Research Article

Survey of *Kudoa* spp. (Myxozoa, Cnidaria) in fishes from the Madeira Archipelago and the Portuguese mainland coast: detection of *Kudoa thyrsites* in new hosts *Scomber colias* and *Micromesistius poutassou*

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Abstract: Myxozoan parasites of the genus *Kudoa* Meglitsch, 1947 are associated with *post-mortem* tissue degradation that causes great financial losses to commercial fisheries. *Kudoa thyrsites* (Gilchrist, 1924) is a species with a very wide host range including commercial tunas, mackerels, salmonids and flatfishes. A sample of 190 fishes of 18 species from the Madeira Archipelago and 30 Atlantic chub mackerel, *Scomber colias* Gmelin, and 30 blue whiting, *Micromesistius poutassou* (Risso), from the Portuguese mainland coast were examined for the presence of species of *Kudoa*. The prevalence of *Kudoa* spp. was 80% in *M. poutassou* and 60% in *S. colias*. No spore was detected in *S. colias* from Madeira, which was confirmed by specific PCR screening of the muscle from all individuals of *S. colias*. SSU rDNA analysis revealed that *M. poutassou* and *S. colias* from the Portuguese mainland coast were infected with *K. thyrsites*, an economically important myxozoan parasite. Both sequences were identical with sequences of the eastern Atlantic *K. thyrsites* genotype, including that from the type host of this parasite. This is the first report of *K. thyrsites* from *M. poutassou* and *S. colias*. The fact that spores of species of *Kudoa* were not detected in fishes screened in the Madeira Archipelago may be explained by various ecological factors, such as the absence of a continental shelf, a short insular shelf, and oceanic waters with low productivity, all resulting in reduced abundance of benthic organisms. Consequently, it is possible that as yet unknown annelid definitive hosts of *Kudoa* spp. are absent or very rare near Madeiran coasts.

Keywords: Atlantic, food safety, parasites, Portugal, scombrid, tuna, Atlantic chub mackerel, blue whiting.

The study of fish parasites has grown over the last decades mainly due to their economic implications in fisheries, aquaculture and, in some cases, risk to human health. Fish parasites that occur in edible parts of the fish are the most relevant for human health. Multivalvulida (Cnidaria: Myxozoa) are widespread and can be associated with mortality or poor flesh quality of commercially important marine fishes (Whipps and Diggles 2006, Kristmundsson and Freeman 2014, Kasai et al. 2016) and, in some cases, foodborne diseases (Suzuki et al. 2015, Takeuchi et al. 2016). *Kudoa* Meglitsch, 1947 is one of the most studied

myxozoan genera whose species infect a wide range of marine fishes from different geographic areas. Around 100 species of *Kudoa* have been described from wild fishes in different regions of the world (Eiras et al. 2014).

In the Eastern Atlantic, they have been reported as parasites of several small pelagic fish species such as *Sardina pilchardus* (Walbaum), *Scomber scombrus* Linnaeus, *Trachurus trachurus* (Linnaeus) and *Trachurus picturatus* (Bowdich) (Gaevskaya and Kovaleva 1985, Cruz et al. 2003, Levsen et al. 2008, Cruz et al. 2011). Spores of species of *Kudoa* have already been detected in the western

Mediterranean, and more southwards alongside the African coast to the Gulf of Guinea in some large pelagic fish species, such as *Thunnus obesus* (Lowe) (Yurakhno and Gorchanok 2011). Wild fishes are not the only suitable intermediate hosts of *Kudoa* spp., because there are also reports of infections in various species from aquaculture. Atlantic salmon, *Salmo salar* Linnaeus, is one of the most affected species, with considerable commercial impact (Goater et al. 2014, Lafferty et al. 2015).

The life cycles of marine myxozoans are poorly known (Eszterbauer et al. 2015), with no data available for species of *Kudoa*. Generally, myxosporean life cycle has two different phases: an actinospore-phase taking place in invertebrate definitive hosts (annelids), where actinospores are produced, and a myxospore-phase that typically occurs in fishes, which are intermediate hosts. The actinospore is released into the water by the annelid and infects fish through direct contact with the gill epithelium or skin (Lom and Dyková 2006, Rangel et al. 2015, Rangel et al. 2016a,b, Atkinson et al. 2019).

Commercial fishes infected by *Kudoa* spp. can be rejected by consumers due to the presence of whitish or black hypertrophic muscular cells, repugnant appearance, or soft texture (*post-mortem* myoliquefaction) induced by these parasites, causing important commercial losses. However, the impact of some species of *Kudoa* on human health is more serious. Several cases of gastrointestinal problems, such as vomiting and diarrhea, were observed after ingestion of raw portions of fish infected with *Kudoa septempunctata* Matsukane, Sato, Tanaka, Kamata et Sugita-Konishi, 2010 (see Kawai et al. 2012, Iwashita et al. 2013). Since 2003 there has been an increase in raw fish consumption and consequently cases of food poisoning caused by *Kudoa* spp. (Kawai et al. 2012).

Myxozoans are generally specific parasites with a few exceptions, which include *Kudoa thyrsites* (Gilchrist, 1924) reported thus far from 40 fish species representing 18 families (Whipps and Kent 2006). This species is associated with *post-mortem* myoliquefaction in a wide range of host species. The analysis of genetic structure of *K. thyrsites* suggested a correlation of genetic samples with their geography and the existence of significant barriers in gene flow at a global scale that may lead to geographic isolation of regionally endemic populations or even emergence of cryptic species (Whipps and Kent 2006).

Scombrids are very important for fisheries in the Northeast Atlantic, with tunas and Atlantic mackerels in the ranking of the most landed species in the Portuguese mainland coast and the Madeira archipelago (INE 2018). However, to the best of our knowledge, studies on the occurrence of *Kudoa* in marine fishes have been carried out only in the Portuguese mainland coast in *T. trachurus* and *S. pilchardus* (Cruz et al. 2003, 2011). There is also a report of the presence of *Kudoa* sp. in *Scomber colias* Gmelin from the Portuguese mainland coast (Alves 2016).

Considering gaps in the knowledge of the occurrence of *Kudoa* spp. in the Northeast Atlantic, the aim of this study was to evaluate the presence and infection levels of these myxozoans in marine fishes from the Portuguese mainland coast and, in particular, from the Madeira Archipelago. We focus mainly on scombrid fish because of their importance to local fisheries (Hermida and Delgado 2016), as well as on a variety of other fish species to provide a better picture of the occurrence of species of *Kudoa* in this region.

MATERIALS AND METHODS

Sampling

A total of 250 fishes from 19 species were sampled for detection of spores of *Kudoa* spp., between May 2016 and October 2017. The main focus of the study were scombrids from the Madeira Archipelago in the North Atlantic Ocean: 22 albacore *Thunnus alalunga* (Bonnaterre), 33 Atlantic chub mackerel *Scomber colias*, 30 bigeye tuna *Thunnus obesus*, and 30 skipjack tuna *Katsuwonus pelamis* (Linnaeus) (Table 1).

Furthermore, a mixed sample of different species of fishes were included in this study to carry out a comprehensive survey of *Kudoa* spp. in important commercial species from the Madeira Archipelago: three grey triggerfish *Balistes capriscus* Gmelin, one barred hogfish *Bodianus scrofa* (Valenciennes), five leafscale gulper shark *Centrophorus squamosus* (Bonaterre), three pink dentex *Dentex gibbosus* (Rafinesque), one sharktooth moray *Gymnothorax maderensis* (Johnson), one island grouper *Mycteroperca fusca* (Lowe), one black gemfish *Nesiarchus nasutus* Johnson, two forkbeard *Phycis phycis* (Linnaeus), one wreckfish *Polyprion americanus* (Bloch et Schneider), 16 greater amberjack *Seriola dumerili* (Risso), 30 longfin yellowtail *Seriola rivoliana* Valenciennes, eight blacktail comber *Serranus atricauda* Günther, two red scorpionfish *Scorpaena scrofa* Linnaeus, and one yellowmouth barracuda *Sphyraena viridensis* Cuvier. In addition, 30 Atlantic chub mackerel *S. colias* and 30 blue whiting *Micromesistius poutassou* (Risso) from the northern part of the Portuguese mainland coast (Matosinhos) were also examined. Total length (TL), fork length (FL) and weight (W) were determined, and muscle samples were collected from each fish.

Parasitological examination

The method used to detect spores of *Kudoa* spp. was the one proposed by Saraiva et al. (2017). One gram of frozen dorsal muscle collected just posterior to the head of each fish was used, except for specimens of *S. colias* and *M. poutassou*, from which one gram of frozen muscle from each region of the fish (anterior, middle and posterior) was used. The muscle was placed on a lip of a Petri dish, moistened with 5 ml of phosphate buffered saline (PBS), macerated with a scalpel and squashed with the base of the Petri dish. The squashed muscle was initially observed under a stereomicroscope for detection of infected hypertrophic muscle cells. Afterwards, the liquid was squeezed out into a centrifuge tube, allowing the spore suspension to settle for 30 minutes. Three drops of liquid (~ 25 µl) were then pipetted from the bottom of the tube to a microscope slide and covered with a coverslip.

Slides were observed under a Differential Interference Contrast (DIC) microscope at 400× magnification. Detected kudoid spores were photographed with a camera integrated into the microscope and measured according to Burger and Adlard (2010). Spore width and thickness and polar capsules width and length were measured in spores in apical view and spore length was measured in spores in lateral view.

Data analysis

Mean and standard deviation were calculated for host parameters (TL, FL, and W). Prevalence, mean intensity and mean abundance of spores were determined based on definitions proposed by Bush et al. (1997), except that the quantification was done not on the host but on a sample of host muscle obtained by the methodology described above.

Statistical analyses were carried out using IBM SPSS 25 statistics software (IBM 2017). Differences in parasite abundance among the three regions of *M. poutassou* and *S. colias* were compared using non-parametric tests for related samples. The Cochran test was used for occurrence comparisons and Friedman's analysis of variance by ranks for abundance comparisons. For all tests, statistical significance was accepted when p < 0.05.

DNA extraction, PCR amplification and sequencing

Total DNA was extracted from samples using a standard phenol-chloroform protocol, after an overnight digestion with proteinase K (50 µg ml⁻¹; Serva, Heidelberg, Germany), at 55 °C (Holzer et al. 2004). DNA was resuspended in 50–100 µl⁻¹ of DNAse-free water and left to dissolve overnight at 4 °C. PCRs were performed using an AccuPower® PCR PreMix (Bioneer,

Daejeon, South Korea) with 25 pmol of each primer, 18 µl of water and 1 µl (approx. 100 ng) of extracted DNA.

Initially, we used a primer pair MyxospecF+18R (Whipps et al. 2003, Fiala 2006). This approach failed to give a positive PCR result for the sample from *M. poutassou* as well as for samples from *S. colias* from Madeira. Therefore, nested PCR was performed with initial primer pair 18e + 18g (Hillis and Dixon 1991) followed by PCR with specific *Kudoa* primer pair KUD1f + KUD2r (Hervio et al. 1997) or semispecific KUD1f + 18g.

Cycling parameters were set up as follows: initial PCR run: denaturation 95 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 58 °C for 1 min s, 72 °C for 2 min and after cycles a terminal extension at 72 °C for 10 min; II run: denaturation 95 °C for 3 min, followed by 35 cycles of 94 °C for 40 s, 55 °C for 50 s, 72 °C for 1 min 40 s with terminal extension at 72 °C for 10 min. PCR products were purified using a Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech Ltd., New Taipei City, Taiwan) and sequenced directly (SeqMe, Dobříš, Czech Republic). Sequences were deposited in GenBank under the accession numbers MT991409 and MT991410.

Phylogenetic analysis

Based on an initial BLAST search of SSU rDNA, sequences from *M. poutassou* and *S. colias* showed a high match score with *K. thyrsites*. Therefore, we retrieved nucleotide sequences of *K. thyrsites* and its related species from GenBank and aligned them with the newly obtained sequence using MAFFT v7.450 algorithm (Katoh et al. 2013) implemented in Geneious v8.0.5 (Kearse et al. 2012) using the E-INS-i multiple alignment meth-
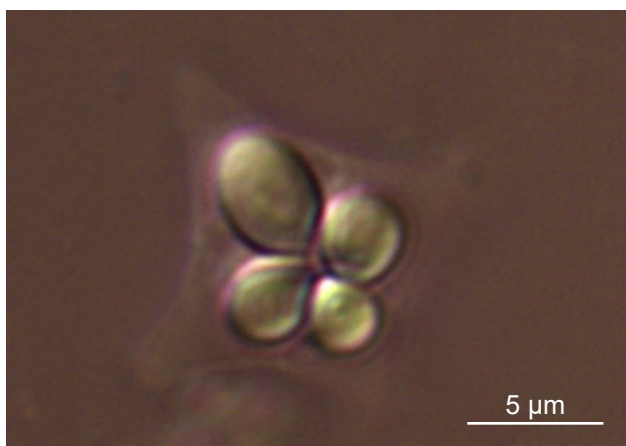

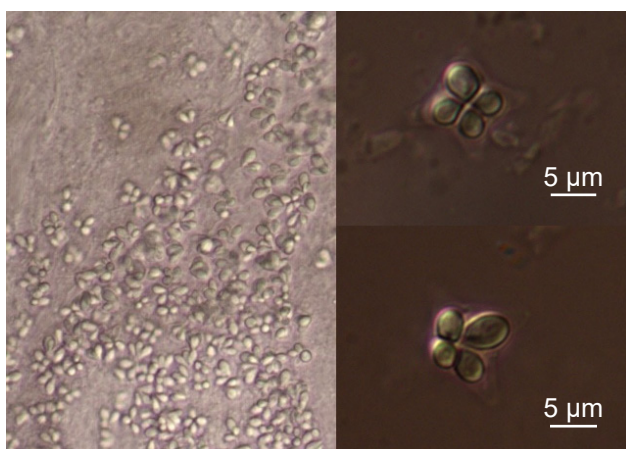**Fig. 1.** Spore of *Kudoa* sp. from *Thunnus obesus* (Lowe) from Madeira Archipelago.

Fig. 2. Spore of *Kudoa* sp. from *Scomber colias* Gmelin from Portuguese mainland coast.
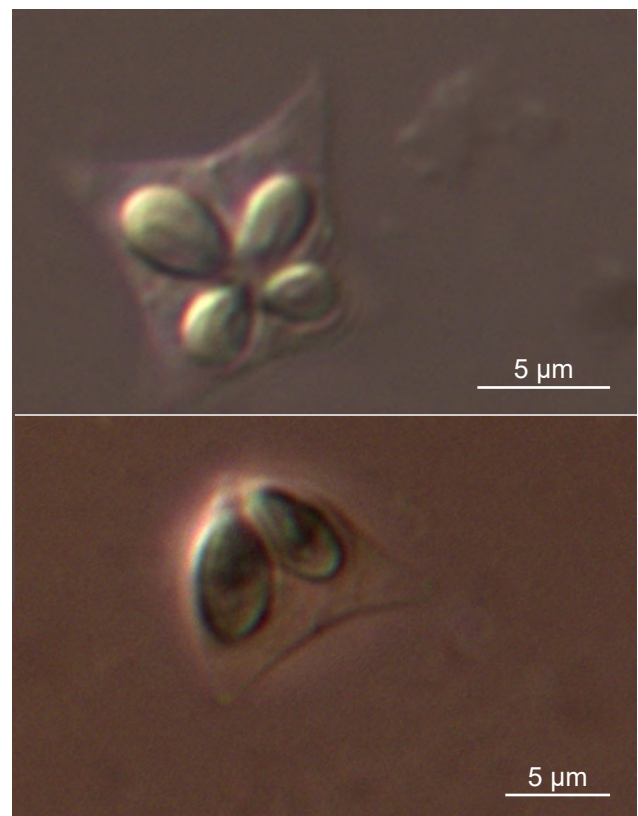

Fig. 3. Spore of *Kudoa* sp. from *Micromesistius poutassou* (Risso) from Portuguese mainland coast.

od, with a gap opening penalty (–op) of 1.5 and gap extension penalty (–ep) of 0.123. Maximum likelihood (ML) analysis was performed in RAxML v7.0.3 (Stamatakis 2006) using the GTR + Γ model of nucleotide substitution. *Kudoa lateolabracis* Yokoyama, Whipps, Kent, Mizuno et Kawakami, 2004 was set as an outgroup. Bootstraps were based on 1,000 replicates. The intraspecific differences were determined from proportional distances of SSU rDNA, which were calculated in Geneious (p-dist) from the dataset previously used for the phylogenetic analysis.

RESULTS

Taxonomic and biometric data of all 250 examined fishes are detailed in Table 1. From all examined specimens from the Madeira Archipelago, no hypertrophic muscle cells were evident macroscopically. Only a single spore of a species of *Kudoa* was found in one specimen of *Thunnus obesus* by light microscopy. The spore was stellate in apical view and had four pyriform capsules of unequal size (Fig. 1). We found 18 out of 30 *Scomber colias* and 24 out of 30 *Micromesistius poutassou* individuals positive for infection with species of *Kudoa* (60% and 80% prevalence, respectively). In both cases, spores were observed in the muscle from all three sampled body regions (anterior, middle and posterior), and infection levels varied between them (Table 2). However, in *S. colias*, no significant differences were observed either in the occurrence or in the abundance (measured as the number of spores in the three drops of liquid examined) among the three regions. Likewise, no significant differences were detected in the occurrence of parasites in *M. poutassou*, but significant differences in abundance were detected among regions (p = 0.040). Spores of *Kudoa* sp. found in *S. colias* (Fig. 2) and *M. poutassou* (Fig. 3) were stellate in apical view and had four pyriform polar capsules of three different sizes: one large, two intermediate and one small. Myxospore measurements from spores detected in *T. obesus* and *M. poutassou* are shown in Table 3.

Both SSU rDNA sequences of *Kudoa* from *S. colias* and *M. poutassou* were identical and revealed 100% identity with *Kudoa thyrsites* (AY078430) from its type species *Thyrsites atun* (Euphrasen) from the coast of South Africa as well as with *K. thyrsites* from *Merluccius capensis* Castelnau from the coast of South Africa (AY941819) and *S. scombrus* from Northern North Sea (EU154349) and the South coast of England, North Sea (AY542482). The comparison was done based on a 1,096 bp and 824 bp long sequence of *Kudoa* sp. from *S. colias* and *M. poutassou*, respectively. Phylogenetic analysis revealed four lineages of *K. thyrsites* genotypes (Fig. 4). *Kudoa* sp. from *S. colias* and *M. poutassou* clustered with sequences of *K. thyrsites* (AY078430, AY941819, EU154349, AY542482, AY542481) with 91% bootstrap support (Fig. 4). Molecular screening of 33 specimens of *S. colias* from the Madeira Archipelago was negative for infection with *Kudoa* spp.
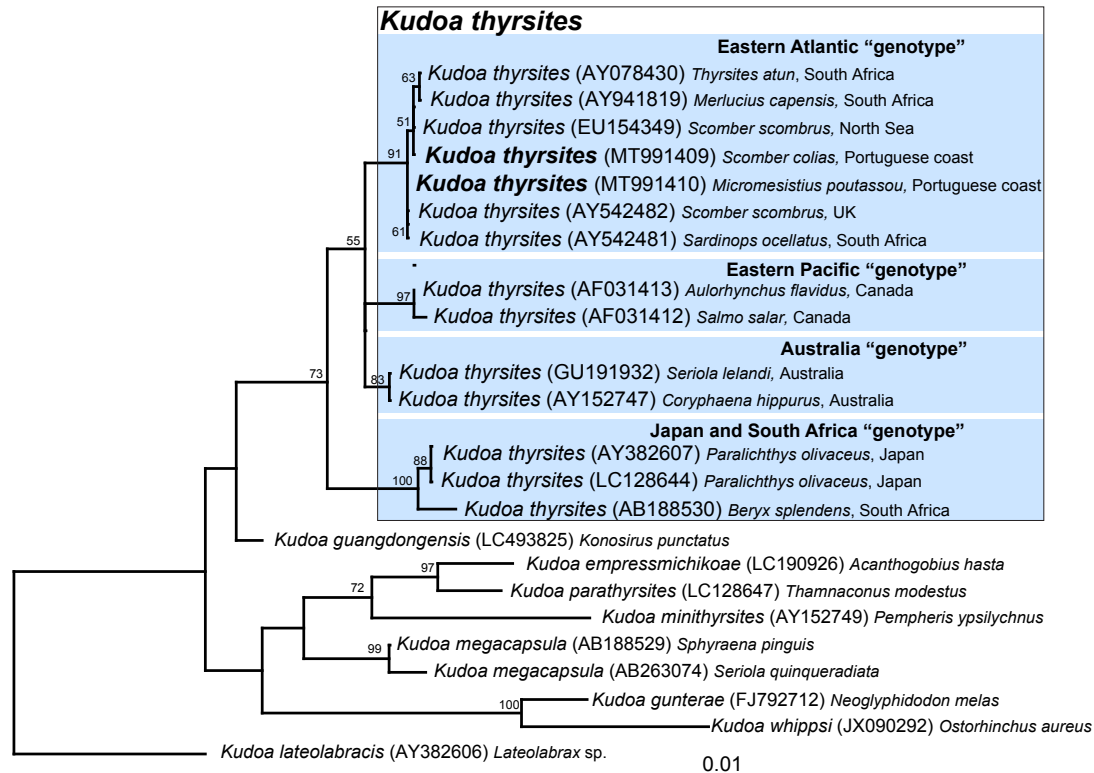

Fig. 4. Maximum likelihood tree of SSU rDNA sequences of *Kudoa thyrsites* (Gilchrist, 1924) and its closely related species. Newly identified sequences are in bold. Maximum likelihood bootstrap nodal supports are shown at every node. GenBank acc. numbers are given after the species names and scale is given under the tree.

Table 1. Fish species examine from the Madeira archipelago and the Portuguese mainland coast, number of specimens (n) and their biological parameters: total length (TL), fork length (FL) and weight (W). Values of measured biological parameters are listed as a mean ± standard deviation.

Species	n	TL (cm)	FL (cm)	W (g)
Madeira Archipelago				
Balistes capriscus	3	48.5 ± 1.6	41.8 ± 3.9	1,822 ± 346
Bodianus scrofa	1	51.0	N/A	2362
Centrophorus squamosus	5	107.0 ± 4.4	N/A	6,205 ± 918
Dentex gibbosus	3	88.4 ± 11.0	79.4 ± 9.7	9,211 ± 2749
Gymnothorax maderensis	1	90.0	a	1589
Katsuwonus pelamis	30	53.6 ± 3.3	50.6 ± 3.1	2,869 ± 642
Mycteroperca fusca	1	69.0	65.7	4533
Nesiarchus nasutus	1	127.0	N/A	5385
Phycis phycis	2	51.9 ± 3.0	N/A	1,576 ± 117
Polyprion americanus	1	64.7	N/A	4565
Scomber colias	33	24.1 ± 4.5	21.9 ± 4.0	128 ± 110
Scorpaena scrofa	2	44.0 ± 3.7	N/A	1,655 ± 208
Seriola dumerili	16	110.5 ± 33.7	99.1 ± 29.6	17,505 ± 9417
Seriola rivoliana	30	52.2 ± 13.6	46.4 ± 11.8	2,344 ± 3842
Serranus atricauda	8	30.6 ± 3.2	N/A	385 ± 178
Sphyraena viridensis	1	44.0	38.4	313
Thunnus alalunga	22	95.1 ± 4.7	87.9 ± 3.8	14,992 ± 2282
Thunnus obesus	30	85.3 ± 8.9	77.6 ± 9.5	10,654 ± 3399
Portuguese Continental Coast				
Micromesistius poutassou	30	19.4 ± 1.0	17.7 ± 0.9	41 ± 9.6
Scomber colias	30	28.4 ± 3.1	26.2 ± 3.0	178 ± 64

N/A - not available

DISCUSSION

Only around ten species of *Kudoa* have been described from the northeast Atlantic Ocean out of approximately 100 nominal species (Eiras et al. 2014). In the Portuguese mainland coast, their occurrence was reported in greater amberjack *Seriola dumerili* (*Kudoa insolita* Kovaleva, Shulman et Yakovlev, 1979), Atlantic horse mackerel *Trachurus trachurus* (*Kudoa* sp.) (Cruz et al. 2003) and European pilchard *Sardina pilchardus* (*Kudoa* sp. with morphology and morphometry consistent with *Kudoa thyrsites*) (Cruz et al. 2011). However, there are no studies on the occurrence of *Kudoa* spp. in the Madeira Archipelago. Our survey of 190 fishes of 18 different species from this archipelago seems to indicate the absence/very rare occurrence of parasites of the genus *Kudoa* in this region.

This is the first reference to the occurrence of the genus *Kudoa* in the blue whiting *Micromesistius poutassou*. Based on spore morphology and molecular analysis, this species was also identified as *K. thyrsites*, confirming the euryxenous host specificity of this parasite at the level of intermediate fish hosts. This *Kudoa* sp. as well as *Kudoa* sp. from *Scomber colias* from the Portuguese mainland coast were morphologically identical with *K. thyrsites*, which was confirmed by molecular analysis that showed 100% identity of both sequences with *K. thyrsites* from the type host. SSU rDNA sequences of our samples clustered with sequences of *K. thyrsites* from the eastern Atlantic genotype *sensu* Whipps and Kent (2006), which includes isolates from fish in the Eastern Atlantic.

Our phylogenetic analysis including the newly identified *K. thyrsites* from *M. poutassou* and *S. colias* from the Portuguese mainland coast supported the idea that the

Table 2. Infection levels of *Kudoa* Meglitsch, 1947 in three muscle regions of Atlantic chub mackerel, *Scomber colias* Gmelin (n = 30) and blue whiting, *Micromesistius poutassou* (Risso) (n = 30) from the Portuguese mainland coast. P (%), CI – 95% confidence interval, SD (range).

Muscle region	Prevalence (CI) %	Mean intensity (±SD)	Mean abundance (±SD)
S. colias			
Anterior	50 (33–70)	88 ± 219 (1–831)	45 ± 159 (0–831)
Middle	43 (27–63)	137 ± 438 (1–1650)	597 ± 301 (0–1,650)
Posterior	33 (17–50)	140 ± 247 (1–734)	47 ± 153 (0–734)
M. poutassou			
Anterior	60 (43–77)	29 ± 43 (1–144)	18 ± 35 (0–144)
Middle	70 (53–87)	41 ± 63 (1–232)	29 ± 56 (0–232)
Posterior	73 (57–87)	63 ± 94 (1–306)	46 ± 85 (0–306)

phylogeny of *K. thyrsites* genotypes correlates with geographic distribution. The host range of *K. thyrsites* is thus broadened to include two economically important fishes, namely Atlantic chub mackerel and blue whiting.

A high prevalence (60%) of *K. thyrsites* detected in *S. colias* from the mainland coast contrasts greatly with no detection of *K. thyrsites* in *S. colias* from the Madeira Archipelago. This divergence is heightened by the almost complete absence of any species of *Kudoa* in fishes from Madeira, which is in accordance with the study of Shukhgalter (2004) who reported *Kudoa* sp. in chub mackerel from Mauritania (a coastal zone) and its absence in specimens from the Azores, where environmental conditions are similar to those in Madeira, which lacks a continental shelf, and where the insular shelf is quite narrow. Results of the present study may also provide additional information about the stock structure of *S. colias* in the Northeastern Atlantic. The presence of *K. thyrsites* in Atlantic chub mackerel from the Portuguese mainland coast and no detection in fish from the Madeira archipelago suggests that the specimens observed may belong to two different populations.

To the best of our knowledge, no life cycle of any species of *Kudoa* has yet been described (Eszterbauer et al. 2015). However, the life cycle is likely similar to that of other genera of the Myxosporea, with the presence of two hosts, annelids (probably a marine polychaete) and fishes. In the Madeira Archipelago, the ocean depth increases steeply at a short distance from the coast; there is no continental shelf, and the insular shelf is quite narrow. In addition, oceanic waters in this region have low productivity (Hermida and Delgado 2016). These conditions may explain the reduced abundance of benthic organisms, and consequently, it is possible that the occurrence of annelids near the Madeiran coasts is very low, or even that the specific hosts of *Kudoa* spp. might not be present in this region.

Thunnus spp. have been reported as hosts of several species of parasites of the genus *Kudoa* in wild and cultured fishes from Japan (Zhang et al. 2010, Meng et al. 2011,

Table 3. Measurements (W – width; T – thickness; L – length) of the spores detected in the muscle of *Micromesistius poutassou* (Risso) and *Thunnus obesus* (Lowe) in µm.

				Apical view				Side view			
	Spore			Large polar capsule		Small polar capsule		Large polar capsule		Small polar capsule	
	W	T	L	L	W	L	W	L	W	L	W
M. poutoussou											
Mean	15.2	8.3	8.4	4.7	3.2	3.7	2.5	6.1	3.2	5.0	2.6
Sd	0.90	0.67	0.53	0.48	0.25	0.51	0.30	0.61	0.30	0.41	0.23
Minimum	14.0	7.5	7.4	3.6	2.5	3.1	1.3	5.3	2.5	4.0	2.4
Maximum	17.8	10.8	9.0	6.3	3.6	5.9	3.0	6.9	3.7	5.6	3.1
n	30	30	15	35	35	35	35	13	13	12	12
T. obesus	-	7.4	-	4.9	2.7	3.0	2.5	-	-	-	-
n		1		1	1	1	1				

Abe and Maehara 2013). Although Japan is also an archipelago, it has an extended continental shelf, which can provide the ideal environmental conditions for the occurrence of benthic annelids, allowing *Kudoa* spp. to complete their life cycle in their waters. This could explain the difference between the high infection rates by *Kudoa* spp. in fishes from Japan and the extremely low prevalence of *Kudoa* spp. in this study.

Furthermore, the presence of *Kudoa* sp. in *T. obesus* does not indicate that this parasite can complete its life cycle in Madeira, since bigeye tuna is a highly migratory species, and thus could have acquired the infection elsewhere. In the Eastern Atlantic, bigeye tuna could acquire the infection, for example, in the African continental shelf, where this parasite was previously reported by Henning et al. (2013).

The results of this study indicate that the occurrence of *Kudoa* spp. in Madeiran waters appears to be uncommon.

This seems to indicate that fishes from the Madeira Archipelago present a limited risk in terms of the danger of *Kudoa* food poisoning. In any case, European Union recommendations of the adequate preparation of fish products should be followed (EFSA 2010).

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