Molecular epidemiological studies on livestock tick-borne parasitic diseases in the Philippines

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フィリピンにおける家畜のマダニ媒介性寄生虫病に関する分子疫学的研究

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Contents

Abbreviations

Abbreviations

A.	AIC	: Akaike information criterion
	Ampliseq	: Amplicon sequencing
	ASV	: Amplicon sequence variant
B.	Bbsl	: *Borrelia burgdorferi* sensu lato
	BLAST	: Basic local alignment search tool
C.	CI	: Confidence interval
D.	DNA	: Deoxyribonucleic acid
E.	EDTA	: Ethylenediaminetetraacetic acid
	EGA	: Equine granuclocytic anaplasmosis
	EMBL	: European Molecular Biology Laboratory
	EP	: Equine piroplasmosis
G.	GPS	: Global positioning system
	groEL	: Heat shock operon
I.	IACUC	: Institutional Animal Care and Use Committee

	IGS	: Intergenic spacer
	ITS	: Internal transcribed spacer
L.	LAMP	: Loop-mediated isothermal amplification
M.	MEGA	: Molecular evolutionary genetics analysis
N.	NCBI	: National Center for Biotechnology Information
	NGS	: Next generation sequencing
O.	OR	: Odds ratio
P.	PCR	: Polymerase chain reaction
R.	R.A.	: Republic Act
	RLB-PCR	: Radioligand binding polymerase chain reaction
	RPA	: Recombinase polymerase amplification
	rrf-rrl	: 5–23S rRNA intergenic spacer
	rRNA	: Ribosomal ribonucleic acid
	RT-PCR	: Real-time polymerase chain reaction
S.	s.l.	: Sensu lato
	SNPs	: Single nucleotide polymorphisms
	s.s.	: Sensu stricto
	ssu	: Small subunit

T. TAE : Tris acetate EDTA

TBD : Tick-borne disease

TBP : Tick-borne pathogen

U. USA : United States of America

UV : Ultraviolet

Unit abbreviations

°C : degree Celsius

hr : hour

µg : microgram

µL : microliter

µm : micrometer

µM : micromolar

mL : milliliter

mM : millimolar

min : minute

% : percentage

sec	: second
U	: units
USD	: United States dollar

General introduction

1. Piroplasma

1.1. Taxonomy

Piroplasma, which originated from the words *pirum* (Latin: pear) and *plàsma* (Greek: figure or image), are so named because of their pear-shaped intra-erythrocytic forms (Mehlhorn and Schein, 1993). These obligatory, dixenous, and tick-transmitted protozoan parasites are classified under the phylum Apicomplexa and class Aconoidasida, making *Plasmodium*, the causative agent of malaria, a close relative (Fig. 1) (Arisue and Hashimoto, 2015). Unlike other apicomplexan parasites, piroplasmids have distinct structural and biological characteristics: pyriform, round, and amoeboid forms lacking conoid and flagella, inability to form oocysts, and presence of large axopodium-like "Strahlen" during the sexual life stage (Adl et al., 2012). The taxonomic and phylogenetic grouping among piroplasma has been repeatedly amended, owing to the complexity and diversity not only of its biology but also of its evolutionary history. The most recent characterization based on the 18S rRNA gene classified piroplasma into ten distinct lineages (Fig. 1). Lineages with official taxonomies include *Babesia* sensu stricto (s.s.) under the family Babesiidae and *Theileria* sensu stricto and *Cytauxzoon* under the family Theileriidae. Meanwhile, the taxonomic nomenclature of the remaining seven lineages, namely species formerly belonging to the *Babesia* sensu lato (s.l.) (Western, Percei, and *Babesia microti*-like) and *Theileria* sensu lato (Monotremata, Marsupialia, and Rhinocerotidae) groups, and the newly separated *Theileria equi* group, are yet to be clarified (Schreeg et al., 2016; Jalovecka et al., 2019).

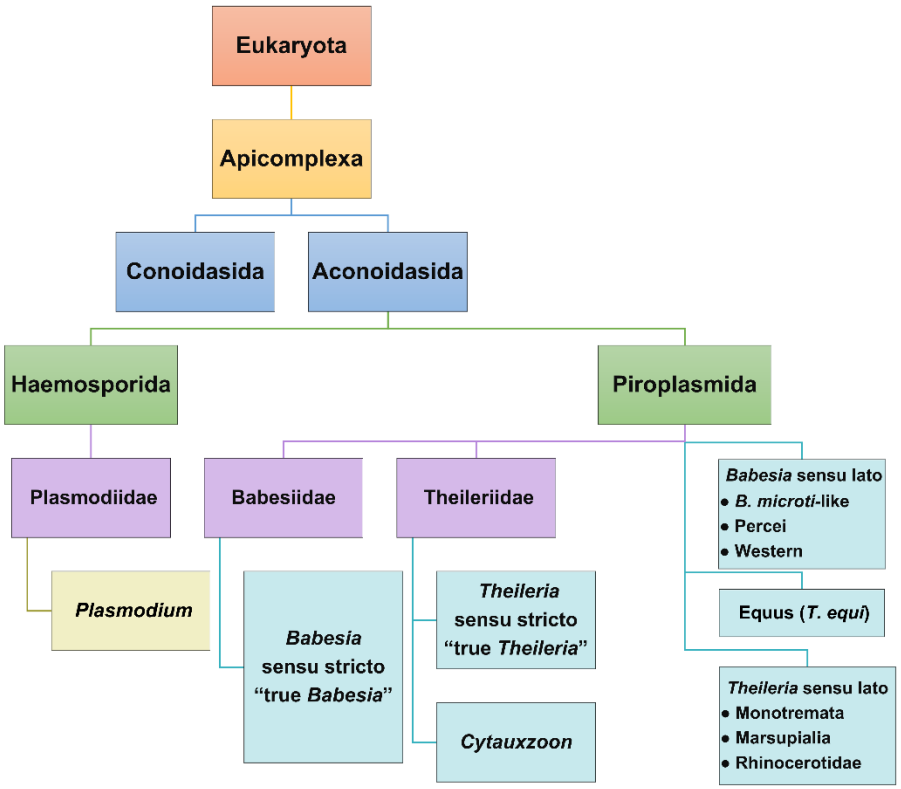

Fig. 1. Taxonomical designations of piroplasmid parasites. Adapted from (Gray, 2017; Jalovecka et al., 2018).

1.2. Biological life cycle of *Babesia* and *Theileria*

The life cycle of the piroplasmid *Babesia* s.s. and *Theileria* parasites (Schnittger et al., 2012; Jalovecka et al., 2018, 2019) are depicted in Fig. 2 and Fig. 3, respectively. In both parasites, the life cycle commences with a blood meal, wherein ticks inject sporozoites that infect leukocytes (*Theileria*) or erythrocytes (*Babesia*). In *Theileria*, the infected leukocytes proceed to schizogony, the phase wherein rapid proliferation gives rise to Koch's bodies or schizonts that infect the erythrocytes (Sugimoto and Fujisaki, 2002; McKeever, 2009). On the other hand, *Babesia* sporozoites directly invade erythrocytes and rapidly multiply (Jalovecka et al., 2019). During merogony, parasites appear as ~3–5 µm or 0.5–2.5 µm forms for *Babesia* and 2×1 µm rod-shaped forms for *Theileria* (Lempereur

et al., 2017). Merozoites can have either of two fates: a.) egress the infected RBCs, then re-infect and replicate inside naïve RBCs, or b.) differentiate into gametocytes, and be ingested by a competent vector in the next tick blood meal. In the tick host, the gametocyte will develop into several sexual forms (gametes, Strahlenkörper or ray bodies, fertilized gametes, and zygotes for *Babesia*; gametes, micro or macrogametes, fertilized gametes, and zygotes for *Theileria*) before invading tick midgut cells. Through meiotic division, primary kinetes are formed and disseminated onto the hemolymph. For *Theileria*, kinetes directly migrate to the salivary glands, whereas *Babesia* kinetes either invade various tick tissues before migrating to salivary glands or infect tick ovaries and eggs for transovarial transmission. The kinetes are transmitted between instars, develop into sporoblasts, then into infectious sporozoites, where they are injected during the next blood meal, which restarts the biological life cycle.

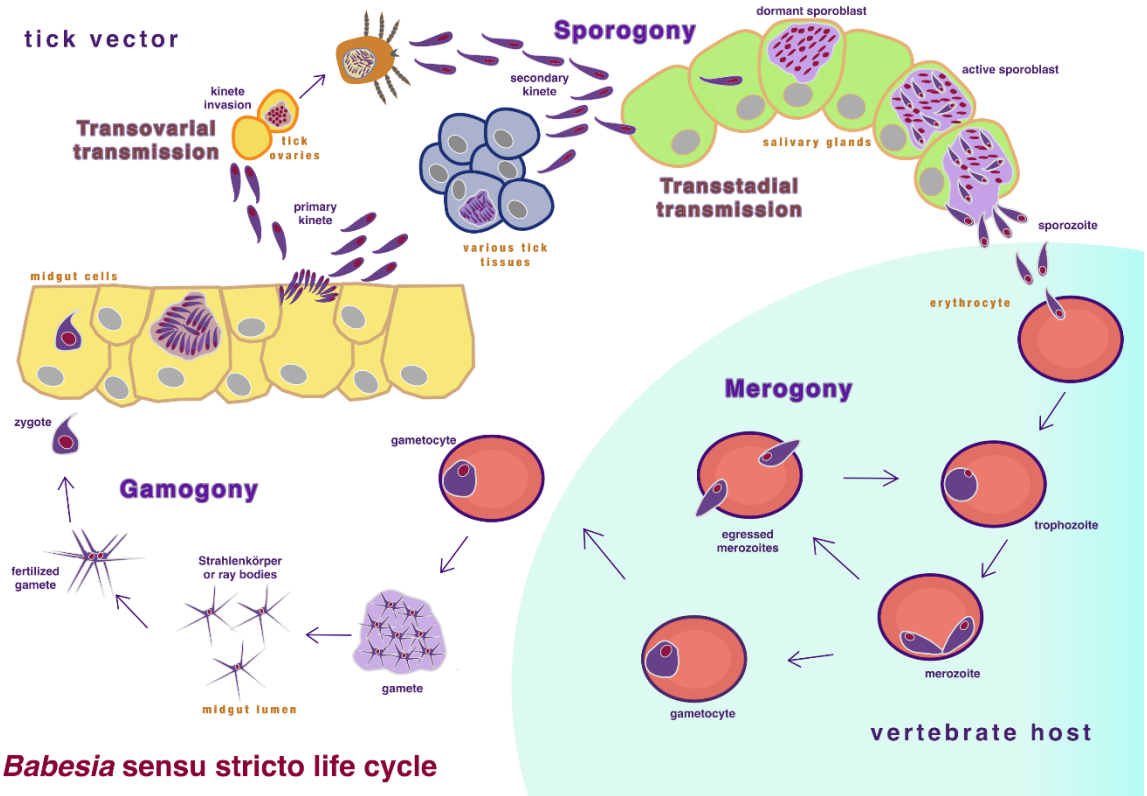

Fig. 2. Life cycle of *Babesia* sensu stricto parasites. Adapted from (Jalovecka et al., 2018, 2019).

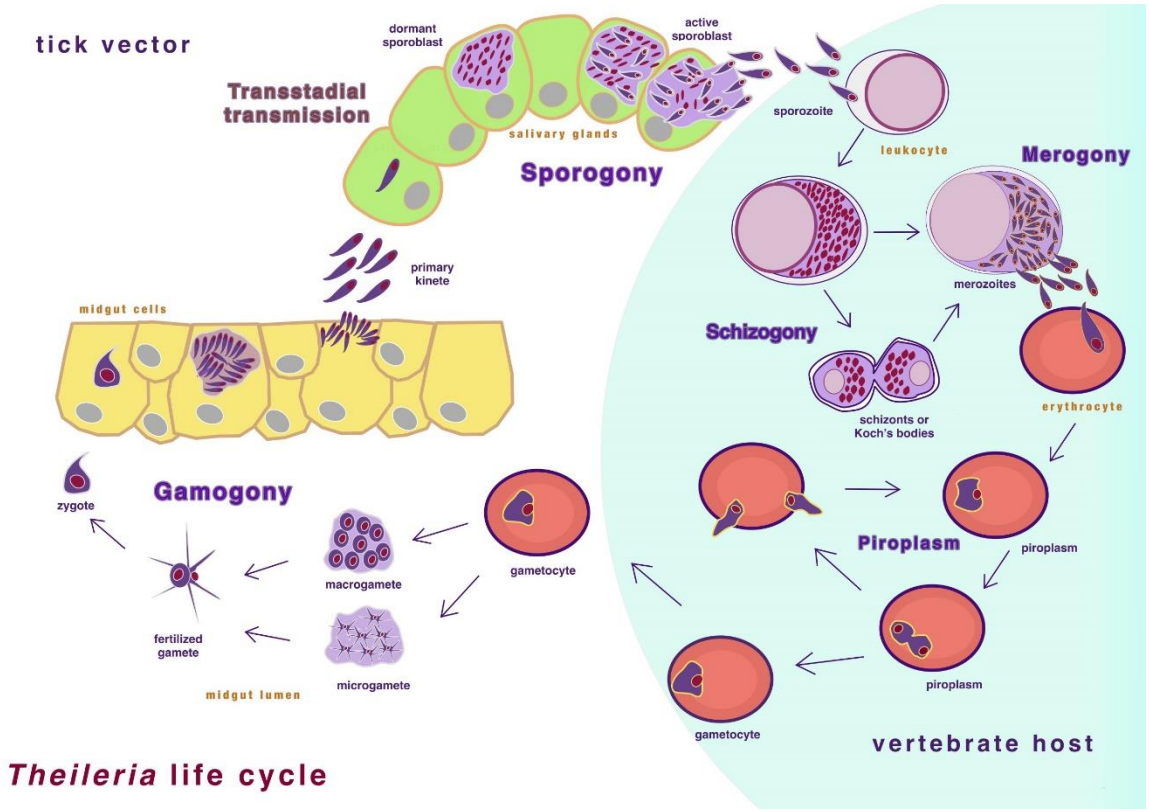

Fig. 3. Life cycle of *Theileria* parasites. Adapted from (Jalovecka et al., 2018, 2019).

1.3. *Babesia* and babesiosis

The first description implicating *Babesia* parasites as the etiologic agent of what is now called babesiosis was reported by Victor Babeş in 1888 (Babes, 1889; Köhler and Köhler, 2003). Since then, the *Babesia* species list has had over 100 species, with the list expanding as more novel species are continuously identified (Schnittger et al., 2012). Species belonging to the genus *Babesia* infect a plethora of hosts, including domestic mammals, wildlife, marsupials, monotremes, and birds (Uilenberg, 2006; Schnittger et al., 2012). Humans can get infected but are considered accidental and dead-end hosts for *Babesia* parasites (Yabsley and Shock, 2013). The ixodid tick genera that transmit important species in livestock include *Rhipicephalus* (*B. bovis, B. bigemina, B. ovis, B. orientalis*), *Ixodes* (*B. divergens, B. motasi*-like), *Haemaphysalis* (*B. ovata, B. major, B.*

motasi, *Babesia* sp. Xinjiang), *Hyalomma* (*B. occultans*, *Babesia* sp. Xinjiang), and *Dermacentor* (*B. motasi*-like) (Schnittger et al., 2022). Clinical manifestations of babesiosis may vary depending on the species, but the most common signs include fever, severe anemia, inappetence, lethargy, and jaundice in animals with acute clinical disease, and can be lethal in serious cases (Schnittger et al., 2012).

1.4. *Theileria* and theileriosis

In many regions of the world, *Theileria* parasites are among the most significant obstacles to livestock production. *Theileria* is distinct from other protozoa as selected species can immortalize infected cells (Dobbelaere and Heussler, 1999). Similar to *Babesia*, it can infect a wide range of hosts, but its impact is most noticeable on cattle, sheep, and goats (Bishop et al., 2004; Ahmed et al., 2011). Several prominent *Theileria* species are transmitted by *Rhipicephalus* (*T. parva*, *T. taurotragi*, *T. lestoquardi*, *T. ovis*, *T. separata*), *Hyalomma* (*T. annulata*, *T. lestoquardi*, *T. ovis*), *Haemaphysalis* (*T. orientalis*, *T. uilenbergi*, *T. luwenshuni*), and *Amblyomma* (*T. mutans*, *T. velifera*) ticks (Schnittger et al., 2022). *Theileria annulata*-infected animals may show fever, hemorrhage, anemia, and jaundice, while those infected with pathogenic isolates of *T. orientalis* may experience lethargy, anemia, paleness, pyrexia, and abortion (Watts et al., 2016; Gebrekidan et al., 2020). In small ruminants, *T. lestoquardi* and *T. uilenbergi* may also cause severe morbidities and mortalities (Ahmed et al., 2011).

2. Molecular diagnostic methods for piroplasmosis

The last half-century has seen an infusion of breakthroughs in molecular technologies that have changed parasitic disease diagnosis. As a result, molecular diagnostics has proven useful in determining the epidemiology of diseases that are relevant

in medical, veterinary, and economic terms (Eybpoosh et al., 2017). Similarly, molecular approaches have led to the discovery of previously unknown pathogens.

Unlike diagnostic platforms that directly detect the presence of parasites (microscopy of blood smears) or assess an animal's exposure to the parasite (enzyme-linked immunosorbent assay, indirect fluorescent antibody test, and immunochromatographic test), nucleic-acid-based diagnostic assays allow highly accurate detection of the parasite's genetic material in field samples, overcoming the aforementioned technologies' sensitivity and specificity concerns (Mosqueda et al., 2012). As such, molecular tools are soon projected to replace the more time-consuming and laborious traditional methods for parasite detection (Mans, 2022).

2.1. Isothermal amplification assays

Isothermal amplification assays are widely used due to their applicability in places where complicated equipment e.g., thermocycler, are unavailable. These types of assays rely on the *in vitro* amplification of parasite nucleic acid. Of these, loop-mediated isothermal amplification (LAMP) is undoubtedly the most regularly used. LAMP is a rapid, highly specific, and efficient DNA detection method that is performed at isothermal conditions. The target sequences are simultaneously amplified with an isothermal DNA polymerase using a set of primers (Mosqueda et al., 2012). Verification of results is done by visual observation or by using a lateral flow dipstick (Alvarez et al., 2019). Due to its ease of use, developed LAMP assays have been widely used for *Babesia* and *Theileria* parasite detection in the field (Mosqueda et al., 2012; Mans et al., 2015). A newer isothermal-based tool, recombinase polymerase amplification (RPA), has recently been employed for parasite detection as well. Compared to LAMP, RPA is quicker, straightforward, and may be performed at a lower temperature without compromising

sensitivity, making it the ideal assay for point-of-care detection (Lobato and O'Sullivan, 2018). Its adaptability to downstream detection is the strong point of RPA. Several RPA assays have been developed for *Babesia* and *Theileria* recently (Yin et al., 2017; Hassan et al., 2018; Lei et al., 2020; An et al., 2021). On the other hand, isothermal assays such as helicase-dependent amplification, rolling circle amplification, nicking enzyme amplification reaction, and others are yet to be developed and utilized for piroplasma detection.

2.2. Polymerase chain reaction (PCR) assays

Polymerase chain reaction (PCR) assays, including variants such as conventional PCR, nested PCR, multiplex PCR, and quantitative RT-PCR assays, are highly useful for amplifying the target DNA fragment to qualitatively or quantitatively confirm the presence of pathogens in blood samples (Mans et al., 2015; Garcia et al., 2022). Conventional PCR assays were initially established for *Babesia* and *Theileria* detection in the early part of the 1990s (Bishop et al., 1992, 1994; Fahrimal et al., 1992; Figueroa et al., 1992). Since then, several dozen PCR assays targeting numerous genes have been extensively used in surveys around the globe (Mosqueda et al., 2012; Mans et al., 2015; Garcia et al., 2022). Moreover, some PCR assays have been improved to enable multiplexing, which further saves time and resources. The quantitative PCR technique is advantageous when highly sensitive and specific quantification of parasites in samples is desired. Thus, it has been used regularly, although to a much lesser extent than conventional and nested PCRs as it requires more sophisticated equipment. Some qPCR assays developed for *Babesia* and *Theileria* include those established by (Chaisi et al., 2013; Yang et al., 2014; Bogema et al., 2015; Pulford et al., 2016; Qurollo et al., 2017).

2.3. Sequencing analysis for species identification

End-point PCR assays are often coupled with several techniques to determine the detected species or to identify the genotypes of the detected pathogen. Classical sequencing using capillary electrophoresis, although a time-consuming and low sample capacity process, is still the typical technique utilized for routine sequencing (Schmitz et al., 2022). However, with the arrival of next-generation sequencing (NGS) technology, molecular diagnostics has been revolutionized by streamlining the bottlenecks of contemporary sequencing techniques. In particular, researchers have exploited its high throughput capabilities to simultaneously detect and identify several parasite species in one run, conserving time and cost, and minimizing losses due to timely diagnosis (Flaherty et al., 2018, 2021; Chaudhry et al., 2019; Huggins et al., 2019; Ghafar et al., 2020; Squarre et al., 2020). Soon, the use of NGS-based parasite sequencing is expected to spread across different countries, as the NGS technology becomes more affordable and accessible.

3. Livestock tick-borne diseases (TBDs) in the Philippines and their impact

In areas where ticks and TBDs are ubiquitous, the decrease in livestock production has a substantial economic impact, especially on resource-poor households that rely on farming for their subsistence and livelihood (Jongejan and Uilenberg, 2004). Recent estimates of global losses incurred through *Rhipicephalus microplus* tick infestation and its accompanying TBDs in cattle are at least 13.9 billion USD annually (Betancur Hurtado and Giraldo-Ríos, 2019). In small ruminants, the annual losses are similarly colossal. For instance, TBD loss amounting to 70 million USD was calculated in China alone (Yin and Luo, 2007).

The livestock and dairy sectors are major contributors to Philippine agriculture (Briones and Espineli, 2022), but TBDs have not been given focus when it comes to the government's disease control program. The most widespread livestock TBDs in the Philippines are babesiosis, theileriosis, and anaplasmosis (Ybañez et al., 2018a). An economic analysis of the effects of cattle tick fever (babesiosis and anaplasmosis) calculated herd fatality rates of 0.1% and annual losses of 0.60 million USD for the Philippines two decades ago (McLeod and Kristjanson, 1999). Hitherto, several molecular studies on livestock TBDs have been conducted in the Philippines, which often investigated cattle and water buffaloes (Ybañez et al., 2018a). Tick-borne pathogen (TBP) species that have been molecularly documented are as follows: *B. bovis*, *B. bigemina*, *B. naoakii*, *T. orientalis*, *Theileria* sp., *A. marginale, A. phagocytophilum*-like, *Ehrlichia minasensis,* and *C. burnetii* in cattle and water buffaloes (Mingala et al., 2009; Ybañez et al., 2013; Yu et al., 2013; Belotindos et al., 2014; Ochirkhuu et al., 2015; Herrera et al., 2017; Galon et al., 2019, 2020; Galay et al., 2020, 2021; Prado et al., 2022) and equine piroplasmas *B. caballi* and *T. equi* (Ybañez et al., 2018b) in horses. Only one TBD study on goats was conducted, wherein all samples were negative (Ybañez et al., 2019a).

4. Objectives of the present study

Molecular techniques have enabled easy-to-use, rapid, and accurate diagnosis of economically important TBDs, which is a prerequisite to establishing disease control. The use of molecular diagnostics for TBDs has been reported in the Philippines before, albeit in a limited capacity and confined areas only. Currently, the molecular epidemiological information on TBDs in the Philippines is scant and sparse, with little data available on animals such as goats and horses; thus, hindering the implementation of appropriate and adequate treatment and prevention measures. Therefore, the general purpose of this

research work was to address this major lacuna by employing molecular tools as diagnostic methods, with a particular focus on the detection of livestock piroplasmid parasites. Specifically, this research aimed to:

1) investigate the status of equine piroplasmosis and other TBDs in racehorses from Cavite, Philippines;
2) evaluate the molecular presence of *Babesia, Theileria,* and *Anaplasma* in goats from different provinces in the Philippines; and
3) assess the utility of a targeted amplicon sequencing approach to characterize bovine piroplasma populations in the Philippines.

Chapter 1

Molecular identification of selected tick-borne protozoan and bacterial pathogens in thoroughbred racehorses in Cavite, Philippines

1-1. Introduction

TBDs are among the biggest obstacles facing the global livestock industry. Common etiological agents of TBDs in horses include *B. caballi*, *T. equi*, *Anaplasma phagocytophilum, Borrelia burgdorferi* sensu lato, *Rickettsia*, and *Coxiella burnetii* (Marenzoni et al., 2013; Stuen et al., 2013; Divers et al., 2018; Freese and Sheats, 2019; Onyiche et al., 2019).

Equine piroplasmosis (EP) is an incapacitating illness caused by piroplasmid species *B. caballi*, *T. equi*, and *T. haneyi* affecting the family Equidae, mostly horses, donkeys, zebras, and, mules (Onyiche et al., 2019). Similar to other TBDs, EP's geographic distribution parallels that of its tick vectors, with only a few countries considered non-endemic (Tirosh-Levy et al., 2020). Four and six genera of hard ticks are recognized as competent vectors for *B. caballi* and *T. equi* complex, respectively (Scoles and Ueti, 2015). Clinical signs of acute EP usually consist of fever, malaise, anemia, dehydration, anorexia, tachycardia, hemoglobinuria, edema, and icterus, and may eventually result in fatalities (Rothschild, 2013; Wise et al., 2013). In contrast, *T. haneyi* causes a subclinical infection (Sears et al., 2019). Besides the adverse health impacts, the blockade of animal international trade is a major issue arising from EP (Friedhoff et al., 1990).

The cosmopolitan *A. phagocytophilum* is the causative agent of granulocytic anaplasmosis in a variety of hosts and of tick-borne fever in ruminants (Stuen et al., 2013). The majority of the clinical signs of equine granulocytic anaplasmosis (EGA) are non-specific, but a pathognomonic characteristic is the presence of thrombocytopenia (Franzén et al., 2007). Serious cases of EGA can be terminal (Dzięgiel et al., 2013). Most EGA clinical cases occur in Canada, Europe, and the USA (Saleem et al., 2018).

Borreliosis is an important zoonotic disease caused by *Ixodes*-transmitted *Bo. burgdorferi* sensu lato (Bbsl) spirochetes. In the field, small mammals like rodents help maintain the Bbsl complex, which consists of 21 species (Eisen, 2020). Humans and domesticated vertebrates such as cattle, dogs, and horses are some of Bbsl's incidental hosts (Basile et al., 2017). Although a definite diagnosis of borreliosis can be challenging, typical clinical features of borreliosis in horses may include neuroborreliosis, uveitis, and cutaneous pseudolymphoma (Divers et al., 2018). Lyme borreliosis in humans is an epidemic in the northern hemisphere countries (Basile et al., 2017; Divers et al., 2018; Swinebroad, 2018). Different Bbsl genospecies have been documented in humans and various animals and tick species in several East and Southeast Asian nations, including China (Masuzawa, 2004), Taiwan (Masuzawa, 2004), Japan (Masuzawa, 2004), Thailand (Trinachartvanit et al., 2016; Takhampunya et al., 2021), and Malaysia (Khoo et al., 2018; Lau et al., 2020).

Diseases caused by *Rickettsia* and *C. burnetii* are also potentially zoonotic. Although *Rickettsia* species are often detected in ticks, only a handful of species, namely *Rickettsia rickettsii*, *R. raoultii*, *R. felis*, and *R. slovaca* have been molecularly detected in horses (Tyrrell et al., 2019; Li et al., 2020b), with *R. rickettsii* recently documented causing clinical disease (Freese and Sheats, 2019). Meanwhile, horses can harbor *C. burnetii*, the bacteria that causes Q fever (Marenzoni et al., 2013). Molecular reports of *C. burnetii* in

horses have been noted in South Korea (Cho et al., 2021), China (Li et al., 2020b), and Iran (Khademi et al., 2020).

An estimated 252,000 horses are raised in the Philippines, mostly for racing, transportation, and meat production (Philippine Statistics Authority, 2020). Of these, horse racing is regarded as the most profitable, contributing about 26 million USD annually (The Manila Times, 2020). In previous studies, microscopy was used to confirm the presence of *B. caballi* and *T. equi* parasites (Cruz-Flores et al., 2010), and both parasites were molecularly detected in horses using PCR assays (Ybañez et al., 2018b). Both investigations showed that horses were exposed to *B. caballi* and *T. equi* using immunochromatographic test strips (Cruz-Flores et al., 2010; Ybañez et al., 2018b). Given the significant impact of EP, surveillance is necessary to preclude future outbreaks, particularly in race parks where naïve horses are introduced frequently. To the author's knowledge, no reports of equine borreliosis, EGA, rickettsiosis, or Q fever in Philippine horses have been made, nor have their respective causal agents been found. The current study's objectives were to identify selected TBPs using molecular assays and to evaluate the risk factors for TBP infections in Philippine thoroughbred racehorses.

1-2. Materials and methods

Sampling location and sampling of animals

Cavite is a province in the southern part of Luzon, Philippines, located southwest of Manila (Fig. 4). The topography of the province is diverse, ranging from coastal plains to upland hilly regions. Cavite’s climate, characterized by the dry season from November to April and the rainy season from May to October, makes the majority of its areas ideal for agricultural production (Philippine Atmospheric, Geophysical and Astronomical Services Administration (PAGASA), 2020). In the Philippines, two major racehorse parks

are situated in Cavite province. Therefore, for this study, 124 racehorses were sampled from a race park in Naic, Cavite. Sex, age, clinical signs, and history of tick infestation of horses were recorded. Approximately 3 mL of whole blood was drawn from the jugular vein and was collected in Vacutainer® EDTA tubes (BD, USA). The blood samples were stored in an ice box and transported to the laboratory.

DNA extraction

Genomic DNA was isolated from 200 µL of whole blood using the QIAmp DNA Blood Mini Kit (Qiagen, Germany), following the manufacturer's instructions. Samples were eluted with 100 µL elution buffer. The DNA concentration of samples was checked using NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, USA), then kept at −20°C until the molecular screening.

PCR assays for the identification of tick-borne pathogens

Previously developed PCR assays were used to screen the samples for TBPs. Table 1 displays an overview of the assays applied in this investigation. For EP, the partial 18S rRNA gene of *B. caballi* (540 bp) and *T. equi*/*T. haneyi* (392 bp) was amplified using a multiplex nested PCR assay (Alhassan et al., 2005). Additionally, *A. phagocytophilum* (Barlough et al., 1996), *Rickettsia* spp. (Simser et al., 2002), and *C. burnetii* (Zheng et al., 2017) PCR assays amplified the partial 16S rRNA gene with target lengths of 928, 426, and 1,450 bp, respectively. For Bbsl, the rrf-rrl (5–23S rRNA intergenic spacer) primer set amplified amplicons of 226–266 bp (Postic et al., 1994). The nested PCR assays' first reactions were run at a volume of 10 µL with final concentrations of 1× ThermoPol® buffer, 2 mM of each dNTP, 0.25 U of Taq DNA polymerase (all from New England Biolabs, USA), and 2 µM of forward and reverse primers. Except for the final primer concentrations of the multiplex PCR for EP: 4 µM of forward primer and 2 µM each of reverse primers, the single assays and second round PCR were carried out with a similar set-up in a final

volume of 25 µL. About 2 µL of DNA was used, whereas 1.5 µL of the first-round product was used for the multiplex or nested assays. The same thermocycling settings were used for each pathogen, except for the extension temperatures (68°C). Double-distilled water was used as a negative control and archived positive DNA samples were used as positive controls. Amplicons were resolved in either 1% or 1.5% agarose in TAE by gel electrophoresis, stained with ethidium bromide, and visualized under UV light.

Sequencing and phylogenetic analyses

Bands were excised from the gel and were purified using the NucleoSpin® Gel and PCR Clean-up Kit (Macherey Nagel, Germany). High-concentration samples underwent direct sequencing, while those with low concentration were cloned. They were ligated onto a pGEM®-T easy vector (Promega Corporation, USA), which were then inserted in DH5α *Escherichia coli* calcium-competent cells. Positive transformants were cultured and purified using NucleoSpin® Plasmid QuickPure Kit (Macherey Nagel). Sequencing was performed using BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

Forward and reverse sequences were manually trimmed and assembled. The accession numbers of sequences deposited to the NCBI GenBank database are MW714970–MW714976 (*B. caballi* 18S rRNA, 575–584 bp); MW714977 (*T. equi* 18S rRNA, 435 bp); MZ150516–MZ150524 (*Anaplasma* sp. 16S rRNA, 924 bp); MZ408312 (*A. marginale groEL*, 855 bp); MZ962640–MZ962656 (*Borrelia* sp. 5-23S rRNA, 236–256 bp). Percent identities of sequences were determined through EMBL's Clustal Omega multiple sequence alignment (Madeira et al., 2019) and nucleotide BLAST search. Clustal W was used for the alignment of sequences, and phylogeny inference was done using Molecular Evolutionary Genetics Analysis (MEGA) X (Kumar et al., 2018). The best

substitution models were determined using maximum likelihood, and the bootstrap method phylogeny test was performed with 1,000 replications.

Statistical analysis

The animal background data comprised animal parameters (age and sex) as independent variables and positivity for *B. caballi*, *A. phagocytophilum*, or Bbsl as dependent variables. Testing positive for the first and second TBP was regarded as independent and dependent variables for coinfections, respectively. Other detected TBPs were excluded in case of low detection rates (Table 2B). Age was divided into three groups: yearling (≤2 years), colt/filly (<4 years), and stallion/mare (≥4 years), while sex was classified as either male or female. Univariable analysis by Pearson's chi-squared or Fisher's exact test was carried out to evaluate the association of positivity with the parameters (age group and sex) and positivity to another pathogen. Subsequently, multivariable logistic regression was run for parameters with a p value of ≤0.20. At first, backward stepwise elimination was conducted to select variables. Then, the models with the lowest Akaike information criterion (AIC) values were chosen. A p value of <0.05 was considered significant. All analyses were performed using the Stats (R Core Team, 2020) and epitools (Aragon, 2020) packages in R.

Ethical clearance

The methodologies and procedures performed in this study were permitted by Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido, Japan (animal experiment permit no. 20–128; DNA experiment permit no. 1723–4). Animal restraining and handling protocols adhered to the Philippine Animal Welfare Act (R.A. 10631) and were approved by the Institutional Animal Care and Use Committee (IACUC) of Cavite State University, Indang, Cavite, Philippines (approval no. 2019–001). The caretakers of the horses and the race park manager were briefed on the purpose of the study.

They gave their verbal consent before sampling was conducted, with due supervision of the park veterinarian.

1-3. Results

Sample population and demographics

A total of 124 horses kept in a horse park in Cavite, Philippines (Fig. 4) were sampled in the present survey. All horses were thoroughbreds that were raised only for racing. The age of the horses ranged from 1 to 20 years (median = 5) and the majority were female (56.45%). The sample population consisted of 12.90% (16/124) yearlings, 17.74% (22/124) colt/filly, and 69.35% (86/124) stallion/mare (Table 2). Ectoparasites such as ticks were not observed in the horses during sampling. According to the animal caretakers, ectoparasite prevention measures include giving the horses daily baths and using ducks as a biological ectoparasite management method. Additionally, none of the studied horses displayed any clinical signs associated with TBDs.

Detected tick-borne pathogens

At least one pathogen was confirmed in 48.39% (60/124) of the samples (Table 3). *Babesia caballi*, *T. equi*, *A. phagocytophilum*, *A. marginale*, Bbsl, and *C. burnetii* were detected in 12.10% (n = 15), 0.81% (n = 1), 10.48% (n = 13), 0.81% (n = 1), 38.71% (n = 48), and 0.81% (n = 1) of the samples, respectively (Table 2A and 2B). Positive samples showed amplicons corresponding to the expected band sizes for *B. caballi* (~ 540 bp), *T. equi* (~ 392 bp), *A. phagocytophilum* (~ 928 bp), *A. marginale* (~768 bp), Bbsl (226–266 bp), and *C. burnetii* (~ 1,450 bp). In contrast, no sample was positive for *Rickettsia* (Table 2B). Single infections were found in 42 samples while coinfections with two or more TBPs were recorded in 18 samples (Table 3). Simultaneous infection of *B. caballi* and Bbsl and *A. phagocytophilum* and Bbsl were noted as the most frequent coinfection types.

Identifying risk factors associated with TBP detection

To evaluate the relationship between PCR-positivity and the various factors, a univariable analysis by Pearson's chi-squared or Fisher's exact test was done (Table 2A and Table 4). Positive samples for *T. equi*, *A. marginale*, and *C. burnetii* were omitted from the analysis due to low detection rates. *Babesia caballi* infection (18.57%) was significantly higher in female horses than in male horses (3.70%) ($p = 0.013$). Sex was not associated with *A. phagocytophilum* ($p = 0.77$) and Bbsl ($p = 0.58$) infections. Meanwhile, *B. caballi* (25%) and *A. phagocytophilum* (18.75%) infections were higher in yearlings, while Bbsl detection rates were comparable across age groups. Nonetheless, the differences among age groups were insignificant, as indicated by *p* values of 0.17, 0.44, and 0.96, respectively.

Multivariable logistic regression analysis was conducted for *B. caballi*, *A. phagocytophilum*, and Bbsl (Table 5). Variables that had *p* values ≤0.20 in the univariable analysis were included in the succeeding analysis: *B. caballi* (sex: $p = 0.013$; age group: $p = 0.17$; Bbsl positivity: $p = 0.09$), *A. phagocytophilum* (Bbsl positivity: $p = 0.13$), and Bbsl (*B. caballi* positivity: $p = 0.09$; *A. phagocytophilum* positivity: $p = 0.13$). The *B. caballi* model, with sex and Bbsl positivity as predictors, gave the lowest AIC value (87.54); hence, it was chosen as the final model. Sex was determined as a significant predictor for *B. caballi* infection ($p = 0.026$), with female horses 5.77 times more likely to test positive for *B. caballi* than male horses (Table 5). Based on the final models for the other TBPs, Bbsl positivity and *B. caballi* and *A. phagocytophilum* positivity were identified as non-significant risk factors for detecting *A. phagocytophilum* and Bbsl in horse samples, respectively (Table 5).

Sequence identities and phylogenetic analysis

Representative sequences for *B. caballi* (n = 7), *T. equi* (n = 1), *A. phagocytophilum* (n = 6), and Bbsl (n = 17) were obtained by sequencing analysis. The *B. caballi* 18S rRNA

isolates (MW714970–MW714976) shared 98.63–100% identity and were 99.15–100% similar to an equine Chinese isolate. The predominant genotype of the *B. caballi* isolates in racehorses from Cavite, Philippines was genotype A (Fig. 5). Meanwhile, the only *T. equi* sequence (MW714977) exhibited the highest identity (99.77%) with isolates from Russia, Mongolia, China, Saudi Arabia, Kazakhstan, South Korea, and Spain. According to the phylogeny, MW714977 was categorized as genotype E and belonged to a sister clade with canine and equine *T. equi* isolates from Saudi Arabia and South Korea, respectively (Fig. 6).

Six PCR-positive samples yielded eight different genotypes of *Anaplasma* 16S rRNA (MZ150516–MZ150518; MZ150520–MZ150524). MZ150516 was 99.03% identical to a novel *Anaplasma* sp. (*A. phagocytophilum*-like) from a Japan sika deer (JN055357). The remaining sequences (n = 7) shared 98.81–99.68% identity and had >99% identities with *A. ovis* isolates from Mongolia, Russia, China, and Kenya. The cluster of the *Anaplasma* sp. (*A. ovis*-like) lineages obtained in the current study deviated from the main *A. ovis* clade. Additionally, two isolates (MZ150523–MZ150524) formed a robust subclade within the aforementioned group (Fig. 7). The alignment analysis showed that the *A.ovis*-like variant MZ150523 displayed nine single nucleotide polymorphisms (SNPs) compared to *A. ovis* sequences from China (KJ639880), Russia (MW600403), and MZ150522, an equine isolate from this study (Fig. 8). On the other hand, MZ150516 formed a phylogenetically distinct subclade with other *A. phagocytophilum*-like isolates from East Asian countries, diverging from the main *A. phagocytophilum*-like group (Fig. 7). Furthermore, 21 SNPs and two deletions were noted by alignment analysis of MZ150516 with U02521, the Webster strain of *A. phagocytophilum* (Fig. 9). The single *A. marginale groEL* sequence (MZ408312) was identical to previous isolates from the

Philippines. Reamplification of the *C. burnetii*-positive sample failed; thus, *C. burnetii* sequence was not generated.

Of the 17 *Borrelia* rrf-rrl sequences, five isolates (MZ962640; MZ962644; MZ962647; MZ962649; MZ962650) shared 98.73–100% identity and had high percent identities (99.36–100%) with *Bo. japonica* isolates from China and Japan, as shown in the phylogenetic tree (Fig. 10). In addition, five sequences (MZ962641; MZ962642; MZ962646; MZ962652; MZ962656) and seven sequences (MZ962643; MZ962645; MZ962648; MZ962651; MZ962653–MZ962653) exhibited identities of 96.05–100% and 95.28–99.61%, respectively. These 12 isolates were most closely related to a Spanish *Borrelia* sp. (MK256778) obtained from *Ixodes ricinus* tick, which was 98–98.40% identical with the isolates in the former group and 95.30–96.50% similar with the isolates in the latter group.

1-4. Discussion

The long-established Philippine horse racing industry is a huge income contributor to the economy. Despite this, barely any progress has been made in equine TBD research in the Philippines in recent years. In this study, I molecularly detected major TBPs in the blood samples of Philippine racehorses.

The equine industry is gravely affected by EP, one of the most devastating diseases (Rothschild, 2013; Wise et al., 2013). In this study, *B. caballi* (12.10%) and *T. equi* (0.81%) detection rates reflect the findings of a previous survey in slaughter and racehorses from Batangas and Manila, wherein the main EP agent found was *B. caballi* (Cruz-Flores et al., 2010). It should be noted that the horse racing operations were moved from the Manila sampling site in 2010 to the current sampling site in Cavite. In contrast, *T. equi* detection in horses was higher than *B. caballi* in Cebu and Bohol provinces (Ybañez et al., 2018). In-

country differences in EP have been documented in several studies and were attributed to variations in extrinsic factors such as geography (Bartolomé Del Pino et al., 2016; Zhao et al., 2020), microclimate (Moretti et al., 2010), and animal management practices (Salim et al., 2008). The positive horses did not manifest any clinical signs related to EP, a hallmark of persistent infections (Scoles and Ueti, 2015). This kind of infection is common in EP-endemic countries, e.g., the Philippines (Tirosh-Levy et al., 2020). Inapparent carriers of EP parasites present a transmission risk as ticks can competently transmit the persistent parasites to naïve horses (Ueti et al., 2008). Despite the lack of overt clinical signs, persistently infected horses used for sports, i.e., racing, may perform inferiorly compared to their healthy counterparts (Rothschild, 2013). Nonetheless, carrier horses should be treated to eradicate parasites as developing clinical EP with evident signs can still occur, particularly in fatigued and immunocompromised horses (Hailat et al., 1997).

To more effectively formulate control measures against EP in a specific horse population, determining important risk factors for EP is essential. In this investigation, sex was found to be a significant risk factor for *B. caballi* infection. A higher *B. caballi* detection rate (18.37%) and higher odds (5.77) for infection were observed for female horses compared to those of male horses, in contrast to the findings of Qablan et al. (2013). However, prior investigations that assessed sex as a risk factor for EP have contradictory and conflicting results (Grandi et al., 2011; Onyiche et al., 2019). Although not significantly different, I similarly observed an indirect relationship between *B. caballi* infection rates and age. This may be linked to the older animals having a stronger immune system that eliminates the parasite (Rüegg et al., 2007; Moretti et al., 2010), which is commonly observed in *B. caballi* persistent infections.

Genotyping based on the 18S rRNA gene has been extensively utilized to characterize EP parasite populations in the field (Tirosh-Levy et al., 2020). The discovery

of *T. equi* genotype E in this study offers crucial information on the *T. equi* population circulating in Philippine horses. Genotype E was previously implicated in mortality cases of equine theileriosis (Kouam et al., 2010) and detected in apparently healthy horses from South Korea (Seo et al., 2013) and China (Hao et al., 2020). To completely understand the composition of *T. equi* populations in horses in the Philippines, additional studies should be carried out in various Philippine provinces. On the other hand, the genotyping of *B. caballi* isolates is relatively limited, with only three genotypes recognized (Tirosh-Levy et al., 2020). I confirmed genotype A as the predominant *B. caballi* in the positive horses. Unlike *T. equi*, *B. caballi* 18S rRNA genotypes provide insufficient insights into its geographical distribution. Therefore, using a variety of gene markers shall be valuable in describing *B. caballi* populations in equines.

I herein report the first molecular detection of *Anaplasma* in horses from the Philippines. *Anaplasma* is relatively more frequently reported in large ruminants in the country (Ybañez et al., 2018), but its presence in other livestock is unknown. Here, I first sought to identify *A. phagocytophilum*, the etiologic agent of EGA. Instead, 16S rRNA sequencing indicated the presence of novel variants of *Anaplasma*. The *Anaplasma* sequences obtained in this study had percent identities of 98.81–99.68% for *A. ovis*-like and 99.03% for *A. phagocytophilum*-like. According to (Kim et al., 2014), these values fall inside the cutoff values of 98.65% for prokaryotic species delineation based on the 16S rRNA gene, suggesting new variants of *A. ovis* and *A. phagocytophilum*. Furthermore, this supports the previously described highly diverse *Anaplasma* populations in the Philippines (Ybañez et al., 2014) and may signify the expansion of different *Anaplasma* species across a variety of hosts.

Given that horses and goats are raised together in the racing park, the discovery of *A. ovis*-like variants in horse samples is particularly intriguing. The two animals were seen

closely interacting during sampling. Cross-species transmission may be a likely explanation for the detection of *A. ovis*-like variants in horses in this study, with goats as the pathogen source. There is no information available about the competency nor of the host specificity of *Anaplasma* vectors in horses in the Philippines. Likewise, *Anaplasma* infections in goats have not been confirmed before. Thus, confirming this premise is necessary to confirm the clinical effects of *A. ovis* -like variants on its incidental hosts and its inter-host transmission. Additionally, an *A. phagocytophilum*-like variant was detected in the present study. In a previous investigation in Cebu province, several *A. phagocytophilum*-like variants in cattle were identified as well (Ybañez et al., 2013). Because of its diverse range of hosts (Atif, 2015), numerous strains and variants of *A. phagocytophilum* with differing abilities to cause diseases have evolved (Woldehiwet, 2010). Notably, the placement of the detected *A. phagocytophilum*-like variant in a subclade with East Asian isolates denotes a geographic grouping. Nevertheless, utilizing the multi-locus characterization may provide a more thorough strain differentiation approach to equine *A. phagocytophilum* (Zeman and Jahn, 2009).

The present study documented the first evidence of *Borrelia* in the Philippines. *Borrelia* has never been reported in the Philippines, and its detection here suggests that non-clinical Bbsl infection in Cavite horses is prevalent. Specifically, I detected *Bo. japonica*, a Bbsl genospecies transmitted by *I. ovatus* ticks and believed to be a non-pathogenic, Japan-exclusive, human Bbsl genospecies (Masuzawa, 2004), and two distinct *Borrelia* sp. genotypes closely related to a *Borrelia* isolate highly similar to *Bo. garinii*. Horses are potential reservoirs of Bbsl genotypes that can cause disease in humans, as documented in Belgium (Marcelis et al., 1987) and South Korea (Bae, 2018). Given its zoonotic nature, Bbsl detection in horses warrants a broader inquiry into the transmission risk to humans who regularly interact with the infected animals.

I detected one horse positive for *C. burnetii*. Recently, *C. burnetii* was molecularly detected in *Rh. microplus* ticks, water buffaloes, and cattle, in the provinces of Rizal and Quezon (Galay et al., 2020), while human and ruminant sera from Northern Samar, General Santos City, and Laguna were *C. burnetii* seropositive (Cardona, 2016). In contrast, all samples were negative for *Rickettsia*. Molecular investigations of *Rickettsia* in the Philippines have been limited. *Rickettsia* was recently detected in dogs from Luzon but was not detected in ruminants and ticks (Galay et al., 2018, 2020). However, this could not rule out probable *Rickettsia* infections as detecting this bacteria from serum and cutaneous samples can be more sensitive than in blood (Renvoisé et al., 2012).

The competent tick vectors of the detected TBPs in this study are still unknown. The tick species that can transmit *B. caballi*, *T. equi*, and *A. ovis*-like variant have not been reported in Cavite province, while possible tick vectors, such as *Rh. microplus* and *Rh. sanguineus* s.l. were previously identified from various hosts in nearby provinces (Goolsby et al., 2016; Galay et al., 2018; Alota et al., 2021). Additionally, the detection of Bbsl genospecies and *A. phagocytophilum*-like variant here should prompt further research into the tick vectors as these pathogens may have the potential to cause zoonotic infections. *Ixodes* ticks are the only recognized competent vectors for *A. phagocytophilum*. However, an alternative epidemiological cycle of *A. phagocytophilum* has been speculated in *Rhipicephalus* ticks (Dugat et al., 2015), which may explain our findings. Similarly, the only established competent tick vectors for *Borrelia* are the *Ixodes* ticks (Eisen, 2020). Detecting these pathogens is not unlikely as the presence of *Ixodes* ticks in Cavite has been reported before, i.e., *I. granulatus* ticks were described in wild rodents by a tick survey performed 50 years ago in Cavite (Parrish, 1971).

Overall, the results of this molecular survey provided information about TBPs in thoroughbred racehorses and their role as possible reservoirs of potential zoonotic TBPs.

Furthermore, this study revealed EP genotypes, novel *Anaplasma* variants, and the molecular presence of *Borrelia* in Philippine racehorses.

1-5. Summary

In this survey, *B. caballi*, *T. equi*, *A. ovis*-like, *A. phagocytophilum*-like, *A. marginale*, *Borrelia burgdorferi* sensu lato genospecies, and *C. burnetii* were molecularly detected in thoroughbred racehorses in Cavite, Philippines. In addition, sex was determined as a significant risk factor for *B. caballi* infection. Furthermore, I performed the first genotyping of EP agents in Philippine horses. These findings provide crucial information on the TBD agents and call for establishing and implementing strategic treatment and control programs for the neglected tick-borne infections in Philippine horses.

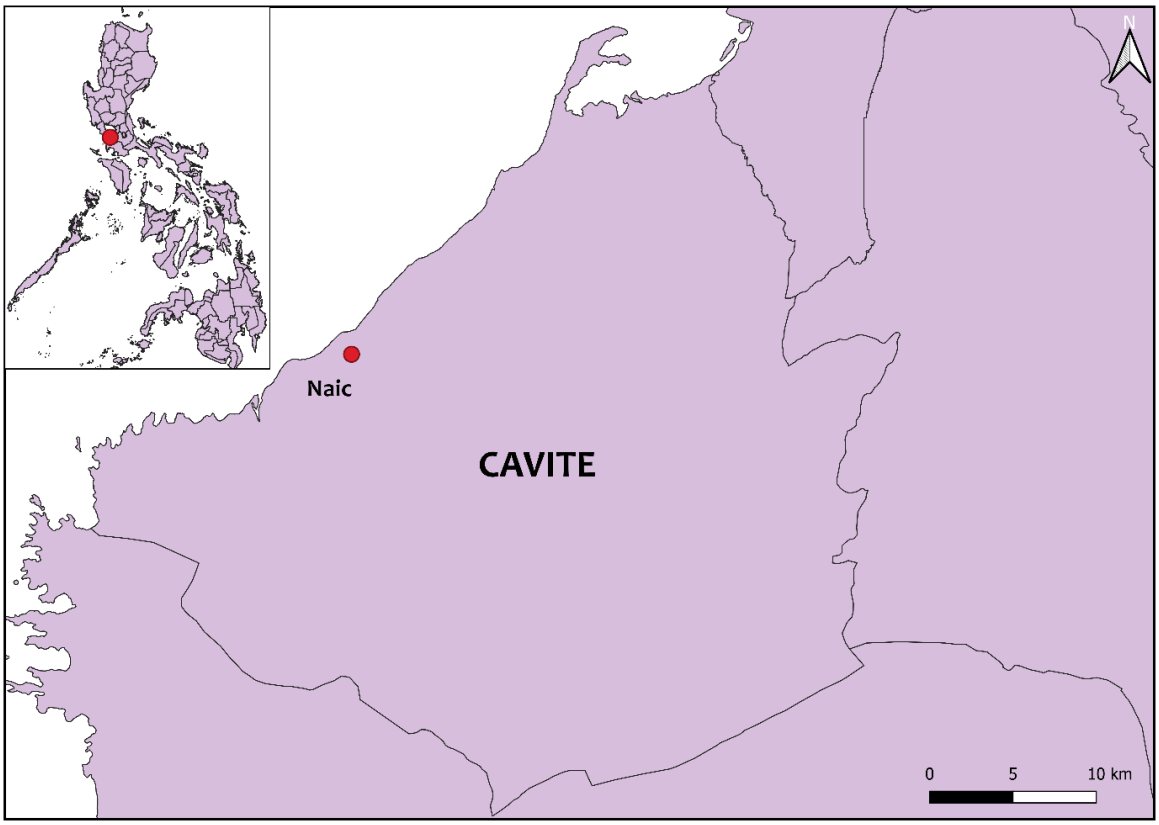

Fig. 4. Location map of the sampling site in Cavite province, Philippines. The Philippine map, with the sampling site indicated in red, is shown in the inset.

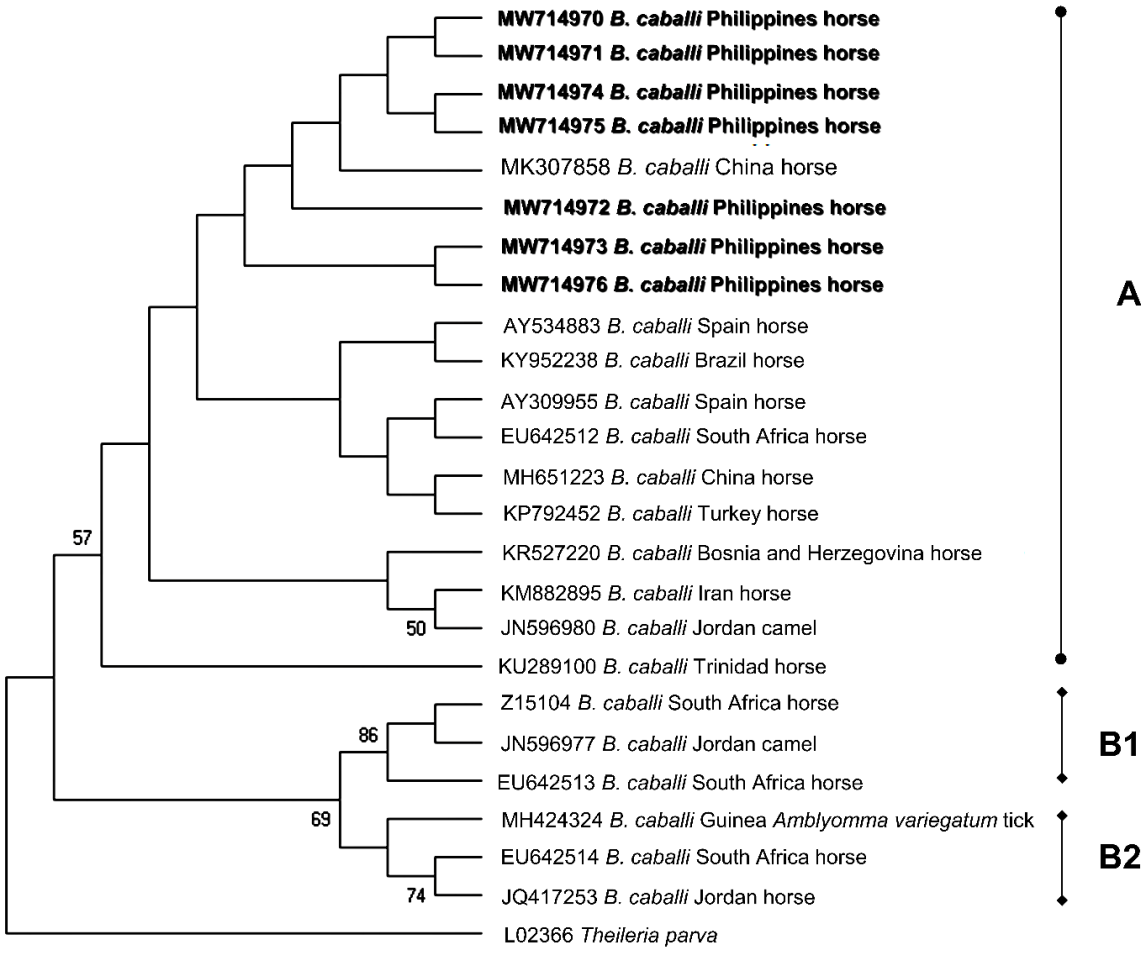

Fig. 5. Phylogenetic analysis of *B. caballi* based on the 18S rRNA gene. The phylogeny was inferred using the maximum likelihood method and Hasegawa-Kishino-Yano model with a discrete Gamma distribution (+G, parameter = 0.3348). The phylogeny test used was the bootstrap method with 1,000 replications. The sequences obtained from the current study are shown in bold. *Theileria parva* was used as an outgroup.

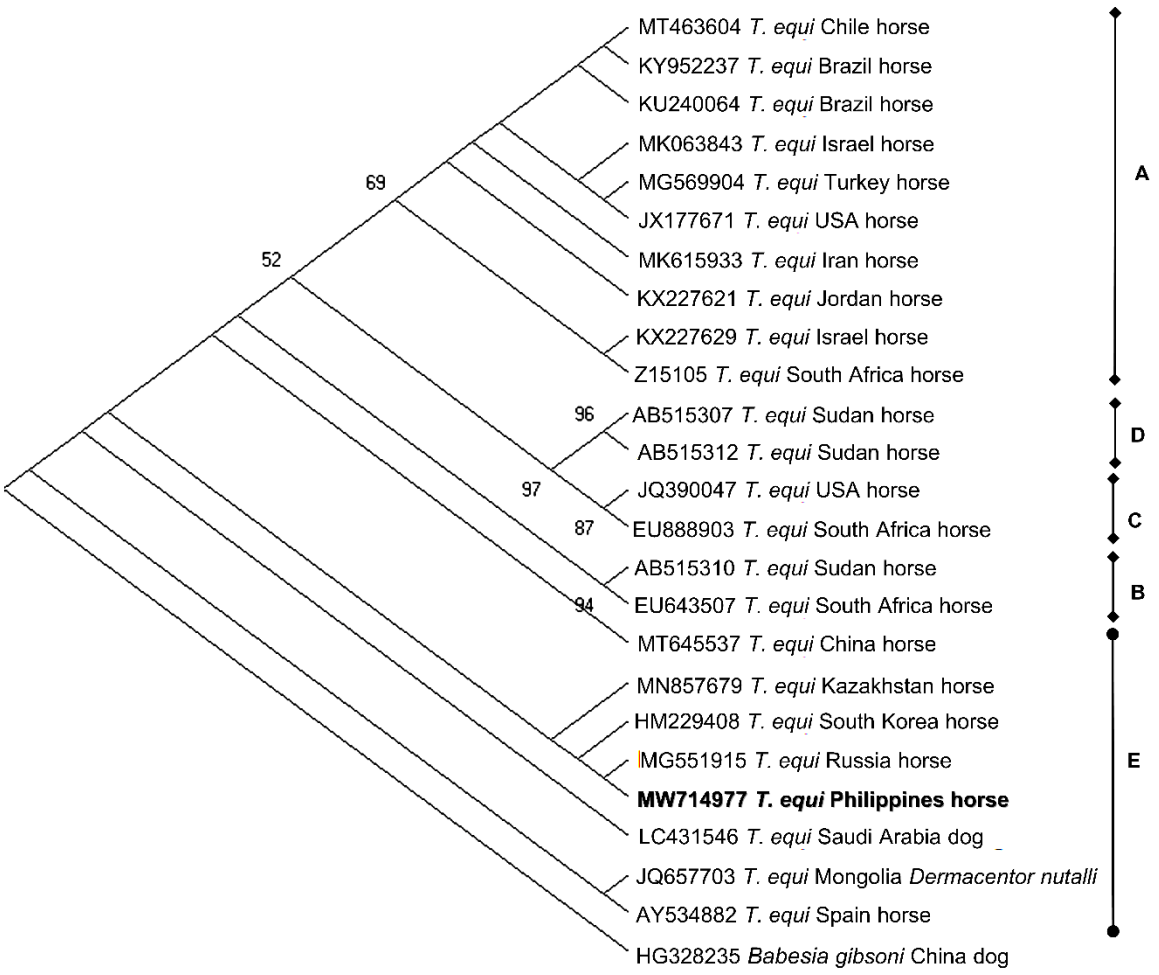

Fig. 6. Phylogenetic analysis of *T. equi* based on the 18S rRNA gene. The phylogeny was inferred using the maximum likelihood method and Tamura-3 parameter model with a discrete Gamma distribution (+G, parameter = 0.3320). The phylogeny test used was the bootstrap method with 1,000 replications. The sequence obtained from the current study is shown in bold. *Babesia gibsoni* was used as an outgroup.

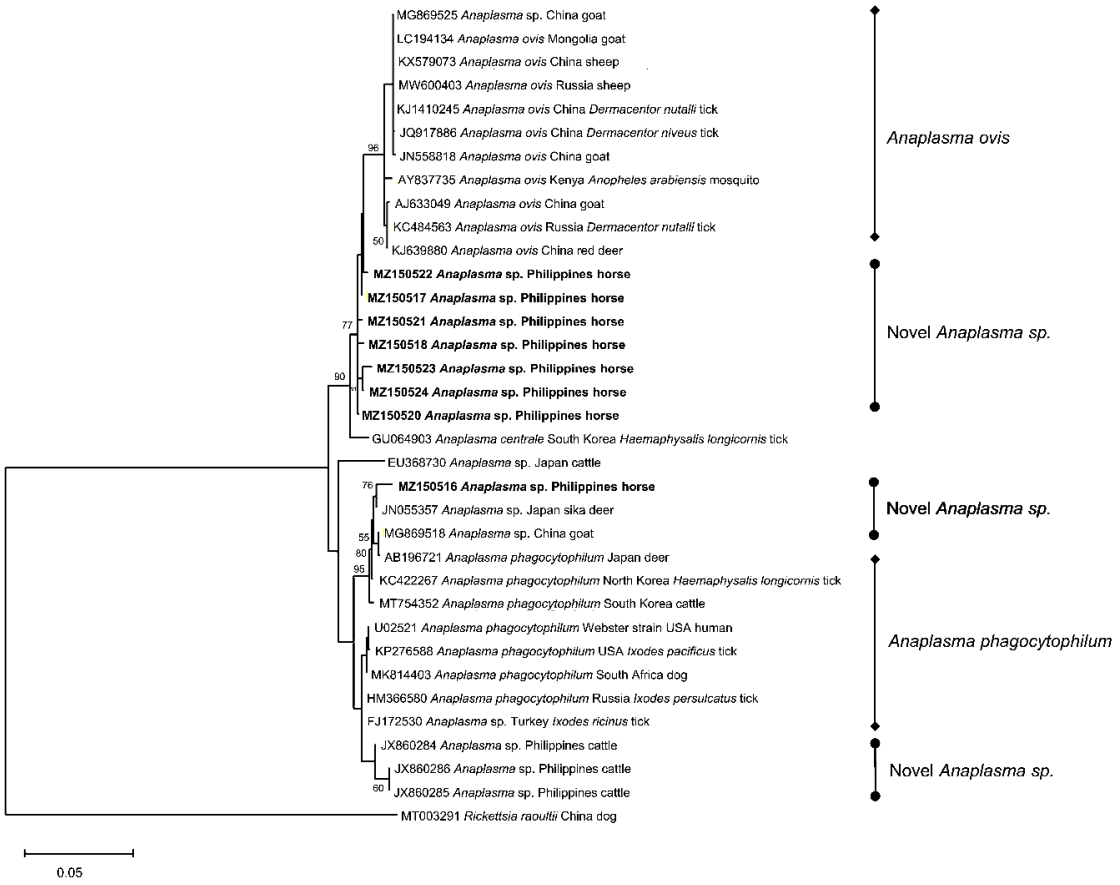

Fig. 7. Phylogenetic analysis of *Anaplasma* spp. based on the 16S rRNA gene. The phylogeny was inferred using the maximum likelihood method and Hasegawa-Kishino-Yano model with a discrete Gamma distribution (+G, parameter = 0.2268). The phylogeny test used was the bootstrap method with 1,000 replications. The sequences obtained from the current study are shown in bold. *Rickettsia raoultii* was used as an outgroup.

Fig. 8. Multiple sequence alignment analysis of *A. ovis*-like 16S rRNA. Sequences obtained from the current study (MZ150522 and MZ150523) were aligned with *A. ovis* sequences obtained from Russia (MW600403) and China (KJ639880). Purple boxes indicate SNPs in MZ150522, while red boxes display the SNPs in MZ150523. The primers are highlighted in yellow.

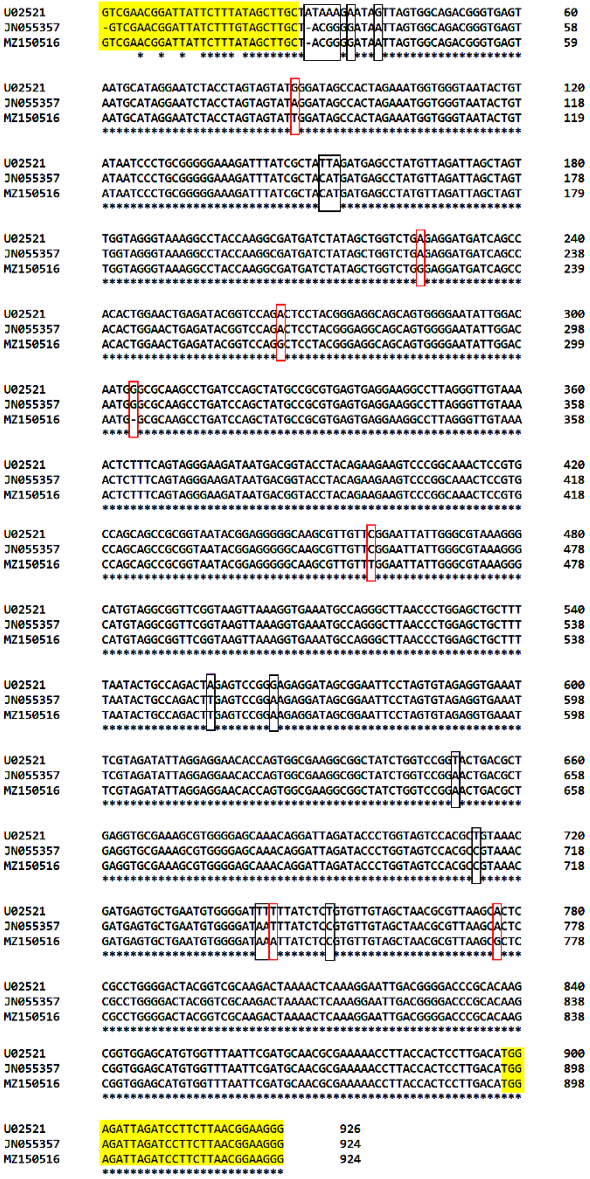

Fig. 9. Multiple sequence alignment analysis of *A. phagocytophilum*-like 16S rRNA. The sequence obtained from the current study (MZ150516) was aligned with *A. phagocytophilum* Webster strain obtained from the U.S.A. (U02521) and *A. phagocytophilum*-like sequence from Japan (JN055357). Purple boxes indicate SNPs and indels with reference to U02521 only, while red boxes display the SNPs and indels with reference to both U02521 and JN055357. The primers are highlighted in yellow.

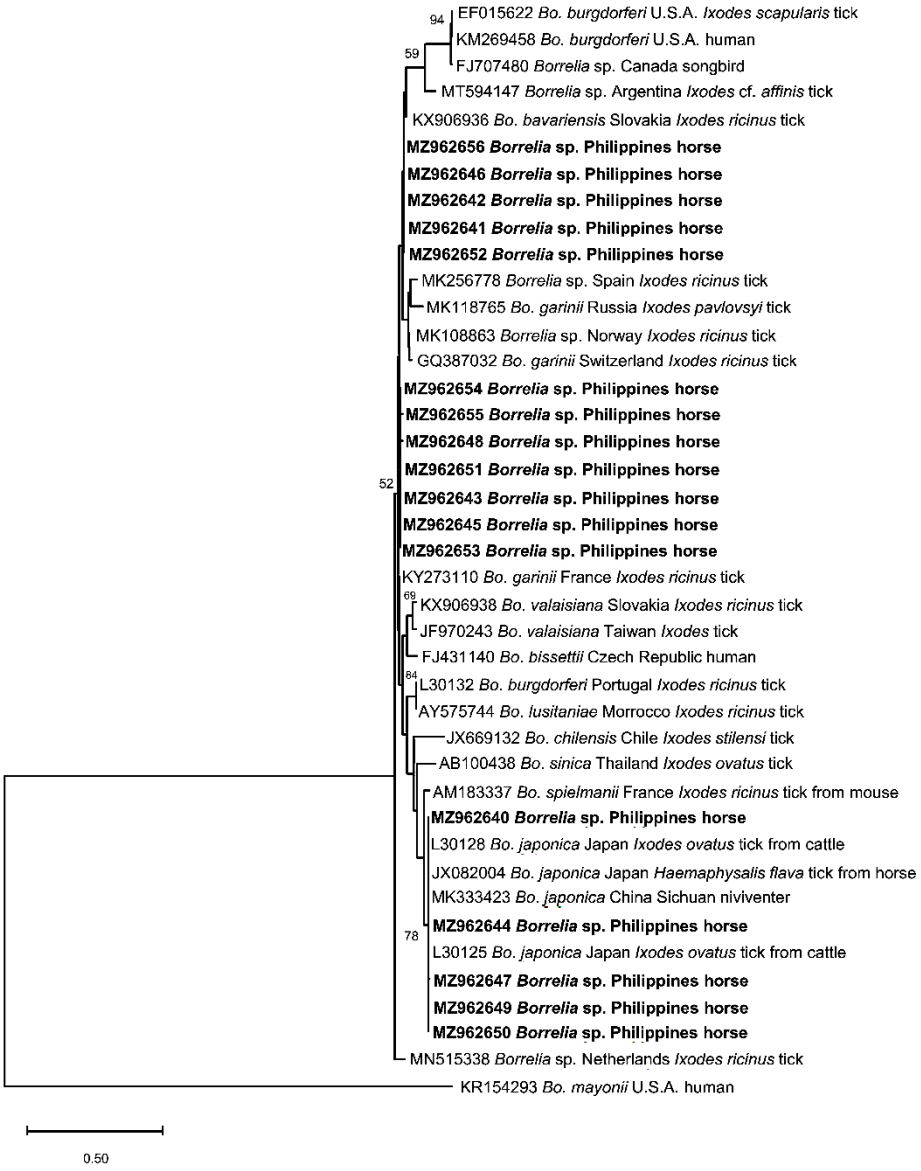

Fig. 10. Phylogenetic analysis of *Borrelia* spp. based on the 5–23S rRNA IGS. The phylogeny was inferred using the maximum likelihood method and Tamura-3 parameter model with a discrete Gamma distribution (+G, parameter = 0.6683). The phylogeny test used was the bootstrap method with 1,000 replications. The sequences obtained from the current study are shown in bold. *Borrelia mayonii* was used as an outgroup.

Table 1. List of primer sets and PCR assay conditions used for detecting TBPs in racehorse samples.

Pathogen	Target gene	PCR assay	Primer sequence (5'–3')	Ta (°C)	Amplicon size (bp)	Reference
Equine piroplasma	18S rRNA	multiplex-nested	GTTGATCCTGCCAGTAGTCA CGGTATCTGATCGTCTTCGA	54	913/867	(Alhassan et al., 2005)
Babesia caballi *Theileria equi*			TCGAAGACGATCAGATACCGTCG CTCGTTCATGATTTAGAATTGCT	54	540	
T.haneyi			TGCCTTAAACTTCCTTGCGAT		392	
Anaplasma phagocytophilum	16S rRNA	nested	TCCTGGCTCAGAACGAACGCTGGCGGC AGTCACTGACCCAACCTTAAATGGCTG	50	1433	(Barlough et al., 1996)
			GTCGAACGGATTATTCTTTATAGCTTGC CCCTTCCGTTAAGAAGGATCTAATCTCC	50	928	
A. marginale	*groEL*	nested-touchdown	TCCTGGCTCAGAACGAACGCTGGCGGC AGTCACTGACCCAACCTTAAATGGCTG	74–65	866	(Ybañez et al., 2014)
			GTCGAACGGATTATTCTTTATAGCTTGC CCCTTCCGTTAAGAAGGATCTAATCTCC	74–68	618–768	
Rickettsia spp.	16S rRNA	single	AACGTCATTATCTTCCTTGC AGAGTTTGATCCTGGCTCAG	59	426	(Simser et al., 2002)
Coxiella burnetii	16S rRNA	touchdown	ATTGAAGAGTTTGATTCTGG CGGCTTCCCGAAGGTTAG	58–48	~1450	(Zheng et al., 2017)
Borrelia burgdorferi sensu lato	rrf-rrl	nested	CGACCTTCTTCGCCTTAAAGC TAAGCTGACTAATACTAATTACCC	57.6	412	(Postic et al., 1994)
			CTGCGAGTTCGCGGGAGA TCCTAGGCATTCACCATA	55	226–266	

PCR: polymerase chain reaction; Ta: Annealing temperature; gDNA: genomic DNA; n.a. not available.

Table 2A. Detection of TBPs and univariable analysis based on age group and sex of the horses.

Variable	n	*Babesia caballi*				*Anaplasma phagocytophilum*				*Borrelia burgdorferi* sensu lato			
		No. of positives (%)	OR	95% CI	*p* value	No. of positives (%)	OR	95% CI	*p* value	No. of positives (%)	OR	95% CI	*p* value
Age group													
Yearling (≤2 years)	16	4 (25.00)		Ref.		3 (18.75)		Ref.		6 (37.50)		Ref.	0.96
Colt/Filly (<4 years)	22	3 (13.64)	0.48	0.06–3.41	0.17#	1 (4.55)	0.22	0.0038–3.00	0.44	9 (40.91)	1.15	0.26–5.37	
Stallion/Mare (≥4 years)	86	8 (9.30)	0.31	0.07–1.64		9 (10.47)	0.51	0.11–3.31		33 (38.37)	1.04	0.31–3.82	
Sex													
Male	54	2 (3.70)		Ref.	0.013#*	5 (9.26)		Ref.	0.77	19 (35.19)		Ref.	0.58
Female	70	13 (18.57)	5.86	1.24–55.90		8 (11.43)	1.26	0.34–5.23		29 (41.43)	1.30	0.59–2.91	
Total	124	15 (12.10)				13 (10.48)				48 (38.71)			

*A *p* value <0.05 was considered significant. #Variables with *p* value ≤0.20 were included in the multivariable analysis. Abbreviations- OR: Odds ratio; CI: confidence intervals; Ref.: reference used; No.: number.

Table 2B. Detection of TBPs based on age group and sex of the horses.

Variable	n	*Theileria equi (%)*	*Anaplasma marginale (%)*	*Coxiella burnetii (%)*	*Rickettsia* spp. (%)
Age group					
Yearling (≤2 years)	16	n.d.	n.d.	n.d.	n.d.
Colt/Filly (<4 years)	22	n.d.	n.d.	1 (4.55)	n.d.
Stallion/Mare (≥4 years)	86	1 (1.16)	1 (1.16)	n.d.	n.d.
Sex					n.d.
Male	54	1 (1.85)	1 (1.85)	1 (1.85)	n.d.
Female	70	n.d.	n.d.	n.d.	n.d.
Total	124	1 (0.81)	1 (0.81)	1 (0.81)	n.d.

n.d.: not detected.

Table 3. Infection type of TBP-positive horse samples.

Species	Number of positives	%
Single infection	42	33.87
Babesia caballi	5	4.03
Theileria equi	1	0.81
Anaplasma phagocytophilum	4	3.23
Anaplasma marginale	1	0.81
Borrelia burgdorferi sensu lato (Bbsl)	31	25
Multiple infections	18	14.52
Bbsl and *B. caballi*	9	7.26
Bbsl and *A. phagocytophilum*	7	5.65
B. caballi and *A. phagocytophilum*	1	0.81
Bbsl, *A. phagocytophilum*, and *C. burnetii*	1	0.81
Total number of positive samples	60	48.39

Table 4. Univariable analysis of coinfection with two pathogens in horses.

Coinfecting pathogens	No. of positives (%)	OR	95% CI	*p* value
Bbsl and *B. caballi*	9 (7.26)	2.67	0.78–9.84	0.09[#]
Bbsl and *A. phagocytophilum*	8 (6.45)	2.81	0.75–11.71	0.13[#]
B. caballi and *A. phagocytophilum*	1 (0.81)	0.58	0.013–4.53	0.61

[#]Variables with *p* value ≤0.20 were included in the multivariable analysis. Abbreviations- OR: Odds ratio; CI: confidence intervals; Bbsl: *Borrelia burgdorferi* sensu lato; No.: number.

Table 5. Multivariable logistic regression analysis of the identified risk factors in TBP-positive horses.

Pathogen	Variable	Category	β	SE	*p* value	OR	95% CI	Final model AIC
B. caballi	Sex	Male			Ref.			87.54
		Female	1.75	0.79	0.026*	5.77	1.23–27.03	
	Bbsl positivity	Negative			Ref.			
		Positive	0.95	0.58	0.10	2.58	0.83–8.03	
A. phagocytophilum	Bbsl positivity	Negative			Ref.			84.13
		Positive	1.04	0.6	0.08	2.84	0.87–9.27	
Borrelia burgdorferi sensu lato (Bbsl)	*B. caballi* positivity	Negative			Ref.			164.88
		Positive	1.06	0.57	0.063	2.89	0.95–8.82	
	A. phagocytophilum positivity	Negative			Ref.			
		Positive	1.12	0.61	0.067	3.06	0.93–10.12	

*A *p* value <0.05 was considered significant. Abbreviations-β: regression coefficient; SE: standard error; OR: odds ratio; CI: confidence intervals; AIC: Akaike information criterion; Ref.: reference used.

Chapter 2

Molecular detection of *Babesia*, *Theileria*, and *Anaplasma* in goats from the Philippines

2-1. Introduction

Babesiosis, theileriosis, and anaplasmosis are caused by tick-borne blood parasites of the genera *Babesia*, *Theileria*, and *Anaplasma*. These TBDs adversely affect livestock through direct and indirect losses in production. In small ruminants, the impact of TBDs has burdened farmers with losses linked to mortalities, less meat, milk, and wool produce, and increased costs for herd health management (Bilgic et al., 2017; Betancur Hurtado and Giraldo-Ríos, 2019) However, in endemic countries, TBDs are often overlooked despite being widespread in small ruminants, due to the lack of severe clinical manifestations during infection and strong tolerance through acquired natural immunity of infected hosts (Torina and Caracappa, 2012).

Several *Babesia* species can infect small ruminants, but the disease that develops varies between goats and sheep. *Babesia ovis* is fatal in sheep (Hashemi-Fesharki, 1997), while other species may have milder (*B. motasi*) (Smith and Sherman, 2011) or low (*B. crassa*) (Hasherni-Fesharki and Uilenberg, 1981) virulence. In goats, *B. ovis* causes subclinical infection (Yeruham et al., 1998; Stuen, 2020), and *B. motasi* infects goats more frequently than sheep (Smith and Sherman, 2011). Additionally, some newly reported species have been identified in particular locations, namely *Babesia* sp. Xinjiang and *B. motasi*-like in China (Liu et al., 2007; Niu et al., 2009) and *Babesia* sp. in Turkey (Ozubek and Aktas, 2017b). The clinical manifestations of babesiosis in small ruminants may

include fever, anemia, jaundice, depression, and hemoglobinuria. In addition, death may occur in severely affected animals (Yeruham et al., 1998). Ticks of the genus *Rhipicephalus*, *Hyalomma*, and *Haemaphysalis* can transmit *Babesia* to small ruminants (Friedhoff, 1997).

Theileriosis in small ruminants is caused by various species of *Theileria*, of which pathogenic species include *T. lestoquardi*, *T. luwenshuni* (*Theileria* sp. 1), and *T. uilenbergi* (*Theileria* sp. 2) (Ahmed et al., 2006). Other *Theileria* species that can infect small ruminants are nonpathogenic (Ahmed et al., 2011), albeit considerably impacting animal production (Stuen, 2020). The clinical disease in small ruminants may be accompanied by fever, lymph node swelling, icterus, hemorrhage, and diarrhea, while anemia, wasting, lack of appetite, and intermittent fever during chronic infection (Stuen, 2020). Similar to babesiosis, infection in goats is less severe (Smith and Sherman, 2011).

Anaplasma is the causative agent of anaplasmosis and is distributed globally, infecting a broad range of hosts. Small ruminants can get infected with several species, including *A. marginale*, *A. ovis*, *A. phagocytophilum*, and the newly discovered emerging pathogen *A. capra* (Woldehiwet, 2010; Torina and Caracappa, 2012; Peng et al., 2021). Infected animals may experience fever, hemolytic anemia, loss of appetite, weight loss, and fatigue, which translate to reduced milk production in dairy small ruminants (Torina and Caracappa, 2012). Biological vectors of *Anaplasma* are ixodid ticks, but mechanical vectors are also involved in the transmission, especially in places where the tick vectors are absent or rarely present (Kocan et al., 2010).

Small ruminant production is an essential agro-socioeconomic activity that sustains agricultural development in many parts of the world by providing meat, milk, skin, and wool. Compared to other livestock, goat and sheep raising is attractive in rural households because of the relatively smaller resources and effort required to maintain them (Pollott and

Wilson, 2009). They can subsist on unpalatable low-quality fodder and browse and still be prolific, owing to the early sexual maturity, brief gestation duration, and short birth intervals (Dar and Faylon, 1996). In the Philippines, goat raising is a pillar of the mixed (crop-livestock) farming systems and provides supplemental income to smallholder farming families (The 2003 Goat Farming Committee, 2004). In 2021, there were 3.2 million heads of goats, and the annual national production value was estimated at 250 million USD (Philippine Statistics Authority, 2022). Despite the huge contribution, goat production is suboptimal, and their full potential is not realized (Terrili, 1986; Pollott and Wilson, 2009). Several factors constrain caprine production in the Philippines, one of which is the high prevalence of parasites from genera *Eimeria* spp., *Fasciola* spp., *Haemonchus* spp., and *Trichostrongylus* spp. (The 2003 Goat Farming Committee, 2004). In contrast, the tick-borne parasites *Babesia, Theileria,* and *Anaplasma* have not been molecularly detected (Ybañez et al., 2019a). Therefore, in this study, I aimed to detect the molecular presence of these tick-borne pathogens in goats and determine the animal parameters associated with the detection.

2-2. Materials and methods

Sample collection and sampling sites

In this study, 396 whole-blood samples from randomly chosen goats were collected. The goats were randomly chosen irrespective of sex, age, and breed from March 2017 to March 2020. Sampling was done in backyards and farms selected by convenience in the provinces of Cavite (n = 42), Quezon (n = 20), Bohol (n = 35), Cebu (n = 74), Leyte (n = 26), and Davao del Sur (n = 199), Philippines. The specific sampling sites and their GPS coordinates are shown in Fig. 11. Approximately 2 mL of blood was collected via

venipuncture of the jugular vein of the goats into sterile EDTA tubes and kept cool until processing in the laboratory.

Genomic DNA isolation

The DNA extraction protocol was similar to that described in the materials and methods section in Chapter 1.

PCR assays for pathogen detection

The PCR conditions performed in this study are referred to in Table 6. The samples were processed using a nested PCR assay targeting the hypervariable V4 region of the 18S rRNA gene of piroplasma (Gubbels et al., 1999; Georges et al., 2001) and 16S rRNA of *Anaplasma* spp. (*A. phagocytophilum*) (Barlough et al., 1996). In addition, a single specific primer set amplifying the *B. ovis* 18S rRNA gene was also used (Aktaş et al., 2005). For the nested PCR assays, both first and final reactions were run to a final volume of 10 µL consisting of 1× ThermoPol® buffer (New England Biolabs), 2 mM of dNTP mix (New England Biolabs) 2 µM of forward and reverse primers, 0.25 U of Taq DNA polymerase (New England Biolabs), and 2 µL of genomic DNA sample for the first assay or 1 µL of the PCR product for the nested assay. For the screening of *B. ovis*, the conventional assay was performed similarly to the aforementioned setup, except for the final concentration of primers (5 µM). The company-provided thermocycling conditions were followed, with the annealing temperature for each assay listed in Table 6. Positive (DNA samples confirmed positive for *Theileria* sp., *B. ovis*, and *Anaplasma* sp. (Tumwebaze et al., 2020)) and negative controls (UltraPure™ DNase/RNase-Free distilled water; Invitrogen, USA) were run alongside the samples in each assay. Visualization of amplicons after exposure to UV light was done after electrophoresis of PCR products in 1.5% agarose gel and staining with ethidium bromide solution.

Sequencing and phylogenetic analysis

Similar procedures for cloning and sequencing analysis followed the protocols provided in Chapter 1, materials and methods section. For the phylogenetic analysis, forward and reverse reads were manually trimmed and overlapped to assemble the sequences. Shared identities between presently obtained and previously deposited sequences were determined by NCBI BLAST nucleotide search, while the identity matrix generated from EMBL Clustal Omega multiple sequence alignment (Madeira et al., 2019) determined the intersequence percentage identities. After nucleotide alignment by Clustal W and the determination of the best DNA model, the maximum likelihood trees were constructed by phylogeny testing using the bootstrap method with 1,000 replications. All analyses related to phylogeny were conducted using the MEGA X software (Kumar et al., 2018). The sequences obtained from this study were banked in the NCBI GenBank with accession numbers MW786647–MW786653 for *Theileria* sp. 18S rRNA (372–426 bp), OP003548 for *B. ovis* 18S rRNA (552 bp), and OP351259–OP351272 for *Anaplasma* sp. 16S rRNA (923–925 bp).

Statistical analyses

The association between *Babesia/Theileria* sp. and *Anaplasma* sp. positivity (dependent variable) and animal parameters (categorical independent variables), namely, sex, age group, breed, and location, were evaluated. Background data of animal samples were available for all except for the breeds of goats from Leyte; thus, I excluded them in the analysis for the breed variable. Fisher's exact test was used to calculate the exact *p* values, whereas if not applicable, Pearson's chi-squared test was employed to calculate the approximate *p* values. A *p* value of <0.05 was considered significant. All statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, USA).

Ethical clearance

Field sampling and animal handling protocols were conducted in accordance with the Philippine Animal Welfare Act (R.A. 10631) and the guidelines set by the IACUC of the University of the Philippines Cebu and Cavite State University. Experimental procedures and methodologies related to this study were permitted as in Chapter 1. The farmers and owners of the animals were oriented regarding the purpose of the study and provided verbal consent prior to the start of the sample collection.

2-3. Results

Sample composition and background information

In this study, samples were collected from 396 randomly selected goats across six provinces in the Philippines (Fig. 11), namely, Cavite (n = 42), Quezon (n=20), Bohol (n = 35), Cebu (n = 74), Leyte (n = 26), and Davao del Sur (n = 199) (Table 7). The goat population was comprised of the following: 60% adult (n = 237) and 40% young (n = 159); 87% female (n = 344) and 13% male (n = 52); 56% purebred (n = 222), 22% crossbred (n = 87), 15.4% Philippine native (n = 61), and unknown breed 6.6% (n = 26). The goats were raised in backyards by smallholder farmers, except those from Cebu and Bohol, which were domesticated in semicommercial and stock farms, respectively. The backyard goats were tethered and/or freely grazed, while goats from semicommercial farms were reared in semi-intensive and intensive systems.

Detection of pathogens and association with host parameters

Using the nested PCR assay targeting the 18S rRNA V4 hypervariable region of *Babesia/Theileria*, piroplasma DNA was detected in 305 (77.02%) samples (Table 7). The highest detection rates were recorded from one year or older adult (191/237; 80.59%), female (273/344; 79.36%), and purebred (179/222; 80.63) goats. Notably, piroplasma was

most frequently detected in samples from Davao del Sur (183/199; 91.96%). Statistical analysis indicated that sex (p = 0.007), breed (p = 0.027), and location (p <0.001) were associated with testing positive for piroplasma (Table 7). On the other hand, 153/396 (38.64%) samples were positive for *Anaplasma* spp. (*A. phagocytophilum*). *Anaplasma* detection rates were higher in young (77/159; 48.43%), male (25/52; 48.08%), and purebred (101/222; 45.50%) goats, with those from Quezon (16/20; 80.00%) showing the highest positivity rate (Table 7). Significant factors associated with *Anaplasma* positivity were age group (p = 0.001), location (p <0.001), and breed (p = 0.026). In addition, six (6/396; 1.52%) samples from Leyte (n = 3) and Cavite (n = 3) showed amplicons corresponding to the target of the *B. ovis* PCR assay.

Sequencing and phylogenetic analysis of representative sequences

I sequenced representative samples that showed strong bands for piroplasma (n = 7), *Anaplasma* sp. (n = 14), and *B. ovis* (n = 1) to determine their sequence identities and to analyze their phylogenetic relationships with previously published sequences in the GenBank sequence database. The seven sequences (MW786647–MW786653) were confirmed as *Theileria* species and exhibited intersequence identities of 89.97–97.74%. As shown in Fig. 12, three isolates (MW786649; MW786651; MW786653) were located in a subclade with other *T. orientalis* isolates from China, Pakistan, India, Bangladesh, and Malaysia. One *Theileria* sp. (MW786653) was most closely related to *Theileria* sp. Thung Song isolate from Thailand (99.30% identity) and formed a sister clade with the Chinese *T. sinensis* isolates, while MW786648 shared 99.53% identity with *T. annulata* isolates from India and Thailand (Fig. 12). MW786650 was similar to cattle isolate of *T. orientalis* from Pakistan, whereas MW786652 was located in a branch solitarily (Fig. 12). The *Anaplasma* sp. sequences (OP351259–OP351272) obtained in this study shared the following identities: 99.68% (OP351261 and OP351271); 98.81–99.89% (OP351262,

OP351263, OP351265–OP351270); 98.38% (OP351260 and OP351264), 98.48% (OP351259 and OP351267); 97.84% (OP351271 and OP351272). Based on the *Anaplasma* 16S rRNA phylogenetic tree, OP351260 and OP351264 clustered with *A. phagocytophilum* in *Rhipicephalus microplus* from Taiwan, *Anaplasma* sp. in cattle from Ethiopia, and *Candidatus* A. boleense in mosquitoes from China, while OP351262, OP351263, and OP351265–OP351270 grouped together and were related to *A. odocoilei* from the US (Fig. 13). In addition, OP351261, OP351271, and OP351272 were phylogenetically related to various *A. platys* isolates. The *B. ovis* isolate from the current study (OP003548) was closely related and had 99.82% identity with sheep, goat, and horse *B. ovis* isolates from Turkey, Iran, Spain, and Portugal (Fig. 14).

2-4. Discussion

Herein, I present the first molecular identification of *Babesia*, *Theileria*, and *Anaplasma* in goats from the Philippines. A high detection rate of piroplasma DNA was recorded in goats (77.02%), which was higher than caprine *Babesia* and *Theileria* rates recorded in Pakistan (5–40.80%) (Iqbal et al., 2013; Nasreen et al., 2020), Turkey (21.40%) (Ozubek and Aktas, 2017a), Italy (11.70%) (Torina and Caracappa, 2012), China (11.90–34.70%) (Wang et al., 2019; Yang et al., 2022a), Ethiopia (1.90%) (Gebrekidan et al., 2014), and Tunisia (4.70%) (M'ghirbi et al., 2013), while comparable to that from Malawi (72.70%) (Chatanga et al., 2021). The detection rate (38.64%) of *Anaplasma* spp. in the current study was higher than what was observed in goats from Bangladesh (15.75%) (Rahman et al., 2022), Thailand (13.50%) (Aung et al., 2022), Pakistan (7.80%) (Niaz et al., 2021), and South Korea (7.62%) (Miranda et al., 2021), but lower than in goats from China (58.50%) (Yang et al., 2022b). The relatively high detection rates may be due to several factors related to the climate, environment, host susceptibility, vector population

density, and production management systems (Genchi and Manfredi, 1999; Torina and Caracappa, 2012; Ozubek and Aktas, 2017a). In addition, *B. ovis* was detected in 1.52% of the goat samples and was present in two provinces (Leyte and Cavite). The current non-detection of *B. ovis* in Cebu goats agrees with the results of a previous molecular investigation where *Babesia* was not detected in caprine blood samples (Ybañez et al., 2019a).

A significant association between host parameters, including sex, age group, breed, and location, and pathogen detection was noted in the present study. Piroplasma positivity in female goats was significantly higher than in male ones, while studies on small ruminants in Ethiopia (Gebrekidan et al., 2014), Turkey (Zhou et al., 2017), and Tunisia (Rjeibi et al., 2014) found goat sex to be negligible. On the other hand, *Anaplasma* spp. detection in young goats was significantly higher than in adults, which is parallel to the findings in Pakistan goats (Niaz et al., 2021). Moreover, the detection of *Anaplasma* spp. and *Babesia/Theileria* were significantly associated with goat breeds, wherein a higher number of purebred or exotic goats tested positive compared to upgraded and native goats. Earlier surveys observed a similar trend, where the indigenous goat breeds were described to have higher resistance to piroplasma (Rjeibi et al., 2014; Islam et al., 2021) and *Anaplasma* (Said et al., 2015). The information available on goat breed resistance to TBDs is scarce, but one possible explanation may be the greater susceptibility of exotic goat breeds to tick bites compared to local breeds (Schwalback et al., 2003). Piroplasma and *Anaplasma* detection rates significantly differed among the location of the goats. The same was observed in goat herds from China (Yang et al., 2022a), Tunisia (Rjeibi et al., 2014), and Oman (Al-Fahdi et al., 2017). Location-specific factors in this study, such as management practices and macroclimatic conditions that affect tick proliferation, may be attributed to the different sampling sites characterized by diverse topography, terrain, microclimate, and fauna.

Sequencing and phylogenetic analysis confirmed that the representative *Theileria* sp. sequences obtained in this study were most closely related to *T. orientalis*, *T. annulata*, and *Theileria* sp. Thung Song isolate. Four *Theileria* isolates in the present study (MW786649; MW786651; MW786653) were similar to *T. orientalis* isolates from other locations. Members of the *T. orientalis* complex have been reported in cattle from the Philippines in previous studies (Belotindos et al., 2014; Ochirkhuu et al., 2015; Prado et al., 2022). More notably, I obtained an isolate highly similar to *T. annulata*. *Theileria annulata* is a species that can infect goats and causes a potentially fatal disease in cattle (Brown et al., 1998). However, it should be confirmed whether the infected goats can be inapparent carriers of various *T. orientalis* genotypes and *T. annulata* and if they can spread the pathogens to bovids, as in sheep (Brown et al., 1998; Khukhuu et al., 2011; Lawrence et al., 2021). *Theileria* sp. Thung Song is an isolate from dairy cattle in Thailand and was genotypically divergent from other benign *T. orientalis* types (*T. buffeli* and *T. sergenti*) (Chansiri et al., 1999; Chansiri and Sarataphan, 2002). Its detection in goats in the current study may indicate host shifting of this parasite. I also obtained an isolate phylogenetically distinct from other analyzed sequences (MW786652; 98.75% identity with *Theileria* sequences in GenBank), which may be a new *Theileria* sp., although more studies are needed to verify this claim. On the other hand, *T. luwenshuni,* which is a common species in goats reported from nearby Southeast Asian countries, namely, Thailand (Kaewhom and Thitasarn, 2017; Tu et al., 2021; Aung et al., 2022; Udonsom et al., 2022), Myanmar (Bawm et al., 2018), and Vietnam (Khukhuu et al., 2011; Sivakumar et al., 2013, 2020), was not confirmed in the obtained sequences.

In this study, *Anaplasma* isolates (OP351259–OP351272) closely related to *A. odocoilei, A. phagocytophilum,* and *A. platys* were confirmed in goats from the Philippines. *A. odocoilei* is a species causing chronic *Anaplasma* infection in white-tailed deer

discovered in the US (Tate et al., 2013). *A. odocoilei* does not cause severe clinical disease in experimentally infected white-tailed deer, and natural infections have only been detected in North America and South America (Yabsley et al., 2005; Ojeda-Chi et al., 2019; Orozco et al., 2020). Additionally, I obtained isolates (OP351261 and OP351271) highly similar to *A. platys* (99.10% and 99.46% identity with other *A. platys* isolates, respectively) and OP351272, a potential novel *A. platys*-like isolate (97.30% highest identity with GenBank *A. platys* isolates). A novel *A. platys*-like species that can be vertically transmitted to the goat's offspring was identified in China recently (Wei et al., 2020). Furthermore, I identified in Chapter 1 that a couple of novel *A. phagocytophilum*-like and *A. ovis*-like variants are circulating in Philippine horses. Thus, the isolates obtained from the current study warrant further probing into their genetic characteristics and the clinical impact of the infections they inflict on goats.

Despite the high rate of positivity, clinical signs associated with TBDs were not observed. This may indicate the endemicity of these pathogens in goats in the Philippines. Likewise, this may correspond to persistent infections, a characteristic of natural infections in places where the disease is presumed endemic (Stuen, 2020). While *B. ovis* causes acute and severe disease in sheep, natural infection with *B. ovis* in goats is rarely clinical (Yeruham et al., 1998). This was evident in the positive goats in the current study. In the case of *Theileria*- and *Anaplasma*-positive goats, the impact of subclinical infections should not be ignored because the pathogenicity of different species and genotypes vary depending on the host (Woldehiwet, 2010; Sivakumar et al., 2014).

Some aspects were outside the scope of this study. For instance, only a few samples were subjected to sequencing analysis and species confirmation was performed by partial amplification of one fragment from one gene. Therefore, additional studies based on species-specific detection should be conducted to elucidate the species diversity of *Babesia*,

Theileria, and *Anaplasma* in goats from the Philippines. In this study, the tick vectors were not determined. Since tethering and freely grazing systems are the more common production system in the Philippines (The 2003 Goat Farming Committee, 2004), there are more opportunities for the ticks to feed on the host as goats are exposed to vegetation where questing ticks are abundant. The most likely vector of piroplasma and *Anaplasma* is the ubiquitous *Rh. microplus* ticks, which were found infesting goats in Bulacan province (Swann and Claveria, 2017). The tick species *Rh. microplus* and *Haemaphysalis bispinosa* infesting goats from neighboring countries Thailand (Aung et al., 2022; Hirunkanokpun et al., 2022) and Malaysia (Lim et al., 2019) were also confirmed to be carrying *T. luwenshuni*. Similarly, *Rh. sanguineus* s.l. ticks, the vector of canine *A. platys* in the Philippines (Ybañez et al., 2012; Galay et al., 2018), may also be implicated as the vector of *A. platys*-like variants detected in the present study. The three-host tick *Rh. sanguineus* s.l. commonly infests dogs, its main host, but humans and other animals may also be incidentally infested (Dantas-Torres, 2010). A majority of goats in this study were raised in backyards and regularly interacted with companion animals (dogs and cats), which may have possibly exposed goats to the former's tick vectors. Further research should be done on other transmission methods, specifically, mechanical transmission by blood-sucking arthropods, such as *Tabanus, Stomoxys,* and mosquitoes (Kocan et al., 2010).

2-5. Summary

In this study, the DNA samples obtained from goats were screened for piroplasma (*Babesia/Theileria*) and *Anaplasma*. Of 396 samples, 77.02% (305/396) and 38.64% (153/396) were positive for piroplasma and *Anaplasma* using PCR assays targeting the 18S rRNA and 16S rRNA genes, respectively. Similarly, *Babesia ovis* was detected in six samples (1.52%). Representative *Babesia/Theileria* sequences shared 89.97–97.74%

identity and were most closely related to *T. orientalis*, *T. annulata*, and *Theileria* spp. Meanwhile, *Anaplasma* 16S rRNA sequences were related to *A. odocoilei*, *A. platys,* and *A. phagocytophilum*. This is the first molecular identification of *B. ovis, Theileria* spp., and *Anaplasma* spp. in goats from the Philippines.

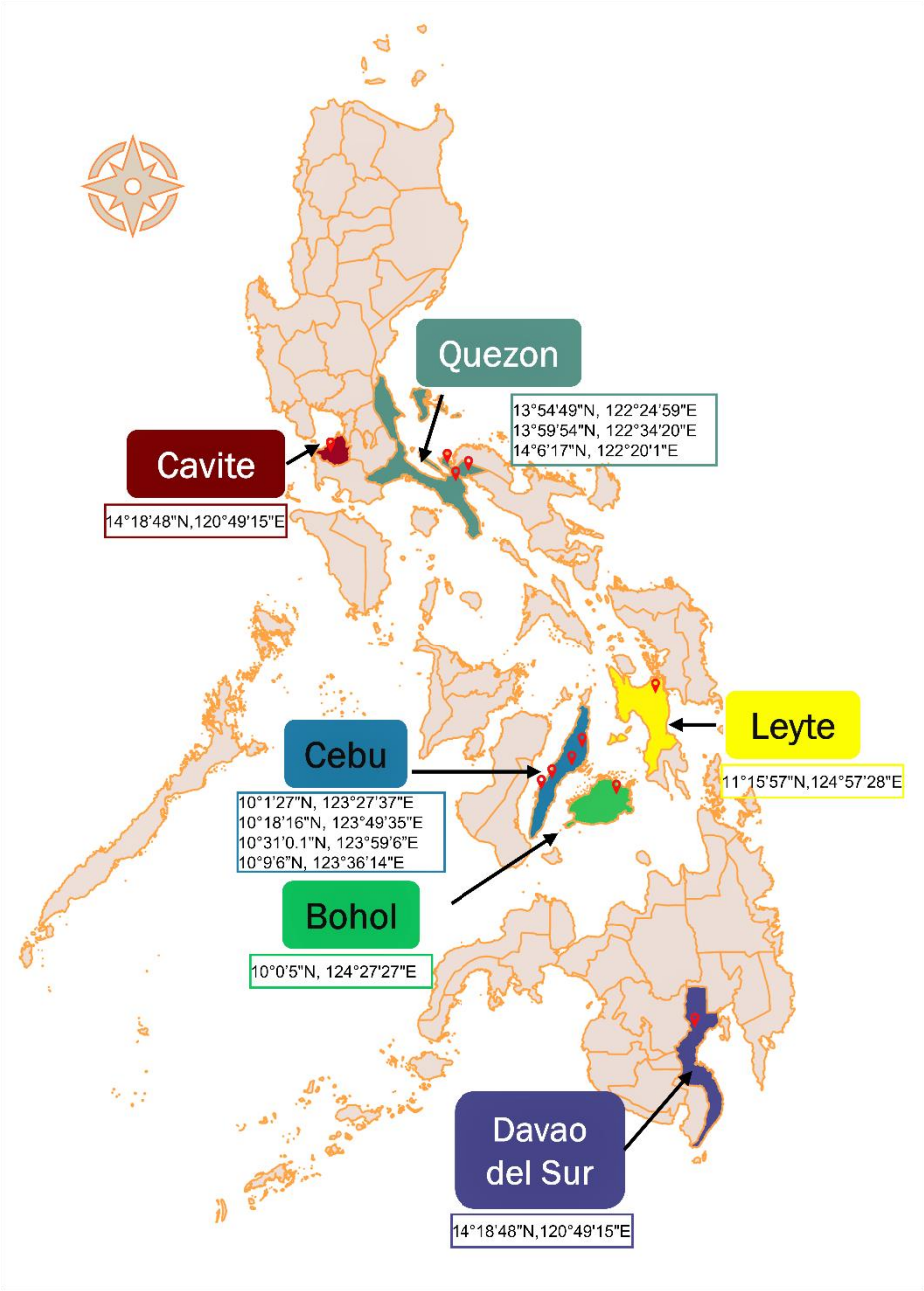

Fig. 11. Sampling locations of goats from the Philippines. The Philippine map shows the six provinces (colored) where samples were collected from, with pinned sampling sites and GPS coordinates. The map was generated using the QGIS software (QGIS Development Team, 2019).

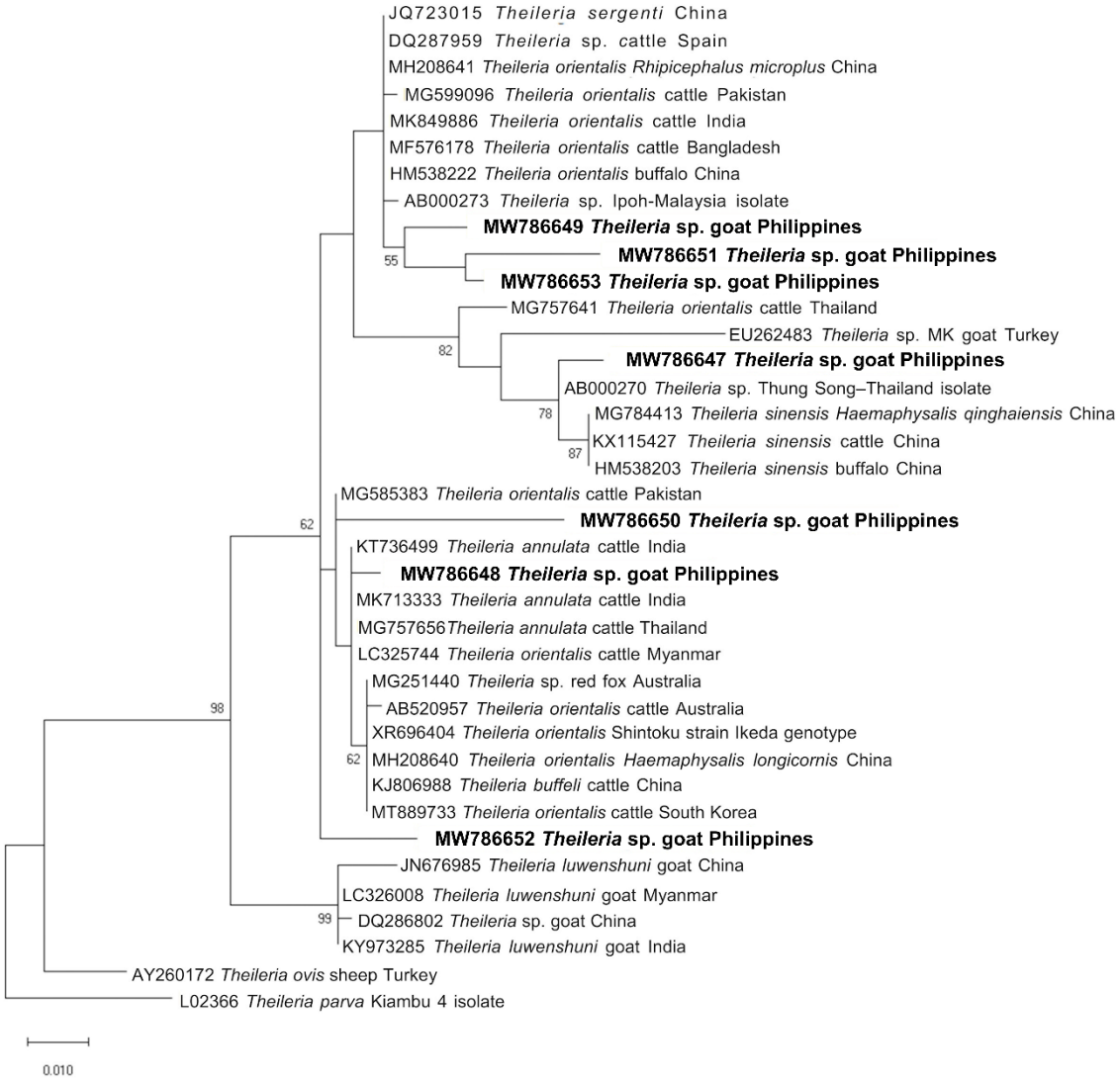

Fig. 12. Phylogenetic analysis of piroplasma sequences based on the 18S rRNA gene. Sequences obtained in this study (MW786647–MW786653) are shown in bold. The maximum likelihood tree was constructed using the Tamura-3 model plus discrete gamma distribution (+G, parameter = 0.3348). The phylogeny test was performed using the bootstrap analysis with 1,000 iterations. The sequences obtained from the current study are shown in bold. *Theileria parva* was designated the outgroup.

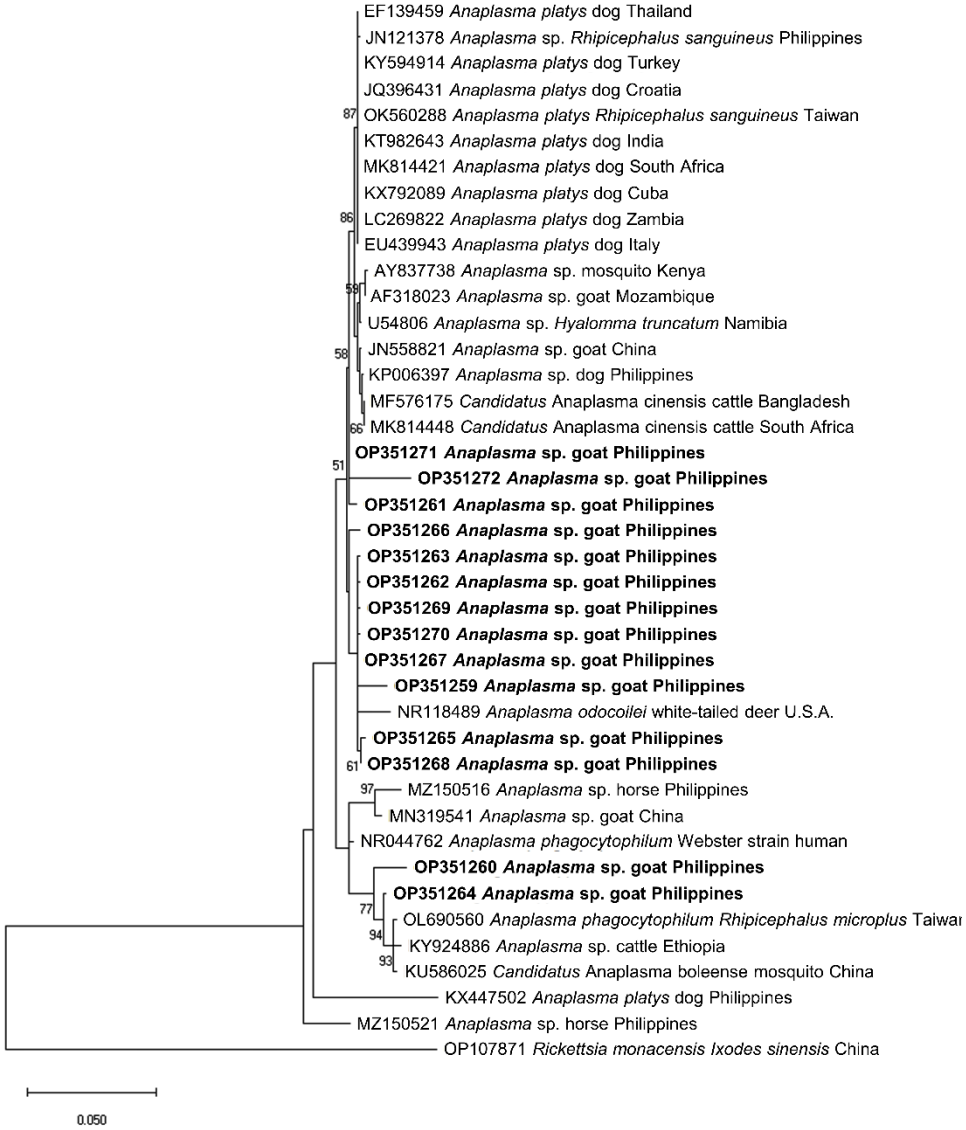

Fig. 13. Phylogenetic analysis of *Anaplasma* spp. sequences based on the 16S rRNA gene. Sequences obtained in this study (OP351259–OP351272) are shown in bold. The maximum likelihood tree was constructed using the Hasegawa-Kishino-Yano model plus discrete gamma distribution (+G, parameter = 0.3915). The phylogeny test was performed using the bootstrap analysis with 1,000 iterations. The sequences obtained from the current study are shown in bold. *Rickettsia monacensis* was designated the outgroup.

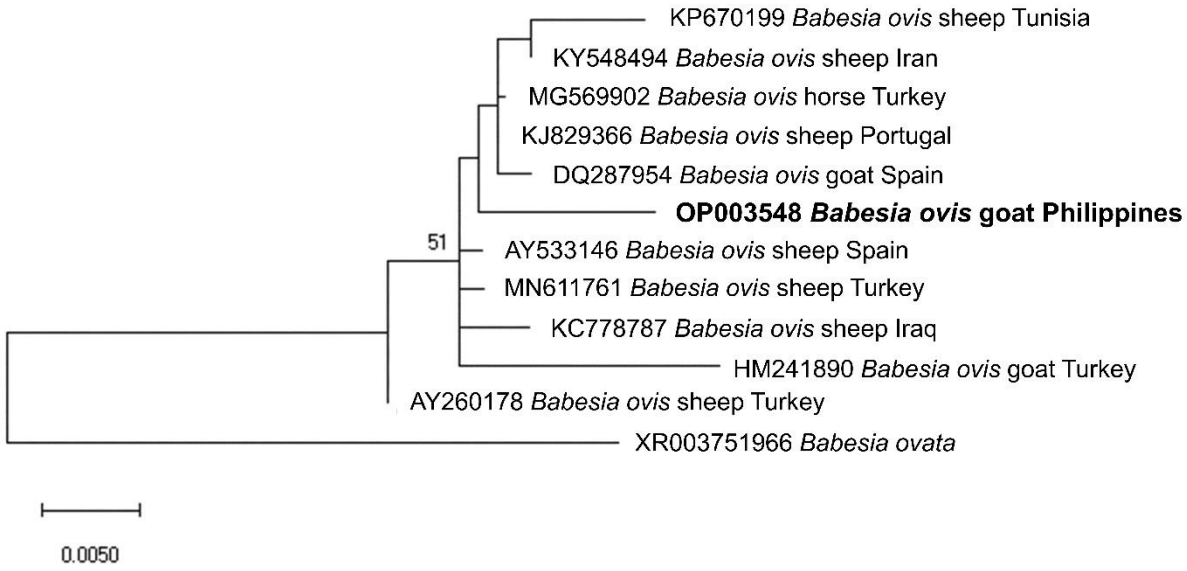

Fig. 14. Phylogenetic analysis of the *B. ovis* sequence based on the ssu rRNA gene. The sequence obtained in this study (OP003548) is shown in bold. The maximum likelihood tree was constructed using the Jukes–Cantor model with uniform rates among sites. The phylogeny test was performed using the bootstrap analysis with 1,000 iterations. The sequence obtained from the current study is shown in bold. *Babesia ovata* was designated the outgroup.

Table 6. List of PCR primers and conditions used for TBP detection in goats.

Pathogen	Target gene	Primer sequence (5'–3')	Annealing temperature (°C)	Target length (bp)	Reference
Piroplasma (*Babesia*/*Theileria*)	18S rRNA (V4 hypervariable region)	GAGGTAGTGACAAGAAATAACAATA TCTTCGATCCCCTAACTTTC	50	~460–520	(Gubbels et al., 1999)
		GACACAGGGAGGTAGTGACAAG CTAAGAATTTCACCTCTGACAGT	60	~390–420	(Georges et al., 2001)
Babesia ovis	Small subunit 18S rRNA	TGGGCAGGACCTTGGTTCTTCT CCGCGTAGCGCCGGCTAAATA	62	~549	(Aktaş et al., 2005)
Anaplasma phagocytophilum (*Anaplasma* spp.)	16S rRNA	TCCTGGCTCAGAACGAACGCTGGCGGC AGTCACTGACCCAACCTTAAATGGCTG	50	~1433	(Barlough et al., 1996)
		GTCGAACGGATTATTCTTTATAGCTTGC CCCTTCCGTTAAGAAGGATCTAATCTCC	50	~925	

Table 7. Molecular detection of *Babesia/Theileria* spp. and *Anaplasma* spp. based on goat parameters.

Variable	n	*Babesia/Theileria* spp.		*Anaplasma* spp.	
		No. of positives (%)	*p* value	No. of positives (%)	*p* value
Age-group					
Young (<1 year)	159	114 (71.70)	0.051	77 (48.43)	0.001 **
Adult (≥1 year)	237	191 (80.59)		76 (32.07)	
Sex					
Male	52	32 (61.54)	0.007 **	25 (48.08)	0.169
Female	344	273 (79.36)		128 (37.21)	
Location					
Davao del Sur	199	183 (91.96)	<0.001 ***	82 (41.21)	<0.001 ***
Cebu	74	35 (47.30)		37 (50.00)	
Bohol	35	16 (45.71)		10 (28.57)	
Quezon	20	8 (40.00)		16 (80.00)	
Leyte	26	24 (92.31)		2 (7.69)	
Cavite	42	39 (92.86)		6 (14.29)	
Breed [#]					
Purebred (Anglo-Nubian or Boer)	222	179 (80.63)	0.027 *	101 (45.50)	0.026 *
Crossbred or upgrades	87	58 (66.67)		25 (28.74)	
Philippine native	61	44 (72.13)		25 (40.98)	
Total	396	305 (77.02)		153 (38.64)	

Asterisks indicate significant differences: * <0.05; ** <0.01; *** <0.001. [#] Goats of unknown breeds (n = 26) were excluded from the analysis.

Chapter 3

Utility of targeted amplicon sequencing (Ampliseq) for the characterization of bovine piroplasma populations in the Philippines

3-1. Introduction

Piroplasmosis significantly affects livestock health, which leads to foregone income and opportunity losses for farmers worldwide. Molecular techniques, such as PCR assays, have revolutionized the surveillance and diagnosis of TBDs, particularly, babesiosis and theileriosis (Mans, 2022). The increased sensitivity and specificity of molecular assays over the more laborious traditional and immunological detection methods have contributed to the former's wide use, attesting to the excellent applicability of molecular methods in the field (Criado-Fornelio, 2007; Mosqueda et al., 2012; Mans et al., 2015; Alvarez et al., 2019). PCR assays coupled with sequence-based characterization using classical sequencing platforms (e.g., capillary electrophoresis) have been the typical approach for molecular-based parasite characterization (Schmitz et al., 2022). Still, the lengthy, arduous, and relatively expensive process stemming from the low sample throughput capacity presents a major bottleneck (Valentini et al., 2009). Fortunately, the onset of the NGS era has facilitated the fine-tuning of contemporary tools by streamlining the previously difficult process. In addition, the recent drastic cost decrease of NGS has ushered in applications such as shotgun metagenomics, which analyzes the diversity of uncultured microorganisms in a sample using whole genomes (Wooley et al., 2010), and marker gene sequencing,

which relies on a particular gene region to uncover the specific microbial communities present in a sample (Pérez-Cobas et al., 2020).

Targeted amplicon sequencing (Ampliseq), sometimes referred to as marker gene sequencing, amplicon deep sequencing, or amplicon-based NGS, leverages the amplification of conserved genes with hypervariable regions (i.e., 16S rRNA, 18S rRNA, ITS) to identify taxonomic groups and determine phylogenetic relationship of the detected microorganisms. It is a fast, high-throughput, and well-tested approach that elucidates microbial phylogenies in a given sample, especially those heavily contaminated with host DNA (Knight et al., 2018). As such, several studies have adopted this tool for the molecular epidemiology of economically important diseases such as TBDs. Notably, the 18S rRNA gene has been used as a marker gene for amplicon sequencing of piroplasma parasites in Cambodia (Huggins et al., 2022), Pakistan (Chaudhry et al., 2019; Ghafar et al., 2020), Thailand (Huggins et al., 2019), and Zambia (Squarre et al., 2020). On the other hand, piroplasma identification in livestock in the Philippines has been conducted before only through the use of PCR assays coupled with Sanger sequencing analysis. Therefore, in this study, I aimed to evaluate the applicability of Ampliseq to identify and characterize the bovine piroplasma populations in the Philippines.

3-2. Materials and methods

Ethical clearance

The sampling of cattle in the current study followed similar protocols in Chapters 1 and 2 and used similar permit numbers.

Blood sample collection and DNA extraction

In this study, approximately 2 mL of whole blood was collected from 162 cattle in two provinces in the Philippines (Fig. 11), namely Cavite (n = 62), Cebu (n = 76), and Bohol (n = 24). Genomic DNA extraction was done as in Chapters 1 and 2.

PCR assays for screening and amplicon tagging and multiplexing

The workflow of this study followed a previously published methodology (Squarre et al., 2020). The bovine DNA samples were initially screened using the RLB-PCR assay (Table 8), which amplifies the 18S rRNA gene (V4 hypervariable region) of piroplasma (Gubbels et al., 1999). This assay was chosen as it satisfied the ideal criterion for assays to be used in amplicon sequencing analysis: amplification of a highly variable region that is flanked by highly conserved regions where primers can bind (Knight et al., 2018). The initial reaction was done in a volume of 20 µL, which consisted of 1× Ex Taq buffer (Takara Bio, Japan), 4 mM dNTP mixture, 4 µM forward and reverse primers, 0.5 U Ex Taq polymerase, 3 µL gDNA, and 12.9 µL UltraPure™ water. Positive and negative controls were run alongside the samples. The thermal cycling condition was set at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 90 sec, and a final extension of 72°C for 10 min. Amplification was checked by gel electrophoresis using 10 µL of the PCR product, then visualized under UV light after staining with ethidium bromide.

The remaining PCR products from positive samples were diluted 100 times with UltraPure™ water and were subjected to a 2-step PCR that introduced Illumina adapters (tail primers) and index primers (metabarcodes) to the amplicons (Fig. 15A and Table 8). For the Illumina tail-tagging PCR, a final volume of 10 µL comprised of the following: 5 µL 2× Ampdirect® Plus (Shimadzu, Japan), 2.5 µM Illumina tailed-RLB forward and reverse primers, 0.25 U BioTaq™ HS DNA polymerase (Meridian Bioscience, USA), 3.95 µL UltraPure™ water, and 0.5 µL diluted PCR product. The PCR condition was similar to

the previous PCR settings, except for the initial denaturation step (10 min) and the fewer amplification cycles (12 cycles).

Thereafter, the indexing PCR was run using the high-fidelity KAPA Taq EXtra PCR kit (Kapa Biosystems, USA), which consisted of 4 μL 5× buffer, 1.4 μL $MgCl_2$ (25 mM), 0.5 μL dNTP mix (10 mM), 1 μL mixed Illumina-index primers (5 μM each), 1 μL 50-times-diluted PCR product from the tailing PCR, 0.675 U KAPA Taq EXtra DNA polymerase, and 11.975 μL UltraPure™ water for a final reaction volume of 20 μL. Eight forward (P5) and twelve reverse (P7) index primers were used for the multiplexing of amplicons from 95 samples and 1 *B. bovis* genomic DNA (positive control). The thermocycling condition for the indexing PCR was performed as follows: initial denaturation at 95°C for 5 min, 15 cycles of denaturation at 92°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 30 sec, and a final extension step at 72°C for 15 min. Then, 2 μL of indexing PCR product was electrophoresed in a 1% agarose gel in TAE buffer, stained with ethidium bromide, and viewed under UV light.

Library preparation and amplicon sequencing

Based on the intensity of the bands, the quantity of each sample that was used for sequencing was estimated. The indexing PCR products were normalized (2–10 μL per sample) and were pooled into a library, which was gel electrophoresed. The gel-suspended amplicon library was carefully excised and purified using Wizard® SV Gel and PCR Clean-Up System (Promega). The amplicon library was sequenced in a 600-cycle format (2 × 300 bp) using MiSeq Sequencing Reagent Kit v3 (Illumina, USA) with a spike-in control (25% PhiX DNA), as per the manufacturer's protocol, in an Illumina MiSeq platform.

Bioinformatics and phylogenetic analysis

The obtained raw reads were demultiplexed and quality checked with Trimmomatic (Bolger et al., 2014) using the filtering criteria of TRAILING (20), SLIDINGWINDOW (4:15), and MINLEN (36). The AMPtk package (Palmer et al., 2018) was leveraged to concatenate both reads (forward and reverse) with the minimum merged length set at 400 bp and produce amplicon sequence variants (ASVs), which were denoised by DADA2 (Callahan et al., 2016) and filtered by LULU (Frøslev et al., 2017). The NCBI BLAST non-redundant database parameters used to assign taxonomy to ASVs were: -max_target_seqs 1, -perc_identity 70, -qcov_hsp_perc 70, and -evalue 1e-20 (Altschul et al., 1990). Amplicon sequence variants (ASVs) were clustered (99% identity) using VSEARCH (Rognes et al., 2016). The summary of the workflow is depicted in Fig. 15B. ASVs with less than 10 reads in each sample were deemed invalid and excluded from further analyses (Ghafar et al., 2020).

The highest percentage identities of ASVs were confirmed by individual BLASTn search. The ASVs of the same genus, except for *Babesia* for which a separate alignment was done for each species, were aligned using Clustal W, and phylogeny was reconstructed using the maximum likelihood method, with the best substitution model applied for phylogeny testing (1,000 bootstrap iterations) in MEGA X (Kumar et al., 2018).

3-3. Results

In total, 95 of 162 (58.64%) blood DNA samples were positive for piroplasma using the RLB-PCR assay. The highest detection rate was recorded in cattle from Bohol (83.33%; 20/24), followed by Cavite (70.97%; 44/62), and Cebu (40.79%; 31/76).

After denoising with DADA2, a total of 2,179 ASVs were obtained, of which 175 had their taxonomy assigned based on the BLASTnr database. After dereplication, chimera

removal, and clustering by VSEARCH, 97 distinct ASVs were generated. Then, ASVs with less than 10 reads in each sample (n = 18) were discarded, leaving 79 valid ASVs. These ASVs belong to the genera *Babesia* (n=58), *Theileria* (n = 17), *Hepatozoon* (n = 2), and *Sarcocystis* (n = 2) (Table 9). The ASVs' top hits corresponded to 10 species: *B. bovis* (n = 37), *B. bigemina* (n = 18), *T. orientalis* (n = 13), *Babesia* sp. (n = 3), *H. canis* (n = 2), *S. cruzi* (n = 2), *T. annulata* (n = 1), *T. equi* (n = 1), *T. mutans* (n = 1), and *Theileria* sp. Thung Song (n = 1). The parasite species were confirmed in 60 samples, while no parasite ASVs were mapped for the remaining 35 samples. These samples are required to be resequenced and reanalyzed. As shown in Table 9, the most frequent piroplasmas detected in cattle were *B. bovis* (38/60; 259,704 reads), *B. bigemina* (33/60; 207,232 reads), and *T. orientalis* (22/60; 347,535 reads). In addition, 5 infection types were observed in the successfully sequenced samples (Table 10). The most common infection types were dual infection with *B. bovis* and *B. bigemina* (15/60), and single infections with *B. bovis* (8/60) and *T. orientalis* (7/60).

ASVs assigned to *B. bovis* (n = 37) and *Babesia* sp. (n = 2) were 90.76–100% identical with GenBank-deposited *B. bovis* isolates and demonstrated three major subclades based on the phylogenetic analysis (Fig. 16). The first subclade consisted of 14 ASVs, which grouped with *B. bovis* isolates from China (JX495403, KP710223, KY805832), South Africa (MH527732), Mexico (EF643469), and Argentina (MH569533). The second distinct subclade contained 15 ASVs and was closely related to a bovine isolate from Mexico (GU906883), while the third subclade clustered with isolates from China (MN252440), Bolivia (LC645224), India (KF928959), Brazil (FJ426364), and USA (HQ264112). On the other hand, ASVs assigned to *B. bigemina* (n = 18) and *Babesia* sp. (n = 1) had 90.23–99.77% identity with isolates from the database and the evolutionary inference indicated that, except for ASV52 and ASV49, most ASVs were phylogenetically

distinct from previously reported *B. bigemina* isolates, as evidenced by the strong statistical support in the subclades (Fig. 17). Phylogenetic analysis of *Theileria* ASVs showed species-specific grouping (Fig. 18). A total of 13 ASVs (91.72–100% identical to GenBank *T. orientalis* isolates) clustered in the *T. orientalis* complex subclade, 4 of which formed a subgroup with high nodal support. In addition, ASV46 and *Theileria* sp. Thung Song (AB000270) formed a sister clade to the *T. orientalis* complex clade. As expected, ASVs taxonomically identified as *T. annulata*, *T. equi*, and *T. mutans* were grouped separately according to their respective species.

Hepatozoon and *Sarcocystis* phylogenies are presented in Fig. 19 and Fig. 20, respectively. The 2 *Hepatozoon* ASVs clustered in a clade with a canine *H. canis* isolate from Nigeria (AB365071) and with isolates Zambia (wild dog and lion; MT814761 and MT814748) and Israel (dog; MK091084). Notably, the phylogenetic placing of the current *H. canis* ASVs were divergent from previously detected Philippine *H. canis* isolates (LC428208 and KP182934) from dogs (Fig. 19). Meanwhile, the phylogenetic reconstruction of *Sarcocystis* ASVs revealed that ASV96 and ASV97 were most closely related to bovine *S. cruzi* isolates from Malaysia (KR155197) and India (KT306829), respectively (Fig. 20).

3-4. Discussion

Babesiosis and theileriosis impose a massive health threat on bovine herds. The impact brought by these diseases causes cattle farmers all around the world to suffer financial losses in the millions. For the longest time, bovine babesiosis and theileriosis were thought to be endemic in the Philippines as clinical cases are rarely observed despite the occurrence of widespread infections. Previous molecular reports and species characterization have relied on the use of qualitative molecular methods i.e., PCR assays

and classical sequencing. In the current study, by employing the high-throughput NGS platform via the Ampliseq technique, I deciphered the composition of piroplasma populations circulating in field cattle raised in three provinces of the Philippines.

Piroplasma DNA was detected in more than half of the cattle samples tested (58.64%), which is higher than a previous survey in four Philippine provinces that noted a 29.9% detection rate (Prado et al., 2022). In contrast, the current finding was lower compared to the rates recorded in cattle from Zambia (63.4%) (Squarre et al., 2020) and Pakistan (85.8%) (Ghafar et al., 2020) using a similar assay. The detection of 79 ASVs belonging to four parasite genera exhibited the extent of parasite diversity in the infected cattle, adding new information on the tick-borne microbial communities in bovines in the Philippines.

The causative agents of bovine babesiosis, *B. bovis* and *B. bigemina*, were the most commonly identified species in the cattle samples in this study. Bovine *Babesia* species have been molecularly detected using PCR assays in at least ten provinces across the country (Foronda et al., 2010; Ybañez et al., 2013; Yu et al., 2013; Ochirkhuu et al., 2015; Herrera et al., 2017; Prado et al., 2022). In addition, infection with at least two species of *Babesia* was the dominant coinfection type recorded. This is frequent in areas where these pathogens coexist and are endemic (Obregón et al., 2019), and in this case, because both *Babesia* species are carried and transmitted by a similar vector, *Rh. microplus* ticks (Bock et al., 2004). *Theileria orientalis* was also present, which was similarly detected in previous surveys of bovines in the Philippines (Belotindos et al., 2014; Ochirkhuu et al., 2015; Prado et al., 2022). The etiologic agent of oriental theileriosis, *T. orientalis*, was previously thought to cause mild infections, but Chitose and Ikeda genotypes have since been determined to be pathogenic and occasionally prompt outbreaks (Gebrekidan et al., 2020). Therefore, genotyping of the currently obtained *T. orientalis* isolates is expected to give

insights into their potential to cause oriental theileriosis, specifically in immunosuppressed and fatigued cattle (Watts et al., 2016).

Phylogenetic analyses revealed numerous possible novel variants of *B. bovis, B. bigemina,* and *T. orientalis* based on the sequences analyzed in the current survey. This is parallel to the findings in piroplasma populations in Pakistan, wherein it was attributed to new population variants or the emergence of cryptic species (Ghafar et al., 2020). The latter may be a more plausible proposition, considering the relatively low percent identities (90–97%) of some ASVs with *Babesia* and *T. orientalis* sequences in the database. However, caution should be heeded when determining new population variants based on the 18S rRNA gene (Ghafar et al., 2020), especially in the present case as I obtained only partial sequences.

In the current investigation, the following species were molecularly detected in cattle for the first time in the country: *T. annulata, Theileria* sp. Thung Song, *T. mutans, T. equi*, *H. canis*, and *S. cruzi.* The detection of *T. annulata* in the current study is a very significant finding because of its potential to cause severe clinical cases and mortalities in cattle (Bishop et al., 2004). Moreover, this confirmation corroborates the detection of *T. annulata* in goats (described in Chapter 2) and warrants additional exploration as it can bring devastating impact if ever an outbreak occurs. Meanwhile, *Theileria* sp. Thung Song and *T. mutans* cause benign infections in cattle (Chansiri et al., 1999; Bishop et al., 2004). *Theileria* sp. Thung Song was likewise detected in goats as reported in Chapter 2. The detection of *T. mutans* is unusual as it is an African species, but cannot be entirely dismissed as a study in nearby Malaysia also detected *T. mutans* in swamp buffaloes (Ramakrishnan and Abas Mazni, 1993).

Interestingly, the detection of *T. equi* and *H. canis* was recorded, both non-conventional parasites in cattle. One of the causative agents of EP, *T. equi* is an important

equine TBP and commonly infects horses in the Philippines (as reported in Chapter 1). Its natural hosts are equids, but has been detected in other mammals such as dogs (Criado-Fornelio, 2004; Beck et al., 2009; Fritz, 2010; Qablan et al., 2012a; Inácio et al., 2019; Salim et al., 2019), camels (Qablan et al., 2012b), and tapirs (de Souza Gonçalves et al., 2020). Meanwhile, the detection of *T. equi* in cattle (Algeria) has only been reported once (Sadeddine et al., 2020). In the same manner, *H. canis*, a known tick-transmitted parasite, has been extensively documented in dogs and cats in the Philippines (Baticados et al., 2011; Adao et al., 2017; Galay et al., 2018; Ybañez et al., 2019b; Colella et al., 2020), and *Rh. sanguineus* s.l. is the recognized tick vector (Galay et al., 2018; Nguyen et al., 2020). One possible explanation of *H. canis* detection in cattle may be through accidental transmission by *Rh. microplus* ticks (de Miranda et al., 2011; Li et al., 2020a), which is the main tick ectoparasite of cattle in the Philippines. Technically not a piroplasmid, ASVs corresponding to *S. cruzi* were also obtained. *Sarcocystis* is an apicomplexan parasite that requires a two-host life cycle (Gjerde, 2016). *Sarcocystis cruzi*, whose definitive hosts include dogs and wolves, is the most pathogenic of the five *Sarcocystis* species that can infect bovines and cause fever, anemia, hair loss, abortion, and stunting (Lindsay and Dubey, 2020). Previously, *S. cruzi* infection in cattle has been reported in the Philippines (Claveria et al., 1999, 2001), and its detection here signifies the importance of surveillance of other anemia-causing parasites in cattle.

3-5. Summary

The results from this study demonstrated the applicability of the Ampliseq technique to elucidate the piroplasma populations of cattle in the Philippines. A relatively high diversity of *B. bovis*, *B. bigemina*, and *T. orientalis* populations were observed, and the pathogenic *T. annulata* was molecularly detected in cattle for the first time. Moreover,

non-conventional parasites for cattle *T. equi* and *H. canis* were identified as well, and the first molecular detection of *S. cruzi* was documented.

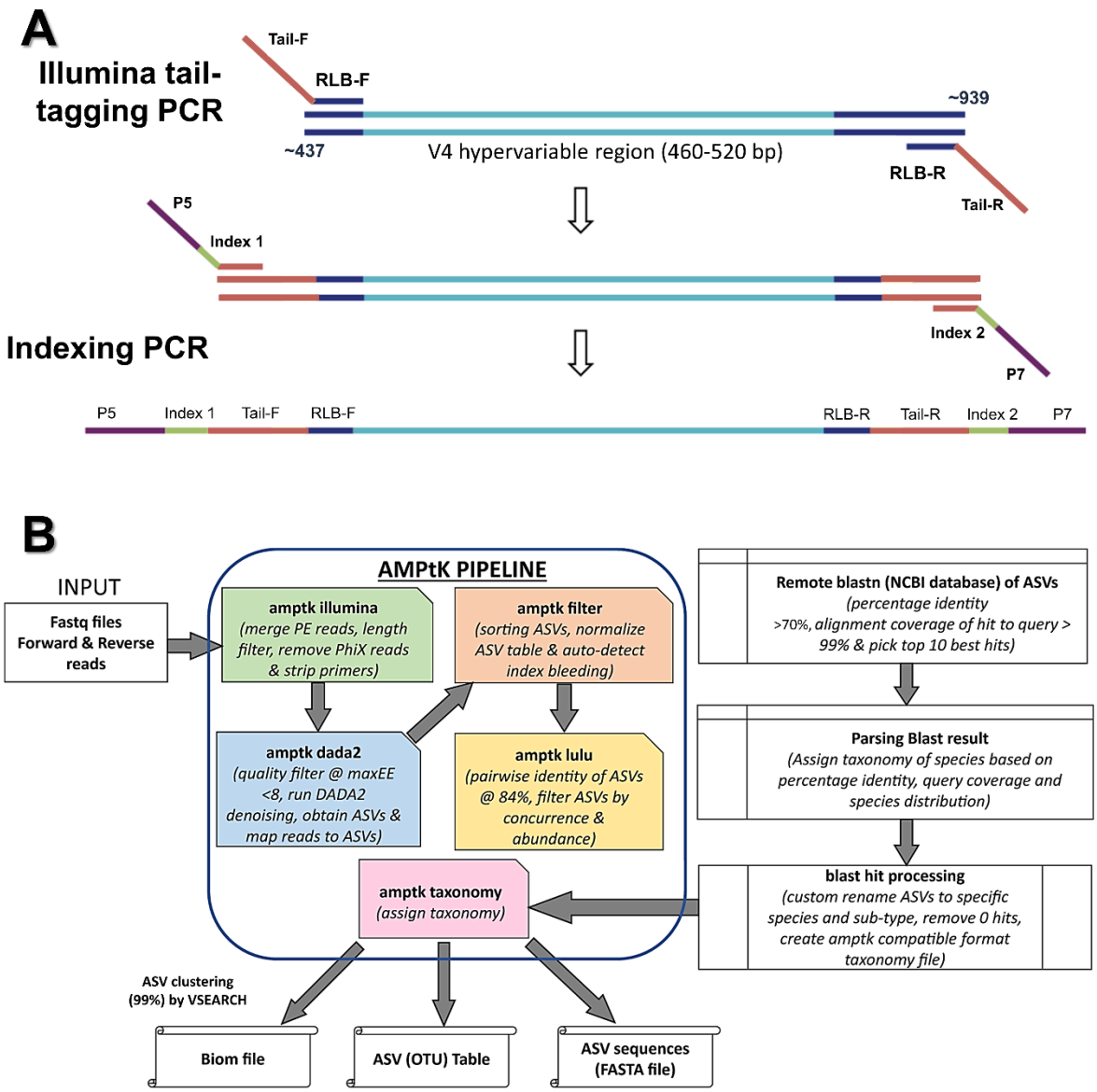

Fig. 15. Amplicon tagging and bioinformatics pipeline used in the study. A.) Schematic diagram of the two-step PCR assay for Illumina-tail tagging and multiplexing of samples. Adapted and modified from (Avramenko et al., 2015). B.) The AMPtk pipeline is employed to produce the ASVs in this study. Adapted and modified from (Gaithuma et al., 2019; Squarre et al., 2020).

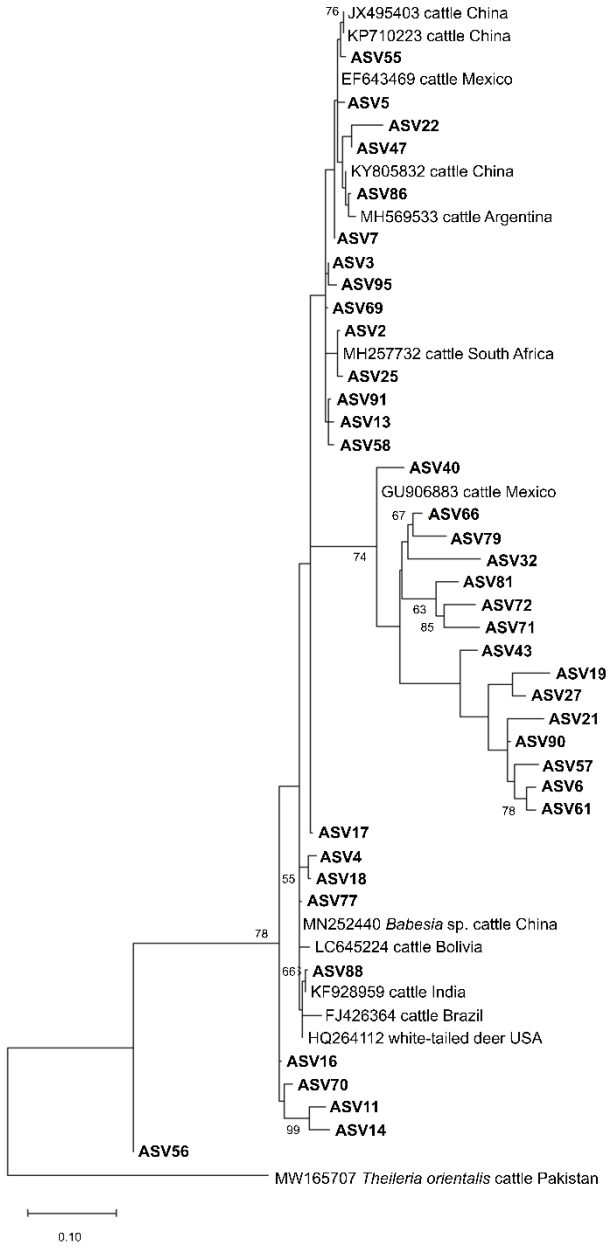

Fig. 16. Phylogenetic tree of *B. bovis* and *Babesia* sp. ASVs. The tree was generated using the maximum likelihood method and Kimura-2 parameter model, with a discrete Gamma distribution (4 categories (+G, parameter = 0.4708)) and a rate variation model for evolutionary invariability ([+I], 33.57% sites). The phylogeny inference was conducted using 1,000 bootstraps. ASVs from the current study are shown in boldface. *Theileria orientalis* was designated the outgroup.

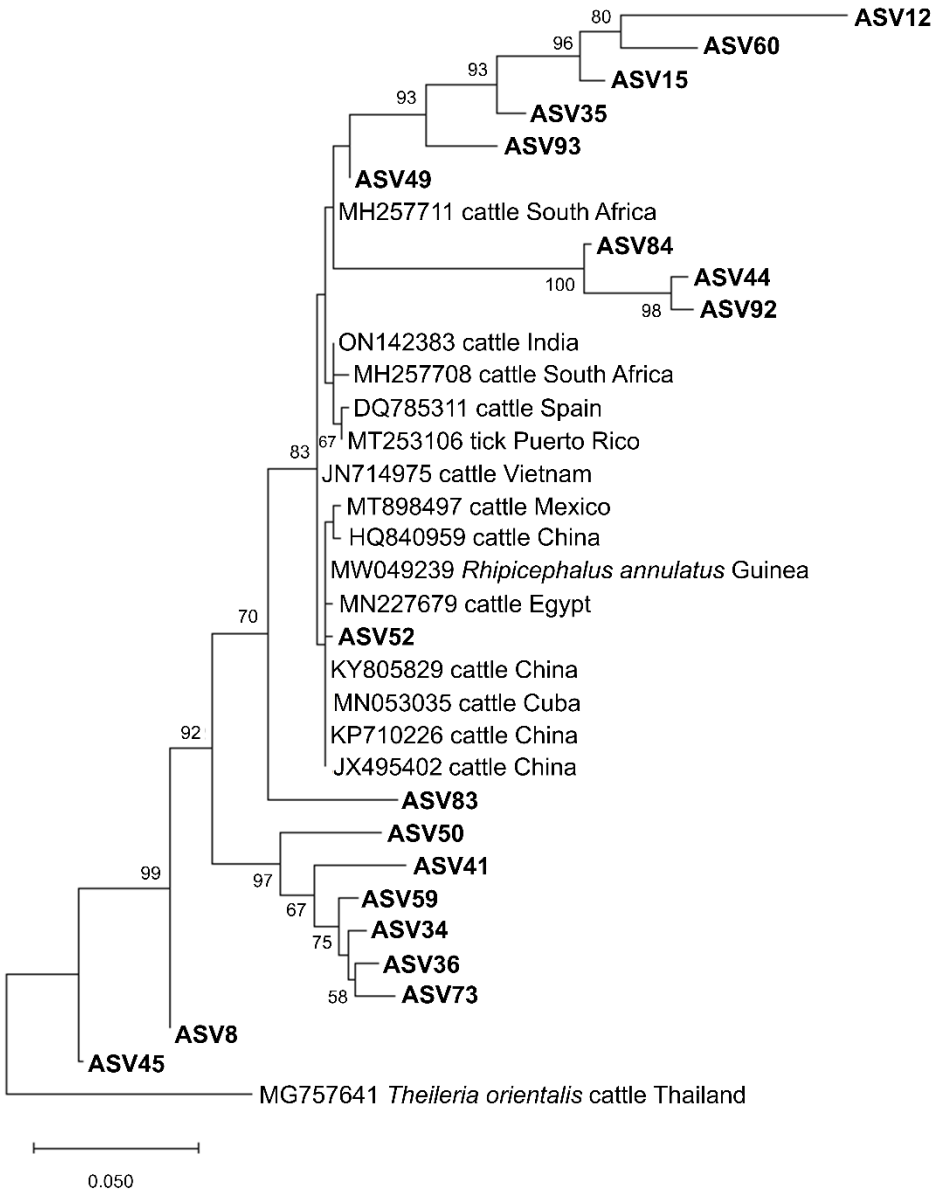

Fig. 17. Phylogenetic tree of *B. bigemina* and *Babesia* sp. ASVs. The tree was generated using the maximum likelihood method and Tamura-3 parameter model, with a discrete Gamma distribution (4 categories (+G, parameter = 0.4859)). The phylogeny inference was conducted using 1,000 bootstraps. ASVs from the current study are shown in boldface. *Theileria orientalis* was designated the outgroup.

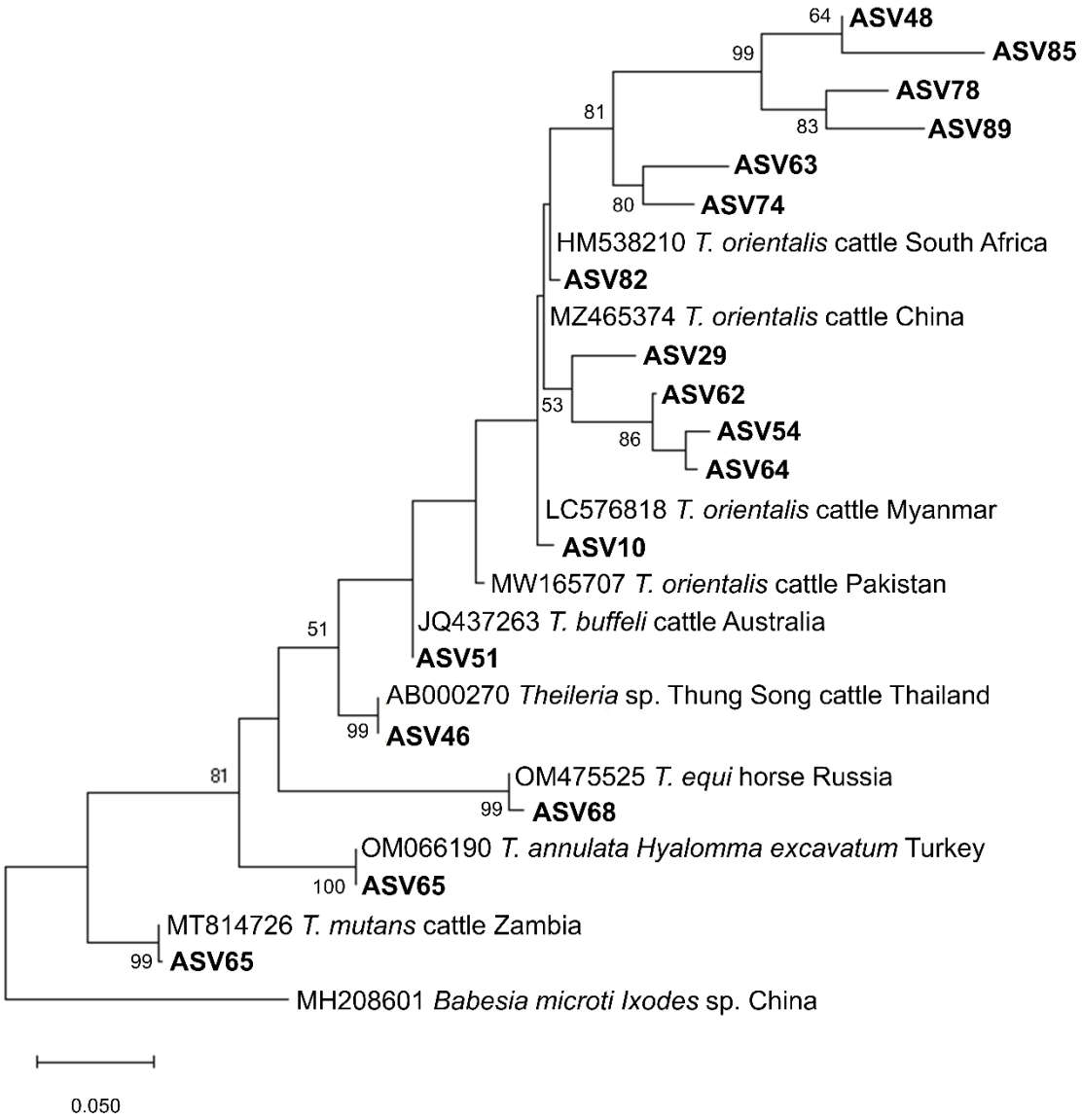

Fig. 18. Phylogenetic tree of *Theileira* ASVs. The tree was generated using the maximum likelihood method and Tamura-3 parameter model, with a discrete Gamma distribution (4 categories (+G, parameter = 0.3974)). The phylogeny inference was conducted using 1,000 bootstraps. ASVs from the current study are shown in boldface. *Babesia microti* was designated the outgroup.

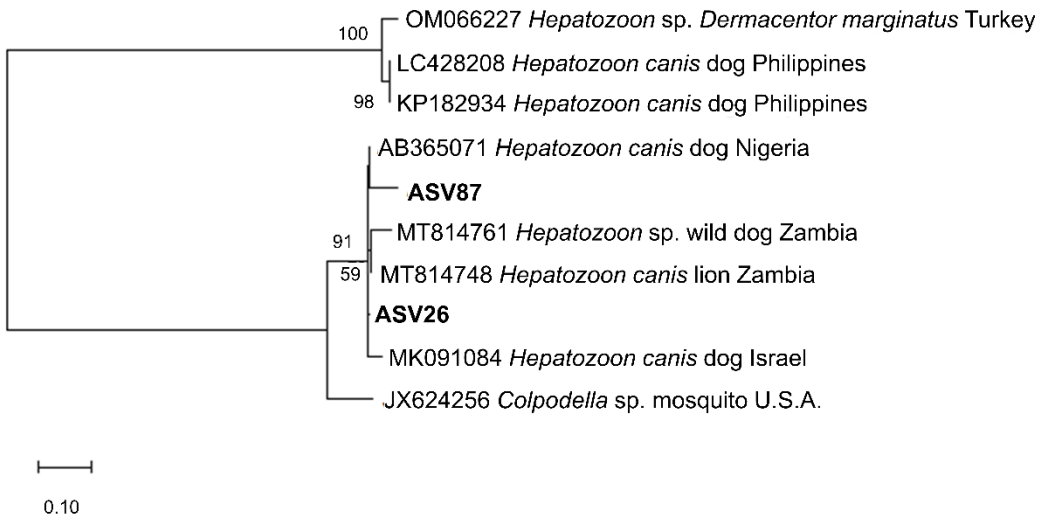

Fig. 19. Phylogenetic tree of *Hepatozoon* ASVs. The tree was generated using the maximum likelihood method and Tamura-3 parameter model, with a discrete Gamma distribution (4 categories (+G, parameter = 0.6804)) and a rate variation model for evolutionary invariability ([+I], 36.04% sites). The phylogeny inference was conducted using 1,000 bootstraps. ASVs from the current study are shown in boldface. *Colpodella* sp. was designated the outgroup.

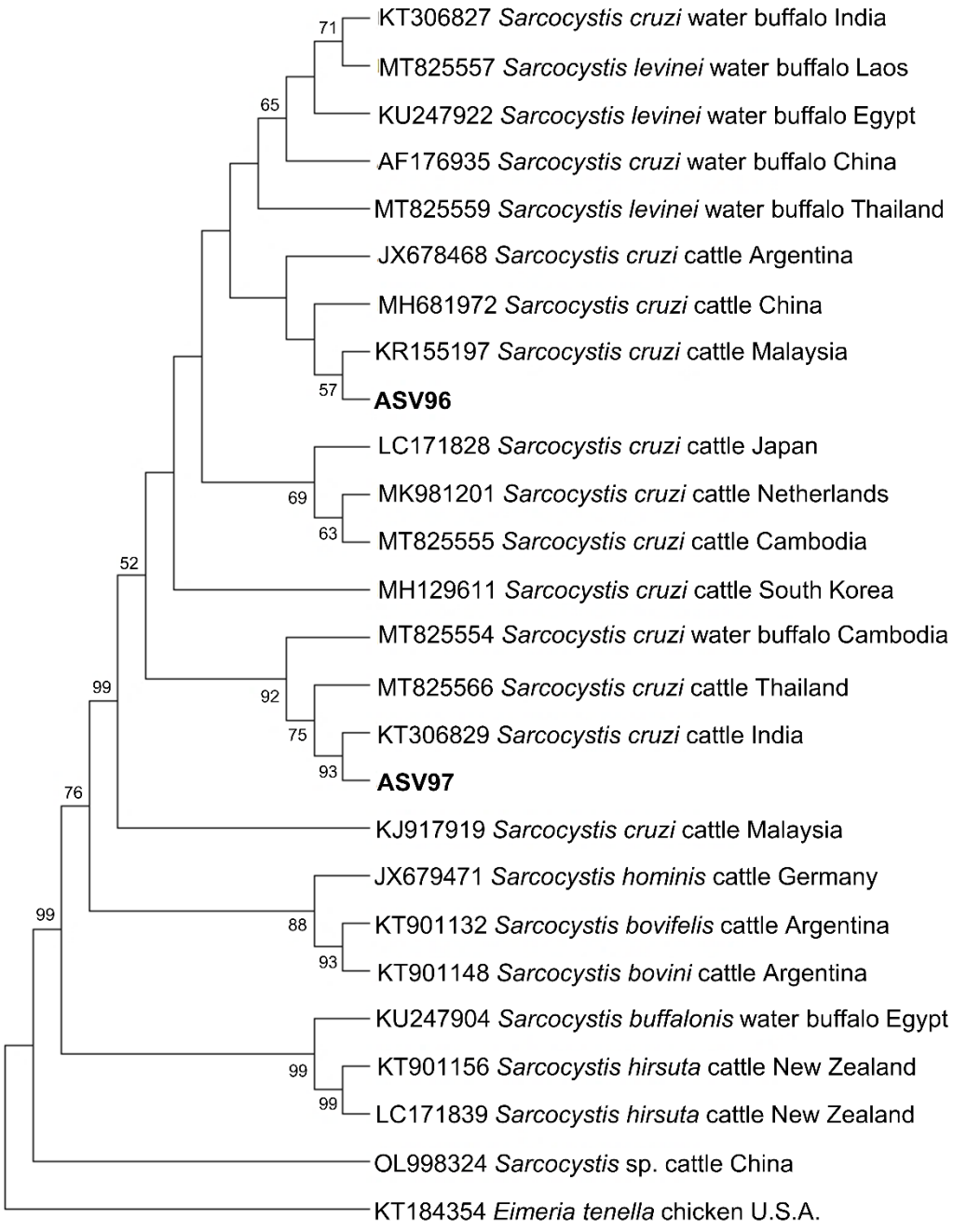

Fig. 20. Phylogenetic tree of *Sarcocystis* ASVs. The tree was generated using the maximum likelihood method and Tamura-3 parameter model, with a discrete Gamma distribution (4 categories (+G, parameter = 1.0235)). The phylogeny inference was conducted using 1,000 bootstraps. ASVs from the current study are shown in boldface. *Eimeria tenella* was designated the outgroup.

Table 8. List of PCR primers for amplicon sequencing of piroplasma.

Description	Primer name	Primer sequence (5'–3')	Legend	References
V4 hypervariable region of the 18S rRNA gene of piroplasma	RLB-F	GAGGTAGTGACAAGAAATAACAATA	{RLB-F}	(Gubbels et al., 1999)
	RLB-R	TCTTCGATCCCCTAACTTTC	{RLB-R}	
RLB primers with Illumina tails	Illumina RLB-F	ACACTCTTTCCCTACACGACGCTCTTCCGATCT{RLB-F}	{IlluminaF}	(Squarre et al., 2020)
	Illumina RLB-R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT{RLB-R}	{IlluminaR}	
Illumina-index primers	Illumina-P5	AATGATACGGCGACCACCGAGATCTACAC{index-D5*}{IlluminaF}		
	Illumina-P7	CAAGCAGAAGACGGCATACGAGAT{index-D7*}{IlluminaR}		

Table 9. Top hits and composition of ASVs identified in this study.

Taxonomy hit	No. of ASVs	Total sequence reads	No. of positives (n = 60)
B. bovis	37	259,704	38
B. bigemina	18	207,232	33
Babesia sp.	3	162	9
T. orientalis	13	347,535	22
T. annulata	1	459	1
T. equi	1	715	2
T. mutans	1	2,188	1
Theileria sp. Thung Song	1	1,100	6
H. canis	2	490	1
S. cruzi	2	818	2
Total	**79**	**820,403**	

Table 10. Infection types detected in cattle.

Taxonomy hit	No. of samples
Single infection	23
B. bovis (*Bbo*)	8
B. bigemina (*Bbi*)	4
T. orientalis (*Tor*)	7
T. annulata	1
T. equi	2
T. mutans	1
Dual infection	25
Bbo and *Bbi*	15
Bbi and *Tor*	4
Tor and *Theileria* sp.	3
Bbo and *S. cruzi*	1
Bbo and *H. canis*	1
Bbo and *Babesia* sp.	1
Triple infection	7
Bbo, *Bbi*, and *Tor*	2
Bbo, *Bbi*, and *Babesia* sp.	2
Bbo, *Bbi*, and *Theileria* sp.	1
Bbo, *Bbi*, and *S. cruzi*	1
Bbo, *Babesia* sp., and *Tor*	1
Quadruple infection	4
Bbo, *Bbi*, *Babesia* sp., and *Tor*	3
Bbo, *Babesia* sp., *Tor*, and *Theileria* sp.	1
Quintuple infection	1
Bbo, *Bbi*, *Babesia* sp., *Tor*, and *Theileria* sp.	1

General discussion

Hemoparasites carried by ticks are a worldwide burden to livestock farming, and thus, require ample attention. Despite its enormous impact on the financial aspect of livestock raising, these diseases are virtually unknown to farmers in endemic countries. Thus, the field of TBDs has received little attention in the Philippines and is often neglected. Consequently, the current status of its epidemiology is inadequate and lacking; hence, impeding control interventions for such diseases. Traditional methods of diagnosing tick-borne infections involve time-consuming and complicated methods, such as microscopy and immunological tools such as serological assays (Mosqueda et al., 2012; Mans et al., 2015), whereas molecular diagnostics provide a more accurate diagnosis in a timely manner, minimizing losses incurred by TBDs (Garcia et al., 2022). Therefore, three epidemiological investigations utilizing molecular tools were performed to assess the TBD status of various livestock animals in the Philippines.

In Chapter 1 of this dissertation, the molecular presence of TBPs and the risk factors for TBDs in racehorses raised in a race park in Cavite, Philippines were evaluated. As one of the most serious TBDs, surveillance for EP is imperative, especially in highly valued animals such as racehorses. The presence of *B. caballi* and *T. equi* was demonstrated in 12.10% and 0.81% of the 124 tested horse blood samples, respectively, confirming the findings of previous studies on the possible endemicity of EP agents in the Philippines (Cruz-Flores et al., 2010; Ybañez et al., 2018b). The horses that tested positive for EP did not display any clinical signs of EP, a hallmark of persistent infections (Scoles and Ueti, 2015), and are commonly observed in endemic countries (Tirosh-Levy et al., 2020). However, as carriers of EP, transmission risk is still a concern as ticks are capable of disseminating the parasites to unexposed horses (Ueti et al., 2008) and relapse can still

occur, especially in fatigued and immunosuppressed horses (Hailat et al., 1997). In this investigation, sex was found to be a significant risk factor for *B. caballi* infection, as supported by a higher positivity (18.37%) and higher odds (5.77) for infection in female horses, which contradicts the findings of Qablan et al. (2013). However, prior studies that looked at sex as a risk factor for EP have produced contradictory and conflicting findings (Grandi et al., 2011; Onyiche et al., 2019). Moreover, genotyping of the parasites revealed that *T. equi* genotype E and *B. caballi* genotype A populations were present in Cavite racehorses.

In addition, *A. phagocytophilum* and *A. ovis* variants were molecularly observed in racehorses, the first report in the Philippines. In horses, the most significant *Anaplasma* species is *A. phagocytophilum*, the causative agent of EGA (Dzięgiel et al., 2013; Saleem et al., 2018). Through sequencing analysis, I detected *A. phagocytophilum*-like species, the clinical impact of which is currently unknown. Due to *A. phagocytophilum*'s wide variety of hosts (Atif, 2015), it has evolved several strains and variations that have vastly different disease-causing potentials (Woldehiwet, 2010). Similarly, the discovery of *A. ovis*-like variants in horses in this study could be explained by cross-species transmission, as goats are raised in the same horse park. Whether a host expansion scenario is occurring with *A. ovis*, whose natural hosts are goats, and whether its clinical effects are same in horses, should be further verified.

To the best of my knowledge, there has never been a verified case of *Borrelia* in the Philippines before, and Bbsl detection in the present survey raises the possibility of equine subclinical infections. Specifically, species closely related to human-infective *Bo. japonica* and *Bo. garinii* were detected. Horses can serve as a possible reservoir of human borreliosis (Marcelis et al., 1987; Bae, 2018). The discovery of zoonotic Bbsl in horses necessitates a more thorough investigation of the potential of the disease spreading to

people who come in touch with the affected animals. Based on the findings in Chapter 1, a treatment regimen for *B. caballi*, *T. equi*, *Anaplasma*, Bbsl, and *C. burnetii*-positive racehorses is proposed and regular molecular surveillance is strongly suggested to monitor TBDs and prevent future outbreaks in racing equines.

Although goats are the most populous ruminants in the Philippines, caprine TBDs have never been molecularly investigated. Hence, in Chapter 2, I set out to explore the TBPs in Philippine goats and found widespread infections in six provinces. Goats had a positivity rate of 77.02% for piroplasma (1.52% *B. ovis*) and 38.64% for *Anaplasma* spp., which were higher than the previously documented detection rates of caprine *Babesia* and *Theileria* (Torina and Caracappa, 2012; Iqbal et al., 2013; Gebrekidan et al., 2014; Ozubek and Aktas, 2017a; Wang et al., 2019; Nasreen et al., 2020; Yang et al., 2022b) and *Anaplasma* (Miranda et al., 2021; Niaz et al., 2021; Aung et al., 2022; Rahman et al., 2022). Varied detection rates among provinces were also observed, which may be explained by certain factors related to the environment, host, vector, and raising practices (Torina and Caracappa, 2012). In addition, more female goats were piroplasma positive and *Anaplasma* was more likely to be detected in young goats than in adults, consistent with a previous finding (Niaz et al., 2021). Additionally, there was a strong correlation between the identification of *Anaplasma* and *Babesia*/*Theileria* and goat breeds. This may be due to the higher resistance of indigenous goat breeds to piroplasma (Rjeibi et al., 2014; Islam et al., 2021) and *Anaplasma* (Said et al., 2015) and the increased vulnerability of exotic breeds to tick infestations (Schwalback et al., 2003).

Species identified through sequencing of representative samples include *T. orientalis*, *T. annulata*, *Theileria* sp. Thung Song, *A. odocoilei*-like, *A. phagocytophilum*-like, and *A. platys*-like. Various *Theileria* species, mostly under the *T. orientalis* complex, have been documented in cattle from the Philippines (Belotindos et al., 2014; Ochirkhuu et

al., 2015; Prado et al., 2022), while this is the first molecular report of *T. annulata* in the country. Known to be fatal to bovines, the present detection of *T. annulata* is highly relevant and implicates goats in the transmission of this significant species in the field. The ability of the infected goats to disseminate the parasites to other bovids, as is the case in sheep, and act as covert carriers of *Theileria* species should be studied further (Brown et al., 1998; Khukhuu et al., 2011; Lawrence et al., 2021). The clinical impact of the possibly novel *Anaplasma* variants obtained in this study also requires further investigation, especially for variants whose goats are non-conventional hosts.

As Chapters 1 and 2 have depicted the usefulness of the typical molecular methods for TBP detection, I aimed to utilize a relatively new molecular tool to unveil the bovine piroplasma populations in the Philippines in Chapter 3. The detection of 79 ASVs, which belong to genera *Babesia*, *Theileria, Hepatozoon,* and *Sarcocystis*, exhibited the magnitude of parasite diversity, mainly of piroplasma species, providing new details on the apicomplexan populations in Philippine cattle. *B. bovis*, *B. bigemina,* and *T. orientalis* were the most frequent species in this study, and coinfection with the *B. bovis* and *B. bigemina* was the most common. Phylogenetic analysis denoted possible novel or cryptic parasite variants (Ghafar et al., 2020). The presence of these parasites may indicate the high endemicity of bovine babesiosis and theileriosis in the sampled areas and necessitates an action plan for the control of cattle piroplasmosis in the Philippines. Noteworthy, *T. mutans, T. equi*, *H. canis,* and *S. cruzi* were also identified in the present study. More importantly, *T. annulata* was demonstrated in cattle for the first time, an extremely important finding, alongside its detection in goats in Chapter 2. *Theileria annulata* infections incur massive economic and veterinary problems in bovine herds (Bishop et al., 2004). Thus, monitoring of *T. annulata* infection is required as it could have disastrous effects in the event of an outbreak. Overall, these findings demonstrated that the Ampliseq technique is applicable

in characterizing the bovine piroplasma species diversity in the field. For this reason, its application as a diagnostic tool for TBDs of other livestock animals and companion animals is eagerly anticipated.

General summary

Babesiosis, theileriosis, and anaplasmosis, alongside other TBDs, are significant parasitic diseases that inflict adverse health effects on livestock animals. This impact directly translates to million worth of financial losses for livestock raisers globally. The use of molecular tools to uncover the epidemiology of TBDs has been instrumental in their prevention and control. Considering the economic impact, elucidating the presence of tick-borne infections in livestock using appropriate diagnostic tools is vital not only in improving herd health but also in formulating and implementing countermeasures, which are expected to help farmers to recuperate the losses from TBDs. In this dissertation, I was able to elucidate the major TBPs of horses (Chapter 1), goats (Chapter 2), and cattle (Chapter 3) in the Philippines through the application of molecular diagnostic tools.

In Chapter 1, the presence of equine tick-borne infections in a racehorse park from Cavite, Philippines, was evaluated. A total of 124 thoroughbred horses specifically raised for racing were sampled. *Babesia caballi* (12.10%; 15/124), *Theileria equi* (0.81%; 1/124), *Anaplasma phagocytophilum* (10.48%;13/124), *Borrelia burgdorferi* sensu lato (Bbsl) (38.71%; 48/124), *A. marginale* (0.81%; 1/124), and *Coxiella burnetii* (0.81%; 1/124) were detected in racehorses. Of the 60 positive samples, 42 were single infections and 18 were multiple infections, the most frequent of which are coinfection with Bbsl and *B. caballi*. Sex ($p = 0.026$) was found to be a significant risk factor for *B. caballi* infection, with female horses 5.77 times more likely to be infected with *B. caballi*. Sequencing analysis revealed that seven partial 18S rRNA *B. caballi* isolates shared 98.63–100% identity and were classified as genotype A, whereas the single *T. equi* sequence had 99.77% identity with GenBank isolates and was confirmed as genotype E. Eight *Anaplasma* 16S rRNA partial sequences were highly identical to *A. phagocytophilum* and *A. ovis*, while partial sequences

of *Borrelia* 5–23S rRNA were most closely related to *Bo. japonica* and *Bo. garinii*-like isolates. The first molecular detection of *Borrelia* and *Anaplasma* is reported in this study, as well as the first genotyping of *B. caballi* and *T. equi* in racehorses in the Philippines.

In Chapter 2, a total of 396 goat samples were collected from six Philippine provinces and were molecularly screened for the presence of *Babesia/Theileria* and *Anaplasma*. A detection rate of 77.02% (305/396) and 38.64% (153/396) were noted for the respective TBPs. Six samples were positive for *B. ovis* (1.52%). Sex and age group were associated with higher *Babesia/Theileria* and *Anaplasma* detection rates, respectively, while significantly higher rates for both pathogens were observed in exotic goat breeds. The representative *Babesia/Theileria* sequences shared 89.97–97.74% identity and were most closely related to *T. orientalis*, *T. annulata*, and *Theileria* sp. On the other hand, *Anaplasma* 16S rRNA sequences were related to *A. odocoilei*, *A. platys*, and *A. phagocytophilum*. This is the first molecular identification of *B. ovis*, *Theileria* spp., and *Anaplasma* spp. in goats from the Philippines.

In Chapter 3, a new molecular tool was evaluated for the characterization of piroplasma species diversity in bovines. Of 162, 58.64% tested positive for piroplasma using a conventional RLB-PCR assay that targets the V4 hypervariable region of the 18S rRNA gene. The positive cattle samples were from Bohol (83.33%; 20/24), Cavite (70.97%; 44/62), and Cebu (40.79%; 31/76). By leveraging the AMPtk pipeline, the merged reads generated a total of 2,179 ASVs. The BLAST non-redundant database assigned the taxonomy of 175 ASVs, which were then dereplicated into 97 ASVs. Further filtering yielded a final count of 79 distinct ASVs. The taxonomy hits of the 79 ASVs corresponding to 10 species were *B. bovis* (n = 37), *B. bigemina* (n = 18), *T. orientalis* (n = 13), *Babesia* sp. (n = 3), *Hepatozoon canis* (n = 2), *Sarcocystis cruzi* (n = 2), *T. annulata* (n = 1), *T. equi* (n = 1), *T. mutans* (n = 1), and *Theileria* sp. Thung Song (n = 1). Based on

the phylogenetic analysis, ASVs assigned to *B. bovis* and *Babesia* sp. showed three major subclades and were 90.76–100% identical with *B. bovis* isolates deposited in GenBank. On the other hand, the evolutionary inference suggested that most of the *B. bigemina* and *Babesia* sp. ASVs were phylogenetically distinct from previously reported isolates, as evidenced by the strong statistical support, and shared 90.23–99.77% identity with isolates from the database. Thirteen *T. orientalis* ASVs (91.72–100% identical to other *T. orientalis* isolates) clustered in the *T. orientalis* complex subclade, 4 of which formed a subgroup with high nodal support. As anticipated, ASVs taxonomically recognized as *T. annulata*, *T. equi*, and *T. mutans* were separated into groups based on the species to which they belong. Furthermore, the *Hepatozoon* ASVs were distinctly divergent from previously detected Philippine canine *H. canis* isolates, while phylogenetic analysis revealed that *Sarcocystis* ASVs were closely related to bovine *S. cruzi* isolates from Malaysia and India.

The fundamental purpose of this research was to map out the different TBPs infecting economically important livestock in the Philippines. The presented findings from the three chapters of this dissertation attest to the attainment of this purpose. The circulating TBP populations and the extent of tick-borne infections in horses, goats, and cattle in the Philippines were revealed through the use of molecular detection tools, proving the applicability of these platforms in identifying tick-borne infections in different livestock. These results are vital in ascertaining the TBD status in the Philippines, where infections are widely present but often overlooked and neglected. The findings from these studies shall be beneficial in crafting and implementing livestock tick and TBD control and prevention programs in the country.

和文要約

バベシア症、タイレリア症、アナプラズマ症は、他のマダニ媒介感染症（TBD）と並んで、家畜の健康に悪影響を与える重要な寄生虫病である。こんら影響は、世界中の畜産農家が被る甚大な経済的損失に直結する。このような経済的損失を低減するために、適切な分子診断手法を用いて家畜のマダニ媒介性感染症の流行実態の一端を明らかにすることは、家畜の健康状態を改善するだけでなく、対策の立案・実施に不可欠である。そこで、本研究ではフィリピンにおけるウマ（第 1 章）、ヤギ（第 2 章）、およびウシ（第 3 章）の主要なマダニ媒介性病原体（TBP）の分子疫学調査を実施した。

第 1 章では、フィリピンカビテ州の競走馬牧場におけるマダニ媒介性感染症について調べた。サラブレッド馬 124 頭から血液サンプルを採集し、各種寄生虫の核酸検出を行ったところ、*Babesia caballi*（12.10％；15/124）、*Theileria equi*（0.81％；1/124）、*Anaplasma phagocytophilum*（10.48％；13/124）、*Borrelia burgdorferi* sensu lato（Bbsl）（38.71％；48/124）、*A. marginale*（0.81％；1/124）、*Coxiella burnetii*（0.81％；1/124)が検出された。陽性 60 検体のうち、42 検体が単独感染，18 検体が混合感染であり、Bbsl と *B. caballi* との重複感染が最も多かった。*B. caballi* と *T. equi* について、遺伝子型を調べたところ、それぞれ A 型と E 型に分類された。これらの結果のなかで、*Borrelia* と *Anaplasma* の検出と *B. caballi* と *T. equi* の遺伝子型の特定は、いずれもフィリピンのウマにおいては初の報告である。

第 2 章では、フィリピン 6 州から計 396 頭のヤギ血液サンプルを採集し、マダニ媒介性病原体の核酸検査を行った。*Babesia*/*Theileria* 属および *Anaplasma* 属の病原体の検出率はそれぞれ 77.02%（305/396）、38.64%（153/396）であった。*Babesia*/*Theileria* 属および *Anaplasma* 属の検出率は、それぞれ外来種と高年齢層で高かった。*Babesia*/*Theileria* の代表的な塩基配列は 89.97-97.74%の同一性を持ち、*T. orientalis*、*T. annulata*、*Theileria* sp.に最も近縁であった。*B. ovis* は 6 検体（1.52%）で陽性であった。一方、*Anaplasma* 16S rRNA 塩基配列は *A. odocoilei*、*A. platys*、*A. phagocytophilum* に近縁であった。これらの結果のなかで、*B. ovis*、 *Theileria* spp.および *Anaplasma* spp.は、フィリピンのヤギにおける初の知見である。

第 3 章では、ウシにおけるピロプラズマの種多様性を特徴付けるための新しい分子ツールを評価した。18S rRNA 遺伝子の V4 超可変領域を標的とした従来の RLB-PCR 法で 162 頭中 58.64%がピロプラズマ（*Babesia* 属/*Theileria* 属）陽性と判定された。陽性ウシは、ボホール（ 83.33%; 20/24）、カビテ（ 70.97%; 44/62）、セブ（ 40.79%; 31/76）由来であった。AMPtk パイプラインを用いると、マージされたリードから合計 2,179 個の ASV が生成された。BLAST non-redundant データベースにより 175 個の ASV が分類され、さらに 97 個の ASV に分割された。フィルタリングを行った結果、最終的に 79 個の異なる ASV を数えることができた。79 個の ASV のうち 10 種に対応する分類のヒットは、*B. bovis*（n = 37）、 *B. bigemina*（n = 18）、 *T. orientalis*（n = 13）、 *Babesia* sp.（n = 3）、*Hepatozoon canis*（n = 2）、*Sarcocystis cruzi*（n = 2）、*T. annulata*（n = 1）、*T. equi*（n = 1）、*T. mutans*（n = 1）および *Theileria* sp. Thung

Song（n = 1）であった。 系統解析の結果、*B. bovis* および *Babesia* sp. に割り当てられた ASV は 3 つの主要なサブクレードを示し、GenBank に登録されている *B. bovis* 分離株と 90.76～100％の同一性を有していた。 一方、進化推論では、*B. bigemina* と *Babesia* sp. の ASV の多くは、既報告分離株とは系統的に異なることが示唆された。 13 種の *T. orientalis* ASV（他の *T. orientalis* 分離株と 91.72～100%同一）は *T. orientalis* complex サブクレードにクラスター化し、そのうち 4 種は高いノードサポートを持つサブグループを形成していた。予想通り、分類学的に *T. annulata*、*T. equi*、*T. mutans* と認識されている ASV は、その属する種に基づきグループに分けられた。さらに、*Hepatozoon* ASVs は、これまでに検出されたフィリピン由来イヌの *H. canis* 分離株とは明確に分岐しており、*Sarcocystis* ASVs はマレーシアおよびインド産由来の *S. cruzi* 分離株に近縁であることが明らかとなった。

本研究の総括的な目的は、フィリピンにおける経済的に重要な家畜に感染している様々なマダニ媒介性病原体を特定することであった。特に第 3 章で得られた知見は、この目的の達成を証明するものである。フィリピンのウマ、ヤギ、およびウシにおけるマダニ媒介性病原体の流行と分布が分子検出ツールによって明らかになり、異なる家畜におけるマダニ媒介性感染症の特定にこれらのプラットフォームが適用可能であることが証明された。これらの結果はフィリピンの家畜でこれまでに見過ごされがちであったマダニ媒介感染症の状況を把握する上で極めて重要である。これらの研究結果は、今後フィリピンにおける家畜のマダニ媒介性感染症の管理・予防プログラムの策定・実施に有益なものとなるであろう。

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Felix, qui potuit rerum cognoscere causas
Fortunate, who was able to know the causes of things
- Virgil

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