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RESEARCH NOTE

Phylogenetic relationships among *Linguatula serrata* isolates from Iran based on 18S rRNA and mitochondrial cox1 gene sequences

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Abstract

The phylogenetic relationships among seven *Linguatula serrata* (*L. serrata*) isolates collected from cattle, goats, sheep, dogs and camels in different geographical locations of Iran were investigated using partial 18S ribosomal RNA (rRNA) and partial mitochondrial cytochrome c oxidase subunit 1 (*cox1*) gene sequences. The nucleotide sequences were analysed in order to determine the phylogenetic relationships between the isolates. Higher sequence diversity and intraspecies variation was observed in the *cox1* gene compared to 18S rRNA sequences. Phylogenetic analysis of the *cox1* gene placed all *L. serrata* isolates in a sister clade to *L. arctica*. The Mantel regression analysis revealed no association between genetic variations and host species or geographical location, perhaps due to the small sample size. However, genetic variations between *L. serrata* isolates in Iran and those isolated in other parts of the world may exist and could reveal possible evolutionary relationships.

Keywords

Linguatula serrata, molecular phylogenetic, 18S rRNA, cytochrome c oxidase subunit 1

Linguatula serrata (*L. serrata*) is an aberrant arthropod of the class Pentastomida (Hendrix 1998). The adult parasite is found in the nasopharynx of canids (Khalil and Schacher 1965). The larval form occurs in visceral organs of herbivores, in which it develops to the infective nymphal stage. Human infection may occur via consumption of raw or undercooked liver or visceral organs, with associated lymph nodes, of infected animals (Beaver 1984; Drabick 1987; el-Hassan *et al*. 1991). Several studies have attempted to determine the prevalence rate of *L. serrata* in the domestic dog (Meshgi and Asgarian 2003; Oluwasina *et al*. 2014), sheep and goats (Gul *et al*. 2009; Khalil 1976; Rezaei 2012; Tavassoli *et al*. 2007), cattle and buffaloes (Alborzi *et al*. 2013; Khalil 1976; Ravindran *et al*. 2008), camels (Khalil 1976; Rezaei 2012; Shakerian *et al*. 2008) and wild animals (Young 1975). There is limited data available on molecular differences between parasites isolated from different animals. This study aimed to compare partial genomic sequences of 18S rRNA and *cox1* gene in *L. serrata* isolates and to investigate the sequence diversity of these genes among the isolates.

The mesenteric lymph nodes (MLNs) from different ruminants including goats, buffaloes, sheep, camels and cattle were collected from abattoirs and examined for *Linguatula serrata* nymphs between September 2012 and June 2013. At least three MLNs from each slaughtered animal were investigated. Parasites collected from dogs or farmed animals were identified to genus using morphological methods (Rezaei 2012) and preserved in 70% ethanol for further investigation (Table I).

Total genomic DNA was extracted from tissue samples of individual *L. serrata* specimens using a DNA extraction kit (Promega, VIC, Australia) according to the manufacturer's instructions. The concentration and quality of extracted DNA was evaluated and adjusted to 5 ng/µl and used in PCR immediately or stored at –20ºC for future use.

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Table I. *L. serrata* isolates used in this study, their origin and GenBank accession numbers of 18S rRNA and *cox1* gene sequences

Sample ID and host	developmental stage	infected organ	geographical location	GenBank acc no.	
				18S rRNA	*cox1*
Cattle-1	nymph	lymph node	Tabriz	KF830128	KF830137
Sheep-4	nymph	lymph node	Tabriz	KF830130	KF830138
Dog-5	adult	sinus cavity	Urmia	KF830131	KF830139
Goat-7	nymph	lymph node	Khoramabad	KF830132	KF830140
Camel-13	nymph	lymph node	Yazd	KF830133	KF830141
Sheep 19-1	nymph	lymph node	Tabriz	KF830134	KF830143
Sheep 19-2	nymph	lymph node	Tabriz	KF830135	KF830144

Two sets of primers for the target genes were designed. The first set partially amplified the 18S small subunit ribosomal RNA (18S rRNA) gene (18S-F 5'-CCATGGTTGTCACGGGTGACG-3' and 18S-R 5'-CTTGCGACGATCCAAGAATTT-3') and was based on complete 18S rRNA sequences available for this genus (GenBank accession no. JX088397). The second set (cox1-F 5'-CAATATACGCCCAGCAAAAT-3' and cox1-R 5'-TGGTAAATAGGAAGATGAAA-3') was based on available mitochondrial cytochrome c oxidase subunit 1 (*cox1*) gene sequences (GenBank accession no. KF029447).

PCR amplifications were performed in 25 µl reaction volumes on a I-Cycler thermal cycler (Bio-Rad). The reaction mixture contained 1 µl extracted genomic DNA (5ng), 25 µM of each primer, 1.5 mM $MgCl_2$, 1250 µM of each dNTP, 1´ GoTaq® Green Flexi Reaction Buffer and 1 U of Go Taq DNA polymerase (Promega, USA). PCR conditions for the 18S rRNA and *cox1* gene amplification were identical and consisted of one cycle of 94 °C for 60 s, 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 10 s, and a final extension cycle of 72°C for 60 s.

PCR amplicons were purified using the QIAquick® PCR Purification Kit (QIAGEN) following the manufacturer's in-

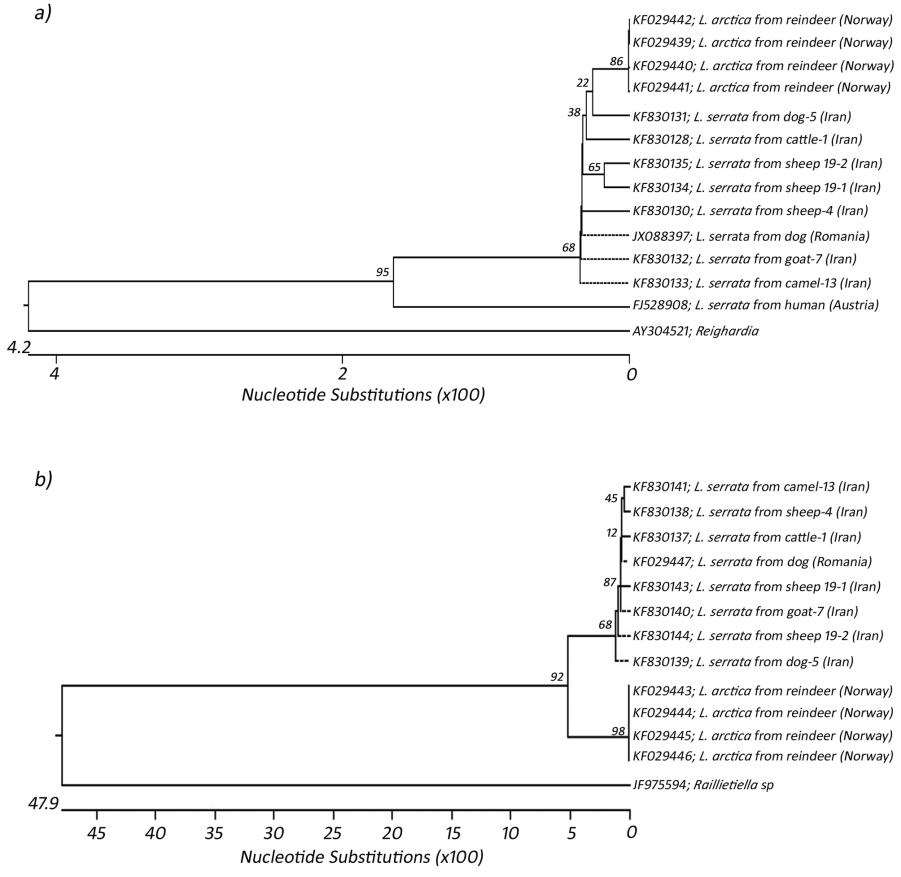

Fig 1. Phylogenetic relationship of *L. serrata* isolates (a) based on partial sequence of 18S rRNA gene and (b) partial sequence of *cox1* gene. The numbers at nodes represent bootstrap values based on 1000 replicates (maximum likelihood)

structions. Purified amplicons were subjected to automated sequencing (BigDye® Terminator v3.1, Applied Biosystems) in both directions, using the same primers as used for PCR. In addition, available sequence data for *Linguatula* was retrieved from GenBank for the 18S rRNA gene (JX088397: *L. serrata* isolated from a dog in Romania; FJ528908: *L. serrata* isolated from a human eye in Austria) and the *cox1* gene (KF029447: *L. serrata* isolated from a dog in Romania) and were included in the sequence analysis. The 18S rRNA gene (KF029439-KF029442) and *cox1* gene (KF029443 – KF029446) sequences from the most closely related *Linguatula* species (*L. arctica* isolated from reindeer in Norway) were also included. Outgroup sequences of the cephalobaenids *Reighardia* (18S rRNA gene) and *Raillietiella* (*cox1* gene) were included. The sequences were aligned using ClustalW (Thompson et al. 1994) within the Geneious software package (version 6.0.5) and BioEdit Sequence Alignment Editor (version 6.0.9.0). Multiple sequence alignment was generated using a gap open penalty of 10 and gap extension penalty of 1. Phylogenetic trees were generated using the maximum likelihood (ML) method implemented in the PhyML plugin in Geneious, with 1000 bootstrap replications (Fig. 1).

PCR amplicons of an expected size for *Linguatula* of approximately 595 bp (for 18S rRNA) and 542 bp (for *cox1*) were generated from the *L. serrata* isolates using described specific primers. Partial 18S rRNA was amplified and sequenced for all *L. serrata* isolates. Nucleotide sequences were deposited in GenBank and accession numbers are illustrated in Table I. Sequence alignment of these isolates and percentage of sequence identity (Table II) showed that the similarity of nucleotide sequences among *L. serrata* isolated in Iran ranged between 97.1–100%. The lowest nucleotide diversity (95.3%) among all *L. serrata* isolates examined was between an Iranian cattle isolate (Cattle-1) and the Austrian human isolate (FJ528908). All *L. arctica* isolates had identical sequences but were different from *L. serrata* isolates, with 97.8 – 99.8% sequence identity between the species. Nucleotide sequences from the *L. serrata* isolated from a human in Austria (Koehsler et al. 2011), however, showed considerably less nucleotide similarity to the *L. serrata* isolates tested in this study (≤97.5%). Two *L. serrata* isolates (sheep-19-1 and sheep-19-2) were collected from a single sheep and showed 99.7% sequence identity. However, an *L. serrata* isolate (sheep-4) from a different sheep had 99.2% and 99.5% sequence identity with the other sheep isolates (sheep-19-1 and sheep-19-2 respectively). The sequence identity between *Linguatula* isolates and *Reighardia* (outgroup) was less than 93%.

A partial fragment of about 542 bp of the *cox1* gene from all *L. serrata* isolates was amplified and sequenced. The GenBank accession numbers for these sequences are shown in Table I.

The *cox1* gene nucleotide sequences were first translated into amino acids and a codon-based multiple alignment was generated and analysed to ensure the validation of sequences. The amino acid sequences were then back-translated into nucleotides and multiple nucleotide alignment was carried out using ClustalW within Geneious software (version 6.0.5) and BioEdit Sequence Alignment Editor (version 6.0.9.0). The percentage of sequence identity of tested samples for *cox1* gene is provided in Table II. The diversity of *cox1* gene sequence identities among *L. serrata* isolates in this study ranged between 96.9 – 99.6%. All *L. arctica* isolates had identical sequences and showed ≥91.1% similarity to all *L. serrata* sequences. Two *L. serrata* isolates from a single sheep (sheep-19-1 and sheep-19-2) were 98.0% identical. However, *L. serrata* isolated from a different sheep (sheep-4) showed 96.9 and 97.5% similarity with those two isolates (sheep-19-1 and sheep-19-2 respectively). Both *Linguatula* species had less than 31% sequence identity with *Raillietiella*, a pentastomid, which was used as an outgroup. All 4 identical *L. arctica* isolates were placed as a sister clade to all *L. serrata* isolates, which formed a clade with reasonable support (68% of bootstraps), while both *L. serrata* and *L. arctica* formed a strongly supported clade relative to *Raillietiella* (JF975594), a pentastomid, which was used as outgroup. Isolation of *L. serrata* from livestock as well as dogs, camels, wildlife and humans shows the broad host range that can be affected by this parasite (Fard *et al*. 2012; Maleky 2001; Meshgi and Asgarian 2003; Tavassoli *et al*. 2007; Young 1975). The comparison of nucleotide sequences of 18S rRNA and *cox1* genes have been used to demonstrate the phylogenetic relationships of *L. serrata* and other pentastomids to other metazoan groups (Gjerde 2013). However, reports describing the genetic variations among *L. serrata* isolated from livestock are very limited.

The nucleotide sequence variation between *L. serrata* isolates in this study were higher in their *cox1* gene compared to the 18S rRNA gene. The two *Linguatula* species showed less than 92% sequence identity in their *cox1* gene and were differentiated by sequence analysis. However, the sequence identity in their 18S rRNA gene was greater than 97%. Due to low sequence variation in the 18S rRNA gene among the other *Linguatula* isolates in this study, the human isolate may not belong to the genus *Linguatula*. This could have been due to misidentification of the parasite isolated from the human eye as has also been indicated in earlier reports (Gjerde 2013). The partial 18S rRNA and *cox1* genes also showed slight intraspecies differences between *L. serrata* isolates. Two *L. serrata* isolated from a single host (sheep) had 99.7% identity in their 18S rRNA gene and clustered together in the phylogenetic analysis. However, these two isolates showed lower sequence similarity in their *cox1* gene (98.0%) and did not form a sister clade in the phylogenetic analysis. This confirms the higher sequence variability among *L. serrata* isolates in the *cox1* gene compared to the 18S rRNA gene and shows the existence of intraspecies differences between isolates from a single host. These two nymph isolates could have been the progeny of two different *L. serrata* eggs that have infected the host at different times. On the other hand, a *L. serrata* isolated from a different sheep showed slightly less sequence identity, 96.9% and 97.5% respectively, in the *cox1* gene to those two

Table II. Percentage of sequence identity between 7 *L. serrata* isolates in their 18S rRNA and cox1 gene

18S rRNA sequence identity															
Isolate	**Sheep-19-1 *L. serrata*-Iran**	**Sheep-19-2 *L. serrata*-Iran**	**Cattle-1 *L. serrata*-Iran**	**Camel-13 *L. serrata*-Iran**	**Dog-5 *L. serrata*-Iran**	**Goat-7 *L. serrata*-Iran**	**Sheep-4 *L. serrata*-Iran**	**FJ528908 *L.serrata* human-Austria**	**JX088397 *L. serrata* – dog – Romania**	**KF029442 *L. arctica* reindeer – Norway**	**KF029439 *L. arctica* reindeer – Norway**	**KF029440 *L. arctica* reindeer – Norway**	**KF029441 *L. arctica* reindeer – Norway**	**AY304521 *Reighardia***	
Sheep-19-2 *L. serrata*-Iran		99.7	97.1	99.5	97.3	99.5	99.2	97.0	99.5	99.3	99.3	99.3	99.3	92.2	**sheep-19-1 *L. serrata*-Iran**
Sheep-19-1 *L. serrata*-Iran	98.0		97.5	99.8	97.6	99.8	99.5	97.3	99.8	99.7	99.7	99.7	99.7	92.6	**sheep-19-2 *L. serrata*-Iran**
Cattle-1 *L. serrata*-Iran	98.2	98.7		98.0	97.4	98.0	97.6	95.3	97.8	97.8	97.8	97.8	97.8	90.4	**Cattle-1 *L. serrata*-Iran**
***L. serrata*-IranCamel-13**	97.3	97.2	97.4		97.8	100.0	99.7	97.5	100.0	99.8	99.8	99.8	99.8	92.7	**Camel-13 *L. serrata*-Iran**
Dog-5 *L. serrata*-Iran	99.3	97.2	97.4	97.3		98.5	98.2	95.8	98.3	98.3	98.3	98.3	98.3	90.6	**Dog-5 *L. serrata*-Iran**
Goat-7 *L. serrata*-Iran	99.6	97.6	97.8	97.7	99.6		99.7	97.5	100.0	99.8	99.8	99.8	99.8	92.7	**Goat-7 *L. serrata*-Iran**
Sheep-4 *L. serrata*-Iran	97.5	96.9	97.0	97.3	97.5	97.8		97.1	99.7	99.5	99.5	99.5	99.5	92.4	**Sheep-4 *L. serrata*-Iran**
KF029447 *L. serrata* –dog – Romania	98.5	99.1	99.3	98.2	98.5	98.9	98.2		97.8	97.6	97.6	97.6	97.6	92.2	**FJ528908 *L. serrata* human – Austria**
KF029446 *L. arctica* reindeer – Norway	90.4	91.0	90.4	89.1	90.0	90.4	89.1	91.1		99.8	99.8	99.8	99.8	92.7	**JX088397 *L. serrata* –dog – Romania**
KF029443 *L. arctica* reindeer – Norway	90.4	91.0	90.4	89.1	90.0	90.4	89.1	91.1	100.0		100.0	100.0	100.0	92.6	**KF029442 *L. arctica* reindeer – Norway**
KF029444 *L. arctica* reindeer – Norway	90.4	91.0	90.4	89.1	90.0	90.4	89.1	91.1	100.0	100.0		100.0	100.0	92.6	**KF029439 *L. arctica* reindeer – Norway**
***L.* KF029445 *arctica* reindeer – Norway**	90.4	91.0	90.4	89.1	90.0	90.4	89.1	91.1	100.0	100.0	100.0		100.0	92.6	**KF029440 *L. arctica* reindeer – Norway**
JF975594 *Raillietiella* sp	28.5	30.4	29.7	29.2	28.5	28.7	29.2	30.1	30.6	30.6	30.6	30.6		92.6	**KF029441 *L. arctica* reindeer – Norway**
	Sheep-19-2 *L. serrata*-Iran	Sheep-19-1 *L. serrata*-Iran	Cattle-1 *L. serrata*-Iran	Camel-13 *L. serrata*-Iran	Dog-5 *L. serrata*-Iran	Goat-7 *L. serrata*-Iran	Sheep-4 *L. serrata*-Iran	KF029447 *L. serrata* – dog – Romania	KF029 *L. arctica* reindeer – Nor-way446	KF029443 *L. arctica* reindeer – Norway	KF029444 *L. arctica* reindeer – Norway	KF029445 *L. arctica* reindeer – Norway	JF975594 *Raillietiella* sp		**AY304521 *Reighardia***
***Cox1* gene sequence identity**															

isolates from the single sheep. The *L. serrata* isolates from sheep were collected from one location (Tabriz) but showed slight sequence differences in their *cox1* gene. However, despite the presence of nucleotide differences in amplicons of both genes among *L. serrata* isolates, there was no association between nucleotide variations, host species and geographical locations when these relationships were tested by Mantel regression analysis.

Amplification of partial 18S rRNA or *cox1* genes using the primer sets described in this study can facilitate the diagnosis and identification of *L. serrata* larval stages in intermediate hosts such as farm animals. In addition, sequencing of amplicons can provide genotypic information that can assist in species differentiation, the discovery of genetic differences between isolates and an understanding of the evolutionary ecology of the parasite.

Phylogenetic analysis of the 18S rRNA and *cox1* genes demonstrated similar relationships among the three different pentastomids used in the analysis. The *L. serrata* and *L. arctica* were placed in two different clades but appeared to be more closely related when compared to *Reighardia* or *Raillietiella*. Incorrect alignment of the 18S rRNA gene using ClustalW may result in comparison of non-homologous nucleotides while the codon-based alignment of *cox1* gene is less subjected to these errors (Gjerde 2013). Therefore, the phylogenetic analysis of *cox1* gene might be more genetically reliable.

Knowledge of genetic diversity among *L. serrata* isolates can provide information for understanding the evolution of the parasite in different regions of the world. This can also help to resolve taxonomic discrepancies. The number of samples and their geographical diversity in this study was not sufficient to successfully analyse the correlation of genetic differences with host species or geographic location. However, we believe that analysing more specimens collected from different hosts and regions would assist in answering the questions concerning the possible dispersal routes of the isolates and their evolution.

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References

Alborzi A., Molayan P.H., Akbari M. 2013. Prevalence of *Linguatula serrata* Nymphs in Mesenteric Lymph Nodes of Cattle and Buffaloes Slaughtered in Ahvaz Abattoir, Iran. *Iranian Journal of Parasitology*, 8, 327–332

Beaver P.C. 1984. *Clinical parasitology*. Philadelphia: Lea & Febiger

Drabick J.J. 1987. Pentastomiasis. *Review of Infectious Diseases*, 9, 1087–1094

el-Hassan A.M., Eltoum I.A., el-Asha B.M. 1991. The Marrara syndrome: isolation of *Linguatula serrata* nymphs from a patient and the viscera of goats. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 85, 309

Fard S.R., Ghalekhani N., Kheirandish R., Fathi S., Asl E.N. 2012. The prevalence of *Linguatula serrata* nymphs in camels slaughtered in Mashhad slaughterhouse, Northeast, Iran. *Asian Pacific Journal of Tropical Biomedicine*, 2, 885–888. DOI:10.1016/S2221-1691(12)60247-0

Gjerde B. 2013. Phylogenetic position of *Linguatula arctica* and *Linguatula serrata* (Pentastomida) as inferred from the nuclear 18S rRNA gene and the mitochondrial cytochrome c oxidase subunit I gene. *Parasitology Research*, 112, 3517–3525. DOI:10.1007/s00436-013-3534-9

Gul A., Deger S., Denizhan V. 2009. The prevalence of *Linguatula serrata* (Frohlich, 1789) nymphs in sheep in the Van province. *Turkiye parazitolojii dergisi / Turkiye Parazitoloji Dernegi – Acta Parasitologica Turcica/Turkish Society for Parasitology*, 33, 25–27

Hendrix C.M. 1998. *Diagnostic veterinary parasitology*. St. Louis, MO: Mosby

Khalil G.M. 1976. Prevalence of *Linguatula serrata* infection in animals from the Cairo abattoir. *The Journal of Parasitology*, 62, 126

Khalil G.M., Schacher J.F. 1965. *Linguatula serrata* in relation to halzoun and the marrara syndrome. *The American Journal of Tropical Medicine and Hygiene*, 14, 736–746

Koehsler M., Walochnik J., Georgopoulos M., Pruente C., Boeckeler W., Auer H., Barisani-Asenbauer T. 2011. *Linguatula serrata* tongue worm in human eye, Austria. *Emerging Infectious Diseases*, 17, 870–872. DOI:10.3201/eid1705.100790

Maleky F. 2001. A case report of *Linguatula serrata* in human throat from Tehran, central Iran. *Indian Journal of Medical Sciences*, 55, 439–441

Meshgi B., Asgarian O. 2003. Prevalence of *Linguatula serrata* infestation in stray dogs of Shahrekord, Iran. *Journal of Veterinary Medicine. B, Infectious diseases and veterinary public health*, 50, 466–467

Oluwasina O.S., Thankgod O.E., Augustine O.O., Gimba F.I. 2014. *Linguatula serrata* (Porocephalida: Linguatulidae) Infection among Client-Owned Dogs in Jalingo, North Eastern Nigeria: Prevalence and Public Health Implications. *Journal of Parasitology Research*, 2014:916120. DOI:10.1155/2014/916120

Ravindran R., Lakshmanan B., Ravishankar C., Subramanian H. 2008. Prevalence of *Linguatula serrata* in domestic ruminants in South India. *The Southeast Asian Journal of Tropical Medicine and Public Health*, 39, 808–812

Rezaei F., Tavassoli M., Javdani M. 2012. Prevalence and morphological characterizations of *Linguatula serrata* nymphs in camels in Isfahan Province, Iran. *Veterinary Research Forum,* 3, 61–65.

Shakerian A., Shekarforoush S.S., Ghafari Rad H. 2008. Prevalence of *Linguatula serrata* nymphs in one-humped camel (*Camelus dromedarius*) in Najaf-Abad, Iran. *Research in Veterinary Science*, 84, 243–245. DOI:10.1016/j.rvsc.2007.04.015

Tavassoli M., Tajic H., Dalir-Naghadeh B., Hariri F. 2007. Prevalence of *Linguatula serrata* nymphs and gross changes of infected mesenteric lymph nodes in sheep in Urmia, Iran. *Small Ruminant Research*, 72, 73–76. DOI 10.1016/j.smallrumres.2006.08.013

Thompson J.D., Higgins D.G., Gibson T.J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, 22, 4673–4680

Young E. 1975. Pentastomiasis (*Armillifer and Linguatula sp.*) infestations of wild animals in the Kruger National Park. *Journal of the South African Veterinary Association*, 46, 335–336

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