Molecular genetic relationships of five *Stemphylium* species pathogenic to alfalfa

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Genomic similarity analyses of geographically diverse isolates of *Stemphylium* species isolated from alfalfa were performed using random amplified polymorphic DNA (RAPD) markers. The RAPD assay detected DNA polymorphisms among 28 monoconidial isolates from five morphology-based taxonomic species of *Stemphylium*, and one isolate each of *Pithomyces chartarum* and *P. atro-olivaceus*. *Pithomyces* is a saprobe that is morphologically similar to *Stemphylium*, and was included as an outgroup. Eleven oligodeoxynucleotide 10-base primers generated 205 RAPD fragments from total genomic DNA. Principal component analysis of RAPD fragment occurrence grouped the 28 *Stemphylium* isolates into two clusters. One cluster included *S. botryosum* and *S. globuliferum*. The second cluster included *S. alfalfae*, *S. herbarum*, and *S. vesicarium*. A separate analysis of the second cluster separated the three species. *Pithomyces chartarum* and *P. atro-olivaceous* were widely separated from *Stemphylium* and from each other. One major RAPD fragment of about 2.5 Kb was common to all *Stemphylium* isolates but was absent in the *Pithomyces* species. Southern analysis revealed strong cross-hybridization of major RAPD fragments across species, inferring that they were of the same nucleotide sequences. No cross-hybridization to the *Pithomyces* fragments was detected. These results supported recent morphologically-based taxonomic revisions and indicated that at least five genetically distinct species of *Stemphylium* can cause leafspot of alfalfa.

Keywords: electrophoresis, RAPD, Stemphylium leaf spot, principal component analysis.

Stemphylium leaf spot of alfalfa (*Medicago sativa* L.) in the United States was first attributed to the *Stemphylium botryosum* Wallr. and its teleomorph, *Pleospora herbarum* (Pers.: Fr.) Rabenh. (Smith, 1940). Later, two distinct Stemphylium leaf spot symptoms, cool-temperature and warm-temperature symptoms, were described on alfalfa, and attributed to ecotypes of the *S. botryosum*/*P. herbarum* holomorph (Cowling & al., 1981). However, Simmons (1985) reclassified *Stemphylium*/*Pleospora* holomorphs and reported that

S. botryosum and *P. herbarum* were not components of the same holomorph. He assigned *P. tarda* as the teleomorph of *S. botryosum* and *S. herbarum* as the anamorph of *P. herbarum*. Simmons (1985) also erected a new holomorph, *S. alfalfae/P. alfalfae*, based on an isolate from alfalfa from Western Australia. The morphology of these three holomorphs, along with the anamorphs *S. globuliferum* and *S. vesicarium* pathogenic to *Medicago* species, recently were described and compared (Gilchrist & Simmons, 1990). However, the degree of genetic distinctiveness of these species is unknown. Our objective was to investigate genomic similarity among and within the five *Stemphylium/Pleospora* holomorphs to better understand pathogen diversity involved in Stemphylium leaf spot of alfalfa.

Materials and methods

The identity and sources of the 30 monoconidial fungal isolates (including 9 monoconidial subcultures) used in this study are shown in Tab. 1. Species of *Pithomyces* were included for comparison. *Pithomyces*, a saprobe, is morphologically similar to *Stemphylium* and often occurs in Stemphylium leaf spot lesions. All isolates were preserved in 15% glycerol at –80 C. Prior to DNA isolation, isolates were grown on V-8 (Campbell Soup Co., Camden, NJ) juice agar (Gilchrist & Simmons, 1990) for 14 days at 25 C under an 8 h photoperiod of cool white fluorescent lighting.

DNA was extracted from mycelia grown in 500 ml diluted V-8 broth [100 ml of centrifuged V-8 juice, 3 g $CaCO_3$ and 900 ml distilled water] in 1 l Erlenmeyer flasks at 25 C, on an orbital shaker at 200 rpm. Flasks were inoculated with 5 ml of 10^6 conidia/ml in 0.5% Tween 20. Mycelial masses were harvested after 3 days by suction filtration and frozen at –80 C. DNA was isolated from mycelia using the CTAB method of Murray & Thompson (1980). Mycelia were homogenized in 2% CTAB extraction buffer with 0.5 mm glass beads (1:1 w/w of mycelia:glass beads) for 2–3 min in a Bead Beater apparatus (Biospec, Bartlesville, OK), and the DNA was purified according to Murray & Thompson (1980).

RAPD reaction mixtures, prepared in 0.5 ml microfuge tubes, consisted of 20.25 µl of sterile nanopure water, 3 µl of 10X Replitherm buffer (Epicenter Technologies, Madison, WI), 4.8 µl of 10 µM solutions of each dATP, dTTP, dGTP, and dCTP (Gibco BRL, Bethesda, MD), 1 µl of 4.5 nM RAPD primer [ssDNA 10-mers of arbitrary sequence (Tab. 2); Operon Technologies, Alameda, CA], 0.06 µl of 5 units/µl (0.3 units) of Replitherm thermostable polymerase (Epicentre Technologies, Madison, WI) and 1 µl containing about 1 µg

Tab. 1. – Identification and origin of fungi used in random amplified polymorphic DNA (RAPD) assay[1].

Isolate Working code		Contributor's code	Anamorph	Geographic origin
I[2]	II[3]			
A	-	37-065s[4]	*Stemphylium vesicarium*	South Africa
B	BS	36-138.2s[4]	*S. herbarum*	India
C	CS	36-088[4]	*S. alfalfae*	Western Australia
D	DS	36-083[4]	*S. alfalfae*	Western Australia
E	ES	04-118C[4]	*S. botryosum*	Ontario, Canada
F	FS	30-181[4]	*S. herbarum*	New Zealand
G	GS	08-069[4]	*S. botryosum*	New Hampshire
H	HS	36-101[4]	*S. globuliferum*	Australia
PV	PVS	914[5]	*S. botryosum*	Pennsylvania
CN1	-	S-CA (Chino-1)	*S. alfalfae*[9]	California
CN2	-	S-CA (Chino-2)	*S. alfalfae*[9]	California
MT	-	MT SH2 SS71[6]	*S. alfalfae*[9]	California
KS8	-	KS83[7]	*S. alfalfae*[9]	Kansas
KS1	-	KS1[7]	*S. alfalfae*[9]	Kansas
AL	-	WI (Arlington)	*S. alfalfae*[9]	Wisconsin
JS	-	UT (Jensen)	*S. alfalfae*[9]	Utah
PS	-	WA (Pasco)	*S. alfalfae*[9]	Washington
IH	IHS	ID-H[8]	*S. alfalfae*[9]	Idaho
WT[10]	WTS	WA-T[6]	*S. alfalfae*[9]	Washington
IW[10]	IWS	IA-W[8]	*Pithomyces chartarum*[9]	Iowa
TD	-	Pi (*T. dubium*)	*P. atro-olivaceus*[9]	Washington

[1] Isolates were from *Medicago sativa* except A, H, and TD were from *Medicago* sp., *M. truncatulata*, and *Trifolium dubium*, respectively.
[2] Transfer from contributor's isolate.
[3] Monoconidial subculture.
[4] Isolates obtained from, and identified by, E. G. Simmons.
[5] Isolate obtained from K. T. Leath.
[6] Isolate obtained from D. G. Gilchrist.
[7] Isolates obtained from D. L. Stuteville.
[8] Isolates obtained from J. I. Edmunds.
[9] Isolates identified by C. Chaisrisook.
[10] Not included in the analysis

total genomic DNA. A no-DNA control was included in each set of reactions. RAPD reactions were covered with 50 µl of sterile mineral oil, and amplified in a COY tempcycler (COY Products, Grass Lake, MI). RAPD conditions were an initial denaturation for 3 min at 92 C, then 50 cycles of denaturation at 92 C for 1 min, primer annealing at 37 C for 1 min, and primer extension at 72 C for 1 min. The procedure was terminated with a primer extension at 72 C for 5 min, then chilling to 4 C. RAPD products were examined by electrophoresis in 1.4% agarose gels in 0.5X TBE. Gels were stained with ethidium bromide, and photographed with transmitted UV light. All RAPD

reactions were replicated at least once; some were replicated as many as nine times. Only fragments occurring in all trials were included in the analysis.

RAPD fragment occurrence data were collected by scoring for the presence or absence of bands (DNA fragments) on the gel photographs of all trials of a given primer. Data were recorded as "1" (present) or "0" (absent). Data analyses consisted of principal component analyses of RAPD fragment occurrence data, and calculations of genetic distances. The principal component analyses were carried out on the 1,0 data with the SAS (1989) procedure PRINCOMP. Genetic similarities were calculated by Nei and Li's (1979) equation: $F = 2N_{xy}/(N_x+N_y)$, where F is the similarity between two populations or species, N_{xy} the number of RAPD fragments in common between individuals X and Y, N_x the number of total RAPD fragments of individual X, and N_y the number of total RAPD fragments in individual Y. These coefficients were calculated with a computer program written locally.

Southern analyses were used to determine whether similarly-sized RAPD products from *Stemphylium* species and one *Pithomyces* species shared significant regions of homology. The DNA probes were purified from gels with the Geneclean Kit (Bio 101 Inc., LaJolla, CA). The Southern blot procedure used was depurination in 0.5 M HCl for 15 min, denaturation with 0.5 M NaOH, 1.5 M NaCl for 30 min, and neutralization in 3M NaCl, 0.5 M TRIS (pH 7.0) for 30 min. Gels were blotted to Nytran nylon membrane (Schleicher & Schuell, Keene, NH) using 5X SSPE as the transfer buffer for at least 2 h. Membranes were dried at 42 C for 1 h and were exposed to UV light for 1 min to cross-link the DNA to the membrane. Membranes were washed with 0.5X SSPE, 0.1% SDS for 30 min before hybridization. Hybridization buffer consisted of 50% deionized formamide, 4X SSPE, 1% SDS, and 0.5% skim milk (w/v). DNA probes were labelled with ^{32}P-dCTP using standard nick-translation procedures (Sambrook & al., 1989). Hybridizations were carried out at 42 C in a hybridization incubator (Eurotherm Model 310, Robbins Scientific, Sunnyvale, CA) for 20 h. Membranes were then washed in 0.5% SSPE, 0.1% SDS; once at room temperature for 10 min, then twice at 60 C for 30 min with agitation. Membranes were blotted briefly on filter paper and were sealed in plastic wrap. Autoradiography was carried out at –80 C with two intensifying screens.

Results

A total of 205 RAPD fragments were reproducibly generated from total genomic DNAs with 11 primers. One RAPD fragment, Y03-0,

Tab. 2. – Random amplified polymorphic DNA (RAPD) products generated from *Stemphylium* total DNA by 11 oligodeoxy nucleotide 10-mers.

Primer code[1]	Primer sequence (5′ to 3′)	Number of RAPD fragments	Species-specific fragments
A03	AGTCAGCCAC	21	A03-5[2], A03-12[2]
A04	AATCGGGCTG	20	A04-0[2]
A05	AGGGGTCTTG	11	A05-5[2], A05-8[3], A05-10[2]
F01	ACGGATCCTG	20	——
F02	GAGGATCCCT	12	F02-6[2], F02-8[4]
Y01	GTGGCATCTC	19	Y01-3[2]
Y02	CATCGCCGCA	29	Y02-4[2], Y02-10[2], Y02-11[2],Y02-18[2]
Y03	ACAGCCTGCT	22	Y03-0[5], Y03-21[2]
Y04	GGCTGCAATG	25	Y04-0[2], Y04-1[3]
Y05	GGCTGCGACA	11	Y05-4[2], Y05-6[2]
Y06	AAGGCTCACC	15	Y06-9[2]

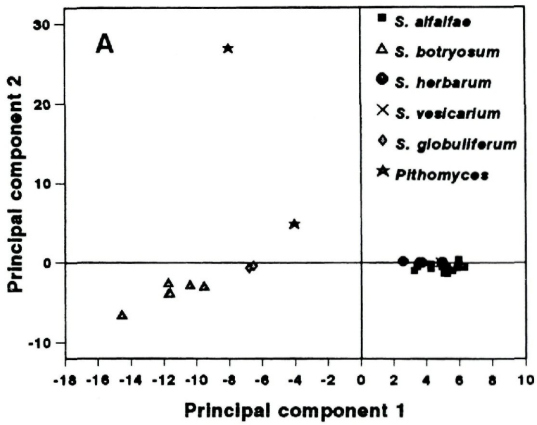
[1] Primer codes were assigned by their manufacturer, Operon technologies, Alameda, CA.
[2] RAPD marker specific for *S. botryosum*.
[3] RAPD marker specific for *S. herbarum*.
[4] RAPD marker specific for *S. alfalfae*.
[5] RAPD marker common to *Stemphylium* spp.

generated with Y03 primer (Tab. 2) was common to all *Stemphylium* isolates but lacking from both *Pithomyces* species. Other primers also provided some RAPD fragments that tended to be species-specific (Tab. 2). The first two components from principal component analysis of RAPD fragment occurrence data explained 39.1% of the variance and grouped *Stemphylium* isolates into two large, distinct clusters (Fig. 1A). The two *Pithomyces* species were well separated from all *Stemphylium* isolates (Fig. 1A). One *Stemphylium* cluster consisted of *S. botryosum* and *S. globuliferum*, the second cluster included *S. alfalfae*, *S. herbarum* and *S. vesicarium* (Fig. 1A). A principal component analysis of the species in the second cluster yielded good separation of *S. alfalfae* and *S. herbarum* (Fig. 1B) suggesting that those isolates represented distinct *Stemphylium* species. The single isolate of *S. vesicarium* did not occur within the other clusters (Fig. 1B) suggesting that it may also represent a distinct taxon. The average levels of genetic similarity within and among the *Stemphylium* species are shown in Tab. 3.

Strong cross-hybridization of similar-sized RAPD products among the *Stemphylium* species was found. This homology indicated that the major RAPD fragments occurring in the *Stemphylium* isolates were likely to be the same genomic sequence. There was no cross-hybridization of any RAPD products from *Stemphylium* to *Pithomyces*. These results confirmed that RAPD products from *Stemphylium* species indicated genomic similarities, and that *Pithomyces* fragments were not similar to *Stemphylium* at the DNA sequence level.

Discussion

Genomic diversity detected by the RAPD analysis among and within *Stemphylium* species from alfalfa corroborated the morphology-based taxonomy (Gilchrist & Simmons, 1990; Simmons, 1985) by distinguishing at least four of the five *Stemphylium* species (Fig. 1). The single isolate of *S. vesicarium* studied may also represent a separate species. These results indicated that the species distinguished on morphological grounds are genetically distinct, and not merely morphological variants. No two species had more than 53.8% of fragments in common. RAPD products used in this study provided genetic markers that may be useful in species identification especially when combined with morphological comparisons. Genetic variation was also found between original and monoconidial isolates of *Stemphylium*, resulting in slightly different positions on the principal component graph. The same phenomenon has been found in



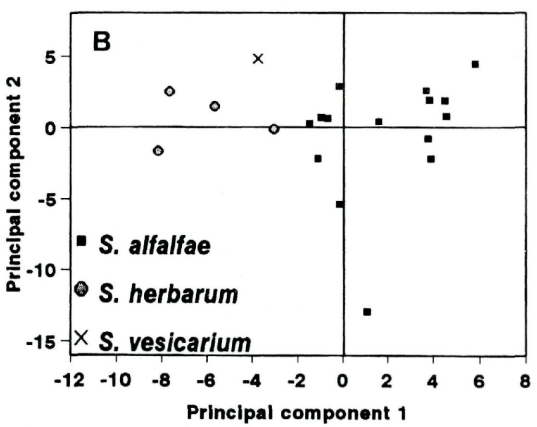


Fig. 1. – Graph of principal component analysis. – A. Analysis of RAPD fragment occurrence among five *Stemphylium* spp. pathogenic to alfalfa and two *Pithomyces* species. – B. Analysis of RAPD fragment occurrence among *S. alfalfae*, *S. herbarum* and *S. vesicarium*.

Tab. 3. – Average levels of genetic similarity among five species of *Stemphylium* pathogenic to alfalfa.

	Average similarity* to			
Species	*S. alfalfae*	*S. botryosum*	*S. globuliferum*	*S. herbarum*
S. alfalfae	76.2			
S. botryosum	11.6	80.1		
S. globuliferum	6.9	32.0	79.5	
S. herbarum	52.3	11.6	7.1	83.3
S. vesicarium	53.8	8.1	4.6	53.8

*Average percentage of RAPD fragments in common based on 205 fragments generated with 11 arbitrary 10-base primers.

the rice blast fungus, *Pyricularia oryzae* (teleomorph: *Magnaporthe grisea*) (Ou, 1985).

The confirmation of genetically distinct fungal taxa involved in Stemphylium leaf spot of alfalfa provides valuable insight into the potential variability in this disease. A survey of 22 *Stemphylium* isolates from across the United States revealed that at least two different species (*S. botryosum* and *S. alfalfae*) are widely distributed (Chaisrisook, 1993). Specific pathogenic ability among these *Stemphylium* species has not been investigated. The extensive variation revealed by RAPD markers, however, indicates substantial genomic differences. It seems unlikely that pathogenic ability would be identical among species, suggesting it may be advisable to evaluate resistance to each fungal species during plant breeding efforts.

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