CANONICAL DISCRIMINANT ANALYSIS OF *ROTYLENCHULUS RENIFORMIS* IN ALABAMA

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ABSTRACT

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The reniform nematode, *Rotylenchulus reniformis*, infests over 300 plant species worldwide and over the last two decades it has emerged as a major cotton pest in the southeastern United States. Nine locations in Alabama and one location in Mississippi were selected for study of *R. reniformis*. Thirteen morphometric measurements were made on 20 male and 20 female nematodes from each population. The sex and location interaction was significant ($P < 0.005$) for all traits except total body length ($P = 0.29$) and the derived ratio trait a (body length / maximum body width, $P = 0.06$). Canonical discriminant analysis effectively separated the 10 sampling locations into three distinct groups; among them, Group 1 and 3 were distinct with an intermediate group (Group 2) differentiating in the middle. Furthermore, both female and male *R. reniformis* based on the morphometrics measured here adhered to this metrics-based grouping. Belle Mina (Limestone County, AL), Huxford (Escambia County, AL), and Mississippi State University, MSU (Oktibbeha County, MS) locations were separated from the remaining seven locations based on the large positive CAN1 centroid means. Eight out of 13 traits had high phenotypic correlations ($r > 0.80$) with CAN 1 for both sexes. Anal width and length of the hyaline portion of the tail measurement accounted for a significant amount ($r > 80\%$) of the variation in total and sex-based canonical structure. Occurrence of the three non-overlapping morphometric groups in cotton-growing fields in close proximity (250 mile radius) suggests a greater biological variation in this species than expected. Cotton cultivars with differential resistance and soil types are among the major factors to be tested for further delineating the causes of morphometric variation in *R. reniformis*.

Key words: Reniform Nematode, Morphological Variation, Canonical Discriminant Analysis, Mahalonobis distance

RESUMEN

Nyaku, S. T, R. V. Kantety, K. S. Lawrence, E. van Santen and G. C. Sharma. 2013. Análisis discriminante canónico de *Rotylenchulus reniformis* en Alabama. Nematropica 43:171-181.

El nematodo reniforme, *Rotylenchulus reniformis*, parasita más de 300 especies de plantas en todo el mundo y durante las dos últimas décadas se ha convertido en una de las principales plagas del algodón en el sureste de Estados Unidos. Para este estudio con *R. reniformis* fueron seleccionadas nueve localidades en Alabama y una en Mississippi. Trece medidas morfométricas fueron tomadas en 20 machos y 20 hembras de cada población. La interacción con el sexo y la ubicación fue significativa ($P < 0.005$) para todos los caracteres, excepto para la longitud total del cuerpo ($P = 0.29$) y su derivado índice a (longitud total del cuerpo/ancho máximo del cuerpo, $P = 0.06$). El análisis discriminante canónico separó efectivamente los 10 lugares de muestreo en tres grupos; el Grupo 1 y 3 fueron diferentes, con un grupo intermedio (Grupo 2). Además, tanto hembras como machos de *R. reniformis* se adhirieron a este agrupamiento basado en mediciones. Las localidades Belle Mina (Limestone County, AL), Huxford (Escambia County, AL) y Mississippi State University, MSU (Oktibbeha County, MS) fueron separadas de las 7 localidades restantes en base a los centroides en el eje positivo CAN1. Ocho de los 13 caracteres tuvieron alta correlación fenotípica ($r > 0.80$) con CAN 1 en ambos sexos. El ancho del ano y la longitud de la porción hialina de la cola representaron una cantidad significativa ($r > 80\%$) de la variación en la estructura canónica total y basada en el sexo. La existencia de tres grupos morfométricos que no se superponen en campos de algodón muy próximos (250 millas de radio) sugiere una variación biológica mayor a la esperada en esta especie. Los cultivares de algodón con resistencia diferencial y los tipos de suelo están entre los principales factores para ser analizados a fin de evaluar las causas de la variación morfométrica de *R. reniformis*.

Palabras clave: nematodo reniforme, variación morfológica, análisis discriminante canónico, distancia de Mahalonobis.

INTRODUCTION

The reniform nematode, *Rotylenchulus reniformis* Linford and Oliveira 1940, is distributed widely in tropical and subtropical regions of the world, and is found throughout the southern U.S. (Heald and Robinson, 1990; Kinloch and Sprenkel, 1994). This devastating pest has a wide host range that includes cotton and a broad range of vegetable and field crops (Robinson *et al*., 1997). This nematode is considered an important pest in upland cotton (*Gossypium hirsutum* L.), especially in the southeastern United States (Davis *et al*., 2003; Koenning *et al*., 2004). Cotton yields are greatly affected by *R. reniformis* (RN) damage in Alabama (Gazaway *et al*., 2001), Louisiana (Overstreet, 1999), and Mississippi (Lawrence and McLean, 1999) and nine other states (Georgia, Florida, Texas, South Carolina, North Carolina, Arkansas) (Heald and Robinson, 1990), Missouri (Wrather *et al*., 1992), Tennessee (Newman, 2005), and Virginia (Eisenback and Hopkins, 2004). This nematode was first discovered in cotton fields in east-central region of Alabama in 1958 (Minton and Hopper, 1959), and was identified as a serious pest of cotton in 1986 (Gazaway and McLean, 2003). The RN is spread from farm to farm through soil particles on farm implements and vehicles, and can survive for more than two years in stored soil (Lawrence *et al*., 2005). Nematode populations in Alabama are well established on croplands because over 90% of cotton is monocultured (Gazaway and McLean, 2003). Cotton yield loss due to RN usually range from 10% to 25% but may be as high as 50% in heavily infested and drought-stressed fields (Gazaway *et al*., 2001; Kirkpatrick and Robbins 1998).

While increasing cotton yield is of importance (Ribera and Landivar, 1999), all upland cotton cultivars presently being marketed are susceptible to RN (Weaver *et al*., 2007).

Traditional management of plant-parasitic nematodes has been mainly through crop rotation, nematicides, and host resistance. Common resistance strategies can be applied to minimize populations of RN if these pests are found to be homogeneously spread in a field. However, if these populations are heterogeneous, then divergent host-plant resistance strategies for RN management may be required; hence more specific studies will need to be developed. Nematode management in cotton is through non-host crop rotations and the use of chemical nematicides, among these are abamectin (Avicta), oxamyl (Vydate), thiodicarb (Aeris), and 1,3dichloropropene (Telone). Other nematicides are applied as seed treatments such as abamectin and thiodicarb and these are effective in improving yield increases in cotton (Lawrence and Lawrence, 2007). New investigations into relationships existing between environmental variables (e.g., water and temperature) and their response to nematicides for yield improvements are underway (Wheeler *et al*., 2013).

Nematicides are being eliminated due to health and environmental issues (Noling and Becker, 1994), despite positive impacts of nematicides on cotton yields. This concern therefore makes host resistance the most efficient and economical method of RN control (Robinson *et al*., 1999; Robinson *et al*., 2007). There have been increased efforts in recent years to develop commercial upland cotton cultivars with resistance to RN (Cook and Robinson, 2005; Robinson *et al*., 2007; Weaver *et al*., 2007). Plant breeders introgress this resistance from the wild relatives into the cultivated species, a time-consuming process. In upland cotton, *Gossypium hirsutum* (2n=52), there has been some success in this regard, through introgression of RN resistance from *G. longicalyx* (Robinson *et al*., 2007, Dighe *et al*., 2009), and from *G. arboreum* and a *G. hirsutum/G. aridum* bridging line (Sacks and Robinson, 2009). Due to inefficiencies in chromosome paring, interspecific crossing among related plant species is always not successful, and therefore limits the transfer of agronomically important traits among species (Beasly, 1940, 1942). Novel and specific strategies are thus needed for controlling RN.

Our objectives were to i) Identify and measure the variation in morphological attributes of female and male RN, ii) Locate the most useful morphometric characters in discriminating among the populations through Canonical Discriminate Analysis.

MATERIALS AND METHODS

Soil Sample Collection, Extraction and Establishment of RN Populations

Samples of RN-infested soil were obtained from nine cotton farms located in four counties in Alabama and one location in Mississippi (Table 1). Each sample was thoroughly mixed and a 150 cm3 subsample was used for the extraction, identification, and quantification of the nematodes. The infested soil was placed in a bucket of running water until the soil was covered by at least two times its volume. The solution was then mixed vigorously until the soil was sufficiently dispersed and then allowed to settle for 3 min. The liquid supernatant was then poured through an 841 µm sieve nested onto a 44 µm sieve. The 44 µm sieve with was washed thoroughly with water until as much clay and other fine particles were washed out of the sieve. The remaining sample with the nematodes was then washed into a 250 ml beaker and allowed to settle for 5 min, afterwards the solution was transferred into a 50 ml centrifuge tube. Centrifugation was carried out at 1,000 x g for 3 min in a Marathon 21 k benchtop centrifuge (Fisher Scientific, Suwanee, GA). The supernatant was discarded and if necessary, successive samples centrifuged until a final pellet obtained from the collective population of nematodes. Two mL of OptiprepTM (Axis-Shield PoS AS, Oslo, Norway) solution was gently added to a 15 mL

Table 1. GIS information for one Mississippi (MS) site and nine Alabama (AL) sites that were sampled for the morphometric analysis of female and male reniform nematodes on cotton farms. Locations are listed in descending order of the CAN 1 centroid mean for females.

Group	Abbreviation	Location	County / State	Latitude	Longitude	Infested since*
I	M	MSU	Oktibbeha, MS	88.78 W	33.48 N	Early 1980s
I	B	Belle Mina	Limestone, AL	86.89 W	34.66 N	Early 1990s
I	X	Huxford	Escambia, AL	87.46 W	31.22 N	Early 1980s
II	S	Shaw	Limestone, AL	86.94 W	34.64 N	Early 1990s
II	R	Murphy	Limestone, AL	86.75 W	34.59 N	Late 1980s
II	L	Lamons	Lawrence, AL	87.12 W	34.63 N	Early 1980s
III	H	Hargrave	Limestone, AL	86.85 W	34.62 N	Late 1980s
III	A	Hamilton	Lawrence, AL	87.18 W	34.61 N	Early 1980s
III	T	Thornton	Lawrence, AL	87.37 W	34.73 N	Early 1980s
III	W	Whitehead	Fayette, AL	87.73 W	33.84 N	Late 1980s

*Personal communication with farmers

Table 2. Phenotypic correlation between original response variables and canonical variates (between canonical structure), Eigenvalues, and percent total variance accounted for by the first two canonical variates for female and male reniform nematodes collected from nine locations in Alabama and one location in Mississippi. The discriminant analysis was performed separately for females and males.

	Females		Males	
Morphometric and derived ratio traits	CAN1*	CAN2	CAN1*	CAN2
Anal Width (AW)	0.93	0.35	0.88	0.37
Length of Hyaline Portion of Tail (TL)	0.92	-0.02	0.99	-0.07
Body Length (BL)	0.91	0.04	0.86	-0.03
Position of Vulva in females / spicule length in males	0.89	0.29	0.89	-0.02
Position of Excretory Pore (EP)	-0.87	0.38	-0.67	0.28
c' = (TL/AW)	-0.86	-0.38	0.92	-0.30
Maximum Body Width (MW)	0.85	0.10	0.34	0.47
Position of Dorsal Esophageal Gland Orifice (DEGO)	0.80	-0.55	0.90	0.39
Stylet Length (SL)	0.77	-0.58	0.92	0.30
c = (BL/TL)	-0.67	0.09	-0.99	0.03
Esophageal Length (EL)	0.47	-0.74	0.18	0.55
a = (BL/MW)	-0.28	0.04	0.30	-0.41
b = (BL/EL)	0.10	0.87	0.32	-0.42
Eigenvalue	4.98	1.45	7.26	0.68
% of total variance	0.66	0.19	0.82	0.08

*Traits are listed in descending order of absolute values for the between CAN 1 structure in females. Absolute correlations $\geq$ 0.92, 0.85, 0.72, and 0.55 are significant at $P \leq 0.0001$, 0.001, 0.01, and 0.05, respectively.

conical centrifuge tube, and the nematode suspension added to the Optiprep solution and centrifuged at 1,000 x g for 1 min. Immediately after centrifugation, half the supernatant without any nematodes was discarded. The solution layer with RN just above the OptiprepTM – water interface was collected into a new 15 mL conical centrifuge tube. The efficiency of the OptiprepTM method for the extraction of nematodes has recently been determined to be 85% or higher using this method compared to the sucrose gradient method (Deng *et al*., 2008). The remaining infested soil samples were place in the greenhouse and RN populations were maintained on cotton cultivar 'Delta and Pineland 425 BG/RR' (DPL 425) for RN multiplication.

Morphometric Measurements

Extracted RN from soil samples were placed on slides which were briefly passed over a flame prior to measurements. Morphometric measurements were determined on 20 individual vermiform female (F) and male (M) nematodes from each field sample using an IMT-2 microscope (Olympus Optical Co. Ltd, Japan). Nine morphometric variables were measured separately for each sex: body length (BL), stylet length (SL), position of vulva (PV), spicule length (PS), length of hyaline portion of tail (TL), position of dorsal esophageal gland orifice (DEGO), position of excretory pore (EP), maximum width (MW), esophageal length (EL), and anal width (AW). In addition, de Man's formula ratios a = body length / maximum body width, b = body length / esophageal length, c = body length / tail length, and, c' = tail length / anal body width were also calculated (Dasgupta *et al*., 1968).

Statistical Analysis

A two-step approach was used to investigate the pattern of morphometric variability among the locations sampled. First, canonical discriminant analysis (CDA) as implemented in SAS® PROC CANDISC, SAS 8.0 (SAS, Cary, NC) was used to collectively observe all morphometric traits with sampling location as the class variable. The *P*-values for pairwise differences among locations were adjusted for multiple comparisons by the Bonferroni method as implemented in SAS® PROC MULTTEST. Individual morphometric traits were then evaluated by ANOVA using location, sex, and location x sex as fixed effects. Residual variation was modeled with the repeated statement in SAS® PROC MIXED using the group = sex option to allow for heterogeneity of variances among sexes. If the likelihood ratio test was significant, then separate residual variances were used for females and males.

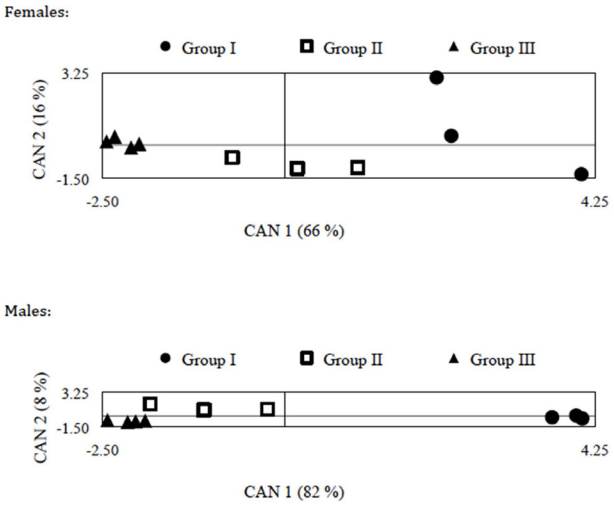

Fig. 1. Centroid means from canonical discriminant analysis of female and male reniform nematodes collected from nine sites in Alabama and one site in Mississippi. The axes in each panel were scaled to reflect the relative contributions to the multi-variance among nine morphometric traits and four derived ratio traits.

RESULTS

Canonical Discriminant Analysis (CDA)

The first two canonical variates (CAN 1 and 2) accounted for 85% and 90% of the multi-variance among the nine measured and four derived traits for female and male RN, respectively (Table 2). The contributions of the first canonical variates (0.66 and 0.82) were 4 and 10-fold higher than the second variates (0.19 and 0.08) in females and males respectively, resulting in a strong differentiation among locations along the first axis.

The magnitude of the phenotypic correlation between class means for original response variables and canonical variate means (between canonical structure in SAS parlance) gives an indication which original variables drive the separation among classes along a particular axis (Table 2). For females, CAN 1 had the highest correlation with anal width (r = 0.93), followed by length of the hyaline portion of tail (r = 0.92) and total body length (r = 0.91). Canonical variate 2 had the greatest correlation with the ratio trait b (r = 0.87), followed by esophageal length (r = -0.74), and stylet length (r = -0.58). For males, CAN 1 had a near perfect negative correlation with length of hyaline portion of tail (r = 0.99) and the derived ratio trait c (r = -0.99), followed by stylet length (r = 0.92) and the derived ratio trait c' (r = 0.92). Canonical variate 2 had the greatest correlation with esophageal length (r = 0.55), followed by maximum body length (r = 0.47).

Canonical discriminant analysis visually separated the 10 locations into three groups for both females and males (Fig. 1). The three locations Belle Mina (Limestone County, AL), Huxford (Escambia County, AL), and MSU

Table 3. Mahalonobis pairwise distances (D) among locations (above the diagonal) and Bonferroni adjusted *P*-values (below the diagonal) for female reniform nematodes. The shaded rectangles indicate the distances and *P*-values within groups (see Fig. 1 and Table 1). Locations are listed in descending order of the CAN 1 centroid mean for females.

		Mahalonobis pairwise distances (D)									
Group	Location	MSU	Belle Mina	Huxford	Shaw	Murphy	Lamons	Hargrave	Hamilton	Thornton	Whitehead
I	MSU		3.5	5.0	4.1	4.5	5.1	6.3	6.4	6.8	6.8
I	Belle Mina	< 0.0001		3.2	2.5	3.1	3.4	4.6	4.8	4.8	5.1
I	Huxford	< 0.0001	< 0.0001		4.4	4.6	4.6	5.1	5.3	5.3	5.5
II	Shaw	< 0.0001	0.0002	< 0.0001		1.3	2.2	3.7	3.7	4.1	4.1
II	Murphy	< 0.0001	< 0.0001	< 0.0001	1.0000		1.6	2.8	2.8	3.3	3.3
II	Lamons	< 0.0001	< 0.0001	< 0.0001	0.0060	1.0000		1.7	1.9	2.2	2.3
III	Hargrave	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	1.0000		1.1	1.1	1.6
III	Hamilton	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.2643	1.0000		1.4	1.6
III	Thornton	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0068	1.0000	1.0000		1.7
III	Whitehead	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0044	1.0000	1.0000	1.0000	

Table 4. Mahalonobis pairwise distances (D) among locations (above the diagonal) and Bonferroni adjusted *P*-values (below the diagonal) for male reniform nematodes. The shaded rectangles indicate the distances and *P*-values within groups (see Fig. 1 and Table 1). Locations are listed in descending order of the CAN1 centroid mean for females.

		Mahalonobis pairwise distances (D)									
Group	Location	MSU	Belle Mina	Huxford	Shaw	Murphy	Lamons	Hargrave	Hamilton	Thornton	Whitehead
I	MSU		2.1	1.2	4.9	6.3	5.4	6.3	6.2	6.2	6.6
I	Belle Mina	0.0461		2.0	4.4	6.0	5.2	6.0	5.8	6.0	6.3
I	Huxford	1.0000	0.1527		4.7	6.2	5.2	6.3	6.1	6.2	6.6
II	Shaw	< 0.0001	< 0.0001	< 0.0001		2.8	2.1	3.0	2.9	3.0	3.2
II	Murphy	< 0.0001	< 0.0001	< 0.0001	< 0.0001		1.7	2.7	2.7	2.6	2.6
II	Lamons	< 0.0001	< 0.0001	< 0.0001	0.0169	1.0000		2.3	2.1	2.1	2.3
III	Hargrave	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0013		1.5	0.6	1.4
III	Hamilton	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0339	1.0000		1.5	1.5
III	Thornton	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0329	1.0000	1.0000		1.4
III	Whitehead	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0034	1.0000	1.0000	1.0000	

Table 5. Location ranges for female and male reniform nematodes for the morphometric and derived ratio traits with phenotypic correlation (between structure in Table 2) ≤ |0.85| with the first canonical variate for females.

	Group	I	I	I	II	II	II	III	III	III	III		
	Location	MSU	Belle Mina	Huxford	Shaw	Murphy	Lamons	Hargrave	Hamilton	Thornton	Whitehead	SEDwithin	SEDbetween
MW	Females	14.3-19.0	14.3-21.4	11.9-19.0	14.3-16.7	14.3-16.7	14.3-16.7	14.3-16.7	11.9-16.7	14.3-16.7	11.9-16.7	0.5	0.30
	Males	14.3-16.7	11.9-14.3	11.9-16.7	14.3-16.7	14.3-16.7	11.9-16.7	14.3-14.3	11.9-14.3	14.3-16.7	11.9-14.3	0.4	
DEGO	Females	30.9 -47.6	30.9-38.1	23.8-40.5	28.6-45.2	26.2-47.6	28.6-38.1	23.8-33.3	28.6-35.7	26.2-35.7	26.2-35.7	1.17	1.12
	Males	28.6-47.6	28.6-40.5	28.6-50	28.6-38.1	26.2-38.1	26.2-38.1	23.8-30.9	26.2-35.7	26.2-31.0	26.2-33.3	1.14	
SL	Females	16.7-23.8	14.3-19	11.9-23.8	14.3-19	14.3-19	14.3-19	14.3-16.7	14.3-16.7	14.3-14.3	11.9-16.7	0.53	0.43
	Males	11.9-16.7	11.9-16.7	11.9-21.4	11.9-14.3	11.9-16.7	11.9-16.6	11.9-11.9	11.9-14.3	11.9-11.9	11.9-11.9	0.48	
EL	Females	100.0-200.0	80.0-160.0	81.2-147.6	110.0-150.0	100.0-140.0	100.0-160.0	100.0-140.0	100-130	100.0-140.0	90.0-140.0	4.96	3.07
	Males	100.0-130.0	100.0-130.0	90.0-120.0	90.0-120.0	100.0-140.0	100.0-140.0	100.0-120.0	100.0-120.0	100.0-120.0	80.0-130.0	4.12	
a	Females	20.0-28.0	17.7-30.1	15.8-32	21.0-28.7	19.8-28.7	21.0-28.0	20.4-28.0	22.4-29.4	18.6-27.3	18.6-28.6	0.81	0.79
	Males	22.2-30.8	25.2-35.3	18.9-31.1	21.0-30.1	20.4-28.0	19.8-33.6	23.8-28.0	23.1-34.5	20.4-28.7	23.1-31.1	0.8	
b	Females	1.1-3.7	2.5-4.2	0.6-1.2	2.3-3.3	2.7-3.4	2.5-3.6	2.3-3.8	2.4-3.5	2.4-3.5	2.6-3.8	0.12	0.11
	Males	3.1-4.4	2.9-3.9	3.0-4.0	3.2-4.2	2.6-3.6	2.5-4	3.2-4.0	2.9-4.0	2.9-4.1	2.9-4.4	0.12	
c	Females	8.9-16.4	8.0-15.1	9.5-17	9.8-14.4	10.7-15.4	8.9-15.3	9.7-15.6	11.0-14.7	11-14.9	10.7-17.3	0.53	0.45
	Males	6-10.1	6.9-10.9	6.9-10.1	9.8-15.9	11.9-16.8	10.1-15.3	12.3-17.7	11.2-18.1	11.6-16.8	12.3-17.2	0.49	
AW	Females	7.1-14.3	9.5-14.3	7.1-16.6	7.1-11.9	7.14-9.52	7.1-9.5	7.1-7.1	7.1-7.1	7.1-9.5	7.1-9.5	0.43	0.31
	Males	7.1-11.9	7.1-11.9	7.1-11.9	7.1-11.9	7.1-9.5	7.1-9.5	7.1-9.5	7.14-7.14	7.1-7.1	7.1-7.1	0.37	
TL	Females	23.8- 42.8	28.6-47.6	24.7-44.5	26.2-35.7	23.8-35.7	23.8-35.7	23.8-33.3	23.8-30.9	26.2-30.9	21.4-30.9	1.2	1.40
	Males	35.7-61.9	35.7-54.7	33.3-57.1	23.8-35.7	23.8-30.9	23.8-35.7	21.4-28.6	23.8-30.9	21.4-31.0	21.4-28.6	1.3	
BL	Females	350.0- 450.0	320.0- 430.0	300.0-430.0	350.0-410.0	320.0-440.0	320.0- 400.0	300.0-400.0	310.0-410.0	310.0-420.0	310.0-390.0	8.44	8.29
	Males	350.0-440.0	350.0-440.0	270.0-420.0	340.0-430.0	310.0-410.0	300.0-420.0	340.0-400.0	320.0-430.0	340.0-410.0	330.0-410.0	8.37	
PV	Females	240.0-380.0	250.0-310.0	261.8-345.8	250.0-300.0	240.0-320.0	220.0-300.0	220.0-300.0	210.0-300.0	210.0-300.0	220.0-280.0	7.2	N/A
PS	Males	14.3-21.4	14.3-21.4	14.3-23.8	11.9-19.0	14.3-21.4	11.9-19.0	14.3-19.0	14.3-16.7	14.8-19.0	14.3-19.0	5.11	
EP	Females	47.6-71.4	47.6-76.2	47.6-83.3	59.5-83.3	47.6-83.3	59.5-83.3	66.6-80.9	64.3-80.9	64.3-83.3	59.5-83.3	2.1	2.0
	Males	54.7-71.4	47.6-76.2	50-83.3	52.4-73.8	59.5-83.3	59.5-76.2	59.5-80.9	59.5-78.5	59.5-71.4	59.5-71.4	2.1	
c'	Females	1.3-3.0	1.4-3.3	1.3-2.9	1.6-2.5	1.6-2.2	1.6-2.5	3.3-4.7	3.3-4.3	2.8-4.3	2.5-4.3	0.11	0.2
	Males	3.8-7.7	3.0- 7.0	3.4-7.3	2.5-5	2.5-4	2.5-5	2.8-4.0	3.3-4.3	3.0-4.3	3.0-4.0	0.16	

(Oktibbeha County, MS) in Group I were separated from the remaining seven populations based on large positive CAN1 values. Members of this group were significantly ($P < 0.0001$) different from the remaining seven locations (Tables 2 and 3). Whereas the centroid means for males in this group are tightly clustered ($P > 0.05$), there was a greater spread for females with Mahalanobis' distances of up to 5.0 (Table 3 and 4). The second location group (Group II), consisting of Shaw (Limestone County, AL), Murphy (Limestone County, AL), and Lamons (Fayette County, AL), had a maximum within group distance of D = 2.8 for males and 2.2 for females (Tables 3 and 4). The last group (Group III) consisting of the North Alabama locations Hargrave (Limestone County), Hamilton (Lawrence County), Thornton (Lawrence County), and Whitehead (Fayette County) was very homogeneous with a maximum distance among locations of 1.7 and a unity *P*-value following the Bonferroni adjustment to account for the increase in Type I error due to the 45 pairwise comparisons that were made.

Analysis of Variance (ANOVA) and Individual Traits

The sex x location interaction was significant at $P < 0.001$ for most morphometric traits (data not shown), except for total body length ($P = 0.293$), position of the excretory pore ($P = 0.014$), maximum body width ($P = 0.005$) and the derived ratio trait a = body length / maximum body width ($P = 0.059$). Trait means, therefore, were presented separately for females and males.

The strong separation of Group III from Group I and II for females along CAN 1 can be seen in the morphometric traits that had the highest absolute phenotypic correlation with CAN 1 centroid means (Table 2). The class means for the first four traits – anal width, length of hyaline portion of tail, body length and position of vulva – generally followed the ranking obtained by CDA, with a few rank changes involving adjacent classes (Table 2). The correlation between the independent variable and the canonical variate can also be interpreted geometrically as the cosine between the two vectors (Anderson, 2003); a high correlation thus is equivalent to an acute angle between these vectors. As shown in Table 2 for males, six of the nine morphometric traits and two out of four derived traits had $r \geq 0.85$ underscoring the stringency of most traits selected for measurements in this study and affirmed by CDA. This value < 0.85 thus became an important determinant of significance.

The location ranges for female and male populations for four morphometric traits and three derived ratio traits with phenotypic correlation (between structure in Table 2) $\leq |0.85|$ with the first canonical variate, CAN1 for female populations are shown in (Table 5). The lowest mean value for MW (13.4 μm) was observed in group III from the male Hamilton population, their measurements fell in the range of (11.9-14.3), and the highest mean value for MW (16.9 μm) was observed from group I from the female Belle Mina population, their range fell within (14.3-21.4). The lowest mean value for DEGO (27.7 μm) was in group III from the male Hargrave population, their measurements were in the range of (23.8-30.9), while the highest mean value for DEGO (39.9 μm) was in group I from the female MSU population, with ranges of (30.9-47.6). The highest value for stylet length SL (18.9 μm) was in group I from the MSU female population, these group had ranges of (16.7-23.8), and the lowest value (11.9 μm) was observed in three male populations within group III. The female population from MSU, in group I had the highest esophageal length of 150.5 μm, measurements ranged from (100.0-200.0). The standard error of a difference (SED) within and between the male and female populations were less than 1 with the exception of values for DEGO and Esophageal length. Female populations from MSU could easily be differentiated from the other populations within group I because of the length of their esophagus. Similarly, the location means for female and male RN for additional 6 morphometric traits (AW, TL, BL, PV, PS, EP) and the derived ratio trait c' (Table 5) differed similarly among the locations and thus contributed to CDA and groupings.

DISCUSSION

Our study utilized nine morphometric characters and four derived ratio traits for canonical discriminant analysis providing clear evidence that morphological differences exist at various locations based on genotypic or environmental variables. The CDA greatly aided in progressively pooling linear combination of variables having the highest multiple correlations with data from each location. Such an approach has also been utilized in discriminant analysis of nine species of *Longidorus* including five new species in Arkansas (Ye and Robbins, 2004). This study is unique because it covers a finite geographic area, where seven of 10 sampling sites were located within a 25-mile radius spanning two counties (Lawrence and Limestone). The remaining sites were approximately 80 miles (Whitehead, AL), 125 miles (MSU, MS) and 250 miles (Huxford AL) away. Our findings and those of Agudelo *et al.* (2005) report high variability in morphometrics within populations across the southern United States. An interesting observation in our study was the specific groups for male and female populations which were not observed in the study by Agudelo *et al*. 2005. Morphometric variability among RN populations has been mentioned by a number of authors (Dasgupta *et al*., 1968; Linford and Oliveira, 1940; Nakasono, 1983; Robbins, 1994; Sivakumar and Seshadri, 1971; Soares *et al*., 2003; Van der Berg, 1978). Larger body lengths of RN than those observed in our study are reported from Hawaiian and Japanese populations (Nakasono, 1983). Anal width and length

of the hyaline portion of the tail measurement also accounted for a significant amount of the variation in total canonical structure as well as between canonical structures in both RN sexes in our study. Agudelo *et al.* (2005), found similar correlations.

Canonical discriminant analysis was used by Cho and Robbins (1991), among 23 *Xiphinema americanum* mixed with seven additional species collected from seven disparate states. In their study, three groups were generated that were closely related to geographic origin of these populations. However, due to overlap among them, no clear distinction was observed among their populations. Based on the analyses conducted in this study, we grouped these ten populations into three distinct groups by centroid means. The first location group (Fig 1: Group I), for female and males include populations from Belle Mina (Limestone County, AL), Huxford (Escambia County, AL), and MSU (Oktibbeha County, MS). The Huxford and Belle Mina locations are believed to be some of the oldest populations in Alabama. These are comparable to the population from MSU because, these populations are believed to have spread laterally from the Mississippi river area and the MSU populations possibly spawned the Alabama region. The second location group (Group II) for females and males, consistently supports high populations of RN independent of varying soil types and crop rotation practices included populations from Shaw (Limestone County, AL), Murphy (Limestone County, AL), and Lamons (Fayette County, AL). The third location group (Group III) made up of the North Alabama locations Hargrave (Limestone County), Hamilton (Lawrence County), Thornton (Lawrence County), and Whitehead (Fayette County), although dissimilar in soil morphology, has typically supported lower populations of RN.

Subbotin *et al.* (1999) identified groups within *Heterodera avenae* species using CDA, the first two canonical variables accounted for 87% of variance. In our study, for the female populations, the first two canonical variables accounted for 85% of variance, for males it was even higher (90%). The first two canonical variates in our study can thus be utilized to identify the most important variables for discriminating among the populations. Other investigations suggest the tail length and the length of the hyaline portion of the tail to be the most correlated (Subbotin *et al*., 1999) of the nine morphometric traits studied. Furthermore, Ching (1969) also noted considerable variation in tail length, tail shape, and tail annule numbers for Alabama and Hawaiian populations, among these, the longest hyaline area in tail and tail lengths were found within the Hawaiian population. Our study also showed canonical variable 1 having the highest correlation with length of hyaline portion of tail (0.99) followed by stylet length (0.92), and the derived ratio trait c' (0.92) for the male populations under the between canonical structure. Within the female populations, anal width (0.93) was highly correlated with canonical variable 1, followed by length of hyaline portion of tail (0.92) under the between canonical structure. This shows the importance of the length of the hyaline part of the tail in discriminating among the male and female RN populations. Significant morphological variation therefore exists for male and female RN populations across Alabama. This was also confirmed in the ANOVA documenting significant variation for the locations ($P < 0.05$) and sexes ($P < 0.05$). Single characters were insufficient in differentiating variability within populations and groups because of the high degree of variability within and between populations for male and female RN. The populations in group I which had the highest Mahalanobis distance values clustered distinctly from groups II and III.

Canonical discriminant analysis was therefore a useful tool for discrimination of RN populations. This could be seen in the three major groups that were observed for the populations; again the important variables discriminating among these populations were identified through the first and second canonical axis. Our study should be considered a prelude to extensive molecular analysis of the 18S and ITS1 rRNA regions in the RN genome and further based on the morphometric groupings generated after CDA was performed, which will potentially identify molecular signatures for variation in RN groups.

Representative populations from each of the three groups may be tested for their pathogenicity using genotypes of variable resistance to explore the presence of biotypes of RN.

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