Parasitology International 70 (2019) 27–32

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Supplementary studies on *Henneguya guanduensis* (Cnidaria: Myxosporea) infecting gills and intestine of *Hoplosternum littorale* in Brazil: Ultrastructural and molecular data

Diego Henrique Mirandola Dias Vieira[a,*], Larissa Sbeghen Pelegrini[a], Vanessa Doro Abdallah[b], Rodney Kozlowiski de Azevedo[b]

[a] Departament of Parasitology, Universidade Estadual Paulista "Júlio de Mesquita Filho" – UNESP, Botucatu, São Paulo, Brazil
[b] Centro Universitário CESMAC, Maceió, Alagoas, Brazil

ARTICLE INFO

Keywords:
Callichthyidae
Catfish
Myxobolidae
Phylogeny
Ultrastructure

ABSTRACT

Henneguya guanduensis was originally described as a gill parasite of the *Hoplosternum litoralle*, a siluriform fish belonging to the Callichthyidae family. A study was undertaken with 58 specimens of *H. litoralle* taken from Batalha River in the state of São Paulo. The fish were collected and examined searching for lesions and/or myxosporean plasmodia. The prevalence of infection was 9.31% in the gills and 5.17% in the intestine. The mature spores had elongated bodies with polar capsules of inequal size and a caudal length greater than body length. Morphological characteristics identified the parasite as *H. guanduensis*. Molecular analysis of the SSU rDNA partials sequences resulted in a 1796 bp and 1712 bp for gills and intestine repectively, demonstrating significant genetic differences with previously described species of *Henneguya* and 99.7% similarity to each other when aligned. Phylogenetic analysis comparing the SSU rDNA sequence of *H. guanduensis* with closest species as indicated by BLASTn Max Score showed *H. guanduensis* as sister species of *H. loretoensis* in a subclade compose by species that parasites fishes from Amazon basin. This is the first report of the finding of *Henneguya guanduensis* spores in the intestine of *Hoplosternum littorale*.

1. Introduction

Hoplosternum littorale (Hancock, 1828) (Siluriformes: Callichthyidae), popularly known as "tamboatá" or "caborja", is an omnivorous catfish. It can be found throughout South America, from the Amazon basin to Prata river basin. It has medium size and is considered a nocturnal benthic fish, inhabiting ponds and rivers of various sizes [1]. According to the FAO [2], this species of fish is among the economically important species that reach a high price in the market of countries like Guyana and Venezuela, including potential for cultivation. Despite its economic importance, little is known about the myxozoans that infect *H. littorale*.

Myxozoans are cnidarians parasites obligate, mainly in fish. The genus *Henneguya* Thélohan 1892 (Myxosporea: Myxobolidae) is a specious genus, with > 200 known species being about 45 described in South America [3,4]. From these, *Henneguya guanduensis* Abdallah, Azevedo, Luque and Bonfim, 2007 was described infecting *H. littorale* gills collected in the Guandu River, state of Rio de Janeiro, Brazil. Myxozoans can induce significant mortality rates in fish farmed, leading to large economic losses [5,6]. Among the problems caused by *Henneguya* are the reduction of respiratory capacity [7] and degenerative cardiomyopathy [8].

The identification of myxozoans was based only on the analysis of spore morphology [9]. However, it was found that this method is flawed, mainly due to the high similarity between species that are found in the same infection site and host, only with some subtle differences between the spores [10]. The molecular analysis, mainly through the SSU rDNA gene, has been used to collaborate in the detection and identification of the species [11].

The present study supplements the original description of *H. guanduensis*, presenting data from the partial sequence of SSU rDNA, phylogenetic analysis and additional information on parasite morphology through ultrastructural analysis.

* Corresponding author at: Universidade Estadual Paulista, R. Prof. Dr. Antônio Celso Wagner Zanin, 250, Rubião Junior, CEP: 18618-689, Botucatu, São Paulo, Brazil.
E-mail address: diego.vieira@unesp.br (D.H.M.D. Vieira).

https://doi.org/10.1016/j.parint.2019.01.009
Received 25 September 2018; Received in revised form 18 January 2019; Accepted 30 January 2019
Available online 31 January 2019
1383-5769/

Table 1
SSU rDNA primers used in this study, sequence, amplicon size and reference.

Primer	Sequence 5′–3′	Amplicon size (bp)	Reference
ERIB1	ACCTGGTTGATCCTGCCAG	≈1900	[24]
ERIB10	CTTCCGCAGGTTCACCTACGG		[24]
MyxospecF	TTCTGCCCTATCAACTTGTTG	≈800	[25]
MyxospecR	GGTTCNCDGRGGGMCCAAC		[20]
Act1R	AATTTCACCTCTCGCTGCCA	≈900	[26]
MyxGen4F	GTGCCTTGAATAAATCAGAG	≈900	[27]

2. Matherial and methods

Between March 2015 and December 2017, 58 specimens of *H. littorale* were collected in the Batalha River (21° 52′ 33.0″S, 49° 14′ 19.9″W), Reginópolis, São Paulo, Brazil. The collections were always carried out in the rainy season. Fish were collected using waiting networks and euthanasied immediately after capture. The gills, heart, liver, gonad, stomach, intestine, bone marrow, kidney, swim bladder and gallbladder were observed in stereomicroscope. Infected gill and intestine samples were collected and fixed according to the protocols for further morphological and molecular analysis.

The length and width of the spore, tail length, total spore length, length and width of the polar capsules and the number of turns of the polar filament were measured following the recommendations of Lom and Arthur [12]. Measurements of 30 spores found in the gills and 30 spores found in the intestine of infected fish were taken from digital images at a magnification of 1000× under light microscopy with the Leica LAS V3.8 software application package (Leica Microsystems, Wetzlar, Alemanha). All measurements are in micrometers and are presented with mean and standard deviation.

For transmission electron microscopy (TEM), a fragment of infected intestine containing plasmodia was fixed in Karnovsky's solution at 4 °C for 10 h. After rinsing, the tissue was post-fixed in 2% osmium tetroxide for 3 h at 4 °C. Subsequently, the tissue was immersed in 0.5% uranyl acetate in distilled water for 2 h. The tissue fragments were dehydrated through an ascending series of ethanol concentration, followed by propylene oxide and embedded in Epon resin. Semi-thin sections were stained with methylene blue-Azure II and observed under a differential interference contrast microscope. Ultrafine sections were double stained with aqueous uranyl acetate and lead citrate and were observed under a JEOL 100CXII (TEM) operated electronic microscope at 60 kV.

Infected intestine and gills were fixed in absolute ethanol for DNA extraction. DNA extraction followed the protocol for animal tissue DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). The SSU rDNA gene was amplified using different combinations of primers and PCR conditions (Table 1). Amplifications were performed using a Bio-Rad Mycycler thermal cycler (Bio-Rad Laboratories Pty Ltd., Gladesville, Australia), initial denaturation at 94 °C for 3 min, followed by 30 cycles of 94 °C for 40 s. A specific annealing temperature of 55 °C for 40 s was used, followed by 72 °C for 2 min and a final extension at 72 °C for 10 min. PCR reactions were performed in 25 µl reactions containing 3 µl of extracted DNA, 1 µl of each PCR primer using Ready-to-Go beads PCR (Pure TaqTMReady-to-GoTM Beads, GE Healthcare, Chicago, USA). The solution consisted of stabilizers, BSA, dATP, dCTP, dGTP, dTTP, ± 2.5 units of puReTaq DNA polymerase and reaction buffer. Each bead was reconstituted to a final volume of 25 µl and the concentration of each dNTP was 200 µM in 10 mM Tris-HCl (pH 9.0 at room temperature), 50 mM KCl and 1.5 mM MgCl 2. After checking the presence of the expected DNA amplicons on a 1% agarose gel in TAE buffer, the PCR products were cleaned using the QIAquick PCR purification kit (Qiagen, CA, USA). Automated sequencing was performed directly on the PCR products purified from samples using BigDye v.3.1 Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA, USA) for cycle sequencing. Sequences were run on an Applied Biosystems ABI 3500 DNA gene analyzer, edited in Sequencher ™ v. 5.2.4 (Gene Codes, Ann Arbor, MI) and subjected to BLAST analysis (http://blast.ncbi.nlm.nih.gov). The newly generated partial sequences of SSU rDNA were aligned using Geneious version 7.1.3 [13] with the ClustalW algorithm [14] and standard configurations with related sequences for recovered *Henneguya* species of GenBank. Bayesian inference (BI) analysis was performed using MrBayes 3.1.2 [15]. Markov Chain Monte Carlo Chains (MCMC) have been run for 10 million generations and loglikelihood scores were plotted. The "burn in" was set to 25%. *Ceratonova shasta* (AF001579) was used as outrgroup. All analyzes were performed using only positions that were unequivocally aligned in all taxa (1111 nt). Phylogenetic trees were generated and edited in FigTree v1.4 [16]. An alignment containing only trimmed sequences of *Henneguya* spp. reported parasitizing fishes from Amazon basin was used to produce a distance matrix.

3. Results

3.1. Additional description of Henneguya guanduensis Abddalah et al, 2007

The prevalence of infection was 9.31% in the gills and 5.17% in the intestine. Infections were not observed in the gill and intestine in the same host specimen. Plasmodia sizes of *H. guanduensis* found in the gills and intestine were approximately 0.5 mm and 0.7 mm, respectively. The spores found in the gills (Fig. 1A) showed as average measures: total length 33.9 ± 1.2 µm, body length 14.8 ± 0.5 µm, body width 6.8 ± 0.3 µm, tail length 19.8 ± 1.0 µm. The polar capsules had different sizes, and the larger polar capsule was 4.3 ± 0.3 µm in length and 2.1 ± 0.2 µm in width and the smaller capsule was 3.8 ± 0.5 µm in length and 2.0 ± 0.3 µm wide (Table 2). The spores found in the

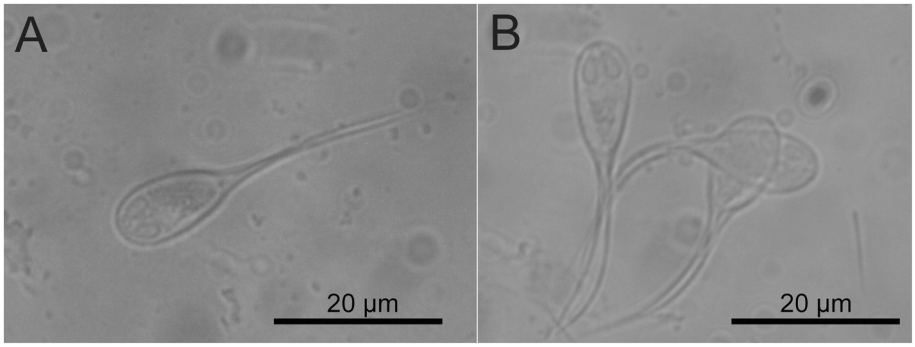

Fig. 1. Images of *Henneguya guanduensis* spores in *Hoplosternum litoralle*. (A): Mature spores found in gills. (B): Mature spores found in intestine.

Table 2
Comparison of the morphometric analysis between the description of *Henneguya guanduensis* and the measures found in this study. All measurements are given in μm.

	Total length	Body length	Body width	Tail length	Larger PC length	Larger PC width	Smaller PC length	Smaller PC width
H. guanduensis (Abdallah et al. 2007)	33.6 (27.3 –38.1)	14.6 (11.4 – 16.7)	6.5 (4.9 – 7.9)	19.0 (15.6 – 22.5)	4.4 (3.3–5.6)	2.0 (1.6 – 2.3)	4.1 (3.3 – 5.3)	2.2 (1.5–2.8)
H. guanduensis (gills)	33.9 ± 1.2	14.8 ± 0.5	6.8 ± 0.3	19.8 ± 1.0	4.3 ± 0.3	2.1 ± 0.2	3.8 ± 0.5	2.0 ± 0.3
H. guanduensis (intestine)	33.4 ± 0.4	14.3 ± 0.9	6.1 ± 0.4	18.5 ± 0.5	4.4 ± 0.8	2.2 ± 0.2	3.9 ± 0.4	2.2 ± 0.1

intestine (Fig. 1B) presented as measures on average: 33.4 ± 0.4 μm of total length, 14.3 ± 0.9 μm of body length, 6.1 ± 0.4 μm of body width and 18.5 ± 0.5 μm of tail length. The polar capsules had different sizes, and the larger capsule was 4.4 ± 0.8 μm in length and 2.2 ± 0.2 μm in width and the smaller capsule was 3.9 ± 0.4 μm in length and 2.2 ± 0.1 μm wide (Table 2). Polar filament allocated in 6–7 coils.

The ultrastructural analysis of *H. guanduensis* showed details of the morphology of the spores that in the description work could not be concluded. Transverse cuts allowed the visualization of one of the two nuclei present in the sporoplasm, as well as the symmetrical valves of the spores (Fig. 2A). These cuts also showed that the polar capsule is formed by two layers, one thin and the other thick and dense (Fig. 2B). The longitudinal cut allowed us to accurately observe the number of turns of the polar filament present inside the polar capsules (Fig. 2C).

Host: *Hoplosternum littorale* (Hancock, 1828), Siluriformes: Callichthyidae.

Location: Batalha River (21° 52′ 33.0″S, 49° 14′ 19.9″W) located in the municipality of Reginópolis, São Paulo, Brazil.

Site of infection: gills and intestine.

Sequence data: The 1796- and 1712-bp of SSU rDNA sequences obtained from infected *H. littorale* gills (GenBank Acc. Num. MK415825) and intestine (GenBank Acc. Num. MK415826) respectively.

Partial sequences of the SSU rDNA gene from the sample extracted from gills and intestine of *H. guanduensis* resulted in sequences of 1796 and 1712 nucleotides repectively. When aligned the sequences presented 99.7% similarity with only five different nucleotides (Table 3). BLAST search using the partial data from the SSU rDNA sequences for *H. guanduensis* did not find any myxozoan whose partial sequence was 100% similar, among those available from GenBank.

Phylogenetic analysis showed that *Henneguya* species were grouped into several major clades and subclades, according to various criteria, but mainly according to the host groups. There is a main clade formed by species that parasitize Siluriformes fish, in addition to clades formed by species of *Henneguya* that parasitize fish of the orders Characiformes, Perciformes and Esociformes. *Henneguya guanduensis* appears as a sister species of *Henneguya loretoensis* Mathews, Naldoni and Adriano, 2018 in a subclade formed by species that parasitize fish of the family Callichthyidae. In addition *H. guanduensis* is within a subclade formed by species described in the Amazon basin (Fig. 3).

4. Discussion

This study showed the partial sequencing of *H. guanduensis* SSU rDNA gene found in *H. littorale* gills and intestine, captured in the Batalha River, State of São Paulo, Brazil, allowing the phylogenetic analysis of this parasite. The morphometric and morphological data obtained in this study clearly confirm the identification of the species as *H. guanduensis*, originally described by Abdallah et al. [17].

Abdallah et al. [17] described *H. guanduensis* based on spore morphology. This was in the past the main method for the characterization and identification of myxozoans [18]. However, Kent et al. [19] and Lom and Dyková [11] have suggested that the amplification of the SSU rDNA gene is fundamental for the description of new species of myxozoans due to the difficulties of morphological characterization of spores. The SSU rDNA gene is used in molecular systematics to determine the relationships between myxozoans, as it is highly variable among closely related species [19].

Fiala [20] indicated that the host preference is very important and that myxozoans species can be grouped according to host fish species. Our phylogenetic analyzes of *H. guanduensis* with the more closely related species of *Henneguya* revealed several clades and subclades that correlated mainly with the family or order of the host fish. *Henneguya*

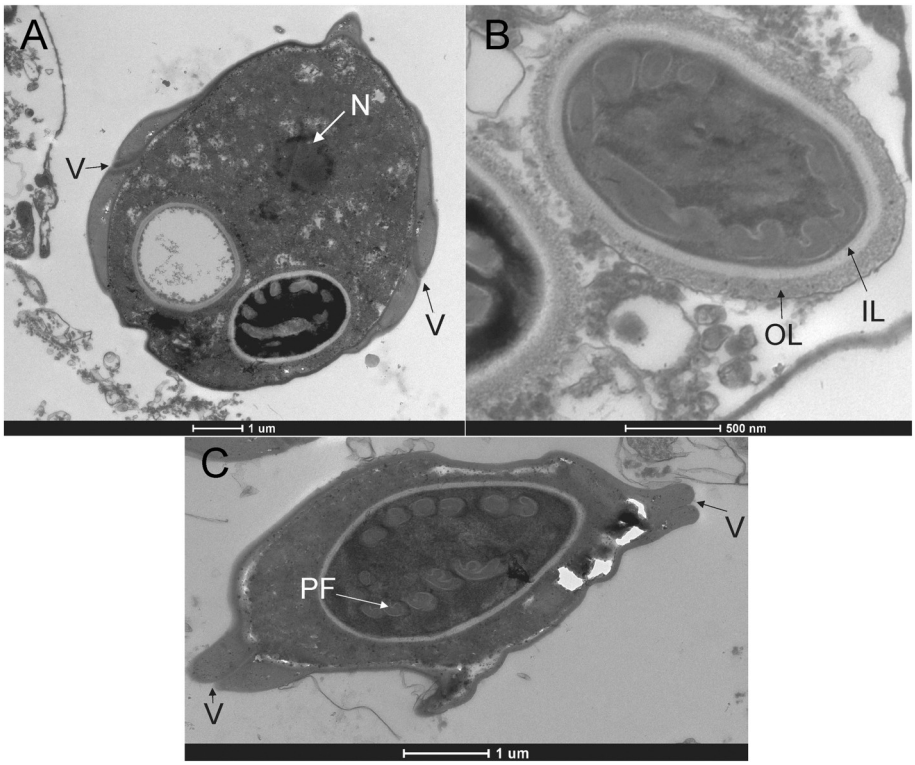

Fig. 2. Transmission electron microscopy images of spores of *Henneguya guanduensis* in the intestine of *Hoplosternum litoralle*. (A): Transversal cut shwoing the valves (V) and nucleus (N). (B): Longitudinal cut showing the polar capsule. Notice the outer electron-dense layer (OL), an inner electron-lucent layer (IL). (C): Longitudinal cut showing the valves (V) and containing coiled polar filament (PF, 6–7 coils).

Table 3
Divergence in SSU rDNA sequences of *Henneguya* spp., parasites of fishes from Amazon basin. Data below the diagonal represent the number of different nucleotides; data above the diagonal represent the percent nucleotide divergence (Tamura-Nei model)

	Species	1	2	3	4	5	6	7
1	*Henneguya guanduensis* (gills)		0.3	6.6	11.2	7.2	8.1	10.3
2	*Henneguya guanduensis* (intestine)	5		6.5	11.0	7.1	8.0	10.3
3	*Henneguya loretoensis*	59	59		11.1	8.0	8.2	10.1
4	*Henneguya tapajoensis*	101	100	100		5.7	5.0	8.9
5	*Henneguya jariensis*	65	64	71	51		3.1	5.8
6	*Henneguya tucunarei*	73	72	73	45	28		5.7
7	*Henneguya paraensis*	93	93	90	80	52	51	

guanduensis is located as a sister species of *H. loretoensis,* the only other species already described parasitizing fish of the family Callichthyidae that we have a partial sequence available.

The vast majority of myxozoans species have a strict specificity of host tissue, although the use of different habitats has been demonstrated in some species. For example, *Myxobolus wulii* Landsberg & Lom, 1991 was first described from the gills of goldfish, *Carassius auratus auratus* (Linnaeus, 1758), and subsequently it was reported parasitizing the hepatopancreas of the same host [21]. As there are no simultaneous infections by *H. guanduensis* in the gill and intestine of *H. litoralle*, this suggests different infection mechanisms for both tissues. Further studies on the life cycle of *H. guanduensis* are required to clarify the infection mechanisms.

Although *H. guanduensis* was described in the Paraíba do Sul river basin and in this work occur in the Paraná River basin, the species was grouped with species described in the Amazon basin. This probably occurred because *H. littorale* is a species native to the Amazon basin and found today in the Southeast region of Brazil [22]. It is known that the relationship between the parasites and their hosts is intimate, dynamic and complex, and the evolution of both is correlated [23]. These data suggest that *H. guanduensis* is also a species native to the Amazon region that migrated along with its host to the rest of Brazil.

In conclusion, additional data on the morphology and partial sequencing of the SSU rDNA gene, as well as phylogenetic analysis involving *H. guanduensis* were provided. The data contained in the present study will facilitate future diagnosis, research on epidemiology, life cycles and understanding of the phylogenetic relationships of the parasite. This is the first time that *He. guanduensis* is recorded in the Batalha River and found parasitizing the intestine of *Ho. littorale*.

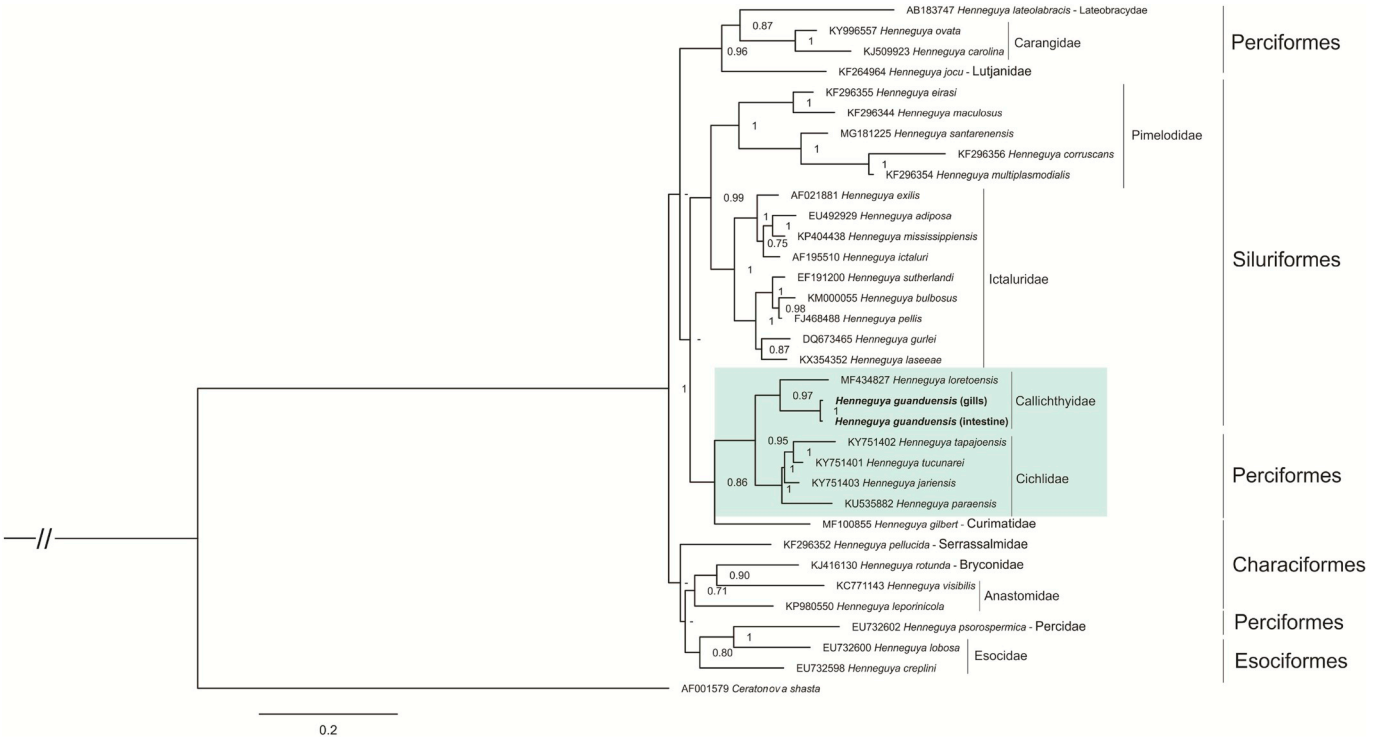

Fig. 3. Phylogenetic tree based on partial SSU rDNA sequences showing the position of *Henneguya guanduensis* among other *Henneguya* spp.. Numbers at the nodes represent Bayesian posterior probability (BI) gaining > 0.7 posterior probability. Values lower than 0.7 are represented by dashes. In the highlighted area the clade formed by species that parasitize fish native to the Amazon Basin. Scale bar is given under the tree.

Acknowledgments

The authors thank the Laboratório de Parasitologia de Animais Silvestres (LAPAS), Department of Parasitology, UNESP, for the support in the carrying out of the molecular biology.

Financial support

This study was funded by the Fundação de Amparo à Pesquisa do Estado de São Paulo – FAPESP (Procs. 2015/24901-2 - 2014/12862-0) to DHMDV and RKA.

Declarations of interest

None.

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