DNA Barcodes of Tetragnathid Spiders (Araneae: Tetragnathidae) in Malaysia

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ABSTRACT

Although diverse groups of spiders are present in tropical countries, very little attention has been given on various types of differences that exist within these groups, particularly for the groups of tropical spiders in Malaysia. The morphological and genitalia characteristics are traditionally used to identify species variants. However, in this study, a molecular approach was utilized to produce a more precise and accurate result in an effort to identify, delineate and verify the species. Mitochondria-encoded cytochrome oxidase I (COI) and nuclear-encoded 18S rRNA (18S) genes were adopted to establish DNA barcodes for 17 species of tetragnathid spiders (Araneae, Tetragnathidae) in Malaysia. Generally, the molecular data of tetragnathid spiders was consistent with their classification based upon morphological characteristics, though species boundary of Opadometa grata and Leucauge decorate could not be resolved by 18S gene.

INTRODUCTION

The long-jawed, orb-weaver, spider family Tetragnathidae, contains 47 genera (World Spider Catalog, 2020) with at least 967 species throughout the world. It comprises three subfamilies, namely the Leucauginae Caporiacco 1955, Metaeinae and Tetragnathinae Menge 1866. The genus Tetranghatha Lateville 1804, is the genus type of this family (Alvarez-Padilla and Hormiga, 2014). Members from this family are diverse in morphological and behavioral characteristics. Many of these characteristics are homoplasius to Araneidae and Nephilidae (Griswold et al., 1998; Alvarez-Padilla and Hormiga, 2011). However, the morphological features within the family vary significantly, with some displaying distinctive characteristics that do not fit into the typical tetragnathid morphology. The body length of tetragnathids range between 2-23 mm. The habitats used by tetragnathid spiders in tropical and subtropical ecosystems are highly diverse, and include in low vegetation areas, in tree buttresses, at cave entrances, and near waterways (e.g. rivers, ponds) (Alvarez-Padilla, 2008; Dzulhelmi et al., 2014a, b; Dzulhelmi et al., 2017). However, some species appear to be confined to specific habitats, such as caves or mangroves (Koh and Ming, 2013; Dzulhelmi, 2016).

As with other tropical rainforests in Southeast Asia, Malaysia is home to many tetragnathid species (Barrion and Litsinger, 1995; Murphy and Murphy, 2000; Song et al., 2002; Jager, 2007; Jager and Praxaysombath, 2009, 2011; Jager et al., 2012; Koh and Ming, 2013; Dzulhelmi, 2016). Currently, there are at least six genera representing 32 species recorded in Malaysia. From these, six genera with 20 species are found in the Peninsular Malaysia (Norma-Rashid and Li, 2009; Dzulhelmi et al., 2014a), eight genera with eight species in Sarawak (Koh et al., 2013), and two genera with four species in Sabah (Dzulhelmi et al., 2014b). Only two known subfamilies, namely the Leucauginae and Tetragnathinae, are found in Malaysia. The subfamily Leucauginae is characterized by some specific modifications to the female genital system, such as a weakly sclerotized spermathecal wall (Alvarez-Padilla et al., 2009). This distinctively identifiable female physical characteristic has been extensively examined by researchers and has been established to be confined only to the species from the genera Leucauge, Opadometa, Mesida and Tylorida. On the other hand, the subfamily Tetragnathinae’s distinguishing characteristic is its lack of a sclerotized
plate and fertilization ducts, where only the copulatory ducts are found to be present (Alvarez-Padilla et al., 2009). In Malaysia, the genus Tetragnatha is the only genus that has been documented within the Tetragnathinae subfamily.

The genetics of tetragnathid species from different parts of the world have been well-studied (i.e. Levi, 1980; Hormiga et al., 1995; Pan et al., 2004; Blackledge et al., 2009; Alvarez-Padilla et al., 2009; Dimitrov and Hormiga, 2011). These previous studies adopted combination sets of different markers to identify the relationships between the studied species, but the determination of the most suitable and reliable markers for taxonomic identification is important to reduce the cost and time (i.e. Fang et al., 2000; Astrin et al., 2006). Nonetheless, the genetic data on Malaysian tetragnathid species is still unknown, with no molecular studies being reported to date.

The objective of this study was to determine the sequence diversity of two gene fragments (mitochondria-encoded cytochrome oxidase I (COI) and the nuclear-encoded 18S rRNA (18S)) in 17 Malaysian tetragnathid species, and thereby attempting to establish DNA barcodes for members of the subfamilies Leucauginae (Leucauge, Leucauginae, and Tylorida) and Tetragnathinae (Tetragnatha). The COI and 18S sequences were analyzed independently. The results were then cross-examined with the morphological characteristics of the regional tetragnathid species to categorize the specimens’ subfamily. The genetic information derived from the results of this molecular analysis would be highly useful for future species identification and taxonomic verification of Malaysian tetragnathids.

**MATERIALS AND METHODS**

**Sample collection and identification**

Random sampling was employed during the sample collection. Tetragnathid spiders were individually caught by hand following visual detection. The sampling was performed throughout the day and night, and was conducted at various types of diverse habitats available throughout Malaysia. The collected specimens were immediately preserved in individual jars containing 70% (v/v) ethanol, and subsequently stored in a freezer at -20°C.

For species identification, the preserved specimens were first observed under a 50× dissecting microscope (Amscope, USA and identified to species based on the morphology descriptions of the Leucauge and Opadometa (Yoshida, 2009; Dzulhelmi et al., 2015), Mesida (Jager and Praxaysombath, 2011), Tylorida (Tanikawa, 2004; Jager and Praxaysombath, 2009; Kulkarni, 2014) and Tetragnatha (Okuma, 1987, 1988) subfamilies, and further supported by other documented morphological references (Barrion and Litsinger, 1995; Murphy and Murphy, 2000; Song et al., 2002; Koh et al., 2013; Dzulhelmi and Suriyanti, 2015).

In this study, a total of 17 tetragnathid species were collected from 10 different locations in Malaysia (Table 1). The Leucauginae subfamily is represented by the genera Leucauge (five out of six species), Opadometa (two out of three species), Mesida (two out of two species) and Tylorida (three out of four species). The Tetragnathinae subfamily is represented by the genus Tetragnatha (five out of 16 species). The genus Larinioides cornutus and Gasteracantha cancriformis were used as the basis and main reference for the COI gene, while the genus Gasteracantha kuhlii and Cyclosa conica were used as the basis and main reference for the 18S gene. Both groups of main references used were obtained from the GenBank and were used as outgroups (Table 1).

**DNA extraction, polymerase chain reaction and sequencing**

For DNA extraction, two or more legs (depending on the spider’s size) were removed from the preserved specimen, rinsed several times with distilled water and then transferred into a 1.5-mL microcentrifuge tube containing lysis buffer and homogenized in liquid nitrogen for at least 5 min. Then, Proteinase K was added and the homogenized sample was incubated overnight at 65°C in a water bath before heating to 95°C for 10 min to deactivate the proteinase K. Finally, the genomic DNA was extracted from the resultant sample using the Qiagen DNeasy Tissue Kit according to the manufacturer’s recommended instructions.

**Amplification of the mitochondrial COI and nuclear 18S gene fragments**

For 18S gene fragment, the full length 18S region was amplified with the universal primer set 18Sα (3’-ATTAAAGTTGGCGGTCA-5’) and 9r (3’-GATTCCTTCGACGTTACCTAC-5’) (Alvarez-Padilla, 2008). The total reaction volume used was 50 µL, consisting of 10 µL of dH2O, 5 µL of each primer (0.5 mM), 25 µL of the master mix (Lucigen, USA) and 5 µL (ca. 250 ng) of the DNA template. ‘Touchdown’ condition for the PCR reaction was set at 94°C for 2 min, followed by 13 cycles of 94°C for 20 s, 60°C for 35 s and 65°C for 30 s and then 21 cycles at 94°C for 15 s, 48°C for 35 s and 65°C for 30 s and then a final 72°C for 3 min (Dzulhelmi, 2016).

For COI, the PCR amplification was performed in the same manner except using the universal forward primer LCO1490 (3’-GGTCACAAAAATCATATTGG-5’) and reverse primer HCO2198 (3’-TAACCTCAAGGGTGACCAAAAATCA-5’) (Alvarez-Padilla, 2008), and the thermal cycling was performed at 94°C for 2 min, followed by 34 cycles of...
94°C for 20 s, 50°C for 35 s and 65°C for 30 s, and then a final 72°C for 3 min (Dzulhelmi, 2016).

Each PCR-amplified sample was resolved by agarose gel electrophoresis mixed with ethidium bromide to enable viewing of the amplified nuclear of the 18S gene under ultra-violet illumination. Finally, the products were then sent to Genomics Bioscience and Technology Co. Ltd., for purification and sequencing.

Table 1.- Seventeen Tetragnathid species, localities, coordinates and GenBank accession numbers of specimens examined in this study.

| Species               | Locality                                | Coordinates                  | COI            | 18S            |
|-----------------------|-----------------------------------------|------------------------------|----------------|----------------|
| Leucauge argentina    | Penang National Park, Pulau Pinang      | 5°26′16″N, 100°17′27″E       | KU836866       | KU836900       |
|                       | Poring Hot Spring Nature Reserve, Ranau | 6°2′35″N, 116°42′7″E         | KU836869       | KU836901       |
|                       | Kubah National Park, Kuching            | 1°36′41″N, 109°11′44″E       | KU836868       | KU836899       |
|                       | Gading National Park, Kuching           | 1°41′27″N, 109°50′45″E       | KU836867       | KU836898       |
| Leucauge celebiana    | Poring Hot Spring Nature Reserve, Ranau | 6°2′35″N, 116°42′7″E         | KU836871       | KU836903       |
|                       | Mesilau Resort Nature Reserve, Ranau    | 6°0′5″N, 116°54′1″E          | KU836872       | KU836904       |
|                       | Kubah National Park, Kuching            | 1°36′41″N, 109°11′44″E       | KU836870       | KU836892       |
| Leucauge decorata     | Crocker Range National Park, Keningau   | 5°58′5″N, 116°08′2″E         | KU836874       | KU836905       |
|                       | Crocker Range National Park, Keningau   | 5°58′5″N, 116°08′2″E         | KU836873       | KU836896       |
| Leucauge sp.          | Gading National Park, Kuching           | 1°41′27″N, 109°50′45″E       | KU836875       | KU836907       |
| Leucauge tessellata   | Ulu Gombak Nature Reserve, Gombak       | 3°22′60″N, 101°47′20″E       | KU836876       | KU836909       |
| Opadometa grata       | Rimba Ilmu Botanical Garden, Kuala Lumpur | 3°7′29″N, 101°39′12″E       | KU836883       | KU836915       |
| Opadometa kuchingensis| Bako National Park, Kuching             | 1°41′8″N, 110°26′10″E        | KU836884       | KU836916       |
| Mesida gemnea         | Kubah National Park, Kuching            | 1°36′41″N, 109°11′44″E       | KU836879       | KU836910       |
|                       | Gading National Park, Lundu             | 1°41′27″N, 109°50′45″E       | KU836878       | -              |
|                       | Ulu Gombak Nature Reserve, Gombak       | 3°22′60″N, 101°47′20″E       | -              | KU836911       |
| Mesida yini           | Universiti Kebangsaan Malaysia, Bangi   | 2°55′47″N, 101°46′44″E       | KU836880       | KU836912       |
|                       | Universiti Kebangsaan Malaysia, Bangi   | 2°55′47″N, 101°46′44″E       | KU836881       | KU836913       |
| Tetragnatha hasselti  | Kubah National Park, Sarawak            | 1°36′41″N, 110°11′44″E       | KU836891       | -              |
|                       | Kubah National Park, Sarawak            | 1°36′41″N, 110°11′44″E       | KU836892       | KU836924       |
| Tetragnatha lauta     | Fraser Hill Forest Reserve, Pahang       | 3°43′7″N, 101°44′25″E        | KU836892       | KU836925       |
| Tetragnatha maxillosa | Fraser Hill Forest Reserve, Pahang       | 3°43′7″N, 101°44′25″E        | KU836893       | KU836926       |
| Tetragnatha pinicola  | Kuala Selangor Nature Park, Kuala Selangor | 3°20′16″N, 101°14′56″E   | KU836895       | KU836928       |
|                       | Kuala Selangor Nature Park, Kuala Selangor | 3°20′16″N, 101°14′56″E   | KU836896       | KU836929       |
| Tetragnatha sp.       | Bako National Park, Kuching             | 1°41′8″N, 110°26′10″E        | KU836897       | KU836930       |
| 1Tylorida striata     | Narativat Province, Thailand            | 5°47′45″N, 101°50′44″E       | EU003309       | -              |
|                       | Universiti Kebangsaan Malaysia, Bangi   | 2°55′47″N, 101°46′44″E       | -              | KU836919       |
| Tylorida tianlin      | Mesilau Resort Nature Reserve, Kungsang | 6°0′2″N, 116°54′1″E          | KU836885       | KU836917       |
|                       | Mesilau Resort Nature Reserve, Kungsang | 6°0′2″N, 116°54′1″E          | KU836886       | KU836918       |
| Tylorida ventralis    | Universiti Kebangsaan Malaysia, Bangi   | 2°55′47″N, 101°46′44″E       | KU836890       | KU836923       |
|                       | Universiti Kebangsaan Malaysia, Bangi   | 2°55′47″N, 101°46′44″E       | KU836891       | KU836924       |
|                       | Kuala Selangor Nature Park, Kuala Selangor | 3°20′16″N, 101°14′56″E   | -              | KU836920       |
|                       | Ulu Gombak Nature Reserve, Gombak       | 3°22′60″N, 101°47′20″E       | KU836888       | -              |
|                       | Fraser Hill Forest Reserve, Pahang       | 3°43′7″N, 101°44′25″E        | -              | KU836921       |
|                       | Bako National Park, Kuching             | 1°41′8″N, 110°26′10″E        | KU836887       | -              |
| 2Larinioides cornutus | Point Pelee, Ontario, Canada             | Unstated                     | JN308507       | -              |
| 3Gasteracantha cancriformis | Heredia province, Costa Rica           | 10°25′53″N, 84′00″13″W      | EU003287       | -              |
| 3Gasteracantha kuhlii | Unstated                                 | Unstated                     | -              | AB910478       |
| 3Cyclosa conica       | Mon Hunoso Lake, Denmark                | Unstated                     | -              | EU003343       |

1Alvarez-Padilla et al. (2009); 2Blagoev et al. (2016); 3Tanikawa et al. (2014).
Table II.- Genetic pairwise distance (%) of the COI gene sequences among the 17 tetragnathid species \textit{(Leucauge} (5 species), \textit{Mesida} (2 species), \textit{Opadometa} (2 species), \textit{Tetragnatha} (5 species) and \textit{Tylorida} (3 species)) plus the two outgroups (\textit{Larinioides cornutus} and \textit{Gasteracantha cancriformis}). The distances were calculated by using the Kimura-two-model (Kimura, 1980).

| Species                         | 1  | 2       | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 |
|---------------------------------|----|---------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| \textit{Tetragnatha pinicola}   | 0.00-| 0.32   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| \textit{Tetragnatha maxillosa}  | 14.24-| 0.00-  | 15.04 | 1.28 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| \textit{Tetragnatha lauta}      | 15.2-| 14.88-   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| \textit{Tetragnatha hasselti}   | 18.4-| 18.88-   | 18.56 | 19.04 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| \textit{Tetragnatha sp.}        | 14.88-| 15.68  | 16.48 | 17.28 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| \textit{Mesida gemmea}          | 17.28-| 18.08-  | 19.84- | 22.24- | 19.68- |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| \textit{Mesida yini}            | 17.76 | 19.36  | 20.00 | 23.84 | 19.84 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| \textit{Tylorida striata}       | 17.44-| 18.72-  | 20.48- | 22.40- | 19.36- | 15.04- | 1.12 |    |    |    |    |    |    |    |    |    |    |    |    |
| \textit{Tylorida ventralis}     | 17.92 | 19.68  | 20.96 | 22.72 | 15.84 |    |    |    |    |    |    |    |    |    |    |    |    |    |    |
| \textit{Leucauge celebesiana}   | 16.60 | 19.52-  | 19.84 | 24.32 | 19.68 | 16.16- | 14.08- | 19.16 | 16.48 | 14.24 |    |    |    |    |    |    |    |    |
| \textit{Leucauge decorata}      | 16.40 | 18.80  | 19.20 | 22.72 | 19.20 | 16.48 | 17.44 | 16.16 | 4.64 |    |    |    |    |    |    |    |    |    |
| \textit{Opadometa grata}        | 14.24-| 15.84-  | 16.64- | 18.88- | 16.16- | 16.32- | 16.00- | 15.52- | 0.16 |    |    |    |    |    |    |    |    |
| \textit{Opadometa kuchingensis} | 14.56 | 16.32-  | 16.80- | 19.20 | 16.48 | 16.80 | 16.64 | 16.32 | 16.16 | 0.32 |    |    |    |    |    |    |    |
| \textit{Leucauge tessellata}    | 16.00 | 14.88-  | 17.28 | 19.84 | 16.96 | 16.32- | 17.44- | 17.60 | 16.00- | 9.44 |    |    |    |    |    |    |    |
| \textit{Leucauge sp.}           | 16.16 | 15.52 | 16.64 | 17.76 | 16.80 | 9.60 |    |    |    |    |    |    |    |    |    |    |    |
| \textit{Tylorida tianlin}       | 14.56-| 16.32-  | 16.96 | 19.36 | 15.52 | 15.84- | 17.12 | 16.64 | 16.80 | 10.40 | 11.68 |    |    |    |    |    |    |
| \textit{Leucauge argentina}     | 14.72 | 16.74 | 16.64 | 16.96 | 19.36 | 15.68 | 16.64 | 17.12 | 16.64 | 16.96 | 10.72 | 11.68 | 0.32 | 0.32 |    |    |
| \textit{Larinioides cornutus}   | 16.48 | 16.80-  | 18.40 | 18.72 | 16.48 | 17.28 | 17.44 | 16.48 | 8.96 | 11.68 | 10.24 | 10.24 |    |    |    |    |    |
| \textit{Larinioides cornutus}   | 16.28 | 18.08 | 18.24 | 18.24 | 9.12 |    |    |    |    |    |    |    |    |    |    |    |    |
| \textit{Leucauge sp.}           | 15.36 | 16.48 | 18.72 | 20.00 | 17.44 | 15.68- | 16.48- | 17.44 | 17.12 | 11.84 | 12.96 | 13.44 | 13.44 | 10.40 |    |    |
| \textit{Tylorida tianlin}       | 15.68 | 16.00 | 17.12 | 17.76 | 12.00 |    |    |    |    |    |    |    |    |    |    |    |    |
| \textit{Leucauge argentina}     | 17.76 | 16.16- | 17.44 | 22.72 | 19.04 | 17.28 | 18.24- | 16.96 | 17.12 | 13.76 | 15.84 | 15.36 | 13.44 | 14.40 |    |
| \textit{Larinioides cornutus}   | 16.96 | 17.28 | 18.88 | 17.76 | 13.92 | 14.08 | 13.60 | 15.52- | 18.08- | 12.48 |    |    |    |    |    |    |
| \textit{Gasteracantha canc.}    | 17.28 | 17.44- | 19.68 | 20.08 | 17.92 | 20.32- | 19.36- | 18.88- | 17.12 | 18.40 | 19.52 | 19.20 | 17.76 | 18.08 | 17.44 | 17.60- | 14.72 |    |
Multiple sequence alignment and sequence analyses

The electrophoragram of forward and reverse sequences was checked manually and assembled using BioEdit Sequence Alignment Editor Version 7.0.5 (Hall, 2005). Multiple alignments of the cleaned sequences were performed using Clustal X version 1.81 (Thompson et al., 1997). The lengths of the COI and 18S gene fragment DNA sequences were 625 bp and 930 bp, respectively. The COI and 18S rRNA sequences obtained from this study were deposited into the National Center for Biotechnology Information (NCBI) GenBank DNA sequence database (Table I).

Phylogenetic tree reconstruction

The phylogenetic trees were constructed based on maximum parsimony (MP) and maximum likelihood (ML) analyses for the 18S and COI gene fragments, respectively, using (PAUP) software version 4.0 (Swofford, 2003). For the ML and MP analysis, the best model was suggested by Akaike Information Criterion (AIC), which was performed using the PhyML 3.0 software (Guindon et al., 2010). The phylogenetic trees were reconstructed using HKY85 and GTR model for COI and 18S, respectively. Pairwise genetic distance and neighbour joining distances of both the COI and 18S genes were calculated using the Kimura-two-parameter model. A full heuristic search was used for the MP analysis. The tree reliability was then estimated by bootstrapping the phylogenetic tree data with 1000 replications of data sets for MP and 100 replications for ML. In addition, Bayesian Inference (BI) analysis, using four chains from the Markov Chain Monte Carlo (MCMC) generations until it reached less than 0.01 was also performed for both the 18S and COI datasets using the MrBayes version 3.1.2 software (Ronquist and Huelsenbeck, 2003).

RESULTS

The phylogenetic analyses produced almost similar topologies for both the COI (BI and ML analyses) and 18S (BI and MP analyses) gene fragments, but displayed different bootstrap support values. Both genes revealed two main clusters, corresponding to the Leucauginae and Tetragnathinae subfamilies. The Leucauginae cluster further formed two separate subclusters and one subcluster consisted of members from the genera Leucauge-Opadometa group (L. argentina, L. celebesiana, L. decorata, L. sabahan, L. tessellata, Opadometa grata and O. kuchingensis), while the second subcluster consisted of members from the genera Mesida-Tylorida group (M. gemmea, M. yini, T. striata, T. tianlin and T. ventralis). On the other hand, the Tetragnathinae cluster consisted of members from the genus Tetragnatha (T. hasselti, T. lauta, T. maxillosa, T. pinicola and Tetragnatha sp.). Overall, the molecular data of tetragnathid spiders was consistent with their classification based upon morphological characteristics, though Opadometa grata and Leucauge decorate could not be resolved by 18S gene.

For COI gene fragment, the aligned sequences had 625 characters, with 25 uninformative and 238 parsimony informative sites. The genetic distances within the genus Leucauge ranged between 8.96-9.12% (L. celebesiana vs. L. tessellata) to 15.52-16.32% (L. argentina vs. L. sabahan). On the other hand, the genetic distances within the genus, Opadometa were 0.00-0.32% (O. grata vs. O. kuchingensis). Interspecific genetic distances within the Leucauge-Opadometa group ranged between 10.08-10.72% (L. celebesiana vs. O. kuchingensis) to 14.08-15.84% (L. argentina vs. O. grata and L. argentina vs. O. kuchingensis). The distances within the genus Mesida were 15.04-15.84% (M. gemmea vs M. yini). As for the genus Tylorida, the genetic distances ranged between 15.52-16.16% (T. striata vs. T. ventralis) to 17.12-17.76% (T. tianlin vs. T. ventralis). Interspecific genetic distances within the Mesida-Tylorida group ranged between 14.08-14.24% (M. yini vs. T. striata) to 18.24-18.88% (M. yini vs. T. tianlin). The genetic distances within the Tetragnatha group ranged between 14.24-15.04% (T. maxillosa vs. T. pinicola) to 20.08% (T. lauta vs. T. hasselti) (Table II).

For 18S rRNA gene, the aligned sequences consisted of 945 characters, of which there were 59 uninformative and 144 parsimony informative sites. The genetic distances within the genus Leucauge ranged from 0.21% (L. decorata vs. L. sabahan) to 1.73% (L. argentina vs. L. celebesiana), while within the Opadometa it was 0.11% (O. grata vs. O. kuchingensis). Interspecific genetic distances within the Leucauge-Opadometa group ranged from 0.11% (L. decorata vs. O. kuchingensis) to 1.51% (L. argentina vs. O. kuchingensis). The distance within the genus Mesida was 0.76% (M. gemmea vs M. yini), while within the genus Tylorida the ranges were between 2.81% (T. tianlin vs. T. ventralis) to 4.54% (T. tianlin vs. T. striata). Interspecific genetic distances within the Mesida-Tylorida group ranged from 1.30% (M. yini vs. T. ventralis) to 3.68% (M. gemmea vs. T. striata). The genetic distances within the Tetragnatha group ranged from 1.73% (T. maxillosa vs. T. pinicola) to 6.66% (T. lauta vs. Tetragnatha sp.) (Table III).

The distance matrix calculated using the COI gene fragment sequences showed a wider range of values for each studied species, compared to 18S gene fragments. The existence of a higher number of informative sites in the COI genes could have been responsible for the wider range of genetic distances garnered compared to the more

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conservative 18S genes. For COI gene fragments, there were greater genetic distances between *T. tianlin* and other species within the *Mesida-Tylorida* group. Additionally, the genetic distance matrix using the 18S gene showed consistency within the *Mesida-Tylorida* group in this species.

**DISCUSSION**

Two distinct and independent sequence alignments of the COI and 18S gene fragments produced two almost identical tree structures with only minor differences. It can be hypothesized that the lineage history of the mitochondrial and nuclear DNA could have caused the slight differences in the tree topology. For the family Tetragnathidae, the two subfamilies and three sub-groups were clearly identified, delineated and resolved within the both the COI and 18S phylogenetic trees (Fig. 1). The *Leucauge-Opadometa* and *Mesida-Tylorida* were both found clustered within the Leucauginae-group, while the *Tetragnatha* were clustered in the Tetragnathinae-group. Some of these groupings were strongly supported by high bootstrap values of greater than 70% (Hillis and Bull, 1993).

These phylogenetic trees, based on the BI and MP analyses of the 18S gene and the BI and ML analyses of the COI gene, were highly comparable and corroborated with the documented internal relationships hypothesis of the Tetragnathidae family group (Alvarez-Padilla et al., 2009; Alvarez-Padilla and Hormiga, 2011). When taken together, the reconstructed phylogenetic trees in this study strongly support the separate groupings of *Leucauge-Opadometa* and *Mesida-Tylorida* within the subfamily Leucauginae are coherent with their morphological characteristics. Both these findings suggest that the COI and 18S genes are reliable genetic markers which can be used to identify, delineate and verify the natural groupings within the Tetragnathidae subfamilies (Alvarez-Padilla et al., 2009), as well as for other spider families, which are theoretically highly similar and strongly related based on their morphological criteria (Astrin et al., 2006). Comparative analysis of both the COI and the 18S gene fragment sequences of *Leucauge* and *Opadometa* were located within the same clade, suggesting they may represent a sister relationship. However, this hypothesis required further verification by adopting multiple gene sequence analyses with larger sample size. Nevertheless, Alvarez-Padilla and Hormiga (2011) showed that *Opadometa* was closely related to *Leucauge* based on the numerous similarities observed in their morphology, behaviour and DNA sequences.

Table III.- Genetic pairwise distance (%) of the 18S gene sequences among the 17 tetragnathid species (Leucauge (5 species), Mesida (2 species), Opadometa (2 species), Tetragnatha (5 species) and Tylorida (3 species)) plus the two outgroups (Larinioides cornutus and Gasteracantha cancriformis). The distances were calculated by using the Kimura-two-model (Kimura, 1980). The distances were calculated by using the Kimura-two-model (Kimura, 1980).

| Species             | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  | 13  | 14  | 15  | 16  | 17  | 18  | 19  |
|---------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Leucauge decorata   | —   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Opadometa kuchingensis | 0.11 | —   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Leucauge celebesiana | 0.43 | 0.54 | —   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Leucauge sp.        | 0.21 | 0.32 | 0.43 | —   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Opadometa grata    | 0.00 | 0.11 | 0.43 | 0.21 | —   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Leucauge tessellata | 0.54 | 0.65 | 0.97 | 0.54 | 0.54 | —   |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Leucauge argentina | 1.40 | 1.51 | 1.73 | 1.30 | 1.40 | 1.30 | —   |     |     |     |     |     |     |     |     |     |     |     |     |
| Tylorida ventralis | 3.89 | 3.78 | 4.00 | 4.00 | 3.89 | 4.00 | 4.43 | —   |     |     |     |     |     |     |     |     |     |     |     |
| Mesida gemmea      | 3.67 | 3.56 | 3.67 | 3.78 | 3.67 | 4.00 | 4.21 | 1.40 | —   |     |     |     |     |     |     |     |     |     |     |
| Mesida yini       | 3.34 | 3.24 | 3.35 | 3.46 | 3.35 | 3.67 | 3.89 | 1.30 | 0.76 | —   |     |     |     |     |     |     |     |     |     |
| Tylorida tianlin  | 4.32 | 4.21 | 4.43 | 4.32 | 4.21 | 4.64 | 2.81 | 2.37 | 2.27 | —   |     |     |     |     |     |     |     |     |     |
| Tylorida striata  | 5.30 | 5.19 | 5.51 | 5.19 | 5.29 | 5.40 | 6.05 | 3.78 | 3.68 | 3.24 | 4.54 | —   |     |     |     |     |     |     |
| Tetragnatha hasselti | 8.29 | 8.18 | 8.50 | 8.40 | 8.29 | 8.07 | 8.62 | 7.51 | 7.74 | 7.42 | 7.41 | 9.00 | —   |     |     |     |     |     |
| Tetragnatha sp.   | 8.71 | 8.81 | 8.93 | 8.82 | 8.71 | 8.49 | 8.93 | 8.04 | 8.59 | 8.27 | 8.06 | 8.56 | 5.53 | —   |     |     |     |     |
| Tetragnatha lauta | 7.15 | 7.04 | 7.15 | 7.25 | 7.15 | 7.04 | 7.26 | 6.60 | 7.26 | 6.93 | 7.03 | 7.55 | 6.50 | 6.66 | —   |     |     |     |
| Tetragnatha maxillosa | 5.21 | 5.32 | 5.43 | 5.32 | 5.21 | 5.10 | 5.32 | 4.55 | 4.88 | 4.56 | 4.88 | 6.06 | 5.44 | 5.34 | 4.98 | —   |     |     |
| Tetragnatha pinicola | 5.52 | 5.63 | 5.84 | 5.63 | 5.52 | 5.41 | 5.63 | 4.98 | 5.42 | 4.96 | 4.68 | 5.86 | 4.89 | 5.53 | 5.07 | 1.73 | —   |     |
| Gasteracantha kuhlii | 8.64 | 8.75 | 8.53 | 8.75 | 8.64 | 8.97 | 9.40 | 9.08 | 8.86 | 8.64 | 9.94 | 10.38 | 11.79 | 11.71 | 8.90 | 9.53 | —   |     |
| Cyclosa conica     | 9.08 | 9.19 | 9.08 | 9.19 | 9.08 | 8.97 | 9.41 | 9.31 | 8.86 | 8.65 | 9.62 | 10.50 | 11.92 | 11.72 | 8.59 | 9.44 | 3.23 | —   |
Fig. 1. Phylogenetic trees of the Tetragnathid spider species from Malaysia as inferred from (LEFT) COI gene analyzed by BI and ML and (RIGHT) 18S gene analyzed by BI and MP. Bootstrap values with 50% majority rule applied above branches while the BI posterior probability values are shown below branches.
To date, the reclassification of *Opadometa* species is still ongoing, but has never been successfully revised due to the difficulty of procuring the scarce male specimen that is necessary for precise species identification. However, the widely accepted view is that the general morphology of male *Opadometa* highly resembles the smaller-sized male of *Leucauge* species (Alvarez-Padilla and Hormiga, 2011). As such, *Opadometa* species is grouped under *Leucauge* species (Yoshida, 2009). Similarly, *L. argentina* was categorized as a sister group to the *Leucauge-Opadometa* family group in this study, it was the only small-sized species (< 6 mm) found in the present study.

Within the *Mesida-Tylorida* group, members share many similar morphological characteristics (Tanikawa, 2001; Alvarez-Padilla and Hormiga, 2011) and so these two genera can only be distinguished by minor differences in their outer appearance (Tanikawa, 2001, 2004; Kulkarni, 2014). *Tylorida striata* was recognized as a sister-clade to *M. argentiopunctata* and the *Mesida* species (Tanikawa, 2001) based on a cladistic analysis of their morphological characteristics. Based on a previous study conducted by comparing their morphological characteristics, behaviour and DNA sequence, *Tylorida* was identified as a sister group to *Orsinome* species (Alvarez-Padilla and Hormiga, 2011). However, since the specimens used in this study failed to include the *Orsinome* species from Malaysia, this observation cannot be fully verified as yet.

Meanwhile, the genus *Tetragnatha* is the only genus from the subfamily Tetragnathinae that has a readily available record in Malaysia. The genus *Tetragnatha* was documented as a sister-clade in the grouping, as inferred from their genetics, morphology and behavior patterns, which were all highly similar to the *Glenognatha* and *Pachygnatha* (Alvarez-Padilla and Hormiga, 2011), albeit both genera are yet to be documented in Malaysia (Dzulhelmi et al., 2014a, b; Norma-Rashid and Li, 2009). In spite of their similarities, the Tetragnathinae-group was found to differ significantly from the Leucauginae-group in terms of their morphological characteristics (Alvarez-Padilla et al., 2009). Nevertheless, the species boundaries among *Tetragnatha* spp. in Malaysia could not be fully resolved. *Tetragnatha pinicola* from the present study was identical to some of the unpublished COI sequences of *T. ceylonica* from Malaysia.

A detailed morphological analysis of these *T. ceylonica* samples would be beneficial in confirming their species identity. There is also a possibility of the shared DNA barcodes among closely related members, hence a comparative genetic analysis between *T. ceylonica* and *T. pinicola* by adopting various genetic markers would be needed to unveil the differences or to identify if they are a species complex. The species status of *T. pinicola* was also complicated by the distinct COI genetic distances (>16%) between Malaysian strain and those from the GenBank (i.e., China, Germany, Italy, Spain Switzerland). This discrepancy also deserves additional research efforts to resolve its species status.

The tree-based taxa clustering in the present study indicated that the molecular evidence provided by the COI gene supports the morphological hypothesis. This DNA identification method has, therefore, been found to be scientifically reliable and useful for spider taxonomic studies. An important point to note is that the current findings indicate that the COI gene has adequate variable regions and is more informative than the 18S gene in resolving intra- and interspecific relationships among the Tetragnathid species. For example, a single marker from the COI gene is already sufficient for studying the genetic relationships in other spider species (i.e. Garb et al., 2004; Tanikawa et al., 2006; Vink et al., 2009; Smith et al., 2012; Musliman et al., 2015). Nonetheless, some groups of spiders achieve better results from using other genetic markers (i.e. Croom et al., 1991; Fang et al., 2000; Astrin et al., 2006), or a combination of several genetic markers (i.e. Benjamin et al., 2008; Alvarez-Padilla et al., 2009; Su et al., 2011; Franzini et al., 2013).

**CONCLUSION**

This study utilized COI and 18S gene sequences to the DNA barcodes of 17 studied tetragnathid spider species in Malaysia. However, their genetic diversity maybe underestimated because the low sample size used in the present study may not sufficient to disclose their intraspecific variation. Therefore, it is highly recommended that a wider range of the Malaysian tetragnathid species with larger sample size should be used in future studies to further compare and contrast the compatibility of these two gene sequences. Testing on other genetic markers is also warranted to resolve the species boundaries among some of the complex taxa.

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Statement of conflict of interest
The authors have declared no conflict of interests.

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