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BACTERIAL, FUNGAL AND VIRUS MOLECULAR BIOLOGY - SHORT COMMUNICATION

Molecular detection and phylogenetic analysis of *Cyprinid herpesvirus 3* in Brazilian ornamental fish

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Abstract

Cyprinid herpesvirus 3 has a worldwide distribution and presents high mortality rates in species of *Cyprinus carpio*, causing serious economic loss to the global aquaculture industry. The description of this infection in other ornamental fish species is still limited. For this purpose, 100 ornamental fish from 24 different species were tested by PCR for *Cyprinid hespesvirus 3* and the positive samples represented 6% of the tested samples. Phylogenetic reconstruction, based on the Thymidine Kinase gene, revealed the existence of two distinct clades. One clade grouped a Brazilian sample with European and Asian genotypes of CyHV-3 and a second clade, containing only Brazilian sequences described in this study. All of the Brazilian sequences showed identity values greater than 97.7% when compared to each other. This is the first report of the occurrence of *Cyprinid herpesvirus 3* in ornamental fish species in Brazil. These results in association with further studies of viral isolation and characterization can help in establishing effective surveillance and disease control program.

Keywords *Alloherpesviridae* · *Cyprinivirus* · Koi herpesvirus · Molecular diagnosis

Introduction

Common carps (*Cyprinus carpio carpio*) are one of the main species marketed worldwide and are considered one of the species with the greatest economic added value in aquaculture [1, 2].

Cyprinid herpesvirus 3 (CyHV-3) (genus *Cyprinivirus*, family *Alloherpesviridae*), also known as Koi herpesvirus (KHV), is a linear and double-stranded DNA virus surrounded by an icosahedral capsid [3, 4]. This virus has been described in several countries around the world since 1998 and the occurrence of outbreaks is associated with high mortality rates mainly in fish of the species *Cyprinus carpio* (common and koi carps) [5]. Therefore, the occurrence of this virus is of mandatory notification by the World Organization for Animal Health (OIE). Infection with CyHV-3 is very common in the spring and occurs mainly through cohabitation between animals carrying the virus and healthy animals, with the skin being the main portal of entry for the virus [4, 6].

The main clinical signs of infection caused by CyHV-3 are anorexia, discoloration, apathy, necrotic gills, and skin lesions [7, 8]. Internally, the animal may present with hepatosplenomegaly [9]. Histological analysis show mass proliferation of gill epithelium and intranuclear inclusions in infected cells [10]. The presumptive diagnosis of the disease includes the observation of the high mortality of Koi and common carps, even with the adoption of measures for treatments against bacteria and external parasites in water with temperature ranging from 18 to 26 °C [9].The confirmation of the diagnosis for Koi herpesvirus infection depends on demonstrating the presence of the virus by isolating it in cell culture, using Koi carp (Koi fin—KF-1) cell lines or other susceptible cells infection, followed by PCR (polymerase chain reaction) of the isolated virus [9, 11, 12].

The aim of this study was to present the occurrence of CyHV-3 for the first time in Brazil in ornamental fish. For

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this purpose, molecular diagnostics and phylogenetic reconstruction were used.

Materials and methods

Samples

Tissues samples (kidney, spleen, and liver) from 100 ornamental fish, including 27 samples from carps (*Cyprinus carpio carpio*), were collected from a wholesaler in São Paulo state, Brazil. The species and the description of the clinical signs are in Table 1. All applicable institutional guidelines for the care and use of animals were followed (CEUA n° 6,782,040,416).

DNA extraction

Fifty milligrams of pooled tissue (spleen, kidney and liver) wAS submitted to DNA extraction using the NucleoSpin Extract II Kit (Macherey-Nalgel, Germany), according manufacturer's instructions. Then, the DNA extracted was solubilized in 50 µL of nuclease-free water (Life Technologies ™/Thermo Fisher Scientific, USA) ant the DNA concentration was measured by spectrophotometry (DS-11, DeNovix, USA) according A260/A280 ratio.

Primers and nested-PCR

Primers were selected to amplify fragments of the Thymidine Kinase gene (TK gene) [13, 14] and the fish β-actin gene was used as the internal control. The primer sequences used were KHV F1: 5'-GGGTTACCTGTA CGAG-3' and KHV R1: 5'- CACCCAGTAGATTATGC-3' (409-bp amplicon); KHV F2: 5'- CGTCTGGAG GAATAC GACG-3' and KHV R2: 5'- ACCGTACAGCTCGTACTG G-3' (348-bp amplicon); β-actin-F: 5′-GTAGATATCCGT AAGGACCT-3′ and Actin-R: 5′-CACATCTGCTGGAAG GTGG-3′ (209-bp amplicon).

Nested-PCR reactions were performed using GoTaqTM Colorless Master Mix (Promega, USA), according to the manufacturer's instructions. Nuclease-free water replaced DNA in negative control reactions. The thermal cycle

Table 1 Results for CyHV-3 by *nested*-PCR

	Common name	Clinical signs	Positive samples/total samples	Sequenced samples
Araipama gigas	Araipama	apathy	0/2	0
Brachdanio albolineatus	Pearl danio	apathy	0/1	0
Carassius auratus	Goldfish	apathy	1/14	0
Chromis viridis	Blue green damselfish	none	0/1	0
Cyprinus carpio	Common carp	body injury	1/27	0
Glossolepsis incises	Redrainbowfish	body injury	0/1	0
Hemiodopsis gracillis	Slender Hemiodus	none	0/1	0
Hyphessobrycon eques	Jewel tetra	none	1/1	0
Hypostomus plecostomus	Suckermouthcatfish	presence of mucus	0/2	0
Macropodus opercularis	Paradisefish	apathy/lethargy	0/5	0
Misgurnos anguilicaudatus	Pond loach	apathy	1/5	0
Moenkhausia costae	Tetra fortune	none	0/4	0
Monodactylus argentus	Silver moony	body injury	0/2	0
Pangasius hypophthalmus	Stripedcatfish	body injury	0/2	0
Poecilia reticulata	Guppy	body injury	0/2	0
Pomacanthus imperator	Emperor angelfish	body injury	0/1	0
Pomacanthus narvachus	Bluegirdled angelfish	anorexia	0/1	0
Pterophyllum scalare	Freshwater angelfish	none	0/3	0
Pygocentrus nattereri	Red piranha	lethargy/abnormal swimming	1/8	0
Serrasalmus gibbus	Piranha	none	0/1	0
Trichogaster lalius	Dwarf gourami	apathy	0/2	0
Trichogaster leeri	Pearl gourami	lethargy	0/2	0
Trichogaster trichopterus	Three spot gourami	body injury	0/10	0
Xiphophorus maculatus	Common platy	apathy	1/2	0
		TOTAL	6/100	4

protocol used for 409-bp fragment was the following: initial denaturation at 94ºC for 5 min, and 40 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min and a final extension at 72 °C for 10 min, in a Swift™ MaxPro Thermal Cycler (Esco Technologies Inc., EUA) [13]. The nested-PCR amplification profile (369-bp fragment) was the same described above but the cycles were reduced to 30 [14]. The thermal cycle protocol used for fish actin was 95 °C for 2 min, followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 15 s, and final extension at 72 °C for 5 min. Amplicons were subjected to 1.5% agarose gel electrophoresis and the gels were stained with SYBRTM Gold nucleic acid gel stain (Life Technologies, USA).

PCR products of the expected size were extracted from the gel and purified using an IllustraTM GFXTM PCR DNA and Gel Band Purification Kit (GE Healthcare, USA), according to the manufacturer's instructions. Sequencing reactions were performed in an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems, Life Technologies, EUA) using a BigDyeTM Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Life Technologies, EUA), according to the manufacturer's instructions.

Phylogenetic analysis and tree topology comparison

A search for similarity of sequences generated and edited with BioEdit Sequence Alignment Editor Software version 7.0.9 [15] was carried out using BLAST software version 2.0 [16]. The ClustalW software version 1.4 was used for alignment and editing of nucleotide and deduced amino acid sequences obtained [17]. Jalview version 2.11.1.4 [18] was used as multiple sequence alignment viewer. The distance matrices from the percentages of similarity and identity among nucleotide and deduced amino acid sequences were generated using the MatGAT software, version 2.0 [19]. The level of nucleotide substitution saturation was evaluated in DAMBE software [20], by plotting transitions and transversions against pairwise genetic distance, and was used for detecting phylogenetic signals. Additionally, substitution saturation was also evaluated with Xia's test [21] in DAMBE. Phylogenetic reconstructions were performed in MEGA version 11 [22] and IQ-TREE version 2.1.3 [23] software by the neighbor joining (NJ) and maximum-likelihood (ML) methods. JC (Jukes and Cantor, 1969) was selected as the best-fitting evolutionary model according to Corrected Akaike Information (AICc) and Bayesian Information (BIC) Criteria implemented in jModelTest version 2.1.10 [24]. For phylogenetic trees, bootstrap nodal support for 1000 pseudo-replicates was used [22]. Additionally, topological analyses among the phylogenetic reconstructions obtained (NJ and ML trees/MEGA and IQ-TREE software) by using the RELL approximation method [25], including bootstrap proportion, and approximately unbiased (AU) [26], Kishino-Hasegawa (KH) [27], and Shimodaira-Hasegawa (SH) [28] tests which were performed in IQ-TREE with 10,000 RELL replicates, aiming to identify differences statistically significant ($p < 0.05$) among the tree topologies.

Results

Nested-PCR

All samples were positive result for the β-actin gene. Six samples were positive for CyHV-3 among the 100 samples analyzed (positivity index = 6%), and four out of the positive samples were confirmed by nucleotide sequencing (Table 1). The positive samples were from ornamental fish of the species *Pygocentrus nattereri*, *Cyprinus carpio*, *Misgurnosanguillicaudatus*, *Carassius auratus*, *Xiphophorus maculatus* and *Hyphessobryconeque*. *Carassius auratus* and *Hyphessobryconeque* were asymptomatic. Specimens of *Xiphophorus maculatus*, *Misgurnusanguillicaudatus* and *Cypriniscarpio* presented apathy as a unique symptom, which, despite being one of the clinical signs described in CyHV-3 infection, is nonspecific and may present in common viral and bacterial infections. *Pygocentrus nattereri* had lethargy and erratic swimming; however, this specimen was co-infected with Megalocytivirus and this signal is characteristic of infections caused by this genus. Four samples were sequenced by the Sanger method and these sequence data were submitted to the GenBank databases. The virus isolates and their accession numbers in Genbank are *Pygocentrus nattereri* (CyHV-3(358); MW015816), *Misgurnusanguillicaudatus* (CyHV-3(417); MW015817); *Hyphessobrycon eques* (CyHV-3(427); MW015818); *Carassius auratus* (CyHV-3(445); MW015819).

Phylogenetic analysis and tree topology tests

The identity analysis of the CyHV-3 nucleotide sequences revealed identity values greater than 97.7%, when the four Brazilian sequenced samples were compared to each other and values greater than 97.4% when the samples obtained in this study were compared with other corresponding nucleotide sequences deposited on GenBank (Table 2).

The level of substitution saturation was evaluated by plotting transitions and transversions against genetic distance (JC) for the dataset (Fig. 1). This procedure showed that the frequency of both transitions and transversions increased linearly along with divergence, with transversions outnumbering transitions. This indicated that the saturation plateau had not been reached, and that data still retained adequate phylogenetic signal. Moreover, Xia's test supported little

Table 2 The similarity and identity of CyHV-3 sequences obtained from four Brazilian fish samples when compared to sequences of other CyHV-3 strains obtained from the GenBank database

Similarity/Identity (%)										
Vírus	CyHV-3(358)	CyHV-3(417)	CyHV-3(427)	CyHV-3(445)	AB375385	AB375386	AB375387	AB375389	AB375390	AB375391
CyHV-3(358)		97.7	97.7	98.7	98.4	98.4	98.4	98.4	98.4	98.4
CyHV-3(417)	98.4		98.0	98.0	97.7	97.7	97.7	97.7	97.7	97.7
CyHV-3(427)	97.7	98.0		97.7	98.4	98.4	98.4	98.4	98.4	98.4
CyHV-3(445)	98	97.4	97.4		97.7	97.7	97.7	97.7	97.7	97.7
AB375385	98.0	97.7	98.4	97.4		100.0	100.0	100.0	100.0	100.0
AB375386	98.0	97.7	98.4	97.4	99.7		100.0	100.0	100.0	100.0
AB375387	98.0	97.7	98.4	97.4	99.7	99.7		100.0	100.0	100.0
AB375389	98.0	97.7	98.4	97.4	99.7	99.7	99.7		100.0	100.0
AB375390	98.0	97.7	98.4	97.4	99.7	99.7	99.7	99.7		100.0
AB375391	98.0	97.7	98.4	97.4	99.7	99.7	99.7	99.7	99.7	
HM347096	98.0	97.7	98.4	97.4	99.7	99.7	99.7	99.7	99.7	99.7
HM347097	98.0	97.7	98.4	97.4	99.7	99.7	99.7	99.7	99.7	99.7
HM347098	98.0	97.7	98.4	97.4	99.7	99.7	99.7	99.7	99.7	99.7
JN180630	98.0	97.4	98.0	97.0	99.3	99.3	99.3	99.3	99.3	99.3
JQ247182	97.7	97.7	98.4	97.4	99.7	99.7	99.7	99.7	99.7	99.7
KP280047	98.0	97.7	98.4	97.4	99.7	99.7	99.7	99.7	99.7	99.7
KT290517	98.0	97.7	98.4	97.4	99.7	99.7	99.7	99.7	99.7	99.7
AP008984	98.0	97.7	98.4	97.4	99.7	99.7	99.7	99.7	99.7	99.7
DQ657948	98.0	97.7	98.4	97.4	99.7	99.7	99.7	99.7	99.7	99.7
DQ177343	98.0	97.7	98.4	97.4	99.7	99.7	99.7	99.7	99.7	99.7

Similarity/Identity (%)										
Vírus	HM347096	HM347097	HM347098	JN180630	JQ247182	KP280047	KT290517	AP008984	DQ657948	DQ177343
CyHV-3(358)	98.4	98.4	98.4	98	98.4	98.4	98.4	98.4	98.4	98.4
CyHV-3(417)	97.7	97.7	97.7	97.4	97.7	97.7	97.7	97.7	97.7	97.7
CyHV-3(427)	98.4	98.4	98.4	98	98.4	98.4	98.4	98.4	98.4	98.4
CyHV-3(445)	97.7	97.7	97.7	97.4	97.7	97.7	97.7	97.7	97.7	97.7
AB375385	100.0	100.0	100.0	99.7	100.0	100.0	100.0	100.0	100.0	100.0
AB375386	100.0	100.0	100.0	99.7	100.0	100.0	100.0	100.0	100.0	100.0
AB375387	100.0	100.0	100.0	99.7	100.0	100.0	100.0	100.0	100.0	100.0
AB375389	100.0	100.0	100.0	99.7	100.0	100.0	100.0	100.0	100.0	100.0
AB375390	100.0	87.8	87.8	99.7	100.0	100.0	100.0	100.0	100.0	100.0
AB375391	100.0	87.8	87.8	99.7	100.0	100.0	100.0	100.0	100.0	100.0
HM347096		87.8	87.8	99.7	100.0	100.0	100.0	100.0	100.0	100.0
HM347097	99.7		100.0	99.7	100.0	100.0	100.0	100.0	100.0	100.0
HM347098	99.7	99.7		99.7	100.0	100.0	100.0	100.0	100.0	100.0

Table 2 (continued)

JN180630	99.3	99.3			99.7	99.7	99.7	99.7	99.7	99.7	
JQ247182	99.7	99.7		99.3		100.0	100.0	100.0	100.0	100.0	
KP280047	99.7	99.7		99.3	99.7		100.0	100.0	100.0	100.0	
KT290517	99.7	99.7		99.3	99.7	99.7		100.0	100.0	100.0	
AP008984	99.7	99.7		99.3	99.7	99.7	99.7		100.0	100.0	
DQ657948	99.7	99.7		99.3	99.7	99.7	99.7	99.7		100.0	
DQ177343	99.7	99.7		99.3	99.7	99.7	99.7	99.7	97.7		

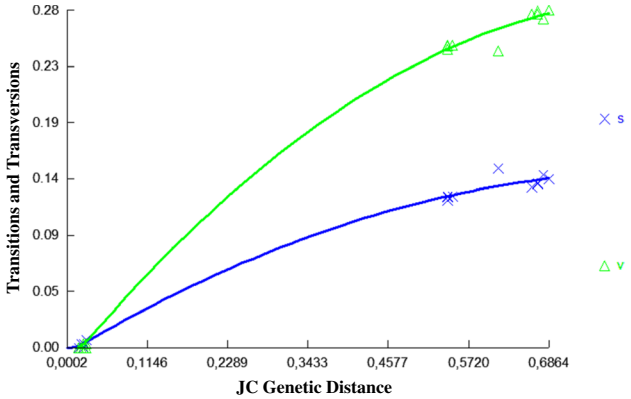

Fig. 1 The number of transitions (×) and transversions (Δ) versus of the genetic distance calculated with the JC among all pairwised nucleotide sequences of CyHV-3 TK gene. Solid lines indicate the best fit found in each mutational type. The "s" and "v" represented the number of transitions (×) and transversions (Δ), respectively

saturation for CyHV-3 sequences (Iss < Iss.c, $p < 0.0005$), supporting graphical results.

A multiple alignment for deduced amino acid partial sequences of the TK gene from Brazilian *Cyprinid herpesvirus* 3 and other sequences retrieved from GenBank is depicted in Fig. 2. Table 3 shows the *p*-values for the tree topology tests carried out. Based on topological analyses, all phylogenetic trees displayed a very similar topology ($p > 0.05$), indicating that neither the reconstruction model (NJ or ML) nor the software (MEGA/IQ-TREE) added greater resolution to the phylogeny. Thus, we selected the ML/IQ-TREE tree to illustrate the phylogenetic relationship among the nucleotide CyHV-3 sequences due to the relatively higher p-values found. The phylogenetic reconstruction for Cyprinid herpesvirus 3 based on the TK gene (Fig. 3), using 348-bp sequences revealed the existence of 2 distinct groupings: one Brazilian sample in association with the European and Asian genotypes of CyHV-3 and a second grouping containing only Brazilian sequences described in this study. Even though, the genetic distances between all sequences are relatively small, corroborating a high degree of conservation of the TK gene.

Discussion

The occurrence of CyHV-3 in carps is a reality around de word and is associated with high morbidity and mortality rates among the affected animals, causing severe economic losses [1, 29]. Because of this, the infection by CyHV-3 has been designated as a notifiable disease by OIE.

This is the first description of CyHV-3 in Brazil. The main cause for the rapid spread of the virus is the intense trade of ornamental fish that most of the time

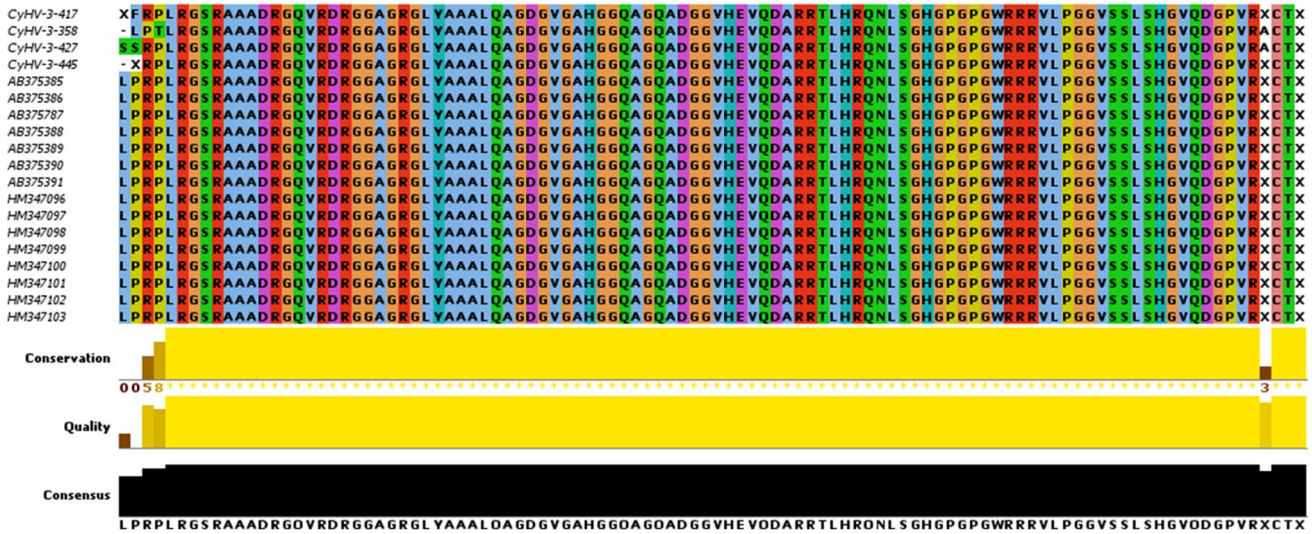

Fig. 2 Multiple alignment among the 102-deduced amino acid sequences of the TK gene from *Cyprinid herpesvirus* 3 from Brazilian samples and from other *Cyprinid herpesvirus 3* recovered from GenBank

happens without the certification of animal health, as well as the carp exhibitions by collectors [13, 30, 31]. In addition, deficiencies in rapid diagnostic methods available on the market today and the absence of stricter laws to prevent the introduction of the virus within different countries also contributes significantly to the spread of the disease [32].

Carp is the main species affected by this virus, in line with other studies such as the one carried out by Rahmati-Holasoo et al. (2016) who also described the occurrence of CyHV-3 infection in fish of this species [29]. In this study, the carp positive to the molecular diagnosis for CYHV-3 showed only lesions on the body, indicating that the animal's internal organs were probably not affected severely. Some studies show that CYHV-3 infection is temperature dependent and in certain seasons of the year, carp have a low viral load, acting as natural reservoirs of the virus [29]. These asymptomatic carriers continuously release the virus into the water making it possible to infect other animals. When these animals are subjected to stressful conditions as low temperatures, the viral titer tends to increase, causing serious episodes of mortality.

Despite predominantly infecting carp, in this study, unprecedented in Brazil, it has been reported the occurrence of CyHV-3 in other species of ornamental fish. Bergmann et al. (2009) also reported the occurrence of virus infection in other species of ornamental fish, including the species *Carassius auratus*; in addition, these fish were asymptomatic and, therefore, apparently healthy, in the same way as the fish analyzed by this work [33]. According to Bergmann et al. (2010), *Carassius auratus* is susceptible to infection by CyHV-3 but, after the occurrence of infection, the fish does not develop the disease, becoming asymptomatic carrier of the virus and, therefore, a potential source of infection of the virus to other fish species [34]. One of the main explanations for the occurrence of CyHV-3 in other species is the cohabitation of these species in tanks containing fish of the species *Cyprinus carpio* infected by CyHV-3 [10, 35].

The phylogenetic reconstruction for Cyprinid herpesvirus 3 based on the TK gene (Fig. 1) revealed the existence of 2 distinct groupings, with one Brazilian sample in association with the European and Asian genotypes of CyHV-3 and a second grouping, containing only Brazilian sequences described in this study. Even though, the genetic distances between all sequences

Table 3 Statistical tests for three competing hypotheses related to phylogenetic reconstruction model (NJ or ML) and software (MEGA/IQ-TREE). Statistical tests of significance ($p < 0.05$) were conducted for different competing phylogenetic trees in IQ-TREE, using combined data sets by the RELL approximation method with 10,000 resamplings ranked by likelihood. The abbreviations used are as follows: *NJ* neighbor joining; *ML* maximum-likelihood; *deltaL* logL difference from the maximal logl in the set; *bp-RELL* bootstrap proportion using RELL method; *AU* approximately unbiased test; *KH* the Kishino–Hasegawa test; *SH* the Shimodaira–Hasegawa test; *: the best tree

Tree (method/software)	deltaL	bp-RELL	*p* values		
			KH	SH	AU
*ML/IQ-TREE	0	0.249	0.547	1.000	0.551
NJ/MEGA	1×10^{-4}	0.334	0.453	0.453	0.449
ML/MEGA	1×10^{-4}	0.417	0.453	0.453	0.449

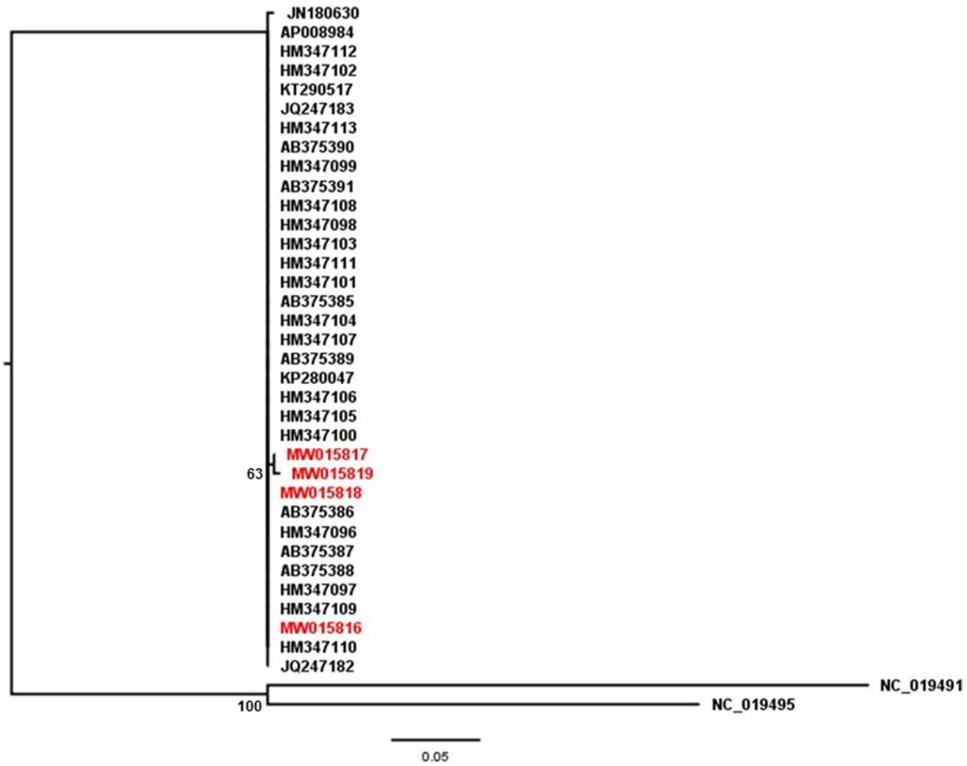

Fig. 3 Phylogram representing a rooted phylogenetic tree of sequences using a 348-bp fragment of the TK gene of *Cyprinid herpesvirus 3* by maximum likelihood method (JC). Bootstrap values greater than 50% obtained from 1,000 pseudoreplicates are shown at the appropriate branch points. The Brazilian sequences obtained in the present study are highlighted in red. The scale bar represents the phylogenetic distance between sequences. NC_019491 (CyHV-1) and NC_019495 (CyHV-2) were used for tree rooting

are relatively small, corroborating a high degree of conservation of the TK gene. Nevertheless, the nucleotide sequence data set still retained adequate phylogenetic signal. Dong et al. (2013), when performing phylogenetic analysis containing part of the sequences obtained from the GenBank described in this study, also observed the formation of 2 clades, in which the CyHV-3 sequences grouped with the Asian genotype and European genotype in separate clades; however, it should be noted that the sequences used for phylogenetic reconstruction were larger (651-bp), allowing possibly a better discriminatory power than that verified in the present study [36].

Conclusion

This is the first report of the occurrence of *Cyprinid hespervirus 3* in Brazilian ornamental fish. In addition, the presence of the virus was observed in other ornamental fish species besides *Cyprinus carpio carpio*, which indicates that these fish can act as potential carriers of the virus. These results contribute for a better understanding of the molecular epidemiology of CyHV-3 in Brazilian ornamental fish and in association with further studies of viral isolation and characterization can help in establishing effective surveillance and disease control program.

Author contribution **Conceived for designed study:** Samara Rita de Lucca Maganha, Pedro Henrique Magalhães Cardoso, Sabrina Ribeiro de Almeida-Queiroz and Ricardo Luiz Moro de Sousa. **Performed research:** Samara Rita de Lucca Maganha. **Analyzed data:** Samara Rita de Lucca Maganha, Sabrina Ribeiro de Almeida-Queiroz and Ricardo Luiz Moro de Sousa. **Contributed new methods or models:** Samara Rita de Lucca Maganhaand Ricardo Luiz Moro de Sousa. **Wrote the paper:** Samara Rita de Lucca Maganha, Pedro Henrique Magalhães Cardoso, Simone de Carvalho Balian, Andrezza Maria Fernandes and Ricardo Luiz Moro de Sousa.

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Data availability The sequences obtained in this study are available in GenBank.

Code availability Not applicable.

Declarations

Ethics approval All applicable institutional guidelines for the care and use of animals were followed (CEUA n° 6782040416).

Consent to participate All authors have agreed to the participate.

Consent for publication All authors have read and agreed to the published version of the manuscript.

Conflict of interest The authors declare no competing interests.

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