Epidemiological and immunological study for intractable infectious diseases in livestock

（家畜の難治性感染症の疫学的および
免疫学的研究）

Nyamsuren Ochirkhuu

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Laboratory of Infectious Diseases

Department of Disease Control

Graduate School of Veterinary Medicine

Hokkaido University

CONTENTS

ABBREVATIONS

aa	aminoacid
Ab	antibodies
Ag	antigen
AMA-1	apical membrane antigen-1
AlHV-1	alcelaphineherpesvirus 1
AL	aleukemic
APCs	antigen-presenting cells
BVDV	bovine viral diarrhea virus
BLV	bovine leukemia virus
BLAST	basic local alignment search tool
bp	base pair
cDNA	complementary DNA
CMEA	council for Mutual Economic Assistance
CP	cytopathogenic
CTL	cytotoxic T-lymphocytes
CTLA-4	cytotoxic T-lymphocyte-associated protein-4
DDW	double distilled water
DDBJ	DNA DataBank of Japan
DNA	deoxyribonucleic acid
DTU	technical university of Denmark
EBL	enzootic bovine leucosis
ELISA	enzyme-linked immunosorbent assay
env	envelope
GAL-9	galectin-9
groEL	the heat-shock protein
HRP	horseradish peroxidase
Ig	immunoglobulin
Ig	immunoglobulin
JD	Johne’s disease
LTR	long terminal repeat
LAG-3	lymphocyte activation gene-3
LB agar	Luria-Bertani agar
MAP	*Mycobacterium avium* subspecies *paratuberculosis*

MCF	malignant catarrhal fever
MEGA 7	molecular evolutionary genetics analysis-7
MPSP	major piroplasm surface protein
msp1b	major surface protein 1
msp4	major surface protein 4
msp5	major surface protein 5
NCP	non-cytopathogenic
ND	neutralizing domain
NK	natural killer cells
OD	optical densities
OvHV2	ovine gammaherpes virus 2
PD-1	programmed cell death-1
PD-L1	programmed cell death-ligand-1
PCR	polymerase chain reaction
PI	persistently infected
PL	persistent lymphocytosis
RAP-1	rhoptry associated protein-1
RNA	ribonucleic acid
RoTat1.2	rode trypanozoon antigen type 1.2
RT-PCR	reverse transcription polymerase chain reaction
SA-MCF	sheep associated-malignant catarrhal fever
TIM-3	T cell immunoglobulin and mucin domain-3
Th cells	T helper cells
Tregs	regulatory T cells
UTR	untranslated region
UV light	ultraviolet light
VSG	variant surface glycoprotein
WA-MCF	wildebeest-associated malignant catarrhal fever

NOTES

The contents of Chapter I have been published in *Veterinary Parasitology.*

- **Ochirkhuu N, Konnai S, Mingala CN, Okagawa T, Villanueva M, Pilapil FM, Murata S, Ohashi K.** 2015. Molecular epidemiological survey and genetic analysis of vector-borne infections of cattle in Luzon island, the Philippines. *Vet Parasitol.* **212**:161-167. © 2015 Elsevier B.V.

The contents of Chapter II have been published in *Japanese Journal of Veterinary Research, Vector-Borne and Zoonotic Diseases*, and *Archives of Virology.*

- **Ochirkhuu N, Konnai S, Odbileg R, Murata S, Ohashi K.** 2015. A preliminary survey of the seroprevalence of *Mycobacterium avium* subspecies *paratuberculosis* in Mongolian cattle. *Jpn J Vet Res.* **63**:191-194. ©
- **Ochirkhuu N, Konnai S, Odbileg R, Nishimori A, Okagawa T, Murata S, Ohashi K.** 2017. Molecular epidemiological survey and genetic characterization of *Anaplasma* species in Mongolian livestock. *Vector Borne Zoonotic Dis*. **17:**539-549. © Mary Ann Liebert, Inc.
- **Ochirkhuu N, Konnai S, Odbileg R, Nishimori A, Okagawa T, Murata S, Ohashi K.** 2016. Detection of bovine leukemia virus and identification of its genotype in Mongolian cattle. *Arch Virol.* **161**:985-991. © Springer-Verlag Wien 2015.
- **Ochirkhuu N, Konnai S, Odbileg R, Odzaya B, Gansukh S, Murata S, Ohashi K.** 2016. Molecular detection and characterization of bovine viral diarrhea virus in Mongolian cattle and yaks. *Arch Virol.* **161**:2279-228. © Springer-Verlag Wien 2016.

PREFACE

Infectious diseases of livestock are a major threat to global animal health and welfare, and their effective control is crucial for raising healthy animals, safeguarding food supplies, and alleviating rural poverty. Animal husbandry is inseperable part of agricultural industry and plays a vital role in social and economic evolution in developing countries such as, the Philippines and Mongolia. In 2016, about 61.5 million heads of livestock, including 25.6 million goats, 27.8 million sheep, 3.6 million horses, 4.1 million cattle, and 0.4 million camels, were reported and about 30% of labor forces belong to the animal husbandry in Mongolia (Mongolian ministry of food, agriculture and light indusrty). In addition, the Philippines is also primarily an agricultural country and agriculture sector is composed of 4 sub-sectors such as, farming, fisheries, livestock, and forestry which together employs about 40% of the labor forces of the country (Philippine statistics authority).

The occurrence of infectious diseases of livestock has been mostly reported from developing countries and causes huge economic loss in the livestock industry (Tomley and Shirley, 2009). Therefore, the survey of pathogens for infectious diseases in livestock is essential as the first step for the control of infections in livestock, and subsequently for public health concern and promotion for animal industry (Tisdell et al., 1999). However, the research and epidemiological surveillances on animal diseases are extremely limited in these countries due to the lack of financial capacity, inconvenient condition of laboratory facility with educated human resourse. Several viral, bacterial and protozoan diseases are likely to be prevalent in livestock of each country because suspected cases have been frequently reported. Thus, this study mainly focused on molecular epidemiological survey of several pathogens that cause intractable infectious diseases of livestock in the Philippines and Mongolia. All of the pathogens that were examined in this study were briefly described in below sections.

Anaplasma marginale is an obligate intra-erythrocytic, rickettsial pathogen, primarily affecting cattle which cause bovine anaplasmosis with serious illness (Aubry and Geale, 2011). As the disease progresses in the host, infected red blood cells are destroyed predominantly in the liver and spleen, resulting in severe anemia, which sometimes causes the death of infected animals (Kosan et al., 2010). *A. marginale* transmits mechanically by biting flies and biologically by ticks; approximately 20 species of ticks have been implicated as vectors of the pathogen (Kocan et al., 2003).

Babesia bovis and *Babesia bigemina* are both obligate intra-erythrocytic, protozoan

parasites and they cause bovine babesiosis in cattle (Hunfeld et al., 2008). Although these pathogens were closely related to each other, they cause remarkably different diseases in cattle due to more virulence of *B. bovis* than *B. bigemina* (Abdela and Jilo, 2016). The clinical signs of the infection usually appear assiociated with the invasion and repeated rounds of asexual multiplication of the parasites in the host's erythrocytes (Gohil et al., 2010). These pathogens are mainly transmitted by ticks such as *Rhipicephalus* (*Boophilus*) *microplus*, which is one of the widely distributed and economically important tick species (Bock et al., 2004).

Theileria spp. are also obligate intracellular hemoprotozoan parasites and cause bovine theileriosis with mild to severe clinical signs in cattle (Mans et al., 2015). The most pathogenic species in cattle are *T. annulata* or *T. parva* which are causative agents of tropical theileriosis or East Coast fever and induce transformation of infected cells including lymphocyte and macrophages/monocytes (Nene et al., 2016). In contrast, *T. orientalis* (historically known as *T. sergenti* and *T. buffeli*) is responsible pathogen for benign theileriosis in cattle and multiplies predominantly within infected erythrocytes (Watts et al., 2016). Therefore, several tick species involve for the transmission of these pathogens and most important tick vectors are the genera of *Hyalomma, Rhipicephalus Amblyomma* and *Haemaphysalis* (Bishop et al., 2004).

Trypanosoma evansi is another important protozoan blood parasite which is causative agent of surra in various animal species (Dabson et al., 2009). The pathogen survives and multiplies in the extracellular fluids such as blood in mammalian hosts, andis transmitted mechanically by various flies such as tabanids and stomoxes (Habila et al., 2012). *T. evansi* develops particular strategies tocause surra with several nonspesific clinical signs such as anaemia, loss of weight, and abortion. The most well-known escape mechanism from host defence is antigenic variation of the pathogen by successively exhibiting various main membrane surface glycoproteins (the variant surface glycoprotein, VSG) (Desquesnes et al., 2013). *T. evansi* causes tenacious infections in cattle with great economic loss through substantial mortality and morbidity, and decrease in milk and meat productions. The similarities of these pathogens such as *T. evansi, A. marginale, B. bovis, B. bigemina,* and *Theileria* spp. are transmitted by vectors such as ticks and flies, and widely distributed in topical and subtropical regions of the world.

Mycobacterium avium subspecies *paratuberculosis* (MAP) is an intracellular bacterium that causes chronic, contagious, invariably fatal enteritis in cattle known as Johne's disease (JD) or paratuberculosis (Möbius et al., 2017). The primary source of the

infection is feces with MAP shedded from infected animals as well as contaminated colostrum or milk. Ingested mycobacteria is taken up by M cells over Peyer's patches in the host and crosses the intestinal epithelial layer and is engulfed by macrophages in which it survives and replicates (Whittington and Sergeant, 2001). The main clinical features of JD in cattle are diarrhea, initially intermittent but becoming persistent and progressive weight loss with normal appetite, which lead to death of the animals (Manning and Collins, 2001). MAP is widely distributed in the world and has been recognized as one of the most costly infectious diseases of dairy cattle (Hasonova and Pavlic, 2006).

Bovine leukemia virus (BLV) belongs to the genus of *Deltaretrovirus* within the family of *Retroviridae*. BLV is closely related to human T-lymphotropic virus type 1, and is a causative agent of enzootic bovine leucosis (EBL) (Sagata et al., 1985). The primary target cell for the pathogen is B lymphocyte in cattle and is integrated into the host genome as a provirus. BLV infection results in a prolonged asymptomatic period but infected cattle are characterized into three disease stages as, aleukemic (AL), persistent lymphocytosis (PL), and leukemia or lymphoma (Kabeya et al., 2001). BLV is transmitted horizontally through blood, colostrum and milk containing infected lymphocytes by insect bites or contaminated items (Ooshiro et al., 2013). In addition, vertical transmission, including perinatal infection *in utero* and in the birth canal, and postnatal infection via colostrum and milk, is also frequently observed (Gutiérrez et al., 2011). BLV infection is widespread over the world except for Western Europian contries, but many countries embark on eradication programmes (Viltrop and Laht, 1996; Nuotio et al., 2003; Acaite et al., 2007; European commission, 2012).

Bovine viral diarrhea virus (BVDV) belongs to the genus of *Pestivirus* within family of *Flaviviridae*, and is classified into two species, namely BVDV 1and BVDV 2 (Liu et al., 2009b). BVDV is transmitted by both horizontally and vertically, and most important source of the virus is persistently infected (PI) cattle that continuously shed the virus in large quantities throughout their life (Wang et al., 2014). BVDV isolates of either genotype can be a cytopathic or non-cytopathic biotype. Although non-cytopathic isolates are responsible for the majority of BVDV infections, cytopathogenicity gives no indication of disease-causing potential (Gamlen et al., 2010). The virus is acquired by the oranasal route, initially replicates in the oranasal mucosa and subsequently, the virus spreads throughout the body byfree in plasma or in association with leukocytes (Lanyon et al., 2013). The clinical presentation can actually manifest in a variety of ways ranging from subclinical to the fatal muscosal disease. BVDV is widely prevalent over the world but many European

countries have initiated control and eradication campaigns in national or regional levels (Ståhl and Alenius, 2012).

Ovine gammaherpesvirus 2 (OvHV2) is classified into the genus of *Macavirus* within the family of *Herpesviridae*, and is a causative agent of sheep associated-malignant catarrhal fever (SA-MCF) in domestic and wild ruminants (Davison et al., 2009). OvHV2 causes inapparent infection in sheep which is a natural host of the virus, but it can cause fatal lympho-proliferative disease in susceptible hosts, such as cattle (Li et al., 2014). The virus is transmitted through respiratory tract and spreads to regional lymph nodes, and viremia is developed after viral multification in infected T lymphocytes (Russell et al., 2009). Clinical signs of the disease in cattle are severe and include necrotizing lesions in the upper respiratory tract and eyes: conjunctivitis and corneal oedema or opacity (O'Toole D and Li, 2014). SA-MCF has a global distribution across many countries, particularly those with a large sheep population or with high levels of consumption of sheep meat (Russell et al., 2009).

Though surveillance of pathogens and understanding of their pathogenic mechanisms are important for the establishment of effective strategy to control of infectious diseases as mentioned above, the other way should focus on the host, and comprehension of host immunity against pathogens is also essential (Whitelaw and Sang, 2005). In general, the host can evolve two types of defence mechanism to increase its fitness when challenged with a pathogen, resistance and tolerance (Schneider and Ayres, 2008). Moreover, it is suspected that Mongolian native animals may be resistant or tolerant against several pathogens compared to other domestic animal breeds or species because there are no visible clinical symptoms in native cattle and yaks during the infection with BVDV, MAP, OvHV2, and *A. ovis* in this study. Therefore, molecular structure of several immunoinhibitory molecules such as programmed cell death 1 (PD-1), programmed cell death-ligand 1 (PD-L1), T-cell immunoglobulin and mucin domain 3 (TIM-3), galectin 9 (GAL-9), lymphocyte activation gene 3 (LAG 3), and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) were identified through cloning and sequencing. These molecules express on several immune cells during chronic infections and cancers, and negatively regulate the immune functions such as the change the manner of homeostasis, activation, and differentiation of effecter T cells (Grosso et al., 2007; Hastings et al., 2009; Gorman and Colgan, 2014; Goldberg and Drake, 2011; Buchbinder and Desai et al., 2016; Das et al., 2017). The comparative investgation of these immunoinhibitory molecules in Mongolian native cattle and yak is important to determine the differences with regards to

their reaction to pathogens.

In this study, molecular epidemiological survey and genetic characterization of several vector-borne pathogens such as *A. marginale, B. bovis, B. bigemina, Theileria* spp. and *T. evansi* in cattle in Luzon island, the Philippines in Chapter I, and other bacterial and viral pathogens such as MAP, *A. ovis*, BLV, BVDV and OvHV2 in cattle, yaks, sheep and goats in Mongolia in Chapter II were performed to provide useful information for the control and prevention strategy against infectious diseases in livestock. In addition, molecular characterization of immunoinhibitory molecules, such as PD-1, PD-L1, TIM-3, GAL-9, LAG 3 and CTLA-4, of Mongolian native cattle and yak were performed in Chapter III as the first step to understand the disease progression during chronic infection.

CHAPTER I

Molecular epidemiological survey and genetic analysis of vector-borne infections of cattle in Luzon island, the Philippines

I.1.Introduction

Vector-borne diseases are various infections that are transmitted by the bite of infected arthropod species, such as mosquitoes, ticks, flies, sandflies, fleas, and bugs. Anaplasmosis, babesiosis, theileriosis, and trypanosomiasis are most common vector-borne diseases in cattle in tropical regions of the world and cause significant economic loss for animal industries.

Bovine anaplasmosis is caused by *Anaplasma marginale* (*A. marginale*) which is a rickettsial gram-negative, intra-erythrocytic pathogen within the *Anaplasma* species (Kocan et al., 2010). *A. marginale* is the most concerning species for cattle regarded as the causing serious illness consequently leading to significant economic loss in cattle industry (Kosan et al., 2015). Infected cattle develop mild to severe clinical signs such as, fever with depression followed by weight loss and progressive anemia, and icterus (Kocan et al., 2010). The pathogenis widely distributed worldwide, North and South America, Europe, Africa, and Asia including the Philippines (Mingala et al., 2009), and can be transmitted mechanically by biting flies and biologically by ticks; about 20 species of ticks have been implicated as vectors of the pathogen (Kocan et al., 2003).

Bovine babesiosis is a tick borne, intra-erythrocytic protozoan disease and caused by *Babesia bovis* (*B. bovis*) and *Babesia bigemina* (*B. bigemina*) within the genus of *Babesia* species (Hunfeld et al., 2008). Infected cattle show severe clinical signs including continuous fever, high parasitemia, anemia, icterus, and often hemoglobinuria (Abdela and Jilo, 2016). The pathogens of the disease are mostly distributed to tropical and subtropical regions of the world and cause great economic losses particularly in developing countries (Abdela and Jilo, 2016). The main vectors of these pathogens are ticks within the genus of *Boophilus* and *Rhipicephalus* (Bock et al., 2004).

Theilerioses is also a tick borne protozoan disease in ruminants caused by blood parasites belonging to the genus of *Theileria* (Dolan et al., 1989). There are a number of *Theileria* species that infect cattle and cause mild to severe disease with fever, lymphadenopathy, leukopenia, anorexia, depression, anemia, jaundice, weight loss, and sometimes death ofinfected cattle (Abdela and Bekele, 2016). The most pathogenic species are *T. annulata* and *T. parva* whereas *T. orientalis* (also known historically as *T. sergenti* and *T. buffeli*) is usually benign disease in infected cattle but it causes heavy production losses in the cattle industry (Mans et al., 2016). Geographically, *T. parva* is only distributed to the African region whereas *T. annulata* is wide spread throughout the Mediterranean basin, the Middle East and Asia. In contrast, *T. orientalis* is mainly found in

Asia and Australia (Sugimoto and Fujisaki, 2002) Therefore, several tick species are involved for the transmission of these pathogens and most important tick vectors are the genera of *Hyalomma*, *Rhipicephalus Amblyomma* and *Haemaphysalis* (Jongejan and Uilenberg, 2004).

Surra, caused by *Trypanosoma evansi* (*T. evansi*), which belongs to the genus of *Trypanosoma*, is an important protozoan blood disease of various species of vertebrate animals (Desquesnes et al., 2013). The pathogen of the disease is mostly distributed to tropical and semi-tropical regions of the world and is mainly transmitted mechanically by various *tabanids* and other flies (Habila et al., 2012). The animals infected with the pathogen show acute to chronic disease with the severity of the clinical signs such as, fever weight loss or wasting, lethargy, signs of anemia and enlargement of the lymph nodes (Reid, 2002). In addition to illness and deaths, surra causes economic losses through decreased productivity in farm animals such as, reduced weight gain, decreased milk yield, disorder of reproduction and spend the treatment costs (Dabson et al., 2009).

The Philippines is an agricultural country located in the Southeast Asia, and several vector-borne diseases have been reported in the livestock populations. In previous surveys conducted in the Philippines, the prevalence of *B. bigemina*, *A. marginale*, and *T. evansi* in the water buffaloes were 10.3%, 4.4%, and 2.9%, respectively (Konnai et al., 2008; Mingala et al., 2009). More recently, surveys for vector-borne pathogens have been conducted in Cebu island (Ybañez et al., 2013b, c). However, the prevalence and genetic diversity of these pathogens have not been studied in Luzon island, which is the main and largest island in the Philippines. Thus, the aims of this study were to detect various bovine tick-borne pathogens, including *A. marginale*, *B. bigemina*, *B. bovis*, *Theileria* spp., and *T. evansi*, and understand the genetic diversity of these pathogens.

I.2. Materials and Methods

I.2.1. Blood sample collection

Three hundred thirty-nine bovine blood samples were collected from two dairy farms on the Luzon island of the Philippines in November and December, 2012. Blood (5 ml) was collected from the jugular vein using a BD K3EDTA Vacutainer tube (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The blood samples were stored at 4 °C until DNA extraction.

I.2.2. DNA extraction

Genomic DNA was extracted from the blood samples using theGenomic DNA Purification kit (Promega, Madison, WI, USA), according to the manufacturer's instructions. Total DNA was diluted with 100 µl conservation buffer and stored at -30 °C until further use.

I.2.3. The polymerase chain reaction assays for detection of pathogens

The primers for polymerase chain reaction (PCR) in this study wereshown in Table I-1-1. *A. marginale* and *B. bovis* were screened by nested PCR assay based on *16S rRNA* (Weisburg et al., 1991; Ybañez et al., 2013a) and *rhoptry-associated protein* (*RAP*)-*1* (Figueroa et al., 1993), whereas *B. bigemina*, *Theileria* spp., and *T. evansi* were detected using single-step PCR based on *apical membrane antigen* (*AMA*)-*1* (Sivakumar et al., 2012), *major piroplasm surface protein* (*MPSP*) (Kakudai et al., 1998) and rode trypanozoon antigen type 1.2 (*RoTat1.2*) (Claes et al., 2004) genes, respectively. All these PCR assays were conducted as previously described methods with slight modifications. Briefly, 1.5 µl (100 ng) of a DNA sample was added to 28.5 µl of reaction mixture that comprised of 3 µl of 10 × buffer (Takara Bio Inc., Shiga, Japan), 2.4 µl of dNTPs (Takara Bio Inc.), 2 µl of 10 µM of each forward and reverse primers (Hokkaido System Science Co. Ltd., Sapporo, Japan), 0.1 µl of DNA Taq polymerase (Takara Bio Inc.), and 21.5 µl of double distilled water. PCR amplifications were performed under the following thermal cycle conditions: initial denaturation at 94 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 30 sec, annealing at each optimal temperature for 30 sec, extension at 72 °C for 1 min, and a final synthesis at 72 °C for 7 min using the GeneAmp PCR System 9700 (Applied Biosystems, USA). The identities of the amplified PCR products (expected sizes of approximately 875 bp, 356 bp, 211 bp, 852 bp, and 205 bp for *A. marginale*, *B. bovis*, *B. bigemina*, *Theileria* spp., and *T. evansi*) were confirmed by

electrophoresis on 1.5% agarose gel and visualized under ultraviolet (UV) light.

I. 2.4.DNA cloning and sequencing

Eight positive samples for each pathogen were subjected to the sequencing analysis. The extracted PCR products by using the FastGene gel/PCR Extraction kit (Nippon Genetics, Tokyo, Japan) were ligated into the pGEM-T Easy vector (Promega), and the plasmid was introduced into the *E. coli* strain DH5α, plated on a Luria–Bertani (LB) agar (Invitrogen, Carlsbad, CA, USA), and cultured in LB broth (Invitrogen). The plasmid DNAs from the positive clones were extracted from the LB culture using the FastGene Plasmid Mini kit (Nippon Genetics). The sequence amplifications of the plasmids were performed using the GeneAmp PCR System 9700 (Applied Biosystems). The quality of the plasmid preparation of each gene of the pathogen were checked by NanoDrop 8000 analytic equipment (Thermo Fisher Scientific Corporation, USA), and the sequencing analysis of the pathogen was carried out with the CEQ8000 DNA analysis system (Beckman Coulter Inc Company, USA).

I. 2.5. Phylogenetic and homology analyses

The obtained sequences were analyzed using the Bio-Edit software (Hall, 1999) and basic local alignment search tool application (BLAST). Phylogenetic trees for each pathogen were constructed by theMEGA 7 software (Tamura et al., 2007) with the neighbor-joining method (Saitou and Nei, 1987) by usingthe sequences which were identified in this study and database sequences from other countries deposited in GenBank.

Table I-1-1. The target genes and primer sequences of the pathogens in this study

Pathogen	Name of target gene	Oligonucleotide sequence (5´-3´)	Amplification size (bp)	Reference
A. marginale	*16S rRNA*	AGAGTTTGATCCTGGCTCAG	1500	Weisburg et al.,1991
		ACGGCTACCTTGTTACGACTT		
		TACGCAGCTTGCTGCGTGTATG	877	Ybanez et al., 2013c
		GCCCTTCTGTTAAGAAGGATCTAG		
B. bovis	*RAP-1*	CACGAGGAAGGAACTACCGATGTTGA	356	Figueroa et al., 1993
		CCAAGGAGCTTCAACGTACGAGGTCA		
		TCAACAAGGTACTCTATATGGCTACC	298	Figueroa et al., 1993
		CTACCGAGCAGAACCTTCTTCACCAT		
B. bigemina	*AMA-1*	TACTGTGACGAGGACGGATC	211	Sivakumar et al., 2012
		CCTCAAAAGCAGATTCGAGT		
Theileria spp.	*MPSP*	CACGCTATGTTGTCCAAGAG	852	Kakuda et al., 1998
		TGTGAGACTCAATGCGCCTA		
T.evansi	*RoTat 1.2*	GCGGGGTGTTTAAAGCAATA	205	Claes et al., 2004
		ATTAGTGCTGCGTGTGTTCG		

I. 3. Results

I.3.1. Prevalence of vector-borne pathogens in cattle

Out of the 339 samples, 324 (95.5%), 154 (45.4%), 209 (61.6%), 140 (41.3%), and 2 (0.6%) were positive for *A. marginale*, *B. bovis*, *B. bigemina*, *Theileria* spp., and *T. evansi* infections, respectively (Table I-1-2). The most prevalent pathogen was *A. marginale* compared to the other pathogens. Furthermore, mixed infections were also detected in 291 (85.8%) samples that include 115 (33.9%) samples with two kinds of pathogens, 144 samples (42.5%) with three kinds of pathogens, and 31 (9.1%) samples with four kinds of pathogens.

I.3.2. Molecular characterizaion of the pathogensdetected in this study

The phylogenetic analysis showed that all of the identified sequences based on the *16S rRNA* gene of *A. marginale* (LC007100) were 100% identical to each other and the isolates from other countries such as China, Japan, the Philippines (Cebu), Israel, and USA (Fig. I-1-1). Three independent sequences for *B. bovis* based on the *RAP-1* gene were identified, and were 92.27%–100% homologies with one another and other reference sequences from several countries. Most sequences as representative (LC006976) were identical to the isolates from South Africa (KC894405 and KC894397), Brazil (FJ588012 and FJ588013), Argentina (AF030062), and the Philippines (Cebu) (LC006976). Other 2 isolates were divergent: one (LC006978) was identical to the sequences from USA, Argentina, and Sri Lanka, and the other one (LC006977) was identical to the isolate from Sri Lanka (AB690859) (Fig. I-1-2). Four independent sequences of *B. bigemina AMA-1* gene with 97.67%–100% homologies to each other were identified. Especially, these sequences were 97.07%–100% identical to the reference sequences derived from Cebu, the Philippines (JX860289, JX860290, JX860293, and JX860294) (Fig. I-1-3). Finally, five independent sequences of *Theileria* spp. based on the *MPSP* gene were identified, and were 83.51%–100% similar to each other. These sequences were genetically classified into *T. orientalis* which is benign group of *Theileria* spp. and were grouped together with isolates from other countries such as Australia, Taiwan, Russia, Japan, Korea, and China (Fig. I-1-4).

Table I-1-2. Prevalence of vector-borne diseases in cattle in the Luzon island

Detected pathogens	**No. of positive cattle[a] (%)**
A. marginale	324 (95.5)
B. bovis	154 (45.4)
B. bigemina	209 (61.6)
Theileria spp.	140 (41.3)
T. evansi	2 (0.6)
Infected with 2 kinds of pathogens	115 (33.9)
A. marginale+*B. bovis*	30 (8.8)
A. marginale+*B. bigemina*	50 (14.7)
A. marginale+*Theileria* spp.	30 (8.8)
B. bigemina+*B. bovis*	3 (0.9)
B. bovis+*Theileria* spp.	1 (0.3)
A. marginale+*T. evansi*	1 (0.3)
Infected with 3 kinds of pathogens	144 (42.5)
A. marginale+*B. bovis*+*B. bigemina*	67 (19.8)
A. marginale+*B. bovis*+*Theileria* spp.	20 (5.9)
A. marginale+*B. bigemina*+*Theileria* spp.	56 (16.5)
B. bovis+*B. bigemina*+*Theileria* spp.	1 (0.3)
Infected with 4 kinds of pathogens	31 (9.1)
A. marginale+*B. bovis*+*B. bigemina*+*Theileria* spp.	31 (9.1)

No. of tested cattle were 339.

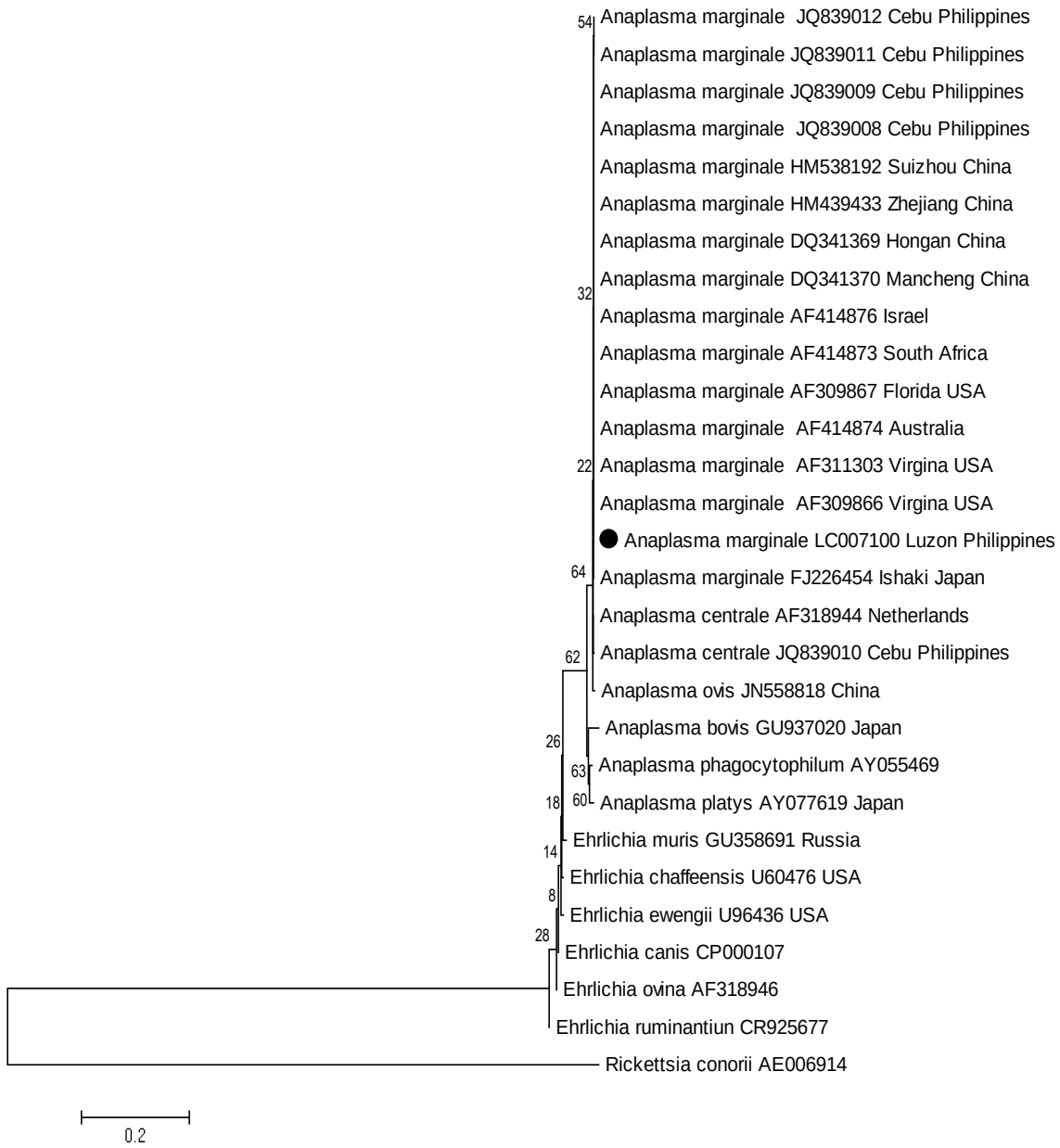


Fig.I-1-1. Phylogenetic relationship of *A. marginale* based on the *16S rRNA* gene. Sequence derived from this study is presented with abullet. The tree was constructed with the neighbor-joining method and was supported by 1,000 bootstrap replications. This figure shows the relationship between 875 bp sequences of the *16S rRNA* gene of *A. marginale* obtained from this study and related other sequences from the GenBank. The DNA sequence identified in this study was deposited to DDBL with accession number LC007100.

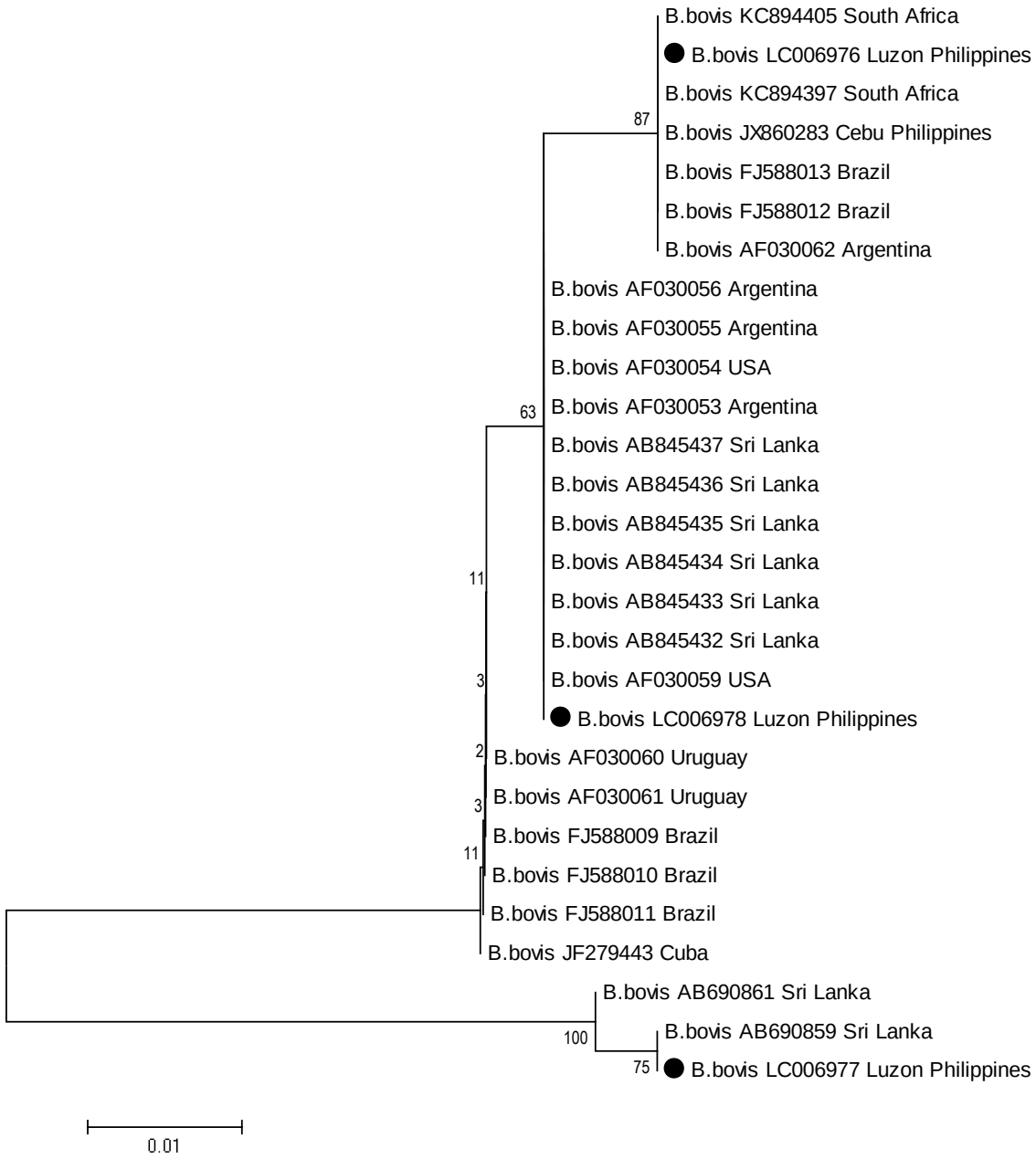


Fig.I-1-2. Phylogenetic relationship of *B. bovis* based on the *RAP-1* gene. Sequences derived from this study are presented with bullets. The tree was constructed with the neighbor-joining method and was supported by 1,000 bootstrap replications. This figure shows the relationship between 298 bp sequences of the *RAP-1* gene of *B. bovis* obtained from this study and related other sequences from the GenBank. The DNA sequences identified in this study were deposited to DDBJ with accession numbers, LC006976, LC006977, LC006978, and LC006979, respectively.

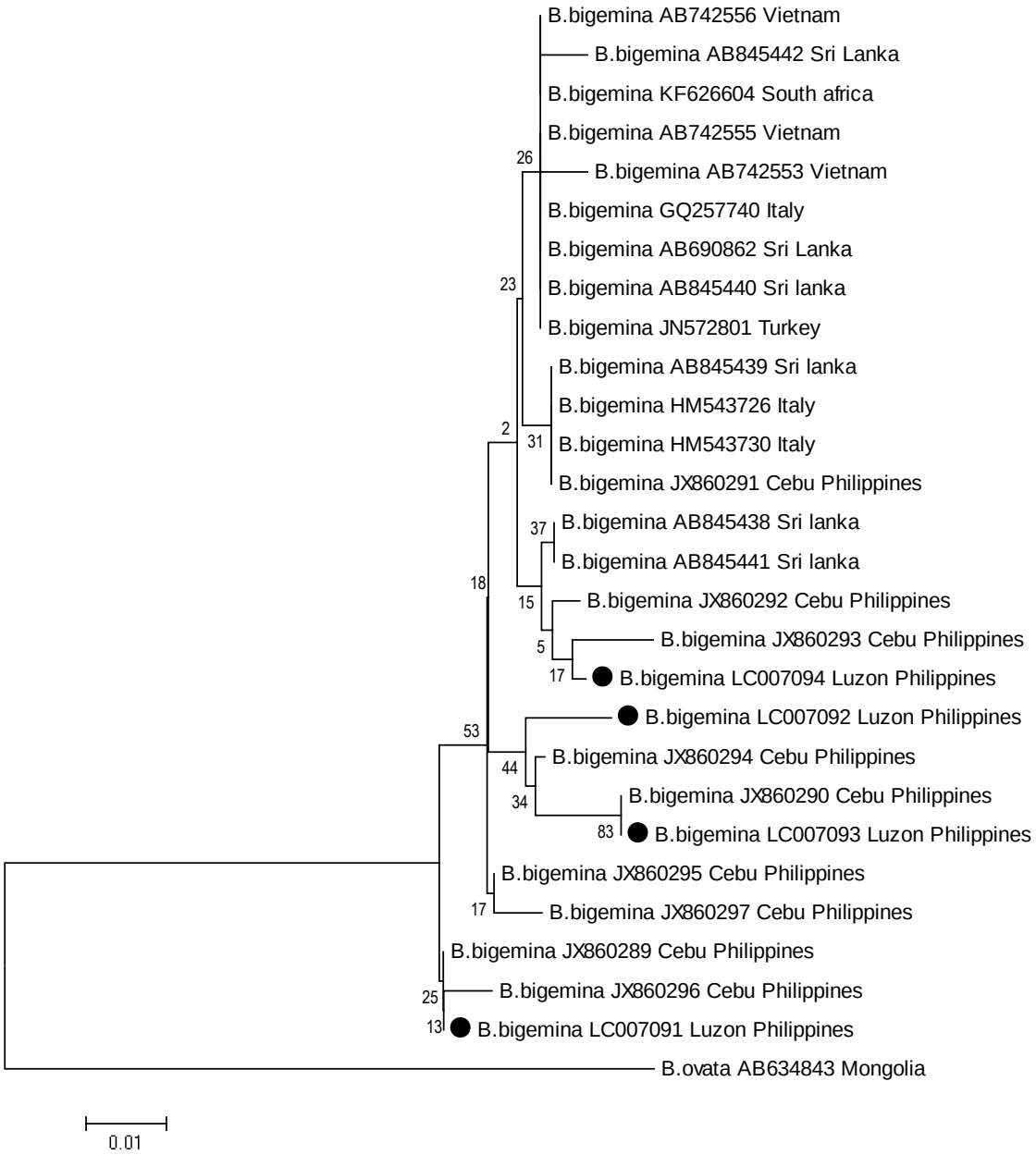


Fig.I-1-3. Phylogenetic relationship of *B. bigemina* based on the *AMA-1* gene. Sequences derived from this study are presented with bullets. The tree was constructed with the neighbor-joining method, and was supported by 1,000 bootstrap replications. This figure shows the relationship between 211 bp sequences of *AMA-1* gene of *B. bigemina* obtained from this study and related other sequences from the GenBank. The DNA sequences identified in this study were deposited to DDBJ with accession numbers, LC007091, LC007092, LC007093, and LC007094, respectively.

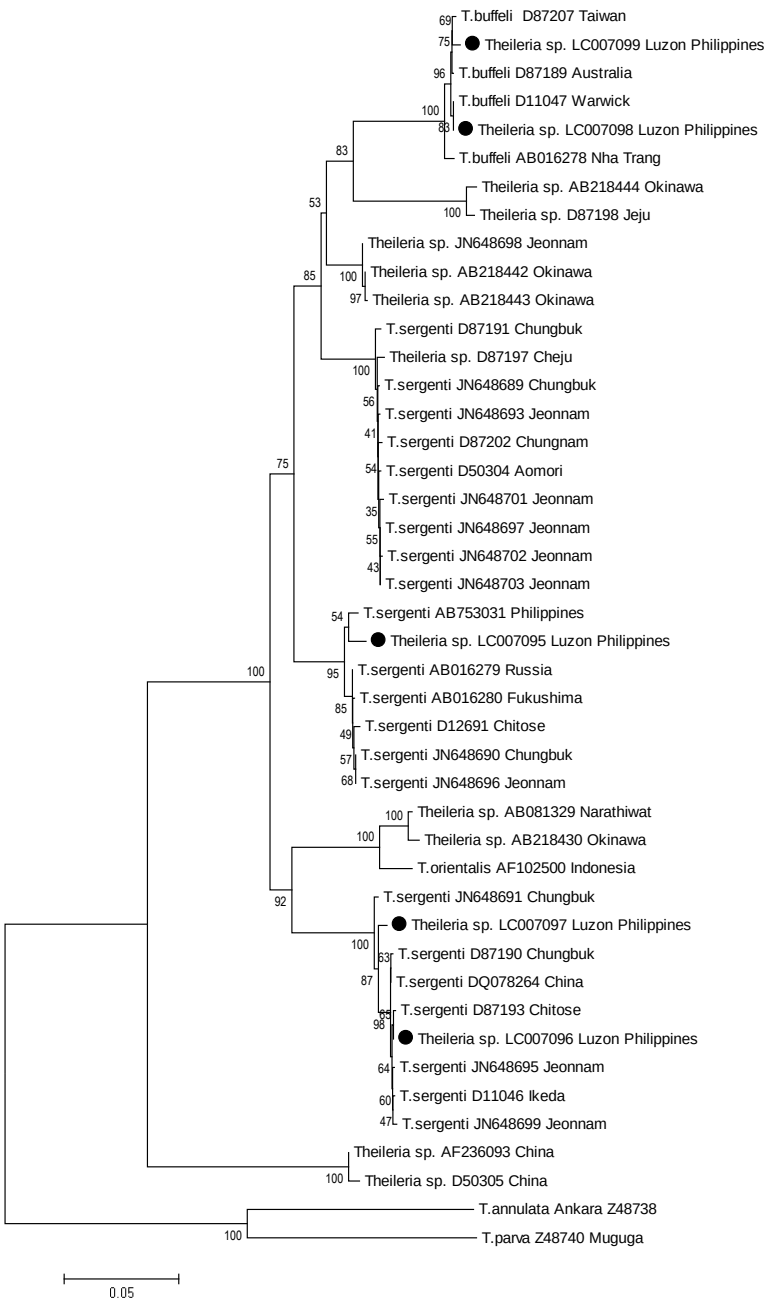


Fig.I-1-4. Phylogenetic relationship of *Theileria* spp. based on the MPSP genes. Sequences derived from this study are presented with bullets. The tree was constructed with theneighbor-joining method, and was supported by 1,000 bootstrap replications. This figure shows the relationship between 852 bp sequences of the *MPSP* gene of *Theileria* spp. obtained from this study and related other sequences from the GenBank. The DNA sequences identified in this study were deposited to DDBJ with accession numbers, LC007095, LC007096, LC007097, LC007098, and LC007098, respectively.

I.4. Discussion

The prevalence and molecular epidemiological survey of several tick-borne pathogens, such as *A. marginale*, *B. bovis*, *B. bigemina*, and *Theileria* spp. in cattle in the Luzon island, the Philippines, were investigated in the study. Although some researchers have already reported the vector-borne infections in several parts of the Philippines, no study in this particular island has been published. In the result, the prevalence of *A. marginale* was very high as 95.5%, and was also higher than previous reports such as 10.3% for water buffalo and 19.8% for cattle (Mingala et al., 2009; Sivakumar et al., 2014). These finding seems to be supported by other reports that cattle were more susceptible than water buffalo during *A. marginale* infection (Rajput et al., 2005). *A. marginale* sequences targetingthe *16S rRNA* gene showed that they were 100% identical with each other, and were formed same lineage to the sequences from countries such as USA, China, Japan, Israel, Australia, and the Philippines.

The prevalences of *B. bovis* and *B. bigemina* were 45.4% and 61.6%, respectively, which were higher than previous epidemiological data for each of the infections (Ybañez et al., 2013c; Yu et al., 2013). The earlier studies reported that the prevalencesof *B. bovis* and *B. bigemina* in cattle were 10.8% and 6.4% in other 5 different regions (Yu et al., 2013) as well as 10% and 15.4% in the Cebu island in the Philippines (Ybañez et al., 2013c). The nucleotide alignment of *B. bovis* based on the *RAP-1* gene showed that 6 sequences were 100% identical and other 2 sequences were 92.27%–99.26% similar to one another. In the phylogenetic tree, these sequences were similar to the isolates from several other countries such as, Brazil, Argentina, South Africa, the Philippines, Sri Lanka, and the USA. In addition, the nucleotide alignment of the *AMA-1* gene of *B. bigemina* showed that 5 sequences were 100% identical and another 3 sequences were 97.67%–98.84% homologyto each other. The phylogenetic analyses showed that they were same and similar lineage as the isolates from the Cebu island of the country.

Furhermore, the prevalence of *Theileria* spp. was 41.3%, which was higher than that of the previous report as 14.7% for cattle in the different region of the Philippines (Belotindos et al., 2014). In addition, a previous study aimed to detect *T. annulata* and *T. orientalis* but no positive samples were identified for these pathogens in cattle in this country (Ybañez et al., 2013c). *MPSP* gene sequences of *Theileria* spp. from cattle in the Philippine showed high divergence among the isolates as 83.51%-100% homology to one another, and were belonged to *T. orientalis*. The previous researches stated that benign groups of *Theileria* spp. were mainly distributed tothe sub-tropical zones, such as *T. orientalis* of Japan and *T.*

buffeli of Australia (Sugimoto and Fujisaki, 2002). This finding was consistent with the phylogenetic tree constructed in this study, and confirmed the distribution sites of the benign groups of *Theileria* spp. that were mainly found in several Asian countries such as, China, Japan, Russia, Korea, Taiwan, Indonesia, and Australia.

Mixed infections containing 2-3 pathogens were observed in the present study (85.2% of the samples). Out of the samples with mixed infections, 33.6% had 2 kinds of pathogens, 42.5% had 3 kinds, and 9.1% had 4 kinds of pathogens. Most of the co-infection and infection with multiple pathogens were caused by *A. marginale* + *B. bigemina*, and *A. marginale* + *B. bovis* + *B. bigemina*, respectively. In addition, *A. marginale* was present with almost all other pathogens. Thus, the mixed infections in previous studies in the Philippines were lower compare to those of the present study: most of the infected samples with two and three kinds of pathogens were *A. marginale* + *B. bigemina* (8.1%) and *Anaplasma* spp. + *B. bovis* + *B. bigemina* (1%), respectively (Ybañez et al., 2013c). Although clinical cases were not observed, it was possible that the infected cattle with multiple pathogens may be involves more pronounced clinical signs or hematological abnormalities than those infected with only a single pathogen (Hofmann-Lehmann et al., 2004). Therefore, it would be interesting to compare the pathological progression between single and mixed infections in the susceptible cattle (Ybañez et al., 2013c). These observations suggested that *A. marginale*, *B. bovis*, *B. bigemina*, and *T. orentalis* are potential pathogens that cause mixed infection.

In addition, many ticks especially, *Riphicephalus microplus* species which is the principal vector of *B. bovis* and *B. bigemina* were observed in forage grasses and cattle skin during the sample collection. Moreover, *A. marginale* can be transmitted by most of thetick species, including *Riphicephalus microplus* (Ybañez et al., 2013b) and through mechanical transmission by biting flies, suggesting that these pathogens would have been transmitted by the same tick species. It is recommended that farmers, local veterinarians, veterinary epidemiologists, and local government units in the Philippines should cooperate in the prevention and control of tick-borne diseases, such as anaplasmosis, babesiosis, and theilerosis, as these may cause considerable economic losses. Therefore, extensive and comparative epidemiological studies of these pathogens are vital to elucidate the geographical distribution in all areas of the Philippines.

I.5. Summary

The vector-borne diseases such as anaplasmosis, babesiosis, theileriosis and surra are the most common infections in cattle of tropical regions of the world and cause significant economic loss for animal industries. Molecular epidemiological survey and genetic characterization of the pathogens of these infectious diseases were performed with 339 samples from cattle in the Luzon island, the Philippines. As the results, 95.5%, 45.4%, 61.6%, 41.3%, and 0.6% were positive for *A. marginale*, *B. bigemina*, *B. bovis*, *Theileria* spp., and *T. evansi* infections, respectively. Mixed infections were detected in 85.5% samples, of which 33.9% had 2 pathogens, 42.5% had 3 pathogens, and 9.1% had 4 kinds of pathogens. *A. marginale* based on the *16S rRNA* gene sequences were 100% identical to one another and the isoltes from other countries. The sequences of the *B. bovisRAP-1* and *B. bigemina AMA-1* genes were 92.27%-100% and 97.07%-100% homology to one another and were same or similar to the isolates mainly from Asia. *Theileria* spp. based on the *MPSP* gene sequences were 83.51%-100% identical with one another and were grouped into the *T. orientalis.* These results indicate that infection rates of the vector-borne pathogens in cattle were extremely high in this area and the control of these infections is needed. These findings provide vital information that can be used for the planning and execution of effective control measures for vector-borne diseases in cattle industries of the Philippines.

CHAPTER II

Molecular epidemiological survey and genetic analysis of intractable infectious diseases in Mongolian livestock

II.1. Seroprevalence of *Mycobacterium avium* subspecies *Paratuberculosis* in Mongolian cattle

II.1. 1. Introduction

Paratuberculosis or Johne's disease (JD) is caused by the infection with *Mycobacterium avium* subspecies *paratuberculosis* (MAP). JD has been mainly reported in various vertebrate species such as, cattle, goats, sheep, deer, bison (Manning and Collins, 2001; Carta et al., 2013). Although calves are more susceptible for the infection, adult cattle get the infection in case of high dosage of MAP exposure. In most case, cattle get infection at early age, but the disease symptoms do not appear until they become adults (Collins 2003). Therefore, MAP infections have been extensively studied in dairy cattle than in other domestic animals because the disease causes significant economic loss in the affected dairy cattle, and there is still no effective treatment (Good et al., 2009). The economic loss due to the JD is associated with decreased milk production concurrent with an increased incidence of mastitis, changes in milk parameters, increased somatic cell counts, reproductive dysfunction, and increased predisposition to other diseases (Manning and Collins 2001).

Main clinical symptoms of JD are persistent diarrhea and progressive weight loss in cattle and primary source of the infection is contaminated feces. In addition, calves get infection from the mother by *in utero* route (Hasonova and Pavlic 2006). Cattle or sheep may also acquire infection through contaminated pastures shared with wildlife (Carta et al., 2013). MAP is widely distributed all over the world and has been recognized as one of the most costly infectious disease of dairy cattle. The prevalence of MAP infection in cattle herd was 21.4% in Ireland (Good et al., 2009), 70.4% in USA (Lombard et al., 2013), 9.8-43.1% in Canada, 80-86% in Denmark, 20-71% in the Netherlands, and 27.6-42.5% in UK (Geraghty et al., 2014). In addition, the reported prevalence in Latin American and Caribbean countries was 16.9%, with 75.8% in cattle, 16% in sheep, and 3.7-4.3% in goats (Fernández-Silva et al., 2014). In the past, several countries, including France, Iceland, Norway, USA, UK, and Australia have initiated programs to control MAP infections in sheep, goats, and cattle. Based on the results of these various eradication programmes, it is evident that successful eradication programmes for sheep, goats, and cattle will only be possible with the use of various diagnostic tests, vaccination, restriction of animal movements, and preventive management measures (Benedictus et al., 2000)

Animal husbandry is one of the major sources for the national economy and

employment to the country and more than 61.5 million livestock, including sheep, goats, cattle, horses, and camels were reported in 2016 (Mongolian ministry of food, agriculture and light industry). Numbers of dangerious animal diseases have been occurredin Mongolian livestock during last 10 years such as, foot-and-mouth disease, pesti des petits ruminants, sheep and goat poxes and equine influenza. Conversely, several bacterial diseases occur in cattle population, such as brucellosis, anthrax, leptospirosis, tuberculosis, pasteurellosis, listeriosis, and blackleg (Odontsetseg et al., 2005). However, there is no information on MAP infection in Mongolian livestock. The main purpose of the present study was to determinethe prevalence and distribution of MAP infection in Mongolian cattle.

II.1.2. Materials and Methods

II.1.2.1. Blood sample collection

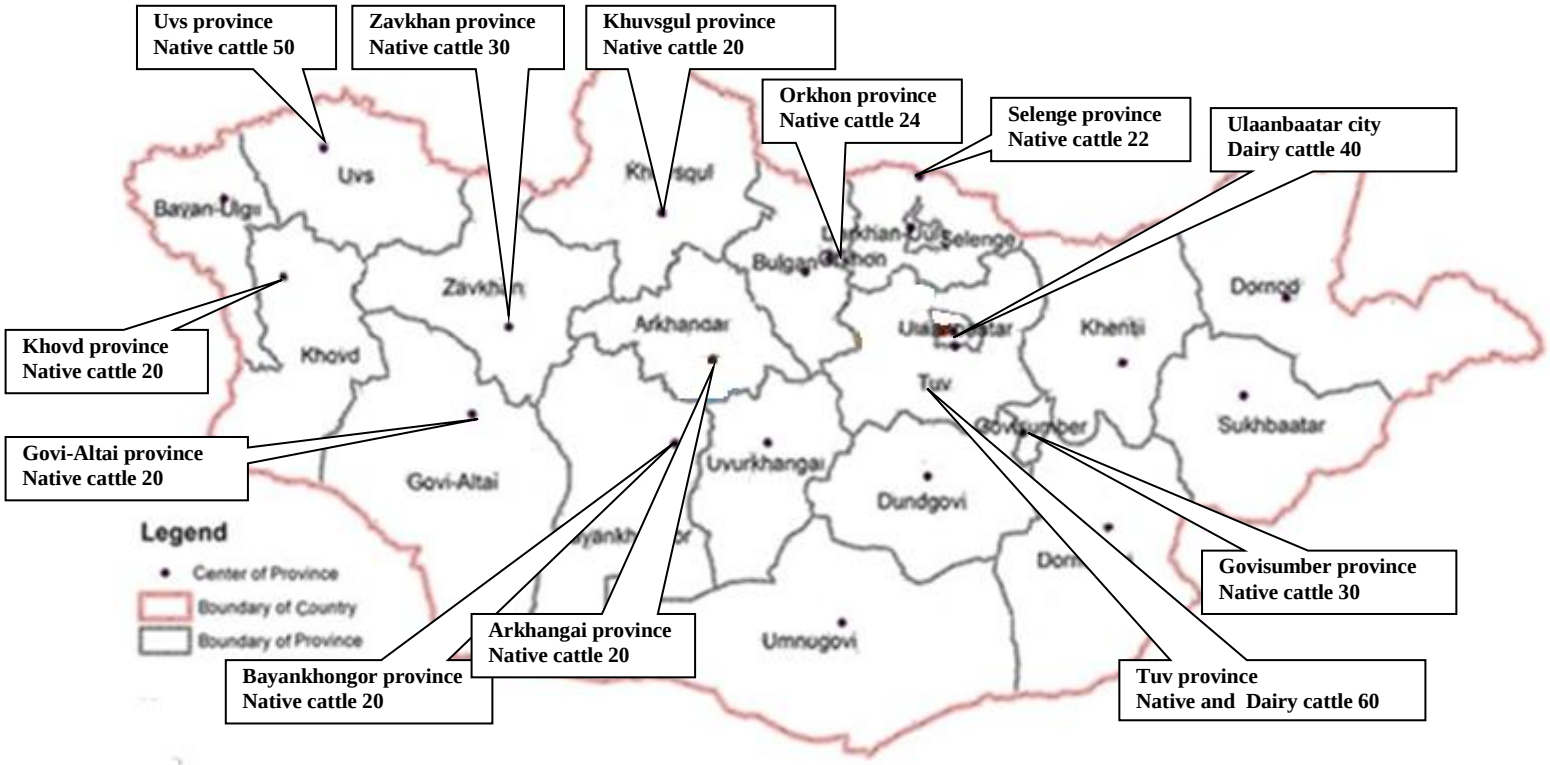
A total of 356 cattle serum samples (20–50 serum samples from each province) was selected for this survey, out of 1805 samples which were kept in Institute of Veterinary Medicine, Mongolian University of Life Science. These serum samples were collected in 2011-2014 in Ulaanbaatar city and other eleven provinces, including Uvs, Khovd, Zavkhan, Govisumber, Govi-Altai, Bayankhongor, Selenge, Arkhangai, Tuv, Khuvsgul and Orkhon (Fig. II-1-1). Most of the cattle used for sampling were 3-8 years old, but several calves which had diarrhea were also included. Blood was collected from the jugular vein of each animal using vacutainer tube with serum clot activator (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Tubes were kept in upright position at room temperature for about 3 hrs after the collection, followed by the separation of serum into 1.5 ml tubes.

II.1.2.2. ELISA assays for MAP detection

The serum samples were tested for specific antibodies against MAP using a commercial ELISA test kit (Kyoritsu Seiyaku Corporation, Tokyo, Japan) according to the manufacturer's protocol. Briefly, the samples were first incubated in a buffer containing *Mycobacterium phlei* extracts in order to absorb non-specific antibodies for 15 minutes at room temperature. Absorbed 100 µl /wells of seraand controls were then incubated on ELISA plate pre-coated with purified whole antigen of MAP for 45 minutes at room temperature. In this step, the antibodies (Ab) specific to MAP present in positive serum samples will bind the antigen (Ag) in the wells. Then, plates were washed 3 times with washing buffer for the elimination of unbound substances. After that, 100 µl/well of anti-bovine IgG antibody conjugated with horseradish peroxidase (HRP) was added and incubated for another 45 minutes at room temperature. Consequently, the excess of this conjugate is eliminated by a second series of washes 3 times same as mentioned aboveand its attachment was revealed with a 100 µl/wells of peroxidase substrate for 15 minutes at room temperature in a dark place. Following this incubation, the conjugate, if present, reacts with the substrate and a blue color develops. The reaction was then stopped by adding 100 µl/well of 2M sulfuric acid and the optical densities (OD) at 450 nm were read by Titertek Multiskan PLUS MK II Microplate Reader Typ 314 (Labsystems, Finland). The required OD values for test validity were confirmed as followed, OD negative is less than 0.25 and OD positive is between 0.8 and 1.6 in successful test.

ELISA value calculation method=$\frac{OD(sample)-OD\ (negative\ control)}{OD(positive\ control)-OD\ (negative\ control)}$

Fig.II-1-1. Map of the sampling areas in Mongolia for ELISA assay



II.1.3. Results

Out of the 336 samples tested, 3 (0.84%) were found positive for MAP. The seropositive bovine samples were derived from 3 independent sampling sites, cattle from Tsenkher soumof Arkhangai province, Murun soum of Khuvsgul province belonging to pasture based cattle herds and the cattle from Bornuur soum of Tuv province belonging to intensive farming cattle herd (Table II-1-1). Out of the 3 positive animals, only six months old a calf showed diarrhea. Distances between these positive sampling areas were approximately five hundred km far from one another. All sampling areas belonged to the forest area of the country.

Table II-1-1. Seroprevalence of *Mycobacterium avium* subspecies *paratuberculosis* in Mongolian cattle

Name of province	Number of tested samples	Number of positive (Mean seroprevalence)	Note of positive animals	SP-value
Uvs	50	0		
Khovd	20	0		
Zavkhan	30	0		
Govisumber	30	0		
Govi-Altai	20	0		
Bayankhongor	20	0		
Selenge	22	0		
Arkhangai	20	1 (5.00%)	MNC[1)], F[2)], 8 years old	1.2
Tuv	60	1 (1.67%)	HS[3)], F, 5 years old	0.6
Ulaanbaatar	40	0		
Khuvsgul	20	1 (5.00%)	MNC, F, 6 months old	0.3
Orkhon	24	0		
Total (12 provinces)	356	3 (0.97%)		

1. Mongolian native cattle
2. Female
3. Holstein breed

II.1.4. Discussion

In this survey, the seroprevalence of MAP in Mongolian cattle was studied for the first time and 3 positive cattle (0.84%) were found from Tsenkher soum of Arkhangai province, Murun soum of Khuvsgul province and Bornuur soum of Tuv province. The prevalence of MAP in Mongolian cattle is much lower than those in other counties such as, 21.4% in cattle herd of Ireland (Good et al., 2009), 70.4% in USA (Lombard et al., 2013), 9.8-43.1% in Canada, and 27.6–42.5% in UK (Geraghty et al., 2014). The positive cattle in this study were six months, five and eight years old, and all of them were female. Interestingly, the youngest cow had diarrhea. Further studies with larger number of samples are necessary to confirm whether this low rate of the infection is consistent in the Mongolian cattle population. Each seropositive case may be the separate infection because there was no correlation and animal movement among areas where those seropositive cattle were idendified.

Furthermore, detailed studies are required to determine the incidence of MAP infection in other animal species and to perform molecular characterization of the Mongolian isolates. This is the first report on the prevalence of MAP infection in Mongolian cattle population. The implementation of systematic control measures against infectious agents, including MAP, in livestock should still be considered important in Mongolia. However, the epidemiology of infectious agents requires further in-depth investigation to provide information that is vital for the planning and execution of effective control measures in the Mongolian dairy and beef industries.

II.1.5. Summary

Johne's disease (JD) is caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP), which is characterized as chronic disease in cattle and causes huge economic losses to cattle industry. The seroprevalence of MAP in cattle of Mongolian was estimated by an ELISA assay using 356 serum samples which were collected from 11 provinces and Ulaanbaatar city. Out of these samples, 3 (0.84%) were found to be seropositive for MAP, originating from Tsenkher soum of Arkhangai province, Murun soum of Khuvsgul province, and Bornuur soum of Tuv province in Mongolia. This study represents thefirst confirmation of JD in Mongolian cattle. These findings provide vital information that can be used for the planning and execution of control measures for Johne's disease in the Mongolian cattle industry.

II. 2. Molecular epidemiological survey and genetic characterization of *Anaplasma* species in Mongolian livestock

II.2.1. Introduction

The genus *Anaplasma* encompasses a group of obligate intracellular pathogens belonging to the family *Anaplasmataceae* in the order *Rickettsiales* (Dumler et al., 2001). Currently, six species of *Anapasma* have been recognized: *Anaplasma marginale, Anaplasma centrale, Anaplasma ovis, Anaplasma phagocytophilum* (formerly *Ehrlichia phagocytophilum), Anaplasma bovis* (formerly *E. bovis*), and *Anaplasma platys* (formerly *E. platys*). (Kocan et al., 2015). Furthermore, a new zoonotic *Anaplasma* species referred to as *A. capra* was reported from China (Li et al., 2015).

Anaplasma marginale is an obligate intra-erythrocytic pathogen, primarily affecting cattle, which causes bovine anaplasmosis (Kocan et al., 2010). Bovine anaplasmosis leads to great economic loss for cattle farms in tropical or subtropical regions of the world (Aubry and Geale, 2011) and can be transmitted mechanically by biting flies and biologically by ticks; approximately 20 species of ticks have been implicated as vectors of the pathogen (Kocan et al., 2003). *Anaplasma ovis* is another intra-erythrocytic pathogen, primarily infects sheep or goats, causing ovine anaplasmosis (Renneker et al., 2013) and has been reported from many regions of the world (Ndung'u et al., 1995; de la Fuente et al., 2007, Renneker et al., 2013, and Yang et al., 2015). In China, a neighbouring country to Mongolia, three biological vectors of *A. ovis* were identified: *Dermacentor nuttalli, Hyalomma asiaticum* and *Rhipicephalus pumilio* (Yin and Luo, 2007). *A. phagocytophilum* is the most concerning *Anaplasma* species due toits invasion to both humans and animals (Woldehiwet, 2010), and replicates mainly in granulocytes of the hosts, and causes tick-borne fever in ruminants or granulocytic anaplasmosis in humans, dogs, and horses (Rikihisa, 2011). *A. phagocytophilum* has been reported in many countries in the Northern hemisphere because its distribution is associated with several members of ticks in genus *Ixodes* (Kawahara et al., 2006; de la Fuente et al., 2005; Sainz et al., 2015; Cao et al., 2006, and Carrade et al., 2009). *A. bovis* infects monocytes and causes monocytic anaplasmosis, primarily in cattle (Rymaszewska and Grenda, 2008), but it has been reported from other animal species including goats (Liu et al., 2012), dogs (Sakamoto et al., 2010) and rabbits (Goethert and Telford, 2003). *A bovis* has been most commonly reported in South Africa (Harrison et al., 2013), Tunisia (Belkahia et al., 2015), India (Nair et al., 2013), China (Liu et al., 2012), Korea (Doan et al., 2013), and Japan (Ooshiro et al., 2008). Currently known

vectors of the pathogen are *Haemaphysalis longicornis* (Doan et al., 2013), *Haemaphysalis punctata* (Palomar et al., 2015), and *Haemaphysalis leporispalustris* (Goethert and Telford, 2003).

To date, three *Anaplasma* species have been detected from Mongolian livestock: *A. marginale* from cattle (Ybañez et al., 2013a), *A. ovis* from sheep, goats, and cattle (Sophia et al., 2012), as well as reindeer (Haigh et al., 2008), and *A. phagocytophilum* from sheep, goats, cattle and horse (Sophia et al., 2012). However, these studies included only few sampling areas in the country, specifically the Khuvsgul or Khentii provinces, and information on genetic characterizations of these *Anaplasma* species was still limited. In addition, an *Anaplasma* species, closely related to *A. ovis* (95.0% identity) was recently identified from Mongolian cattle based on the *groEL* gene (Ybañez et al., 2013a) but further studies are needed for its characterization. Despite little information on the prevalence of *Anaplasma* infection in Mongolian livestock, pathogens such as *A. ovis* were known to cause a severe disease in reindeer in the Khuvsgul province (Haigh et al., 2008). Thus, additional studies are necessary to examine the prevalence and molecular identity of *Anaplasma* infection in various species of Mongolian livestock in different areas.

The purpose of this study was to investigate the presence of *Anaplasma* species such as *A. marginale, A. bovis, A. ovis,* and *A. phagocytophilum* in cattle, yak, sheep, and goats from five different sampling areas of Mongolia, and todetermine the molecular characterizaion of detected pathogens by sequence analysis.

II.2.2. Material and methods

II.2.2.1. Samples and study areas

Totally, 928 whole blood samples were collected using BD K_3 EDTA vacutainer blood collection tubes (Becton, Dickinson and Company, USA) from randomly selected free range Mongolian livestock between June and July of 2014. Out of them, 517 blood samples were collected from bovine species: 100 samples from yaks (*Bos grunniens*) in Bulgan soum of Arkhangai Province or Songinokhairkhan District of Ulaanbaatar City, 117 samples from native cattle (*Bos taurus*) in Tsenkher soum of Arkhangai Province or Lun soum of Tuv Province, and 300 samples from dairy breed cattle (Holstein, Simmental and Alatau) (*Bos taurus*) in Bornuur soum of Tuv Province or Songinokhairkhan District of Ulaanbaatar City. In addition, 411 blood samples were collected from sheep (*Ovis aries)* and goats (*Capra aegagrus hircus*): 211 samples from sheep in Lun soum of the Tuv Province or Tsenkher soum of the Arkhangai Province, and 200 samples from goat in Lun soum of the Tuv Province (Fig. II-2-1). All of these animals were apparently healthy and showed no visible clinical symptoms during the sampling time. In addition, no tick attachments were detected in all of the animals used inthis study and this may be associated with seasonal influence for lifecycle of the ticks.

II.2.2.2. DNA extraction

Genomic DNA was extracted from the blood samples soon after the sample collection using a Genomic DNA Purification kit (Promega Corporation, USA) according to the manufacturer's instructions. Total DNA was eluted with 100 µl of conservation buffer and stored at -30°C until further use.

II.2.2.3. Diagnostic polymerase chain reaction assays for detection of *Anaplasma* species

A. phagocytophilum and *A. bovis* were screened by nested PCR assay based on the *16S rRNA* gene (Kawahara et al., 2006) while *A. marginale* was checked by nested PCR assays targeting the *16S rRNA* (Weisburg et al., 1991; Ybañez et al., 2013a), major surface protein 1 (*msp1b*) (Molad et al., 2006), and major surface protein 5 (*msp5*) genes (Ybañez et al., 2013a). In contrast, *A. ovis* was detected using single-step PCR targeting *major surface protein 4* (*msp4*) (de la Fuente et al., 2007) and nested PCR based on the *16S rRNA* (Barlough et al., 1997) or *heat-shock protein* (*groEL*) (Ybañez et al., 2013a) genes. The primers used in this study are shown in Table II-2-1. The *βeta-globin* (*B. globin*) gene was

amplified as an internal control to confirm the presence of DNA in the templates according to the previous report (Tajima et al., 2003).

Briefly, 1.5 µl of extracted sample DNA, 1.5 µl positive control DNA or 1.5 µl double distilled water (DDW) as negative control was added to 28.5 µl of reaction mixture that comprised of3 µl of 10×buffer (Takara Bio Inc., Japan), 2.4 µl of dNTPs (Takara Bio Inc., Japan), 1 µl (10 µM concentration) of forward and reverse primers (Hokkaido System Science Company, Japan), 0.1 µl of DNA *Taq* polymerase (Takara Bio Inc., Japan), and 21 µl of DDW. The PCR amplification was performed under the following thermal cycle condition: initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at each optimal temperature for 30 sec, extension at 72°C for 1.5 min, and a final synthesis at 72°C for 7 min using the GeneAmp PCR System 9700 (Applied Biosystems, USA). The amplified PCR products were separated by electrophoresis on a 2% agarose gel and visualized under ultraviolet (UV) light.

II.2.2.4. DNA cloning and sequencing

A total of 72 PCR products: 24 positive samples for each of the three genes were extracted using FastGene gel/PCR Extraction kit (Nippon Genetics Company, Japan) for sequencing. The extracted PCR products were ligated into the pGEM-T Easy vector (Promega Corporation, USA), and the plasmid was transformed into *Escherichia coli* strain DH5α, plated on a Luria-Bertani (LB) agar (Thermo Fisher Scientific Corporation, USA), and cultured in LB broth (Thermo Fisher Scientific Corporation, USA). The plasmid DNAs from the positive clones were extracted from the LB culture using FastGene Plasmid Mini kit (Nippon Genetics Company, Japan). The plasmids were amplified using the GeneAmp PCR System 9700 (Thermo Fisher Scientific Corporation USA).

The quality of the plasmid preparation of each gene of the pathogen were checked by NanoDrop 8000 analytic equipment (Thermo Fisher Scientific Corporation, USA), and the sequencing analysis of the pathogen was carried out using the CEQ8000 DNA analysis system (Beckman Coulter Inc Company, USA).

II.2.2.5. Phylogenetic and homology analyses

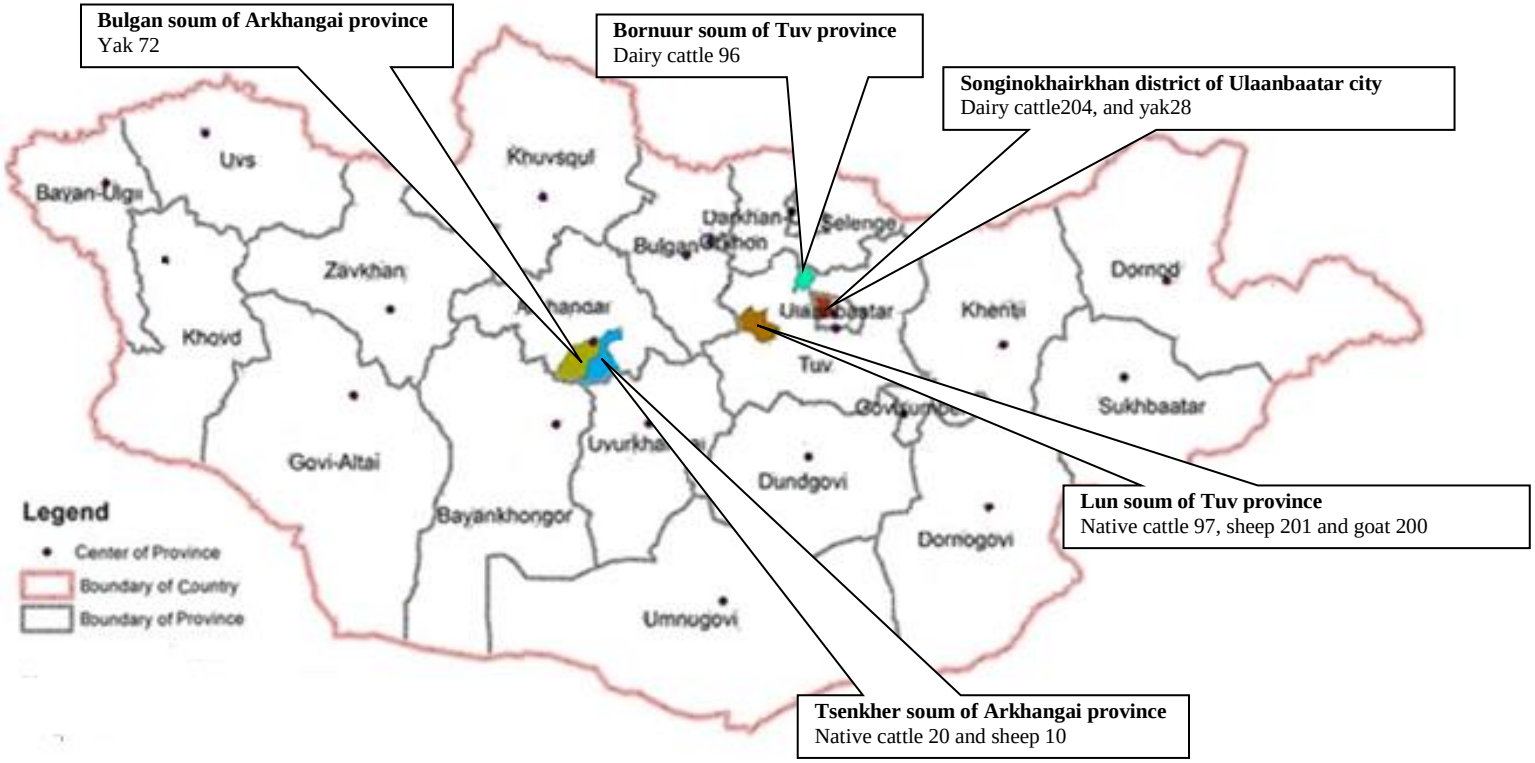
The obtained sequences were analyzed using the Bio-Edit software (Hall, 1999) and basic local alignment search tool application (BLAST). The phylogenetic trees were constructed by MEGA 7 software (Tamura et al., 2007) with the neighbor-joining method (Saitou and Nei, 1987) with 1,000 bootstrap replications.

Table II-2-1. Primers for detection and characterization of *Anaplasma* species in Mongolian livestock

Pathogens	Target gene	Primer	Oligonucleotide sequences (5´-3´)	Size	Reference
A. marginale	*msp1b*	AM-456	CCATCTCGGCCGTATTCCAGCGCA	732	Molad et al., 2006
		AM-1164	CTGCCTTCGCGTCGATTGCTGTGC		
		AM-634	CGAGAGCGTGGGACTACGTGC	291	This study
		AM-925	TGGCCTTCCGCGAGCATGTG		
	msp5	AM-49F1	GTGTTCCTGGGGTACTCCTATGTGAACAAG	547	Ybanez et al., 2013a
		AM-595R1	AAGCATGTGACCGCTGACAAACTTAAACAG		
		AM-211F2	AAGCACATGTTGGTAATATTCGGCTTCTCA	195	Ybanez et al., 2013a
		AM-376R2	AATTCTCGCATCAAAAGACTTGTGGTACTC		
	16S rRNA	fD1	AGAGTTTGATCCTGGCTCAG	1500	Weisburg et al.,1991
		Rp2	ACGGCTACCTTGTTACGACTT		
		AM-87F	TACGCAGCTTGCTGCGTGTATG	877	Ybanez et al., 2013a
		AM-936R	GCCCTTCTGTTAAGAAGGATCTAG		
A. phagocytophilum	*16S rRNA*	EE1	TCCTGGCTCAGAACGAACGCTGGCG	1430	Barlough et al., 1996
		EE2	AGTCACTGACCCAACCTTAAATGGCTG		
		SSAP2f	GCTGAATGTGGGGATAATTTAT	641	Kawahara et al., 2006
		SSAP2r	ATGGCTGCTTCCTTTCGGTTA		
A. bovis	*16S rRNA*	EE1	TCCTGGCTCAGAACGAACGCTGGCG	1430	Barlough et al., 1996
		EE2	AGTCACTGACCCAACCTTAAATGGCTG		
		AB1f	CTCGTAGCTTGCTATGAGAAC	551	Kawahara et al., 2006
		AB1r	TCTCCCGGACTCCAGTCTG		
A. ovis	*msp4*	MSP45	GGGAGCTCCTATGAATTACAGAGAATTGTTTAC	870	de la Fuente et al., 2007
		MSP43	CCGGATCCTTAGCTGAACAGGAATCTTGC		
	groEL	AMgroES-111F1	AGAGCTCGAAGGAAAGAAGTTCATAGT	1668	Ybanez et al., 2013a
		AMgroEL1557R1	CATGAATACAGCTGCR*AGTGACACAGCCA		
		AMgroES67F2	TAATCGCTAAGGAGGCGTAGTC	580	Ybanez et al., 2013a
		AMgroEL513R2	GTCTTTGCCAACTTCCCTTACGCACTGTG		
	16S rRNA	EE1	TCCTGGCTCAGAACGAACGCTGGCG	1430	Barlough et al., 1996
		EE2	AGTCACTGACCCAACCTTAAATGGCTG		
		297E	ACACGGTCCAGACTCCTACG	848	This study
		1144R	CTTGACATCATCCCCACCTT		

*Degenerate primer: R= A or G.

Fig.II-2-1. The map of sampling areas in Mongolia



II.2.3. Results

II.2.3.1. Prevalence of the pathogen in Mongolian livestock

A total of 928 samples collected from Mongolian livestock were screened by the single step or nested PCR assay for the detection of several *Anaplasma* species.Asthe result, *A. ovis* was successfully detected from all animal species, sheep, goats, cattle and yaks, and all 5 sampling areas (Songinokhairkhan district of Ulaanbaatar city, Lun or Bornuur soum of Tuv Province and Tsenkher or Bulgan soum of Arkhangai Province) in the country whereas *A. phagocytophilum, A. bovis* and *A. marginale* were not detected in this study. The overall infection rate of *A. ovis* determined by PCR targeting the *groEL* and *msp4* genes were 44.5% and 33.2% in the livestock, respectively, and prevalence of the pathogen in different hosts or different sampling areas in the country was shown in details in Table II-2-2.

II.2.3.2. Molecular characterizaion of the pathogen

By the sequencing analysis, 24 positive samples for each different three genes such as *groEL*, *msp4*, and *16S rRNA* were identified and each of the sequenced samples were associated with the host animal species and geographical locations as well as some identified samples for each gene originated from the same animals. Consequently, 17 sequences for the *groEL* gene with 95.5%-100% homology, 15 sequences for the *msp4* gene with 94.0%-100% homology, and 3 sequences for *16S rRNA* with99.3%-100% homology to one another were identified and the sample sources, numbers, as well as accession numbers for each of the sequences were shown in details in Table II-2-2.

In the phylogenetic tree, *A. ovis* sequences based on the *groEL* gene were separated into 2 distinct clusters. Six independent sequences derived mainly from ovine species such as 6 sheep, 3 goats and 1 yak were clustered together, and were similar to the isolates from several countries whereas another 11 independent sequences only obtained from bovine species such as 12 cattle and 2 yaks were similar to one another and separated into new cluster with an isolate previously identified from Mongolian cattle (Fig. II-2-2). The alignment of the representative sequences for the *groEL* gene of the pathogen from Mongolian livestock were compared with the sequences from other countries such as South Africa, Cyprus and China, showing that they are very similar to one another with a few substitutions (Fig. II-2-3).

In addition, the *msp4* gene of *A. ovis* by using same and different samples with the *groEL* gene sequence showed that twelve sequences derived from both bovine and ovine

species such as 11 cattle, 1 yak, 6 sheep and 3 goats were clustered together and were genetically same or similar to the isolates from several countries but 3 sequences derived only from bovine species such as 2 yaks and 1 cattle were separated into another separate branch (Fig. II-2-4). The alignment of the representative sequences for the *msp4* gene of the pathogen from Mongolian livestock was compared with the sequences from other countries such as Hungary, Italy, Spain, USA and China, showing that they are very similar to one another with a few substitutions (Fig. II-2-5).

The relationships of these divergent clusters of *A. ovis* were elucidated by targeting the *16S rRNA* gene and three independent sequences were identified, and nucleotide polymorphism or divergence of them were shown in details in Table II-2-3. Two independent sequences (LC191433 and LC191434) derived mainly fromovine species, such as 6 sheep, 3 goats and 1 yak, were similar to one another and were same or similar to the isolates from China whereas 1 independent sequence (LC191432) derived from bovine species, such as 12 cattle and 2 yaks, was divergent from the other sequences in the phylogenetic tree (Fig. II-2-6).

Table II-2-2. The prevalence of *A. ovis* andaccession number of the DNA sequences according to sampling areas, numbers and animal species

Province/ City	Soum/ Districts	Animal species	No of animals	*groEL* gene			*msp4* gene			16S rRNA gene	
				Infection rates	ID of animals	Accession No	Infection rates	ID of animals	Accession No	ID of animals	Accession No
Tuv	Lun	Native cattle	97	48 (49.5%)	6	LC141098[d]	12 (12.4 %)	2	LC141086[c]	6	LC194132[d]
					10	LC141100[d]		3	LC141087[c]	10	LC194132[d]
					15	LC141099[d]		15	LC141088[c]	15	LC194132[d]
		Sheep	201	191 (95.0%)	2	LC194131[c]	189 (94.0%)	3	LC141077[c]	2	LC194133[c]
					3	LC194129[c]		18	LC141077[c]	3	LC194133[c]
					4	LC194127[c]		27	LC141077[c]	4	LC194133[c]
		Goat	200	115 (57.5%)	5	LC194128[c]	73 (36.5%)	16	LC141081[c]	5	LC194134[c]
					16	LC194130[c]		18	LC141080[c]	16	LC194134[c]
					28	LC194129[c]		32	LC141078[c]	28	LC194134[c]
	Bornuur	Dairy cattle	96	5 (5.2%)	2	LC141093[d]	4 (4.2%)	2	LC141079[c]	2	LC194132[d]
					27	LC141092[d]		27	LC141078[c]	15	LC194132[d]
					32	LC141092[d]		32	LC141078[c]	27	LC194132[d]
Arkhangai,	Tsenkher	Native cattle	20	7 (35.0%)	9	LC141101[d]	7 (35.0%)	4	LC141082[c]	9	LC194132[d]
					18	LC141094[d]		14	LC141077[c]	18	LC194132[d]
					20	LC141092[d]		20	LC141083[c]	20	LC194132[d]
		Sheep	10	10 (100%)	2	LC194129[c]	10 (100%)	1	LC141078[c]	2	LC194133[c]
					3	LC194129[c]		2	LC141077[c]	3	LC194133[c]
					4	LC194131[c]		3	LC141077[c]	4	LC194133[c]
	Bulgan	Yak	72	20 (27.8%)	17	LC141092[d]	3 (4.2%)	37	LC141089[d]	37	LC194132[d]
					37	LC141102[d]		61	LC141090[d]	61	LC194132[d]
					66	LC141103[c]		66	LC141091[c]	66	LC194133[c]
Ulaanbaatar	Songino-khairkhan	Dairy cattle	204	17 (8.3%)	50	LC141095[d]	10 (4.9%)	50	LC141084[c]	50	LC194132[d]
					155	LC141096[d]		155	LC141078[c]	155	LC194132[d]
					190	LC141097[d]		169	LC141085[d]	190	LC194132[d]
		Yak	28	0 (0%)			0 (0%)				
		Cattle and Yak	517		97 (18.8%)			36 (7.0%)			
		Sheep and Goats	411		316 (79.9%)			272 (66.2%)			
		Total	**928**		**413 (44.5%)**			**308 (33.2%)**			

c: common.The sequences were similar with the sequences from other countries.

d: divergent.The sequences were divergent compared to the sequences from other countries.

Table II-2-3. Nucleiotide polymorphisms of *A. ovis* based on their 16S rRNA gene sequences

DDBJ Acc. number		Nucleotide and its position according to *A.ovis* [Genbank:KJ639881]								
		60	61	62	77	80	194	248	693	1224
A.ovis	LC194132 [d]	T	A	T	A	C	C	G	A	C
A.ovis	LC194133 [c]	C	G	C	G	T	T	T	G	T
A.ovis	LC194134 [c]	C	G	C	G	T	C	T	A	T

[d: divergent] The sequences which were divergent compare to the sequences from other countries.

[c: common] The sequences which were similar with the sequences from other countries.

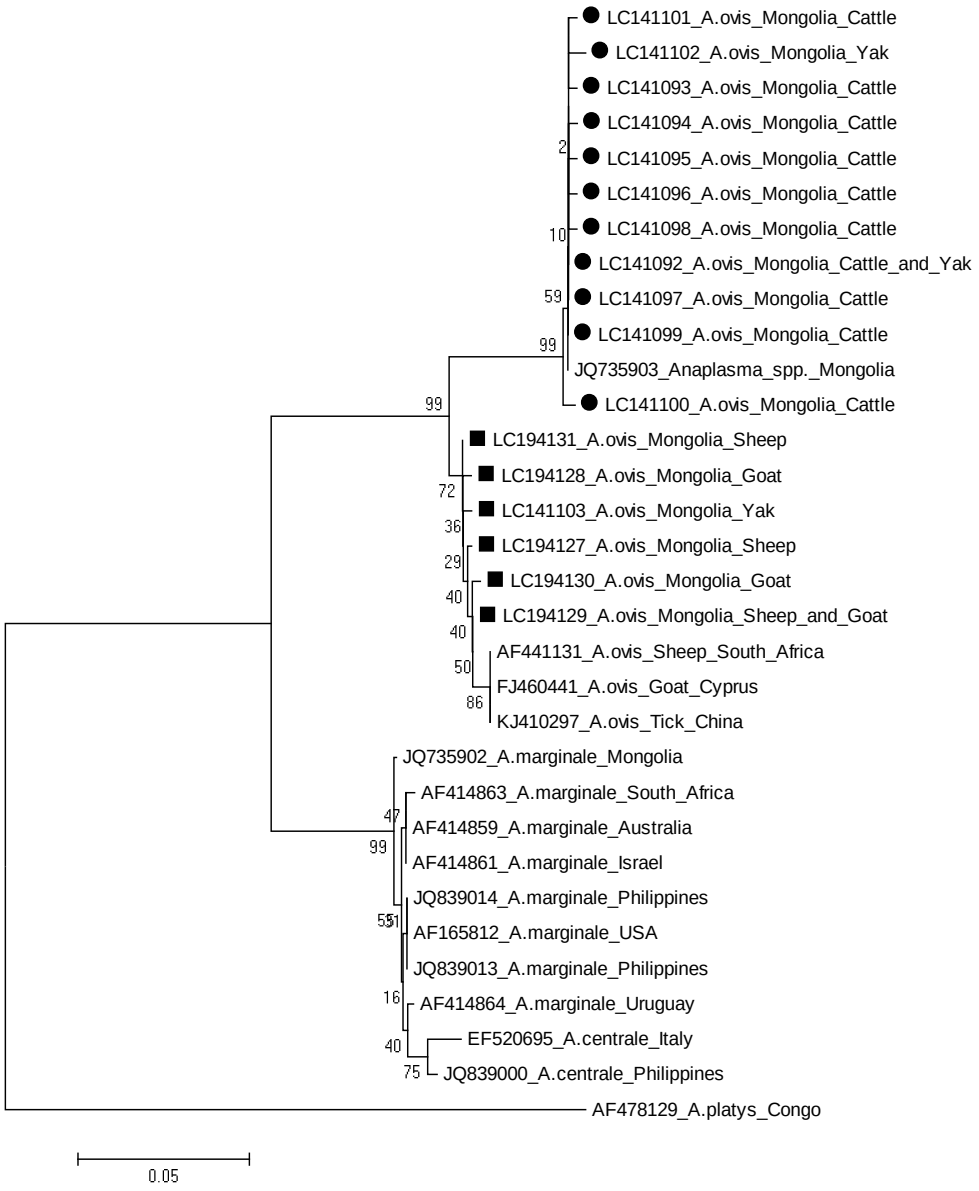


Fig.II-2-2. Phylogenetic relationship of *A. ovis* based on the *groEL* gene. Sequences derived from this study are highlighted with square and circular bullets regarding to their sequence divergence. The tree was constructed with the neighbor-joining method and was supported by 1,000 bootstrap replications. This figure shows the relationship between 580 bp sequences of the *groEL* gene of *A. ovis* obtained from this study and related other sequences from the GenBank. The DNA sequences identified in this study were deposited to DDBJ with accession numbers, LC141092, LC141093, LC141094, LC141095, LC141096, LC141097, LC141098, LC141099, LC141100LC141101, LC141102, LC141103, LC194127, LC194128, LC194129, LC194130, and LC194131, respectively.

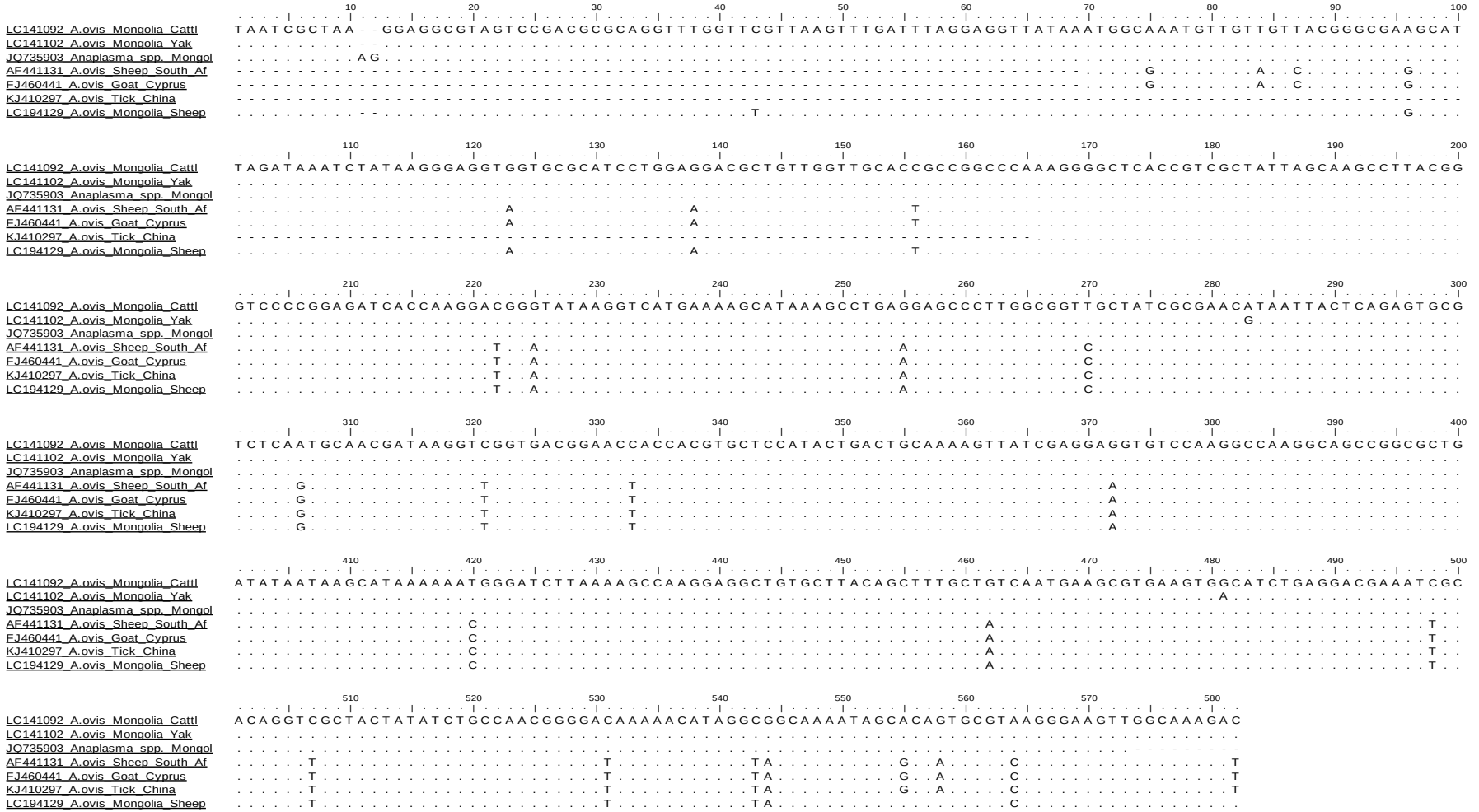


Fig. II-2-3. The alignment nucleotide comparison of the representative sequences for *groEL* gene of *A. ovis*. The alignment of representative sequence for *groEL* genes of the pathogen from Mongolian livestock were compared with the sequences from other countries such as South Africa, Cyprus and China.

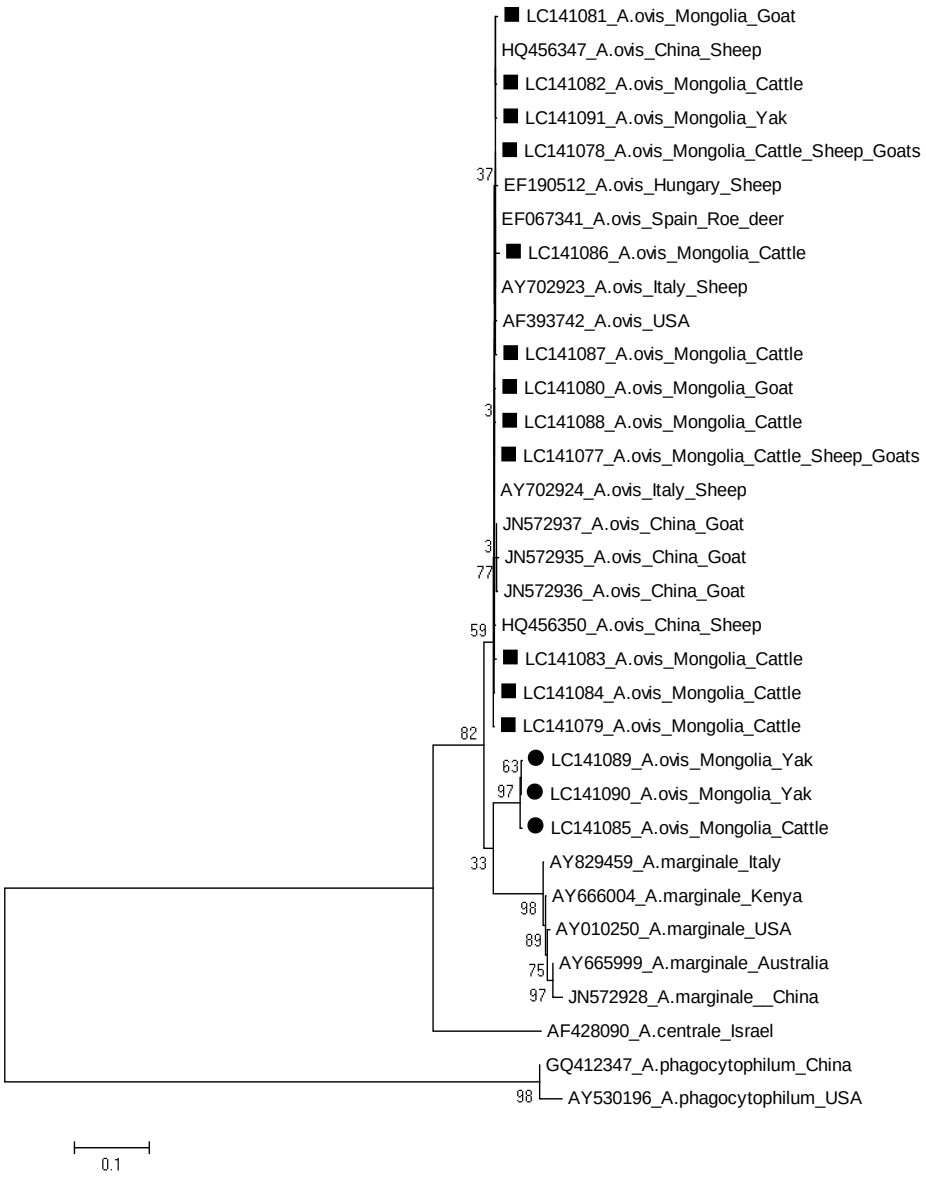


Fig. II-2-4. Phylogenetic relationship of *A. ovis* based on the *msp4* gene. Sequences derived from this study are highlighted with square and circular bullets regarding to their sequence divergence. The tree was constructed with the neighbor-joining method and was supported by 1,000 bootstrap replications. This figure shows the relationship between 870 bp sequences of the *msp4* gene of *A. ovis* obtained from this study and related other sequences from the GenBank. The DNA sequences identified in this studywere deposited to DDBJ with accession numbers, LC141077, LC141078, LC141079, LC141080, LC141081, LC141082, LC141083, LC141084, LC141085, LC141086, LC141087, LC141088, LC141089, LC141090, and LC141091, respectively.

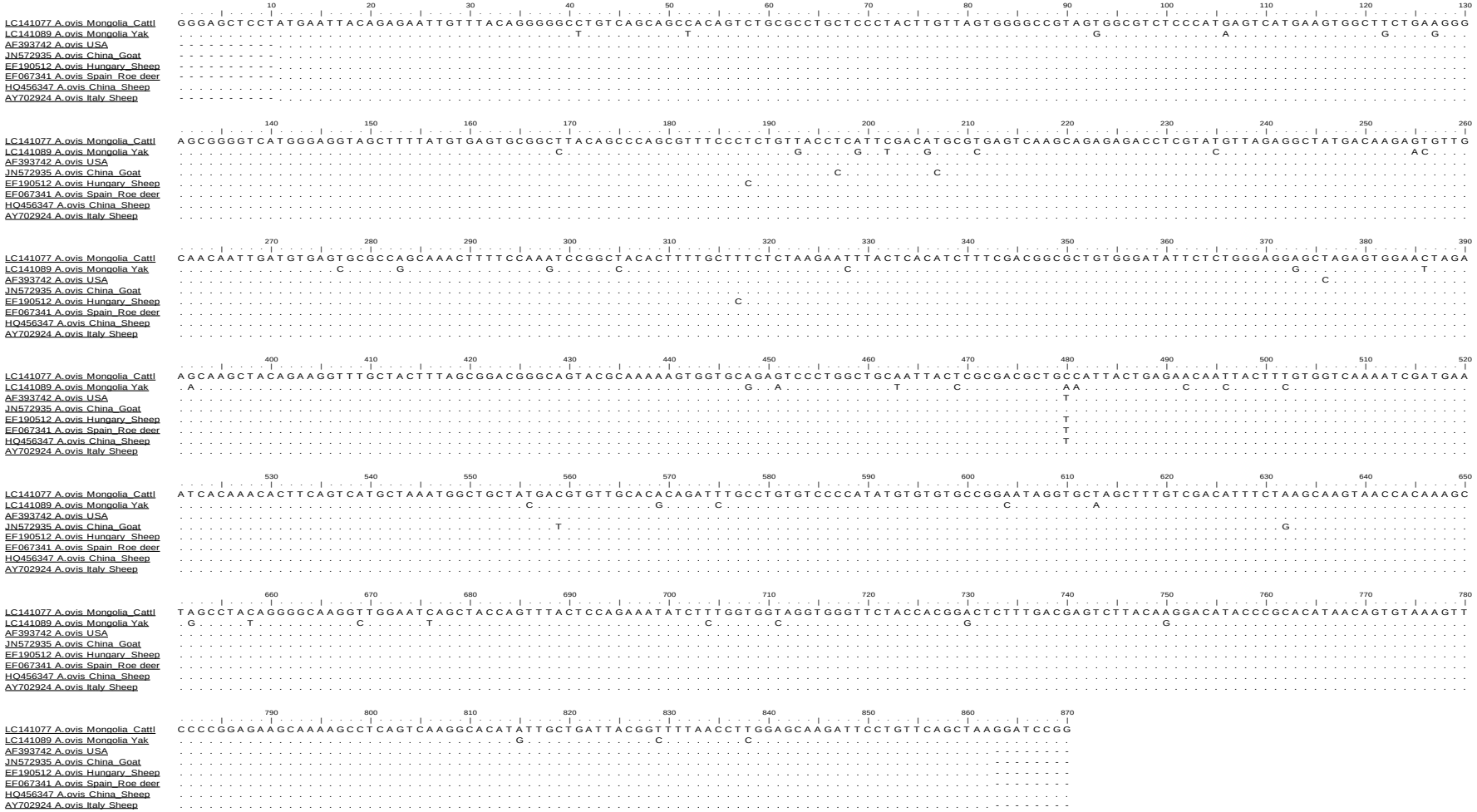


Fig. II-2-5. The alignment nucleotide comparison of the representative sequences for *msp4* gene of *A. ovis.* The alignment of representative sequences for *msp4* gene of the pathogen from Mongolian livestock was compared with the sequences from other countries such as Hungary, Italy, Spain, USA and China.

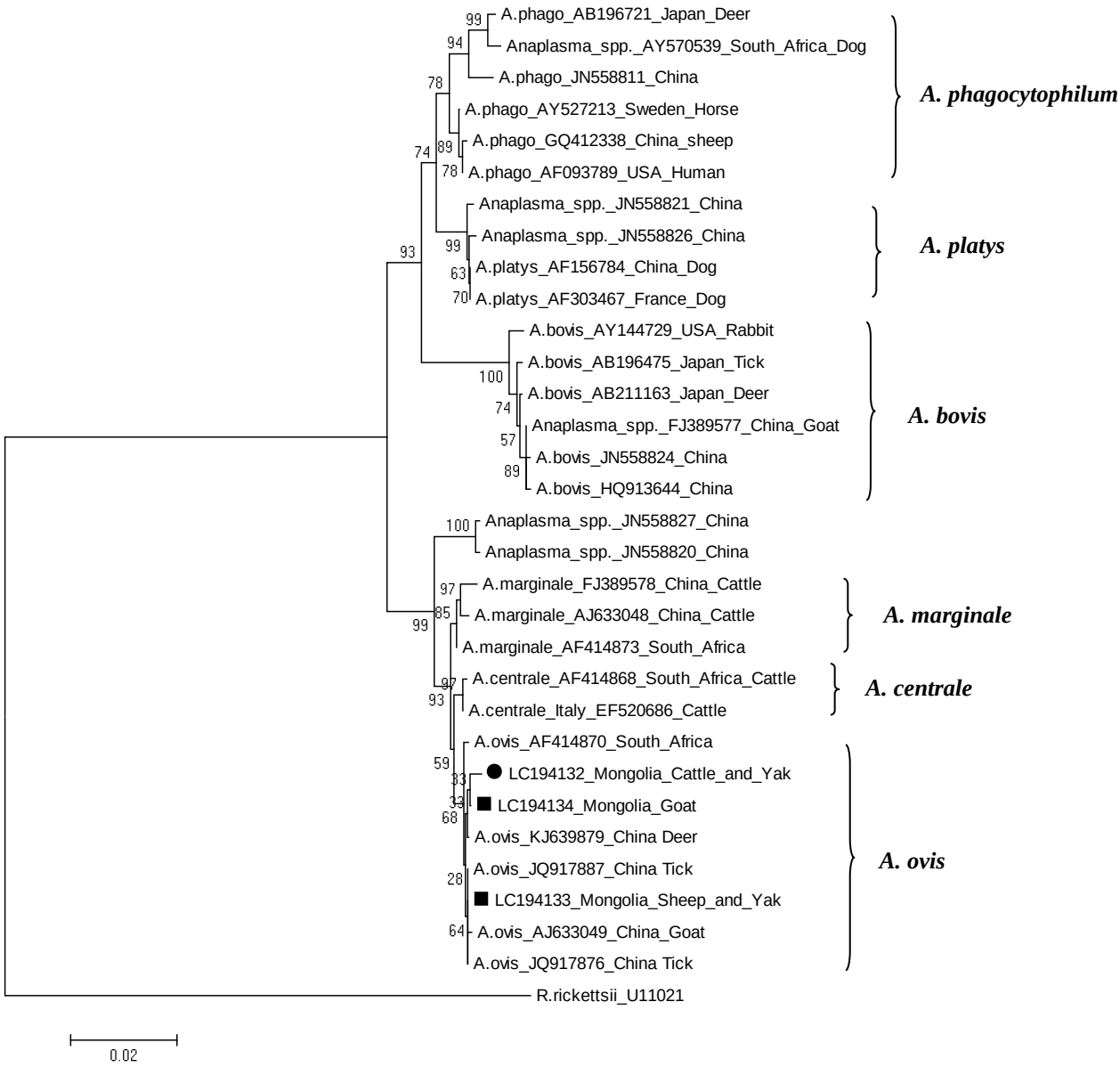


Fig.II-2-6. Phylogenetic relationship of *A. ovis* based on the *16S rRNA* gene. Sequences derived from this study are highlighted with square and circular bullets regarding to their sequence divergence. The tree was constructed with the neighbor-joining method and was supported by 1,000 bootstrap replications. This figure shows the relationship between 1,430 bp sequences of the *16S rRNA* gene of *A. ovis* obtained from this study and related other sequences from the GenBank. The DNA sequences identified in this study were deposited to DDBJ with accession numbers, LC194132, LC194133, and LC194134, respectively.

II.2.4. Discussion

The occurrence of the infectious diseases in Mongolian livestock has been increasing year after year and causing great economic loss into livestock industry, which is one of the major income sources in the economy (Batsukh et al., 2012; Beard et al., 2010; Wieland et al., 2015; Undrakhbayar et al., 2016). *Anaplasma* species such as *A. marginale* (Ybañez et al., 2013a), *A. ovis* and *A. phagocytophilum* (Sophia et al., 2012) have been already reported in Mongolian livestock, but the prevalence or molecular characterization of the pathogens in the livestock from various regions of the country were still unclear. Occasional occurrence of the diseases caused by *Anaplasma* species in the livestock seems to have agreat potentiality to cause huge economic loss to animal industry and to give impact on public health concern (Haigh et al., 2008; Sophia et al., 2012). It was suspected that *Anaplasma* species may have been expanding through animal populations of the country or those newly introduced from neighbouring countries to Mongolian livestock associated with geographical expansion or distribution of vector ticks of *Anaplasma* pathogen due to the trend of global warming (Kocan et al., 2003).

According to the results from nested or single step PCR targeting both the *groEL* and *msp4* genes, *A. ovis* was detected from all of the animal species or all of thesampling areas. The higher infection rates were especially observed from Mongolian native animals including native cattle, sheep and goats than those of yaks and dairy breed cattle based on the *groEL* gene, such as 95.2% for sheep, 57.5% for goats, 47.0% for native cattle, 7.3% for dairy breed cattle and 27.8% for yaks. The difference in the results of PCR targeting these 2 genes of the pathogen may be associated with genetic variability of the pathogen, or the different sensitivity of single step or nested PCR, or the condition of amplification for PCR such as unsuitable annealing temperature.The prevalence of *A. ovis* in Mongolian livestock was quite higher than that of a previous report showing that the infection rate of the pathogen was 47.2% in goats, 39.8% in sheep, and 13.0% in cattle (Sophia et al., 2012) in the Khuvsgul and Khentii Provinces, as well as 80.0% in reindeer in the Khuvsgul Province (Haigh et al., 2008). Regarding to otherprevious reports, *A. ovis* has been mostly detected from small ruminants including goat from Kenya (Ndung'u et al., 1995), sheep and deer from USA (de la Fuente et al., 2007), sheep from Iraq, Turkey, Sudan, and Portugal (Renneker et al., 2013), sheep from China (Yang et al., 2015) and sheep, goats and cattle from Mongolia (Sophia et al., 2012). Although *A. ovis* seems to infect to small ruminants and is host-specific, Mongolian cattle and yaks are shown to be infected with *A. ovis*, as demonstrated in this study with a relatively high infection rates (13.0%), and this

study may be the first report that *A. ovis* has been found from the yaks. The overall prevalence of *A. ovis* with 95.2% for sheep, 57.5% for goats from Mongolia was quite higher prevalence than thatin Turkey (31.4%), Sudan (41.6%) (Renneker et al., 2013), China (40.5%) (Yang et al., 2015), USA (37%) (de la Fuente et al., 2007), and similar or lower prevalence than those of reported in Iraq (66.6%) and Portugal (82.5%) (Renneker et al., 2013). These results indicate that *A. ovis* is widely distributed to Mongolian livestock, especially native animals, including native cattle, sheep, and goats, which were more affected to the pathogen than those of dairy breed cattle and yaks.

In addition, *A. ovis* causes mild clinical symptoms in the infected animals but stress factors, such as drought and heavy tick infestations, induce the virulence of the pathogen (Renneker et al., 2013). Haigh et al., (2008) reported that reindeer naturally-infected with *A. ovis* showed varied clinical symptoms such as fever, lethargy or pale mucous membranes and sometimes caused sudden death in Khuvsgul province of Mongolia. However,in this study,all of these *A. ovis*-infected animals (cattle, yak, sheep, and goats) seemed to be healthy and did not show any visible clinical symptom during the sampling time. It is likely that Mongolian animals may have a tolerance or resistance to *A. ovis* infection associated with wide prevalence and occurrence of the pathogen through many animal species in the country.

Other *Anaplasm*a species were already reported in Mongolian livestock such as *A. marginale* was 8.7% for cattle (Ybañez et al., 2013a) in Khentii, Uvurkhangai and Uvs provinces, and *A. phagocytophilum* was 35.8% for varied animal species in Khentii or Khuvsgul Provinces of the country (Sophia et al., 2012) and the vectors of *A. phagocytophilum* were *Ixodes persulcatus* and *Dermacentor nuttalli* from Selenge province in Mongolia (Javkhlan et al., 2014). In contrast, there was no report for *A. bovis* in Mongolian livestock but the transmission of the pathogen is possible because it has been already reported from China (Yang et al., 2015), which is a neighbouring country. However, these *Anaplasma* species were not detected in this study. *A. marginale* was screened targeting 3 different genes by the same methods described in chapter I with a positive control, whreas the detection of *A. phagocytophilum* and *A. bovis* was conducted in the absence of a positive control but it was performed with several different conditions such as changing annealing temperatures except for pursuing the previously described methods. These findings showed that *A. marginale, A. phagocytophilum* and *A. bovis* may not be prevalent in the livestock in the sampling areas.

The phylogenetic analysis targeting the *groEL* gene sequences of *A. ovis* from

Mongolian livestock showed the presence of 2 divergent types of *A. ovis*. The first branch of the sequences from sheep and goats were genetically similar to the common isolates from other countries such as China, South Africa, and Cyprus. Interestingly, the second cluster of the sequences from cattle and yak were quite distinct compared to those of the sequences from sheep and goats in this study as well as the available sequences in the Genbank except for apreviously published single sequence (Ybañez et al., 2013a), which was identified as *Anaplasma* species (JQ735903) from Mongolian cattle, but it was not declared as *A. ovis* because of limited study at that time point (Fig. II-2-2).

Further sequencing analysis of the pathogen based on the *msp4* gene by using both same and different samples in the case of the *gro EL* gene showed that all of thesequences derived from both ovine and bovine species except for three sequences derived from 2 yaks and 1 cattle were same or similar to one another and were clustered into the same group with several isolates from other countries such as China, Italy, Spain, Hungary, and USA (Fig. II-2-4). Interestingly, one sequence derived from the same yak source showed that this sequence was divergent based on both the *groEL* and *msp4* genes (LC141112 and LC141089). However, other two divergent sequences based on the *msp4* gene (LC141085 and LC141090) derived from one cattle and yak were not sequenced for the *groEL* gene due to mismatching PCR results but they also may belong to the divergent cluster for the *groEL* gene because all of the sequences derived from bovine species except for 1 yak (LC141103) were grouped into the divergent cluster in the phylogenetic tree (Fig. II-2-2).

In addition, the genetically different *A. ovis* from Mongolian animals was confirmed by the analysis of the *16S rRNA* gene, which is a well conserved region of the bacteria and has been used for the classification of the *Anaplasma* species (Dumler et al., 2001). The sequences from sheep (LC194133) and goats (LC194134) were similar (99.8%) to each other but they were divergent as 99.3% homology to the sequences derived from cattle. In addition, the sequences from yaks showed that two of them were identical with the sequences from cattle and another one was identical to the sequences from sheep (Fig. II-2-6). These results showed that there were genetically two different *A. ovis* in Mongolian livestock based on the *groEL* and *16S rRNA* genes as up to 95.5% and 99.3% homology: one was identified from sheep and goats, and another one was derived only from cattle but each genetically divergent *A. ovis* were identified from yaks. In addition, these genetically different *A. ovis* were same or similar with each other based on the *msp4* gene whereas three of them derived from yaks and cattle were divergent as up to 94.0% homology to others.

Infectious diseases threaten to animal husbandry through causing huge economic loss. *Anaplasma* species, including *A. ovis* should be considered by veterinary authority as one of the widely prevalent pathogenin Mongolian livestock. Furthermore, additional studies of molecular traits, pathogenesis, and tick vectors of all of *Anaplasma* species in Mongolian livestock are required to generate additional information for more comprehensive understanding of the pathogens.

II.2.5. Summary

Anaplasma species are obligate intracellular rickettsial pathogens that cause great economic loss to the animal industry. Thus far, few studies on *Anaplasma* infections in Mongolian livestock have been carried out. This study was conducted to investigate the prevalence of *A. marginale, A. ovis, A. phagocytophilum,* and *A. bovis* by PCR assay in 928 blood samples from native cattle and dairy cattle (*Bos grunniens*), yaks (*Bos grunniens*), sheep (*Ovis aries)*, and goats (*Capra aegagrus hircus)* in 4 Provinces or Ulaanbaatar city in Mongolia. The positive samples were genetically characterized by sequencing analysis based on the heat-shock protein (*groEL*), major surface protein 4 (*msp4*), and 16S rRNA genes. The results showed that only *A. ovis* was detected in Mongolian livestock (cattle, yaks, sheep and goats) and itspositive rates were 44.5% for *groEL* and 33.2% for *msp4* genes. In the phylogenetic tree, *A. ovis* sequences based on the *groEL* gene were separated into two distinct clusters. One cluster was composed of the sequences derived mainly from sheep and goats, and was similar to the selected *A. ovis* isolates from several countries. Another divergent cluster was composed of the sequences derived from cattle and yaks, and was branched newly with previously published single isolates from Mongolian cattle. In addition, the *msp4* gene of *A. ovis* by using same and different samples in the case of the *groEL* gene of the pathogen showed that all ofthe sequences derived from all animal species, except for 3 sequences derived from cattle and yak, were clustered together, and were same or similar with the isolates from many countries. Then, the genetically divergent *A. ovis* was investigated by using the *16S rRNA* gene sequences, and they showed high homology to one another as 99.3%-100%, but the sequences derived from cattle were not match to the sequences from sheep and goats. The study on theprevalence and molecular characterization of *A. ovis* in Mongolian livestock can be useful information for processing the control method of infectious diseases in the country.

II.3. Detection of bovine leukemia virus and identification of its genotype in Mongolian cattle

II.3.1. Introduction

Bovine leukemia virus (BLV) belongs to the genus *Deltaretrovirus* within the family *Retroviridae*. It is closely related to human T-lymphotropic virus type 1 and is a causative agent of enzootic bovine leukosis (EBL) (Schwartz and Lévy, 1994). BLV-infected animals can be characterized into three disease stages, namely, aleukemic (AL), persistent lymphocytosis (PL), and leukemia or lymphoma (Mirsky et al., 1996). Approximately 30 % of infected animals progress to PL, characterized by a polyclonal expansion of B cells, whereas only 0.1–10 % develops malignant lymphosarcoma. Typically, long duration is required between these disease stages as BLV modulates the immune system of the host (Kabeya et al., 2001). BLV is transmitted vertically or horizontally through the transfer of infected cells via several potential routes. Previous studies showed one of the major routes of horizontal transmission to be through bites of blood-feeding insects (Ooshiro et al., 2013), and BLV-infected pregnant cattle containing a viral load show a high risk for vertical transmission (Mekata et al., 2015). In particular, infected cattle with high viral loads or PL are considered a major source of infection within a herd (Ooshiro et al., 2013; Mekata et al., 2015; Mekata et al., 2015). Thus, the elimination of infected cattle showing a high risk is important for the control of this infection.

BLV infection can affect cells of both the innate and adaptive immune systems and lead to an increased susceptibility to other infections (Frie and Coussens, 2015). Increased prevalence of BLV within dairy herds was found to be associated with decreased milk production and longevity of cows (Da et al., 1993; Bartlett et al., 2013). Furthermore, the annual economic loss to the dairy industry in USA due to BLV infection was estimated at $525 million during 2003 (Ott et al., 2003). In Western Europe, BLV infection has been successfully eradicated, and the European commission has officially declared most of its member states to be free of EBL (Viltrop and Laht, 1996; Nuotio et al., 2003; Acaite et al., 2007; European commission, 2012). Likewise, attempts to eradicate BLV infection in Australia and New Zealand have been successful. In contrast, several epidemiological studies have indicated that BLV infection is globally distributed, with high prevalence in USA (Ott et al., 2003), Japan (Murakami et al., 2011, 2013), Canada (VanLeeuwen et al., 2001), Russia (Zinovieva et al., 2012), Iran (Morovati et al., 2012), the Philippines (Polat et al., 2015), and South American countries (Gutiérrez et al., 2011; Benavides et al., 2013)

and has resulted in major economic losses due to decreased cattle production and export. However, no reports regarding BLV infections in Mongolian cattle are currently available.

Eight BLV genotypes have been identified on the BLV *env* sequences from several geographical areas in the world (Rodriguez et al., 2009; Balić et al., 2012**)**. Phylogenetic analysis based on the partial sequences of BLV *env* has indicated that genotypes 1 and 3 are mostly found in USA and Japan. In contrast, genotypes 2, 5, and 6 have been isolated exclusively from South American countries, whereas the genotypes 4 and 7 were most commonly isolated in Russia and Eastern European countries. Additionally, the newly identified genotype 8 has been identified in Russia and the Eastern European counties (Rola-Łuszczak et al., 2013). However, information concerning the distribution of the BLV genotypes in Mongolia is not available. Therefore, the purpose of the present study was to identify the genotypes and distribution of BLV infection in the cattle population of Mongolia.

II.3.2. Materials and Methods

II.3.2.1. Samples and study areas

Totally, 517 bovine samples including native cattle, dairy breed cattle and yaks were screened in this study and were described in detail in II.2.2.1 of this chapter.

II.3.2.2. DNA extraction

Total cellular DNA was extracted by the same method as described in II.2.2.2 of this chapter.

II.3.2.3. Diagnostic polymerase chain reaction assays for BLV detection

BLV infection was tested using a nested PCR assay targeting the *LTR* gene (198 bp) (Tajima et al., 1998), and the viral genotypes of the positive samples were further identified using nested PCR targeting *env* (444 bp) as described previously (Fechner et al., 1997).To detect BLV, nested PCR targeting *LTR* was conducted using rTaq polymerase (Takara Bio Inc., Shiga, Japan) as described previously (Tajima et al., 1998). BLV genotyping was conducted as described previously with slight modifications (Fechner et al., 1997). The techinque and each of the details for the nested PCR assays to detect BLV was the same as described in II.2.2.3 of this chapter.

II. 3.2.4. DNA cloning and sequencing

Totally, 19 positive samples were subjected to the sequencing analysis, and the procedure for cloning and sequencing were the same as described in II.2.2.4 of this chapter.

II.3.2.5 Phylogenetic and homology analyses

Phylogenetic and homology analyses were performed by the same methods as described in II.2.2.5 of this chapter.

II.3.3. Results

II. 3.3.1. Prevalence of BLV in Mongolian cattle

In the present study, out of the 517 samples tested, 20 were positive for BLV. The infection rate was 2.6 % and 14.6 % in cattle from Songinokhairkhan district of Ulaanbaatar city and Bornuur soum of Tuv province, respectively, and both sites were in the areas of intensive cattle farming (Table II-3-1). The distance between these two areas was approximately 60 km and cross-infection between these sampling areas is possible. In addition, the infection rate was variable over the sampling sites, and BLV infection was isolated in 5 and 7 farms from the Songinokhairkhan district of Ulaanbaatar city and Bornuur soum of Tuv province, respectively. The BLV-positive cattle were all dairy breeds and, 13, 3 and 4 individuals belonged to the Holstein, Alatau, and Simmental breeds, respectively. In contrast, no cases of infection were isolated in native Mongolian cattle and yaks. Among BLV-positive cattle, only one individual was under 1 year old, whereas other individuals were 4-17 years old.

II. 3.3.2. Molecular characterizaion of the pathogen

Out of 20 LTR-positive samples, 19 samples contained the amplified *env* gene. Out of 19 sequences, 11 independent sequences were identified, and these showed 96.4 %–100 % sequence identity to one another and 95.7 %–100 % identity to viral sequences from several other countries. Eleven isolates were classified into genotype 4, one into genotype 7, and a further four into genotype 1 (Fig. II-3-1). Mongolian isolates of genotypes 4 and 7 were clustered together with isolates from Russia and the Eastern European countries. Genetic analysis showed that the Mongolian isolate LC060792, which belonged to the genotype 4, was 100 % identical to isolates from Russia (KC886628), Ukraine (HM563781), and Poland (EU262581). Another isolate was found to be 100 % identical to a genotype 4 isolate from Poland (EU262575). In addition, one remaining group belonging to the genotype 1 was clustered with isolates from several counties; however, it was closely related to Iranian isolates.

Table II-3-1.The detection of BLV in Mongolian cattle

Province/City	Soum/District	Farm ID	Breed name	Sample number	Positive samples	Genotype
Tuv	Bornuur	A	Holstein	6	2	4
			Simmental	3	1	1
		B	Holstein	11		
		C	Holstein	15	5	1, 4
			Simmental	1		
		D	Holstein	9	3	4
			Simmental	3	1	4
		E	Holstein	2	1	4
			Simmental	11		
		F	Holstein	1	1	1
			Simmetal	5		
			Alatau	9		
		G	Holstein	8		
			Simmental	12		
	Lun	A	Mongolian native	31		
		B	Mongolian native	20		
		C	Mongolian native	22		
		D	Mongolian native	24		
Arkhangai	Bulgan	A	Yak	15		
		B	Yak	9		
		C	Yak	12		
		D	Yak	9		
		E	Yak	10		
		F	Yak	10		
		G	Yak	7		
Ulaanbaatar	Tsenkher	A	Mongolian native	20		
	Songinokhairkhan	A	Holstein	4		
			Simmetal	1	1	1
			Alatau	12	1	ND
		B	Holstein	7		
			Simmetal	3		
			Alatau	1		
		C	Holstein	9		
			Simmetal	5		

Province/City	Soum/District	Farm ID	Breed name	Sample number	Positive samples	Genotype
Ulaanbaatar	Songinokhairkhan		Alatau	5		
		D	Holstein	12		
			Alatau	6		
		E	Holstein	9		
			Alatau	6		
		F	Holstein	18		
			Alatau	2		
		G	Yak	28		
			Holstein	10	1	1
			Simmental	8		
			Alatau	6		
		H	Holstein	**22**		
			Simmental	2		
			Alatau	7	2	7
		I	Holstein	14		
			Simmental	1		
			Alatau	3		
		J	Holstein	1		
			Simmental	5		
			Alatau	5		
		K	Holstein	2		
			Simmental	1	1	4
			Alatau	17		
Total (%)				517	20 (3.9 %)	

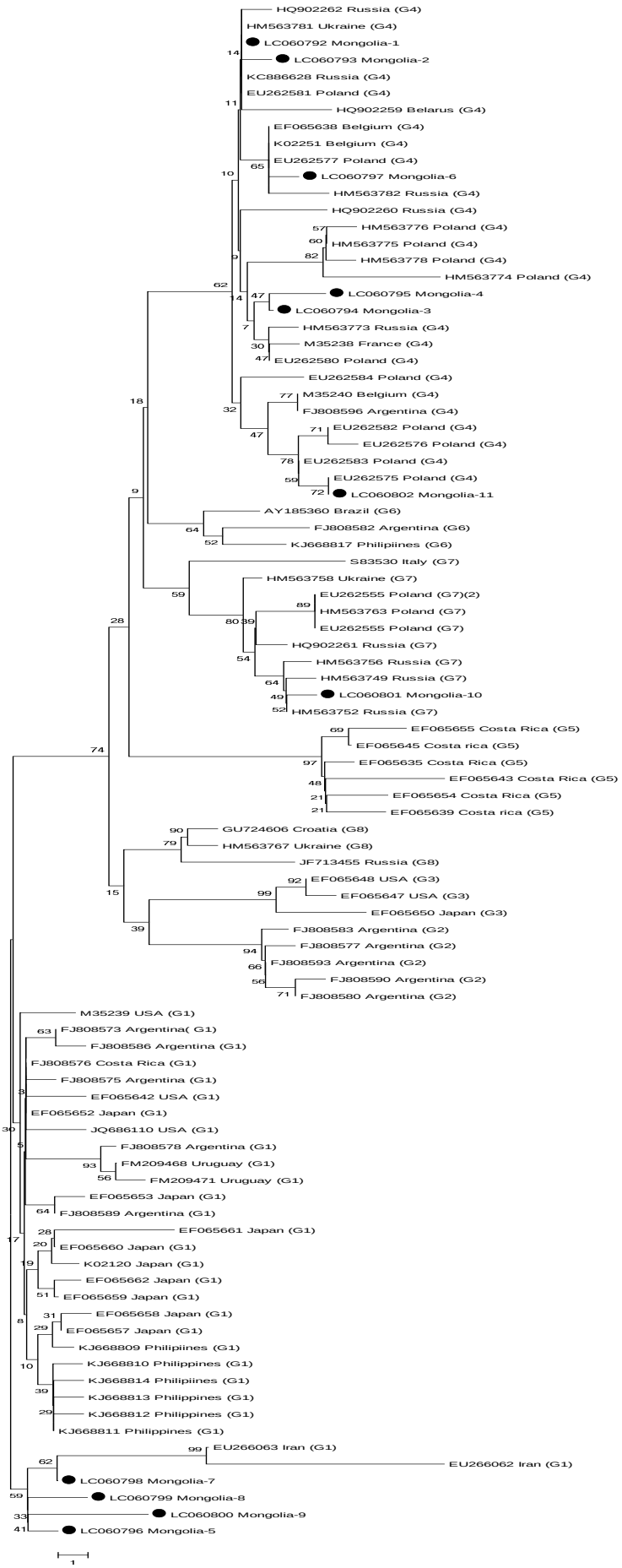

HQ902262 Russia (G4)
HM563781 Ukraine (G4)
LC060792 Mongolia-1
LC060793 Mongolia-2
KC886628 Russia (G4)
EU262581 Poland (G4)
HQ902259 Belarus (G4)
EF065638 Belgium (G4)
K02251 Belgium (G4)
EU262577 Poland (G4)
LC060797 Mongolia-6
HM563782 Russia (G4)
HQ902260 Russia (G4)
HM563776 Poland (G4)
HM563775 Poland (G4)
HM563778 Poland (G4)
HM563774 Poland (G4)
LC060795 Mongolia-4
LC060794 Mongolia-3
HM563773 Russia (G4)
M35238 France (G4)
EU262580 Poland (G4)
EU262584 Poland (G4)
M35240 Belgium (G4)
FJ808596 Argentina (G4)
EU262582 Poland (G4)
EU262576 Poland (G4)
EU262583 Poland (G4)
EU262575 Poland (G4)
LC060802 Mongolia-11
AY185360 Brazil (G6)
FJ808582 Argentina (G6)
KJ668817 Philipiines (G6)
S83530 Italy (G7)
HM563758 Ukraine (G7)
EU262555 Poland (G7)(2)
HM563763 Poland (G7)
EU262555 Poland (G7)
HQ902261 Russia (G7)
HM563756 Russia (G7)
HM563749 Russia (G7)
LC060801 Mongolia-10
HM563752 Russia (G7)
EF065655 Costa Rica (G5)
EF065645 Costa rica (G5)
EF065635 Costa Rica (G5)
EF065643 Costa Rica (G5)
EF065654 Costa Rica (G5)
EF065639 Costa rica (G5)
GU724606 Croatia (G8)
HM563767 Ukraine (G8)
JF713455 Russia (G8)
EF065648 USA (G3)
EF065647 USA (G3)
EF065650 Japan (G3)
FJ808583 Argentina (G2)
FJ808577 Argentina (G2)
FJ808593 Argentina (G2)
FJ808590 Argentina (G2)
FJ808580 Argentina (G2)
M35239 USA (G1)
FJ808573 Argentina(G1)
FJ808586 Argentina (G1)
FJ808576 Costa Rica (G1)
FJ808575 Argentina (G1)
EF065642 USA (G1)
EF065652 Japan (G1)
JQ686110 USA (G1)
FJ808578 Argentina (G1)
FM209468 Uruguay (G1)
FM209471 Uruguay (G1)
EF065653 Japan (G1)
FJ808589 Argentina (G1)
EF065661 Japan (G1)
EF065660 Japan (G1)
K02120 Japan (G1)
EF065662 Japan (G1)
EF065659 Japan (G1)
EF065658 Japan (G1)
EF065657 Japan (G1)
KJ668809 Philipiines (G1)
KJ668810 Philippines (G1)
KJ668814 Philipiines (G1)
KJ668813 Philippines (G1)
KJ668812 Philippines (G1)
KJ668811 Philippines (G1)
EU266063 Iran (G1)
EU266062 Iran (G1)
LC060798 Mongolia-7
LC060799 Mongolia-8
LC060800 Mongolia-9
LC060796 Mongolia-5
1

Fig. II-3-1. Phylogenetic relationship of BLV based on the *env* gene. Sequences derived from this study are highlighted with circular bullets (●) regarding to their sequence divergence. The tree was constructed with the neighbor-joining method and was supported by 1,000 bootstrap replications. This figure shows the relationship between 444 bp sequences of the *env* gene for BLV obtained from this study and related other sequences from the GenBank. Genotypes and geographical origins of each isolates are indicated by the numbers. The DNA sequences identified in this study were deposited to DDBJ with accession numbers, LC060792, LC060793, LC060794, LC060795, LC060796 LC060797, LC060798, LC060799, LC060800, LC060701, and LC060802, respectively.

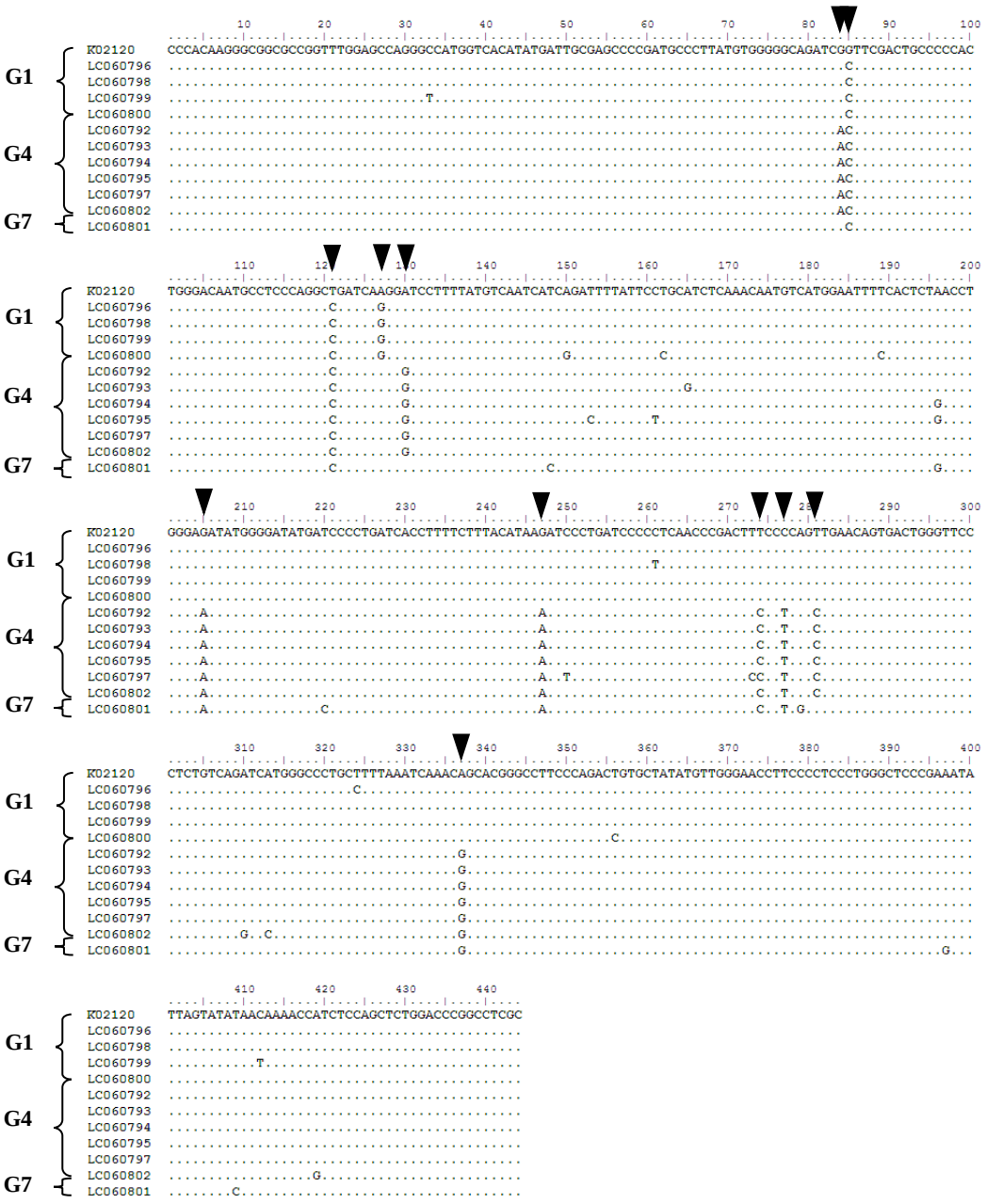


Fig. II-3-2. Nucleotide sequence alignment of Mongolian BLV isolates based on the *env* gene. One typical BLV isolate from each identical sample was submitted to GenBank. Unique nucleotide substitutions are indicated by numbers and filled triangle (▼). Genotypes (G-1, G4 and G7) are indicated by the black bars at the far left of the figure. Japanese isolate K02120 was used as a reference in this study.

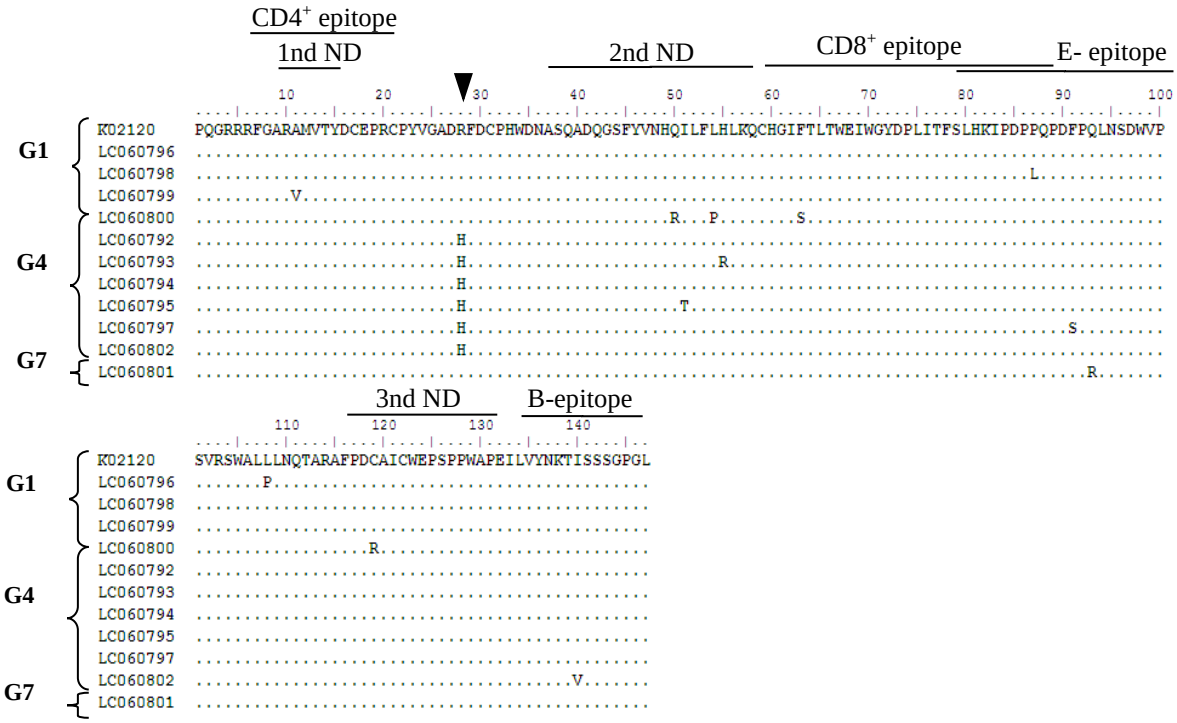


Fig. II-3-3. Amino acid alignment of Mongolian BLV isolates based on the *env* gene. One typical BLV isolate from each identical sample was submitted to GenBank. Unique nucleotide substitutions are indicated by numbers and filled triangle (▼). The first, second and third neutralizing domain (ND) and other epitopes are shown at the top of the alignment. Genotypes (G-1, G4 and G7) are indicated by the black bars at the far left of the figure. Japanese isolate K02120 was used as a reference in this study.

II.3.4. Discussion

Out of 517 bovine samples from 5 sampling areas, 20 positive cattle were found for BLV from only 2 sampling areas and the overall infection rate was 3.9 % but infections were only found in dairy breed cattle such as Holstein, Simmental and Alatau. In contrast, no infections were found in Mongolian native cattle and yaks. The infection rate was varied in each of the sampling areas and highest infection rate as 14.6% was observed from Bornuur soum of Tuv province, in which historical BLV infection of the country was reported. Thus, the BLV prevalence in Mongolian cattle was lower than those in several other countries such as Turkey 11 % (Uysal et al., 1998), Japan 35.2 % (Murakami et al., 2013), Iran 29.9 % (Morovati et al., 2012), the Philippines 4.8–9.7 % (Polat et al., 2015), USA 46 % (Ott et al., 2003), Canada 30.9 % (VanLeeuwen et al., 2001), Colombia 19.8 %, (Benavides et al., 2013), Argentina 85 % (Gutiérrez et al., 2011), and Russia 28.5–36.1 % (Zinovieva et al., 2012).

In addition, 19 positive samples based on the *env* gene were subjected to the sequencing analysis and 11 independent sequences with 96.4 %–100 % homology to one another were identified in this study. In the phylogenetic tree, 4 independent sequences belonged to the genotype 1, six to genotype 4, and one to genotype 7, respectively. Interestingly, Mongolian sequences belonging to genotypes 4 and 7 were the same or similar lineage to the several sequences from Russia and Eastern European countries such as Poland and Ukraine. These findings suggest that BLV infection in Mongolian dairy cattle was probably introduced into Mongolia from dairy cattle impoted from Russia for the establishment and progression of intensive cattle farming. Historically, Mongolian dairy cattle were imported from the former Soviet Union. BLV genotypes 4 and 7 were widely distributed to Russia, Belarus, Ukraine, Poland through cattle trading between countries belonging to Council for Mutual Economic Assistance (CMEA) in the 1970s and 1980s (Rola-Łuszczak et al., 2013). In contrast, the Mongolian isolates belonging to genotype 1 were independently clustered with Iranian isolates. Further study is required to determine the origin of this genotype 1.

These 11 independent nucleotide or deduced amino acid sequences were aligned with the Japanese K02120 strain belonging to the genotype 1 as a reference sequence (Sagata et al., 1985). There were several substitutions in Mongolian sequences when compared to the reference sequence of genotype 1 showed one common substitution at a nucleotide position 127, and all of the sequences that clustered into genotype 4 showed eight common substitutions at nucleotide positions 84, 130, 205, 246, 274, 277, 281 and 337. By contrast,

all of the sequences belonging to genotypes 1, 4, and 7 showed two common substitutions at nucleotide positions 85 and 121 (Fig. II-3-2). In addition, the amino acid alignment which includes the several regions of the *env* gene of BLV, the first neutralizing domain (ND) (amino acid positions 8–12), the second ND (amino acid positions 38–57), and the third ND (amino acid position 117–132), a portion of the $CD4^+$ T-cell epitope (amino acid positions 8–19), the $CD8^+$ T-cell epitope (amino acid positions 61–89), and the viral G (amino acid position 28), E (amino acid positions 80–100), and B (amino acid positions 134–150) epitopes (Balić et al., 2012), showed that high nucleotide similarity to that of the Japanese K02120 strain, 13 different amino acid substitutions were identified among the Mongolian BLV strains (Fig. II-3-3). Of these substitutions, seven belonged specifically to genotype 1 strain, and five belonged to genotype 4 strains. By contrast, only one amino acid substitution was found in the genotype 7 strains. The substitutions were mainly found in antigenic determinants, such as the $CD4^+$ and $CD8^+$ T-cell epitopes, the second neutralizing domain, and the G, B, and E epitopes. However, whether the Mongolian isolates have different pathogenic properties when compared to other isolates remains unclear. The current observations are preliminary, and the accurate rate of BLV infection requires further confirmation using a larger number of samples from several provinces in Mongolia. The implementation of systematic control measures against infectious agents such as BLV in livestock should be maintained in Mongolia.

II.3.5. Summary

Bovine leukemia virus (BLV) belongs to the genus *Deltaretrovirus* within the family *Retroviridae*. BLV infection results in a prolonged asymptomatic period and infected animals can be characterized into three disease stages, namely, aleukemic, persistent lymphocytosis, and leukemia or lymphoma. Increased prevalence of BLV within dairy herds was found to be associated with decreased milk production and longevity of cows. Epidemiological studies have indicated that BLV infection is globally distributed. However, no information regarding the disease and genetic diversity of BLV in the cattle of Mongolia is currently available. In this study, the prevalence of BLV was assessed using PCR, and the genetic diversity was analyzed through DNA sequencing. Of the 517 samples tested, 20 positives were identified. Phylogenetic analysis showed that 4, 6, and 1 isolates were classified into genotypes1, 4, and 7, respectively. Most isolates were clustered with isolates from Eastern Europe and Russia. This study is the first reportto investigate the BLV genotypes in Mongolia.

II.4. Molecular epidemiological survey and genetic characterization of sheep-associated malignant catarrhal fever in Mongolian livestock

II.4.1. Introduction

Malignant catarrhal fever (MCF) is a dramatic and mostly fatal disease in domestic and wild ruminants, which is characterized by low morbidity but high mortality (Li et al., 2014). The causative agents of the disease are several *herpesviruses* in the MCF virus group belonging to the genus *Macavirus* in the subfamily *Gammaherpesvirinae,* the family of *Herpesviridae* (Davison et al., 2009). At least 10 members of the MCF virus group have been identified, six of which are associated with the clinical disease in natural conditions. Of these, *alcelaphine herpesvirus* 1 (AlHV-1) and *ovine gammaherpesvirus* 2 (OvHV-2) are the major causative agents, and are responsible for wildebeest-associated MCF (WA-MCF) and sheep-associated MCF (SA-MCF), respectively (O'Toole and Li 2014). These viruses cause inapparent infection in their respective natural hosts, but can cause fatal lympho-proliferative disease when they infect various other susceptible hosts (Li et al., 2014). For instance, SA-MCF has been known to cause severe disease in several animal species, including cattle (Plowright et al., 1960), white-tailed deer (Palmer et al., 2013), rabbit (Li et al., 2011), pig (Alcaraz et al., 2009), water buffalo (Stahel et al., 2013), bison (Cunha et al., 2012) and mule deer (Schultheiss et al., 2007).

WA-MCF was the first disease to be identified in the MCF group (Plowright et al., 1960) and occurs predominantly on the African continent, particularly in the sub-Saharan regions (Wambua et al., 2016). By contrast, SA-MCF has been reported in many countries, including Turkey (Yildirim et al., 2012), Japan (Giangaspero et al. 2013), Croatia (Turk et al., 2010), Egypt (Zaki et al., 2016), Saudi Arabia (Abu Elzein et al., 2003), India (Sood et al., 2014), America (O'Toole et al., 2002), Indonesia (Wiyono et al., 1994), New Zealand (Wilson, 2002),Spain (Yus et al., 1999), Belgium (Pardon et al., 2009), Brazil (Costa et al., 2009), Italy (Campolo et al., 2008) and Norwey (Loken et al., 2009). Furthermore, SA-MCF has been causing economic and welfare issues in Bali cattle in Indonesia (Daniels et al., 1988) and bison in USA (O'Toole et al., 2002) for many years.

OvHV-2, the causative agent of SA-MCF is predominantly transmitted horizontally (Russell et al., 2009) via the respiratory tract of susceptible animals, mainly through nasal shedding (Taus et al., 2006, 2010; Li et al., 2001, 2008). However, vertical transmission of the virus from infected cow to fetus has also been reported recently (Headley et al., 2015).

The clinical manifestations of SA-MCF are diverse, depending on the susceptibility of

the infected animals to the virus. Most commonly, the gastrointestinal tract, eyes, or central nervous system (Russell et al., 2009) are affected, and infected individuals display severe clinical symptoms such as depression, anorexia, high fever, lymphadenopathy, conjunctivitis, corneal opacity, ulceration, and exudation of the digestive or upper respiratory tract, diarrhea, and neurological deficiencies. Moreover, cattle or other susceptible animals develop mild to severe clinical symptoms ending in death (Sood et al., 2013; O'Toole and Li, 2014).

Preliminary diagnoses for SA-MCF in cattle have been reported in Mongolia (Sugar et al., 2012), but the etiological agent has not been detected as well as the disease has not been confirmed with molecular diagnostic assays such as PCR or sequencing analysis. Therefore, there is a need to examine the prevalence of the infection in several areas and to molecularly characterize the pathogen to better understand the disease epidemiology across various species of livestock in Mongolia. Thus, the purpose of the study was to survey theprevalence of OvHV2, the causative agent of SA-MCF in cattle, yaks, sheep, and goats from five different sampling areas of the country as well as to clarify the molecular characterization of the pathogen.

II.4.2. Material and Methods

II.4.2.1. Samples and study areas

Totally, 928 animal samples including cattle, yaks, sheep and goats were screened in this study and were described in detail in II.2.2.1 of this chapter.

II.4.2.2. DNA extraction

Total cellular DNA was extracted by the same method as described in II.2.2.2 of this chapter.

II.4.2.3. Diagnostic polymerase chain reaction assays for SA-MCF detection

All samples were screened with semi-nested PCR based on theamplification of the tegument protein gene (238 bp), as previously described (Baxter et al., 1993). The primers used in this study were 556 (5'-AGTCTGGGGTATATGAATCCAGATGGCTCTC-3') and 775 (5'-AAGATAAGCACCAGTTATGCATCTGATAAA-3') for the first stage, and 556 and 555 (5'-TTCTGGGGTAGTGGCGAGCGAAGGCTTC-3') for the second stage. The techinque and process for the semi-nested PCR assay to detect the pathogen was the same as described in II.2.2.3 of this chapter.

II.4.2.4. DNA cloning and sequencing

All 14 positive samples were subjected to the sequencing analysis and the technique for cloning and sequencing were the same as described in II.2.2.4 of this chapter.

II.4.2.5 Phylogenetic and homology analyses

Phylogenetic and homology analyses were performed by the same methods as described in II.2.2.5 of this chapter.

II.4.3. Results

II.4.3.1. The prevalence of SA-MCF in Mongolian livestock

The semi-nested PCR assay with 928 samples screened for SA-MCF in Mongolian livestock such as cattle, yaks, sheep and goats in five different areas of the country. Most important sampling area in this study was in Tsenkher soum of Arkhangai Province because SA-MCF outbreak was reported in cattle in this sampling site previously. Asthe result, 14 samples were positive for SA-MCF, 9 out of 10 sheep (90.0%) and 2 out of 20 cattle (10.0%) samples from Tsenkher soum of Arkhangai Province, and 3 out of 201 sheep (1.5%) samples from Lun soum of Tuv Province, respectively. In contrast, no positive samples were detected among the livestock from other areas in Mongolia. All 12 positive sheep were adults and 2 positive cattle were 7- and 11-year-old females (Table II-4-1).

II.4.3.2. Molecular characterization of the pathogen

All 14 positive samples for SA-MCF, representative for hosts or geographical locations were subjected to sequencing analysis targeting the same gene as the PCR assay for the screening of the pathogen. All of the sequences from both cattle and sheep were 100% identical to one another and one representative sequence of this study was deposited to DDBJ as accession number LC203437. The representative sequences from Mongolian livestock was genetically sameas the isolates from India and Egypt whereas slightly divergent as 98.0% homology when compared to the isolates from Germany and Brazil (Fig. II-4-1).

Table II-4-1. Prevalence of SA-MCF in Mongolian sheep and cattle

Province/ City	Soum/ Districts	Animal species	Positive / Tested animals	ID of animals	Age	Gender	Accession No
Arkhangai,	Tsenkher	Sheep	9/10 (90.0%)	1	Adult	F	LC203437
				2	(ND)	M	
				3		F	
				4		F	
				5		F	
				6		F	
				7		F	
				8		M	
				9		F	
		Native cattle	2/20 (10.0%)	8	7Y	F	
				18	11Y	F	
	Bulgan	Yaks	0/72	-	-	-	
Tuv	Lun	Sheep	3/201 (1.5%)	34	2Y	M	
				40	2Y	M	
				67	3Y	M	
		Cattle	0/97	-	-	-	
		Goats	0/200	-	-	-	
	Bornuur	Dairy breed cattle	0/96	-	-	-	
Ulaanbaatar	Songinokhairkhan	Dairy breed cattle	0/204	-	-	-	
		Yaks	0/28	-	-	-	
		Bovine samples 517 Sheep and goats 411					
		Totally 928					

M: male

ND: not determined

Y: year

F: female

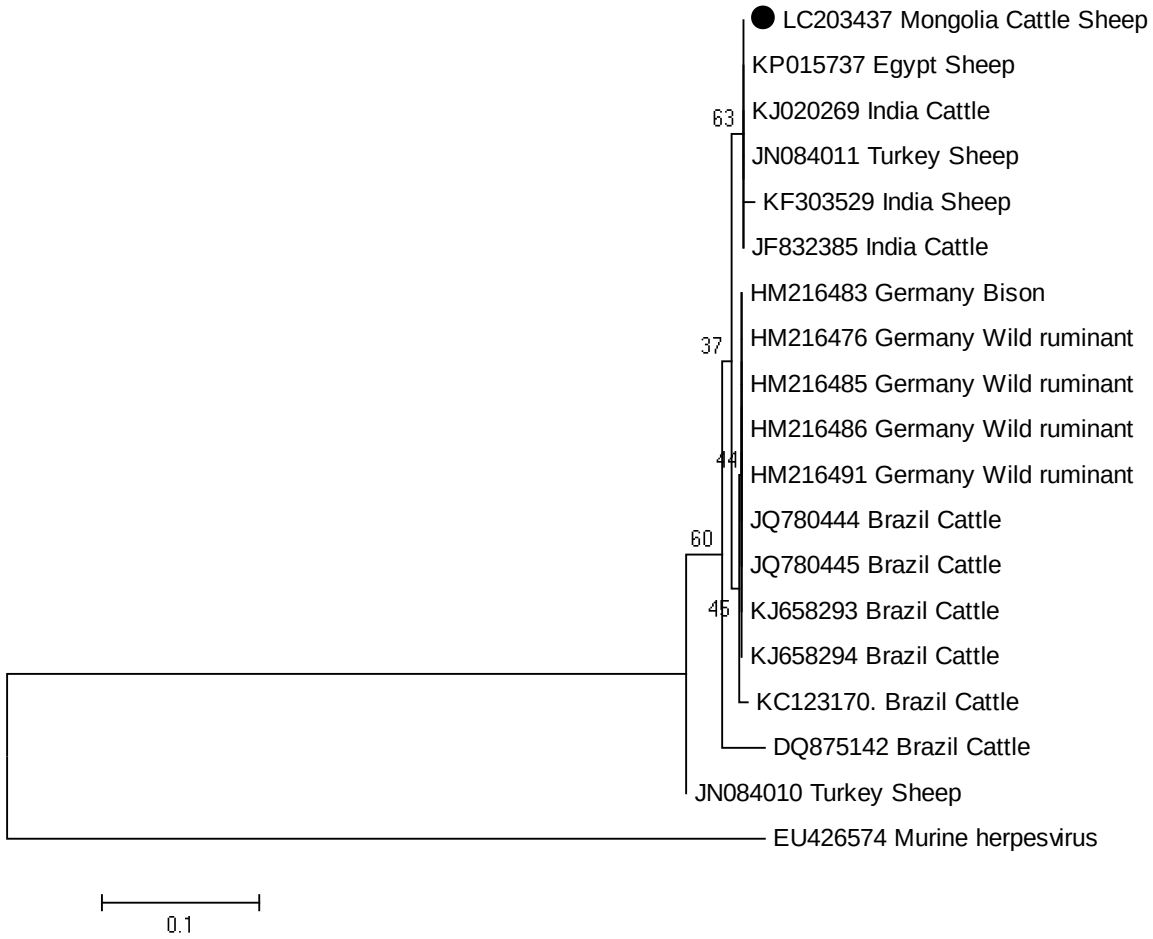


Fig. II-4-1. Phylogenetic relationship of OvHV2 based on the tagument protein gene. The sequence derived from this study is highlighted with a circular bullet (●) regarding to its sequence divergence. The tree was constructed with the neighbor-joining method and was supported by 1,000 bootstrap replications. This figure shows the relationship between 238 bp sequences of the tagument protein gene for OvHV2 obtained from this study and related other sequences from the GenBank. Genotypes and geographical origins of each isolates are indicated by the numbers. The DNA sequences identified in this study were deposited to DDBJ with accession number, LC203437.

II.4.4. Discussion

SA-MCF outbreaks have been reported in many counties but the etiological agent was never detected and the disease has not been confirmed to date even though MCF-like signs have been occasionally observed in Mongolian cattle. Therefore, it was suspected that SA-MCF may be prevalent in Mongolian livestock because both reservoir host sheep and susceptible cattle are kept in close proximity, sharing a stockyard and pasture. The small number of the sample (sheep 10, cattle 20) was collected from Tsenkher soum of Arkhangai Province in which historical SA-MCF-like infection was reported. The pathogen of the disease was detected from both cattle and sheep, and infection rate was extremely high in sheep. Interestingly, the two infected cattle did not exhibit any clinical symptoms at the time of sampling, appearing to be healthy. According to the anamnesis from the owner of these animals, 17 out of 32 cattle that were housed and grazed with sheep and goats suddenly succumbed to an unknown illness with fever, nasal discharge, and eye cataracts between February and August 2012. Consequently, 14 cattle (nine 2-year-old, two 3-year-old, two adult cattle, and one adult bull) died and only 3 cattle recovered from the illness, following no further incidence of the infection in this herd. The infection was diagnosed as SA-MCF by the State Central Veterinary Laboratory of Mongolia based on the clinical symptoms of the affected cattle such as the long-term high fever, the loss of eye sight due to cataracts, and the formation of scaly ulcers in the nose and mouth mucus. In addition, post-mortem examination showed that mucosal ulcerations and hemorrhage were common in the affected organs, particularly in the parietal pericardium, the epithelial lining of the urinary bladder had characteristic ecchymotic hemorrhages, the upper respiratory tract had catarrhal exudates or erosions, and the gastrointestinal tract displayed hemorrhagic or diphtheritic ulcers with clotted blood-like secretions (Sugar et al., 2012).

By contrast, in Lun soum of Tuv Province, in which SA-MCF has not previously been reported in any animals, the pathogen was detected in sheep but not in goats or cattle in this study. Since Lun soum and Tsenkher soum are approximately 350 km away from each other and there was no correlation between disease incidence and animal movements across these areas, animals from these sampling sites may have contracted the infectious diseases independently.

The sequencing analysis for the positive samples showed that the sequences obtained from the 8 sheep and 2 cattle in Tsenkher soum of Arkhangai Province, and 3 sheep in Lun soum of Tuv Province had 100% overlapping identity with one another. The BLAST

searching tool showed that most of the overlapping sequences originated from Brazil, Turkey, Egypt, Germany, and India, whereas none of the sequences had previously been found in China or Russia, which are border of Mongolia. There was not much diversity among the similar sequences, and the sequences from Egypt (KP015737; JF832385), India (KJ020269), and Turkey (JN084011) were 100% identical whereas the sequences from Germany and Brazil were about 98.0% homology to the Mongolian sequences in the phylogenetic tree (Fig. II-4-1).

Therefore, SA-MCF usually appears sporadically in susceptible hosts, but frequent outbreaks of the disease result in significant economic loss due to the high mortality rate. Epidemiological studies have shown that SA-MCF has a global distribution across many countries, particularly those with a large sheep population or high levels of sheep meat consumption such as New Zealand (Wilson et al., 2002), Turkey (Yildirim et al., 2012), India (Sood et al., 2014), USA (O'Toole et al., 2002), Brazil (Costa et al., 2009), Egypt (Zaki et al., 2016) and Saudi Arabia (Abu Elzein et al., 2003).

Our findings indicate that Mongolian sheep are the primary host for OvHV2 and that this pathogen may be widely prevalent in the sheep population. Infected sheep are known to be a major source of SA-MCF, with the infection being transmitted horizontally between animals in close contact with each other (Li et al 2000). However, bison flocks that were separated by up to 5 km have also been reported to be infected (Li et al., 2008). Infected sheep of all ages can shed the virus continuously through nasal secretions, but high levels of virus shedding are predominantly found in 6–9-month-old lambs (Li et al., 2001). Under natural flock conditions, the majority of lambs is not infected with the pathogen until at least 2 months of age (Li et al., 1998) and can remain uninfected as adults if they avoid contact with infected sheep from an early age (Li et al 1999). Therefore, this strategy has been used by sheep producers and zoos in America and Europe to maintain OvHV-2-free sheep populations (Cooley et al., 2008). However, because most Mongolian herders conduct traditional livestock herding practices, where the cattle are kept in the same backyard or pasture as other animals such as sheep or goats, there is a high potential risk of disease outbreak in cattle or other susceptible hosts in prevalent areas of the pathogen.

This study represents the first attempt to undertake a detailed investigation of SA-MCF in Mongolian livestock. Therefore, further studies are required to determine the prevalence of the infection in other parts of Mongolia and to identify the genetic diversity of the pathogen between regions to develop control strategies for the disease in this country.

II.4.5. Summary

Sheep-associated malignant catarrhal fever (SA-MCF) is a fatal *herpesvirus* infection of domestic or wild ruminants with dramatic clinical symptoms, resulting in death of cattle. The recently, SA-MCF has been reported sporadically in Mongolian cattle by local veterinarians based on clinical symptoms but prevalence and molecular characterization of the pathogen is unknown in Mongolia. In this study, the survelliance of SA-MCF in cattle, yak, sheep and goats from 5 different areas in the country was conducted by PCR assay with 928 sampes. As the result, 14 positive samples for SA-MCF were identified from both sheep and cattle in Tsenkher soum and among sheep from Lun soum, Mongolia, but no positive samples were detected from the other sampling areas. The phylogenetic analysis revealed that the Mongolian sequence of SA-MCF was identical to sequences from Egypt, India, and Turkey, and 98.0% similar to sequences from Germany and Brazil. This is the first confirmed report that SA-MCF is prevalent in Mongolian sheep and cattle, and these findings are important for further study and process for the control program of the infection in the country.

II.5. Detection of bovine viral diarrhea virus and identification of its genotype in Mongolian cattle

II.5.1. Introduction

Bovine viral diarrhea virus (BVDV) is classified into two species, namely Bovine viral diarrhea virus 1 (BVDV-1) and Bovine viral diarrhea virus 2 (BVDV-2), within the genus *Pestivirus* and family *Flaviviridae*, together classical swine fever virus and border disease virus (Liu et al., 2009b). Furthermore, new bovine pestiviruses have been reported in several countries, including Thailand (Liu et al., 2009a), Italy (Decaro et al., 2011), Brazil (Ståhl and Alenius, 2012) and China (Mao et al., 2012), and these are referred to as HoBi-like or atypical bovine pestivirus. However, these newly discovered viruses have not yet received official recognition. The diversity of BVDV includes both genetic and antigenic differences, which have an impact on both diagnostic testing and protective effects of vaccination (Fulton et al., 2005).

According to sequence comparison studies that were typically based on the 5'-UTR region, 21 distinct subtypes of BVDV-1 (1a–1u) (Deng et al., 2015), and two subtypes of BVDV-2 (2a-2b) have been recognized to date (Polak et al., 2014; Ren et al., 2013). Although some researchers suggested that BVDV-2 should be classified into three subtypes (2a-2c) (Luzzago et al., 2001), this has not been universally adopted, and most researchs still use the former classification. Although the natural hosts of BVDV are cattle, this virus can infect both domestic and wild ruminants, including deer, pigs, sheep, goats, bison and camelids. The transmission of BVDV occurs by direct and indirect routes and most important sources of BVDV infections are persistently infected (PI) hosts that shed alarge amount of viruses throughout their lives, because the virus can be much more efficiently transmitted from these hosts than from non-PI animals(Wang et al., 2014).

BVDV is also biologically divided into a non-cytopathogenic (NCP) biotype and a cytopathogenic (CP) biotype based on their effects on cultured cells. The CP biotype induces apoptosis in cultured cells, whereas the NCP biotype does not, and only the NCP biotype of BVDV induces persistent infection. The CP biotype of BVDV is relatively rare (Lanyon et al., 2013). The clinical manifestations that are associated with BVDV infections are diverse, with the most common clinical signs in infected cattle being an affected respiratory system, abortion, and diarrhea.

These clinical signs of BVDV are more frequently reported with BVDV-2 than BVDV-1 (Ahn et al., 2005). BVDV is a widely-distributed worldwide and BVD has been reported

in the cattle populations of many countries, including China (Deng et al., 2015; Mao et al., 2012; Ridpath et al., 2000), Korea (Oem et al., 2009), Japan (Nagai et al., 2008), Thailand (Kampa et al., 2004), Poland (Polak et al., 2014), Turkey (Workman et al., 2015), Brazil (Fernandes et al., 2015), Australia (Mahony et al., 2005), and USA (Schirrmeier et al., 2004). However, studies regarding the prevalence and genotype diversity of this virus in animals in Mongolia remain rare. Thus, the aim of this study was to detect BVDV and determine its genotypes in different bovine species in Mongolia, including native cattle, dairy-breed cattle, and yaks.

II.5.2. Materials and Methods

II.5.2.1. Sample collection

During 2014, a total of 127 blood samples, including, 68 samples from yaks, 40 from native cattle, and 19 from dairy cattle, were collected from the Songinokhairkhan district of the city of Ulaanbaatar, Bulgan and Tsenkher soums of Arkhangai province, and Bornuur and Lun soums of Tuv province of Mongolia. The samples were collected from the jugular vein of each animal using a BD K3EDTA Vacutainer tube (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and were stored at 4°C until RNA extraction.

II.5.2.2. RNA extraction and cDNA synthesis

Total RNA was extracted using TRIzol Reagent (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The synthesis of cDNA was performed with random hexamers in a total reaction volume of 20 µL.

II.5.2.3.Reverse transcription polymerase chain reaction (RT-PCR) assay

BVDV infections were detected using a reverse transcription polymerase chain reaction (RT-PCR) assay targeting the 5'-UTR region, and positive samples were characterized further. The PCR primers used in this study were 324/5'-ATGCCCWTAGTAGGACTAGCA-3' and 326/5'-TCAACTCCATGTGCCATGTAC-3' as described previously (Vilcek et al., 2001). RT-PCR amplification was conducted in a total volume of 30 µL containing 1 µL (100 ng) of the cDNA sample that was added to 29 µL of a reaction mixture containing 0.15 µL of Ex Taq polymerase (Takara Bio Inc.), 2.4 µL of dNTPs, 1 µL of 10 µM forward primer, 1 µL of 10 µM reverse primer, 3 µL of Ex Taq buffer, and 21.45 µL of double-distilled water. PCR amplification was performed under the following thermal cycling conditions: initial denaturation at 94 °C for 5 min, followed by 50 cycles of denaturation at 98 °C for 15 sec, annealing at 55 °C for 30 sec, extension at 72 °C for 45 sec, and a final synthesis at 72 °C for 1 min in a GeneAmp PCR System 9700 (Applied Biosystems, USA). The amplified PCR products were confirmed using a Mupid-exU Electrophoresis System (Takara Bio Inc.) on a 2.0 % agarose gel and were visualized under an ultraviolet light print-graph AE-6905CF (Atto, Tokyo, Japan). The *βeta-actin* (*B-actin*) gene was amplified as an internal control to confirm the presence of cDNA in the templates (Okagawa et al., 2012).

II.5.2.4. DNA cloning and sequencing

All 11 positive samples were subjected to the sequecing analysis and the technique for cloning and sequencing were the same as described in II.2.2.4 of this chapter.

II.5.2.5. Phylogenetic and homology analyses

Phylogenetic and homology analyses were performed by the same methods as described in II.2.2.5 of this chapter.

II.5.3.Results

II.5.3.1. Prevalence of the BVDV in Mongolian cattle and yaks

The infection rate of BVDV-positive samples was 15.8 % (3/19) in cattle from Bornuur soum of Tuv province, and 20.0 % (8/40) in yaks from the Bulgan soumof Arkhangai province. In contrast, no positive samples were detected from Mongolian native cattle in Lun soum of Tuv province, Tsenkher soum of Arkhangai province, or yak samples from the Songinokhairkhan district of the city of Ulaanbaatar. The ages of the BVDV-positive animals ranged from 1 month to 17 years old, and all of them were female cattle and yaks, except for one 2-year-old male yak (Table II-5-1). Most of the animals did not exhibit any clinical symptoms at the time of sampling, and only the youngest yak had diarrhea containing blood, but the exact cause of the diarrhea was unknown.

II.5.3.2. Molecular characterizaion of BVDV from Mongolian cattle and yaks

All of the positive samples were subjected to further sequence analysis targeting the 5'-UTR region. Out of 11 sequences, eight were identified as either BVDV-1 or BVDV-2. The nucleotide sequences were 78.3 %-100 % homology to one another, with the sequences of BVDV-1 being 95.2 %–100 % homology and those of BVDV 2 being 91.1 %–100% homology to one another. In the phylogenetic analysis, four sequences (LC099927, LC099928, LC099930, and LC099931) obtained from three yaks in Bulgan soum of Arkhangai province and two cattle in Bornuur soum of Tuv province were found to belong to BVDV-1a and were subclustered with Japanese isolates, with 97.9 %-98.95 % sequence identity (Fig. II-5-1). In contrast, four other sequences (LC099925, LC099926, LC099929, and LC099932) obtained from five yaks in Bulgan soum of Arkhangai province and one cattle in Bornuur soum of Tuv province were clustered in BVDV-2a and branched with isolates from Germany, the USA, and Japan. Of these, two sequences (LC099929 and LC099932) derived from each of the positive sampling sites were 100 % identical to one other, whereas two other sequences (LC099925 and LC099926) obtained from four yaks in Bulgan soum of Arkhangai province were quite divergent from the others (Fig. 5-1).

Table II-5-1. BVDV detection results in Mongolian cattle and yaks

Province/City	Soum/District	Positive rates/ Tested animals (%)	Breed	Positive sample ID	Age	Sex	Genotype	Accession number
Tuv	Bornuur	3/19 (15.8%)	Simmental	37	17 Y	F	1 a	LC099930
			Holstein	40	7 Y	F	1 a	LC099931
			Holstein	91	6 Y	F	2 a	LC099932
	Lun	0/20	Mongolian native	-	-	-	-	-
Arkhangai	Bulgan	8/40 (20.0%)	Yak	1	7 Y	F	2 a	LC099925
				2	8 Y	F	2 a	LC099926
				8	8 Y	F	2 a	LC099926
				10	8 Y	F	1 a	LC099927
				15	1 M	F	1 a	LC099927
				27	2 Y	M	1 a	LC099928
				40	5 Y	F	2 a	LC099929
				42	4 Y	F	2 a	LC099926
	Tsenkher	0/20	Mongolian native	-	-	-	-	-
Ulaanbaatar	Songinokhairkhan	0/28	Yak	-	-	-	-	-
Total		11/127 (8.7%)						

Y: years
M: months

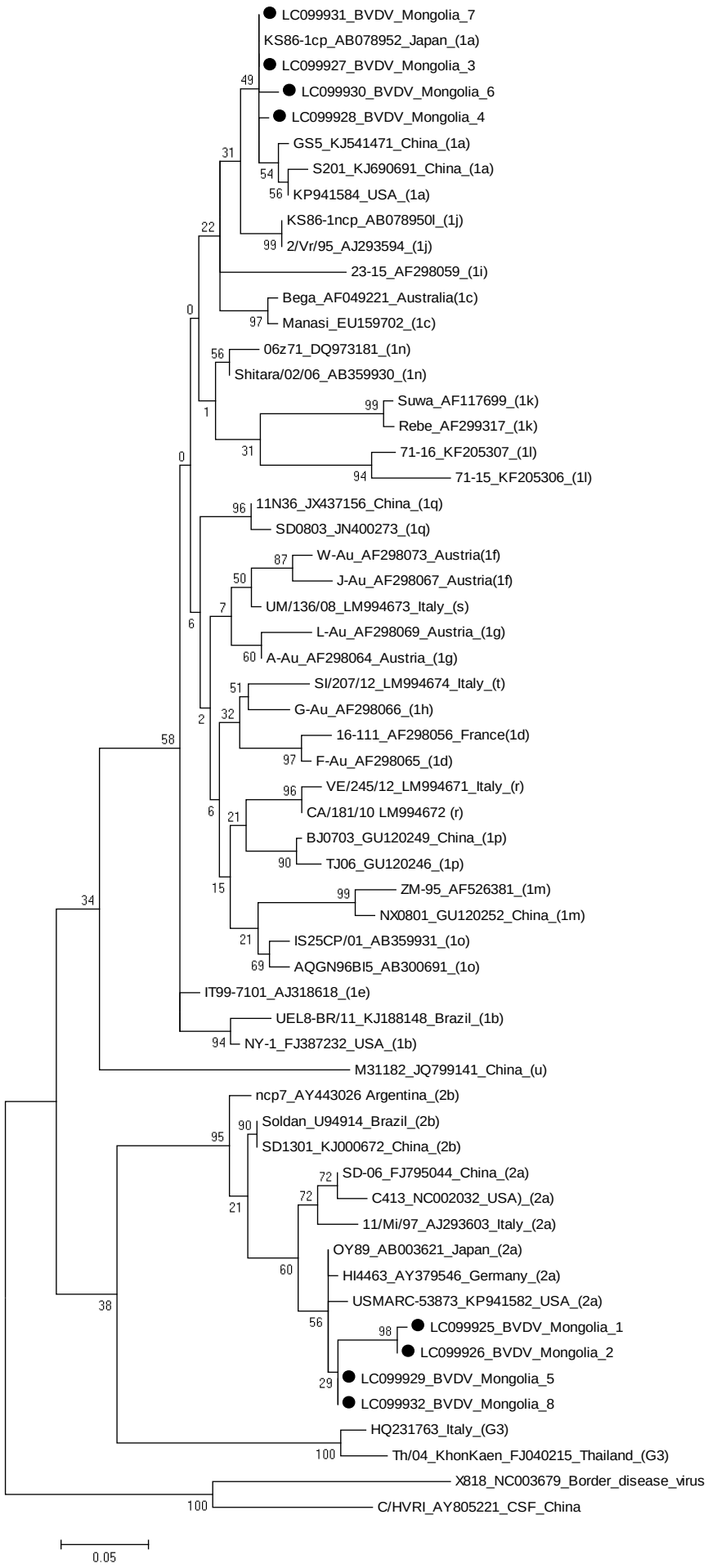

LC099931_BVDV_Mongolia_7
KS86-1cp_AB078952_Japan_(1a)
LC099927_BVDV_Mongolia_3
LC099930_BVDV_Mongolia_6
LC099928_BVDV_Mongolia_4
GS5_KJ541471_China_(1a)
S201_KJ690691_China_(1a)
KP941584_USA_(1a)
KS86-1ncp_AB078950l_(1j)
2/Vr/95_AJ293594_(1j)
23-15_AF298059_(1i)
Bega_AF049221_Australia(1c)
Manasi_EU159702_(1c)
06z71_DQ973181_(1n)
Shitara/02/06_AB359930_(1n)
Suwa_AF117699_(1k)
Rebe_AF299317_(1k)
71-16_KF205307_(1l)
71-15_KF205306_(1l)
11N36_JX437156_China_(1q)
SD0803_JN400273_(1q)
W-Au_AF298073_Austria(1f)
J-Au_AF298067_Austria(1f)
UM/136/08_LM994673_Italy_(s)
L-Au_AF298069_Austria_(1g)
A-Au_AF298064_Austria_(1g)
SI/207/12_LM994674_Italy_(t)
G-Au_AF298066_(1h)
16-111_AF298056_France(1d)
F-Au_AF298065_(1d)
VE/245/12_LM994671_Italy_(r)
CA/181/10 LM994672 (r)
BJ0703_GU120249_China_(1p)
TJ06_GU120246_(1p)
ZM-95_AF526381_(1m)
NX0801_GU120252_China_(1m)
IS25CP/01_AB359931_(1o)
AQGN96BI5_AB300691_(1o)
IT99-7101_AJ318618_(1e)
UEL8-BR/11_KJ188148_Brazil_(1b)
NY-1_FJ387232_USA_(1b)
M31182_JQ799141_China_(u)
ncp7_AY443026 Argentina_(2b)
Soldan_U94914_Brazil_(2b)
SD1301_KJ000672_China_(2b)
SD-06_FJ795044_China_(2a)
C413_NC002032_USA)_(2a)
11/Mi/97_AJ293603_Italy_(2a)
OY89_AB003621_Japan_(2a)
HI4463_AY379546_Germany_(2a)
USMARC-53873_KP941582_USA_(2a)
LC099925_BVDV_Mongolia_1
LC099926_BVDV_Mongolia_2
LC099929_BVDV_Mongolia_5
LC099932_BVDV_Mongolia_8
HQ231763_Italy_(G3)
Th/04_KhonKaen_FJ040215_Thailand_(G3)
X818_NC003679_Border_disease_virus
C/HVRI_AY805221_CSF_China
0.05

Fig. 5-1. Phylogenetic relationship of BVDV based on 5'-UTR region. Sequences derived from this study are highlighted with circular bullets (●) regarding to their sequence divergence. The tree was constructed with the neighbor-joining method and was supported by 1,000 bootstrap replications. This figure shows the relationship in the 287 to 290 bp sequences of the 5'-UTR region of BVDV obtained from this study and related other sequences from the GenBank. Genotypes and geographical origins of each isolates are indicated by small alphabets. The DNA sequences identified in this study were deposited to DDBJ with accession numbers, LC099931, LC099927, LC099930, LC099928, LC099925, LC099926, LC099929, and LC099932, respectively.

II.5.4. Discussion

The RT-PCR assay with 127 cDNA samples were screened for BVDV, and found 11 positive samples from dairy breed cattle in Bornuur soum of Tuv province and yaks in Bulgan soum of Arkhangai province. This is the first report that BVDV is prevalent in Mongolian cattle and yaks though epidemiological studies have shown that BVDV is globally distributed in the cattle populations of many countries. The overall positive rate of BVDV infection in the two sampling areas in Mongolia was 8.7 % and was lower than that in China where BVDV is highest prevalent as the infection rate is 22.6 % for RT-PCR detection and 58.0 % for antibody detection in several ruminant species, including cattle, yaks, and water buffalo (Deng et al., 2015) and 23.1 %-33.6 % in pigs (Deng et al., 2012). Thus, the results of these studies were consistent with our results showing that yaks are more susceptible to BVDV infection. However, the infection rate in Mongolia is likely to be higher, and a more realistic rate could be obtained if a large number of cattle and yaks were screened for the virus, particularly among dairy-breed cattle in the country, because the number of samples from the infected areas was small. In general, each of the sampling sites may have separately contracted this infectious disease, because there was no correlation with animal movement between these areas.

The investigation for PI hosts is very important because they shed alarge amount of viruses throughout their lives and the virus can be much more efficiently transmitted fromthese hosts than from non-PI animals. Therefore, there are several tests for BVDV diagnosis, including virus isolation, RT-PCR, immunohistochemistry, and antigen enzyme-linked immunosorbent assay (Ag ELISA), but these tests require time and repeated sampling to clarify whether the infection is persistent or not. In this study, other diagnostic tests other than RT-PCR were not conducteddue to the difficulties in sample transportation between Mongolia and Japan, and also because of a lack of informationon the prevalence of the infection in the country. The current study was primarily a molecular survey of BVDV infection in Mongolian animals together with a description of the host animals and their geographical location.

In addition, all ofthese RT-PCR positive samples were subjected to the sequencing analysis based onthe 5'-UTR region of the virus, and eight independent sequences were identified as 78.3 %–100 % homology to one another in this study. In the phylogenetic tree, four independent sequences as up to 95.2 % homology, derived from both cattle and yaks belonged to the gentotype BVDV-1a and were clustered together with the isolates from Japan and China. Another four independent sequences as up to 91.1 % homology, derived

from also both cattle and yaks belonged to the genotype BVDV-2a and branched together with the isolates from Japan, USA and Germany.The habitats of dairy cattle and yaks are different in the country, with dairy cattle mostly distributed around the city of Ulaanbaatar, whereas yaks are only distributed to high mountainous areas, including Arkhangai province, but both BVDV 1 and BVDV 2 were identified from each of the animal species in this study. This indicates that BVDV is likely to be prevalent widelyin Mongolian livestock, including dairy cattle and yaks.

The recent phylogenetic analysis of BVDV revealed the existence of at least 21 subtypes within BVDV-1 (1a−1u) (Deng et al., 2015). BVDV 1a has been reported in several countries, including Canada, France, Germany, New Zealand, Mozambique, Spain, Sweden, the UK, the USA (Walz et al., 2010), Australia (Mahony et al., 2005), China (Deng et al., 2012), Korea (Oem et al., 2009) and Japan (Nagai et al., 1998). In contrast, two subtypes of BVDV-2 have been identified, namely, BVDV-2a and BVDV-2b (Yilmaz et al., 2012). Subtype 2a appears to be prevalent in USA (Fulton et al., 2005), Italy (Luzzago et al., 2001), Korea (Oem et al., 2009), Poland (Ren et al., 2013), Japan (Luzzago et al., 2001) and Turkey (Oguzoglu et al., 2010). In contrast, 2b has been identified in China (Yilmaz et al., 2012), Brazil (Canal et al., 1998) and Argentina (Jones et al., 2004). However, some European countries have successfully implemented thecontrol and eradication programs for BVDV infections since the 1990s, and the Scandinavian countries are now considered free from the disease, but the control program is still underway in some European countries (Vilcek et al., 1994).

There are three important aspects of BVDV prevention and control: 1) identifying and eliminating PI animals; 2) enhancing immunity by vaccination, and 3) implementing biosecurity measures to prevent exposure of BVDV to susceptible animals (Wang et al., 2014). The animal industry in Mongolia is considered one of the major sectors that influence economic development of the country. However, epidemiological studies of infectious diseases, including BVDV, have been limited Mongolian livestock. However, the prevalence of BVDV infection and identification of its genotypes in Mongolian cattle and yaks were investigated in this study, further studies are required to determine the accurate prevalence and genetic diversity of BVDV infections in a larger number of susceptible animals, including cattle and yaks, because the information gained would be important for the processing of the control programs.

5.5. Summary

Bovine viral diarrhea virus (BVDV) is classified into two species, namely, BVDV-1 and BVDV-2, and affects cattle worldwide, resulting in significant economic loss. The prevalence of BVDV-1 and BVDV-2 infections and its genotypes in Mongolian animals has not been studied. In this study, the molecular survey of BVDV infection in dairy cattle and yaks from Bornuur and Bulgan soums was performed by RT-PCR, and the average infection rates in the sampling sites were 15.8 % and 20.0 %, respectively. In addition, molecular features of the 5'-UTR region of the BVDV genome in Mongolian cattle and yaks were identified as belonging to the subtypes BVDV-1a and BVDV-2a, respectively. Determining the prevalence, geographical distribution, and molecular diversity of BVDV-1 and BVDV-2 in various host species in Mongolia is important for further studies and process of the control programs.

CHAPTER III

Study on identification of immunoinhibitory molecules of Mongolian native cattle and yaks

III.1. Introduction

The strategies to control of infectious diseases focus on either the pathogen or the host. Therefore, understand the host immune system against certain pathogens is essential for the control of infectious diseases (Whitelaw and Sang, 2005). The host can evolve two types of defense mechanisms to increase its fitness when challenged with pathogens: resistance and tolerance (Schneider and Ayres 2008). The immunoinhibitory molecules such as programmed cell death 1 (PD-1), programmed cell death-ligand 1 (PD-L1), T-cell immunoglobulin and mucin domain 3 (TIM-3), galectin 9 (GAL-9), lymphocyte activation gene 3 (LAG 3), and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) are expressed on several immune cells during chronic infection and cancers, and these molecules negatively regulate immune function such as, change the manner of homeostasis, activation, and differentiation of effecter T cells (Grosso et al., 2007; Hastings et al., 2009; Gorman and Colgan, 2014; Goldberg and Drake, 2011; Buchbinder and Desai et al., 2016; Das et al., 2017). Therefore, the comparative in-dept study of molecular structures of these moleculesamong bovine species is very important to determine differences with regards to their reaction against pathogens (Workman et al., 2002; Zhu et al., 2005; Blank and Mackensen, 2007; Anderson, 2014; Arasanz et al., 2017; Walker et al., 2011).

PD-1is a member of the immunoglobulin (Ig) superfamily, and has been identified as a cell surface receptor upon theinteraction with its ligand, PD-L1, and can inhibit the function of antigen-specific T cells (Blank and Mackensen, 2007). PD-1 is expressed mostly on the surface of T cells, but it is also expressed on B cells, natural killerT cells, activated monocytes, and dendritic cells, whereas PD-L1 is constitutively expressed on various cells such as, T and B cells, dendritic cells, macrophages, mesenchymal stem cells, and bone marrow–derived mast cells (Keir et al., 2008). Moreover, the PD-1/PD-L1 pathway is involved in immune dysfunction associated with several tumors and chronic infections through the transmission of an inhibitory signal which reduces cytokine production and suppresses T cell proliferation (Sharpe et al., 2007).

TIM-3 is a member of TIM family, and has been identified as a transmembrane protein, which contains an immunoglobulin and a mucin-like domain and is persistently expressed in dysfunctional T cells during chronic infection and cancer (Anderson, 2014). It is mostly found on various cells such as $CD4^{+}$Thelper (Th) 1 cells, cytotoxic T-lymphocytes (CTL), monocytes and natural killer (NK) cells. TIM-3 is characterized as a negative regulator for immune responses (Das et al., 2017), contains a predicted intracellular tyrosine-kinase phosphorylation motif, and acts as a functional receptor that transduces signals through the

phosphorylated tyrosine residue (Monney et al., 2002; Kuchroo et al., 2003). Gal-9,a member of the galectin family (also called S-type lectins) is secreted by several cells and has been identified as the TIM-3 ligand (Zhuet al., 2005).GAL-9 consists of N- and C-terminal carbohydrate-binding domains, which are connected by a link peptide and binds to TIM-3 via a carbohydrate chain (Wada and Kanwar, 1997; Zhu et al., 2005). Gal-9-induced intracellular calcium flux, aggregation, inhibition of cell proliferation and cytokine production as well as death of T cells are known to be TIM-3-dependent (Hastings et al., 2009; Sabatos et al., 2003). Therefore, theTIM-3/GAL-9 pathway hasbeen closely associated with immune exhaustion and disease progression in chronic infectious diseases and tumors (Barber et al., 2006; Tieu et al., 2014).

CTLA-4 and LAG-3 are member of the immunoglobulin superfamily, and have been identified as a membrane protein. CTLA-4 is expressed on activated effector cells and regulatory T cells (Tregs), that binds to CD80 (B7-1)/CD86 (B7-2) on antigen-presenting cells (APCs), while LAG-3 is expressed on various immune cells such as T lymphocytes, NK cells, eosinophils, monocytes and dendritic cells(Mulley and Nikolic-Paterson, 2008; Wing et al., 2011, Workman et al., 2002). LAG-3 has four extracellular Ig-like domains with conserved structural similarities between domains 1 and 3, as well as between domains 2 and 4 (Huard et al., 1994). CTLA-4 and LAG-3 takes part in the mechanisms involved in the downregulation of immune responses during the progression of chronic diseases as well as in facilitating immune evasion by several pathogens causing chronic infections and tumors (Leng et al., 2002; Kaufmann and Walter, 2009, Grosso et al., 2007; Khaitan and Unutmaz, 2011).

Mongolians have been engaged with the animal hubsandry for long time and the all of the livestock graze in free range pasture land, which is associated with great risks of the exposure to infectious agents from the wildlifes. Several pathogens were detected from Mongolian livestock as described in chapter II, but none of the Mongolian native animal showed any visible clinical signs during the infection with BVDV, MAP, OvHV2, or *A. ovis*.However, there is no detailed information for playing role of immunoinhibitory molecules in Mongolian native animals during disease progression. Therefore, molecular identification of immunoinhibitory molecules such as, PD-1, PD-L1, TIM-3, GAL-9, LAG-3, and CTLA-4 in Mongolian native cattle and yak were characterized through cloning and sequencing analysis in this study.

III.2. Materials and Methods

III.2.1. Sample collection

Total of 127 blood samples were taken for RNA extraction, including, 68 samples from yaks, and 40 from native cattle from the Songinokhairkhan district of the city of Ulaanbaatar, Bulgan and Tsenkher soums of the province of Arkhangai, and Lun soum of Tuv province of Mongolia in 2014. In addition, 18 representative samples for each of the animal species inlcuding 3 samples for one specific gene each were used for the amplification and identification of the molecules. Blood was collected from the jugular vein of each animal using a BD K3EDTA Vacutainer tube (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and was stored at 4°C until RNA extraction.

III.2.2. RNA extraction and complementary DNA synthesis

Total RNA was isolated from whole blood using TRIzol® reagent (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The RNA samples were stored at -80°C until further use.

The complementary DNA (cDNA) synthesis was performed using cDNA synthesis kit (Takara Bio Inc., Otsu, Shiga, Japan) following the manufacturer's procedure. The primer mixture of RNA was prepared using 1 µL of random 6 mers, 1 µL of dNTP, 3 µL of extracted RNA templates and 5 µL of RNase-free distilled water. The mixture was incubated in a PCR machine at 65°C for 5 min and was cooled immediately on ice.Then, the reaction mixture was added for each sample as containing 4 µL of 5× PrimeScript Buffer, 0.5 µL of RNase Inhibitor, 1 µL of PrimeScript RTase and 4.5 µL of RNase-free distilled water. The combining 20 µL of reaction mixture was first incubated at 30°C for 10 min. The temperature was then raised to 50°C for 45 min and raised further to 95°C for another 4 min. After incubation, total reaction mixture was stored at -20°C until subsequent reactions.

III.2.3.Polymerase chain reaction (PCR) for the amplification of the *β-actin* gene

The *β-actin* primers, forward 5'-TCTTCCAGCCTTCCTTCCTG-3' and reverse 5'-ACCGTGTTGGCGTAGAGGTC-3, were used (Okagawaet al., 2012). One µL of a cDNA sample was added to 29 µL of a reaction mixture containing 0.1 µL of rTaq polymerase (Takara Bio Inc.), 2.4 µL of dNTPs, 1 µL of 10 µM forward primer, 1 µL of 10 µM reverse primer, 3 µL of Ex Taq buffer, and 21.5 µL of double-distilled water. A total of 30 µL of the reaction micture were subjected to PCR consisting of initial denaturation for

5 min at 94°C, 35 cycles of denaturation step for 30 sec at 94°C, annealing step for 30 sec at 55°C, and extension step for 30 sec at 72°C followed by the final extension step for 5 min at 72°C that completed the reaction, using the GeneAmp PCR System 9700 (Applied Biosystems, USA). The amplicon size was 112 base pairs. The amplified PCR products were separated by electrophoresis on a 2% agarose gel and visualized under UV light print-graph AE-6905CF (Atto, Tokyo, Japan). Only samples positive for the *β-actin* gene were further subjected to PCR for the amplification of immunoinhibitory molecules.

III.2.4. PCR amplification of immunoinhibitory molecules

Immunoinhibitory receptor molecules such as PD-1, PD-L1, TIM-3, GAL-9, LAG 3, and CTLA-4 were amplified by PCR. The primers used in this study are shown in Table III-1. Briefly, 5 µl of synthesised cDNA samples was added to 45µl of reaction mixture that is comprised 5µl of 10xEx *taq* buffer (Takara Bio Inc, Japan), 4 µl of dNTPs (Takara Bio Inc, Japan), 4µl (10 µM concentration) of forward and reverse primers (Hokkaido System Science Company, Japan), 10 µl of 5M betain, 0.25µl of Ex *taq* polymerase (Takara Bio Inc., Japan), 17.75 µl of DDW. The PCR was performed with initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at each optimal temperature for 30 sec, extension at 72°C for 1 min, and a final synthesis at 72°C for 7 min using the GeneAmp PCR System 9700 (Applied Biosystems, USA). The amplified PCR products were separated by electrophoresis and visualized under UV light as described above.

III.2.5. DNA cloning and sequencing

Three positive samples for each of the molecules of Mongolian native cattle and yaks were subjected to the sequecing analysis and the technique for cloning and sequencing were the same as described in II.2.2.4 of chapter II.

III.2.7 Phylogenetic and homology analyses

Phylogenetic and homology analyses were performed by the same methods as described in II.2.2.5 of Chapter II. In addition, signalP4.1 server (DTU, Copenhagen, Denmark), TMHMM server version 2.0 (DTU), and NetNGlyc 1.0 server (DTU) were used to predict the cleavage sites, transmembrane helices, and possible N-linked glycosylation sites in the deduced protein sequences of each genes.

Table III-1. Primers for detection and characterization of immunoinhibitory molecules in Mongolian native cattle and yak

Gene	Product size		Primers (5´-3´)	References
PD-1	513	849	ATG GGG ACC CCG CGG GCG CT GAT GAC CAG GCT CTG CAT CT	Mingala et al., 2011
	504		AAT GAC AGC GGC GTC TAC TT TCA GAG GGG CCA GGA GCA GT	
PDL-1	870		CGG CAG GTC ATT CCA GAA A CCA AAC CAC AGG CTG AGA A	This study
CTLA-4	666		ATG GCT TGC TCT GGA TTC CA TCA ATT GAT GGG AAT AAA ATA AGG C	Mingala et al., 2011
GAL-9	972		GGG AGA AGT GGC AGT GGC TAC AGA ATC CAG ATA GCA GCA CAG GGC AG	Okagawa et al., 2012
LAG-3	1031	1551	CCT GAT CTG TCT GGC TTT CC GAG AAG ACT GGA TCC CCA CA	This study
	773		TGT GGG GAT CCA GTC TTC TC AGC TGA GGA GAT GAG GAT GG	
TIM-3	843		AAA CGG CAC CTA AAC AGA GC GAC AAC ACC AAG CCC CTA GA	Okagawa et al., 2012

III.3. Results

III.3.1. Nucleotide and amino acid alignments of the immunoinhibitory molecules

Six immunoinhibitory molecules were succesfully cloned and identified from both Mongolian native cattle and yak. The amplicon sizes of them were same for each of the animals as, 843 bp encoding for 280 aa for TIM-3, 972 bp encoding for 323 aa for GAL-9, 1,551 bp encoding for 516 aa for LAG-3, 666 bp encoding for 221 aa for CTLA-4, 849 bp encoding for 282 aa for PD-1, and 870 bp encoding for 298 aa sequences for PD-L1 genes.

The identities of nucleotide and amino acid sequences were 82.9-100% and 73.4-100% for the TIM-3 gene (Tables III-2), 69.1-100% and 94.1-100% for the GAL-9 gene (Tables III-3), 87.3-99.9% and 80.2-99.6% for the LAG-3 gene (Tables III-4), 97.6-100% and 95.9-100% for the CTLA-4 gene (Tables III-5), 80.5-100% and 71.6-100% for the PD-1 gene (Tables III-6), and 82.0-100% and 82.5-100% for the PD-L1 gene (Tables III-7) in Mongolian native cattle compared to the sequences from other artiodactyl species. The identities of nucleotide and amino acid sequences were 81.9-97.9% and 72.0-96.5% for the TIM-3 gene (Tables III-2), 95.8-100% and 94.1-100% for GAL-9 gene (Tables III-3), 87.4-99.6% and 70.8-99.4% for the LAG-3 gene (Tables III-4), 97.3-99.7% and 95.9-99.5% for the CTLA-4 gene (Tables III-5), 80.8-99.3% and 72.8-99.6% for the PD-1 gene (Tables III-6), 88.1-99% and 82.9 -99.7% for the PD-L1 genes (Tables III-7) in yak compared to the sequences from other *artiodactyla* species.

III.3.2. Prediction of structure of the immunoinhibitory molecules

The amino acid sequences of those immunoinhibitory molecules were subjected to the prediction server of protein structure as the Center for Biological Sequence Analysis (CBS) at the Technical University of Denmark (DTU). According to the prediction, amino acid residues of TIM-3 at positions 1-23 with 23 amino acid (aa), 24-191 with 168 aa, 192-214 with 23 aa and 215-280 with 67 aa were found to correspond to the signal peptide, extracellular domain containing immunoglobulin and mucin domains, transmembrane region, and intracellular domain (Fig. III-1a), whereas amino acid residues of GAL-9 at positions 1-148 with 148 amino acid (aa), 149-197 with 49 aa, and 198-323 with 126 aa were found tocorrespond to the N-terminal carbohydrate-recognition domain, a linker peptide, and C-terminal carbohydrate-recognition domain (Fig. III-2a).

Amino acid residues of LAG-3 at positions 1-23 with 23 amino acid (aa), 24-433 with 410 aa, 434-456 with 23 aa and 457-516 with 60 aa were found to correspond to signal peptide, extracellular domain containg 4 regions as domain 1-4, transmembrane domain,

and intracellular domain (Fig. III-3a) whereas amino acid residues of CTLA-4 at position 1-35 with 35 aa, 36-161 with 125 aa, 162-182 with 21 aa and 185-221 with 36 aa correspond to signal peptide, extracellular domain, transmembrane region, and intracellular domain (Fig. III-4a).

Amino acid residues of PD-1 at positions 1-20 with 20 amino acid (aa), 21-170 with 149 aa, 171-191 with 21 aa, and 192-282 with 91 aa were predicted as the signal peptide, extracellular domain, transmembrane region, and intracellular domain (Fig. III-5a) whereas amino acid residues of PD-L1 at positions 1-24 with 24 amino acid (aa), 25-238 with 214 aa, 239-259 with 21 aa, and 260-289 with 30 aa were predicted as the signal peptide, extracellular domain containing IgV and IgC domain, transmembrane region, and intracellular domain, respectively (Fig. III-6a).

III.3.3. Predicted functional domains and motifs of the immunoinhibitory molecules

The amino acid alignment of the immunoinhibitory molecules obtained from Mongolian native cattle and yak showed that all cysteine residues including4 aa for GAL-9, 6 aa for PD-1, 8 aa for TIM-3, LAG-3, and CTLA-4, and 10 aa for PD-L1 were intact in their respective locations except for 1 aa substitution at position 9 in TIM-3 for yak (Y). In addition, potential N-linked glycosylation sites were also intact for these genes and were found as 2 aa for TIM-3 at positions 73 (NVT) and 99 (NVT), 1 aa for GAL-9 at position 54 (NDS), 2 aa for LAG-3 at positions 166 (NCS) and 225 (NVS), 3 aa for CTLA-4 at positions 37 (NVT), 111 (NLT) and 143 (NGT), 1 aa for PD-1 at positions 49 (NAT), and 3 aa for PD-L1 at positions 35 (NVT), 192 (NVT) and 200 (NTT), respectively. The potential tyrosine-kinase phosphorylation motif (HPVENIY) was conserved in intracellular domain of TIM-3 for all bovine species. In addition, possible cleavage site by metalloproteases at positions 404-413 with 10 aa, and an immunoinhibitory motif at positions 473-478 with 6 aa as 'KTGELE' were found for LAG-3, respectively.

III.3.4. Amino acid substitutions of the immunoinhibitory molecules

Amino acid alignment showed that immunoinhibitory molecules among different cattle breeds were high homology to one another, and there was no amino acid substitution in GAL-9, CTLA-4, PD-1 and PD-L1 in Mongolian native cattle compared to the both Holstein and Hereford breed cattle. In TIM-3 of Mongolian native cattle, 3 aa substitutions (1 aa for extracellular doma for intracellular domain) and none of amino amino acid substitution were observed compared to those of Holstein and Hereford breed cattle,

respectively. In addition, 2 aa substitutions (1 aa for extracellular domain and another for intracellular domain) were found in the amino acid residues of LAG-3 of Mongolian native cattle compared to the both Holstein and Hereford breed cattle.

Whereas amino acid alignment of immuninhibitory molecules between yak and cattle showed that 8 aa substitutions (1 aa for the signal peptide, 4 aa for extracellular domain, 1 aa for trabsmembrane region, and 2 aa for intracellular domain) for TIM-3, 2 aa substitutions for LAG-3 (both 2 aa for extracellular domain), 1 aa substitution for intracellular domain of CTLA-4, 1 aa substitution for extracellular domain of PD-1, and 1 aa substitution for intracellular domain of PD-L1 were found, respectively.

III.3.5. Phylogenetic analysis of the immunoinhibitory molecules

The phylogenetic analysis revealed that immunoinhibitory molecules of Mongolian native cattle and yak belonged to the artiodactyla cluster including the sequences from several species such as cattle, buffalo, sheep, goat and pig.

The deducedamino acid sequences of these genes in Mongolian native cattle showed high homologies to the sequences from other bovine species such as, 96.8-97.5% for swamp- or riverine-type buffaloes and 98.9-100% for Holstein or Hereford breed cattle in TIM-3 (Fig. III-1b); 89.3-93.1% for swamp-or riverine-type buffaloes and 99.6-99.8% for Holstein, Hereford or Japanese black cattle in LAG-3 (Fig. III-3b); 96.0% for riverine-type buffalo and 100% for Holstein or Hereford cattle in GAL-9 (Fig. III-2b); 99.1% for swamp-type buffalo and 100% for Holstein, Hereford or Japanese black cattle in CTLA-4 (Fig. III-4b); 98.2% for wild yak (*Bos mutus*), 97.2% for both swamp- or riverine-type buffaloes, and 100% for Holstein or Hereford cattle in PD-1 (Fig. III-5b); 97.9% for swamp-type buffalo and 100% for Holstein or Hereford cattle in PD-L1 (Fig. III-6b).

The deduced amino acid sequences of immunoinhibitory molecules in yak also showed high homoloies to the sequences from other bovine species: 95.1-94.7% for swamp-or riverine-type buffaloes and 96.1-96.5% for Hereford or Holstein cattle in TIM-3 (Fig. III-1b); 96.0% for riverine-type buffalo and 100% for both Holstein and Hereford breed cattle in GAL-9 (Fig. III-2b); 89.5-93.1% for swamp- or riverine-type buffaloes, and 99.2-99.4% for Holstein, Hereford or Japanese black cattle in LAG-3 (Fig. III-3b); 98.6% for swamp-type buffalo and 99.5% for Holstein, Hereford or Japanese black cattle in CTLA-4 (Fig. III-4b); 99.6% for wild yak (*Bos mutus*), 97.2% for swamp- or riverine-type buffaloes, and 99.6% for Holstein or Hereford cattle in PD-1 (Fig. III-5b); and 98.3% for swamp-type buffalo and 99.7% for Holstein or Hereford cattle in PD-L1 (Fig. III-6b).

Table III-2. Comparison of the nucleotide and amino acid sequences of TIM-3 among Mongolian native cattle, yak and different species

Species	Nucleotide and amino acid homology percentage			
	Mongolian native cattle		Yak	
	Nucleotide	Amino acid	Nucleotide	Amino acid
Mongolian native cattle (LC271182)	-	-	97.9	96.1
Yak (LC271183)	97.9	96.1	-	-
Holstein Cattle (AB689695)	99.1	98.9	97.6	96.5
Hereford Cattle (NM001077105)	100.0	100.0	97.9	96.1
Swamp-type Buffalo (LC002530)	98.2	97.5	97.0	95.1
Riverine-type Buffalo (LC002529)	97.7	96.8	96.6	94.7
Sheep (XM004009016)	96.8	94.0	95.1	90.8
Goat (XM005683223)	97.2	95.4	95.7	93.0
Pig (XM003134109)	82.9	73.4	81.9	72.0
Horse (XM003362865)	84.4	75.4	83.2	73.7
Dog (XM536456)	83.2	75.8	82.5	73.3
Chimpanzee (XM518059)	77.9	64.9	76.9	62.8
Human (NM032782)	78.0	65.2	76.9	63.2
Rat (NM001100762)	73.1	60.7	72.5	59.3
Mouse (NM134250)	74.2	62.8	73.7	61.8

Table III-3. Comparison of the nucleotide and amino acid sequences of GAL-9 among Mongolian native cattle, yak and different species

Species	Nucleotide and amino acid homology percentage			
	Mongolian native cattle		Yak	
	Nucleotide	Amino acid	Nucleotide	Amino acid
Mongolian native cattle (LC271178)	-	-	99.7	100.0
Yak (LC271179)	99.7	100.0	-	-
Holstein Cattle (AB689697)	100.0	100.0	99.7	100.0
Hereford Cattle (NM001015570)	99.9	100.0	99.6	100.0
Riverine-type Buffalo (LC002527)	97.6	96.0	97.3	96.0
Sheep (XM004012486)	96.1	94.1	95.8	94.1
Rabbit XM002718781	78.0	71.9	78.0	71.9
Horse (XM001504075)	80.6	72.5	80.7	72.5
Dog (NM001003345)	82.2	74.1	82.1	74.1
Mouse(NM001159301)	75.5	67.3	75.4	67.3
Rat (NM012977)	75.9	70.1	75.7	70.1
Chimpanzee (XM001156415)	82.2	73.8	82.1	82.2
Human NM002308)	83.0	75.6	82.9	75.6

Table III-4. Comparison of the nucleotide and amino acid sequences of CTLA-4 among Mongolian native cattle, yak and different species

Species	Nucleotide and amino acid homology percentage			
	Mongolian native cattle		Yak	
	Nucleotide	Amino acid	Nucleotide	Amino acid
Mongolian native cattle (LC271176)	-	-	99.7	99.5
Yak (LC271177)	99.7	99.5	-	-
Holstein Cattle (AB910936)	100.0	100.0	99.7	99.5
Japanese-black Cattle (AB910937)	99.8	100.0	99.5	99.5
Hereford Cattle (NM174297)	100.0	100.0	99.7	99.5
Swamp-type Buffalo (FJ827142)	98.9	99.1	98.6	98.6
Sheep (AF092740)	97.6	95.9	97.3	95.9
Dog (AF154843)	87.0	87.5	86.7	87.1
Cat (AF170725)	87.8	86.2	87.5	85.7
Monkey (AF344846)	84.6	83.0	84.3	82.6
Human (AY209009)	85.4	84.4	85.1	83.9
Mouse (BC042741)	77.3	73.7	77.1	73.2

Table III-5. Comparison of the nucleotide and amino acid sequences of PD-1 among Mongolian native cattle, yak and different species

Species	Nucleotide and amino acid homology percentage			
	Mongolian native cattle		Yak	
	Nucleotide	Amino acid	Nucleotide	Amino acid
Mongolian native cattle (LC271172)	-	-	99.3	99.6
Yak (LC271173)	99.3	99.6	-	-
Holstein Cattle (AB510901)	100.0	100.0	99.3	99.6
Hereford Cattle (NM001083506)	100.0	100.0	99.3	99.6
Swamp-type Buffalo (FJ827144)	97.4	97.2	96.9	97.2
Riverine-type Buffalo (FJ827145)	97.5	97.2	97.1	97.2
Wild_yak (Bos_mutus) (XM005901507)	98.4	98.2	99.7	99.6
Goat_(XM013963098)	93.0	88.7	92.7	88.7
Mouflon (XM012157336)	94.7	92.3	94.5	92.3
Pig_(NM_001204379)	80.5	71.6	80.8	72.8
Horse (XM005610777)	76.5	65.7	76.4	66.1
Dog (NAB898677)	78.3	69.9	77.5	69.8
Cat (NM001145510)	77.6	69.6	78.0	70.0
Monkey (NM001114358)	74.5	64.6	74.3	64.6
Human (NM0050182)	74.6	63.9	74.4	63.9
Rat (NM001106927)	66.6	56.3	67.4	56.3
Mouse (NM008798)	66.0	51.5	66.3	51.5

Table III-6. Comparison of the nucleotide and amino acid sequences of PD-L1 among Mongolian native cattle, yak and different species

Species	Nucleotide and amino acid homology percentage			
	Mongolian native cattle		Yak	
	Nucleotide	Amino acid	Nucleotide	Amino acid
Mongolian native cattle (LC271174)	-	-	99.9	99.7
Yak (LC271175)	99.9	99.7	-	-
Holstein Cattle (AB510902)	100.0	100.0	99.9	99.7
Hereford Cattle (NM001163412)	100.0	100.0	99.9	99.7
Swamp-type Buffalo (FJ827146)	98.3	97.9	98.4	98.3
Pig (AY837780)	88.0	82.5	88.1	82.9
Horse (XM001492842)	87.5	81.5	87.5	81.5
Dog (XM541302)	83.9	79.5	84.0	79.5
Monkey (EF444816)	82.0	71.9	82.1	72.3
Human (NM014143)	82.9	73.3	83.0	73.6
Mouse (NM021893)	73.4	65.8	73.5	65.9

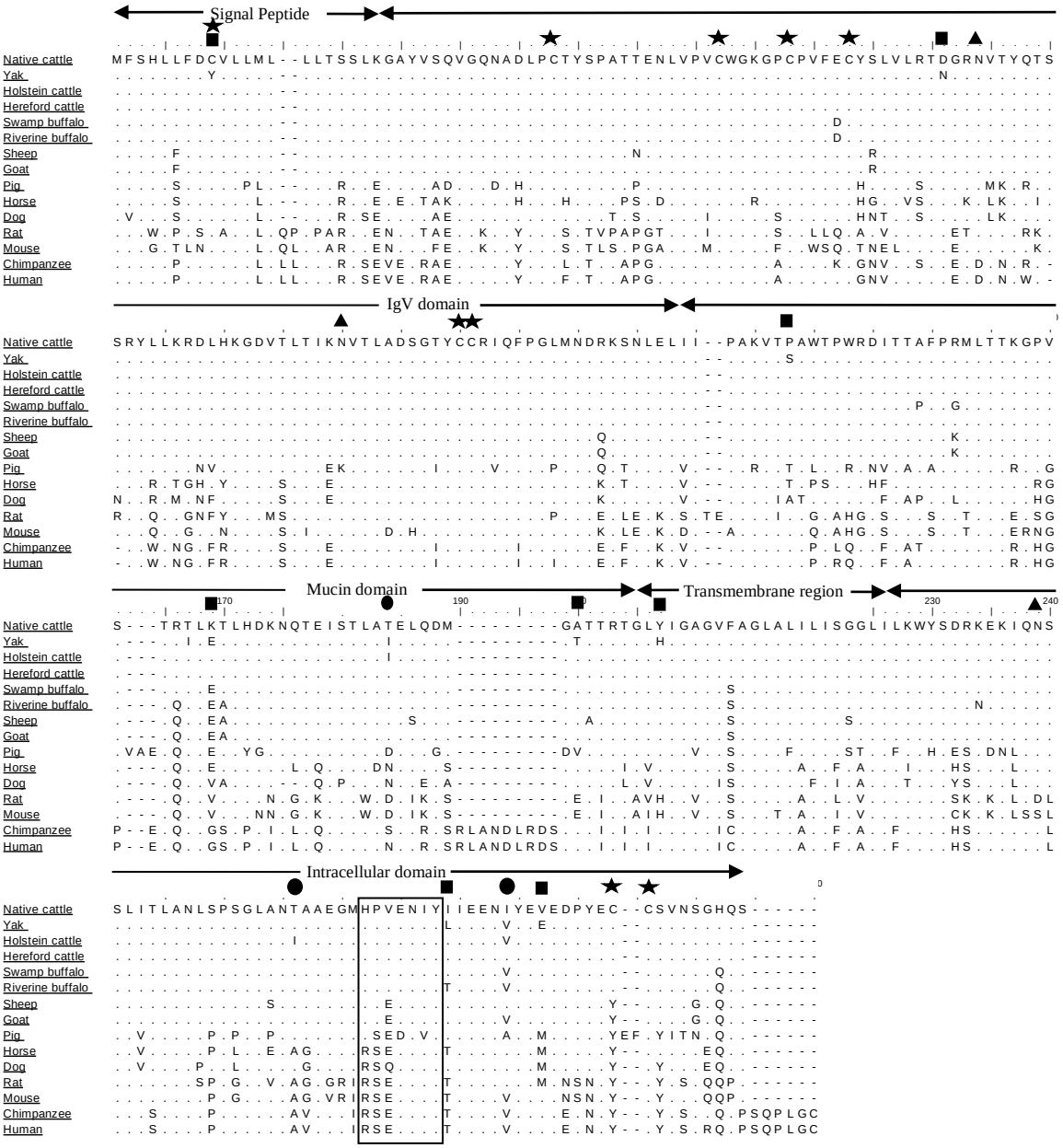


Fig. III-1a. Alignment of deduced amino acid sequences of TIM-3 from Mongolian native cattle and yak compared to other species. A solid star indicates the cystein residue, and solid triangle indicates the potential N-linked glycosylation sites. Solid square indicates the amino acid difference between yak and cattle species. Solid circle indicates the amino acid substitution between Mongolian native cattle and other cattle species. The tyrosin-kinase phosphorylation motif was enclosed in a box.

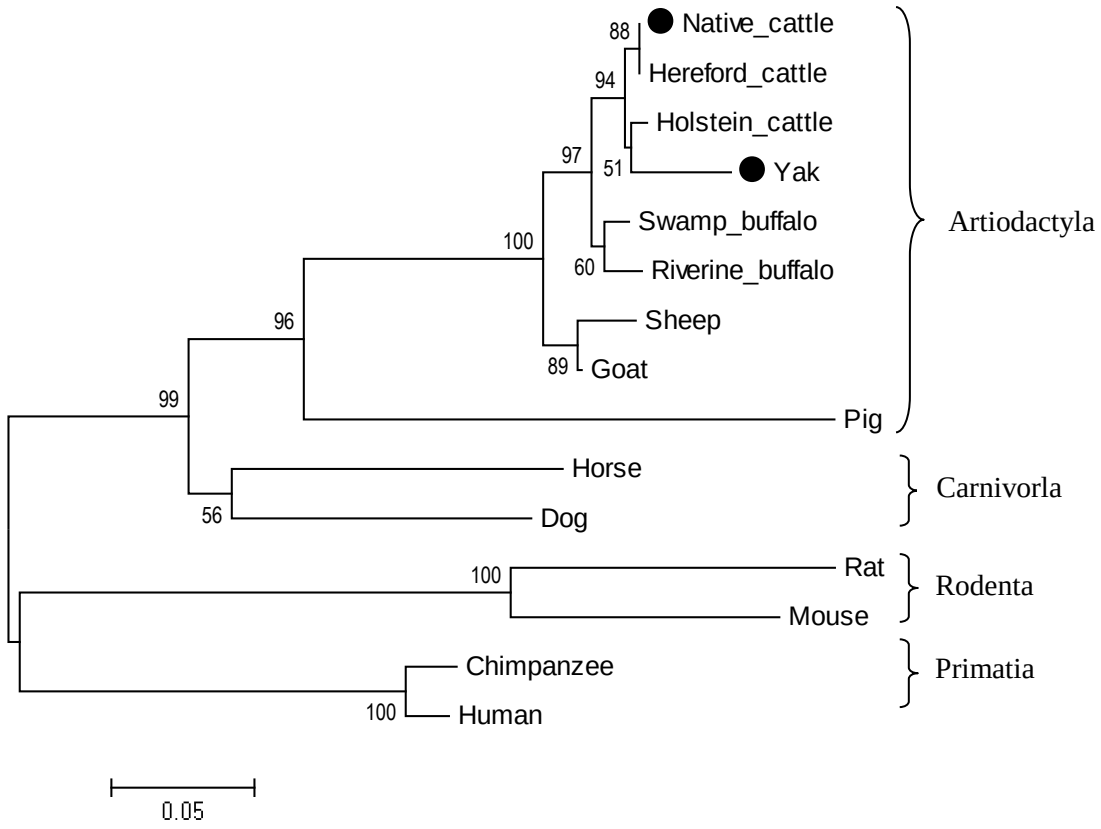


Fig. III-1b.A phylogenetic tree constructed based on the amino acid sequences of TIM3 from Mongolian native cattle and yak compared to other species. Sequences derived from this study are highlighted with circular bullets (●) regarding to their sequence divergence. The tree was constructed with the neighbor-joining method and was supported by 1,000 bootstrap replications.

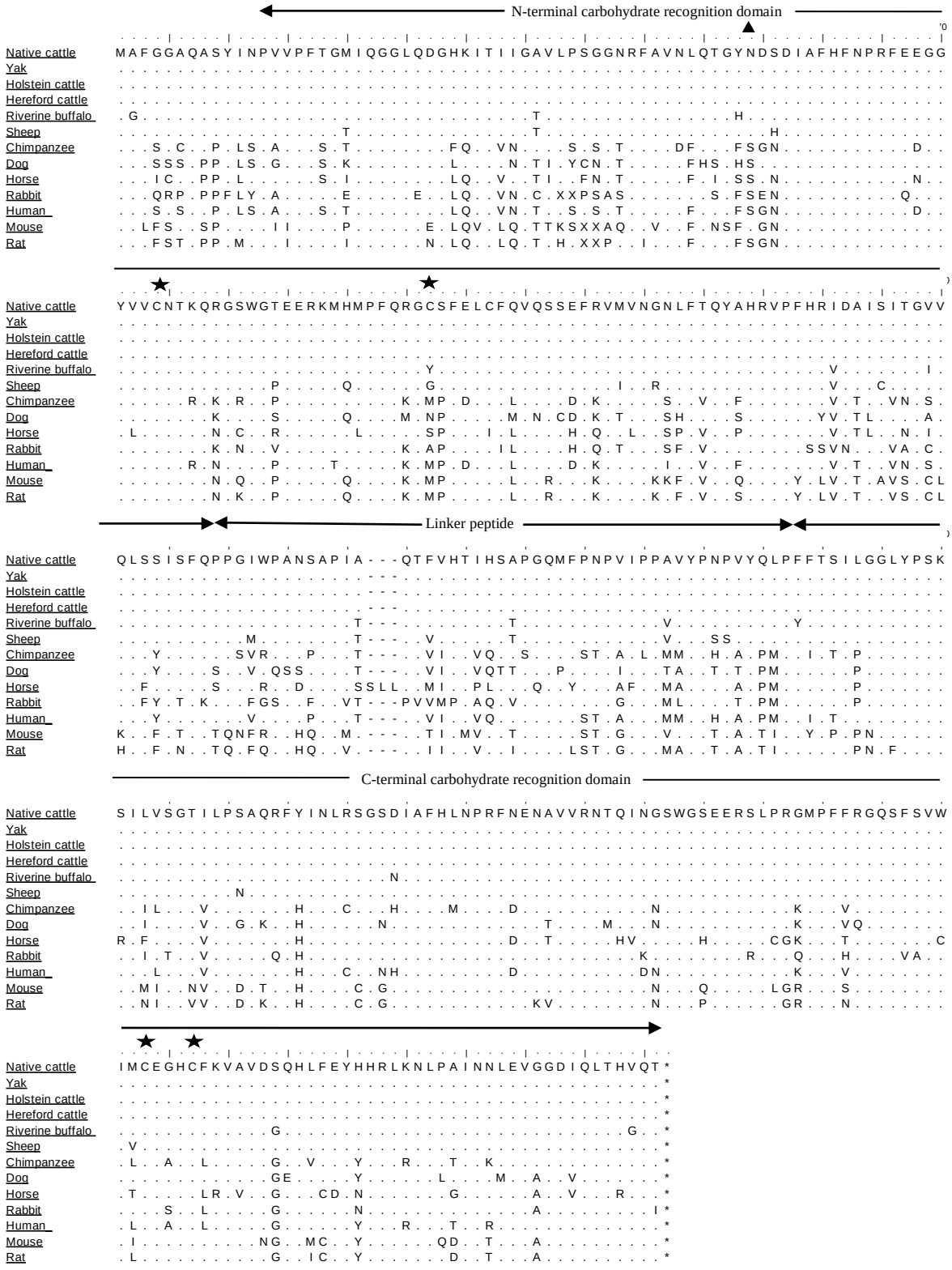


Fig. III-2a. Alignment of deduced amino acid sequences of GAL-9 from Mongolian native cattle and yak compared to other species. A solid star indicates the cystein residue, and a solid triangle indicates the potential N-linked glycosylation site.

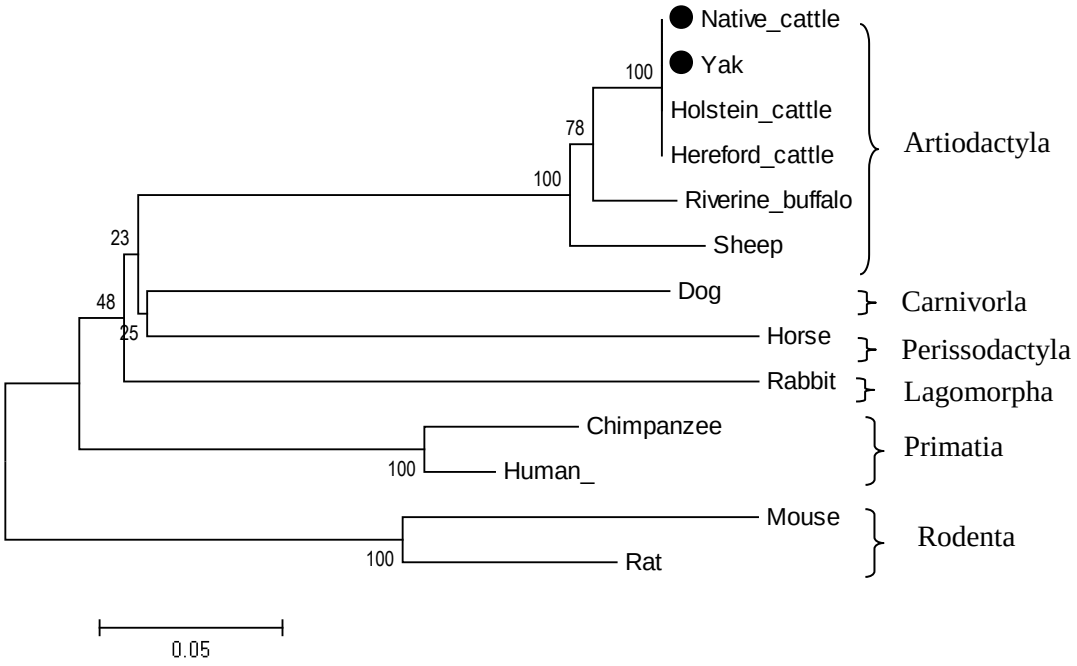


Fig. III-2b. A phylogenetic tree constructed based on the amino acid sequences of GAL-9 from Mongolian native cattle and yak compared to other species. Sequences derived from this study are highlighted with circular bullets (●) regarding to their sequence divergence. The tree was constructed with the neighbor-joining method and was supported by 1,000 bootstrap replications.

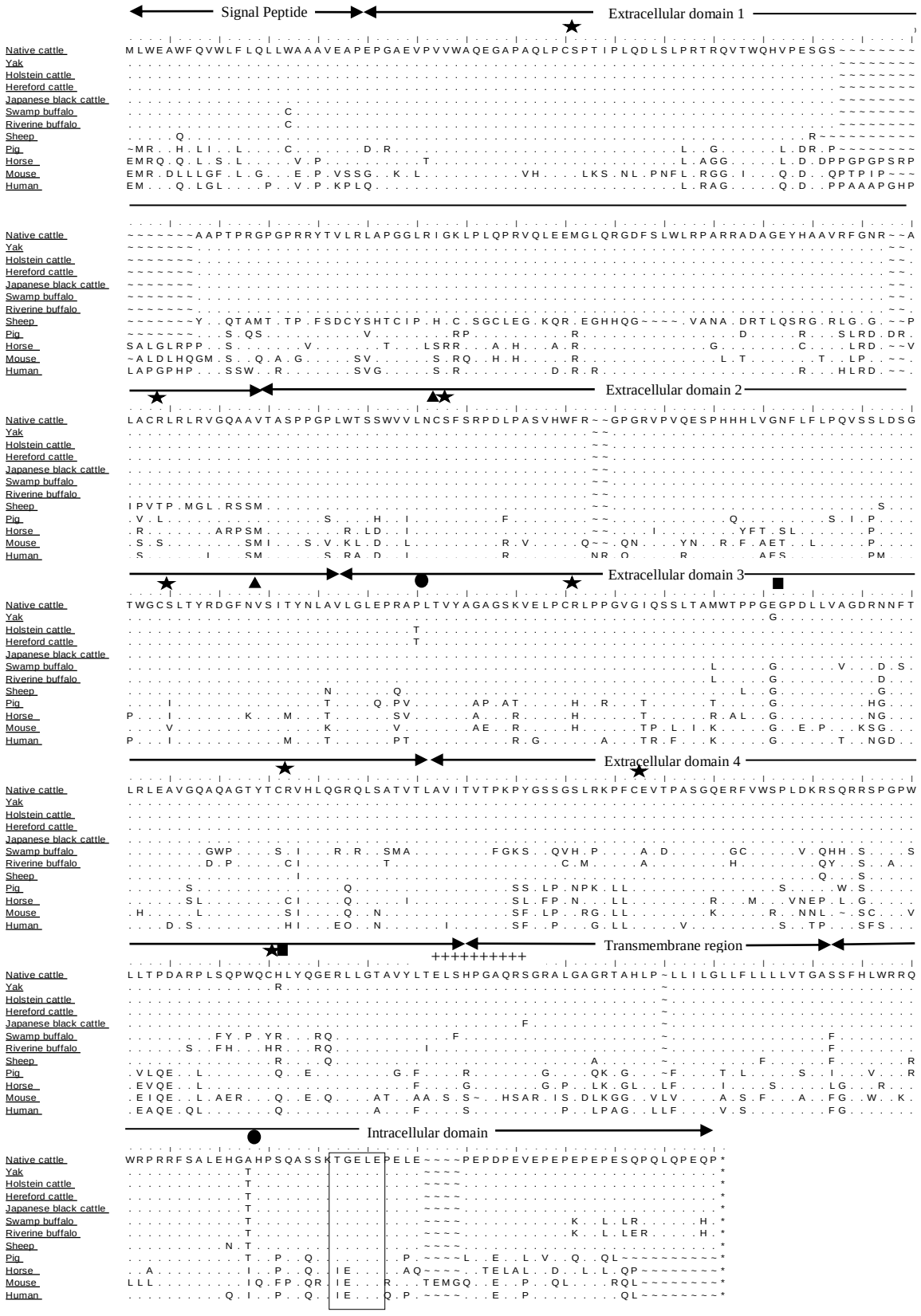


Fig. III-3a. Alignment of deduced amino acid sequences of LAG-3 from Mongolian native cattle and yak compared to other species. A solid star indicates the cystein residue, and a solid square indicates the amino acid difference between yak and cattle species. A solid circle indicates the amino acid substitution between Mongolian native cattle and other cattle species. Amino acid residue at positions 404-413, indicated by plus (+) are possible cleavage sites by metalloproteases. Enclosed in the box is the KTGELE inhibitory motif. Solid triangle indicates the potential N-linked glycosylation sites.

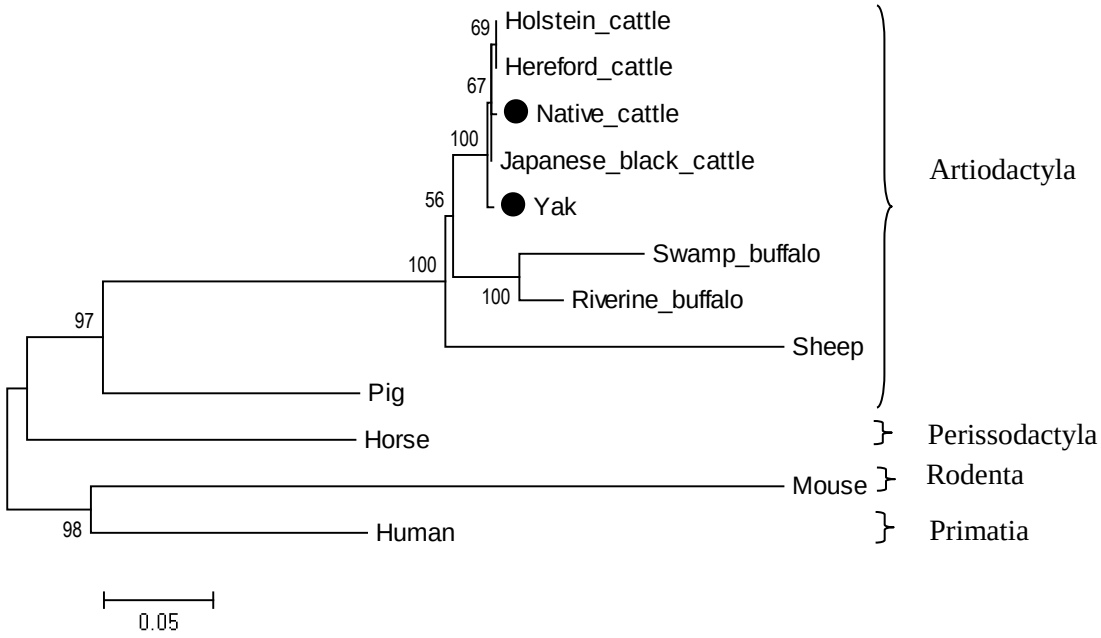


Fig. III-3b. A phylogenetic tree constructed based on the amino acid sequences of LAG-3 from Mongolian native cattle and yak compared to other species. Sequences derived from this study are highlighted with circular bullets (●) regarding to their sequence divergence. The tree was constructed with the neighbor-joining method and was supported by 1,000 bootstrap replications.

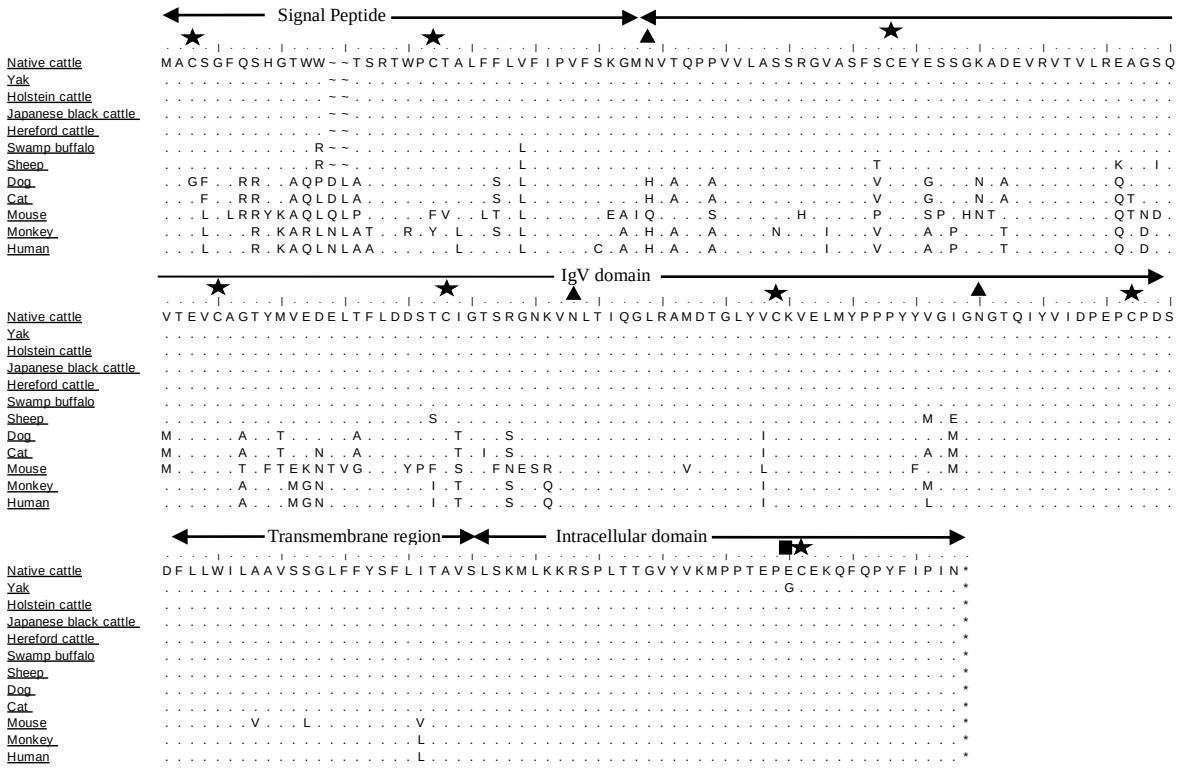


Fig. III-4a. Alignment of deduced amino acid sequences of CTLA-4 from Mongolian native cattle and yak compared to other species. A solid star indicates the cystein residue, and solid triangle indicates the potential N-linked glycosylation sites. Solid square indicates the amino acid difference between yak and cattle species.

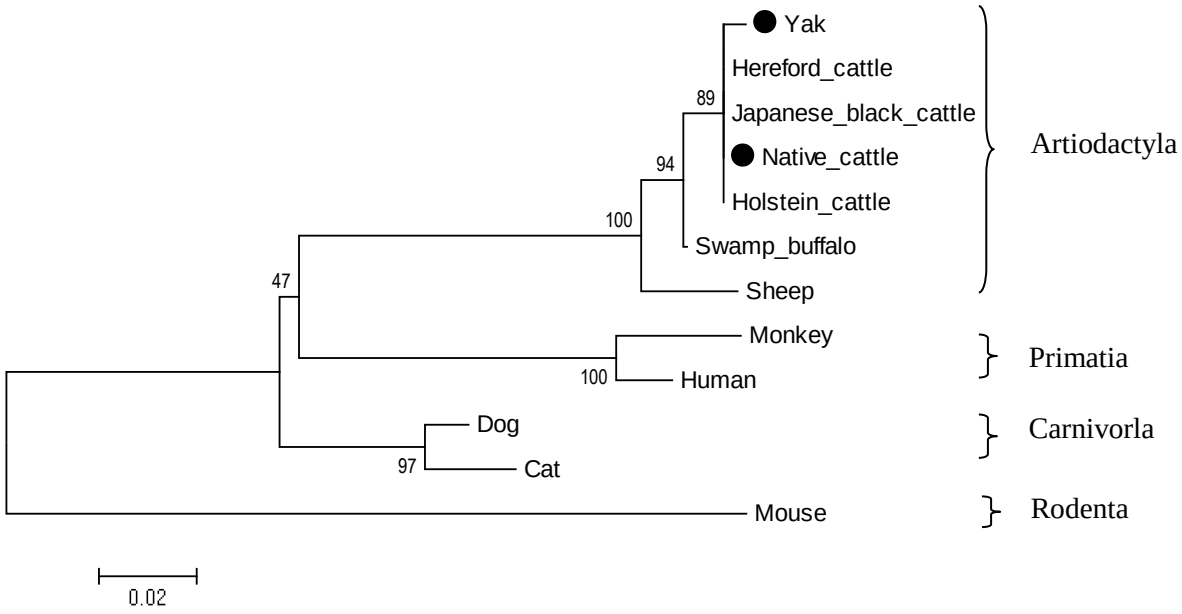


Fig. III-4b. A phylogenetic tree constructed based on the amino acid sequences of CTLA-4 from Mongolian native cattle and yak compared to other species. Sequences derived from this study are highlighted with circular bullets (●) regarding to their sequence divergence. The tree was constructed with the neighbor-joining method and was supported by 1,000 bootstrap replications.

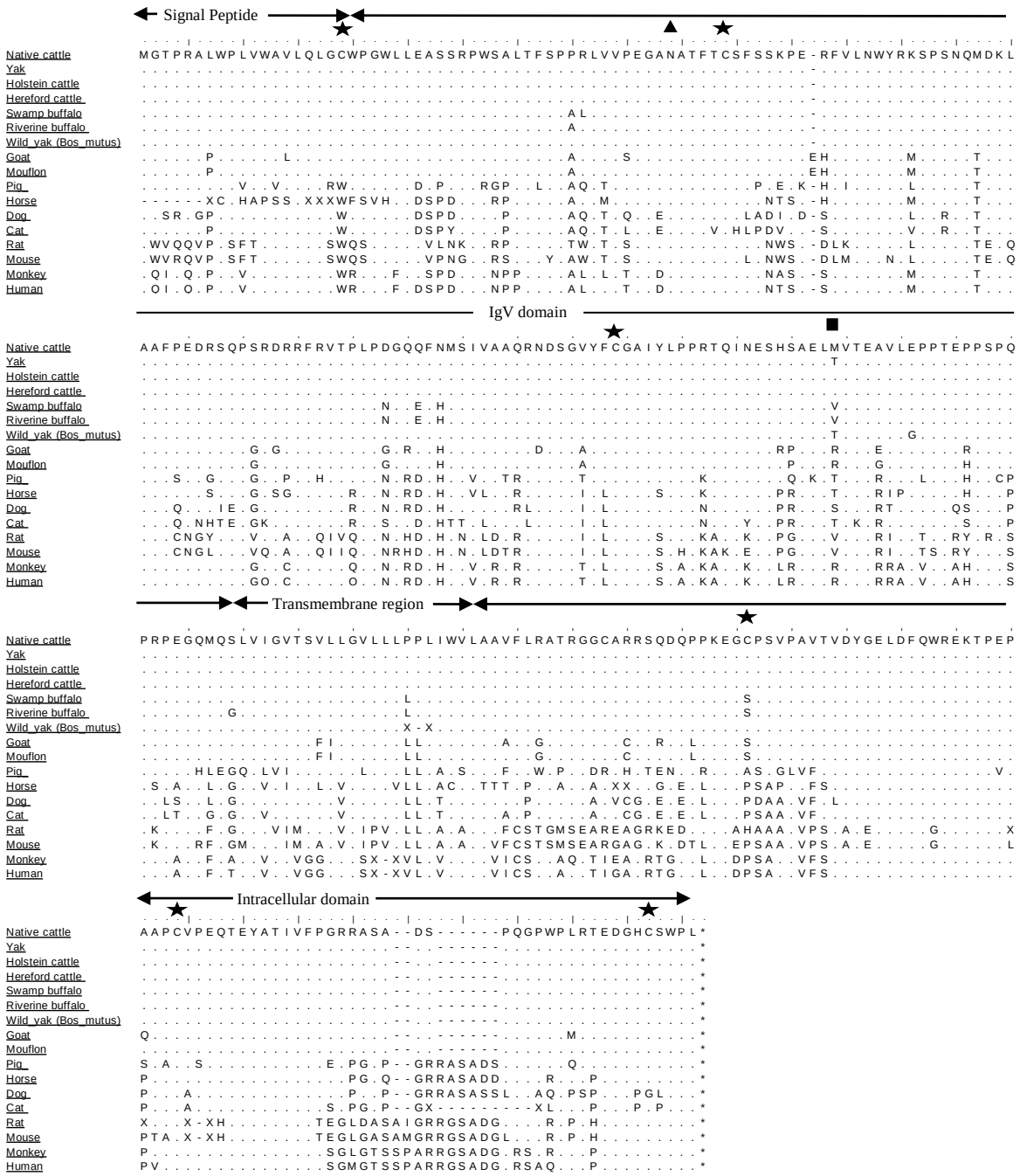


Fig. III-5a. Alignment of deduced amino acid sequences of PD-1 from Mongolian native cattle and yak compared to other species. A solid star indicates the cystein residue, and a solid triangle indicates the potential N-linked glycosylation sites. A solid square indicates the amino acid differences between yak and cattle species.

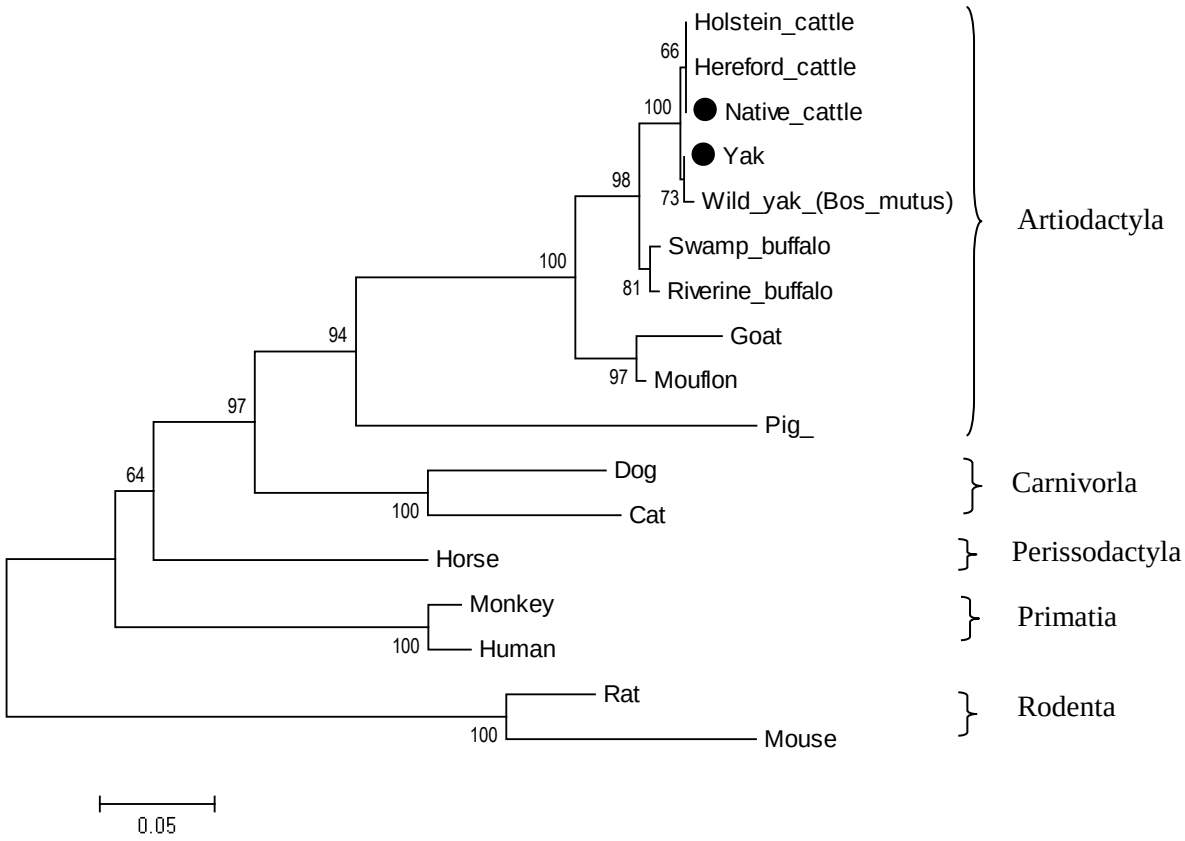


Fig. III-5b. A phylogenetic tree constructed based on the amino acid sequences of PD-1from Mongolian native cattle and yak compared to other species. Sequences derived from this study are highlighted with circular bullets (●) regarding to their sequence divergence. The tree was constructed with the neighbor-joining method and was supported by 1,000 bootstrap replications.

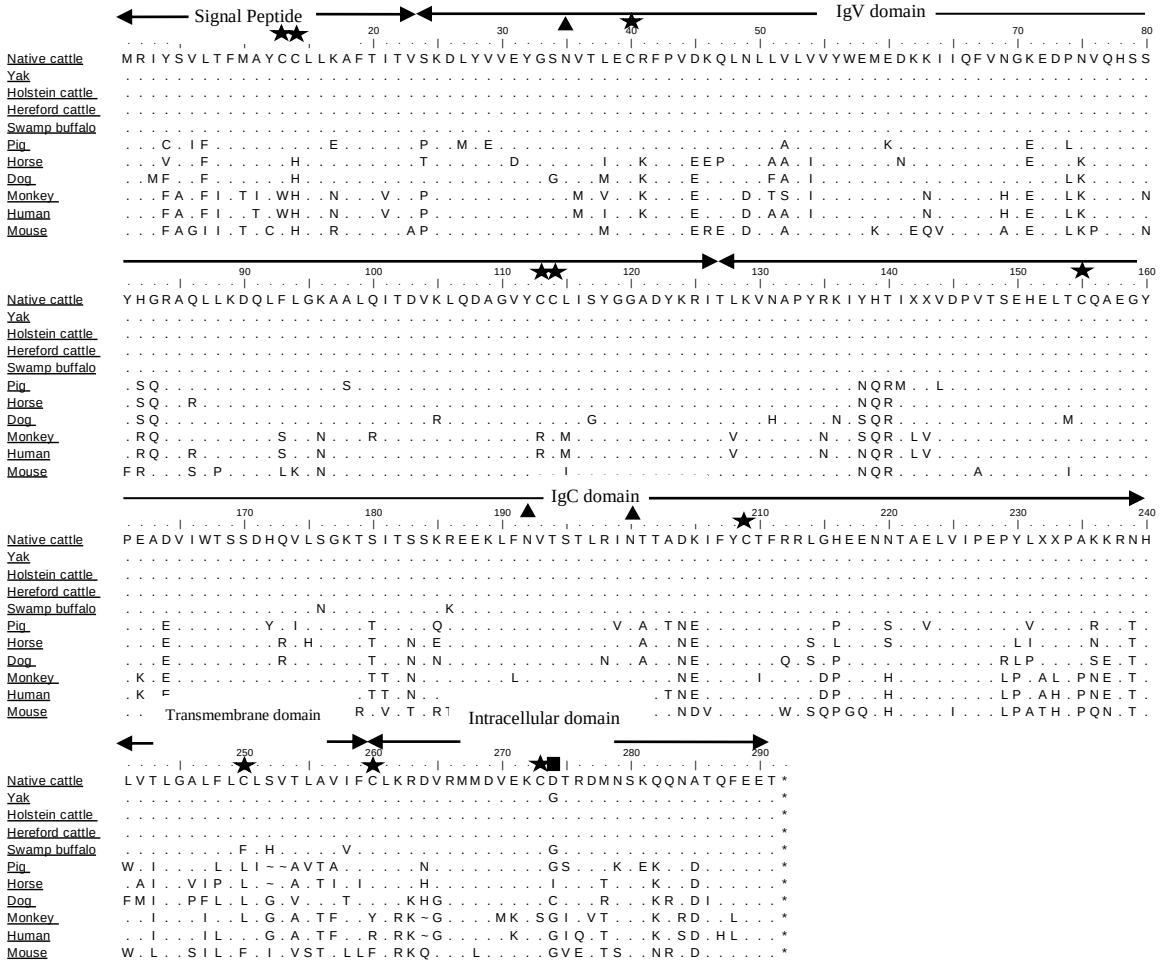


Fig. III-6a. Alignment of deduced amino acid sequences of PD-L1 from Mongolian native cattle and yak compared to other species. A solid star indicates the cystein residue, and a solid triangle indicates the potential N-linked glycosylation sites. A solid square indicates the amino acid difference between yak and cattle species.

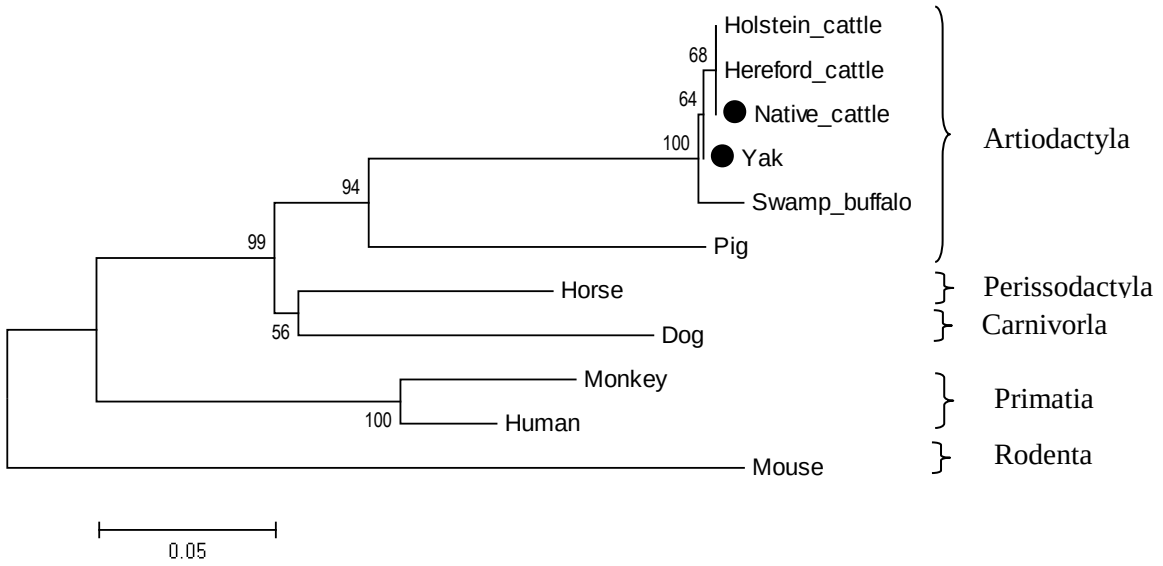


Fig. III-6b. A phylogenetic tree constructed based on the amino acid sequences of PD-L1from Mongolian native cattle and yak compared to other species. Sequences derived from this study are highlighted with circular bullets (●) regarding to their sequence divergence. The tree was constructed with the neighbor-joining method and was supported by 1,000 bootstrap replications.

III.4. Discussion

Molecular characterization by sequencing and phylogenetic analysis of immune-inhibitory molecules such as PD-1, PD-L1, TIM-3, GAL-9, LAG-4 and CTLA-4 in Mongolian native cattle and yak were described in this chapter. The obtained sequences were compared with the sequences of various *artiodactyla* species such as cattle, buffaloes, sheep, goat and pig and other animals which belong to order of *perissodactyla*, *carnivora*, *primatia*, and *rodentia*, respectively.

The genes of the immunoinhibitory molecules of tested animals showed high homologies to those of all of the compared species belonging to order *artiodactyla*, indicating a closer phylogenetic relationship between members of this order. The analyzed genes except for GAL-9 were predicted to encode signal peptide, extracellular domain, transmembrane region and intracellular domain. On the other hands, GAL-9 was structurally different and was predicted to comprise of N- and C-terminal carbohydrate-binding domains connected by a link peptide, because GAL-9 is a soluble molecule secreted by several cells and was originally characterized as an eosinophil chemoattractant (Matsumoto et al.,1998).

Cysteine residues are important for protein stability and function because they are often involved in disulfide bonds that stabilize protein structure as well as in binding of metallic ions for function (Miseta and Csutora, 2000). The cystein residues in the analyzed genes were almost all conserved in their respective locations in all *bovidae* family. Single amino acid substitutions were found in TIM-3 of yak, and GAL-9 of riverine-type buffalo, LAG-3 of riverine-type buffalo, PD-1 of both swamp- and riverine-type buffaloes, PD-L1 of swamp-type buffalo, and in addition, 2 substitutions were only found in LAG-3 of swamp-type buffalo.

N-linked glycosylation sites also play an essential role in the folding and the quality control of proteins in the endoplasmic reticulum. Therefore, a tightly controlled and conserved biosynthetic pathway ensures the correct assembly of the glycan, and the specificity of the oligosaccharyltransferase guarantees that only mature oligosaccharides are transferred to substrate polypeptides (Burda et al., 1999; Helenius and Aebi, 2004). Numerous diseases associated with defects in the N-glycosylation pathway underline its importance in humans (Haeuptle and Hennet, 2009; Hennet, 2012). Potential N-linked glycosylation sites such as 1 for GAL-9 and PD-1, 2 for TIM-3 and LAG-3, and 3 for CTLA-4 and PD-L1 were found and were all intact in their respective locations.

The tyrosine-kinase phosphorylation motif (HPVENIY) in the predicted intracellular

domain of TIM-3 of Mongolian native cattle and yak were identical to that of other *bovidae* family members. In general, the motif for tyrosine-kinase phosphorylation transduces signals through the phosphorylated tyrosine by acting as a functional receptor which is involved in many important physiological processes including cellular growth, differentiation, and intracellular signalling (Yarden and Ullrich, 1988; Ullrich and Schlessinger, 1990; Kiyokawa et al.,1994; Monney et al.,2002; Kuchroo et al.,2003). The inhibitory motif in cytoplasmic region (KTGELE/KIEELE) of LAG-3 was conserved in both Mongolian native cattle and yak, and this inhibitory motif is known to mediate the intracellular signal transduction by downregulating the T cell expansion (Iouzalen et al., 2001).

The alignment of deduced amino acid sequences showed that a substitution for CTLA-4, PD-1, and PD-L1, 2 substitutions for LAG-3, and 8 substitutions for TIM-3 were found between yak and cattle sequences, whereas no subsitiution for GAL-9 were found. These amino acid substitutions found in these receptors may alter their functions, though significance of these substitutions remains to be established. Detailed understanding of the negative signals that this molecule transduce and of their interplay in T-lymphocytes may facilitate the genesis of more powerful and specific strategies for therapeutic manipulation of the immune system (Parry et al.,2005; Mingala et al., 2011; Duran et al., 2015; Anderson, 2014; Arasanz et al., 2017). The immunoinhibitory molecules and their ligands play a crucial role in immune system because they allow adjacent and distant cells to communicate with each other, and this mechanism could result in the disease progression during the infection (Blank and Mackensen, 2007; Okagawa et al., 2012; Gorman and Colgan, 2014). This is the first report which described the genetic characteristic of the immunoinhibitory molecules in Mongolian native cattle and yak. This study showed that the related genes of Mongolian native cattle and yak had high homologies to each other and to those from other cattle breeds. Therefore, further in-detail investigations of immunoinhibitory molecules through elucidate the playing role in immune responses during chronic infection in these animal species are very important.

III.5. Summary

Immunoinhibitory molecules such as PD-1, PD-L1, TIM-3, GAL-9, LAG-3, and CTLA-4 from Mongolian native cattle and yak were characterized through their cloning and sequencing, because these genes involved in the cell-mediated immune responses may be important to determine the differences in their immune reactions against the pathogens among bovine species. The deduced amino acid sequences of these genes were predicted and functional domains such as signal peptide, extracellular domain, transmembrane region and intracellular domain were found in each molecule, whereas GAL-9 was structurally different from other molecules and was composed N- and C-terminal carbohydrate-binding domains connected by a link peptide.

Amino acid alignment of these immunoinhibitory molecules in Mongolian native cattle and yak were high homologies to the sequences from other bovine species belong to order *artiodactyla*. This study isthe first report to characterize the structure of these immunoinhibitory molecules in Mongolian native cattle and yak, and further studies are necessary to assess whether these molecules play a role in the disease progression during chronic infection in these animals.

CONCLUSION

Infectious diseases caused by viruses, bacteria, and parasites in animals have been increasing in recent years and causing great economic loss in livestock industry, which is one of the major income source for the economy in many countries. Therefore, the effective control of infectious disease is important to keep healthy animals, safeguard food supplies, and alleviate rural poverty by boosting the quality and quantity of animal products. The molecular epidemiological survey of several pathogens that cause intractable infectious diseases in livestock in the Philippines and Mongolia was performed to understand their prevalence and to utilize for the prevention and control of infectious diseases in the field.

In addition, the control strategies of infectious diseases also focus on immune responses of the hosts instead of pathogens because genetic background of the animals is one of the important factors for the outcome of many infectious diseases. Therefore, immunoinhibitory molecules in Mongolian native cattle and yak were also characterized for the future assessment of the relationship between disease progression and chronic infection in these animals. The brief summary for the prevalence of investigated pathogens in each animal species were concluded in Table-conclusion 1 and also brief description of each chapter were shown in below.

CHAPTER I: Vector-borne diseases, which include anaplasmosis, babesiosis and theileriosis have been reported in the livestock in the Philippines, and cause significant economic loss in cattle industry. However, the prevalence and genetic diversity of these pathogens have not been studied in details in the country. Thus, molecular epidemiological survey and genetic characterization of *Anaplasma marginale, Babesia bigemina, Babesia bovis, Theileria* spp. and *Trypanosoma evansi* were performed in 339 samples from cattle in Luzon island, the Philippines. As the results, high prevalence of *A. marginale, B. bigemina, B. bovis* and *Theileria* spp. were observed, whereas the prevalence of *Trypanosoma evansi* was low in the sampling areas. In addition, the mixed infections were detected in the most of the samples. The molecular characterization of the *16S rRNA* gene for *A. marginale, RAP-1* gene for *B. bovis, AMA-1* gene for *B. bigemina*, and *MPSP* gene for *Theileria* spp. showed that their pathogens were genetically close to those from other countries such as Sri Lanka, Korea, Japan, China, Australia and USA. These results indicate that the infections of the vector-borne pathogens in cattle were extremely high prevalence in Luzon island, and therefore, the practical control of these infections are necessary in the areas.

CHAPTER II: Animal husbandry is a special and inseparable sector for Mongolia and is very important for national economy and employment. However, many infectious diseases have been occurring and cause huge economic loss in the livestock industry. Therefore, it is needed to survey the prevalence of the pathogens and identify their molecular characteristics to understand the epidemiological situation of infectious diseases in Mongolian livestock. Several bacterial and viral pathogens were surveyed in 928 samples from cattle, yak, sheep and goats in 5 different areas in the country. *Mycobacterium avium* subspecies *partuberculosis* (MAP), *Anaplasma ovis*, bovine leukemia virus (BLV), bovine viral diarrhea virus (BVDV), and ovine gammaherpesvirus 2 (OvHV2) were successfully detected and their genetic characteristics were analyzed. Seroprevalence of MAP was low in cattle. In contrast, the infection rate of *A. ovis* was high in sheep, goats, cattle and yak, and sequencing analysis identified that these isolates were genetically unique. BLV infection was detected from dairy breed cattle, and the nucleotide sequences were closely related to those of Russian isolates, suggesting that the BLV was transported from Russia into Mongolia. BVDV was detected in dairy breed cattle and yaks, and the Mongolian isolates were classified into genotypes 1 and 2. OvHV2, a causative agent of sheep-associated malignant catarrhal fever, was also detected in sheep and cattle, and the phylogenetic analysis revealed that the Mongolian isolates of OvHV2 were identical to those from Asia and the Middle East. These results show that numbers of pathogens which have a risk causing huge economic losses are distributed to Mongolian livestock, but the infection rates were dependent on the geographical locations and animal species. Thus, epidemiological survey should be continued to know the current distribution of the pathogens, and could provide useful information on the establishment of the effective control methods of the infectious diseases.

CHAPTER III: The immunoinhibitory molecules such as programmed cell death 1 (PD-1), programmed cell death-ligand 1 (PD-L1), T-cell immunoglobulin and mucin domain 3 (TIM-3), galectin 9 (GAL-9), Lymphocyte activation gene 3 (LAG 3), and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) in Mongolian native cattle and yak were identified through cloning and sequencing for understand the playing role during chronic infections in these animals. Based on the sequence data, the immunoinhibitory molecules, except for GAL-9, in Mongolian native cattle and yak were predicted to carry the signal peptide, the extracellular domain, the transmembrane region and the intracellular domain, and they were the same as those of other species. On the other hands, GAL-9 was structurally different from the immunoinhibitory molecules and was composed of N- and

C-terminal carbohydrate-binding domains connected by a link peptide. The genes of the immunoinhibitory molecules in tested animals showed high homologies to those of all bovine species. These results suggest that the immunoinhibitory molecules in Mongolian native cattle and yaks have similar functions as previously reported in other bovine species. This study is the first report for genetic characterization of immunoinhibitory molecules in Mongolian native cattle and yak, and further studies including the assessment of the role of these genes in the disease progression during chronic infection is imperative.

Healthy animals promote livestock industry and influence the economic development of the countries. Molecular epidemiological study of several intractable infectious diseases in cattle in the Philippines and livestock in Mongolia were performed and these findings can be used for the planning and execution of effective control measures for infectious diseases in the livestock in each of the country. In addition, molecular characterization of immunoinhibitory molecules in Mongolian native cattle and yak were performed, and it will be the first step to assess the disease progression in these animals.

Table-conclusion 1. Prevalence of the investigated pathogens in each animal species

Animal breeds/species	**MAP**	***A. ovis***	**BLV**	**OvHV2**	**BVDV**
Native cattle	5 .0%	47.0%	0%	1.7%	0%
Dairy cattle	1.6%	7.3%	6.6%	0%	15.7%
Yak	N/D	20.0%	0%	0%	20.0%
Sheep	N/D	95.2%	-	0%	N/D
Goat	N/D	57.5%	-	0%	ND

ND: not determined

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SUMMARY IN JAPANESE

（和文要旨）

ウイルス、細菌および寄生虫等の病原体により引き起こされる動物の感染症は、近年、増加傾向にあり、畜産業を主要な収入源とする多くの国々において、多大な経済的被害を及ぼしている。それ故に、これらの感染症を効果的に防除して、畜産物の品質向上や安定供給をはかることは、安全な食糧の生産維持や畜産業の発展による貧困の解消に重要である。そこで本研究では、フィリピンやモンゴルにおいて、難治性感染症を引き起こす種々の病原体の疫学情報を入手して、それらの感染症の防除法確立に応用するために、分子疫学調査を行った。また一方で、感染症の制御には、病原体の性状のみならず、免疫抑制を含む宿主の免疫応答の解析も重要であり、宿主の遺伝的背景は多くの感染症の転帰を決定する重要な因子のひとつである。以上の理由より、モンゴル在来牛やヤクにおいて、慢性感染症の病態進行おける免疫抑制因子の役割を解明するために、これらの因子の遺伝子解析も併せて行った。

第1章： フィリピンでは、家畜においてアナプラズマ病、バベシア病やタイレリア病などのベクター媒介性感染症が多く報告されており、大きな経済的被害を及ぼしている。しかしこれらの病原体の疫学情報や遺伝的背景は詳細には解析されていない。そこで、ルソン島でウシから採取した 339 個の試料を用いて、*Anaplasma marginale*、*Babesia bigemina*、*Babesia bovis*、*Theileria*spp 及び *Trypanosoma evansi* の分子疫学調査を行い、検出された病原体の遺伝子多型を解析した。その結果、*T. evansi* を除いて、他の病原体は非常に高率に検出され、多くの試料で混合感染が認められた。*A. marginale* の *16S rRNA* 遺伝子、*B. bovis* の *RAP-1* 遺伝子、*B. bigemina* の *AMA-1* 遺伝子、*Theileria*spp の *MPSP* 遺伝子を用いた系統樹解析により、検出された病原体は、スリランカ、韓国、日本、中国、オーストラリアや米国などで検出されたものと近縁であることが示された。以上より、ルソン島では、これらのベクター媒介性病原体が家畜に非常に高率に分布しており、今後その制御が必要である。

第2章： 畜産は、モンゴルにおいて必要不可欠な産業であり、国家の経済や雇用の創出に重要である。しかし、依然として家畜の感染症が多く発生しており、甚大な被害をもたらしている。そのため、モンゴルの家畜において、これまで詳細な調査がなされていない多くの病原体の分布・疫学性状やそれらの分子生物学的性状を明らかにすることが必

要である。そこで、国内の異なる5ヶ所の地域でウシ、ヤク、ヒツジやヤギから採集した 928 個の試料を用いて、種々の細菌・ウイルスの疫学調査を実施した。その結果、これらの試料から、ヨーネ菌(*Mycobacterium avium* subspecies *partuberculosis*、MAP)、*Anaplasma ovis*、牛白血病ウイルス(BLV)、牛ウイルス性下痢ウイルス(BVDV)及び羊ガンマヘルペスウイルス2型(OvHV2)が検出された。MAP の検出率(血清疫学調査)は低かったが、*A. ovis* は、ヒツジ、ヤギ、ウシおよびヤクから高率に検出され、塩基配列解析の結果、検出された *A. ovis* は、遺伝子的にモンゴル固有なものであることが示された。BLV は乳用牛から検出され、ロシアで検出されたものと近縁であり、BLV はロシアからモンゴルに導入されたことが示唆された。BVDV もまた乳用牛やヤクで検出され、遺伝子型 1 及び2に分類された。またヒツジ随伴型悪性カタル熱の病原である OvHV2 はヒツジやウシから検出され、系統樹解析の結果、アジアや中近東由来のものと同じであることが示された。これらの結果より、モンゴルの家畜には、大きな経済的被害をもたらす可能性がある病原体が多く分布しており、その感染率は地域や動物種に依存していることが明らかとなった。今後も病原体の分布をより詳細に把握するために疫学調査を継続する必要があり、モンゴルにおける感染症の防除法を確立するために有用な情報基盤を提供できると思われる。

第3章: モンゴル在来牛やヤクの免疫学的性状を明らかにするために、免疫抑制因子である programmed cell death 1 (PD-1)、programmed cell death-ligand 1 (PD-L1)、T-cell immunoglobulin and mucin domain 3 (TIM-3)、galectin 9 (GAL-9)、Lymphocyte activation gene 3 (LAG 3)、及び cytotoxic T-lymphocyte-associated protein 4 (CTLA-4)の遺伝子クローニングを行った。塩基配列解析の結果、モンゴル在来牛やヤク由来のこれらの因子は、GAL-9 を除いて、他の動物種由来の因子と同様に、シグナル配列、細胞外領域、細胞膜貫通領域及び細胞内領域を有していることが予想され、一方 GAL-9 には、リンカーに接続して N 及び C 末端に糖鎖結合領域が存在していた。今回解析したモンゴル在来牛やヤクの免疫抑制因子の遺伝子は、他の偶蹄類のものと高い相同性を示したので、モンゴル在来牛やヤクの免疫抑制因子も他の牛種と同様の機能を有している可能性が示唆された。本研究は、モンゴル在来牛やヤクの免疫抑制因子についての最初の報告であり、慢性感染症の病態進行における役割を解析していく必要がある。

家畜の健康維持及び飼育は、畜産業の発展に重要であり、国の経済成長に大きな影響を及ぼす。本研究では、フィリピンやモンゴルのウシにおける種々の難治性感染症の分子疫学調査を実施したが、得られた知見は、それぞれの国における家畜の感染症の

効果的な防除法を確立に有用である。さらにモンゴル在来牛やヤクの免疫抑制因子の性状解析は、今後これらの動物種における病態進行の分子機構を解析するための第1歩となると考えられる。