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**MOLECULAR DATA CONFIRM THE SPECIES STATUS
OF *NEOECHINORHYNCHUS PERSONATUS* AND *N. YAMAGUTII*
(ACANTHOCEPHALA, NEOECHINORHYNCHIDAE)
FROM THE ATLANTIC AND PACIFIC GREY MULLET
(TELEOSTEI, MUGILIDAE)**

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Molecular Data Confirm the Species Status of *Neoechinorhynchus personatus* and *N. yamagutii* (Acanthocephala, Neoechinorhynchidae) from the Atlantic and Pacific Grey Mullet (Teleostei, Mugilidae). Sarabeev, V., Tkach, Ie., Sueiro, R. A., Leiro, J. — *Neoechinorhynchus* is known to be the most diverse acanthocephalan taxon with worldwide distribution; its species are characterized by uniformity of anatomical organization. The taxonomic status of *Neoechinorhynchus agilis* s. l. from grey mullets was recently reviewed that resulted in description of two new species, *Neoechinorhynchus (Hebesoma) personatus* and *Neoechinorhynchus (Hebesoma) yamagutii*. In the current study, 18S rRNA partial gene sequences were obtained to molecularly characterize *N. (N.) agilis*, *N. (H.) personatus* and *N. (H.) yamagutii* from grey mullets, *Chelon labrosus* and *Mugil cephalus*, in the North-East Atlantic, the Japan, Azov and Mediterranean Seas. The universal (F-566 and R-1200) and specific eukaryotic set primers were used to amplify specimens from each species of *Neoechinorhynchus*. It has been found that three species can be clearly recognized using universal primers, which allow to obtain sequences of 590 to 664 bp in length. The genetic divergences estimated between three species sequenced here were relatively high, at ranged between 2.08 and 6.57 %. Phylogenetic analysis demonstrates that the studied species of *Neoechinorhynchus* from grey mullet fish share common ancestor, despite their different geographic location, and are closely related. The terminal position of *N. (N.) agilis* and *N. (H.) personatus* on the evolution tree and the low genetic divergence found between them suggests the recent emergence of this group and that the colonization of the North-East Atlantic and the Mediterranean regions could represent a single event. Our phylogenetic analysis, which included several species of the subgenera *Neoechinorhynchus* and *Hebesoma*, showed that the latter is a polyphyletic taxon.
Key words: 18S rRNA partial gene sequences, *Chelon labrosus*, *Mugil cephalus*, *Neoechinorhynchus agilis*, *Neoechinorhynchus personatus*, *Neoechinorhynchus yamagutii*.

Introduction

The taxonomic status of *Neoechinorhynchus agilis* s. l. (Rudolphi, 1819) from grey mullets (Mugilidae) across localities in the North-East Atlantic and the North-West Pacific areas was recently reviewed by Tkach et al. (2014). This taxonomic review performed on morphological features showed that there were three different species of *Neoechinorhynchus* occurring in mullet hosts, two of those were recorded in the Atlantic and one in the Pacific waters (*Neoechinorhynchus (Neoechinorhynchus) agilis* (Rudolphi, 1819) and *Neoechinorhynchus (Hebesoma) personatus* Tkach, Sarabeev et Shvetsova, 2014, *Neoechinorhynchus (Hebesoma) yamagutii* Tkach, Sarabeev et Shvetsova, 2014, respectively). Morphological identification of *Neoechinorhynchus* spp. from grey mullets is based on the combination of a set of specific characters describing the number of hypodermal nuclei, features of the hooks, lemnisci and genital organs. While the conventional microscopy applied to identification of *Neoechinorhynchus* is complicated because of high species diversity and specific morphology of the group, which commonly requires experienced researcher, accurate taxonomic identification is essential for biological and ecological studies. DNA barcoding techniques are the most technological and precise methods for the identification of a specimens bulk to the genus and species level (Morand, 2018). Biodiversity assessment method of metabarcoding uses a short section of DNA from a standardized region of the genome (Hebert et al., 2003). In the present study, we test the pair of the universal primers, F-566 and R-1200 to amplify variable regions of 18S rRNA gene (Hadziavdic et al., 2014) of three species of *Neoechinorhynchus* from grey mullets. We also designed a set of specific primers that were used to obtain longer sequences of the studied species and to perform comparative phylogenetic analysis. Some regions of the 18S rRNA gene are highly conserved, others are variable or even highly variable (Hadziavdic et al., 2014); thus this gene can provide a basis for resolving relationships at the genus and species levels of studied here acanthocephalan parasites.

Although *Neoechinorhynchus* is the most diverse acanthocephalan group with 121 known species, a few taxa of the genus were analysed using molecular data (Amin, 2013; Smales, 2013; Pinacho-Pinacho et al., 2014; Tkach et al., 2014; Melo et al., 2015; Gautam et al., 2018). There is much fewer data available on 18S rRNA sequences for *Neoechinorhynchus* spp. Malyarchuk et al. (2014) investigated molecular phylogenetic relationships of six *Neoechinorhynchus* species from fishes collected in freshwater localities of North-East Asia using DNA sequences of two genes, cytochrome CO1 of the mitochondrial DNA and 18S rRNA. Two additional almost complete 18S rRNA sequences were obtained by Near et al. (1998) and García-Varela & Nadler (2005) for *Neoechinorhynchus (Neoechinorhynchus) crassus* Van Cleave, 1919 and *Neoechinorhynchus saginatus* Van Cleave et Bangham, 1949, respectively, in their studies of acanthocephalan phylogeny. Finally, three sequences of the target regions of 18S rRNA for *Neoechinorhynchus (Neoechinorhynchus) pseudemydis* Cable et Hopp, 1954, *N. (N.) cylindratus* (Van Cleave, 1914) and *N. (N.) dimorphospinus* Amin et Sey, 1996 were found as deposited in GenBank (table 1).

Here, 18S rRNA partial gene sequences of *N. (N.) agilis*, *N. (H.) personatus* and *N. (H.) yamagutii* were obtained from grey mullet hosts in localities across the North-East Atlantic and North-West Pacific regions, allowing us to molecularly characterize them, reveal interspecific relationships and test the systematic position within the genus.

Materials and methods

Material collection

Grey mullets were collected from five localities in the North-East Atlantic and the Japan, Azov and Mediterranean Seas (table 1). In the current study, acanthocephalan parasites were sampled from two fish species, *Chelon labrosus* (Risso) and *Mugil cephalus* L. Fish intestine and pyloric caeca were examined for parasites within the day of capture, or refrigerated, and surveyed for infections with acanthocephalans under a stereomicroscope. The worms were isolated from the intestine, washed in 0.8 % saline water and after eversion of the proboscis fixed and stored in 70 % or absolute ethanol prior to examination. The specimens of *Neoechinorhynchus* collected in this study were identified as *N. (N.) agilis*, *N. (H.) personatus* and *N. (H.) yamagutii* based on morphological features and host-geographic information (Tkach et al., 2014).

DNA extraction, amplification, sequencing and analysis

Specimens of acanthocephalans were placed individually in tubes and sonicated in TE buffer for several seconds. Then sodium dodecylsulfate and proteinase K were added to the mixture and digested 2 hours at 37 °C with subsequent phenol-chloroform deproteinization, isopropanol precipitation, and ethanol washing. DNA was analysed to estimate its purity and concentration with the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, USA). PCR amplification was performed as previously described by Leiro et al. (2000) and De Felipe et al. (2017). The universal eukaryotic primers (F-566:5'-CAG CAG CCGCGTAATTC C-3' and R-1200:5'-CCC GTGTTGAGTCAA ATT AAG C-3') proposed by Hadziavdic et al. (2014) were used to amplify V4 and V5 variable region of 18S rRNA gene as regions with high taxonomic information. Based on available sequences of 18S rRNA gene in GenBank (www.ncbi.nlm.nih.gov/genbank/) and new ones obtained in the current study for specimens of *Neoechinorhynchus*, the specific primer set (FNeo1086:5'-ATA GCCATG CAT GTG CAG TT-3' and RNeo1086:5'-GCC TTGCGACCATACTCC C-3') was designed and optimized by

Table 1. Isolate identification number (ID), species analysed, sea/region and locality name, host species, sequence length, GenBank accession number and source for specimens of *Neoechinorhynchus* studied in this work

Isolate ID	Species	Sea/region	Locality	Host	Length (bp)	GenBank accession no.	Source
V1	<i>Neoechinorhynchus (Hebesoma) personatus</i> (Tkach, Sarabeev et Shvetsova, 2014)	Mediterranean	Valencia coastal waters, Spain	<i>Mugil cephalus</i> L.	990	MN149066	The present study
V3	<i>N. (H.) personatus</i>	«	«	«	1025	MN149067	«
G42	<i>N. (H.) personatus</i>	Azov Sea	Sivash Lake, Ukraine	«	1042	MN149068	«
G44	<i>N. (H.) personatus</i>	«	«	«	1018	MN149069	«
G45	<i>N. (H.) personatus</i>	«	«	«	1034	MN149070	«
G49	<i>N. (H.) personatus</i>	«	«	«	597	MN149071	«
G55	<i>N. (H.) personatus</i>	«	«	«	1031	MN149072	«
I1	<i>N. (H.) violentus</i> (Van Cleave, 1928)	North-East Asia	Primorye region, Russia	<i>Perccottus glenii</i> Dybowski, 1877	931	KF156881.1	Malyarchuk et al., 2014
A2	<i>Neoechinorhynchus (H.) yamagutii</i> (Tkach, Sarabeev et Shvetsova, 2014)	Japan Sea	Amur Bay, Russia	<i>M. cephalus</i>	590	MN149219	The present study
A3	<i>N. (H.) yamagutii</i>	«	«	«	593	MN149220	«
A31	<i>Neoechinorhynchus (Neoechinorhynchus) agilis</i> (Rudolphi, 1819)	North-East Atlantic	Arousa River, Spain	<i>Chelon labrosus</i> (Risso, 1827)	664	MN148893	«
A12	<i>N. (N.) agilis</i>	«	«	«	616	MN148894	«
A5	<i>N. (N.) agilis</i>	«	«	«	651	MN148895	«
A1	<i>N. (N.) agilis</i>	«	«	«	651	MN148896	«

C9	<i>N. (N.) agilis</i>	«	Caldebarcos coastal waters, Spain	«	1034	MN148897	«
C12	<i>N. (N.) agilis</i>	«	«	«	994	MN148898	«
B1	<i>N. (N.) beringianus</i> Mikhailova et Atrashkevich, 2008	North-East Asia	Chernoe Lake, Russia	<i>Pungitius pungitius</i> (Linnaeus, 1758)	893	KF156875.1	Malyarchuk et al., 2014
-	<i>N. (N.) crassus</i> Van Cleave, 1919	North America	-	<i>Catostomus commersonii</i> (Lacepède, 1803)	1773	AF001842.1	Near et al., 1998
-	<i>N. (N.) cylindricus</i> (Van Cleave, 1914)	North America	Back Bay	<i>Micropterus salmoides</i> (Lacepède, 1802)	1501	MF974925.1	Blubaugh, Gauthier, 2017 www.ncbi.nlm.nih.gov/nuccore/MF974925.1
-	<i>N. (N.) dimorphospinus</i> Amin et Sey, 1996	South China Sea	KienGiang Gulf, Wietnam	<i>Planiliza subviridis</i> (Valenciennes, 1836)	1673	MK510080.1	Amin et al., 2019 www.ncbi.nlm.nih.gov/nuccore/MK510080.1
-	<i>N. (N.) pseudemydis</i> Cable et Hopp, 1954	South-West Asia	Gandoman Lagoon, Iran	<i>Capoeta aculeata</i> (Valenciennes, 1844)	1761	KU363973.1	Dadar, Adel, 2015 www.ncbi.nlm.nih.gov/nuccore/KU363973.1
E4a	<i>N. (N.) salmonis</i> Ching, 1984	North-East Asia	Chistoe Lake, Russia	<i>Salvelinus malma</i> (Walbaum, 1792)	929	KF156878.1	Malyarchuk et al., 2014
C1	<i>N. (N.) tumidus</i> Van Cleave et Bangham, 1949	North-East Asia	Indigirka River, Russia	<i>Coregonus nasus</i> (Pallas, 1776)	896	KF156876.1	Malyarchuk et al., 2014
-	<i>N. saginatus</i> Van Cleave et Bangham, 1949	North America	-	Freshwater fish	1745	AY830150.1	García-Varela and Nadler, 2005
E1	<i>N. simansularis</i> Roitman, 1961	North-East Asia	Engteri Lake, Russia	<i>Salvelinus alpinus</i> (Linnaeus, 1758)	930	KF156877.1	Malyarchuk et al., 2014
-	<i>Floridosisentis mugilis</i> (Machado Filho, 1951)	North America, Mexico	-	<i>Mugil cephalus</i>	1760	AF064811.1	García-Varela et al., 2000

use of the Primer 3Plus program (www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi), with default parameters.

The PCR mixtures (25 µL) contained reaction buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂), 0.2 mM of each deoxynucleoside triphosphate (dNTPs, Roche), 0.4 µM of each primer; 0.5 units of high fidelity Taq polymerase and 50 ng of genomic DNA. The reactions were run in an automatic thermocycler (Mastercycler, Eppendorf, Germany) as follows: initial denaturing at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, annealing at 57 and 55 °C (for F-566 and R-1200 and FNeo1086 and RNeo1086 primers, respectively) for 45 s, and 72 °C for 1 min; and finally, a 7 min extension phase at 72 °C. The PCR products were separated on a 4 % agarose gel in Tris acetate ethylenediaminetetraacetic acid (TAE) buffer containing Sybr Green at 1× concentration, to verify the presence of bands of the correct size under a variable-intensity UV transilluminator and auto image capture software (Alpha Innotech, USA). The PCR product was purified with Thermo Scientific GeneJET PCR Purification Kit and sequenced in complementary directions using Sanger sequencing service (GATC Biotech, Germany).

The sequences were aligned using Clustal Omega online service (Madeira et al., 2019). All positions containing missing data were eliminated and phylogenetic analyses were performed. Trees were obtained using maximum likelihood (ML) with Tamura-Nei model, neighbour joining (NJ) and minimum evolution (ME) methods as applied in MEGA-X (Kumar et al., 2018). Clade support was assessed by bootstrap resampling with 100 replicates. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980).

Estimates of evolutionary divergence based on the number of base differences per site from averaging over all sequence pairs between and within groups (uncorrected p distances) were conducted in MEGA-X and expressed in percentages (table 2). For comparative analysis, the following DNA sequences of *Neoechinorhynchus* spp. were retrieved from GenBank: *N. (N.) beringianus* Mikhailova et Atrashkevich, 2008; *N. (N.) tumidus* Van Cleave and Bangham, 1949; *N. simansularis* Roitman, 1961; *N. (N.) salmonis* Ching, 1984; *N. (H.) violentus* (Van Cleave, 1928); *N. saginatus*; *N. (N.) crassus*; *N. (N.) pseudemydis*; *N. (N.) cylindratus*; *N. (N.) dimorphospinus*. The complete 18S sequence of *Floridosentis mugilis* (Machado-Filho, 1951) was used as outgroup based on its sister relationship to *Neoechinorhynchus* (Malyarchuk et al., 2014; Rosas-Valdez et al., 2012).

Results

Genetic divergence of *Neoechinorhynchus* from grey mullets

Seven specimens of *N. (H.) personatus*, 6 of *N. (N.) agilis*, and 2 of *N. (H.) yamagutii* were sequenced and used for phylogenetic analysis here (table 1). All species were amplified and sequenced using both universal and specific set of primers (F-566 and R-1200; and FNeo1086 and RNeo1086, respectively), excepting for *N. (H.) yamagutii* for which sequences were obtained only with universal primers. PCR products varied from 590 to 664 bp for

Table 2. Estimates of evolutionary divergence between sequences encoding 18S rRNA gene of specimens of *Neoechinorhynchus* from grey mullets across localities in the North-East Atlantic, Mediterranean and Japan Sea

No	*	1	2	3	4	5	6	7	8	9
1	<i>N. (H.) yamagutii</i> A2 and A3									
2	<i>N. (H.) personatus</i> V1	6.56								
3	<i>N. (H.) personatus</i> V3	6.35	0.19							
4	<i>N. (H.) personatus</i> G42	6.36	0.56	0.37						
5	<i>N. (H.) personatus</i> G44	6.56	0.75	0.56	0.19					
6	<i>N. (H.) personatus</i> G45, G49 and G55	6.16	0.37	0.19	0.19	0.37				
7	<i>N. (N.) agilis</i> A31	6.57	2.85	2.66	2.66	2.85	2.47			
8	<i>N. (N.) agilis</i> A1, A5 and A12	6.35	2.65	2.46	2.46	2.65	2.27	0.19		
9	<i>N. (N.) agilis</i> C9 and C12	6.15	2.46	2.27	2.27	2.46	2.08	0.37	0.19	

Note. Analyses were conducted using the Kimura 2-parameter model (Kimura, 1980) also, the evolutionary rates when the divergence times are known. Uncorrected p distances are shown as percentages. There were a total of 541 bp in the final dataset

* Sequences of A2 and A3 of *N. (H.) yamaguti*, G45, G49 and G55 of *N. (N.) personatus*, A1, A5 and A12, and C9 and C12 of *N. (N.) agilis* were combined because their 18S partial sequences were identical.

the universal set of primers and from 990 to 1042 bp for specific ones. The aligned data set included 15 sequences with a length of 541 bp and occupying position from 551 to 1092 nucleotides along the 18S rRNA gene of *F. mugilis*. The genetic divergence estimated among specimens of the same species ranged from 0 to 0.75 % (table 2), while distances between taxa of *Neoechinorhynchus* were much higher (2.08–6.57 %). The 18S sequence divergence between *N. (H.) yamagutii* and both Atlantic species, *N. (H.) personatus* and *N. (N.) agilis*, were higher than that between the latter two (6.15–6.57 % vs. 2.08–2.85 %, respectively). A comparison of long sequences of *N. (H.) personatus* and *N. (N.) agilis* (positions from 96 to 550 nucleotides along the 18S rRNA gene of *F. mugilis*) supplied additional divergence in 3 bp between these species and revealed difference in 2 bp between specimens of C9 and C12 of *N. (N.) agilis*, and in 1 bp between G49 and other specimens of *N. (H.) personatus*.

Phylogenetic analysis of *Neoechinorhynchus* from grey mullets

The phylogenetic analysis of specimens of *Neoechinorhynchus* from grey mullets revealed that 497 out of 541 characters were constant, 41 were parsimony-informative, and 3 variable characters were parsimony-uninformative. The ML analysis yielded a single tree with the highest log likelihood = -1242.96. Trees obtained with NJ and ME methods showed the same topology. The bootstrap support was high for all branches of the phylogenetic tree (fig. 1). All trees comprise of 3 major monophyletic clades, which correspond to *N. (H.) yamagutii*, *N. (N.) agilis* and *N. (H.) personatus*. Clades *N. (N.) agilis* and *N. (H.) personatus* are subsequently subdivided into 2 and 3 subclades, respectively, mainly reflecting different localities.

Phylogenetic relationships of *Neoechinorhynchus* from grey mullets with other members of the genus

The longest sequence of each species obtained in the present study was combined with published sequences from GenBank, including 10 *Neoechinorhynchus* species and *F. mugilis* as outgroup taxa. The aligned data set included 14 sequences, with 442 characters, of which 86 were parsimony-informative. The ML tree with the highest log likelihood (-1619.55) is shown in figure 2. Trees obtained with NJ and ME methods showed a similar topology. This phylogenetic analysis revealed two main clades with strong bootstrap support. One includes *Neoechinorhynchus* spp. associated with freshwater and brackish water fishes; another is

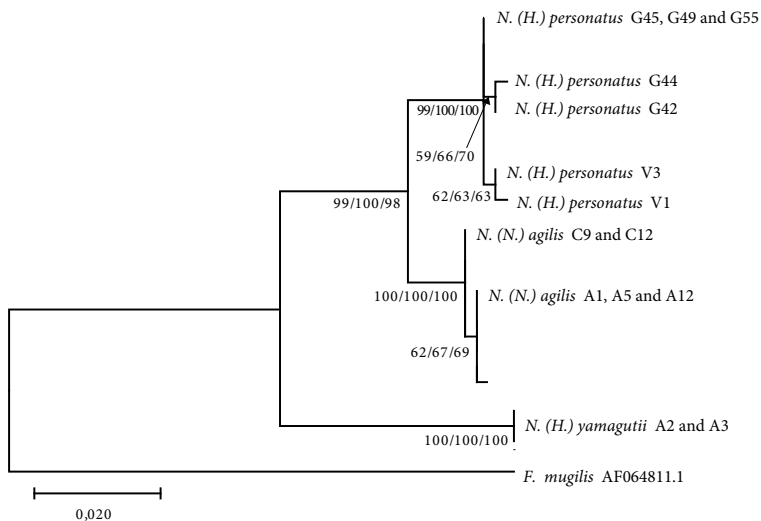


Fig. 1 Phylogenetic tree of *Neoechinorhynchus* from grey mullets and across localities in the North-East Atlantic, Mediterranean and Japan Sea obtained with maximum likelihood (ML) method (-ln likelihood 1242.96) based on the 18S rRNA partial gene sequences. *Floridosentis mugilis* was used as an outgroup. The ML/neighbor joining/minimum evolution bootstrap support is shown at each internal node. Identification number of isolates is as in table 1.

composed by *N. (H.) violentus* and monophyletic subclade containing species from marine grey mullets. This subclade formed by the species *N. (N.) dimorphospinus* — *N. (N.) agilis* is phylogenetically distant from other species of the genus and strongly supported by bootstrap values (100 % for all methods of tree reconstruction). The bootstrap analysis also supports the sister relationship of *N. (N.) dimorphospinus* and *N. (H.) yamagutii* with the two terminal Atlantic species *N. (N.) agilis* and *N. (H.) personatus*. *N. (N.) dimorphospinus* and *N. (H.) yamagutii* are grouped together in one clade with moderate bootstrap support (49 %) in tree reconstructed by ME method (the result is not shown).

Discussion

The phylogenetic analysis inferred from partial sequences of the 18S rRNA gene supports the previous morphological observation of Tkach et al. (2014) indicating that *N. (N.) agilis*, *N. (H.) personatus* and *N. (H.) yamagutii* represent three independent species. The genetic divergences estimated between three species sequenced here were relatively high, at the range between 2.08 % and 6.57 %. These levels of genetic divergence among species are similar or even higher to those exhibited within *Neoechinorhynchus* and ranging between 0.37 % and 5.55 % for the 18S rRNA gene (Malyarchuk et al., 2014). Our data, consistent with results of Malyarchuk et al. (2014), demonstrate the existence of genetic heterogeneity in the species within *Neoechinorhynchus*. The closeness of species representing different clades of the phylogenetic tree can be explained by their common origin in a certain geographic region or in evolutionary related definitive hosts. The monophyletic clade formed with species of *Neoechinorhynchus* from grey mullets, *N. (N.) dimorphospinus*, *N. (H.) yamagutii*, *N. (N.) agilis* and *N. (H.) personatus*, indicates that all these species share a common ancestor. The terminal position of *N. (N.) agilis* and *N. (H.) personatus* and the low genetic divergence found between them suggests the recent emergence of this group and that the colonization of the North-East Atlantic and the Mediterranean regions could represent a single event. One possible explanation of the presence of two closely related species of *Neoechinorhynchus* in the Mediterranean is an evolutionary host-switching, as *N. (N.) agilis* and *N. (H.) personatus* tend to parasitize two different host species, *C. labrosus* and *M. cephalus*, respectively (Tkach et al., 2014). Geographic barriers, such as land masses of Africa and India, prevent migration and consequent gene flow among acanthocephalans,

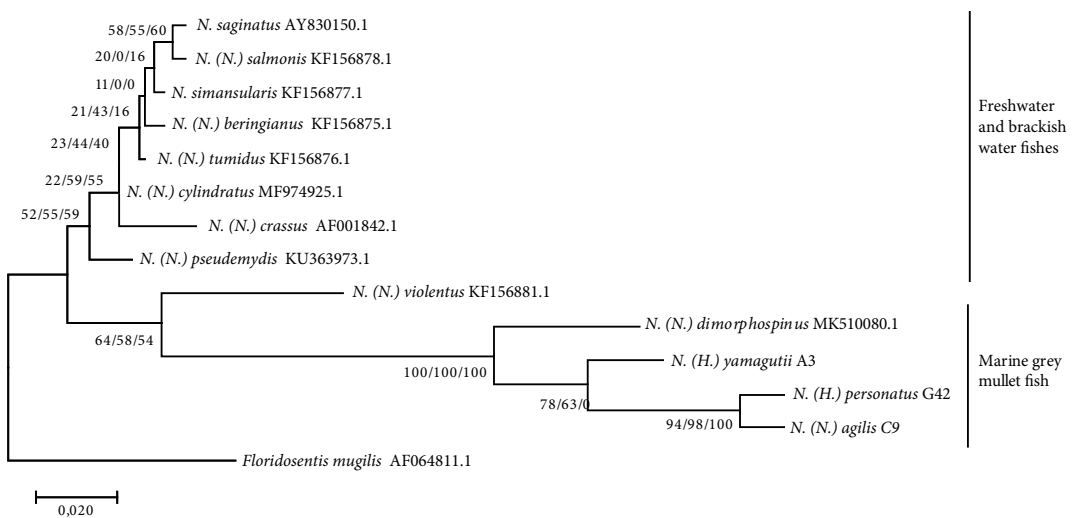


Fig. 2 Phylogenetic tree of *Neoechinorhynchus* species obtained with maximum likelihood (ML) method (– In likelihood 1619.55) based on the 18S rRNA partial gene sequences. *Floridosentis mugilis* was used as an out-group. The ML/neighbor joining/minimum evolution bootstrap support is shown at each internal node.

may explain a high divergence level between *N. (H.) yamagutii* and both Atlantic species, *N. (N.) agilis* and *N. (H.) personatus*. The basal position of *N. (N.) dimorphospinus* and *N. (H.) yamagutii* in relation to *N. (N.) agilis* and *N. (H.) personatus* indicates that the origin of the ancestor of the latter ones, most likely, related with colonization of the North-East Atlantic region by the Pacific species with subsequent vicariance event. Our results suppose that colonization of *N. (H.) yamagutii* of the Sea of Japan is associated with grey mullet fish rather than with freshwater and brackish water fishes of North-East Asia, since the species of *Neoechinorhynchus* from these groups of hosts represent different lineages.

Although Hadziavdic et al. (2014) suggested that acanthocephalans could not be amplified using the primer pair F-566 and R-1200, the PCR product and sequences for all three species of *Neoechinorhynchus* studied here were successfully obtained by applying this set of universal primers. The amplicons of acanthocephalan individuals generated using this universal set of primers averaged 623 nucleotides in length, whereas the specific primer pair supplied fragments with the average length in 1021 nucleotides covering the variable regions V4-V5 and V2-V5 of the 18S rRNA gene, respectively. In agreement with the results of Hadziavdic et al. (2014), our study demonstrates that the variable region V4-V5 yielded higher taxonomic information than the region V2-V3 providing 11 informative characters vs. 3 when comparing sequences of *N. (N.) agilis* and *N. (H.) personatus*. Thus, the universal primer pair F-566 and R-1200 can be effectively used to amplify acanthocephalans for assessing their diversity in field-based studies.

Another important finding of the present study is that the subgenus *Hebesoma* is the polyphyletic group. While all three species of the subgenus *Hebesoma* are assigned to one clade, two species of the subgenus *Neoechinorhynchus* are nested within the same clade (see fig. 2). In addition, the genetic divergence was higher between *N. (H.) yamagutii* and *N. (H.) personatus* than between the latter species and *N. (N.) agilis*. These results match those inferred from CO1 gene by Malyarchuk et al. (2014) for the species of *Neoechinorhynchus* and *Hebesoma* from North-East Asia. Van Cleave (1928) erected the genus and the family Hebesomidae for *Hebesoma violentum*. In the diagnosis of the species he indicated that the eggs had globular polar enlargements of middle membrane, lemnisci were short, and the giant subcuticular nuclei were unnoticeable. Meyer (1932) transferred *Hebesoma* to Neoechinorhynchidae, leaving diagnoses of the genus, and of *H. violentum* without changes. The validity of *Hebesoma* was accepted by Petrochenko (1956) and Yamaguti (1963), they both provided more concise diagnosis of the genus. Salgado-Maldonado (1978) dismissed the importance of the features listed in *Hebesoma* diagnosis, showing their presence in the descriptions of many *Neoechinorhynchus* species and proposed the synonymy of these genera. Amin (2002) argued against monophyly of *Neoechinorhynchus*. He proposed to combine the concepts of both genera and to lower the status of the *Neoechinorhynchus* (sensu stricto) and *Hebesoma* emphasizing on subgeneric importance of the egg structure. *Neoechinorhynchus* is characterized by uniformity of anatomical organization (Malyarchuk et al., 2014), while the specific egg morphology observed in some species provides an important morphologic feature useful for species discrimination. At the same time, erection of a species group to a higher level taxon based on the polar prolongations of the inner envelopes of an egg is not consistent with the molecular results arguing that *Hebesoma* is supposed to be an unnatural taxon.

In conclusion, morphologically similar species *N. (N.) agilis* and *N. (H.) personatus* and *N. (H.) yamagutii* can be clearly recognized using universal primers (F-566 and R-1200) proposed by Hadziavdic et al. (2014) for 18S rRNA gene. Phylogenetic analysis demonstrates that the studied here species of *Neoechinorhynchus* from grey mullet fish share common ancestor, despite their different geographic location, and are closely related. *Hebesoma* is shown to be a polyphyletic taxon. In future investigations, it might be possible to use additional genomic markers to assess the validity of the phylogenetic hypotheses inferred in the present study.

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Author contributions

IeT, JL and VS conceived the ideas. IeT collected the data from the Azov Sea, VS from the North-East Atlantic. RAS designed methodology of DNA extraction and amplification. JL designed primers set. VS obtained PCR product. JL and VS analysed the data. VS led the writing and IeT contributed to the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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