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ORIGINAL ARTICLE

MORPHOLOGICAL AND MOLECULAR STUDY OF THREE SPECIES OF *ACROBELOIDES* (COBB, 1924) THORNE, 1937 (RHABDITIDA, CEPHALOBIDAE) AS NEW RECORDS IN IRAQ

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ABSTRACT

Three species of nematodes within *Acrobeloides* (Cobb, 1924); Thorne, 1937 (Rhabditida, Cephalobidae) were collected and recorded for the first time in Iraq, based on morphometric and molecular data, these species, *A. saeedi* Siddiqi, Ley & Khan, 1992, *A. apiculatus* (Thorne, 1925), and *A. bodenheimeri* (Steiner, 1936), were molecularly characterized using the partial 28S rRNA gene sequences. The phylogenetic tree has been constructed to separate *Acrobeloides* species from closely related species.

Keywords: *Acrobeloides*, Molecular, Morphometrics, Nematodes, New record.

INTRODUCTION

Despite having a vast variety of habitats and about 354 genera, the family Cephalobidae Filipjev (1934) is challenging to recognize and taxonomically describe based solely on morphological characteristics (De Ley and Blaxter, 2004). The morphology of the lip and stoma can be used to distinguish between different genera and species of Cephalobidae (Carta *et al.*, 2022). Cephalobidae includes *Acrobeloides* (Cobb, 1924), which was raised to the rank of genus by Thorne (1937). These nematodes feed on bacteria and are found in almost all sand dunes across all land-dwelling environments, excluding steamy rainforests, agricultural land, deserts, and the barren soils of Antarctica (Timm, 1971; Waceke *et al.*, 2005; Nadler *et al.*, 2006; Rana *et al.*, 2020; Bhat *et al.*, 2021; Loulou *et al.*, 2022). Sequence analysis suggests that the 26 valid species of *Acrobeloides* and the 5 species with uncertain taxonomic status represent at least two phylogenetically distinct groups that interact with species of *Cephalobus* and *Chiloplacus*, among other organisms (Smythe and Nadler, 2006; Nadler *et al.*, 2006; Holovachov *et al.*, 2009).

Acrobeloides and other taxa can be difficult to distinguish morphologically from one another at times because of highly confusing patterns of intra- and interspecific variability (Janssen *et al.*, 2017). Cobb (1924) made the first mention of the *Acrobeloides* taxonomy.

Morphological and molecular study

Some *Acrobeloides* species have been shown to exhibit insect association behavior when soil nematodes are isolated using the *Galleria* soil baiting method described by Bedding and Akhurst (1975), and Azizoglu *et al.* (2016). Grewal *et al*. (2003) have noted that in addition to their association with insects, there has been an infestation of some annelids, arthropods, and molluscs. While previously identified by Saeed *et al.* (1988) as *Cephalobus litoralis* (Akhtar, 1962). Bhat *et al*. (2021) and Rana *et al*. (2021) confirmed that *A. saeedi* was discovered by Siddiqi *et al*. (1992) in Pakistan. Nematodes that feed on soil-dwelling bacteria and fungus, parasitize plants and insects, and even eat other nematodes are among the species in this category that have been researched in great detail.

The Cephalobidae are the most prevalent among them in fields of agriculture. Understanding and communicating each organism's ecological importance requires accurate species-level identification (Andrássy, 1967). According to previous observations (Bedding and Akhurst, 1975; Azizoɪglu *et al*., 2016; Rana *et al*., 2021), numerous *Acrobeloides* species have been found to exhibit the insect association nature when they are isolated from soil samples employing the larvae of *Galleria*. For example, earthworm cocoons are infested by *Acrobeloides nanus* Anderson, 1968, (Kraglund and Ekelund, 2002). It has been earlier reported that they are complicated in the soil nitrogen cycle and soil mineralization. Anderson *et al.* (1981) and Hao *et al.* (2010) report that during these activities, they engage with a range of arthropods and invertebrate species, some of which can be phoretic and necronemic. *A. saeedi* and other morphologically similar species, like *A. bodenheimeri*, were to be included in the new genus *Rafiqius*, which Khan and Hussain (1991) proposed (Steiner, 1936; Thorne, 1937). Based on the morphology of the lip area and the presence of seta-like structures at the labial primary axils, this recently proposed genus was distinguished from *Acrobeloides*. Nevertheless, De Ley *et al*. (1999) felt that the genus's creation was not warranted. There is a description of two Indian cultures of *A. saeedi*. This specimen is consistent both morphologically and morphometrically with *A. bodenheimeri*, *A. longiuterus*, and *A. maximus*, as well as other species in the Maximus-group, particularly *A. longiuterus*. But according to Bharti *et al*. (2020), the Indian material can be more clearly distinguished from each of these species by molecular analyses using ITS, 28 S, and 18 S rDNA.

To the best of our knowledge, no research has documented the morphological and molecular characteristics of the genus *Acrobeloides* in Iraq, thus, the aim of the current investigation was to use molecular, morphometric, and morphological data to culture and recognize nematode species that were taken from bitter gourd fields. The 28S ribosomal gene-based phylogenetic analysis was used to estimate their evolutionary relationship. The molecular and morphological database of *Acrobeloides* species.

MATERIALS AND METHODS

Isolation, cultivation, and processing of nematodes: Between January 2022 and April 2023, soil samples were gathered from several locations in the middle of Iraq (Map 1, Pl. 1); sampling was done according to Orozco (2014), and their nematode content was examined (Tab. 1). It is advisable to allocate a minimum of 2 - 4 m2 for every sampling site. At least five arbitrary soil samples should be taken in this area, with samples being taken at a

minimum depth of 15 cm. Using *Galleria mellonella* larvae in their last instar as bait, nematode specimens were extracted from five soil samples. The cadavers and afflicted larvae were subsequently moved to a white trap (White, 1927), following a thorough cleaning with distilled water and sterilization, with 1% NaOH. After being extracted from cadavers in a white trap, the nematodes were kept in 250 ml tissue culture flasks in an incubator at 8-10°C as described by Bhat *et al.* (2019).

Map (1): Sampling areas.

Plate (1): Soils sampling.

Morphological and molecular study

Galleria mellonella (Linnaeus, 1758) larvae were inoculated with third-stage juveniles (≥100) for morphometric measurements and observations. After the deceased larvae were moved to a white trap, the adult stage (4-5 days) and third stage juveniles (7-8 days) were removed from the trap. After being killed with hot water, the specimens were placed in TAF (a solution of 2 % triethanolamine with 7 % formaldehyde) to be fixed. The fixed nematodes were mounted in pure glycerine on permanent glass slides after being processed to dehydrate glycerine according to Seinhorst's 1959 instructions (Siddiqi, 1964).The following details were recorded for each soil sample obtained: the location of the GPS coordinates, sample collection, the number of samples collected, the name of the strain, the insect host of the several nematode strains isolated, and the nematode species identified.

Table (1): Geographical location of nematode sampling sites.

Sampling site	GPS coordinates	Number of soil sample	Nematodes species isolated	Accession number of Design strain name	Insect host
Wasit, Al-Suwaira	32°56′25″N 044°38′04″E	10	*Acrobeloides saeedi*	OR272345	*Galleria mellonella*
Baghdad, Al-Zafaraniya	33° 15' 2" N 44° 28' 58" E	10	*Acrobeloides* apiculatus	OR363452	*G. mellonella*
Salahuddin / samarra	34°11'32.4" N 43°52'16.1" E	10	*Acrobeloides* bodenheimeri	OR363692	*G. mellonella*
Wasit, Al-Hafriya	32°58'59" N 44°49'60" E	10	none	-	-
Wasit, Badra	33°06'60.0" N 45°56'59.99" E	10	none	-	-
Baghdad, Al-Yusufiyah	33° 3' 29" N, 44° 22' 11" E	10	none	-	-
Baghdad, Al-Mansour	33.3114° N, 44.3497° E	10	none	-	-

Characteristics of Morphology: Nematodes were studied either alive or cooked to 60°C in Ringer's solution for morphological examinations. Every nematode employed in this investigation was raised as *G. mellonella* larvae in the final stage. A temperature-controlled (25±3◦C) petri-dish containing two moist filter sheets was used to expose ten *G. mellonella* larvae to roughly 1000 IJ. To separate the mature females of the first and second generations, the infected larvae were dissected in Ringer's solution 4 and 7 days after infection, respectively.

Following heat-killing, the nematodes were preserved in triethanolamine formalin (TAF) fixative before being processed using a gradual evaporation method with anhydrous glycerin for mounting (Poinar, 1976; Kaya and Stock, 1997). A drawing tube and differential interference components were added to an Olympus BX41 microscope for use in

morphometric and morphology research. The nematode specimens were photographed using a Canon camera (Japan) with magnifications of 10x and 40x. Male and female measurements were made for the following characters: Overall body length, maximum body diameter, anal body diameter, excretory pore position, and distance between the anterior end and position of the nerve ring, pharynx position, tail length, and vulva positions.

Ratios a, b, c, D, and E (Tab. 2), a represent (total body length divided by maximum body diam.); b represents (all body length divided by space from the anterior end to the base of the pharynx); c represents (the entire body length divided by tail length); D represents (the excretory pore position divided by distance from the anterior end to the base of the throat), and E represents (excretory pore position divided by tail length). Using the taxonomic standards recommended by Stock and Kaya (1996), morphological identification was completed. Additionally, scanning electron microscopy was used to analyze the morphological characteristics of female representative isolates of each species group. Specimens were handled in accordance with Nguyen and Smart's (1995) methods for this purpose.

Characterization via morphology and morphometry: An optical microscope fitted with a micrometric eyepiece was used for morphological and morphometric identification. The IJs were taken from the white trap (Pl. 2), and the nematodes were handled by Nguyen and Smart (1996). Wax worm larvae were dissected.

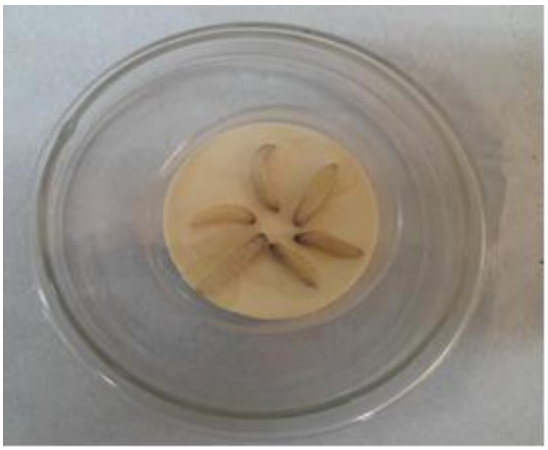

Plate (2): White trap.

Nematode molecular characterization: Using a genomic DNA isolation kit from Geneiad BioTEK and according to the manufacturer's instructions, DNA was extracted from the four isolated worms. PCR was utilized to amplify distinct sections of the rRNA genes and mtCO1 using isolated genomic DNA. ITS regions.

Polymerase Chain Reaction is used for DNA amplification: PCR Using primer forward (F2), the isolated DNA was utilized as a template for PCR amplification of a fragment

containing the D2 and D3 regions of the 28S rR A gene. Reverse rDNA 5'-CGATAGCGAACAAGTACCGAGAG-3 (R2) 5-(900 bp amplification size) CCTGCTCAGGCATAGTTCACCATC-3 28S rDNA (Joyce *et al*., 1994).

PCR cycling conditions used were: A heated lid Techne PHC-3 thermocycler was used to do amplifications. In the thermocycler, the samples were put in and heated to 95 °C. They were then incubated for 2 minutes at 94 °C, then for 40 cycles at 94 °C for 30 seconds, 50 °C for 1 minute, and 72 °C for 1.5 minutes. To make sure all of the final amplification products were full length, a last step of 5 min at 72 °C was added (Yadav *et al*., 2022). The total amount of the 40 µl PCR reaction consisted of 20 µl of the 2xTaq PCR Master mix that was ready to use, 1 µl of each forward and reverse primer (10 pml), 10 µl of genomic DNA (10–20 µg/µl), and 8 µl of sterilized water.

Electrophoresis was used to separate the PCR products in a 1% TAE (Tris-acetic acid-EDTA) buffered agarose gel, stained with GelRed nucleic acid gel dye (Biotium), for 45 minutes at 100 V. The Macrogene firm received the PCR products for Sanger sequencing. Sequences were cut and curated by hand. Every sequence was uploaded to the NCBI databank (National Center for Biotechnology Information). The phylogenetic trees provide accession numbers.

Abbreviations: The following terms are shortened in the tables or text: L is the body length, ABD is the anal or cloacal body diameter, EP is the excretory pore position, ES is the pharynx length, GS is the GuL/SpL, GuL is the gubernaculum length, MBD is the maximum body diameter, NR is the nerve ring position, and the ratios are a = L/MBD, b = L/ES, c = L/T, D% is the EP/ES ×100, E% is the EP/TL × 100, SpL is the spicule length (measured along the curved median line), SW is SpL/ABD, T is the tail length, and ratio v indicates the distance from head end to vulva/ L x 100.

RESULTS AND DISCUSSION

Three species of the genus *Acrobeloides* were found in soil samples taken from several locations in the center of Iraq. Isolates exhibit the physical traits of the genus Acrobeloides, as reported by Thorne (1937) and Steiner (1936), according to microscopic investigations. An explanation of the three new records from Iraq (*A. saeedi*, *A. apiculatus*, and *A. bodenheimeri*) is given.

Measurements: (Tab. 2). The ventral length of the female is slightly bent. The cuticle annulated; the inner labial papillae are six and four outer cephalic papillae and are present in the lip's region, which is continuous with the body's contour. In *A. saeedi*, the body length varied from1050 to 1448 µm, the maximum body width was 75 to 112, and the tail length was 48 to 62 µm, a value of 13.7, b value of 6.7, and c value of 22.2. While in *Acrobeloides bodenheimeri*, the body length was from 635 to 775 µm, the maximum body width was 30 to 40 µm, and the tail length was 35 to 44 µm. a value of 19.8, b value of 5.1, and c value of 17.7, on the other hand, in *Acrobeloides apiculatus* The body length ranged from 445 to 655

µm, the maximum body width was 12 to 16.6µm, and the tail length was 41 to 51 µm, a value of 36.5, b value of 3.7, and c value of 11.1 (Pls. 3, 4, 5).

Sequencing of 28S and phylogenetic tree: Selected specimens were subjected to DNA analysis to verify the morphological identification of the isolated nematodes. A large subunit ribosomal RNA gene (28S) partial sequence of Acrobeloides isolates sequenced revealed 902 bp of unique DNA fragments. The sequences were then matched to rDNA sequences found in Genbank using the BLAST search algorithm. The results of the phylogenetic analysis revealed that 98% of the sequences had similarity with a bootstrap phylogenetic tree based on 28S sequences from local Acrobeloides isolates constructed with morphological structure supported 28S gene identification and was constructed using the neighbor joining (NJ) method.

Table (2): Morphometric data for female of *Acrobeloides* spp. included in this study and isolated from Galleria culture. [All measurements are in µm (except ratio, and percentage) and in the form: mean ± SD (range)].

Characters	*A. apiculatus*	*A. bodenheimeri*	*A. saeedi*
Body length	502.77 ±72.49 (445-655)	690.13±54.79 (635-775)	1.189.37±140.16 (1055-1448)
Mid body diameter	13.76 ± 1.47 (12-16.6)	34.9± 4.14 (30-44)	86.46 ±12.48 (75-112)
EP	91.28 ±10.56 (83-115)	134.29±13.43 (118-151)	144.27 ±17.04 (125.6-171)
ES	133.27 ±10.56 (122-155)	133.53± 3.99 (128-140)	170.42 ±10.38 (155.5-185)
NR	88.87 ±7.28 (82-104)	113.58±6.66 (107-126)	112.07 ±8.12(105-128)
T	44.89 ±3.48 (41-51)	39.75± 3.14 (35-44)	53.50 ±4.62 (48.2-62)
ABD	11.24 ±1.17 (10.5-13.5)	19.49±1.29 (18-22)	27.97±4.24 (22.8-34.0)
a =L/MBD	36.50 ±1.61 (34.92-39.46)	19.86± 1.06 (17.61-21.17)	13.78 ±0.38 (13.45-14.28)
v= vulva distance/L * 100	37.58 ±2.10 (33.47-39.69)	76.44±5.54 (68.68-83.44)	76.01 ±2.37 (72.77-78.96)
b = L/ES	3.76 ±0.26 (3.54-4.23)	5.16± 0.27 (4.91-5.54)	6.97±0.48 (6.39-7.83)
c= L/T	11.17 ±0.72 (10.70-12.84)	17.37± 0.51 (16.20-18.14)	22.20 ±0.96 (20.61-23.85)
D=EP/ES *100	68.45 ±3.19 (65.58-74.19)	100.39± 7.25 (92.19-109.16)	84.48 ±5.56 (76.60-92.43)
E%= EP/T * 100	203.18 ±8.78 (195.83-225.49)	337.39± 9.57 (320.53-352.25)	269.11 ±11.08 (249.02-283.45)
Vulva distance	187.98 ±25.66 (176-260)	528.8±67.96 (445-631)	902.17 ±81.73 (833-1045)

The tree (Diag. 1) shows a strong relationship among *Acrobeloides saeedi* and *A. apiculatus* isolates collected from different regions in Iraq more than *A. bodenheimeri* and clustered together with high similarity with *A. saeedi* from India (MN101167.1,

MK935147.1), while *A. bodenheimeri* clustered together with *A. bodenheimeri* isolates from the USA (AF147065.1 and DQ145625.1). Thakar *et al*. (2022) showed the same result with the participation of species *A. apiculatus* and *A. bodenheimeri* within the same clade. This result was achieved by the 18S small subunit rRNA gene partial sequence. According to phylogenetic analyses based on 18S rDNA sequences published by Rana *et al*. (2020), isolates of *A. saeedi* and other unidentified *Acrobeloides* species from Iran formed a group that was clearly monophyly. These isolates were likely conspecific isolates within a clade that had 100% support, and they joined forces to form a sister clade with other species of the "Maximus" group that were from different geographical regions, including A. maximus and *A. bodenheimeri*. A comparative phylogenetic framework of the morphological characteristics of *Acrobeloides* was presented by Smythe and Nadler (2006); however, during their investigation, they were unable to identify a common ancestor (Smythe *et al*., 2006). By using ribosomal small subunit sequence alignment, visually comparable specimens of *A. saeedi* and *A. maximus* were shown to be conspecific in another molecular characterization of *A. saeedi* (Rana *et al.*, 2020). Phylogenetic analysis can be particularly useful in identifying conspecific or closely related worm species, based on these findings (Thakar *et al*., 2022).

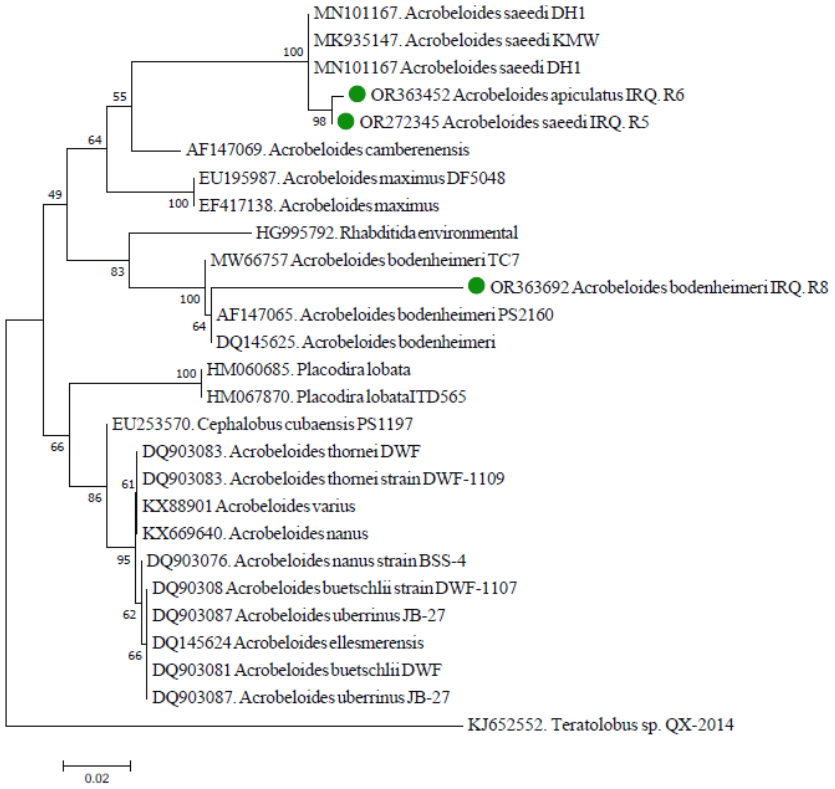

Diagram (1): Phylogenetic tree of *Acrobeloides* species collected from different localities of Iraq based on partial 28S rRNA gene sequences and other genera and species from GenBank.

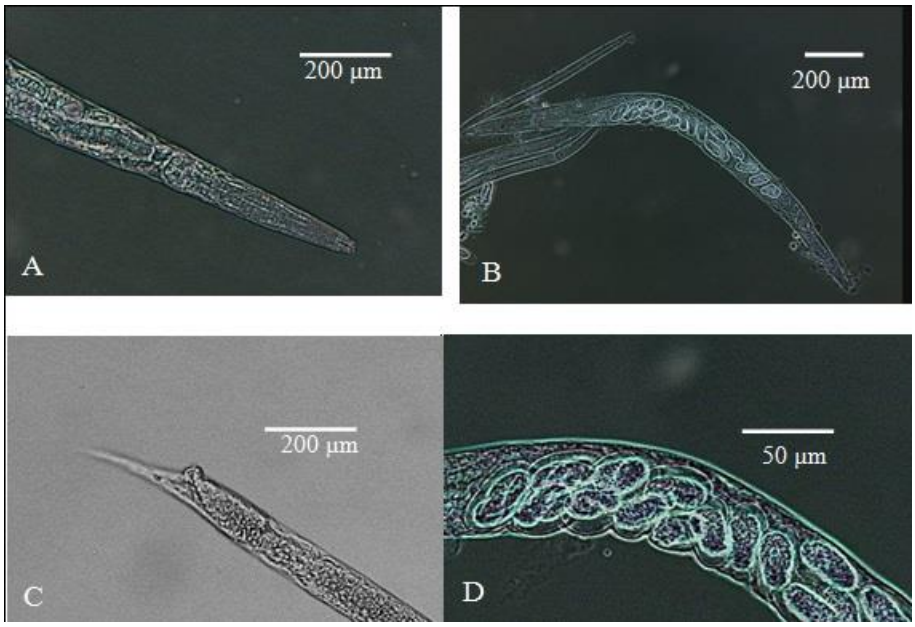

Plate (3): *Acrobeloides saeedi*; (A) Anterior end of female, (B) Entire female, (C) Female posterior end, (D) Vulva of female.

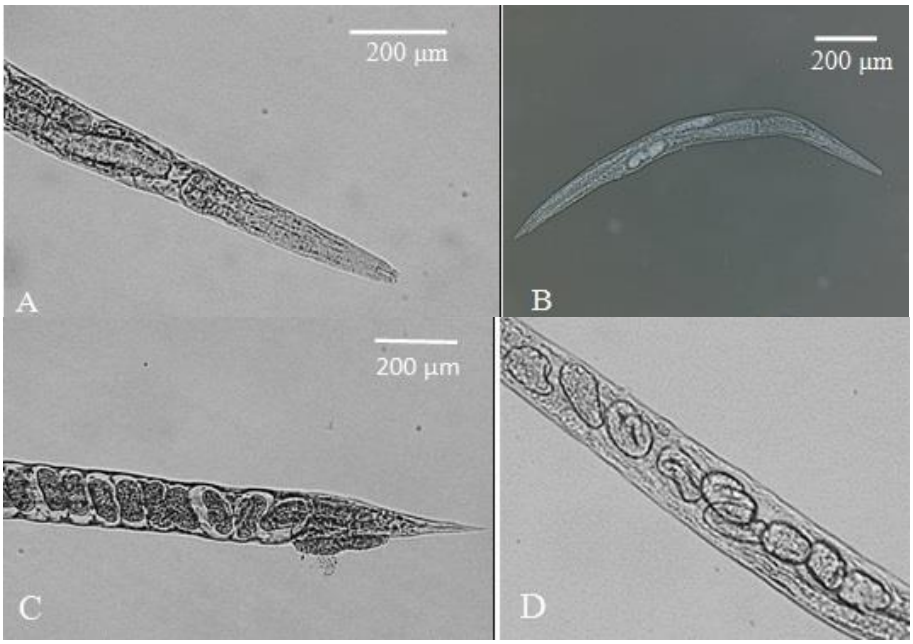

Plate (4): *A. bodenheimeri*; (A) Anterior end of female, (B) Entire female, (C) Female posterior end, (D) Vulva of female.

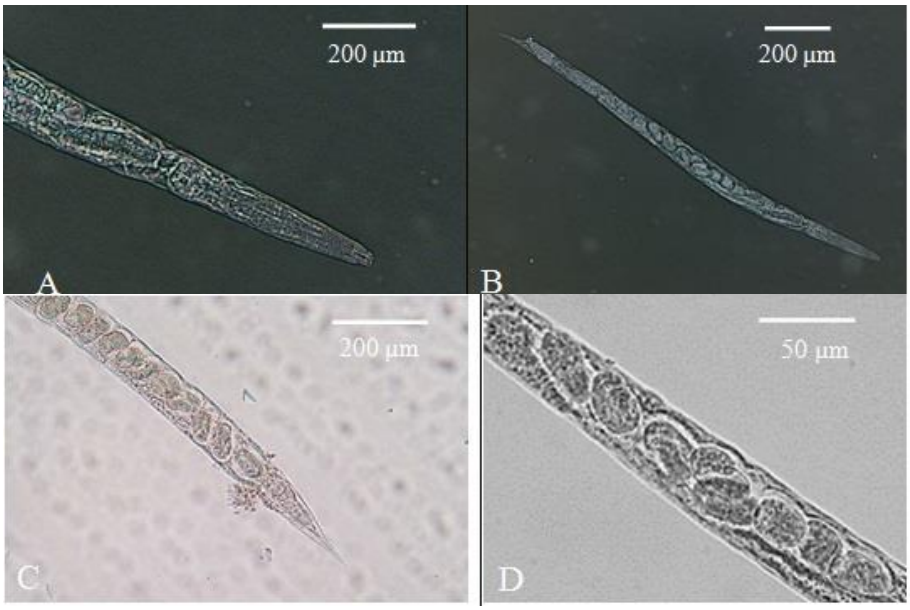

Plate (5): *A. apiculatus*; *(*A) Anterior end of female, (B) Entire female, (C) Female posterior end, (D) Vulva of female**.**

CONCLUSIONS

In the present article, three nematode species from the genus *Acrobeloides* (Cobb, 1924); Thorne, (1937) are described for the first time in Iraqi fauna. This study characterizes the first molecular study of these species in Iraq. The phylogenetic relationship is determined by the sequence of the partial 28S rDNA gene, and a phylogenetic tree was also constructed. The analysis resulted in recording of the species *A. saeedi*, *A. apiculatus*, and *A. bodenheimeri* for the first time in Iraq.

CONFLICT OF INTEREST STATEMENT

We would like to point out that the practical research procedures were carried out by the first and second researchers, while the third researcher analyzed the genetic tree and registered it in GenBank. The results of this study are part of the requirements of the Ph. D. in Zoology, Department of Biology, College of Science-University of Baghdad for the first author.

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دراسة مظهرية و جزيئية لثلاث انواع من جنس *Acrobeloides* (COBB, 1924) Thorne, 1937 (Rhabditida, Cephalobidae) كتسجيلات لاول مرة في العراق

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الخلاصة

عُزِلتْ ثلاثة أنواع من الديدان الخيطية الجنس *Acrobeloides* (Cobb, 1924) Thorne, 1937 (Rhabditida, Cephalobidae) وسُجلتْ لأول مرة في العراق. استنادًا إلى البيانات المورفومترية والجزيئية، وصِفَتْ الأنواع التالية:

A. saeedi Siddiqi, Ley & Khan, 1992

A. apiculatus (Thorne, 1925)

A. bodenheimeri (Steiner, 1936)

جزيئيًا باستخدام تسلسل جينات الرنا الرايبوسي 28 S الجزئي. و أُنشِأتْ شجرة النشوء لفصل أنواع جنس *Acrobeloides* المسجلة عن الأنواع ذات الصلة الوثيقة بها.