

Relatedness of *Macrophomina phaseolina* isolates from tallgrass prairie, maize, soybean and sorghum

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Abstract

Agricultural and wild ecosystems may interact through shared pathogens such as *Macrophomina phaseolina*, a generalist clonal fungus with more than 284 plant hosts that is likely to become more important under climate change scenarios of increased heat and drought stress. To evaluate the degree of subdivision in populations of *M. phaseolina* in Kansas agriculture and wildlands, we compared 143 isolates from maize fields adjacent to tallgrass prairie, nearby sorghum fields, widely dispersed soybean fields and isolates from eight plant species in tallgrass prairie. Isolate growth phenotypes were evaluated on a medium containing chlorate. Genetic characteristics were analysed based on amplified fragment length polymorphisms and the sequence of the rDNA-internal transcribed spacer (ITS) region. The average genetic similarity was 58% among isolates in the tallgrass prairie, 71% in the maize fields, 75% in the sorghum fields and 80% in the dispersed soybean fields. The isolates were divided into four clusters: one containing most of the isolates from maize and soybean, two others containing isolates from wild plants and sorghum, and a fourth containing a single isolate recovered from *Solidago canadensis* in the tallgrass prairie. Most of the sorghum isolates had the dense phenotype on media containing chlorate, while those from other hosts had either feathery or restricted phenotypes. These results suggest that the tallgrass prairie supports a more diverse population of *M. phaseolina* per area than do any of the crop species. Subpopulations show incomplete specialization by host. These results also suggest that inoculum produced in agriculture may influence tallgrass prairie communities, and conversely that different pathogen subpopulations in tallgrass prairie can interact there to generate 'hybrids' with novel genetic profiles and pathogenic capabilities.

Keywords: agricultural matrix, agriculture–wildlands interface, amplified fragment length polymorphism, belowground ecology, generalist pathogen, landscape, *Macrophomina phaseolina*, rDNA, soil-borne pathogen

Received 9 April 2009; revision received 29 September 2009; accepted 15 October 2009

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Introduction

Several generalist pathogen species occur in both naturally established and agricultural plant populations. A critical trait of these pathosystems is the degree to which the same pathogen populations are present in both agricultural and natural ecosystems. Analysis of the population genetic structure of generalist pathogens is one approach to understanding this dynamic and may be simpler if the pathogen reproduces clonally (Anderson & Kohn 1995). For example, the distribution

of mitochondrial DNA haplotypes of the generalist *Sclerotinia sclerotiorum* in agricultural sites and native plant populations indicated that the more common types could be recovered from agricultural systems while there were more haplotypes recovered from native species even at smaller spatial scales (Kohli & Kohn 1996). Gordon *et al.* (1992) concluded that *Fusarium oxysporum* isolates, collected in an uncultivated area and an agricultural field, were part of the same population, although there was more genetic variation in the strains from the uncultivated area. Barley yellow dwarf virus (BYDV) maintained in an invasive weed, wild oats, had very damaging effects on wild grass populations in California (Malmstrom *et al.* 2006), an example of pathogen spillover where pathogens adapted to agriculture move to native systems (Power & Mitchell 2004). In contrast, BYDV strains recovered from Kansas tallgrass prairie did not include the dominant strain in Kansas wheat production (Garrett *et al.* 2004). While plant diversity in wildland systems can provide ecosystem services through regulation of plant disease, more careful evaluation is needed in cases where generalist pathogens can infect both wild and agricultural species (Cheatham *et al.* 2009).

Macrophomina phaseolina (Tassi) Goid. is a soil- and seed-borne generalist fungal pathogen that has a global distribution and can infect more than 284 plant species including monocot and dicot plant hosts (Farr *et al.* 1995). In the United States, *M. phaseolina* causes charcoal rot disease of many agronomically important crops such as sorghum (*Sorghum bicolor* Moench), maize (*Zea mays* L.), soybean (*Glycine max* L. Merrill) and cotton (*Gossypium arborum* L. and *G. hirsutum* L.) (Baird & Brock 1999; Smith & Wyllie 1999; Stack 2002; Bradley & del Río 2003; Yang & Navi 2005). *Macrophomina phaseolina* also can infect many wild plants and is an opportunistic pathogen of humans (Tan *et al.* 2008). Drought and high temperature stress increase crop losses because of *M. phaseolina* (Mihail 1989). In contrast to the many pathogens favoured by change to moister conditions (Garrett *et al.* 2006), *M. phaseolina* may become more problematic in agricultural areas where climate change results in longer drought periods and higher temperatures (Mihail 1989, 1992), such as predicted for the US Great Plains.

As has been the case for many pathogens, especially generalists and pathogens that reproduce clonally, the taxonomic identity of *M. phaseolina* has challenged researchers. *Macrophomina phaseolina* has many synonyms, including *M. phaseoli* (Maubl.) Ashby, *Macrophoma corchori* Sawada, *Macrophoma cajani* Syd. & Butl., *Sclerotium bataticola* Taub., *Rhizoctonia bataticola* (Taub.) Britton-Jones and *Tiarosporella phaseolina* (Tassi) van der Aa (Crous *et al.* 2006; Mihail 1992; Holliday and Punithalingam 1970). Crous *et al.* (2006) used morphological

characters and 28S rDNA sequences to confirm the identity of *M. phaseolina* (anamorph stage) and included it in the ascomycete family *Botryosphaeriaceae*, indicating that *Rhizoctonia bataticola* and *Tiarosporella phaseolina* are inappropriate names for this fungus.

Although only one species has been identified in the genus *Macrophomina*, isolates from different hosts and soils differ in their morphology, e.g. formation of sclerotia, production of pycnidia, colony morphology, pathogenicity and growth on media containing KClO₃. Pearson *et al.* (1986, 1987a) used chlorate resistance to divide isolates into three phenotypic groups that differentially infect various host plants. Isolates with a dense phenotype were more commonly recovered from maize, rice, sorghum and onion, whereas isolates with feathery or restricted phenotypes were more typically recovered from soybean, sunflower, mungbean and okra (Pearson *et al.* 1986, 1987a,b; Cloud & Rupe 1991; Mihail & Taylor 1995; Purkayastha *et al.* 2006; Aboshosha *et al.* 2007). The distribution of strains of these phenotypes also may be affected by crop rotation and heat and drought stress (Pearson *et al.* 1986, 1987a,b; Singh *et al.* 1990; Purkayastha *et al.* 2006).

Molecular markers, e.g. random amplified polymorphic DNA (RAPD), amplified fragment length polymorphic DNA (AFLP) and restriction fragment length polymorphisms in the rDNA region (RFLP-rDNA), have been used to characterize populations of *M. phaseolina* from various plant hosts and geographic locations. For example, in the United States, *M. phaseolina* isolates from four different hosts (maize, soybean, sorghum and cotton) were distinguished by RAPD genotyping, while chlorate phenotype was less predictive of host (Su *et al.* 2001). Similarly, in India, *M. phaseolina* isolates from cluster bean were genetically distinct from those isolates recovered from other crops such as cotton and soybean regardless of their geographic origin or chlorate phenotype (Purkayastha *et al.* 2006). There was a clear association between isolate genotype and host of origin, and the chlorate resistant phenotypes were genetically distinct from the chlorate sensitive ones within each host (Su *et al.* 2001; Purkayastha *et al.* 2006). AFLP fingerprints of *M. phaseolina* isolates from Mexico also were associated with geographic origin and source host species (Mayek-Pérez *et al.* 2001). In contrast, a number of studies have found no association between DNA genotypes and geographic origin or host origin in populations from the United States or elsewhere (Vandemark *et al.* 2000; Reyes-Franco *et al.* 2006). However, when Reyes-Franco *et al.* (2006) re-analysed the AFLP subgroups of *M. phaseolina*, they could discriminate genetically between the isolates from Mexico and isolates from other countries. Although molecular markers have not been used to identify significant differences in pathogenicity of isolates, some investigators

report that isolates with dense chlorate phenotype were more aggressive than those with restricted or feathery phenotypes (Purkayastha *et al.* 2006; Reyes-Franco *et al.* 2006).

We evaluated and compared populations of *M. phaseolina* from agricultural systems in Kansas and from the indigenous tallgrass prairie. The objective of this study was to determine the genetic diversity and differentiation of these populations, which were recovered from 11 host species and five geographic locations. We tested the hypothesis that populations of *M. phaseolina* are host specialized and that the level of genetic diversity in and differentiation of populations varies by host and location. We also determined whether chlorate phenotypes were consistent with previous studies, in which maize and sorghum tended to yield chlorate-resistant (dense) phenotypes while soybean tended to yield chlorate-sensitive (feathery or restricted) phenotypes (Pearson *et al.* 1986, 1987a; Su *et al.* 2001), and whether isolates with the same chlorate phenotype were more closely related than those with different phenotypes. This study provides new perspectives on a generalist pathogen common to native and agro-ecosystems and the extent to which these populations are genetically differentiated.

Materials and methods

Fungal isolate collection

Macrophomina phaseolina isolates were collected from agricultural fields (maize, sorghum and soybean) in

Kansas and from tallgrass prairie in the Konza Prairie Biological Station (KPBS) near Manhattan, Kansas. Maize plants were collected from two fields that were adjacent to the main gate of KPBS, where maize has rarely been grown. Sorghum plants were collected from two fields at the Kansas State University Agronomy Department's Ashland Bottoms Research Farm (ABRF) a few kilometres from the main gate of KPBS. For maize and sorghum fields, symptomatic plant tissues were collected in a systematic sampling grid and *M. phaseolina* isolates were recovered from plants with microscle-rotia visible in stems or leaves. Soybean fields were not common in this area; soybean plants were collected in systematic sampling grids from three sites that were 56 (Rossville), 180 (Leavenworth) and 402 (Columbus) km from KPBS. Native tallgrass prairie plants were sampled from three undisturbed tallgrass prairie sites within KPBS dominated by the grass *Andropogon gerardii*. Isolates were recovered from eight different native prairie plant species (Tables 1 and S1). For soybean and native prairie plant samples, *M. phaseolina* isolates were collected from roots.

Fungal isolation and identification

Fungal isolates from roots were collected by removing infected sections (0.5 cm in length) after rinsing with tap water. Symptoms on the generally smaller roots of the prairie species were difficult to interpret. The root sections were surface sterilized with a 0.5% NaOCl solution for 5–10 min and then dried with paper

Table 1 Isolates of *Macrophomina phaseolina* included in the analyses and their sources

Population/family	Sampling location	Host(s)	Isolates/AFLP haplotypes
Natural	KPBS		17/17
Asteraceae		<i>Ambrosia</i> sp. (AM)*	1/1
Asclepiadaceae		<i>Asclepias</i> sp. (AS)	2/2
Cornaceae		<i>Cornus drummondii</i> (D)	1/1
Asteraceae		<i>Helianthus</i> sp. (H)	3/3
Fabaceae		<i>Lespedeza capitata</i> (L)	1/1
Poaceae		<i>Panicum virgatum</i> (PA)	2/2
Solanaceae		<i>Physalis</i> sp. (PH)	1/1
Asteraceae		<i>Solidago canadensis</i> (SO)	6/6
Maize	KPBS		
Poaceae		<i>Zea mays</i> (C)	20/20
Soybean			44/42
Fabaceae	Rossville	<i>Glycine max</i> (SR)	15/14
	Leavenworth	<i>Glycine max</i> (SL)	20/20
	Columbus	<i>Glycine max</i> (SC)	9/8
Sorghum	ABRF		
Poaceae		<i>Sorghum bicolor</i> (G)	62/57

The number of clones is not additive because some haplotypes contain isolates from more than one population.

*The abbreviation of species name at the end of isolate designation.

towels. Surface-sterilized root pieces were placed in Petri dishes containing a semiselective medium (39 g potato dextrose broth powder (Difco, Detroit, MI, USA), 10 g agar, 250 mg streptomycin sulphate (Sigma-Aldrich, St Louis, MO, USA) and 160 mg Chloroneb (Sigma-Aldrich), in 1 L distilled water (Singleton *et al.* 1992), and incubated for a week at 22°C. The protocol for strain isolation from maize and sorghum stems was similar, except that 0.5 cm sections of symptomatic stem tissue were collected and surface sterilized prior to incubation on the semiselective medium. To isolate *M. phaseolina* from sorghum, Lima bean agar (Singleton *et al.* 1992) was used to reduce contamination by *Fusarium* spp. We identified 143 isolates of *M. phaseolina* based on the production of microsclerotia in culture, with 126 isolates from agricultural fields and 17 from tallgrass prairie (Table 1). One *M. phaseolina* isolate from Arizona was included as a standard (Mihail & Taylor 1995). Three to five small agar pieces containing fungal mycelia were transferred to 125-mL flasks containing 40 mL of a complete medium broth (Leslie & Summerell 2006). The flasks were incubated at 22°C for 24 h without agitation and then transferred to an orbital shaker (150 rpm) and incubated for 3–4 days at room temperature (22–25°C). Mycelia were harvested by filtering the medium through milk filter discs (Ken AG, Ashland, OH, USA), dried by blotting with paper towels and kept in aluminium foil at –20°C until ground under liquid nitrogen.

The chlorate sensitivity/resistance of the *M. phaseolina* isolates was determined on minimal media (MM) containing 1.5% KClO₃ (Pearson *et al.* 1986). Colony growth phenotypes were scored after incubation in the dark for 1 week at room temperature (22–25°C). Three phenotypic classes were identified: dense, feathery and restricted (Pearson *et al.* 1986).

DNA extraction and AFLP analysis

Frozen mycelia were ground to a powder under liquid nitrogen in a mortar and pestle. The ground mycelia were transferred to 1.5 mL microcentrifuge tubes. Fungal DNA was extracted by the CTAB method (Murray & Thompson 1980) as modified by Leslie & Summerell (2006).

AFLPs (Vos *et al.* 1995) were generated as previously described (Leslie & Summerell 2006) with minor modifications. Four AFLP primer-pairs were used, *EcoRI*-AA/*MseI*-AA, *EcoRI*-AA/*MseI*-AC, *EcoRI*-AA/*MseI*-AG and *EcoRI*-AA/*MseI*-AT (Reyes-Franco *et al.* 2006). The *EcoRI*-AA primer was fluorescently labelled with 6-carboxyfluorescein (IDT, Coralville, IA, USA). An ABI DNA 3730 sequencer was used to identify the AFLP PCR-amplicons (KSU Genotyping Laboratory, Manhattan,

KS, USA). Each AFLP reaction contained a Genescan™ 500 LIZ™ size standard (Applied Biosystems, Foster City, CA, USA). GeneMarker version 1.6 software (Soft-Genetics, State College, PA, USA) was used to score and analyse the AFLP data. We recorded the presence/absence of each AFLP band ≥200 bp in length.

Sequencing of rDNA-ITS region

The internal transcribed spacer (ITS) region of the rDNA repeat was amplified from 28 isolates representing the four AFLP groups identified using the following forward and reverse primers: ITS5 (SR6R) 5'-GGAAG-TAAAAGTCGTAACAAGG-3' and LR5 5'-TCCTGAGG-GAAACTTCG-3' (Vilgalys & Hester 1990; White *et al.* 1990). The PCR reaction contained 1 ng/μL genomic DNA, 1× PCR buffer (Bioline, Randolph, MA, USA), 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.25 μM of each primer and 1.0 unit BIOLASE™ DNA polymerase (Bioline) in a 30-μL reaction volume. The PCR program was: one cycle at 94°C for 3 min, 31 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and one cycle of 72°C for 5 min, and then held at 4°C. PCR amplicons were purified with a QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA) and then sequenced directly at the KSU sequencing facility. The DNA sequences were edited and aligned with BioEdit software (<http://www.mbio.ncsu.edu/Bioedit/bioedit.html>). PAUP version 4.0b10 (Swofford 2003) was used to identify haplotypes and determine phylogenetic relationships among the 28 sequences. Other rDNA sequences of *M. phaseolina* previously deposited in GenBank were analysed along with those identified in this study.

Genetic data analyses

Unique and clonal AFLP haplotypes within and among the various populations of *M. phaseolina* were identified by using the unweighted pair-group method with arithmetic mean algorithm (UPGMA) implemented in PAUP. The Dice coefficient (Nei & Li 1979) was used to calculate pairwise genetic distances within and among the populations using SAS software release 9.1 (SAS Institute, Cary, NC, USA).

To determine the level of genetic variability and genetic differentiation within and among populations of *M. phaseolina*, the allele frequencies of polymorphic AFLP loci, and the genetic diversity within and between different populations of *M. phaseolina* (Nei 1973), were calculated with POPGENE version 1.32 (available at: <http://www.ualberta.ca/~fyeh/index.htm>; Yeh & Boyle 1997). POPGENE also was used to estimate: Nei's unbiased genetic identity and the genetic

distance (Nei 1978), the genetic fixation index (G_{st}) (McDermott & McDonald 1993) and the effective migration rate (Nm) (Nei 1987). A locus was considered polymorphic when the band was present at a frequency between 5% and 95%.

Structure software version 2.0 (Pritchard *et al.* 2000) was used to detect population genetic structure of *M. phaseolina* and identify individuals that were admixed or had migrated. Each isolate was assigned to one or more of K clusters. Cluster numbers from $K = 1$ to 10 were tested (each simulation of K was replicated five times) based on the statistics described by Evanno *et al.* (2005) to select the number of clusters that best matched the population structure of *M. phaseolina*.

The variation in AFLP marker patterns among isolates was examined further using principal component analysis (SAS). Major principal components were subjected to analysis of variance to test for effects of host, chlorate phenotype within host and location within host.

Results

Chlorate growth phenotypes

The growth of *M. phaseolina* on MM amended with chlorate divided the isolates into three phenotype classes: dense, feathery and restricted. All of the isolates recovered from the soybeans and the KPBS-prairie were either feathery or restricted. Most of the isolates from KPBS-maize also were either feathery or restricted, but two isolates had the dense phenotype. Eighty per cent of the isolates from ABRF-sorghum had the dense phenotype.

AFLP genotyping

The four AFLP primer-pairs were equally informative. One hundred and fifty AFLP markers were scored across the 144 isolates, including the reference isolate from Arizona. No monomorphic markers were observed. The greatest genetic diversity (Table 2) occurred amongst isolates collected from KPBS-prairie, with an average genetic similarity of 58%, followed by the KPBS-maize population with an average genetic similarity of 71%. The least genetic diversity was seen amongst isolates from soybean fields with an average genetic similarity of 80%. The highest pairwise genetic similarity was between the KPBS-maize and soybean populations. ABRF-sorghum and soybean populations were the most dissimilar (Table 2).

Unique and repeated AFLP haplotypes within and amongst different populations of *M. phaseolina* were identified with PAUP software. Based on the 150 AFLP

Table 2 The pairwise average genetic similarities using the Dice coefficient (between populations above the diagonal and within populations on the diagonal) and pairwise calculations of Nei's unbiased genetic identity (below the diagonal) for four different populations of *Macrophomina phaseolina*

	KPBS-Maize	Soybean	ABRF-Sorghum	KPBS-prairie
KPBS-Maize	0.712	0.767	0.643	0.611
Soybean	0.989	0.797	0.601	0.691
ABRF-Sorghum	0.732	0.656	0.746	0.623
KPBS-prairie	0.908	0.882	0.718	0.584

markers, there were 134 unique AFLP haplotypes (Tables 1 and S1). Most of the AFLP haplotypes were represented by a single isolate; however, eight haplotypes were represented by more than one isolate (Fig. 1). Of these eight haplotypes, three were recovered from different hosts. One haplotype was recovered once from KPBS-maize and twice from ABRF-sorghum. Another haplotype was recovered once from KPBS-maize and once from KPBS-*Helianthus* sp. The third haplotype was recovered once from KPBS-maize and twice from soybeans collected from Rossville, KS (Figs 1 and 2).

Based on the UPGMA clustering analysis, the isolates were grouped in four clusters with bootstrap support of 58–100% (Fig. 1). The first cluster contained most of the isolates from KPBS-maize and soybean, and a few isolates from ABRF-sorghum and KPBS-prairie. The *M. phaseolina* isolate from Arizona also was in this cluster (Fig. 1). All isolates in cluster 1 had either feathery or restricted growth on chlorate medium. Eleven of the 17 isolates from KPBS-prairie grouped in the second cluster and most of the ABRF-sorghum isolates grouped in cluster 3. All isolates in cluster 3 had the dense phenotype on chlorate medium, except for two isolates that grew poorly on minimal medium with or without chlorate. Cluster 4 contained a single feathery isolate collected from KPBS-Solidago. This isolate was genetically distant from the other isolates (Fig. 1).

rDNA-ITS sequencing

To confirm the identity of *M. phaseolina* isolates, the rDNA-ITS region and a portion of the rDNA 28S gene were amplified and sequenced from 28 isolates representing the four AFLP clusters (Fig. 1 and Table S1). The 28 rDNA sequences were deposited in the GenBank database under the accession nos FJ395220–FJ395247. The size of the PCR products ranged from 1420 to 1423 bp. Ten single nucleotide polymorphisms (SNPs) were identified and used to group the 28 isolates into five rDNA haplotypes (Fig. 1 and Table S3). Most of

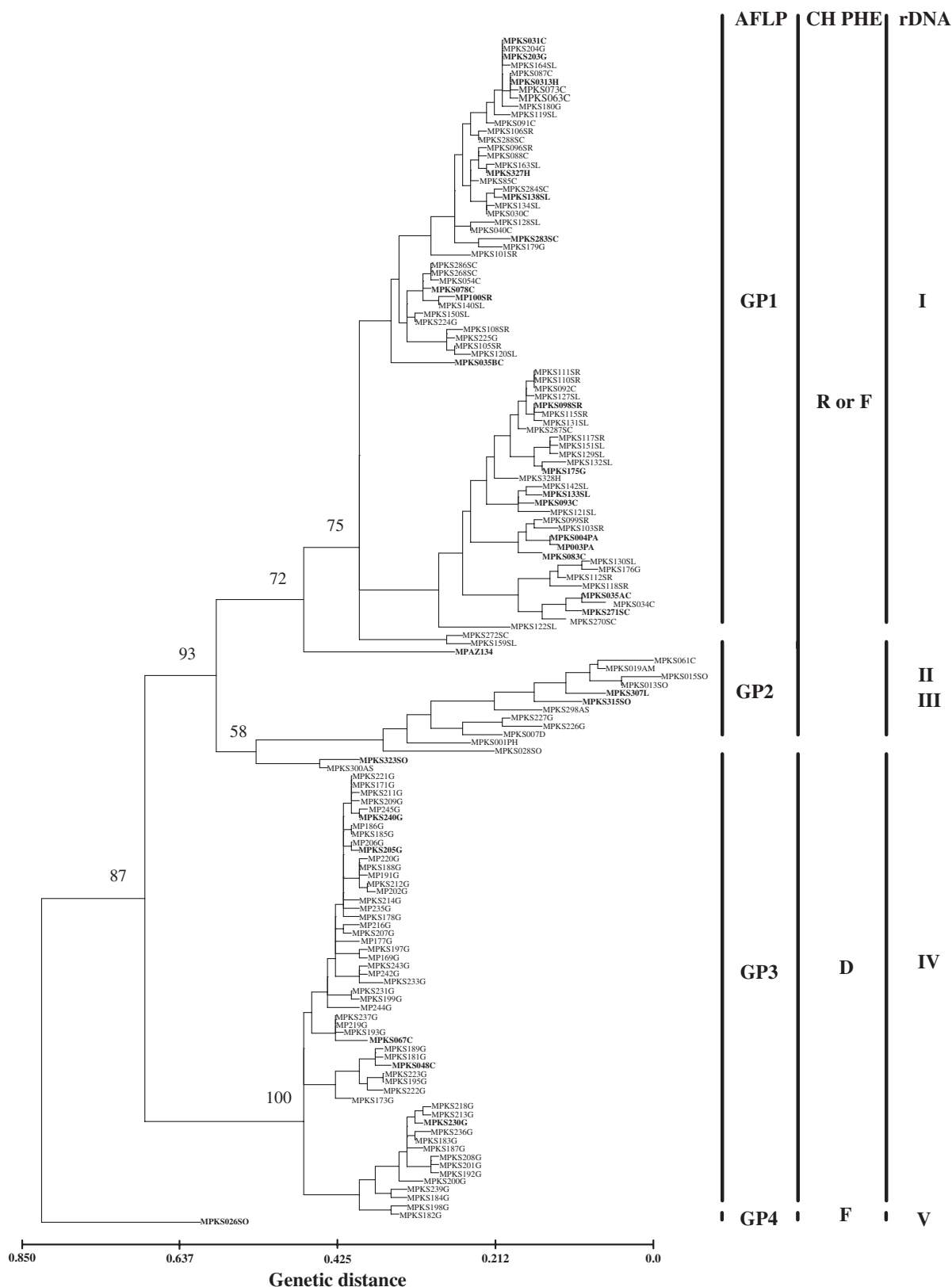


Fig. 1 Phylogenetic tree constructed by UPGMA method based on 150 AFLP markers for the 144 isolates of *Macrophomina phaseolina*. The numbers above the branches represent the bootstrap values for 1000 replicates. The last letters of isolate names indicate the source host from which the isolates were collected (see Table 1). The ITS-rDNA region was amplified from the isolates indicated in bold font. The three chlorate phenotypes, F – feathery, R – restricted and D – dense (CH PHE); and five rDNA haplotypes are indicated.

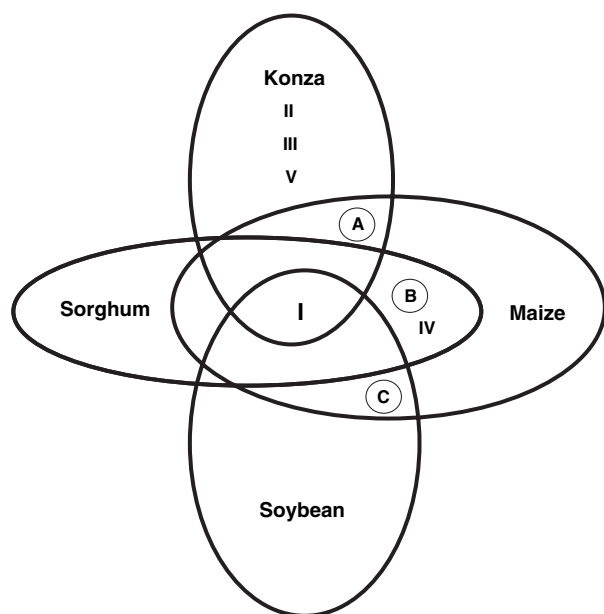


Fig. 2 Venn diagram illustrating the overlap of the four groups of *Macrophomina phaseolina* from different plant hosts and locations. Shared rDNA (in roman numerals) and AFLP (letters in circles) haplotypes among groups are in intersections.

the SNPs (7/10) were located in the ITS-1 region. Only one SNP was detected in the ITS-2 region and no SNPs were detected in the 5.8S nrRNA gene. Two SNPs were detected in the portion of the 28S nrRNA gene. Two haplotypes were represented by only one isolate each (MPKS026 and MPKS323), both of which were collected from KPBS-Solidago. The third and fourth haplotypes were represented by two (collected from KPBS-Lespedeza and KPBS-Solidago) and five isolates (collected from ABRF-sorghum and KPBS-maize), respectively. The fifth haplotype was represented by 18 isolates that were collected from KPBS-maize, ABRF-sorghum, soybean, KPBS-Helianthus and KPBS-Panicum, in addition to the standard Arizona isolate (Fig. 1).

The four AFLP groups were correlated with the five rDNA haplotypes. AFLP cluster 2, which contained most of the *M. phaseolina* isolates collected from KPBS-prairie, was associated with two rDNA haplotypes (Fig. 1). When the five rDNA haplotypes were compared with previously deposited rDNA sequences of *M. phaseolina*, most of the deposited rDNA sequences belonged to a single haplotype, which was recovered at all six of our sample sites and was the most common in GenBank (Table S4). This common haplotype also was recovered from other countries such as Australia, Canada, China, India and Spain in addition to the United States. The sequence of the rDNA haplotype isolated from KPBS-Lespedeza and KPBS-Solidago, was identical

to that of a *M. phaseolina* isolate reported to infect humans (GenBank accession no. EF570500). The other three rDNA haplotypes were unique to Kansas populations (GenBank accession nos FJ395220, FJ395226 and FJ395246). There were a few rDNA sequences deposited in Genbank and attributed to *M. phaseolina* with ITS regions that could not be aligned with the *M. phaseolina* ITS region obtained in this study and with more than six SNPs in the 5.8S nuclear rRNA gene when aligned with the *M. phaseolina* 5.8S nuclear rRNA gene (e.g. GenBank accession nos DQ314733, DQ233663, DQ233665, DQ359740, DQ682587).

When we compared restriction maps of rDNA regions, generated by the NEBcutter program (Vincze *et al.* 2003), of the ITS-rDNA sequences from the present study with the five RFLP patterns reported by Purkayastha *et al.* (2006) for *M. phaseolina* from India, we found significant differences (Table S2). The average total length of the amplified ITS-rDNA sequence was ~1422 bp from the Kansas isolates and ~1600 bp from the Indian isolates. The restriction enzymes used by Purkayastha *et al.* (2006) would result in a single RFLP-rDNA banding pattern for all of the Kansas sequences, although five banding patterns were reported from the Indian population (Table S2). Some of these discrepancies can be explained by errant size estimates from the gels, e.g. *EcoRI*, *TaqI*, *HaeIII*, *MboI* and *RsaI*, but the *HindIII* site in the Indian isolates is completely missing from the ITS-rDNA sequences of the Kansas isolates. Su *et al.* (2001) also amplified the same region from 45 isolates collected from four plant hosts from Louisiana, USA, and used restriction enzymes *EcoRI*, *TaqI*, *HaeIII*, *MboI* and *RsaI*. They identified only one RFLP-rDNA haplotype, which is identical to the one reported in this study. If the Indian rDNA sequences are used to search GenBank, then three of the unique RFLP-rDNA haplotypes match with sequences of fungi other than *M. phaseolina*. In particular, the rDNA sequence of isolate OS-5467-D (GenBank accession no. DQ233665) was the same as that of *Dothideomycete* sp. (GenBank accession no. EU680526), rDNA from isolate CB-100-D (GenBank accession no. DQ233663) was the same as that of *Cochliobolus lunatus* (GenBank accession no. DQ836799) and rDNA from isolate GH-50-D (GenBank accession no. DQ314733) was the same as that of *Alternaria* sp. (GenBank accession no. FJ176475). There also are other cases where fungal isolates were misidentified as *Macrophomina* based on rDNA sequences (e.g. Posada *et al.* 2007).

Population genetic differentiation

POPGENE was used to evaluate the genetic variation within and among *M. phaseolina* populations and to

determine if these populations were genetically differentiated. When the isolates from KPBS-prairie, KPBS-maize, ABRF-sorghum and soybean fields were compared, the overall estimated genetic fixation index was $G_{st} = 0.367$. This high fixation index suggests great genetic differentiation amongst the four location-host groups, i.e. 37% of the total genetic variation could be attributed to genetic differences because of group. The overall effective migration rate (Nm) across the four groups was <1 , indicating that gene flow between these populations is not extensive.

When the groups were compared pairwise, the KPBS-maize and soybean groups had the lowest fixation index ($G_{st} = 0.04$) and the highest effective migration number ($Nm = 13$). These values suggest that significant genetic exchange occurs between these two groups and that there are few barriers to genetic exchange (Table 3). The highest G_{st} values separated the ABRF-sorghum group from the other three groups

Table 3 Pairwise estimates of fixation index (G_{st}) (above diagonal) and effective migration number (Nm) (below diagonal) based on the 88 AFLP markers from four populations of *Macrophomina phaseolina*

Population	Maize	Soybean	Sorghum	Native prairie
Maize	—	0.04	0.34	0.15
Soybean	13.0	—	0.45	0.21
Sorghum	1.0	0.61	—	0.34
Native prairie	2.8	1.9	0.98	—

and ranged from 0.34 to 0.45 (Table 3). Thus, the *M. phaseolina* population from ABRF-sorghum is genetically differentiated from the other groups and is the most genetically divergent of these four groups. The pairwise estimate for G_{st} between the groups from the KPBS-prairie grasses and KPBS-maize was $G_{st} = 0.15$, which indicates that some genetic exchange occurs between these two groups ($Nm < 3$). The pairwise genetic identity between KPBS-prairie grasses and the KPBS-maize groups also was $>90\%$, which is consistent with a hypothesis of genetic exchange between these groups (Table 2).

Structure 2.0 also was used to evaluate the population genetic structure of the 144 analysed isolates and to identify migrant and admixed individuals. ΔK (Evanno *et al.* 2005), which is based on the rate of change in the log-likelihood between successive K values ($K = 1-10$), was used to determine the likely number of clusters. For $K > 3$, the number of populated clusters did not increase, although the number of admixed individuals did. Thus, three clusters suffice to circumscribe the genetic structure of the *M. phaseolina* populations. The results of the Structure analysis were consistent with the results of the AFLP and rDNA analyses in which all isolates from soybeans and most of those from KPBS-maize were assigned to the same cluster that also contained a few individuals from ABRF-sorghum and KPBS-prairie (Fig. 3). Most of the ABRF-sorghum isolates were assigned to a second cluster (Fig. 3). The isolates from KPBS-prairie were assigned to two clusters (Fig. 3). Admixed individuals were detected in soybean/KPBS-maize (8 of 64 isolates), ABRF-sorghum (2 of 62) and KPBS-prairie (5 of 17) clusters. Interestingly,

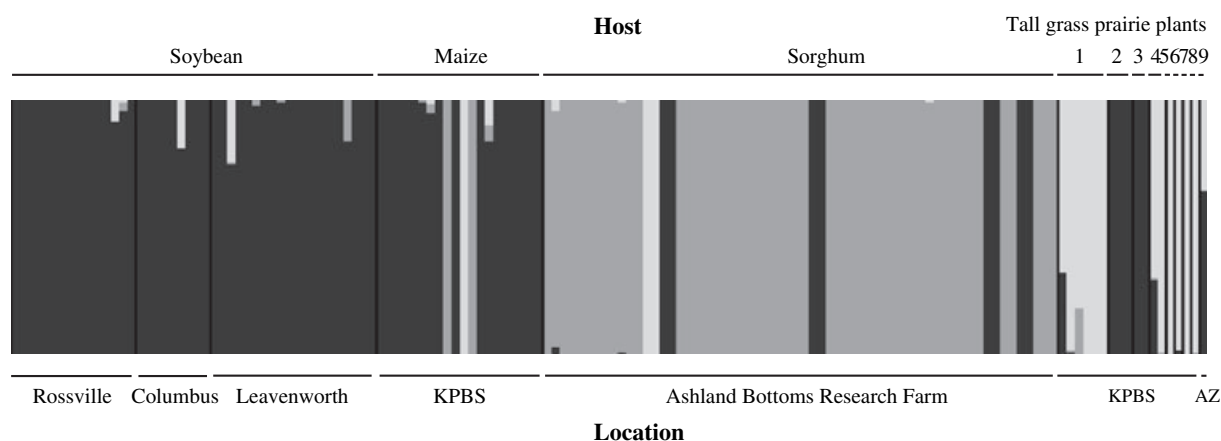


Fig. 3 Structure summary blot showing the assignment of *Macrophomina phaseolina* isolates from different locations/hosts to $K = 3$ clusters, where each cluster is indicated by a different shading. Each fungal isolate is represented by a thin vertical region partitioned into membership fractions of the three assigned clusters. Vertical black lines separate groups of fungal isolates from different locations/hosts. Arabic numbers, 1–8, indicate the following hosts: 1 – *Solidago*, 2 – *Helianthus*, 3 – *Panicum*, 4 – *Asclepias*, 5 – *Cornus*, 6 – *Lespedeza*, 7 – *Ambrosia* and 8 – *Physalis*. Number 9 indicates the standard isolate from Arizona.

Table 4 Analysis of variance of major principal components for AFLP patterns of *Macrophomina phaseolina* isolates from Kansas

Effect	Mean square			
	PC1 (24.3)†	PC2 (39.4)	PC3 (50.5)	PC4 (57.8)
Host	96.92*	24.79	89.52*	129.67*
Phenotype (host)	87.90*	22.65	10.67	1.49*
Location (host)	0.27	0.07	0.14	0.31
Isolate	4.63	16.26	9.53	0.39

*Statistically significant for $\alpha = 0.001$.

†Cumulative % variance.

the standard Arizona isolate was one of the admixed individuals (Fig. 3).

Principal component analysis

The first four principal components of AFLP data explained 58% of the genetic variation among isolates. The first, third and fourth components displayed significant ($P \leq 0.001$) host effects, while the first and fourth components varied ($P \leq 0.001$) among chlorate phenotypes within a given host (Table 4). Significant location within host (for soybean) effects were not observed for any of the major principal components.

Host means are plotted for the first and third principal components in Fig. 4A. The first principal component primarily separated ABRF-sorghum isolates from the other isolates, while the third component separated isolates into two groups, with one group comprised entirely of KPBS-prairie isolates and the other group comprised of isolates from agronomic plants plus those from *Helianthus* sp., *Panicum virgatum* and Arizona soil. The fourth principal component separated the Arizona isolate from all other isolates (data not shown).

Chlorate phenotypes for isolates from KPBS-maize, ABRF-sorghum and soybean were separated by the first and fourth principal components (Fig. 4B). Isolates generally clustered by chlorate phenotype, with dense isolates separated from feathery and restricted isolates by the first component and feathery isolates separated from restricted isolates by the fourth component.

Discussion

Populations of pathogens that infect multiple host species may be partitioned geographically and with respect to host, and the degree of partitioning may differ for native or agricultural populations. For example, Kohn (1995) and Kohli & Kohn (1996) reported more widespread distribution of haplotypes of *Sclerotinia sclerotio-*

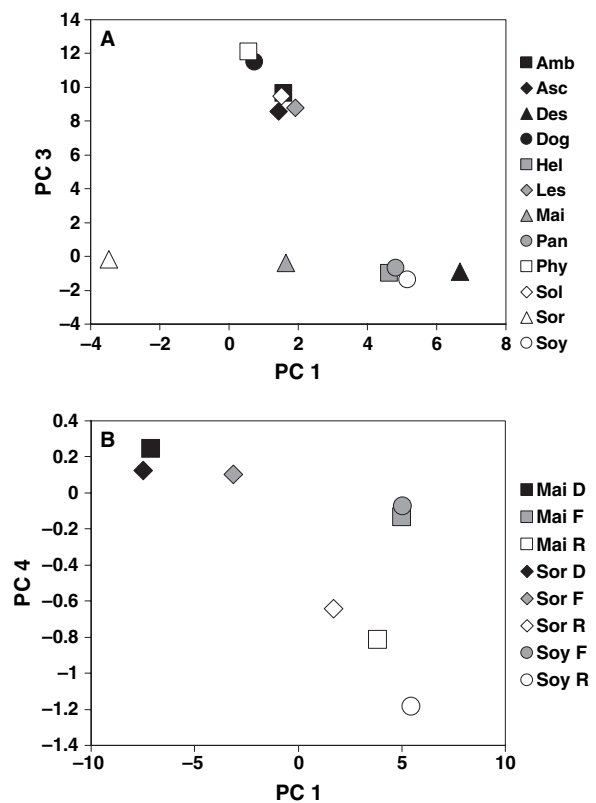


Fig. 4 Scatterplot for (A) host species means of the first and third principal components and (B) chlorate phenotype means of the first and fourth principal components for *Macrophomina phaseolina* AFLP patterns.

rum in agricultural populations than in non-agricultural populations. For *M. phaseolina* in Kansas, we found a common rDNA haplotype of *M. phaseolina* across all the locations and the three crop species sampled. However, there also were rDNA haplotypes that were recovered from only a subset of locations and hosts. Higher dissimilarity was found amongst isolates from tallgrass prairie plants, although this may be explained at least in part by the greater number of host species sampled there (Gordon *et al.* 1992). Unlike the apparent separation in serotypes observed for BYDV between tallgrass prairie and wheat in Kansas (Garrett *et al.* 2004), the results from the present study suggest that a portion of the *M. phaseolina* haplotypes in Kansas are shared between tallgrass prairie plants and crop species cultivated in the region. The most common rDNA haplotype, which was recovered from all six sampling sites, also was the most common in GenBank, and has been recovered from Australia, Canada, China, India and Spain in addition to the United States.

Macrophomina phaseolina is a complex and widely dispersed fungal species in terms of both host and geographic distribution. For a putatively asexual species, it

also is quite diverse when evaluated for a number of genetic and physiological characters. In general, isolates from the same location–host clustered together in all analyses. Although the soybean isolates were collected from three widely dispersed locations, they were the most homogeneous group, with an average genetic similarity of 80%, and all of these isolates belonged to the same AFLP cluster and had the same ITS-rDNA sequence. Most of the isolates from ABRF-sorghum also belonged to a single AFLP cluster with an average similarity of 75%. These results are similar to those of others, e.g. Purkayastha *et al.* (2006) and Su *et al.* (2001), who found that *M. phaseolina* isolates from the same host were genetically more similar to one another and genetically more distinct from isolates from other hosts.

The KPBS-prairie population was genetically the most diverse. These isolates were recovered from different hosts, as there were too few isolates from each host species to treat each group as a separate population. Yet most of the KPBS-prairie isolates, regardless of the host, grouped in the same AFLP cluster. Native tallgrass prairie plants could serve as a reservoir for diversity within *M. phaseolina*. Crop acreage may be a greater overall source of inoculum for *M. phaseolina* in the region, but tallgrass prairie provides an environment in which isolates from different hosts may be in immediate contact as host roots intertwine. Heat and drought stress broaden the effective host range of *M. phaseolina* (Pearson *et al.* 1986, 1987a). The impact of *M. phaseolina* on tallgrass prairie plant communities is not known, but to the extent that climate change increases heat and drought stress, native prairie plant communities should be more heavily impacted by this pathogen.

The uniqueness of the sorghum population was unexpected, but could result if the *M. phaseolina* isolates that colonize this crop originated in Africa and accompanied sorghum to the United States. However, isolates capable of acting as sorghum pathogens also might be recovered from weedy sorghum relatives, e.g. Johnson grass and shattercane that were not sampled in this study. The recovery of clonal isolates from multiple locations and the presence of presumably genetically admixed isolates suggest that both geographic and genetic mixing is occurring in the populations of this fungal pathogen. Thus, selectively advantageous genetic changes could be retained and shared across all of the *M. phaseolina* populations.

The KPBS-maize population was the most complex, with isolates belonging to three AFLP clusters, two ITS-rDNA sequences and three chlorate phenotype groups. This variation could result from several factors, including the location of the sampled field next to tallgrass prairie, with much higher plant diversity than typical agricultural systems and crop rotation within the field.

Crop rotation may affect the proportion of various strain types recovered (Pearson *et al.* 1986, 1987a), probably by altering the selection pressure as different hosts are present. For example, Su *et al.* (2001) reported more colonization of maize plants by *M. phaseolina* occurred when the field in which the maize was growing was infested by *M. phaseolina* isolates from maize compared with when the infesting fungal isolates were from sorghum, soybean or cotton.

The three population subdivisions of the *M. phaseolina* isolates identified in Structure were consistent with the grouping of the isolates based on the molecular, AFLP and ITS-rDNA, and chlorate phenotype data. Isolates carrying the most common ITS-rDNA sequence clustered in the same AFLP cluster and Structure subpopulation regardless of their host or geographic origin, suggesting that members of this group were the oldest and most cosmopolitan. Admixed isolates, i.e. isolates that may have mixed ancestry, also were detected (Fig. 3). The presence of admixed individuals provides evidence for genetic exchange amongst different isolates of *M. phaseolina*, perhaps through hyphal fusion followed by a parasexual recombination process (Mihail & Taylor 1995; Jones *et al.* 1998).

While subdivision of *M. phaseolina* into more than one species has been considered, there generally has not been sufficient evidence to support splitting (Crous *et al.* 2006; Rossman & Palm-Hernández 2008). Numerous studies have used diverse molecular markers to evaluate strains from even more diverse hosts and geographic locations and have identified tremendous levels of genetic variation, but there still are no data that suggest that this species should be subdivided into multiple species (Jones *et al.* 1998; Su *et al.* 2001; Jana *et al.* 2005; Crous *et al.* 2006; Reyes-Franco *et al.* 2006). Our results are consistent with these reports in that the lowest genetic similarity between two isolates based on AFLPs in this study was 58%, a value consistent with that for a single species in other fungi in which inter- and intraspecific comparisons of AFLP diversity have been evaluated (e.g. Leslie *et al.* 2001; Marasas *et al.* 2001; Zeller *et al.* 2003). Moreover, the rDNA showed that it is much conserved amongst the strains used in this study. Consequently, we suggest that the isolates evaluated in this study probably belong to a single fungal species with a global distribution.

Interpretation of the population genetic structure of *M. phaseolina* is challenging, in part because of a general lack of knowledge about dispersal processes for soil-borne pathogens. The hypothesis that expanding populations will be less genetically diverse at their frontiers than in the centre of their range (e.g. Milgroom *et al.* 2008) would be interesting to test in this system, but information about the historical range of *M. phaseolina*

in the central United States is not available. All the haplotypes recovered might have been present throughout the region sampled prior to the introduction of agriculture, followed by selection imposed by cropping systems for particular haplotypes. There also may be current movement between the systems studied; characterizing the connectivity of agricultural and natural components of the landscape for a generalist pathogen such as *M. phaseolina* will be an important step in understanding landscape connectivity, while host specialists are much simpler to address (e.g. Margosian *et al.* 2009). Another change resulting from agricultural cultivation is the much greater potential for dispersal of soil-borne pathogens through wind erosion of soils, or for wind dispersal of infected plant debris. In contrast, tallgrass prairie provides ecosystem services such as protection from wind erosion of soil and a reduced probability of long-distance dispersal of soil-borne pathogens, through extensive perennial root systems.

The importance of *M. phaseolina* is likely to increase in both agricultural and natural ecosystems where climate change scenarios include greater heat and drought stress. An increase in infection by *M. phaseolina* in native ecosystems may alter the relative frequency of plant species in the community and could even result in the extinction of species that are particularly sensitive to this fungus. A larger fungus biomass should also increase the diversity of *M. phaseolina* in both the natural setting and agriculture, and so increase the likelihood of developing more pathogenic lines of agricultural importance. Thus, the abundance and diversity of *M. phaseolina* may become an important indicator of natural and agro-ecosystem health in response to global climate change.

Acknowledgements

The authors thank S. Dendy for technical support and W. Bockus, C. Little and C. Toomajian for helpful discussions. This work was supported in part by the US National Science Foundation through Grants DEB-0130692, EPS-9874732, EF-0525712 (as part of the joint NSF-NIH Ecology of Infectious Disease programme) and DEB-0516046, by the NSF Long Term Ecological Research Programme at Konza Prairie, by USAID INTSORMIL and by the Kansas Agricultural Experiment Station (Contribution 09-236-J).

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Supporting Information

Additional supporting information may be found in the online version of this article:

Table S1 Individual *Macrophomina phaseolina* isolates and their genetic and morphological characteristics

Table S2 Estimated DNA length of restriction-digestion fragments of ITS-rDNA region for *M. phaseolina* isolates from Kansas and India

Table S3 Alignment of the ribosomal DNA ITS region from 28 *M. phaseolina* isolates representing the four AFLP groups

Table S4 Alignment of rDNA haplotypes recovered from this study along with different haplotypes retrieved from GenBank database

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