

## Redescription, systematic status and molecular characterisation of *Multicaecum heterotis* Petter, Vassiliadès et Marchand, 1979 (Nematoda: Heterocheilidae), an intestinal parasite of *Heterotis niloticus* (Osteichthyes: Arapaimidae) in Africa

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**Abstract:** Ascaridoid nematodes referable to *Brevimulticaecum heterotis* (Petter, Vassiliadès et Marchand, 1979) Khalil, 1984 were recorded from the intestine of the African bonytongue, *Heterotis niloticus* (Cuvier) (Arapaimidae, Osteoglossiformes), from the Mare Simenti in the Niokolo Koba National Park, East Senegal and from Kosti, Sudan. Their examination using light microscopy and for the first time both environmental scanning electron microscopy and scanning electron microscopy revealed some previously unreported morphological features and made possible a detailed redescription of this species. The most important finding is the presence of denticular ridges on the inner edges of the lips, which confirms the attribution of this species to *Multicaecum* Baylis, 1923, where it was originally placed, and not to *Brevimulticaecum* Mozgovoy in Skryabin, Shikhobalova et Mozgovoy, 1951 where it had subsequently been transferred. A key to *Brevimulticaecum* and *Multicaecum* species is provided. *Multicaecum heterotis* is the first species of the genus to be sequenced. Partial sequences of the small ribosomal subunit (18S) and internal transcribed spacer 2 region (ITS2) of nuclear ribosomal DNA (rDNA) have been analysed and compared with other nematode species.

**Keywords:** Nematoda, *Brevimulticaecum*, *Multicaecum*, Senegal, Sudan, Africa, *Heterotis*, barcoding, 18S rDNA sequences, ITS2 sequences

*Heterotis niloticus* (Cuvier) (Arapaimidae, Osteoglossiformes) is an opportunistic omnivorous freshwater fish. Olaosebikan and Raji (1998) stated that its standard length is 100 cm and its greatest published weight is 10.2 kg (Paugy et al. 2003). It is a foodfish and profitably cultured in Africa. *Heterotis niloticus* occurs in large rivers and lakes of the Nilo-Sudanian area in Central and West Africa and has been introduced into many rivers and aquaculture stations in Africa (Monentcham et al. 2009).

During a survey of fish parasites in the Niokolo Koba National Park (East Senegal) in the area of Mare Simenti and in Kosti, Sudan, ascaridoid nematode referable to *Brevimulticaecum heterotis* (Petter, Vassiliadès et Marchand, 1979) Khalil, 1984 were obtained from the intestine of the African bonytongue, *Heterotis niloticus*. Petter et al. (1979) described this species as *Multicaecum heterotis* from the same host in Senegal. However, because no denticular ridges on lips were reported in the original description, Khalil (1984) transferred this species to *Brevi-*

*multicaecum* Mozgovoy in Skryabin, Shikhobalova et Mozgovoy, 1951.

The present study of these nematodes, using light microscopy (LM) and, for the first time, scanning electron microscopy (SEM) and environmental scanning electron microscopy (ESEM), revealed some previously unreported morphological features of taxonomic importance and made possible a detailed redescription of this species and the clarification of its generic allocation.

The partial sequences of the small ribosomal subunit (18S) and the internal transcribed spacer 2 (ITS2) region of nuclear ribosomal DNA (rDNA) were chosen as molecular markers for the taxonomic identification of this species. The 18S rDNA gene represents a well-conserved gene that evolves relatively slowly (Hillis and Dixon 1991) and seems to be an effective marker for the barcoding of nematode worms (Floyd et al. 2002). The ITS2 region is considered as an excellent tool for DNA diagnosis (Blouin 2002), because of its high variability, and

has become a popular choice for phylogenetic analysis of closely related species and populations.

## MATERIALS AND METHODS

Four specimens of the African bonytongue, *Heterotis niloticus* (Cuvier), were examined. One of them was collected and dissected at Simenti (13°01'33.4"N; 13°17'40.4"W) in the Niokolo Koba National Park, East Senegal in 2004 and the other three at Kosti (13°10'0"N; 32°40'0"E) in the Sudan in 2006. A total of 92 nematode specimens were collected from the intestines at the first locality and 8 nematode specimens at the second. Recovered nematodes were washed in physiological saline (0.9%) and immediately fixed in hot 4% formaldehyde solution. For molecular study, 4 specimens were fixed in 96% ethanol. For light microscopy, the nematodes were cleared with glycerine, after which they were examined using a light microscope equipped with differential interference contrast (DIC) optics, digital image analysis system (analySIS auto 5.0) and a drawing attachment. After examination, the specimens were stored in vials with 70% ethanol. Measurements are given in micrometres (µm) unless otherwise stated, with the means in parentheses.

For SEM, 2 males and 3 females were used. Fixed specimens were dehydrated through a graded ethanol series, critical-point dried (in a Bal-Tec CPD 030 Critical Point Dryer) using liquid CO<sub>2</sub>, mounted on aluminium stubs with double-sided adhesive disc and sputter-coated with gold (in a Balzers SCD 040). The samples were examined using a Quanta™ 3D FEG scanning electron microscope at an accelerating voltage of 15 kV.

For ESEM, 1 male and 3 females were used. Fixed specimens were hydrated with water; desiccation was not carried out. A Quanta™ 250 FEG at accelerating voltage of 15 kV was used for specimen examination. ESEM observation was carried out at 100% relative humidity in the specimen chamber – where the sample was cooled down to 2°C in an atmosphere of water vapour at a pressure of 700–750 Pa.

For the purposes of DNA barcoding, the partial sequences of the small ribosomal subunit (18S) and the internal transcribed spacer 2 (ITS2) region of nuclear ribosomal DNA (rDNA) were analysed. Genomic DNA was extracted from 4 ethanol-preserved specimens (2 from Senegal and 2 from the Sudan) using QIAamp DNA Mini Kit (Qiagen) and resuspended in 100 µl AE buffer. PCR was carried out in 30 µl of PCR reaction buffer with 2.5 mM MgCl<sub>2</sub>, 200 µM dNTPs, 0.5 unit of *Taq* DNA Polymerase (Fermentas) and 0.5 µM of each primer (partial 18S or ITS2) using the Mastercycler ep gradient S (Eppendorf). The primers and the conditions used for PCR were as follows.

Partial 18S rDNA was amplified using forward primer Nem\_18S\_F and reverse primer Nem\_18S\_R designed by Floyd et al. (2005). The PCR mixture was denatured at 94°C for 5 min, followed by 39 cycles at 94°C for 30 sec, 52°C for 30 sec and 72°C for 1 min, and the final extension at 72°C for 10 min.

The ITS2 region was amplified using primers LC1-F and HC2-R (Navajas et al. 1992). The PCR mixture was denatured at 95°C for 2 min, followed by 30 cycles at 95°C for 1 min, 56°C for 45 sec and 72°C for 3 min, followed by final extension at 72°C for 10 min.

PCR products were checked on GoldView-stained 1.5% agarose gel and were purified by the High Pure PCR Product Purification Kit (Roche). Sequencing was carried out using BigDye® Terminator v3.1 Cycle Sequencing Kit and an Applied

Biosystems 3130 Genetic Analyzer. Sequencing for each sample was carried out for both strands. Nucleotide sequences of the partial 18S and ITS2 were edited using Sequencher software (Gene Codes Corporation, Ann Arbor, MI, USA) and aligned in BioEdit v. 7.0.9.0. (Hall 1999) using Clustal W multiple alignment (Thompson et al. 1994). BLAST searches were performed at <http://www.ncbi.nlm.nih.gov> to look for related or similar species of nematodes.

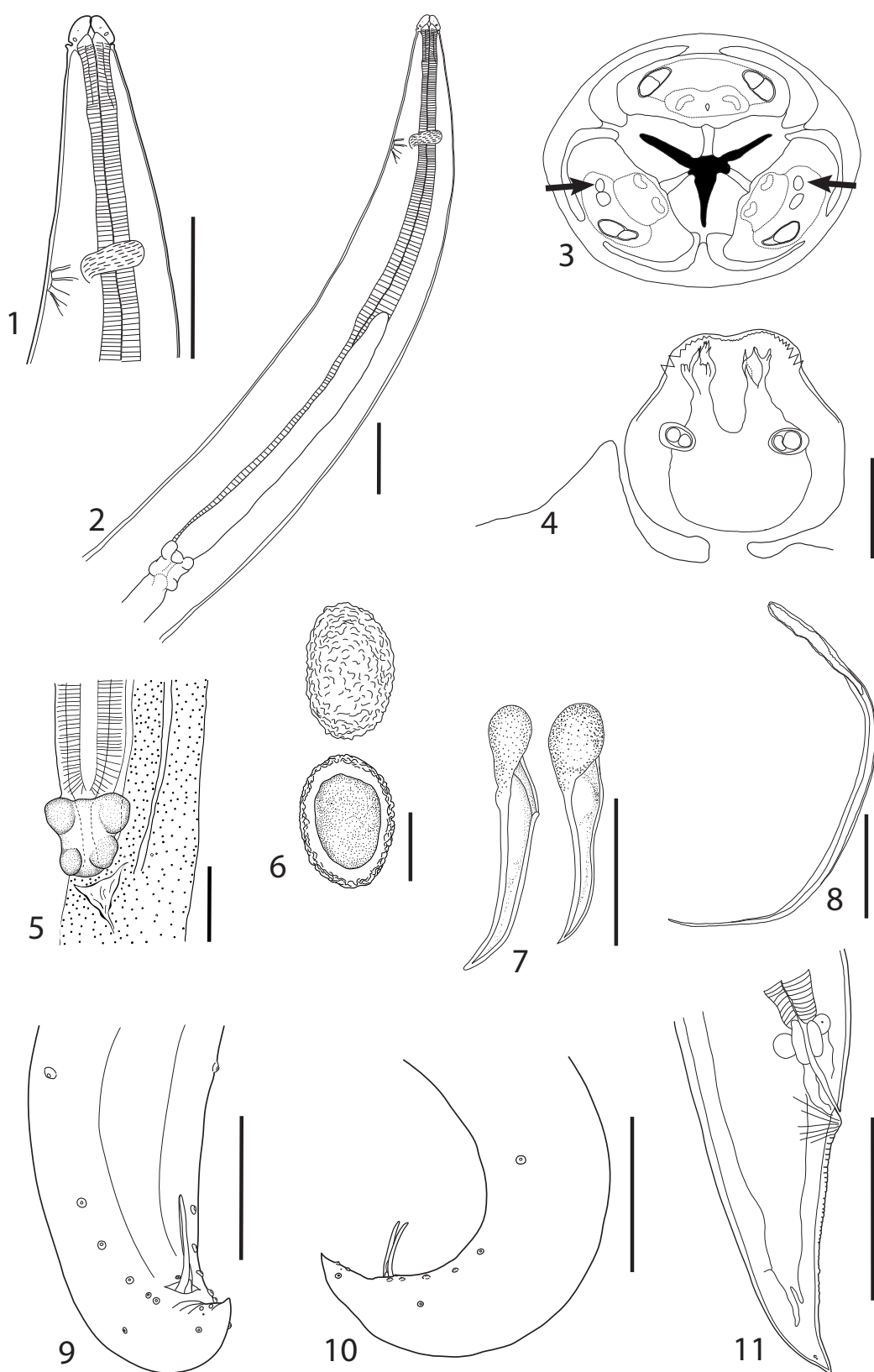
Voucher specimens are deposited in the Helminthological Collection of the Institute of Parasitology, BC ASCR, in České Budějovice, Czech Republic (Cat. No. N-947) and other specimens in the Department of Botany and Zoology, Faculty of Science, Masaryk University, Brno, Czech Republic. Fish name follows FishBase (Froese and Pauly 2009).

## RESULTS

*Multicaecum heterotis* Petter, Vassiliadès et Marchand, 1979 Figs. 1–24

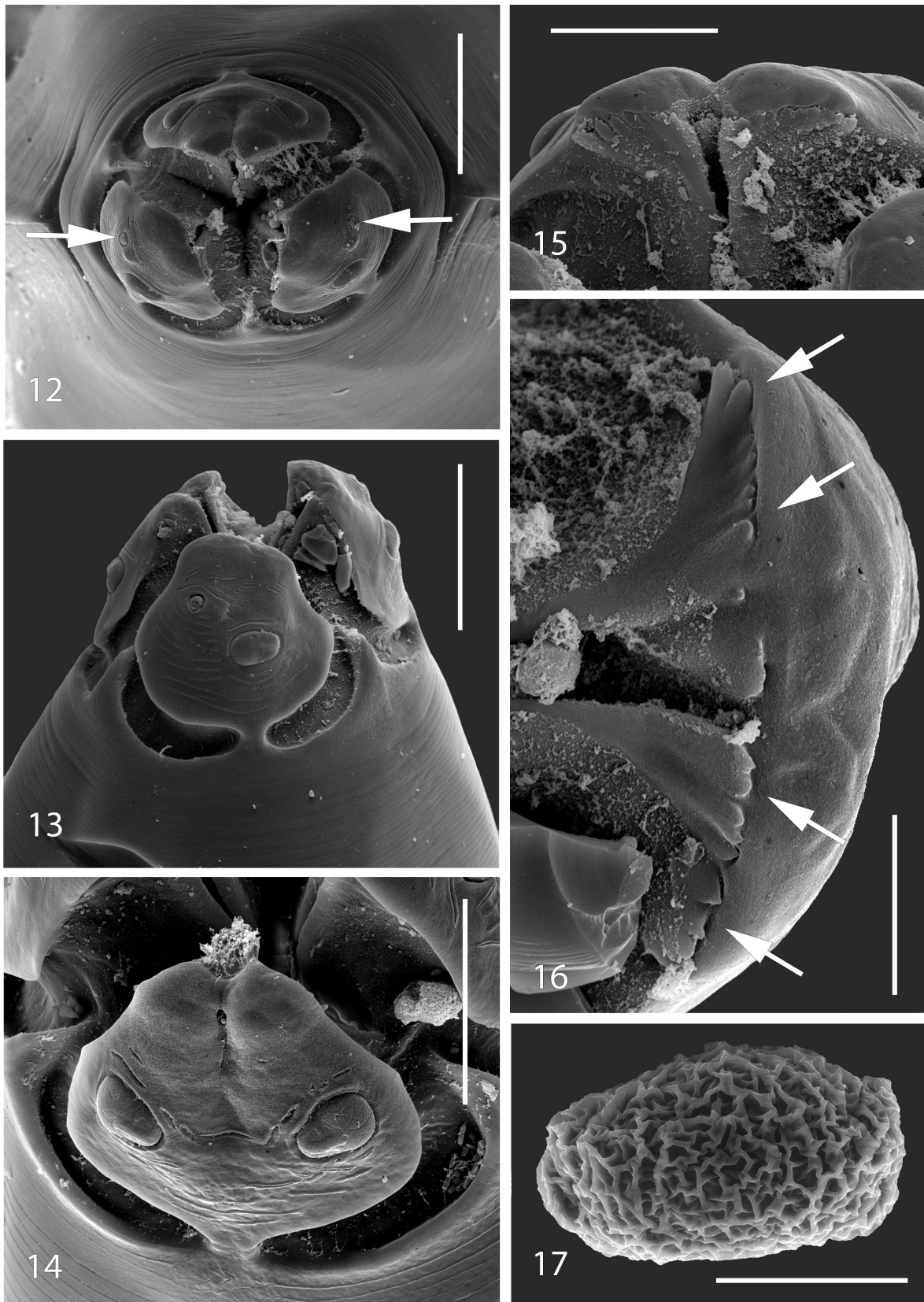
**Description:** Medium-sized, whitish nematodes with 3 lips (Figs. 3 and 12–16); males not markedly smaller than females. Dorsal lip with two elliptical double papillae; each of two subventral lips with one elliptical single papilla and amphid, same wide as long, rounded margins and narrow postlabial grooves. Lower part of each lip with conspicuous cuticular striations; anterior part slightly striated. Labial pulp of each lip with two main branches ending anteriorly in short round cauliflower-like projections (Fig. 4). Small obtuse denticles arranged along inner margin of antero-median border of lip; series of small denticles ends with one or two large denticles on both sides of lip (Fig. 16). Number of denticles 18 or 19; thickness of denticles 0.5–3.4 (1.3). Interlabia pyramidal with undivided tip, approximately half length of lips. Wide lateral cuticular alae reach back to small distance posterior to oesophagus. Excretory pore at level or slightly posterior to nerve ring (Fig. 1); cervical papillae not observed. Oesophagus straight, terminating in ventriculus with two anterior and three short, knob-like posterior appendices (Figs. 2, 5). Intestinal caecum approximately half length of oesophagus.

**Male** (10 specimens from Senegal; measurements of 3 specimens from Sudan in brackets; means in parentheses): Length of body not markedly smaller than female, but more slender. Lips and interlabia 59–88 (72) [63–92 (75)] and 20–34 (27) [20–31 (27)] in length, respectively. Cervical alae 71–136 (109) [128–150 (139)], oesophagus 63–174 (107) [101–165 (132)] and intestinal caecum 78–109 (96) [95–106 (100)] in maximum width. Ventriculus 115–204 (167) [157–191 (171)] long and 87–172 (123) [127–158 (146)] wide. Ventricular appendices 33–72 (50) [34–65 (55)] long. Two alate filiform spicules equal in form and length; distal tip non-alate and sharp (Figs. 8, 19, 20). Proximal and medial parts of spicule 23–27 (25) [21–25 (23)] and 11–17 (14) [10–13 (12)] wide, respectively. Gubernaculum slender in lateral view, triangular in



**Figs. 1–11.** *Multicaecum heterotis* Petter, Vassiliadès et Marchand, 1979; line drawings. **Fig. 1.** Lateral view of anterior end. **Fig. 2.** Oesophageal region with intestinal caecum and ventriculus. **Fig. 3.** Apical view of cephalic end; arrows indicate amphids. **Fig. 4.** Dorsal lip with interlabium. **Fig. 5.** Ventriculus. **Fig. 6.** Egg. **Fig. 7.** Gubernaculum. **Fig. 8.** Spicule. **Fig. 9.** Ventral view of male caudal end with papillae. **Fig. 10.** Lateral view of male caudal end with papillae. **Fig. 11.** Lateral view of female caudal end. Scale bars: Figs. 1, 2, 9 = 300 µm; Figs. 3, 4, 6 = 50 µm; Figs. 5, 8 = 200 µm; Fig. 7 = 100 µm; Figs. 10, 11 = 400 µm.





**Figs. 12–17.** Cephalic region of *Multicaecum heterotis* Petter, Vassiliadès et Marchand, 1979; scanning electron micrographs. **Fig. 12.** Apical view of cephalic end; arrows indicate amphids. **Fig. 13.** Lateral view of cephalic end. **Fig. 14.** Dorsal lip. **Fig. 15.** Inner view of lateroventral lip with teeth. **Fig. 16.** Detail of teeth of dorsal lip; arrows indicate teeth. **Fig. 17.** Egg. Scale bars: Figs. 12, 13 = 50 µm; Fig. 14 = 30 µm; Figs. 15, 17 = 20 µm; Fig. 16 = 10 µm.

**Table 1.** Comparison of measurements of *Multicaecum heterotis* males (upper values) and females (lower values) from Senegal and Sudan with original description. Measurements are in micrometres unless otherwise stated.

Features	♂ n = 1 ♀ n = 1	♂ n = 10 ♀ n = 5		♂ n = 3 ♀ n = 3	
		mean	range	mean	range
Body length [mm]	20	20.92	15.27–31.00	18.99	16.32–21.32
	28.9	26.62	19.28–36.91	16.53	14.09–18.96
Body width	400	526	406–668	486	405–575
	500	612	460–851	393	372–419
Cervical alae from anterior	130	141	117–196	159	146–168
	200	166	120–194	145	120–169
Length of cervical alae [mm]	3.4	2.69	1.73–3.67	3.49	3.06–4.24
	4	2.99	2.01–4.02	3.90	3.00–5.26
Nerve ring from anterior	600	573	456–735	634	545–791
	1 mm	623	506–812	503	444–562
Excretory pore from anterior	800	624	491–815	664	583–820
	1.5 mm	711	551–995	566	505–627
Oesophagus length [mm]	3.8	3.04	2.22–4.02	3.32	2.77–3.65
	5	3.57	2.66–4.88	2.88	2.64–3.11
Intestinal caecum length [mm]	2.1	2.44	1.29–3.65	2.78	2.28–3.08
	2.8	3.29	2.19–4.33	2.48	2.15–2.71
Tail length	250	215	161–291	193	187–200
	600	621	461–802	391	316–467
Spicule length	1080	1038	920–1115	1025	919–1105
Gubernaculum	—	—	—	—	—
	230	212	181–238	230	220–243
Vulva from anterior [mm]	—	—	—	—	—
	12.8	11.32	8.36–14.98	7.93	7.11–8.75
Eggs	—	—	—	—	—
	90 × 74	96 × 66	88–101 × 63–70	90 × 61	82–102 × 54–67
Locality	Richard Toll (Senegal)	Mare Simenti, Niokolo Koba National Park (Senegal)		Kosti (Sudan)	
Authors	Petter et al. 1979	Our data			

ventral view, with tapered rounded tip (Figs. 7, 20). Ten pairs of single caudal papillae and one papilla on upper cloacal lip (Figs. 9, 10, 18, 21–24). Five pairs of precloacal papillae (one pair lateral, four pairs subventral); two pairs of paracloacal papillae and three pairs of postcloacal papillae (one pair lateral, two pairs subventral). Sublateral phasmids present between both subventral postcloacal papillae. Tail tapered with conical tip (Fig. 24). Ratios: oesophagus 13–16% [17–18%] of total body length; ventriculus 5–7% [5–6%] of oesophageal length; spicule 3–7% [4–6%] of body length. For other measurements see Table 1.

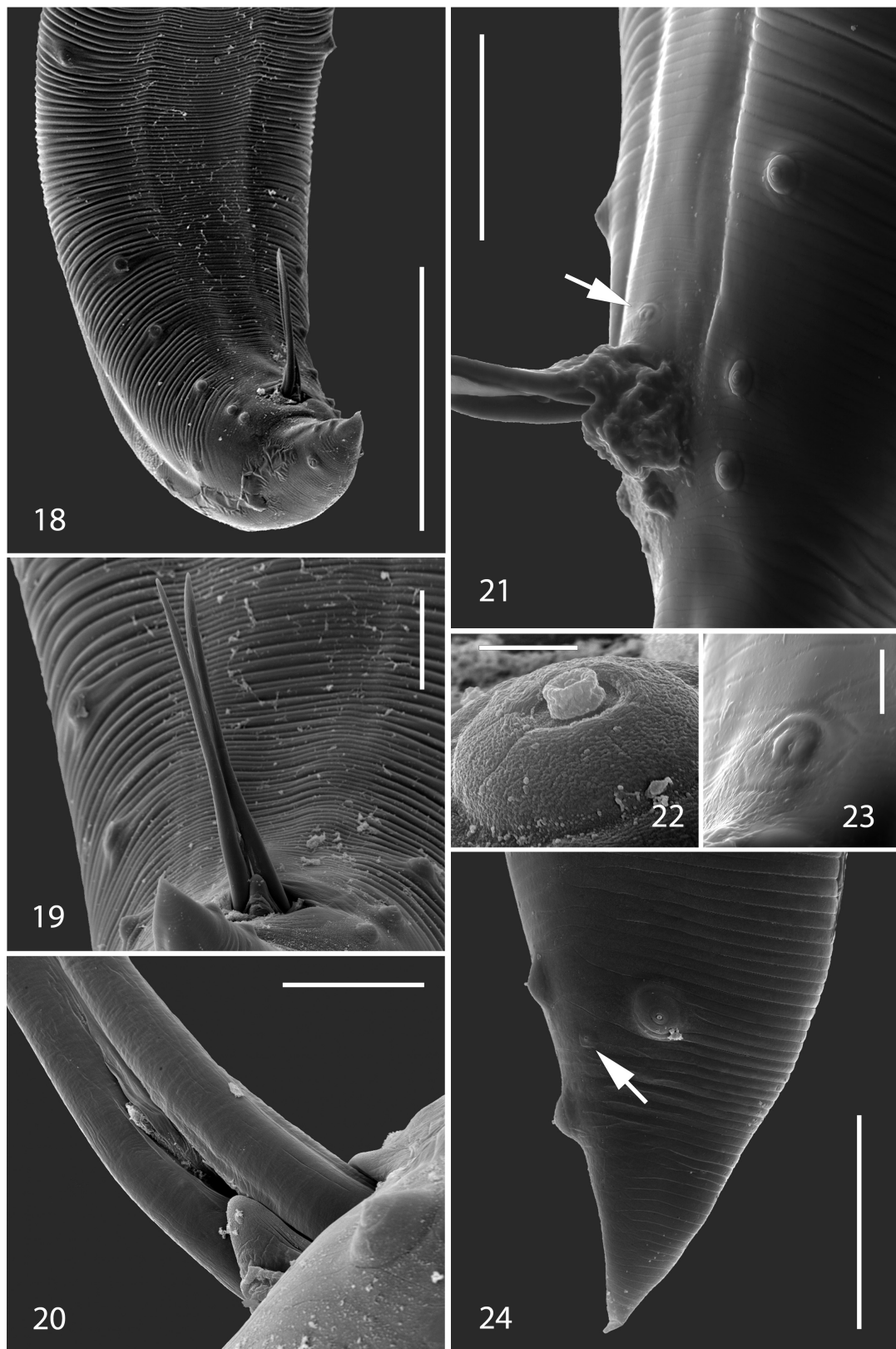
**Female** (5 specimens from Senegal; measurements of 3 specimens from Sudan in brackets; means in parentheses): Body tapers gradually anteriorly; maximum width near middle of body. Lips and interlabium 74–99 (85) [65–66 (66)] and 21–44 (31) [24–25 (25)] long, respectively. Maximum width of cervical alae 103–132 (117) [75–83 (79)]. Oesophagus straight, with maximum width 128–180 (153) [85–133 (109)], continues as small ventriculus 142–252 (188) [138–149 (145)] in length and 116–209 (151) [114–142 (127)] in width; short obtuse ventricular appendices 41–86 (61) [43–58 (52)] long. Intestinal caecum 103–147 (122) [59–82 (71)] in maximum width. Vulva without salient lips or papilla, situated

anterior to middle of body; vagina stout, relatively short, extends anteriorly and reflexes from undivided portion of uterus about same length as vagina; uterus divides into two branches. Vagina 1.15 mm long (length of vagina and undivided uterus 2.41 mm) in Senegal female 21.72 mm long. In females 19.28–36.91 (26.62) mm [14.09–18.96 (16.53)] long, vulva situated at 41–45% [44–50%] of body length from anterior end. Tail tapered with conical tip; phasmids situated laterally near caudal end; tail represents 2–3% [2%] of total body length. Ratios: oesophagus 13–14% [16–19%] of body length; ventriculus 5–6% [5%] of oesophageal length; intestinal caecum 82–97% [81–87%] of oesophageal length. Eggs oval, with fine reticulate surface (Fig. 17). For other measurements see Table 1.

## DNA characterisation

**Partial 18S region.** The sequences obtained of the partial 18S rDNA were 854 bp in length and show no intraspecific nucleotide variability. The similarity between the partial 18S sequence of *M. heterotis* and other nematode sequences included in the alignment produced in BLAST varies from 92% to 98%. Distance trees using fast minimum evolution and neighbour-joining methods constructed by BLAST clustered *M. heterotis* together with





**Figs. 18–24.** Male of *Multicaecum heterotis* Petter, Vassiliadès et Marchand, 1979; scanning electron micrographs. **Fig. 18.** Caudal end with papillae. **Fig. 19.** Spicules. **Fig. 20.** Detail of spicules and distal tip of gubernaculum. **Fig. 21.** Arrow indicates location of precloacal papilla (ESEM). **Fig. 22.** Detail of caudal papilla. **Fig. 23.** Detail of precloacal papilla (ESEM). **Fig. 24.** Tail with post-cloacal papillae and phasmid (arrow). Scale bars: Fig. 18 = 300 µm; Figs. 19, 20, 24 = 50 µm; Fig. 21 = 100 µm; Fig. 22 = 2 µm; Fig. 23 = 10 µm.

*Heterocheilus tunicatus* Diesing, 1839 (accession number U94373.1) and showed that this species is related to other species of the Ascaridoidea available in GenBank. The partial 18S sequence of *M. heterotis* was deposited in GenBank under accession numbers HM370518 and HM370519.

**ITS2 region.** A 418 bp portion of the ITS2 region was amplified and sequenced. The resulting ITS2 sequence showed 88–100% homology, but only 8–27% coverage in relation to previously published nematode sequences using BLAST. Distance trees using fast minimum evolution and neighbour-joining methods produced by BLAST clustered *M. heterotis* together with a sub-cluster of 45 nematode sequences belonging to species of *Anisakis* Mozgovoy, 1951, *Ascaris* Linnaeus, 1758, *Contracaecum* Raillet et Henry, 1912, *Hysterothylacium* Ward et Magath, 1917, *Porrocaecum* Raillet et Henry, 1912, *Raphidascaris* Raillet et Henry, 1915 and *Toxocara* Stiles, 1905. The ITS2 sequence of *M. heterotis* was deposited in GenBank under accession numbers HM370520 and HM370521.

## DISCUSSION

Baylis (1923) established the monotypic ascaridoid genus *Multicaecum* Baylis, 1923 to accommodate *M. agile* (Wedl, 1861) from *Crocodylus niloticus* Laurenti in the White Nile, Egypt. His generic diagnosis included the presence of dentigerous ridges on lips, small interlabia, and the oesophageal ventriculus with anterior and posterior oesophageal appendices. Later, several other species, mainly from crocodiles and gharials, were added to the genus (Skryabin et al. 1951, Mozgovoy 1953, Hartwich 1957). Travassos (1933a, b) pointed out that not all species of *Multicaecum* have dentigerous ridges on the lips. Based on this, Mozgovoy in Skryabin et al. (1951) divided this genus into two subgenera, the nominotypical subgenus *Multicaecum* (dentigerous ridges on lips present, ventricular appendices long and well developed, vulva anterior to middle of body) and *Brevimulticaecum* Mozgovoy in Skryabin et al., 1951 (dentigerous ridges absent, ventricular appendices reduced, vulva usually posterior to middle of body), with *M. (B.) stekhoveni* Baylis, 1937 as the type species. This was followed by Mozgovoy (1953). Later, Sprent (1979) redefined *Brevimulticaecum* and raised it to full generic status, incorrectly reporting *B. baylisi* (Travassos, 1933) as the type species.

Both *Brevimulticaecum* and *Multicaecum* contain species mainly parasitizing crocodiles, caimans and alligators. The first species parasitizing fishes was described from the African osteoglossiform *Heterotis niloticus* in Senegal as *Multicaecum heterotis* by Petter et al. (1979), which was subsequently transferred to *Brevimulticaecum* by Khalil (1984). Study of the present material from the type host and *terra typica* (Senegal) shows that the mor-

phology of male and female specimens corresponds to the original description by Petter et al. (1979), with one major exception, which is the presence of dentigerous ridges on the internal edge of lips. The finding of this feature was enabled by the use of SEM and ESEM, and the subsequent detailed examination of the inner side of preparations of the lips under the light microscope. This difference from the original description can be explained by the fact that this taxon was not studied by SEM either by Petter et al. (1979), due to the small number of available specimens (2 males, 3 females), or by Sprent (1979), who had only one paratype female for study. Further findings of this species by Khalil (1984), Akinsanya (2007) and Akinsanya et al. (2007) were not studied using this method and the nematodes were not redescribed. The species *M. agile* was studied using SEM by Sprent (1979) and the presence of dentigerous ridges was demonstrated. Dentigerous ridges were not detected in *B. baylisi* (Travassos, 1933), *B. stekhoveni* (Baylis, 1947), *B. tenuicolle* (Rudolphi, 1819), *Brevimulticaecum* sp. – larvae, *B. australiensis* (Baylis, 1931), *B. scleropagi* Khalil, 1984, *B. vandenbrandeni* (Baylis, 1929) and *B. regoi* Sprent, 1990, which were also studied using SEM by Sprent (1978, 1979, 1990), Khalil (1984) and Reyda (2008). SEM was not used for the study of *B. gibsoni* (Sprent, 1979) and *B. pinto* (Sprent, 1979).

Apparently, there are two groups of heterocheilid undoubtedly valid genera, one with dentigerous ridges (2 species) and the second one without them (9 species), corresponding to *Multicaecum* and *Brevimulticaecum* according to Sprent (1979). The weight of this feature is clearly of generic importance. In our opinion, the length of the ventricular appendices and the vulva placed anterior or posterior to the middle of body are not generic features. *Gedoelestascaris* Sprent, 1978 was regarded by Sprent (1990) as a synonym of *Brevimulticaecum*. The presence of dentigerous ridges in the species described by Petter et al. (1979) confirms that it actually belongs to *Multicaecum*, as originally designated by Baylis (1923).

Three species of *Multicaecum* and *Brevimulticaecum* are parasites of fishes and elasmobranchs: one of them has dentigerous ridges on the lips (*M. heterotis*, distribution in Africa: Senegal, Nigeria, Sudan described by Petter et al. 1979, Khalil 1984, Akinsanya 2007, Akinsanya et al. 2007) and two species lack this structure (*B. scleropagi*, hosts: *Scleropages jardini* (Saville-Kent) and *S. leichardti* Günther, Osteoglossidae, distribution Papua New Guinea and Australia – Khalil 1984, Sprent 1990; *B. regoi*, host: *Potamotrygon motoro* (Müller et Henle), Potamotrygonidae, distribution South America: Brazil – Sprent 1990, Reyda 2008).

*Multicaecum heterotis* significantly differs from *B. scleropagi* and *B. regoi* in the presence of dentigerous ridges, but also in possessing relatively broad cervi-

cal alae (lateral alae absent in *B. scleropagi* and *B. regoi*) and in the spicule length (*B. regoi* 3.8 mm; *B. scleropagi* 0.89–1.3 mm; *M. heterotis* 0.919–1.115 mm).

It should be noted that Maplestone (1930) described from Indian gharials *Polycaecum gangeticum* Maplestone, 1930. Travassos (1933a, b), Baylis (1936) and Hartwich (1957) considered this species a juvenile stage that should be included within *Multicaecum*. Among nematodes known from crocodilians in India and Ceylon, it is also necessary to mention *Ascaris quadrata* Linstow, 1904 from *Crocodylus porosus* (Schneider). In this nematode, the interlabia and dentigerous ridges on the lips were absent. Mozgovoy in Skryabin et al. (1951) transferred *A. quadrata* to *Terranova* Leiper et Atkinson, 1915. We respect Sprent's (1979) contention that both these taxons (*P. gangeticum*, *A. quadrata*) should be regarded as *species inquirendae incertae sedis* and not be included in *Multicaecum*.

With regard to geographical distribution, *Multicaecum* species have so far been found only in the Old World, extending from Africa, through India, to Australia. *Brevimulticaecum* species have so far been described from the New World, with the exception of one species parasitizing fishes in Papua New Guinea and Australia.

*Multicaecum heterotis* is the first species of the genus to have its small ribosomal subunit rDNA gene region and internal transcribed spacer 2 region sequenced for the purposes of species discrimination and identification. DNA analysis confirms the placement of *M. heterotis* within the Ascaridida and, moreover, it shows the position of *M. heterotis* in the molecular phylogenetic tree reconstructed in BLAST and supports the position of this nematode species based on morphological observations. Sprent (1979) considered the key features for the determination of species of *Brevimulticaecum* to be the spicule length (as a percentage of body length), the nature of the cuticular striation in the anterior region and, as a supplementary feature, the distance of the vulva from the anterior extremity (as a percentage of body length), the host and the distribution. Five species are considered in his key (*B. baylisi*, *B. pinto*, *B. tenuicollis*, *B. gibsoni* and *B. stekhoveni*). In addition to the spicule length, we also use other morphological and metrical features in our key to determining the species of *Multicaecum* and *Brevimulticaecum* (lateral alae, gubernaculum length, female tail length, egg size). In our key, 11 valid species (2 *Multicaecum* and 9 *Brevimulticaecum*) are included.

#### Key to *Multicaecum* and *Brevimulticaecum* species

- 1 Dentigerous ridges on lips present ..... *Multicaecum* 2
  - Dentigerous ridges on lips absent ... *Brevimulticaecum* 3
- 2 Lateral cervical alae very narrow (<40 µm); anterior prolongation of pulp short, obtuse and wide; posterior ventricular appendices longer than length of ventriculus; parasites of crocodiles in Africa, India and Australia ..... *M. agile*
- Lateral cervical alae wide (71–150 µm); anterior prolongation of pulp almost reaches end of lip, pulp has two main branches ending anteriorly in short round projections; ventricular appendices shorter than length of ventriculus; parasites of fishes (*Heterotis*, Arapaimidae) in Africa ..... *M. heterotis*
- 3 Narrow lateral cervical alae present ..... 4
  - Lateral cervical alae absent ..... 5
- 4 Vulva at 21–26% of body length from anterior end. In *Melanosuchus niger*; Brazil ..... *B. gibsoni*
  - Vulva at 43–52% of body length from anterior end. In *Caiman latirostris* and *C. crocodylus*; Brazil ..... *B. pinto*
- 5 Spicule length > 3 mm ..... 6
  - Spicule length < 2 mm ..... 7
- 6 Spicule 3.8 mm long; eggs 55–71 × 49–55 µm. In freshwater stingray *Potamotrygon motoro*; Brazil ..... *B. regoi*
  - Spicule 5.1–6.5 mm long; eggs 58–82 × 42–71 µm. In crocodilians (*Caiman*, *Crocodylus*); Brazil, Venezuela, Texas ..... *B. baylisi*
- 7 Eggs >100 µm (102–114 × 76–83); spicule 0.89–1.30 mm long; parasites of fishes *Scleropages jardini* and *S. leichardti*; Papua New Guinea, Australia ..... *B. scleropagi*
  - Eggs <95 µm; parasites of crocodiles in New World, Africa and Australia ..... 8
- 8 Parasitic in African crocodiles (*C. niloticus*) (Angola, Republic of Congo, Zambia); spicules 1.2–1.3 mm long; gubernaculum 0.16 mm long; eggs 72–89 × 58–79 µm .. *B. vandenbrandeni*
  - Parasitic in Australian and New World crocodiles ..... 9
- 9 Parasitic in Australian crocodiles *C. johnstoni* and *C. porosus*; spicules 1.0–1.7 mm long; gubernaculum 0.19–0.21 mm long; eggs 54–79 × 41–65 µm ..... *B. australiensis*
  - Parasitic in New World crocodiles ..... 10
- 10 Parasitic in *Alligator mississippiensis*, USA; spicules 1.23–1.34 mm long; gubernaculum 0.17–0.18 mm long; eggs 53–81 × 40–65 µm; spicules 7–11% of body length; vulva at 44–53% of body length from anterior end ..... *B. tenuicollis*
  - Parasitic in *Melanosuchus niger*, Brazil; spicules 0.5–1.0 mm long; gubernaculum 0.15 mm long; eggs 71–93 × 56–82 µm; spicules 3.3–4.9% of body length; vulva at 36–53% of body length from anterior end ..... *B. stekhoveni*



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