Original

Morphological and Genetic Analysis of *Diploscapter coronatus*: Insights into Identification Challenges and Species Relationships

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ABSTRACT

Objective: This study aims to address the challenges of identifying *Diploscapter coronatus* by morphological and genetic analysis. The nematode was previously identified morphologically in fecal samples from a patient with Henoch-Schönlein purpura. We used genetic methods to complement and refine species identification.
Materials and Methods: Nematodes isolated from the patient's feces were fixed in formalin, and morphological observations were made under a light microscope. PCR amplification of the SSU rRNA and Hsp90 genes was performed on DNA extracted from the nematodes during the same period. The resulting DNA was cloned, sequenced, and subjected to phylogenetic analysis using the neighbor-joining method.
Results: Morphological observations confirmed that the nematode shared key features with *D. coronatus*. The SSU rRNA analysis showed 99% similarity with 11 *Diploscapter* species, including *D. coronatus*. However, Hsp90 gene analysis placed the nematode in the *D. lycostoma* cluster, revealing inconsistencies between morphological and genetic data.
Conclusion: The present study highlights the complexity of species identification within the genus *Diploscapter* that results from the limited genetic information and overlapping morphological characters. The results underscore the need for more extensive genetic data and comprehensive phylogenetic analyses to resolve these identification challenges and better understand species relationships within the genus.
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Key Words
Diploscapter coronatus, *Diploscapter* spp., genetic analysis, small subunit ribosomal RNA (SSU rRNA), heat shock protein 90 (Hsp90)

I. Introduction

Diploscapter coronatus was first described by Cobb in 1893 as a free-living nematode typically found in soil surrounding decaying plants and roots[1]. Its detection in human samples is very rare, although its presence has been noted in past cases of anoxia and alkaline urine[2),3)]. In addition, recent reports have identified nematodes suspected of being this species in clinical samples such as urine and feces[4),5)]. Several species, including *D. coronatus*, have been documented within the genus *Diploscapter*. In 2002, we detected this nematode in fecal samples from a patient with Henoch-Schönlein purpura, identified by its morphological features[6)]. Recently, both morphological and genetic methods have been used for species identification in nematode studies[7)]. In this study, we report the analysis of a sample of *D. coronatus*, previously identified by morphological observations and now complemented by genetic identification methods.

II. Materials and Methods

1. Samples

Nematodes found in patient feces in 2002 were cultured in nematode growth medium (NGM), then fixed in 10% buffered formalin and stored at room temperature. Morphological observations were carried out on these formalin-fixed nematodes. During the same period, genomic DNA was extracted from *D. coronatus* nematodes derived from the patient and cultured in NGM using a QIAamp DNA Micro Kit (Qiagen, Hilden, Germany) and stored frozen at −80℃. This study was approved by the Ethics Committee of Kochi University Medical School, Japan (No.30-87).

2. Morphology

Morphological observations and measurements of the formalin-fixed nematodes were made using a light microscope and considered in conjunction with the observations we have previously reported[6]. Using De Man's formula[8], specific ratios and indices were calculated based on measurements of different parts of the nematode's body to describe its morphological characteristics.

3. Polymerase Chain Reaction (PCR) and Cloning

The primers used for the small subunit ribosomal RNA (SSU rRNA) gene were obtained from Holterman et al[9]. (2006), and those for the 90 kDa heat shock protein (Hsp90) gene were obtained from Zeng Qi Zhao[7] (2013), respectively. The 20 μL PCR reactions contained 10 μL KAPA2G Fast PCR Kit (Kapa Biosystems, USA), 1 μL (0.05 μM) of each forward and reverse primer, and 2 μL of DNA template. The thermal cycling program was as follows: denaturation at 95℃ for 3 min, followed by 30 cycles of denaturation at 94℃ for 30 s, annealing at 55℃ for 30 s, and extension at 72℃ for 45 s. A final extension was performed at 72℃ for 10 min. The amplified DNA was purified using a QIAGEN Gel Extraction Kit (QIAGEN, Valencia, CA, USA). The cDNA was ligated into a T-vector using the DynaExpress TA PCR Cloning Kit (BioDynamics Laboratory, Tokyo, Japan) and then transformed into competent *Escherichia coli* DH5α cells (FUJIFILM Wako, Tokyo, Japan) using the heat shock method. Finally, the recombinant plasmid was extracted from the transformed *E. coli*, using EZ-10 Spin Column Plasmid DNA Miniprep Kit (BIO BASIC, Ontario, Canada).

4. Sequencing and Phylogenetic Analysis

Purified plasmids were sequenced using the Big Dye™ Terminator Cycle Sequencing Ready Reaction Mix v3.1 Kit. Cycle sequencing products were purified using the FastGene Dye Terminator Removal Kit (NIPPON Genetics, Tokyo, Japan) and analyzed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The genetic sequences obtained were subjected

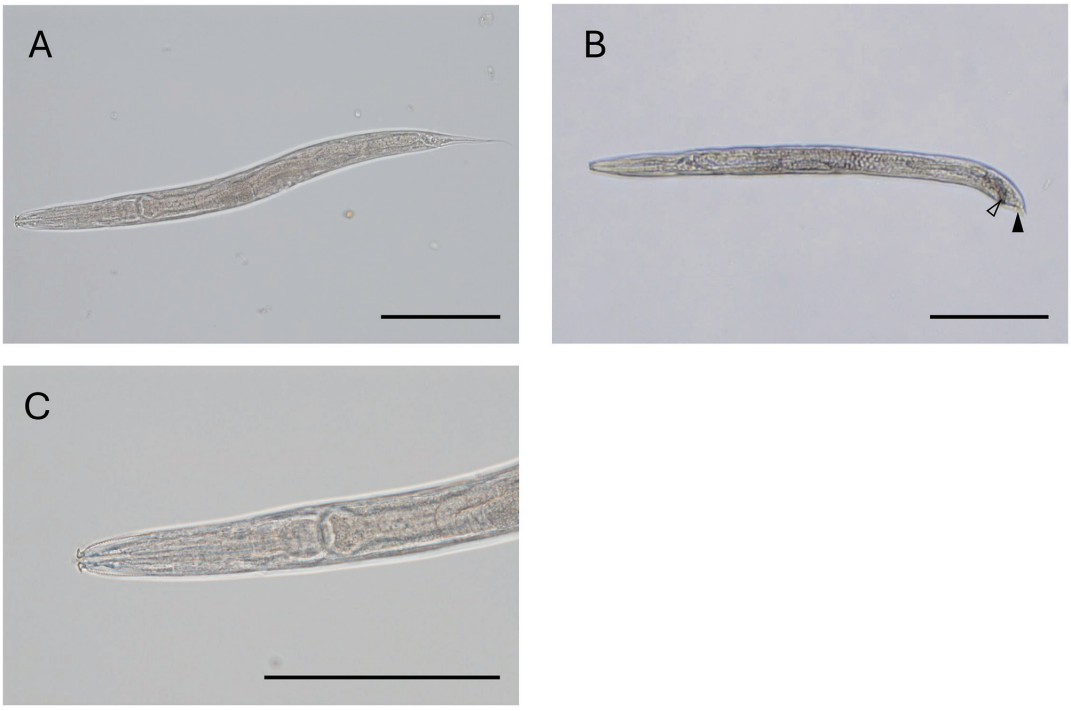

Figure1 Microscopic morphology of adult nematodes.
Whole body of both adult female (A) and male exhibiting the presence of spicules (△) and caudal alae (▲) in the tail (B). Enlarged view of the head of an adult female (C). Scale bar indicating 100 μm.

to similarity searches using BLAST[10] from the National Center for Biotechnology Information (NCBI). DNA sequences were aligned using Clustal OMEGA[11] and a phylogenetic tree was constructed using the Neighbor-Joining method with MEGA 11[12]. The root of the phylogenetic tree was constructed using the nucleotide sequence of *Rhabditis myriophila* (U81588) for SSU, and *Acrobeloides amurensis* (DQ340377) and *Cephalobus cubaensis* (DQ340378) for HSP90.

III. Results

1. Morphological Observations

The microscopic images of the male and female nematodes in this study are shown in **Figure 1**. The length of the adult worms ranged from 344 to 522 μm, with a width of 22 to 39 μm (**Table 1**). They had two characteristic pairs of lips on the head, with the dorsal and ventral lips being hook-shaped and the second pair branching anteriorly, consistent with the characteristics of the genus *Diploscapter* (**Figure 1B**). To compare this nematode with the genus *Diploscapter*, coefficients calculated from the De Man measurements are presented in **Table 1**. Morphologically, both the size and the De Man ratios were similar to those of *D. coronatus*.

2. Genetic Findings

A homology search using BLAST showed that the SSU rRNA region of this nematode exhibited 99% similarity to 11 *Diploscapter* species. The similarity to the two included *D. coronatus* sequences was 99.3%. In the Hsp90 region, similarity ranged from 89.8% to 99.4% for *D. lycostoma* and from 86.8% to 89.7% for *D. coronatus* (**Table 2**). Phylogenetic analysis classified the *Diploscapter* species into clusters based on the four SSU regions, with the nematode in this study placed in a cluster that includ-

Table 1 Comparative measurements of adluts *Diploscapter* spp.*

Author(s)	*D. coronatus*					*D. lycostoma*
	This case	Morimoto et al (2006)	Loof (1964)	Abebe (1998)	Abolafia&Pena-Santiago (2007)	Wahab (1962)
Length body**	344 - 522	348 - 394	250 - 400	350 - 427	358 - 504	519 - 546
a	13.4 - 17.3		14 - 18	16 - 20	15 - 18	15.2 - 16.7
b	4.4 - 5.6		3.5 - 4.5	3.6 - 4.4	4.0 - 5.3	6.3 - 6.5
c	5.3 - 8.4		5.9 - 8.2	5.8 - 9.6	5.7 - 7.4	9.4 - 12.1
c'	4.0 - 7.1			3.5 - 6.5	4.4 - 6.8	
V	45 - 53		51 - 57	50 - 58	48 - 57	56

*The De Man ratios (a to c'+V) Favoured by plant and soil nematologists are as follows: a=body length/maximun width; b=body length/length of esophagus; c=body length/tail length; V= Percentage of body length from the head to the vulva.
**Length body (μm).

Table 2 Homology search in the SSU rRNA region using BLAST

Nematodes	Assession #	Max Score	Total Score	Sequence Coverage (%)	E value	Sequence identity (%)
Diploscapter sp.	EU196003.1	1572	1572	100%	0.0	99.65%
Diploscapter sp.	U81586.1	1572	1572	100%	0.0	99.65%
Diploscapter sp.	AF083009.1	1565	1565	100%	0.0	99.42%
Diploscapter coronatus	KJ636377.1	1557	1557	100%	0.0	99.30%
Diploscapter coronatus	AY593921.1	1555	1555	100%	0.0	99.30%
Diploscapter sp.	OP964516.1	1550	1550	100%	0.0	99.19%
Diploscapter sp.	OP964515.1	1550	1550	100%	0.0	99.19%
Diploscapter sp.	OP964514.1	1550	1550	100%	0.0	99.19%
Diploscapter formicidae	JQ838254.1	1550	1550	100%	0.0	99.19%
Protorhabditis sp.	EU196002.1	1428	1428	100%	0.0	96.64%
Protorhabditis sp.	AF083024.1	1356	1356	100%	0.0	95.13%
Heterorhabditis indica	LN611143.1	1166	1166	100%	0.0	91.35%

Homology search using BLAST was performed with the nucleotide sequences obtained from sequencing the plasmid DNA, which contained the PCR product of the SSU region of this nematode.

ed *D. coronatus* (**Figure 2**). In contrast, using the Hsp90 data, the *Diploscapter* species were classified into clusters corresponding to *D. coronatus*, *D. formicidae* and *D. lycostoma* with the nematode in this study placed in the *D. lycostoma* cluster (**Figure 3**).

IV. Discussion

Morphologically, it has been estimated that there are approximately 15 species within the genus *Diploscapter*[13]. However, to date, comprehensive genetic analyses and electron microscopic evaluations have not been carried out for most species. The nematode analyzed in this study matched the morphological characteristics of *D. coronatus* observed in previous studies[14)-17)]. In addition, the b-ratio (body length/esophagus length) of *D. coronatus* differed from that of *D. lycostoma* in the De Man ratios referenced for nematodes[17]. From these findings, the nematode in this case demonstrated a b-ratio of 4.4-5.6,

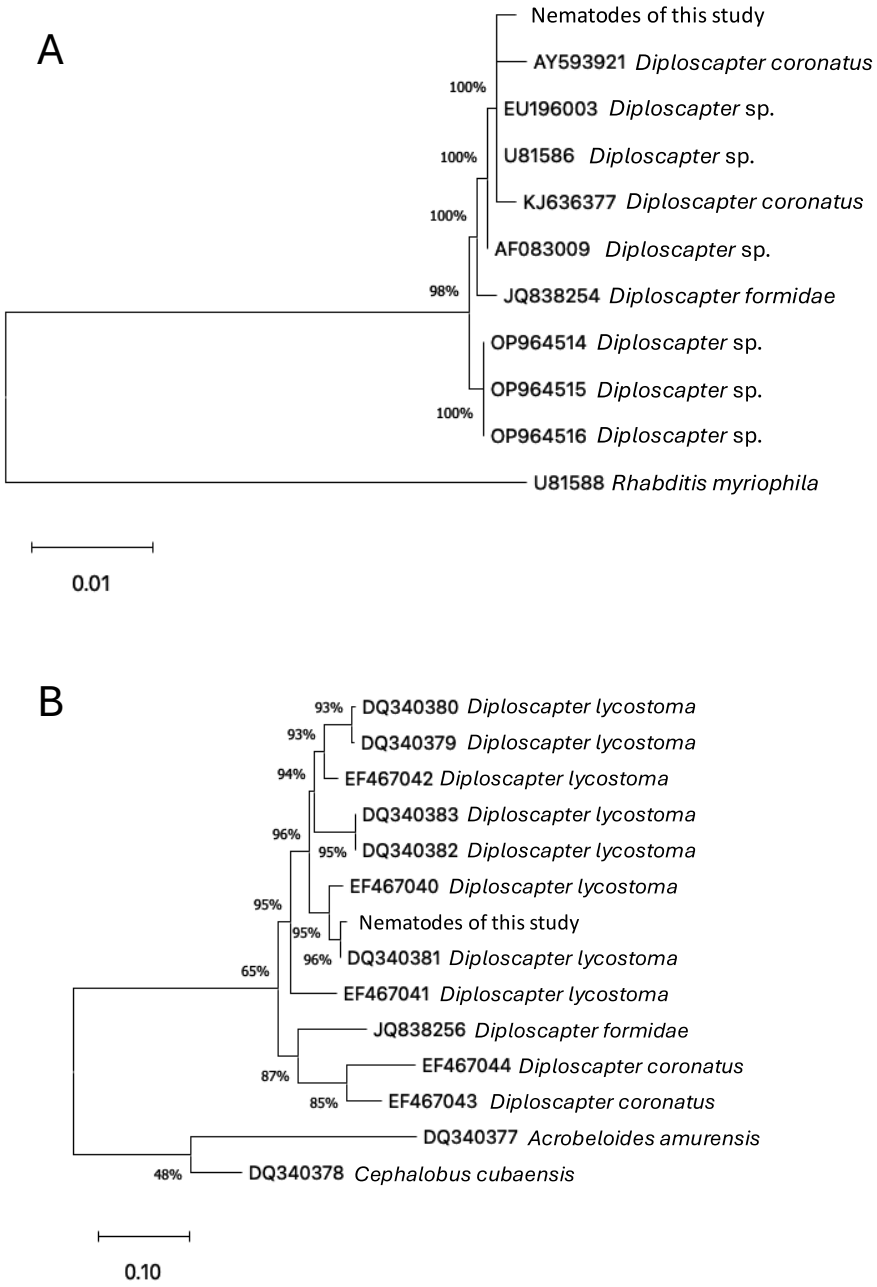

Figure 2 Neighbor-joining phylogenetic trees inferred from SSU rRNA gene (A) and HSP90 gene (B).
The phylogenetic trees include sequences from 9 species for SSU rRNA DNA and from 11 species for the HSP90 gene within the genus *Diploscapter*, along with sequences from other related taxa. Bootstrap values are shown at the nodes. The scale bar indicates the number of nucleotide substitutions per site. Sequences from *Rhabditis myriophila* and other outgroup species were used to root the tree.

Table 3 Homology search in the HSP90 region using BLAST

Nematodes	Assession #	Max Score	Total Score	Sequence Coverage (%)	E value	Sequence identity (%)
Diploscapter lycostoma	DQ340381	1286	1286	99%	0.0	99.44%
Diploscapter lycostoma	EF467040	1168	1168	100%	0.0	96.48%
Diploscapter lycostoma	EF467042	1088	1088	100%	0.0	94.52%
Diploscapter lycostoma	DQ340382	1088	1088	99%	0.0	94.63%
Diploscapter lycostoma	EF467041	1050	1050	100%	0.0	93.58%
Diploscapter lycostoma	DQ340379	907	907	99%	0.0	90.31%
Diploscapter formicidae	JQ838256	885	885	96%	0.0	90.39%
Diploscapter lycostoma	DQ340380	881	881	99%	0.0	89.75%
Diploscapter coronatus	EF467044	765	765	100%	0.0	86.76%
Diploscapter coronatus	EF467043	702	702	79%	0.0	89.72%
Leguminivora glycinivorella	MK343468	174	174	20%	1e-38	88.28%

Homology search using BLAST was performed with the nucleotide sequences obtained from sequencing the plasmid DNA, which contained the PCR product of the HSP90 region of this nematode.

which morphologically identified it as *D. coronatus*.

The information available in NCBI on the genus *Diploscapter* is particularly limited, and only three species are fully documented: *D. coronatus*, *D. lycostoma* and the newly described *D. formicidae*. The nematode in this study showed high similarity to *D. coronatus* in the SSU rRNA gene, while in the Hsp90 region, it exhibited higher similarity to *D. lycostoma*. We currently lack sufficient genetic data on the SSU rRNA region in *D. lycostoma*. In addition, the genetic information available for the Hsp90 region lacks corresponding, detailed morphological descriptions. These factors likely contribute to the inconsistencies observed using genetic identification methods. In practice, when performing a BLAST search for the Hsp90 gene sequence of *D. lycostoma* (EF467042), the similarity to other *D. lycostoma* sequences ranges from 91.4% to 93.6%, and to *D. coronatus* sequences ranges from 85.8% to 85.9%. Identification by genetic methods is therefore challenging.

Interestingly, G. Markin *et al*[18] reported that *D. coronatus* (Cobb 1893[1]) is synonymous with *D. lycostoma* (Volk 1950)[19]. The paucity of samples available for phylogenetic analysis may suggest that the resulting phylogenetic tree does not accurately reflect the true evolutionary relationships between the classified groups.

To date, while *D. coronatus* has been detected in human samples[4, 5], there have been no reports of other species within the genus *Diploscapter* being detected in humans.

In addition, the detection of *Diploscapter formicidae*[7] and *Diploscapter lycostoma*[19, 20] in association with ants suggests a possible correlation between species and host specificity. In general, when a rare parasite is detected in humans, species identification is often performed based on the similarity of gene sequences from a particular genetic region, and genetic identification for various organisms has advanced in clinical examination. However, for the genus *Diploscapter*, the current genetic information available is insufficient, leading to uncertainties in species identification. In fact, Arai et al[21] reported that although they attempted to genetically identify *Diploscapter* from human samples, a definitive identification of *D. coronatus* could not be achieved because of limited nucleotide sequence information.

Interestingly, although males are thought to be absent in the genus *Diploscapter*, we have reported the presence of males in this genus[6]. Furthermore, the presence of males in *D. coronatus* has also been reported by other researchers[4, 22]. If males could be found in other species of the genus *Diploscapter*, it might be possible to identify species based on characteristics such as male reproductive organs.

V. Conclusion

The identification of species within the genus *Diploscapter* requires more extensive morphological and molecular data, with particular emphasis on comparing genetic information among different species to advance phylogenetic analyses. In addition, the ability to specifically detect species will likely provide important insights into host specificity.

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Authorship contributions

All authors were involved in the preparation of the manuscript and reviewed the final manuscript.

Disclosure of Conflicts of Interest

We declare that we have no conflicts of interest.

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