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Genetic diversity and phylogenetic relations of salmon trematode *Nanophyetus japonensis*

Anastasia Voronova, Galina N. Chelomina*

Federal Scientific Center of the East Asia Terrestrial Biodiversity FEB RAS, 100-letiya Street, 159, Vladivostok 690022, Russia

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ABSTRACT

Nanophyetiasis is the severe zoonotic disease caused by parasitic worms from the genus *Nanophyetus*. Humans and carnivorous animals become infected when they ingest raw fish containing metacercariae, especially Pacific salmonids. Nanophyetiasis is detected in limited geographical areas which include the coastal regions of the North Pacific: the United States of America, Russian Federation and Japan. Despite the epidemiological significance, *Nanophyetus* species have not been well studied genetically. In this research, we for the first time explored genetic diversity of *Nanophyetus japonensis* from Japan in comparison with those of related species, *N. salmincola* from North America and *N. schikhobalowi* from the Russian Far East, based on sequence variation in the nuclear ribosomal gene family (18S, ITS1–5.8S–ITS2 and 28S) and mitochondrial *nad1* gene, encoding subunit I of the respiratory chain NADH dehydrogenase. The results confirmed the independent species status for the compared flukes, demonstrated a greater genetic similarity of Asian species between themselves than each of them with the North American one, suggesting that *N. japonensis* and *N. schikhobalowi* are close sister species, and also revealed discrepancy between the levels of morphological and genetic differentiation.

1. Introduction

Species of the genus *Nanophyetus* Chapin, 1927 (Digenea: Troglotremtidae) are parasitic in the small intestine of many species of piscivorous mammals and birds [1–4]. The genus is consisted of four nominal species: *Nanophyetus salmincola* [5] Chapin, 1927 in western North America; *Nanophyetus schikhobalowi* Skrjabin and Podiapolskaia, 1931 in northeastern Asia; *Nanophyetus japonensis* Saito, Saito, Yamashita, Watanabe, and Sekikawa, 1982 in Japan; and *Nanophyetus asadai* (Yamaguti, 1971) n. comb. in Japan [6]. Yamaguti (1971) created a new genus and species, *Pseudotroglotrema asadai*, from Japan. Blair et al. [7] synonymized *Pseudotroglotrema* with *Nanophyetus*, but they did not make a new combination for the type and only species *Ps. asadai*. We here propose the above new combination.

The life cycle and geographical distribution have been given in detail for *N. salmincola*, *N. schikhobalowi*, and *N. japonensis* [1–4,6,8]. *Nanophyetus japonensis* was described by Saito et al. (1982) on the basis of adult specimens found in the small intestine of mammals from Japan: a dog (the holotype), to which metacercariae isolated from the Japanese common char *Salvelinus pluvius* Hilgendorf, 1876 (synonym of *Salvelinus leucomaenis* Pallas, 1814 (Salmonidae)) had been experimentally fed before; the Japanese badger *Meles anakuma* Temminck, 1844 (Carnivora: Mustelidae) and Japanese water shrew *Chimarrogale platycephala* Temminck, 1842 (Soricomorpha: Soricidae) caught in Yamagata Prefecture [6]. Later, Saito (1985) elucidated the life cycle of *N. japonensis* in the field and laboratory [8]. Unfortunately, all the specimens of Saito et al. (1982) and Saito (1985) are missing (Susumu Saito, 2015, personal communication).

Molecular studies have already been made of *N. salmincola* [18] and of *N. schikhobalowi* [9] but not yet of *N. japonensis*, using mitochondrial and nuclear DNA markers. In this paper, we analyse the intraspecific genetic variability of *N. japonensis* based on mitochondrial *nad1* gene, nuclear ribosomal DNA sequence data (18S, ITS1, 5.8S, ITS2, 28S) and predicted secondary structures of both internal transcribed spacers (ITS1 and ITS2); reconstruct the intrageneric phylogeny of the genus *Nanophyetus*; give a taxonomic interpretation of the results.

2. Material and methods

2.1. Isolation of DNA, PCR and sequencing

Metacercariae were obtained (by Hideto Kino) from the gills, kidneys, and body muscles of one individual of *Salvelinus leucomaenis pluvius* Hilgendorf, 1876 (Salmonidae), collected (by Yasuo Araki) in Shirabuzawa (a mountain stream) at Iritazawa, Yonezawa City, Yamagata Prefecture, Japan in 16 of December 2015 (Fig. 1). Isolated

* Corresponding author.
E-mail address: chelomina@ibss.dvo.ru (G.N. Chelomina).

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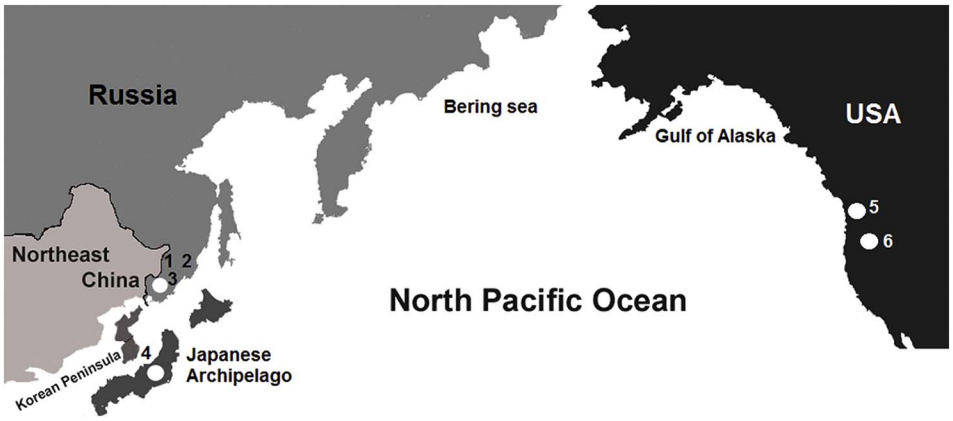

Fig. 1. Specimen collecting areas: Russian Far East: 1 - Komissarovka River (Pogranichnyi district), 2 - Ilistaya River (Khankaysky District), 3 - Komarovka River (Ussuriysky District); 4 - Yamagata Prefecture: Shirabuzawa at. Iritazawa, Yonezawa City (Honshu Island); Rivers sampled in Pacific Northwest of the United States of America: 5 - Bingham Creek (Washington), 6 - West Fork Smith River and Winchuck River (Oregon) (Criscione and Blouin, 2004).

metacercariae were experimentally fed to Syrian golden hamster (*Mesocricetus auratus*), and adults were recovered from its small intestine 7 days after infection (by Kino). Metacercarial and adult specimens were fixed in 80% ethanol for molecular and morphological studies. The specimens were identified (by Takeshi Shimazu) as *Nanophyetus japonensis* based on their morphological characteristics.

Genomic DNA was extracted from 2 individual adult worms and 14 metacercariae using the HotSHOT technique [10].The complete 18S rDNA, ITS1-5.8S-ITS2 rDNA, and 28S rDNA regions and sequences of partial mtDNA *nad1* gene were amplified by traditional polymerase chain reaction (PCR) using several sets of primers and Thermo Scientific PCR Master Mix (2 ×) (California, USA) according to the manufacturer's instructions.

The amplification of the complete 18S rDNA of *N. japonensis* was done in three fragments using primers: 18S-E + 18S27; 892 + 18S4; 18S2 + 18SF, annealing temperature of 58 °C and 35 cycles [11,12]. The amplification of the ITS1 + 5.8S rDNA was performed using BD1 [13] and specific designed by us primers TS700 - (5′ -CTCAGTCTAGC CCAGGATA - 3′) - reverse, NJTS2_1149-R - (5′ -CCACAAAGGCACA AGA- 3′) - reverse, and annealing temperature of 54 °C, 35 cycles. The amplification of the ITS2 region was performed using primers 3S [14] and BD2 [13], and annealing temperature of 54 °C, 30 cycles. The amplification of the complete 28S rDNA was performed using three pairs of external primers: 178 and 1642, U1148 and L2450, U1846 and L3449, and annealing temperature of 55 °C in all cases [15]. The amplification of partial mtDNA *nad1* gene sequences was performed using specific designed by us primers: nds-new2 - (5′-AGAGGTTTATTACAA AGGTT-3′) - forward, nds-newr - (5′-GCTAATGAAACCAATAACACT-3′) - reverse, with annealing temperature of 49 °C and 30 cycles.

ExoSAP-IT PCR Product Cleanup Reagent from Thermo Scientific was used for enzymatic cleanup of amplified PCR products. PCR products (2 complete 18S rDNA, 16 complete ITS1-5.8S-ITS2 rDNA, 2 complete 28S rDNA, and 8 partial *nad1* gene sequences) were sequenced directly on an ABI 3130 Genetic Analyzer using the ABI BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The sequencing primers were the same as those used for PCR, except additional internal primers: one for ITS1 region designed by us NJTS1_663-F - (5′-ACAAAATCCCATATTACACTGT-3′), and for 28S rDNA: 300F, 900F, 1200F, 1600R [16]; U1148, U2229, U2562, L2630, U2771, L2984, U3139, L3449 [9,15]. Contiguous sequences were assembled using MEGA 5.1 [17], and submitted to GenBank under accession numbers: LT796167 - LT796168 for 18S rDNA; LT796171 - LT796186 for ITS1-5.8S-ITS2 rDNA, LT796169 - LT796170 for 28S rDNA, and LT796187 - LT796194 for partial mtDNA *nad1* gene.

2.2. Statistic and phylogenetic analyses

The newly-generated sequences obtained in this study (see above), sequences of *Nanophyetus* which we previously deposited in GenBank (*N. salmincola*: AY222138, KX990282 - KX990286, AY116873; *N. schikhobalowi*: LN852660 - LN852663, LN871800 - LN871821) [9], and 91 sequences of *nad1* for *N. salmincola* (AY269600 - AY269690) [18], as well as sequences of closely related taxa from GenBank (family Paragonimidae: *Paragonimus kellicotti* HQ900670, *P. miyazakii* HM172620, *P. iloktsuenesis* AY116875; AY222141, *P. westermani* JN656178; AJ287556, *P. heterotremus* LT855188, *P. pseudoheterotremus* HM004210, *P. vietnamiensis* LT855189; family Troglotrematidae: *Nephrotrema truncatum* AY222139; AF151936, *Skrjabinophyetus neomidis* AF184252; family Haploporidae: *Haplorchis pumilio* AY245706, HM004191, *H. taichui* KF214770; family Opisthorchiidae: *Opisthorchis felineus* EU921260, *Clonorchis sinensis* DQ116944, family Diplostomidae: *Diplostomum pseudospathaceum* KR269766; family Paramphistomidae: *Paramphistomum cervi* KJ459938; family Heterophyidae: *Euryhelmis costaricensis* AB521797; family Collyriclidae: *Collyriclum faba* JQ231122; family Echinostomatidae: *Isthmiophora hortensis* AB189982; family Schistosomatidae: *Trichobilharzia ocellata* AY157243, *Schistosoma malayensis* AY157252, *S. haematobium* AY157263, *S. japonicum* Z46504; family Sanguinicolidae: *Dendritobilharzia pulverulenta* AY157241) were aligned using the Clustal W program [19].

Genetic distances between species and phylogenetic relationships among taxa using Maximum Likelihood (ML) method with branch support values estimated by 1000 bootstrap replicates were obtained using MEGA 5.1 [17]. Alignment gaps were included in the p-distance calculations. Phylogenetic inferences were also obtained through Bayesian Inference (BI) as implemented in MrBayes 3.1 with 10 million generations of MCMC chains (Markov Chain Monte Carlo), where 25% of generations were discarded as burn-in [20]. Modeltest 3.7 software [21] was used to select the nucleotide substitution model; TPM2uf + G for ITS + 5.8S matrix, for 18S and 28S TrN + I and TPM3uf + G models were chosen, respectively, and GTR + G for *nad1* gene and tree based on the full-length 28S rDNA sequences (3917 bp) was used. Obtained trees were rooted using appropriate genes and spacers (18S rDNA, 28S rDNA and ITS1-5.8S-ITS2 rDNA) of *Haplorchis pumilio* and *H. taichui* (family Heterophyidae). Species of genus *Haplorchis* were chosen as a members of the family that is basal, but not too distant, from the Troglotrematidae to be the outgroup [16].

2.3. Predicted ITS1 and ITS2 RNA secondary structures

The consensus structures for a set of aligned ITS1 and ITS2 sequences were computed using the program RNAalifold [22]. RNAalifold supports prediction of the minimum free energy structures [23] and extends standard dynamic programming algorithms, then averages the energy contributions over all sequences and incorporates covariation terms into the energy model to reward compensatory mutations and penalize non-compatible base-pairs. The input was a single multiple sequence alignment in FASTA format. The server output contained the predicted MFE secondary structure in the usual dot-bracket notation.

RNA was folded at a fixed temperature of 37 °C. Resulted structures of ITS1 and ITS2 regions were compared with those previously obtained for *N. schikhobalowi* and *N. salmincola* [9], non-conserved base pairs and variable sites were manually detected within the structures. Additionally structure drawings proceeded in CorelDRAW X6 graphic designer.

3. Results

3.1. Molecular analysis and characterization of 18S and 28S genes

The size of the complete 18S rRNA gene of two *Nanophyetus japonensis* specimens was 1893 bp long. To analyse the 18S rDNA, sequences of three studied populations were aligned and trimmed together to final length 1793 bp. The 18S rDNA sequences of *N. japonensis* differed from those of *N. schikhobalowi* and *N. salmincola* by 3 (d = 0.17%) and 4 (d = 0.22%) nucleotides, respectively.

We sequenced the complete 28S rRNA gene 3885 bp long of two *N. japonensis* specimens and there were no nucleotide differences between them. However, between *N. japonensis* and *N. schikhobalowi* we observed 4 nucleotide substitutions which make up 0.1% of divergence. 28S rDNA sequences of four *N. schikhobalowi* specimens, one *N. salmincola* and two *N. japonensis* were trimmed to the shortest length 1321 bp. These partial sequences of *N. japonensis* differed in 1 (d = 0.07%) and 8 (d = 0.6%) nucleotide positions with those of *N. schikhobalowi* and *N. salmincola*, respectively.

3.2. Molecular analysis and characterization of ITS1-5.8S-ITS2 rDNA

Complete ITS1-5.8S-ITS2 rDNA regions dataset included in total 42 sequences: 16 sequences belonging to *N. japonensis* (1238 bp), 20 sequences of *N. schikhobalowi* (1219 bp) and 4 - belonging to *N. salmincola* obtained from GenBank (1186 bp). The resulted alignment was 1339 bp long and had several indel gaps (Fig. 2). The *N. japonensis* specimens differed by 3 nucleotides (d = 0.1–0.2%), representing three types of substitutions: A → G transition localized in the ITS1, G → C and A → T transversions localized in 5.8S rDNA gene and in ITS2, respectively. The divergence of the ITS1-5.8S-ITS2 rDNA sequences between *N. japonensis* and *N. schikhobalowi* was 2.6%. Differences included 16 nucleotide substitutions and one 19 bp long insertion in ITS1 of the *N. japonensis*. The divergence between *N. salmincola* and *N. japonensis* reached 13%, including 41 nucleotide substitutons and 14 different sized indels, with the total length of 121 bp.

The variation in the ITS1 region between sequences of *Nanophyetus* spp. resulted from both, nucleotide polymorphisms and size variation, with ITS1 containing the majority of mutations in ribosomal cluster. The size of the ITS1 sequences of the *N. japonensis* specimens were 825 bp and 791 bp long with and without indel gaps, respectively. The ITS1 sequences of *N. japonensis* and *N. schikhobalowi* differed by 10 nucleotide substitutions (1% of alignment length), while *N. japonensis* and *N. salmincola* differed by 33 nucleotides (4% of alignment length). The most common substitutions between *N. japonensis* and *N. schikhobalowi* were transitions A ↔ G (40%) and transversions C ↔ G (40%) and between *N. salmincola* and *N. japonensis* - only transitions: A ↔ G (42%) and T ↔ C (24%).

The size of ITS2 sequences in specimens from all three populations were 290 bp long, i.e. the variation of ITS2 sequences of compared species was only due to nucleotide polymorphisms. There was one nucleotide substitution A → T transversion (d = 0.3%) between *N. japonensis* and *N. schikhobalowi*, whilst 7 substitutions (d = 2.4%) were detected between *N. salmincola* and *N. japonensis*, of them four were transitions T↔C and A↔G (both ~29%) and three -transversions A ↔ T (~29%) and G → T (14%).

3.3. Molecular analysis and characterization of nad1 gene

The total dataset contained 119 sequences of *nad1* gene fragments 622 bp in length, 8 of which belonging to *N. japonensis* were sequenced in this study, 20 sequences belonging to *N. schikhobalowi* we sequenced previously (LT555380 - LT555399), and 91 *N. salmincola* sequences we obtained from GenBank [18]. There were 109 variable sites (of which 88 were parsimony-informative) and 146 variable sites (of which 115 were parsimony-informative) between *N. schikhobalowi* and *N. japonensis*, and *N. salmincola* and *N. japonensis*, respectively. The pair-wise genetic distances within population of *N. japonensis* were estimated to be relatively low (d = 0.2%). The average pair-wise genetic distances between three geographical populations were estimated to be significantly higher: d = 13% for the *N. japonensis* and *N. schikhobalowi* populations and d = 22% for the *N. salmincola* and *N. japonensis* populations.

3.4. N. japonensis ITS1 and ITS2 secondary structure analysis

In this study, consensus Secondary Structures (SS) for the sixteen complete ITS1 and ITS2 transcripts were reconstructed. SS of ITS1 region had a tree-like conformation and consisted of two branches each with different number of helical domains, separated by a helix with 2 medium-sized hairpins (Fig. 3A). The 19 bp insertion almost completely formed the third hairpin localized in Branch I. The RNAalifold free energy DG required for formation of the optimal secondary structure for *N. japonensis* was − 436.95 kcal/mol. Intra- and inter-specific variations were detected within the structure (Fig. 3A). On the SS for *N. japonensis* common with *N. salmincola* and *N. schikhobalowi* features were marked by stars and crosses, respectively (Fig. 3A). In this way, *N. japonensis* had a hairpin with 19 bp insertion localized in Branch I as that of *N. salmincola* and 2 medium-sized hairpins in the helix connecting two branches like to *N. schikhobalowi.* Large trident-like helical structure in the Branch II was highly conserved across species.

The DG required for formation of the ITS2 secondary structure in RNAalifold fixed at − 157.7 kcal/mol. The ITS2 secondary structure of *N. japonensis* shared well-known and typical for digenea model, i.e. a palm with four fingers, first described in detail by Michot et al. [24] (Fig. 3B). SS for *N. japonensis* showed only minor differences; one substitution localized in A domain, and two in C and D (Fig. 3B).

3.5. Phylogenetic reconstructions

The phylogenetic tree based on the 18S rRNA sequence data clearly separated the family Troglotrematidae including *Nanophyetus* spp. and *Nephrotrema truncatum* and the family Paragonimidae represented by *Paragonimus* species. Within the Troglotrematidae clade, we observed high differentiation between genera *Nanophyetus* and *Nephrotrema*. Specimens of the *Nanophyetus* genus from Asia and Northern America formed common subclade with a strong support (100% bootstrap support in ML and 0.94 posterior probability in BI) (Fig. 4A). Within *Nanophyetus* subclade *N. japonensis* formed well supported branch (91% bootstrap support in ML and 0. 81 posterior probabilities in BI) separately from statistically supported *N. schikhobalowi* and *N. salmincola* branches.

The phylogenetic tree based on the complete sequences of the 28S rRNA gene (3917 bp), including representatives of different trematode families (orders Strigeida, Echinostomida and Plagiorchiida) not only well distinguished them, but generated a subclade for *N. schikhobalowi* and *N. japonensis* with maximum values of support (100% bootstrap support in ML and 1.0 posterior probability in BI), where species of *N. japonensis* represented a separate phyletic line (90% bootstrap support in ML/1.0 posterior probability in BI) (Fig. 4B). The tree based on another sample set consisting of partial 28S rRNA gene sequences (1321 bp) was unable to resolve the relationship between *N. schikhobalowi* and *N. japonensis*, combining them into a common branch,

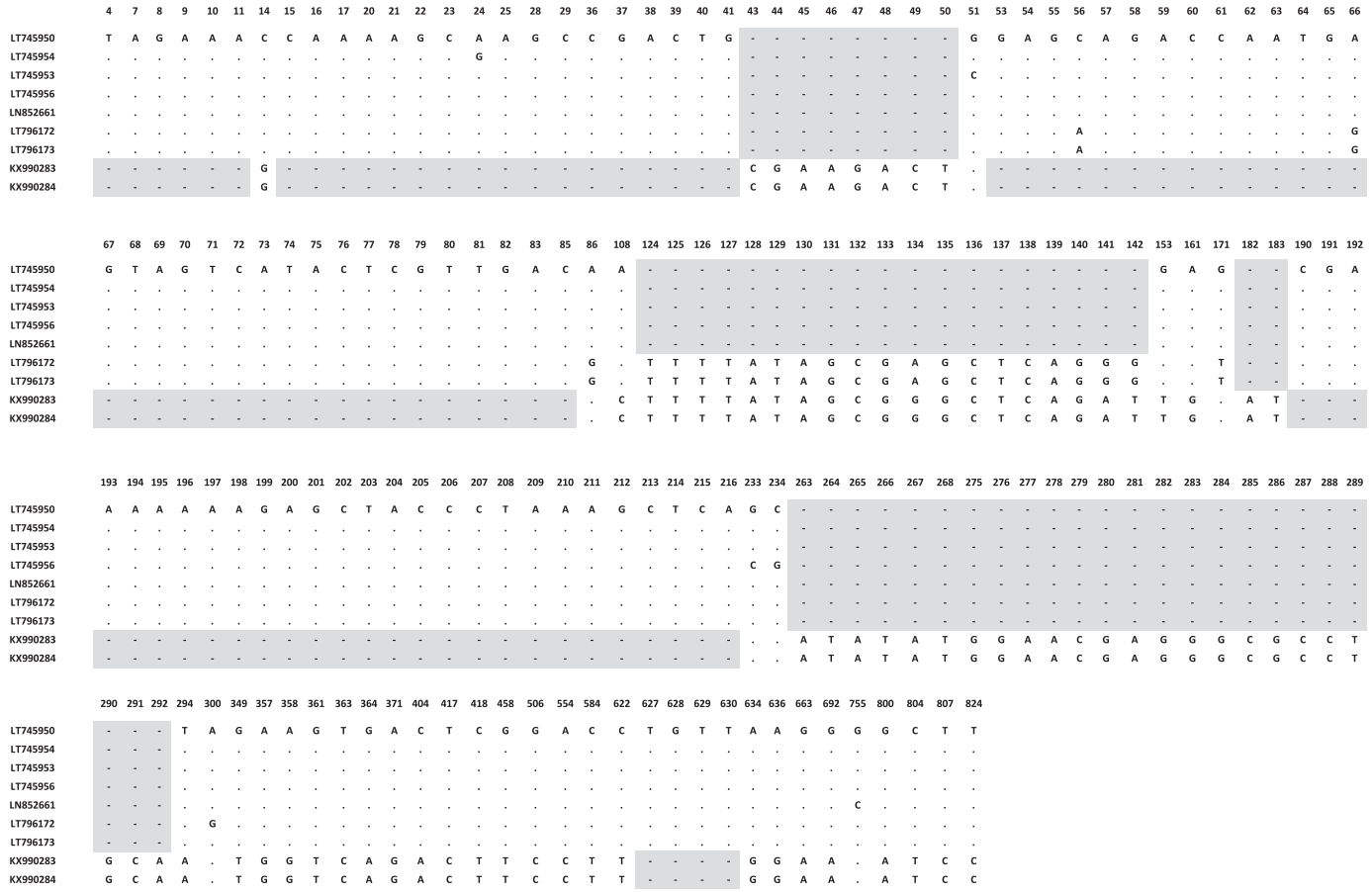

Fig. 2. Ribotypes of the complete ITS1 sequences of studied flukes: *N. schikhobalowi*, *N. japonensis* and *N. salmincola*. The dots indicate identical nucleotides; dashes indicate missing nucleotides (indels). Sites containing indels are shaded.

differentiated from *N. salmincola* (Fig. 4C).

The inferred phylogenetic tree from the sequences of complete ITS1-5.8S-ITS2 rDNA region of three forms of *Nanophyetus* fall into two well supported (100% bootstrap values in ML; 1.0 posterior probabilities in BI) monophyletic lineages (Fig. 5). First consisted of the two subclades, where one, incorporated species of *N. schikhobalowi* (94% bootstrap values in ML/1.0 posterior probabilities in BI), and the other - *N. japonensis*(100% bootstrap values in ML/0.97 posterior probabilities in BI). Second clade accurately formed by *N. salmincola* was also very strongly supported (100% bootstrap values in ML/1.0 posterior probabilities in BI).

The phylogenetic tree obtained from the mtDNA *nad1*dataset is well resolved, with high support values for each species of the genus *Nanophyetus* (Fig. 6). Two major clades can be distinguished (98% bootstrap values in ML/1.0 posterior probabilities in BI), first comprised the sequences of *N. salmincola*, and the second, divided *N. schikhobalowi* and *N. japonensis* into two individual subclades (statistical support 97% bootstrap values in ML/1.0 posterior probabilities in BI and 99% bootstrap values in ML/1.0 posterior probabilities in BI, respectively).

4. Discussion

In the present molecular study analysis of *N. japonensis* was performed using multi-locus nuclear DNA sequence data (18S, ITS1 + 5.8S + ITS2, 28S rDNA) and partial mitochondrial (*nad1*) gene, and also the genetic variability in secondary structures of internal transcribed spacers was explored. P-distances based on 18S and 28S rDNA sequences more often begin to use for species discrimination [11,12,15]. The p-distance values between the *N. japonensis* specimens and *N. schikhobalowi* and *N. salmincola* ones obtained in this study for the 18S rDNA were four time higher (0.2%) than that previously described for *N. schikhobalowi* and *N. salmincola* (0.05%), but it does not contradict the data obtained for other trematodes [25–27].

The 28S rRNA gene showed divergence between the *N. japonensis* and *N. schikhobalowi* and *N. japonensis* and *N. salmincola* specimens (0.1% and 0.6%, respectively) which corresponds well with the previously described values for *N. schikhobalowi* and *N. salmincola* (d = 0.5%). These divergence values are compatible with the literature data corresponding to the lower limits of interspecies differences of trematodes within the same genus of 0.3–1.8% [28–30]. The upper limits of interspecific divergence often ranged from 3.1% to 5.9% [28,31,32] and can reach 10% [27].

The internal transcribed spacers (ITS1 and ITS2) of the nuclear ribosomal DNA region are commonly used markers in differentiation among digenean species or as criterion for intraspecific genetic differentiation [33–36]. Intraspecific variability observed within the *N. japonensis* specimens in our study in the ITS1-5.8S-ITS2 rDNA region was small (d = 0.1–0.2%) which corresponds to most literature data, where this variability usually does not exceed 1% [37–39]. The levels of interspecific genetic divergence based on the complete ITS1-5.8S-ITS2 rDNA region between the *N. japonensis* and *N. schikhobalowi* specimens were approximately 4–5 times lower (d = 3%), than that between the *N. japonensis* and *N. salmincola* (d = 13%), or *N. schikhobalowi* and *N. salmincola* ones (d = 14%). One must admit that levels of interspecific differences observed in various digenean taxa vary within a wide range [38–44]. For example, mean interspecific genetic divergence for the ITS sequences of *Tylodelphys* species and *Plagiorchis* spp. ranged from 0.7 to 8.3% [32] and from 11.1 to 16.5% [29], respectively, and in the genus *Echinostoma* are known to be among highest ones 22% [40].

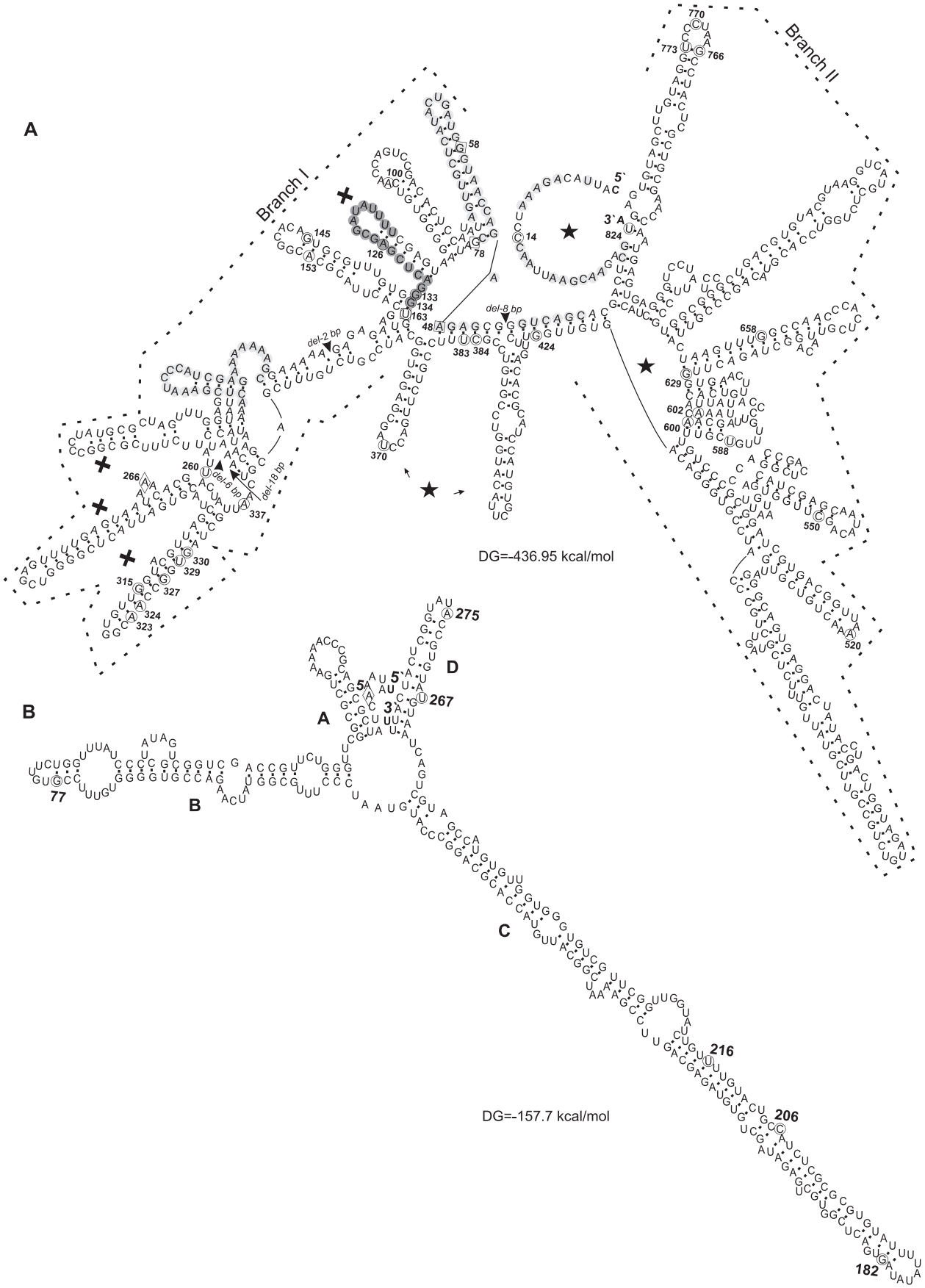

Fig. 3. De novo modeled predicted consensus secondary structures of ITS1 (A) and ITS2 (B) regions reconstructed in RNAalifold for specimens from the Japanese population. Interspecific variable sites between the Japanese and Russian populations are in squares, between the Japanese and American populations are in circles; intraspecific variable sites are in rhombus, insertions are colored grey, deletions marked with triangles. Common features for *N. japonensis* and *N. schikhobalowi* marked by stars, for *N. japonensis* and *N. salmincola* by crosses.

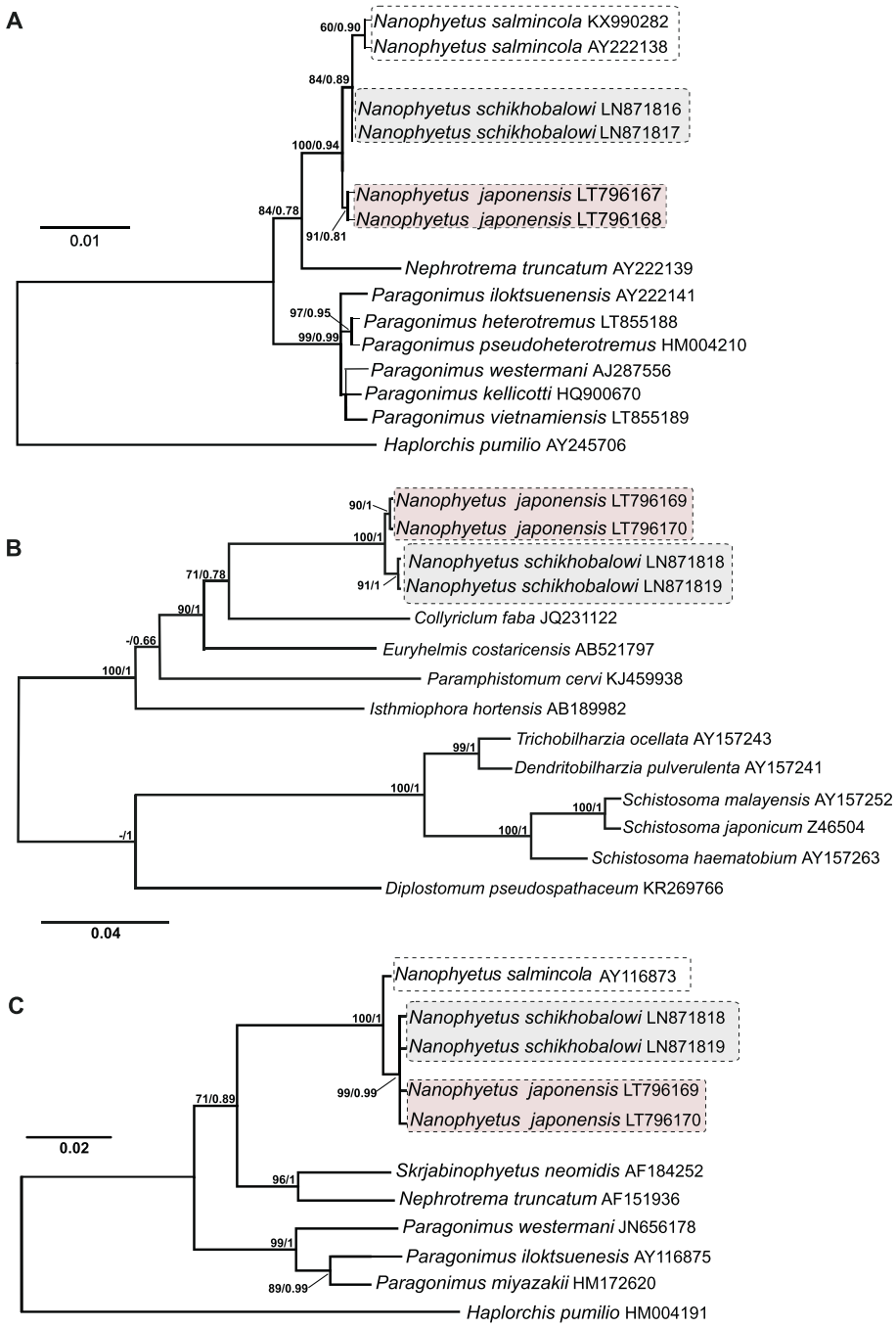

Fig. 4. Maximum Likelihood phylogeny of *Nanophyetus* based on 18S rDNA sequences (A), full 28S rDNA sequences (B), trimmed 28S rDNA sequences (C). Statistical branch support values are as follows: Maximum Likelihood bootstrap values/Bayesian inference posterior probabilities (ML/BI). Only bootstrap support values > 50% in ML and posterior probabilities > 0.7 in BI are shown.

Interspecies differences in ITS1 rDNA in our study (d = 1% and 4%) were significantly lower than that obtained for *N. salmincola* and *N. schikhobalowi* (d = 20%), but corresponds with the Digenea's previously published distances. So, the divergence between the species of *Curtuteria*, *Acanthoparyphium* and seven cryptic species of *Crassicutis cichlasomae* was 1.8%, 0.6–6% [28,45], and 1.0–5.2% [27], respectively.

The interspecific divergence based on the ITS2 rDNA between *N. japonensis* and *N. schikhobalowi* was almost an order of magnitude lower (d = 0.3%) than that of *N. salmincola* with *N. japonensis* (d = 2.4%), as well as with *N. schikhobalowi* (d = 2%). According to the literature data, the range of interspecific genetic variability in ITS2 rDNA in trematodes is extremely wide, 0.3–21% [27]. However, it is believed that generally ITS2 revealed slower evolutionary rate and sometimes demonstrated a complete absence of any substitutions not only within but also between species [26,27,46]. Thus, we observed a wide range of genetic distances among three species of *Nanophyetus*, suggested that the relationship of *N. japonensis* and *N. schikhobalowi* is much closer than each of them with *N. salmincola*.

Analysis of mitochondrial DNA (mtDNA) sequence variation has been used for more than a decade as a tool to understand the phylogeny of species as well as the geographical distribution of genetic variation [47–51]. The average pairwise genetic distances between the *N. japonensis* and *N. schikhobalowi*, as well as the *N. japonensis* and *N. salmincola* specimens (d = 13% and d = 22%, respectively) were comparable with that for the *N. schikhobalowi* and *N. salmincola*

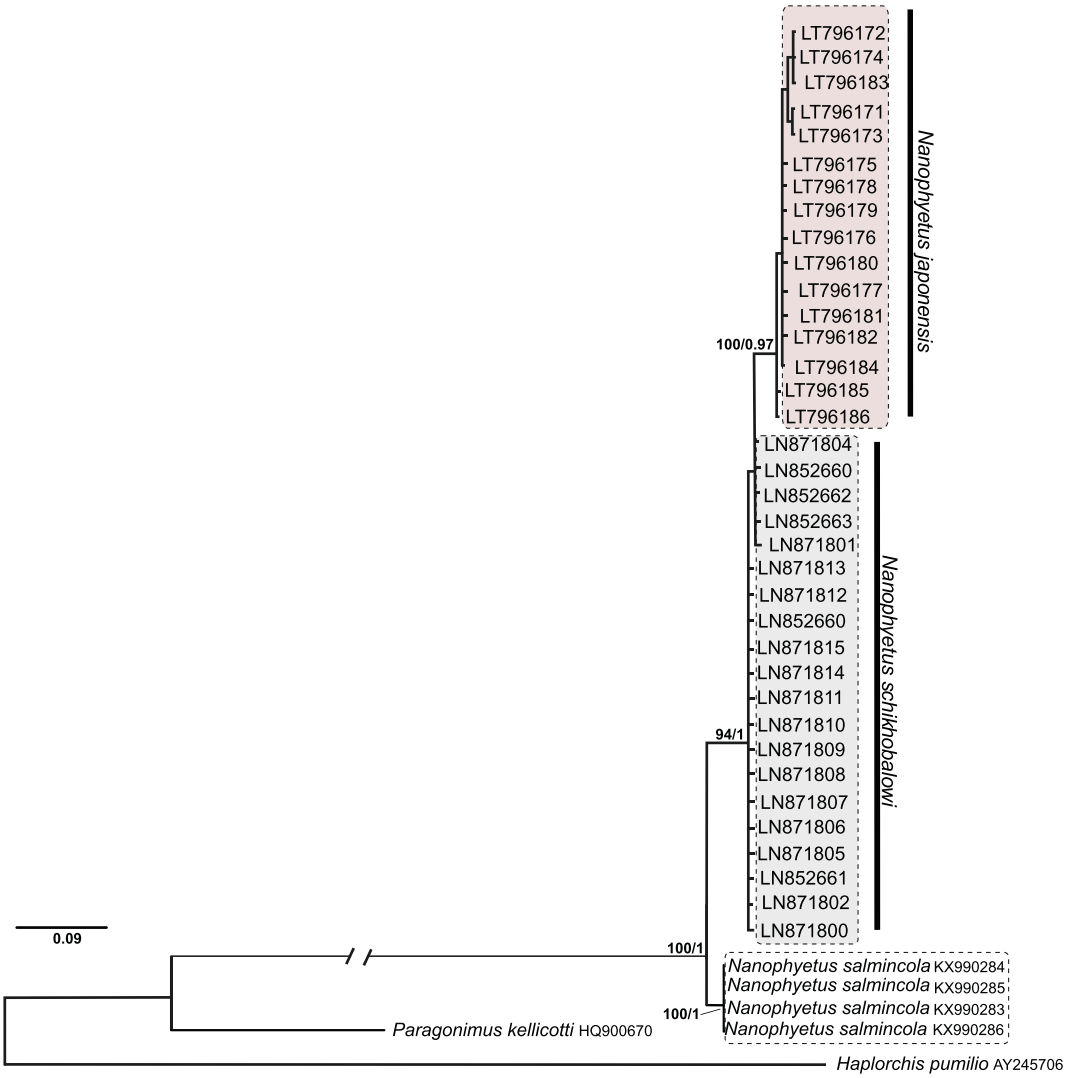

Fig. 5. Maximum Likelihood phylogeny of *Nanophyetus* based on ITS1 + 5.8S + ITS2 rDNA sequences. Statistical branch support values are as follows: Maximum Likelihood bootstrap values/Bayesian inference posterior probabilities (ML/BI). Only bootstrap support values > 50% in ML and posterior probabilities > 0.7 in BI are shown.

(d = 14.2–16.7%). High value of genetic distances obtained in our study for *Nanophyetus* ssp., well consistent with those of other congeneric species within taxa of Platyhelminthes [27,33,50]. An average sequence divergence of the *cox1* gene among *Echinostoma* species was 8% [40], and *Drepanocephalus* sp. - 11% [53]. In cryptic species, mtDNA divergence showed up to 21% [40], with the mean pairwise values between congeners at about 19% (3.9–25%) [52]. It is very clear from these estimations that even the mild genetic distances between *N. japonensis* and *N. schikhobalowi* correspond to the level of interspecific differentiation. The intraspecific variability of trematodes is often due to single nucleotide substitutions in mtDNA genes. For large population sets, the maximum intraspecific differences ranged from 0.3 to 4% and do not overlap with interspecific divergence [49,50]. This observation is in accordance with our results demonstrating 0.2% of nucleotide differences for *N. japonensis* mtDNA although such low value can be partially due to restricted sample set.

In all eukaryotic groups, the secondary structure for transcripts of ITS spacers is essential for correct processing of the rRNA primary transcript [54]. The larger size and high level of variation within the ITS1 effectively precluded the identification of common folding patterns for this region [55–57]. The level of variability detected during comparison of the primary sequences of *N. japonensis* ITS1 and ITS2 regions with that of *N. schikhobalowi* and *N. salmincola* implied that there might be consequences for their secondary structures. Secondary structure model of ITS1 for *N. japonensis* contained elements of SS which are common for both congeners (Fig. 3A), which may reflect the peculiarity of speciation process. Most nucleotide substitutions could be shown to be compensatory. The sites of compensatory mutations are important to preserve the secondary structure pattern and aren't involved in any other interactions. Presumably, most ribosomal genes in any individuals encode rRNA similar in a possible number of nucleotides that could be unpaired [58,59]. The detection of such ITS1 folds for *N. japonensis*, *N. schikhobalowi*, *N. salmincola* may be explained by the operation of mechanisms of evolutionary selection after species divergence from a common ancestor, having all described elements of secondary structures. Interestingly, minimal free energy for the ITS1 (DG = − 436 kcal/mol), and ITS2 (DG = − 157 kcal/mol) formation for *N. japonensis* is almost 1.5 times smaller than required for secondary structures reconstruction of these spacers for *N. schikhobalowi* and *N. salmincola* [9].

The lower level of nucleotide variability and smaller size of the ITS2 permitted the identification of common features in predicted secondary structures for this region across the range of digenean species [24,40,57]. The sequence identity of ITS2 of *Nanophyetus* spp. reflected on the resulting secondary structure of *N. japonensis*, showing only minor differences from the secondary structures of *N. schikhobalowi* and

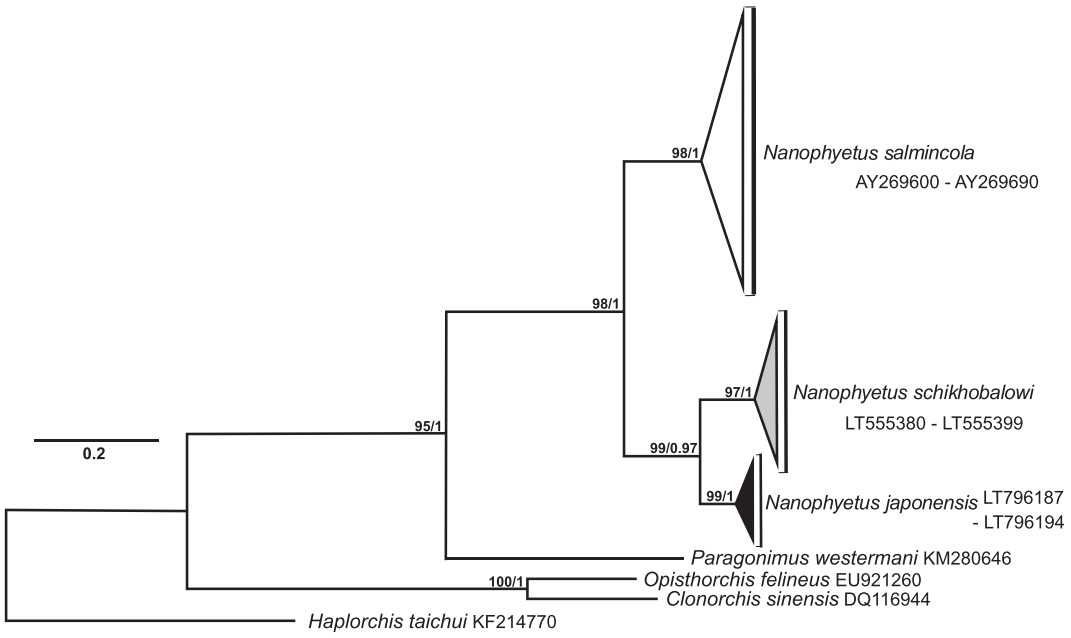

Fig. 6. Phylogenetic relationships between populations of *Nanophyetus* based on *nad1* gene sequences. Branch support values of > 70% are given in the following order ML/BI.

N. salmincola (Fig. 3B). While the conformation of domains A and B was stable, domain C and domain D had several substitutions. In both the trematodes and the monogenean, domain C is always longer than domain D, and although both are highly variable, in the majority of families they fold as unbranched stems (presumably a primitive state) [40]. Only two exceptions to this rule were described: in the families Echinostomidae, where the appearance of an additional branch in domain C is the result of an internal repeat [40], and Lecithodendriidae, where the structure is more difficult to interpret. Although species within the family are potentially capable of remaining unbranched through domain C, usually the optimum minimum-energy models supported the branch [40]. The stem of the domain C of *N. japonensis* was straight and in contrast to *N. salmincola* and *N. schikhobalowi*, contained less number of unique internal loops, which tended to lower the free energy, so energetically it was favorable (see above) (Fig. 3B).

Phylogenetic trees based on different regions of nuclear rDNA (Figs. 4 and 5) and partial *nad1* gene sequences (Fig. 6) used in our analyses produced similar tree topologies with a reliable level of statistical support for the phyletic lineages of the *Nanophyetus* species. Thus, *N. japonensis* was in one clade with *N. schikhobalowi*, and *N. salmincola* usually clustered separately, but all species differed well from each other. Incongruence in topology corresponded only in tree built using partial 28S rDNA sequences, where *N. japonensis* and *N. schikhobalowi* localized on the same branch. 28S rRNA gene has very conservative nature with slow mutation rate, so it may evolve later than the other members of ribosomal family [15]. When using sequences of the complete 28S rRNA gene, the problem was successfully solved and phylogenetic tree had a normal structure, confirming the fact that it is better to utilize maximum length sequences for molecular genetic analysis.

A comparison of the three *Nanophyetus* species revealed the discrepancies between the levels of morphological distinctions published earlier [1–8] and molecular divergence described in this study and previously [9] but showed the positive correlation between the geographical and genetic distances. So, in morphology *N. salmincola* is almost identical to *N. schikhobalowi* but well differed from the *N. japonensis* while genetically it highly diverged from both species. In morphology, *N. japonensis* differs from *N. salmincola* and *N. schikhobalowi* almost equally; however, based on molecular markers it is much closer to the last. From the other hand, the geographically close Asian species *N. japonensis* and *N. schikhobalowi* are genetically more similar between themselves than each of them with congeneric species on the opposite side of Pacific Ocean. In addition, the *Nanophyetus* species appeared to be different in their infectious capability. *N. schikhobalowi* is able to infect humans [1], *N. salmincola* transmit a rickettsia *Neorickettsia helmintheca* (which is the reason of salmon-poisoning disease frequently fatal to dogs and other canids) [60–62], while neither human infection nor salmon-poisoning disease is known for *N. japonensis* [6]. Thus, further studies are needed to test the hypotheses of speciation, diversification and evolution of host-parasite interactions for the genus *Nanophyetus*.

5. Conclusions

Data obtained demonstrated uniqueness of *N. japonensis* and a reliable level of its divergence with other *Nanophyetus* species, especially *N. salmincola*, and quite low intraspecific variation. The resulting phylogenetic trees with strong statistically support demonstrated three monophyletic lineages within the genus *Nanophyetus*: 1 - *N. salmincola*, 2 - *N. schikhobalowi*, and *N. japonensis*, and identified the Asian *Nanophyetus* as sister-species. *N. salmincola* first separated from the common ancestor, and then *N. schikhobalowi*, and *N. japonensis* were formed, that is associated with the division of the American and Asian continents with the subsequent isolation of the Japanese islands. The results provide a useful framework to facilitate identification of worms, involved in gastric diseases – nanophyetiasis and background for their future investigations.

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