BIOLOGICAL CHARACTERIZATION OF FIVE ISOLATES OF *BELONOLAIMUS LONGICAUDATUS*[1]

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ABSTRACT

Han, H-R., D. W. Dickson, and D. P. Weingartner. 2006. Biological Characterization of Five Isolates of *Belonolaimus longicaudatus*. Nematropica 36:25-35.

Belonolamius longicaudatus is a highly virulent nematode pathogen of many important crops in the southeastern United States, yet there have been few comparative studies among different populations. The objective of this study was to determine the population behavior and developmental biology among several isolates obtained from varying geographical locations. These investigations were carried out with nematodes cultured on excised corn roots grown on Gamborg medium at a constant temperature. Females began laying eggs in pairs 2 to 3 days after they were placed on excised corn roots. The period from embryogenesis to hatch of second-stage juveniles for all isolates was 3 to 4 days. Egg development required approximately 9 days at 18°C, 5 days at 23°C, and 3.7 days at 28°C, whereas no eggs were laid at 33°C. At 28°C the Georgia isolate had the fastest rate of development, the North Carolina isolate had the slowest rate, and the three Florida isolates were intermediate. Although second-stage juveniles of all isolates were highly active, their feeding time was less than that for other juvenile stages.

Key words: behavior, *Belonolaimus longicadatus*, development, embryogenesis, nematode, sting nematode.

RESUMEN

Han, H-R., D. W. Dickson, and D. P. Weingartner. 2006. Caracterización Biológica de Cinco Aislamientos de *Belonolaimus longicaudatus*. Nematropica 36:25-35.

Belonolamius longicaudatus es un nematodo altamente virulento y patógeno de muchos cultivos importantes en el sureste de Estados Unidos. Sin embargo, existen pocos estudios comparativos de diferentes poblaciones de este organismo. El objetivo de este estudio fue determinar el comportamiento poblacional y la biología del desarrollo de diferentes aislamientos obtenidos de diversos orígenes geográficos. Estas investigaciones se llevaron a cabo con nematodos cultivados en raíces de maíz en medio Gamborg a temperatura constante. Las hembras iniciaron oviposición en pares 2 a 3 días después de ser puestas en las raíces de maíz. El período de embriogénesis a eclosión del juvenil de segundo estadio fue de 3 a 4 días para todos los aislamientos. Para el desarrollo de los huevos, se requirieron aproximadamente 9 días a 18°C, 5 días a 23°C y 3.7 días a 28°C, mientras que a 33°C no hubo oviposición. A 28°C, el aislamiento de Georgia tuvo la tasa de desarrollo más rápida, el aislamiento de North Carolina tuvo la tasa más lenta, y los tres aislamientos de Florida tuvieron tasas de desarrollo intermedias. Aunque los juveniles de segundo estadio de todos los aislamientos fueron altamente activos, su tiempo de alimentación fue menor que el de otros estadios juveniles.

Palabras clave: Belonolaimus longicadatus, comportamiento, desarrollo, embriogénesis, nematodo.

INTRODUCTION

Belonolaimus longicaudatus Rau is an economically important plant pathogen that attacks many varieties of agronomic and horticultural crops (Christie *et al.*, 1952; Esser, 1976; Holdeman, 1955; Robbins and Barker, 1973). The significance of *B. longicaudatus* in agriculture was first recognized following its report of ectoparasitism and pathogenicity on strawberry, celery, and sweet corn (Christie *et al.*, 1952).

Details on the nematode's biology, development, and behavior have been inadequately studied due to difficulties in rearing and observing them *in vitro*. Previous biological studies conducted under greenhouse and field conditions have resulted in reports on the life cycle and reproductive rate of Florida, Georgia, and North Carolina populations (Boyd and Perry, 1971; Perry, 1964; Robbins and Barker, 1974; Smart and Nguyen, 1991). In Florida field studies, *B. longicaudatus* reproduced better at 29.4°C than at 26.7°C (Boyd and Perry, 1971; Perry, 1964). North Carolina and Georgia populations reproduced well in the range between 25°C and 30°C. The reproduction of the Georgia population was greatest at 30°C, whereas reproduction of the North Carolina population was reduced at 30°C (Robbins and Barker, 1974). In a greenhouse study, the life cycle *of B. longicaudatus* was completed in about 28 days (Smart and Nguyen, 1991). Recently, *in-vitro* cultivation on excised corn roots made it possible to complete more detailed studies on the life cycle and behavioral characteristics of a California isolate of *B. longicaudatus* (Huang and Becker, 1997; 1999). The life cycle was completed in 30 days at 26°C to 27°C (Huang and Becker, 1997) and 24 days at 28°C. Mating occurred after the females completed their last molt and lasted 6 to 10 minutes (Huang and Becker, 1999). All juvenile stages, as well as the adults, fed on the root meristem (Huang and Becker, 1997).

The objective of this study was to determine whether there are differences in development among different isolates of *B. longicaudatus* from the southern United States.

MATERIALS AND METHODS

Nematode Isolates

Five isolates of *B. longicaudatus* were collected from different geographical locations and different hosts. The isolate designation, previous host crops, and location were: HA isolate from potato grown at the Yelvinton farm, Research and Education Center, University of Florida, Hastings, FL; GV isolate from bermudagrass grown at the Gainesville Golf and Country Club, Gainesville, FL; LA isolate from citrus grown in block four, Citrus Research and Education Center, University of Florida, Lake Alfred, FL; GA isolate from cotton grown at the Rural Developmental Center, University of Georgia, Tifton, GA; and NC isolate from corn grown in Scotland County, NC.

Greenhouse Culture

Each isolate was established on bermudagrass (*Cynodon dactylon* (L.) Pers) in the greenhouse. Bermudagrass was grown vegetatively in 25 cm-diameter clay pots filled with pasteurized sandy soil (95.5% sand, 2.0% silt, and 2.5% clay). Nematodes were extracted by Baermann funnel (Ayoub, 1977), and females and males were selected manually. The inoculation level was 100 to 200 nematodes per pot. Each isolate was maintained in a greenhouse at 25 ± 5°C and fertilized once a week with 100 mls of a 20-20-20 N-P-K soluble fertilizer.

Nematode Preparation for Cultivation

Approximately 100 nematodes from each isolate were placed in a 1.5 ml microcentrifuge tube filled with sterile distilled water, and centrifuged three times with fresh water added each time for 2 minutes each at $10{,}000_g$. After centrifugation, the nematodes were transferred onto a sieve with 28-μm-pore opening that had been autoclaved previously, and washed with 1 liter of sterile distilled water.

Nematode Axenic Culture

Each isolate of *B. longicaudatus* was propagated on sweet corn (*Zea mays* L. cv. Silver Queen) cultured on Gamborg B-5 medium (Huettel and Rebois, 1985). Fifteen females and 10 males from each isolate were individually transferred onto sterilize disposable dishes (15 × 60 mm). Corn seed were placed in sterile disposable dishes (100 × 15 mm), surface sterilized with 95% ethanol for 3 minutes and 0.5% NaOCl for 10 minutes, and then placed onto 1.2% water agar and incubated at 28°C (Walker *et al.*, 1993). Two days after corn seed germination, corn roots were cut into 3 to 4 cm pieces with a sterile knife and 3 or 4 pieces were transferred onto the Gamborg B-5 medium (a mixture of agar [1.3%] and Gamborg B-5 media [23%]; pH adjusted to 5.2 to 5.6). The medium-agar mixture was autoclaved for 15 minutes at 253°C and 117 kPa before use. All culture plates were maintained at 28°C (Walker *et al.*, 1993).

Comparison of Developmental Period

Once females started to lay eggs, the developmental period (from egg to adult) was observed using an inverted microscope, and recorded daily. The observations on nematode development for each isolate were replicated 10 times. The developmental time for each stage and total developmental time were subjected to statistical analysis and means were compared by Duncan's multiple-range test.

The time required for egg development (single-cell egg to hatch) was compared among each isolate at 18°C, 23°C, and 28°C. Five eggs of each isolate were arbitrarily selected in five different dishes at each temperature, and developmental time for each was recorded.

Behavioral Characteristics

Feeding, oviposition, and mating behavior of *B. longicaudatus* were observed, recorded, and photographed on excised corn roots cultured in Gamborg-B-5 media with the use of an inverted microscope at magnifications of 20×, 100×, and 400×.

RESULTS

Development and Behavior

Females started to lay eggs 2 or 3 days after inoculation on excised corn roots. The eggs were extruded from the didelphic, amphidelphic female gonad via the vulva to the outside and deposited singly (Fig. 1A-J, top). Deposited eggs were usually one-celled. After 2 to 3 hours, the one-celled stage divided to the two-celled stage, and 3 to 4 hours later, the two-celled stage divided to the four-celled stage (Fig. 1A-I, bottom). The gastrulation stage required approximately 24 to 36 hours and the length of time varied among the nematode isolates. After the gastrulation stage, the tadpole stage was observed. Females laid two eggs, each produced separately from the paired gonads at 30-second to 20-minute intervals. A single female was observed to lay 9 to 10 eggs over a 10 to 15 hour period. The J1 was observed 48 to 60 hours following egg deposition. The time required for single egg deposition from

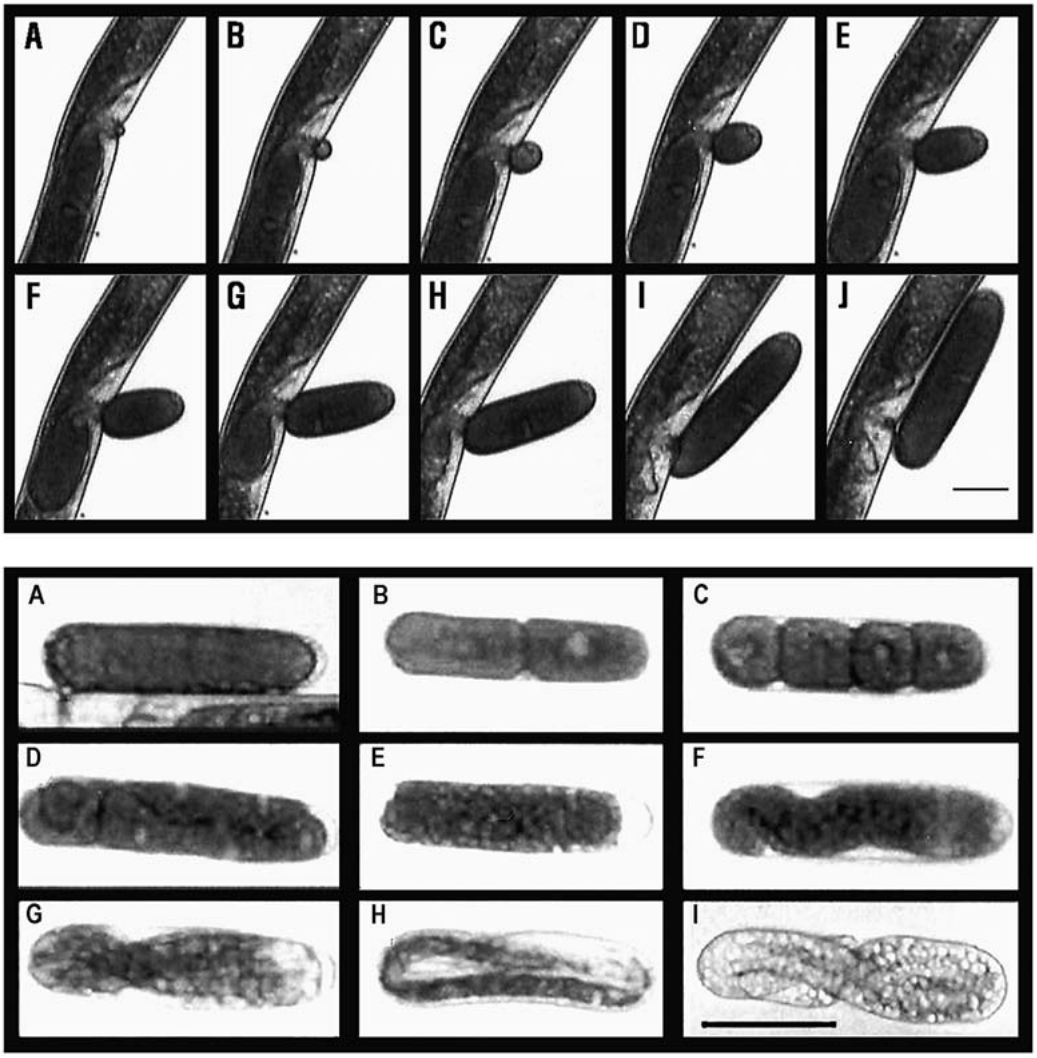

Fig. 1. Photographs showing oviposition in a *Belonolaimus longicaudatus* female (top) and nine stages of egg embryogenesis of *B. longicaudatus* (bottom). Top—A-J) Egg is extruded from uterus. J) A deposited single-celled egg. Scale bar is 10 µm. Bottom—A) Single-cell stage. B) Two-cell stage. C) Four-cell stage. D) Multi-cell stage. E) Gastrulation stage. F) Tadpole stage. G) First-stage juvenile. H) First molt. I) Second-stage juvenile. Scale bar = 20 µm.

each uterus was approximately 80 to 100 seconds, but in some cases it took only 35 to 40 seconds. The first molt occurred inside the eggshell, and took less than 24 hours. The total time of embryogenesis from the one-celled stage to J2 hatch ranged from 70 to 96 hours.

Eggs were laid randomly throughout the culture media, however after egg hatch, the J2 moved quickly to the roots where they congregated around root hairs (Fig. 2A). The J2 were more active in their body movement, but their feeding activity was less than that observed for other juve-

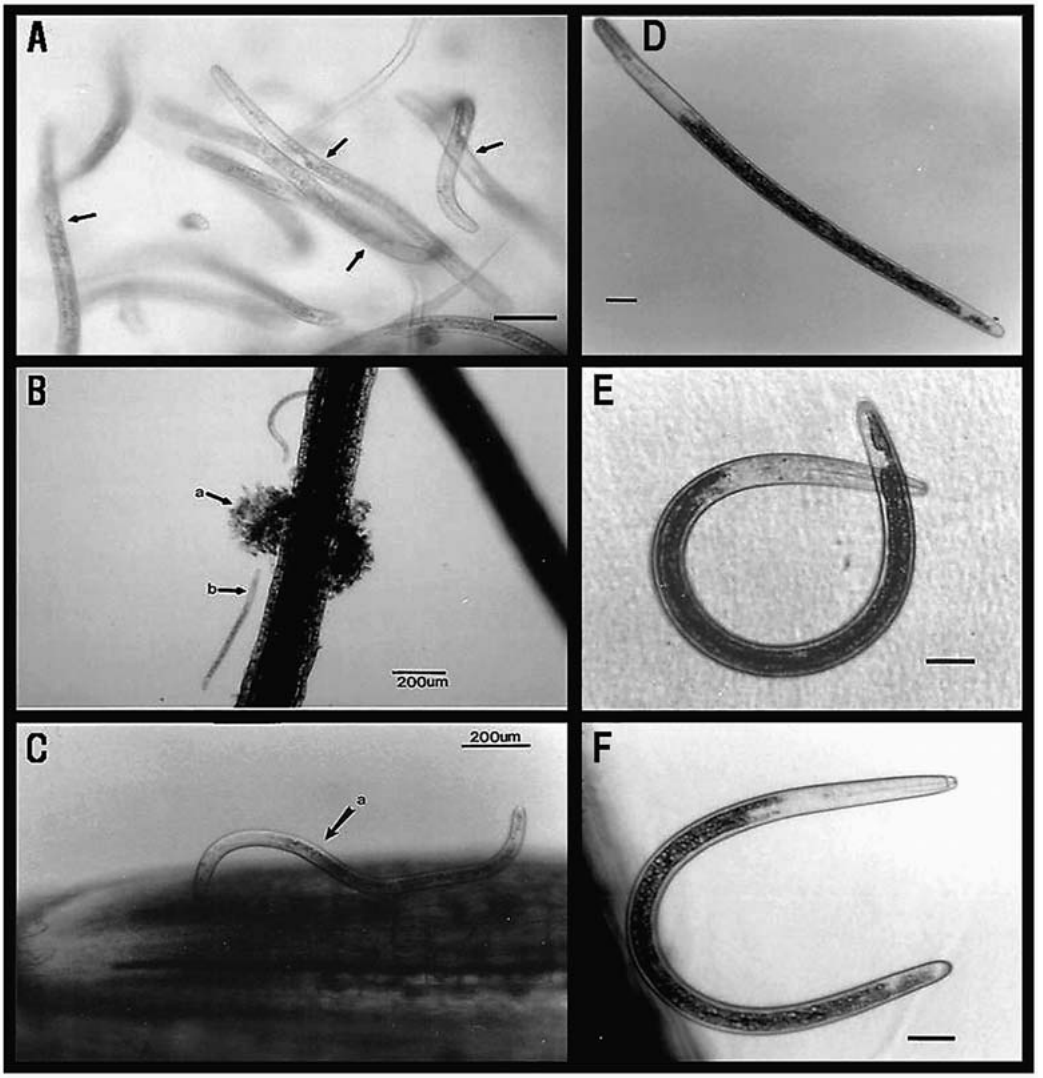

Fig. 2. Behavioral characteristics of second-stage juveniles of *Belonolaimus longicaudatus*. A) Congregated second-stage juveniles around root hairs (arrows indicate second-stage juveniles). B) Second-stage juveniles attracted by root-cell debris. a) Root-cell debris. b) Nematode. C) Second-stage juvenile feeding at root meristem. a) Nematode. D) Straight form. E) Full circle. F) C-shaped. Scale bar = 30 µm.

nile stages. The J2 were more attracted to root hairs (Fig. 2A) or root-cell debris (Fig. 2B) than by root meristems (Fig. 2C). It was not determined whether feeding was essential for the J2 to develop to the J3. After 3 to 5 days, most J2 began the second molt. The J2 were easily distinguished by their dark color, and wider-body shape. Their body movement slowed and stopped completely, which was the initial sign the second molt was beginning. During the molting process their bodies lay in a straight plane (Fig. 2D), were fully curved or slightly curved (Fig. 2E, F). The stylet

cone was replaced with a new one, and the old stylet cone was shed with the cuticle (Fig. 3A). The formation of the stylet shaft and knobs was never observed. The second molt took 2 days for completion for all isolates of *B. longicaudatus*.

The J3 moved immediately to root meristems of major roots or lateral roots. Their body movement was slower than J2 and remained so for the duration of the developmental period. Feeding was observed, frequently lasting 6 hours to approximately 10 hours. The J3 stage lasted a comparatively shorter time than other developmental stages. Two to 3 days passed before the third molt occurred. The distinctive molting sequence was identical to that for the second molt. The J3 became plumper in shape, dark in color, and their movement ceased. During the third molt, the stylet cone was shed with the old cuticle, and most J3 had a full-circle body shape. The period for the third molt lasted 2 days.

The J4 stage lasted for 4 to 6 days until the initiation of the fourth molt. The J4 fed actively during this developmental stage. Females and males were distinguished by the formation of genital primordia. The final molt was similar in process to that of the second and third molts with the exception of the formation of the reproduction system (Fig. 3B-G). The vulva and vagina of the female, and the bursa and spicules of the male were fully developed at the end of the fourth molt. This molting process took more than 2 days, which was longer than the second and third molts.

Mature males and females were observed feeding on cells along the root

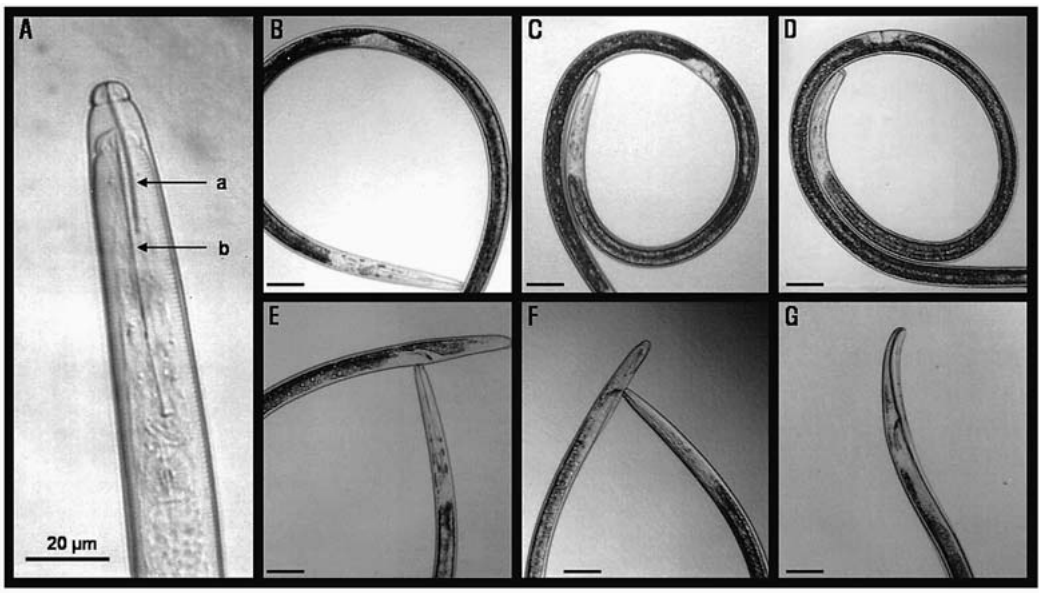

Fig. 3. Molting characteristics of *Belonolaimus longicaudatus*. A) Old stylet cone is shed together with the old cuticle, and new stylet cone is replaced. a) Old stylet cone. b) New stylet cone. B-D) Formation of female reproductive organs during fourth molt. B) Early stage of fourth molt. C) Vagina becomes visible in the middle of the fourth molt. D) Fully developed vulva and vagina at the end of fourth molt. E-G) Formation of male reproductive organs during fourth molt. E) Early stage of fourth molt. F) Middle of the fourth molt. Spicule is visible. G) End of fourth molt. Spicule and bursa are fully developed. Scale bar = 40 µm.

meristems (Fig. 4). All developmental stages except J2 of *B. longicaudatus* were more attracted to root meristems along major (Fig. 4A) and lateral roots (Fig. 4B,C), although a few nematodes were observed feeding along the cell-elongation region. Cell necrosis and cessation of root growth followed nematode feeding, which resulted in roots with a stubby or abbreviated appearance. These symptoms were more pronounced after multiple feedings by many nematodes. Nine to 10 nematodes (both adults and juveniles) were observed feeding along a single root (Fig. 4E). During feeding, the stylet penetrated deeply into the root cells reaching the endodermis near the vascular system of young corn roots. During feeding, the medium bulb was estimated to pulsate at the rate of 167 to 174 beats per minute.

Immediately after the fourth molt, there was attraction between opposite sexes. Distinctive mating behavior such as strong rubbing, touching, and twisting

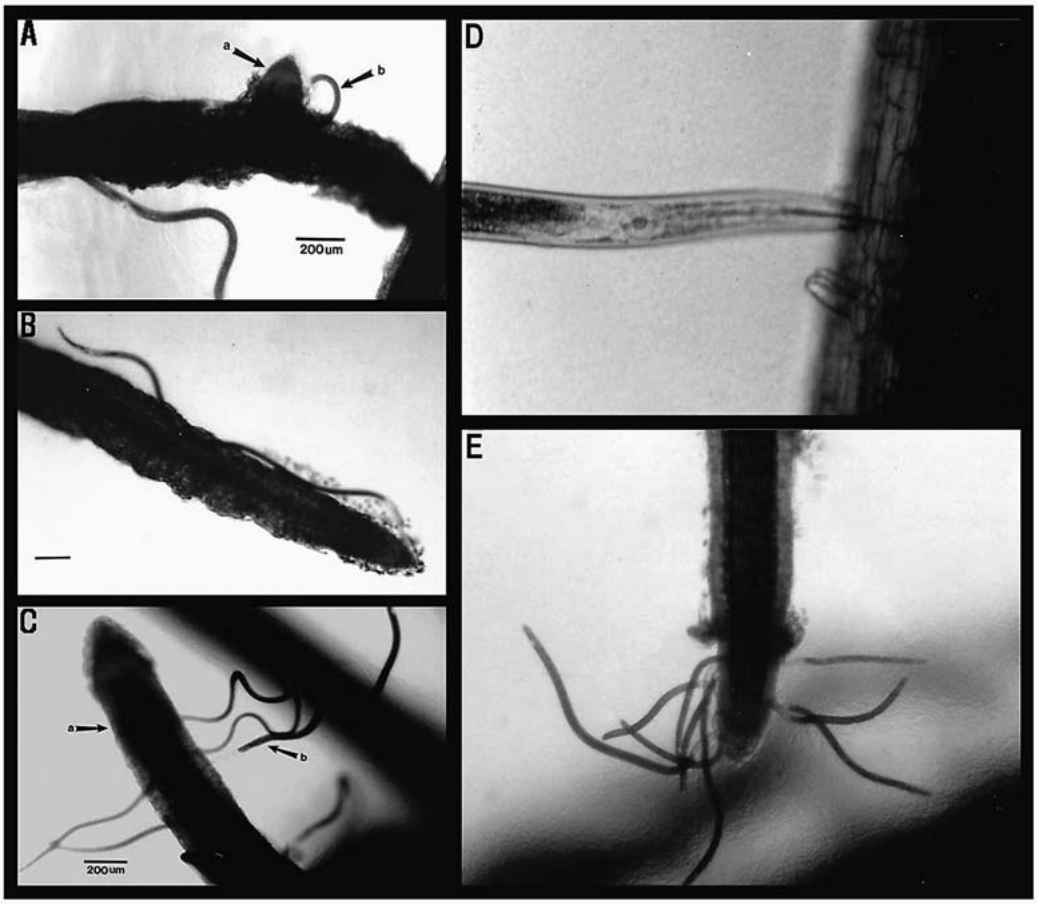

Fig. 4. Characteristics of feeding behavior in *Belonolaimus longicaudatus*. Feeding locations of *Belonolaimus longicaudatus* on corn roots. A) Lateral root a) Corn lateral root b) Nematode B) Major root meristem C) Young lateral root a) Root meristem b) Nematode D) Feeding occurring in higher location of root growth region. E) Multiple feeding by nine nematodes on one root meristem. Scale bar on A and B = 50 µm and 30 µm, respectively.

were observed between males and females with a duration lasting no more than 20 minutes. Mating was observed immediately after the fourth molt and occurred in a very limited timeframe.

Variation of Developmental Periods Among Isolates of B. longicaudatus

The NC isolate required 25 days to complete development from egg to adult, which was the longest period for any isolate ($P \leq 0.05$) (Table 1). The second longest period was 22.9 days for the LA isolate ($P \leq 0.05$). Among the GA, GV, and HA isolates, there were no differences in developmental times with each lasting 18.1, 19.5, and 19.2 days, respectively. Both NC and LA isolates had longer J2 periods than those of the GA, GV, and HA isolates ($P \leq 0.05$). There were no differences among isolates in molting times, although the fourth molt took longer than the second and third molts. There was a greater range of variation in egg developmental time and the J4 period than for the J2 and J3 periods, and the J3 period showed the smallest variation among all developmental stages (Table 1).

At 28°C, the length of time for egg development was different among NC, LA, and GA isolates ($P \leq 0.05$) (Table 2). The eggs of the NC isolate took 4.6 days to develop to J2, which was the longest time among all *B. longicaudatus* isolates ($P \leq 0.05$). The LA isolate had the second longest egg development time with an average of 4 days, whereas the GA isolate had the shortest egg developmental time of 3.2 days. At 18°C, the GA isolate required 8 days for development, which was shorter than any other isolate ($P \leq 0.05$). There were no differences in development among *B. longicaudatus* isolates at 23°C, and the mean egg developmental time ranged from 4.8 days (GA) to 5.6 days (LA). At 33°C, approximately 90% of nematodes died within 7 to 10 days and no egg laying was observed.

DISCUSSION

The developmental time (egg to adult) of *B. longicaudatus* on excised sweet corn roots varied among five isolates collected from different hosts and regions of the southern United States. Differences in the reproductive rate between Georgia and North Carolina isolates have been reported (Robbins and Barker, 1973). According to their report, three North Carolina isolates had a lower rate of reproduction than a Georgia isolate when tested on several different host plants. In addition, the reproductive rate of North Carolina isolates was slightly reduced at 30°C, whereas the Georgia isolate increased in population numbers at this temperature. Although it appears that geographically separated isolates of *B. longicaudatus* have different reproductive rates and temperature preferences, one can not determine from the literature how much variation exists within regions.

Recently, the life cycle of a *B. longicaudatus* isolate collected from turfgrass in Rancho Mirage, California was reported (Huang and Becker, 1999). This isolate completed its life cycle in 24 days at 28°C in gnotobiotic culture. The developmental time of the California (CA) isolate ranged from 20 to 22 days, which was similar to that for the GV, HA, and LA isolates used in our study. It has been hypothesized that the California *B. longicaudatus* was introduced from Florida based on PCR-RFLP analysis of ITS1 (Cherry *et al.*, 1997). The California isolate was distinguished by a longer egg developmental time (5 days) than occurred for our Florida and Georgia isolates, and it had a shorter J2 period (2 days) than all the other isolates. However,

Table 1. Comparisons of the developmental times (days) among five different isolates of *Belonolaimus longicaudatus* cultured on excised corn roots grown in Gamborg B-5 medium at 28 °C.

	Isolates					
Developmental stages	GV	HA	LA	GA	NC	CA[z]
Egg to J2	3.5 ± 0.53 bc[z]	3.5 ± 0.53 bc	4.0 ± 0.67 b	3.2 ± 0.42 c	4.6 ± 0.52 a	5
J2	2.5 ± 0.53 b	2.7 ± 0.48 b	4.3 ± 0.67 a	2.5 ± 0.53 b	4.7 ± 0.48 a	2
2nd molt	2.0 ± 0.00 a	2.0 ± 0.00 a	2.0 ± 0.00 a	2.0 ± 0.00 a	2.0 ± 0.00 a	2
J3	2.4 ± 0.52 ab	2.5 ± 0.53 ab	2.6 ± 0.52 a	2.1 ± 0.32 b	2.8 ± 0.42 a	3
3rd molt	2.0 ± 0.00 a	2.0 ± 0.00 a	2.0 ± 0.00 a	2.0 ± 0.00 a	2.0 ± 0.00 a	2
J4	4.7 ± 1.25 bc	4.0 ± 1.15 c	5.4 ± 0.97 ab	3.9 ± 1.20 c	6.2 ± 1.03 a	4-5
4th molt to adult	2.4 ± 0.52 a	2.5 ± 0.53 a	2.6 ± 0.52 a	2.4 ± 0.52 a	2.7 ± 0.48 a	2-3
Total time	19.5 ± 1.43 c	19.2 ± 1.75 c	22.9 ± 1.52 b	18.1 ± 2.02 c	25.0 ± 1.89 a	20-22

Isolates were collected from bermudagrass in Gainesville, FL (GV), potato in Hastings, FL (HA), citrus in Lake Alfred, FL (LA), cotton in Tifton, GA (GA), and corn in Scotland County, NC (NC). Means within rows followed by common letters are not significantly different according to Duncan's multiple-range test ($P \leq 0.05$).

[y]Mean number of days and the standard deviation for each developmental stage.

[z]The developmental time for the California (CA) isolate was described by Huang and Becker (1999).

Table 2. Comparisons of the egg developmental time (days) for five different isolates of *Belonolaimus longicaudatus* cultured on excised corn roots grown in Gamborg B-5 medium at different temperatures.

	Isolates[y]				
Temperature	CV	HA	LA	GA	NC
10°C	10.0 ± 0.71 a	9.2 ± 0.45 a	9.4 ± 0.55 a	8.0 ± 0.71 b	9.6 ± 0.55 a
23°C	5.2 ± 0.45 a	5.0 ± 0.71 a	5.6 ± 0.55 a	4.8 ± 0.84 a	5.4 ± 0.55 a
28°C	3.5 ± 0.53 bc	3.5 ± 0.53 bc	4.0 ± 0.67 b	3.2 ± 0.42 c	4.6 ± 0.52 a
33°C	—[z]	—	—	—	—

The isolates were collected from bermudagrass in Gainesville, FL (GV), potato in Hastings, FL (HA), citrus in Lake Alfred, FL (LA), cotton in Tifton, GA (GA), and corn in Scotland County, NC (NC). Means within rows followed by common letters are not significantly different according to Duncan's multiple-range test ($P \leq 0.05$). Lack of letter denotes non-significance.

[y]Mean number of days and standard deviation for each developmental stage.

[z]No eggs were observed.

there were no differences in molting time among any of the isolates.

The total developmental time of *B. longicaudatus* ranged from 18 to 25 days at 28°C, which was comparatively shorter than for other reports on ectoparasitic nematodes. *Hoplolaimus indicus* completed their development in 24 to 33 days under in-vitro condition at 28°C to 32°C (Dasgupta *et al.*, 1970). The developmental time of *Criconemoides xenoplax* was 23 to 31 days under laboratory conditions (Seshadri, 1964).

There is no information on embryogenesis of *B. longicaudatus*. In the current study, development of a single-celled egg was observed through two-celled, four-celled, multi-celled, gastrulation, and tadpole stages, first-stage juvenile, and first molt. The gastrulation stage was not only the longest period among the embryonic stages but also the most varied. Approximately 4 to 5 days were required for egg development of *B. longicaudatus*. *Helicotylenchus multicinctus* required 4 to 6 days for egg development, which was close to that for *B. longicaudatus* (Orion and Bar-Eyal, 1995), whereas the embryogenesis of *H. indicus* lasted 8 to 9 days (Dasgupta *et al.*, 1970), and *C. xenoplax* lasted 11 to 13 days (Seshadri, 1964). There are similarities in some developmental characteristics between *H. indicus* and *B. longicaudatus*. For instance, feeding was essential in both species for development of juveniles and necessary for egg laying in females. Emerged J2 of *B. longicaudatus* were found to congregate along young root hairs, which was similar to that reported for *H. indicus* (Dasgupta *et al.*, 1970). However, the time interval between laying the first and second egg was shorter in *B. longicaudatus* (30 seconds to 20 minutes) than for *H. indicus* (15 minutes to 3 hours), and the feeding time was longer in *H. indicus* (more than 72 hours) than in *B. longicaudatus* (6 to 10 hours). For *H. indicus*, sometimes cell division in the eggs started within the uterus and often J2 hatched within the body of the female. This was never observed among any of the *B. longicaudatus* isolates. Another ectoparasitic nematode, *C. xenoplax*, is different from *B. longicaudatus* in that cleavage of eggs in the uterus was common and feeding

was unnecessary for the laying of eggs (Seshadri, 1964).

Success of in vitro culture of ectoparasitic nematodes enables following nematode development from embryogenesis, post embryogenesis, to gametogenesis and observing detailed biological characteristics including feeding behavior. This experimental protocol also makes it possible to compare biological variation among different isolates of *B. longicaudatus* under consistent environmental conditions. The observed biological variations of sting nematode will be useful information for future comparisons of morphology and genetic characteristics among various isolates.

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