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VERSITA

Morphometrical and genetic comparison of two nematode species: *H. spumosa* and *H. dahomensis* (Nematoda, Heterakidae)

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Abstract

Heterakis is a genus of parasitic nematodes, the majority of which are found in ground-feeding birds and only rarely in mammals. The best-known species is *Heterakis spumosa*, a parasite associated with the cosmopolitan invasive rodent *Rattus rattus* of Asiatic origin. *Heterakis dahomensis* was described in 1911 as a parasite of the Gambian giant rat (*Cricetomys gambianus*) from Benin (Africa), subsequently synonymized to *H. spumosa* by Hall (1916). The study of helminths in African rodents is scarce and patchy. Since the original description of *H. dahomensis*, there have been only a few reports from Africa of species belonging to the genus *Heterakis* and the validity of this species has never in fact been confirmed or rejected. In the present study individual *Heterakis* spp. were collected from *C. gambianus* from Senegal. The morphological data taken point to differences between *Heterakis dahomensis* and *H. spumosa*, specifically in the number of tail papillae in males and in the vulva cuticular processes of females. In addition, molecular data revealed differences between these taxa and so *H. dahomensis* should be considered as a valid species. Moreover, recent changes in the systematics of the genus *Cricetomys* mean that it is now necessary to study the morphology and genetics of the *Heterakis* specimens collected from *Cricetomys* spp. (previously assigned to *C. gambianus*) in order to determine their taxonomic status as either *H. dahomensis* o *H. spumosa*.

Keywords

Heterakis dahomensis; *Cricetomys gambianus*; Heterakidae; molecular; Nematoda; SEM

Introduction

The genus *Heterakis* (Nematoda, Heterakidae) was established by Dujardin (1845) for the parasitic helminths that are found largely in ground-feeding birds (mainly Galliformes) and occasionally in mammals (mainly rodents) (Chabaud, 1978). This study focuses on the latter group.

The review by Smales (1996) encompasses five taxa of the genus *Heterakis*: *Heterakis fieldingi*, from the Australian water-rat (*Hydromys chrysogaster* Geoffroy, 1804, Rodentia); *Heterakis spumosa* Schneider, 1886 found in several rodents; *Heterakis inglisi* (Gupta & Trivedi, 1982) found in the Indian desert jird (*Meriones hurrianae* Jordon, 1867, Rodentia) from India (syn. *Heterakis yamaguti*); and *Heterakis pandei* (Gupta & Trivedi, 1982), found in the greater Asiatic yellow bat (*Scotophilus heathi* Horsfield, 1831, Chiroptera) from India (see Tables 1 and 2 for details of localities).

Heterakis dahomensis (Gendre, 1911) was described from the Gambian giant pouched rat *Cricetomys gambianus* Waterhouse, 1840 from West Africa. Gedoelst (1916) also found this nematode in an undetermined rodent in Kivu (nowadays part of the Democratic Republic of Congo) that, according to the author, probably corresponded to *C. gambianus*. Subsequently, Boulenger (1923) reported this nematode from Zanzibar (Tanzania) in *C. gambianus* (although Olayaemi *et al*. (2012) cast doubt on the identification of the *Cricetomys* species in question) and provides measurements and drawings. Hall (1916) synonymised *H. dahomensis* with *H. spumosa*, which was cited posteriorly by Baylis (1928) as *H. spumosa* from *C. gambianus* (*C. gambianus* or, according to Olayemi *et al*. (2012), *Crice-*

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Table I. Measurements of male *Heterakis* spp. reported from other studies versus the measurements obtained in the present study. Bs.As.: Buenos Aires. Dfae:distance from anterior end. Dfpe: distance from posterior end. Mm = millimeters. *According to Olayemi *et al*. (2012), the host of the study by Boulenger *et al.* (1923) would be *C. ansorgei* instead of *C. gambianus*

Species	***H. inglisi***	***H. yamaguti***	***H. pandei***	***H. fieldingi***	***H. spumosa***	***H. spumosa***	***H. spumosa***	***H. dahomensis***	***H. dahomensis***	***H. dahomensis***
Reference	Gupta & Trivedi, 1982	Gupta & Trivedi, 1982	Tewari, 1982	Smales, 1996	Singh & Krishnasamy, 1979	Cabrera & Mendoza, 2001	Robles *et al.*, 2008	Gendre, 1911	Boulenger, 1923	This work
Host	*Scotophilus heathi*	*Meriones lurinae*	*Rattus rattus*	*Hydromys chrysogaster*	Several rodents	*Rattus norvegicus*	*Rattus norvegicus*	*Cricetomys gambianus*	*Cricetomys gambianus**	*Cricetomys gambianus*
Locality	Udaipur, India	Udaipur, India	Lucknow, India	Queensland, Australia	Malaysia	Parcona, Peru	Bs.As., Argentina	Abomey, Benin	Zanzibar, Tanzania	Senegal
Measurements (in µm if not expressed otherwise)										
Body length (mm)	9.78	8.03	9.64	4.4	5.6–11.5	7.62	6.24	9.28	8.60	9.98
Body width	365	360	315	169	210 –430	165	300	390	350	
Body width at esophageal bulb level	–	–	–	–	–	–	–	–	–	302
Anterior esophagus	880	590	820	473	–	441	680	–	–	734
Esophageal bulb length	205	175	170	145	–	144	220	–	–	161
Esophageal bulb width	215	155	185	108	–	–	–	–	–	174
Entire esophagus	1085	765	950	618	770 –1010	585	800	884	900	195
Excretory pore (dfae)	420	405	350	289	240–500	–	–	–	–	479
Cervical alae (dfae)	–	105	–	–	–	76.5	130	–	80	44
Sucker length	90	95	130	44	50–120	–	80	–	–	118
Sucker width	90	95	130	44	60–140	–	70	–	–	125
Sucker (dfpe)	–	–	–	–	450–680	230	420	–	530	689
Left spicule	2440	240	365	308	210–360	245	240	–	345	357
Right spicule	1175	290	440	308	210–360	230	230	–	345	374
Tail length	615	285	410	178	220–390	–	290	359	310	371

Table II. Measurements of female *Heterakis* spp. reported from other studies versus the measurements obtained in the present study. Bs.As.: Buenos Aires. Dfae: distance from anterior end. Mm = millimeters. *See Table I

Species	***H. inglisi***	***H. yamaguti***	***H. pandei***	***H. fieldingi***	***H. spumosa***	***H. spumosa***	***H. spumosa***	***H. dahomensis***	***H. dahomensis***	***H. dahomensis***
Reference	Gupta & Trivedi, 1982	Gupta & Trivedi, 1982	Tewari, 1982	Smales, 1996	Singh & Krishnasamy, 1979	Cabrera & Mendoza, 2001	Robles *et al*., 2008	Gendre, 1911	Boulenger, 1923	This work
Host	*Scotophilus heathi*	*Meriones lurinae*	*Rattus rattus*	*Hydromys chrysogaster*	Several rodents	*Rattus norvegicus*	*Rattus norvegicus*	*Cricetomys gambianus*	*Cricetomys gambianus*	*Cricetomys gambianus*
Locality	Udaipur, India	Udaipur, India	Lucknow, India	Queensland, Australia	Malaysia	Parcona, Peru	Bs.As., Argentina	Abomey, Benin	Zanzibar, Tanzania	Senegal
Measurements (in µm if not expressed otherwise)										
Body length (mm)	11.41	10.95	11.7	5.1	7.2–14.3	10.05	8.9	11.56	11.25	12.09
Body width	335	360	325	172	250–430	175	320	500	400	
Body width (at esophageal bulb level)	–	–	–	–	–	–	–	–	–	311
Body width (at vulva level)	–	–	–	–	–	–	–	–	–	439
Anterior esophagus	905	1050	1065	1065	–	442	215	–	–	754
Esophageal bulb length	220	185	195	153	–	148	230	–	–	171
Esophageal bulb width	390	140	180	111	–	–	–	–	–	178
Entire esophagus	1125	1235	1260	622	770–1080	590	930	924	–	925
Cervical alae (dfae)	–	120	–	–	–	75.5	140	–	–	39
Excretory pore (dfae)	450	515	545	301	230–470	–	420	–	–	491
Vulva (dfae) (mm)	5.59	5.32	5.15	2.53	3.8–6,9	3.44	4.65	–	5.41	6.26
Tail length	1010	775	975	713	430–990	410	760	1051	–	1130
Eggs length	57.5	55	57.5	65	50–80	56	60	64	56	61.41
Eggs width	50.5	45	42.5	36	30–60	40	40	44	45	45.39

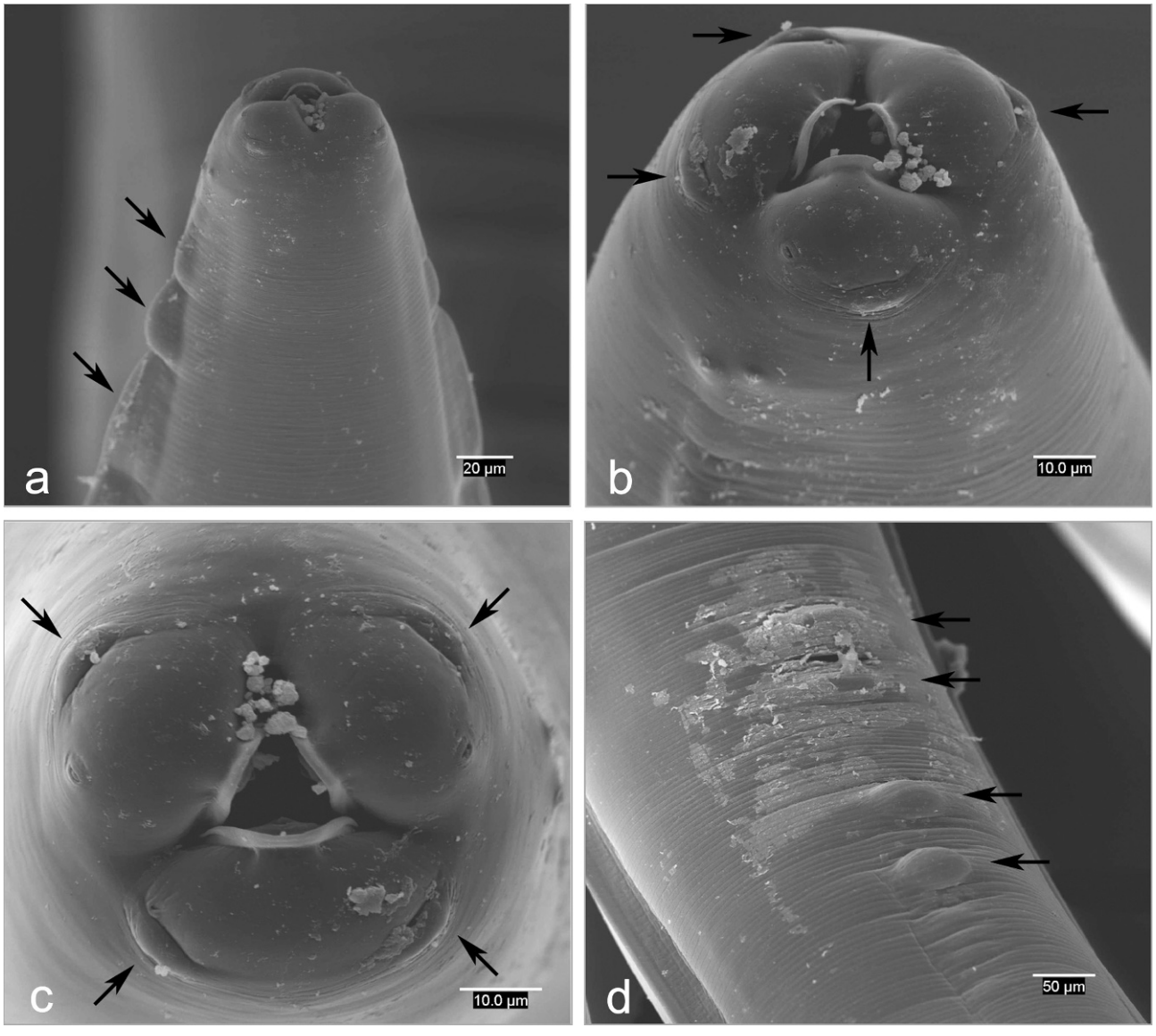

Fig. 1. (a-d) Scanning electron micrographs of *Heterakis dahomensis* from Senegal. (a) General anterior view, arrows show lateral alae. (b) Detail of anterior end. Arrows show papillae. (c) Detail of mouth. Arrows show papillae. (d) Female, vulva with four cuticular processes (in arrows)

tomys sp.1) from life in Nigeria. In a bibliographical review of helminths from African vertebrates, Canaris and Gardner (2003) mention *H. dahomensis* as well as *H. macrospiculum,* although Inglis (1991) placed this latter species in a new genus *Mammalakis* and considers *H. dahomensis* as a synonym of *H. spumosa*, probably because this author was not aware of the work of Boulenger (1923). Smales (1996) may have overlooked the fact that *H. dahomensis* is accepted as a synonym of *H. spumosa* in the literature. Another species, *Heterakis boueti* (Gendre, 1911), was initially described from the squirrel *Xerus erythropus* Desmarest, 1817 from Nigeria, but was subsequently placed in the genus *Oxynema* (Inglis, 1955). The validity of *H. dahomensis* as a valid species thus varies according to the author, one of the facts that motivated the present study.

Heterakis spumosa is found in Africa, having been introduced into the continent with the black rat *Rattus rattus* Linnaeus, 1758 and the brown rat *Rattus norvegicus* Berkenhout, 1769. This genus originated in Southern Asia and, although native to the Indian Peninsula, *R. rattus* currently has a worldwide distribution. Specifically, despite being found in both *Rattus* species in Egypt (Abo-Shady *et al*., 1983), *H. spumosa* is probably much more widely distributed, given the lack of helminthological studies of these rodents from Africa and despite its wide distribution (Musser and Carleton, 2005). Neither Udonsi (1989) in a study of *R. rattus* (n=3694) from the Niger Delta nor Mafiana *et al*. (1997) (n=612) detected this nematode despite their large sample sizes. In the past two decades *Heterakis spumosa* has also

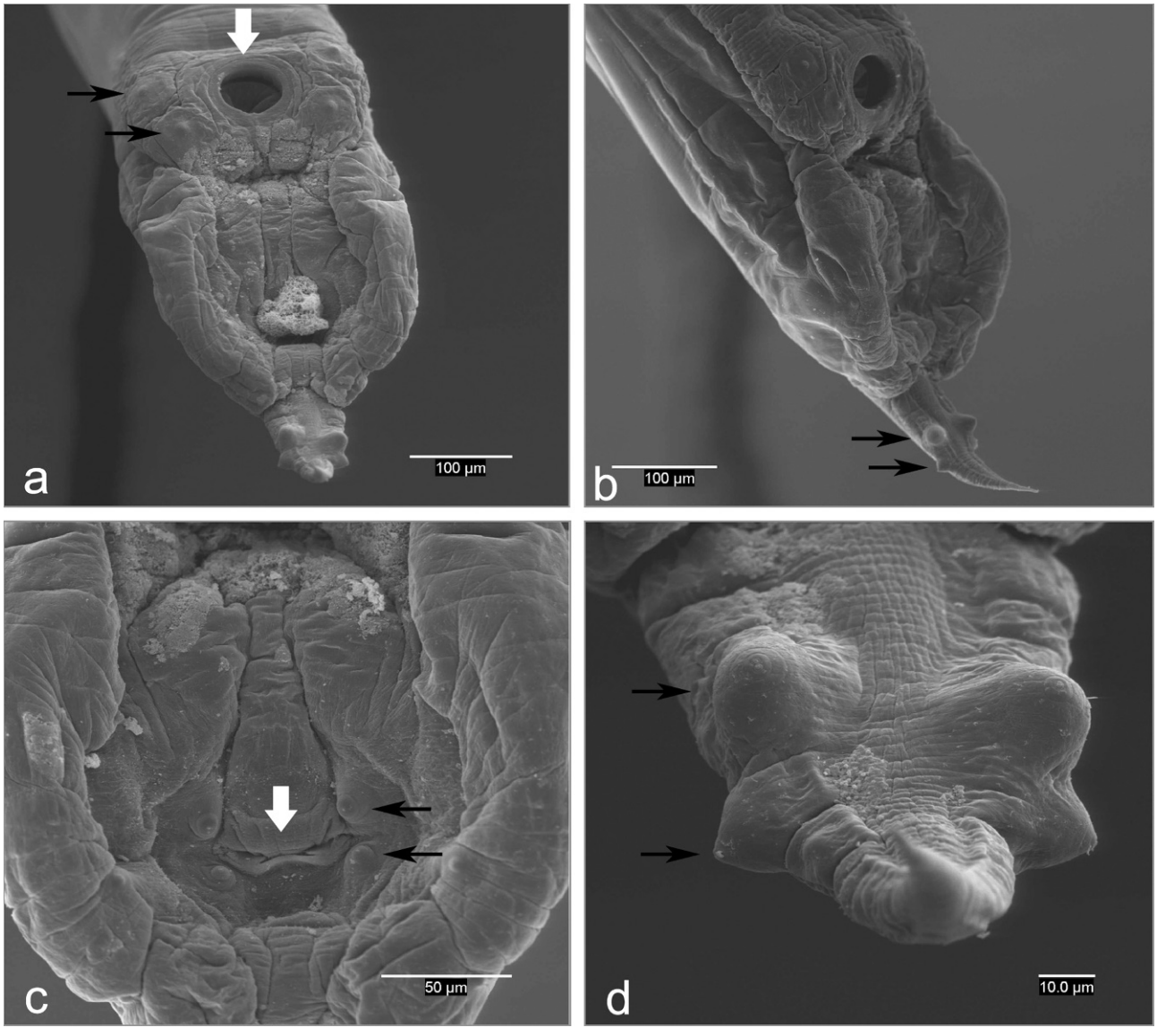

Fig. 2. (a-d) Scanning electron micrographs of male *Heterakis dahomensis* from Senegal. (a) General posterior view. Black arrows show two pairs of cuticular processes around the sucker (white arrow). (b) Lateral view of posterior end. Arrows show two pairs of tail papillae. (c) Detail of posterior end. Black arrows show two pairs of cuticular processes around the cloaca (white arrow). (d) Detail of tail. Arrows show two pairs of tail papillae.

been reported in *Cricetomys gambianus* from Nigeria (Ibrahim *et al*. 1984; Nmorsi and Egwunyenga, 2001) and Zimbabwe (Jooste, 1990). Nevertheless, caution should be taken with the specific identification of *C. gambianus* in light of the review by Olayemi *et al*. (2012), who reports several hitherto undescribed species of this genus. Aside from *Cricetomys* spp., other native African rodents such as *Arvicanthis niloticus* Desmarest, 1822, *Lemniscomys striatus* Linnaeus, 1758 and *Mastomys natalensis* Smith, 1834 in Nigeria (Ugbomoiko and Obiamiwe, 1991) have been found to be infected by *H. spumosa*. The taxonomic validity at species level of this heterakids is uncertain, largely due to the fact that metrical and morphometrical data are not given in the previously cited studies.

Previous molecular studies of the genus *Heterakis* from rodents are limited to work on *H. spumosa* by Zaleśny *et al*. (2010), who analyzed the partial sequence of the small subunit (18S) of the ribosomal DNA (rDNA) of three different rodent hosts in Europe. Multiple alignment showed that the nucleotide composition of DNA from all the hosts was identical, thus indicating the existence of a single species.

The aim of this study was to disentangle and clarify the morphological and genetic characteristics that distinguish two species of *Heterakis*, commonly confused during the past century, via the study of specimens from *Cricetomys gambianus*, the type host in the original description by Gendre (1911). We emphasize that future research on *Heterakis* spp. from the genus *Cricetomys* is still required.

Materials and Methods

A single individual of the Gambian pouched rat *Cricetomys gambianus* was captured in December 2010 in Cheikh Anta Diop University Campus in Dakar, Senegal. The specimen could be identified *in situ* as it is easily separated from the other rodents present in the area by its morphological and metrical features (Olayemi *et al*., 2012). This species is known to be widespread in the African savanna (including the Dakar area) and, according to Olayemi *et al*. (2012), no other species of the genus *Cricetomys* are found in the study area.

The dissection and isolation of the animals' helminths was conducted *in situ*. The cecum was dissected and examined separately under a binocular stereomicroscope according to standard protocols (Ribas *et al*., 2011).

Of the thirteen helminths found in the *C. gambianus* from Senegal, six were studied with a scanning electron microscope (SEM), six were used for taking morphometrical measurements using optical microscopy and one was used for molecular analysis.

For the optical microscopy, the specimens were first cleared with lactophenol. Measurements (given in micrometers and listed as range and mean) were taken using a microscope-mounted camera. All known measurements of *Heterakis* species found in mammals were compiled from other publications to compare with the measurements obtained from the Dakar specimens (see Tables 1 and 2). Illustrations were drawn using a camera lucida (Fig. 4).

For the SEM study, samples were fixed in hot ethanol 70° in the field treated with 1% osmium tetroxide, dehydrated by an

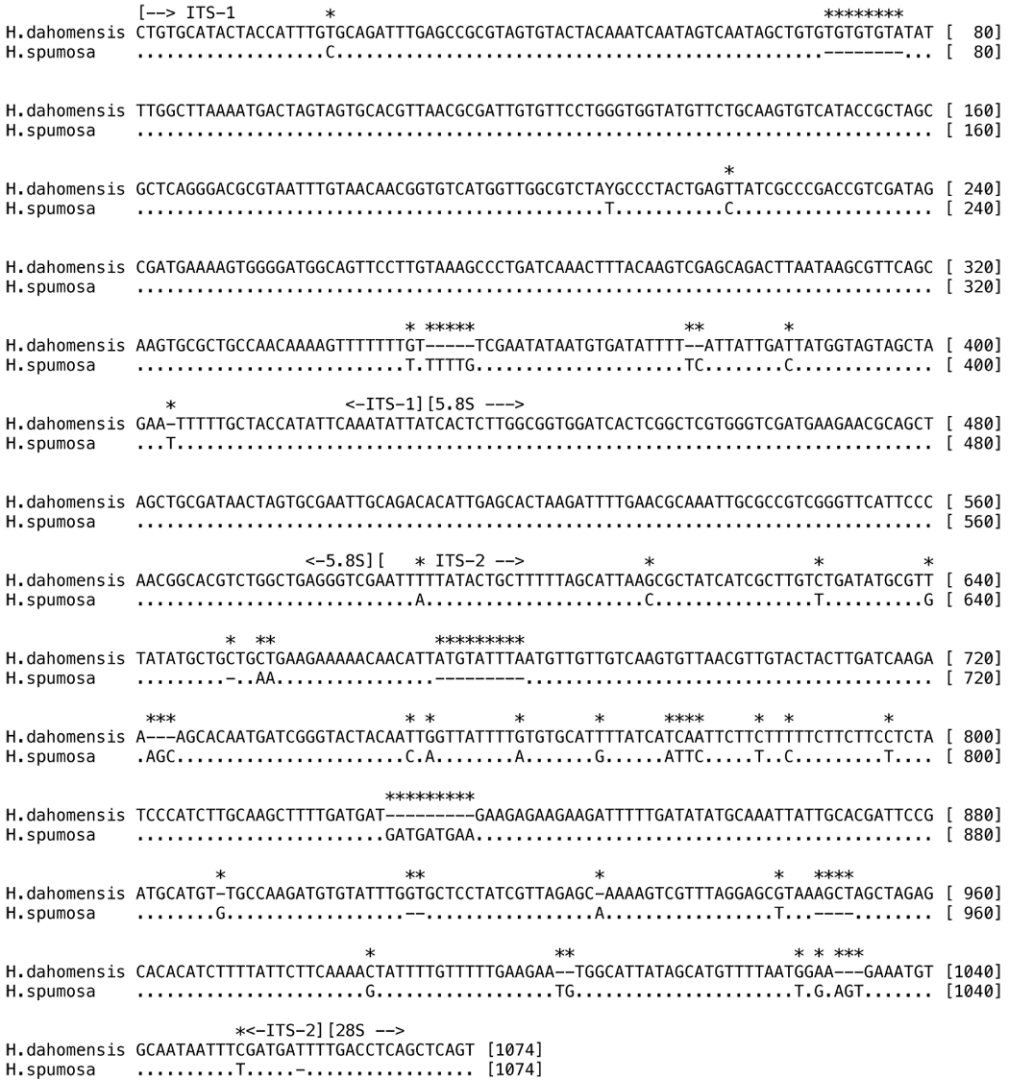

Fig. 3. Alignment of the first internal transcribed spacer (ITS-1), 5.8S, and second internal transcribed spacer (ITS-2) sequences for *Heterakis dahomensis* found in *Cricetomys gambianus,* and *Heterakis spumosa* found in *Mus musculus domesticus*. The numbers refer to alignment positions. Asterisks indicate nucleotide differences.

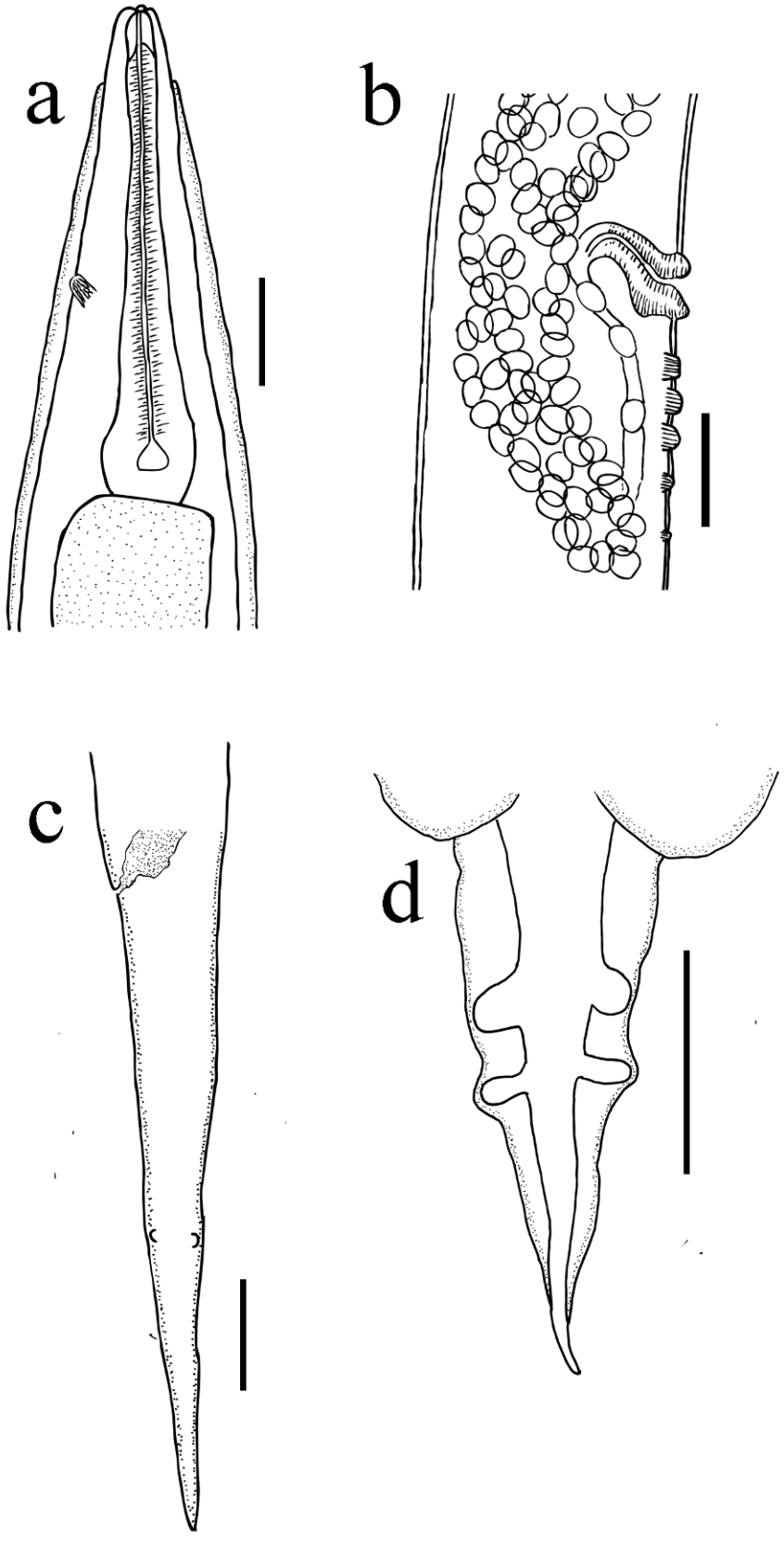

Fig. 4. a. Cephalic end. **b.** Vulval region of female. **c.** Female tail. **d**. Male tail

ethanol series and then critical point dried with carbon dioxide in a Polaron CPD 7501. Finally, specimens were mounted on stubs with adhesive tape and colloidal silver, sputter-coated with gold in a Fisons Instrument SC 510, and examined using a Zeiss DSM 940A scanning electron microscope at 15 kV.

Two *Heterakis* worms were used for the molecular analyses: one from a *Mus musculus domesticus* from Spain, morphometrically identified as *H. spumosa,* and one from a *C. gambianus* from Senegal, morphometrically identified as *H. dahomensis*. The total DNA was extracted from individual worms using the DNeasy tissue kit (Qiagen) and eluted in 50 µL of AE buffer. Polymerase chain reaction (PCR) amplification was performed in 20 µl volume containing 0.2 µM of each primer, 0.2 mM of each dNTP, 1.25 mM $MgCl_2$, 1X DreamTaq buffer, 0.6 unit of DreamTaq DNA Polymerase (Fermentas) and 2 µl of DNA template. The thermal cycling profile was as follows: an initial denaturing step at 94°C for 3 min, followed by 40 cycles at 94°C for 45 s, 53°C for 30 s and 72°C for 1 min, and ending with an extension step of 72°C for 10 min. The region encompassing the ITS-1, 5.8S and ITS-2 of the ribosomal DNA gene was amplified using the NC5 and NC2 primers (Gasser and Hoste, 1995). PCR products were visualized on a 1.4% agarose gel and were purified and sequenced by the VIB Genetic Service Facility (University of Antwerp, Belgium) using the same primers as generated the PCR products. The rDNA sequences have been deposited in GenBank (AN: JX845277-JX845278).

Sequences were aligned using Geneious Pro 5.5.6 (Drummond *et al*., 2010). We estimated the evolutionary divergence using the p-distance method in MEGA 5.05 (Tamura *et al*., 2011) with the standard error estimates obtained by a bootstrap procedure (1000 replicates) and all positions containing gaps eliminated from the dataset.

Results

Morphometrical data

The measurements of the specimens from Senegal, along with those of other *Heterakis* species reported by previous authors, are given in Tables 1 and 2 for males and females, respectively. The morphological details observed by SEM are given in Figures 1 and 2.

The measurements of the *Heterakis* individuals obtained from *Cricetomys gambianus* in Senegal are as follows:

Male (n = 2) (Figs 2, 4)

Body length 9.83–10.12 mm, width (at oesophagus-intestine junction) 277–326 µm. Anterior oesophagus length 721–747 µm, entire oesophagus length 884-907 µm; oesophageal bulb length 59–163 µm, width 158–190 µm. Cervical alae 35–54 µm and excretory pore 479 µm from anterior end. Lateral alae extend along the whole body. Sucker 652–726 µm from posterior end, length 103-134 µm, width 125 µm; left spicule 348-366 µm, right spicule 352-395 µm. Caudal papillae are arranged in nine pairs: two pairs on sides of suckers, three pairs lateral to cloaca, two pairs on sides of cloacal opening and two pairs on terminal spike of tail. Tail length is 371 (366-375) µm.

Female (n=4) (Figs 1, 2, 4)

Body length 12.09 (10.61–13.39) mm, width (at oesophagus-intestine junction) 311 (275-337) µm and 439 (419–474) µm (at vulva). Anterior oesophagus length 754 (731–779) µm, entire oesophagus length 925 (899–958) µm; oesophageal bulb length 171 (157-193) µm, width 178 (167–189) µm. Cervical alae 39 (30–46) µm and excretory pore 491 (476–533) µm from anterior end. Lateral alae extend along the whole body. Vulva is 6.26 (5.66–6.80) mm from anterior end, with cuticular processes (2–5 in number) situated posterior to the vulvar opening. Tail length 1130 (1072–1180) µm. Eggs oval, length 61.4 (52.14–69.19) µm, width 45.4 (40.89–49.85) µm.

Molecular data

We obtained two different sequences of the ITS-1-5.8S-ITS-2 of the rDNA region from the species studied. The ITS-2 region was the most divergent of the two and had an average estimate of evolutionary divergence of 3.12±0.83% base differences per site (Figure 3). The ITS1 region showed only 0.97 ± 0.47% base differences per site. Finally, the 5.8S region was found to be completely monomorphic (Figure 4). These differences in ITS-1 and ITS-2 support the assertion that these *Heterakis* sequences correspond to two different species.

Discussion

No previous studies of the helminths of *C. gambianus* from Senegal exist. The present study thus represents the first attempt to recover and study *Heterakis* sp. from *C. gambianus* since their original description by Gendre (1911).

Caution should be taken with the validity of the assignation of *H. dahomensis* to the individuals from Zanzibar by Boulenger *et al*. (1923) given that the review of the genus, *Cricetomys* by Olayemi *et al*. (2012) reports that its Tanzanian populations in fact correspond to *C. ansorgei* rather than *C. gambianus,* as in the original description by Gendre (1911). As *Heterakis* from both hosts and localities present morphological similarities (two pairs of papillae in the tail and the same arrangement in the females' cuticular processes) and overlapping metrical characters (spicule length) (Table 1), two different hypotheses regarding the identity of these species are possible: either the two worms belong to same species (*H. dahomensis*) or the individuals described by Boulenger *et al*. (1923) represent a close but different species occurring as a

result of co-speciation in the rodent genus *Cricetomys*. Future studies of new morphological, metrical and molecular material from Zanzibar are required to determine the systematic position of these parasites.

Caution should also be taken with the systematic position of individuals assigned to *H. dahomensis* by Gedoelst (1916) from the Belgian Congo (Democratic Republic of Congo) since Olayemi *et al*. (2012) consider that the *Cricetomys* species concerned is in fact *C. emini* and not *C. gambianus*. As no metrical or morphological data are provided by Gedoelst (1916), the systematic status remains uncertain.

Our study confirms that *H. dahomensis* can be differentiated genetically from *H. spumosa* (Fig. 4). Also, the papillae on the male's tail are arranged differently: we found two pairs of papillae in *H. dahomensis* and three in *H. spumosa*, as shown in the SEM images in the study by Robles *et al*. (2008) Unfortunately, in the work by Tenora and Baruš (1983) only SEM images of the anterior end are given.

On the other hand, the possible diagnostic metrical characters of male *H. dahomensis* overlap with those of *H. spumosa* (spicule and sucker lengths and width and length of the esophagus) (Singh & Krishnasamy, 1979; Table 1). Likewise, in females significant metrical characters such as egg length and width and esophagus length overlap (Singh & Krishnasamy, 1979; Table 2). Consequently, individuals from *Cricetomys* spp. identified as *Heterakis spumosa* should be re-evaluated on the basis of both morphology and genetics.

In conclusion, this study finally clarifies the validity of *H. dahomensis* as a good species given that the original description did not provide enough morphometrical data for its separation. As well, it reveals the need to increase the number of studies on *Heterakis* spp. from African rodents.

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