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▣ BRIEF COMMUNICATION

Detection of Tick-Borne Pathogens in the Korean Water Deer (*Hydropotes inermis argyropus*) from Jeonbuk Province, Korea

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Abstract: The objective of this study was to investigate the prevalence of tick-borne pathogens in the Korean water deer (*Hydropotes inermis argyropus*). Pathogens were identified using PCR which included *Anaplasma*, *Ehrlichia*, *Rickettsia*, and *Theileria*. *Rickettsia* was not detected, whereas *Anaplasma*, *Ehrlichia*, and *Theileria* infections were detected in 4, 2, and 8 animals, respectively. The most prevalent pathogen was *Theileria*. Of the 8 *Theileria*-positive animals, 2 were mixed-infected with 3 pathogens (*Anaplasma*, *Ehrlichia*, and *Theileria*) and another 2 animals showed mixed-infection with 2 pathogens (*Anaplasma* and *Theileria*). Sequencing analysis was used to verify the PCR results. The pathogens found in this study were identified as *Anaplasma phagocytophilum*, *Ehrlichia canis*, and *Theileria* sp. To the best of our knowledge, this is the first report identifying these 3 pathogens in the Korean water deer. Our results suggest that the Korean water deer may serve as a major reservoir for these tick-borne pathogens, leading to spread of tick-borne diseases to domestic animals, livestock, and humans. Further studies are needed to investigate their roles in this respect.

Key words: *Anaplasma*, *Ehrlichia*, *Theileria*, tick-borne pathogen, Korean water deer, reservoir

Tick infestations in animals are of increasing concern, mainly because the epidemiology and geographical distribution of these infestations are constantly changing due to climate change, abundance of wildlife animals as hosts, and decrease in environmental biodiversity [1]. As a result, the incidence of tick-borne diseases (TBDs) has increased [2]. Anaplasmosis and theileriosis, considered to be the most important TBDs, have resulted in animal health problems and extensive economic losses causing management-related problems in the livestock industry [3,4]. However, there have been relatively few studies on tick-borne pathogens from wild ungulates in Korea [5-7], and therefore, little information is available on how infections caused by these pathogens might affect the population.

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Korean water deer (*Hydropotes inermis argyropus*) are one of the most widely distributed wild ungulates in Korea, and are known to be important natural reservoir hosts of TBDs [5,6]. The Korean water deer has been classified as a vulnerable species and included in the International Union for Conservation of Nature and Natural Resources Red List, due to a serious decline in their population as a result of poaching and habitat destruction [8]. Recently, however, wild Korean water deer populations have been growing in Korea [9], and because of their close proximity to domestic animals, they may act as a disease transmission of tick-borne pathogens to these animals. Therefore, the objective of this study was to investigate the pathogens of TBDs in Korean water deer and to evaluate their roles as potential reservoirs for TBDs circulating in Korea.

The capture of wild Korean water deer and sample collection were performed with permission from the Wildlife Rescue Center located in the Jeonbuk Province, Korea. Between March and June 2014, 5 animals were captured and whole blood samples were collected. Five more animals were killed in the traffic accidents, found dead and transferred to the Wildlife

Rescue Center by local residents, and their spleens were collected. No ticks were found in the first 5 animals. The blood samples and spleens were immediately frozen at -80˚C until DNA extraction was performed.

Genomic DNA was extracted from whole blood and spleen samples using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, California, USA) according to the manufacturer's instructions. Diagnosis of *Theileria* infection was performed using an AccuPower® *Theileria* PCR kit (Bioneer, Daejeon, Korea) using 2 specific primer sets targeting the 18S ribosomal RNA (F, 5′-GTTATAAATCGCAAGGAAGTTTAAGGC-3′; R, 5′-GTGTACAAAGGGCAGGGACGTA-3′). The predicted size of the amplified PCR product was 239 bp under the following cycling conditions: 94˚C for 5 min, followed by 40 cycles of 94˚C for 20 sec and 65˚C for 35 sec, and a final extension of 72˚C for 5 min. AccuPower® *Rickettsia*les 3-Plex PCR kit (Bioneer) was used to detect *Anaplasma* (F, 5′- TACCTCTGTGTTGTAGCTAACGC-3′; R, 5′-CTTGCGACATTGCAACCTATTGT-3′), *Ehrlichia* (F, 5′-CGGAATTCCTAGTGTAGAGG-3′; R, 5′-AGGAGGGATACGACCTTC AT-3′), and *Rickettsia* (F, 5′-TAGGGGATGATGGAATTCCTA-3′; R, 5′-CCCCCGTCA ATTCCTTTGAG-3′). These PCRs were performed using specific primer sets that targeted the 16S ribosomal RNA. The predicted sizes of the amplified PCR products for *Anaplasma*, *Ehrlichia*, and *Rickettsia* were 429 bp, 340 bp, and 252 bp, respectively, under the following cycling conditions: 95˚C for 15 min, followed by 40 cycles of 95˚C for 10 sec, 58˚C for 30 sec, and 72˚C for 30 sec, and then a final extension of 72˚C for 5 min. PCR products were separated by gel electrophoresis on 1.5% agarose gels and visualized by staining with ethidium bromide.

The PCR products were purified with a QIAquick PCR purification kit (Qiagen). The nucleotide sequences were determined by direct sequencing of the PCR products using a BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, California, USA) and analyzed on an ABI PRISM® DNA analyzer (Applied Biosystems). The sequence data were aligned using the Clustal X (version 1.8) [10]. Additional sequences from representative isolates of anaplasmosis, ehrlichiosis, and theileriosis were obtained from the GenBank database and included with each set of alignments. A phylogenetic tree based on nucleotide alignments was constructed using the neighbor-joining method [11]. Bootstrap analysis was carried out using 1,000 replications, and the tree was visualized using Treeview [12].

The prevalence of TBD pathogens was analyzed from blood and spleen samples of 10 Korean water deer by PCR (Table 1). Of the 10 animals, 4 were positive for *Anaplasma* infection, and 2 were positive for *Ehrlichia* infection, and 8 were positive for *Theileria* infection. Also, of these animals, 2 were found to have mixed-infections with 2 pathogens (*Anaplasma* and *Theileria*, n=2) and another 2 animals were found to have mixed-infections with 3 pathogens (*Anaplasma*, *Ehrlichia*, and *Theileria*, n=2) (Table 1). *Rickettsia* spp. were not detected in any of the samples. According to our results, *Theileria* infection is the most prevalent in wild Korean water deer. These results demonstrate the possibility that wild Korean water deer may serve as a major reservoir for these TBD pathogens.

Table 1. Summary of tick-borne pathogens detected from Korean water deer by PCR in this study

Sample no.	Specimen	*Anaplasma*	*Ehrlichia*	*Rickettisa*	*Theileria*
1	Blood	-	-	-	+
2	Blood	-	-	-	-
3	Spleen	-	-	-	-
4	Spleen	-	-	-	+
5	Spleen	+	-	-	+
6	Blood	+	-	-	+
7	Blood	+	+	-	+
8	Spleen	+	+	-	+
9	Spleen	-	-	-	+
10	Blood	-	-	-	+

"+", positive; "-", negative.

PCR analysis revealed that 4 animals were positive for infection with *Anaplasma* (Table 1). The nucleotide sequences of the 4 amplified samples showed 100% identity, and we submitted 1 sequence to GenBank (accession no. KR045609). *Anaplasma* infections in the Korean water deer were determined to be of the species, *A. phagocytophilum*. Phylogenetic analysis revealed that the Korean isolate (KR045609) detected in this study was in the same clade as US human isolates, a Swedish horse isolate, a wild game isolate, a brown bear isolate, and tick isolates from several different countries, which showed 98.7-99.5% identity to each other. However, isolates from small ruminants (KJ183079, KF569916, and JN558816) and deer (AB196720 and AB196721) were identified as belonging to a different clade (Fig. 1). Interestingly, *A. phagocytophilum* identified in Chinese cattle (KF569910) was distinct from all other isolates.

Ehrlichia canis infections were found in 2 Korean water deer. Two amplicons were sequenced on both strands, and then aligned and analyzed for sequence identity. The 2 isolates were 100% identical, and we submitted 1 sequence to the GenBank (accession no. KR045610). The Korean isolate (KR045610)

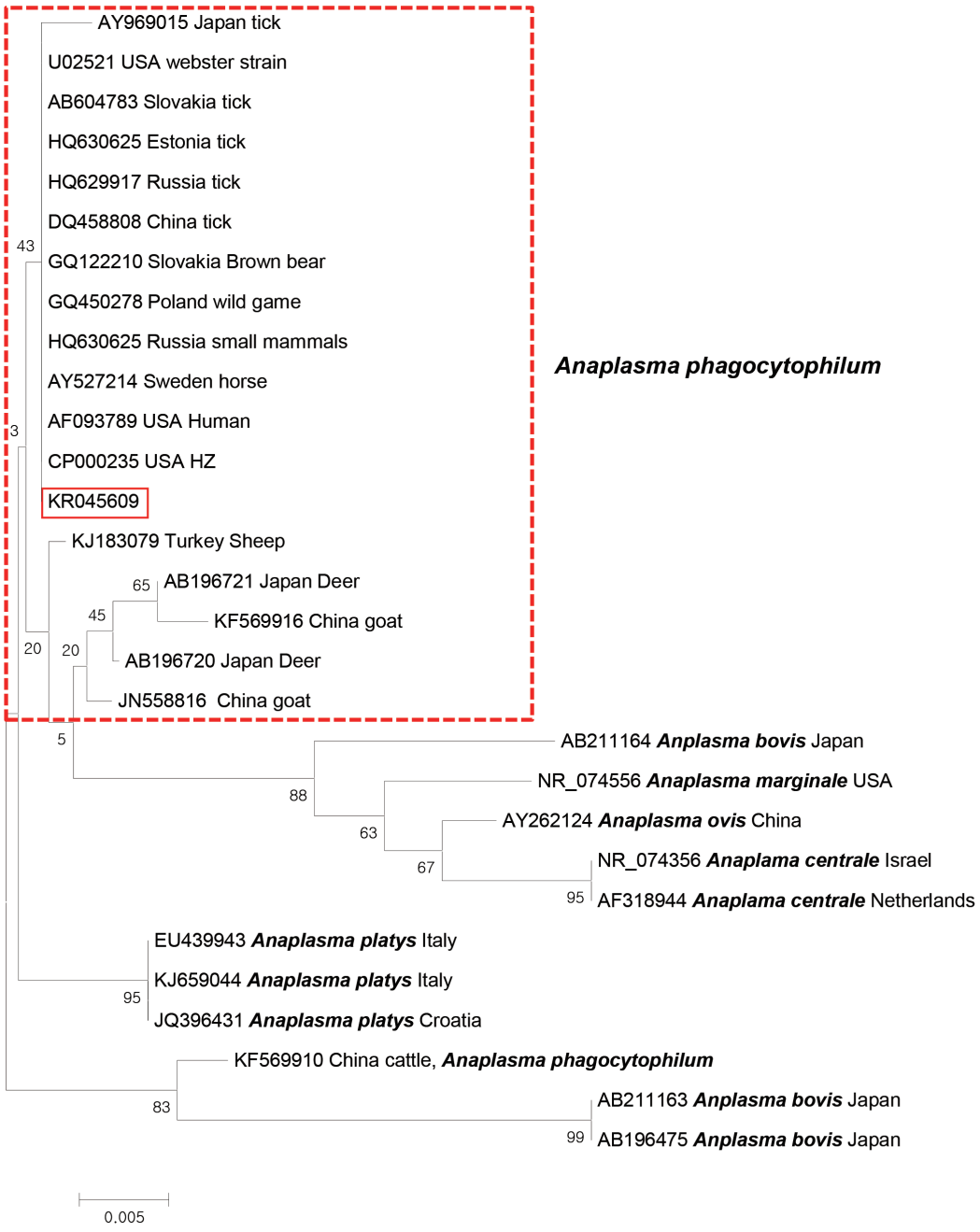

Fig. 1. Phylogenetic analysis of 16S rRNA genes of *Anaplasma phagocytophilum* identified in Korean water deer. The red-faced box indicates the sequence determined in this study. Bootstrapping was carried out using 1,000 replications.

showed 98.6-98.9% homology in nucleotide sequences with *E. canis* isolates from humans, dogs, and ticks, and 97.5-98.2% homology with *E. ruminantium* by sequencing comparison. Phylogenetic analysis of the 16S rRNA gene revealed that the KR045610 isolate identified in this study belongs to the same clade as the previously reported *E. canis* strains, and is closely related to isolates from ticks in Japan (Fig. 2). KR045610 belongs to a different clade with *E. ruminantium*, *E. muris*, and *E. chaffensis*, which is completely separate from *Ehrlichia* sp.

Of the 8 *Theileria*-positive animals, we randomly selected 3 PCR-positive samples for partial sequencing of the 18S rRNA genes, and found that these newly identified sequences shared 99.8% homology. Based on sequencing analysis of the 18S rRNA, there were no obvious differences in the nucleotide se-

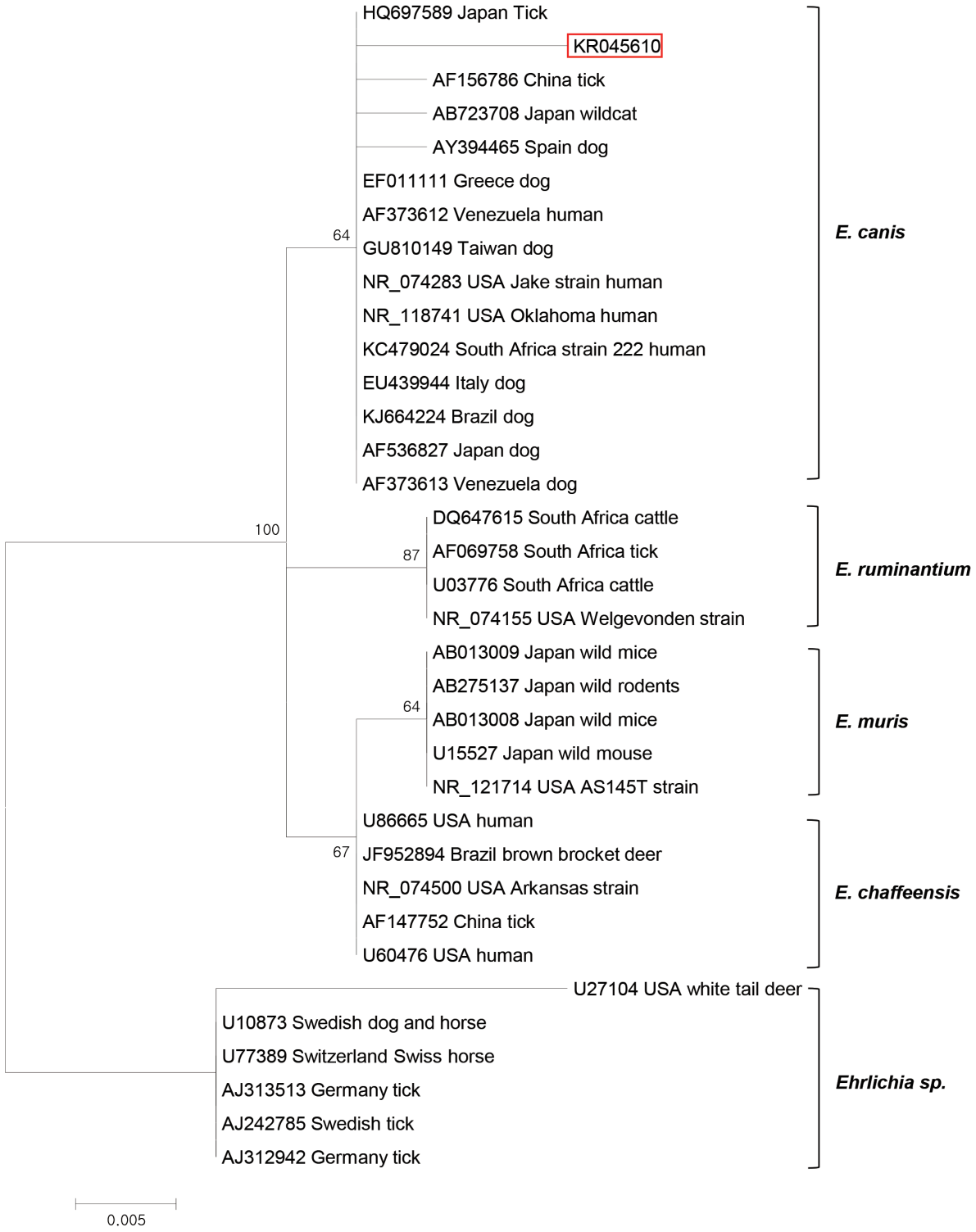

Fig. 2. Phylogenetic analysis of 16S rRNA genes of *Ehrlichia canis* identified in Korean water deer. The red-faced box indicates the sequence determined in this study. Bootstrapping was carried out using 1,000 replications.

quences between *Theileria* sp. and *T. luwenshuni*. The phylogenetic tree showed that the 3 isolates (KT261643-KT2616445) obtained in this study were classified as *Theileria* sp., and were identical to the *Theileria* sp. from small ruminants and wild

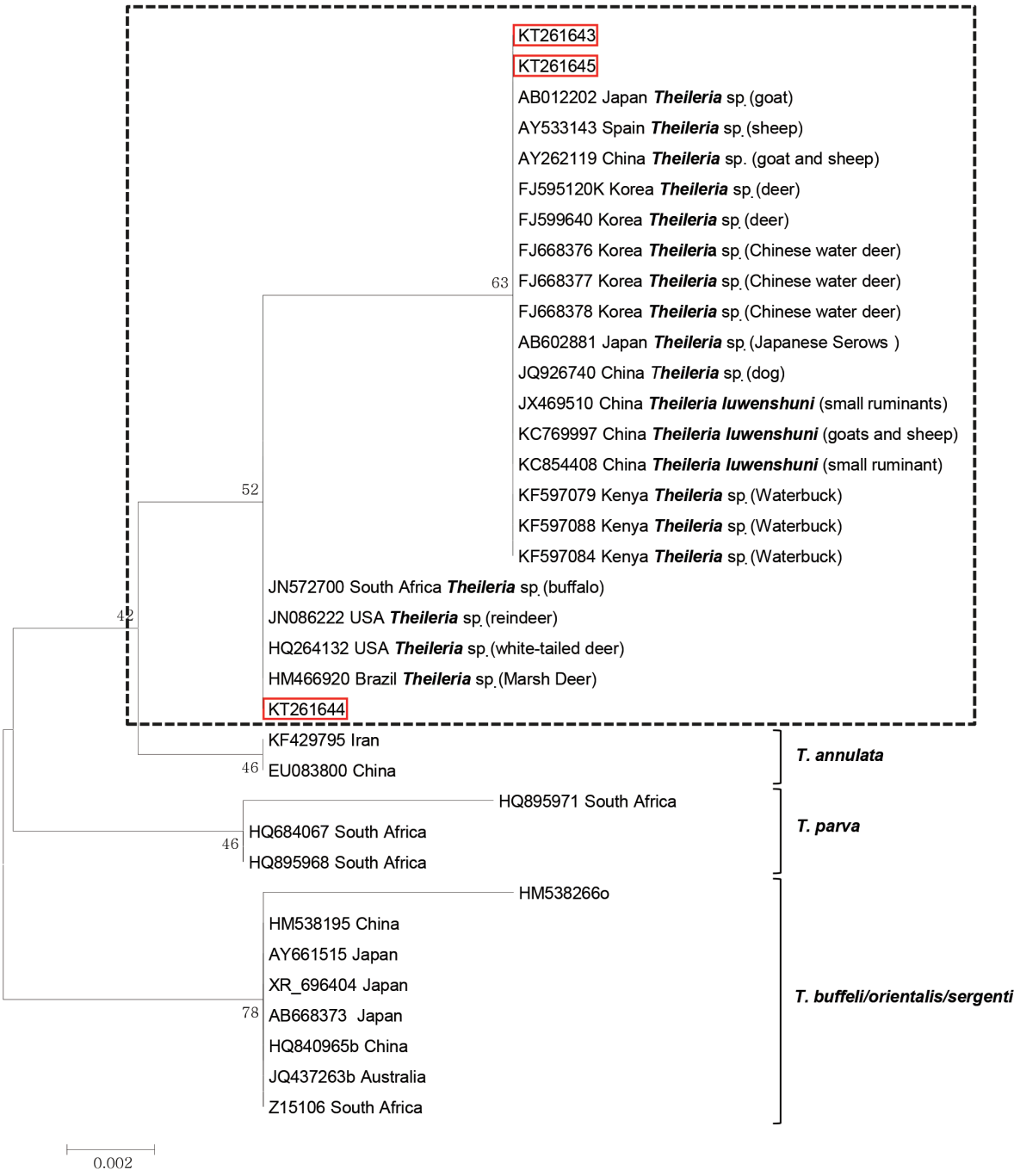

Fig. 3. Phylogenetic analysis based on the alignment of 18S rRNA gene sequences of *Theileria* sp. detected in Korean water deer, together with previously registered sequences from *Theileria* sp. The unrooted phylogenetic tree was constructed using neighbor-joining method. Bootstrapping was carried out using 1,000 replications. The 18S rRNA gene sequences identified in this study are shown in red-faced type.

ungulates in several countries, including China, Japan, Spain, and Kenya. Two (KT261643 and KT261645) of 3 isolates were in the same clade as *T. luwenshuni* (Fig. 3), and were similar to *Theileria* sp. of the wild Chinese water deer that had been isolated in Korea [13]. One isolate (KT261644) was divergent from the other 2 isolates and belonged to the same group as *Theileria* sp. isolated from wild ungulates of USA, Brazil, and South Africa. However, these isolates were obviously different

from other species of *Theileria* such as *T. buffeli/orientalis/sergenti* and *T. annulata/parva*.

Korean water deer populations have been growing to the extent that they are now commonly found even in urban areas. This has led to disturbances, of varying degrees, to the human and livestock populations in Korea. Several studies have demonstrated that Korean water deer may be infected with *Anaplasma* spp. and *Bartonella* spp. [5,6]. So far, very little is known about the incidences of TBDs in Korean water deer in Korea. The present study reports the prevalence of TBDs in the Korean water deer. Using PCR, we demonstrated that mixed-infections of 2 species of pathogens (*A. phagocytophilum* and *Theileria* sp.), and 3 species of pathogens (*A. phagocytophilum*, *E. canis*, and *Theileria* sp.) were found in Korean water deer, and that *Theileria* sp. is the most prevalent pathogen. This is the first study, to our knowledge, reporting *A. phagocytophilum*, *E. canis*, and *Theileria* sp. infections in the Korean water deer. This indicates that Korean water deer may be a reservoir for transmission of TBDs to domestic animals and livestock.

The results of the present study demonstrate that *A. phagocytophilum* infections are the second most prevalent in the Korean water deer. Phylogenetic analysis indicated that the KR045609 isolate identified in this study was found to be more closely related to isolates from humans and ticks, rather than isolates from Asian small ruminants. In addition, 16S rRNA gene sequencing showed that human and tick isolates including KR045609 were almost identical. *A. phagocytophilum* has been reported to be present in various animals, as well as in ticks [14-16]. *A. phagocytophilum* infection found in the Korean water deer in this study may have been a result of tick bites or contact with other animals. These results suggest that Korean water deer play a role in the natural maintenance cycle of *A. phagocytophilum* and increase the chances of this infection being transmitted to humans. Consequently, Korean water deer may act as an important host for the transmission of *A. phagocytophilum* infection, and therefore are a potential threat to animals, livestock, and humans.

E. canis causes canine monocytic ehrlichiosis, which is transmitted by *Rhipicephalus sanguineus* commonly known as the brown dog tick [17]. *R. sanguineus* ticks, which can be found on wild mammals, represents a major threats to dogs, felids, and humans [18,19]. According to several studies, *E. canis* infection has been detected mostly in blood samples from carnivores [19-22]. However, in this study, *E. canis* was detected in the Korean water deer, a wild ungulate, for the first time. Phylogenetic analysis revealed KR045610 was closely related to a tick isolate from Japan. This indicates that there may be a tick that transmits *E. canis* in Korea, and consequently, the *E. canis* infections in the Korean water deer may be a result of tick bites. Further investigation is needed to identify the species of tick that is responsible for transmitting *E. canis* to Korean water deer, and more epidemiological surveys of wild animals will be needed to understand the ecology of this zoonotic pathogen.

In this study, we identified that *Theileria* infections are the most prevalent in Korean water deer by PCR analysis of the 18S rRNA gene. The phylogenetic tree showed that all of the newly isolated, unidentified *Theileria* were classified as *Theileria* sp. These isolates belonged to 2 different clades. KT261643 and KT261645 were identical to those from small ruminants and wild ungulates, including the waterbuck, Chinese water deer, and deer; KT261644 was closely related to isolates from the deer and buffalo. These results suggest that there are at least 2 species of *Theileria* sp. circulating among Korean water deer in Korea. The *Theileria* sp. found in China (AY262119) is considered to be highly pathogenic in sheep [23] and is identical to 2 of our isolates. This finding raises the possibility that pathogenic *Theileria* sp. may be present in Korean water deer, posing a threat to small ruminants.

These results expand our knowledge of the prevalence of tick-borne pathogens. The present work emphasizes the role of Korean water deer in the dissemination of livestock pathogens. Since infected Korean water deer share the same home ranges as cattle and other domestic animals, the causative pathogens of TBDs may be transmitted to domestic animals, livestock, and humans through infected tick bites. Korean water deer are the most commonly rescued wild animal in Korea, and recently their population has been growing, resulting in increased contact with both humans and domestic animals. Human TBDs are often transmitted by domestic animals, such as cattle and goats, rather than wild animals. Our results indicate the significance of Korean water deer as a reservoir host for pathogens that can cause severe TBDs in domestic animals, livestock, and humans. The role of Korean water deer as a reservoir host for TBDs and their effect on public health should be investigated further in Korea.

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CONFLICT OF INTEREST

We declare that we have no conflicts of interest.

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