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Morphological identification, molecular characterization and optimization of cultivation for Acrobeloides maximus species extracted from bitter gourd (Momordica charantia) field at Gujarat, India.

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Abstract

Background

Free-living nematodes are very important part of agricultural soil ecosystems, especially members of the *Cephalobidae* family. Morphological and morphometric analysis is efficient for genus-level classification of *Cephalobidae* members, but to achieve species-level identification, molecular characterization is imperative.

Methods and Results

The nematodes extracted from the collected soil of the bitter gourd field were cultivated on NGM (nutrient growth medium) agar plates under standardized laboratory conditions. The morphological characteristics of 42 females and 6 males were captured under a digital microscope and processed using the ImageJ analysis tool for De-Man measurements and compared with previously submitted databases. DNA barcoding was carried out with the 18S small RNA subunit gene, and the resulting sequence was then processed to BLASTN after alignment and annotation. The phylogenetic tree was constructed from the processed data files and further evaluated by branch lengths and bootstrap values. Blast analysis showed 98% similarity with the *A. maximus* strain, which was also supported by high bootstrap values (96%). *Acrobeloides maximus* was identified based on morphological features described by Throne, 1925 and confirmed by molecular characterization.

Conclusion

The nematodes collected from the soil sample were cultivated and identified as *A. maximus* by a combined approach of morphological, pictorial, morphometric, and molecular analysis, which may further be used in the future identification of *Acrobeloides spp.* To our knowledge, this is the first recorded *A. maximus* from the bitter gourd field of Gujarat, India. Pictogram and measurement of eggs inside the female body is also shown for *A. maximus for the first time.*

Introduction

At all layers of the agricultural ecosystem, a wide range of nematodes can be found. These include nematodes that feed on bacteria and fungi in the soil as free living, parasitize on plants and insects, and even consume other nematodes. Among them, the *Cephalobidae* family is most abundant in agricultural fields. Precision in species level identification is critical for comprehension and communication of any organism's ecological role. Andrássy (1974) coined suborder *Cephalobina* to comprise exceptionally diversified bacterivores of free-living nematodes [1–2]. Family *Cephalobidae* includes 4 subfamilies: *Cephalobinae, Acrobelinae, Kirjanoviinae* and *Panagrocephalinae*, with approximately 275 species, including 24 genera. Following their report, taxonomy of the genus *Acrobeles* has been questioned from

the beginning. Von Linstow based his theory on the identification of *Acrobeloides ciliatus* as a new species with similar characteristics to *Cephalobus* [3]. In the same way, De Man (1884), Steiner (1914), Cobb (1924) and others grouped *Acrobeloides* and similar species with *Cephalobus*, and classified it as a subgenus of *Cephalobus* [3–6]. Cobb's new study shows *Acrobeles*as a distinct genus and proposes the subgenera *Acrobeles* and *Acrobeloides*, a separation that appears to be highly appropriate. Regarding characteristics, cheilorhabdion are wider than stomas in *Cephalobinae*. The family *Cephalobidae* includes a large number of organisms and a wide range of habitats, but they are difficult to identify and classify taxonomically based on morphological features. The genus and species of *Cephalobidae* can be differentiated by the anatomy of the lip, stoma [7], probolae and posterior region characters, such as the length, position and shape of the tail [3]. Among these, probolae is an important feature for identifying most *Cephalobidae* members, but in the case of *Acrobeloides* and *Cephalobus*, it is difficult to distinguish between them because both share similar features, including simple and low probolae and tail shapes. Such morphological and morphometric characters can lead to erroneous identification of species, which could be authenticated by a molecular phylogenetic approach for identification purposes. In addition, the knowledge from phylogenetic analysis contributes to the basic (e.g., evolutionary history of species, evolution of genes, and identification of sampled species) as well as applied biology (e.g., investigation of the route of pathogenic microorganism infection).

Traditionally, morphological identification is performed by pictorial and morphological diagnostic keys. The taxonomy of the genus *Acrobeloides* was first mentioned by Cobb in 1924 [5]. After that, this group recorded many morphological variations, such as body length, shape of the tail, position of metacorpus, and shape of reproductive organs [8–11]. In general, species identification, selective morphometric data were compared as mentioned in de Man Indices, a method given by De Man in 1876 and 1880 and then revised by Cobb (1914) and Thorne (1949), and these data were then matched with a nematode database, popularly known as nemaplex [12]. All characters were confirmed with various identification keys and public databases [3, 9, 13–14]. In the field of nematology, traditional identification has been widely accepted for years, but it requires experience and expertise, with the ability to identify species-level discriminatory characters. Moreover, in some cases, revision of identification was reported due to lack of accuracy in identification of characters [15]. Recent developments in technology, on the other hand, may question human-dependent image analysis. Consequently, the sequencing-based method is influencing the field of nematode taxonomy to overcome the limitations posed by morphological identification. Holterman (2008), and his coworkers performed ribosomal DNA sequencing and employed that data for evolutionary and identification studies [16]. Recently, PCR-based DNA barcoding of the internal transcribed spacer (ITS) region using selective primers has become a highly efficient technology in the identification field [17–18]. Despite the fact that the genome database of nematodes has improved in the current period, some nematode groups, including *Acrobeloides* have not been widely, studied and data of partial fragments only are available in INSDC (GenBank). *A. maximus* has been studied for its role in soil decomposing activity, biocontrol by entomopathogenic nematode (EPN) activity, evaluation of parasitism and microbial relationships, which shows its crucial role in the agricultural food web, even though only 29 partial sequences of *A. maximus* are submitted to GenBank [14, 19–20]. Phylogenetic studies can lead us

in the direction of the relationship of nematodes with their surrounding ecosystem. Mitochondrial DNA-based phylogeny proved to be a good molecular marker and it can provide us with insight into the close ancestry of nematodes [21]. One such phylogenetic study of 33 *Acrobeloides* and *Cephalobus* samples disclosed the core of 22 related clades, which did not describe any monophyletic relationship between *Acrobeloides* and *Cephalobus*, since it was not clear from morphological studies alone [19].

To the best of our knowledge, only a few studies have reported the molecular characterization of *A. maximus* [2, 9]. Thus, the aim of the present investigation was to cultivate and identify nematode species extracted from bitter gourd fields with the help of molecular, morphometric and morphological data. Their evolutionary relation was inferred with the help of phylogenetic analysis based on the 18S ribosomal gene. This study will improve the molecular and morphological database of *Acrobeloides* species which includes species specific primers for identification, pipeline of molecular taxonomy of nematodes, pictorial and morphometrics information of *A. maximus*, which will contribute towards accurate identification of *A. maximus* species. In addition phylogenetic analysis of extracted species improves our understanding regarding *A. maximus* specie's ecological role in agricultural fields.

Material And Methods

Sample Collection

Agricultural field soil samples were collected from bitter gourd farms in Devda Village (23°580726204810233N, 72° 58412992582637E) of Gujarat, India, twice in June and December 2019. Using a sterile spatula, soil approximately 2-2.5 cm deep around the roots of bitter gourd (***Momordica charantia)*** was collected into sterile sample bags, as nematodes do not survive in the upper layer of the soil because of extreme environmental factors. The collected soil samples were sprayed with water to maintain humidity until further processing. Roots, leaves, stems and fruits from the field were also collected to check the parasitic activity of nematodes. All bags were tagged with date of collection, code for sample, and location. The map of the sampling site created with QGIS is provided in Figure 1.

Extraction of nematodes

From the collected soil, a sample labeled Nema2BG showed the presence of nematodes during primary screening, which was then extracted by modified Cobb's [22-23] filtration, followed by placing soil onto muslin cloth attached to a sieve, as shown in Figure 2. The sieve with soil was placed on top of a water field petri plate for 2-3 days, which led to crawling of nematodes into the water due to gravity. Based on the principle of density, the nematodes can be separated from mixed organisms present in water-filled petri plates. For that, water from the Petri dishes was collected into centrifuge tubes after incubation and centrifuged at 3000 rpm for 5 minutes, after that, the pellet was collected into small Petri plates and visualized under a stereoscopic microscope to confirm the presence of nematodes.

Morphological and morphometric characterization

The pellets of nematodes were washed with M9 buffer at 2000 rpm before slide preparation. Morphological and morphometric image analysis was performed with extracted nematodes by transferring a 10 µm nematode pellet onto the cavity slides, and dilute methanol was used as a paralyzing agent. Thereafter, slides were visualized with a digital light microscope (Panthera L with built-in image system), and all morphological observations were recorded with inbuilt microscope software (motic image) in the form of images for further analysis. In addition, measurements of total body length, width, tail length, distance from the anterior part to the esophageal bulb and spicule length were recorded with the help of image analysis software Fiji in micrometers. The obtained ratios were calculated as described by De manian indices [24]. The recorded data were compared and analysed with online databases and pictorial identification keys [9, 25-26]. A micrograph of the important morphological characteristics of *A. maximus* described by Thorne [3] is shown in Figure 3. Species-specific characters, such as body length, width and % vulva position, as described by Throne [3], were used for morphometric comparison. A list of characters and abbreviations considered are listed below:

n - Number of organisms used for the study

L - Total Body Length

W - Body Width

AV - anterior to Metacarpus valve distance

S - Spicule length

T – Tail length

V - % Position of Vulva from Anterior end

a -L/Greatest body length

b -L/AV

c -L/T

Cultivation of nematodes

NGM medium was prepared in 100 ml distilled water containing 4% agar, 0.24% peptone, 1.2% NaCl in 2.5% phosphate buffer (pH 8) along with a natural nutrient mixture (5%) prepared in our laboratory. The prepared media was autoclaved and poured into petri plates. After solidification of NGM medium, extracted nematodes were inoculated on NGM plates. Later, growth was observed for 10 days under a stereoscopic microscope. The plates were stored at 25°C in an incubator. Nematodes were inoculated into new NGM plates every 7 days to maintain fresh culture.

Molecular characterization

Genomic DNA was extracted from fresh nematode samples by a DNA Extraction Kit: 'Genomic DNA Purification Kit' (SCMR006) manufactured by SLS research, based on Spin Column Technology. A fragment of the gene was amplified by PCR with Primers FP GCAAGTCTGGTGCCAGCAGC - RP CCGTGTTGAGTCAAATTAAG. The following thermal conditions for cycling profiles were used for PCR: 95°C for 5 minutes, 35cycles at 95°C for 30 seconds (sec.) 50°C for 30 sec, 20°C for 10 min, and a final extension at 72°C for 7 minutes with primers that amplified approximately 650 bp of product [27]. The quality of the PCR product was evaluated by1.0% agarose gel, which resolved a single discrete PCR amplicon band on gel. The PCR amplicon was purified by column purification to remove contaminants. Furthermore, DNA sequencing of the PCR amplicon was carried out with both primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic analyser. The acquired forward and reverse sequences were aligned to obtain a consensus query sequence. The BioEdit tool was used to visualize sequences [28]. Finally, the altered sequence was submitted to the National Center for Biotechnology Information (NCBI) GenBank database with the ON533625.1 accession number.

Sequence alignment and phylogenetic analyses

The consensus sequence of Sample Nema2BG was converted to FASTA file format using the BioEdit tool. These FASTA files were uploaded to the NCBI Basic Local Alignment Search Tool (BLASTN). Afterwards, the NCBI BLASTIN results were filtered based on the maximum identity score and lower E value, and the first 20 sequences (Table 1) were aligned with the Nema2BG query sequence using the ClustalW alignment tool of MEGA 11. All gaps were removed from the aligned sequences, and the file was converted into mega format. The best model for analyses, named Hasegawa-Kishino-Yano, was suggested by the 'Finding the best DNA' tool. Using this Hasegawa-Kishino-Yano model (the best model for analysis in the NJ method), a 1000 bootstrap replicated phylogenetic tree was constructed. The evolutionary history was inferred by using the maximum likelihood method and Hasegawa-Kishino-Yano model [29]. The tree with the highest log likelihood (-10423.43) was obtained as a result. The initial tree for the heuristic search was obtained automatically by applying the neighbor-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Hasegawa-Kishino-Yano and then selecting the topology with a superior log likelihood value. The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 26.55% sites). This analysis involved 21 nucleotide sequences, and there were a total of 1957 positions in the final dataset. Likewise, the evolutionary analyses were also conducted in MEGA11 [30].

Results

Morphological identification

Image analysis of micrographs shown in Figures 4a and 4b showed similar characteristics like mouth structure, shape of metacorpus, lip structure of vulva and tail shape to *A. maximus* species as described by Thorne, 1925.

The current morphological observations revealed that the anterior end starts with a circular arrangement of papillae surrounding the lips, which are isolated by axils followed by three circular, sleek, thin plates surrounding the mouth in the labial probolae. The pharynx has extensive cuticle impregnation in several areas followed by cylindrical shaped esophagus with midway swelling. However, the vulva is visible in adult females. *Acrobeloides* females showed a large difference between the two lip sizes, one facing the tail was larger in size compared to one facing the mouth. Although the anal cavity is surrounded by lips that merge inside the body cavity, the anus was clearly visible at the tail region. Conoid-shaped tails showed minute differences in morphology, as the tip of the male tails wasventrally arcuate, while that of the female tails was blunt. Spicules and gueraculum were present midway to the tail in males. The occurrence of males was found to be rare in the population.

Description of *A. maximus* (Thorne, 1925) Thorne, 1937 (Figs. 3-4)

Morphometrics

The body length of nematodes varies rapidly from juvenile to adult age. A thick layer of cuticle was found on the body wall. Body measurements of females were recorded in 10x lenses, whereas 4x lenses were used for males. As per de Man's measurement metrics, important body measurements of 42 females and 6 males were recorded, as listed in Table 2.

Forty-two females and six males were selected for current morphemetric studies. Different characters, such as body length, width, tail length and anterior to metacarpus valve distance, were measured. The ratios, standard deviations and mean values were calculated in Microsoft Excel 2007 as mentioned in Table 2 According to the findings of this study, female body size is comparatively larger than male body size. The largest female in the study was 1965 µm long, whereas the largest male was 1066 µm long. The shortest female was found to be 688 µm long, and the male was 894 µm long. In the current study, female tail length varied from 77 µm to 118 µm, and male tail length ranged from 73 µm to 116 µm. In comparison, the male tail was found to be longer than the female tail length. The values of a(L/GBD), b (L/AV)and c (L/Tail) also fall within the range of Throne,1925 studies, as mentioned in Table 1, total egg size of 141 µm was recorded in the female body during image analysis (Fig. 4b).

Table 1 Comparative measurement of various body parts of *Acrobeloides maximus* as reported by Thorne (1925) and Pervez (2011) with Nema2BG measurements. All measurements are in micrometers (except n, ratio and percentage) and in the form: mean± standard deviation.

Characters	Female (□)	Female (□)	Male (□)	Male (□)
n	16	43	1	6
Body Length(L)	1200	1250 ± 305	900	1066 ± 101
Width	**	72 ± 13	**	57 ± 6
Egg size(1)	**	141	**	**
A-V length	**	376 ± 63	**	298 ± 56
Tail Length	66	118 ± 24	**	92 ± 16
a(Ls/GBD)	20	11 ± 2.8	27	16 ± 1.5
b(LS/AV)	5.7	3 ± 0.39	7.2	3 ± 0.72
c(LS/Tail)	18	10 ± 3	18	11 ± 1.4
Spicule length	**	**	**	62 ± 12
References	Thorne, 1925 [3]		Pervez,2011 [31]	

Table 2 List of the top 20 BLASTs used in phylogenetic studies.

Sr. No.	Description	Scientific name	Percentage	Accession No.
1	*A. maximus* strain RT2-R25A	*Acrobeloidesmaximus*	98%	JQ237850.1
2	*A. maximus* strain RT1-R15C	*Acrobeloidesmaximus*	98%	JQ237849.1
3	*A. maximus* isolate wb4	*Acrobeloidesmaximus*	98%	EU306344.1
4	*A. maximus* strain DF5048	*Acrobeloidesmaximus*	98%	EU196016.1
5	*A. bodenheimeri* strain PS2160	*Acrobeloidesbodenheimeri*	97%	AF202162.1
6	*A. bodenheimeri* strain PS1158	*Acrobeloidesbodenheimeri*	97%	AF202159.1
7	*Pseudacrobeles sp*. OH-2016	*Pseudacrobeles sp. OH-2016*	97%	KU180672.1
8	*Dicelisussuriensis*	*Dicelisussuriensis*	97%	EU573706.1
9	*A. thornei strain* DWF1109	*Acrobeloidesthornei*	97%	EU543175.1
10	*A. varius* strain LKC27	*Acrobeloidesvarius*	97%	MK636581.1
11	*A varius* strain PS1959	*Acrobeloidesvarius*	97%	MK636580.1
12	*A.varius* strain LKC52	*Acrobeloidesvarius*	97%	MK636579.1
13	*A. nanus*	*Acrobeloidesnanus*	97%	KX669638.1
14	*A. varius*	*Acrobeloidesvarius*	97%	KX889085.1
15	*Acrobeloides cf. buetschlii* 1 JH-2012	*Acrobeloides*cf.*buetschlii 1* JH-2012	97%	JQ957905.1
16	*Acrobeloides cf. buetschlii* 2 JH-2012 isolate AcLoBue2Z	*Acrobeloides*cf*. buetschlii 2* JH-2012	97%	JQ957904.1
17	*Acrobeloides sp*. KJA	*Acrobeloides sp.* KJA	97%	HQ130506.1
18	*A. buetschlii* strain DWF1107	*Acrobeloidesbuetschlii*	97%	EU543174.1
19	*A. apiculatus* isolate AcLoApi	*Acrobeloidesapiculatus*	97%	AY284673.1
20	*A. nanus*	*Acrobeloidesnanus*	97%	DQ102707.1

Molecular Analysis

Molecular characterization of *A. maximus* was performed by the 18S small subunit rRNA gene partial sequence. A BLASTN search of the query sequence showed 98% identity score with previously submitted *A. maximus* sequences to GenBank. The top 21 sequences (Table 3) along with Nema2BG sequences were selected from the BLASTN search results for annotation and trimming. The resulting 654 bp sequence with 50.3% GC content was accepted by the NCBI gene bank and released publicly with accession numberON533625 for the 18Sribosomal region of *A. maximus* species.

Phylogenetic Analysis

GenBank BLASTN nucleotide sequence analysis showing 97-98% sequence match with *Acrobeloides* species as listed (Table 2). Among them *A. maximus* is on the top of the list with highest species identity high as 98%. Top 20 sequences were selected from BLASTN results for the phylogenetic analysis. The maximum likelihood tree (Fig.5) representing the top 18S rRNA sequences with the highest identity score for the Nema2BG sequence (ON533625) showed that the query sequence was closely related to the *A. maximus* strain RT2-R25A sequence, which also showed 91% bootstrap support. Sequences within the sister clade included three *A. maximus* species (EU196016.1, EU306344.1 and JQ237849.1) highlighted with red dots in the phylogenetic tree (Fig.5), which showed 96%,95% and 48% bootstrap support, respectively, in the bootstrap phylogeny. This phylogenetic tree also displayed other *Acroeloides* strains that are part of sister clades. The clade with three *A. maximus* species shares a clade with *A. varius* (MK636580.1, KX889085.1, MK636579.1 and MK636581.1), *A. apiculatus* (AY284673.1), *A. bodenheimeri* (AF202162.1 and AF202159.1), and *A. buetschlii* (JQ957905.1), and Clade with Nema2BG shares a clade with *A. maximus* (JQ237850.1), *Acrobeloides nanus* (KX669638.1 and DQ102707.1), *A. buetschlii* (EU543174.1) and *A. thoruei* (EU543175.1). From the sister clades, JQ237850.1 showed the highest support (100%), followed by AY284673.1 (96%) and EU306344.1 (95%). The interpretation of the above mentioned phylogenetic tree represents utmost support values with nodes of the *A. maximus* species. Bootstrap analysis values also exhibited very high support values of 100% (JQ237849.1), 96% (EU196016.1), and 95 % (EU306344.1) and 91 % (JQ237850.1) for *A. maximus* species clades (Bootstrap tree in the supplementary file). ME tree inferred a close relationship of Nema2BG samples with *A. maximus* species present in the NCBI GenBank.

Discussion

The *Cephalobidae* family has a substantial number of bacterial feeding nematodes with diverse morphologies and worldwide ranges [32]. Multiple reports have suggested the association of *Acrobeloides* with insects and bacterial species in the past [14, 33–34]. In the present study, nematodes were extracted from bitter gourd soil fields and cultivated on modified NGM agar plates. The samples were identified as *A. maximus* on the basis of morphometric and molecular characterization. *Acrobeloides spp*. has previously been recorded in various agricultural fields around the world; *A. ishraqi* and *A. mushtaqi* from chickpea fields and *A. maximus* from decaying pea roots [35]. Nicholas (1962) cultivated *Acrobeloides* species with some modification in *Rhabditidae* species culture media, and axenic cultivation was successfully performed for *A. buetschlii* [36]. Considering some critical factors, such as temperature, pH and essential elements for the survival and growth of nematodes, large-scale cultivation of nematodes can be preferred by effectual *in vivo* techniques based on solid-state culture [37, 38]. In the current cultivation experiment, different pH, temperature and nutrient media were standardized for the best growth conditions of *A. maximus.*

A. maximus belongs to the *Cephaloboidea* family [eol.org/pages/463236]. *Cephaloboidea* includes large numbers of microbe feeder nematode species with diverse labial morphology. It has been studied over the

last half century, but to date, some genera from this family are facing systematic is suesdue to their labial morphological variations [7]. Nematodes belonging to the *Cephaloboidea* family show variation in their labial morphology, and labial taxonomy is one of the important characters for taxonomists to identify *Acrobeloides* species [19]. Since Thorne's pioneering research, this exceptional labial variety has been progressively emphasized in systematic investigations of *Cephalobidae*, often comprising various elaborations of body wall cuticle, enclosing labial probolae and cephalic probolae. Because of the same labial morphology, *Acrobeloides* is often misdiagnosed as *Cephalobus* [3–5]. Accordingly, Neuyen (2016) transferred *Cephalobus* to the *Acrobeloides* genus [39]. In some species, including *A. maximus* and *A. saeedi*, the body size was almost equal [9], whereas the body designs of *A. saeedi* and *A. bodoenheimeri* were most similar [40]. In such cases, *A. maximus* can be identified on the bases of the absence of hair-like structures on lips [9].In recent studies, many new species based on morphological changes in *Acrobeloides* (Cobb, 1924) have been recorded, but due to the lack of molecular data, the identification efficacy of the genus *Acrobeloides* was reduced. In the present investigation, the morphometry and morphology were matched with the Nemaplex database, and the comparative morphometrics of *Acrobeloides* species with cultivated *A. maximus* are described in the results. The findings suggested that the labial morphology was the same as that mentioned by Throne (1925) [3]. Based on the morphological characters listed below, the *A. maximus* species was partially confirmed: two separated lips, a thick cuticle layer inside the mouth cavity, heavily cuticularized outer body layer, a considerably developed digestive tract with a bulged metacorpus attached to a cylindrically shaped esophagus, and an elongated intestine. The posterior end of the nematodes has vulva in females and spicules in males followed by conoid-shaped tails. The female tail shape is slightly blunt, while males have conoid but point and curve at the end. The posterior lip of the vulva is larger than that of other *Acrobeloides spp*. females. Morphometric analysis also showed similarity with body length reported in earlier species. Different studies have reported that the average body length and body width of *Acrobeloides spp.* range between 610–1570 µm and 160–370 µm, respectively [3, 41]. In our morphometric analysis, *A. maximus* showed 1250 µm body length and 72 µm widths in female populations and 1066 µm body length and 57 µm widths in males. Female body measurements were the same as those mentioned by Throne 1925, but the males were not reported in his study. Thereafter, Pervez (2011) recorded *A. maximus male* body measurements [31], which are congruent with our records. However, in a study by Anderson (1965) during the cultivation of *A. uberrinus* on agar plates, there was a slight difference in body measurements in comparison to soil cultures [8]. In the same way, changes in the morphology of the tail and mouth regions during cultivation have also been reported during studies [42]. To troubleshoot identification errors due to morphological and morphometric analysis, evolutionary and molecular analysis is the latest requirement to improve nematode systematic. The DNA barcoding technique is one such technique that can potentially be used to identify each species based on the knowledge that all organisms have unique nucleotide sequences. It is a fast and economical genomic tool for identification and evolutionary studies [43]. Most studies of molecular characterizations select mitochondrial cytochrome c oxidase subunit I (*COX1*) and rDNA, of which the small subunit ribosomal DNA gene in invertebrates, such as nematodes, is a popular choice due to its highly conserved nature and favorable PCR amplification [44–47].

Phylogenetic analysis based on ribosomal genes provides high-resolution phylogenetic trees and elevated bootstrap values for taxonomical purposes [48]. Smythe and Nadler (2006) showed a comparative phylogenetic framework of morphological characters of *Cephalobidae* and *Acrobeloides*, but they did not find any common ancestor during their study [19]. Another molecular characterization of *A. saeedi* revealed that morphologically similar samples of *A. saeedi* and *A. maximus* were proven to be conspecific with the help of ribosomal small subunit sequence alignment [9]. According to these findings, phylogenetic analysis can be very helpful for identifying conspecific or closely related nematode species. Cultured *A. maximus* samples were subjected to 18S small RNA gene sequencing, and the obtained sequence was submitted to GenBank, which contributed immensely to the enhancement of the *A. maximus* molecular database for identification. Blast analysis of the submitted sequence showed 98% similarity with 4 previously submitted *A. maximus* sequences. This sequence was employed for phylogenetic analysis by plotting the maximum likelihood tree (Fig. 5) of nematode species listed in Table 2 with the highest log likelihood(-10423.43). Bootstrap values (1000 replicates) were also performed for the same dataset used in the ME tree and showed high bootstrap values (91%-100% for *A. maximus* species). Moreover, both analyses represented evolutionary closeness with other 18S ribosomal RNA gene sequences of *Acrobeloides spp.*, apart from *Pseudoacrobeles spp*. and *Dicelisussuriensis*, as they both belong to different genera. The results of the study also suggested that the morphological features such as lip, shape of the tail and digestive tract of the Nema2BG sample show a polyphyletic relation with other species presented in the phylogenetic tree. The ME tree showed a strong support value (100%) with the *A. maximus* strain (JQ237850.1). Finally, the collected nematode species was identified as *A. maximus* by combining morphological data with molecular analysis.

Conclusions

In the present study, nematodes were extracted from bitter gourd agricultural field soil located in Gujarat, India, and cultivated on agar plate medium under laboratory conditions. Morphological data were collected as pictographs and further processed to obtain morphometric De man measurements. The results conclude that the labial morphology, shape and length of the tail, position and measurements of the A-V valve, and shape of the vulva are key characteristics for *A. maximus* identification. However, then early comparable morphological traits of the same genus and the paucity of research on this species make morphological identification less efficient. Thus, molecular and phylogenetic analyses were employed to troubleshoot such uncertainties. The obtained sequence was submitted to NCBI GenBank with the ON533625.1 accession number, and BLAST analysis showed 98% similarity with *A. maximus* species. These results were validated with phylogenetic studies. In summary, our study offered details on cultivation methods and morphologic, pictorial, morphometric, and molecular identification features for *A. maximus* species found in agricultural soil populations and this information would be extremely pertinent for future evaluation studies. Future prospects for adding accuracy to nematode systematic include SEM image analysis for morphological parameters and whole-genome sequencing for further downstream molecular characterization.

Declarations

Compliance with ethical standards

Conflict of interest

The authors declare that they have no competing conflict of interest regarding the content of this article.

Ethics approval

No approval of research ethics committees was required to accomplish the goals of this study because experimental work was conducted with an unregulated invertebrate species. This article does not contain any studies with human participants.

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Data availability

The nucleotide sequence (654 bp) of the small subunit ribosomal RNA gene of *Acrobeloides maximus* species was submitted to NCBI GenBank with accession no ON533625.

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Author Contributions

The study's conception and design involved input from all authors. Shweta Thakar carried out sample collection, morphological identification, image analysis, molecular analysis, sequence alignment, and phylogenetic analyses, Sunita Chauhan assisted with morphological identification and laboratory setup. With their superior intellectual abilities, Drs. Linz-Buoy George and Hyacinth Highland conceived the study, supervised the data analysis and checked the manuscript. All authors offered feedback on earlier draughts of the manuscript. The final manuscript was read and approved by all authors.

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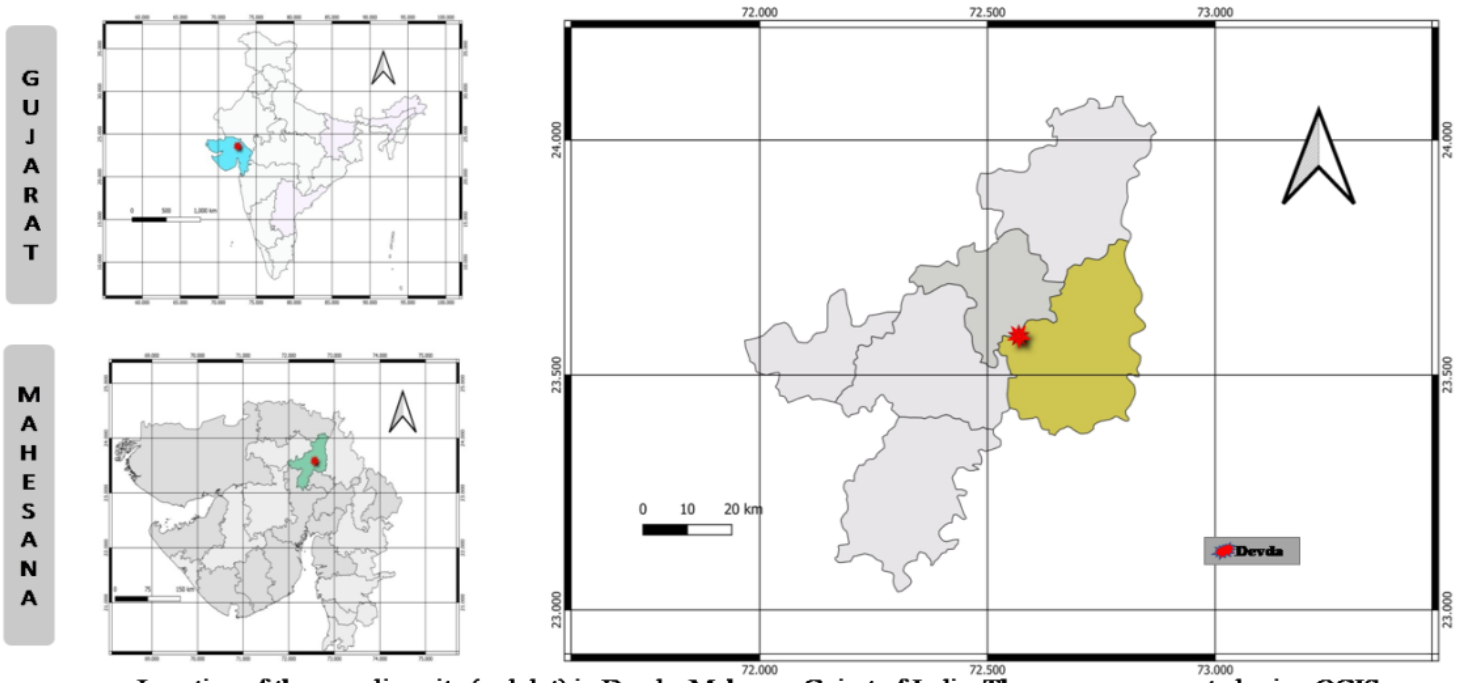
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Figures



Location of the sampling site (red dot) in Devda, Mehsana, Gujrat of India. The maps were created using QGIS.

Figure 1

QGIS map of sampling site

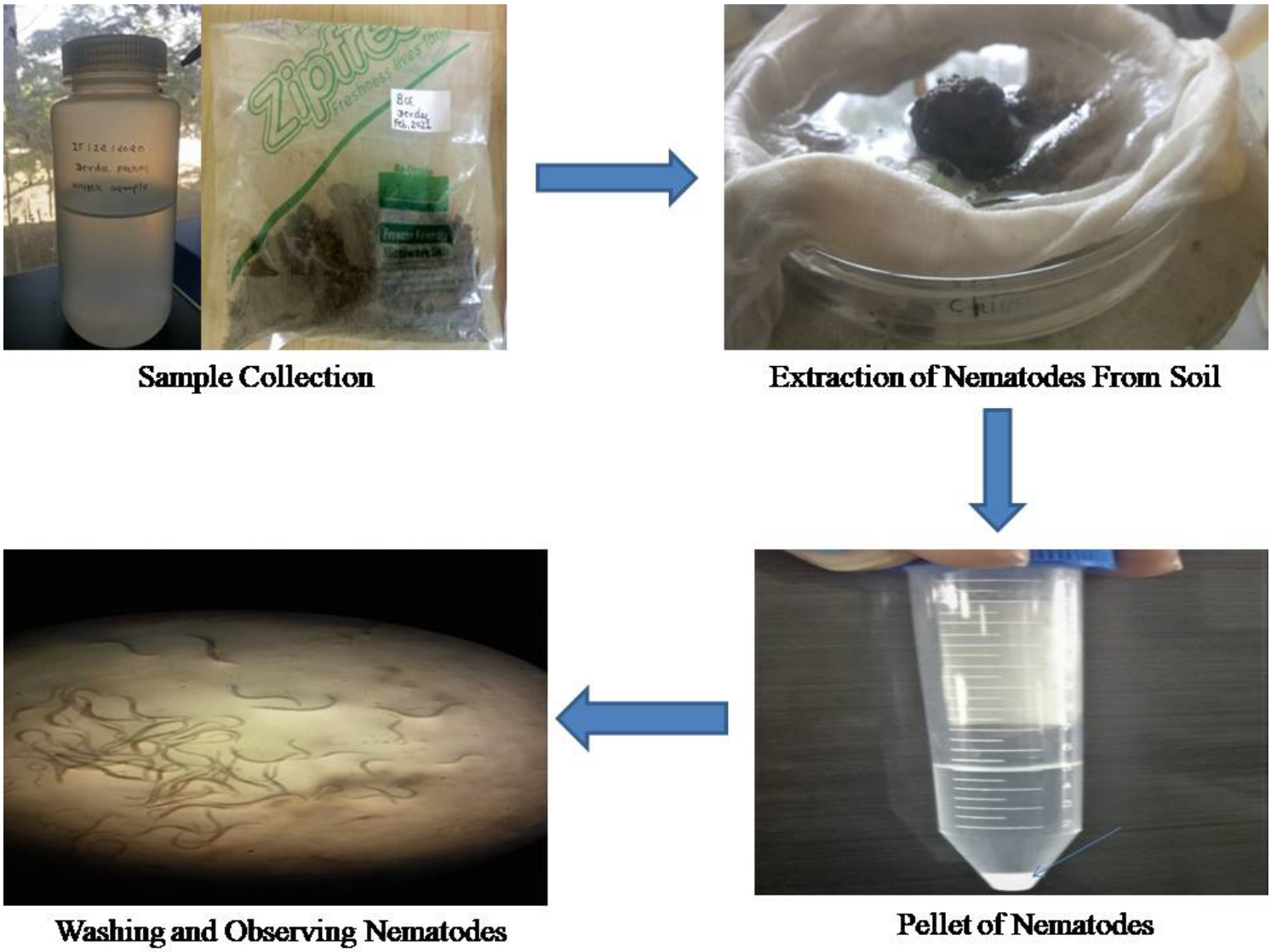


Figure 2

Steps for nematode extraction from soil samples

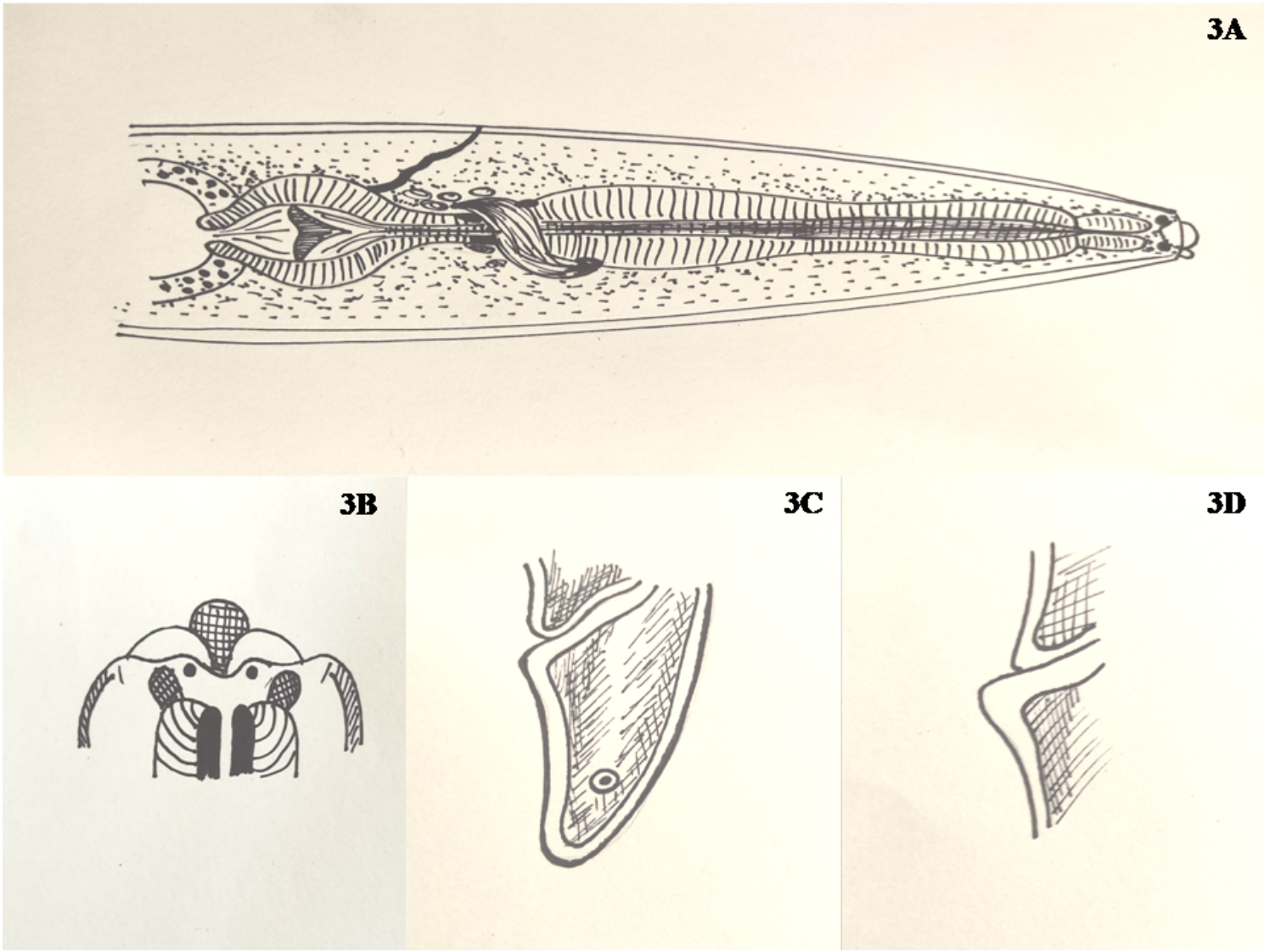
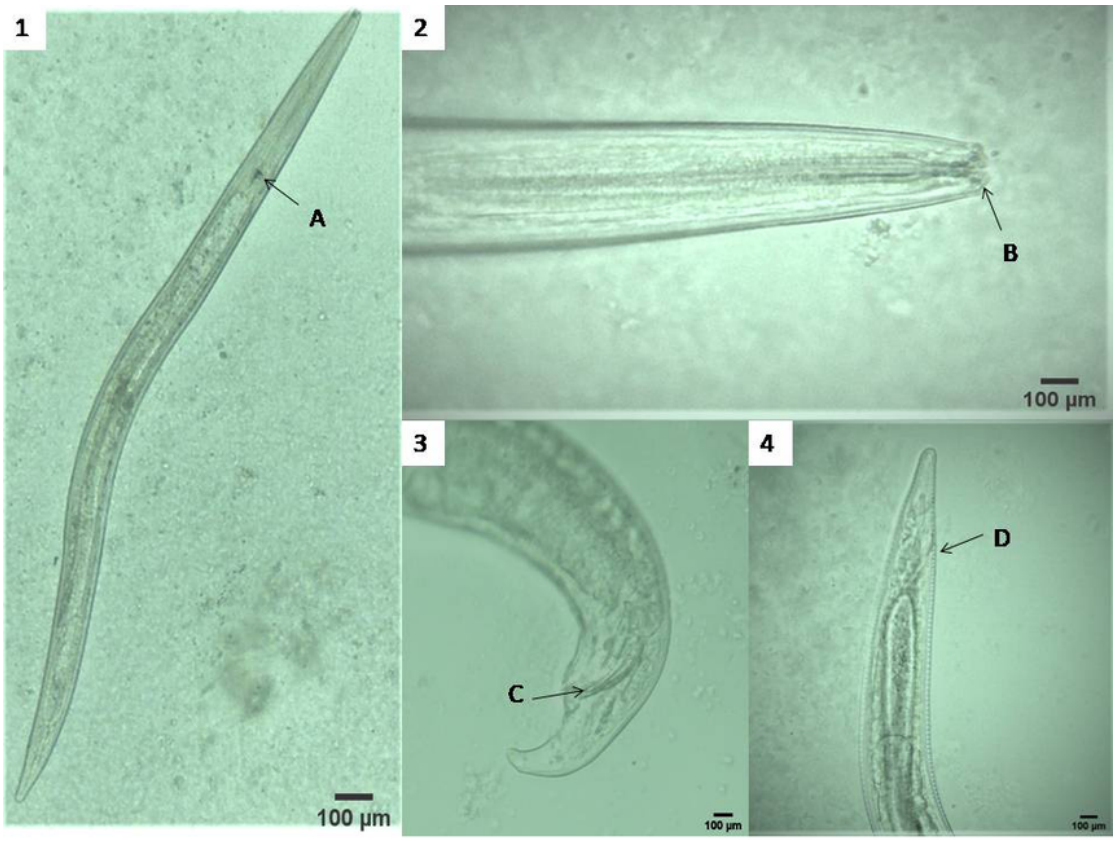
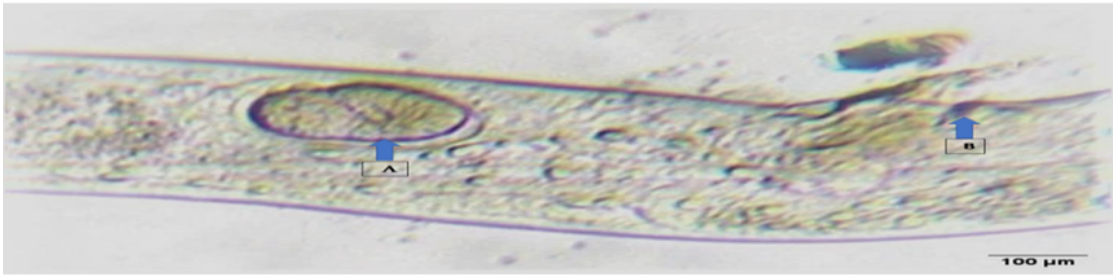


Figure 3

A) Neck and head, B) Lip region, C) Tail, D) Vulva (Thorne, 1925)



A



B

Figure 4

a Morphological characteristics of *Acrobeloides maximus*. 1. Entire male A) Round metacorpus, 2. Head Region B) Duplex lips, 3. Male posterior end C) Spicules, 4. Female posterior D) Anus

b A) Egg B) Vulva



Figure 5

Bayesian consensus tree with the highest log likelihood (-10423.43) based on the maximum likelihood method and Hasegawa-Kishino-Yano model from the top 20 sequences from the database and the newly submitted sequences of *A. maximus*. The percentage of trees in which the associated taxa clustered

together is shown above the branches. Red dots represent the *A. maximus* species. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (Bootstrap support tree was showed in supplementary files).

Supplementary Files

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