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Original article

First detection and genetic identification of *Hepatozoon canis* in *Rhipicephalus sanguineus* sensu lato ticks collected from dogs of Taiwan

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ABSTRACT

We determined the prevalence of infection and genetic identity of *Hepatozoon* spp. harbored by *Rhipicephalus sanguineus* sensu lato ticks in Taiwan. A total of 1082 ticks were collected from dogs and DNA extraction was performed from individual tick specimens. *Hepatozoon* infection was detected by performing a nested-PCR assay based on the 18S small subunit ribosomal RNA (ssrRNA) gene. The genetic identity of detected *Hepatozoon* was identified by gene sequencing and phylogenetic analysis. *Hepatozoon* infection was detected in nymphs, males and females of *R. sanguineus* s. l. ticks with an infection rate of 20.8%, 22.8% and 29.4%, respectively. Sequence and phylogenetic analysis revealed that these *Hepatozoon* spp. of Taiwan were genetically affiliated to the same clade within the genospecies of *H. canis* and can be discriminated from other genospecies of *H. americanum* and *H. felis*. Intraspecies analysis based on the genetic distance (GD) values indicates a lower level (GD < 0.005) genetic divergence within the same genospecies of *H. canis* detected in Taiwan, Brazil and Spain. Interspecies analysis also reveals a higher heterogeneity of Taiwan strains distinguished from other genospecies of *H. felis* (GD > 0.040) and *H. americanum* (GD > 0.056). This study provides the first molecular evidence of *H. canis* detected and identified in various stages of *R. sanguineus* s. l. ticks in Taiwan. Detection of *H. canis* in unfed male ticks may imply the possible mechanism of transstadial survival in *R. sanguineus* s. l. ticks. Further investigations on *Hepatozoon* spp. harbored by various vector ticks in Taiwan may illustrate the epidemiological significance of this parasite.

1. Introduction

Hepatozoonosis is a common tick-borne protozoan disease of a wide range of vertebrate hosts (Smith, 1996). *Hepatozoon canis* was firstly described in India as the agent of canine hepatozoonosis (James, 1905), and is commonly found associated with chronic infection of hepatozoonosis in dogs from Europe, Asia, Africa and South America (McCully et al., 1975; Rajamanickam et al., 1985; Murata et al., 1991; Baneth et al., 1995; Oyamada et al., 2005; Karagenc et al., 2006; Jittapalapong et al., 2006; Forlano et al., 2007; Tsachev et al., 2008; Rubini et al., 2008; Cadoso et al., 2010). However, *H. americanum* has been identified as the causative agent for an emerging lethal infection affecting dogs in North America (Vincent-Johnson et al., 1997; Baneth et al., 2003). Although *H. canis* has been incriminated as the agent for canine hepatozoonosis, there has been no clinical case among dogs in Taiwan and no tick vector has been reported in Taiwan.

Rhipicephalus sanguineus s. l. is the most widespread tick species around the world and is recognized as the dominant ectoparasite on dogs that can occasionally parasitize other hosts, including humans (Dantas-Torres, 2010). This tick species has been recognized as the main vector for maintaining the infection of *H. canis* between the invertebrate host (ticks) and vertebrate host (dogs) (Baneth et al., 2007; Demoner et al., 2013; Giannelli et al., 2013; Aktas and Ozubek, 2017). Based on the morphological and genetic analysis, the taxonomic status of *R. sanguineus* s. l. can be discriminated at least two distinct lineages around the world (Dantas-Torres et al., 2013, 2018; Nava et al., 2018). In addition to *R. sanguineus* s. l., several other tick species have also been recognized as vectors of *H. canis*. Indeed, *Amblyomma ovale* is known as the vector for *H. canis* in Brazil (Forlano et al., 2005; Rubini et al., 2009) as well as the *Haemaphysalis* spp. for *H. canis* in Japan (Murata et al., 1995). Our previous studies indicate that *R. sanguineus* s. l. is the most abundant tick species that infests dogs in Taiwan and the *Babesia* infections were detected in these ticks (Chao et al., 2016, 2017a). However, the vector competence of *R. sanguineus* s. l. for

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Hepatozoon protozoa from Taiwan has never been demonstrated.

Due to the advances of molecular methodologies, molecular assays by PCR and sequence analysis have made possible the detection and phylogenetic analysis of *Hepatozoon* infection in canine hosts (Baneth et al., 2000; Inokuma et al., 2002; Oyamada et al., 2005; Karagenc et al., 2006; Criado-Fornelio et al., 2007; Rubini et al., 2008) and in vector ticks (Murata et al., 1995; Forlano et al., 2005; Baneth et al., 2007; Rubini et al., 2009; Demoner et al., 2013; Giannelli et al., 2013; Aktas and Ozubek, 2017). Their sensitivity and specificity have contributed to the detection of *Hepatozoon* infection and the genetic differentiation of *Hepatozoon* species. Thus, the objectives of the present study were to detect the *Hepatozoon* infection and to clarify the genospecies of *Hepatozoon* protozoa in *R. sanguineus* s. l. ticks collected from dogs of Taiwan by analyzing the sequence similarity of the PCR-amplified 18S ssrRNA genes. In addition, the phylogenetic relationships of these *Hepatozoon* spp. detected in Taiwan were compared with other *Hepatozoon* species identified from various biological and geographical sources which have been documented in GenBank.

2. Materials and methods

2.1. Tick collection and species identification

All specimens of adult and nymphal ticks of *R. sanguineus* s. l. were carefully removed from dogs captured at various localities of Taipei City in northern Taiwan (25°09′N, 121°53′E; 25°09′N, 121°51′E; 25°11′N, 121°55′E; 25°07′N, 121°54′E; 25°07′N, 121°56′E; 25°05′N, 121°50′E; 25°05′N, 121°51′E; 25°02′N, 121°50′E; 25°01′N, 121°49′E; 25°03′N, 121°48′E; 25°03′N, 121°53′E; 25°14′N, 121°51′E; 25°13′N, 121°50′E; 25°03′N, 121°62′E; 25°08′N, 121°60′E; 25°07′N, 121°56′E; 24°98′N, 121°57′E). A total of 1082 ticks (177 partially engorged females, 578 males and 327 partially engorged nymphs) were collected from 138 stray dogs, and all these ticks were subsequently stored in separate mesh-covered and plaster-bottomed vials. All tick specimens of *R. sanguineus* s. l. were identified according to their morphological characteristics, as described previously (Chao and Shih, 2012; Chao et al., 2017b). Briefly, tick specimens were cleaned by sonication in 75% ethanol solution for 5–10 min and then washed twice in sterile distilled water. Afterwards, each stage of tick specimen was placed on a glass slide and photographed using a stereo-microscope (SMZ 1500, Nikon, Tokyo, Japan) equipped with a fiber lamp. The external features of the *R. sanguineus* s. l. ticks were recorded for species identification.

2.2. Extraction of DNA from tick specimen

Total genomic DNA was extracted from individual tick specimen used in this study. Briefly, tick specimens were sonicated for 3–5 min in 75% ethanol solution and then washed twice in sterile distilled water. Afterwards, the individual tick specimen dissected into pieces was placed in a microcentrifuge tube filled with 180-μL lysing buffer solution supplied with a DNeasy Tissue Kit (catalogue no. 69506, Qiagen, Taipei, Taiwan) and then homogenized with a TissueLyser II (catalogue no. 85300, Qiagen, Germany), as instructed by the manufacturer. The homogenate was centrifuged at room temperature and the supernatant fluid was further processed by a DNeasy Tissue Kit, as instructed by the manufacturer. After filtration, the filtrated solution was collected and the DNA concentration was determined spectrophotometrically with a DNA calculator (Nanovue Plus Spectrophotometer), as described previously (Chao et al., 2009).

2.3. DNA amplification by nested-polymerase chain reaction (nested-PCR)

DNA samples extracted from the tick specimens were used as a template for PCR amplification. For screening of infection, a nested PCR was performed with primers designed to amplify the specific fragment encoding the nuclear small subunit ribosomal RNA (18S ssrRNA) gene of *Hepatozoon* protozoa. A universal primer set of 2867F (5′-AACCTG GTTGATCCTGCCAG-3′) and 2868R (5′-TGATCCTTCTGCAGGTTCACC TAC-3′) was designed and applied for the primary amplification, as described previously (Mathew et al., 2000). In the nested PCR, a primer set of HepF (5′-ATACATGAGCAAATCTCAAC-3′) and HepR (5′-CTTAT TATTCCATGCTGCAG-3′) was used and expected to yield approximately a 600-bp fragment depending on the *Hepatozoon* strain (Inokuma et al., 2002). All PCR reagents and Taq polymerase were obtained and used as recommended by the supplier (Takara Shuzo Co., Ltd., Japan). Briefly, a total of 0.2 μmol of the appropriate primer set and adequate amounts of template DNA were used in each 50-μL reaction mixture. The PCR amplification was performed with a Perkin-Elmer Cetus thermocycler (GeneAmp system 9700, Applied Bioosystems, Taipei, Taiwan), and the primary amplification included 3 min denaturation at 94 °C followed by 35 cycles of the following conditions: 94 °C for 1 min, annealing at 55 °C for 2 min, and extension at 72 °C for 2 min. The nested amplification included 1 min denaturation at 94 °C followed by 35 cycles of the following conditions: denaturation at 94 °C for 1 min, annealing at 57 °C for 1 min, and extension at 72 °C for 2 min., as described previously (Inokuma et al., 2002). Thereafter, amplified DNA products were electrophoresed on 2% agarose gels in Tris-Borate-EDTA (TBE) buffer and visualized under ultraviolet (UV) light after staining with ethidium bromide. A DNA ladder (1-kb plus, catalogue no. 10787-018, Invitrogen, Taipei, Taiwan) was used as the standard marker for comparison. A negative control of distilled water was included in parallel with each amplification.

2.4. Sequence and phylogenetic analysis

After purification (QIAquick PCR Purification Kit, catalog No. 28104), sequencing reaction was performed with 25 cycles under the same conditions and same primer set (primer HepF and HepR) of nested amplification by dye-deoxy terminator reaction method using the Big Dye Terminator Cycle Sequencing Kit in an ABI Prism 377-96 DNA Sequencer (Applied Biosystems, Foster City, CA, USA). The resulting sequence was initially edited by BioEdit software (V5.3) and aligned with the CLUSTAL W software (Thompson et al., 1994). Thereafter, the aligned sequence was further analyzed by comparing with other *Hepatozoon* sequences based on the different genospecies and different geographical/biological origin of *Hepatozoon* protozoa that are available in GenBank. Phylogenetic analysis was performed by neighbour-joining (NJ) compared with maximum likelihood (ML) methods to estimate the phylogeny of the entire alignment using MEGA 6.0 software package (Tamura et al., 2013). The genetic distance values of inter- and intra-species variations of *Hepatozoon* protozoa were also analyzed by the Kimura two-parameter model (Kimura, 1980). All phylogenetic trees were constructed and performed with 1000 bootstrap replications to evaluate the reliability of the construction, as described previously (Felsenstein, 1985).

2.5. Nucleotide sequence accession numbers

The nucleotide sequences of PCR-amplified 18S ssrRNA genes from 20 *Hepatozoon* strains of Taiwan determined in this study have been registered and assigned the following GenBank accession numbers: strains 97-TP-DT11-sd02-M7 (MH595892), 97-TP-DT12-sd06-EN5 (MH595893), 97-TP-SL06-sd07-PEA (MH595894), 97-TP-SL08-sd04-PEA (MH595895), 97-TP-SL08-sd07-PEA (MH595896), 97-TP-ZS06-sd03-M1 (MH595897), 97-TP-ZS06-sd03-M4 (MH595898), 97-TP-ZS06-sd03-PEA5 (MH595899), 98-TP-DA09-sd02-M (MH595900), 98-TP-NG09-sd01-M (MH595901), 98-TP-SL11-sd01-PEA1 (MH595902), 98-TP-SL11-sd02-PEA8 (MH595903), 98-TP-SL11-sd04-PEA3 (MH595904), 98-TP-SL11-sd05-PEA2 (MH595905), 98-TP-SL11-sd06-M3 (MH595906), 98-TP-SL11-sd07-EN1 (MH595907), 98-TP-SL11-sd07-M9 (MH595908), 98-TP-SL11-sd07-PEA5 (MH595909), 98-TP-SL11-sd08-M6 (MH595910) and 98-TP-SL11-sd08-M9 (MH595911),

Table 1
Species and strains of *Hepatozoon* used for phylogenetic analysis in this study.

Species and strain	Origin of *Hepatozoon* strain		18S rRNA gene accession number[a]
	Biological	Geographic	
Taiwan strains			
97-TP-DT11-sd02-M7	Tick (*Rhipicephalus sanguineus* sensu lato), male	Taiwan	**MH595892**
97-TP-DT12-sd06-EN5	Tick (*Rhipicephalus sanguineus* s. l.), nymph	Taiwan	**MH595893**
97-TP-SL06-sd07-PEA	Tick (*Rhipicephalus sanguineus* s. l.), female	Taiwan	**MH595894**
97-TP-SL08-sd04-PEA	Tick (*Rhipicephalus sanguineus* s. l.), female	Taiwan	**MH595895**
97-TP-SL08-sd07-PEA	Tick (*Rhipicephalus sanguineus* s. l.), female	Taiwan	**MH595896**
97-TP-ZS06-sd03-M1	Tick (*Rhipicephalus sanguineus* s. l.), male	Taiwan	**MH595897**
97-TP-ZS06-sd03-M4	Tick (*Rhipicephalus sanguineus* s. l.), male	Taiwan	**MH595898**
97-TP-ZS06-sd03-PEA5	Tick (*Rhipicephalus sanguineus* s. l.), female	Taiwan	**MH595899**
98-TP-DA09-sd02-M	Tick (*Rhipicephalus sanguineus* s. l.), male	Taiwan	**MH595900**
98-TP-NG09-sd01-M	Tick (*Rhipicephalus sanguineus* s. l.), male	Taiwan	**MH595901**
98-TP-SL11-sd01-PEA1	Tick (*Rhipicephalus sanguineus* s. l.), female	Taiwan	**MH595902**
98-TP-SL11-sd02-PEA8	Tick (*Rhipicephalus sanguineus* s. l.), female	Taiwan	**MH595903**
98-TP-SL11-sd04-PEA3	Tick (*Rhipicephalus sanguineus* s. l.), female	Taiwan	**MH595904**
98-TP-SL11-sd05-PEA2	Tick (*Rhipicephalus sanguineus* s. l.), female	Taiwan	**MH595905**
98-TP-SL11-sd06-M3	Tick (*Rhipicephalus sanguineus* s. l.), male	Taiwan	**MH595906**
98-TP-SL11-sd07-EN1	Tick (*Rhipicephalus sanguineus* s. l.), nymph	Taiwan	**MH595907**
98-TP-SL11-sd07-M9	Tick (*Rhipicephalus sanguineus* s. l.), male	Taiwan	**MH595908**
98-TP-SL11-sd07-PEA5	Tick (*Rhipicephalus sanguineus* s. l.), female	Taiwan	**MH595909**
98-TP-SL11-sd08-M6	Tick (*Rhipicephalus sanguineus* s. l.), male	Taiwan	**MH595910**
98-TP-SL11-sd08-M9	Tick (*Rhipicephalus sanguineus* s. l.), male	Taiwan	**MH595911**
Hepatozoon canis	Tick (unknown)	Brazil	KF972445
H. canis	Fox (*Pseudalopex gymnocecus*)	Brazil	AY471615
H. canis	Dog blood	Spain	AY731062
H. canis	Dog blood	Spain	AY461378
H. canis	Fox	Spain	AY150067
H. canis	Blood (*Hydrochaeris hydrochoerus*)	Spain	EF622096
H. canis	Dog blood	Taiwan	EU289222
H. canis	Tick (*Rhipicephalus sanguineus* s. l.)	Egypt	MG564216
H. canis	Fox (*Vulpes vulpes*)	Italy	GU371447
H. americanum	Dog blood	USA	AF176836
H. felis	Blood, Asiatic lion	India	HQ829438
H. felis	Blood, Asiatic lion	India	HQ829439

[a] GenBank accession numbers (**MH595892–MH595911**) were submitted by this study.

respectively. For phylogenetic analysis, the nucleotide sequences of 18S ssrRNA genes from other 12 strains of *Hepatozoon* sp. were included for comparison and their GenBank accession numbers are shown in Table 1.

2.6. Ethical statement

This study was approved and carried out within strict accordance of the guidelines by the Ethical Committee of the Institutional Review Board of National Defense Medical Center (IACUC-09-183). All animal process was operated by the Taipei City Animal Protection Office and was adhered to the Animal Protection Law of the Taipei City Government. This study did not involve endangered species and no specific permissions were required for these collections.

3. Results

3.1. Hepatozoon infection detected in various stages of R. sanguineus s. l. ticks

To verify *Hepatozoon* infection in *R. sanguineus* s. l. ticks of Taiwan, all collected ticks were examined for the evidence of *Hepatozoon* infection by nested-PCR using specific primers targeting the 18S ssrRNA genes of *Hepatozoon* protozoa. Results indicate that *Hepatozoon* infections were detected in males, females and nymphs of *R. sanguineus* s. l. ticks with an infection rate of 22.8% (132/578), 29.4% (52/177) and 20.8% (68/327), respectively (Table 2). Altogether *Hepatozoon* infection was detected in 23.3% (252/1082) of *R. sanguineus* s. l. ticks.

Table 2
Nested-PCR detection of *Hepatozoon* infection in various life-cycle stages of *Rhipicephalus sanguineus* sensu lato ticks collected from dogs of Taiwan.

Life-stage of tick	Detection of *Hepatozoon* infection by nested-PCR		% Infected
	No. tested	No. infected	
Male	578	132	22.8
Female (partially-engorged)	177	52	29.4
Nymph (partially-engorged)	327	68	20.8
Total	1082	252	23.3

3.2. Sequence analysis and genetic identification of detected Hepatozoon strains

To clarify the genetic identity of *Hepatozoon* spp. detected in *R. sanguineus* s. l. ticks, all sequences of 18S ssrRNA gene fragments of detected *Hepatozoon* from Taipei City of Taiwan were aligned and compared with the downloaded sequences of known genospecies of 12 strains of *Hepatozoon* species. Results indicate that these detected *Hepatozoon* strains of Taiwan were genetically affiliated to the genospecies of *H. canis* and were verified with a high sequence identity (99.5–100%) within the same genospecies of *H. canis*, and can be discriminated from other genospecies of *Hepatozoon* protozoa. Intraspecies analysis based on the genetic distance (GD) values indicates a lower level (GD $<$ 0.005) of genetic divergence within the same genospecies of *H. canis* detected in various geographical areas (Taiwan, Brazil and

Table 3
Intra- and inter-species analysis of genetic distance values[a] based on the 18S ssrRNA gene sequences among *Hepatozoon* strains detected in *Rhipicephalus sanguineus* sensu lato ticks from Taiwan and other sources of *Hepatozoon* strains around the world.

Genospecies/strain source	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. 98-TP-DA09-sd02-M tick/Taiwan	-													
2. 98-TP-SL11-sd02-PEA8 tick/Taiwan	0.000	-												
3. 97-TP-DT12-sd06-EN5 tick/Taiwan	0.002	0.002	-											
4. 97-TP-ZS06-sd03-PEA5 tick/Taiwan	0.002	0.002	0.000	-										
5. 98-TP-NG09-sd01-M tick/Taiwan	0.002	0.002	0.000	0.000	-									
6. 98-TP-SL11-sd08-M6 tick/Taiwan	0.003	0.003	0.002	0.002	0.002	-								
7. 97-TP-ZS06-sd03-M1 tick/Taiwan	0.005	0.005	0.003	0.003	0.003	0.002	-							
8. *H. canis* dog/Taiwan (EU289222)	0.002	0.002	0.000	0.000	0.000	0.002	0.003	-						
9. *H. canis* tick/Brazil (KF972445)	0.000	0.000	0.002	0.002	0.002	0.003	0.005	0.002	-					
10. *H. canis* dog/Spain (AY731062)	0.002	0.002	0.000	0.000	0.000	0.002	0.003	0.000	0.002	-				
11. *H. canis* tick/Egypt (MG564216)	0.018	0.018	0.016	0.016	0.016	0.014	0.012	0.016	0.018	0.016	-			
12. *H. canis* fox/Italy (GU371447)	0.018	0.018	0.016	0.016	0.016	0.014	0.012	0.016	0.018	0.016	0.000	-		
13. *H. americanum* dog/USA (AF176836)	0.062	0.062	0.060	0.060	0.060	0.058	0.056	0.060	0.062	0.060	0.058	0.058	-	
14. *H. felis* lion/India (HQ829439)	0.046	0.046	0.044	0.044	0.044	0.042	0.040	0.044	0.046	0.044	0.040	0.040	0.031	-

[a] The pairwise distance calculation was performed by the method of Kimura 2-parameter, as implemented in MEGA 6 (Tamura et al., 2013).

Spain). However, all these Taiwan strains were genetically affiliated but more distant (GD = 0.012–0.018) to the *H. canis* detected from Egypt and Italy. Interspecies analysis also reveals that these Taiwan strains have a higher heterogeneity distinguished from other genospecies of *H. felis* (GD > 0.040) and *H. americanum* (GD > 0.056) (Table 3).

3.3. Phylogenetic analysis of detected Hepatozoon strains

Phylogenetic relationships based on the alignment of 18S ssrRNA gene sequences were also performed to analyze the genetic divergence among 20 *Hepatozoon* strains of Taiwan and 12 other *Hepatozoon* strains investigated in this study. Phylogenetic trees constructed by maximum likelihood (ML) (Fig. 1) and neighbour-joining (NJ) (data not shown) analyses showed congruent basal topologies with seven major branches of distinguished clades. All *Hepatozoon* strains detected in Taiwan represent one monophyletic group closely affiliated to the genospecies of *H. canis*, and can be distinguished clearly from other *Hepatozoon* genospecies by both ML and NJ methods. The phylogenetic analysis of ML and NJ trees strongly support the separation of different lineages between the *Hepatozoon* strains from Taiwan and other genospecies of *Hepatozoon* with a bootstrap value of 99, respectively.

4. Discussion

This study conducts the first survey for detecting of *Hepatozoon* infection and provides the first convincing evidence of *H. canis* identified from various stages of *R. sanguineus* s. l. ticks removed from dogs in northern Taiwan. In previous studies, molecular detection of *H. canis* has been identified from dogs of Europe, Africa, Asia and South America (Baneth et al., 2000; Inokuma et al., 2002; Oyamada et al., 2005; Karagenc et al., 2006; Jittapalapong et al., 2006; Rubini et al., 2008; Aktas et al., 2013, 2015) and from various vector ticks (Murata et al., 1995; Forlano et al., 2005; Baneth et al., 2007; Rubini et al., 2009; Aktas, 2014). Although *R. sanguineus* s. l. tick was recognized as the main vector tick for *H. canis* in dogs (Baneth et al., 2001, 2007), other vector ticks may contribute to the perpetuation of *Hepatozoon* spp. in nature. Indeed, previous studies in Brazil and Turkey revealed that *R. sanguineus* s. l. tick was highly associated with *Hepatozoon* transmission in urban areas (O'Dwyer et al., 2001; Aktas et al., 2013) and *Amblyomma* ticks were infested on infected dogs of rural areas (Forlano et al., 2005). In Japan, *Haemaphysalis longicornis* and *H. flava* ticks were suspected to be the vector for *H. canis* (Murata et al., 1995; Oyamada et al., 2005). However, there is no evidence indicating the vector competence of *R. sanguineus* s. l. for *H. canis* and no verified GenBank sequences of *H. canis* from *R. sanguineus* s. l. ticks of Taiwan. Thus, our study demonstrates the first molecular evidence confirming *H. canis* within *R. sanguineus* s. l. ticks collected in northern Taiwan and provides the first convincing sequences (GenBank accession numbers: MH595892˜ MH595911) of *H. canis* detected from *R. sanguineus* s. l. ticks in Taiwan.

Transmission mechanisms for *Hepatozoon* to canine hosts may occur in various routes. It is said that *R. sanguineus* s. l. is considered as an effective vector for *H. canis* and the most familiar pathway for the transmission of *Hepatozoon* to dogs is mediated by ingestion of infected ticks. Indeed, it is postulated that the infected ticks containing mature oocysts acquired from the blood of *Hepatozoon*-infected vertebrate host (dogs) will serve as the definitive invertebrate host where the *Hepatozoon* protozoa develops the sexual phase of its life cycle (Smith, 1996; Baneth et al., 2007). Other mechanism for the transmission of *Hepatozoon* by predation of one vertebrate upon another infected-host was observed on lizard, snake and wild mammals (Smith et al., 1994; Johnson et al., 2009). In addition, the distinct (tropical/temperate) linage group of *R. sanguineus* s. l. may also contribute to the variant *Hepatozoon* transmission around the world (Baneth et al., 2003; Rubini et al., 2008; Cadoso et al., 2010; Aktas et al., 2013). In this study, the dogs were commonly infested by *R. sanguineus* s. l. ticks and the infection of *H. canis* was detected in various stages of *R. sanguineus* s. l. ticks (Table 2). These observations reveal a high prevalence of *H. canis* infection of *R. sanguineus* s. l. ticks in Taiwan. In addition, detection of *H. canis* in male tick may be related to the dogs that were infested by these ticks or may imply the possible mechanism of transstadial survival of *H. canis* in *R. sanguineus* s. l. tick. Although the possibility of transovarial transmission of *H. canis* by the remaining oocyst of *Hepatozoon* within the haemocoel of ticks (Baneth et al., 2007) was plausible, there were no convincing evidence of transovarial transmission detected in *Rh. sanguineus* ticks (Baneth et al., 2007; Giannelli et al., 2013; Aktas and Ozubek, 2017).

Genetic identiity and phylogenetic relationships among *Hepatozoon* species can be determined by analyzing their sequence homogeneity of the 18S ssrDNA. Genetic heterogeneity of *Hepatozoon* strains was firstly demonstrated on the basis of genetic variation between *H. canis* and *H. americanum* (Vincent-Johnson et al., 1997; Baneth et al., 2003). Recently, PCR-based molecular analysis is particularly useful for the detection of *Hepatozoon* infection in dogs with a low parasitemia levels and for differentiation of *Hepatozoon* species from canine hosts. Indeed, PCR amplification of the 18S ssrDNA gene had been proved useful for the detection of *Hepatozoon* infection and to evaluate the genetic relationship of *Hepatozoon* species from canine and vector ticks (Baneth et al., 2000; Inokuma et al., 2002; Forlano et al., 2005; Jittapalapong et al., 2006; Baneth et al., 2007; Rubini et al., 2008). Results from the present study also reveal that the genetic identity of *Hepatozoon* strains detected in *R. sanguineus* s. l. ticks of Taiwan is highly homogeneous

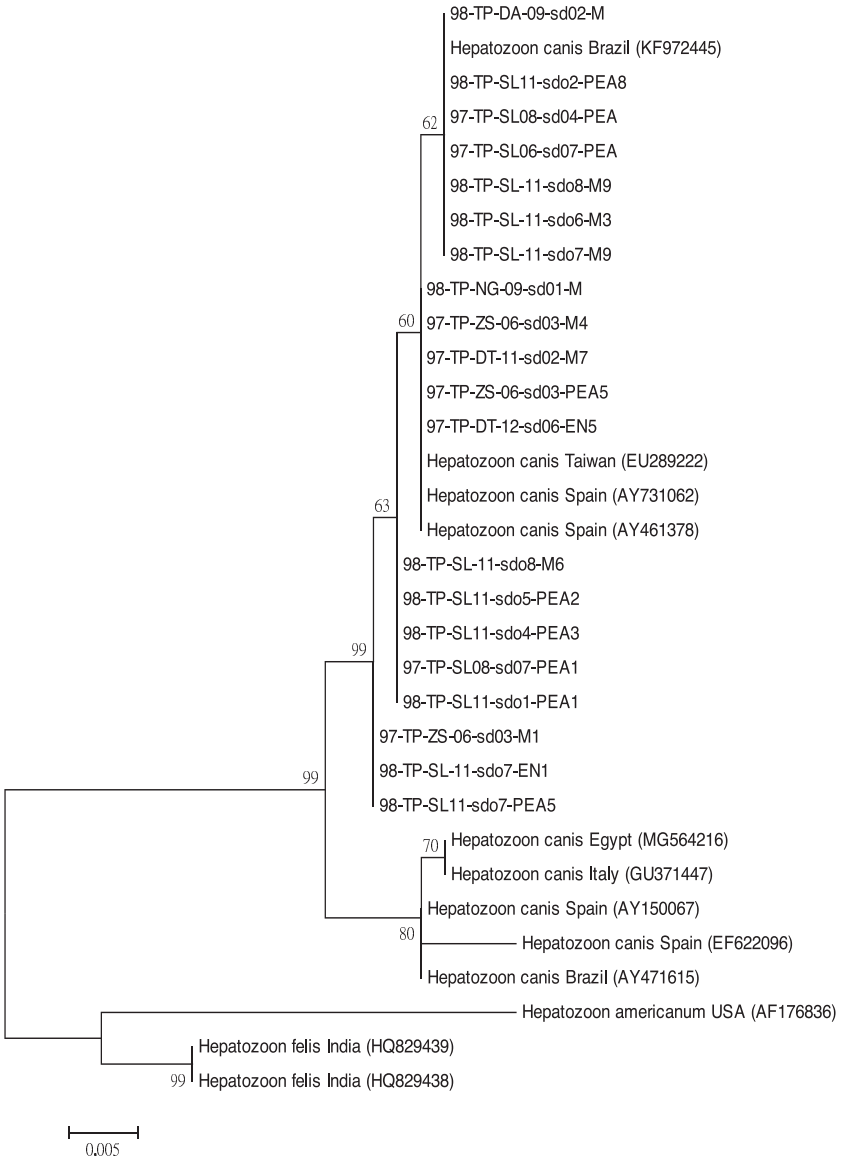

Fig. 1. Phylogenetic relationships based on the 18S small subunit-ribosomal RNA (18S ssrDNA) gene sequences between twenty *Hepatozoon* strains detected in *R. sanguineus* sensu lato ticks of Taiwan compared with 12 other strains belonging to three genospecies of *Hepatozoon* species. The trees were constructed and analyzed by maximum likelihood (ML) method using 1000 bootstraps replicates. Numbers at the nodes indicate the percentages of reliability of each branch of the tree. Branch lengths are drawn proportional to the estimated sequence divergence.

within the genospecies of *H. canis*, and was clearly distinguished from other species of *Hepatozoon* protozoas (Table 3). The phylogenetic trees constructed by either ML or NJ analysis also strongly support the discrimination recognizing the separation of different lineages between the *Hepatozoon* strains from Taiwan and other genospecies of *Hepatozoon* detected from various biological and geographical sources (Fig. 1). Accordingly, these results demonstrate that the genetic identities of *Hepatozoon* strains detected within *R. sanguineus* s. l. ticks collected from northern Taiwan were genetically affiliated to the genospecies of *H. canis*.

5. Conclusions

This report provides the first molecular evidence regarding the existence of *Hepatozoon canis* in *R. sanguineus* s. l. ticks collected from dogs in Taiwan. *Hepatozoon* detected in the present study in Taiwan revealed a high sequence identity with *H. canis*. Because of the ability of dogs serving as host for infected ticks, determination of the prevalence of *Hepatozoon* infection in *R. sanguineus* s. l. ticks is pre-requisite for the prevention of canine hepatozoonosis. Further investigations focusing on the detection of *Hepatozoon* species in different vector ticks parasitizing stray and domestic dogs would help to clarify the significance of genetic diversity of *Hepatozoon* species in Taiwan.

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