Molecular identification of hard ticks (Ixodes sp.) infesting rodents in Selangor, Malaysia

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Siti Nabilah Ishak, Lim Fang Shiang, Farah Shafawati Mohd Taib, Khoo Jing Jing, Shukor Md Nor, Muhammad Afif Yusof, Shahrul Anuar Mohd Sah, Frankie Thomas Sitam, and Jeffrine Rovie Ryan Japning

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Molecular Identification of Hard Ticks (Ixodes sp.) Infesting Rodents in Selangor, Malaysia

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Abstract. This study aims to identify hard ticks (Ixodes sp.) infesting rodents in three different sites in Selangor, Malaysia using a molecular approach. A total of 11 individual ticks infesting four different host species (Rattus tiomanicus, Rattus rattus, Maxomys surifer and Sundamys muelleri) were examined based on its morphological features, followed by molecular identification using mitochondrial 16S rDNA gene. Confirmation of the species identity was accomplished by using BLAST program. Clustering analysis based on 16S rDNA sequences was carried out by constructing Neighbour-joining (NJ) and Maximum parsimony (MP) tree using MEGA 7 to clarify the genetic identity of Ixodes sp. Based on morphological features, all individual ticks were only able to be identified up to genus level as most of the samples were fully engorged, damaged and lacked morphological characters. However, molecular analysis of samples revealed 99% similarity with Ixodes granulatus from the GenBank database. Thus, the result of this study showed that all these ticks (Ixodes granulatus) were genetically affiliated to a monophyletic group with highly homogenous sequences.

Keys: Species identification, Ticks, Ixodes sp., Mitochondrial 16S rDNA, Ixodes granulatus

INTRODUCTION

Ticks are very important and harmful blood-feeding ectoparasites that have a worldwide distribution [1,2]. They parasitize a wide range of vertebrate hosts including mammals, birds and reptiles [3]. Ticks are important in order Acarina [4] and they have the ability to transmit a variety of pathogenic agent to humans and animals [5]. Ticks are divided into two major groups which are Ixodidae (hard bodied ticks) and Argasidae (soft bodied ticks) [6]. Today, there are approximately 899 tick species of Argasidae (185 species) and Ixodidae (713 species) [7]. The abundance and geographical distribution of Ixodes tick species have been recorded from various countries in Southeast Asia, Taiwan, Japan, and China [8-10]. The most recognized local species in Asian country is Ixodes granulatus Supino 1897 [8] and the distribution of this species has been reported from various countries including Malaysia [11-12]. This species may act not only as vector but also a reservoirs of tick transmitted pathogens including bacteria, rickettsia and protozoan [13]. Rodents (Order Rodentia) are small size mammals that comprise more species than any other mammal order [14]. Their abundance and distribution are widespread and often associated with ticks [15]. Because of their role as tick hosts, rodent serve as reservoir of tick-borne pathogens and other important diseases.
such as Lyme borreliosis, rickettsiosis, babesiosis, ehrlichiosis or tularaemia [16-17]. Recent studies have shown that small rodents are important hosts for several species of Ixodidae ticks including in Malaysia [11-12]. From their reservoir host perspective, rodents are known to act as a key ecological links in the transmission of tick-borne diseases [18]. Thus, ticks species identification and determination is crucial in order to develop a strategic control measures of ticks-borne diseases.

**MATERIALS AND METHODS**

**Ticks Sampling and Morphological Identification**

A total of 11 individual ticks were collected from three different sites in Selangor, Malaysia which are Hulu Perdik, Kg. Semungkis, and Sg. Congkak (TABLE (1)). One hundred collapsible cage traps were used to capture wild rodents in each sites. Traps were baited with oil palm fruits, sweet potatoes with peanut butter, salted fish and a special type of aromatic banana as this was shown to be the most effective bait to attract small mammals such as rodents, squirrels and tree shrews [19]. Trappings were checked once daily for 5 consecutive nights. All caught animals were placed in a sampling box and brought back to Animal House (Universiti Kebangsaan Malaysia) for screening process. All ticks were collected using sterile forceps and kept individually in vials containing 70% ethanol. The collected ticks were examined based on external morphological characteristics following identification keys [20-21].

**DNA Extraction**

Ethanol-preserved ticks underwent washing process where each individual ticks were rinsed with sterile distilled water. Before proceeding to the DNA extraction, all selected ticks were observed under stereo microscope to study its morphological characteristics. Extraction of DNA using MN Nucleospin Tissue Kit (Germany) was performed according to manufacturer’s protocol. DNA of ticks was extracted by adding 500 μl of phosphate buffered saline (PBS) into the sample. The samples were incubated at 56 ºC overnight. The following steps were conducted following the manufacturer’s protocols.

**PCR Amplification and DNA Purification**

A pair of specific primer set of 16S+1 (5’CTGCTCAATGATTITTTAAATTGCTGTGG-3’) and 16S-1(5’CCGGTCTGAACCTACAGATCAAGT-3’) were designed by [22] to target the mitochondrial 16S rDNA of ticks. The PCR reactions were conducted in a final volume of 20 μl containing 10 μl of PCR Master Mix (Promega, US), 6 μl of nuclease free water, 1 μl of 10 μmol/L of each primer and 2 μl of DNA template. The PCR was carried out using a Thermo Scientific Arktik Thermal Cycler (TC A0096). The protocol for the 16S rDNA gene amplification using 16+1/16S-1 was: initial denaturation (95 ºC for 5 min); followed by 10 cycles of 92 ºC for 1 min, 48 ºC for 1 min and 72 ºC for 1.5 min; 32 cycles of 92 ºC for 1 min, 54 ºC for 35 seconds, 72 ºC for 1.5 min, followed by final extension of 72 ºC for 7 min (460bp). For each PCR reaction, a negative control containing deionized distilled water was included. The PCR products were visualized in 1.4% agarose gels in Tris-Acetate-EDTA (TAE Buffer) and were visualized under UV light after staining with Gel Red Nucleic Acid Stain. The 100bp DNA ladder was used as a standard marker. The PCR products were then purified using QIAquick PCR Purification Kit (Qiagen, Germany) according to manufacturer’s protocol.

**Sequencing and Alignment Analyses**

Alignments of the sequences were done by using the MEGA software version 7 [23]. Pair wise distance analysis using the Kimura two-parameter was done to estimate the genetic distances among the sequences as in MEGA 7. Phylogenetic trees were constructed for 16S rDNA gene by using neighbour-joining (NJ) and maximum parsimony (MP) method as implemented in MEGA 7. Sequences of the *R. sanguineus*, *H. inermis* and the representatives of other species within the genus *Ixodes* were also obtained from the GenBank.
TABLE 1. List of study sites, host species and stages of ticks used in this study.

<table>
<thead>
<tr>
<th>ID Code</th>
<th>Study Sites</th>
<th>Host Species</th>
<th>Tick Species (Morphological)</th>
<th>Stages</th>
<th>Engorged</th>
</tr>
</thead>
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<td>426-HP05</td>
<td>Hulu Perdik</td>
<td>Rattus tiomanicus</td>
<td>Ixodes sp.</td>
<td>Adult</td>
<td>Engorged</td>
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<td>434-HP08</td>
<td>Hulu Perdik</td>
<td>Sundamys muelleri</td>
<td>Ixodes sp.</td>
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<td>Maxomys surifer</td>
<td>Ixodes sp.</td>
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<td>Rattus rattus</td>
<td>Ixodes sp.</td>
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<td>Engorged</td>
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<td>359-SC017</td>
<td>Sg. Congkak</td>
<td>Sundamys muelleri</td>
<td>Ixodes sp.</td>
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<td>Engorged</td>
</tr>
<tr>
<td>425-SC008</td>
<td>Sg. Congkak</td>
<td>Sundamys muelleri</td>
<td>Ixodes sp.</td>
<td>Adult</td>
<td>Engorged</td>
</tr>
</tbody>
</table>

RESULTS

In order to clarify the genetic identity of hard ticks (Ixodes sp.) in Selangor, the sequences of mitochondrial 16S rDNA fragments of 11 ticks specimens used in this study were aligned and compared with the downloaded sequences from the GenBank: (I. granulatus Taiwan DQ093287, I. dammini DQ093298, I. scapularis L48363, I. persulcatus L34295, R. sanguineus DQ093297 and H. inermis U95872). BLAST results for all specimens showed 99% similarity to Ixodes granulatus as showed in TABLE (2).

TABLE 2. BLAST sequences result against available sequences in the GenBank.

<table>
<thead>
<tr>
<th>ID Code</th>
<th>Study Sites</th>
<th>Host Species</th>
<th>Tick Species (Morphological)</th>
<th>% Similarity with GenBank (Species)</th>
</tr>
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<td>Ixodes sp.</td>
<td>I. granulatus (99%)</td>
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<td>Sundamys muelleri</td>
<td>Ixodes sp.</td>
<td>I. granulatus (99%)</td>
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</tbody>
</table>

The lengths of the aligned sequences varied from 405 to 454 bp and the nucleotide components indicate that 16S rDNA of these ticks is highly A-T rich with average nucleotide frequencies of Thymine (38.5%), Cytosine (8.1%), Adenine (37%) and Guanine (16.4%). The nucleotide sequences between the 11 I. granulatus of Selangor were highly conserved (67%) and the nucleotide variations within I. granulatus of Selangor were measured from 0 to 1.4% as shown in TABLE (3). Based on clustering analysis, NJ tree (Figure 1) revealed a distinction of 100% bootstrap value for I. granulatus and 99% in the MP tree analysis (Figure 2). Both NJ and MP tree showed that genus Ixodes formed its own monophyletic clade, separated from the R. sanguineus and H. inermis. Interspecific variation analysed by the pairwise genetic distance values reveal that populations of I. granulatus ticks can be distinguished from the other Ixodes ticks species and genetically distinct from the outgroup species R. sanguineus and H. inermis.
TABLE 3. Genetic distance values in percent of 16S rDNA sequences.

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<th>HP008</th>
<th>KS011</th>
<th>KS017</th>
<th>KS018</th>
<th>KS029</th>
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DISCUSSION

Molecular markers have been developed over the years to detect variation between taxa and proven their utility in systematic and evolutionary acarology [23]. Genetic and phylogenetic analyses based on DNA sequences could reveal species groups and assign unknown individuals to species [24], and infer the evolutionary relationship of ticks within and between species. DNA sequences have been successfully used to study phylogeny of Ixodid ticks [25-26]. Morphological examination identified the ticks collected in three different sites to one genus in the family Ixodidae (Ixodes sp.). Phylogenetic analysis of 16S rDNA gene verified the species identities of these ticks and further revealed the relationships of Ixodes granulatus. These findings are similar with the previous investigation where sequence analysis of 16S rDNA gene have been used to distinguished closely related tick species and to assess the phylogenetic relationships of the diverse Lyme disease vector ticks [9, 23, 27].

Regarding the genetic distance, a low intraspecific variation was observed among I. granulatus ticks collected from different sites (0%-1.42 %), but high interspecific value (1.14%-29.9%). Thus, these observations suggest that the genetic variation of I. granulatus of Selangor can be determined either interspecies or intraspecies among tick populations by analysing the mitochondrial 16S rDNA gene. Previous findings also found lower intraspecific genetic divergence among I. granulatus collected from Taiwan [9]. Because of the genetically high conservation and strictly maternal inheritance, the 16S rDNA gene sequences appear to provide a reliable and convenient method for distinguishing the lineages among diverse populations of Ixodes ticks [9,27].
FIGURE 1: Phylogenetic relationships of 17 mitochondrial 16S rDNA gene of four *Ixodes* species, rooted with the sequences of *Rhipicephalus sanguineus* and *Haemaphysalis inermis*.

FIGURE 2: Phylogenetic relationships of 17 mitochondrial 16S rDNA gene of four *Ixodes* species, rooted with the sequences of *Rhipicephalus sanguineus* and *Haemaphysalis inermis*. 
Findings from this study also demonstrated a high genetic heterogeneity between *I. granulatus* and other species of *Ixodes* ticks based on the formation of different major clades. The clustering patterns of these ticks is based on different geographical collections. Even though a low intraspecific variation was observed within the same species of *I. granulatus* ticks, all 11 sample ticks represented as a monophyletic group that can be distinguished from *I. granulatus* ticks from Taiwan. The phylogenetic trees constructed by NJ and MP analysis strongly support the discrimination in recognizing the separation of different lineage between *I. granulatus* ticks and other species of *Ixodes* ticks.

In conclusion, this report provides the genetic characterization of 16S rDNA gene of *I. granulatus* ticks collected from different study sites in Selangor. Based on the phylogenetic analysis of the 16S rDNA gene, all these *I. granulatus* of Selangor and Taiwan were genetically related to monophyletic group and were represented as a unique lineage distinguished from other *Ixodes* ticks. The results also showed that *I. granulatus* infested different species of rodents and host identified were *Rattus tiomanicus*, *Rattus ratus*, *Maxomys surifer* and *Sundamys muelleri*. Previous findings conducted in Malaysia also found that small rodents serve as important host of these species [11-12]. For further studies, it is recommended to use other molecular marker from wider study area to better understand the genetic identity of a local species, *I. granulatus* and population control of the host itself need to be considered to prevent any tick-borne infection and diseases.

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