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MOLECULAR CHARACTERIZATION OF *KUDOA NOVA* (MYXOSPOREA: MULTIVALVULIDA) INFECTING THE ROUND GOBY *NEOGOBIUS MELANOSTOMUS* FROM THE SEA OF AZOV

Diagnosis of *Kudoa nova* infecting the skeletal muscle tissue of round gobiid *Neogobius melanostomus* collected in the Sea of Azov was done by adding molecular data to the demographic and morphological characterizations of the parasite. The small subunit (SU) ribosomal DNA sequence analysis revealed *Kudoa nova* cluster within clade that includes *Kudoa paniformis*, *Kudoa dianae*, *Kudoa* sp., *Kudoa miniauriculata*, *Kudoa alliaria* and *Kudoa rosenbuschi*. Results on the molecular sequence data of *Kudoa nova* provide new evidence to discuss the hypothesis of tissue tropism playing an important role in the radiation of kudoids.

Key words: Myxozoa, *Kudoa nova, Neogobius melanostomus*, Gobiidae, the Sea of Azov, molecular biology

Currently, there are over 75 described species belonging to genus *Kudoa* Meglitsch, 1947 [3, 25, 29] (Myxozoa: Myxosporea: Multivalvulida), all infecting marine or estuarine fishes worldwide. The majority of species are located in skeletal muscle, but also other target tissues in the gills, brain, heart, kidney, spleen, ovary, gall bladder, urinary bladder, oesophagus, intestine, mesentery, and smooth muscle. Heavy infections with some *Kudoa* species can cause unsightly parasite cysts or post-harvest soft flesh in fish meat, creating serious economic losses for the fisheries industry [25].

Kudoa nova Naidenova, 1975 [26] has been described as forming macroscopic cysts in skeletal muscles of 23 fish species, including 14 ones belonging to Gobiidae and the other ones from Sparidae, Scombridae, Carangidae and Pomatomidae. All the infected gobies inhabit the Black Sea and the Sea of Azov, whereas all non-gobiid species were collected from the Mediterranean Sea (France and Italy) and the Atlantic Ocean (Guinean Gulf, equatorial part of the open ocean and the seacoast of north-western Africa). This parasite has found also on gills of one Cottidae fish species in the Pacific Ocean [40].

Kovaleva et al. [19] suggested that *K. nova* may be introduced by horse-mackerel from the Mediterranean Sea into the Black and Azov seas, where this species spread infecting various gobiid species. In our opinion, *K. nova* is an endemic of the Black Sea and the Sea of Azov and meets exclusively in Gobiidae. Thus *K. nova* is a complex of several related species and requires in molecular biology research from all hosts in various regions of its area [40]. We have done microscopic and molecular analyses of *K. nova* infecting the round goby *Neogobius melanostomus* (Pallas) from the Sea of Azov in order to provide new insights on the taxonomic diagnosis of the species.

Materials and methods. Parasite sampling and morphological examinations. Fresh caught gobiids from a coastal zone of the Sea of Azov were purchased at local fish market of Dnepropetrovsk (Ukraine) in 2004 – 2005. These fishes were frozen and transported to the Department of Parasitology of the Institute of Biology of the southern Seas (Sevastopol, Ukraine). A total of 161 specimens of round goby *Neogobius melanostomus* were examined for parasites.

After necropsy, muscle tissues were examined macroscopically and followed by wet-mount preparations of muscle lesions using gelatin-glycerin slides for light microscopy under 600 – 1350x magnifications [6]. Descriptions and measurements of spores were made according to [21, 22]. Cysts of *K. nova* from muscles of fresh round goby catched in the Sea of Azov (Berdyansk) were fixed in 95 % ethanol for subsequent DNA analyses and transported to the Instituto de Investigaciones Marinas-CSIC (Vigo, Spain).

Genomic DNA extraction and PCR amplification. DNA extractions were performed employing the DNAzol reagent® (Invitrogen Life Technologies™) according to the manufactures' instructions. Conserved primers 16S-A (5'-AAC CTG GTT GAT CCT GCC AGT-3') and 16S-B (5'- GAT CCT TCC GCA GGT TCA CCT AC-3') [24] were used to amplify the small subunit (SSU) ribosomal DNA (rDNA). PCR reactions were performed in a total volume of 25 μl containing 1 μl of genomic DNA (200 ng), PCR buffer at 1x concentration, 1.5 mM $MgCl_2$, 0.2 mM nucleotides (Roche Applied Science), 0.3 μM primers and 0.025 U/μl Taq DNA polymerase (Roche Applied Science). The cycling protocol was 94 ºC for 2 min, 35 cycles of 94 ºC for 30 sec, 55 ºC for 1 min and 72 ºC for 2 min, followed by 72 ºC for 7 min. A negative control (no DNA) was included in all PCR reactions. PCR products were separated on a 2% agarose (in 1X Tris-acetic EDTA buffer) gel, stained with ethidium bromide and scanned in a GelDoc XR documentation system (Bio-Rad Laboratories).

DNA sequencing and phylogenetic analysis. PCR products were cleaned for sequencing using the commercial kit Rapid PCR Purification system (Marligen Biosciences, Inc.) according to the manufactures' instructions. The sequence reaction was performed on an ABI PRISM™ 3100 (Applied Biosystems) using a **BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems)** as supplied by the manufacturer. Based on the obtained sequences specific primers KN-1 (5'-CCA AGG TTG TGA CGG GTA AC-3') and KN-2 (5'- AAC TAA GAA CGG CCA TGC AC-3') were synthesized using Primer-3 program [28], which were employed to obtain the entire sequence.

The chromatograms were analysed using ChromasPro version 1.32 Technelysium Pty LtdA. All sequences generated were searched for similarity using BLAST (Basic Local Alignment Search Tool) at web servers of the National Center of Biotechnology Information and then were aligned using the Clustal W algorithm [30] to produce the entire SSU rDNA fragment. The sequence obtained was aligned with other 29 *Kudoa* sequences which are available on GenBank (Table 1).

Sequences of *Zschokkella mugilis* and *Henneguya salminicola* were used as out-group. Alignment was accomplished with Clustal W algorithm in MEGA version 3.1 software [20], with settings at defaults: gap opening / gap extension penalties = 15/ 6.66 for both pair wise and multiple alignments, and with transitions weighted of 0.5. Pairwise distance estimation was calculated using Kimura 2-parameter correction. For the phylogenetic tree minimum evolution and maximum parsimony analysis were performed using MEGA version 3.1 software. Maximum parsimony analysis was conducted using the close neighbour interchange (CNI) heuristic option with initial trees by random addition of 1000 replicates, a search level of 1 and the bootstrap values were calculated over 100 replicates. Minimum evolution analysis was performed using the Kimura-2 parameters model, using the CNI heuristic option with a search level of 2, and the bootstrap values were calculated over 1000 replicates.

Results. Demographic and morphological characterization. Demographic values of *K. nova* in *N. melanostomus* from the Sea of Azov showed high variability in prevalence and intensity of infection in relation to sampling date and locality (Table 2). Also, there were quite different pseudocysts, from white strictly formed spine- or sometimes worm-like cysts (0.2 – 0.75 x 1.1 – 4.5 mm) to more transparent cysts with white and narrow stripes of band shapes (0.2 – 0.3 x 4.0 – 7.0 mm). Cysts are located between muscle fibres and surrounded by thin tissue-uniting host capsule, containing a great number of spores. Spores are square, four-side pyramid, front pole rounded, back one – widened and flattened (Fig. 1). Rounded top of every valve drops out a bit. Pear-shaped polar capsules are large. Individual sizes of spores and especially polar capsules can differ considerably even in one fish (Table 3).

Sequence analysis and phylogeny**.** A fragment of 1.556 bp of the SSU rDNA was amplified, sequenced and deposited in GenBank accession no. EF644198.

Table 1 Myxosporean species used in molecular analysis
Табл. 1 Виды миксоспоридий, использованные в молекулярном анализе

Species	Host	**Accession no.**	Reference
Kudoa alliaria	*Macruronus magellanicus*	**DQ182561**	[33]
Kudoa amamiensis	*Seriola quinqueradiata*	**AF034638**	[16]
Kudoa chaetodoni	*Chaetodon unimaculatus*	**DQ519387**	[3]
Kudoa ciliatae	*Sillago ciliata*	**DQ519390**	[3]
Kudoa crumena	*Thunnus albacares*	**AF378347**	[18]
Kudoa dianae	*Sphoeroides annulatus*	**AF414692**	[7]
Kudoa grammatorcyni	*Grammatorcynus bicarinatus*	**AY302739**	[34]
Kudoa hypoepicardialis	*Nomeus gronovii*	**AY302722**	[2]
Kudoa iwatai	*Pagrus major*	**AY641571**	[5]
Kudoa lateolabracis	*Lateolabrax sp.*	**AY382606**	[36]
Kudoa lethrini	*Gymnocranius audleyi*	**DQ519388**	[3]
Kudoa megacapsula	*Seriola quinqueradiata*	**AB188529**	[35]
Kudoa miniauriculata	*Sebastes paucispinis*	**AF034639**	[16]
Kudoa minithyrsites	*Pempheris ypsilychnus*	**AY152749**	[31]
Kudoa monodactyli	*Monodactylus argenteus*	**DQ439814**	[15]
Kudoa neurophila	*Latris lineata*	**AY172511**	[34]
Kudoa ovivora	*Thalassoma bifascialum*	**AY152750**	[32]
Kudoa paniformis	*Merluccius productus*	**AF034640**	[16]
Kudoa permulticapsula	*Scomberomorus commerson*	**AY078429**	[15]
Kudoa quadricornis	*Carangoides fulvoguttatus*	**AY078428**	[15]
Kudoa rosenbuschi	*Merluccius hubbsi*	**AY623795**	[1]
Kudoa scomberomori	*Scomberomorus commerson*	**AY302737**	[34]
Kudoa shiomitsui	*Paralichthys rubripes*	**AB183718**	Direct submission
Kudoa sp.	*Paralichthys lethostigma*	**AY302723**	[2]
Kudoa thalassomi	*Thalassoma lunare*	**AY302738**	[34]
Kudoa thyrsites	*Scomber scombrus*	**AY542482**	[34]
Kudoa trifolia	*Liza aurata*	**AM183300**	[17]
Kudoa unicapsula	*Liza ramada*	**AM490334**	Direct submission, [41]
Kudoa yasunagai	*Paralichthys olivaceus*	**AY302741**	[34]
Henneguya salminicola	*Oncorhynchus nerka*	**AF031411**	[16]
Zschokkella mugilis	Mugilidae fish	**AF411336**	[27]

Table 2 Infection values with *Kudoa nova* in *N. melanostomus* from the Sea of Azov (*number of cysts counted in 21 cm^2 of smashed muscles)
Табл. 2 Показатели заражённости *Kudoa nova* в *N. melanostomus* из Азовского моря (* количество цист в 21 $см^2$ расплющенных мышц)

Localities	Date	N	Prevalence (%)	Number of cysts*
Kazantip	August 2004	86	52	3 – 30
Berdyansk	December 2004	1	100	17
	January 2005	14	79	3 – 45
Kirillovka	February 2005	31	90	1 – 120
Kerch	February 2005	29	83	4 – 13

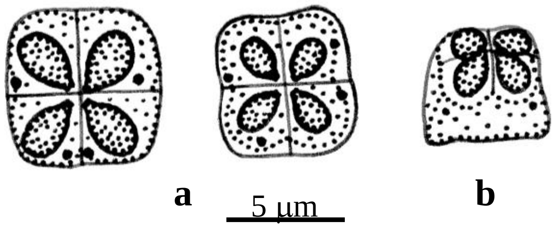

Fig. 1 *Kudoa nova*. Spores from *N. melanostomus* sampled at the Sea of Azov (a – sutural view; b – lateral view) (original)
Рис. 1 *Kudoa nova*. Споры из *N. melanostomus*, выловленного в Азовском море (a – вид сверху; b – вид сбоку) (оригинал)

Character (μm)	(mean ± SD)	(range)
Spore length	5.0 ± 0.44	4.5 – 5.5
Spore width	7.2 ± 0.31	6.8 – 8.0
Spore thickness	6.1 ± 0.32	5.5 – 6.8
Polar capsule length	2.1 ± 0.26	2.0 – 3.0
Polar capsule width	1.3 ± 0.18	1.2 – 1.8

Table 3 Measurements of *Kudoa nova* spores from *N. melanostomus* (glycerin-gelatin preparations)
Табл. 3 Размеры спор *Kudoa nova* из *N. melanostomus* (глицерин-желатиновые препараты)

GenBank Blast search showed the closest identity with *K. permulticapsula*, *K. alliaria*, *K. rosenbuschi*, *K. thyrsites*, *K. scomberomori*, *K. grammatorcyni*, and *K. lateolabracis*, differing at 74 – 77 nucleotides of 1556 bp. The lowest nucleotide identity values were observed with *K. shiomitsui, K. crumena, K. amamiensis* and *K. hypoepicardialis* with identity values at 88 %. The trees constructed with maximum parsimony (MP) (Fig. 2) and minimum evolution (ME) methods (not shown) revealed the same topology. *K. nova* clustered in a clade with *K. paniformis*, *K. dianae*, *Kudoa* sp., *K. miniauriculata*, *K. alliaria* and *K. rosenbuschi* with low bootstrap values of 51 % and 39 % for MP and ME analysis, respectively. The minimum genetic distance observed in this clade was 0.1474 % between *K. alliaria* and *K. rosenbuschi*, and the maximum genetic distance was 3.4722 % between *K. nova* and *K. rosenbuschi* (Table 4). The distances observed between *K. nova* and its closest relatives, *Kudoa* sp. was 2.701 7%.

Table 4 Genetic distances (%) between SSU rDNA sequences of *Kudoa nova* and related species
Табл. 4 Генетические расстояния (%) между секвенциями SSU rDNA *Kudoa nova* и связанных с ней видов

	K. nova	*Kudoa* sp.	*K. alliaria*	*K. dianae*	*K. miniauriculata*	*K. paniformis*
Kudoa sp	2.7017	-	-	-	-	-
K. alliaria	3.3188	1.2632	-	-	-	-
K. dianae	3.1670	1.4987	2.3241	-	-	-
K. miniauriculata	3.1628	0.8891	1.7148	1.9432	-	-
K. paniformis	3.3945	2.1682	2.8556	2.3988	2.1711	-
K. rosenbuschi	3.4722	1.4129	0.1474	2.4755	1.8653	3.0082

Discussion. Demographic parameters of infection by *K. nova* in *N. melanostomus* from the Sea of Azov undergo considerable variations which are likely due to variations in host species and fish size, as well as on the region and season of investigations. This argument has been supported by previous authors who provided well-illustrated examples in the Central Eastern Atlantic [4, 8 – 12, 38]. Also, morphological description of *K. nova* showed high variability in shape and size of pseudocysts and spores. Such high changeability in morphological and structural characters is typical for this species [13, 14, 23, 26, 37, 39]. In fact, a high phenotypic plasticity, a wide host range and a broad geographical distribution have often complicated the taxonomic diagnosis of this species. In this paper, genetic study of the small subunit (SSU) ribosomal DNA (rDNA) of *K. nova* has been performed successfully to provide molecular evidences of a SSU rDNA sequence phylogenetically close to other *Kudoa* species (*Kudoa* sp., *K. paniformis, K. dianae, K. miniauriculata, K. alliaria, K. rosenbuschi*) having four polar capsules. Regarding to the geographic distribution, these *Kudoa* species form a heterogeneous clade, since three species were recorded in the Pacific Ocean (*K. paniformis, K. dianae* and *K. miniauriculata*), two in the Atlantic Ocean (*K. alliaria* and *K. rosenbuschi*) and one (*K. nova*) in the Sea of Azov. The high variability of the morphological characters does not exclude the possibility that *K. nova* can be a complex of several related species rather than one species infecting a large range of fishes around the European waters**.**

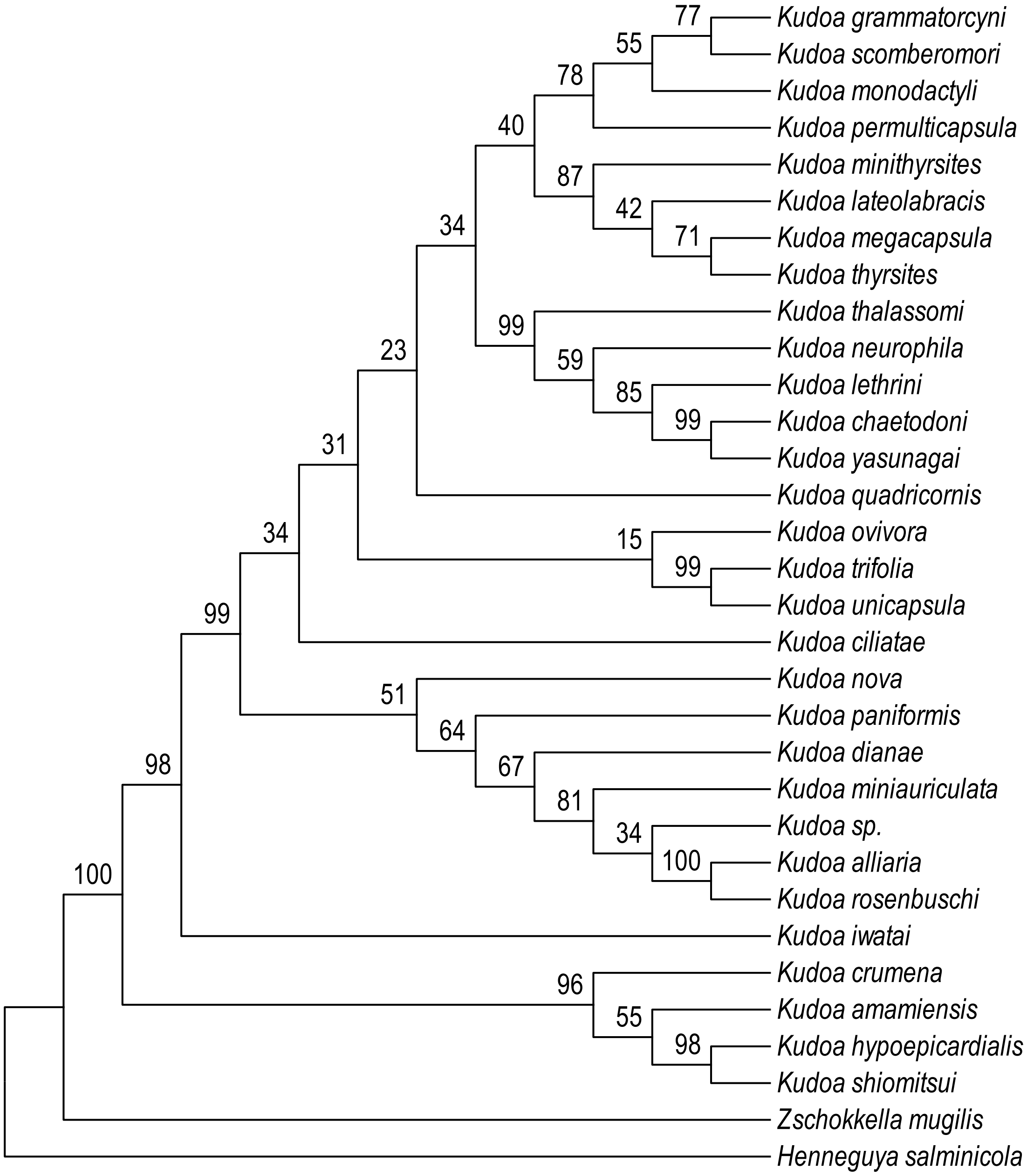

Fig. 2 *Kudoa nova.* Maximum Parsimony analysis showing the taxonomic position of *K. nova* in relation to compared myxosporeans. Numbers at branch nodes indicate bootstrap confidence values in percent. The analysis was conducted using 1365 aligned nucleotide positions.

Рис. 2 *Kudoa nova.* Использование метода анализа максимальной экономии (Maximum Parsimony) позволяет показать таксономическое положение *K. nova* среди сравниваемых миксоспоридий. Номера в отделениях узлов филогенетического древа показывают статистическую надежность в процентах, рассчитанную с применением бутстреп-анализа. Анализ был проведен путем сравнения 1365 нуклеотидных позиций

The use of molecular markers applied to *K. nova* morphotypes found in different fish hosts and geographical areas is thus desirable to provide higher limits of resolution to investigate the host range, geographical distribution and validity of this species. Otherwise, recently Burger et al. [3] have hypothesized that the tissue tropism of some kudoids correlates well with genetic relatedness. The phylogenetic analysis and genetic distances herein reported, defined the taxonomic position of this species in a clade with other skeletal muscle-infecting species (e.g., *K. paniformis*, *K. miniauriculata*, *K. alliaria, K. rosenbuschi*). In contrast, the molecular phylogenetic analyses revealed that *K. nova* sequence largely differs from that of other non muscle-inhabiting species, namely *K. shiomitsui* or *K. hypoepicardialis*. Our data support the Burger´s hypothesis of multiple clades, although as it occurred in the above author's work there were also significant departures represented in our case by *K. dianae*.

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Молекулярна характеристика *Kudoa nova* (Myxosporea: Multivalvulida), що паразитує в бичка-кругляка *Neogobius melanostomus* з Азовського моря. С. Паскуаль, Е. Аболло, В. Юрахно, А. Гаєвська. Опис *Kudoa nova* з скелетної мускулатури бичка-кругляка *Neogobius melanostomus*, виловленого в Азовському морі, виконаний на основі молекулярно-біологічного дослідження, доповнив демографічну та морфологічну характеристики паразита. Аналіз секвенцій малої частини (SU) рибосомальної ДНК показав, що *Kudoa nova* знаходиться в кластері разом з *Kudoa paniformis, Kudoa dianae, Kudoa* sp., *Kudoa miniauriculata, Kudoa alliaria* і *Kudoa rosenbuschi*. Результати молекулярно-біологічного дослідження *Kudoa nova* представили нові докази для обговорення гіпотези тканинного тропізму, що грає важливу роль в радіальної структурі представників роду *Kudoa*.

Ключові слова: Myxozoa, *Kudoa nova, Neogobius melanostomus*, Gobiidae, Азовське море, молекулярна біологія

Молекулярная характеристика *Kudoa nova* (Myxosporea: Multivalvulida), паразитирующего в бычке-кругляке *Neogobius melanostomus* из Азовского моря. С. Паскуаль, Е. Аболло, В. Юрахно, А. Гаевская. Описание *Kudoa nova* из скелетной мускулатуры бычка-кругляка *Neogobius melanostomus,* выловленного в Азовском море, выполнено на основе молекулярно-биологического исследования, дополнившего демографическую и морфологическую характеристики паразита. Анализ секвенций малой части (SU) рибосомальной ДНК показал, что *Kudoa nova* находится в кластере вместе с *Kudoa paniformis, Kudoa dianae, Kudoa* sp., *Kudoa miniauriculata, Kudoa alliaria* и *Kudoa rosenbuschi*. Результаты молекулярно-биологического исследования *Kudoa nova* представили новые доказательства для обсуждения гипотезы тканевого тропизма, играющего важную роль в радиальной структуре представителей рода *Kudoa.*

Ключевые слова: Myxozoa, *Kudoa nova, Neogobius melanostomus*, Gobiidae, Азовское море, молекулярная биология