



First zootiological survey of *Amblyomma geoemydae* ticks (Acari: Ixodidae) infesting a wild turtle (*Cuora flavomarginata*) in northern Taiwan

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Abstract

The prevalence of *Amblyomma geoemydae* infesting yellow-margined box turtle (*Cuora flavomarginata*) was determined for the first time in northern Taiwan. In total, 352 ticks (140 adults, 61 nymphs and 151 larvae) were collected from 56 yellow-margined box turtles. The overall infestation was observed with an average density of 6.3 ticks per turtle and the highest seasonal prevalence was observed on March with an average tick density of 14.3 ticks per turtle. The higher seasonal prevalence was observed on April, May, July and October with an average density of 4.8, 8.3, 5.7 and 8.5 ticks per turtle, respectively. The seasonal activity of these turtles may contribute to the variation in the number of collected ticks. The genetic identity was determined by comparing the sequences of ITS2 genes obtained from seven Taiwan species of *A. geoemydae* and nine other species representing eight *Amblyomma* species and *Ixodes persulcatus* as outgroup. Our results provide the first zootiological survey of *A. geoemydae* infesting wild turtles in northern Taiwan and highlight the importance of this tick species in the study of parasite dynamics on testudines in Taiwan. Further study focused on the seasonality pattern of turtle ticks will help reveal the ecology of this host-parasite system and its impact on animal/human health.

Keywords *Amblyomma geoemydae* · Reptilian tick · *Cuora flavomarginata* · Turtle · Taiwan

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Introduction

Ticks are obligate blood-sucking arthropods that parasitize terrestrial vertebrate groups around the world (Guglielmone et al. 2014). Systematics and nomenclature studies of ticks have shown that the genus *Amblyomma* is the third largest genus of hard ticks (Guglielmone et al. 2020). Although ticks have been known to be associated with both humans and animals for hundreds of years (Balashov 1972), the global impact of ticks has been recognized by their ability to transmit a variety of tick-borne pathogens and by direct injury to livestock because of their feeding behavior (Jongejan and Uilenberg 2004). Their detrimental impact on humans and animals has become a major concern in medical and veterinary health.

Amblyomma geoemydae is a tick species first described from the tortoises of Bengal (Cantor 1847) and is widely distributed throughout Asia, ranging from South India to the Philippines, Vietnam, Myanmar, Japan China and Taiwan (Yamaguti et al. 1971; Robbins et al. 2006; Robbins and Platt 2011; Sun et al. 2016; Takahashi et al. 2017; Chao et al 2022; Amarga et al. 2022). It has been reported as a common ectoparasite of several species of reptiles, including elongated tortoise (*Indotestudo elongata*), Asian yellow pond turtles (*Mauremys mutica kami*) and yellow-margined box turtles (*Cuora flavomarginata*) (Takano et al. 2012; Bilbija et al. 2019; Kaenkan et al. 2020; Qiu et al. 2021; Chao et al 2022; Amarga et al. 2022). Although this reptilian tick has been recognized as a potential vector for the natural transmission of *Rickettsia*, *Ehrlichia* and *Borrelia* pathogens in wild environment (Takano et al. 2012; Kaenkan et al. 2020; Qiu et al. 2021), very little is known about its biology or life cycle in Taiwan.

The yellow-margined box turtle (*Cuora flavomarginata*) was first described as *Cistoclemmys flavomarginata* from material collected from China and Taiwan (listed as Formosa) (Gray 1863) and is found in various provinces along with the Yangtze drainage of Central China (Hsu 1930). Ernst and Lovich (1990) reported this species as *Cuora evelynae*, a new species of *Cuora* from the Ryukyu Islands of Japan, but this name was later moved as synonym of *C. flavomarginata*. This species is an ectothermic terrestrial reptile that commonly habitats the low-elevation forested hills (Zhao 1998; Lin et al. 2010). In Taiwan, *C. flavomarginata* mainly feeds on earthworms, snails, slugs and mealworms (Chen and Lue 1999, 2002). The adult yellow-margined box turtle has a highly domed shell, the carapace and plastron of which are dark brown with a cream-yellow strip on the vertebral keel. The edge of the plastron is lightly pigmented due to the marginal and plastral scutes near their edges. The external difference between male and female *C. flavomarginata* is slight, males have a broader tail, whereas females have a thinner tail that is almost triangular in shape. This species is recognized as endangered and protected species in Taiwan as well as listed as endangered under the criteria of the IUCN (<https://www.iucnredlist.org>).

Because of climatic and geographical factors, the tick species infesting wild hosts may not be the same around the world (Guglielmone et al. 2014). Although the broad geographical distribution of *A. geoemydae* has been known to exist from southeastern to eastern Asia, the prevalence of this tick species feeding on specific host has never been investigated in Taiwan. Thus, the objectives of this study intend to demonstrate the seasonal prevalence of *A. geoemydae* infesting *C. flavomarginata* in northern Taiwan and identify the collected turtle ticks using the internal transcribed spacer gene (ITS2).

Materials and methods

Collection of tick specimens from turtles

Ticks infesting yellow-margined box turtle were collected every month from April 2021 to March 2022 at the reservation area around the Fei-tsui Reservoir located in the Shiding District of New Taipei City in northern Taiwan (Fig. 1). In general, ticks were collected from the legs, head, body and shell of the turtles and cleaned by sonication in 75% ethanol solution for 5–10 min and then washed twice in sterile distilled water. All cleaned ticks were stored at -20°C until further analysis (Chao et al. 2022).

Species identification of ticks

Each tick developmental stage was placed on a glass slide and photographed using a SMZ 1500 Nikon stereomicroscope equipped with a fiber lamp (Chao et al. 2013). The external features of the *Ambloymma* ticks were recorded for species identification, as previously described (Yamaguti et al. 1971; Teng and Jiang 1991; Chao et al. 2022). In addition, molecular identification of collected ticks was also performed by targeting the ITS2 gene, as previously used by Chao et al. (2011).

DNA extraction from tick specimens

Genomic DNA was extracted from individual ticks. Briefly, the individual tick was homogenized in a microcentrifuge tube filled with 180- μl lysing buffer solution (DNeasy Blood & Tissue Kit; Qiagen, Taipei, Taiwan) and then homogenized with a

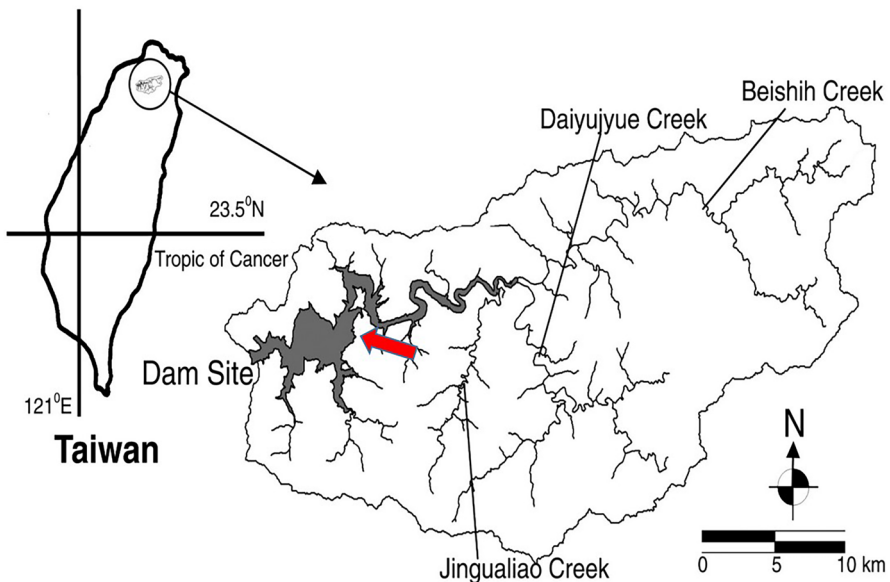


Fig. 1 Map of Taiwan showing the tick collection site of the Fei-tsui Reservoir in northern Taiwan

TissueLyser II apparatus (Qiagen, Hilden, 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. After filtration, the filtrated fluid was collected and the DNA concentration was determined spectrophotometrically with a DNA calculator (Epoch, microplate spectrophotometer; BioTek-Agilent, Santa Clara, CA, USA) and the extracted DNA is stored at $-80\text{ }^{\circ}\text{C}$ for further investigations.

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 3SA (50-CTAAGCGGTGGATCACTCGG-30) corresponding to the 3' end of the 5.8S rDNA and JB9A (50-GCACTATCAAGCAACACGACTC-30) corresponding to the 5' end of the 28S rDNA was designed to target the ITS2 rDNA gene, as described previously (Barker 1998). All PCR reagents and Taq polymerase were obtained and used as recommended by the supplier (Takara Shuzo, Kyoto, Japan). Briefly, each 25- μl reaction mixture contained 3- μl DNA template, 1.5- μl forward and reverse primers, 2.5- μl 10 \times PCR buffer (Mg^{2+}), 2- μl dNTP mixture (10 mM each), 1 unit of Taq DNA polymerase and filled-up with adequate volume of ddH₂O. In contrast, adequate amounts of sterile distilled water were added for serving as a negative control. PCR amplification was performed with a Veriti thermocycler (Applied Biosystems, Taipei, Taiwan) and was amplified for 35 cycles with the conditions of denaturation at 95 $^{\circ}\text{C}$ for 30 s, annealing at 50 $^{\circ}\text{C}$ for 30 s, and extension at 68 $^{\circ}\text{C}$ for 2 min., as described previously (Chao et al. 2011). 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 100-bp GeneRuler DNA ladder (Thermo Fisher Scientific, 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

Approximately 10- μl of each selected samples with clear bands on the agarose gel was submitted for DNA sequencing (Mission Biotech, Taipei, Taiwan). After purification (QIAquick PCR Purification Kit), sequencing reaction was performed with 25 cycles under the same conditions and same primer set 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 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 ITS2 genes from seven tick species of Taiwan were analyzed by comparing with nine other tick species from different biological and geographical origins available from GenBank. Phylogenetic analysis was performed by neighbour-joining method to estimate the phylogeny of the entire alignment using MEGA X software package (Kumar et al. 2018). The phylogenetic tree was constructed and performed with 1000 bootstrap replications to evaluate the reliability of the construction, as described previously (Felsenstein 1985).

Results

Collection of various life-stages of *Amblyomma geoemydae* from turtles

In total, 352 tick specimens representing 99 females, 41 males, 61 nymphs and 151 larvae were collected from 56 turtles (Table 1). Initially, yellow-margined box turtles were captured from the wet-bottomed forest (Fig. 2A) at the reservation area around the Feitsui Reservoir in northern Taiwan. The attached ticks were found on the turtles' carapace (Fig. 2B), neck (Fig. 2C), edge of the plastron (Fig. 2D) and leg (Fig. 2E) of the turtle. A magnified view of a female tick revealed a body length of 8–9 mm with the scutum covering less than half of the body (Fig. 2F).

Monthly prevalence and infestation of *Amblyomma geoemydae* on turtles

The monthly prevalence of *A. geoemydae* infesting turtles was recorded from April 2021 to March 2022 (Fig. 3). The total monthly number of collected ticks ranged from 114 (March; 59 larvae, 24 nymphs, and 31 adults) to two (December; 2 adults) (Table 2; Fig. 3). No ticks were collected in the winter months (January and February) to prevent disturbance of the turtles (Fig. 3). The overall average infestation was 6.29 ticks per turtle (all stages included), ranging from 14.25 ticks per turtle (March) to two ticks per turtle (December, but just a single turtle was examined this month) (Table 2). Higher seasonal mean abundance was observed in April, May, July and October with an average density of 4.82, 8.33, 5.67 and 8.50 ticks per turtle, respectively (Table 2).

Genetic and phylogenetic analysis of tick specimens

The sequences of ITS2 rDNA fragments of seven representative samples used in the study were aligned and compared with the downloaded sequences of eight *Amblyomma* species and one *Ixodes* outgroup species (*Ixodes persulcatus*) from GenBank. The aligned nucleotide sequences of 789 bp long of *A. geoemydae* were highly conserved between the seven Taiwan samples with only a few point mutations/substitutions. All these *A. geoemydae* of Taiwan were genetically affiliated to a monophyletic group with highly homogeneous sequences (99.2–100% similarity), and can be distinguished from the other lineage group of *Amblyomma* spp. and the outgroup *Ixodes persulcatus* (Fig. 4).

Table 1 Field collection of various life-stage of *Amblyomma geoemydae* ticks from yellow-margined box turtles (*Cuora flavomarginata*) in northern Taiwan

Sex of turtle	No. of live ticks collected				Total (%)
	Larval	Nymph	Adult		
			Male	Female	
Male	44	17	20	32	113 (32.1)
Female	107	44	21	67	239 (67.9)
Total (%)	151 (42.9)	61 (17.3)	41 (11.7)	99 (28.1)	352 (100)



Fig. 2 Field micrographs of *Amblyomma geoemydae* ticks observed on the yellow-margined box turtle, *Cuora flavomarginata*. **A** A turtle in the wet-bottomed forest at the Fei-tsui Reservoir. **B** Adult ticks attached to the turtle's carapace. **C** A partially engorged adult female tick on the neck of a box turtle. **D** Adult ticks attached to the edge of the plastron, **E** Adult tick attached to a leg. **F** An attached female with the scutum covering less than half of the body

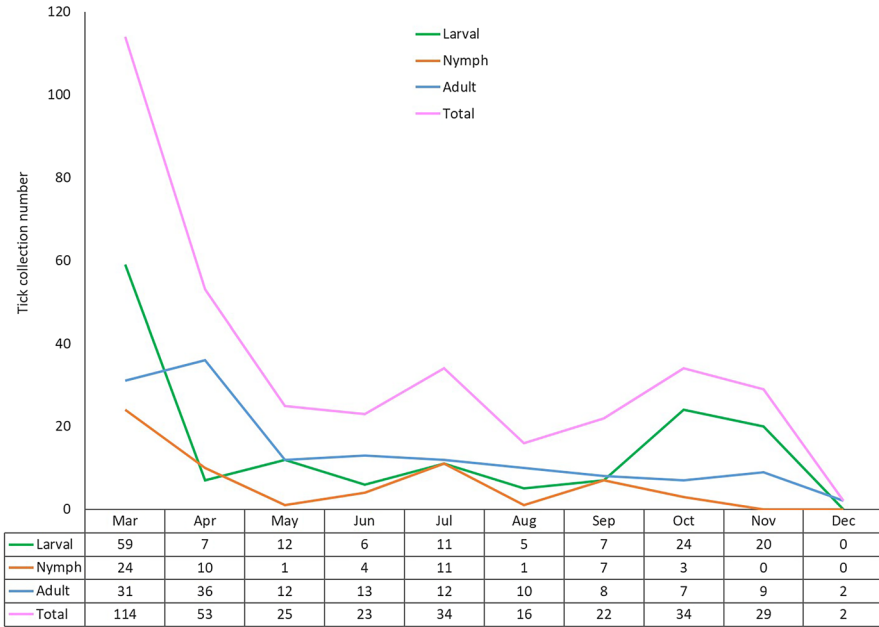


Fig. 3 Monthly prevalence of various life-stages of *Amblyomma geoemydae* ticks infesting wild *Cuora flavomarginata* turtles in northern Taiwan

Table 2 Monthly infestation and density of *Amblyomma geoemydae* ticks infested on the yellow-margined box turtles (*Cuora flavomarginata*) in northern Taiwan

Collection month ^a	Tick infestation and density on turtle host		
	No. of ticks collected	No. of turtles examined	Average density (No. ticks/turtle)
March	114	8	14.25
April	53	11	4.82
May	25	3	8.33
June	23	7	3.29
July	34	6	5.67
August	16	4	4.00
September	22	5	4.40
October	34	4	8.50
November	29	7	4.14
December	2	1	2.00
Total	352	56	6.29

^aTo prevent disturbance of turtles, no ticks were collected during the winter months (January and February)

Nucleotide sequence accession numbers

The nucleotide sequences of PCR-amplified ITS2 genes of seven strains of *A. geoemydae* of Taiwan determined in this study have been registered and assigned the following GenBank

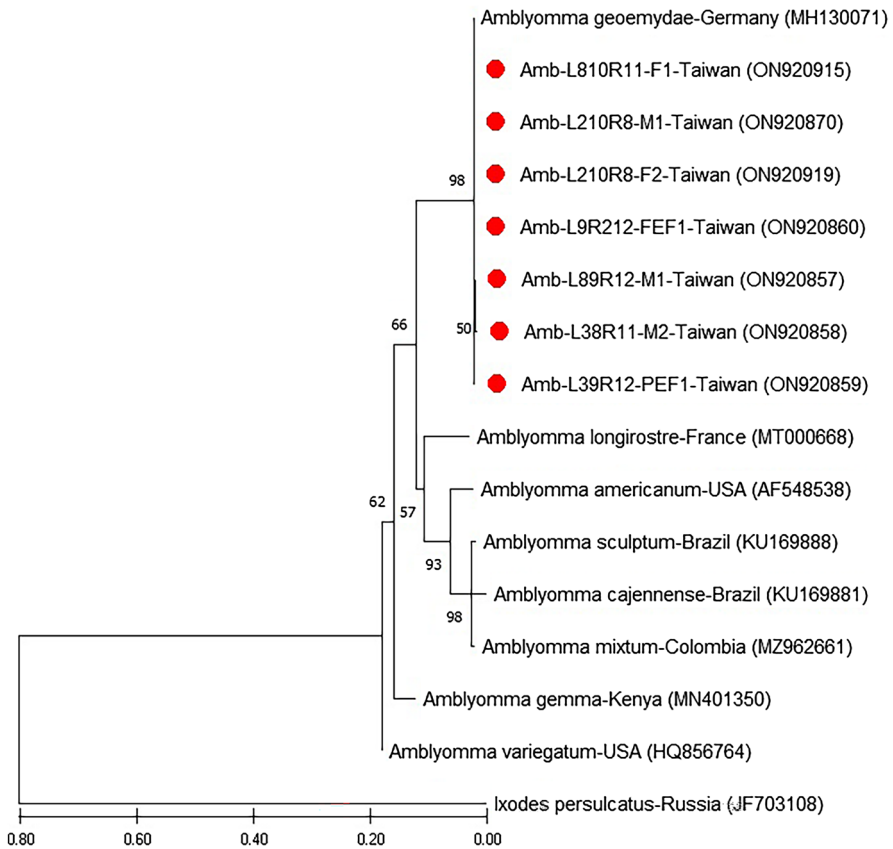


Fig. 4 Phylogenetic relationships based on ITS2 gene sequences between seven Taiwan strains (samples of *Amblyomma geoemydae* ticks from northern Taiwan and nine other species belonging to (eight *Amblyomma* spp. and one *Ixodes* species as outgroup). The tree was constructed and analyzed by neighbor-joining method using 1000 bootstraps replicates. Numbers at the nodes indicate the reliability (%) of each branch of the tree. Branch lengths are drawn in proportion to the estimated sequence divergence

accession numbers: Amb-L810R11-F1 (ON920915), Amb-L210R8-M1 (ON920870), Amb-L210R8-F2 (ON920919), Amb-L89R12-M1 (ON920857), Amb-L38R11-M2 (ON920858), Amb-L39R12-PEF1 (ON920859), and Amb-L9R212-PEF1 (ON920860). The GenBank accession numbers of the eight other *Amblyomma* species and the outgroup *Ixodes* are shown in Fig. 4.

Discussion

This study described the first year-round survey of *A. geoemydae* infesting *C. flavomarginata* in northern Taiwan. All life-stages of this tick could be collected almost every month from this turtle host. The highest collection of larval, nymph, and adult ticks was observed in March and April, which is the spring season in northern Taiwan. This observation may be attributed to the availability of turtle hosts in the spring which is highly favorable for the tick feeding. Reduced activity of these turtles in the field was

observed during the winter season (December to February), which probably explains the low/no collection of *A. geoemydae* in this survey in December. Previous studies indicated that one family of snake and three families of tortoises are parasitized by *A. geoemydae* (Takano et al. 2012; Takahashi et al. 2017; Bilbija et al. 2019; Kaenkan et al. 2020; Qiu et al. 2021). Thus, further investigation on the diverse hosts for *A. geoemydae* in the field would help to reveal the natural life cycle of *A. geoemydae* in northern Taiwan.

Although *A. geoemydae* is known as a naturally inhabited species in Taiwan, the ecology and distribution of this tick species need to be further identified. In previous reports, the yellow-margined box turtle has been recognized as a suitable host for *A. geoemydae* in various countries (Yamaguti et al 1971; Robbins et al 2006; Takano et al 2012; Tsai et al 2012; Bilbija et al 2019; Qiu et al 2021; Chao et al 2022; Amarga et al 2022). However, this turtle is restricted to low-elevation forest hills in northern Taiwan, and the populations in lowland areas may have been extirpated by land development and habitat fragmentation (Lin et al 2010). Because of extensive habitat loss and commercial exploitation (such as burgeoning demand in the food and pet markets), *C. flavomarginata* is categorized as endangered species on the ICUN Red List. Further study focused on the seasonal dynamics of *A. geoemydae* on reptilian hosts will help reveal the ecology of this host-parasite system in Taiwan.

Although morphological features of the adult stage of hard ticks have traditionally been recognized in pictorial keys for species identification (Keirans and Clifford 1978; Sonenshine 1991), a DNA-based investigation provides the opportunity to discriminate the genetic variance at the individual base-pair level and gives much more direct measures of the genetic diversity between and within species of Ixodidae (Black and Roehrdanz 1998; Crosbie et al. 1998; Chigagure et al. 2000). Indeed, previous phylogenetic studies based on rDNA sequences of ITS2 region also demonstrated the heterogeneity among the closely related tick species (Zahler et al. 1997; Rich et al. 1997; Barker 1998; Fukunaga et al. 2000). Through genetic analysis, the turtle ticks collected in this study were identified as *A. geoemydae* with highly homogeneous sequences (99.2–100% similarity) associated with *A. geoemydae* from Germany (GenBank no. MH130071), and could be distinguished from the other *Amblyomma* spp. and the outgroup *Ixodes* sp. (Fig. 4). Thus, molecular analysis based on the genetic analysis of ITS2 region has made particular success for the identification and discrimination of taxonomically similar tick species.

In conclusion, this year-round survey reveals the seasonality pattern of *A. geoemydae* infesting yellow-margined box turtle in northern Taiwan. Further investigations focused on various tick-borne pathogens in this tick species would help to illustrate the epidemiological and geographical impacts of *A. geoemydae* on human/animal health in northern Taiwan.

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Author contributions LLC and CMS wrote the main manuscript text and prepared the figures and tables, THC worked on the field collection of ticks, and LLC performed the molecular analysis. All authors have read and agree its submission.

Declarations

Competing interests The authors declare no competing interests.

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