

The High Divergence of Two Morphologically Similar Trematode Species of the Genus *Nanophyetus* of Salmonids from the Data of mtDNA *nad1* Gene Sequences

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Abstract—Intestinal flukes of fish (mainly salmonids) belonging to the genus *Nanophyetus* (Trematoda: Troglotrematidae) are the causative agents of nanophyetiasis, a zoonotic disease of animals and humans, which is widespread in countries in the northern Pacific. Two geographical forms, one from North America and the other from the eastern Eurasia were described within this genus; however, their taxonomic status was debatable. A multilocus analysis of nuclear rDNA sequences applied in this study has shown that these forms are independent nominal species: *Nanophyetus salmincola* and *Nanophyetus schikhobalowi*. This study, based on sequencing the mtDNA *nad1* gene, has evaluated the genetic variability of *N. schikhobalowi* from eastern Eurasia (Russia) and compared our data with the results obtained for *N. salmincola* from North America (United States). The genetic differentiation within the Eurasian sample was 1.4%, that for the North American sample was 0.8%, and differentiation between the samples was 15.5%. High values of genetic divergence and completed sorting of mitochondrial haplotypes confirmed the species independence of the compared geographic forms of *Nanophyetus*.

Keywords: Trematoda, *Nanophyetus*, *nad1*, haplotypes, molecular phylogenetics, taxonomy

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INTRODUCTION

Nanophyetiasis or “fish flu” is an invasive disease of humans, as well as domestic and wild carnivores, caused by trematodes of the genus *Nanophyetus* (salmon flukes). Infection of the final hosts occurs through consumption of fish infected with parasites. The disease is known for its association with “salmon poisoning disease” (SPD), which is deadly for dogs and other canines [13, 27]. Nanophyetiasis occurs in the United States and in Russia, where the spread of the infection among the local indigenous population can reach 95–98% [2, 3, 14]; the disease occurs also in Japan [6].

N. salmincola is described for North America [9], and *N. schikhobalowi* for the eastern part of Eurasia [23]. Despite a smaller egg size, *N. schikhobalowi* was synonymized with *N. salmincola*, because of their high morphological similarity [30]. Morphologically, these trematodes differ only in the size of eggs, the location of the genital pores and the shape of the cirrus sac [4, 6]. To show the biological and geographical differences of these worms and their minor morphological

differences, *N. schikhobalowi* began to be considered as a subspecies of *N. salmincola* [5, 12].

A taxonomic problem cannot always be solved without genetic approaches. A comparative analysis of North American and Eurasian representatives of the genus *Nanophyetus* performed on multilocus markers of nuclear rDNA made it possible for the first time to establish the interspecific level of genetic differentiation [29]. The aim of this study was to confirm the species independence of the species *N. schikhobalowi* using a molecular marker with a different type of inheritance. To do this, based on the sequences of the mtDNA *nad1* gene, which encodes the subunit I of the respiratory-chain NADH dehydrogenase, we evaluated the genetic variability of *N. schikhobalowi* samples from the Russian Far East and the results were compared with the data published for North America [11]. The molecular markers of mtDNA have several advantages over nuclear marker and are recognized as highly informative for the study of a large group of trematodes (including morphologically hardly distinguishable sibling, sister and cryptic species) both at the

intra-species and at the interspecific levels [7, 10, 18, 19, 22, 28].

MATERIALS AND METHODS

Metacercariae were isolated from infected fish, that is, the salmon (*Brachymystax lenok*) and carp (*Phoxinus oxycephalus*) that were caught in the southern areas of the Russian Far East. The fish muscle tissues with the larvae were fed to laboratory rats under experimental conditions. At 1 month after infection, the adult flukes were recovered from the small intestines of rats, thoroughly rinsed in physiological saline and fixed in 70% ethanol. Samples were identified by their morphological characteristics.

Genomic DNA was isolated from 20 individuals of *Nanophyetus schikhobalowi* by alkaline lysis [26]. Partial sequences of the *nad1* gene were amplified using primers f-MB352 (5'-CGTAAGGGKCCCTAAYAAG-3') and r-MB399 (5'-CTTACAAAATAGTCATAGCG-3') [11]. The polymerase chain reaction was carried out in accordance with the described scheme [11]. Sequencing was performed with the same primers using Sanger's method on an ABI 3130 Genetic Analyzer using the ABI BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

The obtained sequences of the *nad1* gene with a length of 639 base pairs of nucleotides (bp) (Accession numbers in the GenBank NCBI DNA sequence database: LT555380-LT555399) and the corresponding sequences of *Nanophyetus salmincola* obtained from the GenBank database (AY269600-AY269690) [11], as well as sequences of *Paragonimus westermani* KM280646 and three species of the external group *Opisthorchis felineus* EU921260, *Clonorchis sinensis* DQ116944 and *Haplorchis taichui* KF214770 were aligned using the Clustal W program [25].

The pairwise intra- and interpopulation nucleotide differences (p-distances, d) were calculated in the MEGA 5.1 program. Phylogenetic relationships were reconstructed using the maximum likelihood (ML) algorithms in MEGA 5.1 [24] and Bayesian Inference (BI) as implemented in MrBayes 3.1 [21]. The optimal model of nucleotide substitutions (GTR + G) was selected using the Akaike and Bayes (BIC) information criteria in the Modeltest 3.7 program [20]. The statistical reliability of molecular tree nodes was assessed using a 1000-replicates (ML) bootstrap analysis or a posteriori probabilities (BI). In BI analysis, 1 mln Markov chains generations were generated and were sampled every 100 generations. The first 500 samples were excluded from the analysis as "burn in."

RESULTS AND DISCUSSION

The data set included 111 sequences of the *nad1* gene fragment with a length of 639 bp, which represented 96 mitochondrial haplotypes (76 of which belonged to the North American sample and 20 to the

Eurasian sample). No common haplotypes have been identified; this may indicate a fully completed sorting of mtDNA lines. The pairwise genetic distances were estimated as being relatively low for both geographical samples; however, the genetic variability of the North American sample (d = 0.2–1.4%) was slightly lower than that of the Eurasian sample (d = 0.2–2.5%), with the average values of 0.8 and 1.4%, respectively. The genetic distances between the two samples were significantly higher: d = 14.2–16.7% with an average value of 15.5%.

The levels of genetic divergence of the species obtained in this study are very consistent with those observed in various digenean taxa. For comparison, the divergence of the sequence of the *nad1* gene in the genera *Echinostoma*, *Fasciola*, *Schistosoma* and *Drepanocephalus* varies from 10.3 to 35.4% [19, 28]. On the other hand, the intraspecific variability of trematodes is often determined by single nucleotide substitutions in the mtDNA genes and usually does not exceed 2–4% [15, 22, 28].

The data set contained 165 variable sites, 129 of which were parsimony-informative. Phylogenetic trees constructed by different methods had identical topology with two reliable clades (statistical support of 100% for ML and 1.0 for BI), one of which was formed by North American representatives of the genus *Nanophyetus*, and the other by Eurasian flukes (see Fig. 1). The internal structures of small phylogenetic clades were mostly polytomic. The lack of intraspecific genetic structuration excludes the specificity of mtDNA haplotypes to fish host species and intra-regional localities, which could be possible for parasites.

According to phylogenetic analysis of mtDNA, salmon flukes form clades associated with the geographic region. Such phylogenetic patterns can characterize units evolving in time separately and, therefore, are useful in distinguishing species [16]. The high degree of divergence of the two mitochondrial lines of *Nanophyetus* is probably related both to the historical formation of the current ranges of trematodes and their parasitofauna [1, 8], and to certain biological peculiarities of the species. As an example, the intermediate and final hosts of *N. salmincola* and *N. schikhobalowi* differ well at species and generic levels [5, 12]. The North American species *N. salmincola* is a vector for the pathogenic bacterium *Neorickettsia helminthoeca* (see: [13, 27]), whereas this bacterium has not been identified in the eastern part of Eurasia [2, 3]. It is known that parasitic taxa, because of a small spectrum of their morphological criteria, often contain cryptic species or sibling species that can differ in features that are important for parasite-host interactions, including host specificity, pathogenesis, and epidemiology [8, 17].

Thus, based on the sequences of the mtDNA *nad1* gene, we have obtained data for the first time on the genetic diversity of salmonid flukes, which are fish

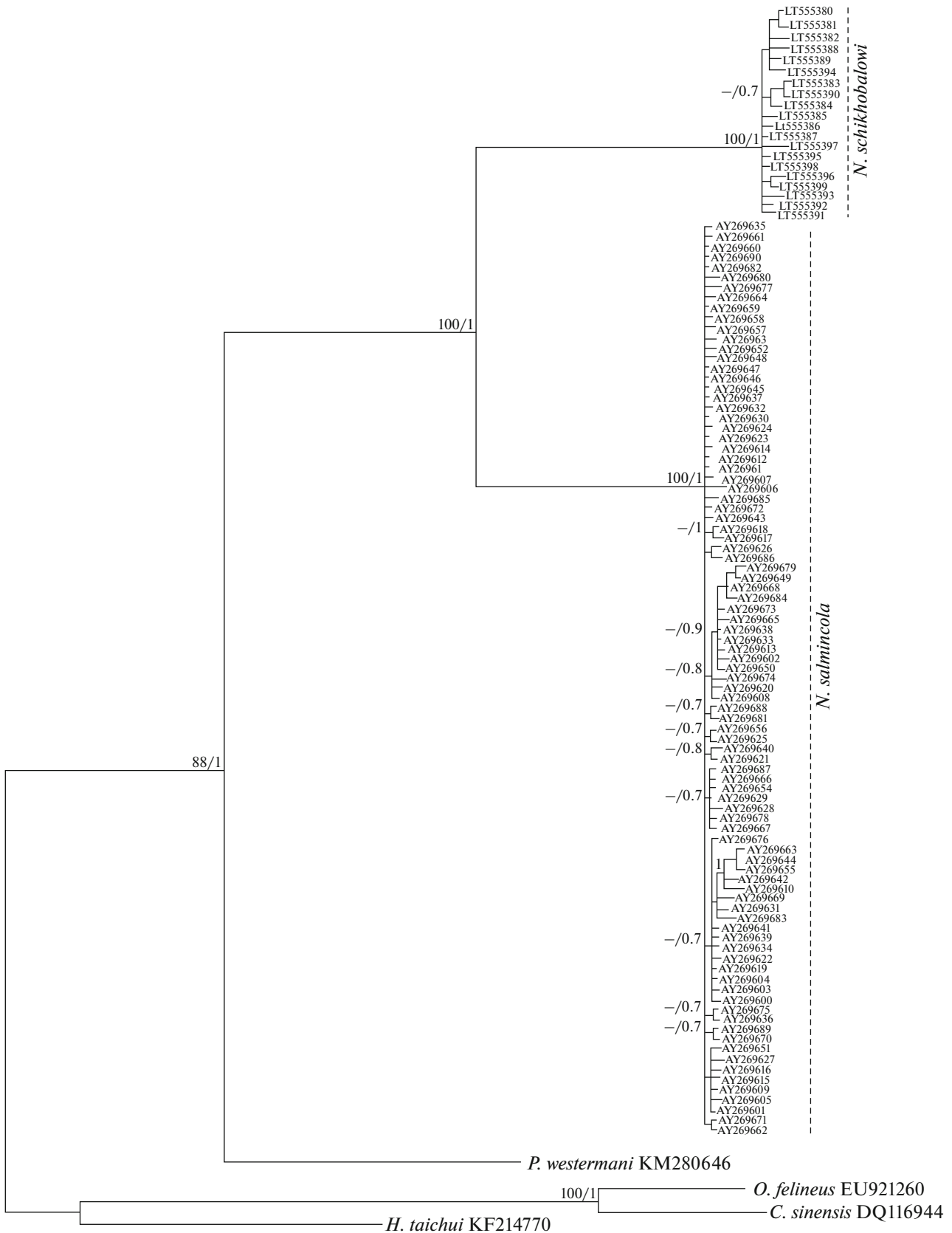


Fig. 1. The phylogenetic connections between populations of the genus *Nanophyetus* studied on the basis of mitochondrial *nadI* gene sequences. The numbers in knots are the values of bootstrap support of branches of more than 70% for ML and posterior probabilities more than 0.7 for BI. The tree is rooted in the sequence of *Haplorchis pumilio* (KF214770).

parasites of the Far East of Russia. These data allow us to consider the morphologically very similar geographic forms of *Nanophyetus* as highly divergent species. This confirms the results of our previous studies and the high efficacy of the selected mitochondrial marker for species identification and phylogenetic analysis at the intra-generic level. These results can be useful for understanding the mechanisms of formation of parasite-host associations and speciation of trematodes.

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