

Phylogenetic Relationships of 3 Korean *Neodiplostomum* Species (Digenea: Neodiplostomidae) Based on Partial CO1 Gene

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Abstract: The phylogenetic relationships of the 3 *Neodiplostomum* spp. (Digenea: Neodiplostomidae) occurring in Korea (*N. seoulense*, *N. leei*, and *N. boryongense*) were analyzed using the partial mitochondrial cytochrome c oxidase subunit 1 (CO1) gene. The adult flukes were recovered from Sprague-Dawley rats (*N. seoulense*) and newborn chicks (*N. leei* and *N. boryongense*) experimentally infected with the neodiplostomula from the grass snake, *Rhabdophis tigrinus tigrinus*. The genomic DNA was amplified using specific primers, and the sequence of CO1 was obtained. According to the results, the pairwise similarity was 96.1% between *N. boryongense* and *N. seoulense*, but was 95.0% between *N. boryongense* and *N. leei* and 94.2% between *N. leei* and *N. seoulense*. The results demonstrated a closer phylogenetic relationship between *N. seoulense* and *N. boryongense*. This high relationship of *N. seoulense* and *N. boryongense* may be related to their similar morphologic features including the limited distribution of vitellaria and the presence of a genital cone. *N. leei* is distinct on the other hand with an extensive distribution of vitellaria and the absence of a genital cone.

Key words: *Neodiplostomum seoulense*, *Neodiplostomum leei*, *Neodiplostomum boryongense*, phylogeny, CO1 gene

The grass snake, *Rhabdophis tigrinus tigrinus* (Boie, 1826), inhabiting the Republic of Korea, has at least 3 species of small-sized neodiplostomula (metacercariae of neodiplostomid flukes), i.e., *Neodiplostomum seoulense*, *Neodiplostomum leei*, and *Neodiplostomum boryongense* [1-4]. These neodiplostomula are indistinguishable based on their morphology; however, the definitive hosts and life cycles are different. *N. seoulense* develops into an adult fluke in the small intestine of rodents, as well as humans, whereas *N. leei* and *N. boryongense* develop in the small intestine of chicks but not in rodents [2,3]. The metacercariae of *N. leei*, when given orally to mice and rats, migrate to the liver of these rodent hosts. On the other hand, *N. seoulense* and *N. boryongense* do not migrate to visceral organs of rodent hosts [2,3]. Moreover, these neodiplostomula have a different destiny in the murine small intestine; *N. seoulense* develops into adult flukes, whereas *N. boryongense* do not

develop into adults, but disappear from the intestine in a few days after infection.

Considering their common second intermediate (or paratenic) host with similar geographical distribution and similar morphologies of metacercariae and even adults, the 3 species of *Neodiplostomum* may have a close phylogenetic relationship. To analyze the degree of relationships between different parasite species, partial mitochondrial genes have been used [5]. In particular, the mitochondrial cytochrome c oxidase subunit 1 (CO1) gene has been used for DNA-based identification of parasites, providing the resolution of diversity based on the conserved mitochondrial gene [6]. The examples of parasites studied include *Echinococcus* spp. [7], *Taenia multiceps* [8], *Taenia taeniaeformis*, and other taeniid cestodes [9], *Echinostoma* species [10], *Plagiorchis* spp. [11], *Ancylostoma duodenale* and *Necator americanus* [12], *Spirometra* and *Diphyllobothrium* [13], and rodent pinworms [14]. Although ITS [15] is also used for taxonomic studies, CO1 gene has been suggested to be useful for general evolutionary studies [16]. The present study was performed to analyze the phylogenetic relationships of the 3 species of *Neodiplostomum* occurring in Korea, using the partial CO1 gene.

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Newborn broilers were purchased from a hatchery in Gyeonggi-do, Korea. Female Sprague-Dawley rats, 6-8-week-old, which were raised under specific pathogen-free (SPF) conditions, were purchased from Samtako (Osan, Gyeonggi-do, Korea). The grass snakes, *R. tigrinus tigrinus*, naturally infected with the neodiplostomula, were purchased from local snake collectors in enzootic areas, such as Osan and Ansong in Gyeonggi-do (Province), Hongcheon and Hoeongsung in Gangwon-do, and Cheonan, Onyang, Hongsung, and Boryong in Chungchongnam-do. Snakes were kept in a cold room for hibernation until used.

Small-sized neodiplostomula were collected by the digestion technique using artificial gastric juice containing 0.5% pepsin (1:10,000, Sigma-Aldrich, St. Louis, Missouri, USA) and 0.8% HCl. Snakes were sacrificed under ether anesthesia, and skinned [3]. The entire visceral mass and carcasses were separated and chopped before incubation in pepsin-HCl solution. The digested mixture was incubated for 90 min at 37°C and washed several times by filtering through coarse and fine stainless steel meshes (US standard testing sieves, 425 µm, 300 µm, 150 µm, and 106 µm, W. S. Tyler Incorp., Mentor, Ohio, USA). After final filtration, the mixture was transferred to a Petri dish, and neodiplostomula were collected using a stereomicroscope. The whole procedure was carried out under cold conditions to maintain the viability of the neodiplostomula. Large-sized diplostomula of *Pharyngostomum cordatum* were carefully collected and removed [3].

Rats were orally infected with the neodiplostomula using a gavage needle and adult flukes of *N. seoulense* were collected from the small intestine at week 2 post-infection (PI). For collection of *N. leei* adults, metacercariae which were recovered from the liver of rats infected with small-sized neodiplostomula, were orally infected to newborn chicks, and adult flukes were recovered from the intestine of chicks at week 2 PI. The procedure of harvesting the neodiplostomula from the rat liver was as described previously [17]. The livers of infected rats were resected, cut into small pieces, digested with artificial gastric juice, and then the neodiplostomula were collected under a stereomicroscope. For collection of *N. boryongense* adult flukes, small-sized neodiplostomula recovered from snakes were directly fed to newborn chicks and the adult flukes were recovered from the intestine of chicks at week 2 PI. To collect the adult flukes, the intestine was taken out and put on a Petri dish containing saline, and then opened longitudinally. Adult flukes were collected under a stereomicroscope.

For cloning of *CO1* gene from the genomic DNA of *Neodiplostomum* species, the primer set was prepared as follows [10]: the forward primer 5'-TTT TTT GGG CAT CCT GAG GTT TAT-3' and the reverse primer 5'-TCA TGA AAA CAC CTT AAT ACC-3'. The genomic DNA of neodiplostomid flukes was individually extracted using the DNA-EASY kit (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions. DNA was amplified with *CO1* specific primers and PCR pre-mix (Bioneer, Seoul, Korea). Amplification was performed in a gradient Master cycler (Eppendorf, New York, USA) under the following conditions: 30 cycles consisting of 1 min at 94°C, 1 min at 49°C, and 1 min at 72°. Unincorporated primers and nucleotides were removed from the PCR product using the QIA quick gel extraction kit (Qiagen, Hilden, Germany). PCR products were ligated into a TA cloning vector in TOPO TA cloning kit (Invitrogen) and the subcloned DNA was sequenced (Cosmo Genetech Inc., Seoul, Korea) and analyzed using a bioinformatics tool (Geneious Pro 5.4.7, Geneious Co., Wellington, New Zealand). Because *CO1* gene products were of different nucleotide sizes, the overlapped *CO1* gene domain among the 3 species of *Neodiplostomum* flukes were used for this phylogenetic analysis.

We obtained partial *CO1* gene sequences of the 3 species of neodiplostomid flukes (Fig. 1). An analysis program (Geneious Pro 5.4.7; Geneious Co.) was used for analysis of the gene alignment and phylogenetic tree. The partial *CO1* gene of *Fasciola hepatica* (GenBank accession no. AB207170.1) was used as an outgroup. For alignment of the 3 *CO1* genes, the sequenced DNA was processed by the Geneious Pro 4.6.2 program (Geneious Co.). The results were as follows: cost matrix was the lowest among the list with 51% similarity, gap open penalty score was 12, gap extension penalty score was 3, and alignment type was global alignment in general. The partial *CO1* gene sequences studied were *N. boryongense* (280 bp), *N. leei* (276 bp), and *N. seoulense* (283 bp).

According to the results, the pairwise similarity between *N. boryongense* and *N. leei* was 95%, and the pairwise similarity between *N. leei* and *N. seoulense* was 94.2%; the identical sites were 270 bp (93.4%) and 268 bp (94.4%) respectively. On the other hand, the pairwise similarity between *N. boryongense* and *N. seoulense* was 96.1% and the identical sites were 272 bp (94.1%). The genetic distance model used was 'Tamura-Nei', and the tree-built method was 'Neighbor-joining'. The results showed that the score using "PATRISTIC DISTANCES MATRIX" between *N. boryongense* and *N. leei* was 0.993, that between *N.*

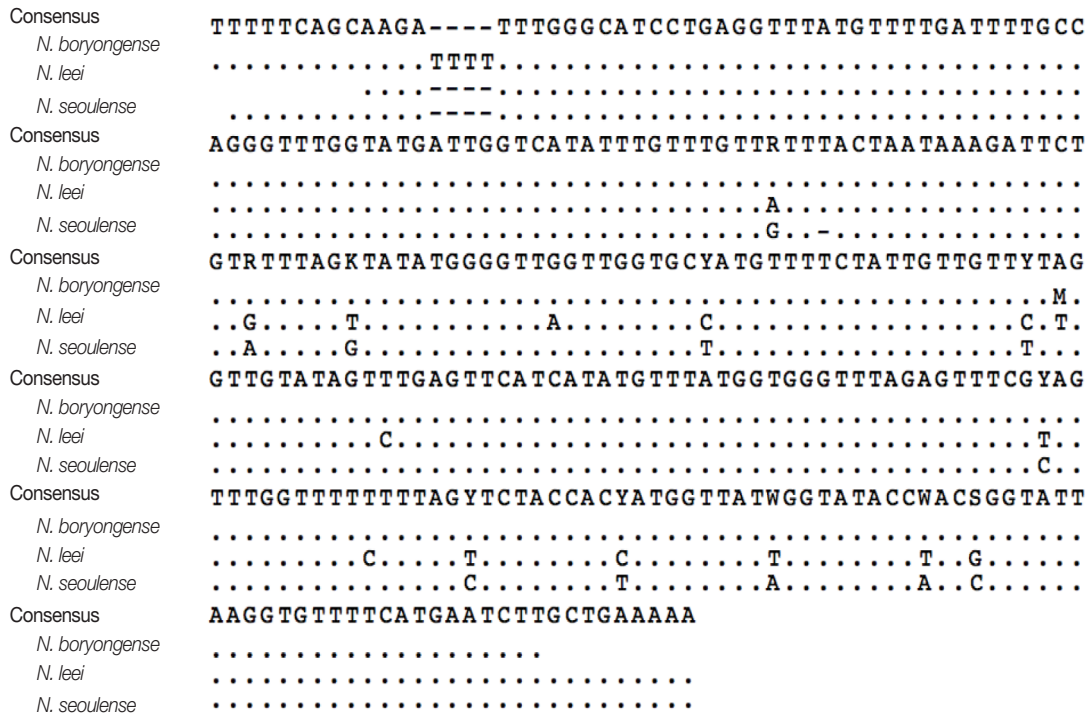


Fig. 1. Partial cytochrome c oxidase subunit 1 (CO1) sequences of 3 *Neodiplostomum* species. Dash indicates gap introduced into alignments among the 3 trematode parasites.

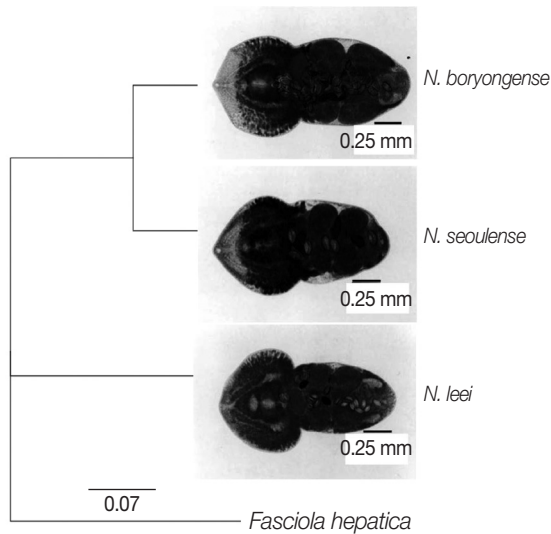


Fig. 2. Phylogenetic tree inferred from the aligned sequence of partial CO1 gene. The sequence of *Fasciola hepatica* (GenBank accession no. AB207170.1) as an outgroup, and sequences of *Neodiplostomum* spp. (*N. boryongense*, *N. seoulense*, and *N. leei*) were compared and aligned using the Geneious Pro 5.4.7 program. The genetic distance represents the dissimilarity among the 4 kinds of nucleotide sequences.

seoulense and *N. leei* was 0.919, and that between *N. seoulense* and *N. boryongense* was 0.073. Therefore, *N. seoulense* and *N.*

boryongense were genetically closer than they were with *N. leei* (Fig. 2).

The present study revealed that the genetic proximity between *N. seoulense* and *N. boryongense* was higher than that between the other 2 combinations. The sequence similarity of CO1 gene was 96.1% between *N. seoulense* and *N. boryongense*, whereas that between *N. leei* and *N. seoulense* was 94.2% and that between *N. leei* and *N. boryongense* was 95.0%. It was highly suggested that all these 3 *Neodiplostomum* spp. have a common ancestry, and *N. seoulense* and *N. boryongense* may have differentiated later than *N. leei*. Neodiplostomid flukes may have progressed to be adapted from birds to mammals through predator and prey interactions [5,18]. Therefore, *N. seoulense* may be the newest clade followed by *N. leei* and *N. boryongense*.

The closer genetic relationship between *N. seoulense* and *N. boryongense* compared to *N. leei* may be phenotypically related to the morphology and life cycles of these neodiplostomid flukes. *N. seoulense* has vitellaria which are less extensive than *N. leei* distributing only up to the level of the ventral sucker, and 2 bilobed testes resembling a butterfly-shaped cuff link [4]. *N. leei* has vitellaria which are very extensive and distributed from the pharynx to near the posterior end of the body; it

has no genital cone but has 2 bilobed testes with a long, prominent median constriction forming a dumbbell shape [2]. *N. boryongense* has vitellaria which are not extensive and distributed chiefly in the forebody without distribution between the pharynx and the ventral sucker [3]. Their testes are severely bilobed and eye-glass shaped.

The genital cone has taxonomic significance and is used to differentiate the genus *Neodiplostomum* into 2 subgenera, *Neodiplostomum* (*Conodiplostomum*) Dubois, 1937 and *Neodiplostomum* (*Neodiplostomum*) Dubois, 1937 [2,3]. *N. seoulense* and *N. boryongense*, which belong to *Neodiplostomum* (*Conodiplostomum*), have a genital cone near the terminal portion of the genital opening, whereas *N. leei* has no genital cone and thus belongs to *Neodiplostomum* (*Neodiplostomum*) [2,3]. Moreover, *N. leei* uses rodents as a paratenic host in which its neodiplostomula migrate to the liver [2]. However, *N. seoulense* and *N. boryongense* do not migrate to the liver of rodents and develop into adults in the intestine of rodents and chicks, respectively [2,3]. These comparative characters support our results of closer genetic proximity between *N. seoulense* and *N. boryongense* than with *N. leei*.

The phylogenetic analysis of only *cox1* gene may not be sufficient to properly explain overall coevolution of parasites and their hosts [5]. However, our results could provide at least some knowledge on genetic proximity among the 3 neodiplostomid flukes occurring in Korea. Further biological and genetic studies will be helpful to support our results.

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CONFLICT OF INTEREST

We have no competing interest related to this work.

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