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Molecular analysis of *Rhipicephalus sanguineus* (Acari: Ixodidae), an incriminated vector tick for *Babesia vogeli* in Taiwan

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Abstract The genetic identity of *Rhipicephalus sanguineus* tick was determined for the first time in Taiwan. The phylogenetic relationships were analyzed by comparing the sequences of mitochondrial 16S ribosomal DNA gene obtained from 32 strains of ticks representing six species of *Rhipicephalus*, two species of *Dermacentor* and two outgroup species (*Haemaphysalis inermis* and *Ixodes ricinus*). Seven major clades can be easily distinguished by neighbour-joining analysis and were congruent by maximum-parsimony method. All *R. sanguineus* ticks of Taiwan were genetically affiliated to the tropical lineage group of *R. sanguineus* sensu lato with highly homogeneous sequence (99.7–100% similarity), and can be discriminated from the temperate lineage group of *Rhipicephalus* sp. II and *R. turanicus* with a sequence divergence ranging from 1.7 to 5.2%. In contrast, the nucleotide variations among other *Rhipicephalus* spp. and other species/genus of ticks compared with the *R. sanguineus* ticks of Taiwan were measured from 10.6 to 25.5%. Moreover, intra- and inter-species analysis based on the genetic distance (GD) values indicated a lower level ($GD < 0.003$) within tropical lineage group compared with temperate lineage group ($GD > 0.055$) of *Rhipicephalus*, as well as other ($GD > 0.129$) and outgroup ($GD > 0.236$) species. Our results provide the first genetic identification of *R. sanguineus* ticks collected from Taiwan and demonstrate that all these *R. sanguineus* of Taiwan affiliated to the tropical lineage group of *R. sanguineus* sensu lato.

Keywords *Rhipicephalus sanguineus* · Tick · Genetic identity · Taiwan

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Introduction

Ticks are obligate hematophagous arthropods that parasitize every class of vertebrates in almost every region of the world and it may act as vectors with the ability to transmit various pathogens including bacteria, rickettsiae, and protozoan (Balashov 1972). The medical and veterinary importance with the recent emergence of human babesiosis (Shih et al. 1997), Lyme borreliosis (Shih and Chao 1998; Chao et al. 2011) and canine babesiosis (Lee et al. 2010) in Taiwan raises the focus of research attention on vector ticks. The brown dog tick, *Rhipicephalus sanguineus*, is the most widespread tick species around the world and is recognized as the dominant ectoparasite of dogs that can occasionally parasitize other vertebrate hosts, including humans (Felz et al. 1996; Dantas-Torres 2010). In addition, *R. sanguineus* has been recognized as the primary vector for the transmission of *Babesia vogeli*, *Ehrlichia canis*, *Rickettsia rickettsii*, and *R. conorii* in humans and animals (Walker et al. 2000; Otranto et al. 2009; Eremeeva et al. 2011; Dantas-Torres et al. 2012). Although the hard tick of *R. sanguineus* had been identified as the incriminated vector tick for the zoonotic transmission of *B. vogeli* in Taiwan (Chao et al. 2016), the genetic identity of *R. sanguineus* collected from endemic sites of Taiwan remain undefined.

Although species determination and differentiation of *Rhipicephalus* ticks have traditionally been based on morphological features of the adult stages of these ticks, the taxonomic status of the *R. sanguineus* ticks has been repeatedly debated (Gray et al. 2013; Dantas-Torres and Otranto 2015; Nava et al. 2015). Because of the high level of morphological similarity among brown dog ticks within the *R. sanguineus* complex, ambiguity in taxonomy of the *R. sanguineus* ticks was reiterated by using molecular tools for phylogenetic analysis (Szabo et al. 2005; Burlini et al. 2010; Moraes-Filho et al. 2011; Levin et al. 2012; Liu et al. 2013). Indeed, a DNA-based approach provides the feasibility to investigate the genetic variance at the individual base-pair level and gives much more direct pathway for measuring the genetic diversity between and within species of Ixodidae (Black and Piesman 1994; Caporale et al. 1995; Black and Roehrdanz 1998). Current studies based on the mitochondrial 16S ribosomal DNA (rDNA) target region have revealed the existence of at least two separate groups (tropical vs. temperate lineage) of *R. sanguineus* ticks (Szabo et al. 2005; Moraes-Filho et al. 2011; Zemtsova et al. 2016). Thus, molecular analysis based on the genetic polymorphism of mitochondrial 16S rDNA gene has made possible in facilitating the identification and discrimination of taxonomically similar *Rhipicephalus* ticks.

It may be that the vector tick of *R. sanguineus* for canine babesiosis in Taiwan is a genetically distinct lineage, as compared with the existing common vector ticks of *Rhipicephalus* species around the world and the potential of genetic variation in relation to the geographical distribution may also exist among these *R. sanguineus* ticks characterized with similar morphology. Thus, the objective of this study intends to investigate the phylogenetic relationships between and within the species of *R. sanguineus* ticks by analyzing the mitochondrial 16S rDNA gene. The genetic divergence of *R. sanguineus* ticks collected from endemic sites of Taiwan was analyzed by their differential nucleotide composition, as compared with other tick species identified from various geographical sources which have been documented in GenBank.

Materials and methods

Collection and identification of tick specimen

All specimens of adult ticks including 28 strains of *Rhipicephalus* ticks, two strains of *Dermacentor* ticks, and two outgroup species (*Haemaphysalis inermis* and *Ixodes ricinus*) were used for genetic analysis in this study (Table 1). Of these, 14 strains of *R. sanguineus*

Table 1 Source of tick specimens used for phylogenetic analysis in this study

Tick strain	Specimen source	GenBank accession numbers[a]
Rhipicephalus sanguineus (Taiwan)		
99KHDS09EN6	Kaohsiung, Taiwan	KX685412
99KHDS04M2	Kaohsiung, Taiwan	KX685413
99KHDS09EN5	Kaohsiung, Taiwan	KX685414
99KHDS04M1	Kaohsiung, Taiwan	KX685415
98KHCJ10PEA	Kaohsiung, Taiwan	KX685416
98KHCJ08M	Kaohsiung, Taiwan	KX685417
100KHAL04PEA1	Kaohsiung, Taiwan	KX685418
100KHAL04M1	Kaohsiung, Taiwan	KX685419
100KHCH07PEA2	Kaohsiung, Taiwan	KX685420
100KHCH07EN1	Kaohsiung, Taiwan	KX685421
99KHYC06PEA2	Kaohsiung, Taiwan	KX685422
99KHYC06M2	Kaohsiung, Taiwan	KX685423
99KHZY01M9	Kaohsiung, Taiwan	KX685424
98KHZY09EN2	Kaohsiung, Taiwan	KX685425
R. sanguineus	American Samoa	KT382446
R. sanguineus	Thailand	JX997387
R. sanguineus	Cuba	JX997389
R. sanguineus	Brazil	GU553075
R. sanguineus	China	KC203362
R. sanguineus	Spain	JX997393
R. sanguineus	Argentina	JX195167
R. sanguineus	Chile	GU553077
R. turanicus	South Africa	GU553080
R. microplus	Brazil	EU918178
R. microplus	South Africa	EU918182
R. australis	Australia	EU918192
R. australis	Indonesia	EU918190
R. appendiculatus	USA	L34301
Dermacentor marginatus	China	KF547985
D. nuttalli	China	KF547991
Haemaphysalis inermis	USA	U95872
Ixodes ricinus	Germany	JF928527

[a] GenBank accession numbers (KX685412 ~ KX685425) were submitted by this study

Fig. 1 Map of Taiwan and its adjacent islands, showing the collection site for tick specimens

were collected from dogs captured at various districts of Kaohsiung City (22°36′N, 120°18′E; 22°39′N, 120°17′E; 22°43′N, 120°25′E; 22°47′N, 120°22′E; 22°53′N, 120°19′E; 22°53′N, 120°28′E) in southern Taiwan (Fig. 1). All these ticks were subsequently stored in separate mesh-covered and plaster-bottomed vials. All tick specimens of *R. sanguineus* were identified to species level on the basis of their morphological characteristics, as described previously (Chao et al. 2016). Ultrastructural observations by stereo-microscope were used to delineate the morphological features of all stages of *R. sanguineus* ticks in Taiwan. Briefly, tick specimens were cleaned by sonication in 70% 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* ticks were recorded for species identification.

DNA extraction from tick specimen

Total genomic DNA was extracted from individual tick specimens used in this study. Briefly, tick specimens were cleaned by sonication for 3–5 min in 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), 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 filtrate was collected and the DNA concentration was determined spectrophotometrically with a DNA calculator (Nanovue Plus Spectrophotometer).

DNA amplification by polymerase chain reaction (PCR)

DNA samples extracted from the tick specimens were used as a template for PCR amplification. A specific primer set of 16S + 1 (5′-CTGCTCAATGATTTTTTAAATT GCTGTGG-3′) corresponding to the 3′ end of the mitochondrial 16S rDNA and 16S − 1 (5′-CCGGTCTGAACTCAGATCAAGT-3′) corresponding to the 5′ end of the mitochondrial 16S rDNA were designed to target the mitochondrial 16S rDNA gene, as described previously (Black and Piesman 1994). All PCR reagents and Taq polymerase were obtained and used as recommended by the supplier (Takara Shuzo, 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. In contrast, adequate amounts of sterile distilled water were added for serving as a negative control. PCR amplification was performed with a Perkin-Elmer Cetus thermocycler (GeneAmp system 9700) and was amplified for 40 cycles with the conditions of denaturation at 92 °C for 1 min, annealing at 54 °C for 35 s, and extension at 72 °C for 90 s., as described previously (Chao et al. 2009). 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.

Sequence alignments 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 of initial 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 sequences were initially edited by BioEdit software (V5.3) and aligned with the CLUSTAL W software (Thompson et al. 1994). Thereafter, the aligned sequences of 14 tick strains of Taiwan were further analyzed by comparing with other 18 strains of tick specimens based on the different genus and different geographical origin of *Rhipicephalus* ticks that are available in GenBank. Phylogenetic analysis was performed by neighbour-joining (NJ) compared with maximum parsimony (MP) 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 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).

Nucleotide sequence accession numbers

The nucleotide sequences of PCR-amplified mitochondrial 16S rDNA genes of 14 strains of *R. sanguineus* ticks determined in this study have been registered and assigned the following GenBank accession numbers: strains 99KHDS09EN6 (KX685412), 99KHDS04M2 (KX685413), 99KHDS09EN5 (KX685414), 99KHDS04M1 (KX685415), 98KHCJ10PEA (KX685416), 98KHCJ08M (KX685417), 100KHAL04PEA1 (KX685418), 100KHAL04M1 (KX685419), 100KHCH07PEA2 (KX685420), 100KHCH07EN1 (KX685421), 99KHYC 06PEA2 (KX685422), 99KHYC06M2 (KX685423), 99KHZY01M9 (KX685424), and

99KHZY09EN2 (KX685425), respectively. For phylogenetic analysis, the nucleotide sequences of 16S rDNA genes from other 14 strains of *Rhipicephalus*, two strains of *Dermacentor*, and two outgroup ticks (i.e. *H. inermis* and *I. ricinus*) were included for comparison and their GenBank accession numbers are shown in Table 1.

Results

Sequence alignment and genetic analysis

To clarify the genetic identity of *R. sanguineus* ticks of Taiwan, the sequences of mitochondrial 16S rDNA fragments of 14 Taiwan strains of *R. sanguineus* performed by this study were aligned and compared with the downloaded sequences of eight different geographical strains of *R. sanguineus*, six strains of *Rhipicephalus*, two strains of *Dermacentor*, and two outgroup strains of *H. inermis* and *I. ricinus* from GenBank. Results indicate that the lengths of the aligned sequences were measured from 369 to 397 bp, and the nucleotide sequences between the 14 strains of *R. sanguineus* of Taiwan were highly conserved with only a few point mutations/substitutions. All these *R. sanguineus* ticks of Taiwan were genetically affiliated to the tropical lineage group of *R. sanguineus* sensu lato with highly homogeneous sequence (99.74–100% similarity), and can be distincted from the temperate lineage group of *Rhipicephalus* sp. II and *R. turanicus* with a sequence divergence ranging from 1.68 to 5.17% (Table 2). In contrast, the nucleotide variations among other *Rhipicephalus* ticks and other species/genus of ticks compared with the *R. sanguineus* ticks of Taiwan were measured from 10.59 to 25.47% (Table 2). In addition, intra- and inter-species analysis based on the genetic distance (GD) values indicated a lower level ($GD < 0.003$) of genetic divergence within the tropical lineage group of *R. sanguineus* ticks as compared with the temperate lineage group ($GD > 0.055$) of *R. sanguineus*, as well as other ($GD > 0.129$) and outgroup ($GD > 0.236$) species of ticks (Table 3).

Phylogenetic analysis of tick specimens

Phylogenetic relationships based on the sequence alignment of mitochondrial 16S rDNA were performed to demonstrate the genetic divergence among 32 strains of ticks investigated in this study. Bootstrap analysis was used to analyze the repeatability of the clustering of specimens represented in phylogenetic trees. Phylogenetic trees constructed by both NJ (Fig. 2) and MP (Fig. 3) analyses showed congruent basal topologies with seven major branch of distinguished clades (Figs. 2, 3). All these *R. sanguineus* ticks of Taiwan constitute a monophyletic clade closely affiliated to the tropical lineage group of *R. sanguineus* ticks, and can be easily discriminated from the temperate lineage group (*Rhipicephalus* sp. II) and *R. turanicus* ticks with a bootstrap value of 97 and 95 in NJ analysis (Fig. 2). The phylogenetic tree of MP analysis was identical to the NJ tree and strongly support the separation of different lineages between the *R. sanguineus* from Taiwan and the temperate lineage group of *Rhipicephalus* ticks with a bootstrap value of 97 (Fig. 3). These results reveal a lower genetic divergence within the same species of *R. sanguineus* ticks from Taiwan, but a higher genetic variations among different lineage or genus of *Rhipicephalus* ticks.

Table 2 The nucleotide divergence of mitochondrial 16S rDNA sequences between various strains and genus of ticks, as compared with the *R. sanguineus* (99KHZY01M9) of Taiwan

Tick strain	Sequence length	Number of variant positions	% of nucleotide divergence
Rhipicephalus sanguineus (Taiwan)			
99KHZY01M9	388	0	0
99KHYC06M2	388	0	0
98KHCJ10PEA	388	0	0
100KHAL04PEA1	388	0	0
100KHCH07EN1	388	0	0
99KHDS04M2	388	1	0.26
R. sanguineus (American Samoa)	387	0	0
R. sanguineus (Thailand)	387	0	0
R. sanguineus (Cuba)	388	0	0
R. sanguineus (Brazil)	358	0	0
R. turanicus (South Africa)	358	6	1.68
R. sanguineus (Argentina)	364	22	6.04
R. sanguineus (Spain)	384	19	4.94
R. sanguineus (Chile)	356	20	5.62
R. sanguineus (China)	387	20	5.17
R. appendiculatus (USA)	387	41	10.59
R. microplus (Brazil)	374	47	12.57
R. australis (Australia)	387	46	11.89
Dermacentor marginatus (China)	397	70	17.63
D. nuttalli (China)	393	68	17.30
Haemaphysalis inermis (USA)	388	60	15.46
Ixodes ricinus (Germany)	369	94	25.47

Discussion

This study describes the first genetic identification of the mitochondrial 16S ribosomal gene among *R. sanguineus* ticks collected on Taiwan. In previous investigations, sequence analysis of the mitochondrial 16S rDNA have been used to distinguish closely related *R. sanguineus* ticks (Burlini et al. 2010; Moraes-Filho et al. 2011; Levin et al. 2012; Nava et al. 2012; Dantas-Torres et al. 2013; Zemtsova et al. 2016) and to assess the phylogenetic relationships of diverse species of *Rhipicephalus* ticks (Erster et al. 2013; Low et al. 2015; Zemtsova et al. 2016) by comparing their nucleotide variations of the mitochondrial 16S rDNA. Indeed, current investigations demonstrate that the existence of at least two distinguished groups of *R. sanguineus* ticks around the world. The tropical lineage group represented by *R. sanguineus* sensu lato collected from the countries of Brazil, Cuba, Colombia, Costa Rica, Japan, Kenya, Marshall island, Mozambique, South Africa, Thailand, and USA-FL. In contrast, the temperate lineage group includes ticks from Chile, Spain, France, Italy, Germany, Argentina, and USA-GA (Dantas-Torres et al. 2013; Zemtsova et al. 2016). Results from this study demonstrate that the nucleotide composition of the mitochondrial 16S rDNA derived from these *R. sanguineus* ticks of Taiwan is highly homogeneous (99.74–100% sequence similarity) with the tropical lineage group of *R.*

Table 3 Intra- and inter-species analysis of genetic distance values[a] based on the mitochondrial 16S rDNA sequences within *Rhipicephalus sanguineus* ticks, and between various strains and genus of ticks analyzed in this study

Tick strains[b]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1. 99KHZY01M9	–																		
2. 99KHYC06M2	0.000	–																	
3. 98KHCJ10PEA	0.000	0.000	–																
4. 100KHAL04PEA1	0.000	0.000	0.000	–															
5. 100KHCH07EN1	0.000	0.000	0.000	0.000	–														
6. 99KHDS04M2	0.003	0.003	0.003	0.003	0.003	–													
7. *Rs*-American Samoa	0.000	0.000	0.000	0.000	0.000	0.003	–												
8. *Rs*-Thailand	0.000	0.000	0.000	0.000	0.000	0.003	0.000	–											
9. *Rs*-Cuba	0.000	0.000	0.000	0.000	0.000	0.003	0.000	0.000	–										
10. *Rs*-Brazil	0.000	0.000	0.000	0.000	0.000	0.003	0.000	0.000	0.000	–									
11. *Rs*-Argentina	0.066	0.066	0.066	0.066	0.066	0.069	0.066	0.066	0.066	0.066	–								
12. *Rs*-Spain	0.055	0.055	0.055	0.055	0.055	0.058	0.055	0.055	0.055	0.055	0.009	–							
13. *Rs*-Chile	0.058	0.058	0.058	0.058	0.058	0.062	0.058	0.058	0.058	0.058	0.006	0.003	–						
14. *Rs*-China	0.055	0.055	0.055	0.055	0.055	0.058	0.055	0.055	0.055	0.055	0.048	0.044	0.048	–					
15. *Rm*-Brazil	0.150	0.150	0.150	0.150	0.150	0.155	0.150	0.150	0.150	0.150	0.145	0.139	0.135	0.190	–				
16. *Ra*-Australia	0.143	0.143	0.143	0.143	0.143	0.148	0.143	0.143	0.143	0.143	0.138	0.133	0.129	0.143	0.015	–			
17. *Dm*-China	0.242	0.242	0.242	0.242	0.242	0.248	0.242	0.242	0.242	0.242	0.231	0.224	0.218	0.254	0.269	0.260	–		
18. *Hi*-USA	0.246	0.246	0.246	0.246	0.246	0.252	0.246	0.246	0.246	0.246	0.293	0.284	0.277	0.310	0.265	0.274	0.236	–	
19. *Ir*-Germany	0.587	0.587	0.587	0.587	0.587	0.595	0.587	0.587	0.587	0.587	0.553	0.536	0.527	0.596	0.504	0.525	0.532	0.382	–

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

[b] Strains: *Rhipicephalus sanguineus* (*Rs*), *R. microplus* (*Rm*), *R. australia* (*Ra*), *Dermacentor marginatus* (*Dm*), *Haemaphysalis inermis* (*Hi*), and *Ixodes ricinus* (*Ir*)

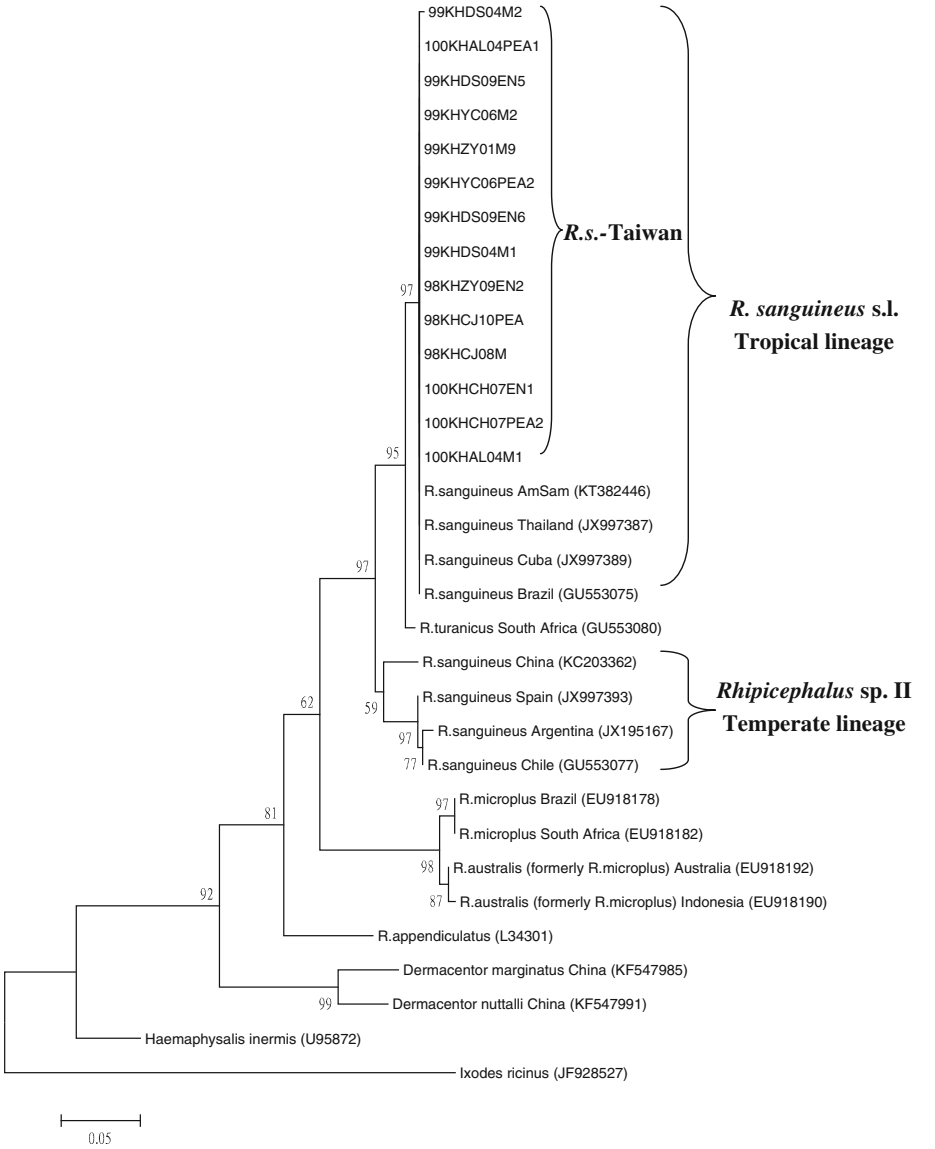

Fig. 2 Phylogenetic relationships based on the 16S ribosomal DNA (rDNA) gene sequences between 14 strains of *R. sanguineus* ticks from southern Taiwan and 18 other strains belonging to five species of *Rhipicephalus*, one species of *Dermacentor* and *Haemaphysalis*, and one strain of *Ixodes ricinus* served as outgroup comparison. The trees were constructed and analyzed by neighbour-joining (NJ) 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

sanguineus ticks. Thus, our study demonstrates the first molecular evidence confirming the genetic identity of *R. sanguineus* ticks collected in southern Taiwan and provides the first convincing sequences (GenBank accession numbers: KX685412 ~ KX685425) of *R. sanguineus* ticks in Taiwan.

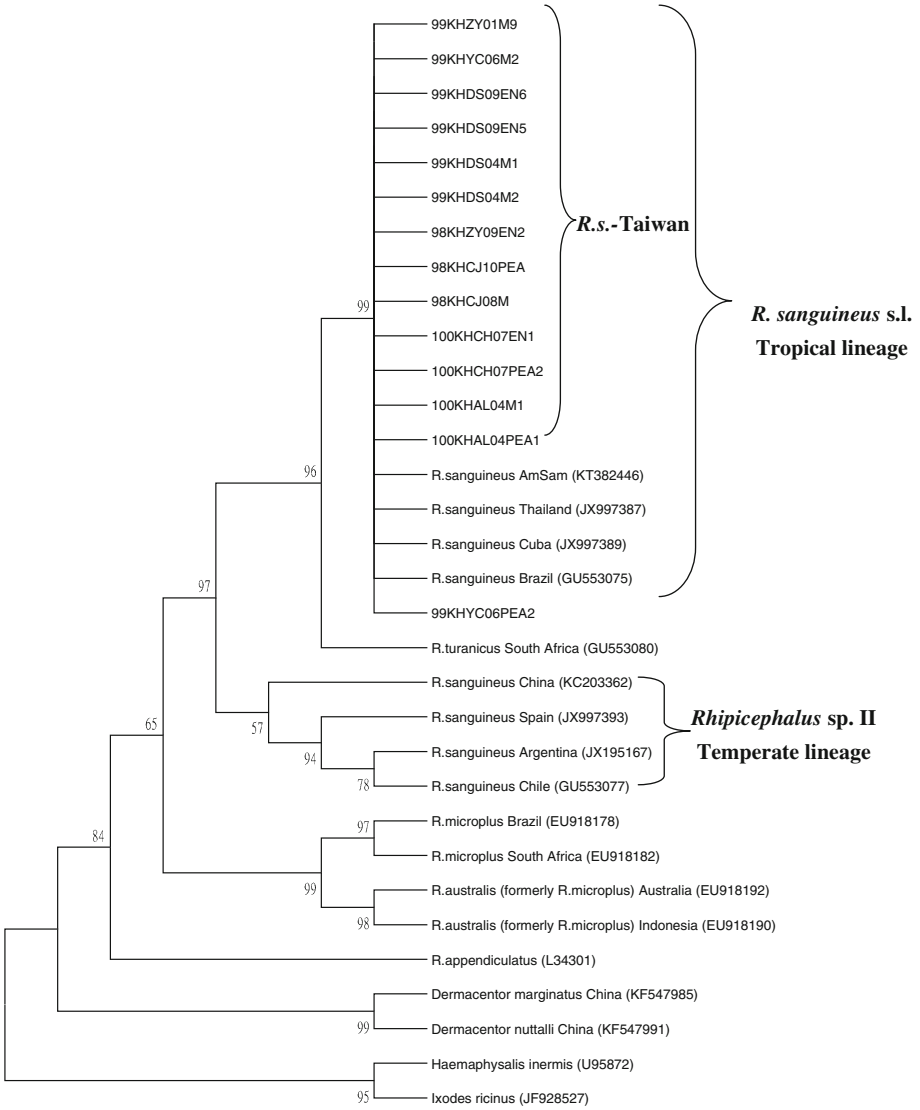

Fig. 3 Phylogenetic relationships based on the 16S ribosomal DNA (rDNA) gene sequences between 14 strains of *R. sanguineus* ticks from southern Taiwan and 18 other strains belonging to five species of *Rhipicephalus*, one species of *Dermacentor* and *Haemaphysalis*, and one strain of *Ixodes ricinus* served as outgroup comparison. The trees were constructed and analyzed by maximum parsimony (MP) 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

Because of the genetically high conservation and strictly maternal inheritance, the mitochondrial 16S rDNA sequences appear to provide a reliable and convenient method for distinguishing the lineages among diverse populations of *Rhipicephalus* ticks. In previous studies, two mitochondrial ribosomal genes, 12S and 16S rDNA, have been sequenced entirely for phylogenetic analysis of ixodid ticks focused on the family and

subfamily levels (Black and Roehrdanz 1998; Campbell and Barker 1999). Indeed, genetic analysis of the mitochondrial 16S rDNA sequences of various species of *Rhipicephalus* ticks also permits quantitative assessment of their relatedness (Moraes-Filho et al. 2011; Nava et al. 2012; Dantas-Torres et al. 2013; Erster et al. 2013; Low et al. 2015; Zemtsova et al. 2016). Results from this study also demonstrate the closely related individuals of *R. sanguineus* ticks of southern Taiwan and the genetic divergence among various species of *Rhipicephalus* ticks based on the genetic variations of 16S rDNA (Table 2; Fig. 1). Intraspecific analysis reveals that nucleotide compositions within Taiwan and the tropical lineage group of *R. sanguineus* ticks averaged less than 0.3% sequence variations may fully represent a distinct species discriminated from the temperate lineage group of *R. sanguineus* ticks (Tables 2, 3). However, interspecific analysis also indicates the nucleotide variations between *R. sanguineus* ticks of Taiwan and other *Rhipicephalus* species or genus of ticks averaged more than 10.59% sequence variations (Tables 2, 3). Further investigation on the sequence divergence based on various targets of the mitochondrial genes of *R. sanguineus* ticks collected from different localities of Taiwan and its adjacent islands would be required to clarify the genetic divergence as well as the evolutionally origin among and within *R. sanguineus* ticks from Taiwan and its adjacent islands.

Phylogenetic relationships among *Rhipicephalus* ticks can be determined by analyzing the sequence heterogeneity of the mitochondrial 16S rDNA. Indeed, sequence analysis of the mitochondrial 16S rDNA among various species of *Rhipicephalus* ticks had been shown to be useful for evaluating the taxonomic relatedness of tick specimens collected from various geographical sources (Moraes-Filho et al. 2011; Nava et al. 2012; Dantas-Torres et al. 2013; Erster et al. 2013; Low et al. 2015; Zemtsova et al. 2016). In previous studies, two distinct lineages of *R. sanguineus* ticks are evident by comparing their mitochondrial 16S rDNA sequences collected from different regions of Latin America (Moraes-Filho et al. 2011; Nava et al. 2012; Zemtsova et al. 2016). Phylogenetic analysis of tick species related to the members of the *Rhipicephalus* complex also revealed intraspecific variation between different geographical collections (Moraes-Filho et al. 2011; Erster et al. 2013; Low et al. 2015). In this study, the phylogenetic analysis based on the mitochondrial 16S rDNA sequences among various tick species demonstrated a high genetic heterogeneity between *R. sanguineus* and other species of ticks (Figs. 2, 3). Although a low intraspecific variation was observed among the same species of *R. sanguineus* ticks, all the 14 strains of *R. sanguineus* ticks from Taiwan represented as a monophyletic group that can be distinguished from the temperate group of *R. sanguineus* and other species/genus ticks (Table 3; Fig. 2). The phylogenetic trees constructed by either NJ or MP analysis strongly support the discrimination recognizing the separation of different lineages between the *R. sanguineus* collected from Taiwan and the temperate group of *R. sanguineus*. Accordingly, these observations demonstrate that genetic identities of *R. sanguineus* ticks collected from southern Taiwan were verified as a unique group affiliated to the tropical lineage of *R. sanguineus* sensu lato.

In conclusion, this report provides the first genetic identification of the mitochondrial 16S rDNA gene of *R. sanguineus* ticks collected from the Taiwan area. Based on the sequence divergence of the mitochondrial 16S rDNA, all these *R. sanguineus* ticks of Taiwan were genetically related to a monophyletic group and were represented as a unique lineage distinguished from the temperate group of *R. sanguineus* ticks as well as other *Rhipicephalus* ticks including the common vector ticks for canine babesiosis. Further application of this molecular tool to investigate the genetic variability of *R. sanguineus* collected from different localities of Taiwan may help to elucidate the phylogenetic

relationships among tick populations in relation to the epidemiological features of tick-borne pathogens in Taiwan.

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