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ORIGINAL ARTICLE

MORPHOLOGICAL AND MOLECULAR DESCRIPTION FOR A NEW RECORD OF NEMATODE *ACROBELOIDES VARIUS* KIM, KIM & PARK, 2017 (RHABDITIDA, CEPHALOBIDAE) FROM IRAQ

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ABSTRACT

This study aimed to identify and describe one of the bacterial feeder nematode *Acrobeloides varius* Kim, Kim and Park, 2017 (Rhabditida, Cephalobidae), which was isolated from soil samples that were collected from Baghdad, central of Iraq, and was classified using both morphological and molecular criteria. All specimens of *A. varius* were cultured, identified and described using morphometric criteria. Selected specimens (Zah. IRQ3 OR994579.1 isolate) of this species were characterized by having the body length of the male ranging from (184.94 – 221.72 µm), the body length of the female ranging (507.38 – 521.92 µm) and the body length of the juvenile ranging from (355.53 – 490.35 µm). Selected specimens of this species were molecularly characterized using the partial 18S rRNA gene sequences. The 18S-rRNA sequence of Zah. IRQ3 OR994579.1 isolate had a range of (100%) sequence homology with the 18S rRNA sequence of *A. varius* available in the NCBI database. A phylogenetic tree was created to separate this species from closely related genera and species. *A. varius* Zah. IRQ3 OR994579.1 isolate represents the first record of this species in Iraq.

Keywords**:** Bacterial feeders' nematode, Iraq, Morphometric, Soil, 18S rRNA gene.

INTRODUCTION

The family Cephalobidae includes members of the genus *Acrobeloides* Cobb, 1924; Thorne, 1937, which are bacterial feeders and among the most prevalent and widespread nematode groups in a variety of terrestrial settings, including sand dunes (Wall *et al*., 2002), hills (Boström, 1993), forest (Háněl, 1999) and agricultural land (Pervez, 2011). Previous investigations have identified morphological and morphometric differences in this group,

including body size, nerve ring and excretory location, and tail form (Anderson, 1965, 1968; De Ley *et al.*, 1999; Abolafia and Peña-Santiago, 2003). These morphological variations frequently cause species-level taxonomy to be misguided by impeding species identification and delimitation. Only 29 of the 40 nominal species that have been described in this genus thus far may be considered valid (Andrássy, 2005), including two species that have been reported in Korea (Kim *et al*., 2016, 2017): *A. nanus* de Man, 1880 (Anderson, 1968) and *A. varius* (Kim *et al*., 2017). Kamal *et al*. (2024) isolated three species of nematodes within *Acrobeloides* (Cephalobidae) from the middle part of Iraq: these species (*A. saeedi* Siddiqi *et al*., 1992, *A. apiculatus* (Thorne, 1925) Thorne, 1937, and *A. bodenheimeri* (Steiner, 1936) Thorne, 1937) were the first to be reported in Iraq.

In the detritus food web, soil nematodes play a key role; examining their taxonomy and feeding habits, allows us to learn a lot about changes in the soil environment (Gupta and Yeates, 1997; Neher, 2001). In soil food webs, microfauna predators such as nematodes and protozoa are essential for connecting primary consumers like bacteria and fungi to higher trophic levels. They contribute to soil nutrient cycling and mineralize elements contained in microbial tissue by preying on micro-organisms (Neidig *et al*., 2010; Kadhim and Mahmood, 2014; Kadhim, 2021, 2022). Because nematodes have unique characteristics other soil organisms lack, their community structure can be an important bioindicator in environmental monitoring (Bongers and Ferris, 1999).

In PCR, amplification is performed using nematode DNA or the worm itself as a template (Seesao *et al*., 2014). Revised nematode classification and identification methods based on 18S rRNA sequence similarities have been proposed by a number of researchers, with a renewed emphasis on the PCR approach (Dawkins and Spencer, 1989). The 18S rRNA gene is frequently used to differentiate between unrelated nematode species and has been shown to develop more conservatively than the COI gene (Prosser *et al*., 2013; Armenteros *et al*., 2014). Despite its inability to distinguish among closely related nematode species, the 18S rRNA gene conserved regions have made it possible to create a number of promising primer sets that may be used to amplify an extensive diversity of worms in a general manner (Porazinska *et al*., 2009; Sapkota and Nicolaisen, 2015; Macheriotou *et al*., 2019). It has been demonstrated that the 18S small subunit ribosomal gene is a useful marker for nematode barcoding (Floyd *et al*., 2002). As a result, an increasing number of nematode databases are being created for use as model organisms in studies on a wide range of issues related to human health. Classification methods vary from those that rely on proteins or DNA to those that employ more advanced techniques (Faraj *et al*., 2019; Faraj and Al- Amery, 2020; Bhat *et al*., 2022).

The study of soil nematodes in Iraq has been a topic of interest in recent years. Additionally, molecular characterizations of bacterial feeder nematodes in Iraq have been conducted through some surveys in different regions. The literature on soil nematodes in Iraq highlights the importance of studying the genetic, phylogenetic relationships and distribution of nematodes in Iraqi soils.

MATERIALS AND METHODS

Collection of soil samples: A total of 54 soil samples were collected from Baghdad Province, in the middle of Iraq, which included three crop fields located in the north of Baghdad City, Al Rashidiya District (33˚25ˈ13.4" N 44˚21ˈ45.3" E), middle of Baghdad, Al Jadriya (33˚16ˈ35.7" N 44°23ˈ26.3" E) and west of Baghdad, Abo Ghareeb (33˚19ˈ15.5" N 44˚11ˈ57.1" E). The samples were collected within two seasons: spring (April–May 2023) and summer (July 2023). Each soil sample was composed of 5-7 subsamples randomly collected around each plant in a square -shaped fashion using a hand spade at a depth of 15-20 cm (Adegbite *et al*., 2006). Sub-samples placed in a plastic bag, mixed thoroughly, tightly closed to prevent drying, and then labeled. The samples were kept away from direct sunlight, then stored at 8-10 ˚C in a cooler container until they were sent to the laboratory for extraction and for estimating the presence of nematodes. Three samples were taken from different parts of each site. The weight of each soil sample was 1.5-2 kg. Three replicates were taken from each homogenized sub- sample to collect nematodes (Coyne *et al*., 2007).

Isolation of nematodes: The Baermann funnel technique was used to isolate nematodes from 250g of soil (Morise *et al*., 2012). Ten mL water suspensions were collected from each replicate and specifically screened by examining 1 mL chosen at random. A dissecting microscope (KRUSS Optronic/ Germany) was used to isolate nematodes.

Cultivation of nematodes and morphological measurements: Last-stage larvae of the greater wax moth *Galleria mellonella* (Linnaeus, 1758) were used to reproduce the isolated nematodes for permanent slides and DNA extraction. *G. mellonella* were killed with a sterilized lancet placed into a Petri dish (9 cm in diameter) with two pieces of filter paper. The isolated nematodes were added to the Petri dish, which was held at room temperature (22 ± 2 ºC) for 5-7 days. The reproduced nematodes were collected and transferred to an Eppendorf tube (1.5 ml) and kept in a fridge at 8-10 ºC. Before killing the nematodes and using the fixative methods, a number of nematodes (males, females or juveniles) were transferred to a microtube (1.5 ml) with sterilized water and kept at refrigerator temperature at 10˚C for molecular purposes. Other nematodes were used for fixation based on the methods reported by De Grisse (1969). For morphological and morphometric studies, permanent slides of collected nematodes were prepared (Al-Zaidawi *et al*., 2019). By randomly selecting 12 individuals (adults and juveniles) from the permanent slides, the morphological identification of preserved specimens was completed using an ocular micrometer (4X, 10X & 40X) with a compound microscope (Olympus, Japan) in accordance with the standard keys (Stock and Hunt, 2005; Nguyen, 2007) after they were fixed using different fixation methods (Al-Zaidawi *et al*., 2019). The body parts and other morphological characteristics of the specimens were observed, measured and photographed by a camera (opto-Edu image view 2021/ China). The following measurements were considered to describe the isolated nematodes: (L) Body length, (a) body length divided by maximum body width, (b) body length divided by oesophageal length, (c) body length divided by tail length, (c') tail length divided by body width at anus, (V%) position of vulva from anterior end as a percentage of

body length, (EP) distance from anterior end to excretory pore, (NR) distance from anterior end to nerve ring, (ES) distance from anterior end to the end of the pharynx, (T) tail length, (ABD) anal body diameter, (D%) distance from anterior end to excretory pore as a percentage of the distance from anterior end to the end of pharynx, (E%) distance from anterior end to excretory pore as a percentage of tail length (Nguyen, 2007).

Scanning electron microscopy (SEM): Morphological features of adults were examined using scanning electron microscopy (FEI Company, Netherland). For examination, the specimens (adults) were rinsed with distilled water three times. Then they were mounted on aluminum SEM stubs, coated with gold (nanoparticles). A plasma sputtering coater (China) was then used to coat the specimens, which were subsequently examined using an Inspect F 50 scanning electron microscope (Forst *et al*., 1997).

DNA extraction, amplification and electrophoresis: About 50 grams of cultured nematodes was used to extract the total genomic DNA. The DNA extraction was done using gSYNC ™ total DNA Extraction kit (Geneaid, Taiwan) in accordance with the manufacturer's instructions. A thermal cycler was used to amplify segments of the 18S region. The primer set of 26R (5`-CAT TCT TGG CAA ATG CTT TCG-3`) and G18S4 (5`-GCT TGT CTC AAA GAT TAA GCC-3`) was used following Rana *et al*. (2020). The PCR profile for all loci included 35 cycles of amplification in an Eppendorf thermocycler (analytikjena/ Germany). The program consisted of 94 °C for 4 minutes of initial denaturation, followed by 35 cycles of 94 °C for 1 minute, 55 °C for 1 minute, and 72 °C for 2 minutes, with a final extension for 10 minutes at 72 °C. Subsequently, the PCR product was electrophoresed on 1% agarose gels for 40 minutes using 10X TBE buffer 5%, and the gel was stained with green-viewer (SYBR). Ultimately, 3 µl of the PCR product and 2.5 µl of DNA ladder were added to each gel well. A 100-bp molecular DNA ladder (Bioneer, Korea) was used to determine the size of the amplified products.

DNA sequencing and analysis: For sequencing, the PCR products were sent to Macrogen Co. in Korea. Next, chromatogram quality was assessed, and consensus sequences were generated using the DNA Baser Assembler (DNA Sequence Assembler v4 (2013), Heracle Bio Soft, www.DnaBaser.com). The NCBI BLAST tool (http://www.ncbi.nlm.nih.gov/) was utilized to perform homology searches for every sequence. The phylogenetic analyses and nucleotide distance were calculated by using MEGA.7 program. In addition to this, the evolutionary history was inferred by using the Maximum likelihood method based on the Tamura 3-parameter model (Tamura, 1992). The tree with the highest log likelihood (-2490.84) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search was obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite likelihood (MCL) approach, and then selecting the topology with the superior log likelihood value. The analysis involved 16 nucleotide sequences in addition to the local isolate, *Caenorhabditis elegans*, which was considered as an outgroup. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2016).

RESULTS AND DISCUSSION

Among the nematode specimens that were gathered from the soil in Baghdad, Iraq, the suspected *Acrobeloides varius* Kim, Kim and Park, 2017, was cultured and identified based on molecular technique and morphological parameters. Morphological examinations of selected individuals (n=12) revealed that all the identified *A. varius* in the present study possessed the typical features of the species *A. varius*. The specimens of adult *A. varius* (Pl. 1) had the following characteristic features: Body cylindrical, usually ventrally curved after fixation but occasionally irregularly contorted. Cuticle annulated. Head region continuous with neck. Lip region with 6 + 4 papillae. Nerve ring position varies from the posterior extremity of the corpus to the posterior region of the isthmus. Excretory pore located at isthmus or infrequently at extremity of posterior corpus. Vulval lips protruding or not, post-vulval sac indistinct, ovary with or without double or fourfold flexure posterior to vulva. Tail conical. Phasmids located before the middle of the tail. All morphological measurements are illustrated in Table (1) and Plate (2).

Male: N=4, Body length(L) ranged from (184.94 – 221.72 µm), a = (14.07 – 15.85), b = (2.39 – 2.65), c = (8.5 – 47.82), c' = (1.23 – 2.05), the maximum body width ranged from (12.59 – 14.59 µm), EP (129.62 – 225.08), NR = (52.66 – 62.12), ES = (74.99 – 86.21), tail length (T) ranged from (3.46 – 18.24 µm), (Move it after the Anal body diameter) Anal body diameter. ranged from (3.02 – 9.24 µm), D% = (167.69 – 269.07), E% = (527.42 – 4695.32).

Female: N=4, Body length (L) ranged from (507.38 – 521.92 µm), a = (16.05 – 18.53), b = (4.28 - 4.34), c = (10.58 – 41.3), c' = (1.25 – 1.89), V% =(54.69 – 66.89), Greatest body diam. ranged from (44.36 – 67.88 µm), EP = (772.77 – 1013.17), NR = (128.2 – 147.94), ES = (181.47 – 201.67), tail length (T) ranged from (82.43 – 100.75 µm), (Move it after the anal body diameter) Anal body diameter. Ranged from (27.54 – 32.26 µm), D% = (406.17 – 415.75), E% = (958 – 4029.64).

Juvenile: N=4, Body length (L) ranged from (355.53 – 490.35 µm), a = (15.2 – 17.18), b = (3.36 – 3.88), c = (13.13 – 24.63), c' = (1.58 – 2.04), Greatest body diameter. ranged from (23.38 - 28.62 µm), EP = (333.28 – 465.5), NR = (84.56 – 101.82), ES = (101.57 – 132.01), Tail length (T) ranged from (17.9 – 29.18 µm), Anal body diameter (ABD). ranged from (11.11 -14.53 µm), D% = (313.44 – 369.72), E% = (1213.06 – 2362.74). The lengths and measurements of the *A. varius* in this study were similar to those described by Kim *et al*. (2017) from South Korea.

Morphological and molecular description

Table (1): Morphometric measurements (Mean ± SEM) of adults female, male and juvenile of *Acrobeloides varius* isolate (in µm).

Measurements	Male (4)	Female (4)	Juvenile (4)
L	203.33 ± 18.39 (184.94 – 221.72)	514.65 ± 7.27 (507.38 – 521.92)	422.94 ± 67.41 (355.53 – 490.35)
A	14.96 ± 0.89 (14.07 – 15.85)	17.29 ± 1.24 (16.05 – 18.53)	16.19 ± 0.99 (15.2 – 17.18)
B	2.52 ± 0.13 (2.39 – 2.65)	4.31 ± 0.03 (4.28 - 4.34)	3.62 ± 0.26 (3.36 – 3.88)
C	28.16 +19.66 (8.5 – 47.82)	25.94+ 15.36 (10.58 – 41.3)	18.88 ± 5.75 (13.13 – 24.63)
c’	1.64 ± 0.41 (1.23 – 2.05)	1.57 ± 0.32 (1.25 – 1.89)	1.81 ± 0.23 (1.58 – 2.04)
V		60.79 ± 6.10 (54.69 – 66.89)	
Greatest body diam.	13.59 ± 1 (12.59 – 14.59)	29.9 ± 2.36 (27.54 – 32.26)	26 ± 2.62 (23.38 - 28.62)
EP	177.35 ± 47.73 (129.62 – 225.08)	490.81 ± 6.21 (484.6 – 497.02)	399.39 ± 66.11 (333.28 – 465.5)
NR	57.39 ± 4.73 (52.66 – 62.12)	93.82 ± 4.05 (89.77 – 97.87)	93.19 ± 8.63 (84.56 – 101.82)
ES	80.6 ± 5.61 (74.99 – 86.21)	119.44 ± 1.61 (117.83 – 121.05)	116.79 ± 15.22 (101.57 – 132.01)
Tail length (T)	10.85 ± 7.39 (3.46 – 18.24)	23.84 ± 9.02 (14.82 – 32.86)	23.54 ± 5.64 (17.9 – 29.18)
Anal body diam. (ABD)	6.13 ± 3.11 (3.02 – 9.24)	14.71 ± 3.45 (11.26 – 18.16)	12.82 ± 1.71 (11.11 -14.53)
D%	218.38 ± 50.69 (167.69 – 269.07)	410.96 ± 4.79 (406.17 – 415.75)	341.58 ± 28.14 (313.44 – 369.72)
E%	2611.37 ± 2083.95 (527.42 – 4695.32)	2494.06 ± 1535.58 (958 – 4029.64)	1787.90 ± 574.84 (1213.06 – 2362.74)

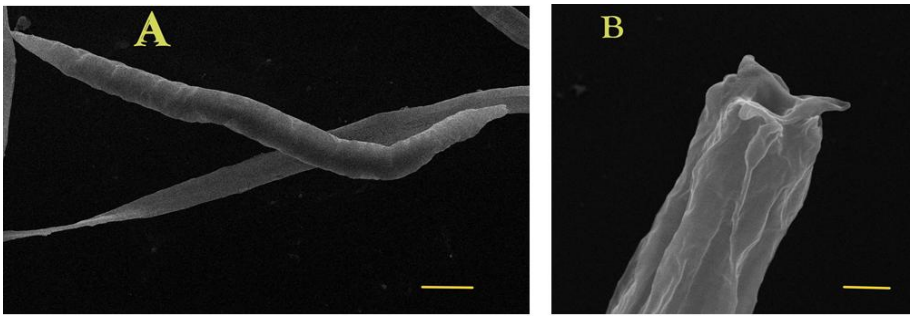

Plate (1): Scanning electron micrographs (SEM) of *Acrobeloides varius* (adult); (A) Whole body, (B) Anterior region. [Scale bars: A= 100 µm, B= 2µm].

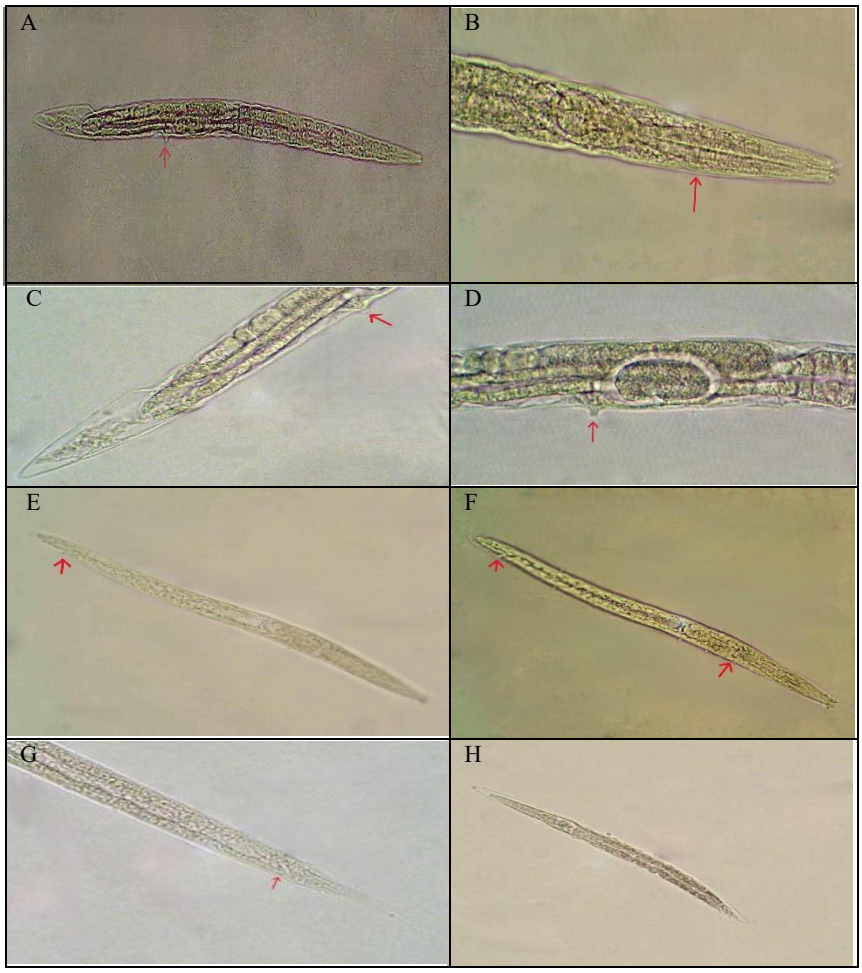

Plate (2): Light microphotographs of *Acrobeloides varius*; (A) Whole body of the female (10X), (B) Anterior region of female NR (40X), (C) Female posterior end (40X), (D) Vulval region of the female (40X), (E) Whole body of the male (40X), (F) Anterior region of male (40X), (G) Tail region of male (40X), (H) whole body of juvenile (10X).

Analysis using 18S sequence for *Acrobloides varius* isolate: Other selected specimens of *A. varius* were subjected to molecular technique using DNA sequence or using molecular techniques based on DNA to verify the morphological identification of the isolated nematodes. The 18S-rRNA (898 bp) amplicon from the selected individual yielded single bands on agarose gels. Nucleotide sequence data reported from this isolate are available in the GenBank database. 18S-rRNA nucleotide sequence data is found under the accession number

OR994579.1. The 18S-rRNA sequences of this isolate had 100% sequence homology with 18S-rRNA sequence of *A. varius* (Accession number MK636581), (Accession number MK636580), (Accession number KX889085), (Accession number MK636579), (Accession number KY119884) and (Accession number EU543174).

The mean inter-specific distance between the *A. varius* Zah.IRQ3 (Accession number OR994579.1) isolate and other isolates of *Acrobeloides* was 0.130 % (range 0.00 % – 1.134 %), which has been calculated using the Tamura 3-parameter model based on the 18S gene. Nucleotide distance between *A. varius* isolates from Iraq and *A. buetschlii* de Man, 1884, DWF1107(Accession number EU543174) was 0.001% (Tab. 2). The tree (Diag.1) shows that *A. varius* (Accession# OR994579) from this current investigation was in the same clade together with *A. apiculatus* (Accession# KY119884) and *A. buetschlii* (Accession# EU543174) isolates from previous investigations. This result agreed with the result illustrated by Rana *et al*. (2020). They showed that *A. varius* had same clade with both *A. apiculatus* and *A. buetschlii.* The current finding also agreed with the results reported by Kamal *et al*. (2024), who showed that *A. varius* was in the same clade with *A. buetschlii.* In accordance with Rana *et al*. (2020) phylogenetic analyses based on 18S rDNA sequences, isolates of *Acrobeloides* species formed a group that was obviously monophyletic. In a clade with 100% support, these isolates were probably conspecific isolates that merged to create a sister clade with "Maximus" group species from various geographic locations.

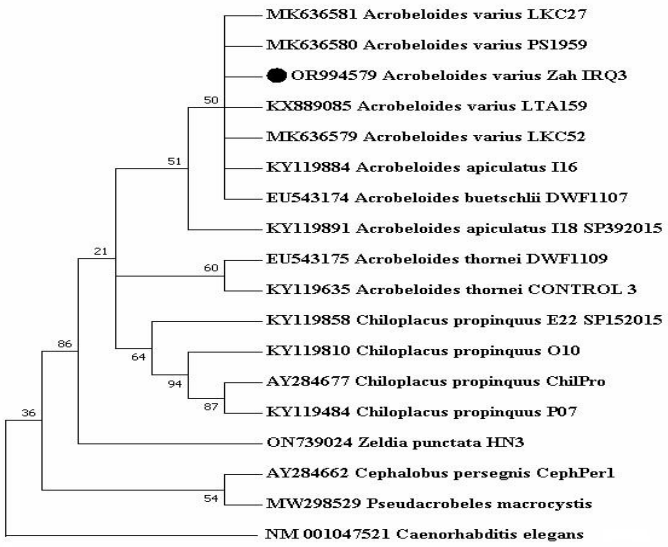

Diagram (1): Phylogenetic relationships of the *A. varius* isolate with 16 isolates of other species related to *Acrobeloides* genus based on 18S-rRNA gene sequences as inferred from neighbor-joining (NJ) analysis, *C. elegans* (NM001047521) was used as an outgroup, Support values are presented near the nodes in the form of bootstrap values in ML.

Table (2): Comparing several *Acrobloides* species and isolates pairwise based on the amount of nucleotide differences with *Acrobloides varius* Zah.IRQ3 based on 18S sequences.

No.	Nematode species	Differences of distances among species											
1	OR994579 *Acrobeloides varius* Zah IRQ3												
2	MK636581 *Acrobeloides varius* LKC27	0.000											
3	MK636579 *Acrobeloides varius* LKC52	0.000	0.000										
4	AY284677 *Chiloplacus propinquus* ChilPro	0.011	0.011	0.011									
5	AY284662 Cephalobus persegnis CephPer1	0.013	0.013	0.013	0.018								
6	EU543174 *Acrobeloides buetschlii* DWF1107	0.001	0.001	0.001	0.013	0.014							
7	KY119884 *Acrobeloides apiculatus* l16	0.001	0.001	0.001	0.013	0.014	0.003						
8	EU543175 *Acrobeloides thornei* DWF1109	0.001	0.001	0.001	0.013	0.014	0.003	0.003					
9	ON739024 *Zeldia punctata* HN3	0.01	0.01	0.01	0.02	0.02	0.01	0.01	0.01				
10	KY119484 *Chiloplacus propinquus* P07	0.013	0.013	0.013	0.001	0.019	0.014	0.014	0.014	0.021			
11	AF202159 *Acrobeloides bodenheimeri* PS1158	0.013	0.013	0.013	0.019	0.013	0.014	0.014	0.014	0.021	0.018		
12	KY119810 *Ciloplacus propinquus* O10	0.014	0.014	0.014	0.008	0.021	0.015	0.015	0.015	0.023	0.009	0.022	
13	MW298529 *Pseudacrobeles macrocystis*	0.019	0.019	0.019	0.022	0.015	0.021	0.021	0.021	0.026	0.021	0.021	0.028
14	NM 001047521 *Caenorhabditis elegans*	1.134	1.134	1.134	1.150	1.153	1.142	1.142	1.142	1.159	1.147	1.121	1.184	1.124

CONCLUSIONS

This study provides the first confirmed record of *Acrobeloides varius* in Iraq, based on an integrated identification approach combining detailed morphometric characterization with molecular analysis of the 18S rRNA gene. The morphological measurements of males, females, and juveniles were consistent with previously published descriptions of the species, while the partial 18S rRNA sequence of the isolate Zah. IRQ3 OR994579.1 showed 100% homology with *A. varius* sequences available in the NCBI database, confirming the accuracy of the identification. The phylogenetic tree further supported the separation of *A. varius* from closely related genera and species. Overall, the findings highlight the importance of this work in documenting the nematode biodiversity in Iraqi soils and provide a foundation for future ecological and taxonomic studies on bacterial-feeding nematodes in the region.

CONFLICT OF INTEREST STATEMENT

It is worth noting that this work is part of a Ph. D. dissertation submitted to the Department of Biology, College of Science, University of Baghdad for the first author.

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الوصف المظهري والجزيئي لسجل جديد للدودة الخيطية *Acrobeloides varius* Kim, (Cephalobidae, Rhabditida) Kim and Park, 2017 من العراق

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الخلاصة

هدفت هذه الدراسة الى تشخيص الدودة الخيطية *Acrobloides varius* Kim, Kim and Park, 2017 من رتبة Rhabditida و عائلة Cephalobidae التي تتغذى على البكتريا وفقاً للصفات المظهرية والجزيئية. عزلت هذا النوع من عينات التربة التي جمعت من بغداد، وسط العراق. تم تربية جميع نماذج *A. varius* لتشخيصها و وصفها باستخدام بعض المعايير المظهرية. تميزت النماذج المختارة لهذا النوع عزلة (Zah. IRQ3 OR994579.1) بطول جسم الذكر الذي يتراوح (184.94–221.72 مايكرومتر) وكان طول جسم الانثى (507.38-521.92 مايكرومتر) وكان طول جسم الصغار (355.53-490.35 مايكرومتر). وصفت العينات المختارة من هذا النوع جزيئيًا باستخدام تسلسلات جين 18S-rRNA الجزيئية. كان لتسلسل 18S-rRNA لعزلة (Zah. IRQ3 OR994579.1) نطاق من التماثل التسلسلي (100%) مع تسلسل 18S-rRNA لـ *A. varius* المتوفر في قاعدة بيانات NCBI. تم انشاء الشجرة التطورية لفصل النوع المدروس عن الاجناس والانواع ذات الصلة الوثيقة. يمثل *A. varius* عزلة (Zah. IRQ3 OR994579.1) أول تسجيل للعراق.