Resolving Species Identification and Distribution Patterns of *Neotrygon* spp. in Thai Waters: Inefficiency of Morphometric Analysis and the Power of *COI* Gene Barcoding and Phylogenetics

Thadsin Panithanarak[1*], Ratima Karuwanjaroen[1], Chanapa Utama[2], Penchan Laongmanee[2], Titirat Meejan[1], Wansuk Senanan[3], Wanlada Klangnurak[4], Jenjit Khudamrongsawat[5] and Tassapon Krajangdara[6]

ABSTRACT

Three species of maskrays in the genus *Neotrygon* have previously been reported in Thai waters. However, the high morphological similarity among species within the blue-spotted maskray complex (*Neotrygon* spp.) makes accurate identification challenging. This study aimed to confirm species identification in *Neotrygon* spp. using morphometric and genetic data and to further examine their phylogenetic relationships in Thai waters by analyzing mitochondrial *COI* (cytochrome *c* oxidase subunit I) sequences. Multivariate analysis (PCA) of 37 morphometric characters from 55 specimens proved insufficient for species-level discrimination. However, molecular phylogenetic analysis of 14 unique *COI* sequences from Thai waters, combined with 61 reference sequences from previously documented related species and closely related species, identified two monophyletic clades. These clades corresponded to two species: *N. varidens*, found exclusively in the Gulf of Thailand, and *N. malaccensis*, recorded for the first time in Thailand, occurring in both the Gulf of Thailand and the Andaman Sea. This DNA-based identification provides clear evidence for species boundaries within Thai *Neotrygon* species and highlights the importance of molecular tools for distinguishing morphologically similar marine species in the Indo-Pacific region.

Keywords: *COI*, Cryptic species complex, Gulf of Thailand, Molecular taxonomy, *Neotrygon kuhlii*

INTRODUCTION

The genus *Neotrygon* (Order Myliobatiformes, Family Dasyatidae), commonly known as maskrays, initially composed of six valid species, including four Australian endemics (*N. annotata, N. leylandi, N. ningalooensis,* and *N. picta*), *N. trigonoides* from the South-West Pacific, and the blue-spotted maskray *N. kuhlii* from the Indo-West Pacific (Last *et al*., 2016). Previously considered a single species, *N. kuhlii* (Müller and Henle, 1841) underwent extensive taxonomic revision, supported by molecular genetic evidence, which has since revealed that it represents a cryptic species complex comprising more than 10 distinct species worldwide (Ward *et al*., 2008; Borsa *et al*., 2012; Naylor *et al*., 2012; Arlyza *et al*., 2013; Puckridge *et al*., 2013; Last *et al*., 2016; Borsa *et al*., 2018).

[1]Institute of Marine Science, Burapha University, Chonburi, Thailand
[2]Faculty of Marine Technology, Burapha University, Chanthaburi Campus, Chanthaburi, Thailand
[3]Department of Aquatic Science, Faculty of Science, Burapha University, Chonburi, Thailand
[4]Department of Animal Production Technology and Fishery, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand
[5]Department of Biology, Faculty of Science, Mahidol University, Bangkok, Thailand
[6]66/27 Soi 5, Thep Thani Village, Moo 2, Rasada, Muang, Phuket, Thailand
[*]Corresponding author. E-mail address: thadsin@buu.ac.th
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The blue-spotted maskray species complex, widely distributed throughout the Indo-West Pacific from eastern Africa to Japan, presents identification challenges due to morphological similarities and overlapping diagnostic characters among its members (Last *et al*., 2016; Borsa *et al*., 2018). Phylogenetic analyses initially revealed nine divergent clades (Puckridge *et al*., 2013), with subsequent studies identifying additional lineages (Arlyza *et al*., 2013; Borsa *et al*., 2016). Currently recognized species within this complex include *N. trigonoides* (Coral Sea), *N. kuhlii* (Vanikoro, Santa Cruz archipelago), *N. australiae* (northern Australia, New Guinea, eastern Indonesia), *N. caeruleopunctata* (Indian Ocean, southern Bali, southern Java), *N. orientalis* (North-West Pacific), and the resurrected *N. varidens* (Indo-West Pacific) (Last *et al*., 2016; Borsa *et al*., 2019). Additional species include *N. bobwardi* (western Sumatra, Aceh), *N. malaccensis* (eastern Andaman Sea, Malacca Strait), *N. moluccensis* (eastern Banda Sea), *N. westpapuensis* (northern West Papua) (Borsa *et al*., 2018), *N. indica* (Indian Ocean) (Pavan-Kumar *et al*., 2018), *N. vali* (Solomon archipelago) (Borsa, 2017), and the recently described *N. yakkoei* (Japan) (Hata and Motomura, 2024).

In Thailand, three *Neotrygon* species have previously been reported (Last *et al*., 2016): *N. caeruleopunctata* from the Andaman Sea and *N. orientalis* from the Gulf of Thailand, both characterized by conspicuous blue ocellated spots on the dorsal disc, and *N. varidens* from the Gulf of Thailand, distinguished by its dark brown or mauve dorsal disc lacking blue ocellated spots. These species are frequently misidentified as *N. kuhlii*, the previous name for this species complex (Krajangdara *et al*., 2022). To resolve species identification and distribution patterns in Thai waters, this study employed both morphometric analyses and *COI* gene barcoding, while also investigating phylogenetic relationships within the species complex through *COI* sequence analysis.

MATERIALS AND METHODS

Morphological examination and morphometric data analysis

Fifty-five frozen maskray specimens were examined for morphological analysis, including 37 specimens from the Gulf of Thailand (Chonburi Province (n = 2), Chanthaburi Province (n = 5), Chumphon Province (n = 4), Surat Thani Province (n = 3), Songkla Province (n = 8), Nakhon Si Thammarat Province (n = 8), and Pattani Province (n = 7)) and 18 specimens from the Andaman Sea (Ranong Province (n = 3), Trang Province (n = 2), Phuket Province (n = 2) and Satun Province (n = 11)). Details of all specimens used in the morphological examination are listed in Supplementary Table S1. Specimens were preliminarily classified following the identification guide by Krajangdara *et al*. (2022), based primarily on the presence or absence of conspicuous blue ocellated spots on the dorsal disc. Additionally, species distribution ranges reported by Last *et al*. (2016) and Borsa *et al*. (2018) were considered for classification.

Thirty-seven morphometric characteristics of the specimens were analyzed based on Last *et al*. (2016). Morphometric data were expressed as a percentage of disc width (%DW), except for disc width itself, which was reported in mm. To isolate size effects from shape, all morphometric data were transformed to proportions relative to disc width before conducting Principal Component Analysis (PCA). PCA was performed to assess similarity among specimens based on these characteristics. Statistical analysis was carried out using R 4.1.0 (R Core Team, 2022), and PCA results were extracted and visualized using the factoextra R package (Kassambara and Mundt, 2020).

DNA extraction, amplification, and sequencing

Fifty-nine tissue specimens (approximately 1×1 cm, preserved in absolute ethanol at 4 °C) were used for genetic analysis, including 38 specimens from the Gulf of Thailand [Chonburi Province (n = 3), Rayong Province (n = 1), Chanthaburi Province (n = 6), Chumphon Province (n = 2), Surat Thani Province (n = 4), Nakhon Si Thammarat Province

(n = 7), Songkla Province (n = 8), Pattani Province (n = 5), and R.V. Chulabhorn (Gulf of Thailand Expedition, lat 7.723832, long 101.797185) (n = 2)] and 21 specimens from the Andaman Sea [Ranong Province (n = 3), Trang Province (n = 5), Phuket Province (n = 2), and Satun Province (n =11)]. Details of all specimens used in the genetic analysis are listed in Supplementary Table S1.

DNA extraction was performed using a commercial DNA extraction kit (DNeasy Blood and Tissue kit, Qiagen, Hilden, Germany). Subsequently, the mitochondrial *COI* fragment was amplified using the universal primers FishF1 (5'- TCAACC AACCACAAAGACATTGGCAC -3') and FishR1 (5'-TAGACTTCTGGGTGGCCAAAGAATCA -3') (Ward *et al*., 2005) with an approximate size of 700 bp. A polymerase chain reaction (PCR) was conducted in a 25 µL volume containing 80 µM dNTP (2 µL) (Invitrogen, Waltham, Massachusetts, USA), 1xPCR buffer (2.5 µL), 0.4 µM primer (1 µL) (Macrogen, Inc., Seoul, South Korea), 2 mM $MgCl_2$ (1 µL), 1 unit *Taq* DNA polymerase (0.2 µL) (Invitrogen) and 1 µg template DNA (1 µL). The PCR procedure consisted of an initial denaturation at 95 ºC for 5 min, then 35 cycles of (1) denaturation at 95 ºC for 30 s, (2) annealing at 50 ºC for 30 s, and (3) extension at 72 ºC for 30 s. The procedure was completed with a final extension at 72 ºC for 10 min. PCR purification was conducted to decontaminate the PCR products using a commercial PCR purification kit (QIAquick PCR purification kit, Qiagen). DNA sequencing by the Sanger method was conducted at the sequencing service (U2Bio (Thailand) Co., ltd., Bangkok, Thailand).

Genetic data analysis

Nucleotide sequences of the *COI* gene were manually checked for accuracy. All sequences were compared and then aligned using MUSCLE implemented in MEGA 11: Molecular Evolutionary Genetic Analysis version 11 (Tamura *et al*., 2021). Furthermore, all sequences were compared with mitochondrial *COI* sequences of the maskrays (genus *Neotrygon*) deposited in GenBank using BLASTN (http://blast.ncbi.nlm.nih.gov).

Genetic distance and phylogenetic analysis

Genetic distance between pairs of the maskray sequences (distance measure = Kimura 2-parameter) was calculated using MEGA11 (Tamura *et al*., 2021). The phylogenetic dataset included *COI* sequences from this study and 61 reference sequences (with at least 95% site coverage when aligned to the sequences from this study, Supplementary Table S2), including best-matched BLASTN sequences, previously recorded species in Thai waters (*Neotrygon varidens*, *N. caeruleopunctata*, and *N. orientalis*), and closely related Indo-West Pacific species (*N. malaccensis, N. bobwardi, N. indica, N. moluccensis, N. australiae, N. westpapuensis*, *N. trigonoides*, and *N. picta*) from GenBank and/or iBOL database. However, a reliable reference sequence for *N. kuhlii* with a verified taxonomic designation and at least 95% coverage was unavailable (Borsa *et al*., 2016; 2018; Pavan-Kumar *et al*., 2018).

As many reference sequences in public databases were assigned to *N. kuhlii* and had not been updated following recent taxonomic revisions, scientific names used in this study were revised according to Borsa *et al*. (2016; 2018) and Pavan-Kumar *et al*. (2018). The dataset also included broad cowtail ray (*Pastinachus ater*, accession number: EU398973) as an outgroup, as it belongs to the family Dasyatidae and is closely related to *Neotrygon*.

Phylogenetic relationships among mitochondrial *COI* haplotypes from this study and sequences from GenBank and/or iBOL database were inferred using the maximum likelihood method. A maximum likelihood tree was constructed using the T92+G model (Tamura 3-parameter model with gamma-distributed rate variation among sites) (Tamura, 1992), which was identified as the best DNA substitution model based on the lowest Bayesian Information Criterion (BIC) scores in MEGA11 (Tamura *et al*., 2021). Bootstrap analysis with 1,000 replicates was performed to assess tree topology.

RESULTS AND DISCUSSION

Morphological study

The maskray specimens were classified into three groups: A, B, and C, based on their distribution and the presence or absence of conspicuous blue ocellated spots on the dorsal disc (Figure 1, Supplementary Table S1 and S3). Notably, due to the ambiguity of morphology-based classification in the blue-spotted maskray species complex, species distribution ranges have been used despite their limitations (Borsa *et al*., 2018). Additionally, the absence of conspicuous blue ocellated spots on the dorsal disc was used to distinguish Group A from Groups B and C, following the identification guide by Krajangdara *et al*. (2022). Group A comprised specimens from the Gulf of Thailand (n = 28) that either lacked blue spots on the dorsal disc or had only a few tiny blue spots, likely representing *Neotrygon varidens*. Group B was recognized by the presence of conspicuous large blue spots scattered across the dorsal disc, with specimens collected from the Gulf of Thailand, specifically from Songkla (n = 1) and Nakhon Si Thammarat (n = 8) Provinces (total, n = 9), likely corresponding to *N. orientalis*. Similarly, Group C exhibited conspicuous large blue spots scattered across the dorsal disc, but all specimens were from the Andaman Sea (n = 18), likely representing *N. caeruleopunctata*.

Morphometric measurements, presented as percentages of disc width (%DW), showed considerable overlap among the pre-classified groups (Supplementary Table S4). Among the 37 morphometric characters examined, only disc thickness and pelvic-fin length distinguished Group B from others, while Groups A and C remained morphometrically indistinguishable. Principal Component Analysis (PCA) revealed two distinct clusters (Figure 2), with Principal Components 1 and 2 explaining 47.63% and 11.52% of the total variance, respectively. One cluster comprised specimens from Group B, while the other included specimens from both Groups A and C.

Genetic study

The *COI* nucleotide sequences of 59 maskrays in this study were 667 bp in length and categorized into 14 haplotypes (HC1-HC14, accession numbers PQ151389-PQ151402, Supplementary Tables S5 and S6). The most common haplotype was HC1 (n = 24), followed by HC7 (n = 12) and HC10 (n = 7). Haplotypes HC2, HC5, HC11, HC12, and HC14 were each observed in two maskrays, while the remaining haplotypes (HC3, HC4, HC6, HC8, HC9, and HC13) were detected in only one specimen each. Haplotypes HC1-HC5 were restricted to the Gulf of Thailand, whereas HC7 and HC10-HC12 were found in both the Gulf of Thailand and the Andaman Sea.

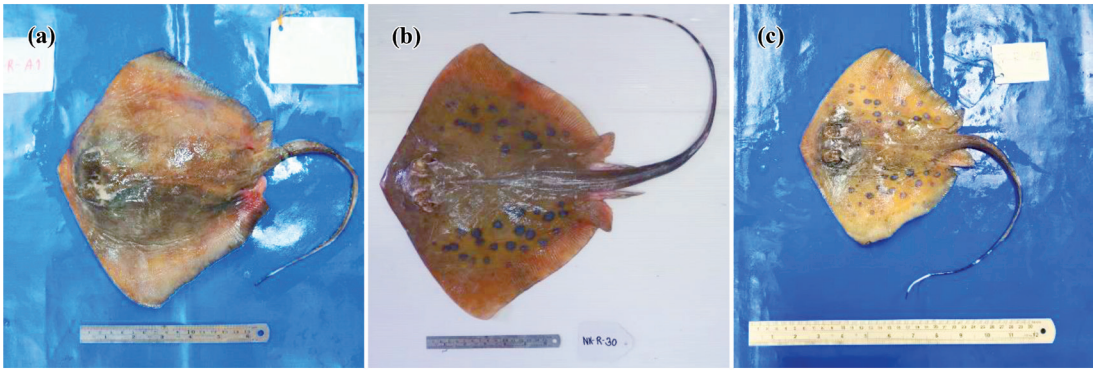

Figure 1. Dorsal view of three groups of blue-spotted maskray (*Neotrygon* spp.) specimens from Thai waters: (a) Group A, (b) Group B, and (c) Group C. Classification was based on their distribution and the presence of conspicuous blue ocellated spots on the dorsal disc.

Phylogenetic analyses based on *COI* gene sequences included 14 novel haplotypes from Thai waters (HC1-HC14, accession numbers PQ151389-PQ151402), 61 reference sequences, and *Pastinachus ater* (EU398973) as an outgroup. Maximum likelihood analysis using the T92+G substitution model (Tamura, 1992) revealed two distinct clades of Thai maskrays. The first clade, supported by moderate bootstrap value (75%), comprised all Andaman Sea haplotypes along with four shared haplotypes from both the Gulf of Thailand and the Andaman Sea (PQ151395, PQ151398–PQ151400), clustering with *Neotrygon malaccensis* sequences. The second clade, with high bootstrap support (95%), included all Gulf of Thailand haplotypes, grouping with *N. varidens*.

Overall, the phylogenetic analysis identified 11 major clades within the maskray species complex, supported by bootstrap values ranging from 54% to 99% (Figure 3, excluding the *N. orientalis* clade). Each major clade corresponded to recognized species, except for *N. picta*, which was paraphyletic within the species complex.

Kimura 2-parameter genetic distances within *Neotrygon* clades were consistently low, ranging from 0.11±0.11% to 1.09±0.29% (*N. australiae*: 0.89±0.24%, *N. varidens*: 0.20±0.09%, *N. caeruleopunctata*: 0.13±0.09%, *N. malaccensis*: 0.28±0.10%, *N. trigonoides*: 0.53±0.23%, *N. orientalis*: 0.46±0.16%, *N. picta*: 0.80±0.37%, *N. indica*: 1.09±0.29%, *N. bobwardi*: 0.90±0.27%, *N. westpapuensis*: 0.21±0.15%, and *N. moluccensis*: 0.11±0.11%). In contrast, between-clade distances were substantially higher, ranging from 1.70±0.46% to 11.35±1.51% (Table 1). These findings align with Borsa *et al.* (2016), who reported

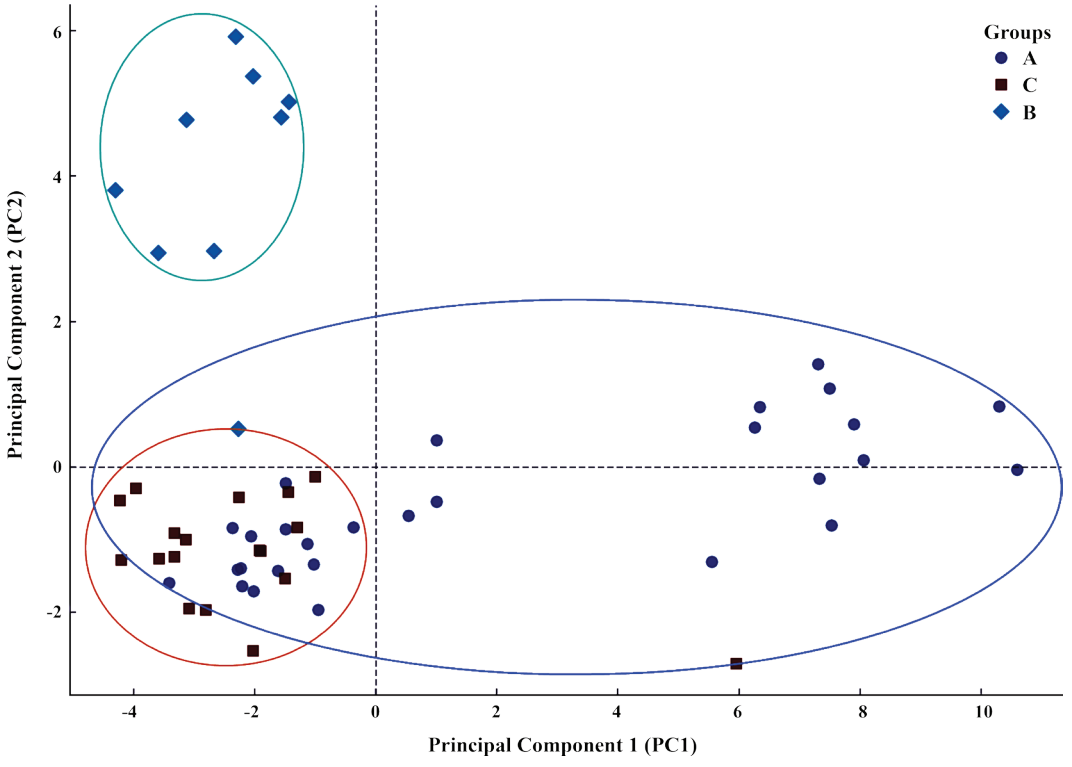

Figure 2. Principal Component Analysis (PCA) plot showing clustering in morphometric data for maskrays (genus *Neotrygon*). Specimens were categorized into three groups: A (no or very small blue spots on the disc, Gulf of Thailand), B (large blue spots on the disc, Gulf of Thailand origin), and C (large blue spots on the disc, Andaman Sea).

Figure 3. Maximum-likelihood tree of blue-spotted maskrays (*Neotrygon* spp.) based on partial *COI* gene sequences, constructed using the T92+G model (Tamura, 1992). Sequences from this study are highlighted in bold (accession numbers PQ151389-PQ151402, followed by place of origin). Reference sequences are annotated with GenBank accession numbers, scientific names, and places of origin. Numbers above branches indicate bootstrap values greater than 50.

Table 1. Mean (±SD) pairwise genetic distances (Kimuara 2-parameter model) within and between clades of the blue-spotted maskray species complex (*Neotrygon* spp.). Genetic distances are presented as percentages (%) with within-clade distances highlighted in bold. Clades were identified based on the maximum-likelihood tree.

	N. australiae	***N. varidens***	***N. caeruleopunctata***	***N. malaccensis***	***N. trigonoides***	***N. orientalis***	***N. picta***	***N. indica***	***N. bobwardi***	***N. westpapuensis***	***N. moluccensis***
N. australiae	**0.89±0.24**										
N. varidens	3.64±0.72	**0.20±0.09**									
N. caeruleopunctata	2.92±0.65	3.49±0.73	**0.13±0.09**								
N. malaccensis	2.98±0.65	3.58±0.73	1.84±0.54	**0.28±0.10**							
N. trigonoides	4.44±0.84	4.67±0.87	3.97±0.80	4.02±0.83	**0.53±0.23**						
N. orientalis	3.04±0.66	1.95±0.52	2.39±0.59	2.15±0.54	3.74±0.74	**0.46±0.16**					
N. picta	10.66±1.43	10.60±1.42	10.96±1.50	10.89±1.50	10.37±1.43	10.28±1.43	**0.80±0.37**				
N. indica	2.65±0.56	3.42±0.56	1.79±0.48	1.77±0.45	3.87±0.76	2.32±0.50	10.69±1.44	**1.09±0.29**			
N. bobwardi	3.06±0.64	3.46±0.64	1.85±0.49	1.70±0.46	3.60±0.73	2.61±0.56	11.35±1.51	1.95±0.44	**0.90±0.27**		
N. westpapuensis	2.96±0.64	3.76±0.64	2.13±0.57	2.48±0.62	3.64±0.80	3.05±0.65	10.24±1.43	2.31±0.54	2.63±0.60	**0.21±0.15**	
N. moluccensis	2.80±0.63	3.59±0.76	2.35±0.63	2.44±0.63	3.22±0.72	2.59±0.61	11.02±1.50	2.28±0.54	2.51±0.60	2.39±0.62	**0.11±0.11**

within-clade Tamura-Nei distances of 0.1–0.7% and between-clade distances that were 2 to 29 times greater (1.4–2.9%) using concatenated *COI* and *cytochrome b* genes. The significantly higher between-clade versus within-clade genetic distances further support previous studies (Ward *et al*., 2008; Naylor *et al*., 2012; Arlyza *et al*., 2013; Puckridge *et al*., 2013; Borsa *et al*., 2016), reinforcing the recognition of *Neotrygon* spp. as a cryptic species complex.

Phylogenetic analysis of *COI* sequences revealed two distinct clades of maskrays in Thai waters, supported by moderate (75%) and high (95%) bootstrap values (Figure 3). The first clade, identified as *N. malaccensis*, comprised all Andaman Sea haplotypes and some haplotypes from the Gulf of Thailand, clustering with reference sequences from the Andaman Coast (Thailand), Myanmar, the west coast of Peninsular Malaysia, and the Malacca Strait. The second clade, identified as *N. varidens*, consisted of haplotypes restricted to the Gulf of Thailand, grouping with reference sequences from Penghu Island (Taiwan), Borneo Island (Malaysia), and the east coast of Peninsular Malaysia.

Intra-specific genetic distances (K2P: 0.11–1.09%) observed in this study were consistent with previous findings for elasmobranchs, including Australasian (average K2P: 0.37%; Ward *et al*., 2008) and Malaysian waters (p-distance: 0–1%; Loh *et al*., 2023). Similarly, inter-specific distances between clades (K2P: 1.70–11.35%) aligned with previously reported values for sharks and rays from Australasian waters (average K2P: 7.48%; Ward *et al*., 2008) and Malaysian waters (p-distance: 3–10%; Loh *et al*., 2023). The clear separation between intra- and inter-specific genetic distances, along with increasing genetic divergence at higher taxonomic ranks, demonstrates the effectiveness of *COI* as a molecular marker for species identification within the *Neotrygon* species complex, supporting previous findings.

Morphometric analyses of 37 morphometric characteristics and phylogenetic studies derived from the *COI* gene were inconsistent. The maskrays in Group A, which were morphologically hypothesized as *N. varidens*, grouped with *N. varidens* reference sequences based on their haplotypes (accession numbers PQ151389–PQ151393). However, specimens in Group C, which exhibited overlapping morphology with Group A and were hypothesized as *N. caeruleopunctata* based on morphological characteristics, instead clustered with *N. malaccensis* reference sequences (accession numbers PQ151394–PQ151402). Similarly, maskrays in Group B, which showed distinct morphometric characteristics compared to the other two groups and were hypothesized as *N. orientalis* based on their morphology, grouped with *N. malaccensis* reference sequences (accession numbers PQ151395 and PQ151398– PQ151400). These findings indicate that the morphologically distinct B and C groups correspond to *N. malaccensis*.

This pattern is unsurprising, as species within the blue-spotted maskray complex in the Indo –West Pacific, such as *N. australiae*, *N. caeruleopunctata*, *N. orientalis*, *N. kuhlii*, *N. bobwardi*, *N. malaccensis*, *N. moluccensis*, and *N. westpapuensis*, are frequently misidentified due to their similar morphological similarity and overlapping diagnostic characters (Borsa *et al*., 2018). The incongruence between morphometric and phylogenetic analyses in this study likely reflects morphological variation and diagnostic overlap within the blue-spotted maskray species complex. For example, overlapping morphological characters have been reported in *N. australiae*, *N. caeruleopunctata*, *N. kuhlii*, and *N. orientalis* (Borsa *et al*., 2018). These findings further suggest that morphological characters alone are insufficient for accurately distinguish closely related species within the species complex, even though they were genetically different.

Two species of the blue-spotted maskray complex were identified in Thai waters: *N. varidens* and *N. malaccensis*. In contrast, *N. caeruleopunctata* and *N. orientalis*, previously reported from this region (Last *et al*., 2016; Krajangdara *et al*., 2022), were not detected in our study, likely due to the challenges in distinguishing diagnostic morphological characters within this species complex (Borsa *et al*., 2018). This study confirmed the presence of *N. varidens* throughout the Gulf of Thailand,

complementing its known distribution in the South China Sea and off Taiwan (Borsa *et al*., 2016; Last *et al*., 2016). Notably, we documented the first occurrence of *N. malaccensis* in the Gulf of Thailand, extending its known range from the northern Malacca Strait and eastern Andaman Sea (Borsa *et al*., 2018) to the western Gulf of Thailand, where it occurs sympatrically with *N. varidens*. These findings highlight the need for accurate species identification in biodiversity assessments and the development of effective conservation strategies for chondrichthyans at both local and global scales.

CONCLUSIONS

This study integrated morphometric analyses with mitochondrial *COI*-based phylogenetics to investigate cryptic species diversity within the blue-spotted maskray (*Neotrygon* spp.) in Thai waters. While morphometric analyses proved insufficient for species discrimination, phylogenetic analysis successfully distinguished two morphologically similar species: *N. varidens*, found only in the Gulf of Thailand, and *N. malaccensis*, a new record in Thailand, occuring in both the Gulf of Thailand and the Andaman Sea. *N. caeruleopunctata* and *N. orientalis*, previously reported from this region, were not detected. These findings underscore the critical role of molecular tools in accurately identifying species within the blue-spotted maskray complex, reinforcing their importance for biodiversity assessments and the formulation of effective conservation strategies for cartilaginous fishes at regional and global levels.

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