-, tri-, tetra- and pentamer repeats was used (Table 2). RAPD assays were performed using 10 random 10-mer oligonucleotide primers (Table 2). PCR amplification was performed in 20 µL reaction containing 1× PCR buffer, 30 ng sample DNA, 2.5 µM primer, 200 µM of each dNTP, 1.5-2.5 mM $MgCl_2$ and 1.5 unit of *Taq* DNA polymerase (Cinnagene, Iran). All amplifications were carried out in an Eppendorf thermocycler as follow: 94ºC for 3 min, followed by 35 cycles of denaturation at 93ºC for 45 s, annealing at optimum Tm for 45 s, and extension at 72ºC for 90 s. A final extension cycle at 72°C for 10 min was also included. PCR products were separated on 1.2% agarose gels, stained with ethidium bromide and scored for the presence (1) or absence (0) of bands.

Nei's genetic distance (16) was determined among the isolates and used for cluster analysis by UNJ (Un-

weighted Neighbor Joining) method as described previously (17). The fit of dendrograms obtained were checked by bootstrapping using 100 replications. NTSYS ver 2.02 (18) and DARwin ver 5.0 (19) were used for clustering. Mantel statistic was used to compare the dissimilarity matrices through NTSYS software. Polymorphic information content (PIC) values were calculated for each ISSR and RAPD primers according to the formula: PIC= 1- Σ $(P_{ij})^2$, where P_{ij} is the frequency of the i^{th} pattern revealed by the j^{th} primer summed across all patterns revealed by the primers (20).

4. Results

ISSR analysis of 34 *P. tritici-repentis* isolates revealed distinct scorable fragments per primer and in total, 112 polymorphic and monomorphic bands were obtained using eight primers (Table 2). The number of amplified fragments varied from 10 to 17, with an average 14 bands per primer. Out of 112 bands, 67 bands were polymorphic and the polymorphism percentage averaged to 0.59 across all the isolates. Maximum number of polymorphic bands (10 out of 15 bands) was obtained for UBC815. The average PIC value was 0.38, ranging from 0.27 to 0.49. The highest PIC value was obtained by UBC807 (0.49; Table 2).

For RAPD, only primers (6 primers) that exhibited unambiguous and reproducible banding patterns were selected for genetic similarity and cluster analysis. A total of 66 bands were detected in 34 *P. tritici-repentis* isolates via RAPD. Out of which 48 were polymorphic (Table 2). Number of polymorphic bands were ranged from 6 (OPF2) to 10 (OPB7) with an average of 8 bands per primer. PIC values ranged from 0.39 to 0.48, with an average value of 0.43 per primer (Table 2).

A dendrogram was constructed based on unweighted neighbor-joining method using the combined data of the two sets of molecular markers that revealed five distinct clusters among the 34 *P. tritici-repentis* isolates (Figure 2). Cophenetic coefficient was considered acceptable in two molecular markers types (ISSR=0.81; RAPD=0.88), indicating good fit for clustering. Cluster I consisted of 6 isolates that 5 of which collected from East Mazandaran and isolate P21 from Kordkuy, Golestan province (Figure 1). Cluster II had seven isolates, all collected from East Golestan. The isolate P20 was the only isolate that was collected from this region, but grouped in cluster V. Cluster III consisted of five isolates that were originated from a region between Behshar and Kordkuy. Cluster IV consisted of seven isolates that was collected from the same region, but was closely related to cluster III. The most disperse cluster was cluster V including nine isolates that were collected from different geographical regions (Figure 2). Isolate P20 that was collected from East of Golestan province grouped in this cluster.

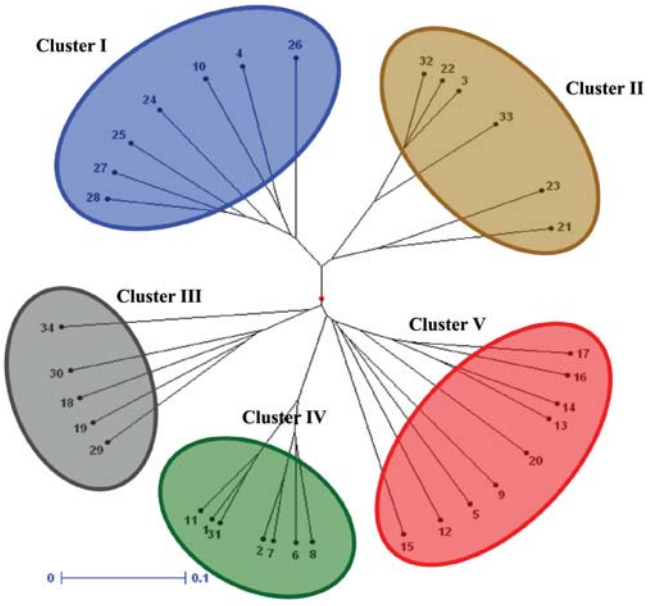

Figure 2. Dendrogram of *Pyrenophora tritici-repentis* isolates drawn using Un-weighted Neighbor Joining algorithm. Five major clusters are identified based on the dissimilarity matrix data developed by combined ISSR and RAPD markers

5. Discussion

The level of genetic diversity within fungal populations is mainly attributed to several major factors including sexual recombination, mutation rate, gene flow, migration and selection pressure (8). *P. tritici-repentis* is a homothallic wheat pathogen that reproduces both sexually and asexually. Sexual reproduction occurs on wheat stubble producing ascospores that are generally thought to be the source of primary inoculum. Asexual reproduction occurs in several cycles during the growing season (21). Sexual recombination in nature is likely the reason for high level of genetic variability among *P. tritici-repentis* isolates (10). Furthermore, *P. tritici-repentis* is seed borne. Therefore, fungal inoculums can travel long distances and disperse over long distances (7). ISSR and RAPD molecular markers were used to determine the level of genetic diversity of *P. tritici-repentis* isolates collected from different wheat growing areas of Northern Iran. A high level of genetic variability among Iranian *P. tritici-repentis* isolates was identified. The results from the

cluster analysis indicated that the genetic similarity among the Iranian *P. tritici-repentis* isolates could be partly explained by geographic origin. Leisova *et al.* (7) showed that the genetic similarity among the isolates of *P. tritici-repentis* from Czech Republic, Slovak Republic, Russia, Canada, USA and Argentina could be partly explained by race classification, host species, geographical origin and the sampling year (7). Lepoint *et al.* (22) studied the genetic diversity of the mating type and toxin production genes in *P. tritici-repentis*. The phylogenetic analysis of the MAT locus revealed two distinct groups, unlinked to geographical origin or ToxA profile (22). Likewise, clustering of *P tritici-repentis* isolates from Russia, Germany, and the Czech Republic showed that groups of isolates from Bashkiria, Dagestan and North Osetia were separated from others and might be considered as different geographical populations (13). Nevertheless, no clear differentiation between isolates from other sites was revealed (13). In contrast, Santos *et al*. (11) showed that although the Brazilian isolates of *P. tritici-repentis* had a high level of polymorphisms, no correlation between RAPD data and geographical origins was identifiable. Likewise, Friesen *et al.* (2) also found that the worldwide population of *P. tritici-repentis* isolates had no genetic grouping of pathotypes or grouping for geographic location. Similarly, Singh and Hughes (10), showed that all isolates had unique banding patterns. However, clustering of isolates was independent of their race designation or geographical origin (10). Moreno *et al.* (12) identified intraspecific variability within *P. tritici-repentis* isolates, but they could not establish a relationship between this variability and the geographical regions and/or wheat cultivars from where *P. tritici-repentis* isolates were obtained (12). They proposed that the occurrence of the sexual state and long distance dispersal of inoculums could have contributed to pathogenic and genetic variability independent of geographic region (12).

Iran located in Fertile Crescent that is considered the centre of origin of most *Triticum* species and their fungal pathogens (14). Accordingly, the relatively high level of differentiation observed among the Iranian *P. tritici-repentis* isolates is something that one can expect. Wheat and *P. tritici-repentis* have been around for thousands of years. Therefore, genetic drift have had greater chance to operate, as opposed to other parts of the world where *P. tritici-repentis* was found not long time ago. The relatively distinct gene pools for the *P. tritici-repentis* isolates collected from North of Iran may also suggest that migration events rarely occurred among these populations. It is possible that the relative abundance of asexual- over sexual reproduction within each geographical region may lead to clonal and local populations that are distinctly different from other populations.

Although limited number of Iranian isolates and limited number of molecular markers were used, this study paved the road for future investigation by providing valuable information on the genetic diversity of Iranian population of *P. tritici-repentis*. A larger number of isolates including those that are collected from more diverse regions in Iran from wheat and its wild relatives as well as a global panel of *P. tritici-repentis* isolates might be needed to have a comparative analysis. It has been shown that fungal isolates from the Middle East where both hosts and their pathogens are co-evolved, had significantly higher gene diversity than populations in America and Europe (23). This issue for *P. tritici-repentis* populations remains to be addressed in future.

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