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Diversity and phylogenetic relationships within the spiral nematodes of *Helicotylenchus* Steiner, 1945 (Tylenchida: Hoplolaimidae) as inferred from analysis of the D2-D3 expansion segments of 28S rRNA gene sequences

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Summary – The spiral nematodes of the genus *Helicotylenchus* are globally distributed and associated with the root system of diverse groups of plants in cultivated and uncultivated areas. Several species are considered serious parasites of crops. The identification of many *Helicotylenchus* species is not always reliable, in part because many species share very similar diagnostic characters and high intraspecific variation. To verify species identification of geographically distant populations of *Helicotylenchus*, we tested monophyly of some classical morphospecies and studied their phylogenetic relationships; specifically, we conducted sequence and phylogenetic analysis of 89 sequences of the D2-D3 expansion segments of 28S rRNA gene sequences from 54 *Helicotylenchus* isolates, including species identified as *H. brevis*, *H. digonicus*, *H. dihystera*, *H. labiodiscinus*, *H. leiocephalus*, *H. martini*, *H. multicinctus*, *H. platyurus*, *H. pseudorobustus* and *H. vulgaris*, together with three outgroup taxa. Phylogenetic analysis distinguished nine highly or moderately supported major clades within *Helicotylenchus.* Using the molecular approach we were able to confirm congruence with morphological-based identification of samples of *H. dihystera* and *H. multicinctus*. However, sequence and phylogenetic analysis using Bayesian inference and maximum parsimony analysis showed that isolates collected in different countries and morphologically identified as *H. pseudorobustus*, *H. digonicus* or *H. vulgaris* were each representative of several different and, sometimes, unrelated lineages. Further detailed comparative morphometrics and morphological studies will help to elucidate if there is some misidentification or if putative species actually comprise a complex of cryptic species. Molecular analysis also revealed that 14 samples were classified as representatives of 11 unidentified species. Molecular characterisation of known *Helicotylenchus* species especially, using samples collected from type localities, is needed for future reliable identification of species of this genus.

Keywords – Bayesian inference, *Helicotylenchus digonicus*, *Helicotylenchus dihystera*, *Helicotylenchus multicinctus*, *Helicotylenchus pseudorobustus*, *Helicotylenchus vulgaris*, maximum parsimony, species delimiting.

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Helicotylenchus Steiner, 1945 is a cosmopolitan genus with more than 200 species which are commonly called spiral nematodes because of their coiled *habitus mortis* (Marais, 2001). These migratory ectoparasitic or semi-endoparasitic nematodes may occur in very high numbers feeding upon roots of diverse plants and may be abundant in soil surrounding host roots (Taylor, 1961; Norton, 1977; Krall, 1978). Species of *Helicotylenchus* are globally distributed, spanning many climates, and are associated with the root system of diverse crops of agricultural importance. Although data are not available to implicate most *Helicotylenchus* as serious parasites, plant growth suppression has been consistently associated with at least five cosmopolitan species: *H. digonicus* Perry *in* Perry, Darling & Thorne, 1959, *H. dihystera* (Cobb, 1896) Sher, 1961, *H. indicus* Siddiqi, 1963, *H. multicinctus* (Cobb, 1893) Golden, 1956 and *H. pseudorobustus* (Steiner, 1914) Golden, 1956. Other species, such as *H. cavenessi* Sher, 1966, *H. erythrinae* (Zimmermann, 1904) Golden, 1956 and *H. microcephalus* Sher, 1966, have also been implicated as potentially damaging pests (O'Bannon & Inserra, 1989). The banana spiral nematode, *H. multicinctus*, is endoparasitic and polyphagous, but it is best known for suppressing growth and yield of banana in many regions of the world (Krall, 1978; McSorley & Parrado, 1986; De Waele & Elsen, 2007). Another less known endoparasite is *H. variocaudatus* Yuen, 1964, which parasitises banana roots in the islands of São Tome and Príncipe (Vovlas *et al.*, 1995) and also in Rwanda (Van den Berg *et al.*, 2003).

Available dichotomous or polytomous identification keys to spiral nematodes (Sher, 1966; Siddiqi, 1972; Boag & Jairajpuri, 1985; Firoza & Maqbool, 1994) are especially helpful in the identification of species that have peculiar morphological characters. Such species include those with a posterior gonad less developed than the anterior one as in the case of *H. multicinctus*, which is distinguished also by a short C-shaped body, a slightly tapering tail, a hemispherical and annulated tail terminus, and numerous males (Vovlas *et al*., 1995). However, the identification of other species is not always reliable, partly because many species share very similar diagnostic characters and species boundaries are not well established. Some features have broad overlapping ranges and intraspecific variability with characters apparently influenced by environmental conditions, including the host plant (Fortuner, 1979, 1984; Fortuner & Quénéhervé, 1980; Fortuner *et al*., 1981). Although multivariate analyses can be useful in reducing the effect of intraspecific variability of morphological characters (Fortuner & Maggenti, 1991), identification of these nematodes by morphology alone often remains unresolved or uncertain due to limitations of the morphological analysis. Application of non-morphological characters such as DNA sequences can help to confirm classical morphology-based identifications and resolve some of the problems experienced in the identification of *Helicotylenchus* species.

Application of rRNA gene sequences provides an attractive solution for quick and reliable nematode diagnostics. Recently, several studies using the ITS-rDNA (Chen *et al.*, 2006), D2-D3 of 28S rRNA (Subbotin *et al.*, 2006, 2007; Bae *et al.*, 2009), and 18S rDNA (Holterman *et al.*, 2009) demonstrated the usefulness of this approach for identification of species of *Helicotylenchus*. Analysis of rRNA gene sequences (Subbotin *et al.*, 2007; Bae *et al.*, 2009; Holterman *et al.*, 2009) also provides a basis for reconstructing phylogenetic relationships within this genus. However, such a phylogeny has not been proposed previously based on morphological or molecular datasets.

The major objectives of the present study were to: *i*) to verify species identification of geographically distant populations of *Helicotylenchus* by analysing their fragments of rRNA gene sequences; *ii*) test monophyly of classical morphospecies and estimate species boundaries using rRNA gene sequences from large numbers of geographically diverse isolates; and *iii*) study phylogenetic relationships within *Helicotylenchus* using sequences from the D2-D3 expansion segments of the 28S rRNA gene as inferred from Bayesian inference and maximum parsimony approaches.

Materials and methods

Nematode populations, species identification and delimiting

Nematode populations used in this study were obtained from soil samples collected from geographically diverse locations (Table 1). The nematodes were extracted from samples using the Baermann funnel, centrifugal flotation or elutriation techniques (Hooper, 1986). Specimens were killed by gentle heat, fixed by 4% formalin, TAF or FPG and mounted in anhydrous glycerin or immobilised by gently heating and then mounting in water agar for examination (Netscher & Seinhorst, 1969; Esser, 1986). All morphological identifications of specimens, except for the South African ones, were done by using identification keys and descriptions provided by Siddiqi

Table 1. Helicotylenchus *populations and species used in the present study.*

Identification based on morphology and molecular data	Preliminary identification based on morphology	Clade (Subclade)	Location	Plant-host	Sample code	GenBank accession number	Reference or source
H. pseudorobustus, type C	*H. pseudorobustus*	I (1)	USA, Kansas, Konza Prairie, Manhattan	*Koeleria pyramidata*	14622	HM014266, HM014267	Present study, T. Powers
H. leiocephalus	*H. leiocephalus*	I (2)	USA, Nebraska, Lincoln, Nine Mile Prairie	*Amorpha canescens*	14620	HM014268, HM014269	Present study, T. Powers
H. pseudorobustus, type D	*H. pseudorobustus*	I (3)	USA, Nebraska, Lincoln, Nine Mile Prairie	*Panicum virgatum*	14619	HM014270, HM014274	Present study, T. Powers
H. digonicus, type B	*H. digonicus*	I (4)	USA, Nebraska, Nine Mile Prairie, Lincoln	*Panicum virgatum*	14623	HM014271, HM014272	Present study, T. Powers
Helicotylenchus spI-5	*Helicotylenchus* sp.	I (5)	USA, Kansas, Konza Prairie, Manhattan	*Koeleria pyramidata*	14630	HM014273	Present study, T. Powers
H. platyurus	*H. platyurus*	I (6)	USA, Nebraska, Nine Mile Prairie, Lincoln	*Amorpha canescens*	14621	HM014265, HM014275	Present study, T. Powers
H. pseudorobustus, type A	*H. pseudorobustus*	I (7)	Germany, Münster, BBA glasshouse	–	590	DQ328751	Subbotin *et al.* (2006)
H. pseudorobustus, type A	*H. labiatus*	I (7)	New Zealand, Rotorua	*Lolium perenne*	CD256	HM014279, HM014280	Present study, G. Yeates
H. pseudorobustus, type A	*Helicotylenchus* sp.	I (7)	New Zealand, MAF farm, Kaitoke	*Lolium perenne*, *Trifolium repens*	N453; CD428	HM014278	Present study, G. Yeates
Helicotylenchus spI-8	*Helicotylenchus* sp.	I (8)	USA, Konza Prairie, Manhattan, Kansas	*Andropogon bladhi*	14628	HM014281, HM014282	Present study, T. Powers
Helicotylenchus spI-9	*Helicotylenchus* sp.	I (9)	USA, California, Death Valley	Grasses	CD363	HM014276, HM014277	Present study, S.A. Subbotin
Helicotylenchus spI-10	*Helicotylenchus* sp.	I (10)	USA, California	–	4I22G4	DQ077794	De Ley *et al.* (2005)
H. pseudorobustus, type B	*H. pseudorobustus*	I (11)	USA, Illinois, University of Illinois	Turfgrass	ILC171	FJ485649	Bae *et al.* (2009)
H. pseudorobustus, type B	*H. pseudorobustus*	I (11)	USA, California, Fresno	*Zea mays*	C599	HM014263, HM014264	Present study, S.A. Subbotin
H. pseudorobustus, type B	*H. pseudorobustus*	I (11)	Italy, Ancona	–	727	DQ328750	Subbotin *et al.* (2007)
H. pseudorobustus, type B	*H. pseudorobustus*	I (11)	China, Beijing	–	718	DQ328747, DQ328749	Subbotin *et al.* (2007)
H. pseudorobustus, type B	*H. pseudorobustus*	I (11)	USA, California, Fresno	–	CA4	DQ328748	Subbotin *et al.* (2007)
H. dihystera	*Helicotylenchus* sp.	II	USA, Florida, Ft Lauderdale	*Schefflera arboricola*	CD603	HM014245	Present study, S.A. Subbotin

Table 1. *(Continued).*

Identification based on morphology and molecular data	Preliminary identification based on morphology	Clade (Subclade)	Location	Plant-host	Sample code	GenBank accession number	Reference or source
H. dihystera	*Helicotylenchus* sp.	II	USA, Florida, Goulds	Bromeliads	CD508	HM014242, HM014246	Present study, R. Inserra
H. dihystera	*Helicotylenchus* sp.	II	USA, Florida, Ft Pierce	*Ficus benjamina*	CD423	HM014247	Present study, S.A. Subbotin
H. dihystera	*Helicotylenchus* sp.	II	USA, Hawaii, Kawai	Grasses	CA157	HM014248	Present study, S.A. Subbotin
H. dihystera	*H. rotundicauda*	II	USA, Georgia, University of Georgia, Athens	–	14627	HM014249, HM014252	Present study, T. Powers
H. dihystera	*Helicotylenchus* sp.	II	USA, Hawaii, Kawai	Grasses	CA152	HM014243, HM014250	Present study, S.A. Subbotin
H. dihystera	*Helicotylenchus* sp.	II	USA, Florida, Lake Worth	*Ficus* sp.	CD359	HM014258, HM014259	Present study, S.A. Subbotin
H. dihystera	*H. dihystera*	II	South Africa, Mpumalanga Province, Nelspruit	*Psidium* sp.	Tv1947; CD385	HM014256, HM014260	Present study, M. Marais
H. dihystera	*H. dihystera*	II	USA, Florida, Apopka	*Neoregelia* sp.	CD616	HM014261, HM014262	Present study, S.A. Subbotin
H. dihystera	*Helicotylenchus* sp.	II	USA, Hawaii, Maui	–	CD617	HM014244, HM014254	Present study, S.A. Subbotin
H. dihystera	*H. rotundicauda*	II	USA, Georgia, University of Georgia, Athens	–	14626	HM014251, HM014253	Present study, T. Powers
H. dihystera	*H. dihystera*	II	USA, Florida, Homestead	Bromeliads	CD600	HM014257	Present study, S.A. Subbotin
H. dihystera	*Helicotylenchus* sp.	II	USA, Hawaii, Kawai	Grasses	CA150	HM014255	Present study, S.A. Subbotin
H. dihystera	*H. dihystera*	II	USA, Georgia, UGA Research Station, Midville	–	GAC177	FJ485651	Bae *et al.* (2009)
Helicotylenchus spIII-1	*H. dihystera*	III (1)	Burkina Faso, Leguéma	*Vigna unguiculata*	CA172	HM014285, HM014286	Present study, P. Roberts
Helicotylenchus spIII-1	*H. dihystera*	III (1)	Burkina Faso, Farako-Bâ	*Vigna unguiculata*	CA175	HM014287, HM014288	Present study, P. Roberts
Helicotylenchus spIII-1	*H. microlobus*	III (1)	USA, Florida, Ft Lauderdale	*Stenotaphrum secundatum*	FLC180	FJ485648	Bae *et al.* (2009)
Helicotylenchus spIII-2	*Helicotylenchus* sp.	III (2)	USA, Florida, Goulds	*Agave* sp.	CD601	HM014289	Present study, S.A. Subbotin
H. multicinctus	*H. multicinctus*	III (3)	Sudan	*Musa* sp.	–	DQ328745, DQ328746	Subbotin *et al.* (2007)
H. multicinctus	*H. multicinctus*	III (3)	South Africa, Limpopo Province, Lambani	*Musa* sp.	Tv1957; CD511	HM014290, HM014291	Present study, M. Marais
H. multicinctus	*H. multicinctus*	III (3)	USA, Florida, Ft Pierce	*Ficus benjamina*	CD423	HM014292	Present study, S.A. Subbotin

Table 1. *(Continued).*

Identification based on morphology and molecular data	Preliminary identification based on morphology	Clade (Subclade)	Location	Plant-host	Sample code	GenBank accession number	Reference or source
Helicotylenchus spIV	*Helicotylenchus* sp.	IV	USA, Hawaii, Kawai	Grasses	CA157	HM014284	Present study, S.A. Subbotin
Helicotylenchus spIV	*Helicotylenchus* sp.	IV	USA, Hawaii, Kawai	Grasses	CA150	HM014283	Present study, S.A. Subbotin
H. vulgaris, type B	*H. vulgaris*	V (1)	South Africa, Limpopo Province, Lambani	Grasses	Tvl1987; CD620	HM014238, HM014239	Present study, M. Marais
H. digonicus, type C	*H. digonicus*	V (2)	South Africa, Limpopo Province, Koedoeskop	*Glycine max*	Tvl1949; CD382	HM014240, HM014241	Present study, M. Marais
H. labiodiscinus	*H. digonicus*	VI	USA, Kansas, Konza Prairie, Manhattan	*Poa pratensis*	14625	HM014293, HM014298	Present study, T. Powers
H. labiodiscinus	*H. labiodiscinus*	VI	USA, Kansas, Konza Prairie, Manhattan	*Schizachyrium scoparium*	14631	HM014295, HM014296	Present study, T. Powers
H. labiodiscinus	*H. labiodiscinus*	VI	USA, Kansas, Konza Prairie, Manhattan	*Andropogon gerardii*	14629	HM014294, HM014297	Present study, T. Powers
H. brevis	*H. brevis*	VII (1)	South Africa, North West Province. Magaliesberg, near Maretlwane River	*Solanum mauritianum*	Tv1969; CD556	HM014299, HM014300	Present study, M. Marais
Helicotylenchus spVII	*Helicotylenchus* sp.	VII (2)	USA	–	CD347	HM014301	Present study, S.A. Subbotin
H. martini	*H. martini*	VIII	South Africa, Limpopo Province, Lambani	Grasses	Tvl1984; CD613	HM014304, HM014305	Present study, M. Marais
Helicotylenchus spIX-1	*Helicotylenchus* sp.	IX (1)	USA, California, Solano, Winters	*Juglans* sp.	CD505	HM014302, HM014303	Present study, S.A. Subbotin
H. digonicus, type A	*H. digonicus*	IX (2)	Italy	–	723	DQ328758	Subbotin *et al.* (2007)
Helicotylenchus spIX-3	*Helicotylenchus* sp.	IX (3)	Belgium, Ghent	–	LG43	DQ328754	Subbotin *et al.* (2007)
Helicotylenchus spIX-4	*Helicotylenchus* sp.	IX (4)	Russia, Moscow region	–	RU21	DQ328755	Subbotin *et al.* (2007)
H. vulgaris, type A	*H. vulgaris*	IX (5)	USA, Arkansas, University of Arkansas	–	KrC210	FJ485650	Bae *et al.* (2009)
H. vulgaris, type A	*H. vulgaris*	IX (5)	Italy, Ancona	–	AI36, AI13	DQ328759-DQ328761	Subbotin *et al.* (2007)

(1972), Krall (1978), Anderson and Eveleigh (1982), Boag and Jairajpuri (1985) and Firoza and Maqbool (1994). The South African materials were identified using the relevant species descriptions without the use of any of published diagnostic keys.

For some populations, species were delimited and defined based on an integrated approach that considered morphological evaluation combined with molecular-based phylogenetic inference (tree based methods) and sequence analyses (genetic distance methods) (Sites & Marshall, 2004).

DNA EXTRACTION, PCR, CLONING AND SEQUENCING

Nematode DNA was extracted from several individuals using proteinase K. Detailed protocols for DNA extraction and PCR were as described by Tanha Maafi *et al.* (2003). The forward D2A (5′-ACAAGTACCGTGAGGGAAAG TTG-3′) and reverse D3B (5′-TCGGAAGGAACCAGCT ACTA-3′) primers were used for amplification and sequencing of the fragment of D2-D3 regions of the 28S rRNA gene (Subbotin *et al.*, 2006). PCR products were purified using QIAquick (Qiagen, Valencia, CA, USA) gel extraction kits and then cloned using pGEM-T Vector System II kit (Promega, Madison, WI, USA). One or two clones were sequenced from each sample. The resulting products were purified and run on a DNA sequencer at the University of California, Riverside, Genomics Center. The newly obtained sequences have been submitted to the GenBank database under accession numbers indicated in Table 1.

SEQUENCE AND PHYLOGENETIC ANALYSES

The newly obtained sequences were aligned using ClustalX (Thompson *et al*., 1997) with default parameters and with sequences published for *Helicotylenchus* in GenBank (De Ley *et al*., 2005; Subbotin *et al.*, 2007; Bae *et al*., 2009) and with *Rotylenchus magnus* Zancada, 1985, *Hoplolaimus galeatus* (Cobb, 1913) Thorne, 1935 and *H. seinhorsti* Luc, 1958 used as outgroup taxa (Subbotin *et al.*, 2007; Bae *et al.*, 2008; Vovlas *et al.*, 2008). Sequence and phylogenetic analysis of the dataset was performed with Bayesian inference (BI) using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001) and maximum parsimony (MP) using PAUP* 4b10 (Swofford, 2003). BI analysis under the GTR + I + G model was initiated with a random starting tree and was run with four chains for 1.0×10^6 generations. The Markov chains were sampled at intervals of 100 generations. Two runs were performed for each analysis. The log-likelihood values of the sample points stabilised after approximately 10^3 generations. The topologies were used to generate a 50% majority rule consensus tree. Posterior probabilities (PP) are given on appropriate clades. For MP we used a heuristic search setting with ten replicates of random taxon addition (max. tree number = 1000), tree bisection-reconnection branch swapping to seek the most parsimonious trees. Gaps were treated as missing data. To obtain an estimate of support for each node, a bootstrap analysis (BS) with 100 replicates (max tree number = 100) was done. Sequence differences between samples were calculated with PAUP* 4b10 as an absolute distance matrix and the percentage was adjusted for missing data.

Results

SPECIES IDENTIFICATION AND DELIMITING

Eighty-six sequences from 54 *Helicotylenchus* isolates were included in the analysis. Sixty-eight sequences were newly obtained in the present study. Using traditional morphological taxonomic characters and molecular criteria (apomorphies and DNA distances), we distinguished the following species within the samples: *Helicotylenchus brevis* (Whitehead, 1958) Fortuner, 1960, *H. digonicus*, *H. dihystera*, *H. labiodiscinus* Sher, 1966, *H. leiocephalus* Sher, 1966, *H. martini* Sher, 1966, *H. multicinctus*, *H. platyurus* Perry *in* Perry, Darling & Thorne, 1959, *H. pseudorobustus* and *H. vulgaris* Yuen, 1964. Several samples, which were identified morphologically as representative of the same species, showed differences in molecular characteristics, and were thus classified as different species types: *H. pseudorobustus* type 'A', 'B', 'C' and 'D', *H. vulgaris* type 'A' and 'B' and *H. digonicus* type 'A' and 'B'. Fourteen samples were classified as representatives of 11 unidentified species. More detailed morphological and molecular analysis is required to further evaluate and identify these samples. Sequence and phylogenetic analysis confirmed that each analysed sample used in the present study contained representatives of a single species only. One exception, collected in Kawaii Island, included a mixture of specimens with *H. dihystera* and *Helicotylenchus* spIV.

Sequence analysis

Amplification of D2-D3 of the 28S rRNA gene using PCR produced a single fragment of *ca* 680 bp for the samples studied. The sequence alignment for *Helicotylenchus* and outgroup taxa included 89 sequences and was 596 bp in length. Sequence diversity within all studied taxa including outgroup taxa reached 20.7% (118 nucleotides (nt)) and for *Helicotylenchus* it reached 19.9% (115 nt). Minimal interspecific sequence variation was observed for taxa belonging to clades I, III, V and IX (Figs 1, 2). Intraspecific sequence diversity varied for *H. pseudorobustus* type A from 0.2-0.5% (1-3 nt), *H. pseudorobustus* type B from 0-0.5% (0-3 nt), *H. labiodiscinus* from 0.5-1.5% (3-9 nt), *H. multicinctus* from 0.5-1.0% (3-6 nt), *H. vulgaris* type A from 0.3-0.9% (2-5 nt), and *H. dihystera* from 0-2.3% (0-13 nt). Heterogeneity was observed for many taxa among sequenced clones originated from the same PCR product. The largest difference was found between two sequenced clones for a *H. martini* sample, which reached 6% (35 nt).

Phylogenetic analysis

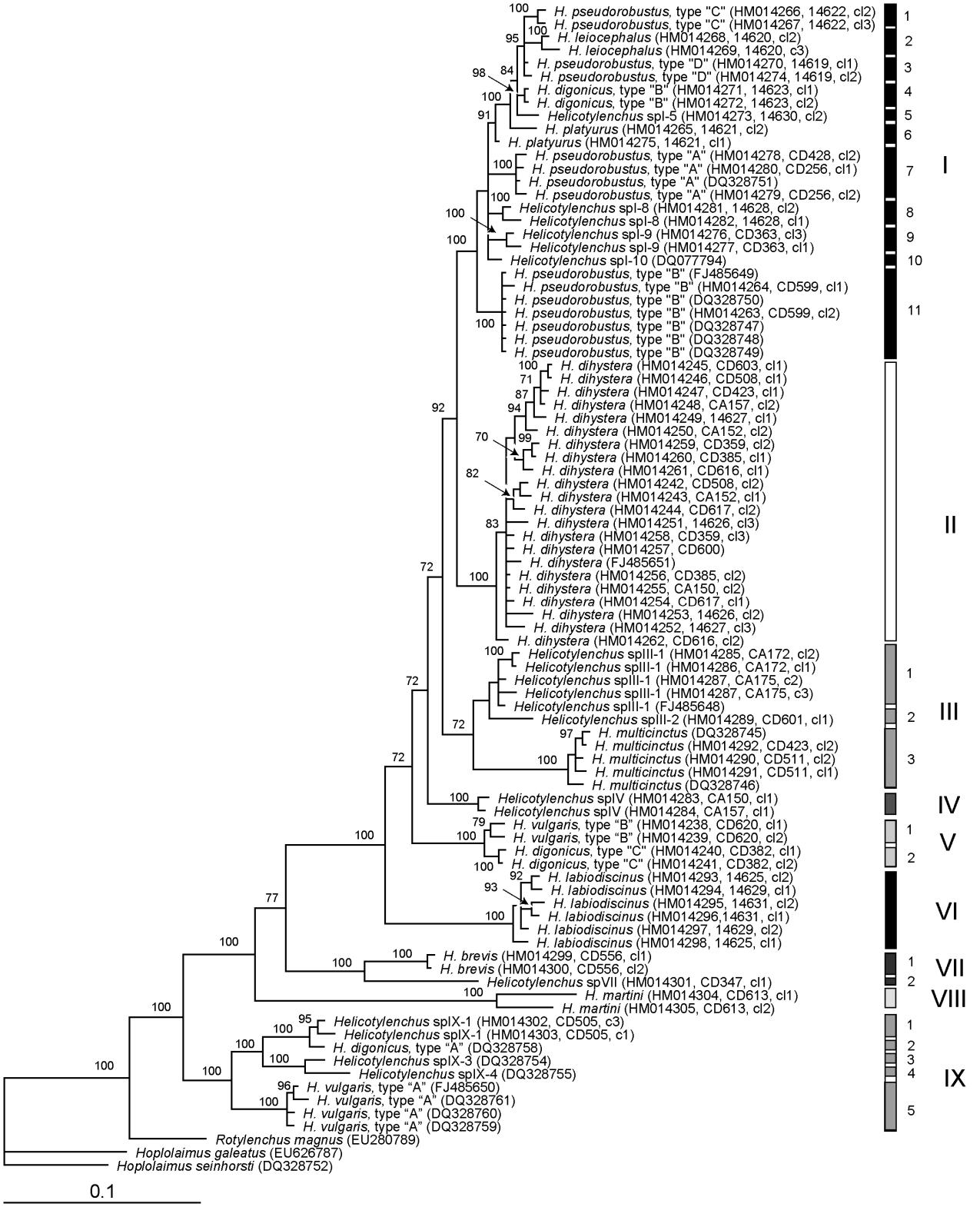
Phylogenetic relationships within *Helicotylenchus* as inferred from Bayesian inference and maximum parsimony are given in Figures 1 and 2, respectively. Topologies of BI and MP trees were congruent, except for positions of some weakly supported clades. Nine highly or moderately supported major clades were distinguished within *Helicotylenchus.* Clade I (PP = 100%, BS = 74%) and included 11 putative taxa as follows: *H. pseudorobustus* type A, B, C and D, *H. leiocephalus*, *H. digonicus* type B, *H. platyurus*, *Helicotylenchus* spI-5, spI-8, spI-9 and spI-10. Clade II (PP = 100%, BS = 97%) consisted of 22 sequences obtained from 14 samples identified here as *H. dihystera*. Clade III (PP = 72%, BS < 50%) included five sequences of *H. multicinctus* and sequences from two unidentified *Helicotylenchus* taxa (*Helicotylenchus* spIII-1 and spIII-2). Clade IV (PP = 100%, BS = 100%) included only one unidentified *Helicotylenchus* sample (*Helicotylenchus* spIV). Clade V (PP = 100%, BS = 100%) contained four sequences from samples identified as *H. vulgaris* type B and *H. digonicus* type C. The highly supported clades VI and VIII each included only a single taxon, *H. labiodiscinus* and *H. martini*, respectively. Clade VII (PP = 100%; BS = 97%) consisted of *H. brevis* and an unidentified *Helicotylenchus* sample (*Helicotylenchus* spVII). Clade IX (PP = 100%; BS = 96%) included *H. vulgaris* type A, *H. digonicus* type A, and three unidentified *Helicotylenchus* samples (*Helicotylenchus* spIX-1, spIX-3, spIX-4).

Discussion

Integrated approach for *Helicotylenchus* systematics

Identification of *Helicotylenchus* species is often not an easy task because of high intra- and interspecific variability and a large number of poorly described species (Fortuner, 1979, 1984; Fortuner & Quénéhervé, 1980). Various authors have published dichotomous keys for *Helicotylenchus*, but none of these keys is reliable for species diagnostics (Fortuner & Wong, 1984). To try to overcome the inherent flaws of dichotomous keys a number of compendia have been published. However, compendia, like keys, rapidly become outdated (Boag & Jairajpuri, 1985; Firoza & Maqbool, 1994; Vovlas *et al*., 1995). The use of such diagnostic keys and compendia can consequently lead to unresolved or uncertain identification of *Helicotylenchus* species.

Phylogenetic and DNA sequence analyses of nematode samples provide additional criteria for identifying and delimiting species within *Helicotylenchus*. Our findings show that there was congruence between the results of the molecular and morphological analyses of *H. dihystera* and *H. multicinctus*. However, the morphological identification of a large number of the spiral nematodes studied seems not to be reliable. For example, the preliminary results comparing morphological identification from different nematology laboratories failed to delimit species boundaries and conflicted with results based on a molecular approach. Common species collected and morphologically identified from different countries as *H. pseudorobustus*, *H. vulgaris* or *H. digonicus* were assessed as being different and often not closely related taxa when they were subjected to molecular analysis. In this study we provisionally distinguished such samples by a letter code: *H. pseudorobustus* type A, B, C and D, *H. vulgaris* type A and B and *H. digonicus* type A and B. Comparative detailed morphometrics and morphological studies can help to elucidate if there is some misidentification or if each of these putative species is actually comprised of a complex of cryptic species. Identification of these samples will be possible after careful molecular and morphological characterisation of type representatives of these species, including new material collected from the type localities. Similarly, several samples each were identified as representatives of *H. leiocephalus*, *H. platyurus*, *H. labiodisci-*


H. pseudorobustus, type "C" (HM014266, 14622, cl2)
H. pseudorobustus, type "C" (HM014267, 14622, cl3)
H. leiocephalus (HM014268, 14620, cl2)
H. leiocephalus (HM014269, 14620, c3)
H. pseudorobustus, type "D" (HM014270, 14619, cl1)
H. pseudorobustus, type "D" (HM014274, 14619, cl2)
H. digonicus, type "B" (HM014271, 14623, cl1)
H. digonicus, type "B" (HM014272, 14623, cl2)
Helicotylenchus spI-5 (HM014273, 14630, cl2)
H. platyurus (HM014265, 14621, cl2)
H. platyurus (HM014275, 14621, cl1)
H. pseudorobustus, type "A" (HM014278, CD428, cl2)
H. pseudorobustus, type "A" (HM014280, CD256, cl1)
H. pseudorobustus, type "A" (DQ328751)
H. pseudorobustus, type "A" (HM014279, CD256, cl2)
Helicotylenchus spI-8 (HM014281, 14628, cl2)
Helicotylenchus spI-8 (HM014282, 14628, cl1)
Helicotylenchus spI-9 (HM014276, CD363, cl3)
Helicotylenchus spI-9 (HM014277, CD363, cl1)
Helicotylenchus spI-10 (DQ077794)
H. pseudorobustus, type "B" (FJ485649)
H. pseudorobustus, type "B" (HM014264, CD599, cl1)
H. pseudorobustus, type "B" (DQ328750)
H. pseudorobustus, type "B" (HM014263, CD599, cl2)
H. pseudorobustus, type "B" (DQ328747)
H. pseudorobustus, type "B" (DQ328748)
H. pseudorobustus, type "B" (DQ328749)
H. dihystera (HM014245, CD603, cl1)
H. dihystera (HM014246, CD508, cl1)
H. dihystera (HM014247, CD423, cl1)
H. dihystera (HM014248, CA157, cl2)
H. dihystera (HM014249, 14627, cl1)
H. dihystera (HM014250, CA152, cl2)
H. dihystera (HM014259, CD359, cl2)
H. dihystera (HM014260, CD385, cl1)
H. dihystera (HM014261, CD616, cl1)
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H. dihystera (HM014243, CA152, cl1)
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H. dihystera (HM014257, CD600)
H. dihystera (FJ485651)
H. dihystera (HM014256, CD385, cl2)
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H. dihystera (HM014254, CD617, cl1)
H. dihystera (HM014253, 14626, cl2)
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H. dihystera (HM014262, CD616, cl2)
Helicotylenchus spIII-1 (HM014285, CA172, cl2)
Helicotylenchus spIII-1 (HM014286, CA172, cl1)
Helicotylenchus spIII-1 (HM014287, CA175, c2)
Helicotylenchus spIII-1 (HM014287, CA175, c3)
Helicotylenchus spIII-1 (FJ485648)
Helicotylenchus spIII-2 (HM014289, CD601, cl1)
H. multicinctus (DQ328745)
H. multicinctus (HM014292, CD423, cl2)
H. multicinctus (HM014290, CD511, cl2)
H. multicinctus (HM014291, CD511, cl1)
H. multicinctus (DQ328746)
Helicotylenchus spIV (HM014283, CA150, cl1)
Helicotylenchus spIV (HM014284, CA157, cl1)
H. vulgaris, type "B" (HM014238, CD620, cl1)
H. vulgaris, type "B" (HM014239, CD620, cl2)
H. digonicus, type "C" (HM014240, CD382, cl1)
H. digonicus, type "C" (HM014241, CD382, cl2)
H. labiodiscinus (HM014293, 14625, cl2)
H. labiodiscinus (HM014294, 14629, cl1)
H. labiodiscinus (HM014295, 14631, cl2)
H. labiodiscinus (HM014296,14631, cl1)
H. labiodiscinus (HM014297, 14629, cl2)
H. labiodiscinus (HM014298, 14625, cl1)
H. brevis (HM014299, CD556, cl1)
H. brevis (HM014300, CD556, cl2)
Helicotylenchus spVII (HM014301, CD347, cl1)
H. martini (HM014304, CD613, cl1)
H. martini (HM014305, CD613, cl2)
Helicotylenchus spIX-1 (HM014302, CD505, c3)
Helicotylenchus spIX-1 (HM014303, CD505, c1)
H. digonicus, type "A" (DQ328758)
Helicotylenchus spIX-3 (DQ328754)
Helicotylenchus spIX-4 (DQ328755)
H. vulgaris, type "A" (FJ485650)
H. vulgaris, type "A" (DQ328761)
H. vulgaris, type "A" (DQ328760)
H. vulgaris, type "A" (DQ328759)
Rotylenchus magnus (EU280789)
Hoplolaimus galeatus (EU626787)
Hoplolaimus seinhorsti (DQ328752)
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nus, *H. brevis* and *H. martini*, although none of these were from the type locality, thereby underscoring the need for further work to confirm these identifications.

In several cases, molecular approaches failed to delimit boundaries of recognised species. For example: *i*) two sequence clones from the D2-D3 rRNA PCR product obtained from a single sample and identified as *H. platyurus* did not cluster together; and *ii*) two sequences of *H. martini* showed a high level of nucleotide differences beyond the level of intraspecific variation common for *Helicotylenchus* species.

These observations, coupled with the indistinct nature of species boundaries, emphasise the importance of using an integrated approach to delimiting species and caution against reliance on any single dataset or method for this purpose. Particularly for groups such as *Helicotylenchus*, these considerations also challenge defining species concepts and how to operationally address such concepts.

Phylogeny and taxonomy of *Helicotylenchus*

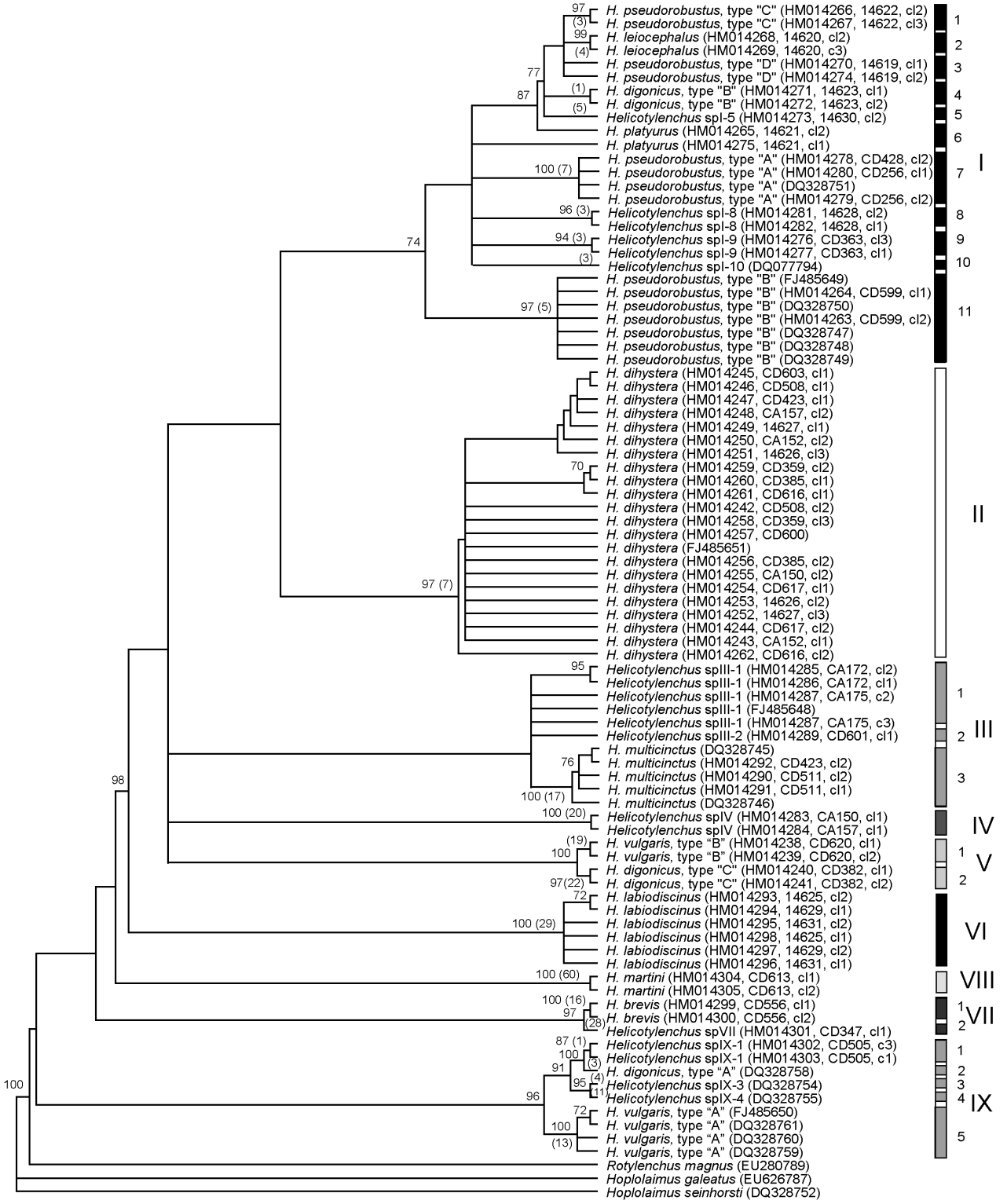
Fortuner (1991) suggested that *Helicotylenchus* most likely originated from ancestral forms close to *Pararotylenchus* and he also noted that it was not known whether *Helicotylenchus* and the other Hoplolaiminae are monophyletic. In phylogenetic analyses using 18S rRNA gene sequences (Holterman *et al.*, 2009; van Megen *et al.*, 2009), *Helicotylenchus* was supported as monophyletic and its representatives formed a single clade but with bootstrap support varying from strong to weak. In the D2-D3 regions of 28S trees reconstructed under the GTR model of DNA evolution, the genus *Helicotylenchus* was paraphyletic (Subbotin *et al.*, 2006; Vovlas *et al.*, 2008; Bae *et al.*, 2009) and composed of two distinct lineages. However, application of the secondary structure model for the same dataset (Subbotin *et al.*, 2006) led to a tree with lower resolution of relationships among the main clades and suggested that the paraphyly was the result of an artefact of the conventional models used. Based on these results, we conclude that the presently available molecular data do not provide convincing evidence in support of a paraphyletic origin of this genus.

Whitehead (1958) proposed the genus *Rotylenchoides* Whitehead, 1958 with *R. brevis* Whitehead, 1958 as the type species. *Rotylenchoides* only differed from *Helicotylenchus* in a single characteristic that is in the regression of the posterior genital branch. *Rotylenchoides* was made a junior synonym of *Helicotylenchus* by Fortuner (1984). Fortuner (1984) did not consider this character as sufficient justification for establishing a genus, because of observation of a transformation series of regression of this organ documented throughout additional species of the genus *Helicotylenchus* or presence of the so called intermediate forms. Siddiqi (1986, 2000) rejected the synonymy but Fortuner's opinion was widely supported and the synonymy of *Rotylenchoides* accepted (Ebsary, 1991; Vovlas *et al.*, 1995; Marais, 1998, 2001; Van den Berg *et al.*, 2003). The results of our phylogenetic analysis show that *H. brevis* clusters within *Helicotylenchus* and thus supports the synonymisation of *Rotylenchoides* with *Helicotylenchus*.

Species complexes within *Helicotylenchus*

Both *H. dihystera* and *H. pseudorobustus* have a worldwide distribution and have been reported from many different host plants. *Helicotylenchus dihystera* is the type species of the genus, whereas *H. pseudorobustus* is considered, after *H. dihystera* and *H. multicinctus*, to be the most frequently reported species of *Helicotylenchus* in the world literature (Fortuner *et al.*, 1984). In our tree, *H. dihystera* was represented by 14 populations which were collected from different plants in subtropical and tropical regions and formed clade II. Fortuner *et al*. (1981) made *H. rotundicauda* Sher, 1966 a junior synonym of *H. dihystera* on the grounds that the species shares the same range of variation as *H. dihystera*. Furthermore, as originally defined, the two species only differ in tail shape and shape of the stylet knobs and he did not consider those sufficient to accept them as distinct species. The synonymy was accepted by some taxonomists (Boag & Jairajpuri, 1985; Ebsary, 1991; Marais, 2001) but rejected by Siddiqi (Siddiqi, 1986, 2000). The results of our phylogenetic analysis show that one of the samples identified by morphological characters as *H. rotundicauda* clusters within *H. dihystera* and thus supports the synonymy of *H. rotundicauda* with *H. dihystera.* Samples from Burkina Faso, West Africa were identified morphologically as *H. dihystera* (Sawadogo *et al.*, 2009) and clustered in Clade III, which includes *H. multicinctus.* These samples also clus-

Fig. 1. *Phylogenetic relationships within* Helicotylenchus *populations and species: Bayesian 50% majority rule consensus tree from two runs as inferred from analysis of D2-D3 of 28 rRNA gene sequence alignment under the GTR + I + G model. Posterior probabilities equal or more than 70% are given for appropriate clades.*


H. pseudorobustus, type "C" (HM014266, 14622, cl2)
H. pseudorobustus, type "C" (HM014267, 14622, cl3)
H. leiocephalus (HM014268, 14620, cl2)
H. leiocephalus (HM014269, 14620, c3)
H. pseudorobustus, type "D" (HM014270, 14619, cl1)
H. pseudorobustus, type "D" (HM014274, 14619, cl2)
H. digonicus, type "B" (HM014271, 14623, cl1)
H. digonicus, type "B" (HM014272, 14623, cl2)
Helicotylenchus spI-5 (HM014273, 14630, cl2)
H. platyurus (HM014265, 14621, cl2)
H. platyurus (HM014275, 14621, cl1)
H. pseudorobustus, type "A" (HM014278, CD428, cl2)
H. pseudorobustus, type "A" (HM014280, CD256, cl1)
H. pseudorobustus, type "A" (DQ328751)
H. pseudorobustus, type "A" (HM014279, CD256, cl2)
Helicotylenchus spI-8 (HM014281, 14628, cl2)
Helicotylenchus spI-8 (HM014282, 14628, cl1)
Helicotylenchus spI-9 (HM014276, CD363, cl3)
Helicotylenchus spI-9 (HM014277, CD363, cl1)
Helicotylenchus spI-10 (DQ077794)
H. pseudorobustus, type "B" (FJ485649)
H. pseudorobustus, type "B" (HM014264, CD599, cl1)
H. pseudorobustus, type "B" (DQ328750)
H. pseudorobustus, type "B" (HM014263, CD599, cl2)
H. pseudorobustus, type "B" (DQ328747)
H. pseudorobustus, type "B" (DQ328748)
H. pseudorobustus, type "B" (DQ328749)
H. dihystera (HM014245, CD603, cl1)
H. dihystera (HM014246, CD508, cl1)
H. dihystera (HM014247, CD423, cl1)
H. dihystera (HM014248, CA157, cl2)
H. dihystera (HM014249, 14627, cl1)
H. dihystera (HM014250, CA152, cl2)
H. dihystera (HM014251, 14626, cl3)
H. dihystera (HM014259, CD359, cl2)
H. dihystera (HM014260, CD385, cl1)
H. dihystera (HM014261, CD616, cl1)
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H. dihystera (HM014258, CD359, cl3)
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H. dihystera (HM014254, CD617, cl1)
H. dihystera (HM014253, 14626, cl2)
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H. dihystera (HM014244, CD617, cl2)
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Helicotylenchus spIII-1 (HM014285, CA172, cl2)
Helicotylenchus spIII-1 (HM014286, CA172, cl1)
Helicotylenchus spIII-1 (HM014287, CA175, c2)
Helicotylenchus spIII-1 (FJ485648)
Helicotylenchus spIII-1 (HM014287, CA175, c3)
Helicotylenchus spIII-2 (HM014289, CD601, cl1)
H. multicinctus (DQ328745)
H. multicinctus (HM014292, CD423, cl2)
H. multicinctus (HM014290, CD511, cl2)
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H. multicinctus (DQ328746)
Helicotylenchus spIV (HM014283, CA150, cl1)
Helicotylenchus spIV (HM014284, CA157, cl1)
H. vulgaris, type “B” (HM014238, CD620, cl1)
H. vulgaris, type “B” (HM014239, CD620, cl2)
H. digonicus, type "C" (HM014240, CD382, cl1)
H. digonicus, type "C" (HM014241, CD382, cl2)
H. labiodiscinus (HM014293, 14625, cl2)
H. labiodiscinus (HM014294, 14629, cl1)
H. labiodiscinus (HM014295, 14631, cl2)
H. labiodiscinus (HM014298, 14625, cl1)
H. labiodiscinus (HM014297, 14629, cl2)
H. labiodiscinus (HM014296, 14631, cl1)
H. martini (HM014304, CD613, cl1)
H. martini (HM014305, CD613, cl2)
H. brevis (HM014299, CD556, cl1)
H. brevis (HM014300, CD556, cl2)
Helicotylenchus spVII (HM014301, CD347, cl1)
Helicotylenchus spIX-1 (HM014302, CD505, c3)
Helicotylenchus spIX-1 (HM014303, CD505, c1)
H. digonicus, type “A” (DQ328758)
Helicotylenchus spIX-3 (DQ328754)
Helicotylenchus spIX-4 (DQ328755)
H. vulgaris, type “A” (FJ485650)
H. vulgaris, type “A” (DQ328761)
H. vulgaris, type “A” (DQ328760)
H. vulgaris, type “A” (DQ328759)
Rotylenchus magnus (EU280789)
Hoplolaimus galeatus (EU626787)
Hoplolaimus seinhorsti (DQ328752)
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tered with samples from Florida, USA, one of which was identified as *H. microlobus* by Bae *et al.* (2009). Sher (1966) synonymised *H. microlobus* with *H. pseudorobustus* because he could not morphologically distinguish the topotypes of *H. microlobus* from those of *H. pseudorobustus*. The opinion of Sher was supported by a number of authors. These Clade III relationships show no obvious interpretive pattern of association based on morphology or geographical distribution, and require further analysis at the morphological and molecular levels.

Since Sher (1966) redescribed *H. pseudorobustus* from topotypes, many populations have been described from different countries. These populations show a high degree of variability in several taxonomic characters, a fact that often confounds differentiation of this species from similar species (Fortuner *et al.*, 1984). Fortuner *et al.* (1984) noted that this may be interpreted as a high degree of intraspecific variability or it may be seen as evidence of several species under the name of *H. pseudorobustus.* Using multivariate analyses of characters for 28 populations identified as *H. pseudorobustus*, Fortuner *et al.* (1984) revealed some morphological differences among the populations of *H. pseudorobustus*, mostly between samples from North America and Western Europe. The differences were most apparent in the pattern of the junction of the inner lines of lateral field on the tail, as well as the position of the phasmids and the dorsal gland opening. They concluded that multivariate analyses are a valuable identification tool that can overcome the problem of intraspecific variability. They also noticed that a few samples originally proposed as *H. pseudorobustus* were, in fact, more similar to *H. dihystera* or could represent another, unidentified species. Against this background it is not surprising that in our study we were not able to identify unambiguously some samples as *H. pseudorobustus* and instead we proposed four possible candidates named here as *H. pseudorobustus* type A, B, C and D. Most likely, the type B found in Europe and having a wider distribution represents the true *H. pseudorobustus.* Future molecular analysis of *H. pseudorobustus* samples collected from the type locality in Switzerland could give a reliable sequence signature for this species and will provide a basis to clarify identification of our samples.

The grouping of *H. pseudorobustus* type A and species, morphologically identified as *H. labiatus*, from New Zealand (clade I (7)), despite the consistent differences in lip region shape and the lateral fields on their tails, clearly raises questions about their distinctness. Yeates and Wouts (1992) found only four *Helicotylenchus* species across the 159 managed soils they sampled, with *H. pseudorobustus* being recorded from 52% of the sites and *H. labiatus* from 35% of the sites and with no males being recognised. However, Wouts and Yeates (1994) found eight *Helicotylenchus* species from native vegetation and undisturbed soils but did not report either *H. pseudorobustus* or *H. labiatus*. Thus, these two nominal species were considered to be apparently introduced to New Zealand, with the probability of multiple introductions. They each have wide distribution within New Zealand and their variability in both morphological and molecular criteria may reflect the global pool of populations from which introductions were derived.

Clade VII consists of a single species, *H. martini*. This species was described from Zimbabwe and had since only been reported from Africa (Ali *et al*., 1973; Marais, 1998). This species has a unique set of characteristics that place it apart from all the other *Helicotylenchus* species. Adults do not have lip annuli and internal fasciculi are described as present. Another interesting feature for females of this species is the relatively long tail ranging from 17 to 49 μm (Van den Berg, 1978; Marais, 1998).

The results of the present study suggest that observed genetic diversity of *Helicotylenchus* is significantly higher than has been shown by morphological observations. Integration of morphological and morphometric studies with molecular analyses may clarify the identification of species within this complex genus. Molecular characterisation of *Helicotylenchus* species using analysis of the D2-D3 expansion segments of 28S rRNA gene sequences and sequences of more variable genes, such as ITS-rRNA gene and *cox*I of mtDNA, can become an important step in verification of identified samples and diagnostics of the spiral nematodes.

Fig. 2. *Phylogenetic relationships within* Helicotylenchus *populations and species: Strict consensus of 1000 maximum parsimony trees as inferred from analysis of D2-D3 of 28 rRNA gene sequence alignment. (Tree length = 744; CI (excl. uninformative characters) = 0.5045; HI (excl. uninformative characters) = 0.4955; RI = 0.8518; RC = 0.4763). Bootstrap values equal or more than 70% are given for appropriate clades. Numbers of apomorphies for a clade representing the same species are given in parentheses.*

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