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Precise identification of different stages of a tick, *Ixodes granulatus* Supino, 1897 (Acari: Ixodidae)

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ABSTRACT

Objective: To identify different stages of *Ixodes granulatus* (*I. granulatus*) based on morphological characters prior to molecular identification which is significant for confirming and identifying the nymphal stages of *I. granulatus*.

Methods: A total of 14 individuals of adult, engorged and nymphal ticks collected from three different localities were examined morphologically using taxonomic keys, followed by PCR using cytochrome oxidase subunit I (*COI*). Clustering analysis based on *COI* sequences was carried out by constructing neighbor-joining and maximum parsimony tree to clarify the genetic variation and diversity of local *I. granulatus*.

Results: Based on external morphological characterizations, nine individuals (64.3%) were successfully identified as *I. granulatus*, while five individuals were recognized only as *Ixodes* sp. due to lack of morphological characters visible and development during that stage. Molecular analysis of local *I. granulatus* using *COI* gene revealed 93%–94% sequence homology from available sequence in GenBank and was in concordance with the morphological identification. Furthermore, a low intraspecific variation was observed among the species of *I. granulatus* collected from different localities (0%–3.7%).

Conclusions: These findings demonstrated for the first time the establishment of *COI* gene for identifying *I. granulatus* nymphal tick which is of paramount importance to the control of potential tick-borne infections in Malaysia. Moreover, this study provides evidence that a combination of morphology and molecular data was corroborated as an accurate tool for tick identification.

1. Introduction

Wherever present, ticks pose a threat to human and animals. In tropical countries, they are considered as the arthropods of medical and veterinary importance only second to mosquitoes [1]. They have the ability to transmit various pathogenic agents that are responsible for diseases and fatalities [2]. Approximately 896 tick species belong to three families, namely, Ixodidae, Argasidae and Nuttalliellidae that are described worldwide [3,4]. The genus *Ixodes* Latreille, 1795 is the largest genus in the Ixodidae, comprising 243 species [5,6]. *Ixodes granulatus* Supino, 1897 (*I. granulatus*) is an exclusively Asian species, ranging from Japan through Southeast Asia and westward to India and China [7]. As a kind of the most widespread species, the distribution of *I. granulatus* has been reported from various countries including Malaysia [8,9]. Further studies over many years in Peninsular Malaysia have also indicated all active stages of *I. granulatus* as the most common and abundant species infesting mammals especially rodents [10,11]. As a vector, *I. granulatus* transmits a number of pathogens that cause infectious diseases such as human babesiosis, disease caused by Langat virus and rickettsia, and tick typhus [12,13].

The conventional method to identify ticks is based on microscopic observation on external morphological

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characteristics [14]. Such an approach works well for adult ticks but not for the immature stages (larvae or nymphs) due to the lack of specific taxonomic keys for some genera and not fully developed characteristics [15]. Apanaskevich and Horak in their study on discrimination of *Hyalomma anatolicum anatolicum* and *Hyalomma anatolicum excavatum* reported that identification based on morphological characters such as color and size of the scutum of different stages of ticks are very weak [16]. In addition, species identification by morphological observation can be difficult especially when the physical characters of specimens are damaged during collection, and when the specimens are engorged with blood, or due to similarity in morphologies across different species [17,18]. To overcome these difficulties, an approach using molecular DNA marker has been examined to evaluate the taxonomic status and identity of ticks. DNA-based methods provide an opportunity to determine intraspecific or intra-individual polymorphism of the sequences [13], providing much more useful information for the genetic characterization and differentiation of morphologically similar tick species [19]. PCR and online sequences databases such as GenBank are often used in molecular systematics and are now found useful for identifying a variety of medically important species of ticks.

Several DNA markers are routinely used for classification of ticks and studies of different species. Cytochrome oxidase subunit I (*COI*) gene was actively used as a molecular marker in the identification process of many arthropods due to the higher rates of molecular evolution that allows differentiation between closely allied species [20]. The performance of DNA barcoding in identifying tick species has been evaluated by many researchers [13,21]. Erster *et al*. tested *COI* gene to identify *Ixodes ricinus* (*I. ricinus*) on beef cattle [22], while Lv *et al*. assessed four DNA fragments including *COI* gene for species identification of Ixodidae [17]. This gene is encoded by mitochondrial genome which is much smaller than nuclear genome and has been considered easier to align because it is a protein coding sequence that has no gaps within alignment [23]. Proper species identification using molecular markers may offer rapid diagnosis of tick-borne infection as different tick species transmit different pathogens. This is an essential first step for preparedness of the nation to face and manage potential outbreaks of tick-borne infections in future.

I. granulatus ticks are commonly identified by the presence of a single morphological feature which is coxa I with two short spurs whereby the internal spur was slightly longer than the external spur [24]. To date, there is no such study on identification of *I. granulatus* ticks using well defined molecular approach in Malaysia. In order to provide a useful tool for accurate identification of tick, the objectives of this study were therefore to identify different stages of *I. granulatus* species according to the morphological characters, and verify the species status using molecular markers. Species differentiation and genetic species variation of *I. granulatus* were determined using clustering analysis based on *COI* data.

2. Materials and methods

2.1. Ticks sampling

On-host ticks at various developmental and feeding stages (nymph, adult, fully-engorged) were collected from three different localities in Peninsular Malaysia. The study sites were Hulu Langat in Selangor, Bukit Tinggi in Pahang and Gunung Tebu in Terengganu. The habitats chosen were mainly pristine tropical rainforest, secondary forests and shrubs. The localities were chosen based on the records from available previous data of high numbers of tick infestation on small animals [25]. Wire traps baited with bananas and oil palm fruits were used to capture wild rodents and tree shrews in each study site. Caught animals were placed in white cloth bags and brought back to the Institute for Medical Research for further processing. All experimental procedures involving animals were conducted in accordance to International Conference of Harmonization Good Clinical Practice Guidelines (Malaysian Research & Ethics Committee) and also approved by the Animal Use Committee of Ministry of Health Malaysia with reference number ACUC/KKM/02(6) 2009. The animals were anesthetized with chloroform before screening and the ticks were collected in the laboratory [11]. Each animal was examined in details under 20× magnification and any ticks found around the eyes, ears and any parts of the body were collected. The epidemiology data such as locality and host were recorded.

2.2. Tick morphological identification

A total of 14 on-host ticks were collected using sterile soft forceps or sharpened wooden applicator sticks. The ticks were then kept individually in vials containing 70% ethanol. The collected ticks were examined based on external morphological characteristics under a stereo microscope, model Stemi DV4 Zeiss (Germany) and the samples were preliminarily identified to genera and species levels where using specific illustrated morphological taxonomic keys is possible [24,26].

2.3. DNA extraction

Considering similar morphological features of these ticks, all different stages of the 14 ticks were subjected to molecular analysis. Prior to DNA extraction, each tick was individually washed three times with sterile distilled water. Extraction of DNA using QIAamp mini kit (Qiagen, Germany) was performed according to manufacturer's protocol. DNA of ticks was extracted by adding 80 μL of phosphate buffered saline and 100 μL of tissue lysis buffer into the sample. The ticks were then macerated using sterile tips for 5 min before adding 20 μL of proteinase K. The samples were incubated at 56 °C (6 h) for complete lysis. The following steps were the same as those in the manufacturer's protocols. The DNA was then used for subsequent PCR [27,28].

2.4. PCR amplification and DNA purification

A pair of universal primers designed by Folmer *et al*., namely, LCO1490 (5′GGTCAACAAATCATAAAGATATTGG3′) and HCO2198 (5′TAAACTTCAGGGTGACCAAAAATCA3′) was used to amplify *COI* gene using PCR for the ticks species [29]. The PCR reactions were conducted in a final volume of 50 μL containing 25 μL of 2× *Taq* PCR Master Mix, 2.5 μL of 0.5 μmol/L of each primer, 10 μL of nuclease free water and 10 μL of DNA template. The PCR was carried out using an Eppendorf Master Cycler Personal machine (Eppendorf, Germany). The amplification program consisted of a total of 35 cycles, denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1.5 min and final extension at

72 °C for 7 min, with an initial denaturation at 96 °C for 1 min. For each PCR reaction, a negative control containing deionized distilled water was included. The PCR products were visualized in 1.5% agarose gels sbtained with ethidium bromide and viewed under an ultraviolet trans-illuminator (wavelength at 254 nm). The PCR product was excised with a sterile gel cutter and purified using 5 Prime PCR Agarose Gel Extract Mini Kit (Hamburg, Germany) according to the manufacturer's protocols.

2.5. Sequencing and alignment analyses

All PCR products were then directly sent to a local sequencing service company, Medigene Sdn Bhd. in Petaling Jaya, Selangor, Malaysia. The sequencing was bi-directional for all specimens and the primers combination for this step was the same as that used in the PCR amplification. Sequencing results were exported as FASTA sequence files. The *COI* gene sequences of samples were aligned using ClustalW multiple alignment of BioEdit to determine the similarity of characters between the sequences [30]. In addition, seven *Ixodes* sequences that were available in GenBank were aligned simultaneously and implemented in dataset of *I. granulatus* as analysis background and species control (Table 1).

Table 1
DNA sequences obtained from GenBank implemented in the clustering analysis.

Sample name	Species	Locality	GenBank accession no.
I. ovatus	*I. ovatus*	Japan	AB231670
I. ricinus 118	*I. ricinus*	Switzerland	AY945422
I. ricinus 277	*I. ricinus*	Switzerland	AY945433
I. granulatus 1	*I. granulatus*	Japan	AB231673
I. granulatus 2	*I. granulatus*	China	JQ625686
I. granulatus 3	*I. granulatus*	China	JQ625690
I. granulatus 4	*I. granulatus*	China	JF758633

I. ovatus: *Ixodes ovatus*.

2.6. Basic local alignment search tool (BLAST) analysis

The obtained sequences were then compared with those available in the GenBank database using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) for identification of the species of ticks and detection of sequence contamination. This approach was reported to be simple and robust for rapid comparison of query sequences with database sequences leading to species identification [31]. The approach enabled the similarity of sequences to be measured depending on several criteria such as expected value, maximum identical, query coverage and maximum score [32].

2.7. Clustering analysis

The clustering analysis for all sequences of ticks was carried out to cluster the *I. granulatus* species using Phylogenetic Analysis Using Parsimony 4.0b10. For distance analysis, a neighbor-joining tree was generated from a Kimura's two-parameter distance matrix. Maximum parsimony (MP) analysis was performed to determine the most parsimonious tree(s) with a heuristic search of 1 000 replications using tree bisection and reconnection option for branch-swapping algorithm. The clustering analyses were set to 1 000 replications for both trees. In this study, *Argas persicus* (GenBank accession No. FN394341.1) was selected as an outgroup. An examination of the pairwise genetic distance was carried out based on Kimura's two-parameter test in Phylogenetic Analysis Using Parsimony.

3. Results

3.1. Morphological identification

A total of 14 on-host ticks (nymph, adult and fully engorged) were successfully collected from four species of hosts comprising *Leopoldamys sabanus* (*L. sabanus*), *Sundamys muelleri* (*S. muelleri*), *Rattus tiomanicus* and *Maxomys surifer* (*M. surifer*) (Table 2).

Table 2
List of locality, host and stages of tick samples used in this study.

ID code of tick	Locality	Host species	Tick species (morphological)	Stages
HL04-17	Malaysia:	*S. muelleri*	*I. granulatus*	Adult
HL04	Selangor,	*S. muelleri*	*Ixodes* sp.	Nymph
HL04-15	Hulu	*S. muelleri*	*Ixodes* sp.	Nymph
HL02-5	Langat	*L. sabanus*	*I. granulatus*	Adult
HL02-1		*L. sabanus*	*Ixodes* sp.	Nymph
GT23-2	Malaysia: Terengganu,	*L. sabanus*	*I. granulatus*	Fully engorged
GT23-8	Gunung Tebu	*L. sabanus*	*I. granulatus*	Fully engorged
BT03-6	Malaysia:	*L. sabanus*	*I. granulatus*	Adult
BT04-2	Pahang,	*S. muelleri*	*I. granulatus*	Adult
BT04-3	Bukit	*S. muelleri*	*I. granulatus*	Adult
BT07-3	Tinggi	*Rattus tiomanicus*	*I. granulatus*	Adult
BT09-2		*M. surifer*	*I. granulatus*	Adult
BT09-3		*M. surifer*	*I. granulatus*	Adult
BT09-4		*M. surifer*	*I. granulatus*	Adult

Most of the collected ticks were found on the upper and lower abdomens of the rodents, and some on the ears. Nine (64.3%) adult individuals of ticks were successfully identified up to the species level (*I. granulatus*) using specific taxonomic keys prior to verification using molecular approach for species confirmation and measuring the species variation. Three ticks at nymphal stages and two fully engorged ticks were only identified up to the genus level (*Ixodes*) based on external morphological characteristics; therefore those individuals were subjected to molecular identification.

3.1.1. External morphological characteristic

In this study, ticks from the genus *Ixodes* were identified primarily according to their distinct anal groove embracing the anus anteriorly, forming an arch (Figure 1A). Additionally, all *Ixodes* ticks lacked eyes and festoons and possessed an inornate scutum. The body shape was teardrop with a tapering at the mouthparts. On the idiosoma, a pair of spiracular plate or stigmata was identified behind the coxa IV (Figure 1B) with pores served as respiratory organ. For adult *Ixodes* ticks, mouthparts (gnathosoma) were visible dorsally. Identification of the examined adult *Ixodes* as *I. granulatus* was made based on the following combination characters: coxa I with two short spurs whereby the internal spur is slightly longer than the external one (Figure 2), and the gnathosoma is with club-shape palps.

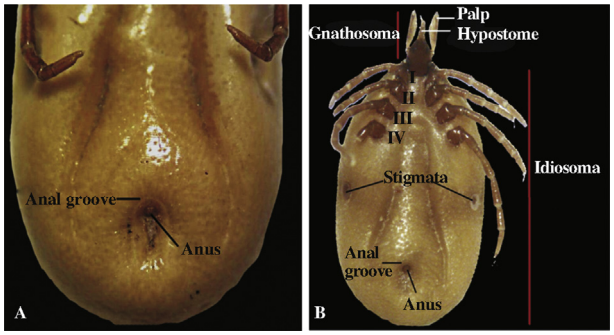

Figure 1. Ventral view of external morphological characteristics of adult tick. A: *Ixodes*; B: *I. granulatus*.

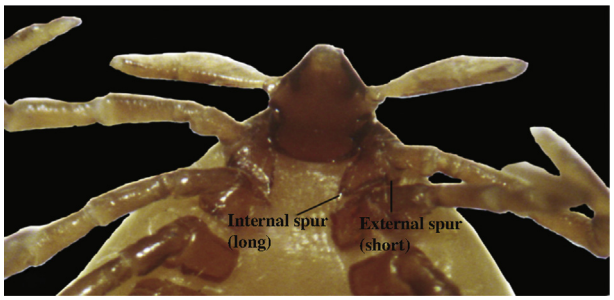

Figure 2. Ventral view of coxa I of *I. granulatus* with two spurs.

3.1.2. Difference in male and female tick

Sexual dimorphism was verified in this species. Male had smaller size than females and the dorsal scutum was well developed, covering almost all dorsal surfaces (Figure 3A). The dorsal shield or scutum of the female was finely granulated, oval, longer than its wide, covering more than half the length of the dorsal (Figure 3B). Partial scutum allows the increase in size for body engorgement in female ticks. A porose area with two small depressions consisting of numerous pores was noticed on the dorsal surface of the scutum. This porose area which is present only in female served as olfactory organs which become active during reproduction period.

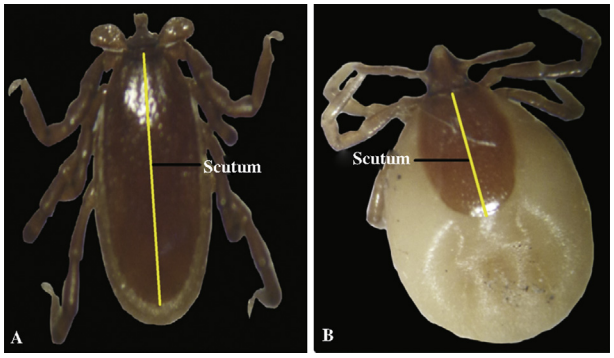

Figure 3. Dorsal view of the scutum of *I. granulatus*. A: Male; B: Female.

3.2. Molecular identification and clustering analysis

After alignment and trim, the *COI* sequences obtained from the ticks in Peninsular Malaysia were approximately 658 bp (including the primers) in length. BLAST results for the ixodid ticks consisted of one genera and one species, namely, *Ixodes* (*I. granulatus*). In general, the percent similarity between queried (unknown) sequences and the closest match in GenBank was between 93% and 94% (Table 3). Eleven out of 14 sequences (78.5%) showed 94% similarity with the online database while three sequences (GT23-2, GT23-8 and BT04-3) matched to *I. granulatus* with 93% similarity. A total of 502 bp fragments were obtained from the multiple alignments of *COI* gene. Sequence analysis indicated that 181 (36.1%) variable sites were detected within the *COI* gene and 86 (47.5%) characters were parsimony informative. Additionally, the conserved sites were constituted by 321 (63.9%) characters showing that *COI* gene is a very conserved gene in the mtDNA. Based on clustering analysis, neighbor-joining tree topology (Figure 4) revealed a distinction with 89% bootstrap value for *I. granulatus*,

Table 3
BLAST results against available sequences in GenBank.

ID code of tick	Tick species (morphology)	Stages	Sex	% Similarity with GenBank (species)
HL04-17	*I. granulatus*	Adult	Male	94 (*I. granulatus*)
HL04	*Ixodes* sp.	Nymph	Female	94 (*I. granulatus*)
HL04-15	*Ixodes* sp.	Nymph	Female	94 (*I. granulatus*)
HL02-5	*I. granulatus*	Adult	Male	94 (*I. granulatus*)
HL02-1	*Ixodes* sp.	Nymph	Female	94 (*I. granulatus*)
GT23-2	*I. granulatus*	Fully engorged	Female	93 (*I. granulatus*)
GT23-8	*I. granulatus*	Fully engorged	Female	93 (*I. granulatus*)
BT03-6	*I. granulatus*	Adult	Female	94 (*I. granulatus*)
BT04-2	*I. granulatus*	Adult	Female	94 (*I. granulatus*)
BT04-3	*I. granulatus*	Adult	Female	93 (*I. granulatus*)
BT07-3	*I. granulatus*	Adult	Female	94 (*I. granulatus*)
BT09-2	*I. granulatus*	Adult	Female	94 (*I. granulatus*)
BT09-3	*I. granulatus*	Adult	Female	94 (*I. granulatus*)
BT09-4	*I. granulatus*	Adult	Female	94 (*I. granulatus*)

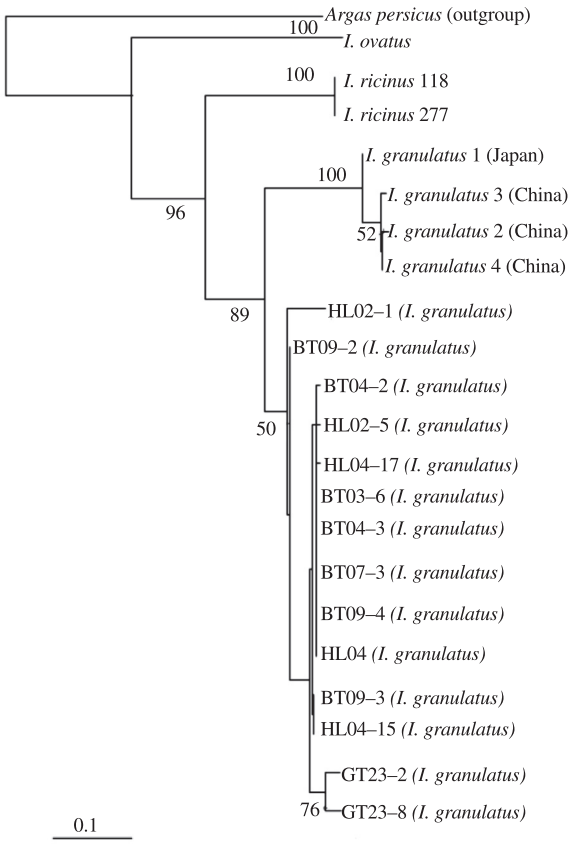

Figure 4. The neighbor-joining tree generated from 22 *COI* sequences (including 1 outgroup) of *I. granulatus* identified in the present study and related sequences from the GenBank.
The numbers at branches stand for bootstrap values of 1 000 replications.

but a higher bootstrap value of 99% was showed by MP analysis (Figure 5) which easily distinguished *I. granulatus* from *I. ovatus* and *I. ricinus*. Significant grouping of China and Japan *I. granulatus* ticks sequences in independent monophyletic subclade was obtained with a bootstrap value of more than 98% in both analyses. The results of this study strongly support the discrimination in recognizing the separation of *I. granulatus* ticks from Malaysia and other countries.

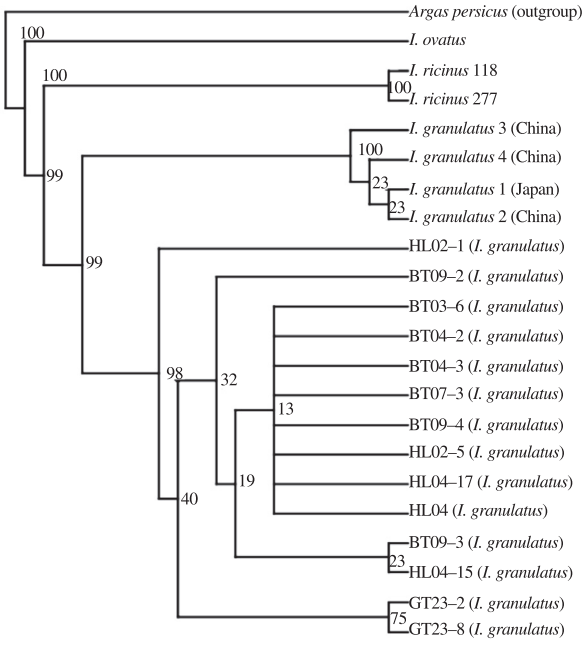

Figure 5. The MP tree generated from 22 *COI* sequences (including 1 outgroup) of *I. granulatus* identified in the present study and related sequences from the GenBank.
The numbers at branches stand for bootstrap values of 1 000 replications.

Pairwise distance analysis of *I. granulatus* ticks showed that the local species is genetically different from Japan and China species with genetic distance value ranged from 4.8% to 10.5% (Table 4). The highest genetic distance (3.7%) between *I. granulatus* was observed between those from Gunung Tebu (GT23-8) and Hulu Langat (HL02-1). There was no significant difference of genetic distance between Hulu Langat and Bukit Tinggi tick populations except for HL02-1 (2.8%). Genetic distance analysis of *I. granulatus* ticks collected from all the three localities indicated a low level of intraspecific value (< 3.7%). Although a low intraspecific variation was observed among the same species of *I. granulatus*, all the 14 local ticks appeared in a monophyletic group and formed a sister clade with *I. granulatus* from Japan and China. However, interspecific distances analyzed by the pairwise comparisons revealed that the local population of *I. granulatus* ticks genetically was closely related to *I. ricinus* compared to *I. ovatus* with level of genetic variation being 7.2%–13.1% and 17.8%–23.6%, respectively.

4. Discussion

4.1. Morphological identification

Morphological characters have been widely used for classifying and identifying diversity in acarology including ticks [33]. The general techniques were direct observation of external phenotype differences between individuals. In the present study, the morphological features observed suggest that ticks collected most probably belonged to the genus *Ixodes* and species *I. granulatus* [34]. The presence of this species in Malaysia was also reported from previous investigations [7,35]. Additionally, the most distinct external feature *i.e.* internal spur longer than external spur on coxa I of adult *I. granulatus* observed is similar to that described by Kohls [24]. The reliability of morphological features as criteria for the identification of adult *I. granulatus* was showed by several other studies [36,37]. However, there are multiple difficulties associated with accurate identification of immature stages and fully engorged tick. In this study, nymphs and fully engorged ticks were unable to be identified up to species level. The fully engorged ticks were difficult to be distinguished with naked eyes due to their appearance and color [14]. Moreover, when full-engorged they appear dark in color, similar in size and significantly distort some features [38]. Besides, species determination for immature stages such as nymph is limited

Table 4
Genetic distance values of the *COI* DNA sequences. %.

Species	*I. ovatus*	*I. ricinus*	*I. granulatus* (China)	*I. granulatus* (Japan)	BT 03-6	BT 04-2	BT 04-3	BT 07-3	BT 09-3	BT 09-4	GT 23-2	HL 02-5	HL 04-15	HL 04-17	HL 04	GT 23-8	HL 02-1	BT 09-2
BT03-6	20.6	11.6	8.4	7.1	–													
BT04-2	20.9	11.8	8.8	7.4	0.0	–												
BT04-3	20.6	11.6	8.4	7.1	0.0	0.0	–											
BT07-3	20.6	11.6	8.4	7.1	0.0	0.0	0.0	–										
BT09-3	20.4	11.3	8.0	6.9	0.0	0.0	0.0	0.0	–									
BT09-4	20.6	11.6	8.4	7.1	0.0	0.0	0.0	0.0	0.0	–								
GT23-2	22.3	12.0	9.9	8.0	1.8	2.0	1.8	1.8	0.0	1.8	–							
HL02-5	20.9	11.8	8.7	7.4	0.0	0.0	0.0	0.0	0.0	0.0	2.0	–						
HL04-15	20.4	11.3	8.0	6.9	0.0	0.0	0.0	0.0	0.0	0.0	2.0	0.0	–					
HL04-17	20.9	11.8	8.7	7.4	0.0	0.0	0.0	0.0	0.0	0.0	1.8	0.0	0.0	–				
HL04	20.6	11.6	8.4	7.1	0.0	0.0	0.0	0.0	0.0	0.0	1.8	0.0	0.0	0.0	–			
GT23-8	23.6	13.1	10.5	8.3	1.4	1.4	1.4	1.4	1.6	1.4	1.4	1.4	1.6	1.6	1.4	–		
HL02-1	20.4	9.4	10.1	7.2	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	3.7	–	
BT09-2	17.8	7.2	4.8	5.0	0.0	0.0	0.0	0.0	0.0	1.2	1.2	0.0	0.0	0.0	0.0	0.0	2.8	–

due to unavailable or lack of taxonomic keys except adults and not fully developed morphological characteristics [39,40]. Consequently, these difficulties and the reliability of morphological features as criteria for the identification of *I. granulatus* was reinforced in this study by using keys based on molecular genetic marker.

4.2. Molecular identification

The universal DNA primers, LCO1490 and HCO2198 [29], are frequently used in species identification and phylogenetic studies due to the ability to amplify successfully a 710 bp region of the mitochondrial *COI* gene from a broad range of metazoan invertebrates [41]. The universal primers for this gene are very robust, enabling recovery of its 5′ regions from representatives of most animals [20]. A similar study of genetic variability of *I. ricinus* based on analysis of *COI* mitochondrial DNA [42], concluded that this gene was considered to provide a better means to study differences between species within the same genus as well as confirm morphological identification. As demonstrated in previous studies [19,21,28], comparison between *COI* sequences is clear and direct because insertions and deletions (indels) are rare, hence the closely related species can easily be confirmed. Obtained *COI* gene sequences are the first reported DNA barcoding sequences of *I. granulatus* ticks collected in Peninsular Malaysia. Prior to this study, there were only four *COI* sequences for *I. granulatus* have been published in GenBank. Therefore, sequences obtained in the present study only revealed 93%–94% sequence homology compensating for this lack of information on genetic data in case of local *I. granulatus*. Some of the differences were probably caused by intraspecific variation and in some cases, it could be due to poor sequence quality, particularly when unassigned nucleotides (Ns) were present in the sequence. Variations in DNA sequences encoding *COI* among individuals of the same species were also common which explain the polymorphism of this marker [18]. It has been suggested that for molecular identification of tick species, sequencing of the *COI* gene should be the first method of choices, and analysis of other genes like 12S or 16S rDNA can be performed as complementary analysis [17,43,44]. The results of this study further demonstrated that the success of ticks DNA barcoding relies heavily on the accurate morphological identification to complement and verify molecular data.

4.3. Clustering analysis

Genetic and clustering analyses have been extensively used to identify species and to understand phylogenetic relationships of ticks in the past two decades [45–48]. The appearance of the adult stage of *I. granulatus* ticks in Southeast Asia provides similar morphological feature which is not visible with naked eyes. Hence, genetic characterizations with regard to the geographical distribution [13] of these species need to be further defined in understanding the nature of ticks for their effective control [18]. Findings of the present study have confirmed the identity of *I. granulatus* ticks as supported by the genetic clade together with the same species in China and Japan. Regarding to the genetic distance, a low intraspecific variation was observed among *I. granulatus* ticks collected from different localities (0%–3.7%), but a high interspecific value (7.2%–23.6%) with other species of the same genus was found. Thus, these observations suggest that genetic variation of *I. granulatus* of Peninsular Malaysia can be determined either by interspecies or intraspecies among tick population by analyzing the mitochondrial *COI* gene. Notably, the Japan and China ticks were separated from local *I. granulatus*, providing evidence that geographical differences could be factors that shape the patterns of genetic structure [28]. The results also showed that *I. granulatus* infested different species of rodents in different localities. Control of these rodents need to be proposed if local *I. granulatus* ticks were identified to cause any tick-borne infections.

The clustering analysis based on the *COI* sequences in this study showed a high genetic heterogeneity between local *I. granulatus* and other species of *Ixodes* ticks with the formation of distinct genetic assemblages. The clustering patterns of these ticks were according to their geographical origins, not the host species. This finding is agreeable with a previous study which reported that *I. granulatus* is not host specific because it can infest several hosts including rodents, as well as shrews, squirrels and also men [8,49]. A short-range migration of *I. granulatus* on host including rodents, could explain the low intraspecific values and the similarity of ticks from some localities in Peninsular Malaysia [7,11,37]. Moreover, tree topologies from different clustering analyses clearly indicated that the *I. granulatus* ticks from Hulu Langat and Bukit Tinggi showed close relationship compared to those from Gunung Tebu. Bukit Tinggi which is nearer to Hulu Langat (~50 km) may probably allow fast mobility pattern of host and contribute to close genetic relation of the ticks' species [50,51]. Fajs *et al*. in his study also reported that ticks and rodents from the same or nearby sampling site shared a high level sequence identity compared to ticks from different locations that showed high sequence divergence [52].

In conclusion, morphological taxonomy of different stages of *I. granulatus* was supported by the molecular data. Our study produced the first *I. granulatus COI* barcoding sequences from different localities in Peninsular Malaysia. Based on the clustering analysis of *COI* gene, both neighbor-joining and MP tree topology showed very clear distinction between *I. granulatus*, *I. ricinus* and *I. ovatus* with a highly supported monophyletic clade. The application of this molecular marker will be useful in studying geographical distribution, intraspecies and interspecies variation of *I. granulatus* in addition to further strengthening of morphological identification. There is a need to examine more samples of *I. granulatus* collected from all states of Malaysia in order to access the significance of its genetic variation and diversity in understanding the epidemiology of potential tick-borne diseases.

Conflict of interest statement

We declare that we have no conflict of interest.

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