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ORIGINAL PAPER

Races, disease symptoms and genetic variability in *Pyrenophora tritici-repentis* isolates from Oklahoma that cause tan spot of winter wheat

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Abstract
In recent years, tan spot of wheat caused by the fungus *Pyrenophora tritici-repentis* has become more prevalent in Oklahoma. Experiments were conducted to investigate the race structure, disease symptoms and genetic variability in *P. tritici-repentis* isolates collected from winter wheat over three decades. Race determination was conducted for 16 isolates based on expression of necrosis and/or chlorosis produced on wheat differentials. Variability in disease symptoms expressed by 12 isolates was determined on 13 hard red winter wheat cultivars grown in Oklahoma. In addition, genetic variability among 17 isolates was determined using amplified fragment length polymorphism-polymerase chain reaction (AFLP-PCR). All isolates except one (El Reno) were classified as race 1. Isolates varied widely in producing necrosis and/or chlorosis symptoms on wheat cultivars, but necrosis with a chlorotic halo was predominant (56.4%). AFLP-PCR analysis using 13 primer pairs produced a total of 494 alleles of which 285 were polymorphic. The overall genetic diversity among the isolates was 25.2%. Genetic relationships based on cluster analysis and principal component analysis showed only minor differences between isolates, and isolates did not form tight clusters or groups. The isolates of *P. tritici-repentis* were predominantly race 1; however, they produced a range of tan spot symptoms on wheat cultivars. The lack of distinct genetic grouping by the AFLP marker study indicates that the isolates used in this study likely originated from a single lineage.

Keywords *Pyrenophora tritici-repentis* · Genetic variability · Tan spot · Wheat

Introduction

Tan spot of wheat (*Triticum aestivum* L.) caused by the necrotrophic ascomycetes fungus *Pyrenophora tritici-repentis* (anamorph: *Dreschlera tritici-repentis* (Died.) Shoemaker) is a major disease in many wheat-growing areas in the world (Francl et al. 1992; Bockus et al. 2010). In the Great Plains of the USA, tan spot causes considerable damage in wheat (Wegulo et al. 2012; Friskop and Liu 2016). A range of 3–50% yield loss in wheat has been observed for tan spot depending on the isolate virulence, cultivar susceptibility and the environmental conditions (Shabeer and Bockus 1988). In Oklahoma in the southern Great Plains of the USA where wheat is grown annually on nearly 3–4 million acres (NAAS 2019), tan spot disease has been reported since the 1970s (Hunger and Brown 1987). Currently, the presence of tan spot in Oklahoma has become more prevalent, and in a field study in Stillwater, a 21% yield loss of wheat has been reported due to tan spot (Kader et al. 2009). The fungus has a sexual stage to its life cycle, which occurs on wheat residue (De Wolf et al. 1998). Shifting cultivation practices from clean tillage to a no-till system, which leaves residue in the field, is favorable for the survival, sexual recombination, and seasonal carry-over of this fungus (Bockus and Classen 1992; Baily 1996).

Variability within plant pathogens is a major problem in achieving disease control, and knowledge of variability within a pathogen species is essential for developing sustainable management practices. *P. tritici-repentis* is able to induce two distinct types of symptoms (necrosis and/or chlorosis) on wheat leaves (Lamari and Bernier 1989). This fungus is classified into eight races based on the necrosis and/or chlorosis symptoms on wheat differentials (Andrie et al. 2007). Race 1 is the most prevalent in the USA, although

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other races (race 2 and 3) are reported occasionally (Ali and Francl 2003). In Canada, race 1 and 2 are predominant although race 3 is also reported (MacLean et al. 2017). As the presence of races of this fungus is a problem in wheat-growing areas, identification of genes to employ a general or race-nonspecific resistance to tan spot in wheat has been emphasized (Faris et al. 2020). Significant research has been accomplished on the identification of quantitative trait loci conferring resistance to tan spot in wheat (Kariyawasam et al. 2018; Singh et al. 2019; Kokhmetova et al. 2020; Phuke et al. 2020).

Measuring the genetic relationship between pathogen isolates based on isolate virulence is often difficult to accomplish because complex host–pathogen-environment interactions affect disease development. Thus, employment of molecular tools is useful and reliable in determining the genetic variability of a species at the genomic level. *P. tritici-repentis* is one of the most extensively studied fungi at the genomic level (Ciuffetti et al. 2014). Researchers have used different molecular markers to study variability in *P. tritici-repentis* or related species, such as, rapid amplified polymorphic DNA (RAPD), inter simple sequence repeats (ISSR), internal transcribed spacer (ITS) sequence analysis, restriction fragment length polymorphism (RFLP) and simple sequence repeats (Faris et al. 1997; Stevens et al. 1998; Santos et al. 2002; Friesen et al. 2005; Singh and Hughes 2006; Aboukhaddour et al. 2011). Amplified fragment length polymorphism (AFLP) is a polymerase chain reaction (PCR)-based marker that is highly reproducible and has been used to estimate genetic relationship in different species of fungi (Vos et al. 1995; Majer et al. 1996; O'Neill et al. 1997; Garzon et al. 2005; Hielmann et al. 2006; Serenius et al. 2007). AFLP was also applied to study population genetic structure of *P. tritici-repentis* isolates (Friesen et al. 2005; Leisova et al. 2008). To date, elucidation of the genetic relationships in *P. tritici-repentis* isolates from Oklahomais lacking. The objectives of this study were to investigate race(s), disease symptoms and to reveal genetic relationship in *P. tritici-repentis* isolates collected over three decades from winter wheat in Oklahoma.

Materials and methods

Fungal isolates

The study was carried out at the Small Grain Pathology Lab at Oklahoma State University from 2008 to 2009 using 17 isolates of *P. tritici-repentis*, derived from single ascospores or single conidia, collected over three decades (the 1980s, 1990s and 2000s) from winter wheat in Oklahoma (Table 1). Isolates were stored in liquid nitrogen for long-term storage. When needed, isolates were removed from liquid nitrogen and were grown on potato dextrose agar (PDA) (200 g potato, 20 g dextrose, 15 g agar in 1L water). Isolates were grown on PDA, and were maintained at 4 °C in the dark during the experiment.

Table 1 Seventeen isolates of tan spot fungus *Pyrenophora tritici-repentis* collected from several counties in Oklahoma and thirteen cultivars of winter wheat included in the study

Isolates	Initial propagule	County	Wheat cultivars[a]
OKA1 (1983)[b]	Ascospore	Garfield	Billings
OKA2 (1983)	Ascospore	Garfield	Chisholm
OKD1 (1983)	Ascospore	Blaine	Deliver
OKD2 (1983)	Ascospore	Blaine	Duster
OKD3 (1983)	Ascospore	Blaine	Endurance
OKD4 (1983)[c]	Ascospore	Blaine	Jagger
OKD5 (1983)	Ascospore	Blaine	Karl 92
RBB6 (1996)	Ascospore	Kay	OK Bullet
GYA3 (1996)	Ascospore	Texas	Okfield
El Reno (2005)	Conidia	Canadian	OK Rising
Guymon (2006)	Conidia	Texas	Pete
Cherokee (2006)	Conidia	Cherokee	Tam 105
OK-06–1 (2006)	Ascospore	Payne	Triumph 64
OK-06–2 (2006)	Ascospore	Payne	
OK-06–3 (2006)	Ascospore	Payne	
Atoka (2007)	Conidia	Atoka	
Kiowa (2007)	Conidia	Pittsburg	

[a]All wheat cultivars are Oklahoma State University releases except for Jagger (Kansas State University) and Tam 105 (Texas A&M University). Source of all wheat cultivars was the Oklahoma Foundation Seed Stocks, Stillwater, OK

[b]Year of collection in parenthesis

[c]OKD4 did not produce conidia on media and was not included in race studies in Table 2

Inoculum preparation

Conidia were produced by each isolate following the procedure of Raymond et al. (1985). A 5-mm diameter mycelial plug, excised with a sterilized cork borer from the edge of an actively growing isolate on fresh PDA, was removed and placed on clarified V8 (CV8) juice agar (150 ml V8 juice, 3 g $CaCO_3$, 15 g agar, 850 ml water) in 90-mm petri plates were maintained in an incubator (Percival I-36LL, Boone, IA) at 23 °C in the dark for 5 days. About 10 drops of sterile water were added and mycelia were matted down using a sterile bent glass rod. Plates were then incubated for 12 h at 23 °C with cool-white fluorescent tubes (40 W, 30 $\mu Es^{-1}\ m^{-1}$) to produce conidiophores followed by 12 h dark at 16 °C to induce conidia production. Conidia were washed from the plate into a beaker using a stream of sterile water. Conidia were adjusted to 2000 per ml before inoculation.

Race identification

Races were determined based on necrosis and/or chlorosis model on wheat differentials (Andrie et al. 2007). Wheat differential lines 6B662, Glenlea, 6B365, and Salamouni were used according to Ali and Francl (2003). Four seeds of each wheat differential were planted separately in plastic containers (15 cm × 3.75 cm) filled with Ready-Earth soil (Sun Gro., Bellevue, WA). Using an atomizer (DeVilbiss Co. Somerset, PA), seedlings with two leaves fully expanded were inoculated with the conidial suspension (2000 conidia/ml) of each isolate until incipient run-off following the procedure of Rodriguez and Bockus (1996). Inoculated plants were allowed to dry for 30 min so that conidia would adhere to leaves and then were placed in a mist chamber for 48 h. Inoculated plants then were placed on a lab bench at 21–23 °C following a cycle of 14 h light (510 μEs^{-1} m^{-1}) and 10 h dark. Seven days after inoculation, symptoms produced by each isolate on the wheat differentials were rated on a scale of 1–5 based on lesion type, where 1 = small, dark brown to black spots without any surrounding chlorosis or tan necrosis (resistant), 2 = small, dark brown to black spots with very little chlorosis or tan necrosis (resistant), 3 = small dark brown to black spots completely surrounded by a distinct chlorotic or necrotic ring, lesions not coalescing (susceptible), 4 = spots completely surrounded by chlorotic or necrotic zones, lesion coalescing (susceptible), 5 = spots may not be distinguishable, lesions coalescing and spread over leaf (susceptible) (Lamari and Bernier 1989). The bottom 2nd leaf from all four seedlings of each differential was rated per isolate. Isolate OKD4 did not produce conidia so was not included in the study.

Symptom variability

To study variability in symptoms, 13 wheat cultivars and 12 isolates were used (Table 3). Seedlings of wheat cultivars were raised, and inoculum of each isolate was prepared as described earlier. At the three-leaf stage, four seedlings of each cultivar were inoculated with 2000 conidia/ml until incipient run-off and then maintained as described earlier. Disease symptoms produced by each isolate (necrosis and/or chlorosis) were recorded visually five days after inoculation from the bottom 2nd leaf.

DNA extraction and ITS sequence

Four day-old fungal mycelia on PDA plates were ground into a fine powder using a mortar and pestle and liquid nitrogen. Genomic DNA was extracted from ~ 0.25 g mycelia powder of each isolate and the purity of DNA was examined on agarose gel following the salt-extraction method (Aljanabi and Martinez 1997).

Nuclear internal transcribed spacer (ITS) regions (ITS1- 5.8S rDNA- ITS2) were amplified by polymerase chain reaction (PCR) using ITS4 and ITS5 primers (White et al. 1990). Electrophoresis of amplified ITS region was performed in a 1% agarose gel. DNA was stained with ethidium bromide and visualized under UV light. Amplified products were purified using Purelink™ PCR Purification Kit (Invitrogen, Carslbad, CA). The ITS region from each isolate was sequenced at the Oklahoma State University (OSU) Core Facility, and the sequences were compared with *P. tritici-repentis* GenBank accessions in the NCBI database (www.ncbi.nih.gov) using the Basic Local Alignment Search Tool (BLAST).

AFLP analysis

AFLP analysis was performed by using an AFLP™ Microbial Fingerprinting kit following manufacturer's instruction (Applied Biosystems, Foster city, CA). In brief, steps in the AFLP performed were DNA digestion by *EcoR*1 and *Mse*1 restriction enzymes, adapter ligation, pre-amplification and selective amplification. The selective amplified products were separated by capillary electrophoresis by an ABI 3730 DNA Analyzer with ROX 500 as internal standard size (Applied Biosystems, Foster city, CA). The chromatograms were converted into a binary data matrix as presence (1) or absence (0) of an allele using GeneMapper 4.0 software (Applied Biosystems, CA) calibrated against 75–500 bp DNA size standards. Thus, a total set of binary data was generated for all 13 primer combinations across all 17 isolates.

Genetic diversity (DI) among the isolates for each primer combination was determined using the POPGENE software (version 1.32, University of Alberta, Edmonton, Canada) as $DI = 1- \sum P^2i$, where P^2i is the frequency of ith allele at a locus in a population (Nei 1978). A principal component analysis (PCA) was carried out using the PRINCOMP procedure of SAS 9.2 (SAS Institute, Cary, NC) to determine genetic relationship among isolates.

Results

Race structure

All isolates except one (El Reno) were identified as race 1 based on the symptoms produced on the wheat differential set (1–5 scale; 1–2 resistant, 3–5 susceptible) (Table 2). No disease symptoms other than dark brown to black spots were induced by El Reno, which therefore was considered as race 4.

Table 2 Reaction of isolates on four wheat differential varieties and identification of races in *Pyrenophora tritici-repentis* collected from 1980s, 1990s and 2000s in Oklahoma

Isolates	Wheat differential				Race[b]
	6B662	Glenlea	6B365	Salamouni	
1980s					
OKA1	1R[a]	4 N	5C	1R	Race 1
OKA2	1R	3 N	5C	1R	Race 1
OKD1	2R	5 N	5C	1R	Race 1
OKD2	2R	4 N	5C	1R	Race 1
OKD3	1R	4 N	5C	1R	Race 1
OKD5	1R	3 N	3C	1R	Race 1
1990s					
RBB6	1R	5 N	5C	1R	Race 1
GYA3	1R	3 N	4C	1R	Race 1
2000s					
El Reno	1R	1R	1R	1R	Race 4
Guymon	1R	5 N	5C	1R	Race 1
Cherokee	2R	4 N	5C	1R	Race 1
OK-06–1	1R	4 N	5C	2R	Race 1
OK-06–2	1R	5 N	5C	2R	Race 1
OK-06–3	1R	5 N	5C	1R	Race 1
Atoka	2R	5 N	5C	2R	Race 1
Kiowa	1R	4 N	5C	1R	Race 1

[a]Lesion type on a 1–5 scale, where 1 = small, dark brown to black spots without any surrounding chlorosis or tan necrosis (resistant), 2 = small, dark brown to black spots with very little chlorosis or tan necrosis (resistant), 3 = small dark brown to black spots completely surrounded by a distinct chlorotic or necrotic ring, lesions not coalescing (susceptible), 4 = spots completely surrounded by chlorotic or necrotic zones, lesion coalescing (susceptible), 5 = spots may not be distinguishable, lesions coalescing and spread over leaf (susceptible) (Lamari and Bernier 1989)

R = resistant, N = necrosis, C = chlorosis. Decade of collection is in bold

[b]Races identified based on symptoms on leaf as necrosis and/or chlorosis (Andrie et al. 2007)

Disease symptoms

All 12 isolates tested were pathogenic and varied widely in symptoms induced on wheat cultivars (Table 3). Single isolates, for example isolate OKA1, were able to produce necrosis with a yellow halo (N), necrosis with extended chlorosis (NC) or only chlorosis extended over the leaf (C) on wheat cultivars. Similarly isolates also produced a range of symptoms on a cultivar. Necrosis was the dominant symptom (56.41%) followed by necrosis with extended chlorosis (32.05%) and chlorosis (11.54%) from the overall combination of 12 cultivars and 13 isolates.

Genetic variability

Results from the AFLP analysis are presented in Table 4. Using 13 primer combinations all 17 isolates of *P. tritici-repentis* yielded a total of 494 alleles of which 285 were polymorphic. On an average each primer combination produced 38 alleles of which ~ 22 were polymorphic. The *EcoR*1-AA and *Mse*1-C combination yielded the highest number of alleles of 70, while the *EcoR*1-AT and *Mse*1-CAG combination produced the fewest number of alleles of 16. The highest number of polymorphic alleles (44 out of 70) was detected by the *EcoR*1-AA and *Mse*1-C combination. The percentage of polymorphism ranged from 26.42 to 100%, with an average of 57.69%. An overall genetic diversity among the isolates was 0.252, with a range of variation from 0.104 to 0.394 for the primer combinations. All the isolates formed one large group by the principal component analysis (PCA) (Fig. 1). In PCA, the first, second and third PC explained 37.3%, 8.02% and 6.45% of the total variability, respectively.

Discussion

In Oklahoma, race 1 was the predominant race present in the three decades of collection time. In this study, 15 out of 16 isolates were race 1, and one was race 4 (Table 2), which is in agreement with previous research. For example, Ali and Francl (2003) tested 22 isolates collected from wheat in the southern Great Plains of the USA and reported that 21 of 22 isolates were race 1 and a single isolate was race 4. In a study with a global collection of *P. tritici-repentis* populations, five isolates included from Oklahoma were determined as race 1 (Friesen et al. 2005). Race 1 is the most virulent and fit race due to the presence of two toxin genes (necrosis and chlorosis). One of the drawbacks of modern wheat monoculture is the lack of genetic diversity among cultivars. Thus, the genetic base in wheat for resistance to tan spot is narrow which favors this pathogen, and as indicated by Ali and Francl (2003), the predominance of race 1 may be due to the lack of selection pressure on this fungus because most of the wheat cultivars are susceptible. Race 4 has also been isolated from alternative non-cereal grasses (Ali and Francl 2003). It is suspected that isolate El Reno might have come into wheat from a non-cereal host. Race 4 likely would not be persistent in wheat because it would not compete as well as the more virulent races.

The presence of specific races of a pathogen can have practical implications on plant disease management studies and on breeding for resistance. For example, in a management study involving tan spot, using a *P. tritici-repentis* race common to an area would be most meaningful, and resistance breeding for a disease such as tan spot should be

Table 3 Different symptoms produced by 13 wheat cultivar in response to 12 isolates of *Pyrenophora tritici-repentis* collected from Oklahoma winter wheat fields

Isolates	Wheat cultivars												
	Billings	Chisholm	Deliver	Duster	Endurance	Jagger	Karl 92	OK Bullet	Okfield	OK Rising	Pete	TAM 105	Triumph 64
OKA1	NC	N	N	N	C	N	N	N	N	N	N	NC	N
OKA2	NC	N	N	N	NC	N	N	N	N	N	N	N	N
OKD1	NC	N	NC	N	C	N	N	N	N	N	N	NC	N
OKD2	NC	NC	NC	N	C	N	N	N	N	N	N	N	N
OKD5	C	N	NC	N	C	N	NC	N	N	N	N	NC	N
RBB6	C	N	NC	NC	C	NC	NC	NC	NC	N	N	NC	NC
Cherokee	C	N	NC	NC	C	N	NC	N	NC	NC	N	N	NC
Guymon	C	NC	NC	NC	C	N	NC	N	N	NC	N	NC	N
OK-06–1	C	NC	NC	N	C	N	NC	N	N	N	N	NC	NC
OK-06–3	C	N	NC	NC	C	NC	N	N	N	N	N	NC	NC
Atoka	NC	N	NC	N	C	N	N	N	N	NC	N	NC	N
Kiowa	C	N	NC	N	C	N	N	N	N	N	N	NC	NC

Cultivars Billings and Endurance produced extended chlorosis with little necrosis

N Necrosis with yellow hallow, *C* Chlorosis extended over leaf, *NC* Both necrosis and chlorosis, usually necrosis with little extended chlorosis

Table 4 Genetic variability in 17 isolates of *Pyrenophora tritici-repentis* following amplified fragment length polymorphism (AFLP) analysis using 13 primer combinations

*EcoR*1-	*Mse*1-	Total allele	Polymorphic allele	Percent polymorphism	Genetic diversity
AA	C	70	44	62.86	0.204
AA	CA	53	14	26.42	0.104
AA	CG	57	32	56.14	0.196
AA	GC	24	23	95.83	0.394
AC	CA	30	15	50.00	0.158
AG	C	57	31	54.39	0.233
AG	GA	21	19	90.48	0.374
AT	C	51	25	49.02	0.193
AT	CA	20	12	60.00	0.256
AT	CAC	19	19	100.00	0.397
AT	CAG	16	16	100.00	0.397
TA	C	27	12	44.44	0.188
TG	C	49	23	46.94	0.183
Total		494	285	–	–
Average		38	21.92	57.69	0.252

focused to select for resistance against the most prevalent races in the area.

Isolates varied considerably in producing necrosis and/or chlorosis symptoms on wheat cultivars (Table 3). For example, isolate OKA1 produced necrosis on cultivar Chisholm but produced chlorosis on cultivar Endurance. A cultivar also produced different symptoms in reaction to isolates. This suggests that collection of isolates should be symptom or cultivar independent. In studying wheat-*Ptr* interaction, other researchers also found that an isolate is able to induce necrosis and chlorosis symptoms on different wheat cultivars (Lamari and Bernier 1991). Conversely, a range of symptoms are produced on a single cultivar when inoculated by different isolates (Moreno et al. 2008). It might be a cultivar has its own genetic and physiological effect in producing disease symptom(s).

AFLP data demonstrated only a slight genetic diversity (0.252) among the isolates (Table 4). This finding is consistent with other marker studies of *P. tritici-repentis* populations where primer combinations revealed a range of 0–0.47 genetic diversity within 97 isolates, with an average genetic diversity of about 0.1 (Friesen et al. 2005). Using AFLP, Leisova et al. (2008) also observed a low genetic diversity (0.096) among 100 isolates of *P. tritici-repentis* in the Czech Republiccollected during 1998–2005. The reasons for genetic diversity in *P. tritici-repentis* could be due to sexual recombination or vegetative compatibility among isolates (Singh and Hughes 2006; Moreno et al 2008). However, as *P. tritici-repentis* is a homothallic fungus, sexual recombination may not contribute to a significant extend in genetic variation as the male and female gametes are coming from the same thallus. Thus, although the isolates were collected over three decades in this study, a greater genetic variation was not observed among the isolates.

Principle component analysis (PCA) revealed no grouping among the isolates of *P. tritici-repentis*, thus, the isolates used in this study are likely derived from a common lineage. In a study with 97 isolates of *P. tritici-repentis*, no genetic grouping was identified by AFLP marker study (Friesen et al. 2005). In PCA, a high value of PC is able to explain the greatest amount of variation (Hielmann et al. 2006). In this

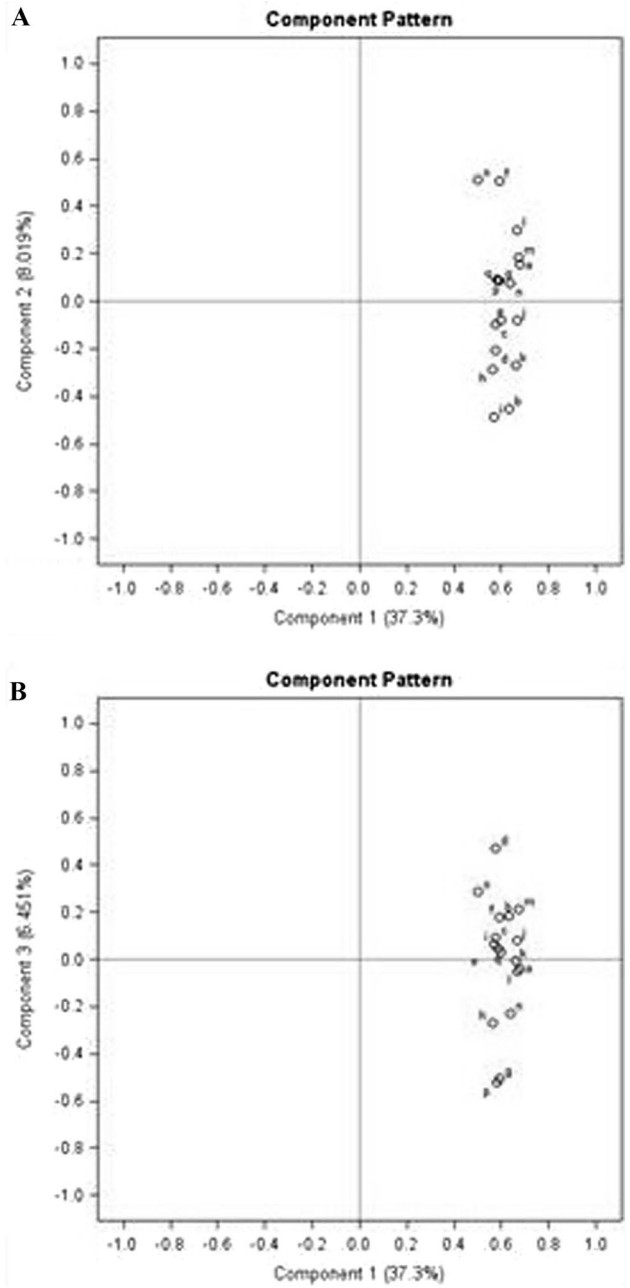

Fig. 1 Principal component analysis based on amplified fragment length polymorphism (AFLP) for 17 isolates of *Pyrenophora tritici-repentis* collected from winter wheat in Oklahoma. Principal component values grouped the isolates in one group (A and B)

study, PC1 explained the highest variability (37.3%) of the total variation and put all the isolates in one group (Fig. 1) which is in agreement with other researchers. In an AFLP analysis with 100 isolates of *P. tritici-repentis*, Leisova et al. (2008) also found that high PC value fit all isolates into one large group. Although isolate El-Reno was race 4, it did not affect the clustering. This is because variation in a single virulence gene determines a race, while in this study, the relationship among the isolates was determined from the variability in whole genome by AFLP analysis.

Race 1 was the predominant race in Oklahoma wheat fields and produced a range of tan spot symptoms. Results in this study also indicate that the isolates originated from a single lineage, and other characteristics such as growth rate, sporulation and virulence that can affect disease epidemiology should be considered in selecting selection of *P. tritici-repentis* isolates for use in wheat resistance screening for tan spot management. Regular monitoring and virulence testing of this pathogen would also benefit tan spot management. This study reveals that a complete understanding of variability in the population of *P. tritici-repentis* is critical to resistance screening of wheat cultivars. Knowledge on the pathogen variability would facilitate resistance breeding programs and the development of management practices, for example, selection of fungicides. A representative isolate from different groups or lineages, if any, should be included in the screening of cultivars to deploy a wider resistance to tan spot.

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