

Alaria alata (Digenea: Diplostomidae) from Korean Raccoon Dog, *Nyctereutes procyonoides koreensis*, in Korea

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Abstract: We report a species of diplostomid fluke recovered from 3 carcasses of wild Korean raccoon dog, *Nyctereutes procyonoides koreensis*, in Korea. A total of 107 diplostomid flukes were recovered from the small intestines of Korean raccoon dogs, which were obtained from the Gangwon Wildlife Medical Rescue Center. Worms fixed with 10% neutral formalin were subjected to microscopic observation and those fixed in 70% ethanol were used for molecular genomic analysis. The worm was divided into 2 separate parts, forebody and hindbody, with a total length of 3,020–4,090 (3,855) μm and a width of 1,210–1,770 (1,562) μm . The boat-shaped forebody has a pair of characteristic tentacular appendage, 2 suckers, holdfast organ, and vitelline follicles. The oval to cylindrical hindbody has reproductive organs. The ovary was round or elliptical and located in the anterior of the testes. Two large testes were slightly segmented and tandemly arranged, occupying almost half of hindbody. The short uterus contained a relatively small number of unembryonated eggs sized 130–140 \times 85–96 μm . The partial sequence of 18S rRNA of this fluke was consistent with *Alaria alata*. Based on the morphological and molecular characteristics, the diplostomid flukes recovered from the small intestine of Korean raccoon dogs were identified as *A. alata* (Digenea: Diplostomidae).

Key words: *Alaria alata*, *Nyctereutes procyonoides koreensis*, Korea

The trematode *Alaria alata* (Goeze, 1782) is cosmopolitan parasite and commonly found in various wild carnivores that feed in water-rich areas such as ponds and lakes [1]. Their life cycle requires 2 intermediate hosts. The first intermediate host is a freshwater snail such as *Helistoma*, *Planorbis*, *Lymnea*, and *Anisus* spp., and the second is an amphibian such as a frog [2]. In addition, many species of omnivorous and carnivorous mammals can also act as paratenic hosts [3,4]. In particular, the wild boar is known as the most common paratenic host [4]. *A. alata* is considered a potential cause of a human disease called alariosis, which is spread through game meat infected with the infective mesocercariae. Alariosis is a food-borne zoonotic helminthiasis [5].

The typical definitive hosts for *A. alata* would be its wild predators; raccoon dog (*Nyctereutes procyonoides*) is one of main species. Raccoon dogs have successfully settled in Eu-

rope, and their numbers are increasing. As a result, they have the potential to pose a threat to human and animal health [6]. There are 5 subspecies of raccoon dogs, among which Korean raccoon dog (*Nyctereutes procyonoides koreensis*) is widely distributed in this country. As in European countries, in Korea, raccoon dogs can pose a threat to public health as they have many opportunities to encounter humans.

The prevalence of *A. alata* in raccoon dog has been reported in Belarus, Estonia, Lithuania, Denmark, and Latvia, with prevalence rates ranging from 32.9% to 91.6% in each country [6–10]. However, only 1 study has been done on *A. alata* in Korea. *A. alata* was detected in 1 out of 30 Korean raccoon dogs, but morphological and molecular characteristics were lacking [11].

From December 2019 to February 2020, 3 deceased Korean raccoon dogs were found in 3 sites in Gangwon-do (province): Manjong-ri (village) (37°20'59.8"N, 127°54'13.1"E), Hojeomyeon (township), Wonju-si (city); Jung-ri (37°22'22.5"N, 128°24'27.1"E), Pyeongchang-eup (town), Pyeongchang-gun (county), Uiam-ri (37°49'57.0"N, 127°40'45.3"E), Sindongmyeon, Chuncheon-si. They were transferred to the Gangwon Wildlife Medical Rescue Center in Kangwon National University. The raccoon dogs were autopsied, and a total of 107 dip-

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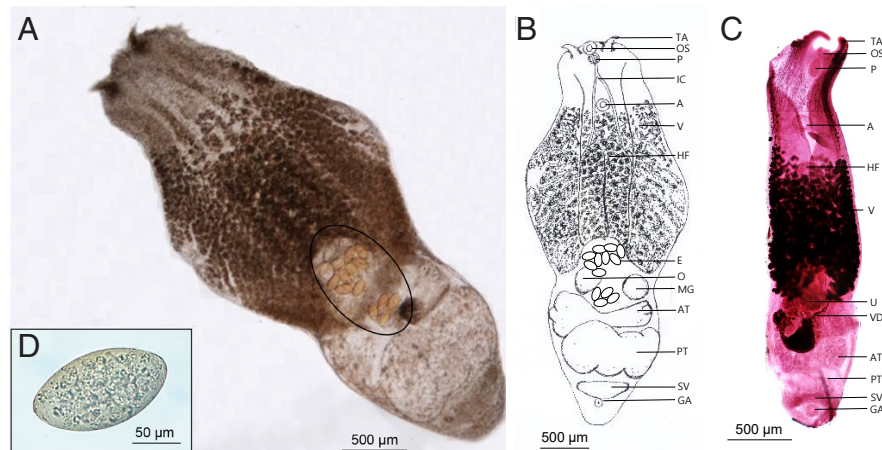


Fig. 1. *Alaria alata* recovered from the intestine of Korean raccoon dog. (A) Unstained adult worm. Note a few eggs (circle). (B) Schematic drawing of the "A" specimen. (C) Early-stage adult worm stained with Semichon's acetocarmine. (D) Egg from feces. A, acetabulum; AT, anterior testis; E, egg; GA, genital atrium; HF, Holdfast organ; IC, intestinal crura; MG, Mehlis's gland; O, ovary; OS, oral sucker; P, pharynx; PT, posterior testis; SV, seminal vesicle; TA, tentacular appendage; U, uterus; V, vitelline follicles; VD, vitelline duct.

lostomid flukes (18, 31, and 58 per raccoon dog) were recovered from the contents of the small intestine. Before the fixation of flukes, we took photograph of flukes stored in saline (Fig. 1A). A part of the flukes was fixed in 10% neutral formaldehyde without flattening procedure, after which stained with Semichon's acetocarmine. The 20 flukes were fixed in 70% ethyl alcohol for the molecular study.

Genomic DNA was extracted from the parasite using the G-spin Total DNA Extraction Mini Kit (INTRON Biotechnology, Seoul, Korea) according to the manufacturer's instructions. Based on the 18S ribosomal RNA gene sequences of *A. alata* (AY222091 and JF769484), primers were designed using the online tool Primer3Plus (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>). The oligonucleotide sequences of primers were as follows: 5'-CCTGTTGCACCTCTGTGATG-3' (forward primer for AY222091), 5'-GCTAAAACAGCACCAATCC-3' (reverse primer for AY222091), and 5'-TAGGTGAACCTGCGGAAG-3' (forward primer for JF769484), 5'-ACCAAAGAGGAACATGACG-3' (reverse primer for JF769484). The primer sets were designed to yield products of 1,628 bp and 985 bp, respectively. PCR was performed using a MyCycler Personal Thermal Cycler (Bio-Rad Laboratories, Hercules, California, USA) and EmeraldAmp GT PCR Master Mix (Takara, Kusatsu, Shiga, Japan) with 1 μ l of DNA aliquot under standard conditions of 95°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 1 min. The PCR products were visualized via electrophoresis on 1.2% agarose gel, and then purified using the QIAquick PCR Purification Kit (Qia-

gen, Alameda, California, USA). PCR amplicons were directly sequenced using the ABI Prism Big Dye terminator ver. 3.0 ready reaction cycle sequencing kits (Applied Biosystems, Foster City, California, USA) with the use of the same primers used in PCR. After trimming the nonspecific sequencing data, we obtained 1,559 bp and 901 bp sequences, respectively. A phylogenetic tree was constructed by the Neighbor-Joining (NJ) method using the BLAST tree. The NJ method was based on a guide tree for pairwise and multiple alignment parameters, including 0.75 Max sequence difference [12]. Different statistical method (Bootstrap consensus tree) was conducted in MEGA 11 [13]. In bootstrap consensus tree, branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test 1,000 replicates are shown next to the branches.

Fig. 1B, C showed the morphological characteristics of the flukes. The worms were distinctly bisegmented with a long boat-shaped forebody and a short cylindrical hindbody with an overall length of 3,020-4,090 (3,855) \times 1,210-1,770 (1,562) μ m. The oral sucker located at the anterior end of the body measured 90-107 (104.5) \times 91-98 (95.7) μ m, almost identical to that of the ventral sucker, 95-109 (103.2) \times 95-103 (98.8) μ m. A pair of distinctive tentacular appendages were present on either side of the oral sucker. They had a well-developed pharynx, 910-1,120 (1,098) \times 530-630 (583) μ m-long holdfast organ, and a short esophagus. Vitellaria extended slightly from the acetabulum to the hindbody and was distributed in the

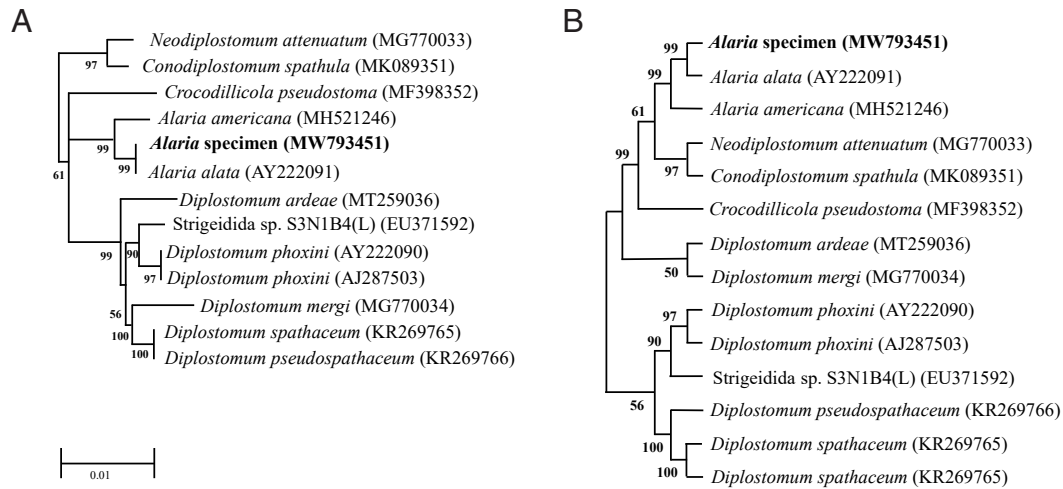


Fig. 2. Phylogenetic tree (Neighbor-Joining) based on *A. alata* sequence analysis. (A) Phylogenetic tree using partial sequence of 18S ribosomal RNA gene. (B) The bootstrap consensus tree inferred from 1,000 replicates.

ventral median line. The cylindrical hind body contained reproductive organs. Two testes, much wider than long, were tandem and slightly segmented. The anterior testis was 353-732 (471.2) × 245-295 (273.4) μm in size, smaller than the posterior testis sized 366-918 (601.9) × 265-310 (297.5) μm. Ovary was located anterior to the testis and measured 95-115 (107.7) × 85-110 (101.8) μm. The uterus was short and contained a small number of relatively large non-embryonated eggs (130-140 μm in length and 85-96 μm in width). The seminal vesicles were located posterior to the testes. The genital pore and atrium were located behind the seminal vesicle and were covered with gonotyle.

Genotype and phylogenetic relationships of this fluke with *A. alata* or Diplostomidae species were determined using the molecular characteristics of the 18S rRNA gene. We registered the sequence information into GenBank under accession numbers MW793451 and MZ057614. As shown in Fig. 2, the 18S rRNA sequence of *Alaria* specimen was compared to those of other parasites, including *A. alata* (AY222091) and *A. americana* (MH521246). The sequence of our specimen (MW793451) was identical to that of *A. alata* (AY222091) (Fig. 2A). Phylogenetic relationships were presented in the topology as distance matrix (scale bar). Furthermore, another sequence of our specimen (MZ057614) contained 24 variable sites of sequences that belonged to the same clade as that of *A. americana* (MH521246) (Fig. 2B). The heuristic search tree was obtained automatically by applying NJ and BioNJ algorithms to a matrix of pairwise distances estimated by the Maximum Composite Likelihood (MCL) (Fig. 2B). We analyzed the topology with superior log

likelihood value using 14 nucleotide sequences. Branches corresponding to partitions reproduced from less than 50% bootstrap replicates were collapsed. In the bootstrap test 1,000 replicates, the percentage of replicate trees in which the associated taxa clustered together was shown next to the branch (Fig. 2B). We eliminated all positions containing gaps and missing data for evolutionary analyses and used 8,300 positions in the final dataset. Based on the evolutionary analysis, adults in the genus *Alaria* Schrank (1788) discovered in the small intestine of Korean raccoon dogs were identified to be *A. alata*.

Family Diplostomidae Poirier (1886) could be characterized by a foliate, spatulate or calyciform forebody and a cylindrical or coniform hindbody [14]. This family Diplostomidae is divided into 2 subfamilies; mammalian parasites Alariinae and avian parasites Diplostominae [14]. The subfamily Alariinae is morphologically characterized by restricted distribution of vitellaria in the forebody and tandem arrangement of testes in opposite directions [14]. Considering the definitive host, distribution of vitellaria, and arrangement of testes, this fluke was identified as belong to the Alariinae.

Subfamily Alariinae Hall and Wigdor, 1918 includes *Alaria*, *Cynodiplostomum*, *Pharyngostomoides*, and *Procyotrema* [14]. Among them, genus *Alaria* has 2 distinct features from other 3 genera. Flukes of the genus *Alaria* have tandem testes on the hindbody and pseudosuckers forming tentacular appendages. Conversely, flukes in the other 3 genera have pseudosucker without unique characteristics and opposite testes on both sides [14]. The flukes in this study have pseudosucker forming tentacular appendages and tandem, multi-lobed testes in the hind-

Table 1. Comparison of the measurements of *Alaria* spp

Types	Species with tentacular appendage					Species with pseudosucker		
	<i>A. alata</i>	<i>A. alata</i>	<i>A. canis</i>	<i>A. marciana</i>	<i>A. arisaemoides</i>	<i>A. intermedia</i>	<i>A. mustalae</i>	<i>A. taxideae</i>
Host	Korean raccoon dog	Red fox	Dog	Striped skunk	Red fox	Dog	Mink, weasel, cat, dog, ferret	Badger
Total length (in mm) ^a	3.0-4.1	2.8-4.0	2.5-4.2	1.56-1.84	7.0-10.6	0.86-1.6	0.82-1.74	1.10-2.27
Oral sucker (L) ^b	90-107	51-122	75-140	95-122	100	76-110	62-116	78-178
Oral sucker (W) ^c	91-98	56-138	110-150	102-136	90	76-110	62-107	78-152
Acetabulum (L) ^b	95-109	72-117	90-120	75-109	100	76-86	53-89	65-106
Acetabulum (W) ^c	95-103	102-133	90-140	88-122	100	76-86	53-89	57-114
Pharynx (L) ^b	115-145	102-138	140-170	150-177	150	100-130	75-125	122-240
Pharynx (W) ^c	89-102	46-112	90-140	109-136	130	67-100	62-116	95-190
Ovary (L) ^b	85-110	150-320	-	-	350	100-160	107-214	102.6-140.6
Ovary (W) ^c	95-115	230-490	-	-	450	40-70	107-214	152-298
Holdfast organ (L) ^b	910-1,120	880-1,630	1,140	540-700	-	280-420	250-580	-
Holdfast organ (W) ^c	530-630	400-970	300	280-570	-	170-320	116-340	-
Anterior testis (L) ^b	245-295	300-500	-	-	700	170-300	-	193.8-240
Anterior testis (W) ^c	353-732	400-700	-	-	1,040	85-170	152-320	247-447
Posterior testis (L) ^b	265-310	400-800	-	-	680	320-460	-	212.8-308
Posterior testis (W) ^c	366-918	600-1,100	-	-	1,500	140-170	187-360	285-744
Eggs (L) ^b	130-140	103-124	107-133	112-129	124-148	125	89-134	106-143
Eggs (W) ^c	85-96	70-80	77-99	65-75	76-94	72	62-89	60-82
References	This study	[15]	[16]	[17]	[18]	[19]	[20]	[21]

^aUnit is micrometer (μm) except for the body length.

^bLength.

^cWidth.

body. We concluded that this fluke belonged to the genus *Alaria*.

Adults of the genus *Alaria* have a scoop shaped forebody and a cylindrical hindbody. Trematodes of the genus *Alaria* are distributed globally. However, despite several morphological studies of flukes in the genus *Alaria*, the validity of each species remains obscure and debatable. The genus *Alaria* includes *A. alata*, *A. arisaemoides* (Augustine and Uribe 1927), *A. canis* (La Rue and Fallis 1936), *A. intermedia* (Olivier and Odlaug 1938), *A. marciana* (La Rue 1917), *A. mustalae* (Bosma 1931), and *A. taxideae* (Swanson and Erickson 1946) [1,3]. *A. alata* is mainly found in Europe including Russia, while the rest of the *Alaria* species are found mainly in North and South America [1]. Three species, i.e., *A. alata*, *A. canis*, and *A. marciana*, have a pair of tentacular appendages on the both sides of oral suckers [15-17]. The worms detected in this study have tentacular appendages on both sides of the oral suckers (Table 1). However, the remaining 4 species, i.e., *A. arisaemoides*, *A. intermedia*, *A. mustalae*, and *A. taxideae*, have a pair of pseudosuckers instead of tentacular appendages on the both sides of oral suckers [18-21].

The measurements of *Alaria* species are summarized in Table 1. In the total body length of *Alaria* species, *A. alata* 2,800-4,000 μm [15], *A. canis* 2,500-4,200 μm [16], and *A. marciana* 1,560-1,840 μm [17]. In this study, body length (3,020-4,090

μm) is larger than that of *A. marciana*, but similar to *A. alata* and *A. canis*. And the vitellaria of *A. canis* is distributed beyond the forebody to the hindbody, while the vitellaria of *A. alata* is distributed only in the forebody [17]. In this study, vitelline follicles are distributed in the forebody, which are similar to those observed in *A. alata* [15].

The flukes of the genus *Alaria* detected in this study had almost no morphological differences from *A. alata* except for very small differences. The *Alaria* species identified in this study had smaller ovary and posterior testis but larger eggs than the species identified in the previous studies [15]. Differences in these measurements may be influenced not only by the size of the individual host, but also by the maturation status of the host and parasite. There can also be significant differences between individuals in each population.

The ribosomal 18S rRNA gene was used to study inter- and intra-specific relationships. We confirmed that the 18S rRNA sequences of *Alaria* specimens were separated from *A. canis* and *Alaria* sp., but were identical to that of *A. alata*. Our diplostomid flukes were identified as *A. alata* based on their morphologic and molecular characteristics.

The *Alaria* spp. from North America is zoonotic; 7 cases of human alariosis have been reported [1]. The pathogenicity of

A. alata has been poorly studied. However, there may have a zoonotic potential because it is closely related to North American *A. canis*. *A. canis* can cause severe disease in humans, including one fatal case of eating inadequately cooked frog legs [1]. Portier et al., [4] indicated that human cases of alariosis have not reported in Europe, but the zoonotic risk can be presumed. The potential danger of this parasite should be taken into consideration. The likelihood of an outbreak due to *A. alata* mesocercaria is also increasing in Korea.

In conclusion, based on the morphological and molecular finding, the diplostomatid flukes recovered from the small intestine of Korean raccoon dog were identified as *A. alata*. Since there is no information on the first and second intermediate hosts of *A. alata* in Korea, further studies on intermediate hosts should be continued.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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