INTRODUCTION

The genus Sarcocystis is recognized in the phylum Apicomplexa that can cause mortality of many animals and impact animal community diversity. These parasites typically infect both intermediate and definitive host animals. Intermediate host animals include herbivores and omnivorous animals that can be infected when ingesting oocysts in the feces of the definitive host animals. In turn, carnivorous animals of definitive hosts are infected when they consume the intermediate host prey infected by Sarcocystis [1-3].

The cysts of Sarcocystis species were prevalent in the cervid and in the bovid. These mammals play as the intermediate hosts in life cycle of the parasites. Recently, several species of Sarcocystis has been found in diverse cervid species [3-10]. Thus, it has been suggested that the host rage of Sarcocystis parasites could be broader than the known animal species [11].

The infection surveys of the Sarcocystis parasites among a domestic cattle population in the Republic of Korea (ROK) reveal the considerably higher infection prevalence of the parasites, especially in the old aged cattle population and also the studies found that dog is the main definitive host animal of the parasite [12-16]. In addition, Sarcocystis grueneri were found in a red deer and Sarcocystis tenella in Korean native goats [17,18]. The water deer (Hydropotes inermis) is a small deer seemingly more similar to a musk deer than a true deer and is the only species of genus Hydropotes which belongs to family Cervidae. There are 2 subspecies: The Chinese Water Deer (H. inermis inermis) and the Korean Water Deer (H. inermis argyropus) [19,20]. In the ROK, the Korean water deer is one of the widely distributed animals and overpopulated in some regions of the ROK [21]. There is no report on Sarcocystis infection in wild Korean water deer in the ROK. Our study first detected and characterized the infection of S. grueneri in wild Korean water deer in the ROK.

MATERIALS AND METHODS

Sample collection and histological study

The cardiac muscle samples were collected from 38 wild Korean water deer (male 23, female 15) at the wild animal rescue centers in Kangwon-do, Gyeonggi-do, Chongcheongbuk-do and Daejeon city (Table 1) in 2004-2012. The divisions of heart
were fixed in 10% neutral buffered formalin, embedded in paraaffin, sectioned at 4 μm, and stained with hematoxylin and eosin for screening by light microscopy (LM).

**DNA extraction and PCR**

Fresh positive myocardium tissues were examined in order that genomic DNA was extracted with frozen after collection and stored at -20°C before examination immediately upon thawing. With tissue cutting of 0.05 g to 0.25 g, both 10 positive samples and 1 negative sample (confirmed by LM) were placed in 1.5 ml microcentrifuge tubes containing with PBS (pH 7.4).

Genomic DNA extraction was carried out using DNeasy Blood

**Table 1.** Prevalence of Sarcocystis from Korean water deer in the Republic of Korea

| Locality and no. of samples | No. positive (%) |
|-----------------------------|-----------------|
| Kangwon-do (n=21)          | 6 (28.6)        |
| Daejeon-si (city) (n=14)   | 4 (28.6)        |
| Chongcheongbuk-do (n=2)    | 0 (0.0)         |
| Gyeonggi-do (n=1)          | 0 (0.0)         |
| Total (n=38)               | 10 (26.3)       |

**Table 2.** Sequence analyzed of the Sarcocystis species used in 18S rRNA genes and GenBank accession no.

| Taxon name/Strain | GenBank accession no. | Intermediate host |
|-------------------|-----------------------|-------------------|
| S. aliciae, SaAa1N| EU282018              | Moose (Alces aliciae) |
| S. aliciae tras, SaAa1C | EU282033               | Moose (Alces aliciae) |
| S. arietanis    | L24382                | Sheep (Ovis aries) |
| S. buffaloni    | AF017121              | Water buffalo (Bubalus bubalis) |
| S. capracanis   | L76472                | Goat (Capra aegagrus hircus) |
| S. comixi, V1   | EU553478              | Hooded crow (Corvus comix) |
| S. cruzi, NSMT: Pr319 | AB662782             | Water buffalo (Bubalus bubalis), Cattle (Bos taurus) |
| S. dispersa     | AF120115              | Mouse (Mus musculus) |
| S. fusiformis, 3f5 | AF176927              | Water buffalo (Bubalus bubalis) |
| S. fusiformis   | U03071                | Water buffalo (Bubalus bubalis) |
| S. gallotae     | AY151112              | Lizard (Gallotia gallot) |
| S. graueri, Sg1RfN | EF0566010             | Reindeer (Rangifer tarandus) |
| S. hardangeri, ShRt1I | EF467654              | Moose (Alces aliciae) |
| S. hirsuta      | AF017122              | Cattle (Bos taurus) |
| S. hjorti, Sd1CeN | GQ250990               | Red deer (Cervus elaphus) |
| S. laceriæ      | AY151113              | Common wall Lizard (Podarcis muralis) |
| S. mucosa       | AF100679              | Rat (Rattus norvegicus) |
| S. muris        | M64244                | Mouse (Mus musculus) |
| S. neuromacites | U07812                | Brown-headed cowbirds (Molothrus ater) |
| S. ovalis, SoAa1N| EU282019              | Moose (Alces aliciae) |
| S. rangi, Sr1Tt1 | EF467655              | Reindeer (Rangifer tarandus) |
| S. rangiferi, Sr1CeN clone 2 | GQ251022          | Red deer (Cervus elaphus) |
| S. nilori, Europe | HM185742          | Mallard duck (Anas platyrhynchos) |
| S. rodentiferis | AY015111              | Reindeer (Rangifer tarandus) |
| S. silva, NoCc11.3 | JN226125            | Roe deer (Capreolus capreolus) |
| S. sinensis, krom | AF266959          | Water buffalo (Bubalus bubalis), Cattle (Bos taurus) |
| S. tarandii, St1RfN | EF056017             | Red deer (Cervus elaphus) |
| S. tarandii, St4RfN clone 3 | GQ250974         | Red deer (Cervus elaphus) |
| S. tarandii, St1GeN clone 3 | GQ251013         | Red deer (Cervus elaphus) |
| S. tarandii, St4RfN clone 1 | GQ250972         | Red deer (Cervus elaphus) |
| S. tarandii, St3RfN clone 4 | GQ250970         | Red deer (Cervus elaphus) |
| Sarcozystis sp. TO-2012 | AB6691780       | Ryukyu Rat (Diplothrix legata) |
| Sarcozystis sp. cyst type I ex) Anser albintrons | EU502869 | Mallard duck (Anas platyrhynchos) |
| Sarcozystis sp. cyst type III ex) Anser albintrons | EU502868 | Hooded crow (Corvus comix) |
| Sarcozystis sp. ex) Larus marinus | JQ733508   | Black-backed gull (Larus marinus) |
| Sarcozystis sp. HM050622 | AB257156 | Sika deer (Cervus nippon yesoensis) |
| Sarcozystis sp. SspEAa1N | EU282017 | Moose (Alces aliciae) |
| Neospora caninum | U17345 | Cattle (Bos taurus) |
| Toxoplasma gondii | U00458 | Vertebrates |
& Tissue Kits (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. DNA was eluted in 30 μl of buffer (10 mM Tris-Cl, pH 8.5) yielding 33.2 ng of the average concentration were measured by Nano spectrophotometer (Nano Photometer, Implen, Munich, Germany). Loads of overlapping region between members of the Sarcocystidae covering 18S rRNA gene were amplified by the polymerase chain reaction (PCR) using primer 18S 2L (F)-GGATAACCGTGGTAATTCTATG and 18S 1H (R)-TATCCCCATCACCATGCATACT [22]. One reaction mixture contained the genomic DNA of 33.2 ng (1 μl/reaction), HiPi PCR PreMix (Elpis Biotech. Inc., Daejeon, Korea)-Taq polymerase in 250 mM Tris-HCl (pH 9.0) of 1 unit, 80 mM (NH₄)₂SO₄, 10% DMSO, 8.75 mM MgCl₂, 0.05% bromophenol blue, 12% glycerol, and the oligonucleotide primers (10 pmol each/reaction of 1 μl), RNase-free water to add to final composition. Reactions were started at 95˚C for 3 min, followed by 35 cycles of 94˚C for 40 sec, 55˚C for 45 sec or 1 min, and 72˚C for 1 min 30 sec, with final incubation at 72˚C for 5 min. The PCR products have been analyzed by electrophoresis of 1.2% agarose gel. The 1,500 bp amplicon of the both species examined proved to be difficult to sequence directly from PCR products; the region was cloned for specific sequences.

**Sequencing and phylogenetic analysis**

The purified PCR amplicons were cloned into the pGEM®-T Easy Vector (Promega, Madison, Wisconsin, USA). Plasmid DNA were purified and sequenced using an automatic sequencer (ABI 3730xl DNA Analyzer, Applied Biosystems, Foster City, California, USA). The achieved sequences were compared with phylogenetic analyses on the SSU rRNA gene from *Sarcocystis* species sequenced as well as with other registered sequences of *Sarcocystis* spp. from intermediate hosts, retrieved from GenBank using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). A score of sequences were aligned by the Align IR (Ver 2.0; http://biosupport.licor.com/support), Clustal X (Ver 2.0; www.clustal.org), and MEGA 4 [23], respectively. Using the
neighbor-joining (NJ) (Kimura 2-parameter distance model) methods was based on a guide tree as pairwise and multiple alignment parameters. The final alignment was comprised of a score of sequences with 39 taxa (Table 2).

RESULTS

Sarcocysts were not found in the liver or lungs (n = 38), but detected from 10 heart muscle samples. All cysts were located

Fig. 3. Phylogram of 18S rRNA sequences from Sarcocystis spp. with evolutionary relationships of 39 taxa. Bootstrap values (>50%) based on 1,000 replications are shown. The S. grueneri detected in this study is red-boxed.
in sarcoplasm of myocardium surrounded by thin and smooth cyst wall. All of the sarcocysts were filled with bradyzoites (Fig. 1). The cysts appeared oval to spherical shape, 110-380 µm long and 90-170 µm wide under LM. The prevalence of Sarcocystis was 26.3% (Table 1). Although it appears that these parasites were the species of S. grueneri, the further molecular identification of these parasites is required.

The cyst samples were amplified using the primers of 18S rRNA and revealed 1.5 kb fragment (Fig. 2) which was a close analogue of S. grueneri found in reindeer (Rangifer tarandus tarandus) [5]. One sequence as a representative of was deposited in GenBank (accession number KC556825). The sample sequences were compared with complete 18S rRNA sequences from other 39 taxa of genus Sarcocystis (Table 2). The 18S rRNA sequence is proposed as a standard gene for the phylogenetic analysis among the species within the protozoa phyla, which is hence used for species identification in this study [24]. The sequence identified between the cyst samples showed 100% homology with S. grueneri and 97% with S. capracanis. In addition, both 53% AT base construction and low level of sequence variations were consistently shown in the phylogenetic analysis (Fig. 3).

DISCUSSION

In previous study, the cysts of S. grueneri were found in the heart muscles, which indicate the specific organ tropism of the parasite [10]. Similar to the previous study, a cervid animal may be infected in heart muscle by similar Sarcocystis species. The Korean water deer may serve as an intermediate host with a similar level of high specificity and prevalence as observed in other studies [9]. For instance, 98.0% of the sister species of a cervid animal in Germany were infected with Sarcocystis parasites [25,26]. Thus, it appears that the infection of Sarcocystis species is quite common to many species of a cervid animal. Although the Korean water deer is widely distributed in ROK, it has not been extensively studies until recent years especially in infection study of the parasite of Sarcocystis species. In this study, we found that the infection rate of the Sarcocystis parasites was 26.3% (10 out of 38 samples of the Korean water deer). Among the 38 collection sites in the ROK, 6 infection muscles (15.8%) were detected in the provinces of Kangwon, Gyeonggi, and Chonbuk, and 4 infection samples in Daejeon city (10.5%), respectively. Furthermore, identification of the parasite that is collected from the Korean water deer was examined by molecular method. In previous study, molecular technique could identify a single species of the parasite from the host animal tissues and distinguish individual species from other parasites of Sarcocystis species [5]. For more accurate morphometry for the species identification of the parasites, the electron microscope has been used and successfully reported key morphological features in several parasites. For instance, the cyst of S. grueneri collected from reindeer are morphologically similar to the cysts collected from either the host animal roe deer (Capreolus capreolus) or red deer (Cervus elaphus) in the protrusion of the cyst wall [8,27].

The general phylogenetic trend of Sarcocystininae has already explained other former studies [22,24,28,29]. In our study, the fundamental composition did not change by including Sarcocystis species from a similar intermediate host. Phylogenetic topology of bootstraps of 1,000 described the 2 species highly similar and their branch length very close from the sequences of 18S rRNA gene in S. grueneri (Genbank accession number EF056010). Thus, the results of phylogenetic analysis indicate that our sample is in the clade of a sister taxa including S. capracanis, S. alces, S. rangi, and S. alceslatrans. Furthermore, these species also share the similar intermediate host animal in the family Cervidae except for S. capracanis (Fig. 3).

In addition, it has been known that the parasites of Sarcocystis species infect both animal hosts such as intermediate host and definitive host, especially in the stage of sporocyst and oocyst. Interestingly, in previous study, it has been shown that humans are susceptible to the infection of sarcocysts [30]. This infection could be caused by water contaminated with feces from the other animal hosts or food washed with unsanitary water [31]. Other unknown animals including human could be a potential target for the infection of Sarcocystis parasites. In this context, the DNA sequences of 18S rRNA gene related to Sarcocystis parasites presented in this study can promote further development of needed diagnostics to prevent a potential infection of Sarcocystis parasite in wild animals and humans.

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CONFLICT OF INTEREST

We have no conflict of interest related to this work.

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