Exploring the Potential in Combining the Two Molecular Approaches, COI Barcoding and PCR-RFLP (COIBar-RFLP) in Identification of Selected Species of the Family Carangidae

LAHIRU MADUSHAN PANDI GAMAGE[1,2,*], DONA HEMALI NANDANA MUNASINGHE[1], MARINGA APSARA MADUMANJAREE RUPASINGHE[1,3]

[1]Department of Zoology, Faculty of Science, University of Ruhuna, Matara, Sri Lanka
[2]College of Medicine, China Medical University, Taichung, Taiwan
[3]Department of Zoology, Faculty of Natural Sciences, Open University, Sri Lanka

*E-mail: madushanlahirugamage@gmail.com | Received: 28/02/2023; Accepted: 20/02/2024

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Abstract

The family Carangidae is considered a markedly diverse, widespread taxon. Due to the characteristic "cryptic diversity" and "hybrid speciation" within the family, there is an exigency for taxonomic approaches that go beyond traditional phenotypic modus in identifying species. Mitochondrial cytochrome c oxidase subunit - I (COI) barcoding gene region plays a significant role over phenotypic characters in identifying species. This study evaluates the combination of the two molecular approaches: the COI DNA barcoding and PCR-RFLP for judicious species discrimination. The partial mitochondrial COI gene region of the selected Carangid fish species was amplified, sequenced and confirmed sequences with a mean length of 655 bp were submitted to the main databases; NCBI and BOLD. Intraspecific and interspecific nucleotide divergences were computed by the Kimura-2-Parameter (K2P) and they ranged between (0.00–2.96 %) and (6.46–21.83 %), respectively. The possibility of acquiring the same RFLP profiles given by the restriction enzymes *Hae*III and *Mbo*II was observed by the theoretical cleavage of 250 reference sequences of the corresponding gene. Hence, major and minority composite haplotypes of each species were obtained based on the fragment types derived by both *Hae*III and *Mbo*II. The resulting divergence values were compatible with the previously reported values for marine fish species. All the species were clearly differentiated by both RFLP banding patterns and the highest probability assumption of getting the same RFLP profile was compatible with the most abundant composite haplotype of each species. This reveals the practicability of the combination of two consolidated molecular approaches, COI barcoding and PCR-RFLP (COIBar-RFLP).

Keywords: composite haplotypes; cryptic species, K2P distance; probability assumption

Introduction

The family Carangidae comprises a mega-diverse group of fishes which are commonly identified as jacks and scads. According to the Eschmeyer's Catalog of Fishes database (http://researcharchive.calacademy.org/research/ichthyology/catalog/fishcatmain.asp), it has been estimated that this family is built up of 39 valid genera and 153 valid species (Eschmeyer et al., 2010). Species of the three genera, *Decapterus*, *Selar* and *Trachurus* are usually considered scads. Trevallies (*Caranx*, *Carangoides* sp.), amberjacks (*Seriola* sp.), pompanos, queen fish (*Scomberoides* sp.), kingfish, pilot fish and runners are collectively known as jacks (Damerau et al., 2018). This family is popular as one of the commercially important fish groups in both the food and ornamental fish industries. Due to the importance of aquaculture and the flexibility in culturing, there is now an international trend to culture them worldwide using modern aquaculture techniques (Rombenso et al., 2016; Kappen et al., 2018).

Cryptic species tend to share similar morphological characteristics with a remarkable discrepancy in the genetic structure avoiding interbreeding (Korshunova et al., 2019). Within the family Carangidae, similarities in morphological and meristic characteristics persist, indicating taxonomic ambiguities at the intra and interspecific levels. In addition to that plasticity in

their body shape, size and pigmentation patterns greatly influence making cryptic diversity within the family (Mat Jaafar et al., 2012). The potential of hybrid speciation among closely related species also alters the evolutionary lineage of the family Carangidae along with increasing the cryptic diversity within the family. For example, two phenotypically mutualistic hybrid species derived from the genus *Caranx*, *C. ignobilis* × *C. melampygus* and *C. melampygus* × *C. sexfasciatus* have been identified from the Hawaiian Islands (Murakami et al., 2007) .

Accurate species identification is the basic as well as the essential step in biodiversity monitoring practices. This information associated with their biology and ecology provides guidelines towards mainly conservation strategies, aquaculture and sustainable fishery management practices (Jordán et al., 2008). Due to the cryptic diversity within the family, individuals of the Carangidae family are identified with several synonyms. Consequently, the taxonomic confusions along with the synonyms cause a considerable effect on a wide area ranging from taxonomic studies to commercial frauds (Ferrito and Pappalardo, 2017). Since the cryptic speciation of the family and the scarcity of either knowledge or scientific reports on Carangid species identification in Sri Lanka, the same synonyms are pronounced for different species. Most of the time they are collectively known as "Parawa/Trevally" and sold mixing together at local fish markets; for example, phenotypic confusion is observed among juveniles of *C. ignobilis* and *C. heberi* (Bennett, 1830).

Compared to the nuclear gene regions, mutations tend to more rapidly accumulate in the mitochondrial genome (mtGenome) resulting in considerable interspecific sequence variations (Hebert et al., 2003; Templonuevo et al., 2018). Therefore, protein-coding genes including cytochrome c oxidase subunit - I (COI) and cytochrome b (Cytb) in the mtGenome are widely used as a powerful molecular marker for genetic diversity analysis at lower categorical levels, including families, genera and species and even for related species identification (Farias et al., 2001; Jaafar, 2013). Both COI and Cytb have similar and greater base substitution rates but amino acid sequence evolution at the 3rd codon position is slower in the COI (Shearer et al., 2002; Tobe et al., 2010). Therefore, the mutation-synonymous properties of the COI gene provide advanced and informative taxonomic clarifications as a barcoding gene in species identification (Hebert et al., 2003).

The PCR-RFLP approach has been identified as a rapid, simple and practically applicable technique and it is not essentially required very good molecular knowledge to interpret results (Partis et al., 2000). Therefore, this technique is widely used in species identification studies including mislabelled/fraudulent and cryptic species identifications (Pappalardo and Ferrito, 2015; Pappalardo et al., 2018). Most of the time mitochondrial Cytb gene region has been considered in PCR-RFLP analyses (Lin and Hwang, 2007; Rea et al., 2009; Hsieh et al., 2010; Besbes et al., 2012). However, the COI barcoding gene region has been used for very few PCR-RFLP studies in fish species identification including Carangid fish (Stefanni et al., 2009; Wong et al., 2014; Ekanayake et al., 2021). The validity of DNA barcoding can be standardized by integrating it with another molecular approach (Pappalardo and Ferrito, 2015). Consequently, the combination of COI barcoding and PCR-RFLP is also referred to as "COIBar-RFLP" (Ferrito and Pappalardo, 2017).

The selected eight Carangid fish species of the current study; *Caranx ignobilis* (Forsskål, 1775), *Caranx heberi* (Bennett, 1830), *Caranx sexfasciatus* Quoy & Gaimard, 1825, *Gnathanodon speciosus* (Forsskål, 1775), *Selar crumenophthalmus* (Bloch, 1793), *Selaroides leptolepis* (Cuvier, 1833), *Carangoides hedlandensis* (Whitley, 1934) and *Carangoides coeruleopinnatus* (Rüppell, 1830) have achieved a higher commercial demand along with their seasonal abundance. Due to the paraphyletic behaviour of the genus *Carangoides*, individuals previously described as *Carangoides* were recently redefined and the three species, *Carangoides malabaricus* (Bloch and Schneider, 1801), *C. coeruleopinnatus* (Rüppell, 1830), and *C. hedlandensis* (Whitley, 1934) described in the current study are systematically valid with the scientific names, *Platycaranx malabaricus*, *Turrum coeruleopinnatum* and *Atropus hedlandensis*, respectively (Kimura et al., 2022).

This study mainly relies on the COIBar-RFLP concept to make a powerful and rapid molecular marker for identifying the selected Carangid species against the cryptic diversity within the family. Furthermore, the validation of the current COIBar-RFLP profiling as a robust molecular marker for accurately identifying selected Carangid species has been scientifically established through comprehensive analysis of samples obtained from diverse geographical regions.

Materials and Methods

Sample collection and DNA extraction

Individuals of the eight fish species belonging to the family Carangidae; *C. ignobilis*, *C. heberi*, *C. sexfasciatus*, *G. speciosus*, *P. malabaricus*, *A. hedlandensis*, *S. crumenophthalmus* and *S. leptolepis* were collected from the marine fish landing sites in Sri Lanka over a period of 4 months from October 2020 to January 2021 (Fig.1).

Digital photographs of all the samples were taken immediately and samples were preserved at - 20 °C. Samples were labelled according to their sample ID for further identification. All the fishes were first identified based on their morphological features using the FAO-Fisheries Identification Guidelines (Fischer and Whitehead, 1974) and identification books published by the Department of Fisheries Malaysia (Mansor et al., 1998; Annie and Albert, 2009).

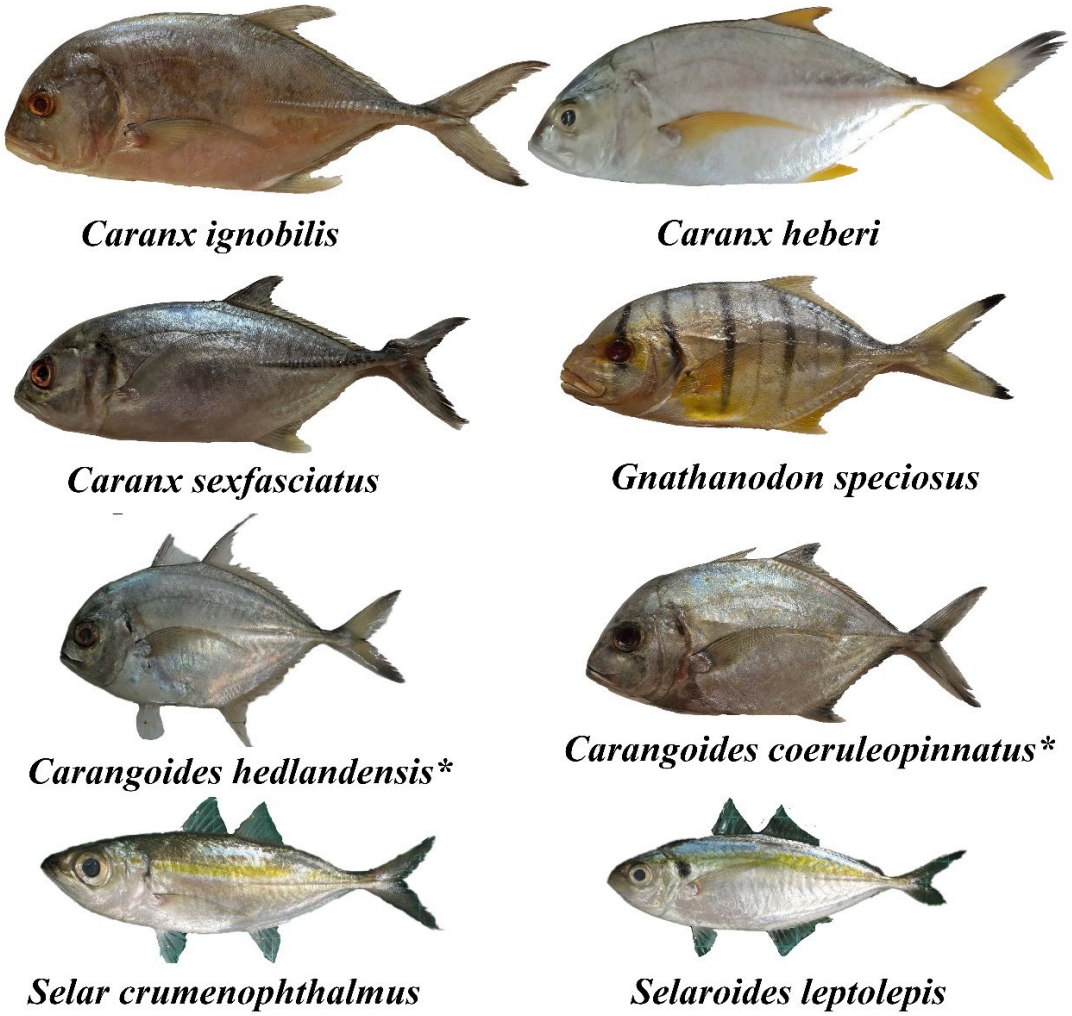

Fig. 1. Selected Carangid species sampled in the current study. **Carangoides hedlandensis* and *Carangoides coeruleopinnatus* redefined as *Atropus hedlandensis* and *Turrum coeruleopinnatum* (Kimura et al., 2022).

Muscle tissues just above the lateral line, close to the operculum of the fish were considered for the DNA extraction. Genomic DNA was extracted according to the protocol given by the DNeasy® Blood and Tissue Kit (Qiagen, USA).

Amplification of the mitochondrial COI partial gene region

Approximately 650 bp length of the mitochondrial COI partial gene region was amplified using a universal fish barcoding primer pair synthesized by Macrogen, South Korea.

FishF1-5'TCAACCAACCACAAAGACATTGGCAC3'
FishR1-5'TAGACTTCTGGGTGGCCAAAGAATCA3' (Ward et al., 2005)

PCR amplification was performed in a 25 µL total reaction volume with 10 µL of 2× GoTaq® Green Master Mix (Promega, USA; contained PCR buffer, $MgCl_2$ solution, dNTPs and Taq Polymerase all together), 0.5 µL of each primer (10 µM), 11 µL of nuclease-free water and 3 µL of DNA template (200 ng.µL^{-1}). An initial denaturation at 95 °C for 7 min followed by 35 cycles of the PCR process including the 3 basic steps; denaturation for 30 sec at 95 °C, primer annealing for 1 min at 58 °C and the extension at 72 °C for 1 min programmed by the GeneAmp PCR Thermal Cycler 2720 (Applied BioSystems, USA). The final extension was programmed at 72 °C for 10 min after the completion of all 35 cycles and the PCR samples were kept at 4 °C until removed from the machine. PCR products having intense and clear bands were selected for the sequencing and RFLP profiling.

Sequence analysis and editing

Selected PCR products were submitted to Macrogen Inc, South Korea and nucleotide sequencing has been performed in the forward and reverse directions using the Next Generation Sequencing. Auto assembler software (ABI Prism, USA) was used to assemble the raw nucleotide sequences (Scott et al., 2002). The consistency of sequence information from both directions was checked by assembling both forward and reverse complementary sequences with the Gene tool Lite software. Sequences were aligned by the ClustalW tool of the MEGA11 software (Tamura et al., 2021). Edited sequences were compared with the

pre-published sequences using the Basic Local Alignment Search Tool (BLASTn) option of the National Center for Biotechnology Information (NCBI) database with the maximum compatibility and similarity to confirm the species level.

Twenty-four (24) COI barcoding sequences having a 655 bp amplicon size were submitted to the NCBI (www.ncbi.nlm.nih.gov/Genbank), the European Molecular Biology Laboratory (EMBL; www.embl.org) and the DNA Data Bank of Japan (DDBJ; www.ddbj.nig.ac.jp) under the same Genbank accession numbers. Subsequently, all the sequences have been uploaded to the Barcode of Life Data (BOLD) database (www.barcodinglife.com) to establish a barcode library. The barcoding project, (LMUOR) has been initiated along with the current study to expand the identification of Carangid fish species encountered in the Indian Ocean further.

Barcoding-based species delimitation analysis

Existing barcode clusters (BINs) were generated for all the sequences of the specimens studied by the BOLD systems tool (Ratnasingham and Hebert, 2007). The taxonomic information, images, sequence and specimen details were all submitted to this platform fulfilling the workflow indicated in the Figure 2.

The BOLD Workbench application (v4) facilitated the calculation of the levels of divergence within and between species, genera, and families, the barcode gap based on the Kimura-2-Parameter (K2P) pairwise nucleotide divergence approach (Kimura, 1980; Tamura et al., 2021) using 1,000 bootstrap pseudoreplicates. In addition, phylogenetic relationships were analysed using the Maximum Likelihood approach. The average nucleotide composition of each species, especially the GC % at the 1st, 2nd, and 3rd codon positions was calculated.

RFLP theoretical analysis / COIBar-RFLP mapping

Twenty-four (24) COI barcoding sequences of the 655 bp mitochondrial COI partial gene region derived from individuals were theoretically cleaved by the Restriction Mapper Version 3 and the NEBcutter v2.0 (New England BioLabs® Inc.) software (Vincze et al., 2003). The RFLP maps of all the sequences were obtained with respect to the restriction endonucleases (*Hae*II, *Hae*III, *Mbo*II, *Nla*III and *Hinc*II) targeting sites along with the nucleotide sequences. The common RFLP maps were illustrated for the eight species with respect to the selected restriction enzymes and their relative target sites.

Restriction enzyme digestion and RFLP profiling

Out of above-mentioned restriction enzymes *Hae*III and *Mbo*II (Fast Gene®, Nippon Genetics EUROPE) were treated to differentiate the eight fish species. According to the standard reaction conditions under the normal protocol recommended by the manufacturer, 50 µL of the total reaction mixture containing 5 µL of 10× FastGene®Buffers (IV/II), 5 µL of PCR products (10 µg) and 1 µL of each restriction

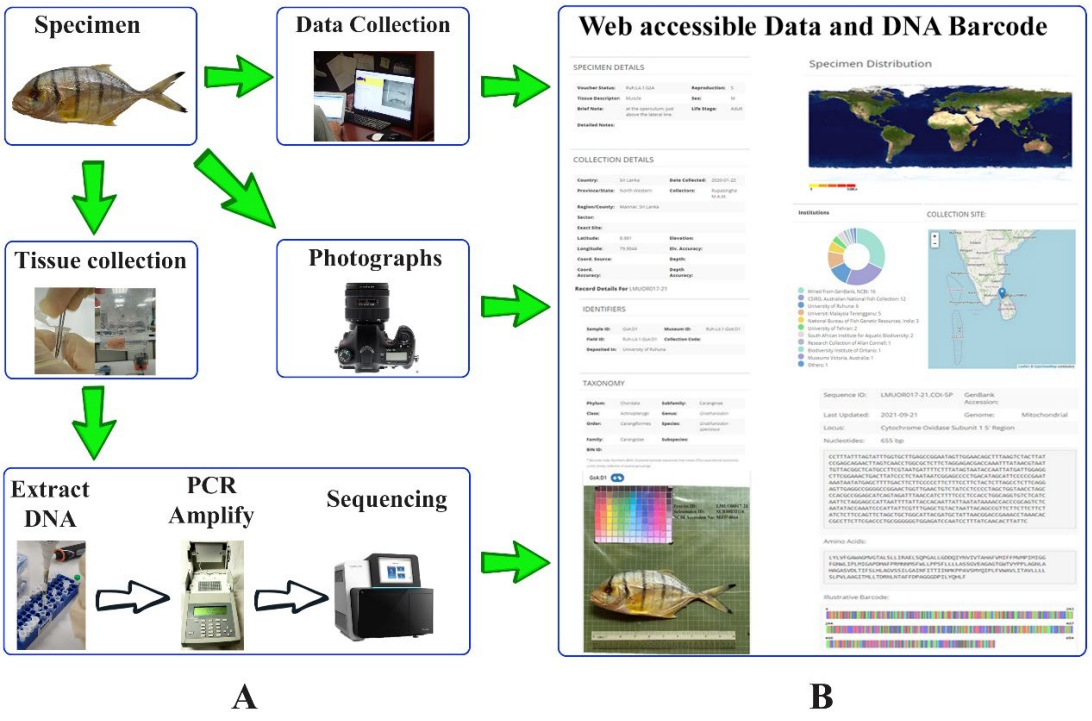

Fig. 2. Overview of the barcode library established under the project name: LMUOR. A: Workflow of the establishment; B: Appearance of the specimen and sequence page.

enzymes; *Hae*III (10 U) / *Mbo*II (5 U) was prepared and incubated at 37 °C for 1 h. The digested fragments were analysed using a 2 % agarose (Sigma-Aldrich, USA) gel electrophoresis for 1 h at 100 V, by comparison with the 100 bp Ladder (Promega, USA). Digital photographs of the RFLP profiles were taken immediately for further interpretations and compared with the predicted fragment patterns.

The accuracy and practicability of the obtained RFLP profiles were theoretically compared with 250 geographically distant COI reference sequences of the eight Carangid species including the 24 COI sequences in the current study available in the BOLD database (Supplementary Table 1). COI reference sequences were first aligned and corresponding sequences of 707 bp length were then theoretically cleaved by the Restriction Mapper Version 3 and the NEBcutter v2.0 software under *Hae*III and *Mbo*II enzymes. Fragment patterns derived from theoretical cleavage was considered for probability assumptions out of 250 Carangid sequences.

Results

COI barcode sequence analysis

The PCR primers of Fish F1/R1 could specifically amplify an average length of 655 bp fragments from the COI gene region of each sample without having stop codons, insertions or deletions. The average nucleotide GC % compositions per codon position are indicated in Table 1. The GC % composition at the 3rd codon position is greater than that of the 1st and 2nd codon positions.

Table 1. Average GC % per codon composition of the selected Carangid species of the current study.

Nucleotide composition	Minimum	Mean	Maximum	SE
GC % at Codon Position 1	31.05	37.44	46.12	0.6125
GC % at Codon Position 2	42.20	42.60	44.04	0.0767
GC % at Codon Position 3	54.13	56.86	58.26	0.1498

SE: Standard error.

GenBank accession numbers and BOLD IDs of the corresponding sequences are available in Table 2.

BLASTn algorithm revealed that 21 out of 24 COI sequences were compatible with the initial phenotypic identification of species. However, the other three individuals which are previously considered as *P. malabaricus* later compatible with *T. coeruleopinnatum* reference sequences available in the GenBank. The average BLASTn comparison of 88.20 % and the average interspecific K2P divergence of 13.70 % derived from the three individuals of the suspected species, *T. coeruleopinnatum* of the study with respect to previously published *P. malabaricus* barcoding sequences reflect the degree of deviation (>2 % speciation criteria) of the two taxa as two species further. The above phenotypic impediments revealed the cryptic diversity of *T. coeruleopinnatum* with the other Carangid members of the family.

The Kimura-2-Parameter (K2P) model revealed a hierarchical increase in nucleotide divergence, with an average divergence of 0.57 % between members of the same species, 6.46 % between species of the same genus, and 12.76 % between species of the same family (Table 3). The lowest mean interspecific divergence was observed between *C. ignobilis* and *C. heberi* (6.46 %), while the highest value was recovered between *S. crumenophthalmus* and *T. coeruleopinnatum* (15.19 %). Generally, the genetic divergence of the given locus, between congeners was 1.9 times greater than that between specimens of the same species, confirming the existence and magnitude of the Barcode Gap (Table 3; Fig. 3).

The Maximum Likelihood tree (Fig. 4) was effective for the separation of all the identified sequences of the different species of the study into 8 clades and the isolation of *P. malabaricus* from *T. coeruleopinnatum* into a completely different clade is a matter of concern (Fig. 4) confirming its previous misidentification during the study.

COIBar-RFLP profiling and composite haplotype frequencies analysis

The common RFLP maps illustrated for the eight species with respect to the selected five restriction enzymes: *Hae*II, *Hae*III, *Mbo*II, *Nla*III and *Hinc*II and their relative target sites available in Figure 5.

Both RFLP profiles derived from the restriction enzymes *Hae*III and *Mbo*II could differentiate the eight Carangid species selected in Sri Lankan waters simultaneously and the RFLP profiles obtained in the current study were compatible with the theoretical assumptions (Table 4). However, the expected bands below 100 bp were difficult to visualize on the 2 % agarose gel (Fig. 6).

The inherent challenge emerged as RFLP profiling, specifically employing *Hae*III and *Mbo*II, yields similar patterns for *P. malabaricus* and *T. coeruleopinnatum*. This unexpected homogeneity in the restriction profiles raises a critical question about the adequacy of these enzymes in discerning genetic differences between the two species (Fig. 7).

Further, the possibility of getting the same fragment pattern from geographically distant samples was able to validate through theoretical cleavage of 250 reference sequences of the selected Carangid species. Polymorphism of the restriction fragments resulted in 11 banding patterns with respect to both *Hae*III and *Mbo*II

Table 2. NCBI accession numbers and BOLD IDs of the Carangid fish species of the current study.

Sample	Sample ID	Scientific name	Redefined scientific name (Kimura et al., 2022)	Common name	Sub group	NCBI accession	BOLD ID
1.	CiA:A_1	*Caranx ignobilis*	-	Giant trevally	Trevally	MZ542428	LMUOR002-21
2.	CiB:A_2	*Caranx ignobilis*	-	Giant trevally	Trevally	MZ542429	LMUOR003-21
3.	CiC:A_3	*Caranx ignobilis*	-	Giant trevally	Trevally	MZ542460	LMUOR004-21
4.	ChA:B_1	*Caranx heberi*	-	Black-tipped trevally	Trevally	MW788376	LMUOR001-21
5.	ChB:B_2	*Caranx heberi*	-	Black-tipped trevally	Trevally	MW817583	LMUOR006-21
6.	ChC:B_3	*Caranx heberi*	-	Black-tipped trevally	Trevally	MW817591	LMUOR007-21
7.	CsA:C_1	*Caranx sexfasciatus*	-	Big eye trevally	Trevally	MZ555707	LMUOR011-21
8.	CsB:C_2	*Caranx sexfasciatus*	-	Big eye trevally	Trevally	MZ555716	LMUOR012-21
9.	CsC:C_3	*Caranx sexfasciatus*	-	Big eye trevally	Trevally	MZ555710	LMUOR013-21
10.	GsA:D_1	*Gnathanodon speciosus*	-	Golden trevally	Trevally	MZ574064	LMUOR017-21
11.	GsB:D_2	*Gnathanodon speciosus*	-	Golden trevally	Trevally	MZ555722	LMUOR018-21
12.	GsC:D_3	*Gnathanodon speciosus*	-	Golden trevally	Trevally	MZ574061	LMUOR019-21
13.	ChA:E_1	*Carangoides hedlandensis*	*Atropus hedlandensis*	Bump nose trevally	Trevally	MZ542451	LMUOR023-21
14.	ChB:E_2	*Carangoides hedlandensis*	*Atropus hedlandensis*	Bump nose trevally	Trevally	MZ542454	LMUOR024-21
15.	ChC:E_3	*Carangoides hedlandensis*	*Atropus hedlandensis*	Bump nose trevally	Trevally	MZ543393	LMUOR025-21
16.	CcA:F_1	*Carangoides coeruleopinnatus*	*Turrum coeruleopinnatum*	Coastal trevally	Trevally	MZ543392	LMUOR026-21
17.	CcB:F_2	*Carangoides coeruleopinnatus*	*Turrum coeruleopinnatum*	Coastal trevally	Trevally	MZ543395	LMUOR027-21
18.	CcC:F_3	*Carangoides coeruleopinnatus*	*Turrum coeruleopinnatum*	Coastal trevally	Trevally	MZ543396	LMUOR028-21
19.	SlA:G_1	*Selaroides leptolepis*	-	Yellow strip scads	Scads	MZ555708	LMUOR030-21
20.	SlB:G_2	*Selaroides leptolepis*	-	Yellow strip scads	Scads	MZ555721	LMUOR031-21
21.	SlC:G_3	*Selaroides leptolepis*	-	Yellow strip scads	Scads	MZ555720	LMUOR032-21
22.	ScA:H_1	*Selar crumenophthalmus*	-	Big eye scads	Scads	MZ555717	LMUOR033-21
23.	ScB:H_2	*Selar crumenophthalmus*	-	Big eye scads	Scads	MZ555712	LMUOR034-21
24.	ScC:H_3	*Selar crumenophthalmus*	-	Big eye scads	Scads	MZ555713	LMUOR035-21

Table 3. Distribution of the K2P nucleotide divergence at each taxonomic level of the selected Carangid species of the current study.

				Nucleotide divergence (%)			
	n	Taxa	Comparisons	Minimum	Mean	Maximum	Standard error
Within species	38	8	78	0.00	0.57	2.96	0.01
Within genus	16	1	84	6.46	9.31	10.78	0.02
Within family	38	1	541	12.76	17.01	21.83	0.00

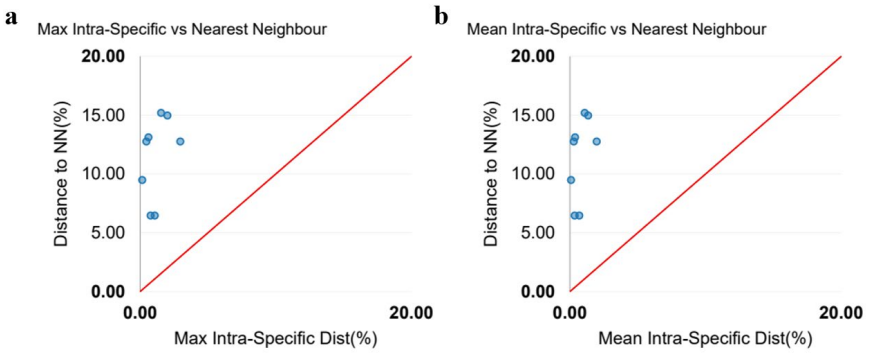

Fig. 3. Barcode gap analysis of the COI sequences of the selected Carangid species sequenced in the current study. Two scatterplots show the overlap of the max (a) and mean (b) intra-specific distances vs. the inter-specific (nearest neighbour) distances.

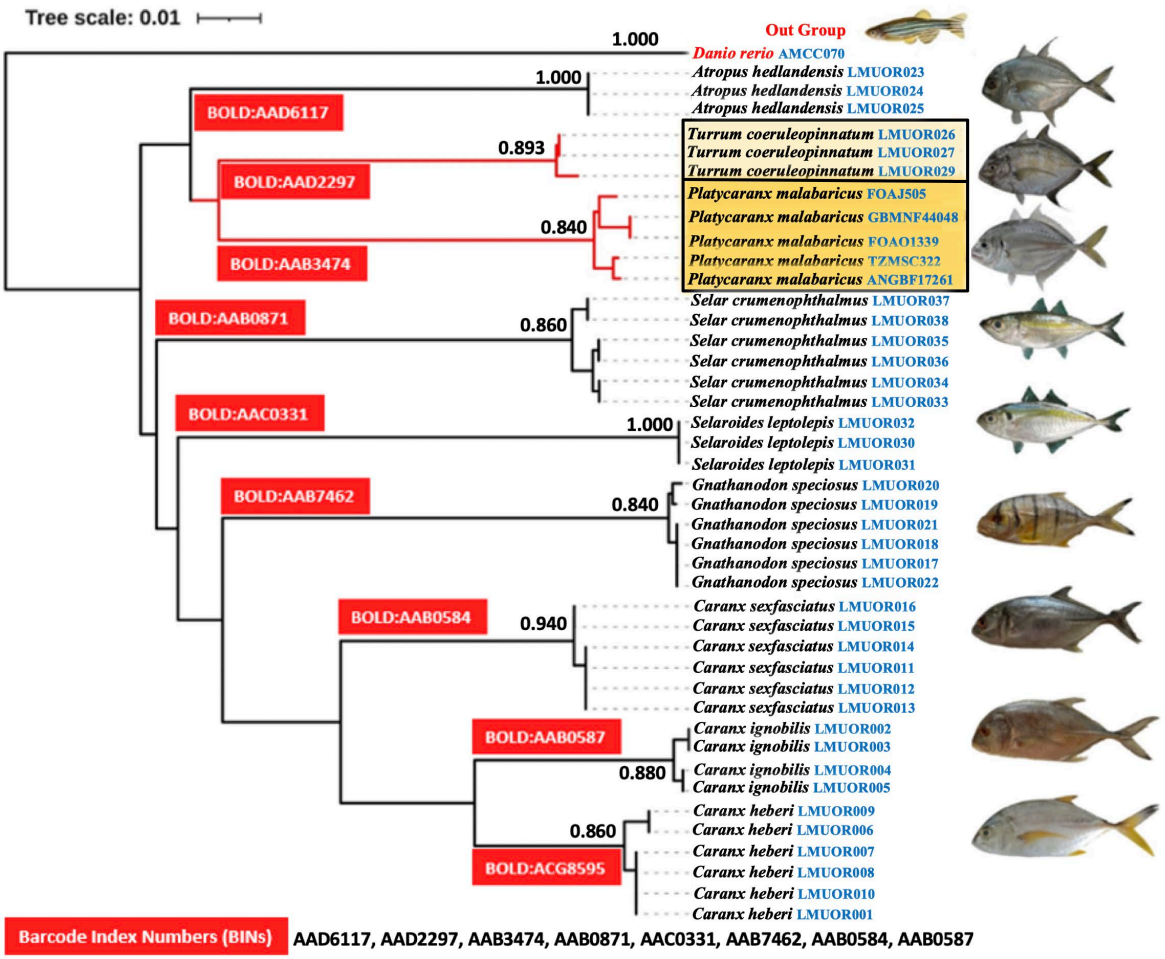

Fig. 4. Maximum Likelihood tree based on the COI barcode, showing the diversity of Carangid fish in this study and reflecting the clade isolation of misidentified *Turrum coeruleopinnatum* with *Platycaranx malabaricus*.

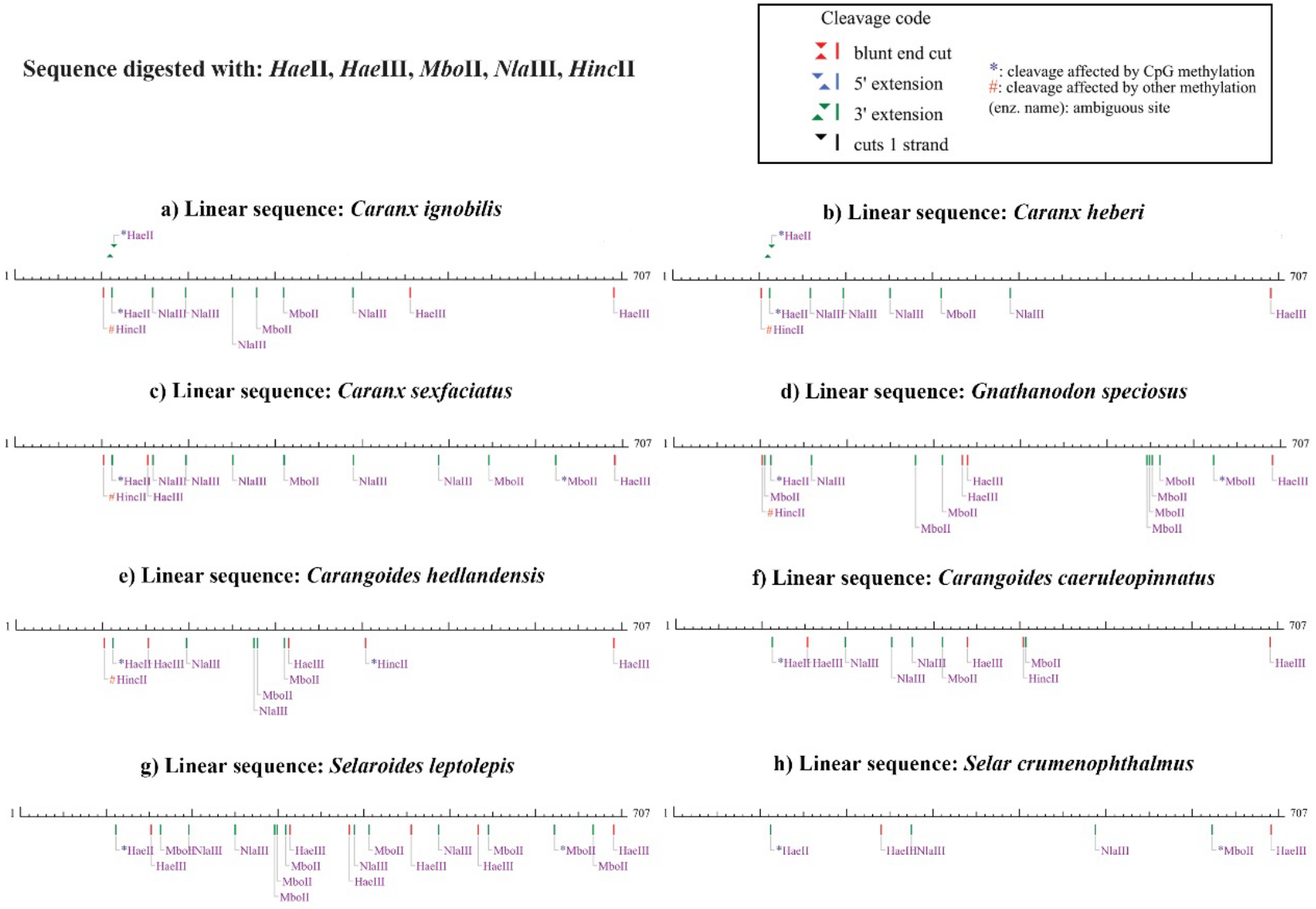

Fig. 5. RFLP maps illustrating relative restriction sites of the eight species under the cleavage of restriction enzymes *Hae*II, *Hae*III, *MbO*II, *Nla*III and *Hinc*II.

Table 4. RFLP product sizes of the 707 bp partial COI gene of the 8 selected Carangid fish with *Hae*III and *Mb*OII.

Species	RFLP product sizes (bp) under *Hae*III	RFLP product sizes (bp) under *Mb*OII
Caranx ignobilis	457+235+15[b]	396+280+31
Caranx heberi	692+15[b]	396+311
Caranx sexfasciatus	538+154+15[b]	311+236+83[b]+77[b]
Gnathanodon speciosus	352[a]+334[a]+15[b]+6[b]	236+174+106[b]+83[b]+62[b]+31[b]+9[b]+3[b]+3[b]
Atropus hedlandensis	376+162[a]+154[a]+15[b]	396+280+31[b]
Turrum coeruleopinnatum	352+186+154+15[b]	311[a]+299[a]+97[b]
Selaroides leptolepis	162[a]+158[a]+153[a]+78[b]+72[b]+69[b]+15[b]	164+139[b]+133[b]+97[b]+77[b]+45[b]+39[b]+10[b]+3[b]
Selar crumenophthalmus	451+241+15[b]	624+83[b]

[a] Appeared as one band.
[b] The low molecular fragments disappeared in the RFLP profile due to this signal being too weak.

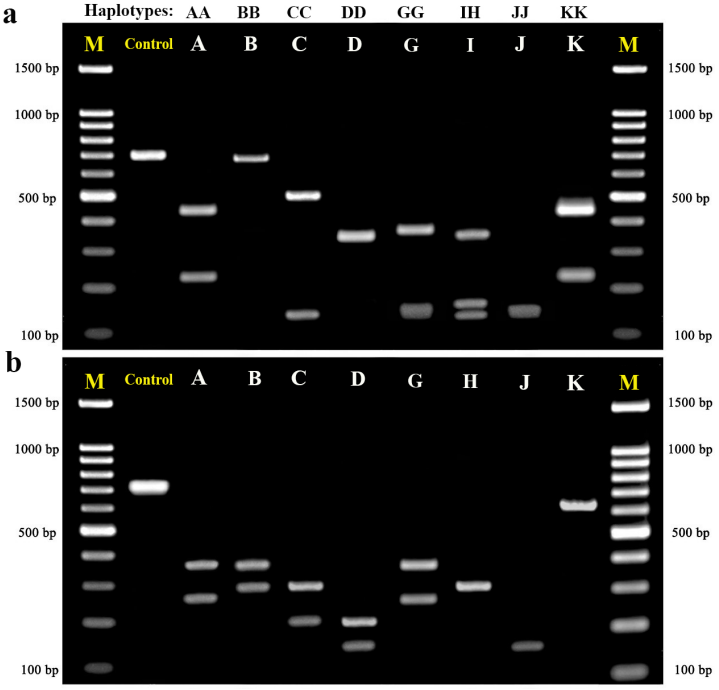

Fig. 6. RFLP profiles obtained under the cleavage of *Hae*III (a) and *Mb*OII (b) illustrating the most common banding patterns/composite haplotypes. Control (uncleaved PCR product); A: *Caranx ignobilis*; B: *Caranx heberi*; C: *Caranx sexfasciatus*; D: *Gnathanodon speciosus*; G: *Atropus hedlandensis*; I/H: *Turrum coeruleopinnatum*; J: *Selaroides leptolepis* K: *Selar crumenophthalmus*; M: 100 bp Ladder.

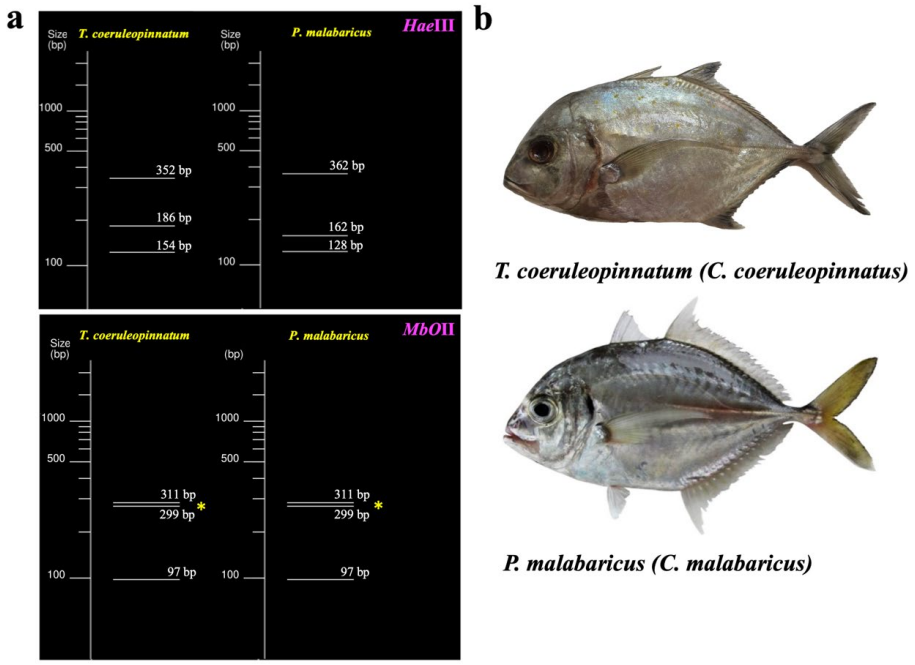

Fig. 7. The crucial significance of differentiation between *Turrum coeruleopinnatum* (formerly *Carangoides coeruleopinnatus*) and *Platycaranx malabaricus* (formerly *Carangoides malabaricus*). a: Similar banding patterns result from theoretical cleavage under *Hae*III and *Mb*OII; b: Phenotypic resemblance between the two species.

(Table 5). For *Hae*III, fragment types A, B, C, I, J and K are derived from *C. ignobilis*, *C. heberi*, *C. sexfasciatus*, *T. coeruleopinnatum*, *S. leptolepis* and *S. crumenophthalmus*, respectively. Fragment types D, E and F are derived from *G. speciosus* and fragment types G and H are derived from *A. hedlandensis*. For *Mbo*II, fragment types A, C, G, J and K are derived from *C. ignobilis*, *C. sexfasciatus*, *A. hedlandensis*, *S. leptolepis* and *S. crumenophthalmus*, respectively. Fragment type B is derived from both *C. ignobilis* and *C. heberi*. Fragment types D, E and F are derived from *G. speciosus* and fragment types H and I are derived from *T. coeruleopinnatum*, respectively.

However, theoretical cleavage revealed the presence of haplotypes out of 250 Carangid individuals. Minor fragment patterns were obtained from both *Hae*III and *Mbo*II cleavage and probability assumptions were therefore made by the frequency of composite haplotypes. Composite haplotypes in combination with both *Hae*III and *Mbo*II fragment types: AA, AB, BB, CC, DD, EE, FF, GG, HG, IH, II, JJ, KK and their frequencies among 250 sequences were identified in the current sample as described by Park and Kijima (2006). The composite haplotypes and their frequencies in the eight Carangid species are shown in Table 6. This reveals the compatibility of getting the combined *Hae*III and *Mbo*II RFLP profiles corresponding to the most abundant composite haplotype of each species and it is labelled in Figure 6 as AA, BB, CC, DD, GG, IH, JJ and KK.

Composite haplotypes AA/AB, BB, CC, DD/EE/FF, GG/HG, II/IH, JJ and KK were found only in *C. ignobilis*, *C. heberi*, *C. sexfasciatus*, *G. speciosus*, *A. hedlandensis*, *T. coeruleopinnatum*, *S. leptolepis* and *S. crumenophthalmus*, respectively. No common haplotypes were found among individuals of the eight species. Therefore, the composite haplotype

Table 5. Fragment patterns produced by the digestion of 250 geographically distant COI reference sequences of the eight Carangid species.

Fragments under *Hae*III (bp)	Fragments under *Mbo*II (bp)
A: 457+235+15	A: 396+280+31
B: 692+15	B: 396+311
C: 538+154+15	C: 311+236+83+77
D: 352+334+15+6	D: 236+174+106+83+62+31+9+3+3
*E: 358+180+154+15	*E: 236+174+106+83+71+31+3+3
*F: 352+180+154+15+6	*F: 242+205+106+83+71
G: 376+162+154+15	G: 396+280+31
*H: 352+162+154+24+15	H: 311+299+97
I: 352+186+154+15	*I: 311+160+139+97
J: 162+158+153+78+72+69+15	J: 164+139+133+97+77+45+39+10+3
K: 451+241+15	K: 624+83

*Minor fragment patterns.

Table 6. Haplotype frequencies of the eight Carangid species using the 250 mitochondrial COI partial gene sequences.

Composite haplotype[a]	1	2	3	4	5	6	7	8
(AA)	0.6897	-	-	-	-	-	-	-
(AB)	0.3103	-	-	-	-	-	-	-
(BB)	-	1.0000	-	-	-	-	-	-
(CC)	-	-	1.0000	-	-	-	-	-
(DD)	-	-	-	0.6667	-	-	-	-
(EE)	-	-	-	0.1852	-	-	-	-
(FF)	-	-	-	0.1481	-	-	-	-
(GG)	-	-	-	-	0.8462	-	-	-
(HG)	-	-	-	-	0.1538	-	-	-
(IH)	-	-	-	-	-	0.8529	-	-
(II)	-	-	-	-	-	0.1471	-	-
(JJ)	-	-	-	-	-	-	1.0000	-
(KK)	-	-	-	-	-	-	-	1.0000

[a]Composite designations are: *Hae*III and *Mbo*II.
1: *Caranx ignobilis*; 2: *Caranx heberi*; 3: *Caranx sexfasciatus*; 4: *Gnathanodon speciosus*; 5: *Atropus hedlandensis*; 6: *Turrum coeruleopinnatum*; 7: *Selaroides leptolepis*; 8: *Selar crumenophthalmus*.

frequencies were obtained as 1.0000 within each species except *C. ignobilis*, *G. speciosus*, *A. hedlandensis* and *T. coeruleopinnatum*. However, the probability of observing the most common composite haplotype frequencies (0.6667, 0.8462 and 0.8529) of *C. ignobilis*, *G. speciosus*, *A. hedlandensis* and *T. coeruleopinnatum* were compared with the haplotypes obtained under the present study ($P < 0.05$). Therefore, the two RFLP profiles obtained in the current study corresponding to *Hae*III and *Mbo*II appear to be closely related to the most common composite haplotype of each species.

Evolutionary assumptions towards the distribution of Carangid haplotypes

Given the geographical distribution of the composite haplotypes identified for each species, it appears that some of them are centred on a particular geographical region. Identified as minority composite haplotypes, *G. speciosus* (EE) is associated with Queensland; Australia, *G. speciosus* (FF), *A. hedlandensis* (HG) and *T. coeruleopinnatum* (II) are native to the Malaysian region. This reveals the key to the evolution of Carangids from their ancestors. Another special observation is that *C. ignobilis* (AB) which is identified as the minority composite haplotype has a wide range of distribution which is further confirmed by migratory encounters in the Indian Ocean, Australia and Hawaiian Islands (USA) (Fig. 8).

Discussion

The three requirements: easily recoverable, analysable and data sufficiency of the target DNA region should be fully filled in an effective DNA-based molecular tool utilized for species-level identification (Persis et al., 2009). All the above requirements were satisfied with the mitochondrial COI barcoding gene region. Based on the simplicity and explicitness of the COI barcoding gene region, it is considered the basic molecular marker in species identification. The average higher GC nucleotide variation obtained in the current study (56.86 %) under the 3rd codon position of the Carangid COI region reflects that it is mostly mutation-synonymous (Persis et al., 2009). This clearly convinces the higher GC variations concentrated at the 3rd codon position of the complete mitochondrial genome of fish (38.40– 43.20 %) and COI region (42.20–47.10 %) (Ward et al., 2005). The current study proves the ability of the COI barcode gene to cluster individuals into congeneric, conspecific groups. Calculated K2P distances of the COI sequences within and among species ranged from (0.00 to 2.96 %) and (6.46 % to 21.83 %), respectively. This revealed the possibility of using barcoding gene regions for the discrimination of phenotypically more or less similar carangid members. The resulting intraspecific and interspecific distance values are consistent with the published values for marine fish species (Bingpeng et al., 2018) and also revealed that

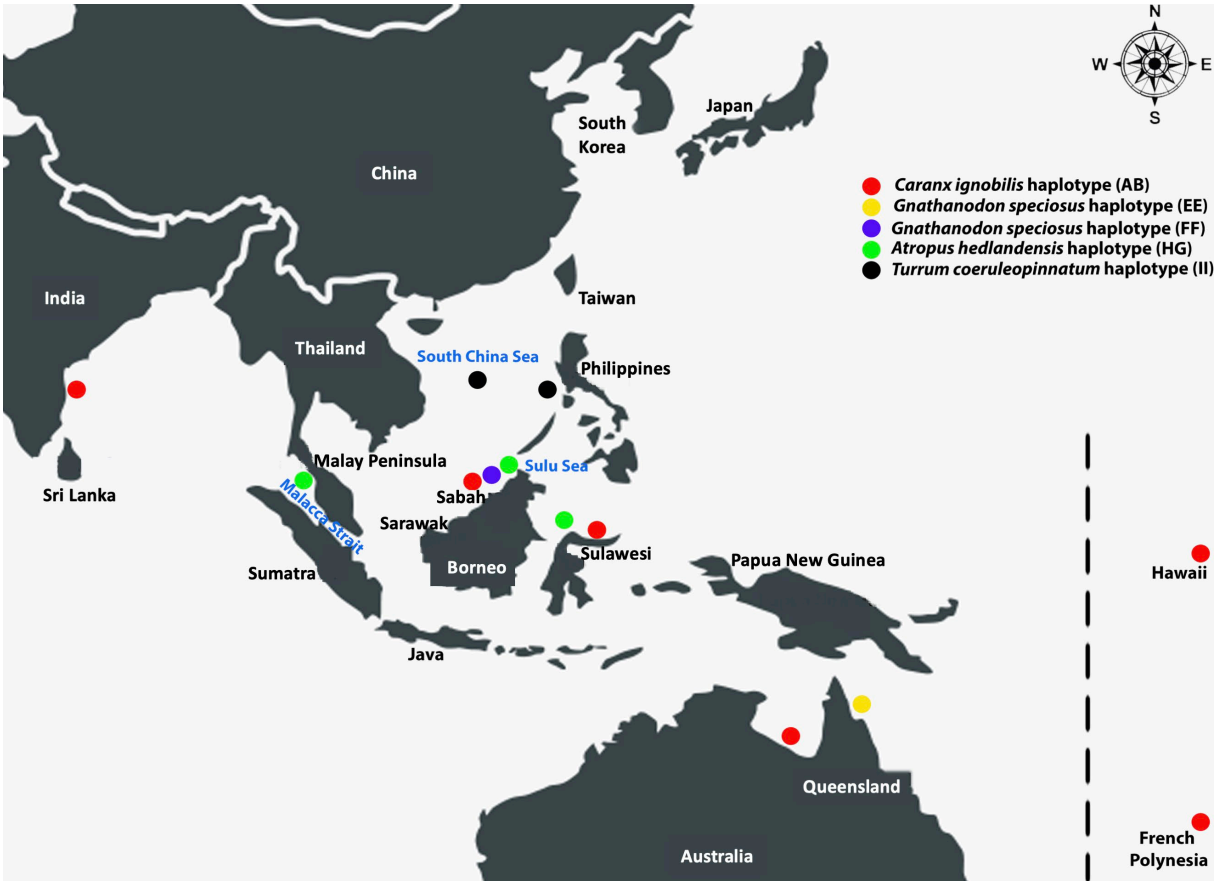

Fig. 8. Distribution of minority composite haplotypes of Carangid species over the Indo-Australian Archipelago (IAA).

COI-DNA barcoding data could be used successfully in discriminating fish species.

The challenges faced in distinguishing between *T. coeruleopinnatum* and *P. malabaricus* highlights the taxonomic ambiguity associated with regional variations of their external phenotypes. Resolving taxonomic misidentification of these two genera may require a combination of morphological, genetic, and ecological data. Despite the external morphological similarities, the identical RFLP patterns suggest a potential convergence at the genetic level, prompting a re-evaluation of the assumed genetic distinctiveness. This unforeseen similarity in the molecular profiles not only challenges the reliability of these particular enzymes in discriminating between *P. malabaricus* and *T. coeruleopinnatum* but also emphasizes the complexity of species differentiation within the Carangidae family. It beckons a comprehensive investigation into integrated molecular markers, such as COI barcoding, to unravel subtler genetic nuances that might elude the discriminatory power of the current RFLP analysis.

The ultimate aim of considering both molecular approaches: COI barcoding and PCR-RFLP together to data validation and increase the accuracy of data interpretation of both molecular tools used in species identification. This concept would be helpful in making a clear lineage from morphological analysis, COI barcoding sequence data analysis, RFLP mapping and interpretation of the RFLP results. Hence, a combination of a consolidated molecular method (PCR-RFLP) and the COI barcoding concept (COIBar-RFLP) enhances the accuracy, effectiveness and reliability of a wide range of species identifications in taxonomic and fraudulent seafood detection (Helyar et al., 2014). In the present study, we found that the application of the COIBar-RFLP profile derived from both *Hae*III and *Mbo*II restriction enzymes together could be useful for the identification of the eight Carangid species. PCR-RFLP strikes a balance between cost-effectiveness and accuracy, making it a favorable choice for large-scale phylogeographic studies. While it may not replace more advanced sequencing methods in all situations, its advantages in terms of cost reduction and efficiency make it a valuable tool, especially when considering the logistical and financial challenges of handling extensive datasets.

However, the restriction patterns did not always agree with those predicted due to several factors. Although low molecular weight bands would be weak and in certain situations such as mobility shifts were detected, major bands coinciding with the predicted fragment patterns, have to be considered. In the current study, only fragments above 100 bp were clearly visualized, hence all the predictions have been proved with the bands above 100 bp. Although the eight Carangid species can be differentiated by both RFLP profiles; *C. ignobilis*, *C. heberi*, *C. sexfasciatus* and *G. speciosus* have demonstrated a conspicuous fragment pattern with *Hae*III, while *Mbo*II made a clear visualisation of *A. hedlandensis*, *T. coeruleopinnatum*, *S. crumenophthalmus* and *S. leptolepis*. Hence, the combination of both profiles made a comparative molecular marker. Furthermore, COIBar-RFLP is considered as a dominant marker and parental recombination would not be interpreted more accurately. Therefore, this marker has limitations in hybrid species identification. In such situations, combining both COIBar-RFLP and random amplified microsatellite polymorphism (RAMP) makes more comparable and transferable results (Liu et al., 2007; Moustafa et al., 2017).

The accuracy and practicality of the RFLP profile are best guaranteed only if it represents a wide range of populations. The validity of the RFLP profiles is determined if any individual of the relevant species followed the fragment pattern corresponding to the current study whatever the geographical distribution they are associated with. A higher resolution of the results would be expected by increasing the sample size further. Along with a large population, various haplotypes would be expected. In such cases, the validity of the current RFLP profiles can be determined by the probability assumptions. A more practical conclusion can be drawn by comparing the RFLP profiles obtained in the present study with composite haplotypes frequently identified in each species under the cleavage of both *Hae*III and *Mbo*II restriction enzymes. In the study of the composite haplotype frequencies obtained according to the restriction enzymes, a higher frequency related to each species was found in all eight species corresponding to the RFLP profiles of the current study is a matter of concern.

The encounter with these haplotypes in relation to the Indo - Australian Archipelago (IAA) and regional remarkable biodiversity including Carangid species can be inferred from various assumptions. Among them, the most acceptable explanations are the "Centre-of-Overlap" and the "Centre-of-Origin" hypotheses (Santini and Winterbottom, 2002; Carpenter and Springer, 2005; Hubert et al., 2012) . The Centre-of-Overlap hypothesis explains geographic isolation at the midpoint of the IAA facilitates considerable changes in the genetic structure of species on each side of the IAA due to limited species distribution. Hence, allopatric speciation and the evolution of cryptic species would be expected. The Centre-of-Origin hypothesis explains geographical interconnection facilitates great species distribution across the midpoint of IAA, with remaining ancestral species at the centre and new species in peripheral geographic areas (Gamage et al., 2022). However, considerable changes in genetic structures would not be expected compared to the Centre-of-overlap hypothesis. Usually, pelagic marine fishes have high fecundity affinities and high dispersal potential from eggs and larvae to the adult stages hence, they can attain large population sizes

over time. Therefore, a limited genetic differentiation would be predicted between geographic populations due to high gene flow rates facilitated by the above traits (Rohfritsch and Borsa, 2005) . Therefore, the origin of the minor composite haplotypes observed in the current study may be a consequence of the phenomenon of "Centre-of-Overlap".

As described by Gamage et al. (2023) in a parallel study, Carangids have a remarkable haplotype distribution away from the regionally isolated Carangid ancestors in the Indo-Malay Archipelago (IMA). Based on the regionally periodic geological incidents and prehistoric events, the IMA region is concentrated with great cryptic Carangid haplotypes (Mat Jaafar et al., 2012; Gamage et al., 2023). High-throughput conventional barcode DNA sequencing can aid in identifying most of the haplogroups more accurately. However, due to high cost, durability, cross-lab validation, taxonomists require a rapid molecular approach for a better interpretation. With all this background and utility, COIBar-RFLP developed in this study would be a reliable laboratory marker to detect the global haplotype distribution of the corresponding Carangid species.

According to the glacial impacts of marine taxa, both terrestrial and marine biogeography has been altered by past climatic and geological events. In the Miocene, marine passages between the Indian and Pacific Oceans were reduced when Asian and Australasian lands collided with each other and completely interrupted in the Pleistocene when sea levels were fallen (Hall, 1998; Voris, 2012). Consequently, the Sunda and Sahul continental shelves were exposed, and the South China Sea was geographically isolated from the Indian Ocean (Rohfritsch and Borsa, 2005). Scientists believe this geographical isolation results in a great phylogeographic breakdown across both the Indian and the Pacific Oceans at species, subspecies or population levels. Also, the Indo-Malay Region has been concentrated with a great species and genetic diversity including the family Carangidae (Hewitt, 2000).

Conclusion

The current study clearly demonstrates the successful identification of Carangid family members by integrating the concept of nucleotide divergences and RFLP (COIBar-RFLP) in the mitochondrial COI barcoding gene region. The utility of the COI region is confirmed by the fact that the COI region shows fewer intraspecific distances compared to interspecific distances, as opposed to the significant cryptic diversity among individuals of the family. The validity of species isolation on the COI divergences values was further increased by the cleavage patterns of the amplified COI regions corresponding to the *Hae*III and *Mbo*II restriction enzymes. A higher resolution of the results would be expected by increasing the sample size further incorporating more Carangid species and testing the approach in other teleost families.

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Supplementary Table 1. Theoretical cleavage of 250 reference sequences (BOLD) of the eight Carangid fish and their composite haplotypes.

Seq. No.	Reference Sequence	Country	Base Pairs	*Hae* III Fragments	*MbO* II Fragments	Composite haplotype
	1. *Caranx ignobilis* (Giant Trevally)					
1	LMUOR002-21	Sri Lanka	655bp	457+235+15	396+280+31	AA
2	LMUOR003-21	Sri Lanka	655bp	457+235+15	396+280+31	AA
3	LMUOR004-21	Sri Lanka	655bp	457+235+15	396+280+31	AA
4	LMUOR005-21	Sri Lanka	655bp	457+235+15	396+280+31	AA
5	TZMSC060-05	South Africa	652bp	457+235+15	396+280+31	AA
6	DSFSE302-07	South Africa	652bp	457+235+15	396+280+31	AA
7	FOAJ697-09	Indonesia	652bp	457+235+15	396+280+31	AA
8	GBMNB5687-20	India	684bp	457+235+15	396+280+31	AA
9	GBMNB5688-20	India	684bp	457+235+15	396+280+31	AA
10	ANGBF17283-19	Source: mined from NCBI	654bp	457+235+15	396+280+31	AA
11	ANGBF17284-19	Source: mined from NCBI	654bp	457+235+15	396+280+31	AA
12	ANGBF17285-19	Source: mined from NCBI	654bp	457+235+15	396+280+31	AA
13	ANGBF17286-19	Source: mined from NCBI	654bp	457+235+15	396+280+31	AA
14	GBMNB5690-20	India	684bp	457+235+15	396+280+31	AA
15	GBMNB5691-20	India	684bp	457+235+15	396+280+31	AA
16	FMVIC740-08	Australia	652bp	457+235+15	396+280+31	AA
17	GAMBA007-12	French Polynesia	655bp	457+235+15	396+280+31	AA
18	DBMF563-10	Malaysia, Sabah	652bp	457+235+15	396+280+31	AA
19	DBMF564-10	Malaysia, Sabah	652bp	457+235+15	396+280+31	AA
20	DBMF705-10	Malaysia, Sabah, Sulawesi Sea	652bp	457+235+15	396+280+31	AA
21	DBMF565-10	Malaysia, Sabah	652bp	457+235+15	396+311	AB
22	DBMF704-10	Malaysia, Sabah, Sulawesi Sea	652bp	457+235+15	396+311	AB
23	UKFBJ906-08	Seychelles	652bp	457+235+15	396+311	AB
24	GBMNB5689-20	India	684bp	457+235+15	396+311	AB
25	FOAF575-07	Australia	655bp	457+235+15	396+311	AB
26	FOAF576-07	Australia	655bp	457+235+15	396+311	AB
27	MBFB037-07	French Polynesia	651bp	457+235+15	396+311	AB
28	GAMBA617-12	French Polynesia	655bp	457+235+15	396+311	AB
29	KANBO26-17	United States - Hawaii	655bp	457+235+15	396+311	AB
	2. *Caranx heberi* (Black-tipped Trevally)					
30	LMUOR001-21	Sri Lanka	655bp	692+15	396+311	BB
31	LMUOR006-21	Sri Lanka	655bp	692+15	396+311	BB
32	LMUOR007-21	Sri Lanka	655bp	692+15	396+311	BB
33	LMUOR008-21	Sri Lanka	655bp	692+15	396+311	BB
34	LMUOR009-21	Sri Lanka	655bp	692+15	396+311	BB
35	LMUOR010-21	Sri Lanka	655bp	692+15	396+311	BB
36	DSFSG944-13	South Africa	652bp	692+15	396+311	BB

Seq. No.	Reference Sequence	Country	Base Pairs	*Hae* III Fragments	*MbO* II Fragments	Composite haplotype
37	DSLAF742-08	South Africa	652bp	692+15	396+311	BB
38	DSLAG626-10	South Africa	652bp	692+15	396+311	BB
39	GOAIL076-17	Israel,Eilat	652bp	692+15	396+311	BB
40	TZSAL432-13	South Africa, Kwazulu-Natal,Ugu	652bp	692+15	396+311	BB
41	TZSAL433-13	South Africa, Kwazulu-Natal,Ugu	652bp	692+15	396+311	BB
42	TZSAL434-13	South Africa, Kwazulu-Natal,Ugu	652bp	692+15	396+311	BB
43	TZSAL435-13	South Africa, Kwazulu-Natal,Ugu	652bp	692+15	396+311	BB
44	DSLAG628-10	South Africa	652bp	692+15	396+311	BB
45	DSLAG627-10	South Africa	652bp	692+15	396+311	BB
46	DSLAF740-08	South Africa	652bp	692+15	396+311	BB
47	DSFSG979-13	South Africa	652bp	692+15	396+311	BB
48	DSLAF743-08	South Africa	652bp	692+15	396+311	BB
49	DSLAF744-08	South Africa	652bp	692+15	396+311	BB
50	DSLAG625-10	South Africa	652bp	692+15	396+311	BB
	3. *Caranx sexfasciatus* (Big eye Trevally)					
51	LMUOR011-21	Sri Lanka	655bp	538+154+15	311+236+83+77	CC
52	LMUOR012-21	Sri Lanka	655bp	538+154+15	311+236+83+77	CC
53	LMUOR013-21	Sri Lanka	655bp	538+154+15	311+236+83+77	CC
54	LMUOR014-21	Sri Lanka	655bp	538+154+15	311+236+83+77	CC
55	LMUOR015-21	Sri Lanka	655bp	538+154+15	311+236+83+77	CC
56	LMUOR016-21	Sri Lanka	655bp	538+154+15	311+236+83+77	CC
57	ABFJ207-07	Japan, Nagasaki	652bp	538+154+15	311+236+83+77	CC
58	ABFJ208-07	Japan, Nagasaki	652bp	538+154+15	311+236+83+77	CC
59	ANGBF17296-19	New Caledonia	655bp	538+154+15	311+236+83+77	CC
60	ANGBF17297-19	New Caledonia	655bp	538+154+15	311+236+83+77	CC
61	ANGBF17298-19	Philippines	652bp	538+154+15	311+236+83+77	CC
62	BIFB247-13	Indonesia, Jawa Barat	652bp	538+154+15	311+236+83+77	CC
63	BIFD1742-14	Indonesia, Jawa Timur	652bp	538+154+15	311+236+83+77	CC
64	BIFZB098-17	Indonesia, Maluku, Ambon Island	652bp	538+154+15	311+236+83+77	CC
65	BIFZB099-17	Indonesia, Maluku, Ambon Island	652bp	538+154+15	311+236+83+77	CC
66	BIFZB100-17	Indonesia, Maluku, Ambon Island	652bp	538+154+15	311+236+83+77	CC
67	BIFZB102-17	Indonesia, Maluku, Ambon Island	652bp	538+154+15	311+236+83+77	CC
68	FOAC447-05	Australia	655bp	538+154+15	311+236+83+77	CC
69	FOAC448-05	Australia	655bp	538+154+15	311+236+83+77	CC
70	FOAC450-05	Australia	655bp	538+154+15	311+236+83+77	CC
71	FOAC451-05	Australia	655bp	538+154+15	311+236+83+77	CC
72	FTW585-09	Taiwan, Pingtung County	652bp	538+154+15	311+236+83+77	CC
73	FTWS241-09	Taiwan, Taichung City	652bp	538+154+15	311+236+83+77	CC
74	RDFCA366-05	Costa Rica, Guanacaste	652bp	538+154+15	311+236+83+77	CC
75	RDFCA394-05	Costa Rica, Guanacaste	652bp	538+154+15	311+236+83+77	CC

Seq. No.	Reference Sequence	Country	Base Pairs	*Hae* III Fragments	*MbO* II Fragments	Composite haplotype
76	RDFCA415-05	Costa Rica, Guanacaste	652bp	538+154+15	311+236+83+77	CC
77	SAIAB488-06	Seychelles, Mahe	652bp	538+154+15	311+236+83+77	CC
78	SAIAB774-08	Tanzania, Tanga	652bp	538+154+15	311+236+83+77	CC
79	SAIAB775-08	Tanzania, Tanga	652bp	538+154+15	311+236+83+77	CC
80	DBMF005-10	Malaysia, Perlis, Malacca Strait	652bp	538+154+15	311+236+83+77	CC
81	DBMF021-10	Malaysia, Perak, Malacca Strait	652bp	538+154+15	311+236+83+77	CC
82	DBMF391-10	Malaysia, Sarawak, South China Sea	652bp	538+154+15	311+236+83+77	CC
83	DBMF030-10	Malaysia, Perak, Malacca Strait	652bp	538+154+15	311+236+83+77	CC
84	DBMF632-10	Malaysia, Subha, Sulu Sea	652bp	538+154+15	311+236+83+77	CC
85	DSFSG934-13	South Africa	652bp	538+154+15	311+236+83+77	CC
86	DSFSG946-13	South Africa	652bp	538+154+15	311+236+83+77	CC
87	DSFSE215-07	South Africa	648bp	538+154+15	311+236+83+77	CC
88	DSFSF026-09	Mozambique	648bp	538+154+15	311+236+83+77	CC
	4. *Gnathanodon speciosus* (Golden Trevally)					
89	LMUOR017-21	Sri Lanka	655bp	352+334+15+6	236+174+106+83+62+31+9+3+3	DD
90	LMUOR018-21	Sri Lanka	655bp	352+334+15+6	236+174+106+83+62+31+9+3+3	DD
91	LMUOR019-21	Sri Lanka	655bp	352+334+15+6	236+174+106+83+62+31+9+3+3	DD
92	LMUOR020-21	Sri Lanka	655bp	352+334+15+6	236+174+106+83+62+31+9+3+3	DD
93	LMUOR021-21	Sri Lanka	655bp	352+334+15+6	236+174+106+83+62+31+9+3+3	DD
94	LMUOR022-21	Sri Lanka	655bp	352+334+15+6	236+174+106+83+62+31+9+3+3	DD
95	WLINDO70-07	India	655bp	352+334+15+6	236+174+106+83+62+31+9+3+3	DD
96	WLINDO71-07	India	655bp	352+334+15+6	236+174+106+83+62+31+9+3+3	DD
97	WLINDO73-07	India	655bp	352+334+15+6	236+174+106+83+62+31+9+3+3	DD
98	GBMNB11805-20	India	663bp	352+334+15+6	236+174+106+83+62+31+9+3+3	DD
99	GBMNB11804-20	India	663bp	352+334+15+6	236+174+106+83+62+31+9+3+3	DD
100	GBMNB11803-20	India	663bp	352+334+15+6	236+174+106+83+62+31+9+3+3	DD
101	GBMNB11802-20	India	663bp	352+334+15+6	236+174+106+83+62+31+9+3+3	DD
102	GBMNB11801-20	India	663bp	352+334+15+6	236+174+106+83+62+31+9+3+3	DD
103	DSFSE776-08	Mozambique	648bp	352+334+15+6	236+174+106+83+62+31+9+3+3	DD
104	DSLAR529-09	Mozambique	648bp	352+334+15+6	236+174+106+83+62+31+9+3+3	DD
105	SAIAB598-07	Seychelles, Mahe	652bp	352+334+15+6	236+174+106+83+62+31+9+3+3	DD
106	FOAM215-10	Indonesia, Nusa Tenggara Barat	652bp	352+334+15+6	236+174+106+83+62+31+9+3+3	DD
107	FOAC413-05	Australia, Queensland	655bp	358+180+154+15	236+174+106+83+71+31+3+3	EE
108	FOAC414-05	Australia, Queensland	655bp	358+180+154+15	236+174+106+83+71+31+3+3	EE
109	FOAC415-05	Australia, Queensland	655bp	358+180+154+15	236+174+106+83+71+31+3+3	EE
110	FOAC416-05	Australia, Queensland	655bp	358+180+154+15	236+174+106+83+71+31+3+3	EE
111	FOAC417-05	Australia, Queensland	655bp	358+180+154+15	236+174+106+83+71+31+3+3	EE
112	DBMF508-10	Malaysia, Sabah	652bp	352+180+154+15+6	242+205+106+83+71	FF
113	DBMF509-10	Malaysia, Sabah	652bp	352+180+154+15+6	242+205+106+83+71	FF
114	DBMF510-10	Malaysia, Sabah	652bp	352+180+154+15+6	242+205+106+83+71	FF

Seq. No.	Reference Sequence	Country	Base Pairs	*Hae* III Fragments	*MbO* II Fragments	Composite haplotype
115	DBMF511-10	Malaysia, Sabah	652bp	352+180+154+15+6	242+205+106+83+71	FF
	5. *Carangoides hedlandensis* (Bump nose Trevally) [Redefined as *Atropus hedlandensis* (Kimura et al., 2022)]					
116	LMUOR023-21	Sri Lanka	655bp	376+162+154+15	396+280+31	GG
117	LMUOR024-21	Sri Lanka	655bp	376+162+154+15	396+280+31	GG
118	LMUOR025-21	Sri Lanka	655bp	376+162+154+15	396+280+31	GG
119	DBMF009-10	Malaysia, Perlis, Malacca Strait	652bp	376+162+154+15	396+280+31	GG
120	DBMF616-10	Malaysia, Sabah, Sulu Sea	651bp	376+162+154+15	396+280+31	GG
121	DBMF615-10	Malaysia, Sabah, Sulu Sea	652bp	376+162+154+15	396+280+31	GG
122	DBMF123-10	Malaysia, Johor, South China Sea	652bp	376+162+154+15	396+280+31	GG
123	DBMF121-10	Malaysia, Johor, South China Sea	652bp	376+162+154+15	396+280+31	GG
124	DBMF119-10	Malaysia, Johor, South China Sea	652bp	376+162+154+15	396+280+31	GG
125	FTW793-09	Taiwan, Hualien City	652bp	376+162+154+15	396+280+31	GG
126	GBMIN131570 - 17	New Caledonia	655bp	376+162+154+15	396+280+31	GG
127	FOAM137-10	Indonesia, Nusa Tenggara Barat	652bp	376+162+154+15	396+280+31	GG
128	FOAM136-10	Indonesia, Nusa Tenggara Barat	652bp	376+162+154+15	396+280+31	GG
129	FOAL061-10	Indonesia, Bali	648bp	376+162+154+15	396+280+31	GG
130	FOAK814-10	Indonesia, Jawa Barat	648bp	376+162+154+15	396+280+31	GG
131	FOAK866-10	Indonesia, Nusa Tenggara Barat	648bp	376+162+154+15	396+280+31	GG
132	FOAM427-10	Indonesia, Jawa Barat	652bp	376+162+154+15	396+280+31	GG
133	FOAM429-10	Indonesia, Jawa Barat	652bp	376+162+154+15	396+280+31	GG
134	FOAM143-10	Indonesia, Nusa Tenggara Barat	652bp	376+162+154+15	396+280+31	GG
135	DBMF122-10	Malaysia, Johor, South China Sea	652bp	376+162+154+15	396+280+31	GG
136	DBMF120-10	Malaysia, Johor, South China Sea	652bp	376+162+154+15	396+280+31	GG
137	DBMF118-10	Malaysia, Johor, South China Sea	652bp	376+162+154+15	396+280+31	GG
138	DBMF018-10	Malaysia, Kedah, Malacca Strait	652bp	352+162+154+24+15	396+280+31	HG
139	DBMF717-10	Malaysia, Sabah, Sulawesi Sea	651bp	352+162+154+24+15	396+280+31	HG
140	DBMF713-10	Malaysia, Sabah, Sulawesi Sea	652bp	352+162+154+24+15	396+280+31	HG
141	DBMF535-10	Malaysia, Sabah	652bp	352+162+154+24+15	396+280+31	HG
	6. *Carangoides coeruleopinnatus* (Coastal Trevally) [Redefined as *Turrum coeruleopinnatum* (Kimura et al., 2022)]					
142	LMUOR026-21	Sri Lanka	655bp	352+186+154+15	311+299+97	IH
143	LMUOR027-21	Sri Lanka	655bp	352+186+154+15	311+299+97	IH
144	LMUOR028-21	Sri Lanka	655bp	352+186+154+15	311+299+97	IH
145	LMUOR029-21	Sri Lanka	655bp	352+186+154+15	311+299+97	IH
146	DSFSF045-09	Mozambique	648bp	352+186+154+15	311+299+97	IH
147	DSFSF057-09	Mozambique	648bp	352+186+154+15	311+299+97	IH
148	DSFSF663-09	South Africa, Kwazulu-Natal	648bp	352+186+154+15	311+299+97	IH
149	DSFSG747-11	South Africa	648bp	352+186+154+15	311+299+97	IH
150	DBMF046-10	Malaysia, Perak, Malacca	652bp	352+186+154+15	311+299+97	IH
151	DBMF048-10	Malaysia, Perak, Malacca	652bp	352+186+154+15	311+299+97	IH
152	DBMF305-10	Malaysia, Terengganu, South China Sea	655bp	352+186+154+15	311+299+97	IH

Seq. No.	Reference Sequence	Country	Base Pairs	*Hae* III Fragments	*MbO* II Fragments	Composite haplotype
153	DBMF721-10	Malaysia, Sabah, Sulawesi Sea	655bp	352+186+154+15	311+299+97	IH
154	DBMF176-10	Malaysia, Pahang, South China Sea	652bp	352+186+154+15	311+299+97	IH
155	DBMF177-10	Malaysia, Pahang, South China Sea	651bp	352+186+154+15	311+299+97	IH
156	DBMF178-10	Malaysia, Pahang, South China Sea	652bp	352+186+154+15	311+299+97	IH
157	DBMF180-10	Malaysia, Pahang, South China Sea	652bp	352+186+154+15	311+299+97	IH
158	DBMF238-10	Malaysia, Terengganu, South China Sea	652bp	352+186+154+15	311+299+97	IH
159	DBMF240-10	Malaysia, Terengganu, South China Sea	652bp	352+186+154+15	311+299+97	IH
160	DBMF398-10	Malaysia, Sarawak, South China Sea	652bp	352+186+154+15	311+299+97	IH
161	DBMF460-10	Malaysia, Sarawak, South China Sea	652bp	352+186+154+15	311+299+97	IH
162	DBMF463-10	Malaysia, Sarawak, South China Sea	652bp	352+186+154+15	311+299+97	IH
163	DBMF537-10	Malaysia, Sabah	652bp	352+186+154+15	311+299+97	IH
164	DBMF540-10	Malaysia, Sabah	652bp	352+186+154+15	311+299+97	IH
165	FOAL914-10	Australia, Queensland, Torres Strait	648bp	352+186+154+15	311+299+97	IH
166	FOAG343-08	Australia, Western	648bp	352+186+154+15	311+299+97	IH
167	FOAG348-08	Australia, Western	648bp	352+186+154+15	311+299+97	IH
168	FOAG350-08	Australia, Western	648bp	352+186+154+15	311+299+97	IH
169	FOAG351-08	Australia, Western	648bp	352+186+154+15	311+299+97	IH
170	FOAO1199-18	Australia, Western	677bp	352+186+154+15	311+299+97	IH
171	DBMF597-10	Malaysia, Sabah, Sulu Sea	652bp	352+186+154+15	311+160+139+97	II
172	DBMF598-10	Malaysia, Sabah, Sulu Sea	652bp	352+186+154+15	311+160+139+97	II
173	DBMF791-10	Malaysia, Sabah, South China Sea	652bp	352+186+154+15	311+160+139+97	II
174	DBMF792-10	Malaysia, Sabah, South China Sea	652bp	352+186+154+15	311+160+139+97	II
175	DBMF795-10	Malaysia, Sabah, South China Sea	652bp	352+186+154+15	311+160+139+97	II
	7. *Selaroides leptolepis* (Yellow-stripped Scad)					
176	LMUOR030-21	Sri Lanka	655bp	162+158+153+78+72+69+15	164+139+133+97+77+45+39+10+3	JJ
177	LMUOR031-21	Sri Lanka	655bp	162+158+153+78+72+69+15	164+139+133+97+77+45+39+10+3	JJ
178	LMUOR032-21	Sri Lanka	655bp	162+158+153+78+72+69+15	164+139+133+97+77+45+39+10+3	JJ
179	ANGBF17513-19	Saudi Arabia	678bp	162+158+153+78+72+69+15	164+139+133+97+77+45+39+10+3	JJ
180	ANGBF17514-19	Saudi Arabia	678bp	162+158+153+78+72+69+15	164+139+133+97+77+45+39+10+3	JJ
181	ANGBF17515-19	Saudi Arabia	678bp	162+158+153+78+72+69+15	164+139+133+97+77+45+39+10+3	JJ
182	ANGBF17516-19	Saudi Arabia	678bp	162+158+153+78+72+69+15	164+139+133+97+77+45+39+10+3	JJ
183	ANGBF17517-19	Saudi Arabia	689bp	162+158+153+78+72+69+15	164+139+133+97+77+45+39+10+3	JJ
184	FOAI136-08	Indonesia, Jawa Timur, East Java	652bp	162+158+153+78+72+69+15	164+139+133+97+77+45+39+10+3	JJ
185	DBMF294-10	Malaysia,Terengganu, South China Sea	652bp	162+158+153+78+72+69+15	164+139+133+97+77+45+39+10+3	JJ
186	DBMF475-10	Malaysia, Sarawak, South China Sea	652bp	162+158+153+78+72+69+15	164+139+133+97+77+45+39+10+3	JJ
187	DBMF519-10	Malaysia, Sabah	652bp	162+158+153+78+72+69+15	164+139+133+97+77+45+39+10+3	JJ
188	GBMIN127813-17	Malaysia	650bp	162+158+153+78+72+69+15	164+139+133+97+77+45+39+10+3	JJ
189	GBMIN127814-17	Malaysia	650bp	162+158+153+78+72+69+15	164+139+133+97+77+45+39+10+3	JJ
190	GBMIN127815-17	Malaysia	650bp	162+158+153+78+72+69+15	164+139+133+97+77+45+39+10+3	JJ

Seq. No.	Reference Sequence	Country	Base Pairs	*Hae* III Fragments	*MbO* II Fragments	Composite haplotype
191	GBMIN127816-17	Malaysia	650bp	162+158+153+78+72+69+15	164+139+133+97+77+45+39+10+3	JJ
192	GBMIN127817-17	Malaysia	650bp	162+158+153+78+72+69+15	164+139+133+97+77+45+39+10+3	JJ
193	FOAL898-10	Australia, Queensland	652bp	162+158+153+78+72+69+15	164+139+133+97+77+45+39+10+3	JJ
194	FSCS084-06	China, Guangdong	652bp	162+158+153+78+72+69+15	164+139+133+97+77+45+39+10+3	JJ
195	FSCS085-06	China, Guangdong	652bp	162+158+153+78+72+69+15	164+139+133+97+77+45+39+10+3	JJ
196	FTWS901-09	Taiwan,Taichung City, Taichong,Wuchi	652bp	162+158+153+78+72+69+15	164+139+133+97+77+45+39+10+3	JJ
197	FTWS259-09	Taiwan, Penghu County, Pescadores	652bp	162+158+153+78+72+69+15	164+139+133+97+77+45+39+10+3	JJ
198	GBMNC15182-20	Pacific Ocean	655bp	162+158+153+78+72+69+15	164+139+133+97+77+45+39+10+3	JJ
	8. *Selar crumenophthalmus* (Big eye Scad)					
199	LMUOR033-21	Sri Lanka	655bp	451+241+15	624+83	KK
200	LMUOR034-21	Sri Lanka	655bp	451+241+15	624+83	KK
201	LMUOR035-21	Sri Lanka	655bp	451+241+15	624+83	KK
202	LMUOR036-21	Sri Lanka	655bp	451+241+15	624+83	KK
203	LMUOR037-21	Sri Lanka	655bp	451+241+15	624+83	KK
204	LMUOR038-21	Sri Lanka	655bp	451+241+15	624+83	KK
205	GBGC8413-09	India	1471bp	451+241+15	624+83	KK
206	GBGC8412-09	India	1471bp	451+241+15	624+83	KK
207	DSFSE554-08	Mozambique	652bp	451+241+15	624+83	KK
208	DSFSE546-08	Mozambique	652bp	451+241+15	624+83	KK
209	DSLAG1147-11	South Africa, KwaZulu Natal	652bp	451+241+15	624+83	KK
210	SAIAB450-06	Seychelles, Mahe, Anse L'Islette	652bp	451+241+15	624+83	KK
211	FOAI214-08	Indonesia, Bali, Bali	652bp	451+241+15	624+83	KK
212	FOAJ507-09	Indonesia, Jawa Timur, West Java	652bp	451+241+15	624+83	KK
213	FOAJ552-09	Indonesia, Jawa Timur, West Java	650bp	451+241+15	624+83	KK
214	FOAN433-11	Indonesia, Bali	651bp	451+241+15	624+83	KK
215	FOAN434-11	Indonesia, Bali	651bp	451+241+15	624+83	KK
216	FOAJ554-09	Indonesia, Jawa Timur, West Java	652bp	451+241+15	624+83	KK
217	ANGBF17504-19	Philippines	684bp	451+241+15	624+83	KK
218	ANGBF54744-19	NCBI	676bp	451+241+15	624+83	KK
219	ANGBF54745-19	NCBI	676bp	451+241+15	624+83	KK
220	ANGBF54756-19	NCBI	676bp	451+241+15	624+83	KK
221	ANGBF54790-19	NCBI	676bp	451+241+15	624+83	KK
222	GBMIN94124-17	China	655bp	451+241+15	624+83	KK
223	FOAO1320-18	Australia Western	677bp	451+241+15	624+83	KK
224	GAMBA004-12	French Polynesia, Tuamotu-Gambier	655bp	451+241+15	624+83	KK
225	GAMBA005-12	French Polynesia, Tuamotu-Gambier	655bp	451+241+15	624+83	KK
226	GAMBA006-12	French Polynesia, Tuamotu-Gambier	655bp	451+241+15	624+83	KK
227	BZLWB357-06	Belize, Stann Creek District	655bp	451+241+15	624+83	KK
228	DBMF130-10	Malaysia, Johor, South China Sea	652bp	451+241+15	624+83	KK
229	DBMF131-10	Malaysia, Johor, South China Sea	652bp	451+241+15	624+83	KK

Seq. No.	Reference Sequence	Country	Base Pairs	*Hae* III Fragments	*MbO* II Fragments	Composite haplotype
230	DBMF192-10	Malaysia, Pahang, South China Sea	652bp	451+241+15	624+83	KK
231	DBMF228-10	Malaysia, Terengganu, South China Sea	652bp	451+241+15	624+83	KK
232	DBMF327-10	Malaysia, Kelantan, South China Sea	652bp	451+241+15	624+83	KK
233	DBMF809-10	Malaysia, Perlis, Malacca Strait	652bp	451+241+15	624+83	KK
234	ANGBF17502-19	Malaysia	650bp	451+241+15	624+83	KK
235	DBMF031-10	Malaysia, Perak, Malacca Strait	652bp	451+241+15	624+83	KK
236	DBMF129-10	Malaysia	652bp	451+241+15	624+83	KK
237	FOAJ553-09	Indonesia, Jawa Timur, West Java	652bp	451+241+15	624+83	KK
238	DBMF001-10	Malaysia	652bp	451+241+15	624+83	KK
239	DBMF013-10	Malaysia	652bp	451+241+15	624+83	KK
240	ANGBF54698-19	NCBI	676bp	451+241+15	624+83	KK
241	ANGBF54717-19	NCBI	676bp	451+241+15	624+83	KK
242	DSFSE551-08	Mozambique	652bp	451+241+15	624+83	KK
243	DSFSE563-08	Mozambique	652bp	451+241+15	624+83	KK
244	NNPF153-10	Iran, Bushehr, Nayband National Park Coast	649bp	451+241+15	624+83	KK
245	NNPF149-10	Iran, Bushehr, Nayband National Park Coast	649bp	451+241+15	624+83	KK
246	NNPF017-10	Iran, Bushehr, Nayband National Park Coast	649bp	451+241+15	624+83	KK
247	NNPF142-10	Iran, Bushehr, Nayband National Park Coast	649bp	451+241+15	624+83	KK
248	NNPF147-10	Iran, Bushehr, Nayband National Park Coast	649bp	451+241+15	624+83	KK
249	BZLWB358-06	Belize, Stann Creek District	653bp	451+241+15	624+83	KK
250	TOBA242-09	Trinidad and Tobago, Tobago	655bp	451+241+15	624+83	KK

The most common composite haplotypes

The minor composite haplotypes