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FISH PARASITOLOGY - ORIGINAL PAPER

Kudoa sp. (Myxozoa, Multivalvulida): first report in five commercial fish species from the Canary Islands-FAO 34 (Macaronesia-Spain)

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Abstract

Kudoid myxozoans have been reported causing serious chronic problems in marine fisheries, by reducing the market value of infected fish through pathological damage to the host musculature. We report here the overall prevalence of a *Kudoa* species in 84/277 (30.3%) fishes from 20 different species of high commercial value captured between October 2011 and December 2013 from the United Nations Food and Agriculture Organization (FAO) 34 commercial fishing area, near the coast of the Canary Islands (Spain). Macroscopic examination showed myxozoan-like cysts in skeletal muscle from 5 of the 20 fish species examined, with the following prevalences: *Pagellus acarne* (86.7%), *Pagellus erythrinus* (46.5%), *Serranus cabrilla* (27.8%), *Spondyliosoma cantharus* (19.4%), and *Sarpa salpa* (28.6%). Infection intensity was determined based on spore counts following muscle tissue digestion. Morphometric studies to characterize the species and DNA sequence analysis results suggest that these infections are attributable to a *Kudoa* species closely related to *Kudoa trachuri*. This paper reports the first study on a multivalvulidan species to be identified from the Canary Islands. Furthermore, this is the first report of *Kudoa* parasites in all of the hosts mentioned above, with the exception of *P. acarne*.

Keywords Canary Islands · Commercial fish · *Kudoa* sp. · FAO 34 · Myxozoa · Parasite

Introduction

The Canary Islands form an archipelago located off the northwest coast of mainland Africa. The Canarian jurisdictional waters have three national maritime borders, with Portugal, Morocco, and Western Sahara. The seafloor bathymetry around the islands is typically abrupt with a narrow shelf and a steep slope plunging to more than 1000-m depth, which produces near-shore conditions similar to the open ocean (Popescu and Ortega Gras 2013).

The archipelago is considered the third Spanish region in aquaculture production (PEACAN 2014) where the estimated

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fishing production for 2020 is around 10,835 tons. Aquaculture installations have the capacity to attract wild fish species due to a constant supply with additional food. Aggregating wild fish may be affected, among other factors, by their parasite load (Tuya et al. 2005). Detecting parasites in commercial wild fish could be useful for knowing the health conditions in Canarian coastal fish populations and its possible impact on fisheries.

The family Kudoidae includes myxosporean parasites that infect various marine teleosts. More than 100 nominal species of genus *Kudoa* have been described (Eiras et al. 2014; Sato and Kasai, 2016) with a wide geographical distribution and a negative effect caused by macroscopic cysts in somatic muscles. Several species also may cause post-mortem myoliquefaction (Abe and Maehara 2013), which have direct impacts in fisheries and aquaculture (Eiras et al. 2014). Moreover, they may induce hazards to human health, with the possibility of food and/or allergic poisoning (Iwashita et al. 2013).

Characterization of *Kudoa* parasites at the species level was initially based on the morphology and morphometry of the spores (Lom and Arthur, 1989). But these morphological characteristics were limited and the characterization cannot depend entirely on the shape and dimensions of the mature spore, due to limited morphological features. Besides some species, that were previously considered as different based on the infection of different hosts species and/or their different geographic distribution, classification has been revised taking into account ribosomal DNA (rDNA) information, with more accurate identification achieved by combining DNA sequence and morphometric data (Adlard et al. 2005; Burger and Adlard 2010, 2011; Heiniger et al. 2013; Whipps et al. 2003, 2004).

In this paper, we report the occurrence of kudoid species in natural seawater fish around the Canary Islands, Atlantic Ocean (Spain), and our attempt to identify the species found in trunk muscle of consumed fish. We present morphological analysis of *Kudoa* sp., with four shell valves and polar capsules, from the skeletal muscle of five different fish species. In addition, genetic analyses were also conducted to clarify the taxonomic relationships of these isolates, as compared with other *Kudoa* species.

Materials and methods

Sample collection

Two hundred seventy-seven individual fishes from 20 different species were collected from Canary Islands coast: Eastern Central Atlantic: Major Fishing Area 34—Subarea 34.1 (FAO 1990–2018), between October 2011 and December 2013 (Table 1). Fishes were caught for consumption. These samples were sent to the Veterinary Faculty of the University of Las Palmas de Gran Canaria (ULPGC). Identification of fish hosts conforms to currently valid names and common names given in FishBase (http://www.fishbase.org/).

The total length of the fish varied between 17 and 34 cm. Each fish was necropsied and, after dissection, all organs were removed and examined under a stereomicroscope. To detect spores, 100 mg of minced muscle sample was processed in a duplicate analyses to obtain a spore suspension in phosphate-buffered saline solution (Samaranayaka et al. 2006) and, if present, the number of spores was counted under a microscope using a Thoma chamber.

When *Kudoa* cysts were found (in 84/277 fish examined), a spore suspension in saline solution was prepared on a glass slide, covered with a glass coverslip and examined under a microscope for recording of morphological characteristics. Kudoid spores were preserved by freezing for DNA extraction. The remaining cysts were frozen in situ with the rest of the fish.

Morphometric analysis

Wet mount preparations from 54 *Kudoa*-positive fishes of five different host species were observed to examine the morphology of cysts (mm) and spores (μm). Photographs were taken with a Leica DM6000 B microscope (Leica Microsistemas S.L.U., Barcelona, Spain) equipped with a Leica DFC 495 digital camera, using differential interference contrast (DIC) microscopy. Morphological measurements of spores followed the guidelines proposed by Lom and Arthur (1989) for species descriptions of Myxosporea and were made as previously described by Adlard et al. (2005). A total of 1620 spores measurements were taken and 50 cysts per fish species.

DNA extraction, polymerase chain reaction

Parasite DNA was extracted from frozen kudoid cysts isolated from five species hosts: *Pagellus acarne* ($n = 10$), *Pagellus erythrinus* ($n = 1$), *Serranus cabrilla* ($n = 2$), *Sarpa salpa* ($n = 1$), and *Spondyliosoma cantharus* ($n = 1$) using an UltraClean™ Tissue DNA Isolation Kit (Mo Bio Laboratories, Qiagen Inc., Madrid, Spain) according to the manufacturer's instructions. The quantity and quality of the DNA were measured by spectrophotometry. Polymerase chain reaction (PCR) amplification of two overlapping fragments of 18S rDNA was performed in a 25-μl volume using a AmpliTaq® DNA Polymerase (Thermo Fisher Scientific Inc.) and primers as listed in Table 3. The PCR conditions were established according to the literature (Hillis and Dixon 1991; Whipps et al. 2003; Whipps et al. 2004). The cycling protocol was 3 min at 95 °C, then 35 cycles of 30 s at 94 °C, 45 s at 53 °C, and 90 s at 72 °C, followed by a final extension at 72 °C for 10 min.

Table 1 Macroscopic examination of fish host species caught during October 2011; October 2012; and April, October, and November 2013

Order	Fish host species	Family	2011			2012			2013			TOTAL		
			N	KM	%	*N*	KM	%	*N*	KM	%	Σ*N*	Σ KM	Σ KM%
Perciformes	*Sparisoma cretense*	*Scaridae*	13	0	0	3	0	0	20	0	0	36	0	0
	Pagellus acarne	*Sparidae*	9	9	100	13	10	84.6	23	20	86.9	45	39	86.7
	Pagellus erythrinus		12	5	41.7	5	2	40	26	13	50	43	20	46.5
	Spondyliosoma cantharus		6	1	16.7	7	2	28.6	18	3	16.7	31	6	19.4
	Pagrus auriga								1	0	0	1	0	0
	Diplodus sargus								4	0	0	4	0	0
	Sarpa salpa		3	2	66.7	5	2	40	41	10	24.4	49	14	28.6
	Serranus cabrilla	*Serranidae*	8	3	37.5				10	2	20	18	5	27.8
	Serranus scriba								1	0	0	1	0	0
	Scomber colias	*Scombridae*	2	0	0							2	0	0
	Acanthocybium solandri								1	0	0	1	0	0
	Katsuwonus pelamis		2	0	0				4	0	0	6	0	0
	Sphyraena viridensis	*Sphyraenidae*							1	0	0	1	0	0
	Pomadasys incisus	*Haemulidae*	10	0	0							10	0	0
	Seriola rivoliana	*Carangidae*	1	0	0							1	0	0
	Mullus surmuletus	*Mullidae*							1	0	0	1	0	0
Tetraodontiformes	*Balistes capriscus*	*Balistidae*				3	0	0	2	0	0	5	0	0
Cupleiformes	*Sardina pilchardus*	*Cupleidae*							12	0	0	12	0	0
	Sardinella aurita								9	0	0	9	0	0
Scorpaenaniformes	*Chelidonichthys lastoviza*	*Triglidae*	1	0	0							1	0	0
Σ*N* = 277 ΣKM = 84			67	20		36	16		174	48		277	84	30.32

N Number of fishes analyzed, *KM* myxozoan-like cysts in skeletal muscle under macroscopic analysis; % percentage of myxozoan-like cysts in skeletal muscle under macroscopic analysis

Sequencing and sequence analysis

PCR were purified following a one-step method based on improved ethanol precipitation and acrylamide published by Fregel et al. (2010). Purified PCR products were sequenced in both directions to ensure reading accuracy, using primers listed in Table 3. The sequencing reaction was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific Inc.), according to the manufacturer's protocol.

The resulting chromatograms were examined in Finch TV (Version 1.4.0; Geospiza Inc.; Seattle, WA, USA; http://www.geospiza.com). The forward sequence and the reverse complemented sequences of the samples were compared to produce a contiguous sequence using Gene Runner (Version 4.0.9.68 Beta; Hastings Software Inc.). Sequences were then aligned automatically with Clustal W (Thompson et al. 1994) and then the alignment was checked and adjusted manually using BioEdit Version 7.2.5 (Hall 1999). This program was also used after a BLAST search (Altschul et al. 1997) to determine the percent identity between the sequences isolated in this study and sequences found in DDBJ/EMBL/GenBank data bases.

Phylogenetic analysis

One from each host of newly obtained 18S rDNA sequences of *Kudoa* sp. in the present study and *Kudoa*-related sequences retrieved from the DDBJ/EMBL/GenBank databases were used for phylogenetic analysis. The accession numbers of the sequences analyzed are given in the figures showing phylogenetic trees. All positions containing gaps and missing data were eliminated. The final datasets were composed of 1424 positions. The sequences were aligned as previously described and the phylogenetic analysis was conducted in Mega 6 (Tamura et al. 2013). The evolutionary history was inferred by using the maximum likelihood method based on the Tamura-Nei model (Tamura and Nei 1993) and the trees shown were the trees with the highest log likelihood. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. *Unicapsula* sp. (Trilosporidae, Multivalvulida) was used as an outgroup for the construction of phylogenetic trees. There were a total of 1472 positions in the final dataset.

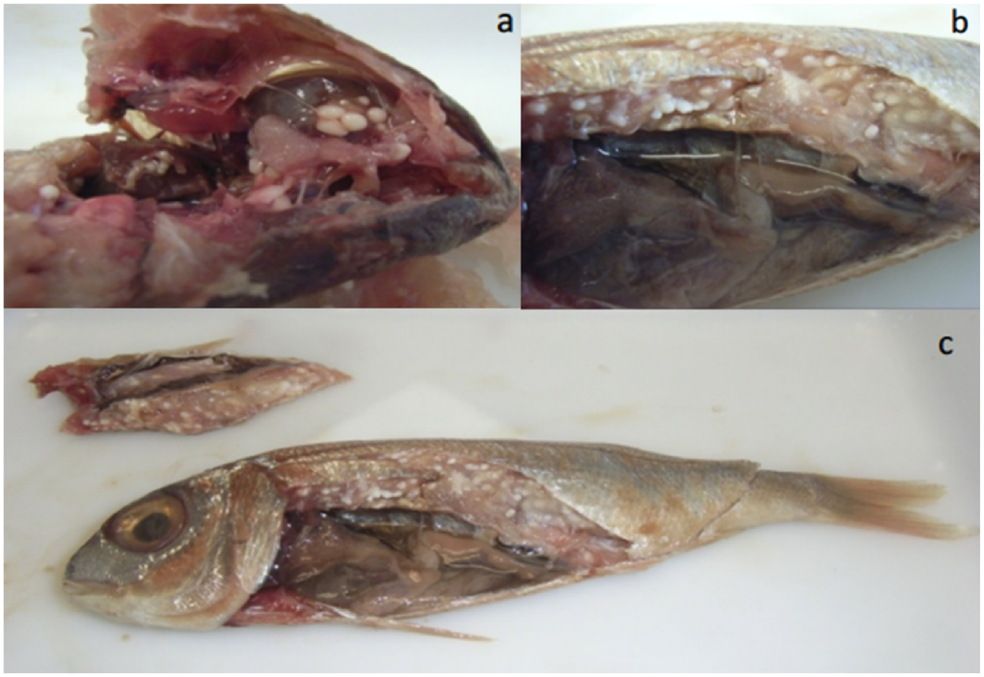

Fig. 1 **a**–**c** Photos of mature *Kudoa* cysts in situ in skeletal muscle of *Pagellus acarne* showing the intensity infection

Results

The present investigation indicated that five (4 Sparidae and 1 Serranidae) out of the 20 fish species sampled had cysts in skeletal muscle (Fig. 1). No other fishes from the total array studied were found to be infected by this parasite. The overall prevalence of infection in all species combined was 30.3% (84/277). More than 50 cysts were counted per infected fish. Cysts were recorded as whitish to creamy in color, subspherical to ovoidal in shape. Cyst size was different in each fish infected, being greater in *Pagellus acarne* (1.3–3.0 mm × 1.1–1.4 mm), and specially smaller and spherical cysts in *Sarpa salpa* with 0.3–0.6 mm in diameter and not easy to detect with the naked eye (Table 2).

Spores

Microscopic examination revealed aggregates of numerous *Kudoa* spores in cysts (Fig. 2). In apical view, spores were rounded to subquadrate with four equal-sized shell valves without ornamentation. Sutural lines were evident; spores normally contained four pyriform and equal-sized polar capsules (Table 2). Aberrant spores with five to seven shell valves and polar capsules were rarely observed.

PCR, sequence analysis, and percent DNA identity

The amplification of the PCR products, using described primers (Table 3), yielded a single product in each case

Table 2 Comparison of dimensions of *Kudoa* spp. cysts and spores with *Kudoa trachuri*. Spores ($n = 1620$) with four polar capsules, infecting five different fishes from Continental Shelf of the Canary Islands

				Cyst (mm)*		Spores (μm)**		Polar Capsules (μm)**	
Kudoa species	Host	*N*	*n*	Length	Width	Thickness	Width	Length	Width
Kudoa sp.	*Pagellus acarne*	17	510	1.3–3.0	1.1–1.4	5.3; 1.4 (4.0–6.6)	7.8; 1.5 (6.0–9.7)	3.5; 0.9 (2.6–4.4)	2.4; 0.6 (1.8–3.1)
Kudoa sp.	*Pagellus erythrinus*	12	360	1–1.3	0.7–1	5.2; 1.6 (3.2–7.3)	7.3; 1.8 (5.6–8.9)	3.6; 0.7 (3.0–4.3)	2.5; 0.4 (2.0–3.0)
Kudoa sp.	*Serranus cabrilla*	10	300	0.5–1.6	0.3–1.1	5.4; 1.2 (3.9–7.0)	7.1; 1.4 (5.4–8.9)	3.6; 0.7 (2.7–4.5)	2.4; 0.5 (1.7–3.1)
Kudoa sp.	*Spondiliosoma cantharus*	7	210	0.3–1.7	0.2–0.9	5.6; 0.7 (4.9–6.2)	7.8; 1.1 (6.4–9.2)	2.9: 0.5 (2.3–3.6)	1.8; 0.4 (1.3–2.4)
Kudoa sp.	*Sarpa salpa*	8	240	0.3–0.6	0.3–0.6	5.2; 1.7 (3.1–7.3)	7.4; 1.6 (5.4–9.4)	3.1; 0.8 (2.1–4.1)	2.0; 0.5 (1.7–2.3)
Kudoa trachuri (Matsukane et al. 2011)	*Trachurus japonicus*			0.5–1.6	0.3–1.1	5.3–6.2	7.0–8.5	2.6–3.5	1.6–2.2

N number of fish of each host analyzed, *n* number of spores measured. *Measurements taken from 50 cysts; **$\bar{x}$; SD (confidence interval at 95%)

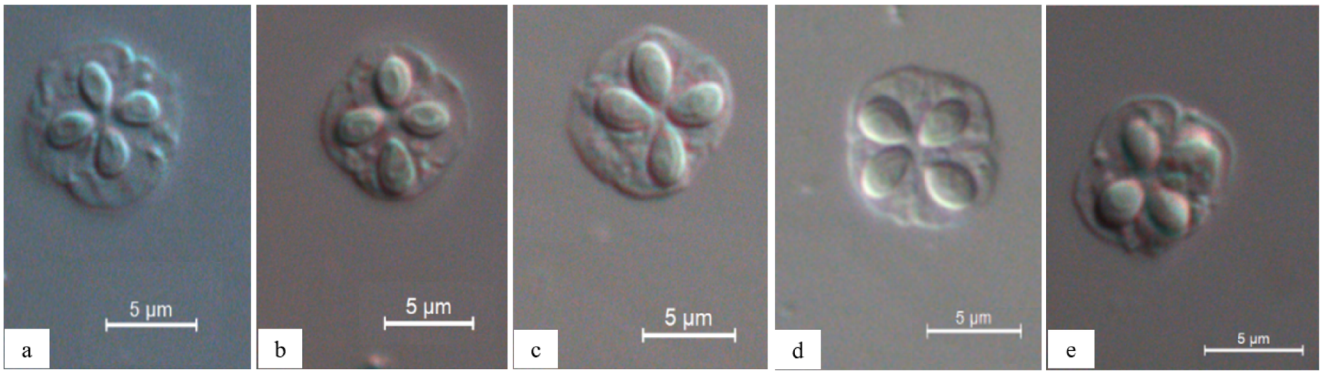

Fig. 2 DIC photomicrographs of fresh spores in apical view of *Kudoa* sp. from *Serranus cabrilla* (**a**), *Sarpa salpa* (**b**), *Spondyliosoma cantharus* (**c**), *Pagellus acarne* (**d**), and *Pagellus erythrinus* (**e**)

and they were successfully sequenced.. Near-complete sequences of the 18S rDNA (1734 bp) were obtained from each fish species and representative sequences submitted to GenBank (Table 4).

The analysis of the near-complete 18S rRNA gene alignment demonstrated no differences between our sequences, regardless of host origins. Where partial sequences (800 bp) were obtained, these corresponded to the first part of the 18S rRNA gene and were identical to each other and to our other sequences where they overlapped. BLAST search analysis showed that the closest *Kudoa* species was *Kudoa trachuri*, with an identity value of 99%. Further BioEdit analyses of the percentages of identity between this *Kudoa* sp. and the range of other myxosporidian species demonstrated a high degree of similarity (>91.0%) with some *Kudoa* spp. The sequences were 98.5% similar to *K. trachuri* (22 nucleotide differences/1534 bases compared), presenting three short indels (insertions-deletions). The next most similar sequences were *Kudoa crumena* and *Kudoa scomberi* (95.2%), and *Kudoa thunni* (94.8%), while the rest of the *Kudoa* spp. analyzed presented a similarity value below 92%.

Phylogenetic analysis

The resulting phylogenic tree inferred from the 18S rDNA sequences by maximum likelihood shows a close association of our *Kudoa* isolates with *K. trachuri*, *K. scomberi*, *K. crumena*, and *K. thunni* (Fig. 3).

Discussion

The *Kudoa* spp. detected in this study infect five hosts belonging to 2 different families from the order Perciformes. In our study, the 18S rDNA sequences are the same for all the samples analyzed, despite some variations in spore size that were detected among the isolates from different fish species. It is known that kudoid species display a degree of morphometric plasticity (Whipps and Kent 2006) and differences in spore morphometry in the same genetic species have also been reported for *K. amamiensis* and *K. hypoepicardialis* (Blaylock et al. 2004; Burger et al. 2008; Burger and Adlard 2011).

Whipps et al. (2003) think that sequence identity alone may be inadequate to identify new myxozoan species and could be very useful when used together with morphological characters. They also used this premise to describe *K. minithyrtites* as a distinct, but sister, species from *K. thyrsites*. Burger and Adlard (2011) agree that because of the morphometric plasticity of kudoid species, an independent character such as DNA sequence should be employed to help establish species boundaries.

In this study, our *Kudoa* spp. are very closely related to *K. trachuri* (Matsukane et al. 2011), with a 18S rDNA similarity of 98.5%, differing in 22 nucleotides from 1534 bases compared. Our phylogenetic analysis placed our *Kudoa* sp. as a sister lineage to *K. trachuri* isolates (Fig. 3). In myxozoans, small rDNA sequence variations may be related to distinct populations from different hosts or related to geographical and ecological separation instead to speciation. Eiras et al.

Table 3 Primers used to amplify and sequence two overlapping segments of 18S rDNA of *Kudoa* spp.

Primer name	Sequence	Reference
18e (Forward)	5′-CTGGTTGATCCTGCCAGT-3′	Hillis and Dixon (1991)
18Rf (Reverse)	5′-CTACGGAAACCTTGTTACG-3′	Whipps et al. 2003
Kud6Fc (Forward)	5′-TCACTATCGGAATGAACG-3′	Whipps et al. 2003
Kud6R (Reverse)	5′-TCCAGTAGCTACTCATCG-3′	Whipps et al. 2004

Table 4 GenBank accession numbers

Samples	Fish species	18S	GenBank accession no.
BE30-13	*Pagellus acarne*	Complete	MG836983
BE34-13	*Pagellus acarne*	Partial	–
BE13-13	*Pagellus acarne*	Partial	–
BE31-12	*Pagellus acarne*	Partial	–
BE30-12	*Pagellus acarne*	Partial	–
BE23-12	*Pagellus acarne*	Partial	–
BE22-12	*Pagellus acarne*	Partial	–
BE50-11	*Pagellus acarne*	Partial	–
BE51-11	*Pagellus acarne*	Partial	–
BE54-11	*Pagellus acarne*	Partial	–
BR14-13	*Pagellus erythrinus*	Complete	MG836984
CA154-13	*Serranus cabrilla*	Complete	MG836985
CA170-13	*Serranus cabrilla*	Complete	MG836986
CH73-13	*Spondyliosoma cantharus*	Complete	MG836987
SL160-13	*Sarpa salpa*	Complete	MG836988

"–" indicates sequences not published. They are identical to MG836983

(2014) published data (cysts, spores, polar capsules) which, compared with the morphological characteristics of the *Kudoa* isolates in this study, allows to conclude that most of the isolates exhibited morphometric data close to *K. trachuri* for the cyst in four of the five species studied (Table 2), and identical in *Serranus cabrilla*. However, cysts detected in *Pagellus acarne* presented the same measurements as those described for *Kudoa iwatai* in Japanese seaperch (*Lateolabrax japonicus*), although its spores were similar to the spores from the other hosts (Fig. 2) and to those of *K. trachuri*, especially if it is considered that polar capsules accounted for approximately 50% or more of total radius of shell valves (Matsukane et al. 2011).

The fishes of the order Perciformes have been described to be parasitized by numerous myxosporeans with four shell valves infecting skeletal muscles (Mansour et al. 2014). From the five fish species analyzed, there are similarities between the 54 isolates analyzed in the present study (Fig. 2) and

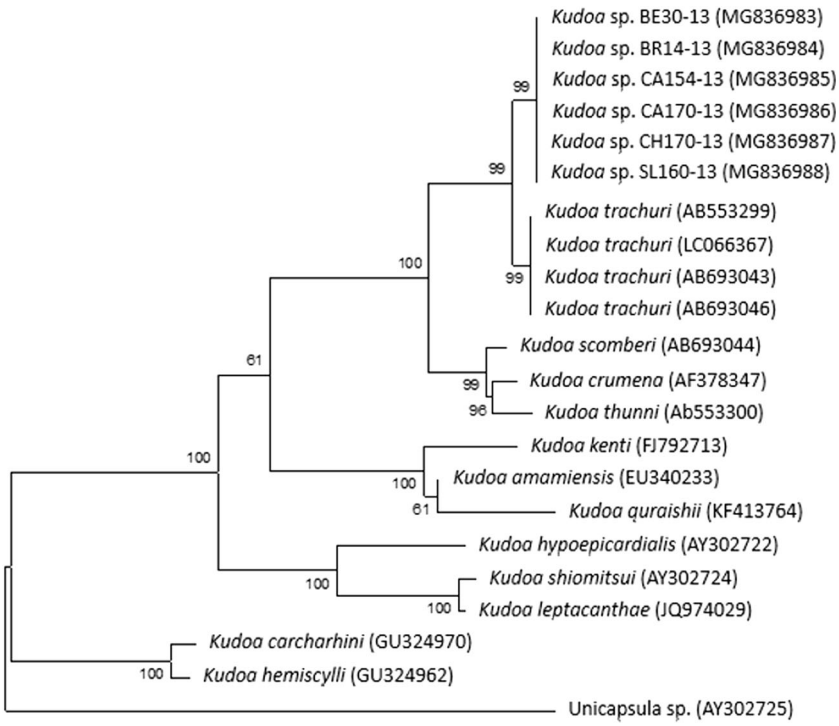

Fig. 3 Phylogenetic relationship showing the taxonomic position of the present isolates in relation to other kudoid species. The tree is based on 18S rDNA sequences. Next to the branches, percentage of trees is shown in which the associated taxa clustered together. *Unicapsula* sp. served as the outgroup

it is possible to conclude that there are no differences in the spore size neither in the size of the polar capsules, resembling every fish studied to *K. trachuri* (Matsukane et al. 2011). Furthermore, differences found in cyst size might be related to the time of infection (Marshall et al. 2016).

The variability required to classify myxozoan organisms as species is currently unknown. Greater base variation in the 28S rDNA sequence than in the 18S rDNA sequence has been shown for several *Kudoa* spp. (Burger and Adlard 2011). In addition, future molecular analysis based on 28S rDNA should be done to confirm the phylogenetic position of *Kudoa* isolates found in this study, which could clarify the identity of these species. In spite of morphology being the foundation of taxonomic classification, there are phenotypical similarities between species and intraspecific morphological variations among kudoid myxozoans, which makes it difficult to distinguish species solely by morphology (Burger and Adlard 2010; Heiniger and Adlard 2012; Heiniger et al. 2013). Moreover, the morphometries of the spores found in this work revealed that the size of these *Kudoa* species was not a distinctive feature that could separate it from all described species having similar morphology. With the exception of the Spanish sea bream (*P. acarne*), which has been reported as host for *Kudoa* sp. (Matsukane et al. 2011), all four kudoid infections in the present study are new host records.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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