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Molecular characterization of *Helicotylenchus multicinctus* and *H. dihystera* (Tylenchida: Hoplolaimidae) from *Theobroma cacao* in Nigeria

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Abstract

The genus *Helicotylenchus* contains cosmopolitan, ubiquitous plant-parasitic nematodes with some species capable of causing significant economic damage to agricultural crops. Accurate species identification in this genus is essential in recognizing the damaging species and establishing effective management options. In a study on cocoa plantations in Nigeria, two species of spiral nematodes were found in high numbers from soil samples obtained from a six-decade old cocoa plantation at the Cocoa Research Institute of Nigeria. An integrated approach involving a combination of morphology, morphometrics, and molecular tools was used to identify the nematode species. Morphological data indicate the presence of both *H. multicinctus* and *H. dihystera*. There is congruence in the morphological and molecular data obtained for *H. multicinctus*. However, phylogenetic analysis of the 28S rDNA expansion segment revealed a high variability in the sequences of the Nigerian population of *H. dihystera,* suggesting the need for a careful appraisal and more comparative studies.

Key words: 28S rDNA expansion section, economic damage, morphology, morphometrics, polymerase chain reaction

Introduction

Cocoa—*Theobroma cacao* L. (Malvales: Malvaceae)—is a perennial tree crop grown in the humid tropics of the world (Yanelis *et al*. 2012) and the largest non-oil foreign exchange earner for producing countries, with more than 70% of production coming from Africa (Simo *et al*. 2018). Crop production is dominated by small-scale farmers who reside and work in the cocoa belt, offering them both jobs and revenue (Ngoh Dooh *et al*. 2015).

In recent times, cocoa production has nosedived, partly due to ageing trees and soil infertility, but mostly due to pests and diseases. New plantings and rehabilitation of moribund farms has increased production, but dieback attributed to a build-up of plant-parasitic nematodes, continues to be a problem. Symptoms such as plant chlorosis, sudden death and seedling growth retardation in nurseries and young plantations, coupled with deteriorating soil fertility has discouraged many farmers, leading to a reduction in crop production (Orisajo & Afolami 2009, Orisajo 2018). Due to their pathogenicity and wide distribution in cocoa-producing regions of the world, species of *Meloidogyne* Göldi, 1889 are considered the most important nematodes of cacao. However, several other plant-parasitic nematodes are often associated with cocoa, with representatives of *Helicotylenchus* Steiner 1945 often recovered in large numbers in Nigeria (Afolami & Caveness 1983, Orisajo 2018).

Helicotylenchus (Tylenchida: Hoplolaimidae) species are ectoparasites or semi-endoparasites that feed on plant roots (Yeates *et al*. 1993) causing plant growth reduction. In Nigeria, these nematodes are ubiquitous and are often found in soil associated with many agricultural crops. Some species have been described as pathogenic, causing damage on several important food crops (Olaniyi 2014, Daramola 2016). Afolami & Caveness (1983) gave a com-

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prehensive report on the occurrence and distribution of plant parasitic nematodes associated with *T. cacao* from six states in Nigeria and reported *H. dihystera* (Cobb, 1893) Sher, 1961 and *H. multicinctus* (Cobb, 1893) Golden, 1956 as the most frequently encountered nematode species in cocoa soils. However, molecular data of the nematode species from Nigeria are yet to be provided.

Helicotylenchus multicinctus is considered the most damaging nematode on banana and plantain and is regarded as the main parasitic nematode species on plantains where temperature and rainfall conditions are sub-optimal for the crop (McSorley & Parrado 1986). *H. multicinctus* was originally described from banana in Suva, Fiji (Cobb 1893) and has been recorded globally on several plant hosts. It is probably the most widespread and abundant nematode with damaging effects on plantains after *Radopholus similis* (Cobb, 1893) Thorne, 1949 (Caveness & Badra 1980, Brentu *et al*. 2004). During establishment, cocoa requires shade provided by plantain and other food plants, which further increases the populations of *Helicotylenchus* in the plant rhizosphere (Afolami 1981, Orisajo *et al*. 2016).

Helicotylenchus dihystera is the type species for the genus *Helicotylenchus* and was described from sugarcane (*Saccharum officinarum* L.) in Australia (Cobb 1893). The species is reported from six continents, including Africa, where it is reported from Angola, Benin, Burkina Faso, Cameroon, Democratic Republic of the Congo, Egypt, Eswatini, Gambia, Ghana, Ivory Coast, Kenya, Liberia, Malawi, Mali, Mauritania, Mozambique, Namibia, Niger, Senegal, South Africa, Sudan, Tanzania Uganda, Zambia, Zimbabwe and Nigeria (Marais 2001, Marais *et al*. 2005, Van den Berg *et al*. 2003, Andrássy 2007).

The habitus of *Helicotylenchus* is ventrally curved, generally creating the distinctive spiral body shape by which most representatives of this genus (spiral nematodes) can be readily differentiated in samples (Wouts & Yeates 1994). However, accurate identification of the species is not always easy, partly because several species share very similar diagnostic characteristics and species boundaries are not well defined (Subbotin *et al*. 2011). Species determination is made more difficult by phenotypic plasticity, which can lead to possible misidentifications. As a consequence, diagnostic problems often arise from under- or over-estimating the intraspecific variability of certain morphological features presently used in the diagnosis of species (Subbotin *et al*. 2015, Fortuner *et al*. 2018). While multivariate analyses may be helpful in decreasing the impact of intra-specific variation in morphological characters (Fortuner & Maggenti 1991), identification of these nematodes using only morphology is often unresolved and can be uncertain owing to morphological analysis constraints. The use of non-morphological characters like DNA sequences has been suggested in verifying the traditional morphological identification of *Helicotylenchus* species and to solve some of the issues encountered in identification (Subbotin *et al*. 2011, 2015).

In this study, molecular tools were used to verify and confirm the identity of the spiral nematodes that were found in association with cocoa in Nigeria.

Methodology

Site description

Nematode populations used in this study were collected from cocoa plantation at the research farm of the Cocoa Research Institute of Nigeria (CRIN), Ibadan situated between 7°13’39.38’’ N latitude and 3°52’5.86’’ E longitude at an altitude of 1222 m above sea level. The site is located in the tropical rain forest ecosystem with mean solar radiation of $18MJm^{-2}day^{-1}$ and an annual average rainfall of 2000 mm with a bimodal pattern. The cocoa plantation, variety F_3 Amazon, was established in 1964 with plantain used as the shade crop during establishment.

Nematode extraction and assessment

Twenty-two trees were randomly selected from the 1ha cocoa plantation. Fifty soil samples were collected from the trees and bulked, from which composite samples were taken. Nematodes were extracted from 100 mL of soil obtained from each composite sample taken within a radius of 50 cm from the base of cocoa trees, using a modified Baermann technique (Coyne *et al*. 2014). Nematodes were counted from sub-aliquots of the extraction under a stereomicroscope and densities calculated according to the method of Coyne *et al*. (2014). Nematodes were then identified to genus level based on morphological features (UNL 2019).

Morphological Examination

Nematodes were fixed in a heated 4 % formaldehyde + 1 % propionic acid (FPG) solution, dehydrated in a glycerine solution and mounted in glycerine on Cobb slides using a wax ring method (Marais *et al.* 2017). Measurements of the mounted specimens were taken with an Olympus BX53F microscope, equipped with a drawing tube. All measurements were made at 1000× magnification. Curved structures were measured along the median line. Morphometrics were calculated according to de Man (1884). All measurements in the descriptions are given in micrometers (µm). The *H. dihystera* specimens are deposited in the National Collection of Nematodes (NCN), Biosystematics, Agricultural Research Council (ARC)—Plant Health and Protection (PHP), Pretoria.

Extraction of DNA and PCR Amplification

DNA was extracted from specimens of each of the two species of spiral nematodes obtained from soil samples taken from the cocoa research farm. Single adult females were hand-picked and cut into 2–3 parts in 10 µL lysis buffer (500 mM MgCl,10 mM DTT, 4.5% Tween20, 0.1% gelatine and 3 µL proteinase K at 600 µg mL^{-1}) which was placed on the side of 0.5 ml Eppendorf tubes. The tubes were kept at -80^{0}C for a period of 15 min to allow for cell lysis and then incubated in a thermocycler at 65^{0}C for 60 min and 95^{0}C for 15 min (Nguyen 2007). DNA samples of the nematodes were kept at -20^{0}C until they were ready for use.

Polymerase chain reaction (PCR) for the amplification of the D2D3 expansion segment of the 28S rDNA gene was performed in a thermocycler using KAPA2G™ Robust Hotstart ReadyMix (KAPA Biosystems) and a primer combination which consisted of forward primer D2A (ACA AGT ACC GTG AGG GAA AGT TG) and reverse primer D3B (TCG GAA GGA ACC AGC TAC TA) as described by Subbotin *et al.* (2006). PCR products were centrifuged at 11,600 rpm at 10^{0}C for 2 min and placed in the thermocycler. The cycling conditions include 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 45 sec and extension at 72°C for 3 min with a final extension at 72°C for 10 min. PCR products were separated on 1.5% agarose gel stained with ethidium bromide and visualised under UV light with a transilluminator imaging system.

Sequencing and phylogenetic analysis

PCR products were purified using the Nucleo-Fast Purification System (Macherey Nagel, Waltham, Massachusetts, USA). Sequencing of the purified DNA was performed in both directions with the Big Dye Terminator V1.3 sequencing kit, followed by the use of electrophoresis on the 3730 X 1DNA Analyser (Applied Biosystems) at the DNA Sequencing Unit (Central Analytical Facilities, Stellenbosch University). Bioedit 7.2 software (Hall 1999) for sequence alignment was used for manual sequence assembly and editing. The newly obtained sequences of *H. multicinctus* and *H. dihystera* have been deposited at the National Centre for Biotechnology Information (NCBI) database with the accession numbers MN650241 and MN650242 for the *H. dihystera* sequences and MN056344 for the *H. multicinctus* sequence.

The newly obtained DNA sequences were used for BLASTN (Altschul et al. 1997) comparison against GenBank sequences. DNA sequences of *Helicotylenchus* from the NCBI database were downloaded and aligned with the newly obtained sequences using the Multiple alignment program for amino acid or nucleotide sequences, MAFFT version 7 (Katoh & Standley 2013). The analysis involved 77 nucleotide sequences of *Helicotylenchus* species with *Caenorhabditis elegans* as the outgroup. Phylogenetic analysis of the dataset was performed using MEGA X (Kumar *et al*. 2018) and the confidence intervals for the various branching points in the trees were estimated using bootstrapping (Felsenstein 1985) with 1000 replicates. Evolutionary history was inferred using the Maximum Parsimony (MP) and Maximum Likelihood (ML) methods. The most parsimonious tree with length = 697 is shown in figure 4. Estimates of evolutionary divergence between sequences of *H. multicinctus* and *H. dihystera,* indicating the number of base differences per sequence between sequences is shown in table 2.

Results

Morphological characterization. Two *Helicotylenchus* species were identified from the sampled cocoa plantation: *H. multicinctus* and *H. dihystera.* Morphological descriptions are presented below and their illustrations are given in Figures 1–3. The morphometry of *H. multicinctus* is similar to the reported type population, while the morphometric values of *H. dihystera* are provided in Table 1.

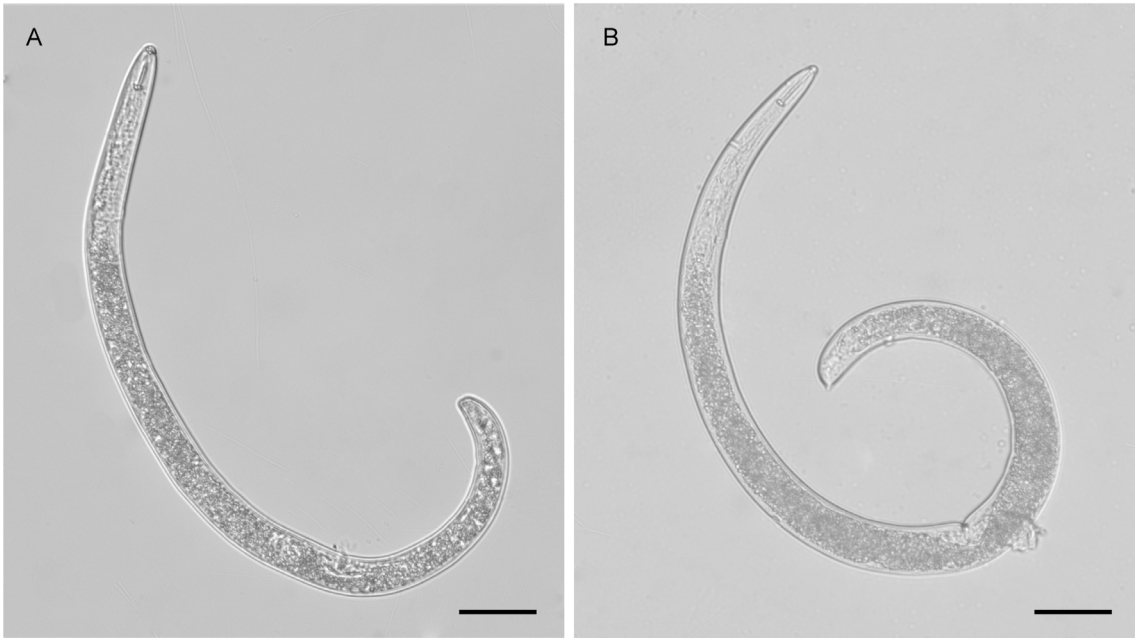

FIGURE 1. Photomicrographs of the entire body of female nematodes A: *Helicotylenchus multicinctus* and B: *H. dihystera* (Scale bar=20 μm).

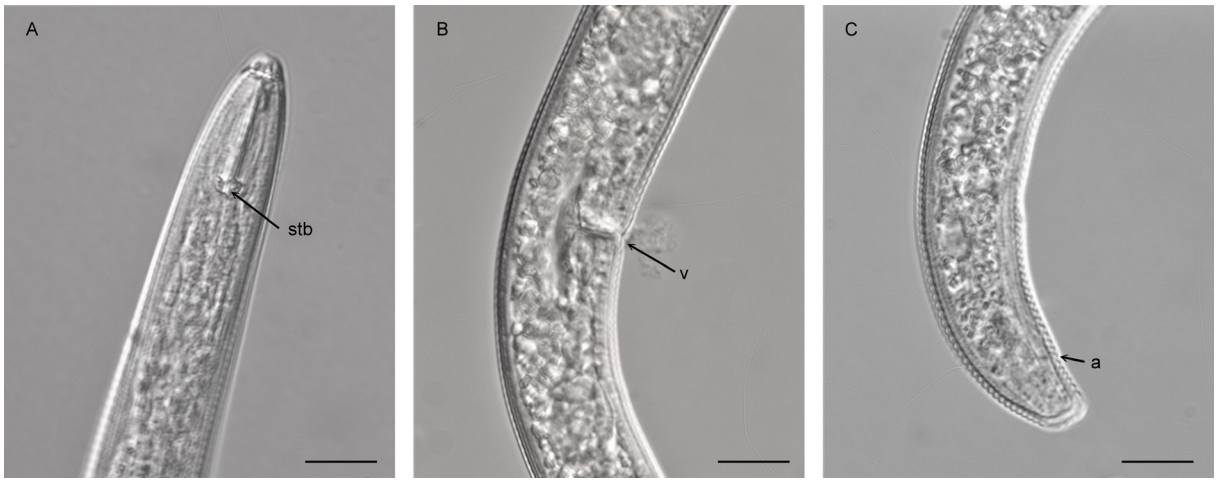

FIGURE 2. Photomicrographs *Helicotylenchus multicinctus* A–C: Head region, tail and female vulva. Stb = stylet knob; v = vulva and a = anus (Scale bar =10 μm).

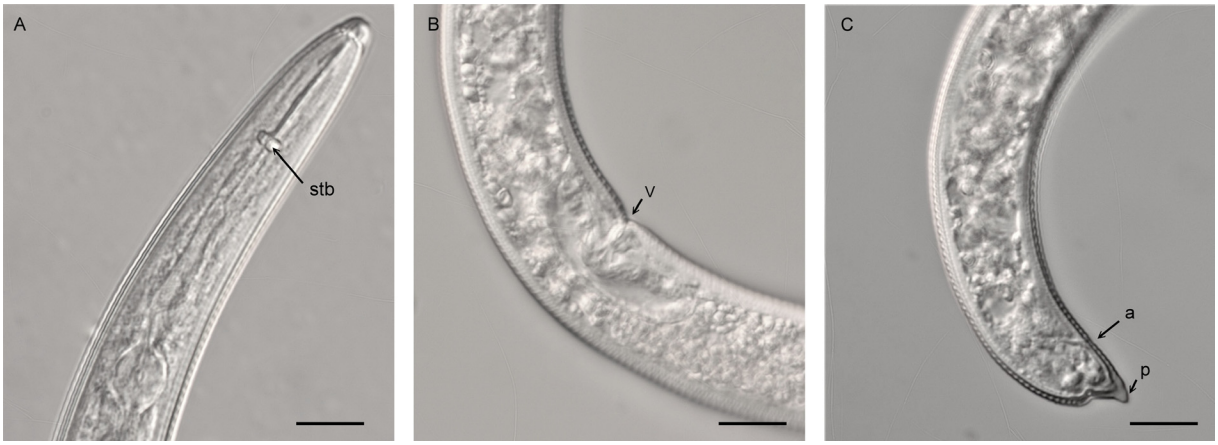

FIGURE 3. *Helicotylenchus dihystera* A–D: Head region, female vulva and tail. Stb=stylet knob; mb = median bulb; v = vulva, a = anus and p = projection (Scale bar =10 μm).

TABLE 1. Morphometrical data of *Helicotylenchus dihystera* from Nigeria. Measurements are in µm, except where stated otherwise, in the form of: mean ± standard deviation (range).

Characters	Measurements
n	10 ♀
L	642 ± 45.4 (591–744)
a	25.2 ± 3.4 (21.1–30.4)
b'	4.7 ± 0.54 (4.2–5.5)
c	41.8 ± 5.9 (35.1–53.8)
c'	1.1 ± 0.1 (0.9–1.3)
o	43 ± 6.2 (33–49)
Stylet length	27 ± 0.6 (26–28)
m	47 ± 0.8 (46–48)
V	63 ± 1.8 (61–66)

Helicotylenchus multicinctus (Cobb, 1893) Golden, 1956

= *Tylenchus multicinctus* Cobb, 1893
= *Tylenchorhynchus multicinctus* (Cobb, 1893) Micoletzky, 1922
= *Anguillulina multicincta* (Cobb, 1893) Goodey, 1932
= *Rotylenchus multicinctus* (Cobb, 1893) Filipjev, 1936
= *Rotylenchus iperoiguensis* Carvalho, 1956
= *Helicotylenchus iperoiguensis* (Carvalho, 1956) Andrássy, 1958

Description. Habitus spiral. Lip region hemispherical, slightly offset, with three to five annuli. Labial framework heavily sclerotized. Outer margins conspicuous and extending posteriorly for 3 to 4 body annules. Cephalids not seen. Stylet well developed (21–24) µm. Stylets knobs prominent (5–6 µm), wide, appearing anteriorly flattened or concave. Position of DGO about 1/4 the total stylet length and posteriorly located behind the stylet knobs. Median bulb round to oval, with a small, centrally placed valve. Glandular part of pharynx overlapping the intestine ventrally. Excretory pore close to pharyngo-intestinal junction. Epiptygma folded into vagina. Hemizonid two to three annules long, located 0–3 annules anterior to excretory pore. Ovaries paired and symmetrical, posterior branch slightly reduced than the anterior. Spermathecae slightly offset, rounded and filled with sperms. Vulva a depressed transverse slit at 67% of the body length. Phasmids pore-like and located from one to six annules anterior to anus level. Tail slightly tapering and curved more dorsally than ventrally, with six to fifteen annules. Ventral caudal projection and mucro absent.

Remarks. The morphological and morphometric characters of this species are consistent with those previously reported in the original description of *H. multicinctus* from Nigeria (Sher 1966, Siddiqi 1972, Ali *et al.* 1973).

Helicotylenchus dihystera (Cobb, 1893) Sher, 1961

= *Tylenchus dihystera* Cobb, 1893
= *Tylenchus olaae* Cobb, 1906
= *Tylenchorhynchus olaae* (Cobb, 1906) Micoletzky, 1922
= *Helicotylenchus olaae* (Cobb, 1906) Sher, 1961
= *Aphelenchus dubius* var. *peruensis* Steiner, 1920
= *Tylenchus spiralis* Cassidy, 1930
= *Helicotylenchus spiralis* (Cassidy, 1930) Sher, 1961
= *Helicotylenchus nannus* Steiner, 1945
= *Helicotylenchus crenatus* Das, 1960
= *Helicotylenchus dihysteroides* Siddiqi, 1972
= *Helicotylenchus flatus* Román, 1965
= *Helicotylenchus glissus* Thorne & Malek, 1968
= *Helicotylenchus rotundicauda* Sher, 1966

= *Helicotylenchus punicae* Swarup & Sethi, 1968
= *Helicotylenchus teleductus* Anderson, 1974
= *Helicotylenchus paraconcavus* Rashid & Khan, 1974
= *Helicotylenchus membranatus* Hui & Zhixin, 1993

Description. *Female* (n = 10): Habitus spiral. Lip 4 ± 0.6 (4–5) µm high and 7 ± 0.8 (6–9) µm wide; rounded anteriorly, with five to six annuli. Outer margins of labial framework extend 4 ±1.9 (3–6) µm posterior from basal plate. Cephalids not seen. Stylet knobs 2 ± 0.7 (2–4) µm high and 5 ± 0.5 (4–5) µm wide, rounded posteriorly, anterior faces indented. Position of DGO 12 ± 1.6 (9–14) µm behind stylet knobs. Median bulb oval, 12 ± 1.3 (10–14) µm long and 9 ± 0.9 (8–10) µm wide; valve 3 µm long and 3 µm wide. Excretory pore 98 ± 4.1 (93–1047) µm from front, i.e. at 15 ± 1.0 (13–16) % of body length. Hemizonid two annuli long, located one annule anterior to excretory pore *(n =1).* Hemizonion and fasciculi not observed. Width of annuli at midbody 1.3 ± 0.2 (1.0–1.7) µm. Body width at excretory pore 19 ± 2.8 (15–23) µm, at midbody 25 ± 3.5 (21–30) µm and at anus 16 ± 2.8 (12–20) µm. Epiptygma folded into vagina. Lateral field 6 ± 1.1 (6–8) µm wide; areolated anterior to excretory pore; inner two lines end on tail in a y-shaped pattern. Caudalid one annulus long located one annulus posterior to anus *(n=1).* Phasmid located from three eleven annuli anterior to anus. Tail 16 ± 1.8 (13–18) µm long, with 10 to 13 ventral annuli; tail asymmetrically rounded, and dorsally curved, with or without ventral projection, if present with rounded end.

Male: Not found.

Remarks. Our specimens were identified as *H. dihystera* because of spiral habitus, stylet length (mean value: 27 µm), body length (mean value: 641 µm), position of vulva (mean V value: 63 %), phasmids anterior to anus, inner lines of lateral field end on tail mostly in y-shaped pattern, tail asymmetrically rounded, with or without terminal projection (if present with rounded end), empty spermatheca, and absence of males (Fortuner *et al.* 1981). The present specimens agree with the amended descriptions of Fortuner *et al.* (1981) and Fortuner (1991). The stylet length for the current specimens exceeds that of the 24–25 µm reported for Nigerian specimens by Ali *et al*. (1973), but still falls in the range for *H. dihystera* (20–31 µm) as reported by Marais (2001).

Phylogenetic analysis

In our DNA sequence analysis, the alignments of the 28S rDNA sequence of the newly obtained sequences of *H. multicinctus* showed 99% similarity to other sequences of *H. multicinctus* that were obtained via NCBI from Florida, China, Vietnam, Sudan, Greece and South Africa**,** differing in 1 to 4 nucleotide bp and with zero indels. However, for the *H. dihystera* specimen*,* high variation was observed when compared with *H. dihystera* sequences that are available from the NCBI database. Our sequence was closest to those obtained from China with similarities ranging from 94.47% to 95.13% with 32 nucleotide bp difference (Table 2). Estimates of the evolutionary divergence between sequences of the Nigerian isolates of *H. multicinctus* and *H. dihystera* with other closely related sequences is given in Table 2.

Phylogenetic relationships within the genus *Helicotylenchus,* as inferred using maximum parsimony (MP) and maximum likelihood (ML), are given in Figures 4 and 5 respectively. The sequence alignment was 720 base pairs long and comprised 78 nucleotide sequences of *Helicotylenchus* spp., with *Caenorhabditis elegans* Maupas, 1900 as the outgroup. Evolutionary history was inferred using the MP method and the most parsimonious tree, with length of 697, was compared with the ML tree. There is congruence between the two trees and both supported nine clades of Subbotin *et al*. (2011).

Our phylogenetic tree contains nine distinct clades, with Clade I containing 23 sequences of *H. dihystera*. Clade II includes *H. pseudorobustus* (Steiner, 1914) Golden, 1956*, H. digonicus* Perry in Perry, Darling & Thorne, 1959*, H. platyurus* Siddiqi, 1972, and *H. leiocephalus* Sher, 1966*.* Clades III and IV include *Helicotylenchus* sp. III (from Spain and Burkina Faso) and *Helicotylenchus* sp. IV respectively. Clade V consists of *H. vulgaris* Yuen, 1964, *H. digonicus* and the Nigerian population of *H. dihystera*. Clade VI is made up of seven sequences of *H. multicinctus*. Clade VII includes six sequences of *H. labiodioscinus* Sher, 1966. Clade VIII includes *H. brevis* (Whitehead, 1958) Fortuner, 1984 and an unidentified *Helicotylenchus* species while clade IX includes *H. martini* Sher, 1966 and an unidentified species from California (Figure 5). The newly obtained *H. multicinctus* and *H. dihystera* sequences are indicated.

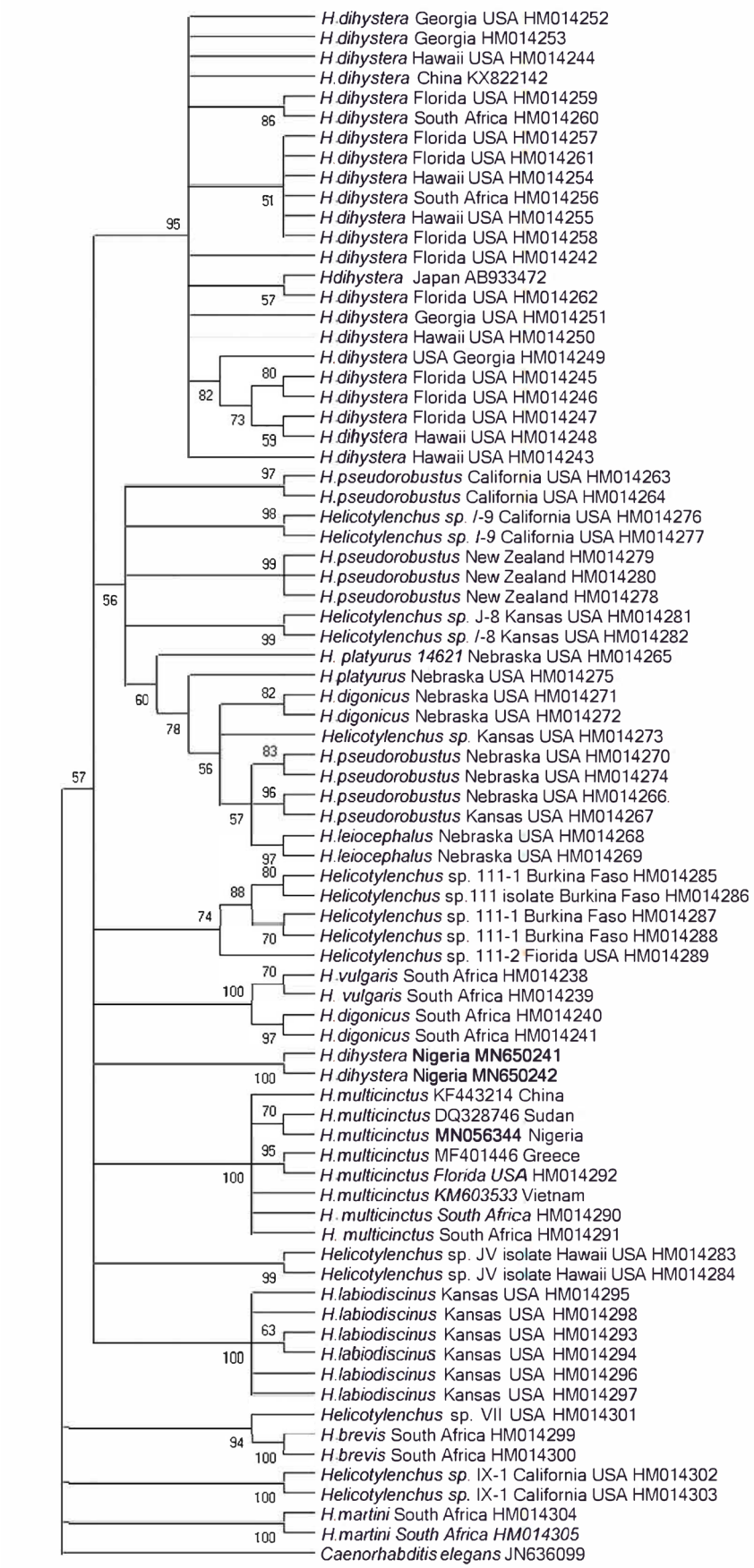

FIGURE 4. Phylogenetic relationships among *Helicotylenchus* species, based on analysis of the 28S D2D3 region with maximum Parsimony (MP), using *Caenorhabditis elegans* as the outgroup. Newly obtained sequences are indicated by bold letters.

TABLE 2. Estimates of evolutionary divergence between sequences of *H. multicinctus* and *H. dihystera*

	Nematode species	D2D3	Expansion region																																					
1	H.dihystera_Nigeria_MN650241	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39
2	H.dihystera_Nigeria_MN650242	1																																						
3	H.dihystera_Hawaii_USA_HM014243	31	32																																					
4	Hdihystera_KX822142_(China)	32	33	3																																				
5	H.dihystera_Hawaii_USA_HM014255	31	32	4	1																																			
6	H.dihystera_South_Africa_HM014256	31	32	4	1	0																																		
7	H.dihystera_Hawaii_USA_HM014254	32	33	5	2	1	1																																	
8	H.dihystera_Florida_USA_HM014258	32	33	5	2	1	1	2																																
9	H.dihystera_Florida_USA_HM014242	32	33	2	3	4	4	5	5																															
10	H.dihystera_Florida_USA_HM014257	32	33	5	2	1	1	2	2	5																														
11	H.pseudorobustus_USA_HM014263	33	33	13	14	13	13	14	14	15	14																													
12	H.dihystera_Georgia_USA_HM014251	33	34	4	3	4	4	5	5	4	5	15																												
13	H.dihystera_Florida_USA_HM014262	33	34	6	5	4	4	5	5	6	5	13	6																											
14	H.digonicus_South_Africa_HM014240	33	33	33	34	33	33	34	34	34	34	26	35	33																										
15	H.vulgaris_South_Africa_HM014238	33	33	32	33	32	32	33	33	33	33	25	34	32	6																									
16	H.vulgaris_South_Africa_HM014239	33	33	34	35	34	34	35	35	35	35	27	36	34	6	4																								
17	H.digonicus_South_Africa_HM014241	33	33	33	34	33	33	34	34	34	34	26	35	33	0	6	6																							
19	H.dihystera_Florida_USA_HM014261	33	34	6	3	2	2	3	3	6	3	15	6	6	35	34	36	35																						
20	H.dihystera_South_Africa_HM014260	34	35	5	2	3	3	4	4	5	4	16	5	7	36	35	37	36	3																					
21	H.dihystera_Hawaii_USA_HM014250	34	35	5	4	5	5	6	6	5	6	14	3	5	32	33	35	32	7	6																				
22	H.dihystera_Florida_USA_HM014259	34	35	5	2	3	3	4	4	5	4	16	5	7	36	35	37	36	3	0	6																			
23	H.dihystera_Hawaii_USA_HM014248	36	37	7	4	5	5	6	6	7	6	18	5	9	36	37	39	36	5	4	4	4																		
24	H.dihystera_Florida_USA_HM014245	37	38	8	5	6	6	7	7	8	7	19	6	10	37	38	40	37	6	5	5	5	1																	
25	H.dihystera_Georgia_USA_HM014252	37	38	8	5	6	6	7	7	8	7	19	6	10	38	37	39	38	8	7	7	7	7	8																
26	Hdihystera_AB933472_(Japan)	38	39	9	8	9	9	10	10	9	10	18	9	7	38	37	39	38	11	10	8	10	12	13	13															
27	H.dihystera_Hawaii_USA_HM014244	35	36	4	3	4	4	5	5	4	5	17	6	8	37	36	38	37	6	5	7	5	7	8	6	11														
29	H.dihystera_USA:_Georgia_HM014249	37	38	8	5	6	6	7	7	8	7	19	6	10	37	38	40	37	6	7	5	7	3	4	8	13	8													
30	H.dihystera_Florida_USA_HM014246	37	38	8	5	6	6	7	7	8	7	19	6	10	37	38	40	37	6	5	5	5	1	0	8	13	8	4												
31	H.dihystera_Florida_USA_HM014247	37	38	8	5	6	6	7	7	8	7	19	6	9	37	38	40	37	6	5	5	5	1	2	8	13	8	4	2											
32	H.dihystera_Georgia_HM014253	39	40	10	7	8	8	9	9	10	9	19	10	10	39	38	40	39	10	9	9	9	11	12	10	13	10	12	12	12										
33	H.multicinctus_KF443214_(China)	39	39	29	30	31	31	32	32	31	32	33	31	31	36	36	38	36	32	32	30	32	34	35	34	34	33	34	35	35	34									
34	H.multicinctus South Africa HM014291	40	40	32	33	34	34	35	35	34	35	36	34	34	39	39	41	39	35	35	33	35	37	38	37	37	36	37	38	38	37	3								
35	H.multicinctus_KM603533_(Vietnam)	41	41	31	32	33	33	34	34	33	34	35	33	33	38	38	40	38	34	34	32	34	36	37	36	36	35	36	37	37	36	2	1							
36	H.multicinctus_DQ328746_(Sudan)	41	41	33	34	35	35	36	36	35	36	37	35	35	38	38	40	38	36	36	34	36	38	39	38	38	37	38	39	39	38	4	5	4						
37	H.multicinctus_MN056344_(Nigeria)	41	41	33	34	35	35	36	36	35	36	37	35	35	38	38	40	38	36	36	34	36	38	39	38	38	37	38	39	39	38	4	3	2	2					
38	H.multicinctus_MF401446_(Greece)	42	42	32	33	34	34	35	35	34	35	36	34	34	39	39	41	39	35	35	33	35	37	38	37	37	36	37	38	38	37	3	2	1	5	3				
39	H.multicinctus_South_Africa_HM014290	42	42	32	33	34	34	35	35	34	35	36	34	34	39	39	41	39	35	35	33	35	37	38	37	37	36	37	38	38	37	5	4	3	7	5	4			
40	H.multicinctus_Florida_USA_HM014292	42	42	32	33	34	34	35	35	34	35	36	34	34	39	39	41	39	35	35	33	35	37	38	37	37	36	37	38	38	37	3	2	1	5	3	0	4		

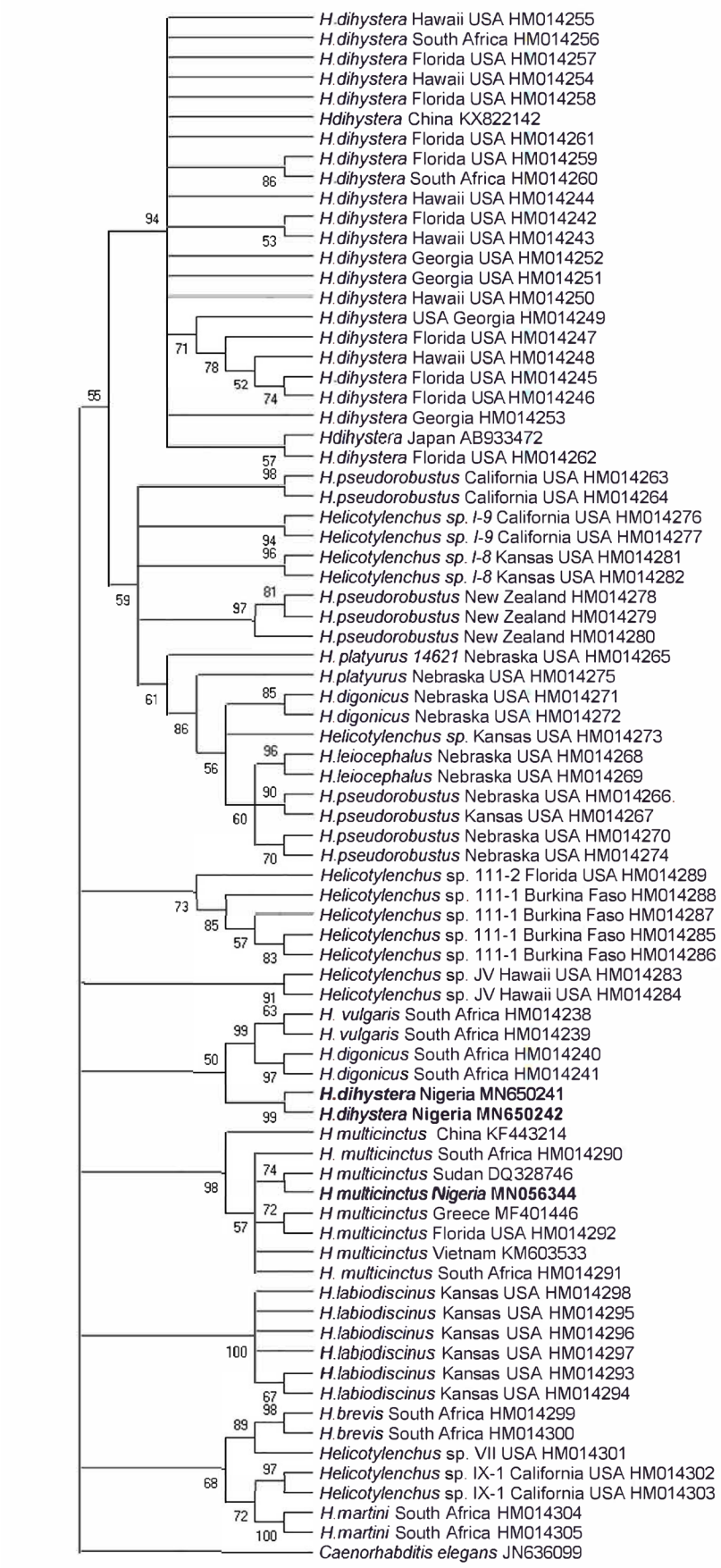

FIGURE 5. Phylogenetic relationship among *Helicotylenchus* species, based on analysis of the 28S D2D3 region with Maximum Likelihood (ML), using *Caenorhabditis elegans* as the outgroup. Newly obtained sequences are indicated by bold letters.

Both MP and ML phylogenetic trees are congruent in the clustering of the Nigerian population of *H. multicinctus* in the same clade with six other sequences of *H. multicinctus* (Subbotin *et al.* 2011). The Nigerian population of *H. dihystera* appeared in a different clade. Twenty-three sequences of *H. dihystera* (Subbotin *et al*. 2011) were used for the phylogenetic analysis, however, the Nigerian population grouped with *H. vulgaris* and *H. digonicus* in clade V (with base pair difference of 26 and 28 respectively). *Helicotylynchus vulgaris* is morphologically different from *H. dihystera* with longer stylet length (30μm) and has a ventral tail projection while tail projections are rarely seen in *H. digonicus*. From the phylogenetic analysis, it is observed that the Nigerian population is morphologically similar but genetically distant to other populations of the *H. dihystera* that are represented in the NCBI database.

Discussion

Accurate species identification is a key diagnostic tool for detecting nematode species of phytopathological importance. It is an important factor to consider when evaluating management options and phytosanitary practices for nematode pests of agricultural crops. Limitations due to inadequate facilities and trained personnel to identify nematodes to the species level has led to misidentification of many nematode species and oftentimes, important, debilitating nematode species are left unreported, especially in developing countries where identification is only taken to the genus level.

Both *H. multicinctus* and *H. dihystera* are widely distributed and have been reported from most countries of the world in association with a wide range of agricultural crops (Fortuner *et al*. 1984, Subbotin *et al*. 2011). In the current study, these two species of spiral nematode were found in high numbers under cocoa plantations that were established almost six decades ago at the Cocoa Research Institute of Nigeria (CRIN). This is consistent with the findings of Afolami and Caveness (1983) where *H. dihystera* and *H. multicinctus* were described as the most frequently encountered nematode species associated with cocoa plantations in Nigeria.

During cocoa establishment in Nigeria, a shade-providing plant is usually required and most frequently, plantain (*Musa* spp.) is used in cocoa nurseries to provide that shade. This could have contributed to the high number of *H. multicinctus* present in the soil and could have resulted in increased populations of spiral nematodes as previously reported (Afolami 1981, Orisajo *et al*. 2016). The increase in the nematode population could also have resulted from the availability of an alternative host plant, the long crop cycle and the age of the plants.

Although significant reduction in the yield of plantain has been reported due to damage caused by *H. multicinctus* (Olaniyi 2014, Tripathi *et al*. 2015), studies on the association of spiral nematodes and their damage potential on cacao has not received much attention in Nigeria. However, Afolami (1981) showed indications of potential damaging effects of the nematodes on cacao seedlings.

From our morphological observations, the two species identified were similar to *H. dihystera* and *H. multicinctus*, which have both been previously reported from Nigeria (Sher 1966, Ali *et al*. 1973, Afolami & Caveness 1983, Orisajo, 2018). There was congruence between the results of the molecular and morphological analyses of *H. multicinctus* (Marais 2001, Subbotin *et al*. 2011). The phylogenetic and DNA sequence analysis confirms the identity of the Nigerian population of *H. multicinctus,* which shows high similarity and clusters with other populations of that species collected from Florida, China, Greece, Sudan and South Africa (Tzortzakakis *et al*. 2017, Subbotin *et al*. 2011).

Sequence analysis of the partial 28S rDNA expansion segment of the Nigerian population of *H. dihystera* from the current study showed high variation in comparison with populations of *H. dihystera* from other countries deposited in GenBank. Subbotin *et al.* (2011) reported some ambiguity in molecular characterization of species within the genus *Helicotylenchus,* due to high inter- and intra- specificity and demonstrated, using a phylogenetic analysis that included 14 populations of *H. dihystera* from several tropical and subtropical countries, the possibility of misidentification of some isolates. They reported that a species initially identified morphologically as *H. dihystera* from Burkina Faso (Sawadogo *et al*. 2009) clustered with *H. multicinctus* sequences, indicating possible inter-species variation in West African populations. Intra-species variation among different populations of *H. digonicus* was also inferred (Subbotin *et al*. 2011) where the sequences formed two separate clades. Shokoohi *et al*. (2018) also reported variation in the phylogenetic position of some species of *Helicotylenchus*. The current phylogenetic analysis placed the Nigerian population of *H. dihystera* in the same clade with *H. digonicus*. *Helicotylenchus dihystera* differs morphologically from *H. digonicus* which has shorter stylet length (25 μm), stylet knob flattened anteriorly, and

hemispherical tail that rarely has a tail projection. The cephalic framework of *H. digonicus* is also well sclerotized and extends into body for 2–3 annuli (Subbotin *et al*. 2015). Stylet knobs of *H. dihystera* are anteriorly indented, the tail is asymmetrically rounded and dorsally curved, and bears a ventral projection.

While we cannot say with certainty that the Nigerian population of *H. dihystera* in the current investigation differs in other morphological characters—apart from the longer stylet length—from the population previously described from Nigeria (Ali *et al.* 1973), it is evident from the phylogenetic and DNA sequence analysis that there is significant variation among this population and those from other countries.

This study further reiterates the importance of using an integrated approach for nematode identification, especially when separating cryptic nematode species, which is often difficult due to overlapping features and plasticity. More detailed comparative studies employing morphology and morphometric data alongside phylogenetic analyses based molecular data from ribosomal and mitochondrial DNA may provide more useful information on the Nigerian population of *H. dihystera* in order to establish the phylogenetic and taxonomic status of this nematode species.

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