



# First molecular assessment of two digenean parasites of the lancehead snake *Bothrops moojeni* Hoge, 1966 (Serpentes, Viperidae) in Brazil

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## Abstract

Two digenean species, *Infidum infidum* Faria, 1910 (Dicrocoeliidae) and *Travtrema stenocotyle* Cohn, 1902 (Plagiorchiidae), were collected in the large pit viper *Bothrops moojeni* Hoge, 1966 from Reserva Particular do Patrimônio Natural Cisalpina, municipality of Brasilândia, Mato Grosso do Sul State, Brazil. In this study, we provide the first molecular characterisation using the 28S rDNA and phylogenetic position data of these two common digeneans from *B. moojeni*. The molecular framework revealed topologies with strongly supported clades using maximum likelihood and Bayesian inference methods, positioned *I. infidum* among Plagiorchiidae and not among Dicrocoeliidae as expected and *T. stenocotyle* (Plagiorchiidae) surprisingly grouped as a sister group to Allasogonoporidae, Microphallidae, Pleurogenidae, and Prosthogonimidae, not related to plagiorchids. Our molecular phylogenetic data showed that these species may not correspond to their assigned families and encourage future studies on the systematic of these understudied groups.

**Keywords** Trematoda · Dicrocoeliidae · Plagiorchiidae · 28S rDNA · Phylogeny

## Introduction

*Bothrops moojeni* Hoge, 1966 (Viperidae) is a large pit viper known as “caíçaca”, with a wide distribution through Brazil, Paraguay, and Argentina (Almeida-Santos et al. 2017; Costa and Bérnils 2018). In Brazil, its distribution comprises the riparian forests and adjacent areas and wetlands in central and southeastern regions, over the Cerrado biome (Wallach

et al. 2014; Almeida-Santos et al. 2017; Costa and Bérnils 2018; Uetz et al. 2020).

Some digenean species were previously reported infecting *B. moojeni* from South America, as follows: *Infidum infidum* Faria, 1910 (Dicrocoeliidae), *Opisthgonimus artigasi* Ruiz and Leão, 1942, *Opisthgonimus fonsecai* Ruiz and Leão, 1942, *Opisthgonimus lecithonotus* Lühe, 1900 (Opisthgonimidae), *Renifer heterocoelium* Travassos, 1921 (Reniferidae), *Sticolecitha serpentis* Prudhoe, 1945, *Styphlodora condita* Faria, 1911, and *Travtrema stenocotyle* Cohn, 1902 (Plagiorchiidae) (Fernandes and Kohn 2014).

Species of the genus *Infidum* Travassos, 1916 (Dicrocoeliidae) occur in the Neotropical realm and parasitize the gall bladder and bile duct of several species of reptiles (Travassos 1916; Lunaschi and Drago 2007, 2010; Martinez-Salazar et al. 2016). In South America, only three species were described for this genus: the type species *I. infidum*, *Infidum luckeri* McIntosh, 1939, and *Infidum similis* Travassos, 1916. Fourteen snakes species from Argentina, Bolivia, and Brazil were previously reported infected with these *Infidum* spp. In Brazil, the infection with *I. infidum* was detected in *B. moojeni*, *Eunectes murinus*

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Linnaeus, 1758 (Boidae), and *Hydrodynastes gigas* Duméril, Bibron and Duméril, 1854 (Colubridae). Additionally, this digenean was also found infecting the anuran *Leptodactylus podicipinus* Cope, 1862 (Leptodactylidae), however, as an accidental infection (Lunaschi and Drago 2007; Campião et al. 2009). The other congeneric species *I. similis* was found infecting *Bothrops jararaca* Wied, 1824 (Viperidae), *Drymarchon corais* Boie, 1827, *Erythrolamprus miliaris* Linnaeus, 1758, *Erythrolamprus poecilogyrus* Wied, 1824, and *Mastigodryas bifossatus* Raddi, 1820 (Colubridae) in Brazil (Fernandes and Kohn 2014).

*Travtrema* Pereira, 1929 (Plagiorchiidae) is also commonly found in reptiles and amphibians (Fernandes and Kohn 2014; Campião et al. 2014) and only *T. stenocotyle* occurs in these hosts in South America. The adult digenean was reported in the intestine of seventeen snake species from Argentina, Brazil, Paraguay, and Uruguay (Fernandes and Kohn 2014) and also in the anuran *L. podicipinus* in Brazil (Campião et al. 2009). Among the Brazilian snakes, *T. stenocotyle* was found in *Bothrops neuwiedi* Wagler in Spix, 1824, *B. moojeni*, *Chironius fuscus* Linnaeus, 1758, *E. miliaris*, *E. poecilogyrus*, *M. bifossatus*, *Philodryas patagoniensis* Girard, 1858, *Thamnodynastes pallidus* Linnaeus, 1758, *Tomodon dorsatus* Duméril, Bibron and Duméril, 1854, and *Xenodon merremii* Wagler in Spix, 1824 (Fernandes and Kohn 2014). Besides, metacercariae of this digenean were found infecting the pharynx, muscles, mesentery, and body cavity of *Leptodactylus chaquensis* Cei, 1950, *Leptodactylus latinasus* Jiménez de la Espada, 1875, *Scinax nasicus* Cope, 1862 (Hamann et al. 2006a, b; 2009, 2010), *Pseudopaludicola boliviana* Parker, 1927 (Duré et al. 2004), *Elachistocleis bicolor* Valenciennes in Guérin-Ménéville, 1838, *Odontophrynus americanus* Duméril and Bibron, 1841, *Physalaemus albonotatus* Steindachner, 1864, and *Physalaemus santafecinus* Barrio, 1965 (Hamann et al. 2009), indicating that amphibians can act as intermediate hosts in the biological cycle of *T. stenocotyle*.

Integrative taxonomy was recently introduced as a comprehensive framework to delimit and describe taxa by integrating information from different types of data and methodologies (Pante et al. 2015). Few digenean parasites of Brazilian snakes were analysed at this viewpoint (Müller et al. 2018). In this study, we provide the first molecular characterisation using the 28S rDNA and phylogenetic position data of two common digeneans from *B. moojeni* in Brazil, which are *I. infidum* and *T. stenocotyle*.

## Material and methods

Three specimens of *B. moojeni* (Fig. 1) (one male, juvenile, snout-vent length (SLV) 49 cm and weight (W) 49.5 g; one female, adult, SLV 78 cm, W 140 g; and one female, juvenile,



**Fig. 1** Specimen of *Bothrops moojeni* Hoge 1966 (Viperidae) from the Reserva Particular do Patrimônio Natural Cisalpina, municipality of Brasilândia, Mato Grosso do Sul State, Brazil

SVL 44 cm, W 37 g) were collected on February 2, 2018, at the Reserva Particular do Patrimônio Natural Cisalpina (21° 17' 20.98" S; 51° 55' 2.76" W), municipality of Brasilândia, Mato Grosso do Sul State, Brazil. During necropsy, we found digeneans in the gall bladder (*I. infidum*) (Fig. 2) and large intestine (*T. stenocotyle*) (Fig. 3). *Infidum infidum* was identified according to Faria (1910), Travassos (1916), and Travassos (1944) while *T. stenocotyle* identification followed Cohn (1902), Mane-Garzon and Gostari (1965), Pereira (1929), and Freitas and Dobbin Jr (1957). The parasites found were fixed in alcohol-formalin-acetic acid solution under the



**Fig. 2** Specimen of *Infidum infidum* Faria 1910, Travassos 1916 (Dicrocoeliidae) parasites of *Bothrops moojeni* Hoge 1966 (Viperidae) from the Reserva Particular do Patrimônio Natural Cisalpina, municipality of Brasilândia, Mato Grosso do Sul State, Brazil





**Fig. 3** Specimen of *Travtrema stenocotyle* Cohn 1902 Goodman, 1951 (Plagiorchiidae) parasites of *Bothrops moojeni* Hoge 1966 (Viperidae) from the Reserva Particular do Patrimônio Natural Cisalpina, municipality of Brasilândia, Mato Grosso do Sul State, Brazil

slight pressure of a coverslip for 10 min and transferred to 70% alcohol for further processing. Two specimens of each parasite were transferred to 96% ethanol for molecular study. Digenean specimens were stained with carmine, cleared with eugenol, analysed in a computerised system for image analysis (V3 Leica Application Suite, Leica Microsystems, Wetzlar, Germany) in a microscope with differential interface contrast for taxonomic identification. The digenean vouchers were deposited in the Helminthological Collection of the Instituto de Biociências, Universidade Estadual Paulista (UNESP), in the municipality of Botucatu, São Paulo State, Brazil, under #CHIBB 9038-90338 for *I. infidum* and #CHIBB 9039-9040 for *T. stenocotyle*. The snake hosts were deposited at the Herpetological Collection of the Universidade Regional do Cariri URCA-H, municipality of Crato, Ceará State, Brazil.

DNA was extracted using a DNeasy® Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, with a final volume of 30  $\mu$ l. PCR amplifications were performed in 25  $\mu$ l reactions containing 5  $\mu$ l of DNA extract with PureTaq™ Ready-to-Go PCR beads (GE Healthcare Life Sciences, UK). The primers and cycling conditions used to amplify and sequence the partial 28S ribosomal DNA (rDNA) (D1–D3) have been previously described (Mendoza-Palmero et al. 2015). Amplicons were visualised on GelRed (Biotium, Fremont, CA, USA) and purified using

a QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Sequencing reactions were performed directly on the purified PCR products using a BigDye v3.1 Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA, USA) and sequences were run on an ABI 3500 DNA genetic analyser (Applied Biosystems).

Contiguous sequences were assembled for phylogenetic analysis, in Sequencher™ 5.2.4 (Gene Codes, Ann Arbor, MI, USA). Sequences of partial 28S rDNA were aligned with representatives of the Plagiorchiida available in GenBank and the chosen outgroups were *Gorgoderia cygnoides* (Zeder 1800) (Gorgoderidae) (AF1519382) and *Nephrotrema truncatum* (Leuckart, 1842) (Troglotrematidae) (AF151936) according to Tkach et al. (2000). Newly obtained sequences were aligned using MUSCLE (Edgar 2004) implemented in Geneious version 11.1.4 (Kearse et al. 2012). To evaluate the occurrence of substitution saturation, we estimated the Iss index in DAMBE 6 (Xia 2013). The best-fit model for nucleotide substitution in the resulting matrix was determined by the Akaike information criterion in jModelTest (Posada 2008). Phylogenetic analyses were performed using Bayesian inference and maximum likelihood (ML) using MrBayes and RaxML analysis was carried out using the computational resource CIPRES (Miller et al. 2010). The Bayesian analysis was run with the nucleotide substitution model GTR+I+G. To search with the Markov chain Monte Carlo method, chains were run with 10,000,000 generations, saving one tree every 1000 generations. On the burn-in, the first 25% of generations were discarded, and the consensus trees were estimated using the remaining trees. Bayesian posterior probabilities (BPP) cutoff was considered > 90%. The supports for ML were determined by performing 1000 bootstrap replicates. The trees were visualised in FigTree v1.3.1 (Rambaut 2009). Genetic divergence was calculated using the Kimura 2-parameter model in MEGA7.0.20 software (Kimura 1980; Tamura et al. 2013).

## Results

Four partial sequences of the 28S rDNA gene (two from *I. infidum* 1268 bp accession number MW317230 and 1182 bp MW317227 and two from *T. stenocotyle* 1269 bp MW317229 and 1264 bp MW317228) were successfully sequenced and aligned with 34 digeneans belonging to Plagiorchiida. The final 28S rDNA alignment consisted of 1262 bp long. The Iss indicated no saturation in either transitions or transversions. Critical index of substitution saturation (Iss.c) values were greater than the Iss values. Genetic divergence among specimens of *I. infidum* presented no variation. Between *I. infidum* and *I. similis*, the divergence was 1%, and between *I. infidum* and *Choledocystus hepaticus* Lutz, 1928

(Plagiorchiidae), it was 2% (Supplementary Table 1). Both *T. stenocotyle* sequences were identical and varied from 11 to 16% among species from Plagiorchiida (Supplementary Table 1).

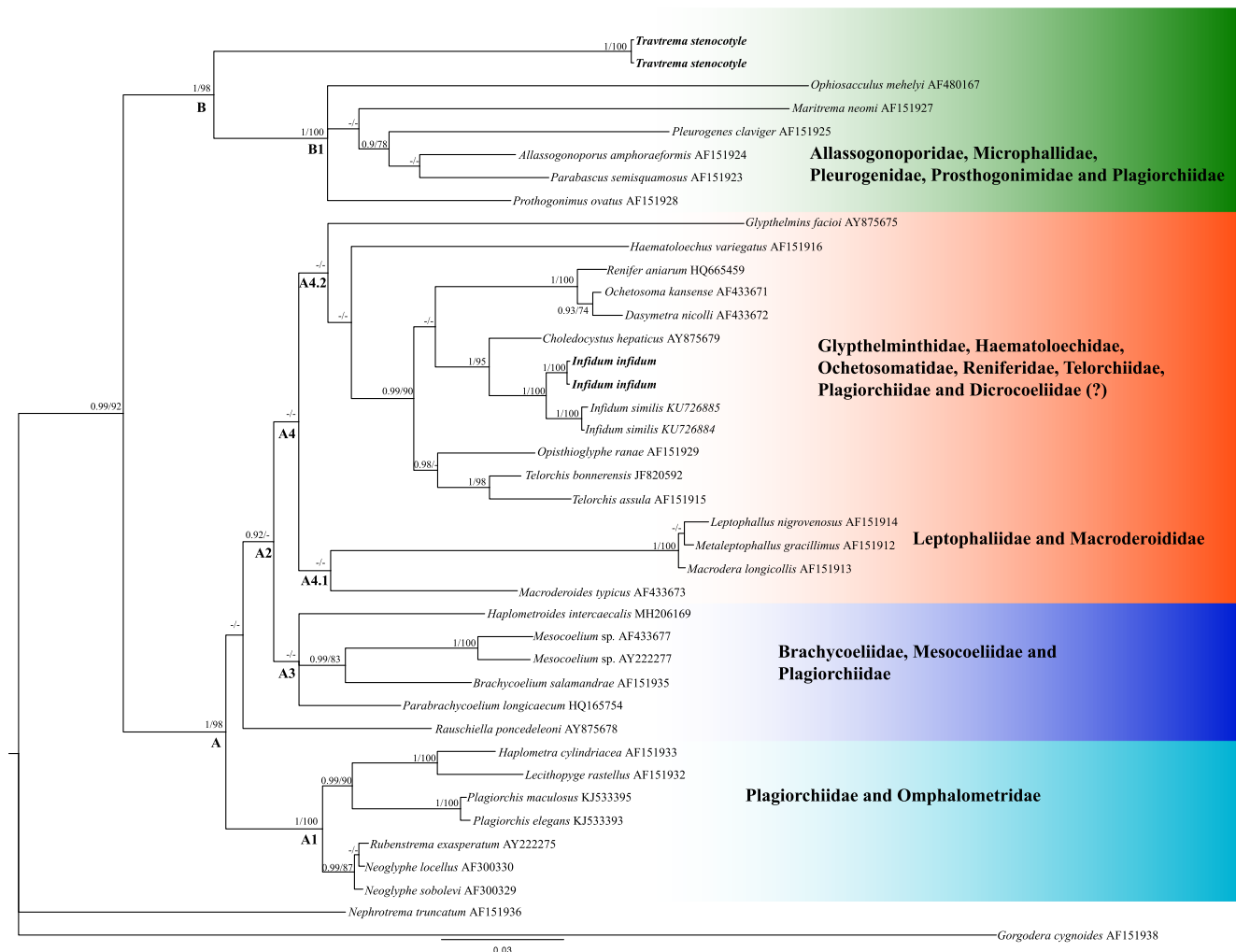
Bayesian and maximum likelihood (ML) inference methods resulted in identical topologies with supported values in the nodes in most clades (Fig. 4). The phylogenetic inference presented two main clades labeled A and B; main clade A represented members from Plagiorchioidea and main clade B, members from Microphalloidea (Fig. 4). Main clade A was strongly supported and divided into clades A1, A2, A3, and A4 (Fig. 4). Clade A1 was highly supported nodes and comprised species from Plagiorchiidae and Omphalometridae. Clade A2 not supported by bootstrap was represented by clades A3 and A4. Clade A3 (not supported) is formed by species from Plagiorchiidae, Brachycoeliidae, and Mesocoeliidae. Clade 4 (not supported) possesses the majority of species and is subdivided into clades A4.1 and A 4.2. Clade

A4.1 is formed by species from Leptophaliidae and Macroderoididae and clade 4.2 is formed by members of the Glythelminthidae, Haematoloechidae, Ochetosomatidae, Reniferidae, Telorchidae, Plagiorchiidae, and Dicrocoeliidae (Fig. 4). *Infidum infidum* cluster as sister species to *I. similis* and closely related to *C. hepaticus* with high PP (posterior probability) and bootstrap support (Fig. 4).

Clade B was composed of *T. stenocotyle* species closely related to species from Allassogonoporidae, Microphallidae, Pleurogenidae, Prosthogonimidae, and Plagiorchiidae, with high nodal support (clade B1) (Fig. 4).

## Discussion

*Infidum infidum* and *T. stenocotyle* are digeneans commonly found in South American snakes, including some Brazilian species. Both species have their morphology well known



**Fig. 4** Bayesian topology based on partial 28S ribosomal DNA sequences of Digenea. GenBank accession numbers are indicated next to species names. Numbers above nodes represent supported nodes by posterior probabilities for Bayesian analyses and bootstrap for maximum

likelihood analyses respectively (posterior probabilities > 0.90 and bootstrap scores > 70). Branch length scale bar indicates number of substitutions per site. Coloured squares represent main clades

since many studies had reported about them (Fernandes and Kohn 2014 and references therein). However, no molecular study was conducted with these parasite species.

According to morphological features, the genus *Infidum* belongs to the family Dicrocoeliidae, subfamily Leiptrematinae (Pojmańska 2008). However, Martínez-Salazar et al. (2016), in their phylogenetic studies using 28S rDNA and COI mtDNA, recovered specimens of *I. similis* clustering among plagiorchids (closely related to *Choledocystus hepaticus*), which led those authors to infer that the phylogenetic placement of this species was not among dicrocoeliids. In our phylogenetic analyses, *I. infidum* was recovered as sister taxa of *I. similis*, as expected, and closely related to *C. hepaticus*, thus corroborating with the findings of Martínez-Salazar et al. (2016).

The taxonomic history of *Infidum* is controversial (Pojmańska 2008; Martínez-Salazar et al. 2016). Travassos (1944) included this genus in the subfamily Infidinae; later, Yamaguti (1958) considered this subfamily invalid and created 2 subfamilies inside Dicrocoeliidae (Leiptrematinae and Stromitrematinae) and placed this genus in the subfamily Leiptrematinae. The last classification and most recent consider the genus among the subfamily Leiptrematinae (Pojmańska 2008).

Molecular phylogenetic analyses provided a framework to discuss the interrelationships among trematodes and have defined classical morphological classification (Hernández-Mena et al. 2016). Clearly, the molecular findings do not agree with morphological classification and perhaps, as Martínez-Salazar et al. (2016) stated, *Infidum* spp. are not Dicrocoeliidae, instead a genus that belongs to Plagiorchidae because of the close relationship to *C. hepaticus*. Despite that our study presents the phylogenetic position of the type species of the genus, *I. infidum*, we might not suggest any modification on the taxonomic classification of the genus by analysing just one marker (28S rDNA). The amplification of 28S rDNA gene is largely used in several taxa around the globe. Among trematodes is perhaps the most common marker used to infer phylogenies (Hernández-Mena et al. 2016; Martínez-Salazar et al. 2016; Müller et al. 2018) and exhibits a great resolution among different families. For example, Tkach et al. (2016) provided a broad diverse phylogeny using 28S rDNA with 80 species, representing 8 families and 40 genera of Echinostomatoidea, and they evaluated the morphological classification consistency with phylogeny based on molecular data.

However, we strongly recommend that, in future studies, more taxa and molecular markers should be added to the phylogeny of *Infidum infidum*, as well as more information on the life cycle and morphological characters, to accommodate these species in the non-monophyletic Plagiorchidae or to assigned them to another taxonomic family. This is the first phylogenetic study of *Travtrema* using molecular data, and

*T. stenocotyle* was first sequenced using 28S rDNA. *Travtrema stenocotyle* (Plagiorchidae) was surprisingly grouped as a sister group to Allassogonoporidae, Microphallidae, Pleurogenidae, and Prosthogonimidae (Fig. 4) in the superfamily Microphalloidea (Tkach et al. 2000) and not within species from Plagiorchioidea as expected. According to Tkach et al. (2000), the suborder Plagiorchiatia sensu stricto comprehends several clades, with two large clades composed with two superfamilies: Plagiorchioidea and Microphalloidea. Plagiorchioidea corresponds to the existing families: Brachycoeliidae, Telorchidae, Plagiorchidae, Haematoloeciidae, and Leptophaliidae (Tkach et al. 2000; Cribb et al. 2003).

Plagiorchidae is the central family in the large superfamily Plagiorchioidea, and despite its systematic and practical importance, it is one of the least understood and understudied groups among Digenea (Ndiaye et al. 2013) and, therefore, there are still many questions and controversies related to the systematic position of several genera and their evolutionary interrelationships (Tkach et al. 2000; Tkach 2008; Ndiaye et al. 2013). The high level of homoplastic characters among Plagiorchiaties, the extreme diversity of taxa placed in the paraphyletic Plagiorchidae, and the lack of agreement of reliable diagnostic criteria might be some characteristics related to the confusion of plagiorchids systematic (Tkach 2008; Zikmundova et al. 2014; Suleman et al. 2019). Poor morphological descriptions, unknown ranges of variability, and low host specificity might also have led to systematics confusion (Zikmundova et al. 2014; Suleman et al. 2019). Several genera which once belonged to Plagiorchidae have been placed in other families, e.g. Glyphthelminthidae and Omphalometridae (Tkach et al. 2001; Razo-Mendivil and Pérez-Ponce de León 2011), or have been waiting for the appropriate placement in another family, e.g. *Haplometroides* spp. (Müller et al. 2018). This might be the case of *T. stenocotyle*, in which more data (systematic, ecological, and evolutionary) and taxa are still necessary to accommodate this genus in a different family. The low support in the nodes recovered in some clades (Fig. 4) might correspond to the low number of available sequences; therefore, more taxa should be sequenced and added to improve these results in the future. In addition, Digenea systematics are controversial, which most of taxa are classified mainly on taxonomic schemes and phylogenetic inference based only on morphology, and some characters are homoplastic (Maldonado et al. 2001; Razo-Mendivil and Pérez-Ponce de León 2011; Müller et al. 2018).

Our study provides molecular phylogenetic position data of two trematodes species *I. infidum*, considered Dicrocoeliidae, and *T. stenocotyle*, considered Plagiorchidae. Our results using molecular phylogenetic data using 28S rDNA showed that these species may not correspond to their assigned families and encourage future studies on the systematic of these understudied groups.



**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s00436-020-07041-3>.

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**Authors' contributions** Designed the study: MIM, RJS; collected data: EE, EPA, LPU, DHM, LHO, RJS; performed research: MIM, MBE, RJS; analyzed data: MIM, RJS; wrote the manuscript: MIM, RJS; revised and approved the final manuscript: MIM, EE, EPA, LPU, LHO, MBE, DHM, RJS

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**Data availability** Permission to collect and transport the snakes was given by the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) under registration 59230-1 (SISBIO).

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflicts of interest.

**Code availability** Not applicable

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