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DOI 10.2478/helm-2022-0035

HELMINTHOLOGIA, 59, 4: 414 – 423, 2022

Research Note

First report of *Aphelenchoides bicaudatus* (Imamura, 1931) Filipjev and Schuurmans Stekhoven, 1941 associated with grass in South Africa

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Article info

Received April 13, 2022
Accepted August 17, 2022

Summary

Aphelenchoides bicaudatus associated with grass in South Africa was identified morphologically and molecularly. This population is characterized by a body length of 409 – 529 µm, a stylet length of 9.5 – 13 µm, a post-vulval uterine sac of 45 – 50 µm, and the characteristic tail bifurcated at the end with one prong longer than the other. Molecular analyses based on the 18S and ITS rDNA data confirmed the primary morphological identification of the *A. bicaudatus* species. The obtained phylogenetic trees revealed a close positioning of the South African population to other representatives of *A. bicaudatus* with the maximum (1.00) posterior probability value. Principal component analysis (PCA) also indicated a variation within the populations of *A. bicaudatus*. This is the first report of *A. bicaudatus* from South Africa.

Keywords: *Aphelenchoides*; grass; morphology; PCA; phylogeny; rDNA; South Africa

Introduction

Grasslands are one of the most critical biomes in South Africa (Le Roux *et al*., 2011; Richardson *et al*., 2020), which cover the northern parts of the Western Cape Province. Grasslands constitute a significant component of the natural vegetation. The interface between grasslands and other biomes contributes substantially to their floristic and faunal diversity and to their important role in the agricultural economy, including livestock. The grasslands of South Africa are also home to most of the human population across the country (Le Roux *et al*., 2011; Richardson *et al*., 2020). *Aphelenchoides* Fischer, 1894 species is large and abundant genus with a worldwide distribution. They are found in a wide range of trophics such as fungal feeder in soil (e.g., *A. pseudogoodeyi* Oliveira, Subbotin, Alvarez-Ortega, Desaeger, Brito, Xavier, Freitas, Vau & Inserra, 2019), mushroom (e.g., *A. composticola* Franklin, 1957), plants (e.g., *A. besseyi* Christie, 1942), and insects (*A. microstylus* Kaisa, 2000) (Nickle, 1970; Handoo *et al*., 2020). *Aphelenchoides bicaudatus* first reported from Japan by Imamura (1931) from a paddy field. This species originally considered as a fungal feeder; however, it has been reported in association with several agricultural crops (Jen *et al*., 2012; Kim *et al*., 2016). *Aphelenchoides bicaudatus* has been reported from India (Das, 1960), Australia (Colbran, 1964), Venezuela (Loof, 1964), USA (Siddiqui and Taylor, 1967), Taiwan (Jen *et al*., 2012), South Korea (Kim *et al*, 2016), and Pakistan (Israr *et al*., 2017). To date, it has been reported in association with more than 200 plant species (Handoo *et al*., 2020). For instance, in South Korea, *A. bicaudatus* has been found with the leaves and shoot tips of chrysanthemum (Kim *et al*., 2016). However, *A. bicaudatus* reported from soil and mushroom (Mcleod, 1967). To date, *A. arachidis* Boss, 1977 and *A. ritzemabosi* (Schwartz, 1911) Steiner and Buhrer, 1932 have been reported from South Africa (Lesufi *et al*., 2015). However, *A. bicaudatus* not yet been reported from South Africa.

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Therefore, the aims of the present work were 1) to study the morphology of *A. bicaudatus*, and 2) to study the molecular characters of *A. bicaudatus* using the 18S and ITS rDNA markers.

Materials and Methods

Nematode extraction, and processing

Specimens were collected at the Kirstenbosch National Botanical Garden in Cape Town (S: 33° 59' 13.19"; E 18° 25' 29.39") and Magoebaskloof (S: 23°52'40.368"; E: 29°56'14.459") from the rhizosphere of grass plants (Family: Poaceae; *Pennisetum clandestinum*). The specimens were extracted using the tray method (Shokoohi, 2021) and were fixed with a hot 4 % formaldehyde solution and transferred to anhydrous glycerin using the De Grisse (1969) method. The classification provided by Handoo *et al*. (2020) was used for the taxonomical study of *Aphelenchoides*. Pictures were taken with a Zeiss Axiolab (Germany) microscope at the Aquaculture Research Unit, equipped with a digital camera. Next, the pictures were used for line illustration. All samples were processed at the Aquaculture Research Unit of the University of Limpopo.

DNA extraction, PCR, and phylogenetic analysis

DNA extraction was done using the Chelex method (Straube & Juen, 2013). Five specimens of the analyzed species were hand-picked with a fine tip needle and transferred to a 1.5 ml Eppendorf tube containing 20 µl double distilled water. The nematodes in the tube were crushed with the tip of a fine needle and vortexed. Thirty microliters of 5 % Chelex® 50 and 2 µL of proteinase K were added to the microcentrifuge tube that contained the crushed nematodes and mixed. These separate microcentrifuge tubes with the nematode lysate were incubated at 56 °C for two hours and then incubated at 95 °C for 10 minutes to deactivate the proteinase K and finally spin for 2 min at 16000 rpm (Shokoohi, 2021). The supernatant was then extracted from the tube and stored at –20 °C. Following this step, the forward and reverse primers, 988F (5'-CTCAAAGATTAAGCCATGC-3') and 1912R (5'-TTTACGGTCAGAACTAGGG-3') for 18S rDNA (Holterman *et al*., 2006), and TW81 (5'-GTTTCCGTAGGTGAACCTGC-3'), and AB28 (5'-ATATGCTTAAGTTCAGCGGGT-3') for ITS rDNA (Joyce *et al*., 1994) were used in the PCR reactions for partial amplification of the 18S and ITS rDNA region, respectively. PCR was conducted with eight µl of the DNA template, 12.5 µl of 2X PCR OneTaq® Quick-Load® 2X Master Mix with Standard Buffer (Inqaba Biotec, South Africa), one µl of each primer (10 pmol μl^{-1}), and ddH_2O for a final volume of 30 µl. The amplification was processed using an Eppendorf Mastercycler gradient (Eppendorf, Hamburg, Germany), with the following program: initial denaturation for 3 min at 94 °C, 37 cycles of denaturation for 45 s at 94°C; 54 °C and 53°C annealing temperatures for 18S rDNA and ITS rDNA for 30 s, respectively; extension for 45 s to 1 min at 72 °C, and finally an extension step of 6 min at 72 °C followed by a temperature on hold at 4 °C. After DNA amplification, four µl of product from each tube was loaded on a 1 % agarose gel in TBE buffer (40 mM Tris, 40 mM boric acid, and one mM EDTA) for evaluation of the DNA bands. The bands were stained with the SafeView™ Classic stain (Applied Biological Materials Inc. (abm); Canada) and visualized and photographed on a UV transilluminator. The PCR products for 18S rDNA and ITS rDNA were stored at –20 °C. Finally, the PCR products were purified and sequenced by Inqaba Biotech (South Africa). The obtained ribosomal DNA sequences were analyzed and edited with BioEdit (Hall, 1999) and aligned using CLUSTAL W (Thompson *et al*., 1994). Phylogenetic trees were generated using the Bayesian inference method as implemented in the program Mr Bayes 3.1.2 (Ronquist & Huelsenbeck, 2003). The HKY+Γ (gamma distribution of rate variation with a proportion of invariable sites) model was selected using jModeltest 2.1.10 (Guindon & Gascuel, 2003; Darriba *et al*., 2012). Analysis using the GTR+G+I model was initiated with a random starting tree and ran with the Markov chain Monte Carlo (MCMC) for 10^6 generations for 18S and ITS rDNA. The trees were visualized with the TreeView program (Page, 1996). Also, as outgroups, *Bursaphelenchus xylophilus* (MF669500, KX856336 for 18S rDNA, and ITS rDNA, respectively) were selected based on Handoo *et al*. (2020). The original partial 18S and ITS rDNA sequences of *A. bicaudatus* were deposited in GenBank under the accession numbers OM883916 and OM910735.

Statistical analysis

To evaluate the morphological variations between the populations of A. bicaudatus, principal component analyses (PCA) were conducted using XLSTAT software (Addinsoft, 2007). Various morphometric features obtained from fixed nematodes, including body length, a (body length/greatest body diameter), b (body length/neck length), c (body length/tail length), c' (tail length/anal body diameter), V (% anterior end to vulva/body length), stylet length, and tail length were included in the PCA analyses (Table 2). The morphometric measurements for the different populations were taken from their original descriptions. The measures were normalized using XLSTAT software before their analysis (Addinsoft, 2007). The scores values were determined for each species based on each of the principal components, and the scores for the first two components were used to form a two-dimensional plot (PC1 and PC2) of each isolate based on the eigenvalues given by the software XLSTAT.

Results

Aphelenchoides bicaudatus (Imamura, 1931) Filipjev & Schuurmans Stekhoven, 1941

Morphological characterization (Eight females in a good state of preservation)

Fig. 1; Table 1

Description

Female: Body slender, tapering slightly anteriorly, and more

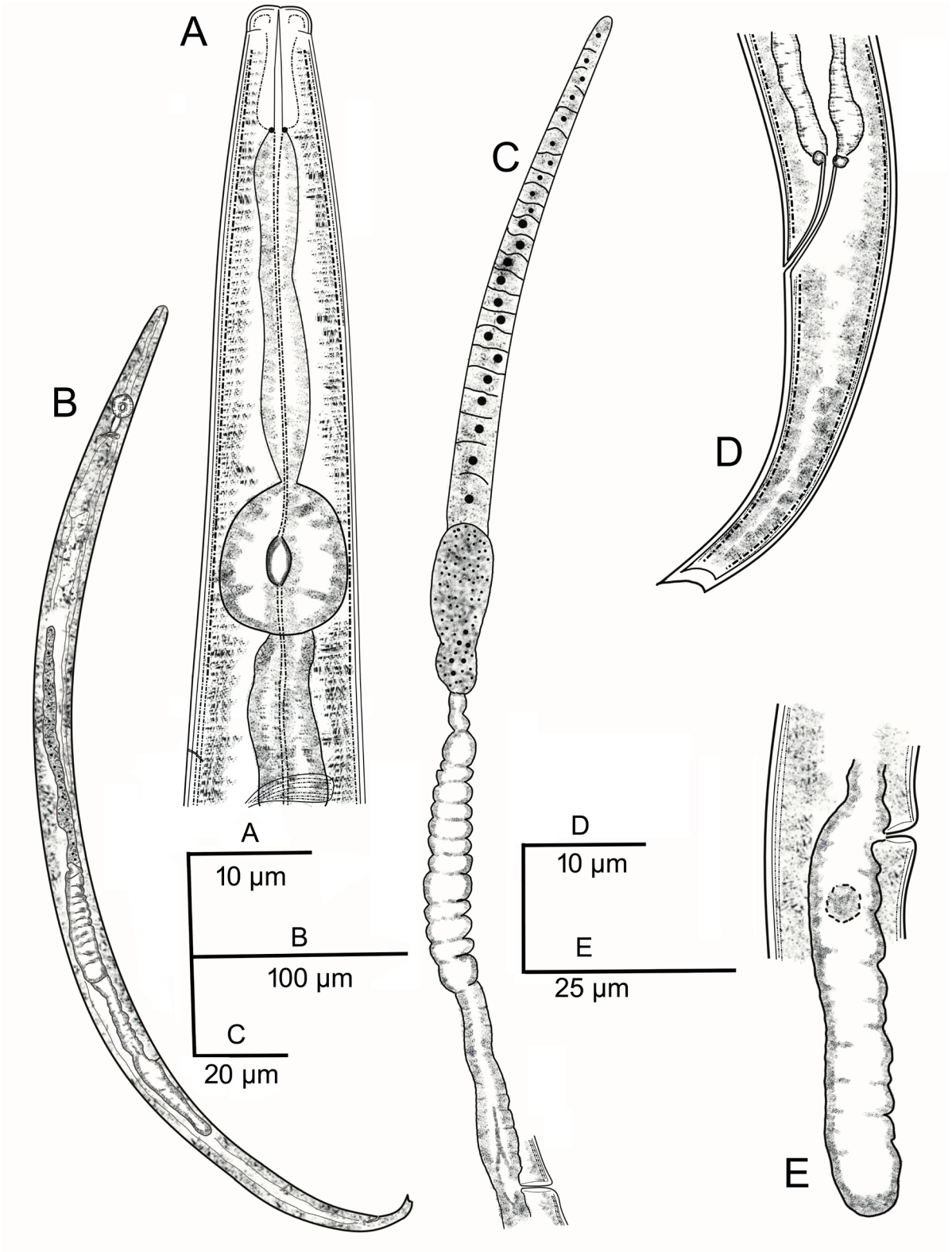

Fig. 1. *Aphelenchoides bicaudatus* (Imamura, 1931) Filipjev and Schuurmans Stekhoven, 1941.
(A) anterior end; (B) entire female; (C) reproductive system; (D) female posterior end; (E) Post-vulval uterine sac.

prominently toward posterior end. Body straight and tail region only slightly curved after heat relaxation. Cuticle annulated; 0.4 – 0.6 µm thick; annuli 0.5 – 0.7 µm wide. Lateral field with two incisures at mid-body region. Lip region rounded, offset, 5.6 – 5.8 µm wide and 2.0 – 3.7 µm high; no annules. Stylet weak, with small basal swellings. Procorpus wider anteriorly, gradually narrowing posteriorly, then widening at median bulb. Median bulb rounded to ovoid, occupying approximately 69 – 78 % of body width, and measuring 8.5 – 10.0 µm wide and 10.7 – 12.0 µm long. Cardia conoid and surrounded by intestinal tissue. Nerve ring about behind median bulb, at 68 – 82 % of the neck length. Excretory pore at isthmus level, at 57 – 73 % of the neck length. Vulva a transverse slit and slightly protruding, at 68 – 71 % of body length from anterior end. Anterior genital branch usually extending to region of pharyngeal gland lobe, 167 – 200 µm long. Post-vulval uterine sac 45 – 50 µm long, and extending for 32 – 38 % of distance from vulva to end of tail. Rectum prominent, straight, near ventral body wall, 12 – 16 µm long. Tail gradually tapering to terminus, which is unevenly bifurcate (Fig 1. D) with one prong longer than the other, 24 – 31 µm long.

Male: not found.

Other material examined: A population from Magoebaskloof, Limpopo Province, was recovered from the rhizosphere of a grass, which resembles the Cape Town population of *A. bicaudatus*.

Remarks: The South African population of *A. bicaudatus* resembles the previous populations studied from Japan (Imamura, 1931 (Filipjev & Schuurmans Stekhoven (1941), the USA (Siddiqui & Taylor, 1967), Taiwan (Jen *et al*., 2012), and South Korea (Kim *et al*., 2016). However, compared with the Japanese population, they differ in the lower range of the body length (409–529 vs 380–470 µm), b (4.1–5.1 vs 6.8–8.4), c (14.1–17.8 vs 9.4–12.6), and the upper range of V (67–71 vs 61.7–90.2). Compared with

Table 1. Measurements of females of *Aphelenchoides bicaudatus* from South Africa. All measurements are in µm and in form: mean ± SD (range), except for ratio.

Province	Western Cape	Limpopo
Locality	Cape Town	Magoebaskloof
n	8 females	4 females
L	455.3 ± 64.5 (409 – 529)	451.7 ± 56.7 (415 – 517)
a	29.1 ± 2.0 (27.1 – 31.1)	28.9 ± 0.7 (28.2 – 29.6)
b	4.6 ± 0.5 (4.1 – 5.1)	4.4 ± 0.3 (4.1 – 4.7)
M	47.9 ± 3.4 (44.1 – 50.6)	49.4 ± 3.6 (45.5 – 52.6)
c	16.3 ± 2.0 (14.1 – 17.8)	15.2 ± 1.3 (14.3 – 16.7)
c’	3.2 ± 0.6 (2.6 – 3.6)	3.3 ± 0.3 (3.1 – 3.6)
V (%)	68.8 ± 1.6 (67 – 71)	68.7 ± 1.7 (67 – 70)
Lip region height	2.8 ± 0.9 (2 – 4)	3.1 ± 0.5 (3 – 4)
Lip region width	5.7 ± 0.1 (5.6 – 5.8)	5.4 ± 0.4 (5.1 – 5.8)
Stylet length	11.1 ± 1.7 (10 – 13)	9.6 ± 0.6 (9.5 – 10)
Conus	4.7 ± 0.3 (4.5 – 5.0)	4.8 ± 0.3 (4.5 – 5.0)
Mid of median bulb to anterior end.	47.3 ± 3.2 (45 – 51)	48.3 ± 2.6 (46 – 51)
Median bulb diameter	9.0 ± 0.8 (8.5 – 10.0)	9.2 ± 0.8 (8.0 – 10.0)
Median bulb length	11.2 ± 0.7 (11 – 12)	10.9 ± 1.0 (10 – 12)
Pharynx length	88.3 ± 11.2 (76 – 98)	93.2 ± 7.3 (86 – 100)
Neck	99.0 ± 8.7 (89 – 104)	103.3 ± 7.5 (95 – 110)
Nerve ring from anterior end	71.7 ± 0.6 (71 – 76)	73.0 ± 2.0 (71 – 75)
Excretory pore from anterior end	61.3 ± 3.2 (60 – 65)	60.3 ± 1.5 (59 – 62)
Body diameter at median bulb	12.5 ± 1.8 (11 – 15)	12.3 ± 1.5 (11 – 14)
Body diameter at mid body	15.6 ± 1.5 (14 – 17)	15.7 ± 2.1 (14 – 18)
Body diameter at anus	8.8 ± 0.7 (8 – 9)	9.1 ± 0.1 (8 – 10)
Anterior branch of reproductive system	179.3 ± 18.0 (167 – 200)	199.7 ± 53.2 (167 – 261)
Post-vulval uterine sac	47.0 ± 2.6 (45 – 50)	46.0 ± 1.0 (45 – 47)
Vagina length	6.3 ± (6 – 7)	6.7 ± 0.6 (6 – 7)
Rectum	14.3 ± (12 – 16)	14.7 ± 1.5 (13 – 16)
Tail length	28.0 ± (24 – 31)	29.7 ± 1.2 (29 – 31)

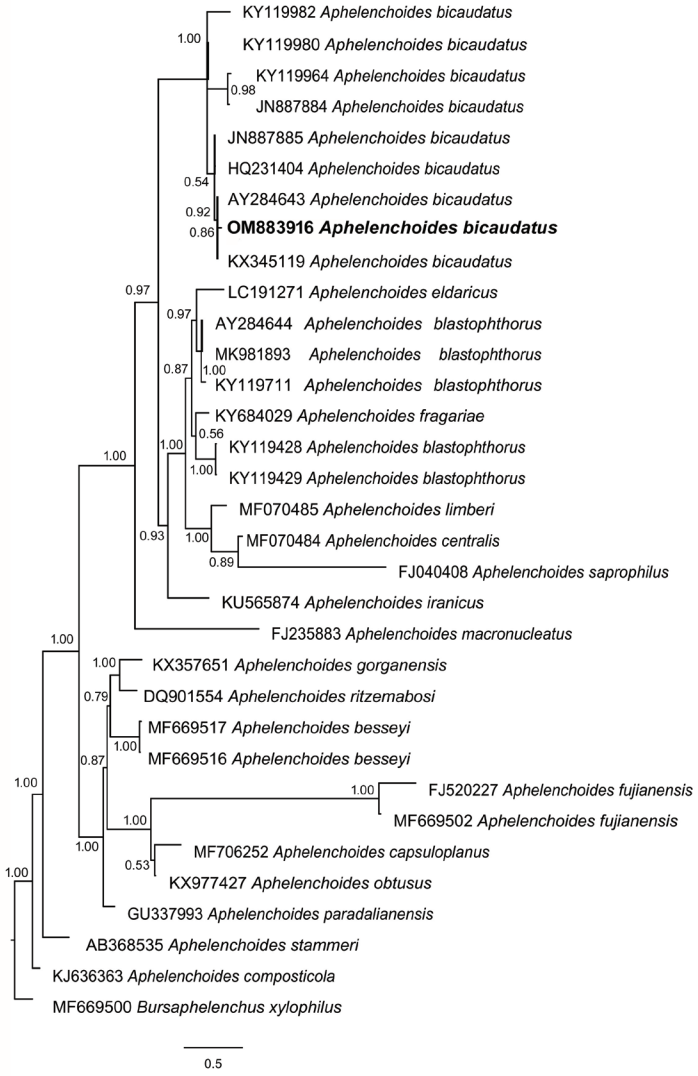

Fig. 2. Phylogenetic position of *Aphelenchoides bicaudatus* from South Africa based on 18S rDNA.

the American population, they differ in b (4.1–5.1 vs 7.3–9.6), c (14.1–17.8 vs 9.8–13.7), and tail length (24–31 vs 41 measurement extracted from original drawing). Compared with South Korean population, they differ in the lower range of body length (409–529 vs 514–523 µm), b (4.1–5.1 vs 7.3–7.4), c (14.1–17.8 vs 10.7–11.9), and tail length (24–31 vs 43–49 µm). Compared with the population from Taiwan, they differ in body length (409–529 vs 376–637 µm), b (4.1–5.1 vs 7.5–10.0), c (14.1–17.8 vs 10.16–14.80), and c' (2.6–3.6 vs 4.13–7.14). However, compared with a population from Pakistan (Israr *et al*., 2017), they differ body length (409–529 vs 360 µm), and b (4.1–5.1 vs 7.2–8.8).

DNA characters

The nBlast test of 18S rDNA showed 98 % similarity of the test population with the South Korean population of *A. bicaudatus* (KX345119), Taiwan (JN887884), and China (MH722388).

The phylogenetic analysis using 18S and ITS rDNA, placed the South African *A. bicaudatus* population in a clade together with other *A. bicaudatus* populations with the maximum posterior probability value (Figs. 2 and 3).

Statistical analysis

An accumulated variability of 74.86 % was observed in female based PCA, specifically, 42.66 % in the PC1 and 32.20 % in the PC2 (Fig. 4). The variables b (r = 0.865), c (r = -0.862), c' (r = 0.866), and tail length (r = 0.811) were responsible for the significant variability of the PC1. Regarding the PC2, body length (r = 0.714), a (r = -0.784), V (r = -0.668), and stylet length (r = 0.852) showed a significant correlation (Table 3). The PCA plot separated different populations of *A. bicaudatus* indicating a morphological variation between the populations (Fig. 4; Table 4). The result categorized the populations of *A. bicaudatus* into three groups,

Table 2. Morphological important characters of females of *Aphelenchoides bicaudatus* from various localities. All measurements are in µm and in form: mean ± SD (range), except for ratio. * = extracted from original drawings.

	Present study	Imamura, 1931 (Filipjev and Schuurmans Stekhoven (1941))	Siddiqui and Taylor (1967)	Jen *et al.*, 2012	Kim *et al.*, 2016	Israr *et al.*, 2017
Population	South Africa	Japan	USA	Taiwan	South Korea	Pakistan
L (µm)	455.3 ± 64.5 (409 – 529)	430 (380 – 470)	460 (410 – 550)	499.12 ± 67.95 (376–637)	517.9 ± 3.8 (513.6 – 522.6)	360
a	29.1 ± 2.0 (27.1 – 31.1)	31.5 (31.3 – 31.7)	28.0 (25 – 31)	33.03 ± 2.42 (27.00–38.64)	28.3 ± 0.5 (27.7 – 28.8)	30.1 – 32.7
b	4.6 ± 0.5 (4.1 – 5.1)	7.4 (6.8 – 8.4)	8.2 (7.3 – 9.6)	9.0 ± 0.7 (7.5–10.0)	7.3 ± 0.0 (7.3 – 7.4)	7.2 – 8.8
c	16.3 ± 2.0 (14.1 – 17.8)	10.6 (9.4 – 12.6)	11.4 (9.8 – 13.7)	11.94 ± 0.93 (10.16–14.80)	11.3 ± 0.5 (10.7 – 11.9)	11.3 – 12.0
c'	3.2 ± 0.6 (2.6 – 3.6)	4.4*	4.7*	5.41 ± 0.56 (4.13–7.14)	4.6 ± 0.1 (4.4 – 4.8)	2.9 – 3.7
Tail (µm)	28.0 ± 3.6 (24 – 31)	44*	41	37 – 43	45.9 ± 2.5 (43.1 – 48.8)	30 – 31
V (%)	68.8 ± 1.6 (67 – 71)	70.4 (61.7 – 90.2)	67.5 (65 – 70)	68.53 ± 1.20 (64.90–71.83)	66.0 ± 0.2 (65.7 – 66.4)	66.8 – 67.2
Stylet (µm)	11.1 ± 1.7 (10 – 13)	10*	11.2 (10 – 12)	10.38 ± 0.63 (9–12)	11.2 ± 0.5 (10.4 – 11.7)	10 – 11

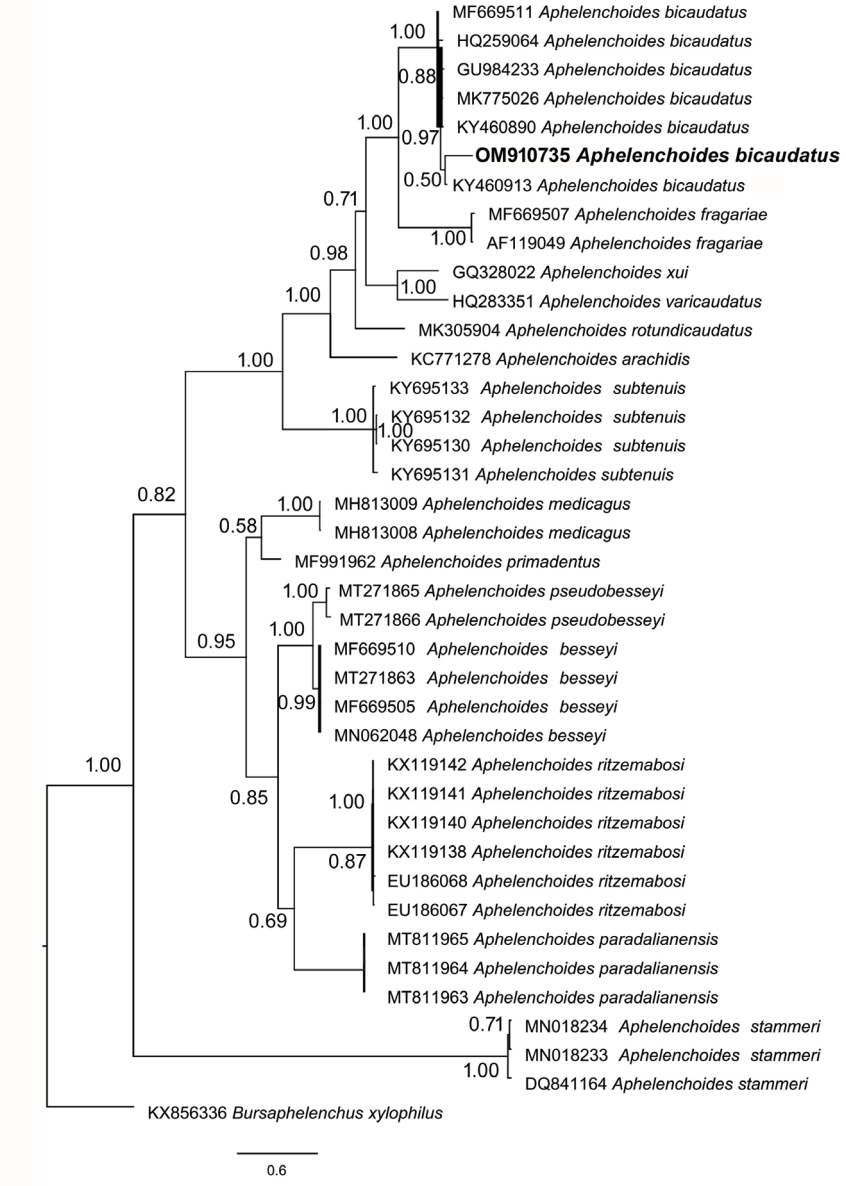

Fig. 3. Phylogenetic position of *Aphelenchoides bicaudatus* from South Africa based on ITS rDNA.

including 1) South Africa and Pakistan, 2) USA and South Korea, and 3) Taiwan and Japan.

Discussion

The genus *Aphelenchoides* comprises a diverse group of species (Handoo *et al*., 2020). The morphological and molecular findings in the current study were in agreement with the mophometrics and phylogenies of *Aphelenchoides* species studied (Jen *et al*., 2012; Kim *et al*., 2016; Handoo *et al*., 2020). Among the species belong to the *Aphelenchoides*, two species, namely *A. bicaudatus* and *A. hainanensis* (Rahm, 1938) Goodey, 1951 having bifurcated tail. However, they distinguished by female body length (360 – 637 µm in *A. bicaudatus* vs 900 – 1300 µm in *A. hainanensis*), a (25.0 – 38.6 in *A. bicaudatus* vs 42.4 – 46.1 in *A. hainanensis*), and tail tip morphology (*A. bicaudatus* with bifurcated in female and conical in male vs *A. hainanensis* with bifurcated in both sexes). Therefore, the data of the present study confirm the South African species as *A. bicaudatus*.

Additionally, principal component analysis using morphometric features of species of *A. bicaudatus,* including the populations from South Africa, showed that *A. bicaudatus* has morphometric variation. The analyzed morphological characters allowed a clear separation between the populations of *A. bicaudatus* of this study.

Table 3. Loading factor of the variables of the different populations of *Aphelenchoides bicaudatus*.

	PC1	PC2
L	0.293	0.714
a	0.435	-0.784
b	0.865	-0.037
c	-0.862	-0.008
c'	0.866	0.323
Tail	0.811	0.417
V	0.073	-0.668
Stylet	-0.483	0.852

Table 4. Factor score of the variables of the different populations of *Aphelenchoides bicaudatus*.

Observation	PC1	PC2
South Africa	-3.296	-0.120
Japan	1.298	-1.668
USA	0.332	1.408
Taiwan	1.912	-0.479
South Korea	0.476	2.367
Pakistan	-0.723	-1.507

The results indicated that there is intraspecific morphological variation across *A. bicaudatus* populations, which depends on the sampling locations. The populations of *A. bicaudatus* from South Africa and Pakistan stand close to each other than other populations. The populations from South Africa and Pakistan similar in tail length, a, c', and V. However, they differ in body length, which indicating a variation between the two populations. PCA showed previously a useful tool to study the variation between the populations of the same species, as mentioned in *Butlerius butleri* (Shokoohi & Abolafia, 2021). Besides, the PCA also indicated a variation between the populations of *Xiphinema hispanum* complex group (Archidona-Yuste *et al*., 2020). The result of the present study is in agreement with the previous studies.

Three permanent microscope slides, containing the females of *A. bicaudatus* were deposited in the Aquaculture Research Unit of the University of Limpopo, South Africa. According to the literature, this is the first record of *A. bicaudatus* in South Africa. In conclusion, the morphological variation exists among the *A. bicaudatus* (e.g., body length, a, b, c, c', vulval position, and tail length,) is due to the geographical location of the population. Besides, the ecological role of *A. bicaudatus* needs to be investigated in the grassland quality of South Africa.

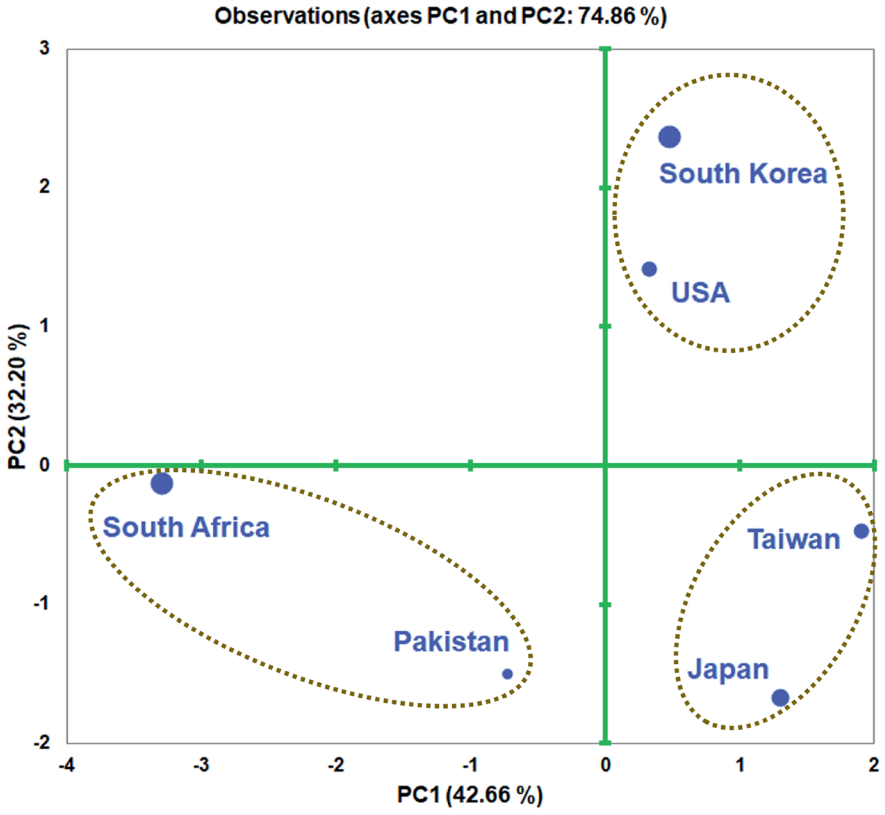

Fig. 4. PCA plot of different populations of *Aphelenchoides bicaudatus* from various locations.

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