A DESSERTATION
FOR THE DEGREE OF MASTER

Molecular detection and identification of *Sarcocystis grueneri* from Korean water deer (*Hydropotes inermis argyropus*)

한국 고라니에서 발견된 *Sarcocystis grueneri*의 검출 및 분자생물학적 동정

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Molecular detection and identification of *Sarcocystis grueneri* from Korean water deer (*Hydropotes inermis argyropus*)

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Abstract

Sarcocystis is a protozoan parasite (Apicomplexa: Coccidia), with a heterogenous 2-host life cycle. Infection of *Sarcocystis* spp. gives an indication as to whether a subclinical lesion or damage to the vascular endothelium associated with immunosuppression.

Cervidae is a known that intermediate host and several species have been isolated from Sarcocystidae. Above *S. grueneri*, *S. rangi, S. tarandivulpes, S. hardangeri, S. rangiferi, and S. tarandi, Sarcocystis*

grueneri–like species have been previously found in a rearing red deer (*Cervus elaphus*) carcasses in Korea.

The purpose of this study was to molecularly characterize *Sarcocystis grueneri* cysts isolated from cardiac muscle of 74 Korean water deer (KWD, *Hydropotes inermis argyropus*).

The samples were examined histologically, analyzed by transmission electron microscopy (TEM), and complete 18S rRNA obtained by polymerase chain reaction (PCR) was sequenced, phylogenetically analyzed. Under light microscopy, *Sarcocystis* cysts were oval to spherical in shape, measuring 90 to 170 x 110 to 380 μm. Histology revealed *Sarcocystis* cysts in the cardiac muscles of 34 out of 74 (45.9%) specimens. Transmission electron microscopy (TEM) showed that has a typical hair–like protrusion of *S. grueneri*.

DNA sequence and alignment were compared with 39 taxa of *Sarcocystis* spp. (include Cervidae) from GenBank database, and was registered at GenBank (KC 556825). Phylogenetic analysis also showed 100% sequence identity of the experimental 18S rRNA sequence with *S. grueneri*. This is the first reported use of molecular techniques to measure the prevalence of *S. grueneri* in the Republic of Korea (ROK).

Keywords : Korean Water Deer, *Hydropotes inermis argyropus,* protozoa, *Sarcocystis grueneri,* Republic of Korea

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Introduction

Sarcocystis, a genus of protozoa, is a parasites from the largest genera of the phylum Apicomplexa that is responsible for disease in various animal species (Soulsby, 1982). However, some infections animals are subclinical. Previous studies have detailed the clinical signs or lesions of *Sarcocystis* infection, however those represent only the pre-cyst stage associated with sarcocystosis (David et al., 1995). *Sarcocystis* spp. have two hosts in which herbivores or omnivores act as intermediate hosts, infected by ingestion of oocysts from the feces of definitive hosts, while the carnivorous, definitive hosts acquires the infection by ingesting sarcocysts in the tissues of intermediate hosts (Soulsby, 1982; Dubey, 1976; Fayer, 1977; Dubey *et al.*, 1982).

Sarcocystis spp. cysts are commonly detected in smooth and striated myocytes, both type I and type Ⅱ muscle fiber such as, Purkinje fibers of the heart, and in neural cells that act in an intermediate host (Powell *et al.*, 1986).

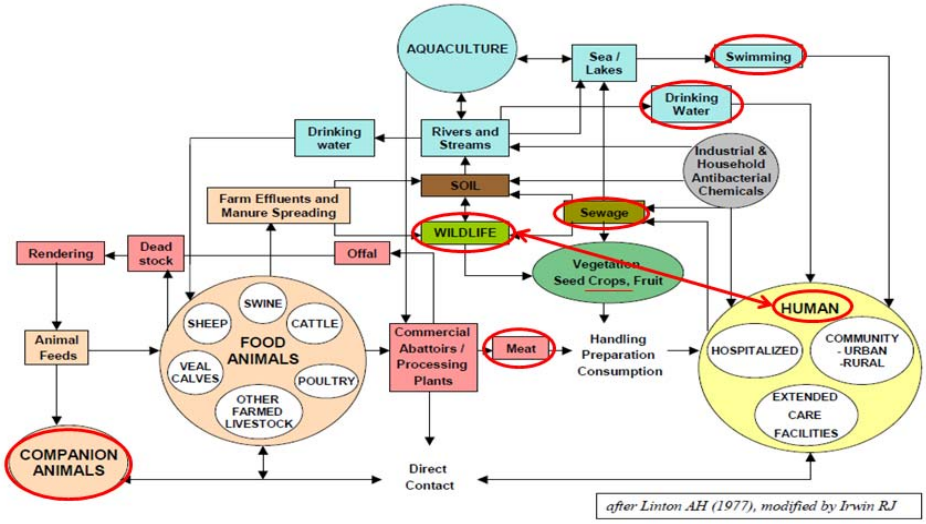

Fig 1. Schematics for possible pathway of infection related to human *Sarcocystis* sp,. The infection that could have been contaminated by feces with sporocysts, including exposure to contact with soil, drinking unrefined water and consuming uncooked vegetable (Linton 1977, modified by Irwin).

Cervidae are well known as typical intermediate hosts for *Sarcocystidae*, including *S. grueneri* and *S. rangi, S. tarandivulpes, S. hardangeri, S. rangiferi, and S. tarandi* (Stina *et al.*, 2008). The sarcocysts in the host species belong to respective species of *Sarcocystis* with similar cyst morphology. Thus, the cyst of *S. grueneri* from reindeer is almost similar in morphological ultrastructure, in red deer, roe deer and moose (Gjerde B, 1985).

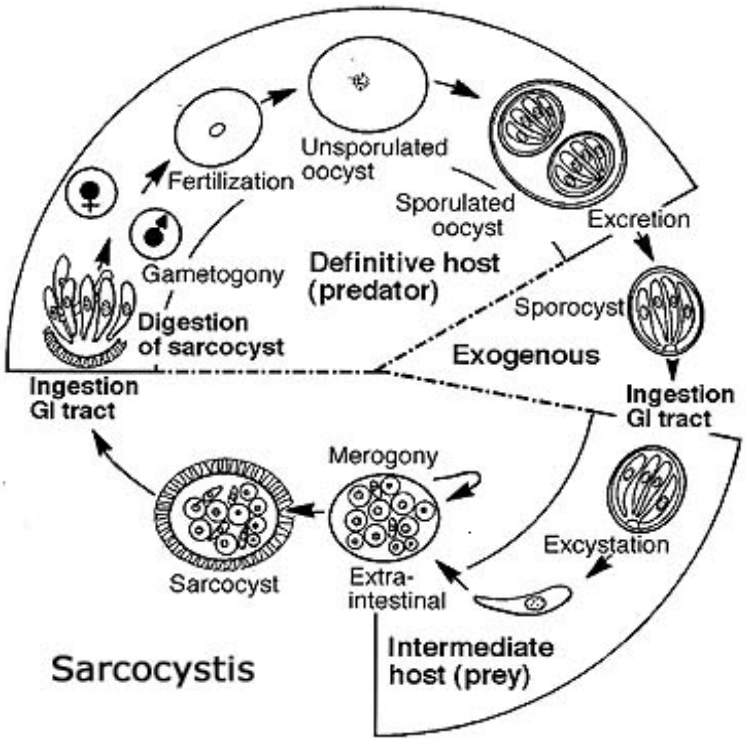

Fig 2. Two hosts life cycle of *Sarcocystis*. The herbivores or omnivorous act as intermediate hosts that are infected after ingesting oocysts in the feces of the definitive hosts. The carnivorous definitive hosts attain the infection by taking Sarcocysts in the tissues of intermediate hosts [Retrieved May 3, 2014, from life cycle of *Sarcocystis*. Image from the http://www.vet.uga.edu/vpp/clerk/rantze/ College of Veterinary Medicine, University of Georgia].

The water deer is the only species of genus *Hydropotes,* which belongs to Family *Cervidae*, Subfamily *Hydropotinae*. Originally found in most parts of the Republic of Korea (ROK), it is native to some parts of eastern China (Cooke and Farrell, 1998). There are two subspecies: the Chinese water deer, *Hydropotes inermis inermis* and the Korean water deer (KWD), *Hydropotes inermis argyropus* (Allen, 1940; Valerius, 1998), both of which have been extensively *Sarcocystis* that are named of several *Sarcocystis* spp. in these species. Despite efforts, the taxomonic classification of this genus is still incomplete (Colwell and Mahrt, 1981; Dahlgren and Gjerde, 2007, 2009; Dubey *et al.*, 1980, 1983; Entzeroth *et al.*, 1982, 1985; Kutkiene, 2003) and is currently under revision. Possibly, the number of species eventually recognized may be reduced with multiple hosts (Atkinson, 1993; Odening, 1998).

Given that the host taxonomy is under revision, gene sequence information could help distinguish specific *Sarcocystis* hosts from among the cervids. The ssu(small subunit) rRNA gene has been commonly used for phylogenetic analyses of species evolution to decipher relationships between similar taxa. The double feature of hypervariable regions interspersed within highly preserved DNA sequences, allows for the

differentiation between eukaryotic species. Therefore, the use of molecular techniques is being widely accepted in case of ambiguous species (Dahlgren and Gjerde, 2007, 2009, Šlapeta, 2001, 2002; Tenter and Johnson, 1997; Morrison *et al*., 2004). The aim of this study was to detect and identify by morphological and molecular methods, infections by *Sarcocystis grueneri* in Korean water deer from ROK as well as study the phylogenetic relationship between founded *S. grueneri* found in wild KWD from ROK and other intermediate hosts, including cervids.

Materials and Methods

1. Sample collection and histological study

The 74 samples (Male 48, Female 26) of wild Korean water deer, *Hydropotes inermis argyropus,* were taken between 2004 and 2014 by Wild Animal Rescue Centers from the cities of Gangwon-do, Gyeonggi-do, Jeollabuk-do and Daejeon in Republic of Korea (Table 1, Fig. 3).

Upon collection preliminary investigation confirmed the presence of *Sarcocystis* in the samples. Heart tissues were fixed in 10% neutral buffered formaline, embedded in paraffin, sectioned at 4um, and stained with hematoxylin and eosin for screening by light microscopy.

Table 1. Sample collection and histological study of *Sarcocystis* of Korean water deer from 2004 to 2014 in Republic of Korea.

Collection provinces and city	Number of Total (n=74)	No. of collected male	No. of collected female
Gangwon-do	**29**	**15**	**14**
Daejeon-si	**41**	**30**	**11**
Gyeonggi-do	**2**	**1**	**1**
Jeollabuk-do	**2**	**2**	**0**

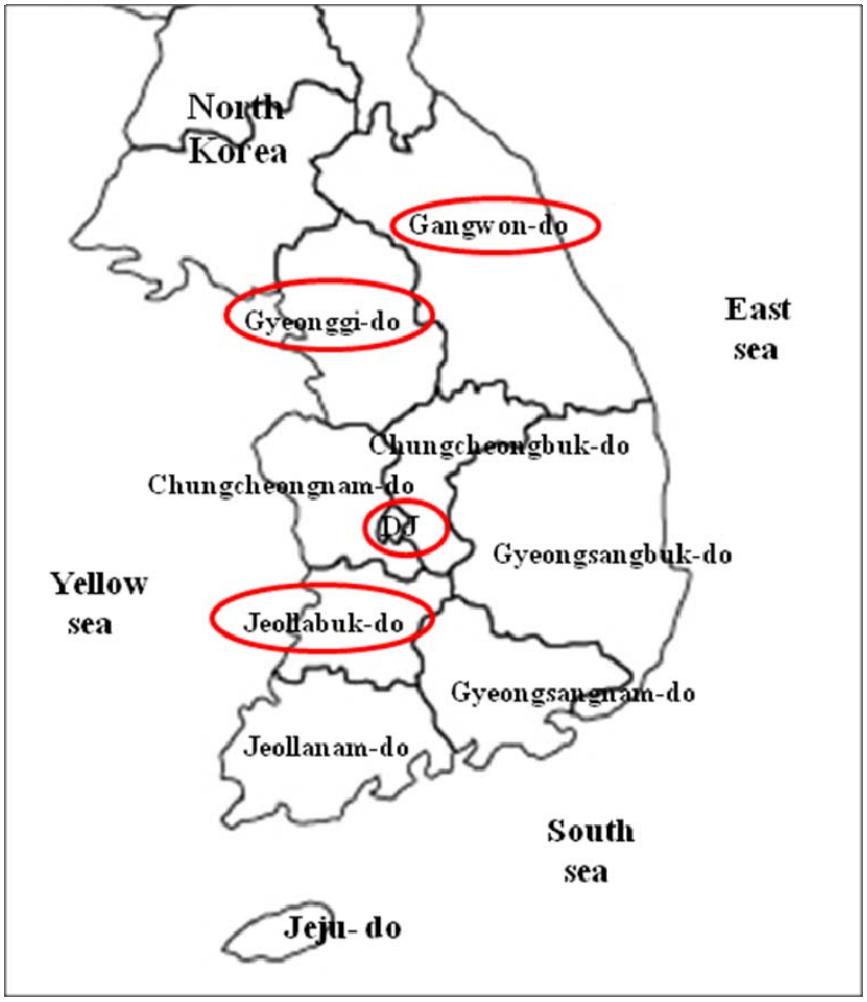

Fig 3. The geographical map of collection sites (○). 74 samples (Male 48, Female 26) of Korean water deer (*Hydropus inermis argyropus*) were taken between 2004 and 2013 by Wild Animal Rescue Centers. The cities of Gangwon-do (n=29), Gyeonggi-do (n=2), Jeollabuk-do (n=2) and, Daejeon-si (n=41) in Republic of Korea.

*DJ; Daejeon-si

2. Transmission electron microscopic examination

A piece of heart muscle containing cyst detected by light microscopy were fixed with 2% glutalaldehyde and 2% paraformaldehyde solution in 0.05 M sodium cacodylate buffer (pH 7.2) for 4 hours. After washing with buffer, the specimens were post-fixed with 1% osmium tetroxide at 4℃ for 4 hours. The specimens were dehydrated in a graded ethyl alcohol series and two changes of propylene oxide, and embedded in epon mixture. The ultrathin sections were stained with 2% uranyl acetate and Reynolds' lead citrate and examined in Tecnai G2 transmission electron microscope 120 kV (FEI, Oregon, USA).

3. DNA extraction and PCR amplification

Genomic DNA was next extracted from *Sarcocystis* positive tissue, frozen, then stored at −20°C before examination immediately upon thawing. With tissue dissections from 0.05 g to 0.25 g (Fig. 4) ten positive samples and one negative sample (confirmed by light microscopy) were placed in 1.5 ml microcentrifuge tubes containing with

PBS (pH 7.4). Genomic DNA extraction was carried out using DNeasy Blood & Tissue Kits (Qiagen, USA) according to the manufacturer's instructions. DNA was eluted in 30 μL of buffer (10 Mm Tris-Cl, pH 8.5) yielding an average of 33.2 ng measured by Nano spectrophotometer (NanoPhotometer™, Implen, Germany)(Fig. 5). Loads of overlapping region between members of the *Sarcocystidae* covering 18S rRNA gene were amplified by the polymerase chain reaction (PCR) using primers 18S 2L (F)-GGATAAACCGTGGTAATTCTATG and 18S 1H (R)-TATCCCCATCACGATGCATAC (Yang *et al.*, 2001). One reaction mixture contained 33.2 ng (1 μL/reaction) of gDNA, HiPi PCR PreMix (Elpis Biotech, Korea)- Taq polymerase in 250 mM Tris-Hcl (pH 9.0) of 1 unit, 80 mM$(NH_4)_2$ SO_4 , 10% DMSO, 8.75 mM $MgCl_2$, 0.05% bromophenol blue, 12% glycerol, oligonucleotide primers (10 pmol each/ reaction of 1 μL), and RNase-free water up to final volume. Initial denaturation at 95°C for 3min was followed by 35 cycles of 94°C for 40s, 55°C for 45s or 1min, and 72°C for 1min 30s, with final extension at 72°C for 5min. The PCR products were visualized via electrophoresis on 1.2% agarose gel. The ~1,500 bp amplicons of the both species examined proved to be difficult to sequence directly from PCR products, and the region was cloned for specific sequences.

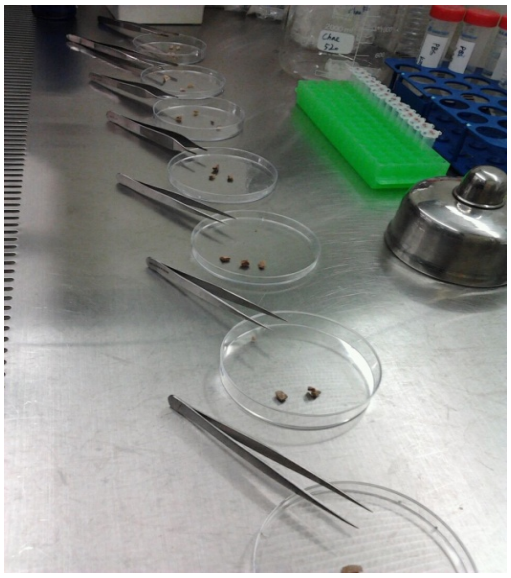

Fig 4. A photograph of tissue section. With tissue dissections in progress from 0.05 g to 0.25 g, ten positive samples and one negative sample (confirmed by light microscopy)

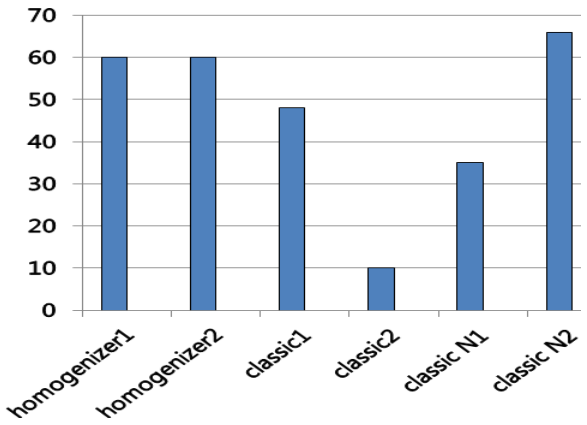

Fig 5. Preliminary study for validation of gDNA quantity. Represented one positive sample that carry out duplicate and measured by Nano spectrophotometer (NanoPhotometer™, Implen, Germany)

Primer	Orientation	Sequence 5'--------3'	Location[a]
Primer 1L	Forward	CCATGCATGTCTAA GTATAAGC	446-469
Primer 1H	Reverse	TATCCCCATCACGA TGCATAC	1650-1670
Primer 2L	Forward	GGATAAACCGTGG TAATTCTATG	156-178
Primer 2H	Reverse	ACCTGTTATTGCCT CAAACTTC	1530-1551
Primer 3L	Forward	CTAGTGATTGGAA TGATGGG	566-586
Primer 3H	Reverse	GGCAAATGCTTTCG CAGTAG	1040-1070
Primer 4H	Reverse	CAGAAACTTGAATG ATCTATCG	348-368

Table 2. Nucleotide each primers for detection of *Sarcocystis* spp. 18S rRNA gene (Yang *et al.*, 2001).

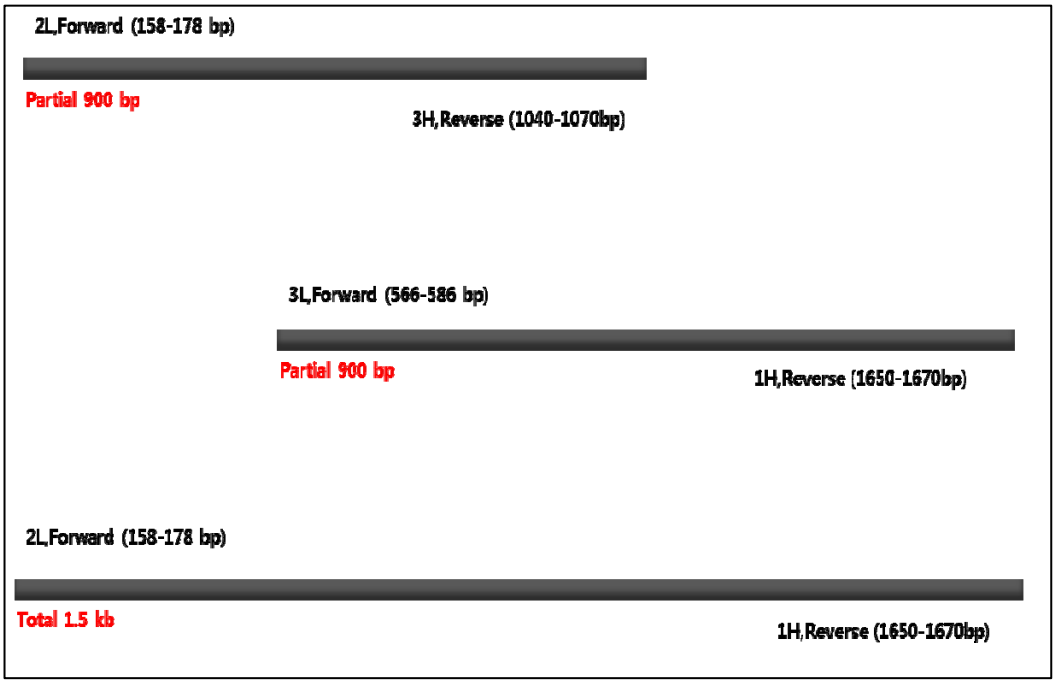

Fig 6. A schematic diagram of *Sarcocystis* spp., PCR primers.

4. Cloning and sequencing

For carry out sequencing, the purified PCR amplicons were cloned by T7 (Forward-TAATACGACTCACTATAGGG) and SP6 (Reverse-TATTTAGGTGACACTATAG) pGEM-T Easy Vector® each strands using TA cloning. After the transformation and the colony PCR that both

insert and vector were amplified by 22 cycles, purified plasmid DNA products from the amplified cloning DNA fragments were sequenced by dideoxy chain termination with an automatic sequencer (ABI 3730xl capillary DNA sequencer, USA).

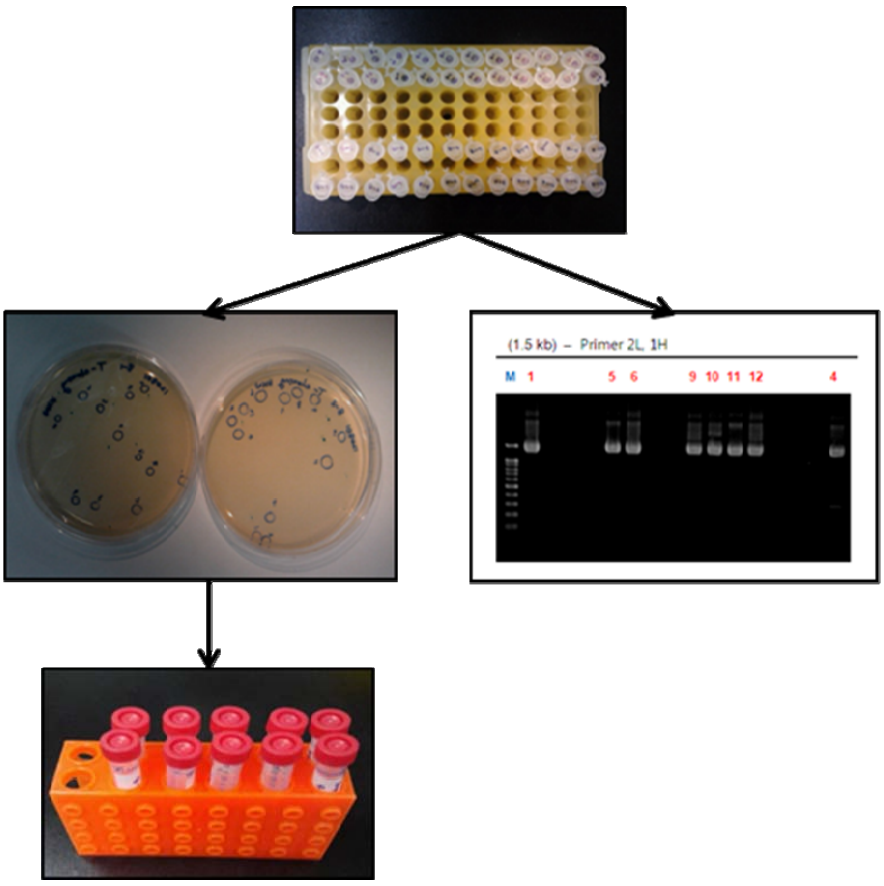

Fig 8. Photos of cloning procedure. PCR amplicons were cloned by T7 and SP6 pGEM-T Easy Vector ® each strands using TA cloning

5. Phylogenetic analyses

The resulting sequences were subjected to phylogenetic analyses on the 18S rRNA gene against previously sequenced *Sarcocystis* species as well as with other registered sequences of *Sarcocystis* spp. from intermediate hosts, retrieved from GenBank database 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), respectively. Phylogenetic tree was determined by using MEGA 4 software (Tamura *et al.*, 2007). Using the neighbor-joining (NJ) (Saitou and Nei, 1987) under the Kimura 2- parameter distance model (Kimura, 1980) methods was based on a guide tree as pairwise and multiple alignment parameters. The final sequence alignment was comprised with 39 taxa (Table. 3).

Table 3. Sequence analyzed of the *Sarcocystis* spp. used in 18S rRNA genes and GenBank accession number

Taxon name/ Strain	Accession Number	Intermediate Host
S. alces, SaAa1N	EU282018	Moose (*Alces alces*)
S. alceslatrans, SalAa1C	EU282033	Moose (*Alces alces*)
S. arieticanis	L24382	Sheep (*Ovis aries*)
S. buffalonis	AF017121	Water buffalo (Huong *et al*., 1997)
S. capracanis	L76472	Goat (*Capra aegagrus hircus*)
S. cornixi, V1	EU553478	Hooded Crow (*Corvus cornix*)
S. cruzi, NSMT: Pr319	AB682782	Water buffalo (*Bubalus bubalis*) , Cattle (*Bos taurus*)
S. dispersa	AF120115	Mouse (Šlapeta et al.,2001)
S. fusiformis, 3f5	AF176927	Water buffalo (*Bubalus bubalis*)
S. fusiformis	U03071	Water buffalo (*Bubalus bubalis*)
S. gallotiae	AY015112	Lizard (*Gallotia galloti*)
S. grueneri, Sg1RtN	EF056010	Reindeer (*Rangifer tarandus tarandus*)
S. hardangeri, ShRt1I	EF467654	Moose (*Alces alces*)

Table 3. Continued

S. hirsuta	AF017122	Cattle (*Bos taurus*)
S. hjorti, Sd1CeN	GQ250990	Red deer (*Cervus elaphus*)
S. lacertae	AY015113	Common wall Lizard (*Podarcis muralis*)
S. mucosa	AF109679	? (Šlapeta *et al*.,2001)
S. muris	M64244	Mouse (Holmdahl *et al*.,1999)
S. neurona, SN5	U07812	Bird (Holmdahl *et al*.,1999)
S. ovalis, SoAa1N	EU282019	Moose (*Alces alces*) (Stina *et al*., 2008)
S. rangi, Sr1RtI	EF467655	Reindeer (*Rangifer tarandus tarandus*)
S. rangiferi, Srf1CeN clone 2	GQ251022	Red deer (*Cervus elaphus*)
S. rileyi, Europe	HM185742	Mallard duck (*Anas platyrhynchos*)
S. rodentifelis	AY015111	Reindeer (*Rangifer tarandus tarandus*)
S. silva, NoCc11.3	JN226125	Roe deer (*Capreolus capreolus*)
S. sinensis, kxmo	AF266959	Water buffalo (*Bubalus bubalis*), Cattle (*Bos taurus*)
S. tarandi, St1RtN	EF056017	Red deer (*Cervus elaphus*)
S. tarandi, St4RtN clone 3	GQ250974	Red deer (*Cervus elaphus*)

Table 3. Continued

S. tarandi, St1CeN clone 3	GQ251013	Red deer (*Cervus elaphus*)
S. tarandi, St4RtN clone 1	GQ250972	Red deer (*Cervus elaphus*)
S. tarandi, St3RtN clone 4	GQ250970	Red deer (*Cervus elaphus*)
Sarcocystis sp. TO-2012	AB691780	Ryukyu Rat (*Diplothrix legata*)
Sarcocystis sp. cyst type I ex Anser albifrons	EU502869	Mallard duck (*Anas platyrhynchos*)
Sarcocystis sp. Cyst type III ex Anser albifrons	EU502868	Hooded Crow (*Corvus cornix*)
Sarcocystis sp. ex Larus marinus	JQ733508	Black-backed gull (*Larus marinus*)
Sarcocystis sp. HM050622	AB257156	Sika deer (*Cervus nippon yesoensis*)
Sarcocystis sp. SspEAa1N	EU282017	Moose (*Alces alces*)
Neospora caninum	U17345	Cattle (Dubey *et al*.,1996)
Toxoplasma gondii	U00458	Many (Holmdahl *et al*.,1999)

Results

1. Morphological characteristics of sarcocysts

By Light microscopy, sarcocysts were found in cardiac muscle from Korean Water Deer. Even though the sarcoplasm of the myocardium presented with bradyzoites enclosed in the thin and smooth cyst wall, the wall of sarcocyst was indiscernible. Morphology of the cysts ranging from oval to spherical were 90 to 170 x 110 to 380 μm in size and contained all the feature of Sarcosporodian cystozoites(Fig. 8 and 9).

Histological examination revealed 45.9% of samples positive that prevalence of *Sarcocystis* from the cardiac muscle was summarized in (Table 4). But it was determined that *S. grueneri* could not be accurately described by histological study. Consequently, it was necessary that examined with transmission electron microscopy (TEM).

Table 4. Prevalence of *Sarcocystis* from Korean water deer in Republic of Korea

Collection provinces and city	**No. of KWD**	**No. of positive male (Infection rates)**	**No. of positive female (Infection rates)**	**No. of positive KWD (Infection rates)**
Gangwon-do	**29**	7 (**9.5%**)	5 (**6.8%**)	12 (**16.2%**)
Daejeon-si	**41**	17 (**22.9%**)	5 (**6.8%**)	22 (**29.7%**)
Gyeonggi-do	**2**	0%	0%	0%
Jeollabuk-do	**2**	0%	0%	0%
Total	**n=74**	**24(32.4%)**	**10 (13.5%)**	**34(45.9%)**

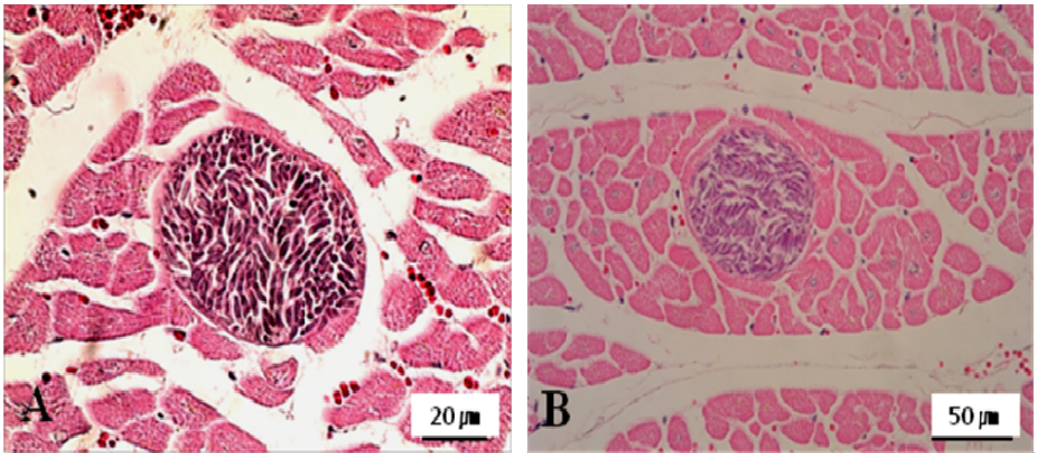

Fig 8. Light microscopic feature of sarcocysts in the cardiac muscle of Korean water deer (cross-section). (A) No specific tissue reactions by the sarcocysts were recognizable, higher magnification of the sarcocyst, Scale bar=20 ㎛. (B) Showing many bradyzoites in cyst. Scale bar=50 ㎛ H&E stain.

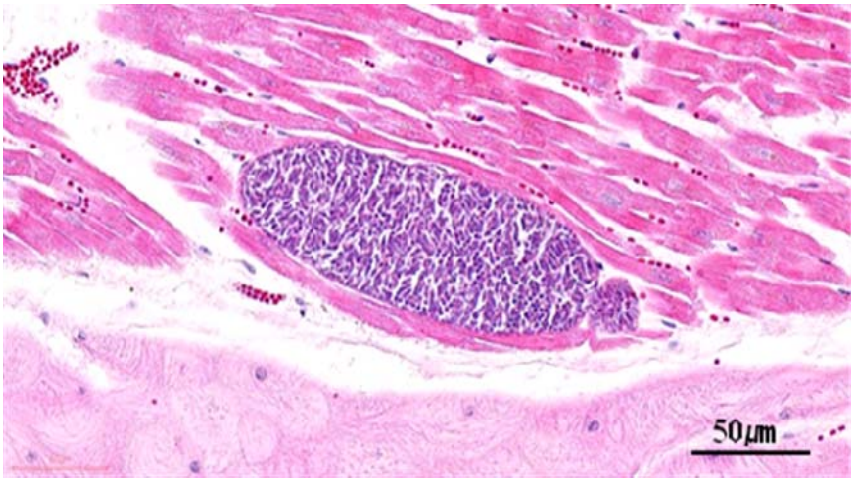

Fig 9. Light microscopic feature of sarcocysts in the cardiac muscle of Korean water deer (longitudinal-section). Scale bar=50 ㎛, H&E stain. The sarcocysts were enclosed by a very thin wall (0.45-0.6 ㎛ thick) that consists of ground substance.

2. Transmission electron microscopic study

All cysts were located within a cardiac muscle fiber. The cyst walls were thin (≤ 1 ㎛ in width) and had knoblike flattened protrusions. The cyst wall consists of secondary layers, ground substance, hair-like flattened protrusion, and sarcoplasm (Fig. 10, A). The protrusions measure about 0.21-1.25 ㎛ in length and 0.05-0.07 ㎛ in width; these arose irregularly from the ground substance, contained a fine granular substance, and lacked internal microfilaments (Fig. 10, B). Variation in the density of protrusion was observed even within a given cyst. The cysts had thin septa (Fig. 10, C), and the cysts was enclosed in an amorphous matrix which contained single fusiform bradyzoites (Fig. 10, D). The bradyzoites consist of containing rhoptries. The number of rhoptry was counted within 6. The several rhoptries appeared in cross-sectioned anterior region of bradyzoite (Fig. 10, E). The last bradyzoites consist of four regions. Four regions of microneme surrounded with abundant amylopectin granules and two rhoptries with having the nucleus (Fig. 10, F).

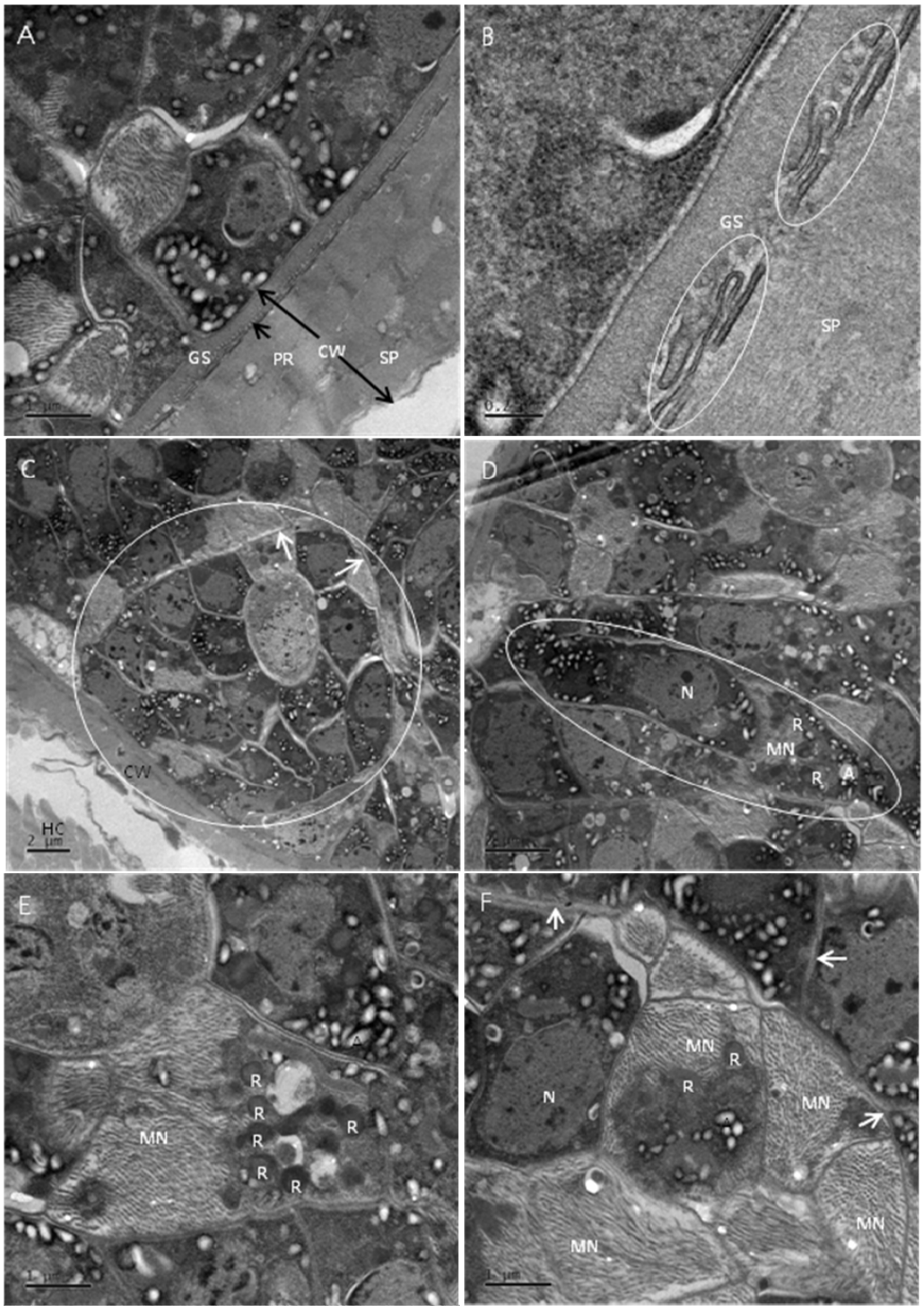
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Fig 10. Transmission electron micrographs of cyst of *S. grueneri* from the cardiac muscle of Korean water deer. (A) The cyst wall. The sarcocyst is enclosed by a thin wall that consist of ground substance (GS), protrusions (PR) and sarcoplasm (SP). (B) The hair-like protrusion (circle). Microfilaments are not found in the protrusion. (C) Bradyzoites in a component (circle). The component separated by thin septa (arrow). (D) A single bradyzoite (circle). (E) Rhoptries. (F) The micronemes and nucleus. Septa (arrow). A, amylopectin granule; CW, cyst wall; GS, ground substance; MN, micronemes; N, nucleus; P, protrusion; R, rhoptry; SP, sarcoplasm; HC, host cell.

3. PCR amplification and alignment analysis

Based on histological results, PCR amplification and gel electrophoresis (Fig. 11) were performed on 18S rRNA , revealing a close analogy of the sarcocysts to *S. grueneri* found in reindeer (Dahlgren and Gjerge, 2007). Direct sequencing revealed a trend of double-peaks or noisy chromatograms in the initial part of the gene sequence, due to the 5'-end half of 18S rRNA gene. By cloning the PCR products before sequencing, single-peak of high quality chromatograms were gained and complete consensus sequence was created from the alignment of two separate chromatograms.

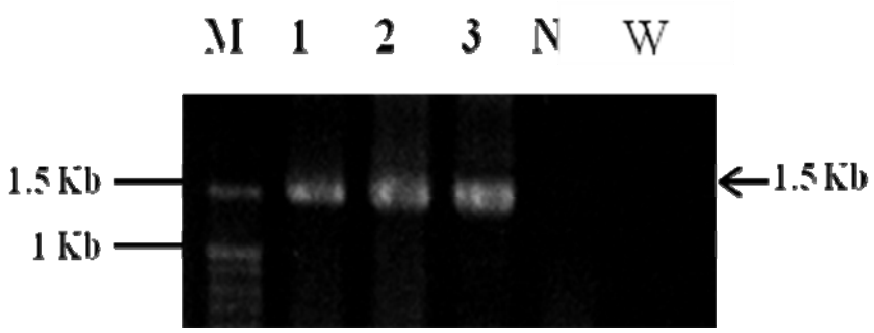

Fig 11. The PCR results of *Sarcocystis* spp. in cardiac muscle. After histological finding by LM, genomic DNA of positive samples (34/74) were amplified by PCR with using primer pairs (2 L/1 H). The PCR results of *Sarcocystis* spp. in cardiac muscle (1.5 kb). M, 100bp Marker (Elpis DNA ladder); N, Negative sample; W, Water Control (Negative control).

4. Phylogenetic analyses

Upon alignment between 1,470 and 1,507 nucleotides flanking the consensus sequence were trimmed. After scoring, the sequences were compared with complete of the 18S rRNA gene from other 39 taxa of *Sarcocystis* spp. (include Cervidae) were deposited in GenBank database. Thus, the resulting sequence has been registered at GenBank (KC 556825, Fig. 12 and 13). According to Morrison *et al*. (2004), the 18S rRNA sequence is an excellent phylogenetic marker within the protozoa and is commonly used for species identification. BLAST searches showed the 18S rRNA sequences shared identity with *S. grueneri* was 100% (Fig. 14). Both exhibited 53% AT base content and little overall variation in base composition that were reflected in a phylogenetic tree (Fig. 15).

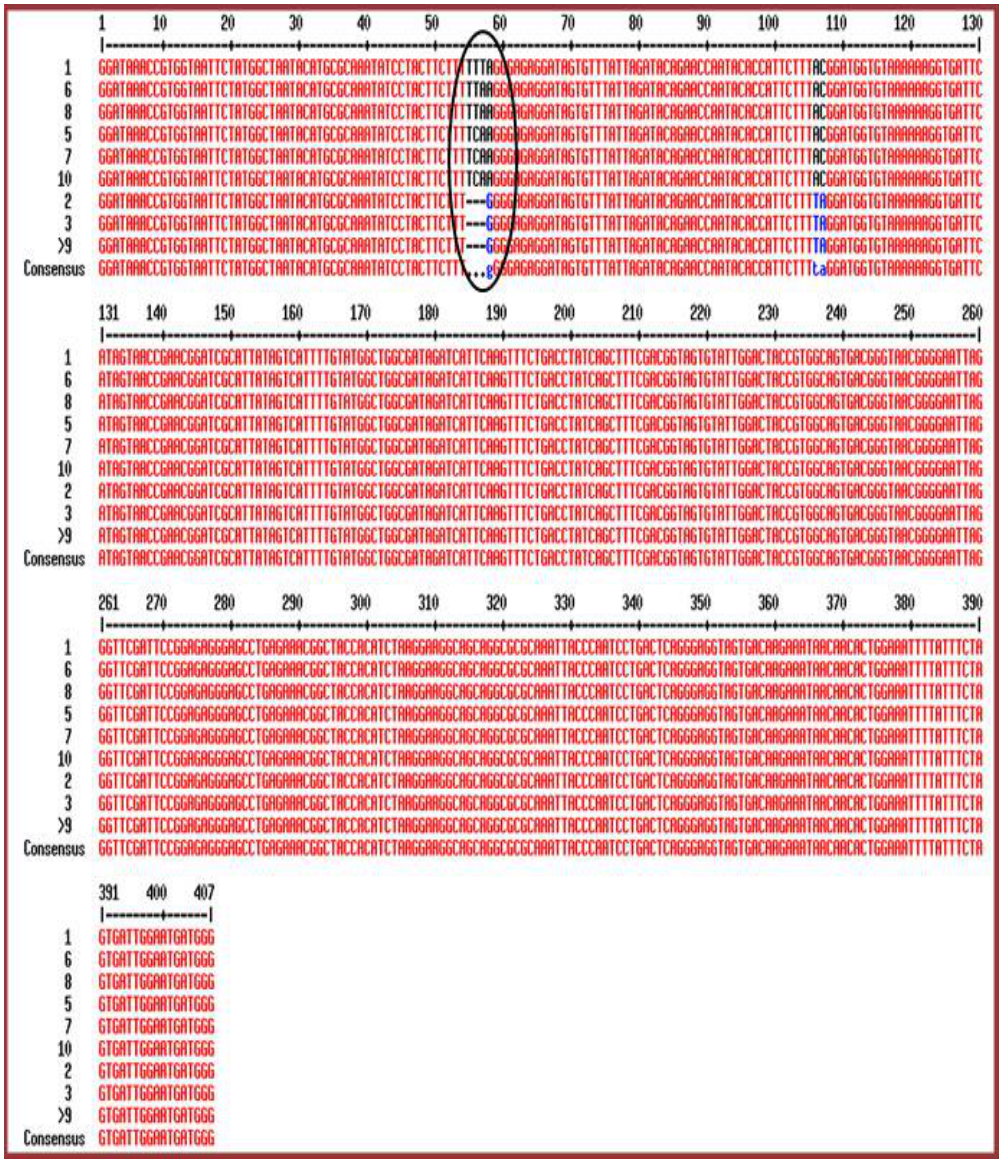

Fig 12. The results of forward sequence analysis (forward and reverse sequence were absent from No.5). After sequencing, BLAST searches revealed the 18S rRNA sequences shared 100% identity with *Sarcocystis grueneri* .

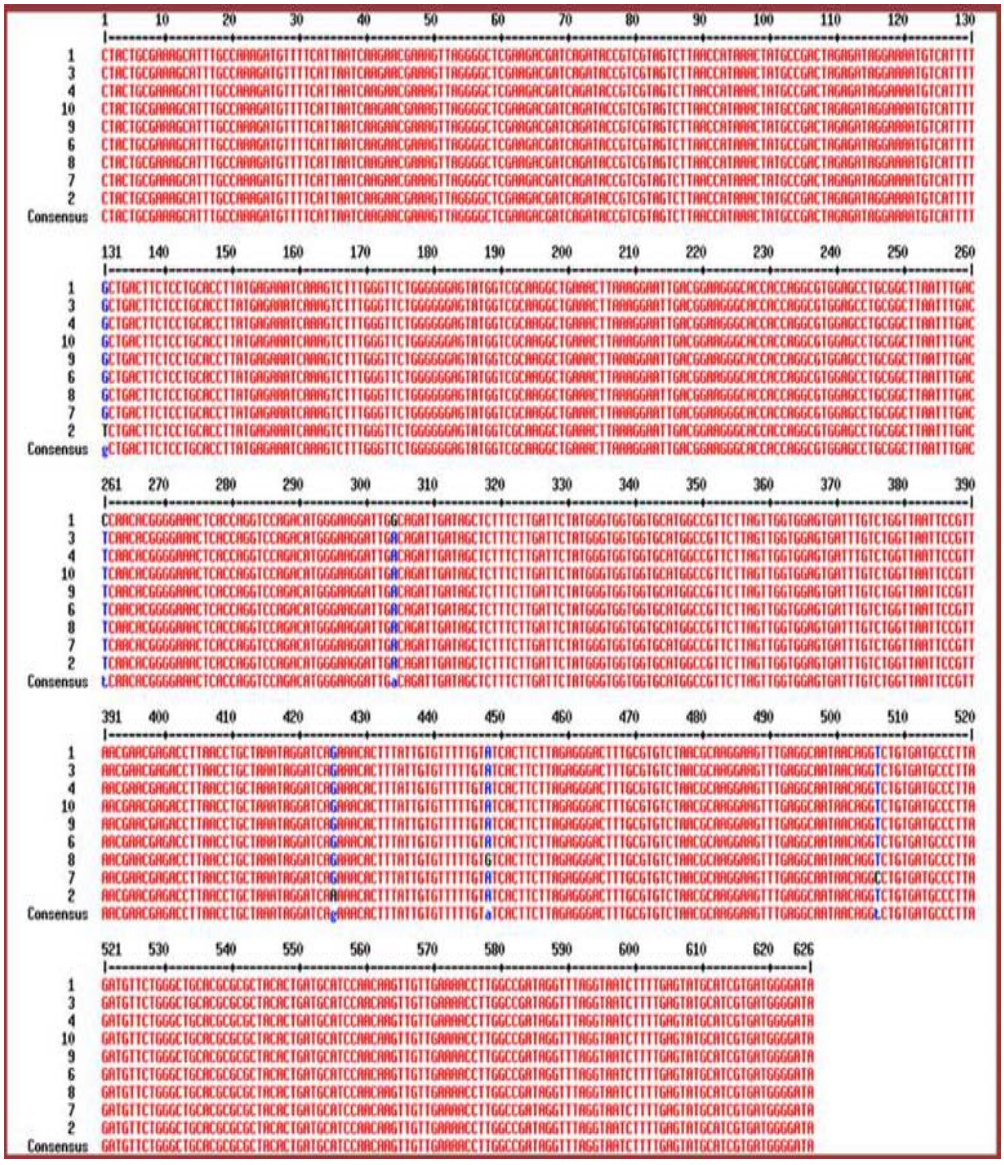

Fig 13. The results of reverse sequence analysis. After sequencing, alignment of the experiment group sequences result (absent No. 5 owing to noisy of peak on Chromas software) from 625 bp to 1 bp with reverse sequence).

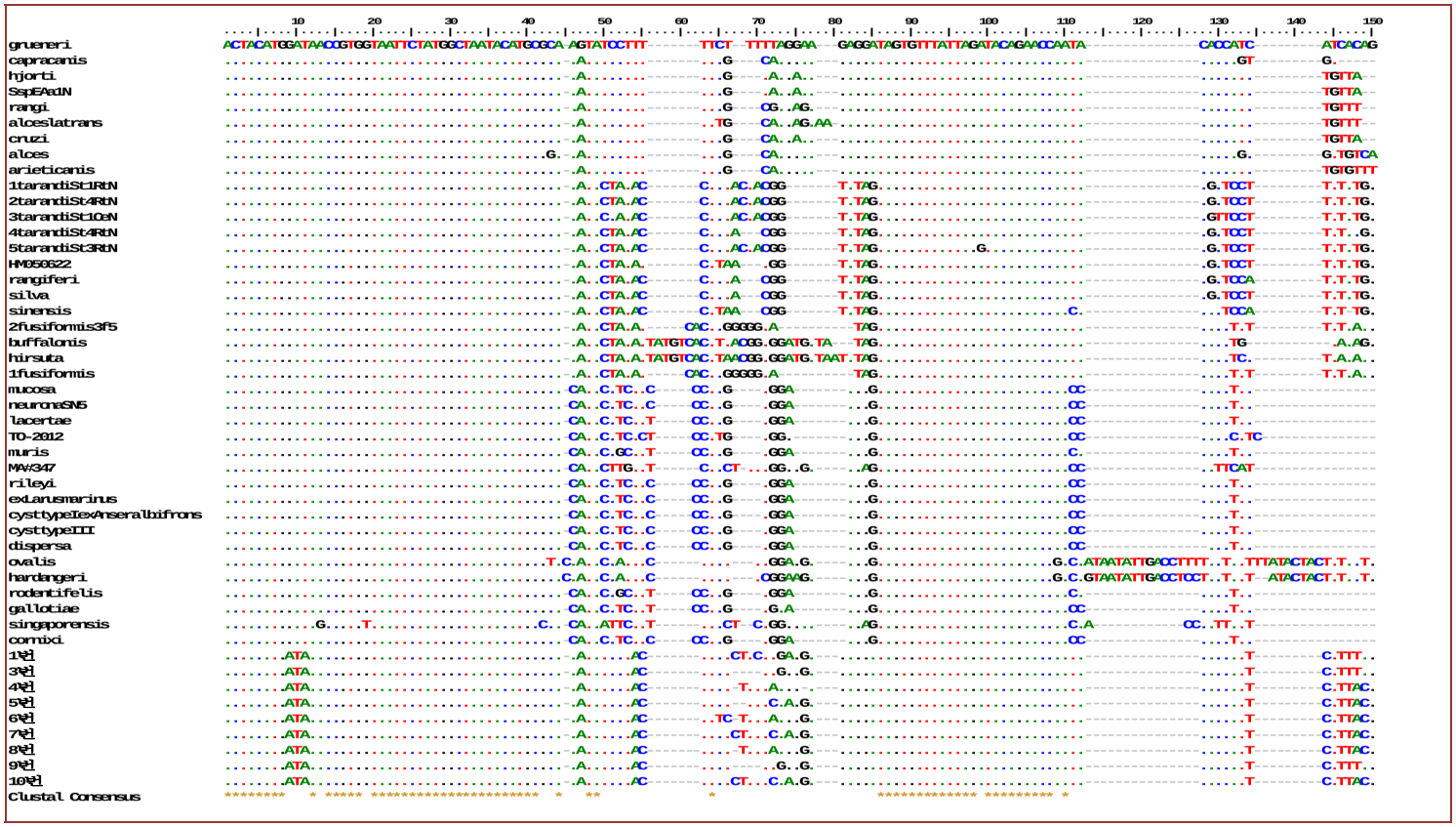

Fig 14. Summary of partial 18S rRNA gene sequences of *Sarcocystis* spp., with corresponding experimental nucleotide sequence.

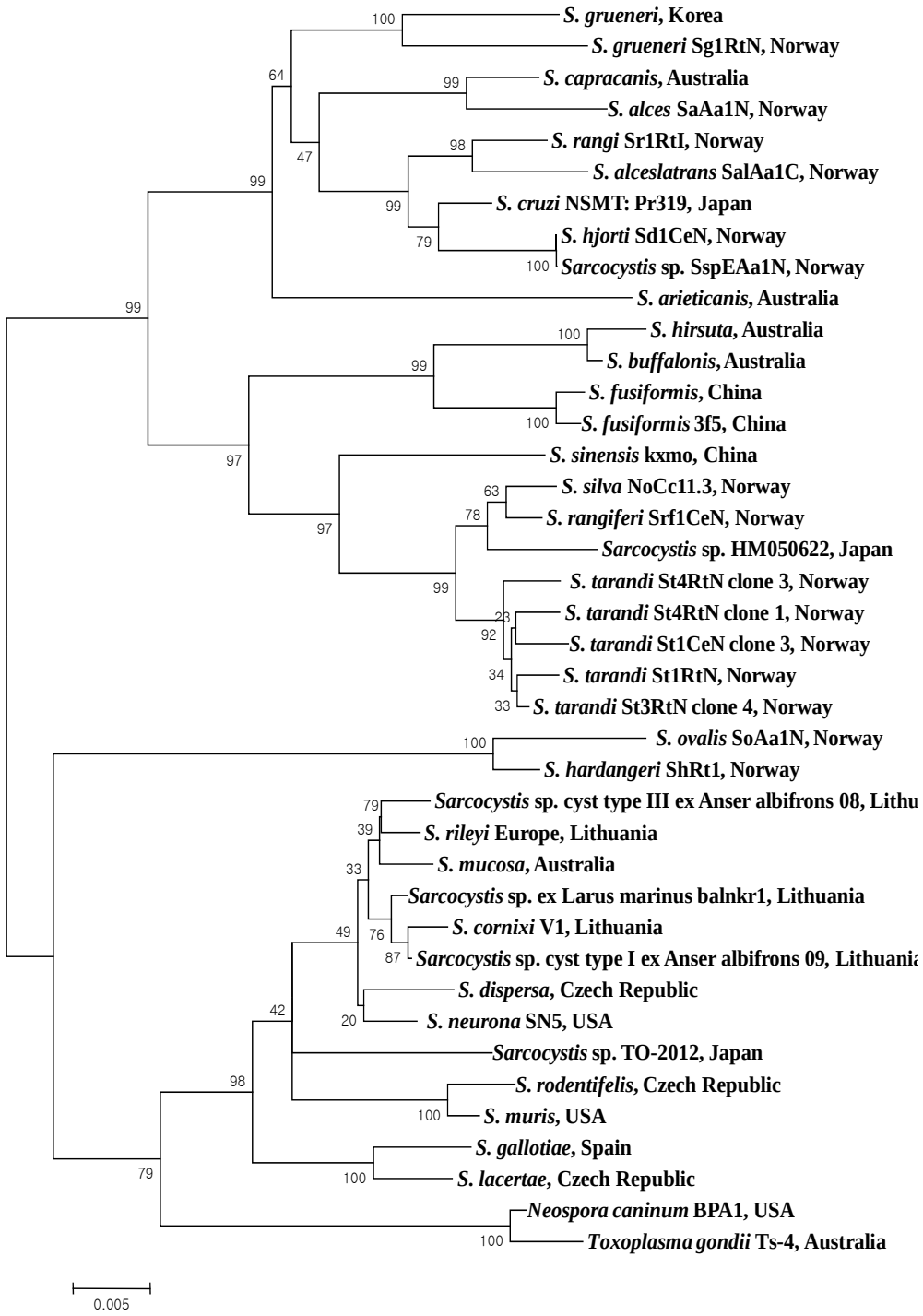
S. grueneri, Korea
S. grueneri Sg1RtN, Norway
S. capracanis, Australia
S. alces SaAa1N, Norway
S. rangi Sr1RtI, Norway
S. alceslatrans SalAa1C, Norway
S. cruzi NSMT: Pr319, Japan
S. hjorti Sd1CeN, Norway
Sarcocystis sp. SspEAa1N, Norway
S. arieticanis, Australia
S. hirsuta, Australia
S. buffalonis, Australia
S. fusiformis, China
S. fusiformis 3f5, China
S. sinensis kxmo, China
S. silva NoCc11.3, Norway
S. rangiferi Srf1CeN, Norway
Sarcocystis sp. HM050622, Japan
S. tarandi St4RtN clone 3, Norway
S. tarandi St4RtN clone 1, Norway
S. tarandi St1CeN clone 3, Norway
S. tarandi St1RtN, Norway
S. tarandi St3RtN clone 4, Norway
S. ovalis SoAa1N, Norway
S. hardangeri ShRt1, Norway
Sarcocystis sp. cyst type III ex Anser albifrons 08, Lithu
S. rileyi Europe, Lithuania
S. mucosa, Australia
Sarcocystis sp. ex Larus marinus balnkr1, Lithuania
S. cornixi V1, Lithuania
Sarcocystis sp. cyst type I ex Anser albifrons 09, Lithuania
S. dispersa, Czech Republic
S. neurona SN5, USA
Sarcocystis sp. TO-2012, Japan
S. rodentifelis, Czech Republic
S. muris, USA
S. gallotiae, Spain
S. lacertae, Czech Republic
Neospora caninum BPA1, USA
Toxoplasma gondii Ts-4, Australia
0.005

Fig 15. Phylogram of the 18S rRNA gene sequences from *Sarcocystis* spp. with evolutionary relationships of 39 taxa (1.5 Kb). The sequences were aligned by the Align IR (Ver 2.0; http:// biosupport.licor.com/support), Clustal X (Ver 2.0; www.clustal.org), respectively. Phylogram was determined by using MEGA 4 software (Tamura *et al.*, 2007). Using the neighbor-joining (NJ) (Kimura, 1980) under the Kimura 2- parameter distance model (Saitou and Nei, 1987) methods a pairwise guide tree was created with multiple alignment parameters. Bootstrap values (>50%) based on 1,000 replications are shown. The tree indicates the position of *Sarcocystis grueneri* detected from cardiac muscles of KWD (*Hydropotes inermis argyropus*).

Discussion

A previous study conducted in Germany revealed that 98.0% of these cervids surveyed were infected with *Sarcocystis* parasites (Erber *et al.*,1978, Partenheimer-Hannemann, 1991) and that 96.7% of the reindeer population in Northern Norway were infected with *S. grueneri* (Malmei *et al.*, 1996). This shows that infection by *Sarcocystis* spp. is quite prevalent in cervids, but it was not until recently that the indigenous eastern populations exhibited *S. grueneri* infection. Futher, Kutkiene (2003) reported that *S. grueneri* was detected in the heart muscle, suggesting a tendency of organ tropism. As *Sarcocystis* infection is common in many cervid species, these results do not rule out high specificity for the intermediate host (Entzeroth *et al.*, 1985).

To understand the direct correlation between *S. grueneri* infection and KWD as the intermediate host, it is essential to employ an adequate population size (Lee, 2003). KWD are widely distributed in ROK, and they tend to live in forest and lowland mountainous areas or riparian zones (Won and Smith, 1999). According to Marques *et al.*, (2001), estimation of wild animal population is related to global climate change;

on this account, it is beneficial to understand the animal's habitat. Most areas of the Korean peninsula are home to KWD, which are less commonly found in large cities or coastal areas (61 to 67%, population size of 1,045,000 to 9,820,000) as compared to 81% of inland areas (Kim *et al.*, 2010).

Previous surveys on *Sarcocystis* infection in host animals in Korea includes prevalence reports of 29.1% identified by Jang *et al.*(1990), up to 36.7% by Yang *et al.*(1990), and up to 43.6% by Park *et al.*(1994), and 41.5% by Kang *et al.*(1988).

This results in ROK similarly show 34 out of 74 (45.9%) positive samples from the four regions of Gangwon-do {n=22 out of 41 (29.7%)}, Daejeon-si {n=12 out of 29 (16.2%)}, Jeollabuk-do {n=2 (0%)} and Gyeonggi-do {n=2 (0%)}. *S. grueneri* isolated from rein deer are morphologically similar to cysts from *Capreolus capreolus* (roe deer) (Entzeroth, 1986) and *Cervus elaphus* (red deer, wapiti) (Speer and Dubey, 1982; Entzeroth *et al.*, 1983). As the shape of the sarcocyst is an essential feature for species identification, accurate morphological identification of these cysts is crucial. This was achieved with TEM that identified a typical sinuous undulating protrusion in *S. grueneri.* Thus,

this study documents the first use of molecular techniques to distinguish *S. grueneri* from other *Sarcocystis* species in KWD. Even though the first direct sequencing failed to yield viable results, cloning results were compensated by unstable sequence data. According to the Šlapeta *et al.* (2002), half ssu rRNA gene on the 5'-end was variable even within a single species. Inevitably, the region was cloned to improved quality of the sequences. General phylogenetic characteristics of Sarcocystinae have been elaborated in previous studies (Tenter and Johnson, 1997; Šlapeta *et al.*, 2002; Yang *et al.*, 2001; Morrison *et al.*, 2004) and the fundamental composition did not affect the classification of *Sarcocystis* species by intermediate host preference.

Phylogenetic topology of 1,000 Bootstraps determined that the two species are highly similar with branch lengths very close to the Genbank sequences of *Sarcocystis grueneri* (EF056010) sequences. Sequence analysis revealed 100% identity with *Sarcocystis grueneri* (Fig.12 and 13). This study supports the importance of molecular identification in the differentiation of species with shared morphology. It is apparent that neither morphological detection nor molecular technique alone can be used to accurately defind the species.

With a life cycle including an intermediate and a definitive host, *Sarcocysts* have been able to transmit sporocyst and oocyst to several species (Dubey *et al.*, 1989; Odening, 1998)and within *Sarcocystis* spp., The hosts are under hormonal control, which means that self-originating steroid hormones are produced during infection with *Sarcocystis* parasites (Spindler, 1988). Additionally, humans are susceptible to potential zoonotic *Sarcocysts* (Heydorn, 1977) from water contaminated with feces from the host or food washed with unsanitary water (Fayer, 2004).

During the past few decades, the KDW species in ROK have been on the verge of extinction with wolf (*Canis lupus chanco*) and tiger species (*Panthera tigris*), Retrieved June 2014 from Wildlife Management Association, http://www.kowaps.or.kr. Corresponding to the decrease in numbers of natural predators, the KWD population gradually increased, recently. Therefore surveillance of *S. grueneri* infection can be used as a preventive measure to conserve KWD number in ROK.

References

Allen GM. Mammals of China and Mongolia. *Natural history of central Asia* (American Museum of Natural History, New York).1940;11: 1126-1261.

Atkinson CT, Wright SD, Telford SR, McLaughlin GS, Forrester DJ, Roelke ME, McCown J W. Morphology, prevalence, and distribution of *Sarcocystis* spp. in white-tailed deer (*Odocoileus virginianus*) from Florida. *J Wildl Dis.* 1993;29:73-84.

Colwell DD, Mahrt JL. Ultrastructure of the cyst wall and merozoites of *Sarcocystis* from moose (*Alces alces*) in Alberta. *Can Z Parasitenkd* . 1981;65:317-329.

Cooke AS, Farrell L. Chinese Water Deer. The Mammal Society and the British Deer Society. 1998;p30.

Dahlgren SS and Gjerde B. 2007. Genetic characterisation of six *Sarcocystis* species from reindeer (*Rangifer tarandus tarandus*) in Norway based on the small subunit rRNA gene. *Vet Parasitol*. 146:204-213.

Dahlgren SS, Gjerde B. *Sarcocystis* in Norwegian roe deer (*Capreolus capreolus*): molecular and morphological identification of *Sarcocystis oviformis* n. sp. and *Sarcocystis gracilis* and their phylogenetic relationship with other *Sarcocystis* species. *Parasitol Res* . 2009;104:993-1003.

David S. Lindsay, Byron L. Blagburn, and Kyle G. Braundw. *Sarcocystis* spp. and Sarcocystosis. BAM. 1995;5:249-254

Dubey JP. A review of *Sarcocystis* of domestic animals and of other coccidia of cats and dogs. *J Am Vet Med Assoc* . 1976;169:1061-1078.

Dubey JP. Coyote as a final host for *Sarcocystis* species of goats, sheep, cattle, elk, bison and moose in Montana. *Am J Vet Res*. 1980;41:1227-1229.

Dubey JP, Jolley WR, Thorne ET. *Sarcocystis sybillensis* sp. nov. from the North America elk(*Cervus elaphus*). *Can J Zool.* 1983;61:737-742.

Dubey JP, Lindsay DS, Adams DS. Serologic responses of cattle and other animals infected with *Neospora caninum. Am J Vet Res*. 1996;57:329-336.

Dubey JP, Speer CA, Fayer R. *Sarcocystis* of animals and man. *CRC Press,* Inc., Boca Raton, Fla. 1989.

Dubey JP, Speer CA , Epling GP. Sarcocystosis in newborn calves fed *Sarcocystis cruzi* sporocysts from coyotes. *Am J Vet Res*. 1982;43: 2147-2164.

Erber M, Boch J, Barth D. Drei Sarkosporidienarten des Rehwildes. Berliner und Münchener Tierärtzliche Wochenschrift. 1978;91:482-486.

Entzeroth R, Chobotar B, Scholtyseck E. Ultrastructure of *Sarcocystis* sp. from the muscle of a white-tailed deer (*Odocoileus virginianus*). *Zeitschrift fur Parasitenkunde* . 1982;68:33-38.

Entzeroth R, Chobotar B, Scholtyseck E. Nemeseri L. Light and electron microscopy study of *Sarcocystis* sp. from the follow deer (Cervus dama). *Zeitschrift fur Parasitenkunde* . 1985;71:33-39

Entzeroth R, Nemeseri L, Scholtyseck E. Prevalence and ultrastructure of *Sarcocystis* sp. from red deer (*Cervus elaphus L*) in Hungary. *Parasitology of Hungary* . 1983;16:47-52.

Entzeroth R. Light, scanning and transmission electron microscope study of the cyst wall of *Sarcocystis gracilis Ratz,* (Sporozoa,

Coccidia) from the roe deer (*Capreolus capreolus L*). *Vet Parasitol.* 1986;129:183-186.

Fayer R. Production of *Sarcocystis cruzi* sporocysts by dogs fed experimentally infected and naturally infected beef . *J Parasitol* . 1977;63:1072-1075.

Fayer R. *Sarcocystis* spp. in Human Infections. *Clin Microbiol Review* . 2004;17:894-902.

Gjerde B. Ultrastructure of the cysts of *Sarcocystis grueneri* from cardiac muscle of reindeer (*Rangifer tarandus tarandus*). *Z Parasitenkd* . 1985;71:189-198.

Heydorn AO, Sarkosporidieninfiziertes Fleisch als mögliche Krankheitsursache für den Menschen. *Archiv Lebensmittelhygiene* 1977;28:27-31.

Holmdahl OJ, Morrison DA, Ellis JT, Huong LT. Evolution of ruminant *Sarcocystis* (Sporozoa) parasites based on small subunit rDNA sequences. *Mol Phylogenet Evol.* 1999;11:27-37.

Huong LT, Dubey JP, Nikkilä T, Uggla A. *Sarcocystis buffalonis* n.sp. (Protozoa: Sarcocystidae) from the water buffalo (*Bubalus bubalis*) in Vietnam. *J Parasitol* . 1997;83:471-474.

Jang H, Kang YB, Wee SH, Choi SH. Survey of *Sarcocystis* infections in cattle in Korea. *Korean J Parasitol* . 1990;32:32-37.

Kang YB, Kim SH, Wee SH. A survey of *Sarcocystis* infections in slaughtered cattle in Korea. *Korean J Vet Res*. 1988;28:387-390.

Kim BJ, Oh DH, Chun SH, Lee SD. Distribution, density, and habitat use of the Korean water deer (*Hydropotes inermis argyropus*) in Korea. *Landscape Ecol Eng.* 2010. DOI 10.1007

Kimura MA. A Simple methods for estimating evolutionary rate of base substitions through comparative studies of nucleotide sequences. *J Mol Evol* . 1980;16:111−120.

Kutkiene L. Investigations of red deer (*Cervus elaphus*) *Sarcocystis* species composition in Lithuania. *Acta Zoologica Lituanica*. 2003;13: 390−395.

Lee BK. Morphological, ecological and DNA taxonomic characteristics of Chinese water deer (*Hydropotes inermis swinhoe*). PhD thesis, Cheongju, Chungbuk National University, Rep. Korea (in Korean) 2003.

Linton AH. Antibiotic resistance: the present situation reviewed. *Vet Record* . 1977;100:354−360.

Malmei R, Seierstad S, Gjerde B. Forekomst av ulike *Sarcocystis−arter*

hos rein i Vest-Finnmark. Degree Project. Norwegian School of Veterinary Science, Oslo. 1996.

Marques FFC, Buckland ST, Goffin D, Dixon CE, Borchers DL, Mayle BA, Peace AJ. Estimating deer abundance form line transect survey of dung: sika deer in southern Scotland. *J Appl Ecol* . 2001;38:349-363

Morrison DA, Bornstein S, Thebo P, Wernery U, Kinne J, Mattsson JG. The current status of the small subunit rRNA phylogeny of the coccidia (Sporozoa). *Int J Parasitol.* 2004;34:501-514.

Odening K. The present state of species-systematics in *Sarcocystis* Lankester (Protista, Sporozoa, Coccidia). *System Parasitol.* 1998;41:209-233.

Park YJ, Kim JS, Joeung DS, Sin MK, Kim KS, Kim TJ. A survey of *Sarcocystis* infections in the slaughtered cattle and identification of *Sarcocystis cruzi*. *Kor J Vet Publ Hlth* . 1994;18:251-259.

Partenheimer-Hannemann C. Untersuchung zum Vorkommen von Sarkosporidien bei Reh-und Rotwild im Raum Bitburg-Prüm area (Rheinland-Pfalz). Inaugural-Dissertation. Hannover, Germany: Tierärztliche Hochschule Hannover. 1991. Accession No.016188692

Powell EC, Pezeshkpour G, Dubey JP, Fayer R. Types of myofibers parasitized in experimentally induced infections with *Sarcocystis cruzi* and *Sarcocystis capracanis*. *Am J Vet Res* . 1986;47:514-517.

Saitou N and Nei M. The neighbor-joining methods: a new methods for reconstructing phylogenetic trees. *Mol Phylogenet Evol*. 1987;4: 406-425.

Spindler KD. Parasites and hormones. In: Mehlhorn H, editor. *Parasitology in focus*. Berlin Heidelberg New York: Springer; 1988:p.465-473.

Šlapeta JR, Iveta Kyselova, Aaron O, Richardson, David Modry, Julius Lukes. Phylogeny and sequence variability of the *Sarcocystis singaporensis* Zaman and Colley. *Parasitol Res*. 2002;88:810-815.

Šlapeta JR, ModryÂ D, VotyÂpka J, Jirku M, Koudela B, Lukes J, Multiple origin of the dihomoxenous life cycle in sarcosporidia. *Int J Parasitol* . 2001;31:413-417.

Son HY, Kim NS, Ryu SY, Kim HC, Rhee JH, Cho JG, Park BK. Ultrastructure of *Sarcocystis grueneri-like* Sarcocysts from cardiac muscle of red deer (*Cervus elaphus*) in Korea. *J Vet Clin*. 2009;26: 595-599.

Stina S, Dahlgren , Bjørn Gjerde. *Sarcocystis* in moose (*Alces alces*): molecular identification and phylogeny of six *Sarcocystis* species in moose, and a morphological description of three new species. *Parasitol Res*. 2008;103:93-110.

Soulsby EJL, Helminths, Arthrophods, protozoa of domesticated animals. 7th ed. London, Balliere Tinadall. 1982;670-691.

Speer CA, Dubey JP. *Sarcocystis wapiti* sp. nov. from the North American wapiti (*Cervus elaphus*). *Can J Zoo.* 1982;60:881-888.

Tamura K, Dundley J, Nei N, Kumar S. MEGA4: Molecular Evolutionary Genetics 110 Analysis (MEGA) software version 4.0. *Mol Biol Evol.* 2007;24:1596-1599.

Tenter AM, Johnson AM. Phylogeny of the tissue cyst-forming coccidia. *Adv Parasitol* . 1997;39:69-139.

Valerius G. Deer of the world. Stackpole books, 1998:pp. 26-28.

Wee SW, Shin SS. Experimental induction of two-host life cycle of *Sarcocystis cruzi* between dogs and Korean native cattle. *Korean J Parasitol* . 2001;39:227-232.

Won CM, Smith KG. History and current status of mammals of the Korean peninsula. *Mammal Rev* . 1999;29:3-36

Yang ZQ, Zuo XY, Yao YG, X.W, Yang GC, Zhang YP. Analysis of the 18S rRNA genes of *Sarcocystis* species suggest that the morphologically similar organisms from cattle and water buffalo should be considered the same species. *Mol Biochem Parasitol*. 2001;115:283-288.

국 문 초 록

한국 고라니에서 발견된 *Sarcocystis grueneri* 의 검출 및 분자생물학적 동정

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*Sarcocystis*는 Coccidea 강의 Apicomplexa 원생생물의 한 속(genus)의 기생충(蟲) 종류 중 하나이다. *Sarcocystis* 종은 중간 숙주와 종 숙주를 가진 외인성(two hosts life)의 생활사를 가지고 있으며, 이 종에 감염이 되면 임상적으로 무증상 이거나 혹은, 면역억제등과 관련되어 조직괴사, 혈관상피세포의 손상 등의 증상이 나타나기도 한다. 사슴과(Cervidae)는 가장 잘 알려진 Sarcocystidae의 전형적인 중간 숙주이며 그 종류로는 *S. grueneri*, *S. rangi, S. tarandivulpes, S. hardangeri, S. rangiferi,* 그리고 *S. tarandi* 등이며 특히, *Sarcocystis*

grueneri-like 종은 이미 한국에서 사육된 붉은 사슴의 사체에서 발견되어 보고 된 적이 있다. 본 연구의 목적은 74마리의 한국 고라니(*Hydropus inermis argyropus*)의 심근에서 분리된 *Sarcocystis greuneri*의 분열체(zoite cyst)를 조직학적 검사를 바탕으로 투과전자현미경(TEM) 관찰을 실시하였고, 18S rRNA 유전자를 이용하여 중합효소연쇄반응을 실시하고, DNA 염기서열 분석(sequencing)을 통해 계통발생학적 분석을 실시하였다. 광학 현미경 관찰을 통해 검출된 *Sarcocystis*의 분열체(zoite cyst)는 타원형에서 구형의 모양으로 그 측정치는 90-170 x 110-380 μm 이며, 조직학적으로 관찰된 심근의 *Sarcocystis* 감염 개체 수는 총 74 마리 중 34마리(45.9%)로 투과전자 현미경으로 확대 관찰하여 전형적인 *S. grueneri* 의 머리카락(hair-like) 모양과 같은 돌출부(protrusion)를 확인할 수 있었다. DNA 염기서열을 통해 얻어진 실험군 *Sarcocystis*의 유전자 서열은 GenBank에 등록시키고 기존에 등록 되어있는 타 종의 중간 숙주(사슴과 포함) 39종의 생물 분류군과 염기서열을 비교한 결과, *S. grueneri* 와는 100%가 *S. capracanis* 와는 97%의 상동성을 보였다. 이러한 18S rRNA 유전자를 바탕으로 실시한 계통발생학적 분석 결과 한국의 고라니에 *Sarcocystis grueneri* 가 감염된 것으로 보이며, 분

자생물학적 방법을 이용한 *Sarcocystis*의 출현률(prevalence)은 처음 보고 되는 것이다.

주 요 어 : 한국 고라니, 기생충, 원생 생물, *Sarcocystis gruneri,* 인수공통전염병

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