Morphological and molecular characterisation of anisakid juveniles from the golden grey mullet of the Black Sea

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Accepted for publication 29 June 2018

Summary. The anisakid juveniles of the genus *Contracaecum* were regularly reported from liver ducts of the golden grey mullet *Chelon auratus* (Risso, 1810) collected in the Black Sea coastal waters of Crimean peninsula. Primarily, these juveniles were identified as *C. rudolphii* (*spiculigerum*) Hartwich, 1964. After additional morphological study and analysis of nucleotide sequences the juveniles were identified as *Contracaecum multipapillatum* (Drasche, 1882) Lucker, 1941. Phylogenetic analysis revealed, that partial sequence of LSU rDNA of *Contracaecum* juveniles from golden grey mullet is identical to the deposited LSU rDNA sequence (AF226574) of *C. multipapillatum* from pelicans in Greece. According to the analysis of ITS rDNA this sequence of *C. multipapillatum* from the Black Sea is similar to those of *C. multipapillatum* from Australian pelicans.

Key words: *Contracaecum*, *Chelon auratus*, IST rDNA, juvenile stages, LSU rDNA, marine fish, phylogeny.

The genus *Contracaecum* Railliet & Henry, 1912 is the largest taxon of a generic level within Anisakidae Railliet & Henry, 1912, with more than 50 valid species. The definitive hosts for the majority of the *Contracaecum* species are fish-eating birds with crustaceans as first-, and fish as second-intermediate hosts. The *Contracaecum* juveniles (the third-stage) were found in liver ducts of golden grey mullet *Chelon auratus* (Risso, 1810) during our examination of these fish in 2001. Primary identification as *C. rudolphii* Hartwich, 1964 by Pronkina and Belofastova (2005) was based on the diagnoses and keys from the monograph describing local fauna of parasites (Gaevskaya *et al.*, 1975). A recent revision of *Contracaecum* by Moravec (2009) demonstrated that this previous identification was probably erroneous for these juveniles. An analysis of nucleotide sequences was applied to identify these *Contracaecum* juveniles from Black sea mullets, which therefore were identified as *Contracaecum multipapillatum*. Morphological and molecular characteristics supporting the identification are presented below.

MATERIAL AND METHODS

More than five hundred of 30-50 mm long golden grey mullet specimens were obtained from Crimea coastal waters in 2001-2017. The nematodes obtained after dissections were fixed in 70% ethanol and mounted in glycerin-lactic acid 1:1 mixture. The middle part of the body of one specimen was fixed in 95% ethanol for DNA extraction, and the remaining parts were mounted to serve as the voucher specimen, or 'hologenophore' (Pleijel *et al.*, 2008). The voucher specimen was deposited in the Collection of marine parasites (CMP IMBR № 1059-1080.N.2q.V 1-22) of the Kovalevskii Institute of Marine Biological Research of the Russian Academy of Sciences (Dmitrieva *et al.*, 2015). All the measurements are in micrometers as (range) mean ± SE.

For molecular studies, a portion of gonadal tube was excised from ethanol fixed nematode. An extraction of DNA was made with 'Promega' columns (Wizard® SV Genomic DNA Purification System). For amplification of LSU rDNA the

 doi:10.24411/0869-6918-2018-10008

following primers proposed by Nadler *et al.* (2006) were used: LSU 391 (5'-AGCGGAGGAAAAGAA ACTAA-3') with LSU 501 (5'-TCGGAAGGAACC AGCTACTA-3'), or LSU 391 with LSU 390 (5'-ATCCGTGTTTCAAGACGGG-3'). PCR cycling parameters for the amplification of partial LSU rDNA included primary denaturation at 94°C for 4 min followed by 35 cycles 94°C for 30 s, 56°C for 30 s and 72°C for 60 s, followed by post-amplification extension at 72°C for 5 min. To amplify ITS rDNA the primer 18S (Vrain *et al.*, 1992) together with primer AB28 (5'-ATATGCTT AAGTTCAGCGGGT-3') (Joyce *et al.*, 1994) were used. PCR cycling parameters for the amplification of ITS rDNA included primary denaturation at 94°C for 5 min followed by 35 cycles 94°C for 30 s, 54°C for 35 s and 72°C for 70 s, followed by post-amplification extension at 72°C for 5 min. The PCR products were purified in 0.8% agarose gel, the bands were excised and DNA was extracted with columns Wizard SV Gel and PCR Clean-Up System (Promega, Madison, USA). After the precipitation (ethanol + ammonium acetate) the samples were directly sequenced by 'Genotech' company (Moscow). Similar sequences were searched for in NCBI GenBank with BLAST algorithm (Altschul *et al.*, 1990). Obtained sequences were analysed in MEGA7.0.14 (Kumar *et al.*, 2016) with three methods: maximum parsimony (MP), neighbour joining (NJ) and maximum likelihood (ML). Obtained sequences were deposited in NCBI GenBank (partial ITS rDNA as MH400190 and partial 28S rDNA as MH398587).

RESULTS

Contracaecum multipapillatum (Drasche, 1882) Lucker, 1941 juveniles

Dissected from the second intermediate host, golden grey mullet *Chelon auratus* (Risso, 1810) (Mugilidae).

Locality. Black Sea coastal water, *i*) near Sevastopol (44º36'24'' N, 33º28'11'' E; 44º30'18'' N, 33º35'54'' E; 44º36'39'' N, 33º29'51'' E; 44º36'31'' N, 33º31'51'' E; 44º36'60'' N, 33º30'44'' E) and *ii*) Karkinitski Bay (45°52'30'' N, 33°32'30'' E).

Localisation in host. Biliary ducts in liver. Up to eight juveniles per fish. The youngest infected fish were two years old.

Material. One juvenile, deposited as the hologenophore and 22 voucher specimens of juveniles (CMP IMBR № 1059-1080.N.2q.V 1-22).

Description (n = 19; intact third-stage juveniles and also the head end and tail of the hologenophore specimen). Body length 14274-29750 (23266 ± 816.3), maximal dia. 575-1125 (849 ± 29); nerve ring dia. 256-425 (343 ± 7.9), anal opening dia. 88-200 (148 ± 8.9). Under light microscope the body surface is transversally annulated. Coarse annulation on head end (Fig. 1A). Cuticle 13-38 (26.8 ± 1.3) thick. Oral opening slit-like, with four flattened protrusions of body surface around (Fig. 1A). Excretory pore opening near head end. Prominent juvenile tooth, 12-25 (17.4 ± 0.7) high (Fig. 1A). Nerve ring 52-96 (76 ± 3) × 129-221 (175 ± 6), in 179-375 (253.3 ± 10.5) from anterior end. Intestinal

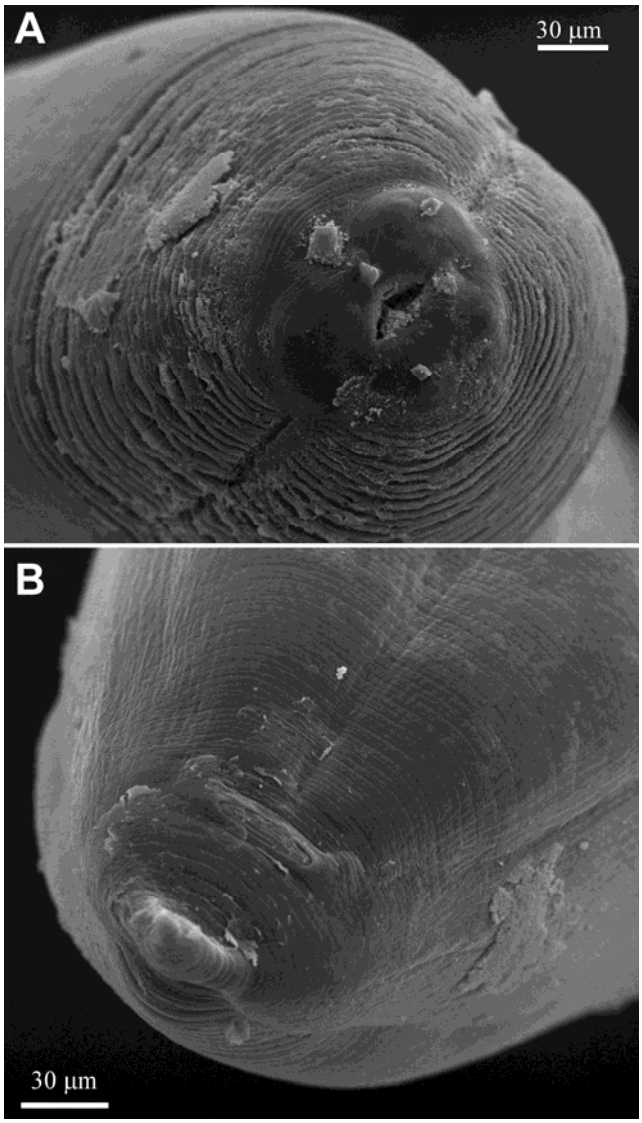

Fig. 1. Surface structures of *Contracaecum multipapillatum* juveniles from golden grey mullet. A – anterior end; B – tail.

caecum well developed, wider than ventricular appendix, 1527-3500 (2227 ± 106) long, 125-550 (327 ± 21) wide. Subglobular ventriculus on pharynx end 88-188 (123 ± 6.0) long and 88-188 (142 ± 6.1) wide with the protruding part 500-760 (658 ± 17.7) long and 100-159.6 (132 ± 3.3) wide. Length ratio of intestinal caecum to pharyngeal ventriculus appendix 3:1. Reproductive system not developed. Rectal gland discernible. Tail end cupola-shaped 125-280 (208 ± 13) long, with conical terminal process (Fig. 1B). Juvenile enclosed into capsule with coarse fibrillar structure of walls.

Primer pairs LSU391-LSU501, and LSU391-LSU390 amplified nearly identical sequences. BLAST search against this sequence revealed a set of close sequences, all of which originated from nematodes of the genus *Contracaecum* and related genera. The length of obtained LSU rDNA sequence was about 700 bp, but because of different lengths of sequences from GenBank the final length of obtained alignment was 669 bp, with 106 informative characters. The topology of the trees obtained with three methods of analysis (MP, NJ and ML) was identical. The sequence obtained for juveniles from golden grey mullet in all three topologies was in the group composed of *Contracaecum multipapillatum* LSU rDNA sequences (Fig. 2). The bootstrap support for the entire group of four sequences and the subgroup of three sequences including that from Black Sea is strong (Fig. 2).

The length of ITS rDNA amplicon obtained with primers 18S and AB28 was about 1000 bp. Alignment of obtained sequence with those found in GenBank was only 451 bp long as some deposited sequences were quite short. The number of informative characters is 198. The topologies of trees constructed with all three methods (MP, NJ and ML) were identical. The ITS rDNA sequence from golden grey mullet clustered with two *C. multipapillatum* sequences and was a part clade with strong support consisting mainly of the sequences belonging to this species. Some sequences of *C. ovserstreeti*, *C. pyripapillatum* and an unidentified *Contracaecum* sp. were also inside this clade (Fig. 3).

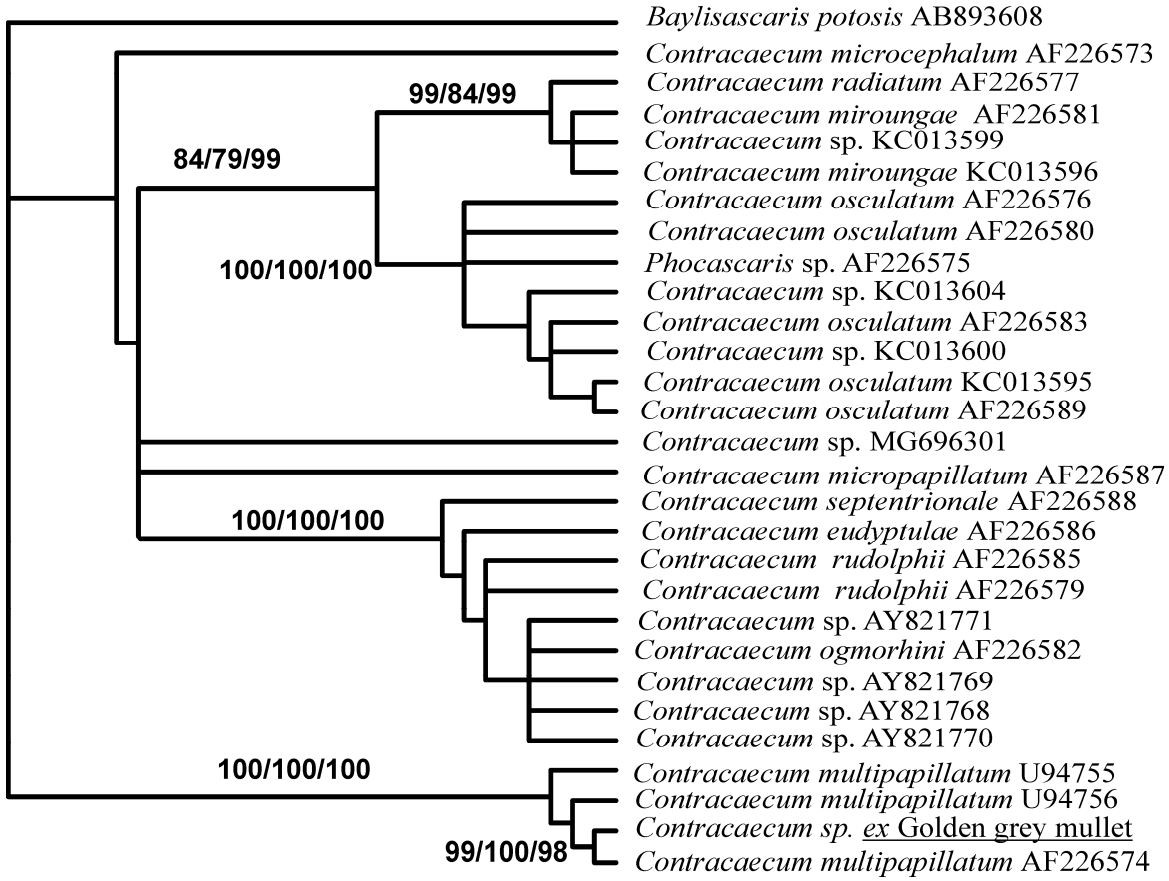

Fig. 2. Phylogenetic relationships of *Contracaecum multipapillatum* from Black Sea golden grey mullet inferred from an analysis of partial LSU rDNA. Bootstrap support values are presented near nodes as MP/NJ/ML. ML analysis (500 bootstrap replications), K2+G model. For MP and NJ – 1000 bootstrap replications.

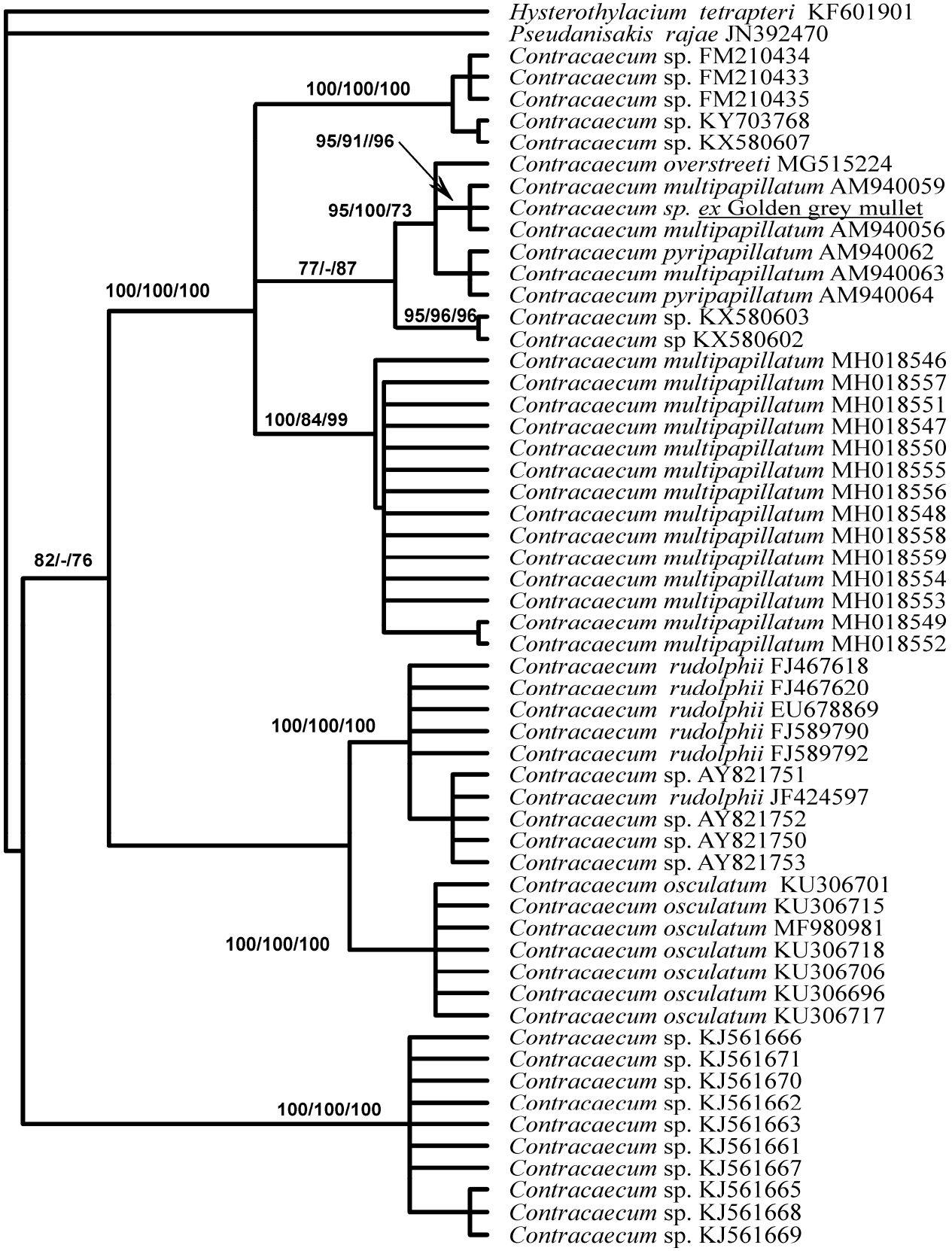

Fig. 3. Phylogenetic relationships of *Contracaecum multipapillatum* from Black Sea golden grey mullet inferred from an analysis of ITS rDNA. Bootstrap support values are presented near nodes as MP/NJ/ML. ML analysis (500 bootstrap replications), K2+G model. For MP and NJ – 1000 bootstrap replications.

DISCUSSION

Previous erroneous identification of juveniles of *Contracaecum* found in golden grey mullet in the Black Sea off Crimea as *C. rudolphii* (earlier was known under the synonym *C. spiculigerum*) (Pronkina & Belofastova, 2005) was based on the incorrect compilation of the data on the morphology of juveniles representative of this genus (Gaevskaya *et al.*, 1975). Measurements of anisakid juveniles obtained in the course of experimental inoculations with these nematodes of dragon-fly larvae and fresh-water fishes (Mozgovoy *et al.*, 1968) were given in this work. These data were illustrated with the figures of anisakid specimens found in the body cavity of catfish from Tumak district in Volga estuary (Dogiel & Bykhovskii, 1939). The same measurements of *Contracaecum* juveniles identified as *C. rudolphii* accompanied with original drawings were given in a monograph by Moravec (1994). The measurements of *Contracaecum* juveniles found in golden grey mullet near Crimea coast (Pronkina & Belofastova, 2005) correspond with the data presented in the above mentioned work, and their morphology to those illustrated by Dogiel and Bykhovskii (1939). Finally, the comparison of *Contracaecum* juveniles from Black Sea golden grey mullet with *C. rudolphii* juveniles described in detail by Moravec (2009) has demonstrated that the former did not fit to the corrected diagnosis of *C. rudolphii*.

Phylogenetic analysis of the sequences of the two nuclear loci obtained from the newly collected specimens of *Contracaecum* from golden grey mullet demonstrated their close similarity with the deposited sequences of *C. multipapillatum*.

Phylogenetic analysis of the obtained sequences demonstrated the affinity of the juveniles studied to the species *C. multipapillatum*. This affinity was unequivocal in the analysis of the partial LSU rDNA (Fig. 2), although only three sequences of this species are deposited in NCBI GenBank. No nucleotide differences in this LSU rDNA sequence was found between Black Sea *Contracaecum* in this study and the deposited sequence AF226574 of adult *C. multipapillatum* from *Pelecanus crispus*, originating from Psatatopi, Greece (Nadler *et al.*, 2000). The difference in 10-11 bp in LSU rDNA was found between juveniles in our material and juveniles of *C. multipapillatum* from *Mugil curema* mullets of the Grand Lagoon, Horn Island, Mississippi, USA.

The ITS rDNA analysis of *Contracaecum multipapillatum* was based on the larger number of sequences deposited and showed that the relationships of the studied *Contracaecum* juveniles from Black Sea mullets were complicated (Fig. 3). Our samples formed the strongly supported clade with two sequences of adult *C. multipapillatum* from pelicans collected and dissected in Australia (Victoria). The difference in 7 bp of ITS rDNA was observed between our samples and the juveniles from USA, whilst all other deposited sequences of *C. multipapillatum* and related species differed at least by 22 bp. The clade of these three sequences of *C. multipapillatum* from mullets was a part of the wider group of deposited sequences with representatives of several identified and unidentified up to species level *Contracaecum* samples (Fig. 3). The phylogenetic relationships of our *C. multipapillatum* sample with other species of the genus revealed the obscure taxonomic status of this species, which was sometimes treated as a 'species complex' (Nadler *et al.*, 2000; Mattiucci *et al.*, 2006; 2010; D'Amelio *et al.*, 2007).

An accumulation of data about the nucleotide diversity of anisakid nematodes in the Black Sea and adjoining waters is needed.

ACKNOWLEDGEMENTS

This work is a part of the state supported studies of Black Sea biota in the Kovalevsky Institute of Marine Biological Research, Russian Academy of Sciences (project no. AAAA-A18-118020890074-2). The work was partially supported by the RFBR grant to S.E.S. (project no. 17-04-00095a).). SEM studies were carried out at the Joint Usage Center "Instrumental Methods in Ecology" at A.N. Severtsov Institute of Ecology and Evolution.

REFERENCES

ALTSCHUL, S.F., GISH, W., MILLER, W., MYERS, E.W. & LIPMAN, D.J. 1990. Basic local alignment search tool. *Journal of Molecular Biology* 215: 403-410.

D'AMELIO, S., BARROS, N.B., INGROSSO, S., FAUQUIER, D.A., RUSSO, R. & PAGGI, L. 2007. Genetic characterization of members of the genus *Contracaecum* (Nematoda: Anisakidae) from fish-eating birds from west-central Florida, USA, with evidence of new species. *Parasitology* 134: 1041-1051.

DMITRIEVA, E.V., LYAKH, A.M., KORNYYCHUK, YU.M., POLYAKOVA, T.A., POPYUK, M.P., YURAKHNO, V.M. & PRONKINA, N.V. 2015. *IMBR Collection of Marine Parasites. Collection of Marine Parasites Maintained by the Institute of Marine Biological Research*. URL: www.marineparasites.org (accessed: January 29, 2018.)

DOGIEL, V.A. & BYKHOVSKII, B.E. 1939. [*Parasites of Fishes of the Caspian Sea*]. USSR, the USSR Academy of Sciences Publishing House. 151 pp. (in Russian).

GAEVSKAYA, A.V, GUSEV, A.V., DELYAMURE, S.L., DONETS, Z.S., ISKOVA, N.I., KORNYUSHIN, V.V., KOVALEVA, A.A., MARGARITOV, N.M., MARKEVICH, A.P., MORDVINOVA, T.N., NAIDENOVA, N.N., NIKOLAEVA, V.M., PARUKHIN, A.M., POGORELTSEVA, T.P, SMOGORZHEVSKAYA, L.A., SOLONCHENKO, A.I., SHTEIN, G.A. & SHULMAN, S.S. 1975. [*Key to the Parasites of Vertebrata of the Black and Azov Seas*]. USSR, Naukova Dumka. 552 pp. (in Russian).

JOYCE, S.A., REID, A., DRIVER, F. & CURRAN, J. 1994. Application of polymerase chain reaction (PCR) methods to the identification of entomopathogenic nematodes. In: *COST 812: Biotechnology. Genetics of Entomopathogenic Nematode – Bacterium Complexes: Proceedings of a Symposium and Workshop Held at St. Patrick's College, Maynooth, Co. Kildare, Ireland and National Reports 1990-1993* (A.M. Burnell, R.-U. Ehlers & J.-P. Masson Eds). pp. 178-187. Brussels, Belgium, Office for Official Publications of the European Communities.

KUMAR, S., STECHER, G. & TAMURA, K. 2016. MEGA7: molecular evolutionary genetics analysis. version 7.0 for bigger datasets. *Molecular Biology and Evolution* 33: 1870-1874.

MATTIUCCI, S., OLIVERO, J., PAOLETTI, M., ARROLLO, B. & NASCETTI, G. 2006. Genetic evidence for new species of *Contracaecum* (Nematoda, Anisakidae) parasites of the brown pelican, *Pelecanus occidentalis*, from Columbia: genetic relationships between congeners and larval identification. In: *Abstracts of the 11th International Congress of Parasitological Associations*. p. 56. Glasgow, Scotland, ICOPA XI.

MATTIUCCI, S., PAOLETTI, M., SOLORZANO, A.C. & NASCETTI, G. 2010. *Contracaecum gibsoni* n. sp. and *C. overstreeti* n. sp. (Nematoda: Anisakidae) from the Dalmatian pelican *Pelecanus crispus* (L.) in Greek waters: genetic and morphological evidence. *Systematic Parasitology* 75: 207-224.

MORAVEC, F. 1994. *Parasitic Nematodes of Freshwater Fishes of Europe*. The Netherlands, Academia and Kluwer Academic Publishers. 473 pp.

MORAVEC, F. 2009. Experimental studies on the development of *Contracaecum rudolphii* (Nematoda: Anisakidae) in copepod and fish paratenic hosts. *Folia Parasitologica* 56: 185-193.

MOZGOVOY, A.A., SHAKHMATOVA, V.I. & SEMENOVA, M.K. 1968. [Life cycle of *Contracaecum spiculigerum* (Ascaridata: Anisakidae), a parasite of domestic and game birds]. *Trudy Gel'mintologicheskoi Laboratorii AN SSSR* 19: 129-136 (in Russian).

NADLER, S.A., D'AMELIO, S., FAGERHOLM, H.P., BERLAND, B. & PAGGI, L. 2000. Phylogenetic relationships among species of *Contracaecum* and *Phocascaris* (Nematoda, Ascaridoida) based on nuclear rDNA. *Parasitology* 121: 455-463.

NADLER, S.A., BOLOTIN, E. & STOCK, S.P. 2006. Phylogenetic relationships of *Steinernema* Travassos, 1927 (Nematoda: Cephalobina: Steinernematidae) based on nuclear, mitochondrial and morphological data. *Systematic Parasitology* 63: 161-181.

PLEIJEL, R., JONDELIUS, U., NORLINDER, E., NYGEREN, A., OXELMAN, B., SCHANDER, C., SUNDBERG, P. & THOLLESSON, M. 2008. Phylogenies without roots? A plea for the use of vouchers in molecular phylogenetic studies. *Molecular Phylogenetics and Evolution* 48: 369-371.

PRONKINA, N.V. & BELOFASTOVA, I.P. 2005. [New data about nematodes of the Black Sea golden grey mullet *Liza aurata* (Pisces: Mugilidae)]. *Ekologiya Morya* 46: 77-82 (in Russian).

VRAIN, T.C., WAKARCHUK, D.A., LEVESQUE, A.C. & HAMILTON, R.J. 1992. Intraspecific rDNA restriction fragment length polymorphism in the *Xiphinema americanum* group. *Fundamental and Applied Nematology* 15: 563-573.

Н.В. Пронькина и С.Э. Спиридонов. Морфологическая и молекулярная характеристика анизакидных личинок от черноморского сингиля.

Резюме. В протоках печени сингиля *Chelon auratus* (Risso, 1810) у побережья Крыма постоянно обнаруживаются личинки паразитических анизакидных нематод рода *Contracaecum*. Первоначально эти личинки были определены как *C. rudolphii* (*spiculigerum*) Hartwich, 1964. После дополнительного морфологического изучения и анализа полученных нуклеотидных последовательностей личинки от сингиля были определены как *Contracaecum multipapillatum* (Drasche, 1882) Lucker, 1941. По нуклеотидным последовательностям LSU rDNA личинки *Contracaecum* от сингиля оказались идентичными депонированной (AF226574) последовательности *C. multipapillatum* от пеликанов из Греции. По ITS-участку rDNA *C. multipapillatum* оказались близки к последовательностям взрослых особей *C. multipapillatum* от пеликанов Австралии.