Pakistan J. Zool., vol. 55(2), pp 881-891, 2023 DOI: https://dx.doi.org/10.17582/journal.pjz/20200910140935

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

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Article Information
Received 10 September 2020
Revised 13 November 2020
Accepted 9 January 2021
Available online 09 May 2022
(early access)
Published 06 January 2023

Authors' Contribution
DMN designed the study, conducted the survey, performed the labwork and prepare the manuscript. SS, LVL and ZM assisted the samplings and experiments. NRY supervised the work.

Key words
Orb-web spider, DNA barcoding, COI, 18S, Diversity.

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 bacodes 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, Metainae and Tetragnathinae Menge 1866. The genus *Tetragnatha* Latreille 1804, is the genus type of this family (Alvarez-Padilla and Hormiga, 2011). 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

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0030-9923/2023/0002-881 $ 9.00/0

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 Tetergnathinae 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 bacodes for members of the subfamilies Leucauginae (*Leucauge*, *Opadometa*, *Mesida* 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 I). 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 I).

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 18Sa (3'-ATTAAAGTTGTTGCGGTTA-5') and 9r (3'-GATCCTTCCGCAGGTTCACCTAC-5') (Alvarez-Padilla, 2008). The total reaction volume used was 50 µL, consisting of 10 µL of dH_2O, 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 LCOI1490 (3'-GGTCAACAAATCATAAAGATATTGG-5') and reverse primer HCOI2198 (3'-TAAACTTCAGGGTGACCAAAAAATCA-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 I.- 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, 110°11'44"E	KU836868	KU836899
	Gading National Park, Kuching	1°41'27"N, 109°50'45"E	KU836867	KU836898
Leucauge celebesiana	Poring Hot Spring Nature Reserve, Ranau	6°2'35"N, 116°42'7"E	KU836871	KU836903
	Mesilau Resort Nature Reserve, Ranau	6°02'5"N, 116°54'1"E	KU836872	KU836904
	Kubah National Park, Kuching	1°36'41"N, 110°11'44"E	KU836870	KU836902
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	KU836906
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
	Ulu Gombak Nature Reserve, Gombak	3°22'60"N, 101°47'20"E	KU836877	KU836908
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
	Bako National Park, Kuching	1°41'8"N, 110°26'10"E	KU836882	KU836914
Mesida gemmea	Kubah National Park, Kuching	1°36'41"N, 110°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	-	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
	Fraser Hill Forest Reserve, Pahang	3°43'7"N, 101°44'25"E	KU836894	KU836927
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
[1]*Tylorida striata*	Naratiwat Province, Thailand	5°47'45"N, 101°50'4"E	EU003309	-
	Universiti Kebangsaan Malaysia, Bangi	2°55'47"N, 101°46'44"E	-	KU836919
Tylorida tianlin	Mesilau Resort Nature Reserve, Kundasang	6°02'5"N, 116°54'1"E	KU836885	KU836917
	Mesilau Resort Nature Reserve, Kundasang	6°02'5"N, 116°54'1"E	KU836886	KU836918
Tylorida ventralis	Universiti Kebangsaan Malaysia, Bangi	2°55'47"N, 101°46'44"E	KU836889	KU836923
	Universiti Kebangsaan Malaysia, Bangi	2°55'47"N, 101°46'44"E	KU836890	KU836922
	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	-
[2]*Larinioides cornutus*	Point Pelee, Ontario, Canada	Unstated	JN308507	-
[1]*Gasteracantha cancriformis*	Heredia province, Costa Rica	10°25'53"N, 84°00'13"W	EU003287	-
[3]*Gasteracantha kuhlii*	-	Unstated	-	AB910478
[1]*Cyclosa conica*	Mon Hunoso Lake, Denmark	Unstated	-	EU003343

[1]Alvarez-Padilla *et al*. (2009); [2]Blagoev *et al*. (2016); [3]Tanikawa *et al*. (2014).

Table II.- Genetic pairwise distance (%) of the COI 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).

Species		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	*Tetragnatha pinicola*	0.00-0.32																		
2	*Tetragnatha maxillosa*	14.24-15.04	0.00-1.28																	
3	*Tetragnatha lauta*	15.2-15.36	14.88-15.04	—																
4	*Tetragnatha hasselti*	18.4-18.56	18.88-19.04	20.8	—															
5	*Tetragnatha* sp.	14.88-15.04	15.68	16.48	17.28	—														
6	*Mesida gemmea*	17.28-17.76	18.08-19.36	19.84-20.00	22.24-23.84	19.68-19.84	5.92													
7	*Mesida yini*	17.44-17.92	18.72-19.68	20.48-20.96	22.40-22.72	19.36	15.04-15.84	1.12												
8	*Tylorida striata*	17.60	19.52-20.16	19.84	24.32	19.68	16.16-16.48	14.08-14.24	—											
9	*Tylorida ventralis*	16.80-18.40	16.32-16.80	18.40-19.20	21.92-22.72	18.88-19.20	15.84-16.48	16.80-17.44	15.52-16.16	0.00-4.64										
10	*Leucauge celebesiana*	14.24-14.56	15.84-16.32	16.64-16.80	18.88-19.20	16.16-16.48	16.32-16.80	16.00-16.64	16.00-16.32	15.52-16.16	0.16-0.32									
11	*Leucauge decorata*	16.00-16.16	14.88-15.52	17.28	19.84	16.96	16.32-16.64	17.44-17.76	17.60	16.00-16.80	9.44-9.60	—								
12	*Opadometa grata*	14.56-14.72	16.32-16.64	16.96	19.36	15.52	15.84-16.64	17.12	16.64	16.80-16.96	10.40-10.72	11.68	—							
13	*Opadometa kuchingensis*	14.56-14.72	16.16-16.64	16.80-16.96	19.20-19.36	15.52-15.68	15.52-16.64	16.80-17.12	16.48-16.64	16.48-16.96	10.08-10.72	11.36-11.68	0.00-0.32	0.00-0.32						
14	*Leucauge tessellata*	16.48	16.80-17.28	18.40	18.72	16.48	17.28-18.08	17.44-18.24	16.48	17.28-18.24	8.96-9.12	11.68	10.24	10.24	—					
15	*Leucauge* sp.	15.36-15.68	16.48	18.72	20.00	17.44	15.68-16.00	16.48-17.12	17.44	17.12-17.76	11.84-12.00	12.96	13.44	13.44	10.40	—				
16	*Tylorida tianlin*	17.76	16.16-16.96	17.44	22.72	19.04	17.28-17.92	18.24-18.88	16.96	17.12-17.76	13.76-13.92	15.84	15.36	15.36	13.44	14.40	—			
17	*Leucauge argentina*	16.64-17.28	15.84-16.96	17.12-18.88	19.20-20.16	17.44-19.20	16.32-16.80	17.44-19.20	16.96-17.92	16.00-16.48	12.80-13.92	12.96-14.08	14.40-15.84	14.08-15.80	13.60-14.72	15.52-16.32	18.08-18.24	0.16-12.48		
18	*Larinioides cornutus*	15.84-16.16	16.64-16.80	17.6	20.64	16.80	19.04-19.68	19.84-20.48	19.04	17.12-17.76	16.00-16.32	14.88	17.76	17.44-17.76	17.12	16.64	18.72	15.68-17.12	—	
19	*Gasteracantha cancriformis*	17.28	17.44-17.76	19.68	20.08	17.92	20.32-20.48	19.36-19.84	19.36	18.88-19.36	17.12-17.28	18.40	19.52	19.20-19.52	17.76	18.08	17.44	17.60-20.64	14.72	—

Multiple sequence alignment and sequence analyses

The electophoragram 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 CO1 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 neigbour 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 sub-cluster 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.). Oveall, 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

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 hypothetically 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
1	*Leucauge decorata*	—																		
2	*Opadometa kuchingensis*	0.11	—																	
3	*Leucauge celebesiana*	0.43	0.54	—																
4	*Leucauge* sp.	0.21	0.32	0.43	—															
5	*Opadometa grata*	0.00	0.11	0.43	0.21	—														
6	*Leucauge tessellata*	0.54	0.65	0.97	0.54	0.54	—													
7	*Leucauge argentina*	1.40	1.51	1.73	1.30	1.40	1.30	—												
8	*Tylorida ventralis*	3.89	3.78	4.00	4.00	3.89	4.00	4.43	—											
9	*Mesida gemmea*	3.67	3.56	3.67	3.78	3.67	4.00	4.21	1.40	—										
10	*Mesida yini*	3.34	3.24	3.35	3.46	3.35	3.67	3.89	1.30	0.76	—									
11	*Tylorida tianlin*	4.32	4.21	4.43	4.43	4.32	4.21	4.64	2.81	2.37	2.27	—								
12	*Tylorida striata*	5.30	5.19	5.51	5.19	5.29	5.40	6.05	3.78	3.68	3.24	4.54	—							
13	*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	—						
14	*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	—					
15	*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	—				
16	*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	—			
17	*Tetragnatha pinicola*	5.52	5.63	5.84	5.63	5.52	5.41	5.63	4.98	5.42	5.09	4.98	6.58	4.89	5.53	5.07	1.73	—		
18	*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	12.74	11.71	8.90	9.53	—	
19	*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	12.65	11.72	8.59	9.44	3.23	—

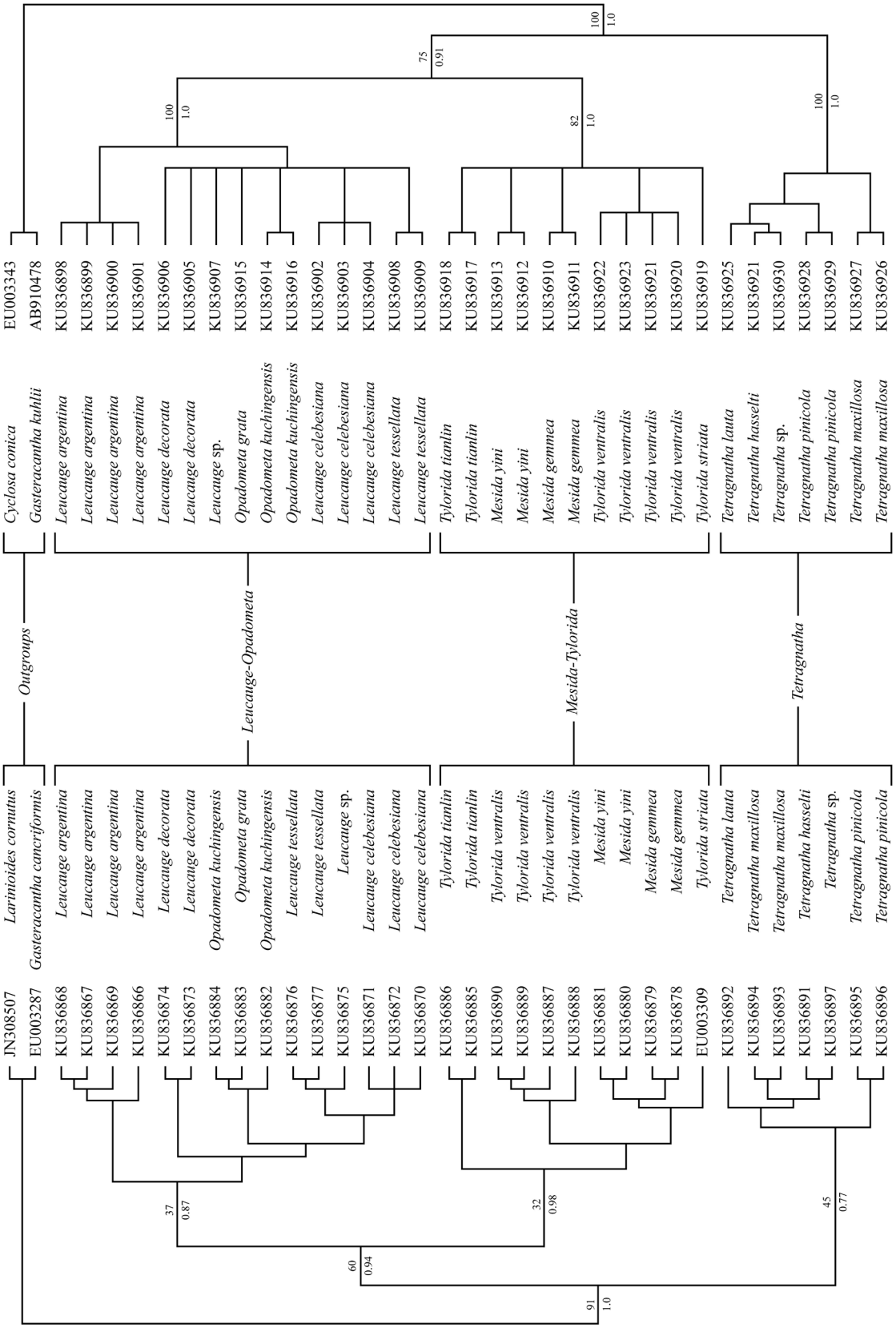

Fig. 1. Phylogenetic trees of the Tetragnathid spider species from Malaysia as inferred from (LEFT) *COI* gene analyzed by BI and ML and (RIGHT) 18S gene analyzed by BI and MP. Bootstrap values with 50% majority rule applied above branches while the BI posterior probability values are shown below branches.

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* formed a separate subclade in the phylogenetic tree (Fig. 1). Although *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 identy 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 inter-specific 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; Muslimin *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.

ACKNOWLEDGEMENTS

We acknowledge the Department of Wildlife and National Parks (PERHILITAN), Sabah Parks and Sarawak Forestry Department for their permission to conduct the research. We are grateful to Ms. Ili Syazwana Abdullah, Mr. Mohd Taufiq Ahmad, Ms. Nurhafiza Dahniar Afandi and Dr. Teh Ser Huy and Dr. Salmah Yaakop for assisting in the molecular technique and explaining important information in phylogenetic analyses. We also thank

anonymous reviewers for their constructive comments to improve the draft of this manuscript. This project was funded by MyBrain15 scholarships and University of Malaya IPPP grant (PG096-2012B) awarded to Dzulhelmi MN.

Statement of conflict of interest

The authors have declared no conflict of interests.

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