

Kudoa inornata sp. n. (Myxosporea: Multivalvulida) from the skeletal muscles of *Cynoscion nebulosus* (Teleostei: Sciaenidae)

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Abstract: A new myxosporean species, *Kudoa inornata* sp. n. (Myxosporea: Multivalvulida), is described from skeletal muscles of the spotted seatrout *Cynoscion nebulosus* (Cuvier), collected in estuarine waters along the coast of South Carolina, USA. Light microscopic and ultrastructural characters rank this species to the group of *Kudoa* species with simple-shaped spores. The uniqueness of the SSU and LSU rDNA sequences justifies its status of a new species with sister relationship to *Kudoa paniformis*. The 100% prevalence in seatrout from four out of five localities sampled and pathogenicity of *K. inornata* recognized in this study should motivate further screening for infections in its host, which is considered a commercially important game fish with a wide distribution in the Western North Atlantic.

Key words: Myxozoa, Myxosporea, *Kudoa inornata*, muscle infections, *Cynoscion nebulosus*

The spotted seatrout *Cynoscion nebulosus* (Cuvier) (Sciaenidae) distributed along the coasts of the Western North Atlantic and through the Gulf of Mexico is considered one of the most important fish species in this area (Bortone 2003). In the southeast, recreational catches of this species average almost 7 million fish per year (Berger 2008). Due to this fact, spotted seatrout was included among indicators of biological integrity of coastal resources (South Carolina Estuarine and Coastal Assessment Program, SCECAP) and numerous studies concern its biology and management (Bortone 2003, James et al. 2007).

In addition to several parasitic metazoans, only two myxosporean species have been reported from *C. nebulosus*: *Henneguya* sp. from the dorsal and caudal fins (Overstreet 1978, 1983) and *Myxidium* sp. from the gallbladder (Blaylock and Overstreet 2003). Among myxosporeans described from other sciaenid hosts, four nominal and one unidentified *Kudoa* species were reported. In addition to *K. sciaenae* Terán, Llicán et Luque, 1990 that was described from the skeletal muscles of Pacific Ocean *Stellifer minor* (Oliva et al. 1992), the other *Kudoa* species were described from the Gulf of Mexico sciaenids: *K. branchiata* Joy, 1972 from the gills and *K. leiostomi* Dyková, Lom et Overstreet, 1994 from the skeletal muscles of *Leiostomus xanthurus* (Joy 1972 and Dyková et

al. 1994, respectively), and *K. hypoepicardialis* Blaylock, Bullard et Whipps, 2004 from the heart of *Pogonias cromis* (Blaylock et al. 2004). An unidentified *Kudoa* sp. was reported from the skeletal muscle tissue of *Cynoscion arenarius* (Overstreet 1983).

Although *Kudoa* species are reported commonly from diverse fish hosts in various parts of the Eastern Atlantic, comparatively few records relate to the Western Atlantic. In the coastal waters where *C. nebulosus* occurs, *K. funduli* (Hahn, 1915) was found in *Fundulus heteroclitus* (Hahn 1915, Meglitsch 1948, Akaishi et al. 2004), *K. clupeidae* (Hahn, 1917) was reported from several clupeids and an eelpout (Hahn 1917, Meglitsch 1947, 1948, Reimschuessel et al. 2003 and Nigrelli 1946, respectively) and *K. cerebralis* Paperna et Zwerner, 1974 from *Morone saxatilis* (Paperna and Zwerner 1974). A *Kudoa* sp. that differed from those species was reported from *Morone americana* and *Brevoortia tyrannus* (Bunton and Poynton 1991, Webb et al. 2005, respectively). *Kudoa crumena* Iversen et van Meter, 1967 was described from the Spanish mackerel *Scomberomorus maculatus* from the Atlantic coast of Florida and was reported also from *Thunnus albacores* in North Carolina (Iversen and Van Meter 1967, Moran et al. 1999, Kent et al. 2001). In addition to *Kudoa* spp. described from sciaenids mentioned above, *K. shkae* Dyková, Lom et Overstreet, 1994 and four unnamed species

were reported from skeletal muscles of various brackish and marine fishes from the Gulf of Mexico (Overstreet 1983, Dyková et al. 1994), which is the southern-most part of the distribution range of *Cynoscion nebulosus*.

The considerable number of unnamed *Kudoa* species reported from various fish host species from all around the world can be attributed to the paucity of features distinguishing the morphology of spores. Because of the lack of distinguishing features, both molecular and morphological approach was taken to describe a *Kudoa* sp. found in the skeletal muscles of *C. nebulosus*. We believe this approach is an effective method to minimize ambiguity in species descriptions of myxozoans.

MATERIALS AND METHODS

A total of 23 spotted seatrout were collected in November and December, 2008 (using trammel netting and electro fishing) in five localities monitored for biological integrity by the South Carolina Department of Natural Resources: locality 1: Romain Harbor (33°01'45"N, 79°22'25"W), salinity 32–35‰, 9 specimens, total length (TL) 400–450 mm; locality 2: upper Ashley River (32°53'0"N, 80°04'50"W), salinity 5‰, 1 specimen, TL 188 mm; locality 3: Ashley River (32°48'0"N, 79°58'20"W), salinity 22‰, 3 specimens, TL 300–450 mm; locality 4: Combahee River that drains in the ACE basin (32°38'32"N, 80°40'29"W), salinity 5.3‰, 1 specimen, TL 163 mm and salinity 2.6‰, 1 specimen, TL 83 mm; locality 5: ACE basin (32°30'0"N, 80°26'18"W), salinity 34–38‰, 8 specimens, TL 250–450 mm.

Samples of epaxial myomeres were excised at the level of the dorsal fin of each spotted seatrout. Small pieces of fresh samples were squashed and examined under the light microscope. Samples of muscle tissue, together with samples of other organs, were fixed in Davidson fixative and processed for histology. *Kudoa* spores found in fresh or gradually decomposing samples of muscle tissue were concentrated by sedimentation and centrifugation for light microscopical and molecular studies. Spores were measured according to the method of Lom and Arthur (1989). Due to the absence of grossly visible lesions in the muscle tissue, *Kudoa*-positive samples could only be separated for electron microscopy from slightly squashed fresh muscle tissue. These were fixed in sucrose-sodium cacodylate-buffered 2% glutaraldehyde for 24 h, stored in holding buffer for three weeks, postfixed in 1% osmium tetroxide and embedded in Spurr resin (Harris 1991). Ultrathin sections were double stained with uranyl acetate and lead citrate and examined under a JEOL JEM-1010 electron microscope at 80 kV accelerating voltage.

DNA extraction, PCR, and cloning. Samples of muscle tissue stored for one week at 4°C were homogenized in PBS. The homogenate was strained and myxospores were left to sediment in vials. Samples from three host individuals were treated separately. Prior to extraction of DNA, myxospores were crushed in beadbeater (FastPrep®-24, M.P. Biomedicals, CA, USA). DNA was extracted using the Jetquick Tissue DNA Spin Kit (Genomed, Germany) according to the manufacturer's protocol. The SSU rRNA gene was amplified using universal eukaryotic primers ERIB1 and ERIB10 (Barta et al. 1997). The LSU rDNA was assembled by two overlapping parts prepared by two nested PCRs. Briefly, the first part of LSU was obtained using the NLF160–NLR1694 and NLF184–NLR1270 primer sets, and

the second part using the NLF1050–NLR3284 and NLFKud–NLR3113 primer sets (Bartošová et al., in press). PCR was carried out in a 25 µl volume using 10 pmol of each primer, 250 µM of each dNTP, 2.5 µl of 10 × PCR Buffer (Top-Bio, Czech Republic), and 1 U of Taq-Purple polymerase (Top-Bio, Czech Republic). The PCR cycling parameters for the primary PCR of both SSU and LSU rDNA were 95°C for 3 min, then 30 cycles of 95°C for 1 min, 48°C for 1 min, 72°C for 2 min, followed by 10 min incubation at 72°C. Amplification for the nested PCR of LSU rDNA consisted of 95°C for 3 min, then 30 cycles of 95°C for 1 min, 52°C for 1 min, 72°C for 1 min, followed by 10 min incubation at 72°C. PCR products were isolated from the 1% agarose gel using the Jetquick Gel Extraction Spin Kit (Genomed, Germany), ligated into pDrive Cloning vector (Qiagen PCR Cloning Kit, Germany) and transformed into competent *E. coli*-strain XL-1. Clones were sequenced from both strands on an ABI PRISM 3130xl automatic sequencer (Applied Biosystems, Czech Republic).

Phylogenetic analysis. SSU and LSU rDNA sequences were aligned together with sequences of *Kudoa* spp. retrieved from GenBank. Accession numbers of sequences included in the study are given in Fig. 15. The SSU and LSU rDNA alignments were generated by the Clustal W algorithm implemented in Clustal X 1.83 (Thompson et al. 1997) with arbitrarily chosen parameters (8.0 for gap opening penalty and 6.0 for gap extension penalty). The alignments were manually edited using the program BioEdit 7.0.5.2 (Hall 1999).

Maximum parsimony (MP) analyses were performed in PAUP* 4.0b10 (Swofford 2001) by heuristic search with random taxa addition (10 replications), the ACCTRAN-option, and the TBR swapping algorithm. Gaps were treated as missing data and Ts/Tv ratio = 1:2. Branch supports were obtained by 1,000 bootstrap replicates with random sequence additions. For the maximum likelihood (ML) analyses, the likelihood ratio test (LRT) implemented in the Modeltest, version 3.06 (Posada and Crandall 1998) was used to determine the best model of evolution. ML was performed with the GTR+Γ+I model for SSU rDNA and combined SSU and LSU rDNA data, whereas GTR+Γ model was applied for LSU rDNA data. Clade support was assessed with bootstrapping (500 bootstrap replicates). For combined SSU + LSU rDNA data, Bayesian inference (BI) was computed in MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). Likelihood parameters were set to nst = 6, rates = invgamma, ngammat = 4 (equivalent to the GTR+Γ+I model, as suggested by Modeltest). Posterior probabilities (PP) were estimated over 1,000,000 generations via two independent runs of four simultaneous MCMC chains with every 100th tree saved. Log-likelihood scores were plotted and the final 95% of trees were used to produce a majority rule consensus tree.

RESULTS

Light microscopy of squash preparations revealed the presence of *Kudoa* spores (Figs. 1–3) in the muscle tissue of 21 out of 23 (91%) examined specimens of *C. nebulosus*. Histological examination revealed the same 91% prevalence of infection (9/9, 1/1, 3/3, 2/2, and 8/8 specimens positive/examined from localities 1, 2, 3 and 5, respectively; 2/2 specimens negative from locality 4). Spores were observed in aggregates, the length of which

reached 200–300 μm . Although aggregates of spores localised between muscle fibres were observed in all specimens under study, histological examination showed that *Kudoa* spores developed inside the muscle fibres (Figs. 4–7). The extent of damage of muscle fibres was consistent with the progress in development of plasmodial stages (Figs. 4–7). The fate of infected muscle fibres, i.e., their possible complete replacement with mature spores was documented (Fig. 7, arrows). Mature spores that substituted sarcoplasm in the long segment of muscle fibre were enveloped by a thin layer of sarcolemma, which separated them from intermysium, suggesting an easy liberation of mature spores from the affected muscle fibres and invasion of intermysium, as often observed. Similarly as in other *Kudoa* spp. infections of fish skeletal muscles, the overall extent of damage important for the quality of flesh evidently depends on intensity of infection, i.e., the number of affected muscle fibres. Host reaction to developing plasmodial stages or aggregates of spores liberated from muscle fibres was not observed in specimens under study. *Kudoa* spores were observed exclusively in skeletal muscles.

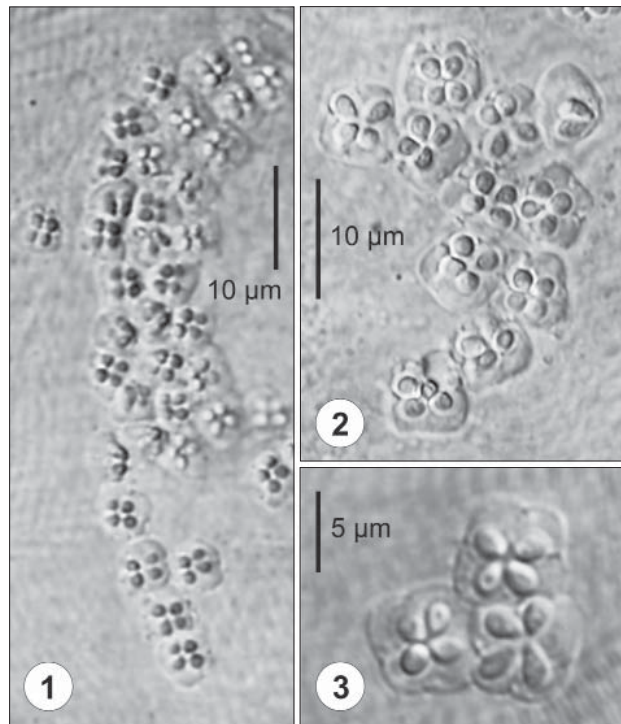
Kudoa inornata sp. n.

Figs. 1–3, 8–12

Morphological description. In the light microscope, shape of spores very simple, both in side and apical views; length 5.4 (5.3–5.5) μm , width 5.9 (5.8–6.0) μm , and thickness 6.0 (5.9–6.1) μm ($n = 20$). In side view, spores rounded posteriorly and slightly pointed in anterior part. Average length of pyriform polar capsules 2.7 μm . Spores in apical view basically quadrate with slightly rounded corners and slightly indicated suture line incisions. Coils of polar filament not seen in fresh spores. Plasmodia polysporic, of spindle-shaped form consistent with shape of muscle fibres, their length and width depending on stage of development.

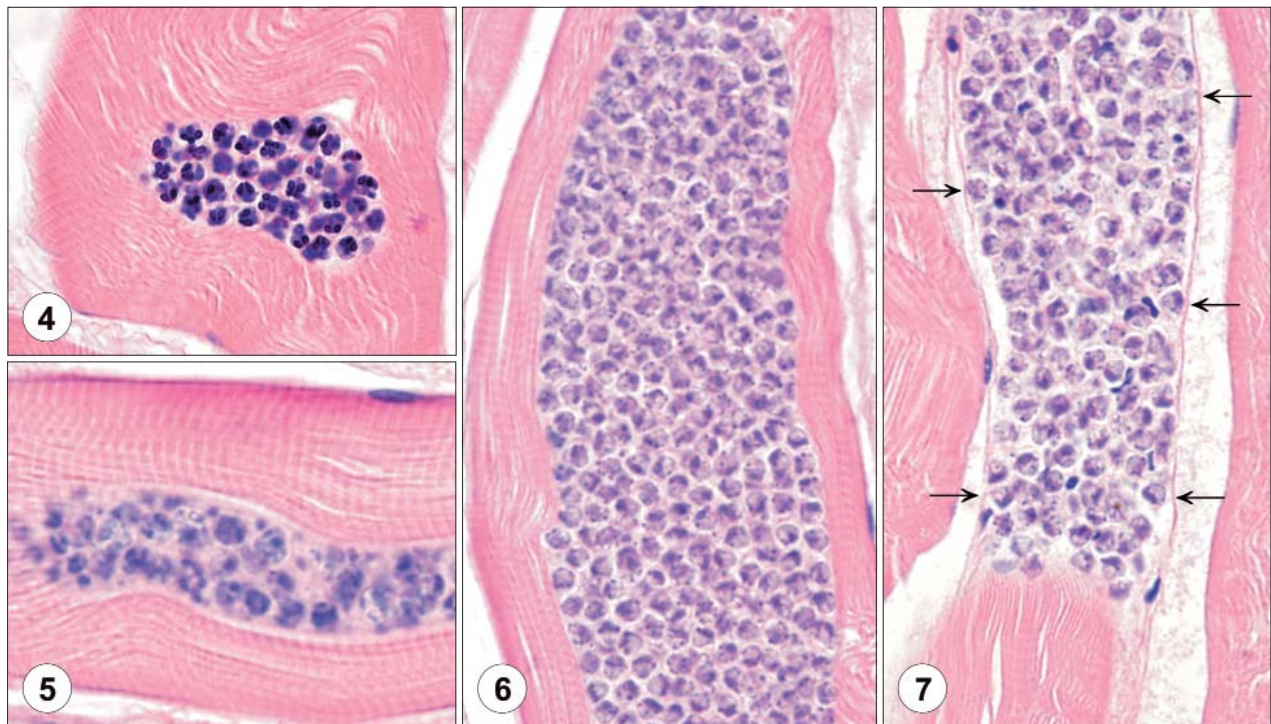
In the transmission electron microscope, inornate type of spores confirmed (Figs. 8, 9). Spores with four shell valves overlapping at suture lines (Fig. 9). Apical part of spore with thickened shell brims (Figs. 8, 12), polar capsules with two turns of polar filament (Fig. 10), sporoplasm in cell-in-cell arrangement (Fig. 8). Capsulogenic cells with electron-dense material of nuclei (Figs. 9, 10). Fibrillar structures connected with spore valves (Fig. 9, arrows) and between two polar capsules (Fig. 11) were observed. Only mature spores were observed within plasmodia and foci of dystrophic muscle tissue. Periphery of plasmodium delimited against host sarcoplasm by thin wall (Fig. 13). As part of muscular disorders, aggregates of host mitochondria with ultrastructural changes were observed (Fig. 14).

Sequence data and phylogenetic analysis. Sequences generated in this study were deposited in GenBank under accession numbers FJ790311 (SSU rDNA) and FJ790312 (LSU rDNA). A marginal difference consisting



Figs. 1–3. Fresh spores of *Kudoa inornata* sp. n. as seen in squash preparations from muscle tissue of spotted seatrout *Cynoscion nebulosus*. Nomarski differential interference contrast.

in one substitution per approximately one thousand base pairs was found among the gene sequences generated from three samples of DNA extracted from three seatrout caught in locality 5 (ACE basin). Sequences for final analyses and deposition in GenBank were selected arbitrarily. Based on both molecular markers (SSU and LSU rDNA), *K. inornata* was found distinct from the hitherto described species with rDNA sequences available in GenBank. Phylogenetic analyses of SSU, LSU, and combined SSU+LSU rDNA sequences revealed a stable sister relationship of *K. inornata* with *K. paniformis* (Fig. 15 and supplementary data). In combined analysis, the nodal support for subclade of the latter species (Fig. 15) was moderate (BI = 0.90, ML = 66% and MP = 78%). The sequence similarity of *K. inornata* and *K. paniformis* was 97.88% when calculated from almost complete SSU rDNA, and 89.83% when calculated from partial LSU rDNA (about 700 nt of 5' end). In both, SSU and SSU+LSU rDNA based analyses, the sequences of *K. inornata* and *K. paniformis* clustered together with sequences of *K. dianae*, *K. mini-auriculata*, *Kudoa* sp. CMW2003, *K. funduli*, *K. clupei-dae*, *K. alliaria*, *K. rosenbuschi* and *Kudoa* sp. MMAG5. Bayesian posterior probabilities and bootstraps calculated for this clade were low (BI = 0.61, ML and MP less than 50%). Phylogenetic analysis of *Kudoa* spp. based on the limited number of LSU rDNA sequences available to date revealed a different topology of tree (see supplementary data at <http://www.mujweb.cz/www/fialaivan/Supplementary.htm>).



Figs. 4–7. Skeletal muscles of spotted seatrout *Cynoscion nebulosus* containing advanced plasmodia of *Kudoa inornata* sp. n. **Fig. 4.** Transverse section through muscle fibre with plasmodium of *K. inornata*. Giemsa $\times 740$. **Fig. 5.** Plasmodium, the shape of which follows the long axis of muscle fibre. HE $\times 1000$. **Fig. 6.** Advanced plasmodium containing almost exclusively mature spores that is enveloped by a thin layer of sarcolemma. HE $\times 760$. **Fig. 7.** Sarcoplasm in a long segment of muscle fibre substituted with mature spores. Sarcolemma marked with arrows. HE $\times 760$.

Taxonomic summary

Type host: *Cynoscion nebulosus* (Cuvier, 1830) (Perciformes: Sciaenidae).

Type locality: Ashepoo-Combahee-Edisto (ACE) basin ($32^{\circ}30'0''\text{N}$, $80^{\circ}26'18''\text{W}$).

Other localities: See in Results.

Site of infection: Skeletal muscles.

Prevalence: 100% in the type locality (8 fish positive/8 examined).

Type material: Phototypes and histological sections deposited in the Institute of Parasitology, Biology Centre AS CR, Česká Budějovice. SSU rDNA sequence deposited in GenBank under Acc. No. FJ790311 and LSU rDNA sequence deposited under Acc. No. FJ790312.

Etymology: The specific name relates to the simple-shaped, inornate spores.

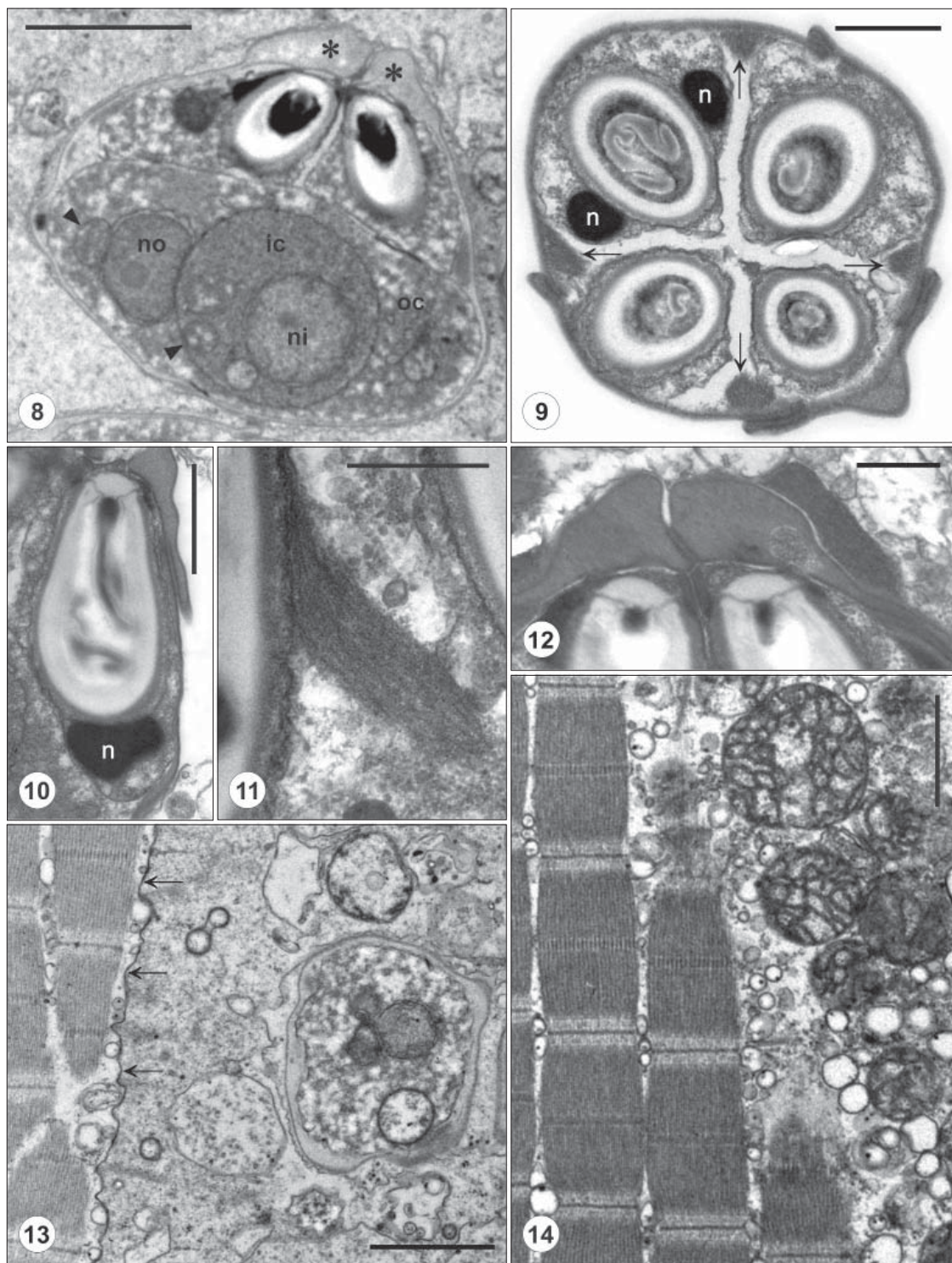
DISCUSSION

The research of *Kudoa* species has been motivated by a number of reasons. Traditional interest of scientists in morphological diversity of parasites along with the more recent interest in relatedness among species through the use of molecular markers is augmented by concerns about the quality of fish meat.

In the past decades, special attention was paid to the impact of *Kudoa* infections on the commercially impor-

tant Pacific hake (*Merluccius productus*) and Atlantic salmon (*Salmo salar*), for which the market value was compromised by *K. paniformis* and *K. thyrsites* infections, respectively (Kudo et al. 1987, Moran and Kent 1999, Moran et al. 1999, Zhou and Li-Chan 2009). In contrast, less effort has been concentrated on other *Kudoa* infections and other fish hosts, even those of commercial or recreational importance. Characterization of any species, irrespective of the importance of its host, is, however, of interest and potential value to fisheries since some *Kudoa* species are considered habitat- rather than host specific (Whipps et al. 2003, Whipps and Kent 2006, Burger et al. 2008). The increasing consumption of fish throughout the world (promoted either for health reasons or because it is an affordable source of proteins), and also the recently discovered potential health risk for humans (Martinez de Velasco et al. 2008) are additional reasons to characterize *Kudoa* species.

In the representatives of *Kudoa* species with inornate, simple-shaped spores that are difficult to distinguish, molecular characterisation is particularly relevant. This is the case of *Kudoa inornata*, the spores of which resemble those of unidentified *Kudoa* spp. from Gulf of Mexico fishes such as *Cyprinodon variegatus*, *Gambusia affinis* and *Menidia beryllina* (Dyková et al. 1994). Unfortunately, molecular data are not available for these *Kudoa* species, the hosts of which are sympatric with *Cynoscion*



Figs. 8–14. *Kudoa inornata* sp. n., ultrastructure of spores and infected host tissue. **Fig. 8.** Mature spore in side view with slightly thickened shell valves in their apical part (*), two polar capsules visible, and cell-in-cell organized sporoplasm (cytoplasm of outer cell = oc, nucleus of outer cell = no, cytoplasm of inner cell = ic, nucleus of inner cell = ni, mitochondria marked with arrowheads). **Fig. 9.** Mature spore in apical view with overlapping shell valves, four symmetrically localised fibrillar structures (arrows) adjoining to valves on the periphery, four polar capsules, including one with electron-dense nuclei of capsulogenic cells (n). **Fig. 10.** Polar capsule of mature spore with two turns of polar filament and electron-dense nucleus (n) of capsulogenic cell at the basal part of polar capsule. **Fig. 11.** Bundle of fibrils between two polar capsules, most probably part of one fibrillar structure marked with arrow in Fig. 9. **Fig. 12.** Apical part of spore in side view. **Fig. 13.** Host-parasite interface with remnant of thin plasmodial wall (arrows). **Fig. 14.** Damage of muscle tissue with a host mitochondrial abnormality involved in the process. Scale bars: Fig. 8 = 2 μ m; Figs. 9, 10 = 1 μ m; Figs. 11, 12 = 500 nm; Figs. 13, 14 = 200 nm.

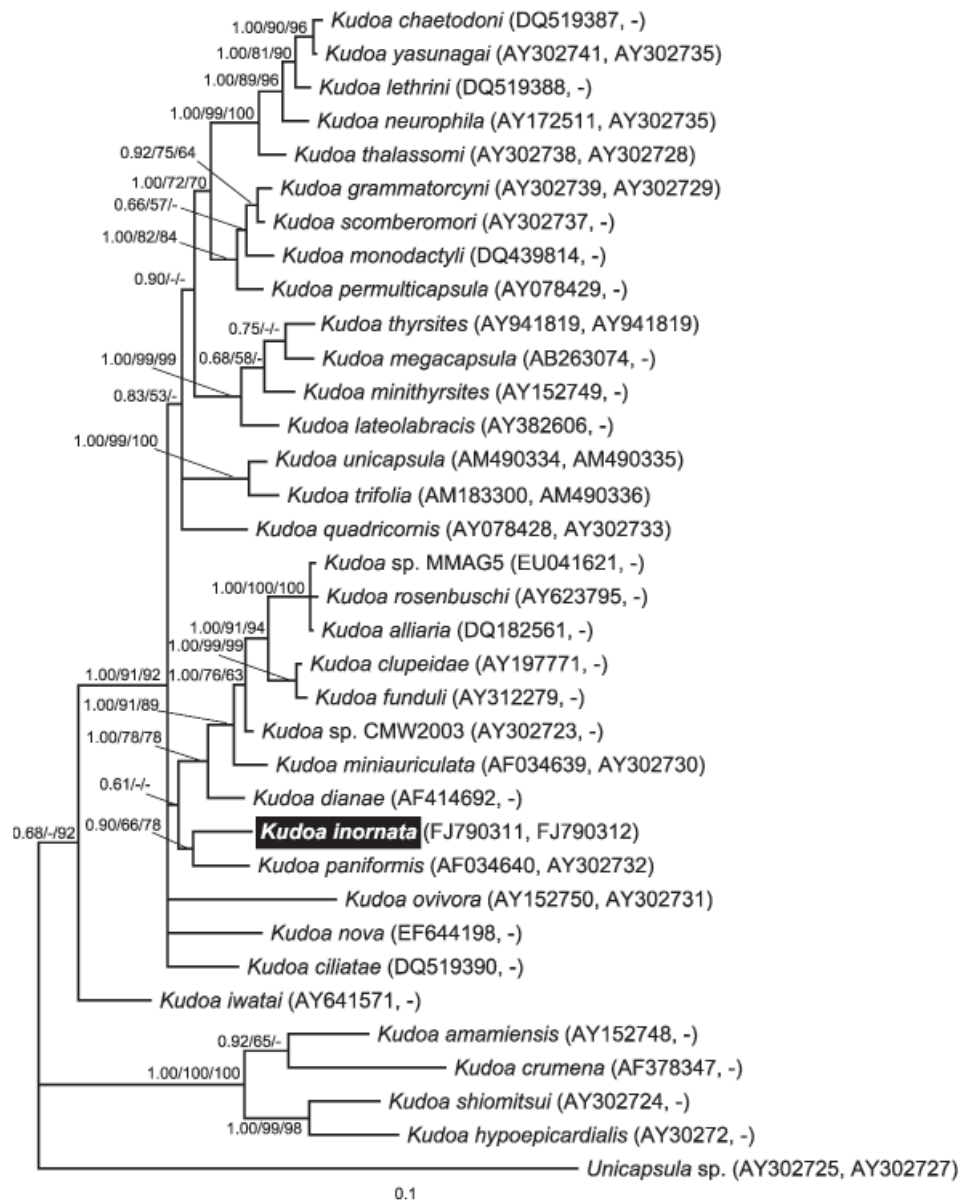


Fig. 15. Bayesian consensus phylogram based on analysis of combined SSU rDNA and LSU rDNA data. *Unicapsula* was set as out-group. Nodal support is indicated for BI (posterior probabilities), ML and MP (only bootstrap values greater than 50% are shown). Species names are supplemented with corresponding GenBank accession numbers for SSU rDNA and LSU rDNA (if the latter were available). All branches are drawn to scale. The number of changes per site is given by scale bar.

nebulosus. To the best of our knowledge, neither morphological nor molecular data are available on the *Kudoa* sp. reported from *Cynoscion arenarius* (Overstreet 1983). Thirty nine specimens of the latter fish species previously studied by Dyková et al. (1994) in the Gulf of Mexico were all *Kudoa* negative.

Comparison of known morphological data of the species phylogenetically most closely related to *K. inornata* revealed that *Kudoa paniformis* Kabata et Whitaker, 1981 described from the Pacific whiting, *Merluccius productus*, also has simple-shaped spores, the size of which does not differ substantially from those of *K. inornata* spores. The

vast difference found in sequence similarity calculated for SSU and LSU rDNA markers of both species can be explained by the presence of two long variable regions in the 5' end of LSU rDNA. The same phenomenon, i.e., a more pronounced variability of LSU rDNA sequences, was recognized by Whipps et al. (2004) for 10 multivalvulid species.

Detrimental effect on the host, known as post-mortem myoliquefaction in *K. paniformis* infections (Patashnik et al. 1982, Stehr 1986, Stehr and Whitaker 1986, Kudo et al. 1987) has not yet been described in spotted seatrout. Nevertheless, a similar effect of *K. inornata* might help

explain the complaints of recreational fishers in South Carolina that spotted seatrout flesh becomes soft and mushy if not consumed promptly after the fish is caught.

The group of species that in our analysis formed a sister subclade to *K. inornata* and *K. paniformis* consisted of those that were treated in detail by Whipps and Diggles (2006). Most of them infect fish in the Western Atlantic. In addition to minute spore differences, these authors also characterized host specificity of individual myxosporean species and pointed out studies containing misidentifications. Compared to a more recent paper on the patterns of relatedness in the Kudoidae (Burger et al. 2007), our study revealed a different phylogenetic position of *K. paniformis*, however, the bootstrap support remained low. In addition, our analysis resolved positions of sequences representing *Kudoa* spores from *Macruronus magellanicus* (EU041621) and *Paralichthys lethostigma* (AY302723). Although these spores were not characterized morphologically, our phylogenetic analyses clarified that spores from the Southern flounder (*P. lethostigma*),

which is sympatric with *C. nebulosus* in estuaries and coastal waters of South Carolina, do not belong to *K. inornata*.

In conclusion, morphological and molecular characterisation of *K. inornata* and the assessment of its pathogenic potential for *C. nebulosus* contributed to the knowledge of an important group of myxosporeans. Further investigations that will involve a study of this species' life cycle, determination of host specificity and the extent of its deleterious effect on its host are deemed necessary to understand its impact on South Carolina estuaries and economy.

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