

Molecular and morphological identification of a human biting tick, *Amblyomma testudinarium* (Acari: Ixodidae), in Taiwan

Li-Lian Chao^{1,2} · Chun-Wei Lu³ · Ying-Fang Lin⁴ · Chien-Ming Shih^{1,2,5}

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Abstract Genetic identity and morphological features of a human biting tick, *Amblyomma testudinarium*, were determined for the first time in Taiwan. Morphological features of adult male and female ticks of *Am. testudinarium* were observed and photographed by a stereo- microscope. The genetic identity was analyzed by comparing the sequences of mitochondrial 16S ribosomal DNA gene obtained from 18 strains of ticks representing 10 species of *Amblyomma*, and four outgroup species of *Dermacentor* and *Rhipicephalus* ticks. Nine major clades could be easily distinguished by neighbour-joining analysis and were congruent by maximum-parsimony method. All these *Am. testudinarium* ticks collected from Taiwan and Japan were genetically affiliated to a monophyletic group with highly homogeneous sequence (99.8–100% similarity), and can be discriminated from other species of *Amblyomma* and other genera of ticks (*Dermacentor* and *Rhipicephalus*) with a sequence divergence ranging from 6.9 to 23.9%. Moreover, intra- and inter-species analysis based on the genetic distance (GD) values indicated a lower level (GD < 0.003) within the same lineage of *Am. testudinarium* ticks collected from Taiwan and Japan, as compared with other lineage groups (GD > 0.108) of *Amblyomma* ticks, as well as outgroup (GD > 0.172) species. Our results provide the first distinguished features of adult *Am. testudinarium* ticks and the first genetic identification of *Am. testudinarium* ticks collected from humans in Taiwan. Seasonal prevalence, host range, and vectorial capacity of this tick species in Taiwan need to be further clarified.

✉ Chien-Ming Shih
cmshih@kmu.edu.tw

¹ M.Sc. Program in Tropical Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung 807, Taiwan, ROC

² Graduate Institute of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan, ROC

³ Department of Dermatology, Chang Gung Memorial Hospital, Linkou, Taiwan, ROC

⁴ Department of Dermatology, Chang Gung Memorial Hospital, Taoyuan, Taiwan, ROC

⁵ Center for Infectious Disease and Cancer Research, Kaohsiung Medical University, Kaohsiung, Taiwan, ROC

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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). There are three generally recognized families of ticks: the Ixodidae, the Argasidae, and the Nuttalliellidae (Oliver 1989; Sonenshine 1991). Based on studies of valid names of tick species, the *Amblyomma* genus comprising 130 species was recognized as the second largest group of Ixodidae ticks (Horak et al. 2002; Barker and Murrell 2008; Guglielmone et al. 2010). In addition, the global importance of ticks was determined by their ability to transmit a variety of tick-borne pathogens associated with human diseases and by direct damage to livestock due to their feeding behaviour (Jongejan and Uilenberg 2004). The impact of ticks and tick-borne pathogens on human and animal health becomes a major concern in public health.

Amblyomma testudinarium is a rare tick species reported only in Asia countries including Malaysia, India, Japan, Korea, Eastern China and Taiwan (Luh and Woo 1950; Nagahana and Matuo 1962; Dhanda and Rao 1964; Maa and Kuo 1966; Suzuki et al. 1990; Robbins 2005). It has previously been reported as a common ectoparasite of several species of reptiles, wild cats, dogs and wild boars (Motoi et al. 2013; Tateno et al. 2015; Suh et al. 2016), and occasionally on humans (Estrada-Pena and Jongejan 1999). This tick species was detected with various pathogens including *Borrelia*, *rickettsia*, *Hepatozoon* and tick-borne virus (Motoi et al. 2013; Ho et al. 2014; Sun et al. 2015; Tateno et al. 2015; Suh et al. 2016; Yun et al. 2016). 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), canine babesiosis (Lee et al. 2010) and rodent babesial infection (Chao et al. 2017b) in Taiwan raises the focus of research attention on vector ticks. Although the hard ticks of *Ixodes granulatus*, *I. ovatus* and *Rhipicephalus sanguineus* had been identified as the incriminated vector ticks for the transmission of *Borrelia* and *Babesia* in Taiwan (Chao et al. 2009, 2016, 2017a), the morphological features and genetic identity of *Am. testudinarium* collected from humans in Taiwan have never been reported.

It may be that the tick species of *Am. testudinarium* collected from Taiwan is a genetically distinct lineage, as compared with the existing common ticks of *Amblyomma* species around the world and the potential of genetic variation in relation to the geographical distribution may also exist among these *Am. testudinarium* ticks characterized with similar morphology. Thus, the objective of this study intends to investigate the genetic identity of *Am. testudinarium* ticks collected from Taiwan, and to analyze the phylogenetic relationships between and within the species of *Amblyomma* ticks by analyzing the mitochondrial 16S rDNA gene. The genetic divergence of *Am. testudinarium* ticks collected from Taiwan was also analyzed by comparing with other tick species identified from various geographical sources which have been documented in GenBank.

Materials and methods

Collection and identification of tick specimen

A total of nine adult *Am. testudinarium* (7 females and 2 males) were collected from attached or infested humans of different localities in Taiwan ($22^{\circ}17'N$, $120^{\circ}52'E$; $23^{\circ}16'N$, $120^{\circ}51'E$; $23^{\circ}04'N$, $120^{\circ}47'E$; $23^{\circ}28'N$, $120^{\circ}54'E$; $24^{\circ}10'N$, $121^{\circ}24'E$; $25^{\circ}06'N$, $121^{\circ}35'E$; $25^{\circ}05'N$, $121^{\circ}35'E$) (Fig. 1). All these adult ticks were removed from attached or infested humans (Table 1; Fig. 2), and were subsequently stored in 70% ethanol or plastic vials until further analysis. All tick specimens of *Am. testudinarium* were identified to species level on the basis of their morphological characteristics, as described previously (Shih and Chao 2011). Ultrastructural observations by stereo-microscope were used to delineate the morphological features of adult *Am. testudinarium* 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 tick specimen was placed on a glass slide and photographed using a stereo-microscope (SMZ 1500, Nikon, Tokyo, Japan) equipped with

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



Table 1 Summary of geographical location, seasonal occurrence, and biting-site on infested humans of adult *Amblyomma testudinarium* ticks collected from various localities of Taiwan

No./Sex	Geographical location	Collection month	Attachment site on human
1/Female	Neihu, Taipei City	June	Left hip
2/Female	Neihu, Taipei City	August	External abdomen
3/Male	Nantou County	September	Right leg
4/Female	Nantou County	October	External abdomen
5/Female	Taoyuan, Kaohsiung City	April	Left arm
6/Female	Taoyuan, Kaohsiung City	November	Left abdomen
7/Male	Taitung County	June	Right leg
8/Female	Taitung County	July	Right arm
9/Female	Hualien County	December	Left waist

**Fig. 2** Light micrograph of an almost fully engorged female of *Amblyomma testudinarium* tick feeding on a human

a fiber lamp. The external features of the *Am. testudinarium* 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- μ L 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'-CTGCTCAATGATTTTT TAAATTGCTGTGG-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- μ mol of the appropriate primer set and adequate amounts of template DNA were used in each 50- μ l 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 10 cycles with the conditions of denaturation at 92 °C for 1 min, annealing at 48 °C for 1 min, and extension at 72 °C for 90 s., then followed by 32 cycles with the conditions of denaturation at 92 °C for 40 s, annealing at 54 °C for 35 s, and extension at 72 °C for 90 s. 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 3 ticks of Taiwan were further analyzed by comparing with other 15 strains of tick specimens based on the different genus and different geographical origin of *Amblyomma*, *Dermacentor*, and *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 three strains of *Am. testudinarium* ticks determined in this study have been registered and assigned the following GenBank accession numbers: strains TWKL-Amt1 (KX685426), TWKL-Amt2 (KX685427), and TWKL-Amt3 (KX685428), respectively. For phylogenetic analysis, a total of 18 strains of ticks representing ten species of *Amblyomma*, and four outgroup species of *Dermacentor* (*D. andersoni* and *D. variabilis*) and *Rhipicephalus* (*R. sanguineus* and *R. appendiculatus*) ticks were included for comparison and their GenBank accession numbers are shown in Table 2.

Results

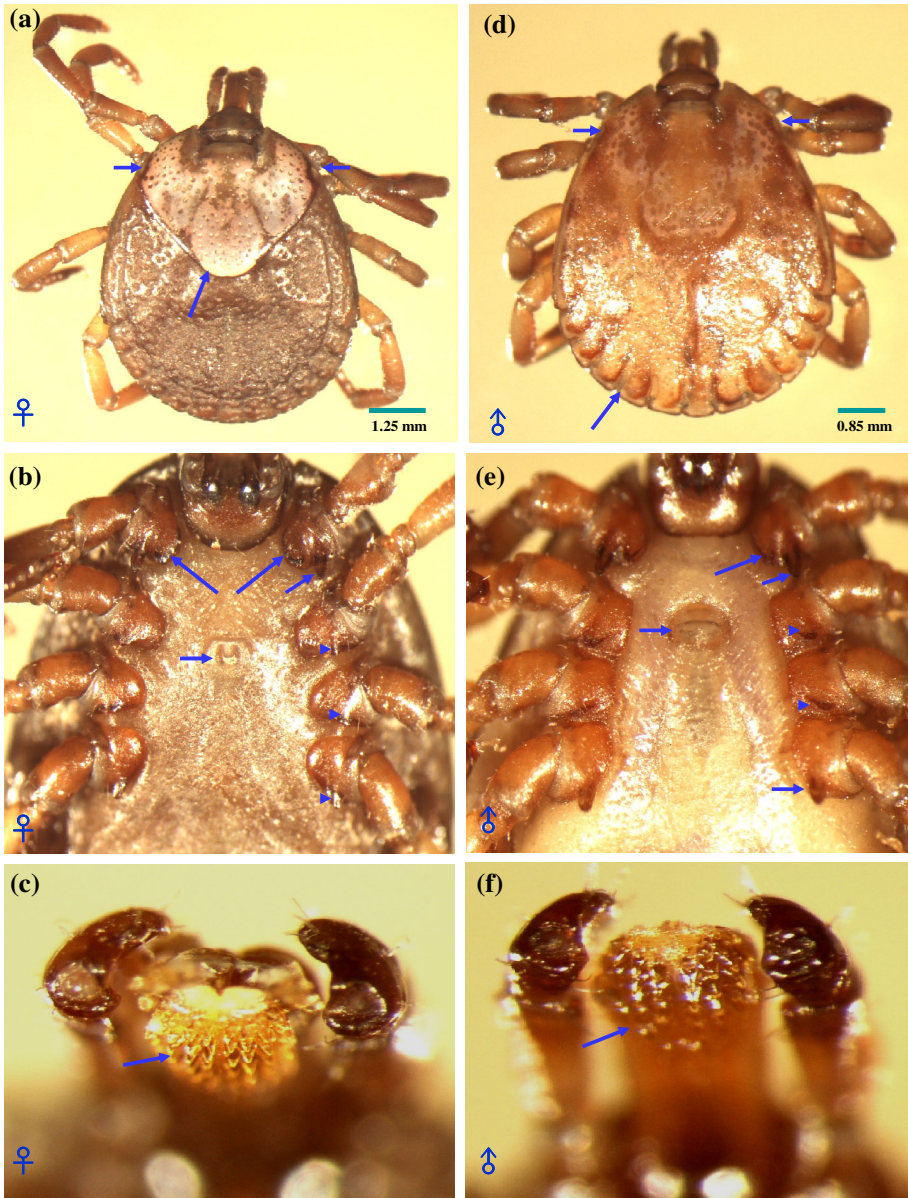
Morphological identification of *Amblyomma testudinarium* ticks collected from attached humans

The ultrastructural features of adult stage of male and female *Am. testudinarium* tick characterized with one pair of long-narrow palp, oval-shaped body, four pairs of legs, and rectangular-shaped basis capituli (Fig. 3a, d). The rhombus-shaped scutum (long-arrow

Table 2 Source of tick specimens used for phylogenetic analysis in this study

Tick species/strain	Source of tick specimen	Genbank accession number ^a 16S gene
<i>Amblyomma testudinarium</i>		
TWKL-Amt1	Taiwan	KX685426
TWKL-Amt2	Taiwan	KX685427
TWKL-Amt3	Taiwan	KX685428
<i>A. testudinarium</i>	Kanagawa, Japan	AB538277
<i>A. testudinarium</i>	Thailand	KC170737
<i>A. helvolum</i>	Thailand	KC170738
<i>A. geoemydae</i>	Japan	AB819162
<i>A. nitidum</i>	Japan	AB819166
<i>A. incisum</i>	Argentina	KM519939
<i>A. brasiliense</i>	Brazil	FJ424399
<i>A. dubitatum</i>	Brazil	KU894374
<i>A. calcaratum</i>	Brazil	JN573302
<i>A. coelebs</i>	Belize	KU001160
<i>A. longirostre</i>	Costa Rica	KF023435
<i>Dermacentor andersoni</i>	Canada	FM955614
<i>D. variabilis</i>	USA	L34300
<i>Rhipicephalus sanguineus</i>	Thailand	KC170744
<i>R. appendiculatus</i>	USA	L34301

^a GenBank accession numbers for 16S gene (KX685426–8) were submitted by this study



indicated on Fig. 3a) of female tick bears metallic yellowish markings with deep punctations and one pair of prolegs (short-arrows indicated on Fig. 3a) located at the lateral margin of rhombus-shaped scutum. In contrast, the oval-shaped scutum covered whole body of male tick bears one pair of prolegs (short-arrows indicated on Fig. 3d) located at the lateral margin near leg and clear festoon located at the bottom of scutum (long-arrow indicated on Fig. 3d). Ventral view of female *Am. testudinarium* shows the prolonged hypostome equipped with numerous sharp teeth of four/four dentition (long-arrow indicated on Fig. 3c), as compared to the curved teeth of male tick (long-arrow

indicated on Fig. 3f). The internal spurs of short triangular observed only on coxae I (long-arrows indicated on Fig. 3b, e) and absent on coxae II–IV of female and male ticks. The external spurs of distinct cone-like were observed on coxa I of female, and coxae I and IV of male (short-arrows indicated on Fig. 3b, e). The external spurs of broad triangular were observed on coxae II–IV of female and coxae II–III of male (arrowheads indicated on Fig. 3b, e). In addition, the genital aperture of female *Am. testudinarium* was situated at level between coxae II and III (short-arrow indicated on Fig. 3b). In contrast, the genital aperture of male *Am. testudinarium* was situated at level between coxae II (short-arrow indicated on Fig. 3e).

Sequence alignment and genetic analysis

To clarify the genetic identity of *Am. testudinarium* ticks of Taiwan, the sequences of mitochondrial 16S rDNA fragments of 3 Taiwan strains of *Am. testudinarium* performed by this study were aligned and compared with the downloaded sequences of 11 different geographical strains of *Amblyomma* ticks, and 4 outgroup strains of *Dermacentor* and *Rhipicephalus* ticks from GenBank. Results indicate that the lengths of the aligned sequences were measured from 365 to 380 bp, and the nucleotide sequences between the 3 strains of *Am. testudinarium* of Taiwan were highly homogeneous (100% similarity). All these *Am. testudinarium* ticks of Taiwan and Japan were genetically affiliated to a monophyletic group with highly homogeneous sequence (99.8–100% similarity), and can be discriminated from other species of *Amblyomma* ticks and other genera (*Dermacentor* and *Rhipicephalus*) ticks with a sequence divergence ranging from 6.9 to 23.9% (Table 3). In addition, intra- and inter-species analysis based on the genetic distance (GD) values indicated a lower level ($GD < 0.003$) within the same lineage of *Am. testudinarium* ticks collected from Taiwan and Japan, as compared with other lineage group ($GD > 0.108$) of *Amblyomma* ticks, as well as outgroup species ($GD > 0.172$) of *Dermacentor* and *Rhipicephalus* 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 18 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. 4) and MP (Fig. 5) analyses showed congruent basal topologies with nine major branch of distinguished clades (Figs. 4, 5). All these *Am. testudinarium* ticks of Taiwan constitute a monophyletic clade closely affiliated to the same lineage group of *Am. testudinarium* ticks, and can be easily discriminated from other lineage groups of *Amblyomma* ticks with a bootstrap value of 98 in NJ analysis (Fig. 4). The phylogenetic tree of MP analysis was identical to the NJ tree and strongly support the separation of different lineages between the *Am. testudinarium* from Taiwan and the other lineage groups of *Amblyomma* ticks with a bootstrap value of 91 (Fig. 5). These results reveal a lower genetic divergence within the same species of *Am. testudinarium* ticks from Taiwan, but a higher genetic variations among different lineage or genus of *Amblyomma* ticks.

◀ **Fig. 3** Light micrographs of female (a–c) and male (d–f) *Amblyomma testudinarium* ticks collected from Taiwan. **a, d** Dorsal view of female and male ticks characterized with one pair of long-narrow palp, oval-shaped body, four pairs of legs, rectangular-shaped basis capituli. **a** The rhombus-shaped scutum (*long-arrow*) of female tick bears metallic yellowish markings with deep punctations and one pair of protrude eyes (*short-arrows*) located at the lateral margin of rhombus-shaped scutum. **d** The oval-shaped scutum covered whole body of male tick bears one pair of protrude eyes (*short-arrows*) located at the lateral margin near leg and clear festoom located at the bottom of scutum (*long-arrow*). **b, e** Ventral view of female and male *Am. testudinarium* shows the internal spurs of short triangular observed only on coxae I (*long-arrows*) and absent on coxae II–IV of female and male ticks. The external spurs of distinct cone-like were observed on coxa I of female, and coxae I and IV of male (*short-arrows*), and the external spurs of broad triangular were observed on coxae II–IV of female and coxae II–III of male (*arrowheads*). In addition, the genital aperture was situated at level between coxae II and III of female, and at level between coxae II of male (*short-arrow*). **c, f** Ventral view of female *Am. testudinarium* shows the prolonged hypostome equipped with numerous sharp teeth of four/four dentition (*long-arrow*), as compared to the curved teeth of male tick (*long-arrow*)

Discussion

This study provides the first distinguished colour figures of adult *Am. testudinarium* ticks in Asia and describes the first genetic identification based on the mitochondrial 16S ribosomal gene among *Am. testudinarium* ticks collected from Taiwan. Although species determination and differentiation of *Amblyomma* ticks have traditionally been based on morphological features of the adult stages of these ticks, 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 *Amblyomma* ticks (Black and Piesman 1994; Burger et al. 2012; Lopes et al. 2016). In previous investigations, sequence analysis of the mitochondrial 16S rDNA have been used to distinguish and to assess the phylogenetic relationships of diverse species of closely related *Ixodes*, *Rhipicephalus* and *Amblyomma* ticks by comparing their nucleotide variations of the mitochondrial 16S rDNA (Black and Piesman 1994; Chao et al. 2009; Burger et al. 2012; Erster et al. 2013; Lopes et al. 2016; Zemtsova et al. 2016). Indeed, results from this study demonstrate that the nucleotide composition of the mitochondrial 16S rDNA derived from these *Am. testudinarium* ticks collected from Taiwan and Japan is genetically affiliated to a monophyletic group with highly homogeneous sequence (99.8–100% similarity), and can be discriminated from the same lineage group of *Am. testudinarium* tick from Thailand, other species of *Amblyomma* ticks and other genera (*Dermacentor* and *Rhipicephalus*) ticks with a sequence divergence ranging from 6.9 to 23.9% (Table 3; Fig. 4). Although this tick species had been recorded in Taiwan (Robbins 2005), no genetic characteristics were available for species identification. Thus, our study demonstrates the first molecular evidence confirming the genetic identity of *Am. testudinarium* ticks collected in Taiwan and provides the first convincing sequences (GenBank accession numbers: KX685426–KX685428) of *Am. testudinarium* ticks in Taiwan.

The geographical distribution of *Am. testudinarium* in Taiwan and eastern Asia needs to be further defined. In previous reports, this tick species has been found majority on reptilian hosts, wild cats and wild boars in northeastern Asia (i.e., Korea and Japan) (Motoi et al. 2013; Tateno et al. 2015; Suh et al. 2016), and human infestation may occasionally occur (Estrada-Pena and Jongejan 1999). In this report, seven female and two male ticks attached or infested on humans were collected from various localities in the lower and higher mountain areas of mainland Taiwan (Fig. 1), and these specimens provide the first evidence regarding the feeding activity of *Am. testudinarium* on humans in Taiwan. Because of increased international trade in wild reptiles in Taiwan (Shiau et al. 2006), accidental transportation of ticks on imported reptiles may contribute to the introduction of

Table 3 Intra- and Inter-species analysis of genetic distance values^a based on the 16S gene sequences among *Amblyomma testudinarium* ticks from infested humans of Taiwan, and strains from other countries and species of ticks

Tick species/strain ^b	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
1. <i>Ai</i> TWKL-Amt1	–																		
2. <i>Ai</i> TWKL-Amt2	0.000	–																	
3. <i>Ai</i> TWKL-Amt3	0.000	0.000	–																
4. <i>Ai</i> Japan	0.003	0.003	0.003	–															
5. <i>Ai</i> Thailand	0.061	0.061	0.061	0.061	–														
6. <i>Ah</i> Thailand	0.111	0.111	0.111	0.111	0.121	–													
7. <i>Ag</i> Japan	0.111	0.111	0.111	0.108	0.125	0.135	–												
8. <i>Ai</i> Costa Rica	0.154	0.154	0.154	0.154	0.154	0.151	0.169	0.150	–										
9. <i>Ac</i> Brazil	0.142	0.142	0.142	0.139	0.153	0.149	0.129	0.168	–										
10. <i>Ad</i> Brazil	0.146	0.146	0.146	0.143	0.161	0.150	0.139	0.153	0.087	–									
11. <i>An</i> Japan	0.121	0.121	0.121	0.118	0.143	0.154	0.091	0.188	0.143	0.165	–								
12. <i>Ai</i> Argentina	0.157	0.157	0.157	0.157	0.146	0.146	0.154	0.111	0.111	0.080	0.188	–							
13. <i>Ab</i> Brazil	0.150	0.150	0.150	0.146	0.153	0.143	0.143	0.132	0.124	0.111	0.180	0.074	–						
14. <i>Ac</i> Belize	0.146	0.146	0.146	0.143	0.157	0.146	0.136	0.169	0.080	0.084	0.150	0.097	0.111	–					
15. <i>Da</i> Canada	0.172	0.172	0.172	0.172	0.172	0.176	0.191	0.169	0.172	0.161	0.196	0.165	0.164	0.165	–				
16. <i>Dv</i> USA	0.183	0.183	0.183	0.184	0.176	0.191	0.196	0.184	0.183	0.183	0.203	0.172	0.183	0.172	0.058	–			
17. <i>Rs</i> Thailand	0.244	0.244	0.244	0.244	0.228	0.227	0.199	0.207	0.248	0.239	0.219	0.236	0.231	0.232	0.136	0.143	–		
18. <i>Ra</i> USA	0.270	0.270	0.270	0.270	0.266	0.215	0.227	0.219	0.232	0.235	0.240	0.215	0.235	0.223	0.147	0.150	0.097	–	

^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: *Ai*, *Amblyomma testudinarium*; *Ah*, *A. helvolum*; *Ag*, *A. geomyzae*; *Al*, *A. longirostre*; *Ac*, *A. calcaratum*; *Ad*, *A. dubitatum*; *An*, *A. nitidum*; *Ai*, *A. incisum*; *Ab*, *A. braziliense*; *Ac*, *A. coelebs*; *Da*, *Dermacentor andersoni*; *Dv*, *D. variabilis*; *Rs*, *Rhipicephalus sanguineus*; *Ra*, *R. appendiculatus*

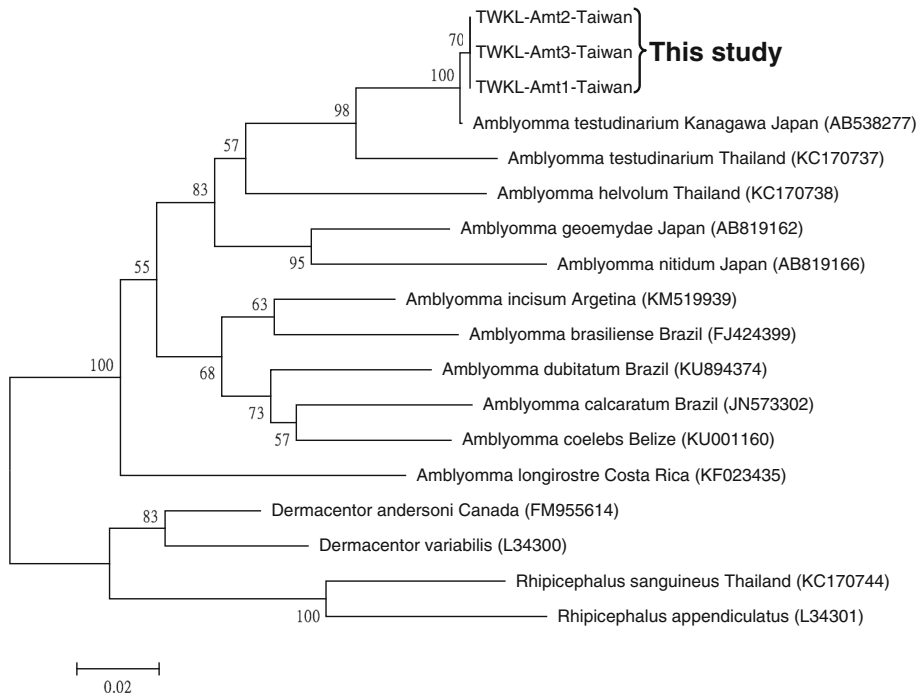


Fig. 4 Phylogenetic relationships based on the 16S ribosomal DNA (rDNA) gene sequences between three strains of *Amblyomma testudinarium* ticks from Taiwan, two strains of *Am. testudinarium* from Japan and Thailand, and nine other strains belonging to nine species of *Amblyomma*. Two species of *Dermacentor* and *Rhipicephalus* ticks 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

exotic ticks into Taiwan from its adjacent Countries. Indeed, accidental transportation of *Am. cordiferum* and *Aponomma varanense* ticks on a king cobra snake from Malaysia to Taiwan has also been reported (Norval et al. 2009). Thus, the geographical dissemination of *Am. testudinarium* tick throughout the eastern Asia is highly anticipated. Further investigation on the identification of *Am. testudinarium* tick in those areas may facilitate our understanding of geographical distribution of *Am. testudinarium* ticks in eastern Asia.

The natural cycle of life stages of *Am. testudinarium* ticks in Taiwan remains unknown. This tick species is known as a reptilian tick in northeastern Asia, and most reported collections are from snakes. Indeed, previous studies indicated that eight species of snakes have been found parasitized by this tick species and this distinctive species is almost exclusively a reptile tick (Suh et al. 2016). In this report, all these adult females and males of *Am. testudinarium* ticks were collected from infested humans throughout the mainland Taiwan. However, there is no collection of larval and nymphal stages of *Am. testudinarium* for further identification. Because the feeding habitat for the snake depends mainly on catching of rodents or small mammal. Thus, it is postulated that reptilian host of snakes and small mammal of rodents may serve as infested hosts for adult and immature stages of *Am. testudinarium* in Taiwan, respectively. Further investigation focused on the seasonal

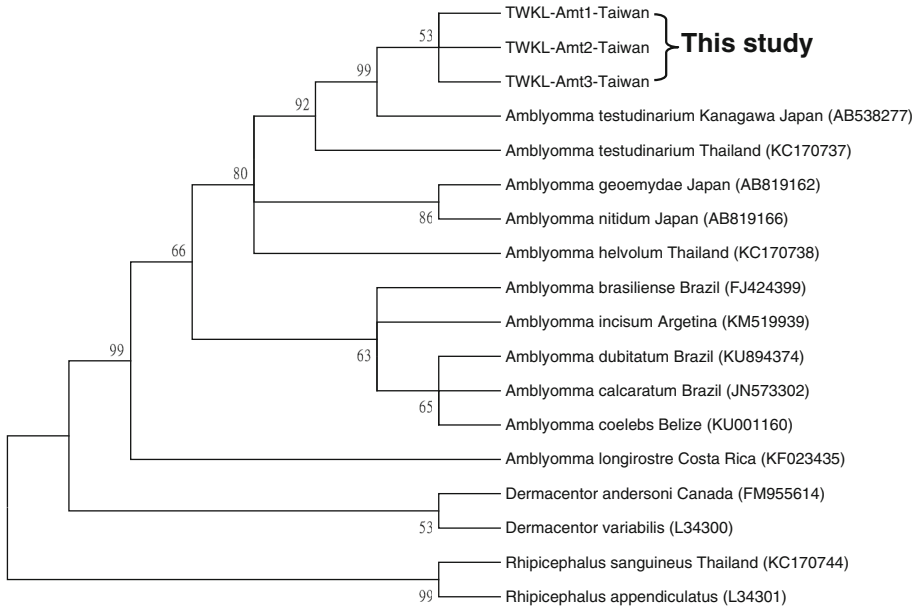


Fig. 5 Phylogenetic relationships based on the 16S ribosomal DNA (rDNA) gene sequences between three strains of *Amblyomma testudinarium* ticks from Taiwan, two strains of *Am. testudinarium* from Japan and Thailand, and nine other strains belonging to nine species of *Amblyomma*. Two species of *Dermacentor* and *Rhipicephalus* ticks 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

prevalence and the host specificity of various stages of *Am. testudinarium* ticks would help to reveal the developmental cycle of *Am. testudinarium* ticks in Taiwan.

In conclusion, this study describes the first report of feeding activity of *Am. testudinarium* tick on humans and provides the first genetic identification of the mitochondrial 16S rDNA gene of *Am. testudinarium* ticks collected from the mainland Taiwan. Further study focused on the feeding habitat and vectorial capacity of this distinctive species of *Am. testudinarium* tick may enhance our understanding of the life cycle and epidemiological feature of tick-borne pathogens transmitted by *Am. testudinarium* tick in Taiwan.

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