Phylogenetic Relationships of *Isognomon* (Lightfoot, 1786) Oysters from North Sulawesi, Indonesia

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Abstract

The *Isognomon* (Lightfoot, 1786) is a genus of oysters found in various coastal ecosystems throughout the world. Along with other bivalves, it performs significant ecological functions in marine ecosystems by providing food and habitat for fish and invertebrate habitats, filtering water, and protecting shorelines. Taxonomic classification of the *Isognomon* oyster can be challenging due to the varied or cryptic phenotypic characters, particularly shell characters. In this study, two specimens with different shell characters of *Isognomon* oyster were collected from mangrove waters in Likupang, North Sulawesi, Indonesia, and subjected to molecular analysis to determine their identity. The mitochondrial cytochrome C oxidase subunit I (COI) gene was utilized as a primer for this purpose, and the genetic distance and phylogenetic position of the two specimens were determined by comparing them with the GenBank database. The Basic Local Alignment Search Tool (BLAST) revealed that the two specimens were of belonged to *Isognomon ephippium*, with a similarity of 99.84%. The genetic distance between the two specimens was calculated using the Tamura Nei model and found to be 0.00, while the genetic distance between *I. ephippium* and other species in the *Isognomon* genus ranged from 0.00 to 0.14. The results of the Neighbor Joining (NJ) tree analyses showed that the two specimens clustered together with *I. ephippium*, which was divided into two distinct clades with a strong bootstrap value of 100 at the node.

Keywords: Bivalvia, COI gene, *isognomon*, oyster, North Sulawesi.

INTRODUCTION

The genus *Isognomon* (Lightfoot, 1786) belongs to marine bivalves of the order Ostreida and superfamily Pterioidae. The current taxonomic classification of the superfamily Pterioidae comprises three recent families; Pteriidae (Gray, 1847), Malleidae (Lamarck, 1818) and Pulvinitidae (Stephenson, 1941) (Benthotage *et al*., 2020). According to Bieler *et al*. (2010), Carter *et al*. (2011) and Benthotage *et al*. (2020), the genus *Isognomon* is no longer classified in the family Isognomonidae, as previously assumed, but is now considered part of the family Pteriidae (Gray, 1847). This family comprises of 16 different species, including *Isognomon alatus* (Gmelin, 1791), *I. albisoror* (Iredale, 1939), *I. australica* (Reeve, 1858), *I. bicolor* (C.B. Adams, 1845), *I. californicum* (Conrad, 1837), *I. dunkeri* (Fischer, 1881), *I. ephippium* (Linnaeus, 1758), *I. incisum* (Conrad, 1837), *I. isognomum* (Linnaeus, 1758), *I. janus* (Carpenter, 1857), *I. legume* (Gmelin, 1791), *I. nucleus* (Lamarck, 1819), *I. perna* (Linnaeus, 1767), *I. radiatus* (Anton, 1838), *I. recognitus* (Mabille, 1895), and *I. vullseloides* Benthotage *et al.* (2020). The bivalve species, including the genus *Isognomon*, perform significant ecological functions in marine ecosystems. They are known to contribute to the formation of reefs made of living assemblages and dead shells, provide a food source for fish and other invertebrates, filter water, and protect shorelines (Dame *et al*., 1984; Meyer *et al*., 1997; Gutiérrez *et al*., 2003; Kirby, 2004). *Isognomon* can be found in a diverse range of habitats, including rocks, mangrove trees, coral reefs, sandy beaches, algal turf, floating debris, and artificial structures, and have a global distribution (Benthotage *et al*., 2020). Despite their abundance and widespread distribution, *Isognomon* remain vastly understudied (Tëmkin and Printrakoon, 2016).

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According to Benthotage *et al*. (2020), species in the genus *Isognomon* may exhibit cryptic characteristics, where two or more species have similar morphologies. Wilk and Bieler (2009) found that *I. alatus* is often misidentified as *I. bicolor* in the field (sympatric species) or as *I. ephippium* in museum collections (allopatric species) due to their morphological similarities. Tëmkin and Printrakoon (2016) also reported that *I. spathulatus* (Reeve, 1858), the mangrove-associated oyster, has been incorrectly synonymized because of its similar morphological characteristics with *I. ephippium*. This ambiguity is not surprising given that the genus *Isognomon* exhibits marked habitat-dependent variability in shell shapes (Coan *et al*., 2000; Wilk and Bieler, 2009) and convergent interspecific morphologies (Benthotage *et al*., 2020). This study aims to utilize molecular analysis to identify two oyster specimens from North Sulawesi Indonesia, examine their molecular divergence, and determine the phylogenetic position of the specimens by biometrical approach.

MATERIAL AND METHODS

In this study, oyster specimens were obtained from the mangrove ecosystem located in Likupang waters (latitude 1.718507488845435, longitude 125.0199707887088), North Sulawesi, Indonesia. Samples for molecular analysis were collected from the foot of the oysters and preserved in 70% ethanol. The foot is located in the anterior surface of the visceral mass, it is tongue-shaped, flattened dorsally, and slightly tapers distally. A total of two oyster specimens (KBL1 and KBL2) were included in the study. DNA was extracted using the innuPREP DNA Mini Kit (Analytic Jena AG, Jena, Germany). The cytochrome oxidase I (COI) region of the mitochondrial DNA was amplified using a universal primer designed by Folmer *et al*. (1994), consisting of the LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HCO2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') primer pairs. The amplification was conducted on a Professional Thermocycler Biometra (manufactured by Analytik Jena) using a reaction with a total volume of 25 µl, which consisted of 1 µl DNA template, 5 µl of 5x Hot fire pool, 1 µl each primer pair and 17 µl ddH2O. The PCR reaction was performed with a specific cycling profile, including 94°C for 3 min, followed by 30 cycles at 94°C for 1 min, 50°C for 30 sec, and 72°C for 1 min, with an additional extension period of 72°C for 10 min during the last cycle. The amplified COI gene was sequenced by FirstBase Co., Selangor, Malaysia. The sequencing procedure was conducted in a bidirectional manner utilizing the BigDye® Terminator v.3.1 Cycles Sequencing Kit manufactured by Applied Biosystems in the United States. The resulting sequences were further read with an ABI PRISM® 377 automatic DNA sequencer. The quality of the obtained sequence was analyzed using the Sequence Scanner version 2.0 Software developed by Applied Biosystem. After analysis, the sequences underwent trimming, assembling, and manual editing using Geneious Prime version 2020, a software developed by Kearse *et al*. (2012) and available at http://www.geneious.com. Finally, the edited sequences were subjected to Basic Local Alignment Tools (BLAST) analysis at The National Center for Biotechnology Information (NCBI), accessible at https://www.ncbi nlm.nih.gov. Phylogenetic analysis was performed using MEGA X software (Kumar *et al*., 2018). The sequences were aligned using Clustal W, with manual editing applied as necessary. Pairwise nucleotide sequence divergences were calculated using the Tamura Nei model, and a Neighbor Joining (NJ) tree method was used to construct a phylogenetic tree. The robustness of the nodes in the NJ analysis was evaluated with 1000 bootstrap replications. The phylogenetic tree was constructed using selected COI sequences of genus *Isognomon* from the GenBank and with the species *Crassostrea* serving as the outgroup

RESULTS AND DISCUSSION

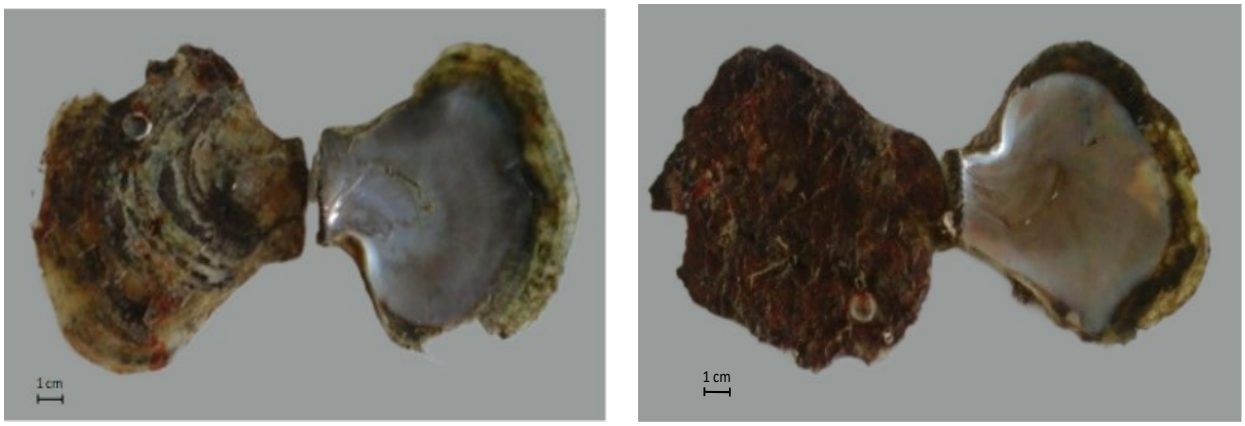
The specimens of *Isognomon* oyster species examined in this study, designated as KBL1 and KBL2, exhibit different shell characteristics. Specifically, KBL1 displays a light brown shell with interspersed dark brown markings, while KBL2 has a dark brown shell with faint and irregular light brown spots (Figure 1). Taxonomic classification of oysters traditionally relies on phenotypic characters, including shell and/or morphological features, as the primary basis (Gosling, 2015). However, several studies have demonstrated that phenotypic characters of *Isognomon* oyster are highly variable and can exhibit cryptic variations, posing challenges for shell-based taxonomic studies (Printrakoon and Tëmkin, 2008; Tëmkin and Printrakoon, 2016; Benthotage *et al*., 2020).

Recently, the World Register of Marine Species (WoRMS) has embraced molecular analysis for oyster identification (Bieler *et al*., 2010; Carter *et al*., 2011; Benthotage *et al*., 2020). As a result of this molecular approach, the taxonomy of the genus *Isognomon* has been revised and it has been included in the family Pteriidae (Gray, 1847) (Phylum: Mollusca, Class: Bivalvia, Order: Ostreida, Superfamily: Pterioidea) (Bieler *et al*., 2010; Carter *et al*., 2011; Benthotage *et al*., 2020). According to the review by Benthotage *et al*. (2020), species in the genus *Isognomon* are widely distributed in various regions around the world, from North America to Asia and Oceania. In Indonesia, species of this genus are found in many islands, including Maluku (Silulu *et al*., 2013), Belitung (Cappenberg and Wulandari, 2019), Sulawesi (Rau *et al*., 2013; Samsi *et al*., 2019), Lombok (Putra *et al*., 2021), Papua (Marey and Maitindom, 2019), Aceh (Mutia *et al*., 2021), Riau (Simarmata and Fajri, 2020), Sumatera (Rinaldi, 2021), and other islands.

In this study, we employed a molecular approach to identify the two oyster specimens from North Sulawesi, Indonesia. The BLAST results using the mitochondrial DNA COI gene database in Genbank, revealed a close relationship between the two specimens based on the amplified COI gene, indicating their affiliation with the genus *Isognomon*. To further analyze the genetic distance and position of the North Sulawesi oyster specimen, we utilized 68 COI gene sequences of species in the genus *Isognomon* and a COI gene sequence of *Crassostrea gigas* from the GenBank database (see Table 1).

The results of the BLAST analysis of the COI gene nucleotide of the two specimens yielded five top hits with close values, including score, query cover, e-value and identity (ranging from 1088-1186, 85-92%, 0.0, and 97.78-99.84%, respectively) matching the species *I. Ephippium* (KY081310.1; MW339756.1; MW339758.1; MW339759.1; MW339757.1) (Table 2). The two *Isognomon* specimens exhibited a percent identity of 97.94-99.84% with the top five hits of *I. ephippium* in the Genbank database. According to various studies by Hebert *et al*. (2003), Barbuto *et al*. (2010), Armani *et al*. (2015), Ratnasingham and Hebert (2007, 2013) and Stahlhut *et al*. (2013), the commonly accepted thresholds for species delimitation based on the COI gene are below 2% for species differentiation and 3% for predicting cryptic or new species.

The results of the genetic distance analysis based on the Tamura Ney model for the 67 COI sequences of the genus *Isognomon* are pesented in Table 3. The pairwise genetic distance values between the two specimens (KBL1 and KBL2) from North Sulawesi were found to be 0.00 indicating their close proximity to eight other vouchers (MW339759.1; MW339758.1; MW339760; MW339756.1; MW339757; MN608258.1; MN608257.1; KY081310.1) of *I. Ephippium* with distances ranging from 0.00 to 0.05. However, the genetic distance values between the two North Sulawesi specimens and



(a) (b)

Figure 1. Specimens KBL1 (a) and KBL2 (b) collected from Likupang, North Sulawesi Indonesia

Table 1. List COI gene sequences of *Isognomon* species an accession numbers in the GenBank

Species	Accession No			References
Isognomon ephippium	MW339756.1;	MN608258.1;	KU341971.1;	Liu *et al*. (2018)
	MW339757.1;	MN608259.1;	KU341972.1;	
	MW339758.1;	MN608260.1;	KU341973.1;	
	MW339759.1;	MN608261.1;	KU341974.1;	
	MW339760.1;	MN608262.1;	KU341975.1;	
	MN608257.1;	KY081310.1;		
Isognomon alatus	KX369031.1;	KU758983.1;	KU758996.1;	Wilk (2016)
	KX369032.1;	KU758984.1;	KU758997.1;	
	KU758978.1;	KU758985.1;	KU758998.1;	
	KU758979.1;	KU758991.1;	KU759005.1;	
	KU758980.1;	KU758993.1;	KU759006.1;	
	KU758981.1;	KU758994.1;		
	KU758982.1;	KU758995.1;		
Isognomon bicolor	KX373613.1			
Isognomon isognomum	MN608263.1	MN608265.1;		Ip *et al*. (2019) Ip *et al*. (2022)
Isognomon legumen	AB076950.1;	MN608275.1;	KU341969.1;	Patoka *et al*. (2020)
	MW284798.1;	KU341965.1;	MT802137.1;	Matsumoto (2003),
	MW284806.1;	KU341966.1;	KX713469.1;	Patoka *et al*. (2020),
	MW284808.1;	KU341967.1;		Combosch *et al*. (2017)
	MW284809.1;	KU341968.1;		
Isognomon nucleus	KU341970.1;	KU759001.1;	KT290125.1	Wilk (2016) Ardura *et al*. (2015)
Isognomon perna	AB076918.1;	MN608271.1;	KU341963.1	Ardura *et al*. (2015)
	MW284794.1;	MN608272.1;	KU341964.1;	Matsumoto (2003)
Isognomon recognitus	KU759007.1;	KT317609.1;		Wilk (2016)
	KT317607.1;	KT317610.1;		Raith (2013)
Isognomon acutirostris	AB076926.1			Matsumoto (2003)
Crassostrea gigas (outgroup)	KF644048.1			Layton *et al*. (2014)

other *I. Ephippium* specimens (MN608262.1; MN608261.1; KU341975.1; MN608260.1; KU341974.1; MN608259.1; KU341973.1; KU341972.1; KU341971.1) were found to be between 0.13 – 0.14%. The pairwise genetic distance value between the two specimens and other species of *Isognomon*, including *I. alatus* (0.39%), *I. bicolor* (0.39%), *I. isognomum* (0.48%), *I. legumen* (0.35-0.49%), *I. nucleus* (0.39-0.42%), *I. perna* (0.49-0.50%) and *I. recognitus* (0.42-0.43%), was significantly different.

A phylogenetic tree showing the relationship between the two Indonesian COI sequences (KBL1 and KBL2) and other *isognomon* species, with *Crassostrea gigas* as outgroup is presented in Figure 2. The tree was constructed using the Neighbor Joining method with 1000 bootstrap replications, following the Tamura-Nei method. The oyster species *I. ephippium, I. recognitus, I. alatus*, and *I. perna* were found to form monophyletic groups, with a high level of support of 98-100% bootstrap. However, *I. legumen* was found to be paraphyletic. *I. recognitus* and *I. nucleus* were determined to be sister group to *I. bicolor* and *I. acutirostris*, respectively. The Indonesian oyster specimens (KBL1 and KBL2) in this study were observed to cluster together with *I. ephippium*, which was divided into two distinct clades with a strong bootstrap value of 100 at the node.

Table 2. Top five hits of the Nucleotide BLAST analysis of the COI gene for the mangrove oyster from North Sulawesi, Indonesia retrieved the NCBI GenBank

Specimen	Species (accession number)	Total Score	Query cover (%)	E-value	Percent identity (%)
KBL1	*Isognomon ephippium* (KY081310.1)	1181	89	0.0	99.54
	Isognomon ephippium (MW339756.1)	1136	85	0.0	99.68
	Isognomon ephippium (MW339758.1)	1131	85	0.0	99.52
	Isognomon ephippium (MW339759.1)	1092	86	0.0	98.09
	Isognomon ephippium (MW339757.1)	1088	86	0.0	97.78
KBL2	*Isognomon ephippium* (KY081310.1)	1186	92	0.0	99.69
	Isognomon ephippium (MW339756.1)	1142	88	0.0	99.84
	Isognomon ephippium (MW339758.1)	1136	88	0.0	99.68
	Isognomon ephippium (MW339759.1)	1098	89	0.0	98.25
	Isognomon ephippium (MW339757.1)	1094	89	0.0	97.94

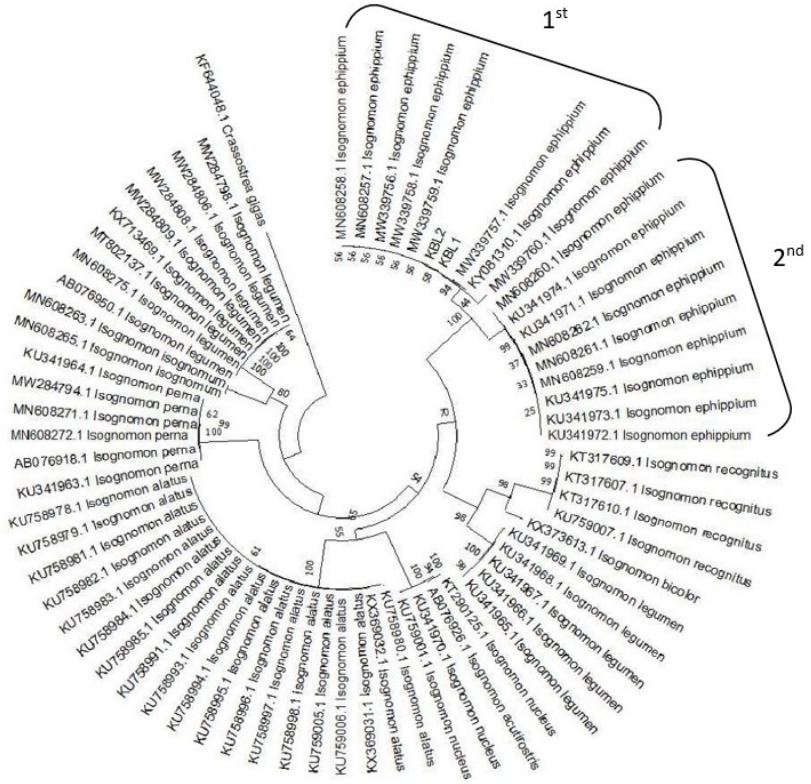


Figure 2. Phylogenetic positioning of mangrove oyster species (KBL1 and KBL2) from North Sulawesi, Indonesia based on Neighbor Joining (NJ) analysis of Tamura Nei distance. NJ bootstrap values are indicated in the branches.

Table 3. Tamura-Nei nucleotide divergences (%) were calculated for 68 sequences representing 10 species within the genus Isognomon.

No	Accession No / Species	Distance	No	Accession No / Species	Distance
1	KBL1	0.00	36	KU758980.1_*Isognomon_alatus*	0.39
2	KBL2	0.00	37	KU758979.1_*Isognomon_alatus*	0.39
3	MW339760.1_*Isognomon_ephippium*	0.05	38	KU758978.1_*Isognomon_alatus*	0.39
4	MW339759.1_*Isognomon_ephippium*	0.00	39	KX373613.1_*Isognomon_bicolor*	0.39
5	MW339758.1_*Isognomon_ephippium*	0.00	40	MN608265.1_*Isognomon_isognomum*	0.48
6	MW339757.1_*Isognomon_ephippium*	0.01	41	MN608263.1_*Isognomon_isognomum*	0.48
7	MW339756.1_*Isognomon_ephippium*	0.00	42	AB076950.1_*Isognomon_legumen*	0.49
8	MN608262.1_*Isognomon_ephippium*	0.13	43	MN608275.1_*Isognomon_legumen*	0.49
9	MN608261.1_*Isognomon_ephippium*	0.13	44	MT802137.1_*Isognomon_legumen*	0.49
10	MN608260.1_*Isognomon_ephippium*	0.13	45	MW284809.1_*Isognomon_legumen*	0.49
11	MN608259.1_*Isognomon_ephippium*	0.13	46	MW284808.1_*Isognomon_legumen*	0.49
12	MN608258.1_*Isognomon_ephippium*	0.00	47	MW284806.1_*Isognomon_legumen*	0.49
13	MN608257.1_*Isognomon_ephippium*	0.00	48	MW284798.1_*Isognomon_legumen*	0.49
14	KY081310.1_*Isognomon_ephippium*	0.00	49	KX713469.1_*Isognomon_legumen*	0.49
15	KU341975.1_*Isognomon_ephippium*	0.13	50	KU341969.1_*Isognomon_legumen*	0.36
16	KU341974.1_*Isognomon_ephippium*	0.13	51	KU341968.1_*Isognomon_legumen*	0.35
17	KU341973.1_*Isognomon_ephippium*	0.13	52	KU341967.1_*Isognomon_legumen*	0.35
18	KU341972.1_*Isognomon_ephippium*	0.13	53	KU341965.1_*Isognomon_legumen*	0.35
19	KU341971.1_*Isognomon_ephippium*	0.14	54	KU341966.1_*Isognomon_legumen*	0.35
20	KX369032.1_*Isognomon_alatus*	0.39	55	KU759001.1_*Isognomon_nucleus*	0.41
21	KX369031.1_*Isognomon_alatus*	0.39	56	KU341970.1_*Isognomon_nucleus*	0.41
22	KU759006.1_*Isognomon_alatus*	0.39	57	KT290125.1_*Isognomon_nucleus*	0.42
23	KU759005.1_*Isognomon_alatus*	0.39	58	AB076918.1_*Isognomon_perna*	0.50
24	KU758998.1_*Isognomon_alatus*	0.39	59	MN608272.1_*Isognomon_perna*	0.50
25	KU758997.1_*Isognomon_alatus*	0.39	60	MN608271.1_*Isognomon_perna*	0.50
26	KU758996.1_*Isognomon_alatus*	0.39	61	MW284794.1_*Isognomon_perna*	0.50
27	KU758995.1_*Isognomon_alatus*	0.39	62	KU341964.1_*Isognomon_perna*	0.50
28	KU758994.1_*Isognomon_alatus*	0.39	63	KU341963.1_*Isognomon_perna*	0.49
29	KU758993.1_*Isognomon_alatus*	0.39	64	KU759007.1_*Isognomon_recognitus*	0.43
30	KU758991.1_*Isognomon_alatus*	0.39	65	KT317610.1_*Isognomon_recognitus*	0.43
31	KU758985.1_*Isognomon_alatus*	0.39	66	KT317609.1_*Isognomon_recognitus*	0.44
32	KU758984.1_*Isognomon_alatus*	0.39	67	KT317607.1_*Isognomon_recognitus*	0.43
33	KU758983.1_*Isognomon_alatus*	0.39	68	AB076926.1_*Isognomon_acutirostris*	0.42
34	KU758982.1_*Isognomon_alatus*	0.39	69	KF644048.1_*Crassostrea_gigas*	0.68

The Indonesian oysters were found to be part of the first clade, which comprised eight *I. ephippium* vouchers originating from Australia (MW339760.1; MW339759.1; MW339758.1; MW339757.1; MW339756.1) (Benthotage *et al*., 2020) and China (MN608258.1; MN608257.1; KY081310.1) (Liu *et al*., 2018). The second clade of *I. ephippium* consisted of nine additional vouchers, all originating from China (MN608262.1; MN608261.1; MN608260.1; MN608259.1; KU341975.1; KU341974.1; KU341973.1; KU341972.1; KU341971.1) (Liu *et al*., 2018). *I. ephippium* was also found to form a distinct clade as reported by Wilk (2016), who used COI and 16S gene sequences and discovered a different clade position of *I. ephippium* from Australia and Thailand.

CONCLUSION

According to our findings, the two *Isognomon* specimens collected from mangrove waters in Likupang, North Minahasa (Indonesia), have been identified as *I. ephippium*, with the highest similarity value of 99.84% in their COI

gene sequences. The genetic distance value between these two specimens was determined to be 0.00, falling within the genetic distance range of 0.00 to 0.14 when compared to *I. ephippium*. Our phylogenetic reconstructions results revealed that these two specimens clustered together with *I. Ephippium* in a clade, and this clade was further devided into two distinct subclades, each with a strong bootstrap value of 100 at the node. Additionally, the two Indonesian specimens were found to be in the same clade as eight *I. ephippium* vouchers originating from Australia and China. The other clade of *I. ephippium* consisted of nine additional vouchers from China, suggesting the presence of potential distinct taxa within the *I. ephippium* species.

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