NOTE
Wildlife Science

Molecular detection and phylogenetic analysis of tick-borne pathogens in wild Korean water deer and farmed elk in Gyeongbuk and Gangwon Provinces of Korea

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ABSTRACT. The purpose of this study was to assess tick-borne pathogenic infections in 42 wild Korean water deer (KWD) and 26 farmed elk in the Gyeongbuk and Gangwon Provinces of Korea. Among the 42 wild KWD tested, the eighteen (42.9%) and five (11.9%) samples tested positive for *Anaplasma phagocytophilum* and *A. bovis*, respectively, by PCR and DNA sequencing. All positive samples were only from wild KWD. All samples were negative for other tick-borne pathogens tested. Detected 16S rRNA sequences of *A. phagocytophilum* and *A. bovis* showed 98.6–99.8% and 94.4–100% identity to those of sequences in GenBank, respectively. Because few studies have examined tick-borne pathogens in wild animals, appropriate control programs and studies are needed to prevent pathogen transmission.

KEY WORDS: *Anaplasma bovis*, *Anaplasma phagocytophilum*, farmed elk, tick-borne pathogens, wild Korean water deer

Tick-borne pathogens occur worldwide, including in Korea [1, 9]. These tick-borne pathogens have a wide range of hosts such as cervids, canine, feline, bovine, equine and humans [8, 12, 16, 20, 31]. Many tick-borne pathogens are zoonotic and cause infections and even diseases to humans and animals [8]. Generally, piroplasms (genera *Babesia* and *Theileria*), rickettsiales (genera *Anaplasma*, *Ehrlichia* and *Rickettsia*), *Coxiella* and *Borrelia* are the most common tick-borne pathogens [25].

Piroplasms, including the genera *Babesia* and *Theileria*, have a wide variety of mammalian hosts, occur worldwide and are transmitted by ticks [4]. Current climate change and global warming has potential to create favorable conditions for tick survival and occurrence of tick-borne disease [10].

Genera *Anaplasma*, *Ehrlichia* and *Rickettsia* belong to the order Rickettsiales [11]. *Ehrlichia* is an obligatory intracellular gram-negative bacterium transmitted by tick bites, and *E. chaffeensis* and *E. ewingii* are zoonotic [11]. *Anaplasma* is also an obligatory intracellular gram-negative bacterium [25]. Five species of *Anaplasma* can infect ruminants, including *A. phagocytophilum*, *A. bovis*, *A. ovis*, *A. centrale* and *A. marginale* [11]. Among these five species, *A. phagocytophilum* is a zoonotic agent of human granulocytic anaplasmosis (HGA) [11]. *Anaplasma phagocytophilum* has a wide range of hosts including horse, dog, deer and ruminant [25]. In animals, its main clinical signs are fever and infection in cervids is generally asymptomatic [16].

Coxiella burnetii, an obligate intracellular bacterium, causes Q fever [22]. Domestic reservoirs include livestock and ticks, which are essential sources of *C. burnetii* transmission [33]. Infected ticks emit high concentrations of *Coxiella* in feces, which pollute the host's skin, and *Coxiella* may steadily exist in the environment [22]. Consequently, ticks may be important for the environmental dissemination of *Coxiella*.

Lyme disease is a prevalent vector-borne disease found in the temperate zone worldwide, and its prevalence and geographic distribution are rapidly expanding [23]. This disease is caused by spirochetes of the *Borrelia burgdorferi* sensu lato group and is transmitted during the bite of infected ticks into vertebrate hosts, which serve as its vectors [23].
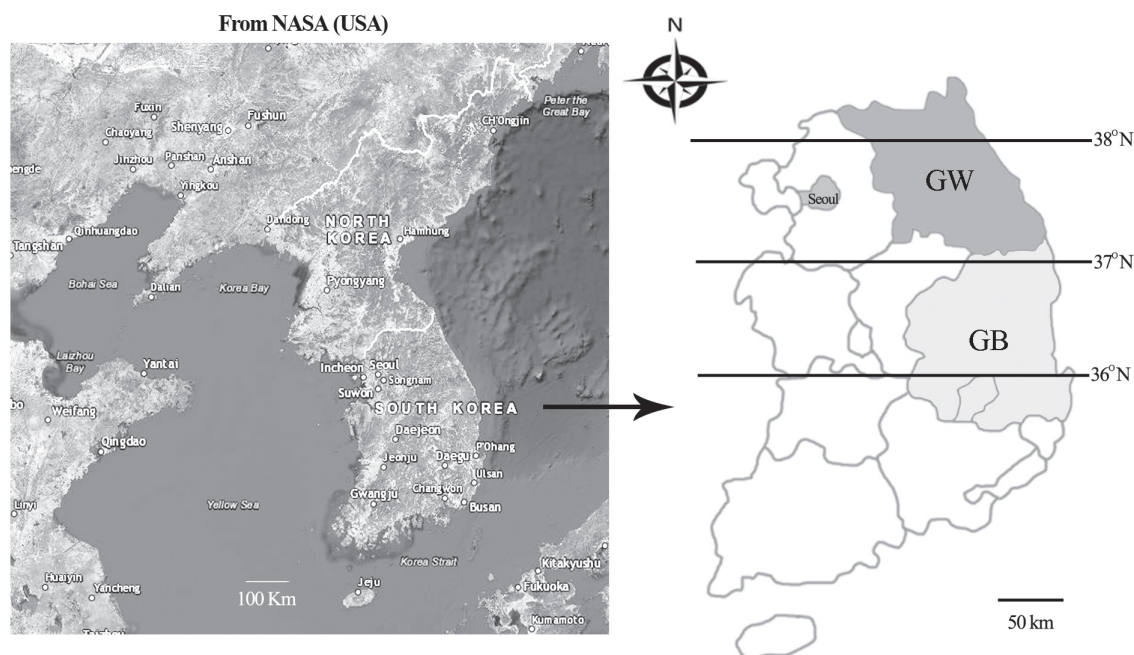
Fig. 1. Map of Korea showing Gyeongbuk (GB) and Gangwon (GW) Provinces where blood samples of the family Cervidae were collected. Two provinces are located between 35 and 39 degrees north latitude. Seoul=capital city of Korea.

Table 1. Prevalence of *Anaplasma phaogocytophilum* and *A. bovis* in wild Korean water deer in Gyeongbuk and Gangwon Provinces of Korea during 2013–2017

Host	Group		Tested	*Anaplasma phaogocytophilum*			*Anaplasma bovis*		
				Positive (%)	95% CI[a]	*P*-value[b]	Positive (%)	95% CI[a]	*P*-value[b]
Wild Korean water deer	Sex	Female	19	12 (63.2)	41.5–84.9	0.0157	5 (26.3)	6.5–46.1	0.0088
		Male	23	6 (26.1)	8.1–44.0		0		
	Province	Gyeongbuk	33	11 (33.3)	17.3–49.4	0.0169	5 (15.2)	2.9–27.4	0.2134
		Gangwon	9	7 (77.8)	50.6–100		0		
	Total		42	18 (42.9)	27.9–57.8	<0.0001	5 (11.9)	2.1–21.7	0.0676

a) CI: Confidence interval. b) Differences were considered significant when *P*<0.05.

In Korea, people consume the raw blood of the family Cervidae because they believe it is good for their health [12]. However, they are not aware of the dangers of exposure to or infection by protozoan and bacterial diseases, which are zoonotic by consuming the infected blood of the family Cervidae [12]. However, few studies have examined ticks and their transmitting diseases in wild animals in Korea, and even fewer studies have evaluated ticks and the diseases they carry in cervids. Therefore, the purpose of this study was to assess the prevalence of tick-borne pathogens from the family Cervidae, including wild Korean water deer (KWD, *Hydropotes inermis argyropus*) and farmed elk (*Cervus elaphus nelsoni*), in the Gyeongbuk and Gangwon Provinces of Korea (Fig. 1).

Among the total 68 blood samples collected from the family Cervidae, 26 were collected from farmed elk in Gyeongbuk and 42 were collected from road-killed wild KWD including 33 in Gyeongbuk and 9 in Gangwon between 2013 and 2017 (Table 1). Blood samples were collected from the jugular vein of live farmed elk after a verbal consent from the owners was obtained or heart puncture from the road-killed cervids.

This study, conducted between 2013 and 2017, did not receive approval from the Institutional Animal Care and Use Committee (IACUC) at Kyungpook National University (KNU), as the IACUC at KNU evaluates laboratory animals maintained in indoor facilities, and not outdoor animals. Under the regulatory "Act on the Prevention of Contagious Animal Disease (Amendment Act 2016)", national and local veterinary institutes in Korea conducted control measures in accordance with annual infectious animal disease control programs. Blood samples were collected from the family Cervidae by practicing veterinarians at local, government-run veterinary institutes during monitoring, surveillance and treatment or during regular check-ups after the receipt of verbal consent from farm owners. Blood collection at the government-run veterinary institutes was carried out according to the administrative rules of the Ministry of Agriculture, Food and Rural Affairs, Korea.

DNA was extracted from whole blood samples by using a commercial G-spin Genonmic DNA Extraction Kit for Blood (Intron

Biotechnology, Seongnam, Korea) according to the manufacturer's instructions. Several different tick-borne diseases were tested with multiple primer sets. A commercial AccuPower HotStart PCR PreMix Kit (Bioneer, Daejeon, Korea) was used for PCR amplification.

For differential diagnosis, several tick-borne pathogens were screened using specific primer sets. For example, infection by rickettsiales (genera *Anaplasma*, *Ehrlichia* and *Rickettsia*) was first screened by PCR using a commercial AccuPower Rickettsiales 3-Plex PCR Kit (Bioneer), which detects the 16S rRNA of rickettsiales. Next, positive samples were further amplified for species identification. For *Anaplasma* spp., two primer sets of EE1/EE2 and EE3/EE4 were reamplified to identify the 16S rRNA genes of *A. phagocytophilum*, *A. bovis* and *A. centrale* with the following program: pre-denaturation at 94°C for 5 min; 40 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 2 min and extension at 72°C for 90 sec; and a final post-extension step at 72°C for 7 min [3, 5, 18]. The 16S rRNA of *E. chaffeensis* was amplified by PCR using the primer set HE1/HE3 with the following program: pre-denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min; and a final post-extension step at 72°C for 10 min [14]. The gene encoding the 17-kDa antigen of *Rickettsia* spp. was amplified by PCR using the genus-specific primer set Rr17.61p/Rr17.492n with the following program: pre-denaturation at 94°C for 3 min; 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 45 sec and extension at 72°C for 1 min; and a final post-extension step at 72°C for 7 min [24]. The 5S (*rrf*)–23S (*rrl*) intergenic spacer gene of *Borrelia* was amplified by nested PCR (nPCR) using two primer sets, Bb23S3/Bb23Sa and Bb23SnF/Bb23SanR with the following program: pre-denaturation at 96°C for 2 min; 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 40 sec; and a final post-extension step at 72°C for 5 min, and the outer surface protein A gene fragment of *B. burgdorferi* was amplified by nPCR using two primer sets, N1/C1c and N2/C2c with the following program: pre-denaturation at 95°C for 3 min; 20 cycles of denaturation at 95°C for 30 sec, annealing at 40°C for 1 min and extension at 72°C for 1 min, which was then followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 50°C for 1 min and extension at 72°C for 1 min; and a final post-extension step at 72°C for 7 min [32]. Multiple primer sets were used to amplify the 16S rRNA of the genus *Coxiella*, including *C. burnetii* and *Coxiella*-like bacteria with the following program: pre-denaturation at 93°C for 3 min; 30 cycles of denaturation at 93°C for 30 sec, annealing at 56°C for 30 sec and extension at 72°C for 1 min; and a final post-extension step at 72°C for 5 min [26]. The 18S rRNA of piroplasms (genera *Babesia* and *Theileria*) was amplified by PCR using a piroplasm universal primer set, BT-F1/BT-R2 with the following program: pre-denaturation at 94°C for 10 min; 30 cycles of denaturation at 95°C for 30 sec, annealing at 65°C for 30 sec and extension at 72°C for 45 sec; and a final post-extension step at 72°C for 10 min [28].

Purified PCR products were sequenced by Macrogen (Seoul, Korea). The results were analyzed using the Clustal Omega (ver. 1.2.1) multiple sequence alignment program, and the alignment was edited in BioEdit (ver. 7.2.5). The sequence alignment was used to construct a similarity matrix, and phylogenetic analysis was performed using the maximum likelihood method in MEGA (ver. 6.0). The stability of the trees obtained was estimated by a bootstrap analysis with 1,000 replicates. The chi-square test was used to analyze significant differences among multiple groups (sex, province and breed), with P-value <0.05 regarded as statistically significant. GraphPad Prism ver. 5.04 (GraphPad Software, Inc., La Jolla, CA, U.S.A.) was used for statistical analyses, and 95% confidence intervals (CI) were calculated for all estimates.

In this study, PCR amplification of several tick-borne pathogens showed that 23 (54.8%) of the 42 wild KWD collected from the Gyeongbuk and Gangwon Provinces of Korea were positive only for rickettsiales. As shown in Table 1, species-specific PCR indicated that *A. phagocytophilum* (18/42, 42.9%, 95% CI: 27.9–57.8) and *A. bovis* (5/42, 11.9%, 95% CI: 2.1–21.7) were detected. No animals were co-infected with multiple *Anaplasma* species. However, *Ehrlichia*, *Rickettsia*, *Coxiella*, *Borrelia*, *Babesia* and *Theileria* pathogens were not detected. None of the farmed elk tested positive in this study.

Anaplasma phagocytophilum (42.9%, 18/42, P<0.0001) and *A. bovis* (11.9%, 5/42, P=0.0676) were found in wild KWD but were not found in farmed elk. Female KWD showed a significantly higher prevalence of *A. phagocytophilum* (63.2%, 12/19) and *A. bovis* (26.3%, 5/19) compared to male KWD (*A. phagocytophilum*: 26.1%, 6/23, P=0.0157; *A. bovis*: 0%, 0/23, P=0.0088). KWD in the Gangwon Province of Korea had a significantly higher prevalence of *A. phagocytophilum* (77.8%, 7/9) compared to those in the Gyeongbuk Province of Korea (33.3%, 11/33, P=0.0169), but the sample size in Gangwon Province was small. *Anaplasma bovis* was only detected in the Gyeongbuk Province of Korea (15.2%, 5/33, P=0.2134).

Nucleotide sequencing of the amplicons produced by species-specific nPCR for multilocus genes showed that 18 and five samples were positive for *A. phagocytophilum* and *A. bovis*, respectively. As *A. phagocytophilum* and *A. bovis* sequences from these positive samples were 100% identical to each other, samples were selected from differently reared regions as representative sequences for phylogenetic analysis.

These included *A. phagocytophilum* 16S rRNA sequences from three KWD (KWD-GB-31, KWD-GW-62 and KWD-GG-63; accession nos. MH338209, MH338210 and MH338211, respectively). Three KWD of *A. bovis* 16S rRNA (KWD-GB-51, KWD-GB-54 and KWD-GB-55; accession nos. MH338212, MH338213 and MH338214, respectively) sequences were also selected. Phylogenetic analysis showed that detected *A. phagocytophilum* and *A. bovis* 16S rRNA sequences clustered with previously deposited sequences (Fig. 2).

Three *A. phagocytophilum* 16S rRNA sequences (KWD-GB-31, KWD-GW-62 and KWD-GG-63) shared 98.6–99.8% identity to several *A. phagocytophilum* sequences from China and Korea. Examples included a dog from Korea (98.7%, KU513794), cow from Korea (98.9%, MG519284) [27], cow from China (99.8%, JN862825) and KWD from Korea (99.3%, GU556621; 98.6%, GU556624) [16].

For *A. bovis* 16S rRNA, three samples (KWD-GB-51, KWD-GB-54 and KWD-GB-55) belonging to clade B shared 94.4–100%
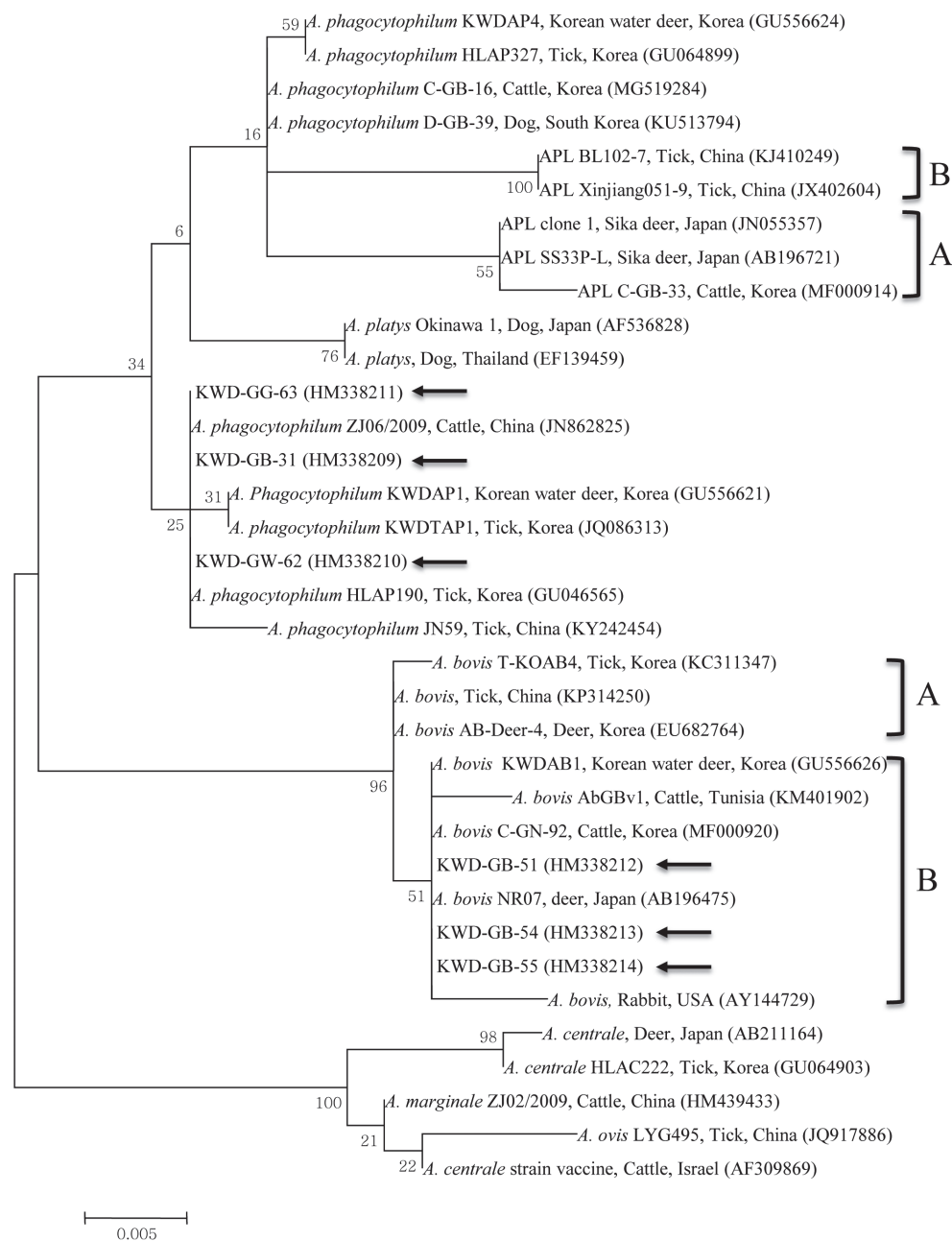
Fig. 2. Phylogenetic tree constructed using the maximum likelihood method based on 16S rRNA nucleotide sequences of *Anaplasma phago-cytophilum* and *A. bovis*. Arrows indicate sequences analyzed in this study. The names and GenBank accession numbers of other sequences are shown. Numbers on branches indicate bootstrap support (1,000 replicates). The scale bar represents the phylogenetic distance between sequences. Clades are denoted as A and B. APL=*Anaplasma* spp. closely related to *A. phagocytophilum*.

identity with clade B samples isolated in Japan and Korea, including deer from Japan (100%, AB196475), KWD from Korea (100%, GU556626) and cow from Tunisia (94.4%, KM401902). Three samples shared 99.3–99.7% identity with clade A samples isolated from Korea, including ticks from Korea (99.3%, KC311347), tick from China (99.7%, KP314250) and deer from Korea (99.4%, EU682764).

Many tick-borne diseases are zoonotic, which can infect humans and cause serious problems [8, 20, 25, 31]. These diseases are transmitted by ticks belonging to the genera *Haemaphysalis*, *Dermacentor*, *Rhipicephalus*, *Hyalomma* and *Boophilus* [8, 12]. Among these varieties of ticks, *Haemaphysalis longicornis* is the most widespread and common tick species found in Korea [12].

Korea has four distinctive seasons; however, because of global warming, the weather has recently changed to a more tropical or sub-tropical climate [29]. In nature, ticks are found from spring to fall, but are currently living longer than they did previously [29]. Tropical weather is a more suitable environment for ticks, which may explain the increase in the number of ticks and greater possibility of animals becoming infected by tick-borne diseases in Korea.

Seventy percent of the Korean peninsula is covered with mountains, providing a suitable habitat for both ticks and wild animals [29]. Especially in geographical locations, Gyeongbuk and Gangwon Provinces are adjacent to the South and North, and are covered with the Taebaek mountain ranges that is the longest mountain range of 600 km. Recently, as the population has increased, development and destruction on mountains has led to the destruction of wild animal habitats, causing these wild animals to approach human habitats including farms. This can expose people and other domestic animals to infections by different diseases and zoonosis.

Koreans are very interested in oriental medicine and a healthy diet [12]. Some Koreans consume large amounts of wild animal products for their health, including deer blood attained from the horns [12]. Wild animals such as KWD are often exposed to ticks in mountain areas, and thus there is a greater possibility that KWD will become infected by tick-borne diseases [13, 16, 31]. Therefore, consuming raw blood from the family Cervidae can be dangerous given the high probability of consuming infected blood, leading to zoonosis [12]. For examples, 10-year-old boy present toxoplasma lymphadenitis that caused by ingestion of raw blood and meat of farmed deer (*Cervus nippon*) in Korea [19], and some patients had extensive exposure to deer blood assumed to be infected with agent of HGA in U.S.A. [2].

In the present study, blood collected from farmed elk showed no positive results for several tick-borne pathogens by PCR. Although there were no positive results in farmed elk, people should be aware of potential infection of tick-borne pathogens in animals and humans.

In previous studies, several *Anaplasma* spp. were detected in cervids from Korea such as *A. phagocytophilum* (89/266 pools, 24.5%) and *A. bovis* (20/266 pools, 5.5%) in ticks collected from KWD [15]; *A. phagocytophilum* (42/66, 63.6%) and *A. bovis* (23/66, 34.8%) in KWD from Chungbuk, Gangwon, Gyeonggi, Jeonnam and Ulsan Provinces [16]; *A. phagocytophilum* (4/10, 40%) in KWD from Jeonbuk Province [30] and *A. bovis* in one Korean spotted deer (*Cervus nippon*) from Joenbuk Province [21]. This is the first molecular detection of *Anaplasma* spp. in KWD from Gyeongbuk Province.

In this study, KWD showed positivity for *A. phagocytophilum* and *A. bovis*. By sex, female cervids showed a higher prevalence of both *A. phagocytophilum* and *A. bovis*. A previous study showed a similar phenomenon, suggesting that immunosuppression of females may occur during lactation and pregnany and persist for up to two years [6].

Phylogenetic analysis of *A. phagocytophilum* using 16S rRNA showed similar homology as previously detected sequences from cattle, ticks, dogs and KWD in Korea. However, it is essential to differentiate between pathogenic *A. phagocytophilum* and closely related *A. phagocytophilum*-like (APL) species that do not cause clinical signs in infected animals, which are presently considered non-pathogenic [7]. For instance, the phylogenetic clades of APL in Japan (APL clade A) [34] and China (APL clade B) [17] differ from those of *A. phagocytophilum*. In Korea, *A. phagocytophilum* (2.1%, 16/764) and APL clade A (2.6%, 20/764) were also detected in cattle [27].

Phylogenetic analysis using 16S rRNA classified *A. bovis* into two clusters; *A. bovis* sequences in this study belonged to clade B, along with those from Korea, U.S.A., Tunisia and Japan, while *A. bovis* sequences belonging to clade A included those from East Asia (Korea and China). Clade B strains were prevalent compared to those from clade A.

In conclusion, our data showed that only wild KWD were positive for *A. phagocytophilum* and *A. bovis*, but not for other tick-borne protozoan and bacterial diseases, and samples from farmed elk were tick-borne disease-free based on our molecular analysis. People in Korea consume the raw blood of the family Cervidae, but there were no positive infection results from farmed elk. The data regarding tick-borne infection in cervids in Korea are limited. Ticks in Korea are likely to prevail as the climate changes towards a more tropical climate. As the number of ticks increases, infection rates also increased. We revealed that the family Cervidae may play an important role in the natural cycle of human and animal anaplasmosis. Moreover, control of potential tick vectors will be valuable to understand the life cycle of these bacteria and the transmission of diseases. Therefore, additional study will be required to determine the relationship among public health, wild and domestic animals and tick-borne disease.

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