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Two novel myxosporean parasites in Black Sea fishes: *Kudoa niluferi* sp. nov. and *Kudoa anatolica* sp. nov. (Cnidaria: Myxosporea)

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ABSTRACT: Members of the genus *Kudoa* are typically histozoic and only a few are coelozoic parasites, mainly in marine fishes. In the present study, 2 novel *Kudoa* species were recovered and described as *Kudoa niluferi* sp. nov. in the musculature of *Neogobius melanostomus* and *Kudoa anatolica* sp. nov. in the musculature, urinary bladder and kidney of *Atherina hepsetus* collected from the coast of Sinop on the Black Sea. Means ± SD (ranges) of mature spores of *K. niluferi* sp. nov. were 5.9 ± 0.1 (5.7–6.1) µm in length, 9.2 ± 0.2 (8.8–9.5) µm in width and 7.5 ± 0.3 (7.0–8.1) µm in thickness, while those of *K. anatolica* sp. nov. were 4.1 ± 0.3 (3.5–4.1) µm in length, 7.1 ± 0.2 (6.7–7.2) µm in width and 5.7 ± 0.2 (5.3–6.0) µm in thickness. In both parasite species, length and width of the 4 polar capsules were not equal and formed 3 distinct size classes, largest (1), intermediate (2) and smallest (1) in size. The prevalence and intensity of infection by *K. niluferi* sp. nov. were 12.8% and 20–29 parasites (per field-of-view, at 200× magnification), respectively, in the musculature of *N. melanostomus.* These values for *K. anatolica* sp. nov. were 32.1% and 10–19 parasites in the musculature as well as 2.9% and 20–29 parasites jointly in the kidney and urinary bladder of *A. hepsetus.* Phylogenetic analysis based on nuclear small subunit rDNA also suggested *K. niluferi* and *K. anatolica* as 2 novel species. These species appeared in the same lineage with *K. nova* and formed a Black Sea lineage.

KEY WORDS: *Kudoa* · Myxozoa · Black Sea · Turkey

INTRODUCTION

Myxosporea represent a major group of fish parasites with a total of about 2200 nominal species classified to 64 genera and 17 families (Fiala et al. 2015). Among myxosporeans, the genus *Kudoa* Meglitsch, 1947 includes a total of 95 nominal species from a wide range of host fishes and geographical areas (Eiras et al. 2014). The majority of species are located in skeletal muscle, but also in other target tissues, including the gills, brain, heart, kidney, spleen, ovary, gall bladder, urinary bladder, oesophagus, intestine, mesentery and smooth muscle (Pascual et al. 2012). Heavy infections by some *Kudoa* species, such as *K. thyrsites* (Gilchrist, 1924), *K. musculoliquefaciens* (Matsumoto & Arai, 1954) and *K. clupeidae* (Hahn, 1917), have been reported to be the cause of post-harvest soft flesh in fish meat, creating serious economic losses for the fisheries industry (Moran et al. 1999, Lom & Dyková 2006, Pascual et al. 2012). *K. septempunctata* Matsukane & Sugita-Konishi, 2010 has recently been reported to be the causative agent of novel food poisoning outbreaks following the consumption of infected raw olive flounder *Paralichthys*

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olivaceus in Japan and Korea (Kawai et al. 2012, Uk Lee 2017). Ohnishi et al. (2013) demonstrated the pathogenicity of *K. septempunctata* in an *in vitro* experiment on human intestinal cells, which were rapidly invaded by sporoplasm of the parasite.

The identification of *Kudoa* spp. is primarily based on the morphology and morphometry of the spores (Lom & Arthur 1989, Burger & Adlard 2010), although molecular characterisations have been included in more recent studies (Matsukane et al. 2010, Kristmundsson & Freeman 2014, Mansour et al. 2015). Prior to our investigation, only 3 species of the genus *Kudoa*, namely *K. nova* Naidenova, 1975, *K. quadratum* (Thélohan, 1895) and *K. stellula* Yurakhno, 1991, were found in Black Sea fishes. *K. nova* parasitizes the muscles of very diverse gobiid fish hosts of 14 species, including giant goby *Gobius cobitis,* black goby *G. niger,* Pinchuk's goby *Ponticola cephalargoides*, flatsnout goby *P. platyrostris,* ratan goby *P. ratan,* syrman goby *P. syrman,* mushroom goby *P. eurycephalus,* round goby *Neogobius melanostomus,* monkey goby *N. fluviatilis,* marbled goby *Pomatoschistus marmoratus,* sand goby *P. minutus,* tubenose goby *Proterorchinus marmoratus,* grass goby *Zosterisessor ophiocephalus* and chameleon goby *Tridentiger trigonocephalus* in the Black Sea (Naidenova 1974, Gaevskaya et al. 1975, Yurakhno 1994, 2009a,b,c, 2012, 2013, 2014, 2016, Gorchanok & Yurakhno 2005, Yurakhno & Gorchanok 2011, Kvach et al. 2014). *K. quadratum* is a parasite of the muscles of Mediterranean horse mackerel *Trachurus mediterraneus*, Zvonimir's blenny *Parablennius zvonimiri*, black-striped pipefish *Syngnathus abaster* and flathead grey mullet *Mugil cephalus* in the Black Sea (Iskov 1989), while *K. stellula* infects only the kidney tubules of Mediterranean sand smelt *Atherina hepsetus* in the Black Sea (Yurakhno 1991, 2013).

In the present study, we provide morphological and molecular identification of 2 novel *Kudoa* species found in round goby *N. melanostomus* and Mediterranean sand smelt *A. hepsetus* collected from the coast of Sinop on the Black Sea.

MATERIALS AND METHODS

Morphological analyses

In the present study, round goby (n = 141) and Mediterranean sand smelt (n = 274) were collected from the coast of Sinop on the Black Sea between January 2016 and February 2018, and examined for their myxosporean parasites using standard methods. Skin, gills, muscles, urinary bladder, gall bladder, stomach, intestine, kidney, gonads and liver were investigated. All organs were dissected and placed separately in Petri dishes to determine infected organs and parasites. A phase contrast Olympus microscope (BX53) equipped with a digital camera (DP50) and differential interference contrast attachment was used at 400× and 1000× magnification for species identification and photography. Measurements of parasite spores were based on 30 fresh individuals. All measurements are in accordance with Lom & Dyková (1992) and are provided as mean ± SD and range (in µm). Infection prevalence (%) was determined according to Bush et al. (1997), and the intensity of infection of myxozoans was semi-quantitatively evaluated following a scale from 1+ to 6+, based on the number of myxosporean parasites per microscopic field at 300×, as described by Alvarez-Pellitero et al. (1995). This protocol was modified for 200× magnification to intensity ranges: 1+ (1–9); 2+ (10–19); 3+ (20–29); 4+ (30–39); 5+ (40–49); 6+ (>50). All applicable international, national and institutional guidelines for the care and use of animals were followed.

Molecular analyses

An Invitrogen PureLink® Genomic DNA Mini Kit was used to extract genomic DNA from infected kidney and muscular tissues of *Atherina hepsetus* (parasite isolates AO-18 and AO-20, respectively) and muscular tissue of *Neogobius melanostomus* (parasite isolate AO-24). Isolated total genomic DNA (containing both fish and parasite DNA) was stored at −20°C prior to use. Nuclear small subunit ribosomal DNA (SSU rDNA), which is the most common marker used for identification of myxozoan parasites, was used for molecular analyses. We used 2 *Kudoa* genus-specific internal primers (designed in this study), Kudoa_SSU_R1 and Kudoa_SSU_F1, paired with universal external primers SR-1 (Nakayama et al. 1996) and NS-8 (White et al. 1990) to solely amplify the full length of myxozoan nuclear SSU rDNA (see Fig. S1A in the Supplement at www.in-res.com/articles/suppl/d128p225_supp.pdf). PCR amplifications were performed using a Techne (TC-Plus) thermal cycler with the conditions given in Fig. S1B, and a 50 µl PCR reaction was prepared as explained in Fig. S1C. The PCR products were electrophoresed on 1% agarose gel (Amresco) prepared in 1× TBE buffer and then visualized with a Vilber Lourmat Imaging System.

Nucleotide sequencing was performed commercially using the same primers as for amplification. The assemblage of the sequences from both strands were made using BioEdit (Hall 1999) and checked manually with the same software. A data set including those *Kudoa* species (Table S1 in the Supplement) that gave the highest BLAST scores with our new haplotypes was compiled. ClustalX (Thompson et al. 1997) was employed for multiple nucleotide sequence alignments with default values (gap opening: 10.00 and gap extension: 0.10; Hall 2004) and optimised by hand with BioEdit. To determine the best fitting evolutionary model(s) to our data set, Akaike's information criterion (AIC; Akaike 1974) and Bayesian information criterion (BIC) tests were performed using jModelTest v. 0.1 (Guindon & Gascuel 2003, Posada 2008). For the neighbour-joining (NJ; Saitou & Nei 1987) and maximum parsimony (MP; Eck & Dayhoff 1966, Fitch 1977) analyses, the software program PAUP* v. 4.0b 10 (Swofford 1998) was employed. An heuristic search approach using the TBR swapping algorithm (10 random repetitions) was performed for MP analyses. PhyML 3.0 (Guindon & Gascuel 2003) software was used for ML analyses. Bootstrap tests (Efron 1982, Felsenstein 1985) were performed with 10000 pseudoreplicates for NJ and 1000 pseudoreplicates for MP and ML analyses with the same software used for phylogenies. To calculate the nucleotide sequence identities and DNA distances between haplotypes, BioEdit and MEGA7 (Kumar et al. 2016) were used, respectively. Our new *Kudoa* nuclear SSU rDNA haplotypes have been deposited in GenBank under accession numbers MH310913–MH310915 (Table S1).

RESULTS

In the present study, 2 myxosporean species were recovered and newly described as *Kudoa niluferi* sp. nov. (Fig. 1A) from the musculature of round goby *Neogobius melanostomus*, and *Kudoa anatolica* sp. nov. (Fig. 1B) from the kidney, urinary bladder and musculature of Mediterranean sand smelt *Atherina hepsetus*. The calculated infection prevalence and intensity levels of *K. niluferi* sp. nov. in the musculature of *N. melanostomus* (n = 141) were 12.8% and 3+, respectively. These values for *K. anatolica* sp. nov. were 32.1% and 2+ in only the musculature (n = 88); 2.6% and 2+ in only the kidney (n = 7); 2.9% and 3+ jointly in the kidney and urinary bladder (n = 8); 1.45% and 5+ jointly in the musculature, kidney and urinary bladder (n = 4) of *A. hepsetus* (n = 274). No host was found to be infected with *K. anatolica* sp. nov. only in the urinary bladder.

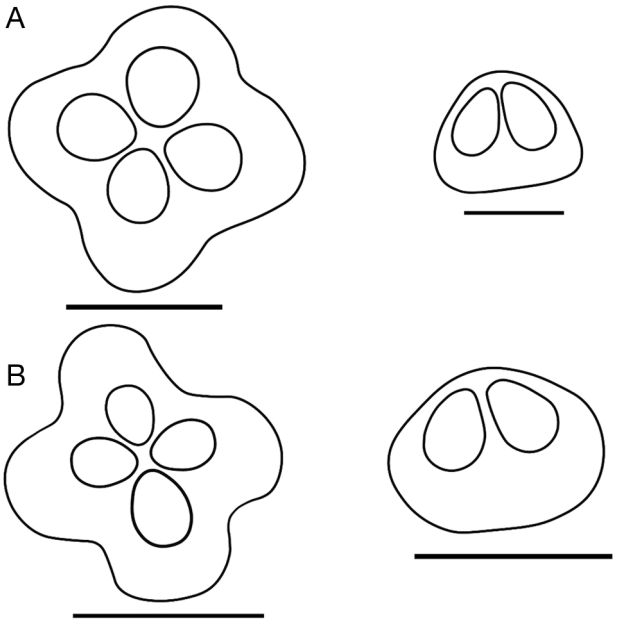

Fig. 1. Apical (left panels) and side views (right panels) of (A) *Kudoa niluferi* sp. nov., and (B) *Kudoa anatolica* sp. nov. Scale bars = 5 µm

Taxonomic summaries

Name: *Kudoa niluferi* sp. nov.
Type host: Round goby *Neogobius melanostomus* (Pallas, 1814) (Perciformes: Gobiidae)
Type locality: Coast of Sinop, Black Sea, Turkey (42° 02′ 51″ N, 35° 02′ 56″ E)
Site of infection: Musculature
Prevalence of infection: 12.8% (n = 141 fish)
Intensity of infection: 20–29 parasites per field-of-view (at 200× magnification)
Type material: One holotype (MyxoKN 2017.1) and 1 paratype (MyxoKN 2017.2) were deposited in the Sinop University, Faculty of Fisheries and Aquatic Sciences Parasitological Collection, Sinop, Turkey.
Etymology: The specific epithet '*niluferi*' recalls the name of the corresponding author's wife Nilufer who has supported his scientific career of over 25 yr.

Name: *Kudoa anatolica* sp. nov.
Type host: Mediterranean sand smelt *Atherina hepsetus* Linnaeus, 1758 (Atheriniformes: Atherinidae)
Type locality: Coast of Sinop, Black Sea, Turkey (42° 02′ 51″ N, 35° 02′ 56″ E)
Site of infection: Musculature, kidney and urinary bladder
Prevalence of infection: 32.1% in only the musculature (n = 88); 2.6% in only the kidney (n = 7); 2.9% jointly in the kidney and urinary bladder (n = 8);

1.5% jointly in the musculature, kidney and urinary bladder (n = 4) (n = 274 fish)

Intensity of infection: 10–19 parasites per field-of-view in only the musculature (n = 88); 10–19 parasites in only the kidney (n = 7); 20–29 parasites jointly in the kidney and urinary bladder (n = 8); 40–49 parasites jointly in the musculature, kidney and urinary bladder (n = 4)

Type material: One holotype (MyxoKY 2017.1) and 1 paratype (MyxoKY 2017.2) were deposited in the Sinop University, Faculty of Fisheries and Aquatic Sciences Parasitological Collection, Sinop, Turkey

Etymology: The specific epithet '*anatolica*' recalls the geographical region of Anatolia, Turkey, where the fish host was collected.

Descriptions

Vegetative stages

No vegetative stages were observed for either new species.

Spores

Kudoa niluferi sp. nov. (Fig. 2A–D). Spores resemble a square pyramid. Anterior pole is rounded, posterior pole flattened and widened. Rounded tips of each valve do not extend to posterior end of spore. Valves are not equal. The 4 polar capsules are large pyriform, and unequal. Length, width and thickness of spores (n = 30) were 5.9 ± 0.1 (5.7–6.1) µm, 9.2 ± 0.2 (8.8–9.5) µm and 7.5 ± 0.3 (7.0–8.1) µm, respectively. Lengths of the polar capsules were unequal and fell into 3 distinct size classes: largest 2.7 ± 0.1 (2.6–2.9) µm, smallest 2.3 ± 0.1 (2.2–2.4) µm and intermediate 2.6 ± 0.1 (2.4–2.8) µm. Similarly, widths of the polar capsules were also not equal and formed distinct size classes: largest 2.3 ± 0.1 (2.1–2.6) µm, smallest 2.1 ± 0.1 (1.9–2.2) µm and intermediate 2.2 ± 0.1 (2.0–2.3) µm.

Kudoa anatolica sp. nov. (Fig. 2E–H). Spores have flattened anterior and rounded posterior poles. Each valve has rounded tips and valves are unequal. Accordingly, the 4 polar capsules also have 3 distinct size classes; of the 2 capsules located at opposite sides, one had largest and the other had the smallest

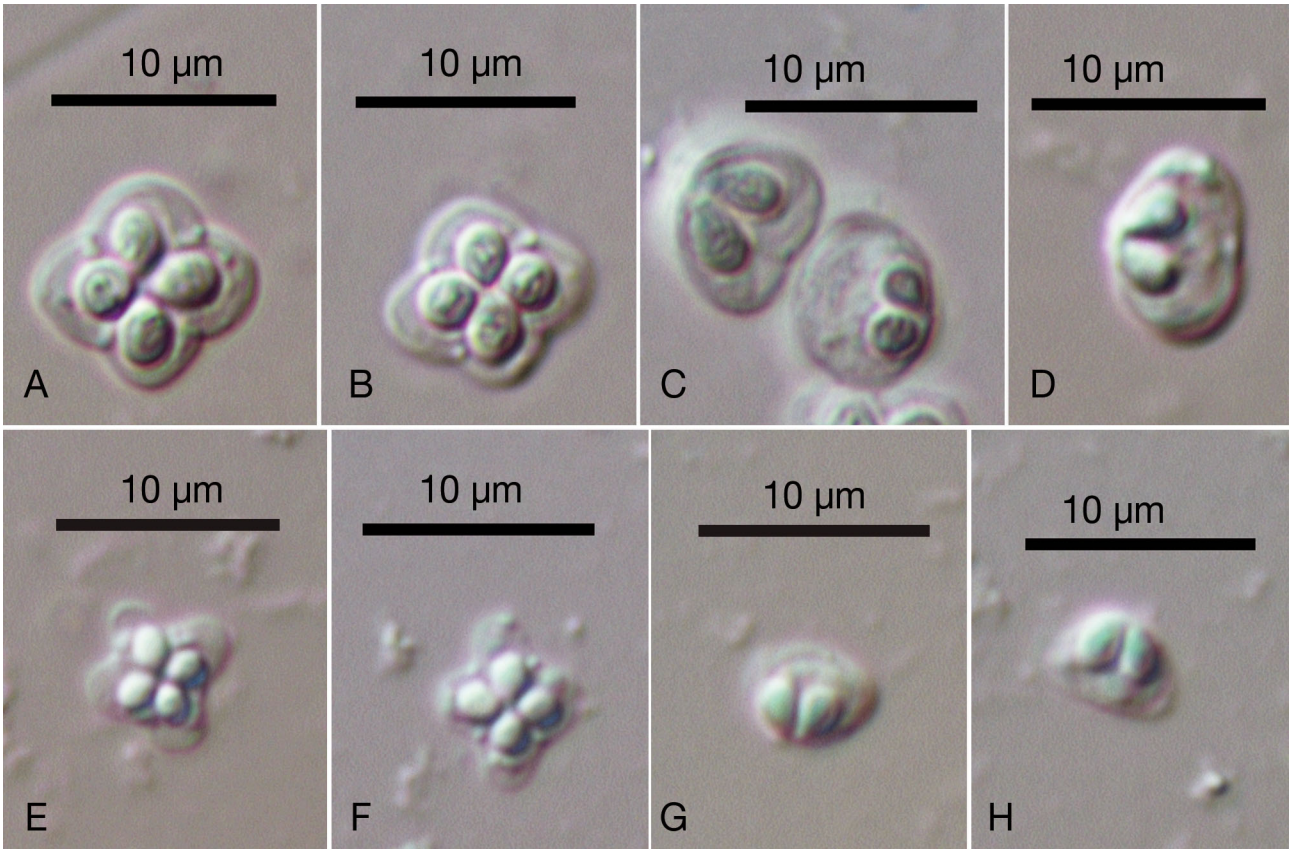

Fig. 2. (A,B) Apical and (C,D) side view of *Kudoa niluferi* sp. nov. from musculature of *Neogobius melanostomus*. (E,F) Apical and (G,H) side view of *Kudoa anatolica* sp. nov. from musculature of *Atherina hepsetus*

dimensions while the other 2 capsules occupying the intermediate position were equal in size. Length, width and thickness of spores (n = 30) were 4.1 ± 0.3 (3.5–4.1) µm, 7.1 ± 0.2 (6.7–7.2) µm and 5.7 ± 0.2 (5.3–6.0) µm, respectively. Lengths of the polar capsules were unequal and fell into distinct size classes: largest 1.8 ± 0.1 (1.7–2.0) µm, smallest 1.5 ± 0.1 (1.3–1.6) µm and intermediate 1.7 ± 0.1 (1.6–1.9) µm. Similarly, widths of the polar capsules were also unequal and fell into distinct size classes: largest 1.6 ± 0.1 (1.5–1.7) µm, smallest 1.3 ± 0.1 (1.2–1.5) µm and intermediate 1.4 ± 0.1 (1.3–1.6) µm.

Differential diagnosis of *Kudoa niluferi* sp. nov. and *K. anatolica* sp. nov.

K. niluferi sp. nov. and *K. anatolica* sp. nov. are similar to each other with respect to the morphology of their unequal spores and polar capsules. On the other hand, *K. niluferi* sp. nov. closely resembled the morphology of *K. nova* spores infecting the skeletal muscle tissue of round goby (Pascual et al. 2012), while *K. anatolica* sp. nov. resembled *K. stellula* infecting the skeletal muscle tissue of the Mediterranean sand smelt (Yurakhno 1991) inhabiting the northern coasts of the Black Sea.

The shape and size of the spores and polar capsules of *K. niluferi* sp. nov. and *K. nova* differ from each other, in that *K. nova* has equal polar capsules whereas *K. niluferi* sp. nov. has unequal polar capsules, with 1 small, 2 intermediate and 1 larger sized (Table 1). Moreover, width, thickness and length of *K. niluferi* sp. nov. spores are also larger than, even double the size of, those of *K. nova* (Table 1). *K. niluferi* sp. nov. and *K. stellula* from *Atherina hepsetus* have unequal

Table 1. Prevalence and site of infection, hosts, geographical localities and dimensions between morphologically close species of the genus *Kudoa* found in marine fish (L: Large; M: Middle; S: Small)

Kudoa species	Spore body (µm) Length	Width	Thickness	Polar capsule (µm) Length	Width	Site of infection	Prevalence (%)	Host	Locality	Reference(s)
K. anatolica sp. nov.	4.1 ± 0.3 (3.5–4.1)	7.1 ± 0.2 (6.7–7.2)	5.7 ± 0.2 (5.3–6.0)	L1.8 ± 0.1 (1.7–2.0) M1.7 ± 0.1 (1.6–1.9) S1.5 ± 0.1 (1.3–1.6)	L1.6 ± 0.1 (1.5–1.7) M1.4 ± 0.1 (1.3–1.6) S1.3 ± 0.1 (1.2–1.5)	Musculature (Mu) Kidney tubules (K) Urinary bladder (U) Joint (Mu)+(K)+(U) Joint (K)+(U)	32.1 2.6 0.0 1.5 2.9	*Atherina hepsetus*	Black Sea (Sinop)	Present study
K. niluferi sp. nov.	5.9 ± 0.1 (5.7–6.1)	9.2 ± 0.2 (8.8–9.5)	7.5 ± 0.3 (7.0–8.1)	L2.7 ± 0.1 (2.6–2.9) M2.6 ± 0.1 (2.4–2.8) S2.3 ± 0.1 (2.2–2.4)	L2.3 ± 0.1 (2.1–2.6) M2.2 ± 0.1 (2.0–2.3) S2.1 ± 0.1 (1.9–2.2)	Musculature	12.8	*Neogobius melanostomus*	Black Sea (Sinop)	Present study
K. alliaria	7.0–8.0	9.0–10.0	8.0–9.0	2.4	1.8	Muscles	25–100	*Micromesistius australis*	Atlantic Ocean	Kovaleva et al. (1979)
	6.3–7.4	6.3–7.4	6.0–6.3	2.1–2.6	2.0	Muscles	40–50	*Patagonotothen ramsayi*	Southern Ocean	
K. dianae	5.0 (4.5–5.5)	6.0 (5.5–6.5)	6.0 (5.5–6.5)	2.0	1.5	Oesophagus	18	*Sphoeroides annulatus*	Pacific Ocean	Dyková et al. (2002)
K. inornata	5.4 (5.3–5.5)	5.9 (5.8–6.0)	6.0 (5.9–6.1)	2.7	–	Muscles	91	*Cynoscion nebulosus*	Atlantic Ocean	Dyková et al. (2009)
K. islandica	4.1–6.8	6.5–9.5	5.0–8.0	1.4–2.5	1.2–1.9	Muscles	90–100	*Cyclopterus, lumpus* *Anarhichas lupus, A. minor*	Atlantic Ocean	Kristmundsson & Freeman (2014)
K. nova	3.1	5.0–6.3	4.0–6.3	2.7–2.9	2.0		5.3	*Neogobius melanostomus*	Black Sea (Zavetnoye)	Yurakhno (1994)
	3.1	5.0–6.3	4.0–6.3	2.0–2.4	1.5–1.7	Muscles	5.3	*Neogobius melanostomus*	Black Sea (Egolitsky Gulf)	Yurakhno (1994)
K. paniformis	5.0 (4.5–6.0)	6.7 (6.0–7.0)	5.9 (5.0–6.5)	2.1 (2.0–2.5)	1.7 (1.5–2.0)	Muscles	25–100	*Merluccius productus*	Pacific Ocean	Kabata & Whitaker (1981, 1986)
K. stellula	–	5.0–6.9	3.4–4.7	L 2.0–2.2 M 1.5–1.9 S 1.3–1.5	L 1.5–1.6 M 0.9–1.4 S 1.3–1.5	Kidney	75	*Atherina hepsetus*	Black Sea (Sevastopol)	Yurakhno (1994)
	–	–	–	–	–	Kidney	5.0	*Atherina hepsetus*	Black Sea (Karadag)	Yurakhno (2013)

polar capsules; however, *K. niluferi* sp. nov. has much larger dimensions of spores than the latter species (Table 1). *K. niluferi* sp. nov. also shows differences in spore dimensions compared to *K. alliaria* (Whipps & Diggles 2006), *K. dianae* (Dyková et al. 2002), *K. inornata* (Dyková et al. 2009), *K. islandica* (Kristmundsson & Freeman 2014) and *K. paniformis* (Kabata & Whitaker 1981) (Table 1) from the Atlantic and Pacific Oceans that have typical spore forms with rounded ends of valves. Moreover, all of the above mentioned species have equally sized polar capsules, in contrast with *K. niluferi* sp. nov. *K. alliaria* also differs from *K. niluferi* sp. nov. by having a rounded anterior end slightly rising in the central part of the spores. *K. niluferi* sp. nov. has longer, wider and thicker spores than *K. dianae, K. paniformis*, *K. inornata* and *K. islandica*. When tissue tropism is considered, *K. niluferi* sp. nov has similar site of infection with the 4 oceanic species mentioned above, but differs from *K. dianae* and *K. stellula*. *K. niluferi* sp. nov has the same site of infection and host species, *N. melanostomus,* as *K. nova*, although the list of hosts for *K. nova* is much wider.

In comparison with the nearly square shape of *K. niluferi* sp. nov. and *K. stellula*, *K. anatolica* sp. nov. has a different form in apical view, with more rounded corners and deeper indentations between quadrants. However, all 3 species have unequal valves. Moreover, *K. anatolica* sp. nov. also differs from *K. stellula* from the same host *Atherina hepsetus* by spore dimensions and site of infection in the kidney and urinary bladder along with muscles of its host (Table 1). *K. anatolica* sp. nov. spores were found mainly in the muscle tissue of the host fish, and kidney and urinary bladder of some host individuals were infected jointly without any infection in the muscles. Despite the differences in the width and thickness of *K. anatolica* sp. nov. and *K. stellula* spores, both species have similarities in the dimensions of 3 length-classes of polar capsules. When compared with *K. nova, K. anatolica* sp. nov. has longer, wider and thicker spores, but smaller polar capsules (Table 1).

Molecular analyses

As the result of nucleotide sequencing, we obtained nearly the full length (approx. 1600 bp) of the nuclear SSU rDNA locus for our samples, AO-18, AO-20 and AO-24. Phylogenetic analyses were carried out using 1399 aligned nucleotides with 257 segregating sites. Both AIC and BIC tests indicated the GTR+I+G (I: 0.637; G: 0.582) substitution model. MP analyses produced 10 most parsimonious trees with 555 steps (consistency index = 0.551, retention index = 0.663, homoplasy index = 0.449). Bootstrap values obtained from ML and MP analyses are shown on the NJ tree (Fig. 3) for each related node. In all phylogenetic trees calculated with NJ (Fig. 3), MP and ML algorithms, sample AO-24 was related to *K. nova* (EF644198) with 99.1% nucleotide sequence similarity, and this relation was supported with 100% bootstrap values in all trees (Table 2). Our other isolates, AO-18 and AO-20, showed the same haplotype and appeared as sister to the lineage above; this relation was supported with 98, 95 and 98% bootstrap values in NJ, MP and ML trees, respectively. The nucleotide sequence similarities between this lineage (AO-18 and AO-20) and AO-24 was 95.3%, whereas it was 96% with *K. nova* (Table 2).

DISCUSSION

Members of the genus *Kudoa* are important pathogens of cultured and wild fish, and some species are detrimental to fish products as a result of their site of infection, primarily the musculature; thus, these parasites are of concern to aquaculture and the wild fish fisheries because of their impact on product quality (Moran et al. 1999). Moreover, 1 species isolated from olive flounder has been reported to be the causative agent of food poisoning in humans consuming raw meat (Kawai et al. 2012, Lee 2017). To date, despite 95 nominal species described from a wide range of host fishes and geographical areas (Eiras et al. 2014), only 3 *Kudoa* species (*K. quadratum*, *K. nova* and *K. stellula*) have been reported from the Black Sea. Of these, *K. quadratum* originated from the Atlantic Ocean and Mediterranean Sea while the latter 2 species were originally described from *Neogobius melanostomus* and *Atherina hepsetus*, respectively, in the Black Sea (Thelohan 1894, Naidenova 1974, Yurakhno 1991). Our 2 specimens, AO-18 and AO-20 from kidney and muscles of *A. hepsetus*, were morphologically (spore shape and dimensions) different from these species and also from the third specimen AO-24 (*Kudoa niluferi* sp. nov.) collected in this study (Table 1). In the phylogenetic analyses of the nucleotide sequences of nuclear SSU rDNA, our samples AO-18 and AO-20 presented a unique lineage and appeared as sister to the lineage comprised of *K. nova* (EF644198) and our specimen AO-24 (*Kudoa niluferi* sp. nov) with significant bootstrap values (Fig. 3). The nucleotide sequence similarity and DNA

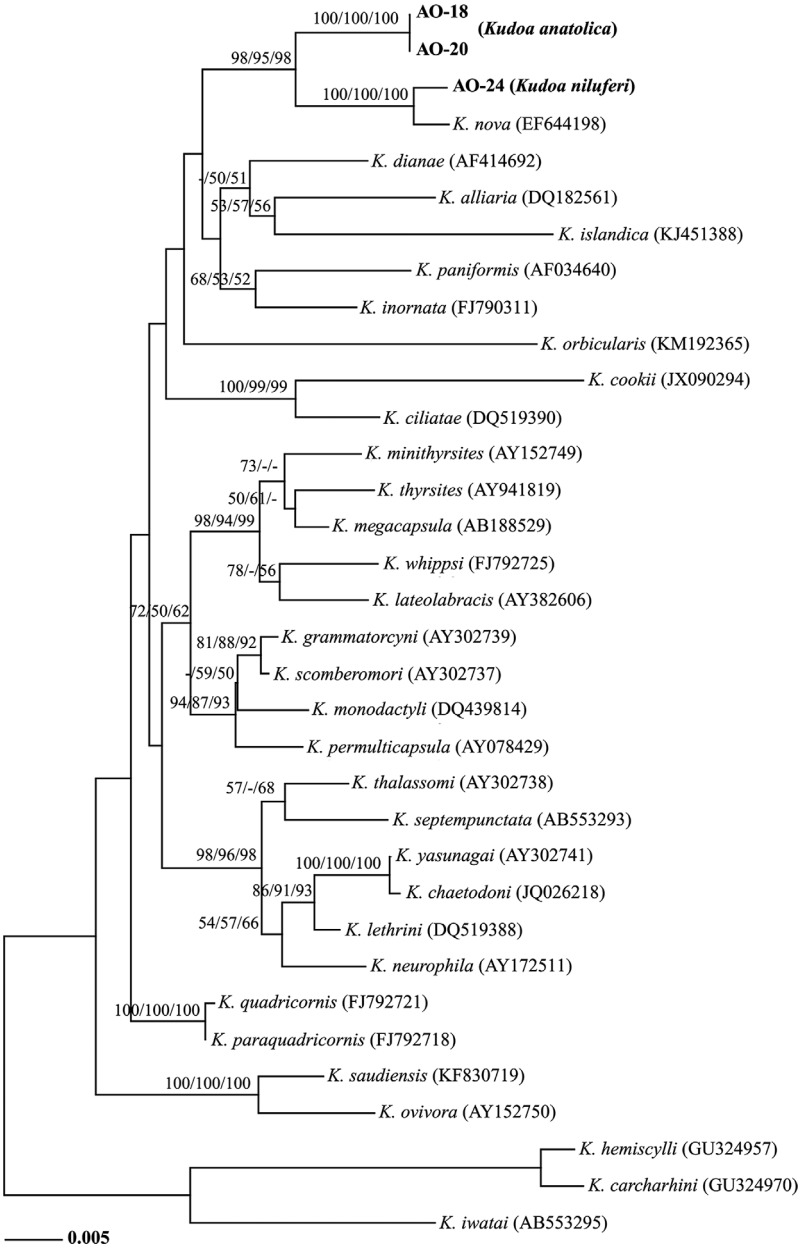

Fig. 3. Neighbour-joining (NJ) tree showing the phylogenetic relationships among small subunit (SSU) rDNA haplotypes of *Kudoa* isolates obtained in this study (AO-18, AO-20 and AO-24, highlighted in **bold**) and haplotypes of closely related *Kudoa* species (obtained from GenBank). The tree was created with the GTR+I+G substitution model. Bootstrap values (≥50%) obtained from NJ, maximum parsimony (MP) and maximum likelihood (ML) analyses are given on each related node in the order NJ/MP/ML

distance between this lineage (AO-18 and AO-24) and *K. nova* and AO-24 were quite high if we consider the nucleotide sequence similarity and DNA distance between other *Kudoa* species (Table 2). Depending on the available morphological and molecular phylogenetic data, we suggest the lineage comprised of our specimens AO-18 and AO-20 as a new *Kudoa* species, namely *Kudoa anatolica* sp. nov.

Our morphological observations (spore types etc.) showed that our third specimen AO-24, recovered from the musculature of *Neogobius melanostomus*, differed in the spore and polar capsule sizes and morphology from *K. nova* reported from the same host in different localities in the Black Sea (Table 1). Additionally, the phylogenetic analyses based on nucleotide sequences of nuclear SSU rDNA supported this result that AO-24 appeared as sister to *K. nova* (Fig. 3). On the other hand, Pascual et al. (2012) suggested that *K. nova* may be a species complex harbouring several closely related species rather than a single species due to its high plasticity in morphology, wide host range, broad geographical distribution and a wide species concept that has been applied for this species. Moreover, complexes of sibling species were determined from many old myxozoan species (Karlsbakk 2001). Our molecular analyses showed that the nucleotide sequence similarity and DNA distance between AO-24 and *K. nova* are 99.1% and 0.008, respectively. Although these values seem relatively high, there are valid species in the genus *Kudoa* which show much higher genetic similarities (Table 2) with their sister taxa than the values we determined between AO-24 and *K. nova*. For instance, nucleotide sequence similarity and DNA distance between *K. quadricornis* and *K. paraquadricornis* were 99.9% and 0.001, respectively, and were 99.8% and 0.001 between *K. yasunagai* and *K. chaetodoni*. From this perspective, although the morphological differences between AO-24 and *K. nova* are relatively minor as a possible result of the wide species concept or its being a cryptic-sibling species, we feel that there is enough genetic divergence between them to consider AO-24 as a new *Kudoa* species, namely *K. niluferi* sp. nov.

Our phylogenetic analyses clearly indicated that the lineage comprised of *K. nova*, *K. anatolica* sp. nov. (AO-18 and AO-20) and *K. niluferi* sp. nov. (AO-24) represents a Black Sea group, since no other species from other seas or oceans appeared here. Of these species, *K. anatolica* sp. nov. and *K. niluferi* sp.

Table 2. SSU rDNA nucleotide sequence similarity percentages and DNA distances between some sister *Kudoa* species. AO-24 (*K. niluferi* sp. nov.) and AO-20 (*K. anatolica* sp. nov.) are isolates obtained in this study

	Similarity (%)	Distance
AO-24 vs. *K. nova*	99.1	0.0064
AO-20 vs. *K. nova*	96.5	0.0264
AO-24 vs. AO-20	96.5	0.0271
K. grammatorcyni vs. *K. scomberomori*	99.3	0.0036
K. yasunagai vs. *K. chaetodoni*	99.8	0.0014
K. quadricornis vs. *K. paraquadricornis*	99.9	0.0007

nov. described from fishes in the Black Sea are possibly endemic to the Black Sea itself. *K. nova* was originally described from the Black Sea and the Sea of Azov by Naidenova (1974), but it is also known from 9 different species of fish (not Gobiidae) from the Mediterranean Sea and Atlantic Ocean (Kovaleva et al. 1979, Yurakhno & Gorchanok 2011). However, our phylogenetic analyses (Fig. 3) showed that *K. nova* originated from a hypothetical ancestor with our new *K. niluferi* sp. nov. and *K. anatolica* sp. nov., which both possibly originated from the Black Sea. In light of this information, suggesting the Black Sea and the Sea of Azov as the origin for *K. nova* is more plausible than the scenario of transfer of this species from the Mediterranean as suggested by Kovaleva et al. (1979). Most likely, *K. nova* is a composite species that needs to be thoroughly re-examined. We also feel that the Black Sea lineage contains more *Kudoa* species, but this presumption needs further studies with more *Kudoa* samples from different hosts.

In conclusion, our study provides morphological and molecular phylogenetic data for 2 novel *Kudoa* species, namely *K. niluferi* sp. nov. and *K. anatolica* sp. nov. isolated from *Neogobius melanostomus* and *Atherina hepsetus*, respectively, collected from the Black Sea. Additionally, our molecular data suggested a *Kudoa* lineage specific to the Black Sea.

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