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Invited article

Occurrence and characterisation of tongue worms, *Linguatula* spp., in South Africa

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ABSTRACT

A total of 509 mammalian vertebrates, belonging to 76 species, were examined for infection with pentastomid parasites. These animals were from 8 of the 9 provinces in South Africa. Linguatulid pentastomes were found only in 7 animals, specifically the African Lion (n = 3) and African Buffalo (n = 4). Adult parasites were found in the lion but nymphs, of various stages, were found in the buffalo. A detailed morphological examination of adult parasites using both light and scanning electron microscopy techniques suggested the specimens were *Linguatula nuttalli* Sambon1922. Sequences of 18S ribosomal DNA and Cox1 regions obtained from both adult and nymph stages suggested they belong to the one species. Phylogenetic analyses of *Linguatula* spp. based on the 18S and Cox1 sequences available in GenBank and obtained in the present study showed a clear distinction between *L. nuttalli, L. arctica* and *L. serrata* (from Europe and Australia). Several specimens from the Palearctic region which were previously assumed to be *L. serrata* formed a distinct group in the phylogenetic tree suggesting they probably belong to a different, and as of yet, unknown species.

1. Introduction

Pentastomid parasites belonging to the family Linguatulidae are of both veterinary and medical significance. They have an indirect life cycle which usually involves herbivorous vertebrates, such as cattle, as intermediate hosts and carnivorous vertebrates, such as dogs and foxes, as definitive host (Basson et al., 1970). Nothing is known about the range of herbivorous vertebrates that are suitable as intermediate host nor what the drivers are for their transmission in the ecosystem between different hosts. Of the species belonging to the Linguatulidae, *Linguatula serrata*, also referred to as the European tongue worm, has been subject to the most attention; however, despite numerous publications on their occurrence across the world, many aspects of their taxonomy, biology and ecology is still unknown.

Linguatula nuttalli, like *L. serrata*, has a complicated and confusing taxonomic history. Sambon (1922) originally described *L. nuttalli* based on 1 male and 2 female specimens obtained from the pharynx of a lion from what is now known as Kenya. The specimens most closely resembled *L. recurvata,* which had been described from a jaguar in Brazil (by Diesing in 1805 as cited in Sambon, 1922), due to its obvious bifid posterior extremity. Haffner et al. (1969), however, considered that the terminal cleft, and subsequent formation of a cloaca in the female, was significant enough to erect a new genus, *Neolinguatula*, consisting of both *N. nuttalli* and *N. recurvata*. Riley (1986), however, considered the possession of a terminal cleft "too trivial" to justify a new genus and returned *nuttalli* and *recurvata* to *Linguatula*. However, authors continued to use the genus *Neolinguatula* (see Christoffersen and De Assis, 2013), although generally only for *N. nuttalli*. Christoffersen and De Assis (2013) also retained *Neolinguatula* in their pentastomid phylogeny as the terminal cleft was considered a potential apomorphic character that distinguished it from *Linguatula*; they stated that the two genera were closely related with this character being the only true distinguishing feature, although *recurvata* was within *Linguatula*.

In South Africa, both larval and adult stages of tongue worms, including *L. serrata, L. nuttalli* and specimens referred to as *Linguatula* sp. have been reported from a range of animals (Table 1). Reports on the occurrence of adult specimens of *Linguatula* in South Africa are particularly rare. A female *L. serrata* was collected from a domestic dog in Makhanda (Grahamstown), Eastern Cape, with a note that it is a common parasite in Europe (Ortlepp, 1934) and Young and Van den Heever (1969) referred to *L. serrata* from the nostrils of lions from Kruger National Park. Adult *L. nuttalli* were also collected from lions in

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Table 1
Previous reports of parasites belonging to *Linguatula* spp. in South Africa.

Parasite	Host (scientific name)	Host (common name)	Locality	Infected organ	Reference
L. serrata	*Canis lupus familiaris*	Dog	Makhanda (Grahamstown)	-#	Ortlepp (1934); Zumpt (1961)
	Panthera leo	Lion	Kruger National Park	Nostrils	Young and Van den Heever (1969)
	Syncerus caffer	Buffalo	Kruger National Park	Heart, Hepatic veins, Pulmonary artery, Liver	Young and Van den Heever (1969); Basson et al. (1970b); Basson et al. (1970a)
	Connochaetes taurinus	Blue Wildebeest	Kruger National Park	–	Young and Van den Heever (1969)
		Impala	Kruger National Park	–	Young and Van den Heever (1969); Young and Wagener (1968)
		Cattle	Not mentioned	–	Young (1975a)
L. nuttalli	*Connochaetes taurinus*	Blue Wildebeest	Kruger National Park	–	Horak et al. (1983)
	Panthera leo	Lion	Kruger National Park	–	Young (1975b)
	Phacochoerus aethiopicus	Warthog	Kruger National Park	–	Horak et al. (1988)
	Tragelaphus strepsiceros	Greater Kudu	Kruger National Park	Heart, Liver, Lung	Horak et al. (1992)
Linguatula sp.	*Damaliscus lunatus*	Common Tsessebe	Kruger National Park	–	Young (1975a)
Linguatula sp.	*Connochaetes taurinus*	Blue Wildebeest	Kruger National Park	Liver, atria and ventricles of the heart as well as some of the larger bloodvessels	McCully et al. (1966)*; Young et al. (1969)
Linguatula sp.	*Giraffa camelopardalis*	Giraffe	Kruger National Park	–	Young (1975b)
Linguatula sp.	*Kobus ellipsiprymnus*	Waterbuck	Kruger National Park	–	Young (1975a)
		Kudu	Kruger National Park	Cardiovascular system and liver	McCully et al. (1966)

* referred to as pentastome
\# found in the vomit.

Kruger National Park (Young, 1975a, b) stating “in several parts of the Kruger Park most or all of the older lions are infested”. Faecal surveys of lions, and other carnivores, throughout Africa have returned predominately negative results for pentastomid infections; Christine (1995) and Bjork et al. (2000) for Tanzania; Kok and Smith (2006) for Namibia; Berentsen et al. (2012) for Zambia. Mukarati et al. (2013) did find linguatulid eggs in the faeces of young captive lions in Zimbabwe; these lions had been fed meat from a variety of sources and Mukarati et al. (2013) suggested that the low incidence of infection may be due to the parasite not being a “true parasite of the lion”. There are many more reports on the occurrence of nymphs and cysts (encapsulated nymphs) of *Linguatula* spp. in various animals in South Africa. McCully et al. (1966) accidentally found numerous small, flat, elongated organisms (3–4 mm) in the hepatic and other veins, beneath Glisson's capsule in the liver, in the chambers of the heart, the pulmonary artery and the aorta, and the lumen of the thoracic portion of the posterior vena cava of a blue wildebeest and subsequently reported 76% infection with nymphs and 90% infection with cysts (n = 21). They also reported nymphs and cysts of pentastomes in kudu and impala. Later, in a targeted study in Kruger National Park, Young and Van den Heever (1969) reported 64.28% of bulls (n = 56), 61.53% of cows and heifers (n = 52), but none of the calves (n = 8), to be infected with *L. serrata*, usually in the liver, the atria and ventriculi of the heart and in some of the larger blood vessels. In a separate study, at the same time period and location, Basson et al. (1970) also reported 69% of African buffalo (n = 97), almost all being more than 2 years old, infected with nymphs of *L. serrata*. Horak et al. (1983) recovered the nymphs of *L. nuttalli* from a fairly large proportion of blue wildebeest (21.8%; n = 55) in the park and later Horak et al. (1988) recovered 91 nymphs of *L. nuttalli* from warthogs (35.3%; n = 51) in the Kruger National Park and concluded that the high proportion of infection in warthogs is due to large number of lions, the final host of this parasite, in the park. Horak et al. (1992) reported 667 nymphs of *L. nuttalli* in kudus (63.2%; n = 95) in the Kruger National Park. It must be noted that in all of these reports, no indication was provided as to how the identification of the nymphs was determined.

Although the review of the literature suggests that *Linguatula* spp. are successfully inhabiting animals in South Africa, many of these reports are old, from limited geographical locations (mainly from Kruger National Park) and lack details on the basis for the specific identification of the parasites. Moreover, to date there has been no molecular work done on any specimen of the African Linguatulidae. The aim of the present study is to provide a more recent report on the occurrence of these parasites from a wide range of potential definitive and intermediate hosts from a broader region; and to provide morphological and genetic characterisation of adult and nymphs of tongue worms found in the present study in South Africa.

2. Materials and methods

2.1. Parasite collection

Animals listed in Table 2 were examined for infection with pentastomid parasites between 2012 and 2018. They were either roadkill animals (permit number ZA/LP/87586), animals found dead or collected from animals hunted during hunting seasons. No animal was killed for the purpose of this study. All animals were examined for infection with adult and larval stages of the linguatulid parasites in accordance with Shamsi et al. (2017). In brief, the skulls of suspected definitive hosts (carnivores) were split into two halves enabling a clear view into the right and left sides of the nasal cavity, which then was extensively searched macroscopically for adult parasites. All parasites collected were rinsed in distilled water before being preserved in ethanol (70%). All other internal organs of all animals, including mesenteric lymph nodes, were subjected to extensive examination for nymphal stages of the parasites. Nymphs, if present, were released from the tissue capsule surrounding them and then preserved in 70% ethanol.

2.2. Parasite examination

Two adults (1 male and 1 female) and nine nymphs from two buffalo were sent to Shamsi's Parasitology Research group at Charles Sturt University, Australia for identification, where all specimens were first examined morphologically. The adult female and one nymph were examined by light microscopy and the adult male and the remaining nymphs were examined by scanning electron microscopy as detailed in (Shamsi et al., 2020). The terminology related to the measurement conventions of the adult hook and fulcrum follows Shamsi et al. (2020). Specimens were deposited in the Australian Museum under accession numbers P.104086 and P.104087.

A small piece of the body of all specimens were cut for DNA

Table 2
List of animals examined in the present study for the presence of *Linguatula* spp.

Order/Family	Host		Locality	No examined (No infected)
	Scientific name	Common name		
Artiodactyla/Bovidae	*Aepyceros melampus*	Impala	Limpopo Province	11 (0)
	Damaliscus pygargus	Blesbok	Limpopo Province	5 (0)
	Bos taurus	Cattle	Limpopo Province	20 (0)
	Capra aegargus	Goat	Limpopo Province	5 (0)
	Connochaetes taurinus	Blue Wildebeest	Limpopo Province	2 (0)
	Ovis aries	Sheep	Limpopo Province	5 (0)
	Raphicerus campestris	Steenbok	Limpopo Province	1 (0)
	Sylvicapra grimmia	Common Duiker	Limpopo Province	1 (0)
	Syncerus caffer	African Buffalo	Mpumalanga Province	8 (4)
	Taurotragus oryx	Common Eland	Limpopo Province	1 (0)
	Tragelaphus angasii	Nyala	Limpopo Province	1 (0)
	T. strepsiceros	Greater Kudu	Limpopo Province	6 (0)
Artiodactyla/Hippopotamidae	*Hippopotamus amphibius*	Hippopotamus	Limpopo Province	9 (0)
Artiodactyla/Suidae	*Phacochoerus africanus*	Warthog	Limpopo Province	2 (0)
	Potamochoerus larvatus	Bushpig	Limpopo Province	1 (0)
	Sus scrofa domestica	Pig	Eastern Cape Province	1 (0)
Carnivora/Canidae	*Canis lupus familiaris*	Dog	Limpopo Province	12 (0)
	C. mesomelas	Black-backed Jackal	Limpopo Province	6 (0)
	Otocyon megalotis	Bat-eared Fox	Limpopo Province	1 (0)
	Vulpes chama	Cape Fox	Free State Province (1); Mpumalanga Province (1)	2 (0)
Carnivora/Felidae	*Caracal caracal*	Caracal	Limpopo Province	2 (0)
	Felis catus	Cat	Limpopo Province	14 (0)
	F.s silvestris lybica	African Wildcat	Limpopo Province	1 (0)
	Leptailurus serval	Serval	Mpumalanga Province	8 (0)
	Panthera leo	Lion	Mpumalanga Province	4 (3)
	P. pardus	Leopard	Mpumalanga Province	3 (0)
Carnivora/Herpestidae	*Atilax paludinosus*	Marsh Mongoose	Limpopo Province	4 (0)
	Galerella sanguinea	Slender Mongoose	Limpopo Province	10 (0)
	Helogale parvula	Common Dwarf Mongoose	Limpopo Province	1 (0)
	Ichneumia albicauda	White-tailed Mongoose	Limpopo Province	12 (0)
	Mungos mungo	Banded Mongoose	Limpopo Province	13 (0)
	Rhynchogale melleri	Meller's Mongoose	Limpopo Province	1 (0)
	Suricata suricatta	Meerkat	North West Province	1 (0)
Carnivora/Hyaenidae	*Crocuta crocuta*	Spotted Hyena	Mpumalanga Province	4 (0)
	Proteles cristata	Aardwolf	Limpopo Province; Mpumalanga Province	2 (0)
Carnivora/Mustelidae	*Aonyx capensis*	African Clawless Otter	Limpopo Province	1 (0)
	Ictonyx striatus	Striped Polecat	Limpopo Province; Mpumalanga Province	4 (0)
	Mellivora capensis	Honey Badger	Limpopo Province	2 (0)
Carnivora/Viverridae	*Civettictis civetta*	African Civet	Limpopo Province	8 (0)
	Genetta genetta	Small-spotted Genet	Gauteng Province (1); Limpopo Province (4)	5 (0)
	G. maculata	Rusty-spotted Genet	Limpopo Province	10 (0)
Erinaceomorpha/Erinaceidae	*Atelerix frontalis*	Southern African Hedgehog	Gauteng Province (1); Limpopo Province (10)	11 (0)
Eulipotyphla/Soricidae	*Crocidura mariquensis*	Swamp Musk Shrew	Limpopo Province	3 (0)
	C. silacea	Shrew	Limpopo Province	1 (0)
Hyracoidea/Procaviidae	*Procavia capensis*	Rock Hyrax	Limpopo Province	1 (0)
Lagomorpha/Leporidae	*Leepus saxatilis*	Scrub Hare	Gauteng Province (1); Mpumalanga Province (1); Limpopo Province (5)	7 (0)
Macroscelidae/Macroscelididae	*Elephantulus myurus*	Eastern Rock Sengi	Limpopo Province	5 (0)
Primates/Cercopithecidae	*Papio ursinus*	Chacma Baboon	Limpopo Province (5); Western Cape Province (5)	10 (0)
	Cercopithecus albogularis	Samango Monkey	Limpopo Province	2 (0)
	Chlorocebus pygerythrus	Vervet Monkey	Limpopo Province	20 (0)
Primates/Galagidae	*Otolemur crassicaudatus*	Thick-tailed Bushbaby	Limpopo Province	12 (0)
	O. moholi	Southern Lesser Bushbaby	Limpopo Province	1 (0)
Rodentia/Gliridae	*Graphiurus murinus*	Woodland Dormouse	Limpopo Province	1 (0)
Rodentia/Hystricidae	*Hystrix africaeaustralis*	Cape Porcupine	Limpopo Province	1 (0)
Rodentia/Muridae	*Aethomys chrysophilus*	Red Rock Rat	Limpopo Province	3 (0)

(*continued on next page*)

Table 2 (*continued*)

Order/Family	Host		Locality	No examined (No infected)
	Scientific name	Common name		
Rodentia/Muridae	*A. namaquensis*	Namaqua Rock Mouse	Limpopo Province	1 (0)
	Gerbilliscus brantsii	Highveld Gerbil	Free State Province	13 (0)
	G. leucogaster	Bushveld Gerbil	Limpopo Province (10); Free State Province (30)	40 (0)
	Lemniscopus rosalia	Single-striped Grass Mouse	Limpopo Province	4 (0)
	Mastomys coucha	Southern Multimammate Mouse	Free State Province (10); Mpumalanga Province (20)	30 (0)
	M. natalensis	Natal Multimammate Mouse	KwaZulu-Natal Province (9); Limpopo Province (5)	14 (0)
	Micaelamys namaquensis	Namaqua Rock Mouse	Limpopo Province	2 (0)
	Mus minutoides	African Pygmy Mouse	Free State Province (4); KwaZulu-Natal Province (3); Limpopo Province (2); Mpumalanga Province (2)	11 (0)
	Otomys auratus		Mpumalanga Province	3 (0)
	Rattus rattus		Limpopo Province	1 (0)
	R. tanezumi	Tanezumi Rat	Limpopo Province	2 (0)
	Rhabdomys chakae		Mpumalanga Province	25 (0)
	R. dilectus	Mesic four-striped grass rat	Limpopo Province	5 (0)
	R. pumilio	Four-striped Grass Mouse	Free State Province (1); Limpopo Province (44)	45 (0)
	Thallomys paedulcus	Acacia Rat	Limpopo Province	1 (0)
Rodentia/Nesomyidae	*Cricetomys ansorgei*	Gambian Pouched Rat	Limpopo Province	7 (0)
Rodentia/Sciuridae	*Paraxerus cepapi*	Tree Squirrel	Limpopo Province	3 (0)
	Xerus inauris	Cape Ground Squirrel	Free State Province	10 (0)
Rodentia/Thryonomyidae	*Thryonomys swinderianus*	Greater Cane Rat	Limpopo Province	1 (0)
Tubulidentata/Orycteropidae	*Orycteropus afer*	Aardvark	Limpopo Province	1 (0)
	Stigmochelys pardalis	Leopard Tortoise	Limpopo Province	2 (0)
			Total	509 (7)

Table 3
Details of sequences used to build phylogenetic trees in the present study.

Species	Localities	Host	COX I	18sRNA	Reference
L. arctica	Norway	Reindeer	KF029443-KF029446	KF029439-KF029442	Gjerde (2013)
L. serrata	Norway	Dog	KF029447	JX088397	Gjerde (2013)
L. serrata	Australia	Dog, Fox	MN893765-MN893769	MN889436-MN889440	Shamsi et al. (2020)
L. serrata	Iran	Cattle, goat, sheep	–	KT581431-KT581433	Unpublished
L. serrata	Iran	Cattle	–	KP100453	Ghorashi et al. (2016)
L serrata	Bangladesh	Zebu	LC150781-LC150784	–	Mohanta and Itagaki (2017)
L serrata	Peru	Vicugna	KY829107-KY829109	–	Gomez-Puerta et al. (2017)
L. nuttali	Africa	Buffalo, Lion	MN905329-MN905338	MN906667-MN906675	This study

extraction using DNeasy Blood & Tissue Kits (Qiagen, Australia). The cytochrome c oxidase subunit I (Cox1) gene and 18S ribosomal RNA (18S rRNA) gene were amplified using the primer sets and conditions in accordance with Gjerde (2013). PCR amplicons were bidirectional sequenced using the PCR primers by Australian Genome Research Facility (Queensland). Sequences of Cox1 and 18S rRNA of *Linguatula* spp. were either generated in the current study, or were obtained from GenBank (Table 3). These sequences were aligned with ClustalW in BioEdit (Hall, 1999). Alignments were manually adjusted and truncated into 941 and 1750 bp, respectively. Pairwise genetic distances among samples shown as percentage of difference were calculated by MEGA7.0.26 (Kumar et al., 2016). Phylogenetic relationship among species was inferred using MrBayes v3.2 (Ronquist and Huelsenbeck, 2003) with Ngen set as 2,000,000. Cox1 and 18S rRNA sequences from *Armillifer agkistrodontis* (FJ607340 and FJ607339, respectively), were used as an outgroup. The best fit evolutionary models for phylogenetic analysis were set as HKY + I and K2P for Cox1 and 18S rRNA as determined by Jmodeltest 2.0, respectively.

3. Results

Of 509 animals, belonging to 72 species, examined in the present study, only seven animals including three African lions (*Panthera leo)* and four African buffalo (*Syncerus caffer*) were found to be infected with tongue worms (Table 2). Although potential hosts were collected and examined from eight out of the nine provinces in South Africa, all infected animals were from the Mpumalanga Province (Table 2). The infected African buffalos were hunted in a nature reserve in Mpumalanga Province (permit number 13582) during spring 2016. Buffaloes were all adult and female. Larvae were yellowish in color and were found in different organs, mostly in the liver under the Glisson's capsule but also in the lung and heart. Intensity was 24–77 (mean 50). The

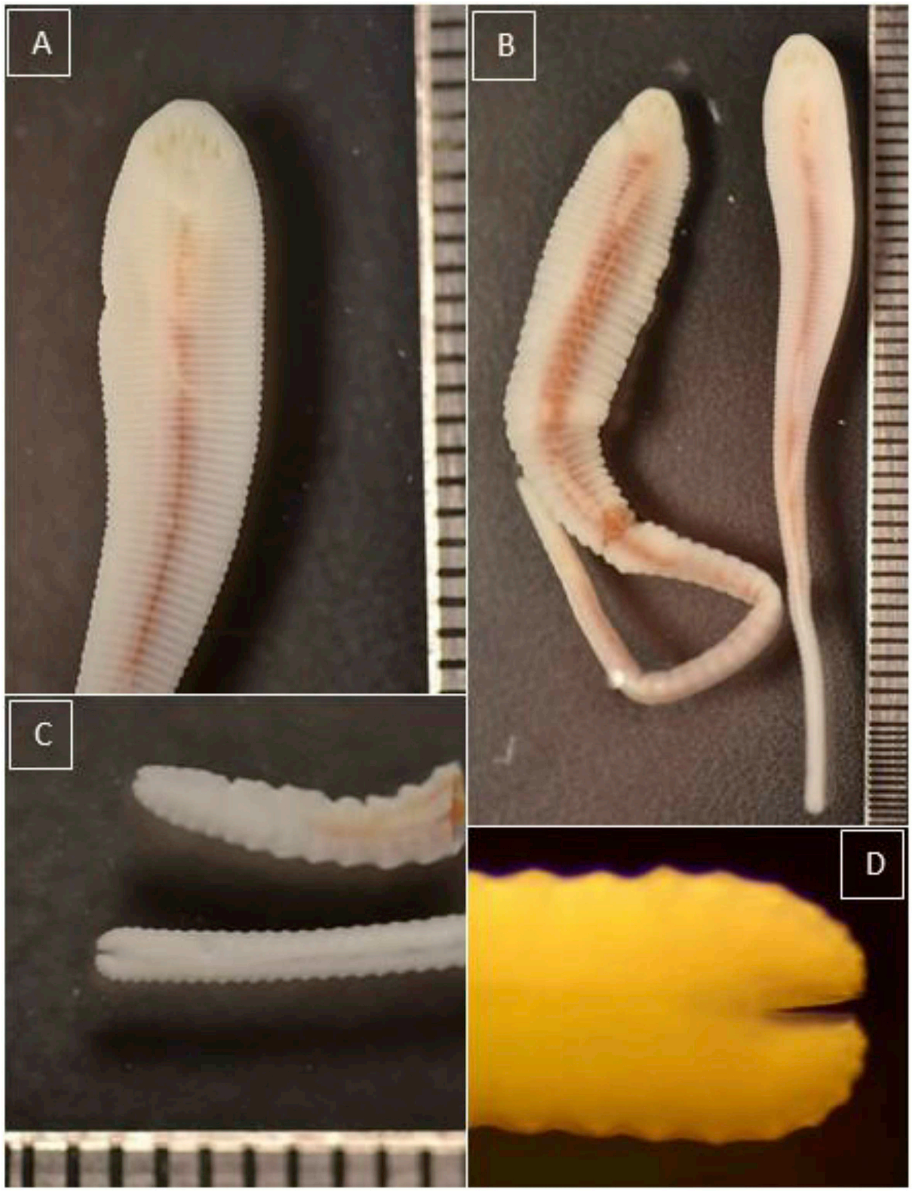

Fig. 1. *Linguatula nuttali* female specimen collected from *Panthera leo.* A) Anterior end, ventral surface. B) *L. nuttali* (on the right) compared to a specimen of *L. serrata* collected from a *Canis familiaris* in Australia (on the left). Note the differences in annuli and overall body shape. C) Posterior ends of *L. nuttali* (on the bottom) compared to a specimen of *L. serrata* collected from a *C. familiaris* in Australia (on the top). Note the deep cleft in the posterior end of *L. nuttali* compared to the posterior end of *L. serrata*. D) The posterior end of *L. nuttali* showing the deep cleft.

three infected lions were infected with adult linguatulid parasites, found in the nostrils and pharynx. One adult male lion had 12 adult pentastomids in the nostrils and three in the pharynx. For the other two lions, one had two adult pentastomids in sinuses and the other one had 9 adult pentastomids in the pharynx.

Description of the adult female (based on light microscopy): body broad, flattened anteriorly (Fig. 1A) but considerably narrowed and attenuated posteriorly (Fig. 1B). To minimize the damage to this specimen, only hooks on the right side of the parasite were dissected and measured. Total body length and width were 47 and 6 mm, respectively. Cephalothorax broadly rounded with mouth was located ventrally (Fig. 1A). Two large pairs of hooks located on each side of the mouth opening (Fig. 1A). Anterior hooks were smaller than posterior ones (Fig. 2B and C), with blade length, hook length, base length, plateau length, hook gape and hook width being 480, 800, 400, 480, 250 and 570 µm for the anterior and 520, 870, 450, 520, 280 and 600 µm for the posterior hook. The body was annulated throughout its length (Fig. 1A) with 128 annuli counted; in comparison to specimens of *L. serrata* from Australia, the annulations are fine (Fig. 1B). Each annulus contained a row of chloride cells, which are external openings of epidermal glands on the anterior region of each annulus, and multiple rows of scale like projections on the posterior margin of each

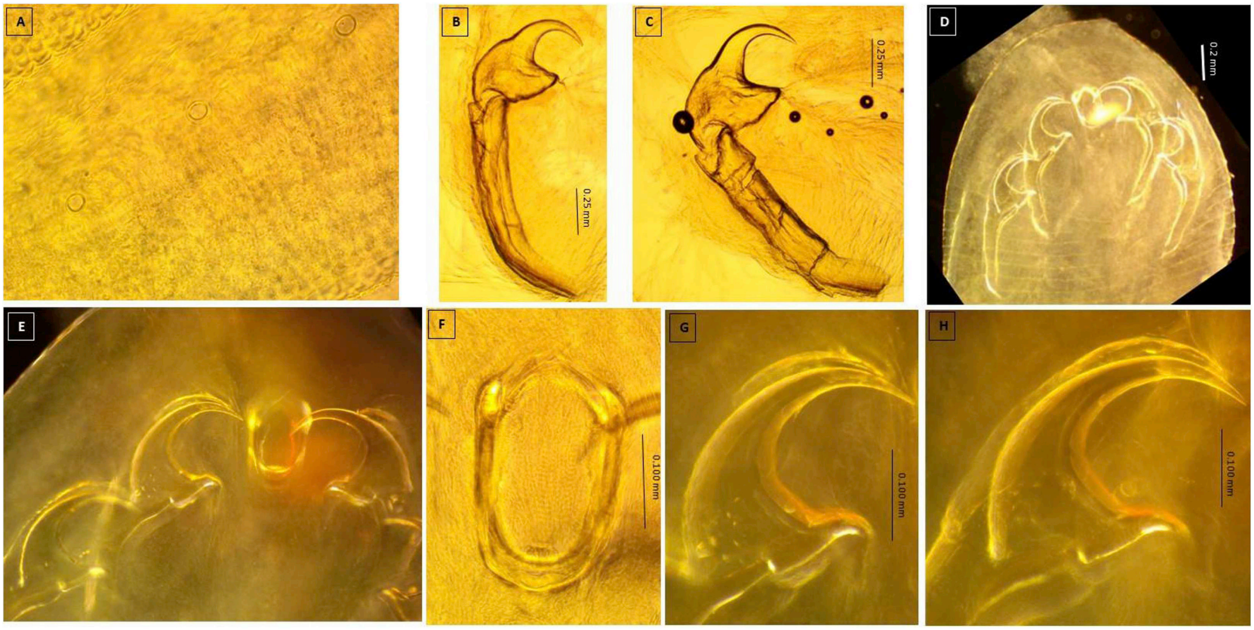

Fig. 2. *Linguatula nuttali* female specimen collected from *Panthera leo* (A to C) and female nymph collected from African buffalo (D to H). A) High magnification of the cuticular annulation. Cuticle taken from near the anterior end. Bottom of annulus has "scales" and a single row of pores across the middle of the annulus. B) Anterior hook and fulcrum. C) Posterior hook and fulcrum. D) Ventral view of the anterior end. E) Magnified view of the buccal cadre and hooks. F) Buccal cadre. G. Anterior hook. H. Posterior hook.

annulus. Body terminated in a marked cleft posteriorly (Fig. 1C and D). Female genital pore not observed externally and may form a cloaca within the terminal cleft as described by Haffner (1969).

Description of the adult male (based on scanning electron microscopy): body broad, flattened anteriorly (Fig. 3A) but narrowed and attenuated posteriorly. Total body length and width not measured prior to preparation for SEM. Cephalothorax broadly rounded with sensory sensillae, gland opening and several other minute projections on each side (Fig. 3B, D). Mouth located ventrally on the cephalothorax. Two large pairs of hooks are located on each side of the mouth opening. Body annulated throughout its length. Each annulus contained a row of chloride cells on the anterior region and multiple rows of scale like projections on the posterior margin (Fig. 3K and L). Body terminated in a cleft posteriorly (Fig. 3M).

Description of nymphs (based on a combination of light and scanning electron microscopy): Measurements of total body length and width and annulus count are provided in Table 4; the measurements for the SEM specimens were based on the SEM images so should be treated with caution. Cephalothorax includes a sub-terminal mouth, guarded by a chitinous framework and two pairs of protractile sharply-curved hooks (Fig. 4). One nymph (#7–5) was determined to be male based on the presence of a genital opening in the 5th annulus; the remaining nymphs were all determined to be female. Body was annulated with number of annuli ranging from about 100 to 145. Each annulus had a posterior border of spines; spines were finely denticulated (Figs. 4G, 5J and 6J) or spatulate (Fig. 7K). Similar to adults, anterior hooks were smaller than posterior hooks, with AC, AD, BC, CD, AB, BD, FL and DAP being 240, 335, 220, 170, 160, 225, 500, 175 μm for the anterior and 265, 360, 230, 140, 180, 235, 510, 200 μm for the posterior hook. Each hook is chitinous and consists of a curved projecting portion, and jointed basal portion embedded in the sac, to which the muscles are attached. A small dorsal accessory piece lying dorsal to the main hook is present on each of the nymphal hooks (Figs. 4D, 5D and 6G, 7B). Length and width of the buccal cadre were 225 and 145 μm.

Sequences of the Cox1 and 18S rRNA were successfully obtained for a number of nymphs and the two adults (GenBank accession numbers: MN905329-MN905338 and MN906667-MN906675, respectively). In the phylogenetic trees built based on these sequences (Fig. 8), specimens in the present study grouped separately from those reported in other parts of the world. All *Linguatula* samples collected in the present study had identical 18S rRNA sequence (Table 5) whereas the bp difference at Cox1 region ranged from 0 to 1% (Table 6).

4. Discussion

Surprisingly we found significantly fewer infected animals compared to previous reports (Young, 1975a). The difference could be due a number of factors. Firstly, our samplings were mostly opportunistic, and mainly performed on roadkill animals that were not necessarily fresh or they had significantly damaged bodies which may have impacted the efficiency of finding the parasites in their hosts. Other reasons could be due to the differences in the studied areas. As Table 1 shows, all previous reports of *Linguatula* spp. from South African wildlife were from Kruger National Park and, indeed, knowledge about the occurrence of these parasites in other regions in the country was unknown. We did not have many host specimens specifically from Kruger National Park for this study. Although a proper comparison cannot be drawn because our sampling was opportunistic, as explained above, it is obvious that even in Kruger National Park, we found significantly less *Linguatula* individuals and fewer infected animals

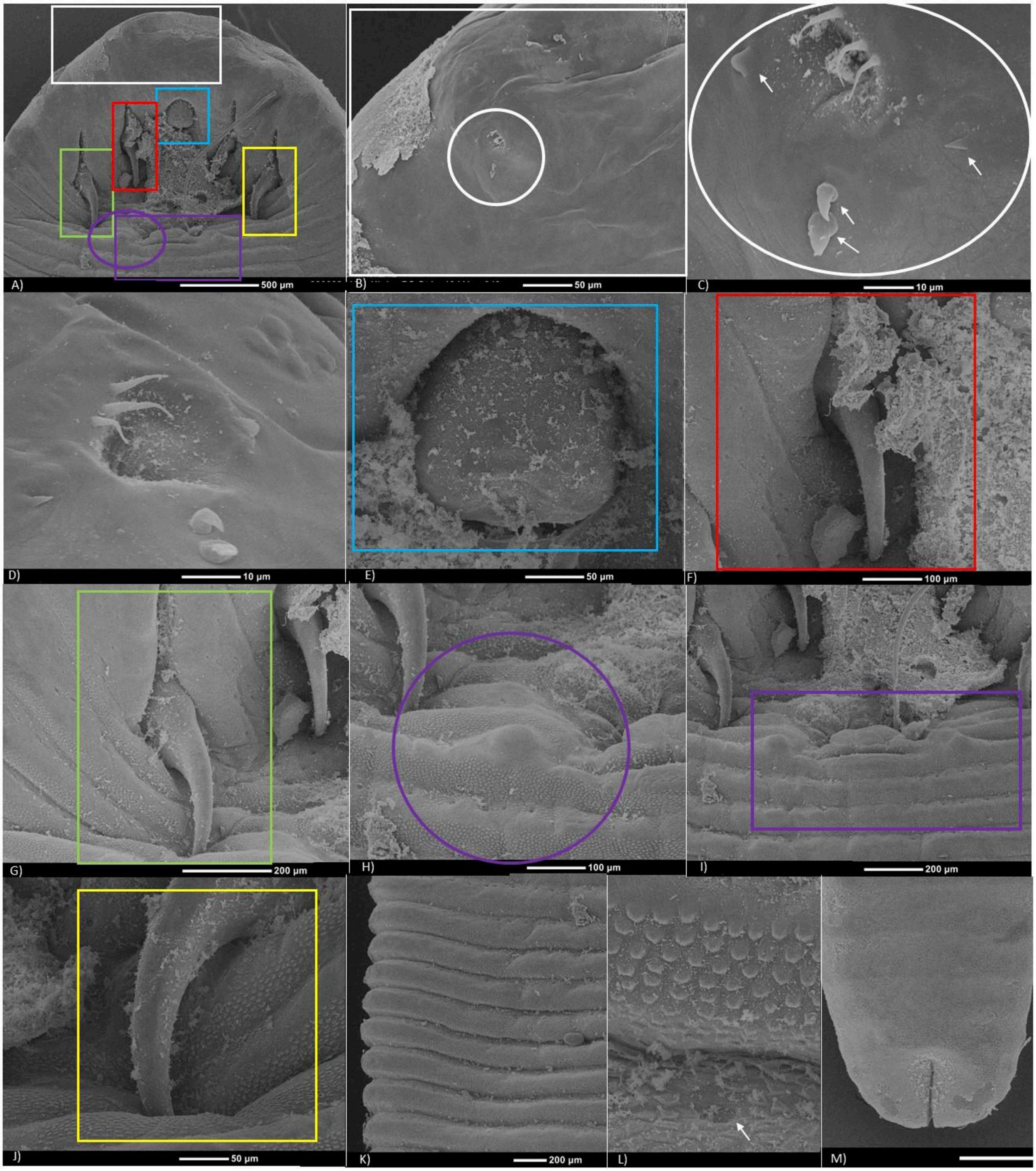

Fig. 3. Scanning electron microscopy of an adult male *Linguatula nuttali* from *Panthera leo*. Colours and shapes in images are enlargement of the corresponding areas in the previous images: A) ventral view of the anterior end of the parasite; B) magnified view of the anterior end indicating location of the minute tentacles on the right side of the parasite; C) magnified view of the right tentacles. Note presence of minute structures around tentacles (arrows); D) Tentacles and the minute structures on the left side of the anterior end; E) mouth; F) magnified view of the most anterior right hook; G) magnified view of the posterior right hook; H) magnified view of a sensory papilla located lateral to the genital pore; I) genital pore; J) magnified view of the posterior left hook; K) arrangement of the annuli on the abdominal region of the parasites; L) magnified view of the border of the annuli in the abdominal region showing presence and arrangements of the scale like projections and pores (arrow); Note presence of multiple rows of scale like projections on the posterior of each annulus and a row of pores on the anterior part of each annulus; M) posterior end showing the terminal cleft (ventral view). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 4
Comparison of the morphometrics of *L. nuttalli* in the present study and the previous reports.

	The present study	Haffner et al. (1969)	The present study	The present study	The present study	The present study	The present study
	Adult Female	Adult female	Female Nymph (#7–3)*	Female Nymph (#7–2)	Female Nymph (#8–2)	Female Nymph (#8–5)	Male Nymph (#7–5)
Total body length (mm)	47	55–65	9.8	6	5	4	4.9
Body width (mm)	6		1.7	1.3	1	1.4	1.7
Number of annuli	128	100–128	145	127	~110	~110	> 100
Anterior hook							
AB – hook gape (μm)	250		160				
AC – blade length (μm)	480		240				
AD – hook length (μm)	800	0.77 mm	335				
BC – base length (μm)	400		220				
BD – hook width (μm)	570		225				
CD – plateau length (μm)	480		170				
FL – fulcrum length (μm)	–	1.44 mm	500				
Posterior hook							
AB – hook gape (μm)	280		180				
AC – blade length (μm)	520		265				
AD – hook length (μm)	870	0.81	360				
BC – base length (μm)	450		230				
BD – hook width (μm)	600		235				
CD – plateau length (μm)	520		140				
FL – fulcrum length (μm)	–	1.44 mm	510				
DAP (μm)			200				

*Specimen examined by light microscopy.

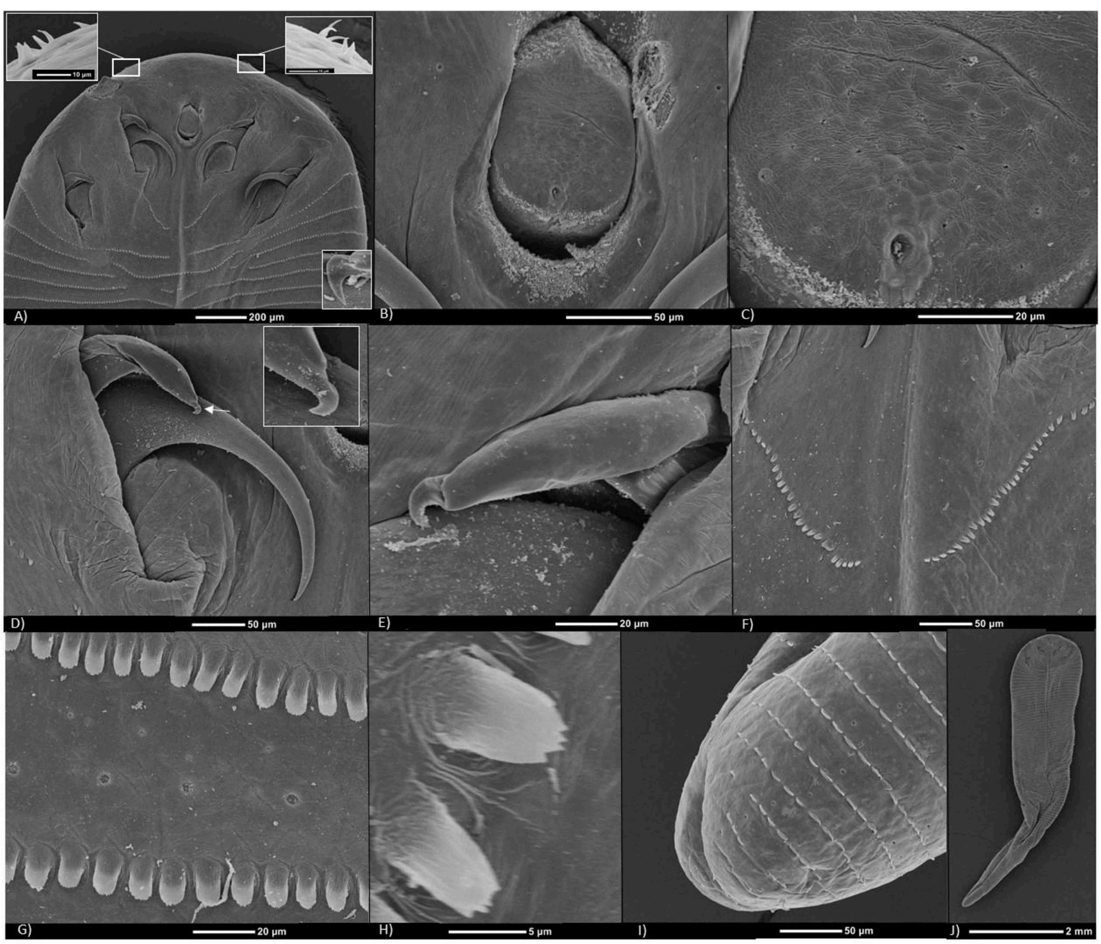

Fig. 4. Scanning electron microscopy of the nymph #7–2 from African buffalo: A) ventral view of the anterior end of the parasite showing the mouth, hooks (including dorsal accessory pieces (and magnified view of the tip of the dorsal accessory piece in the white square at the bottom right of 4A)), sensillae (the white squares at the top of the image); B) view of the outline of the buccal capsule with the oral papilla; C) magnified view of the oral papilla; D) right anterior hook and dorsal accessory piece (arrow) with magnified view of the tip of the latter shown in the white square box; E) dorsal accessory piece of the left anterior hook; F) arrangement of the first row of the annular spines located between the posterior hooks; G) pores and annular spines in mid body region (ventral); H) tips of the annular spines; I) oblique/lateral view of the posterior end of the parasite; J) full view of the parasite.

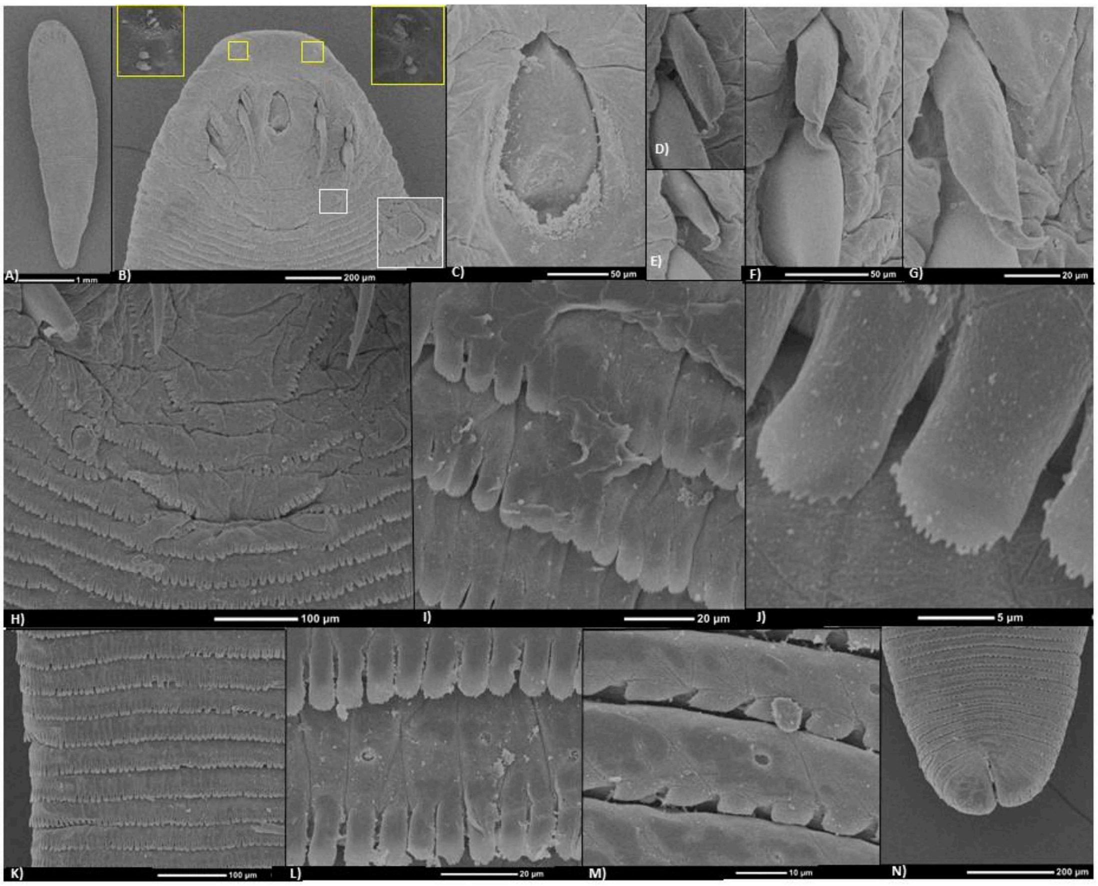

Fig. 5. Scanning electron microscopy of the nymph #7–5 from African buffalo: A) full view of the parasite; B) ventral view of the anterior end of the parasite showing the mouth, hooks, sensillae (the squares at the top of the image) and the sensory papilla (white square); C) mouth; D to G) right anterior, right posterior, left anterior and left posterior dorsal accessory pieces, respectively; H) arrangement of the first rows of the annular spines located between the posterior hooks; I) annular spines on the anterior ventral region; J) tips of the annular spines; K) rows of annular spines (mid-body region); L) pores and annular spines in mid body region (ventral); M) pores and annular spine in posterior region (ventral); N) posterior end of the parasite (ventral view).

compared to previous studies in that region (Horak et al., 1988; Horak et al., 1983; Riley, 1986; Young, 1975b; Young and Van den Heever, 1969; Young and Wagener, 1968). It is possible that these parasites never occurred in those regions previously. Another reason could be due to the dramatic changes in the global climatic conditions, including in South Africa. Although knowledge of the ecology of linguatulid parasites and conditions for survival and completion of their life cycle in the South African ecosystems is very poor, one hypothesis to explain this difference in infections, could be due to South Africa being subjected to alarming weather changes with the observed rate of warming being at least 2 °C per century – more than twice the global rate of temperature increase for the western parts and the northeast of Africa (Anonymous, 2019). Lastly, populations of the definitive hosts of these parasites, the African Lion, have undergone significant decline which could be another contributing factor in finding fewer parasites in the present study.

Another significance of our findings is that the combined use of sequence data and morphological examination allowed us to investigate the life cycle of the parasite and gain some insights into their taxonomic status. This study successfully obtained sequences for the 18S rRNA and Cox1 regions of several nymphs and two adult *Linguatula* in South Africa. All samples had identical sequences in the 18S rRNA region suggesting that adults and larvae found in the present study belong to the one species. Similarly, the intraspecific variation in the 18S rDNA region was 0% among *Linguatula* spp. from other parts of the world (Table 5) except for four sequences (KP100453 and KT581431-3) from Iran for which 18S rRNA genetic matrix showed a much higher interspecific genetic distance (0.2–2.5%) compared to the intraspecific genetic distance (0%) suggesting either a misidentification in the identity of the specimens in particular that Ghorashi et al. (2016) did not provide any justification for identification of the specimens as *L. serrata*. Compared to the 18S region, Cox1 sequences were more variable among specimens. However, still the base pair difference among sequences obtained in the present study was lower than the difference observed between species (Table 6). The pairwise genetic matrix of Cox1 region among *Linguatula* spp. showed an intraspecific genetic distance ranging from 0 to 1%, and an interspecific genetic distance ranging from 9.7 to 12%.

In the phylogenetic tree based on the 18S sequences belonging to *Linguatula* spp. (Fig. 8) there were clear groupings of *L. nuttalli* from

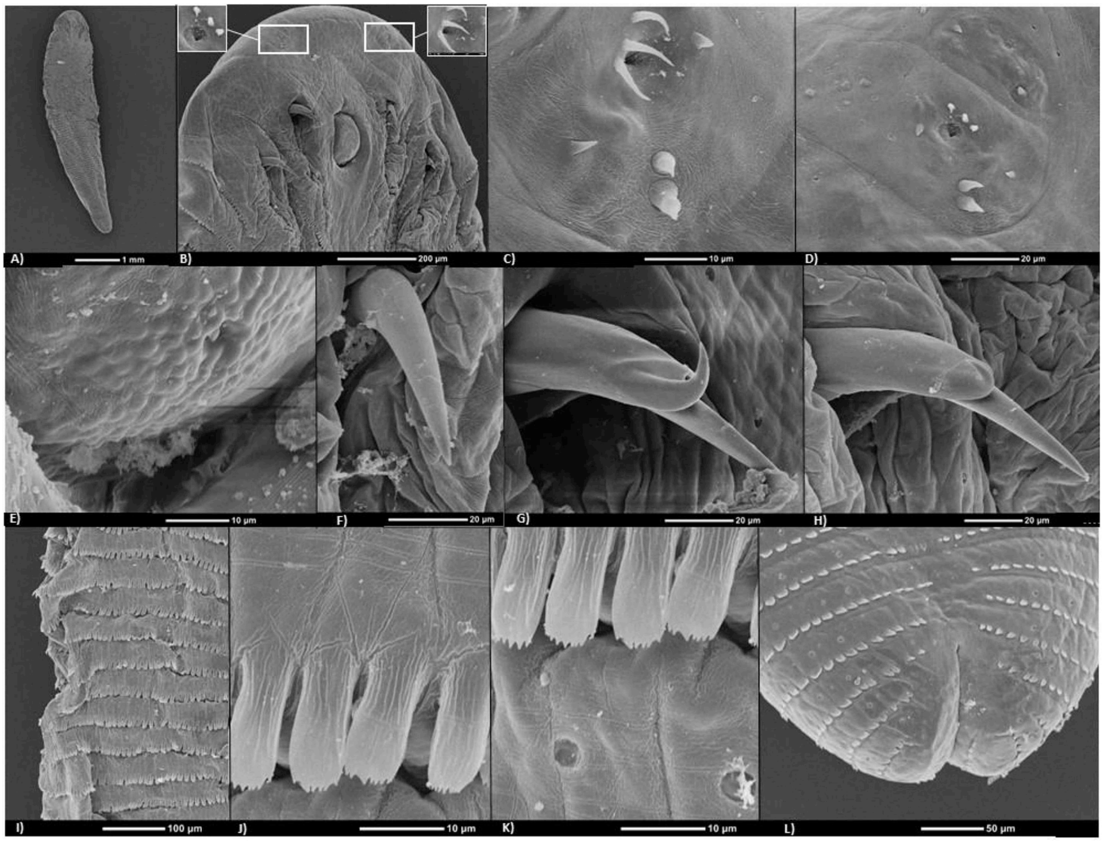

Fig. 6. Scanning electron microscopy of the nymph #8–2 from African buffalo: A) full view of the parasite; B) anterior end of the parasite showing the mouth, hooks, sensillae (the squares at the top of the image) and the sensory papilla (white square); C) & D) magnified view of the right and left sensillae, respectively; E) structure of the surface of the oral papilla; F to H) left anterior, right anterior and right posterior hooks, respectively; I) annular spines on the anterior ventral region; J) tips of the annular spines; K) pores and annular spine in mid body region (ventral); L) posterior end of the parasite (ventral view).

South Africa, *L. arctica* from Norway and *L. serrata* from Australia and Norway. The genetic results in this study, however, cannot confirm if these specimens should belong to a different genus. Until the taxonomy of all species of *Linguatula* is determined with good morphological identification and comparative molecular sequences, we cannot truly say if the differences are enough to support generic level differences. As previously found (Shamsi et al., 2020), specimens identified as *Linguatula* from Iran (GenBank accession numbers: KT581431-KT581433 and KP100453) formed a distinct group suggesting that their identification as *L. serrata* (Ghorashi et al., 2016) was erroneous and that they belong to a different, as yet unknown, species. The grouping of *Linguatula* spp. based on the Cox1, also confirmed the distinction of *L. nuttalli* from South Africa and *L. arctica* from Norway.

In the present study 18S rRNA and Cox1 sequences were obtained for specific identification of the *Linguatula* samples in combination with morphological features. Currently, 18S rRNA and Cox1 sequences are the only available comparable sequences in the GenBank. As these two regions are sourced from two independent genomes of nuclear and mitochondrial, they provide independent views of the phylogenetic relationships among species. The genetic variations in 18S rRNA sequences were less compared to the Cox1 region. Therefore, analyzing one or more nuclear gene regions such as 28S rRNA and ITS sequences would be interesting in the future research on this parasite.

Morphological examination of the adult specimens in the present study suggested they belong to *L. nuttalli* which has been previously reported from the African lion and has been described in detail by Haffner et al. (1969). In terms of the possible impact of the preservative on the appearance of the annuli and overall body shape, to the best of our knowledge there is no information available for pentastomids. Most taxonomic studies of pentastomids have been based on few specimens at a time. There have been no good systematic studies of pentastomes that have incorporated different fixative methodologies. It is certainly an aspect that needs to be studied in the future. With respect to the specific differences noted between *L. nuttali* from the lion and *L. serrata* from the wild dog, the consistent differences in these features across a number of specimens (for *L. serrata*) suggest that they are specific level differences.

No previous study has provided detailed morphological descriptions of the nymphs of *L. nuttalli*. Although the nymphs found in the present study showed overall similar morphology, they differed significantly in body size, as well as in the morphology, pattern and arrangement of the

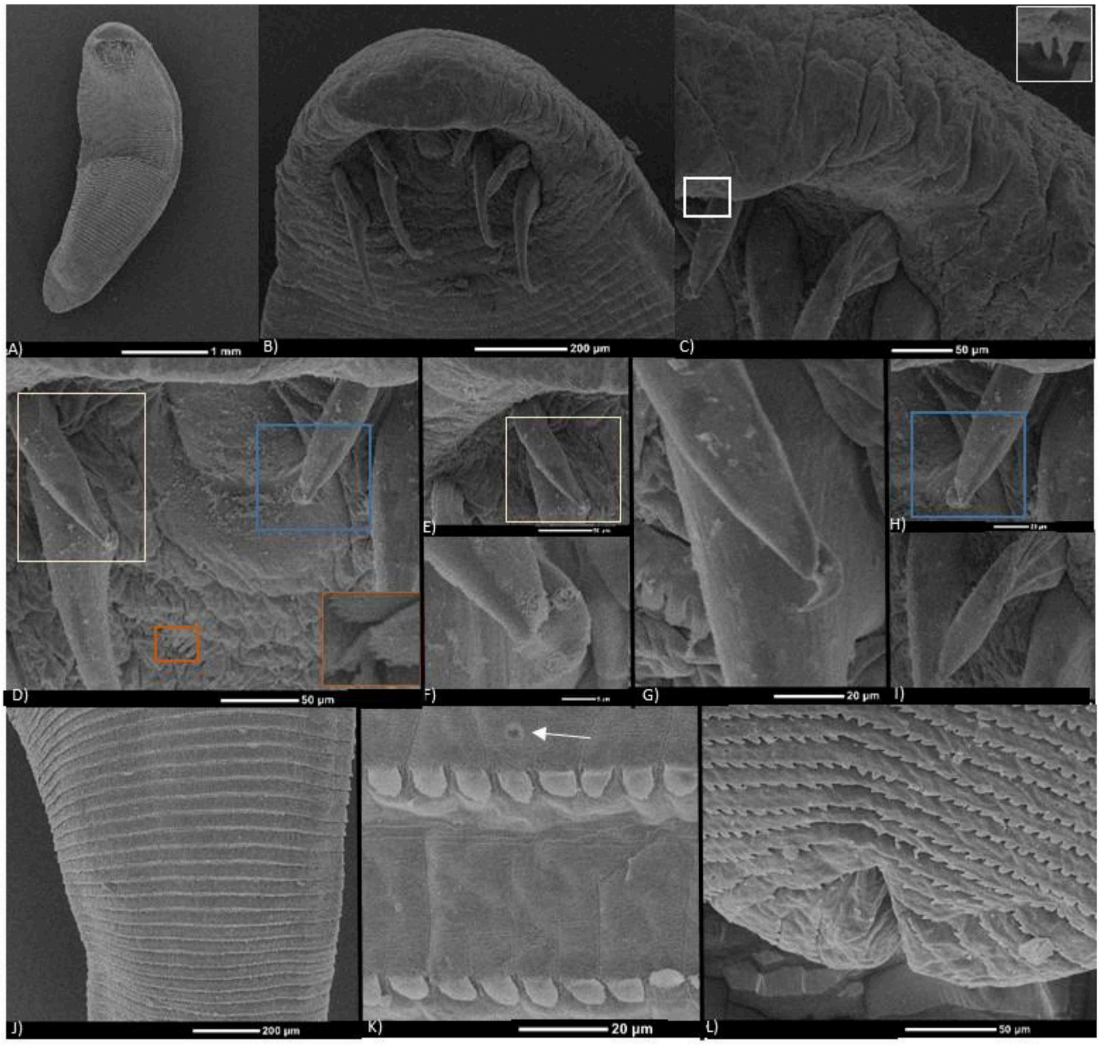

Fig. 7. Scanning electron microscopy of the nymph #8–5 from African buffalo: A) full view of the parasite; B) anterior end of the parasite showing the mouth and hooks; C) location of sensillae; D) right anterior dorsal accessory piece (white box), left anterior dorsal accessory piece (blue box) and annular spines (orange boxes); E & F) tip of the right anterior dorsal accessory piece; G) tip of the right posterior dorsal accessory piece; H) tip of the left anterior dorsal accessory piece; I) tip of the left posterior dorsal accessory piece; J) rows of annular spines on the mid-body region (ventral); K) pore (arrow) and annular spines in mid body region (ventral view); L) posterior end of the parasite (ventral view). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

annular spines (Figs. 4–7) suggesting that they could be different developmental stages. Given that some pentastomids are known to have up to nine nymphal stages (Riley, 1986) this morphological variation in the nymphs supports the need to undertake combined morphological and molecular studies for the correct identification of the species involved.

Pentastomids are potentially zoonotic parasites (Koehsler et al., 2011; Ylmaz et al., 2011) and infections have been reported in other African countries (Lapierre et al., 1976; Le Corroller and Pierre, 1959; Morsy et al., 1999; Ragab and Samuel, 1955; Sellier et al., 2004). In South Africa, there are two reports of human infection with pentastomids, both attributed to *Armillifer armillatus* (Du Plessis et al., 2007; Porter, 1928) but none yet due to *Linguatula* spp. The presence of *Linguatula* spp. in herbivores and carnivores in the country, however, shows the established life cycle of these parasites and the potential risk factor for human infection. These parasites may also be of significance in the conservation of lions. As the population of lions is decreasing and they are listed as vulnerable (Henschel et al., 2015), understanding the direct and indirect impact of infection with parasites could be of value for these animals.

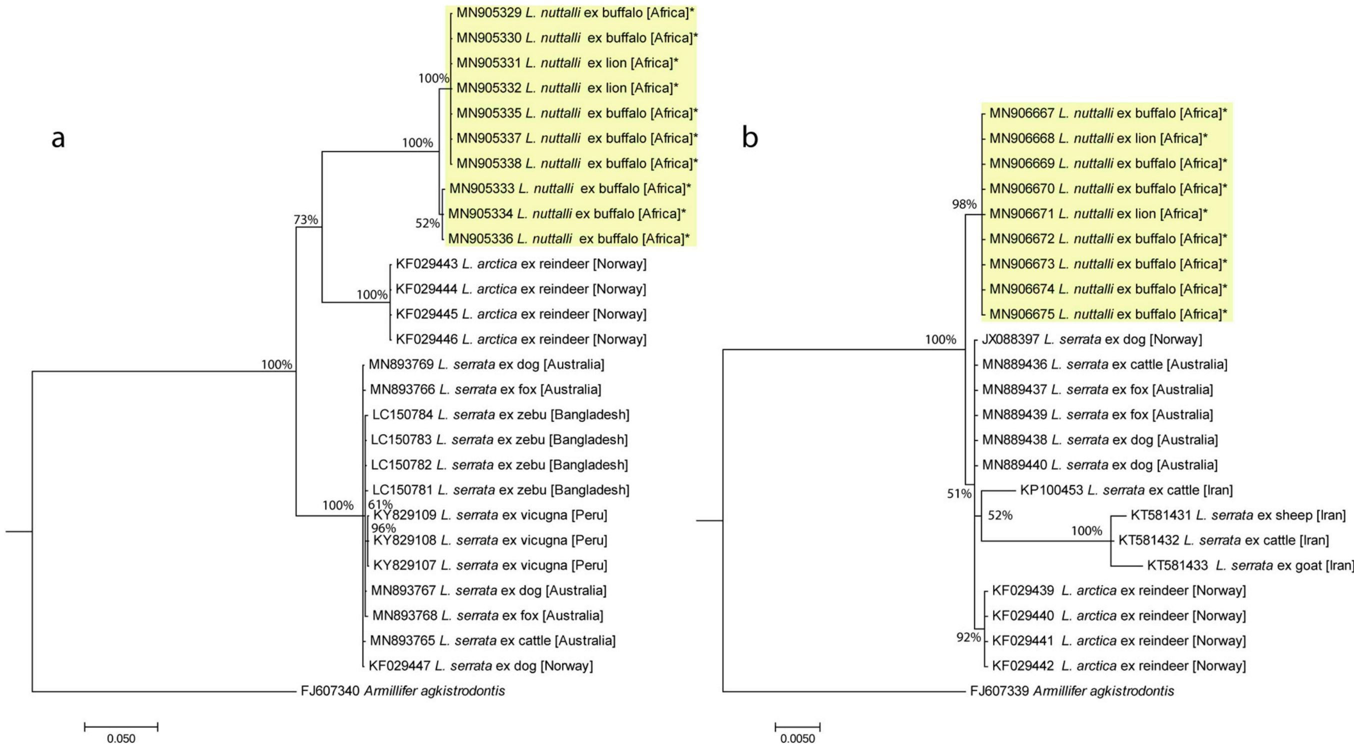

Fig. 8. Phylogenetic analysis of Cox1 and 18sRNA sequences for *Linguatula* spp., *with Armillifer agkistrodontis* as an outgroup for Cox1 (a) and 18sRNA (b) sequences, respectively. Bayesian posterior probabilities values are indicated on the branches.

Table 5
Genetic distances shown as % of difference of 18S sequences among specimens. Asterisk denotes specimens obtained in the present study.

Sample Name	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1_MN906667 *L. nuttalli* ex buffalo [Africa]*																						
2_MN906668 *L. nuttalli* ex lion [Africa]*	0.0																					
3_MN906669 *L. nuttalli* ex buffalo [Africa]*	0.0	0.0																				
4_MN906670 *L. nuttalli* ex buffalo [Africa]*	0.0	0.0	0.0																			
5_MN906671 *L. nuttalli* ex lion [Africa]*	0.0	0.0	0.0	0.0																		
6_MN906672 *L. nuttalli* ex buffalo [Africa]*	0.0	0.0	0.0	0.0	0.0																	
7_MN906673 *L. nuttalli* ex buffalo [Africa]*	0.0	0.0	0.0	0.0	0.0	0.0																
8_MN906674 *L. nuttalli* ex buffalo [Africa]*	0.0	0.0	0.0	0.0	0.0	0.0	0.0															
9_MN906675 *L. nuttalli* ex buffalo [Africa]*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0														
10_JX088397 *L. serrata* ex dog [Norway]	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2													
11_MN889436 *L. serrata* ex cattle [Australia]	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.0												
12_MN889437 *L. serrata* ex fox [Australia]	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.0	0.0											
13_MN889438 *L. serrata* ex dog [Australia]	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.0	0.0	0.0										
14_MN889439 *L. serrata* ex fox[Australia]	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.0	0.0	0.0	0.0									
15_MN889440 *L. serrata* ex dog [Australia]	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.0	0.0	0.0	0.0	0.0								
16_KP100453 *L. serrata* ex cattle [Iran]	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.5	0.5	0.5	0.5	0.5	0.5							
17_KT581431 *L. serrata* ex sheep [Iran]	2.2	2.2	2.2	2.2	2.2	2.2	2.2	2.2	2.2	2.0	2.0	2.0	2.0	2.0	2.0	2.3						
18_KT581432 *L. serrata* ex cattle [Iran]	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	1.8	1.8	1.8	1.8	1.8	1.8	2.2	0.2					
19_KT581433 *L. serrata* ex goat [Iran]	2.4	2.4	2.4	2.4	2.4	2.4	2.4	2.4	2.4	2.2	2.2	2.2	2.2	2.2	2.2	2.5	0.6	0.4				
20_KF029439 *L. arctica* ex reindeer [Norway]	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.1	0.1	0.1	0.1	0.1	0.1	0.6	2.1	1.9	2.3			
21_KF029440 *L. arctica* ex reindeer [Norway]	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.1	0.1	0.1	0.1	0.1	0.1	0.6	2.1	1.9	2.3	0.0		
22_KF029441 *L. arctica* ex reindeer [Norway]	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.1	0.1	0.1	0.1	0.1	0.1	0.6	2.1	1.9	2.3	0.0	0.0	
23_KF029442 *L. arctica* ex reindeer [Norway]	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.1	0.1	0.1	0.1	0.1	0.1	0.6	2.1	1.9	2.3	0.0	0.0	0.0

Table 6
Base pair difference shown as % of difference of CoxI sequences among specimens. Asterisk denotes specimens obtained in the present study.

Sample Name	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1_MN905329 *L. nuttalli* ex buffalo [Africa]*																										
2_MN905330 *L. nuttalli* ex buffalo [Africa]*	0.0																									
3_MN905331 *L. nuttalli* ex lion [Africa]*	0.0	0.0																								
4_MN905332 *L. nuttalli* ex lion [Africa]*	0.0	0.0	0.0																							
5_MN905335 *L. nuttalli* ex buffalo [Africa]*	0.0	0.0	0.0	0.0																						
6_MN905337 *L. nuttalli* ex buffalo [Africa]*	0.0	0.0	0.0	0.0	0.0																					
7_MN905338 *L. nuttalli* ex buffalo [Africa]*	0.0	0.0	0.0	0.0	0.0	0.0																				
8_MN905333 *L. nuttalli* ex buffalo [Africa]*	1.0	1.0	1.0	1.0	1.0	1.0	1.0																			
9_MN905334 *L. nuttalli* ex buffalo [Africa]*	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.1																		
10_MN905336 *L. nuttalli* ex buffalo [Africa]*	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.1	0.0																	
11_KF029447 *L. serrata* ex dog [Norrway]	12.0	12.0	12.0	12.0	12.0	12.0	12.0	12.0	11.9	11.9																
12_MN893765 *L. serrata* ex cattle [Australia]	11.9	11.9	11.9	11.9	11.9	11.9	11.9	11.9	11.8	11.8	0.1															
13_MN893768 *L. serrata* ex fox [Australia]	11.9	11.9	11.9	11.9	11.9	11.9	11.9	11.9	11.8	11.8	0.2	0.3														
14_MN893766 *L. serrata* ex fox [Australia]	11.9	11.9	11.9	11.9	11.9	11.9	11.9	11.9	11.8	11.8	0.1	0.2	0.3													
15_MN893769 *L. serrata* ex dog [Australia]	12.0	12.0	12.0	12.0	12.0	12.0	12.0	12.0	11.9	11.9	0.0	0.1	0.2	0.1												
16_MN893767 *L. serrata* ex dog [Australia]	11.9	11.9	11.9	11.9	11.9	11.9	11.9	11.9	11.8	11.8	0.1	0.2	0.1	0.2	0.1											
17_KY829107 *L. serrata* ex vicugna [Peru]	12.0	12.0	12.0	12.0	12.0	12.0	12.0	12.0	11.9	11.9	0.2	0.3	0.2	0.3	0.2	0.1										
18_KY829108 *L. serrata* ex vicugna [Peru]	12.0	12.0	12.0	12.0	12.0	12.0	12.0	12.0	11.9	11.9	0.2	0.3	0.2	0.3	0.2	0.1	0.0									
19_KY829109 *L. serrata* ex vicugna [Peru]	12.0	12.0	12.0	12.0	12.0	12.0	12.0	12.0	11.9	11.9	0.2	0.3	0.2	0.3	0.2	0.1	0.0	0.0								
20_LC150781 *L. serrata* ex zebu [Bangladesh]	11.9	11.9	11.9	11.9	11.9	11.9	11.9	11.9	11.8	11.8	0.2	0.3	0.2	0.3	0.2	0.1	0.2	0.2	0.2							
21_LC150782 *L. serrata* ex zebu [Bangladesh]	11.9	11.9	11.9	11.9	11.9	11.9	11.9	11.9	11.8	11.8	0.1	0.2	0.1	0.2	0.1	0.0	0.1	0.1	0.1	0.1						
22_LC150783 *L. serrata* ex zebu [Bangladesh]	11.9	11.9	11.9	11.9	11.9	11.9	11.9	11.9	11.8	11.8	0.1	0.2	0.1	0.2	0.1	0.0	0.1	0.1	0.1	0.1	0.0					
23_LC150784 *L. serrata* ex zebu [Bangladesh]	12.0	12.0	12.0	12.0	12.0	12.0	12.0	12.0	11.9	11.9	0.2	0.3	0.2	0.3	0.2	0.1	0.2	0.2	0.2	0.2	0.1	0.1				
24_KF029443 *L. arctica* ex reindeer [Norway]	11.8	11.8	11.8	11.8	11.8	11.8	11.8	11.4	11.3	11.3	9.7	9.8	9.8	9.8	9.7	9.8	9.9	9.9	9.9	9.9	9.8	9.8	9.9			
25_KF029444 *L. arctica* ex reindeer [Norway]	11.8	11.8	11.8	11.8	11.8	11.8	11.8	11.4	11.3	11.3	9.7	9.8	9.8	9.8	9.7	9.8	9.9	9.9	9.9	9.9	9.8	9.8	9.9	0.0		
26_KF029445 *L. arctica* ex reindeer [Norway]	11.8	11.8	11.8	11.8	11.8	11.8	11.8	11.4	11.3	11.3	9.7	9.8	9.8	9.8	9.7	9.8	9.9	9.9	9.9	9.9	9.8	9.8	9.9	0.0	0.0	
27_KF029446 *L. arctica* ex reindeer [Norway]	11.8	11.8	11.8	11.8	11.8	11.8	11.8	11.4	11.3	11.3	9.7	9.8	9.8	9.8	9.7	9.8	9.9	9.9	9.9	9.9	9.8	9.8	9.9	0.0	0.0	0.0

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