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Sequence Analysis of the Cytochrome C Oxidase Subunit I Gene of *Pseudagrion pilidorsum* (Odonata: Coenagrionidae)

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Abstract

Pseudagrion pilidorsum is 1 of over 140 species of *Pseudagrion* (in the family Coenagrionidae), the largest genus of damselfly. This species exhibits dimorphism due to the different body colorations of males and females, making them difficult to distinguish from other congeneric species. This study analyzed the cytochrome C oxidase subunit I (COI) gene sequence of *P. pilidorsum* found in Bogani Nani Wartabone National Park (North Sulawesi) and compared it with other sequences of *P. pilidorsum* from distinct geographical locations in Asia. The COI gene for the Sulawesi specimen was amplified using the universal primer pair LCO1490 and HCO2198. A sequence homology search was conducted through BLAST. Multiple sequence alignment was executed using CLUSTAL O (1.2.1). A phylogenetic tree was constructed using the Neighbor-Joining method, and genetic distance was calculated using the Kimura 2-parameter. The COI gene sequence of the Sulawesi specimen lies in the range of 83.99-89.10% with other *P. pilidorsum* deposited at GenBank, namely KF369526 (Sarawak specimen), AB708543, AB708544, and AB708545 (Japan specimens). The genetic distance falls in the range of 0.146-0.149 between the Sarawak specimen and the Japan specimen; 0.122-0.125 between the Sulawesi and Japan specimens; and 0.185 between the Sulawesi specimen and the Sarawak specimen. It can thus be inferred that the Sarawak and Japan specimens may not belong to the same species; the Sulawesi and Japan specimens may not belong to the same species; and the Sarawak specimen and Sulawesi specimens might be placed in different genera.

Abstrak

Analisis Sequence Gen Cytochrome C Oxidase Subunit I *Pseudagrion pilidorsum* (Odonata: Coenagrionidae). *Pseudagrion pilidorsum* merupakan anggota salah satu dari lebih dari 140 spesies *Pseudagrion* (dalam famili: Coenagrionidae) yang merupakan genus terbesar dari capung jarum. Spesies ini memperlihatkan dimorfisme karena memiliki warna yang berbeda antara jantan dan betina sehingga sulit membedakannya dengan capung jarum dari genus yang sama. Penelitian ini bertujuan untuk menganalisis *sequence* gen *cytochrome C oxidase subunit I* (COI) dari *P. pilidorsum* yang ditemukan di Taman Nasional Bogani Nani Wartabone (Sulawesi Utara) dan membandingkannya dengan *sequence* COI *P. pilidorsum* dari lokasi geografis yang berbeda di Asia. Gen COI dari spesimen Sulawesi diamplifikasi menggunakan pasangan primer universal LCO1490 dan HCO2198. Pencarian homologi *sequence* dilakukan dengan program BLAST. Penjajaran *sequence* gen COI dilakukan dengan program CLUSTAL O(1.2.1). Pohon filogenetik dikonstruksi menggunakan metode Neighbor-Joining (NJ) dan jarak genetik dihitung menggunakan metode Kimura 2-parameter. *Sequence* gen COI *P. pilidorsum* spesimen Sulawesi memiliki nilai identitas berkisar antara 83,99-89,10% dengan *P. pilidorsum* lainnya yang terdapat di GenBank, yaitu KF369526 (spesimen Sarawak), AB708543, AB708544, dan AB708545 (spesimen Jepang). Jarak genetik berkisar 0,146-0,149 antara spesimen Sarawak dengan spesimen Jepang; 0,122-0,125 antara spesimen Sulawesi dengan spesimen Jepang; dan 0,185 antara spesimen Sulawesi dengan spesimen Sarawak. Berdasarkan hasil studi ini kami menyimpulkan bahwa spesimen Sarawak dengan spesimen Jepang dan spesimen Jepang dengan spesimen Sulawesi kemungkinan merupakan spesies yang berbeda, dan spesimen Sarawak dengan spesimen Sulawesi kemungkinan merupakan genus yang berbeda. Analisis spesimen tambahan dari Sulawesi diperlukan untuk menguatkan dugaan tersebut.

Keywords: COI, damselfly, DNA barcode, interspecific, intraspecific

Introduction

There are more than 5,875 species of dragonflies worldwide; 2,739 are from the suborder Zygoptera (damselfly), and 2,941 are from suborder Anisoptera (dragonfly) [1]. Eight hundred and seventy species are in the Australasian region that includes Sulawesi, Moluccas, Papua, and Australia. Approximately 175-250 species in these regions have not been described [2]. An estimated 1,500 species of dragonflies still await description around the world, so the number of dragonfly species that exists today could approach 7,000 [2]. One damselfly species found in North Sulawesi is *Pseudagrion pilidorsum* (Brauer 1868) from the family Coenagrionidae. This type of damselfly is difficult to distinguish from others among the same genera due to the variety of color and also the similarity of morphological characteristics. The existing methods for identifying unknown insects rely on morphological characteristics, which presents a problem if organisms alter themselves physiologically and morphologically due to unfavorable environmental conditions. This situation often may lead to the incorrect identification of species. A tool that enables both fast and accurate identification based on DNA sequence is regarded as one taxonomic method to analyze species diversity [3]. Therefore, the DNA barcode was developed for easy identification [4].

Some studies have used DNA barcodes to calculate the genetic distance threshold e.g., for butterflies [5], crustaceans [6], corals [7], and fishes [8], but this distance cannot be generally applied to the identification of the species because there are some inconsistencies of intra- and interspecific species diversity, which are highly dependent on the organism observed [9]. The latest advances in the fields of statistics and geographical genetics have enabled investigation into how environmental factors affect the geographical organization and population structure of molecular genetic diversity within species [10]. The DNA barcode recommended for animal species identification is the cytochrome C oxidase subunit I (COI) gene, the most important protein-coding gene in mitochondrial DNA (mtDNA). This gene has been widely used in molecular evolution research and for evaluating inter- and intraspecific diversity in ciliates [11] and insects [12]. It may also be used in conjunction with traditional morphology-based identification to more accurately identify and classify species.

Factors that influence the distribution of the diversity of dragonflies are either historical (geological) or ecological factors. Both define the current species diversity, but the compositions of the family and genus level are mainly determined by geological factors [13]. Local biodiversity is strongly influenced by speciation and extinction caused by ecological and non-ecological factors. Many studies have shown that climate patterns also have an impact on radiation and the diversification of species [14]. Some

studies have investigated the habitat, distribution, abundance, and morphology of the damselfly in Bogani Nani Wartabone National Park (BNWB-NP)[15], but there has been no research on their genetic aspects to date, especially in *P. pilidorsum*.

In this study, we used the COI gene sequence data of the damselfly *P. pilidorsum* obtained from Bogani Nani Wartabone National Park. The data were compared with other available COI gene data of other *P. pilidorsum* obtained from GenBank. This study aimed to analyze variation in the COI gene sequences of *P. pilidorsum* from various places in Asia to provide information on the intraspecific variation of COI sequences based on different geographic locations.

Materials and Methods

Study Area. *P. pilidorsum* specimens were obtained in March 2014 on river banks in the primary forest of Bogani Nani Wartabone National Park in North Sulawesi at the coordinates 00°34'33.87"N and 123°53'58.02"E. The study area is situated at an altitude of about 241-289 meters above sea level with a temperature between 26-30 °C, 65-74% humidity, and 60-85% vegetation cover.

Procedure. The damselflies were captured using swing nets. Identification of their morphological characteristics was carried out by observing wing venation, color patterns, and genitalia using identification keys [13]. The coxae were removed carefully from the body of the male damselfly along with the flesh. The coxae and flesh were kept in 90% ethanol until their use. Extraction of DNA from the flesh was performed using a universal innuPrep DNA Micro Kit (Analytik Jena) according to the protocol provided by the manufacturer. Amplification of the DNA was done using 5x Firepol PCR Master Mix Ready-to-Load (Solis BioDyne) with the universal primer set LCO1490 (5'-GGTCAACA-AATCATAAAGATATTGG-3') dan HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') [38]. Amplification of the DNA was carried out according to the method used by Kairupan et al. [5] as follows: 5 minutes initial denaturation at 94 °C followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 54 °C for 45 seconds, elongation at 72 °C for 1 minute and 30 seconds, and final extension for 5 minutes at 72 °C. The amplicons were sent to 1st BASE Malaysia for sequencing using the primer set LCO1490 and HCO2198.

Data analysis. The chromatograms were processed using Geneious v5.6 according to the method described by Tallei and Kolondam [16]. Briefly, the sequences were pairwise aligned using global alignment with free end gaps to identify regions of 93% similarity. Approximately 30 nucleotides at the beginning of the DNA sequences were trimmed, and erroneous nucleotide readings were corrected. Other COI gene sequences of

P. pilidorsum and other members of Coenagrionidae were obtained from GenBank. All sequences were subsequently trimmed accordingly to avoid ambiguous alignment and to reach the core area. The sequences were aligned using Clustal O (1.2.1) multiple sequence alignment (www.ebi.ac.uk/Tools/msa/clustalo). The phylogenetic tree was inferred using the Neighbor-Joining (NJ) method [41]. The evolutionary distances were computed using the Kimura 2-parameter approach [40]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches [43]. Evolutionary analyses were conducted in MEGA 6.0 [42].

Results and Discussion

Damselfly specimens *P. pilidorsum* that were obtained from TN-BNWB are displayed in Figures 1A and 1 B. Table 1 shows all specimens used in this study. The identity percentage matrix that was created using

Clustal O (1.2.1) is shown in Table 2, while the genetic distance of COI based on the Kimura 2-parameter is presented in Table 3. The COI gene of *P. pilidorsum* has been deposited in GenBank with accession number KX447495 (Sulawesi specimen). It has an identity in the range of 83.99–89.10% with other *P. pilidorsum* obtained from GenBank, namely KF369526, AB708543, AB708544, and AB708545. KF369526 is a specimen from Sarawak, Malaysia (voucher RMNH.INS.228961), while AB708543, AB708544, and AB708545 are specimens from Japan.

Based on its identity, the Sulawesi specimen is closer to the Japan specimens (88.86–89.10%) than to the Sarawak specimen (83.99%) (Table 2). This conclusion was supported by the genetic distance between the Sulawesi and Japan specimens, which ranged from 0.122–0.124 (Table 3). The identity of all *P. pilidorsum* ranged from 84.92–100.0%. The genetic distances of all *P. pilidorsum* specimens varied from 0–0.185. There was no genetic distance between AB708543 and AB708544,



Figure 1. *Pseudagrion pilidorsum* (A) male, (B) female

Table 1. List of Specimens (Famili Coenagrionidae) Used in This Study

Nucleotide Accession No.	Protein Accession No.	Species	Location
KX447495	ANT96818	<i>P. pilidorsum</i>	North Sulawesi, Indonesia
KF369526	AGY95255	<i>P. pilidorsum</i>	Sarawak, Malaysia
AB708543	BAM21814	<i>P. pilidorsum</i>	Japan
AB708544	BAM21815	<i>P. pilidorsum</i>	Japan
AB708545	BAM21816	<i>P. pilidorsum</i>	Japan
FJ812818	ADG65303	<i>P. ignifer</i>	-
AB708537	BAM21808	<i>P. microcephalum</i>	Japan
KU220870	AMB62087	<i>Ceriagrion fallax</i>	Bhutan

which are both Japan specimens. This similarity is in contrast with the high genetic distance between the Sulawesi specimen and the Sarawak specimen, which was 0.185. In contrast, the genetic distance between the Sarawak specimen and the Japan specimens is 0.145–0.148. The Sarawak and Sulawesi specimens have a distance (0.185) that is greater than that between *P. ignifer* (FJ812818) and all *P. Pilidorsum* (0.151–0.173) specimens. The distance between *Pseudagrion* and *Ceriagrion* (KU220870) is 0.187–0.222. However, the distance between *P. microcephalum* and other *Pseudagrion* is 0.205–0.235. This shows that the intergeneric distance between *Pseudagrion* and *Ceriagrion* overlaps with the interspecific distance among *Pseudagrion*.

The number of base substitutions per site from between sequences is shown in Table 3. Standard error estimate(s) are displayed above the diagonal. The sequence alignment reveals a high intraspecific variation within *P. pilidorsum* (Figure 2), although only a single amino acid substitution is produced, where Ser becomes Ala (Figure 3). With a length of 431 bp, the COI sequences of these specimens reveal a GC content of 42.9% for KX447495, 39.7% for KF369526, 38.5% for AB708543 and AB708544, and 38.7% for AB708545. Figure 4 shows the evolutionary history inferred using the NJ method [41]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The bar at the bottom represents the amount of genetic change of 0.02 nucleotide substitutions per site calculated using the Kimura two-parameter method. This is the number of substitutions or changes divided by the length of the sequence. The tree supports

the result that the Sulawesi specimen is more closely related to the Japan specimen.

P. pilidorsum has a total body length of 4.7 cm with a wingspan of 1.8 cm. The difference between males and females is largely based on the body color of the head and thorax. The male is generally dark red and orange (Figure 1A), while the female is a dull orange to yellow color (Figure 1B). The abdomens of both males and females are yellowish. This species is found along riverbanks in primary and secondary forests as well as on farmland with a moderate to light intensity [15].

A complete understanding of the diversity of morphology, ecology, behavior, and distribution of dragonflies/damselflies must involve their evolutionary historical factors; future study of the evolution of these traits must be based on a phylogenetic framework [17]. Monitoring the biodiversity in an area that is ecologically significant for conservation will depend on reliable species identification. Molecular methods, such as DNA barcodes, provide consistent results that can be used in conjunction with traditional morphological identification as a taxonomic tool for evaluating species identification and diversity [18,19]. Mitochondrial DNA is also a reasonably accurate genetic marker because it is matrilineally derived [20].

The sequence alignment of the COI gene of *P. pilidorsum* has significantly high intraspecific nucleotide variation (Figure 2) despite its single intra-specific amino acid substitution from Ser to Ala in the Sarawak specimen. There are also several inter-specific/intra-generic amino acid variations within *Pseudagrion* and

Table 2. Percent Identity Matrix using Clustal O (1.2.1)

	KX447495	KF369526	AB708543	AB708544	AB708545	FJ812818	AB708537	KU220870
KX447495	100.00							
KF369526	83.99	100.00						
AB708543	89.10	87.01	100.00					
AB708544	89.10	87.01	100.00	100.00				
AB708545	88.86	86.77	99.77	99.77	100.00			
FJ812818	84.92	86.08	86.31	86.31	86.54	100.00		
AB708537	80.28	81.90	82.13	82.13	82.37	81.21	100.00	
KU220870	80.97	81.67	82.60	82.60	82.37	83.53	81.67	100.00

Table 3. Estimation of Evolutionary Divergence between Sequences using Kimura 2-parameter Model

	KX447495	KF369526	AB708544	AB708543	AB708545	FJ812818	AB708537	KU220870
KX447495		[0.023]	[0.017]	[0.017]	[0.018]	[0.021]	[0.026]	[0.026]
KF369526	0.185		[0.020]	[0.020]	[0.020]	[0.021]	[0.025]	[0.023]
AB708544	0.122	0.145		[0.000]	[0.002]	[0.020]	[0.024]	[0.024]
AB708543	0.122	0.145	0.000		[0.002]	[0.020]	[0.024]	[0.024]
AB708545	0.124	0.148	0.002	0.002		[0.019]	[0.024]	[0.024]
FJ812818	0.173	0.157	0.154	0.154	0.151		[0.025]	[0.023]
AB708537	0.235	0.211	0.208	0.208	0.205	0.219		[0.024]
KU220870	0.222	0.212	0.199	0.199	0.203	0.187	0.213	

AB708537	ATTAGTTC	CCCTAATATTAGGGGCC	CCAGATATGGCCTTCC	CACGCCTTAATAATA	TATAAG	60
KU220870	ATTAGTCC	CTTTAATATTAGGAG	CGCCTGATATAGCTT	TCCACGATTAAATA	ATAATAAG	60
FJ812818	ATTAGTACC	ACTAATATTAGGAG	CTCCAGATATAGCATT	TCCACGACTTAATA	ACATAAG	60
KF369526	GCTAGTGC	CATTGATACTAGGAG	CACCAGATATAGCATT	TCCACGACTTAATA	ATAATAAG	60
KX447495	ATTAGTCC	CCCTAATGTTAGGGG	CCCCAGACATAGCCT	TCCACGGCTTAATA	ACATGAG	60
AB708544	ATTAGTCC	CCCTAATGCTAGGAG	CACCGATATAGCATT	TCCACGACTCAATA	ATAATAAG	60
AB708543	ATTAGTCC	CCCTAATGCTAGGAG	CACCGATATAGCATT	TCCACGACTCAATA	ATAATAAG	60
AB708545	ATTAGTCC	CCCTAATGCTAGGAG	CACCGATATAGCATT	TCCACGACTCAATA	ATAATAAG	60
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AB708537	ATTTTGACT	ACTACCTCCATCGT	TAACGCTATTACTAG	CAAGTAGTCTGGT	TAGAAAAGTGG	120
KU220870	ATTTTGACT	ATTACCTCCTTCAT	TAAACATTATTATT	AGCAAGAAGTTT	TAGTAGAAAAGAGG	120
FJ812818	ATTTTGACT	TCTGCCTCCTTCAT	TAAACTCCTTCTT	TGCAAGAAGAAT	TAGTAGAAAAGAGG	120
KF369526	ATTTTGGCT	TTTTGCCCTGCACT	TAACACTCCTACT	AGCAAGAAGTAT	TAGTAGAAAAGAGG	120
KX447495	ATTTTGACT	CCTACCGCCTTCT	TAACACTTCTATT	AGCCAGAAGAAT	TGGTAGAAAAGAGG	120
AB708544	ATTTTGACT	TCTACCGCCTCCT	TAACACTTCTATT	AGCAAGAAGAAT	TAGTAGAAAAGAGG	120
AB708543	ATTTTGACT	TCTACCGCCTCCT	TAACACTTCTATT	AGCAAGAAGAAT	TAGTAGAAAAGAGG	120
AB708545	ATTTTGACT	TCTACCGCCTCCT	TAACACTTCTATT	AGCAAGAAGAAT	TAGTAGAAAAGAGG	120
	*****	** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * **				
AB708537	AGCGGGA	ACAGGGTGAACCG	TTTACCCCCATT	AGCCGAGCCATT	TGCACACGCAGGAGG	180
KU220870	AGCAGGT	ACTGGTTGAACAG	TATACCCCCACT	GGCAGGAGCTAT	TGCACATGCAGGGGG	180
FJ812818	GGCAGGA	ACAGGATGAACAG	TGTATCCCCATT	GGCAGGAGCAAT	TGCCCATGCAGGAGG	180
KF369526	GGCCGGA	ACGGGTGAACAG	TTTACCCTCATT	GGCTGGAGTAT	TGCACACGCAGGAGG	180
KX447495	GGCAGGA	ACAGGATGGACAG	TATATCCTCCATT	GGCTGGGGCTAT	CGCCCATGCGGGGGG	180
AB708544	AGCAGGA	ACGGGTGAACCT	GTATATCCTCCATT	AGCCGAGCTAT	CGCTCATGCAGGAGG	180
AB708543	AGCAGGA	ACGGGTGAACCT	GTATATCCTCCATT	AGCCGAGCTAT	CGCTCATGCAGGAGG	180
AB708545	AGCAGGA	ACGGGTGAACCT	GTATATCCTCCATT	AGCCGAGCTAT	CGCTCATGCAGGAGG	180
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AB708537	ATCAGTAG	ATCTTACCATTTT	TCTACTGCCTTGG	CAGGTGTCTCAT	CAATTTTGGGGGC	240
KU220870	TTCTGTT	GATTTAACAATTT	TCTCATTACATCT	AGCTGGTGTAT	CTTCAATCTTGGGAGC	240
FJ812818	ATCTGTT	GACTTAACTATCT	TCTCATTACACCT	AGCAGGTGTGT	CATCAATCTTAGGAGC	240
KF369526	GTCTGT	AGATTTAACCAT	CTTCTCATTACACT	TAGCAGGAGTAT	CCTCAATTTTGGGGGC	240
KX447495	GTCTGT	GGATTTAACTAT	CTTCTCATTACAC	CTAGCAGGTGT	ATCATCAATCTGGGTGC	240
AB708544	GTCTGT	AGATTTAACTAT	CTTCTCATTACAC	CTAGCAGGTGT	ATCATCAATCTGGGTGC	240
AB708543	GTCTGT	AGATTTAACTAT	CTTCTCATTACAC	CTAGCAGGTGT	ATCATCAATCTGGGTGC	240
AB708545	GTCTGT	AGATTTAACTAT	CTTCTCATTACAC	CTAGCAGGTGT	ATCATCAATCTGGGTGC	240
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AB708537	AATTAAT	TTTATACCACCACA	ATTAATAAAGTCCC	CAGGAATAAAAAT	TAGATCAAAT	300
KU220870	AATCAAT	TTTCATTACTACAG	TAATTAATAAAAT	TCCAGGAATAAAA	ACTAGATCAAAT	300
FJ812818	AATTAAT	TTTCAATACAAC	TAACTAAATAAAT	TCCAGGAATAAAA	ATAGATCAAAT	300
KF369526	AATTAAT	TTTCAATACAAC	TAACTAAATAAAT	TCCAGGAATAAAA	ATAGATCAAAT	300
KX447495	AATTAAT	TTTCAATACAAC	TAACTAAATAAAT	TCCAGGAATAAAA	ATAGATCAAAT	300
AB708544	AATTAAT	TTTCAATACAAC	TAACTAAATAAAT	TCCAGGAATAAAA	ATAGATCAAAT	300
AB708543	AATTAAT	TTTCAATACAAC	TAACTAAATAAAT	TCCAGGAATAAAA	ATAGATCAAAT	300
AB708545	AATTAAT	TTTCAATACAAC	TAACTAAATAAAT	TCCAGGAATAAAA	ATAGATCAAAT	300
	*** ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** *					
AB708537	ACCCCTG	TTTGTATGAGCAG	TAGTCAATTACAG	CAGTATTATTACT	ACTCTCTACCAGT	360
KU220870	GCCACTA	TTTGTATGAGCAG	TAGTAACTACTGC	AGTTCATTATTAT	TATCATACCAGT	360
FJ812818	ACCTTTA	TGTCTGAGCTGT	AGTTATTACAGCA	ATTCTACTATTAC	TACTACTACCTGT	360
KF369526	ACCCTTG	TTTGTGTTGAGCT	GTAGTTATTACAG	CAGTTTTATTATT	TGTTATCATACCCTGT	360
KX447495	ACCCTAT	TTTGTGTTGGGCT	GTAGTTATTACAG	CAGTTCATTATT	TATTATCACTACCAGT	360
AB708544	ACCATTAT	TTGTTGGGCTGT	AGTTATTACAGC	AGTTTTACTATT	TATTATCACTACCAGT	360
AB708543	ACCATTAT	TTGTTGGGCTGT	AGTTATTACAGC	AGTTTTACTATT	TATTATCACTACCAGT	360
AB708545	ACCATTAT	TTGTTGGGCTGT	AGTTATTACAGC	AGTTTTACTATT	TATTATCACTACCAGT	360
	** * ***** ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** *					
AB708537	ATTAGCAG	GGGCTATTACTATA	CTATTAAACAGACC	GAAATATTAATAC	ATCATCATTCTTTGA	420
KU220870	ATTAGCC	GGTGCTATTACA	ACTATTAACTGACC	GAAATATTAATAC	GCATCTTTTGA	420
FJ812818	ATTAGCAG	GTGCTATTACTATA	TACTAACGGACCG	AAACATTAACAC	GCCTTTTGA	420
KF369526	ACTAGCAG	GAGCCATTACTATA	CTACTAACGGATC	GAAATATTAATAC	ATCCTTTTGA	420
KX447495	ATTGGCAG	GAGCCATCACCAT	GTACTACAGATC	GAAATATCAAC	ACATCCTTTTGA	420
AB708544	GTTAGCAG	GAGCTATCACTATA	CTACTTACAGATC	GAAATATTAATAC	ATCCTTTTGA	420
AB708543	GTTAGCAG	GAGCTATCACTATA	CTACTTACAGATC	GAAATATTAATAC	ATCCTTTTGA	420
AB708545	GTTAGCAG	GAGCTATCACTATA	CTACTTACAGATC	GAAATATTAATAC	ATCCTTTTGA	420
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AB708537	CCCAGCAG	GAG	431			
KU220870	CCCAGCAG	GAG	431			
FJ812818	CCCAGCAG	GTG	431			
KF369526	TCCAGCGG	GGG	431			
KX447495	CCCGCAG	GGG	431			
AB708544	TCCAGCAG	GAG	431			
AB708543	TCCAGCAG	GAG	431			
AB708545	TCCAGCAG	GAG	431			
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Figure 2. The COI Nucleotide Sequence Alignment using CLUSTAL O(1.2.1) Multiple Sequence Alignment

ANT96818	LVPLMLGAPDMAFPRLNNMSFWLLPSSLTLLASSMVESGAGTGWTVYPPLAGAIAHAGG	60
BAM21814	LVPLMLGAPDMAFPRLNNMSFWLLPSSLTLLASSMVESGAGTGWTVYPPLAGAIAHAGG	60
BAM21815	LVPLMLGAPDMAFPRLNNMSFWLLPSSLTLLASSMVESGAGTGWTVYPPLAGAIAHAGG	60
BAM21816	LVPLMLGAPDMAFPRLNNMSFWLLPSSLTLLASSMVESGAGTGWTVYPPLAGAIAHAGG	60
AGY95255	LVPLMLGAPDMAFPRLNNMSFWLLPSSLTLLASSMVESGAGTGWTVYPPLAGAIAHAGG	60
ADG65303	LVPLMLGAPDMAFPRLNNMSFWLLPSSLTLLASSMVESGAGTGWTVYPPLAGAIAHAGG	60
BAM21808	LVPLMLGAPDMAFPRLNNMSFWLLPSSLTLLASSMVESGAGTGWTVYPPLAGAIAHAGG	60
AMB62087	LVPLMLGAPDMAFPRLNNMSFWLLPSSLTLLASSMVESGAGTGWTVYPPLAGAIAHAGG	60
	*****.*****.*****.*****.*****.*****.*****.*****.*****.*****	
ANT96818	SVDLTIFSLHLAGVSSILGAINFITTTINMKSPGMKMDQMLFVWAVVITAVLLLLSLPV	120
BAM21814	SVDLTIFSLHLAGVSSILGAINFITTTINMKSPGMKMDQMLFVWAVVITAVLLLLSLPV	120
BAM21815	SVDLTIFSLHLAGVSSILGAINFITTTINMKSPGMKMDQMLFVWAVVITAVLLLLSLPV	120
BAM21816	SVDLTIFSLHLAGVSSILGAINFITTTINMKSPGMKMDQMLFVWAVVITAVLLLLSLPV	120
AGY95255	SVDLTIFSLHLAGVSSILGAINFITTTINMKSPGMKMDQMLFVWAVVITAVLLLLSLPV	120
ADG65303	SVDLTIFSLHLAGVSSILGAINFITTTINMKSPGMKMDQMLFVWAVVITAVLLLLSLPV	120
BAM21808	SVDLTIFSLHLAGVSSILGAINFITTTINMKSPGMKMDQMLFVWAVVITAVLLLLSLPV	120
AMB62087	SVDLTIFSLHLAGVSSILGAINFITTTINMKSPGMKMDQMLFVWAVVITAVLLLLSLPV	120
	*****.*****.*****.*****.*****.*****.*****.*****.*****.*****	
ANT96818	LAGAITMLLTDNRNINTSFFDPAG	143
BAM21814	LAGAITMLLTDNRNINTSFFDPAG	143
BAM21815	LAGAITMLLTDNRNINTSFFDPAG	143
BAM21816	LAGAITMLLTDNRNINTSFFDPAG	143
AGY95255	LAGAITMLLTDNRNINTSFFDPAG	143
ADG65303	LAGAITMLLTDNRNINTSFFDPAG	143
BAM21808	LAGAITMLLTDNRNINTSFFDPAG	143
AMB62087	LAGAITMLLTDNRNINTSFFDPAG	143
	*****.*****.*****.*****.*****.*****.*****.*****.*****.*****	

Figure 3. Amino Acid Sequence Alignment of COI using CLUSTAL O(1.2.1)

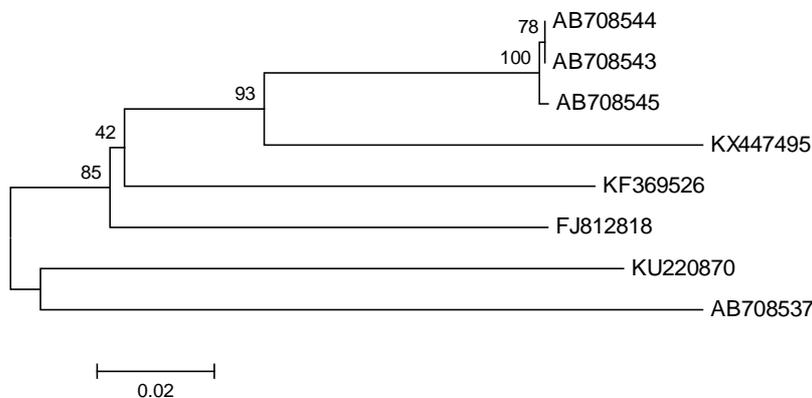


Figure 4. The Phylogenetic Tree of Coenagrionidae Inferred from COI Gene using the Neighbor-Joining Method with Kimura 2-parameters. The Number on the Nodes are the Bootstrap Support Generated after 1,000 Replications

also between *Pseudagrion* and *Ceriagrion* (Figure 3). If the amino acid substitution is at an active site and causes a structural change of the protein, then the protein will have a functional alteration [21]. Serine differs from alanine in that one of its methyl hydrogens has been replaced by a hydroxyl group. Most molecular variations both among and between species are essentially neutral and do not result from selection [22]. Neutral mutations are also the basis for the use of a molecular clock to identify evolutionary events, such as speciation and adaptive or evolutionary radiations. An error in inserting nucleotides during replication or transcription in a coding region may impact amino acid

sequences. There is still a need for further research to determine whether a change from serine to alanine occurs at the active site of COI in the Sarawak specimen and if it would impact the function of the protein.

With its 431-bp length, the COI sequence has a GC content in each *P. pilidorsum* specimen as follows: 42.9% KX447495, 39.7% KF369526, 38.5% AB708543, 38.5% AB708544, and 38.7% AB708545. This indicates that the COI gene sequences used in this research are from mtDNA and not nuclear mitochondrial pseudogenes (numts), such as those found in bird *tyrant flycatchers* (family Tyrannidae) in North America. Numts are

nonfunctional copies of mitochondrial genes that have been translocated into the nuclear genome [23]. In animals, the GC content for all mtDNA ranges between 13–54%, while the sequences used for the DNA barcode range from 22–53% [24]. Numts have higher GC contents compared to orthologous mtDNA [25]. DNA with a high GC content is assumed to have a heat-resistant helix structure, thus providing a selective advantage for animals with high metabolic regulation induced by the environment (e.g., light, temperature, salinity, oxygen, and pH) [26].

Sequence divergences are also present in the mitochondrial COI gene of insects. In the four-spotted skimmern *Libellula quadrimacular*, the sequence divergence is <1% (0.01) in Europe, 0.002–0.023 in North America, and >0.008 in Japan. It is estimated that the substitution rate for insect mtDNA sequences, including the COI region, ranges between 0.015–0.013 per million years [27]. For COI, the intraspecific p-distance for the dragonfly *Orthetrum melania* varies between 0.00–0.03999, while the interspecific p-distance is from 0.033–0.1729 [28]. The inter-subspecific (intraspecific) Kimura 2-parameter genetic distance based on COI genes amongst broad-winged damselflies *Psolodesmus mandarinus kuroiwae*, *P. m. mandarinus*, and *P. m. dorothea* ranged from 0–0.076. The genetic divergence between *Psolodesmus mandarinus kuroiwae* and *P. m. mandarinus* (0.067–0.076) is far higher than that within *P. m. kuroiwae* (0–0.011) or *P. m. mandarinus* (0–0.022). Based on this evidence, *P. m. kuroiwae* was proposed to be classified as a distinct species [39].

Awise (2000 in Herbert et al. [44]) stated that intraspecific divergences of COI genes are rarely greater than 2%; in fact, most are less than 1%. The average percentage of the sequence divergence of congeneric organisms in arthropoda is $10.1\% \pm 4.9$ [44]. Intraspecific Kimura 2-parameter distances range from between 0.122–0.124 (12.2–12.4%) for Sulawesi (KX447495) and Japan specimens, 0.185 (18.5%) for Sulawesi and Sarawak specimens, between 0.145–0.148 (14.5–14.8%) in Sarawak and Japan specimens, and 0–0.002 (0–0.2%) amongst Japan specimens themselves. This distance represents the proportion (p) of nucleotide sites where the two sequences being compared are different. This value is obtained by dividing the number of nucleotide differences by the total number of nucleotides being compared [29]. According to Yong et al. [28], this result indicates that the Sarawak and Japan specimens (0.145–0.148), as well as the Japan and Sulawesi specimens (0.122–0.124), are different species or even possibly different genera because the Kimura 2-parameter distance ranges are very high. The distance between the Sarawak and Sulawesi specimens is 0.185, which is greater than the distance between *P. ignifer* (FJ812818) and the rest of *P. pilidorsum*. Therefore, the Sarawak specimen needs to be revisited. The Sarawak and Sulawesi

specimens can be considered for reclassification as different genera with a distance greater than 17.29% if referring to Yong et al. [28] and Awise [45]. Interestingly, the distance between *P. ignifer* and all *P. pilidorsum* (0.151–0.173) was lower than that found between the Sulawesi and Sarawak specimens (0.185). The distance between all *Pseudagrion* and *Ceragrion* (KU220870) is 0.187–0.222, and this distance has some overlap with the distance between *P. microcephalum* and other *Pseudagrion* (0.205–0.235).

The highest intra-specific distance for the damselfly according to Lin et al. [39] is 0.076 (7.6%). As a comparison, the Asian *Pseudagrion* species studied using the COI gene is more closely related to Asian *Archibasis* compared to two African *Pseudagrion*, so the damselfly taxa in Asia need to be reclassified [30]. Our findings show overlapping intra- and interspecific variations in *Pseudagrion*. For Diptera, for example, there is an overlapping intra- and interspecific variation in COI gene sequences, namely between 0–15.5%, so this result could represent an error in identifying the species [31]. Freshwater crayfish *Cherax preissii* showed the highest divergence value with a maximum genetic distance of 4.45% (patristic distance: 0.5) between the two main populations in North and South Australia [26]. Using the COI gene, Lin et al. [39] showed that the genetic divergence between *Psolodesmus mandarinus kuroiwae* and *P. m. mandarinus* is 7.0–7.6%, which was higher than that for *P. m. kuroiwae* (0–1.1%) or *P. m. mandarinus* (0–2.2%). Based on this evidence, *P. m. kuroiwae* should be classified as a distinct species. The greater the genetic distance between populations or individuals, the more isolated they are from one another due to the less interbreeding that takes place between them. The degree of isolation and demographic history of populations are key factors that affect the population's or individual's potential to adapt to different environmental conditions. Changes in the biogeography may have an impact on genetic variation both within and between populations [32]. The COI gene is widely used as a tool in the study of phylogeography and history at the intra- and interspecific levels because of its low or absence of recombination, uniparental (maternal) mode of inheritance, conserved structure, and relatively high rate of evolution; this gene has been used extensively to study the evolution of insects [12, 20, 32].

Nucleotide diversity is the average number of nucleotide differences per nucleotide sites being compared between DNA sequences [34]. High nucleotide variations in the data presented indicate geographical isolation, which suggests that gene flow between populations is limited in these three locations. Thus, the populations can be regarded as different conservation units because they have substantial differences in the level of DNA [33]. Therefore, these three groups of the population have evolved independently over a given period, so they may

have unique adaptations that ought to be preserved, although the types of adaptations are not yet clear. A population with a low level of genetic diversity is less able to adapt to new selection pressures because the limited set of genes (gene pool) in a particular group will decrease the tendency for the presence of adaptive alleles in a group or population [33]. A population or group will not only differ in adaptive loci as a direct result of natural selection but also will contain variations at neutral loci due to genetic drift, which indirectly results from selection through a reproductive barrier. This pattern was identified in a semi-natural experiment that produced the population or group *Anthoxanthum odoratum*, which was previously thought to be different as a result of adaptation due to the addition of different nutrients to the diet. This is an example that illustrates how local adaptation can shape the genetic diversity and differences between populations [33].

The NJ phylogenetic tree (Figure 4) indicates that *P. pilidorsum* and *P. ignifer* formed a monophyletic group. The position of the Sulawesi specimen is close to the Japan specimens, and this trend is supported by the Kimura 2-parameter genetic distance calculation. The phylogenetic relationship represents kinship among the study groups. The horizontal dimension is the number of genetic changes indicating the evolutionary lineage that changes over time. The longer the branches in the horizontal dimension, the greater the number of changes that have occurred. By assuming one generation per year, the value of pairwise sequence divergence is 2.3% per year for COI so that the neutral mutation rate per nucleotide site per generation (α) is 1.15×10^{-8} [20].

The combination of fragments of COI and ND1 is better compared to only one fragment alone [35]. Several studies that have used more than one fragment for the DNA barcode have provided information on the level of difference and variation patterns for phylogenetic studies by examining the divergence in DNA sequences [36, 37]. A character-based approach provides a higher resolution than the distance-based method in Odonata, especially on a closely related taxonomic entity. Character-based DNA barcodes can be used to characterize species through a unique combination of diagnostic characteristics that are not genetic distance-based. In this way, the species barriers may be determined using a series of diagnostic evaluations about the characters that can be improved at any stage of resolution using multiple genes [18].

Conclusions

Our results indicate that the identity of the COI gene in the Sulawesi specimen and in other *P. pilidorsum* from GenBank (Sarawak and Japan specimens) is low, ranging from 83.99–89.10%. Moreover, the genetic distances are high enough to suggest that the Sulawesi specimen

differs from the Japan specimen and the Sarawak specimen also differs from the Japan specimen. This finding also indicates that the Sarawak and Sulawesi specimens are of different genera. However, more specimens from Sulawesi are needed for analysis to clarify their taxonomic identity. Although this finding suggests the need to reclassify *P. pilidorsum*, in-depth study involving more than one marker for the DNA barcode as well as a character-based DNA barcode must first be undertaken.

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