BOLETIM DO INSTITUTO DE PESCA
Short Communication

Morphological and molecular analysis of *Eustrongylides* sp. (Nematoda: Dioctophymatidae) in zebrafish (*Danio rerio*) from Brazil

Melissa Querido Cárdenas[1], Gabriel de Farias Araujo[2,3], Jucicleide Ramos de Souza[4], Amanda Gleyce Lima de Oliveira[2]*, Enrico Mendes Saggioro[2]

[1]Fundação Oswaldo Cruz – Instituto Oswaldo Cruz – Laboratório de Helmintos Parasitos de Peixes – Rio de Janeiro (RJ), Brazil.
[2]Fundação Oswaldo Cruz – Instituto Oswaldo Cruz – Laboratório de Avaliação e Promoção da Saúde Ambiental – Rio de Janeiro (RJ), Brazil.
[3]Fundação Oswaldo Cruz – Escola Nacional de Saúde Pública – Programa de Pós-Graduação em Saúde Pública e Meio Ambiente – Rio de Janeiro (RJ), Brazil.
[4]Fundação Oswaldo Cruz – Instituto Oswaldo Cruz – Laboratório de Malacologia – Rio de Janeiro (RJ), Brazil.
*Corresponding author: amandagleycelo@gmail.com

ABSTRACT

The zebrafish, *Danio rerio*, is an important vertebrate model organism across various scientific disciplines. The current study reports the nematode larva, *Eustrongylides* sp., presence, in zebrafish from a pet shop, acquired by Fundação Oswaldo Cruz for toxicity studies. Morphological and molecular data from this nematode are given herein, contributing to the understanding of this helminth and aiding future studies. The occurrence of this larva demonstrates the importance of controlling the management of *D. rerio*, since this fish is widely used as an experimental model.

Keywords: Fish nematode; 18S rRNA; Parasitism; Health monitoring.

Análises morfológicas e moleculares de *Eustrongylides* sp. (Nematoda: Dioctophymatidae) em *zebrafish* (*Danio rerio*) do Brasil

RESUMO

O *zebrafish*, *Danio rerio*, é um importante modelo de organismo vertebrado em diversas disciplinas científicas. O presente estudo relata a presença da larva do nematoide *Eustrongylides* sp. em *zebrafish* de um *pet shop*, adquirido pela Fundação Oswaldo Cruz para estudos de toxicidade. Dados morfológicos e moleculares desse Nematoda são apresentados, contribuindo para a compreensão desse helminto e auxiliando estudos futuros. A ocorrência dessa larva demonstra a importância do controle do manejo do *D. rerio*, uma vez que esse peixe é amplamente utilizado como modelo experimental.

Palavras-chave: Nematoides de peixe; 18S rRNA; Parasitismo; Monitoramento da saúde.

Received: September 18, 2024 | **Approved:** January 17, 2025
Section editor: Leonardo Tachibana

INTRODUCTION

The zebrafish, *Danio rerio* (Hamilton, 1822), is one of the most crucial vertebrate model organisms across various scientific disciplines, including genetics, developmental biology, neurophysiology, and biomedicine (Amsterdam & Hopkins, 2006; Grunwald & Eisen, 2002; Rubinstein, 2003; Vascotto et al., 1997). Originating from the Ganges River, Burma, the Malakka peninsula, and Sumatra, this tropical teleost fish belongs to the Cyprinidae family (Braunbeck & Lammer, 2006). Its adaptation to experimental manipulation is highlighted by several distinct characteristics. Because it is small and robust, it can be housed in large numbers and economically in laboratories, where it reproduces consistently throughout the year. With a short generation time, usually three to four months, zebrafish are suitable for selection experiments. It develops rapidly, such that the larvae exhibit active foraging and avoidance behaviors in five days of fertilization (Kimmel et al., 1995). In addition, around 70% of gene expression in the zebrafish model mirrors that of humans (Howe et al., 2013), facilitating validation in developmental genotoxicity and toxicity studies using this model system (Haque & Ward, 2018).

Although the phylum Nematoda includes approximately 30,000 known species, the actual number is estimated to be around 500,000, with approximately half of these being parasitic. It ranks as the fifth most diverse metazoan phylum (Hodda, 2022a, 2022b). This immense diversity poses significant challenges in studying these parasites, particularly in the fields of taxonomy and systematics (Moravec, 1998; Padial et al., 2010).

Even though *D. rerio* are widely used as an experimental model, for their use to be reliable, it is essential that the zebrafish are not infected by any type of parasite. The current study reports the presence of Nematoda in zebrafish from a pet shop, acquired by Fundação Oswaldo Cruz (Fiocruz) for toxicity studies.

MATERIAL AND METHODS

Eighty-six adult *D. rerio* (2 cm ± 1 cm) were obtained from specialized breeders and acclimatized for two weeks in maintenance tanks (150 L, 100 × 40 × 40 cm). Tank water was dechlorinated by natural evaporation and checked for total chlorine levels (Cl_2 < 0.1 mg·L^{-1}) using a Pocket II colorimeter (Hach Company, United States of America). The reference parameters for the acclimatization period were set as follows: pH 7–7.6, temperature 23–27°C, water hardness at 10–60 mg $CaCO_3$ L^{-1}, NH_4^+ < 0.05 mg·L^{-1}, constant aeration, maximum fish density of 0.8 g·L^{-1} (grams of fish: liters of water), a 12/12-hour photoperiod, and feeding twice a day (adapted from ABNT NBR 15088/16 and OECD 203/92).

As some of the fish showed severe ventral distension, all the specimens in the aquarium were euthanized according to internationally recognized animal care guidelines and ethics, through immersion in ice cold water (0 to 4°C) for 10 min to avoid contamination with an anesthetic (AVMA, 2013; Borski & Hodson, 2003; CCAC, 2005; CONCEA, 2015). The current study received ethical approval from the Fiocruz Ethics Committee on the Use of Animals (CEUA), under protocol P-14/15.5, license LM-3/18.

The nematodes found in the visceral cavities were washed in Petri dishes containing 0.65% NaCl and fixed in ethanol 70%. For light microscope study, the nematodes were stained with lactophenol and observed using a Zeiss Axioscope 2 microscope with differential interference contrast. All measurements were given in millimeters; range values were followed by means. A fragment from central region of the body was used for DNA extraction. The extraction was performed using a QIAamp DNA mini kit (QIAGEN) according to the manufacturer's instructions. For partial gene sequencing of the nuclear small subunit ribosomal (18S rRNA), primers SSU18A (5'-AAAGATTAAGCCATGCATG-3') and SSU26R (5'-CATTCTTGGCAAATGCTTTC-3') were used (Blaxter et al., 1998). For this region, the cycling conditions were 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 52°C for 1 min, and 68°C for 1.5 min, then 68°C for 10 min (Floyd et al., 2002). For ITS rDNA region, the primers 18SF (5'- TTGGATGATTCGGTGAGGT- 3') and 28SR (5' –AACCGCTTAGTAATATGCT- 3') were used (Xiong et al., 2013). For this region, the cycling conditions were 94°C for 5 min, followed by 29 cycles of 94°C for 4 sec, 56°C for 30 sec, and 72°C for 90 sec, and then a final extension phase at 72°C for 10 min (Xiong et al., 2013).

The polymerase chain reaction (PCR) products were analyzed by electrophoresis in 1.5% agarose in Tris-borate EDTA gels, stained with SyberGreen (Invitrogen, Eugene, OR, United States of America), and photographed under ultraviolet transillumination. The amplified products were purified using ExoSap-IT (USBR Products Affymetrix Inc., Cleveland, OH, United States of America). DNA cycle sequencing reactions were performed using the BigDye Terminator v.3.1 (Applied Biosystems, Foster City, CA, United States of America), and automated sequencing was performed in an ABI 3730 DNA analyzer at the sequencing platform, subunit RPT01A, of the Fiocruz, city of Rio de Janeiro (RJ), Brazil. Sequences of both

strands were generated, edited, and aligned by using the MEGA software version 11 (Tamura et al., 2021).

The sequences were compared to others available in the GenBank database using the Basic Local Alignment Search Tool (BLAST) program from the National Center for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov/BLAST) (Altschul et al., 1990). Evolutionary divergence estimates between sequences were conducted in MEGA11 using the Kimura 2-parameter (K2p) model.

Paraxonchium laetificans (Andrássy, 1956) (AY284809) and *Tylencholaimus mirabilis* (Bütschli, 1873) (OQ918676) sequences were used as outgroups. The GenBank sequences used for the phylogenetic tree construction are listed in Table 1.

RESULTS

From 86 *D. rerio* examined, seven were infected with one specimen of *Eustrongylides* sp. fourth-stage larva in the visceral cavity. These nematodes are long and reddish with tapered ends (Figs. 1a and 1b). The morphology of the genital primordium allowed the larvae to be distinguished into males and females. The larvae presented a cephalic end bearing 12 labial papillae, disposed in two concentric circles around the oral cavity. Each circle is composed of six labial papillae, which are arranged in pairs. The papillae in the inner circle have spine-like apexes and narrow bases, while the papillae in the outer circle have nipple-like apexes and wide bases. The measurements are based on three males and three females (Figs. 1c–1f). Male body length is 69.18–100.00 (85.39) and female body length is 125.60–132.56 (129.08). A nerve ring is presented in the beginning of the esophagus, just posterior to the buccal cavity, being 0.160–0.290 (0.225) from the anterior extremity in males and 0.185–0.250 (0.217) in females. The width of the body at the height of the nerve ring is 0.365–0.470 (0.405) in males and 0.350–0.375 (0.362) in females. The buccal cavity

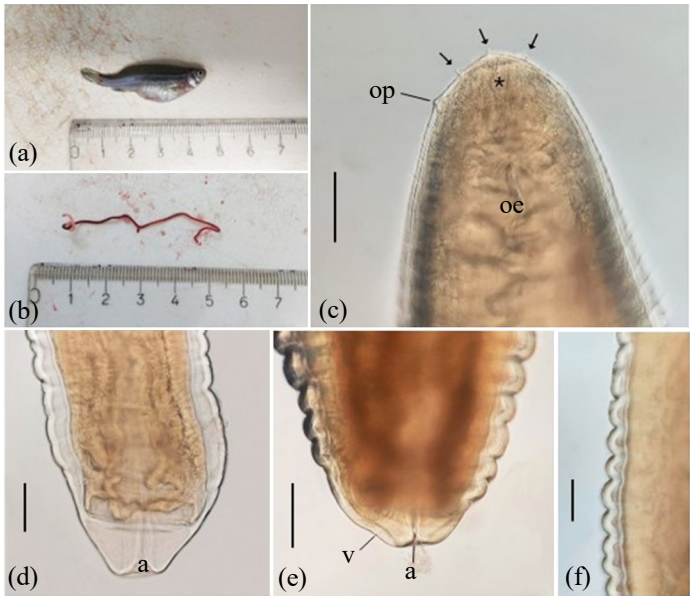


Figure 1. (a) *Danio rerio* infected with *Eustrongylides* sp. presenting an abdominal swelling. (b) *Estrongylides* sp. (fourth-stage larva) from *D. rerio*. (c–f) *Eustrongylides* sp. fourth-stage larvae. (c) Anterior region of female fourth-stage larva showing the inner papillae (arrow), outer papillae (op), buccal cavity (asterisk) and oesophagus (oe). Bar 0.10 mm. (d) Male caudal end primordia of bursa showing the anus (a). Bar 0.10 mm. (e) Female caudal end showing the anus (a) and the vulva (v). Bar 0.20 mm. (f) Detail of cuticle showing three layers. Bar 0.05 mm.

Table 1. List of the species used in the phylogenetic analyses of *Eustrongylides* sp. with respective GenBank accession numbers, location and host.

Species	18S rRNA	Location	Host	Reference
Dioctophymatidae				
Dioctophyme renale	LC389871	Chuo Ward, Tokyo, Japan	*Rattus norvegicus*	Banzai et al. (2018)
Dioctophyme renale	OQ933019	Bishnupur-1, West Bengal, India	*Channa punctata*	Kumar et al. (2024)
Eustrongylides sp.	PP236907	Anhui Province, China	*Alligator sinensis*	unpublished
Eustrongylides sp.	PP934668	Unknown	Unknown	unpublished
Eustrongylides sp.	MG696298	India	*Channa gachua*	unpublished
Eustrongylides sp.	MG696303	India	*Channa punctata*	unpublished
Outgroup				
Paraxonchium laetificans	AY284809	Unknown	Unknown	Holterman et al. (2006)
Tylencholaimus mirabilis	OQ918676	Bishnupur-1, West Bengal, India	*Channa punctata*	Kumar et al. (2024)

is narrow, measuring 0.10–0.21 (0.14) in males and 0.12–0.15 (0.14) in females. The glandular esophagus is long, beginning in the end of buccal cavity, measuring 12.48–21.00 (17.49) in males and 16.20–17.46 (16.83) in females. The width of the body at the height of esophageal-intestinal junction is 0.77–0.85 (0.82) in males and 0.94–1.00 (0.97) in females. The anus is terminal in both sexes. The distance of the rectum from the posterior end is 0.20–0.42 (0.29) in males and 0.32–0.45 (0.39) in females. The width at the posterior end is 0.42–0.47 (0.45) in males and 0.41–0.45 (0.43) in females. Genital primordia sustained by a well-developed process in the body wall. Some small papillae are visible in the posterior end of both larvae (male and female). The cuticle presents three layers.

In total, two partial 18S rRNA sequences from two *D. rerio* larvae were obtained, with 833 and 862 bp (accession numbers PQ303798 and PQ303799, respectively). A partial ITS1 sequence with 155 bp was also obtained (accession number PQ304637).

The first partial 18S rRNA sequence of *Eustrongylides* sp. indicated 99.26 and 99.16% similarity to *Eustrongylides* sp. (PP236907) and *Dioctophyme renale* (Goeze, 1782) Collet-Meygret, 1802 (OQ933019), respectively. The K2p distance between *Eustrongylides* sp. larvae from the present study and *Eustrongylides* sp. (PP236907) was 0.617%, with six divergent nucleotides in 850 bp. In relation to *D. renale* (OQ933019) the K2p distance was 0.616% with five divergent nucleotides in an 850 bp (Fig. 2). The partial ITS sequence indicated 97.45% similarity to *Eustrongylides* sp. (MK650418), with four divergent nucleotides in 155 bp.

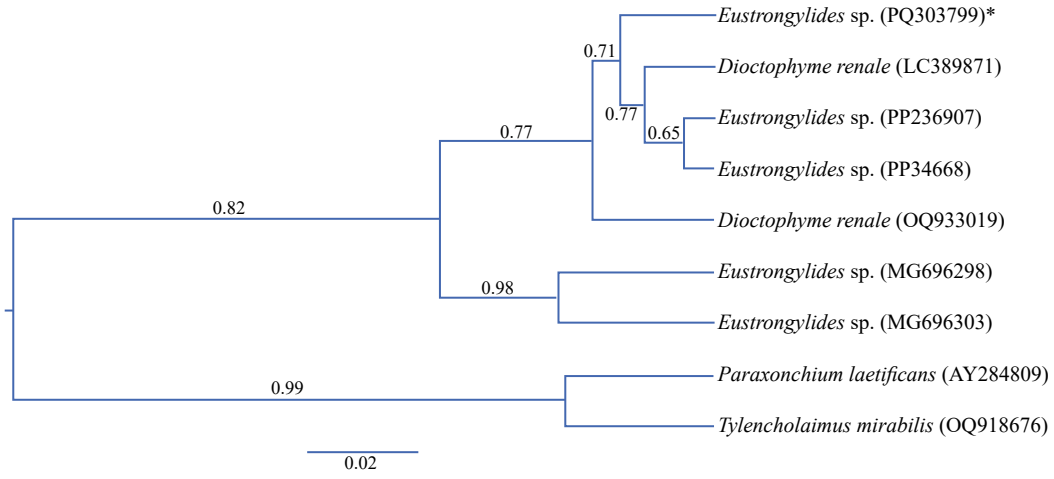
DISCUSSION

To date, the genus *Eustrongylides* Jägerskiöld, 1909 includes four known species:

- *Eustrongylides excisus* Jägerskiöld, 1909;
- *Eustrongylides ignotus* Jägerskiöld, 1909;
- *Eustrongylides mergorum* (Rudolphi, 1809);
- *Eustrongylides tubifex* (Nitzsch in Rudolphi, 1819) Jägerskiöld, 1909 (WoRMS Editorial Board, 2024).

These nematodes, with veterinary importance, undergo an indirect life cycle, utilizing freshwater oligochaetes as their initial intermediate hosts, followed by freshwater fishes, amphibians, and/or reptiles as their secondary intermediate hosts, and finally piscivorous birds as their definitive hosts. Predatory fish, amphibians, and reptiles can also act as paratenic hosts, able to infect other fish-eating animals (Honcharov et al., 2022; Mazzone et al., 2019). Occasionally, these parasites also parasitize human and thus have zoonotic potential (Eiras et al., 2016). Although *Eustrongylides* sp. larvae have been reported from different freshwater fishes from Brazil (Barros et al., 2009; Gueretz et al., 2020; Kuraiem et al., 2020; Lignon et al., 2023; Lima et al., 2019; Martins et al., 2009), up to now no human infections have been reported in this country.

The morphology of the *Eustrongylides* sp. larvae in this study agrees with that previously observed by Kuraiem et al. (2020) and Lima et al. (2019). Fusco et al. (2023) reported the occurrence of this larval nematode in *D. rerio*. Therefore, the current report is the second record of *Eustrongylides* sp. in this fish. The presence of this nematode in the fishes destined for



*New sequence data.

Figure 2. Bayesian phylogenetic topology with posterior probabilities indicating node support based on the 18S region to show their relationships with other Dioctophymatidae. The GenBank accession numbers are shown, and the scale bar indicates the nucleotide mutations per site.

ecotoxicology experiments could have interfered with their results. The fish which had already been contaminated probably ingested oligochaetes containing *Eustrongylides* sp. larvae, which infected the *D. rerio*. A preventive management could use a sanitary controlled feeding. It is also important to quarantine the zebrafish before using them in studies.

Although some authors have studied *Eustrongylides* spp. molecularly (Franceschini et al., 2022; Kuraiem et al., 2020; Mazzone et al., 2019; Pekmezci & Bolukbas, 2021; Shamsi et al., 2023), there are few genetic studies involving this genus. A partial 18S rDNA sequence of *Eustrongylides* sp. from *Hoplias malabaricus* (Bloch, 1794) from northern Brazil is available on GenBank (Correa et al., 2023). However, the sequence region did not match with primer target area used in the present study, and it was not possible to include it in the phylogenetic analysis. In the present work, a partial 18S rRNA and a partial ITS1 sequence were generated and made available in GenBank, contributing to the understanding of this helminth and aiding future studies. Further morphological description of adults and larvae, combined with the molecular analysis, can support the accurate identification at species level of *Eutrongylides* that occur in Brazil.

CONCLUSION

The present work provides morphological and molecular data on an important nematode larva, *Eustrongylides* sp. The occurrence of this nematode demonstrates the importance of controlling the management of *D. rerio*, since this fish is used as an experimental model in several areas. The use of fish feed and rearing in controlled environments are alternatives to avoid contamination with this nematode and other helminths. Future more detailed morphological and molecular studies of *Eustrongylides* and *Dioctophyme* are suggested, to provide better understanding of the relationship between these genera.

CONFLICT OF INTEREST

Nothing to declare.

DATA AVAILABILITY STATEMENT

All data sets were generated or analyzed in the current study.

AUTHORS' CONTRIBUTIONS

Conceptualization: Cárdenas, M.Q., Saggioro, E.M.; **Investigation:** Cárdenas, M.Q.; **Methodology:** Cárdenas, M.Q., Araujo, G.F., Souza, J.R., Oliveira, A.G.L.; **Writing – first draft:** Cárdenas, M.Q.; **Writing – review & editing:** Cárdenas, M.Q., Souza, J.R., Oliveira, A.G.L., Saggioro, E.M.; **Project Administration:** Araujo, G.F., Saggioro, E.M.; **Resources:** Souza, J.R., Oliveira, A.G.L., Saggioro, E.M.; **Funding:** Saggioro, E.M.; **Final approval:** Oliveira, A.G.L.

FUNDING

Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro

Grants No.: E-21/201.271/2021 and E-21/210.298/2022

Conselho Nacional de Desenvolvimento Científico e Tecnológico

Grant No.: 304571/2021-0

ACKNOWLEDGMENTS

The authors are grateful to Dr. Claudia Portes Santos for her valuable suggestions.

REFERENCES

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, 215(3), 403-410. https://doi.org/10.1016/S0022-2836(05)80360-2

American Veterinary Medical Association (AVMA). (2013). *AVMA guidelines for the euthanasia of animals*. AVMA.

Amsterdam, A., & Hopkins, N. (2006). Mutagenesis strategies in zebrafish for identifying genes involved in development and disease. *Trends in Genetics*, 22(9), 473-478. https://doi.org/10.1016/j.tig.2006.06.011

Associação Brasileira de Normas Técnicas (ABNT) (2016). *NBR 15088/16. Ecotoxicologia aquática - Toxicidade aguda - Método de ensaio com peixes (Cyprinidae)* (3ª ed.). ABNT.

Banzai, A., Tanikawa, T., Kimura, G., Sasaki, T., & Kawakami, Y. (2018). Parasitic helminths collected from the brown rat, *Rattus norvegicus*, in Chuo Ward, Tokyo, Japan. *Medical Entomology and Zoology*, 69(4), 171-176. https://doi.org/10.7601/mez.69.171

Barros, L. A., Oliveira, R. D., Moraes Filho, J., Justino, C. D. S., & Mateus, L. D. F. (2009). Análise do parasitismo por *Contracaecum* sp. e *Eustrongylides* sp. em cacharas, *Pseudoplatystoma fasciatum* (Linnaeus, 1766) (Pisces: Pimelodidae) provenientes do rio Cuiabá, Mato Grosso, Brasil. *Revista Brasileira de Ciência Veterinária*, 16(2), 58-61. https://doi.org/10.4322/rbcv.2014.170

Blaxter, M. L., De Ley, P., Garey, J., Liu, L. X., Scheldeman, P., Vierstraete, A., Vanfleteren, J. R., Mackey, L. Y., Dorris, M., Frisse, L. M., Vida, J. T., & Thomas, W. K. (1998). A molecular evolutionary framework for the phylum Nematoda. *Nature*, 392, 71-75. https://doi.org/10.1038/32160

Borski, R. J., & Hodson, R. G. (2003). Fish Research and the Institutional Animal Care and Use Committee. *ILAR Journal*, 44(4), 286-294. https://doi.org/10.1093/ilar.44.4.286

Braunbeck, T., & Lammer, E. (2006). Fish embryo toxicity assays. *Contract*, 20, 725-731.

Canadian Council on Animal Care (CCAC) (2005). *Guidelines on the care and use of fish in research, teaching and testing*. CCAC.

Conselho Nacional de Controle de Experimentação Animal (CONCEA) (2015). *Diretriz da prática de eutanásia do CONCEA. Anexo I - Diretriz referente a procedimentos de eutanásia realizados em animais incluídos em atividades de ensino ou de pesquisa científica, Brasil*. CONCEA.

Correa, L. L., Soares, G. B., Müller, M. I., & Adriano, E. A. (2023). First morphological and molecular characterization of the genus *Eustrongylides* nematode larvae infecting fish in the Brazilian Amazon. *Biologia*, 78(10), 2855-2862. https://doi.org/10.1007/s11756-023-01422-6

Eiras, J. C., Pavanelli, G. C., Takemoto, R. M., Yamaguchi, M. U., Karkling, L. C., & Nawa, Y. (2016). Potential risk of fish-borne nematode infections in humans in Brazil–current status based on a literature review. *Food and Waterborne Parasitology*, 5, 1-6. https://doi.org/10.1016/j.fawpar.2016.08.002

Floyd, R., Abebe, E., Papert, A., & Blaxter, M. (2002). Molecular barcodes for soil nematode identification. *Molecular Ecology*, 11(4), 839-850. https://doi.org/10.1046/j.1365-294X.2002.01485.x

Franceschini, R., Guardone, L., Armani, A., Ranucci, D., Roila, R., Valiani, A., Susini, F., & Branciari, R. (2022). Five-years management of an emerging parasite risk (*Eustrongylides* sp., Nematoda) in a fishery supply chain located on Trasimeno Lake (Italy). *Food Control*, 136, 108858. https://doi.org/10.1016/j.foodcont.2022.108858

Fusco, M. A., Rizzo-Valente, V. D. S., Vizzoni, V. F., Miranda, R. S. D., Aguiar, C. C. D., & Escaleira, R. D. C. (2023). An outbreak of the nematode parasite *Eustrongylides* spp. (Nematoda: Dioctophymatidae) in a zebrafish (*Danio rerio*) facility. *Zebrafish*, 20(3), 126-130. https://doi.org/10.1089/zeb.2022.0041

Grunwald, D. J., & Eisen, J. S. (2002). Headwaters of the zebrafish: emergence of a new model vertebrate. *Nature Reviews Genetics*, 3(9), 717-724. https://doi.org/10.1038/nrg892

Gueretz, J. S., Senger, S. B., & Claus, M. P. (2020). Ocorrência de *Ithyoclinostomum* sp. e *Eustrongylides* sp., parasitos de *Hoplias* aff. *malabaricus* Bloch, 1794 (Characiformes: Erythrinidae), no litoral de Santa Catarina, Brasil. *Brazilian Journal of Development*, 6(4), 21565-21575. https://doi.org/10.34117/bjdv6n4-359

Haque, E., & Ward, A. C. (2018). Zebrafish as a model to evaluate nanoparticle toxicity. *Nanomaterials*, 8(7), 561. https://doi.org/10.3390/nano8070561

Hodda, M. (2022a). Phylum Nematoda: a classification, catalogue and index of valid genera, with a census of valid species. *Zootaxa*, 5114(1), 1-289. https://doi.org/10.11646/zootaxa.5114.1.1

Hodda, M. (2022b). Phylum Nematoda: trends in species descriptions, the documentation of diversity, systematics, and the species concept. *Zootaxa*, 5114(1), 290-317. https://doi.org/10.11646/zootaxa.5114.1.2

Holterman, M., van der Wurff, A., van den Elsen, S., van Megen, H., Bongers, T., Holovachov, O., Bakker, J., & Helder, J. (2006). Phylum-wide analysis of SSU rDNA reveals deep phylogenetic relationships among nematodes and accelerated evolution toward crown Clades. *Molecular Biology and Evolution*, 23(9), 1792-1800. https://doi.org/10.1093/molbev/msl044

Honcharov, S. L., Soroka, N. M., Galat, M. V., Zhurenko, O. V., Dubovyi, A. I., & Dzhmil, V. I. (2022). *Eustrongylides* (Nematoda: Dioctophymatidae): Epizootology and special characteristics of the development biology. *Helminthologia*, 59(2), 127-142. https://doi.org/10.2478/helm-2022-0013

Howe, K., Clark, M., Torroja, C., Torrance, J., Berthelot, C., Muffato, M., Collins, J. E., Humphray, S., McLaren, K., Matthews, L., McLaren, S., Sealy, I., Caccamo, M., Churcher, C., Scott, C., Barrett, J. C., … & Stemple, D. L. (2013). The zebrafish reference genome sequence and its relationship to the human genome. *Nature*, *496*, 498-503. https://doi.org/10.1038/nature12111

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B., & Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Developmental Dynamics*, 203(3), 253-310. https://doi.org/10.1002/aja.1002030302

Kumar, V., Roy, S., Parida, S. N., Bisai, K., Dhar, S., Jana, A. K., & Das, B. K. (2024). Deciphering the impact of endoparasitic infection on immune response and gut microbial composition of *Channa punctata*. *Frontiers in Cellular and Infection Microbiology*, 14, 1296769. https://doi.org/10.3389/fcimb.2024.1296769

Kuraiem, B. P., Knoff, M., Telleria, E. L., Fonseca, M. C. G., Machado, L. S., Cunha, N. C., Nascimento, E. R., Fontenelle, G., Gomes, D. C., & São Clemente, S. C. (2020). *Eustrongylides* sp. (Nematoda, Dioctophymatoidea) parasitizing *Hoplias malabaricus* (Actinopterygii: Erythrinidae) collected from the state of Rio de Janeiro, Brazil. *Revista Brasileira de Parasitologia Veterinária*, 29(1), e014519. https://doi.org/10.1590/S1984-29612019094

Lignon, J. S., Stainki, T. M., & Monteiro, S. G. (2023). *Eustrongylides* spp. (Nematoda: Eustrongylidae) in *Polypterus endlicheri* in central Rio Grande do Sul, Brazil. *Ciência Animal Brasileira*, 24, e-74213. https://doi.org/10.1590/1809-6891v24e-74213E

Lima, F., Pozza, A., & Lehmann, P. (2019). *Contracaecum* spp. (Nematoda: Anisakidae) and *Eustrongylides* spp. (Nematoda: Dioctophymatidae) nematode larvae with zoonotic potencial found in two fish species from Tramandaí river basin, southern Brazil. *Boletim do Instituto de Pesca*, 45(3), e495. https://doi.org/10.20950/1678-2305.2019.45.3.495

Martins, M. L., de Souza Santos, R., Marengoni, N. G., Takahashi, H. K., & Onaka, E. M. (2009). Sazonalidade de larvas de E*ustrongylides* sp. (Nematoda: Dioctophymatidae) em peixes do Rio Paraná, Sudeste do Brasil. *Boletim do Instituto de Pesca*, 35(1), 29-37.

Mazzone, A., Cafara, M., Gustinelli, A., Agnetti, F., Sgariglia, E., Vaglio, G. L., Quaglio, F., & Fioravanti, M. L. (2019). Morphological and molecular characterization of larval and adult stages of *Eustrongylides excisus* (Nematoda: Dioctophymatoidea) with histopathological observations. *Journal of Parasitology*, 105(6), 882-889. https://doi.org/10.1645/19-44

Moravec, F. (1998). *Nematodes of freshwater fishes of the Neotropical Region*. Academia.

Organisation for Economic Co-Operation and Development (OECD) (1992). *OECD 203/92 Guideline for testing of chemicals. Fish, acute toxicity test*. OECD.

Padial, J. M., Miralles, A., De la Riva, I., & Vences, M. (2010). The integrative future of taxonomy. *Frontiers in Zoology*, *7*, 16. https://doi.org/10.1186/1742-9994-7-16

Pekmezci, G. Z., & Bolukbas, C. S. (2021). Morphological and molecular characterization of *Eustrongylides excisus* larvae (Nematoda: Dioctophymatidae) in *Sander lucioperca* (L.) from Northern Turkey. *Parasitology Research*, 120(6), 2269-2274. https://doi.org/10.1007/s00436-021-07187-8

Rubinstein, A. L. (2003). Zebrafish: from disease modelling to drug discovery. *Current Opinion in Drug Discovery Development*, 6(2), 218-223.

Shamsi, S., Francis, N., Masiga, J., Barton, D. P., Zhu, X., Pearce, L., & McLellan, M. (2023). Occurrence and characterisation of *Eustrongylides* species in Australian native birds and fish. *Food and Waterborne Parasitology*, 30, e00189. https://doi.org/10.1016/j.fawpar.2023.e00189

Tamura, K., Stecher, G., & Kumar, S. (2021). MEGA11: molecular evolutionary genetics analysis version 11. *Molecular Biology and Evolution*, 38(7), 3022-3027. https://doi.org/10.1093/molbev/msab120

Vascotto, S. G., Beckham, Y., & Kelly, G. M. (1997). The zebrafish's swim to fame as an experimental model in biology. *Biochemistry and Cell Biology*, 75(5), 479-485. https://doi.org/10.1139/o97-081

WoRMS Editorial Board (2024). *World Register of Marine Species*. WoRMS Editorial Board. https://doi.org/10.14284/170

Xiong, F., Li, W. X., Wu, S. G., Zou, H., & Wang, G. T. (2013). Molecular phylogeny and host specificity of the larval *Eustrongylides* (Nematoda: Dioctophmidae) from freshwater fish in China. *Journal of Parasitology*, 99(1), 137-144. https://doi.org/10.1645/GE-3163.1