Isolation, Characterization, and Distribution of a Biocontrol Fungus from Cysts of *Heterodera glycines*

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ABSTRACT

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Seventy-six populations of *Heterodera glycines* were collected from 33 counties in 10 states of the United States along the Mississippi and Missouri Rivers in 1992 and 1993. A sterile hyphomycete fungus of an unnamed taxon, designated ARF18 and shown to be a parasite of eggs of *H. glycines*, was isolated from eggs and cysts of 10 of the populations from Kentucky, Louisiana, Mississippi, and Tennessee. Ten isolates of ARF18 obtained in this study and seven isolates obtained in earlier studies were characterized for cultural morphology on several growth media, the ability to produce sclerotium-like structures (SLS) on cornmeal agar, growth rates, pathogenicity to eggs of *H. glycines* in vitro, and mitochondrial (mt) DNA restriction fragment length polymorphisms (RFLPs). All 17 isolates of ARF18 readily grew on potato dextrose agar, cornmeal agar, and nutrient agar. Based on colony morphology and SLS appearance on cornmeal agar, the isolates could be grouped into two morphological phenotypes. Isolates that produced SLS that were composed of a compact mass of hyphae were designated ARF18-C, whereas isolates that produced SLS

composed of a mass of loosely clumped hyphae were designated ARF18-L. Only minor differences in growth rates were detected among the ARF18-C and ARF18-L isolates. All 10 ARF18-C isolates, which were from Arkansas, Louisiana, Mississippi, and Tennessee, belonged to a single mtDNA RFLP haplotype. The seven ARF18-L isolates shared many comigrating mtDNA restriction fragments with one another, but belonged to three distinct mtDNA RFLP haplotypes. Ability to infect eggs of *H. glycines* in vitro varied considerably among the various isolates of ARF18. In particular, several of the ARF18-C isolates were consistently able to infect over 50% (mean = 70.0%, standard deviation = 16%) of the eggs of *H. glycines*, whereas ARF18-L infected eggs to a lesser degree (mean = 25%, standard deviation = 27%). ARF18-C was isolated only from *H. glycines* populations from below 37° N latitude. The presence of ARF18 was associated with soils with Mg levels <314 kg/ha, cyst numbers >4.5 per 100 cm³, and iron levels >203.5 kg/ha; or with Mg levels >314 kg/ha and Na levels <121 kg/ha. The widespread distribution of ARF18 and the ability of some isolates to aggressively colonize eggs of *H. glycines* are indications that it has potential as a biological control agent for *H. glycines*.

Additional keywords: races, soil factors, soybean cyst nematode.

The soybean cyst nematode (SCN) *Heterodera glycines* Ichinohe was first detected in the Mississippi River Valley in 1956 from Tennessee and Missouri (7,11) and subsequently has been observed in many other soybean-growing areas (21). In 1994, SCN infestations of *H. glycines* were found in more than 500 counties in 11 states along the Mississippi and Missouri Rivers (R. D. Riggs, unpublished data). Concerns about the availability and risk of chemical pesticides to control nematodes have increased the interest in alternative control strategies. Biological control with fungi that are parasitic on nematode eggs and females have the potential to replace control strategies that are no longer available (13,24,27).

An unnamed sterile hyphomycete fungus, designated ARF18, was isolated from nematode-suppressive soils in Arkansas in 1987 (15) and has shown promise as a biocontrol agent of *H. glycines* (18). ARF18 is unique among nematopathogenic fungi in that it penetrates directly through the cyst wall and kills eggs (19). Direct penetration is particularly important for suppression of cyst nematodes, because the cyst wall is a protective barrier for the eggs.

Although ARF18 was isolated in Arkansas in 1987, its distribution in other states and taxonomic identity remained unclear. ARF18 has resisted extensive efforts to induce sporulation and, thus, few characters are available for identification. Cultural and morphological characteristics of ARF18 include clear, septate hyphae (4.0 µm wide) with Woronin bodies and the formation of sclerotium-

like structures (SLS), more or less closely packed cells that were obviously different from typical filamentous hyphae (Fig. 1), on cornmeal agar (CMA) (14,15). DNA restriction fragment length polymorphisms (RFLPs) are being used increasingly to characterize fungi (1,5). These techniques can be particularly useful for characterizing genotypes of fungi of unknown or uncertain taxa.

The objectives of these studies were to determine the geographic distribution of ARF18 parasitizing cysts of *H. glycines* in the central United States; characterize isolates for growth, cultural characteristics on various media, pathogenicity, and mitochondrial (mt) DNA RFLPs; and characterize the properties of soils from fields where ARF18 was isolated.

MATERIALS AND METHODS

Soil sampling and nematode extraction. Soil samples were collected from soybean fields in the central United States from Louisiana to Minnesota during the fall of 1992 and 1993 (Fig. 2). Only fields infested with *H. glycines*, determined through the use of a portable cyst extractor (17), were sampled. To determine presence or absence of ARF18, approximately 5 kg of soil was collected to a depth of 15 cm after removing the uppermost layer of plant debris. The samples were stored in plastic bags at 4°C and were processed within a month. Each soil sample was divided into four parts: 1 kg of soil was used to determine the number of eggs of *H. glycines* in the soil by the sieving and centrifugation-flotation method (25); 100 g of soil was sent to the Arkansas Soil Testing Laboratory (University of Arkansas, Fayetteville) for chemical analyses; 200 g of soil was used in the particle analysis in the

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Soils Laboratory at the University of Arkansas-Fayetteville; and the remainder was used for isolation of the fungus from cysts.

The race of each nematode population was determined by transplanting three seedlings of soybean cultivar Lee 74, susceptible to all races of *H. glycines*, and three seedlings of each of the race differentials, 'Pickett', 'Peking', 'PI 88788', and 'PI 90763', into soil infested with each of the populations separately. After 30 days, cysts and females were extracted and counted. Race was determined based on the number of females and cysts on each differential cultivar relative to the number of females and cysts on 'Lee 74' (22).

Isolation of ARF18. A sample of soil from each of the 76 collections was processed to extract the cysts of *H. glycines*. About 30 cysts were selected from each population of *H. glycines* for isolation of ARF18. The cysts collected directly from field soil

were typically in poor condition, contained few eggs, and yielded predominantly common saprophytic fungi.

Because few parasites were isolated from cysts taken directly from field soil, isolations were performed on greenhouse-produced cysts as follows. About 250 cm³ of soil from each site was placed in a 15-cm-diameter clay pot in a greenhouse, and two seedlings of 'Lee 74' were transplanted into each pot. After 2 to 4 months, females and cysts of *H. glycines* were extracted by sieving and centrifugation-flotation. Thirty cysts were picked individually from the soil/root extract from each pot. Cysts were selected that had symptoms or signs of infection by ARF18 including black or pink discoloration, and mycelium growing from the anterior or posterior openings; or smaller than normal size and had pale yellow or grayish coloration with clumps of mycelium visible (15,16).

Fig. 1. Sclerotium-like structures (SLS) formed on cornmeal agar (CMA). **A and B**, ARF18-C: mycelia on the surface of SLS appear compact. Structure in **A** is a higher magnification of the boxed area in **B**. **C and D**, ARF18-L: mycelia on the surface of SLS appear loose. Structure in **C** is a higher magnification of the boxed area in **D**. Scale bar: **A and C** = 25 µm; **B** = 100 µm; and **D** = 50 µm.

To isolate fungi from eggs, cysts were surface-disinfested with 0.5% NaOCl (The Chlorox Co., Oakland, CA) for 10 min, washed in sterile water, and the cyst wall broken to release the eggs. The eggs were then surface-disinfested with 0.5% NaOCl for 5 min, washed twice with sterile water, and spread evenly on the surface of 1.5% water agar. The cultures were incubated at $21 \pm 2^\circ\text{C}$ for 7 days and inspected daily (16). Fungal colonies that grew from an egg were transferred to CMA to establish pure cultures.

Growth and colony morphology of isolates of ARF18. To measure growth rate, a 4-mm-diameter plug cut from an actively growing colony of each of the test isolates was transferred to the center of a petri plate (100×15 mm) containing potato dextrose agar (PDA) (Difco Laboratories, Detroit), CMA (Difco Laboratories), or nutrient agar (NA) (Difco Laboratories). The cultures were incubated in the dark at $22 \pm 2^\circ\text{C}$, and the colony size was measured with a metric ruler after marking the edges on the petri dish every 3 days for 18 days. Three replicate plates arranged in a randomized complete block design were used, and the experiment was repeated once. Production of SLS on CMA was observed for a period of 90 days and their characteristics described including their diameter measurements and numbers produced.

Pathogenicity of ARF18 isolates. Pathogenicity of the fungal isolates to eggs of *H. glycines* was determined by growing each isolate on PDA, CMA, and NA. A 4-mm-diameter plug cut from an actively growing colony of each medium was transferred colony-side-up to the center of a 60×15 -mm petri plate containing 1.5% water agar. Five freshly extracted cysts of *H. glycines* race 3 similar in size and color were surface-disinfested (16) and placed directly on top of each inoculum plug. The plates were placed in a plastic bag to prevent drying and incubated in the dark at $22 \pm 2^\circ\text{C}$ for 10 days. Two plates were used per isolate per medium and arranged

in a randomized complete block design; the test was repeated for a total of 20 cysts per isolate per medium. Cysts placed on a plug of 1.5% water agar, a total of 10 cysts per isolate per medium, served as controls. Cysts used in these studies each contained about 350 eggs and a few second-stage juveniles (J2). After 10 days, each cyst was collected from the mycelial mat and placed in a drop of modified lactoglycerol solution (lactic acid, 85%; glycerol, 99.5%; and distilled water [2:2:1, vol/vol]) on a glass slide (16) and crushed to release the eggs. The difference in permeability of the shell of asymptomatic and symptomatic eggs to the stain facilitated detection of colonized eggs. Symptomatic eggs quickly became clear (in 1 min), whereas asymptomatic eggs remained unchanged for >5 min (16). Apparently, the lactoglycerol is able to penetrate the egg shell of infected eggs more rapidly than uninfected eggs. Not all symptomatic eggs were infected. Dead eggs also appear clear in lactophenol and were also rated as symptomatic. The number of symptomatic and asymptomatic eggs and juveniles in each cyst were counted at $\times 30$ magnification using a stereoscopic microscope. Percentage of eggs parasitized was based on the total number of eggs plus juveniles.

mtDNA RFLP analysis. Seventeen isolates were examined for mtDNA RFLPs. Ten of the isolates examined were collected as part of this study and seven isolates were collected previously (15). Mycelium was produced in liquid shake culture using pea juice medium (juice of canned sweet pea/water [1:3, vol/vol]) for 10 days at $22 \pm 2^\circ\text{C}$ under ambient light. Total DNA from mycelium of each isolate was extracted using a 'mini-prep' procedure, whereby lyophilized mycelium was ground in liquid nitrogen, extracted with chloroform and phenol, precipitated, and redissolved in buffer (6). Total DNA was digested with one of three restriction enzymes *Hae*III, *Eco*RI, or *Msp*I (New England Biolabs, Inc., Beverly, MA)

Fig. 2. Sampling area (Δ) and incidence of ARF18-C ($\blacksquare$) and ARF18-L ($\blacktriangle$) in the central United States. ARF18-C designates compact sclerotium-like structures (SLS) and ARF18-L designates loose SLS. Arabic numerals with ($\rightarrow$) indicate strain number: 1, Washington County, AR; 2, Lee County, AR; 3, McCracken County, KY; 4, Avoyelles Parish County, LA; 5, Tate County, MS; 6, Desoto County, MS; 7, Grenada County, MS; 8, Lake County, TN; 9, Lake County, TN; 10, Lauderdale County, TN; 11, Lee County, AR; 12, Pope County, AR; 13, Pope County, AR; 14, Johnson County, AR; 15, Washington County, NC; 16, Dyers County, TN; and 17, Lauderdale County, TN.

according to the manufacturer's recommendations. DNA fragments were separated electrophoretically on 0.7% agarose gels in 0.5× Tris-borate-EDTA buffer. The blots were then probed with two mtDNA clones (2μ18 and 4μ40) obtained from *Colletotrichum orbiculare* (6). An enhanced chemiluminescence DNA labeling kit (ECL; Amersham Corp., Arlington Height, IL) was used to label the mtDNA clones. Isolates were then assigned a mtDNA haplotype by visually examining the resulting mtDNA RFLP pattern.

Soil analysis. The possible association of soil factors with the presence of ARF18 was investigated by a comparison of the chemical and physical factors of soil samples with and without ARF18. To determine the association of soil factors to the presence of the fungus, logistic regression analysis was applied to all 76 samples or to only the 48 samples collected south of 37° N latitude. SAS PROC LOGISTIC is specifically designed to fit linear logistic regression models for the categorical variable by the method of maximum likelihood. Similar tests have been used successfully for the study of soil fungal populations (10,13). Finally, the Classification Tree procedure was used as an additional test for associations. This procedure produces a classification tree showing the particular characteristics and levels that were selected from among the chemical and physical soil sample measurements for predicting presence or absence of ARF18 (23).

RESULTS

Nematode extraction. Populations of *H. glycines* were extracted from 76 soil collections from 33 counties and parishes in 10 states along the Mississippi and Missouri Rivers (Fig. 2). Each *H. glycines* population from Illinois (IL), Iowa (IA), Kansas (KS), Minnesota (MN), Missouri (MO), or Nebraska (NE) was either race 3 or race 1, but most were race 3. Populations from the southern states of Kentucky (KY), Louisiana (LA), Mississippi (MS), and Tennessee (TN) were characterized as race 2, 3, 4, 5, 6, 9, or 14, but only one population was race 3.

Isolation of ARF18 from cysts. When surface-disinfested eggs were placed on water agar, fungal isolates resembling ARF18 grew from infected eggs within 5 days. ARF18 was isolated from 10 of the 76 *H. glycines* populations (Table 1, Fig. 2). The 10 *H. glycines* populations were identified as either race 5 (TN), 6 (KY and MS), 9 (LA and MS), or 14 (MS). However, of the >3,000 eggs from each population that were placed on water agar, ARF18 grew from <50 eggs from each of the 10 populations. All locations from

which ARF18 was isolated were south of 37° N latitude (Fig. 2). A number of other fungi grew from eggs during the isolation procedure, but most were recognized as commonly occurring saprophytes.

Growth and colony morphology of ARF18. The 10 isolates of ARF18 obtained in this survey and the 7 isolates previously obtained grew well on PDA, CMA, and NA. Minor but significant (*P* = 0.01) differences in growth among media were observed. Average growth was greatest on CMA (3.0 to 3.5 cm after 18 days), followed by PDA and NA.

All 17 isolates of ARF18 produced SLS on CMA. SLS averaged 0.20 (range 0.05 to 1.00) mm in diameter and numbered as many as 350 per cm². Among the 17 isolates examined, two morphological SLS phenotypes were observed on CMA (Table 2). SLS of one type, designated ARF18-C, consisted of compact interwoven mycelium that resembled parenchyma tissue of plants (Fig. 1A and B). The distribution of SLS on CMA among the ARF18-C isolates was apparently random over the surface of the medium. In contrast, SLS of a second type, designated ARF18-L, consisted of loosely interwoven mycelium (Fig. 1C and D). Among isolates with the ARF18-L phenotype, the SLS on CMA formed in radial striations on the surface of the medium. The ARF18-C and ARF18-L SLS phenotypes correspond to ARF18-S (smooth) and ARF18-R (rough), respectively, which previously had been described (15). The new acronyms more accurately depict the appearance of the SLS.

Pathogenicity tests. Control plates had 96% asymptomatic and 4% symptomatic eggs and J2 (Table 3). Dead eggs not infected by ARF18 appeared clear when exposed to lactophenol and were rated as symptomatic. For all three media and two experiments, cysts on plates with inoculum averaged 34% asymptomatic eggs and 66% parasitized eggs for the 10 ARF18-C isolates (Table 3). Asymptomatic eggs, parasitized eggs, and J2 for both experiments averaged 5, 71, and 24% on CMA; 6, 74, and 20% on PDA; and 8, 64, and 28% on NA, respectively. The seven ARF18-L isolates averaged less infection of SCN eggs than did ARF18-C isolates. With ARF18-L isolates, asymptomatic eggs, parasitized eggs, and J2 averaged 78, 14, and 8% on CMA; 47, 40, and 13% on PDA; and 76, 13, and 10% on NA, respectively.

Data from the two experiments were analyzed separately, and significant differences in colonization frequencies (*P* = 0.01) were found among media, among isolates, and in the interaction between

TABLE 1. Fields sampled, nematode population levels, and numbers of cysts and eggs tested for ARF18 in a survey of soybean fields in the central United States infested with *Heterodera glycines*

Field site ^a	No. of units from field sample ^b		No. of units from pots ^c		<i>H. glycines</i> race
	Cysts	Eggs	Cysts	Eggs	
KY-1	22	2,910	30	>3,000	6
LA-5	17	4,104	30	>3,000	9
MS-1	10	2,520	30	>3,000	9
MS-3	5	312	30	>3,000	14
MS-5	19	1,848	30	>3,000	6
MS-8	7	888	30	>3,000	6
TN-1	41	4,272	30	>3,000	5
TN-10	10	864	30	>3,000	5
TN-12	26	1,944	30	>3,000	6
TN-14	4	576	30	>3,000	6

^a Fields from which ARF18 was isolated. Cysts from 76 fields were examined for presence of the fungus. The letter and number combinations are field designations and ARF18 isolate designations. KY = Kentucky, LA = Louisiana, MS = Mississippi, and TN = Tennessee.

^b Cysts or eggs (not second-stage juveniles) per 100 cm³ of soil.

^c Nematodes were cultured in a greenhouse for 2 to 4 months before isolations were made. Thirty symptomatic cysts that contained >3,000 eggs were hand-picked for isolation of fungi. Clay pots were 15 cm in diameter.

TABLE 2. Isolates of ARF18, their geographic origin, type of sclerotium-like structure, and mitochondrial (mt) DNA restriction fragment length polymorphism haplotype

ARF18 isolate ^a	Geographical origin	SLS type ^b	mtDNA haplotype ^c
BG2	Arkansas	C	I
903	Arkansas	C	I
KY-1	Kentucky	C	I
LA-5	Louisiana	C	I
MS-1	Mississippi	C	I
MS-3	Mississippi	C	I
MS-5	Mississippi	C	I
MS-8	Mississippi	C	I
TN-1	Tennessee	C	I
TN-12	Tennessee	C	I
887	Arkansas	L	II
907	Arkansas	L	II
908	Arkansas	L	II
Johnson	Arkansas	L	II
NC-15	North Carolina	L	III
TN-10	Tennessee	L	IV
TN-14	Tennessee	L	IV

^a Isolates from Arkansas and North Carolina are original isolates; those from other states are from this study.

^b SLS are sclerotium-like structures that are produced by ARF18 on cornmeal agar. They are compact (ARF18-C) or loose (ARF18-L) in appearance.

^c A mtDNA haplotype is a fungal isolate (in this case) that displays a unique combination of comigrating mtDNA restriction fragments when its mtDNA is digested with a specific set of enzymes.

media and isolates (Table 4). Pathogenicity of eggs by ARF18-C isolates was significantly higher than by ARF18-L isolates in both experiments (Table 4). The estimated difference between the parasitism by ARF18-C isolates and ARF18-L isolates was 48% (± 1.4 standard error), which was statistically significant ($P = 0.01$). The estimated difference between the parasitism of *H. glycines* eggs by isolates collected during the survey and the original isolates was 29.6% (± 1.4 standard error), which was not significant.

mtDNA RFLP analysis. Four mtDNA haplotypes were observed among the 17 ARF18 isolates when genomic DNA was digested with *Hae*III, *Msp*I, and *Eco*RI, and probed with two mtDNA clones (Fig. 3). No polymorphisms were detected among the 10 ARF18-C isolates, and isolates were assigned a single haplotype (haplotype I) (Table 2). Three mtDNA RFLP haplotypes (designated II, III, and IV) were observed among the seven ARF18-L isolates with each of the three enzymes used (Fig. 3, Table 2). The seven ARF18-L isolates in haplotypes II, III, and IV shared many comigrating mtDNA fragments with one another, but shared few comigrating fragments with the ARF18-C isolates in haplotype I.

Soil relationship. The biological, chemical, and physical factors varied greatly among samples (Table 5). The soil types were silt loam (41%), silty clay loam (24%), loam (13%), clay (5%), silt (5%), silty clay (4%), sandy loam (4%), clay loam (3%), and loamy sand (1%). ARF18 was found only below 37° N latitude and soil factors differed significantly between northern and southern states, but soil factor data from all samples were used in the statistical analysis.

SAS PROC LOGISTIC with the stepwise option selected Mg and P as the predictive factors. Tree-based models provide an alternative to linear regression for classification problems (23). The tree-based model fitted to all soil collections indicated that soils containing ARF18 could be classified 93.7% of the time. ARF18 was predicted to be present when the Mg level was <314 kg/ha, cyst number was >4.5/100 cm³, and Fe level was >203.5 kg/ha; or when the Mg level was >314 kg/ha and Na level was <121 kg/ha (Fig. 4).

DISCUSSION

A number of sterile fungi have been isolated from nematode cysts and eggs (2,3,4,8,9,20,26,27) including a sterile hyphomycete fungus of an unknown taxon previously designated as ARF18

(15). ARF18 has been shown to have biological control potential for *H. glycines* (15), but its identity, distribution, and overall biology have not been examined in detail. Furthermore, little is known about pathogenicity or aggressiveness among isolates of ARF18 with regard to infecting cysts of *H. glycines*.

The 17 isolates used in the current study were obtained from various geographical locations from symptomatic cysts of *H. glycines* and putatively identified as ARF18. The isolate from Lee County, AR (Fig. 2), was the first ARF18 to be recovered. ARF18 could be isolated from surface-disinfested symptomatic eggs or J2 of *H. glycines*, and common characteristics include growth on standard media at a rate of 1.7 to 1.9 mm/day, production of sterile septate mycelia with Woronin bodies, production of light brown colonies on PDA, and production of SLS on CMA after 18 days (14). SLS were useful as a diagnostic feature of ARF18. Based on the appearance of the SLS, isolates could be divided into two phenotypes. Those isolates that produced SLS with compact interwoven hyphae were designated ARF18-C, whereas those that produced SLS with loosely interwoven hyphae were designated ARF18-L. In addition, the ARF18-C isolates formed SLS randomly throughout the CMA plates, whereas ARF18-L formed SLS in radial striations on CMA plates.

Analysis of mtDNA RFLPs indicated that there were two distinct groups, which corresponded with the two SLS phenotypes. No

TABLE 4. Analysis of variance of the egg pathogenicity test with 17 isolates of ARF18

Source of variation	Experiment 1		Experiment 2	
	df	Mean square	df	Mean square
Isolates	16	332,109**a	16	262,474**a
C vs. L ^b	1	284,973**	1	215,545**
O vs. S ^c	1	107,979	1	58,363
Media	2	43,526**	2	23,744**
Media $\times$ isolate	32	88,186**	32	38,023**
Error	459	255	459	244
Coefficient of variation (%)		28.8		33.0

a ** Represents significance at $P = 0.01$.
b C = compact (mycelia forming a compact mass) and L = loose (mycelia forming a loose aggregation) sclerotium-like structure.
c O = original isolates obtained before this survey and S = isolates obtained during this survey.

TABLE 3. Percentage of eggs of *Heterodera glycines* infected by isolates of the nematophagous fungus ARF18-C in vitro

Isolate	Experiment 1				Experiment 2			
	CMA ^a	NA ^a	PDA ^a	Mean	CMA	NA	PDA	Mean
Control	5 ^b	5	6	6	2	3	3	2
BG2	68	72	89	77	71	70	74	72
903	70	60	75	68	65	47	73	62
KY-1	78	79	60	72	67	59	64	63
LA-5	78	82	84	82	54	51	58	55
MS-1	93	92	81	89	75	67	79	75
MS-3	64	38	83	62	62	60	69	64
MS-5	77	70	78	75	58	51	73	60
MS-8	80	72	71	74	70	61	66	66
TN-1	84	55	88	76	77	81	60	73
TN-10	69	67	63	67	59	51	64	58
887	5	7	11	8	1	3	2	2
907	4	6	76	29	1	1	45	16
908	15	2	82	33	46	28	68	47
Johnson	21	6	39	22	28	26	25	26
NC-15	3	23	62	29	5	0	47	17
TN-12	9	35	16	20	29	1	31	20
TN-14	28	46	73	49	49	21	14	46
Mean	47	43	59	52	44	37	53	46
Waller ^c	12	11	13		13	9	12	

a CMA = cornmeal agar, NA = nutrient agar, and PDA = potato dextrose agar.
b Values are means of 10 replications. Because of the interaction between isolates and media, differences in numbers of eggs infected are not reported.
c Waller-Duncan k ratio t test, $k = 100$, $P = 0.05$ was used to calculate the minimum difference in parasitism necessary for a significant difference between isolates within a column.

Fig. 3. Mitochondrial (mt) DNA restriction fragment length polymorphisms (RFLPs) among isolates of ARF18. A Southern blot of total DNA was digested with **A**, *Hae*III; **B**, *Msp*I; or **C**, *Eco*RI and probed with two mtDNA probes from *Colletotrichum orbiculare*. Lanes 1 and 19 contain lambda DNA digested with *Hind*III as a size marker in kilobases. Lanes 2 through 18 are isolates BG2, 903, KY-1, LA-5, MS-1, MS-3, MS-5, MS-8, TN-1, and TN-12 (all ARF18-C), and 887, 907, 908, Johnson, NC-15, TN-10, and TN-14 (all ARF18-L).

polymorphisms were detected in the mtDNA among the 10 ARF18-C isolates, which were obtained from Arkansas, Kentucky, Louisiana, Mississippi, and Tennessee, when they were digested with three different endonucleases. These data indicate that isolates identified as ARF18-C represent a single mtDNA RFLP haplotype and apparently represent a single, though unidentified, taxon with a widespread distribution in several states in the southern soybean-growing region of the United States. The seven ARF18-L isolates shared many comigrating fragments, but belonged to three additional haplotypes. Four isolates from Arkansas, one from North Carolina, and two from Tennessee represented mtDNA RFLP haplotypes II, III, and IV, respectively. The ARF18-C and ARF18-L isolates were distinct and shared few comigrating mtDNA fragments.

mtDNA has been shown to be very useful in characterizing genetic diversity within and between fungal species. In some fungi, a considerable degree of mtDNA RFLP diversity occurs among isolates within a species, whereas for some taxa, few or no mtDNA RFLPs exist among isolates within a species (1,5). Although mtDNA RFLPs cannot be used to identify ARF18 to a specific taxon, they appear to be very useful for characterizing genetic diversity. Moreover, the genetic diversity identified by the mtDNA haplotypes

TABLE 5. Means (ranges) of biological, chemical, and physical factor measurements in soils from four southern states where ARF18 was present or absent

Factors ^a	ARF18 present	ARF18 absent
J2	108.8 (24–302)	80.1 (0–312)
Eggs	23.4 (3–43)	25.7 (0–195)
pH	6.8 (6–8)	6.4 (5–8)
% organic matter	1.2 (1–2)	1.1 (1–2)
K	323.1 (98–742)	296.3 (104–926)
Ca	3,952.1 (1,705–7,313)	3,884.7 (1,230–11,208)
Na	149.0 (96–254)	170.0 (96–456)
Mg	348.6 (186–755)	544.7 (95–1,917)
Fe	314.3 (121–549)	265.0 (121–590)
Mn	176.4 (66–383)	148.1 (24–376)
Cu	4.4 (2–7)	4.5 (2–10)
Zn	14.9 (3–59)	6.7 (2–16)
P	153.1 (45–537)	75.2 (27–200)
CEC	13.0 (9–19)	14.3 (7–31)
Sand %	3.8 (1–13)	7.4 (1–62)
Silt %	72.6 (62–80)	64.9 (27–88)
Clay %	23.5 (17–28)	28.1 (8–71)

^a J2 = number of second-stage juveniles per 100 g of soil; Eggs = number per g of soil; Mineral quantities are extractable kg/ha; CEC = cation exchange capacity in meq/100 g of soil.

Fig. 4. Classification tree of the relationship between the soil factors (Table 4) measured and the occurrence of the biocontrol fungus ARF18. In the tree diagram, the point of connection of a vertical line with a horizontal line marks the level of the factor named; to the right of that point is more than that level, and to the left is less than that level. This Classification Tree analysis correctly selected soil samples containing ARF18 >90% of the time.

appears to correspond with features of the morphology and general life cycle of this organism. For example, the phenotypes of the SLS correspond with the mtDNA haplotypes identified. Also, isolates identified as ARF18-C more readily infected eggs of *H. glycines* than did isolates of ARF18-L. These data indicate that, regardless of the taxonomic identities of ARF18-C and ARF18-L, fundamental differences occur in their biology and ecology. mtDNA RFLPs could serve as useful neutral markers to establish reference isolates for comparison in future work with this organism.

The southern distribution of ARF18 could be due to many factors including the longer history of *H. glycines* in this region, the differences in soybean cultivars grown, or generally warmer soil temperatures. *H. glycines* was first found in Tennessee in 1956; Arkansas, Kentucky, and Mississippi in 1957; and Louisiana in 1967. Although *H. glycines* has been known to occur in Missouri since 1956 and Illinois since 1959, it was found more recently in Iowa (1978), Kansas (1985), and Minnesota (1979). Because ARF18 is a parasite of *H. glycines* and would likely be associated with the occurrence of the nematode, the longer history of *H. glycines* in the southern states would favor the more widespread occurrence of the fungus in that area. A more extensive survey may be necessary to determine the precise distribution of the fungus.

Although statistically significant, variation in virulence among the 10 isolates of ARF18-C was relatively small. The percentage of eggs parasitized among 10 isolates of ARF18-C and 7 isolates of ARF18-L was 64 to 82% and 14 to 40%, respectively. In contrast, six isolates of *Verticillium chlamydosporium* on *H. avenae* and *H. schachtii* showed a wider variation in virulence among isolates (12). The percentage of eggs parasitized by *V. chlamydosporium* varied between 3 to 63% on *H. avenae* and 3 to 41% on *H. schachtii* (12).

The influence of soil factors on nematophagous fungi is not well understood. Previous studies have shown that the presence of conidia-forming endoparasites was influenced by high levels of organic matter in the soil, but the presence of predatory fungi was influenced more by pH and moisture (10). However, in the current study, no correlation was found between organic matter or pH and the presence of ARF18. ARF18 is a female and egg parasite that grows well on artificial media and may grow well on organic matter in the soil. The logistic regression analyses varied in their predictive probability values and that may indicate that the soil factors were not suitable for predicting the presence of ARF18 in a particular soil, at least in the current survey. The STEPWISE procedure is reported to be effective in selecting predictive factors. The best model for selection of soils where ARF18 is present is the Classification Tree in which, with only four variables, it correctly classified the presence of ARF18 >90% of the time. The misclassification rate was only 6.7%. The mean Mg level in samples from which ARF18 was isolated was significantly ($P = 0.05$) different from the mean level in samples from which ARF18 was not isolated; the same was true for Fe and Na levels. The significance of the differences may indicate that a relationship between the levels of these elements in the soil and the occurrence of ARF18 may actually exist. However, this survey was very limited and the model is being tested with additional samples.

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