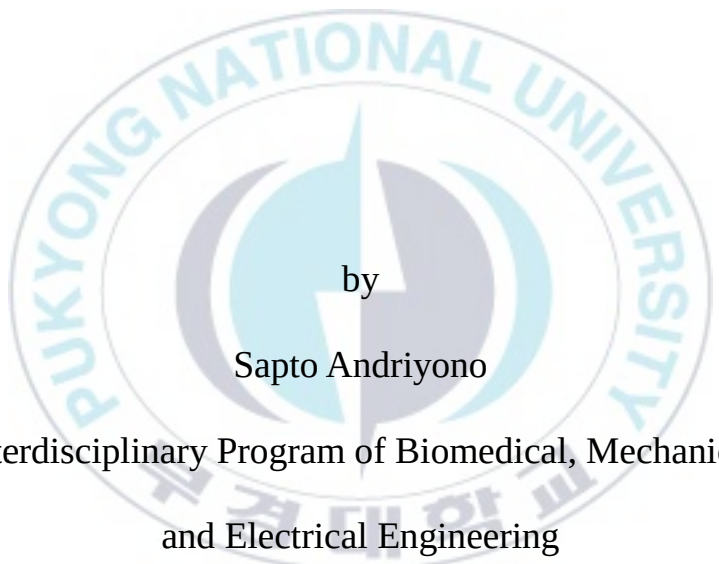
Thesis for the Degree of Doctor of Engineering

Molecular studies on marine fish diversity in Java and Bali, Indonesia



by

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Interdisciplinary Program of Biomedical, Mechanical and Electrical Engineering

The Graduate School

Pukyong National University

August 2019

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(인도네시아 자바와 발리의 해양 어류 다양성에 관한 분자생물학적 연구)

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by

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A thesis submitted in partial fulfillment of the requirements

for the degree of

Doctor of Engineering

in Interdisciplinary Program of Biomedical, Mechanical and Electrical Engineering, The graduate School,

Pukyong National University

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August 23, 2019

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Abstract

Marine ecosystem reflects a substantial contribution to genetic biodiversity than the freshwater and terrestrial ecosystem. The economic benefit is also generated from the marine ecosystem with the presence of aquatic biota that is used in fisheries and management of this valuable resources. Furthermore, numerous products are also capable of producing other derivatives those are more beneficial for the human being (e.g., for food and medicine) and for industrial purposes. Fish diversity in the specific marine ecosystem can be reflected in the health condition of the marine ecosystem, thus requiring the regular in monitoring and observation. Indonesia is the center of marine biodiversity which espouses with the extraordinary wealth of coral reef ecosystem.

The previous study recorded 2,057 species in the marine ecosystem of Indonesia; then the latest of the FishBase database had been recorded 3,611 species, which confirms that Indonesia is the wealthiest country for coral reef fisheries. Traditionally, for estimating marine biodiversity has some limitations in a particular area, which uses bottom trawls and rotenone poisoning. Then, the lack of local taxonomist may stymie morphological-based species identification, which ubiquitous faces in developing countries. The previous study,

combining between conventional survey and geographical information system (GIS) techniques, have been performed to comprehend fish distribution and spatial analysis.

Presently, a sophisticated method had been developed to monitor biodiversity in aquatic ecosystems through environmental DNA metabarcoding analysis. The extraction and analysis of genetic materials obtained directly from the environment by collecting these living particles as an alternative approach to monitor and analyze marine fish and performed as time and cost-effective survey method can be applied as the alternative method in biodiversity assessment. The accuracy on detection of some endangered species, invasive species, and marine fish distribution was performed by environmental DNA metabarcoding had been performed by numerous researchers. In this research, environmental DNA metabarcoding has been conducted by marine water sample from Java and Bali Island, Indonesia, by the MiFish pipeline.

The environmental DNA analysis in four sampling locations revealed 333 species in 405 representative haplotype (99-100% identity) and 52 putative species (95-98% identity). Alpha biodiversity in Bali, both south and north part, have higher than Java sampling site. The Shannon-Wiener Index and Margalef Index in north Bali are highest. In this study we found the fish species under the order Perciformes are dominat, we identified 295 haplotypes (72.84%) under this order, followed by Clupeiformes 29 haplotypes (7.16%) and Tetaraodontiformes 19 haplotypes (4.69%). The Perciformes most diverse order than others, with 44 families had been identified by eDNA metabarcoding analysis. The coral reefs, seagrass and mangrove ecosystem in both north Bali and south Bali was supported marine biodiversity on the Bali Island. That result revealed that the surrounding Lovina beach in the northern part has higher biodiversity.

In conclusion, tropical marine fish around the Bali strait provides an overview of the

effectiveness and sensitiveness of the method of environmental DNA metabarcoding in collecting primary data about the fish biodiversity of the given area. The next-generation sequencing (NGS) based tropical marine analysis fish will allow us to apply biodiversity information beside improvement of the GenBank database, especially in cryptic species for coral-reef fish species. Therefore, proper monitoring and regular survey should be taken, which may increase the number of marine fish detection along this region. Biodiversity study in this region is very crucial for the policy makers in sustainable marine water resources management.

Chapter 1

General introduction

1.1 Introduction

Indonesia has a significantly large sea water with approximately seventeen-thousand islands scattered in the archipelago region. Being a country of islands, the surrounding marine water has great economic importance of both biological and non-biological values in the maritime sector (Nuryadin et al., 2016). In the biological aspect, Indonesia includes The Indo-Malay-Philippines Archipelago (IMPA) and or Indo-Austalia Archipelago (IAA) has long been considered to be highest marine biodiversity, supporting mega-biodiversity (Carpenter and Springer, 2005; Hutama et al., 2017; Roberts et al., 2002). Distributed in tropical regions which have geographic complexity in nearly 2,000 islands, Indonesia is divided into three parts, that includes Sundaland in the West, Wallacea in the middle and Sahul in the East (Hutama et al., 2017).

The Indo-West Pacific (IWP) is one of the centers of maximum marine biodiversity, where many species in this region overlapped by their distribution (Hoeksema, 2007). The Sundaland has an essential contribution to genetic biodiversity even though high exploitation by human also occurred here. The Economic benefits generated from marine ecosystem with the presence of aquatic biota used in fisheries and numerous products that are also capable for producing other important derivatives (Costanza, 1999; FAO, 2016) those are more beneficial for human being (e.g., for food, medicine etc.) and for the industrial purposes (Kadam and Prabhasankar, 2010; Lordan et al., 2011; Ngo et al., 2011). The health of the marine ecosystem can be monitored by estimating the fish diversity; but unfortunately, the presence of these marine fishes subjected to considerable pressure, and the situation has

decreased due to excessive human exploitation (Collette et al., 2011; Hutchings, 2000; Jackson et al., 2001; Pauly et al., 2002).

The bottom trawls and rotenone poisoning are a common method for estimating marine biodiversity, which is very limited to certain areas (Lang and Baldwin, 1996). Then, direct observation of the specimen facing difficulties due to several reasons such as anatomic and morphometric analysis from the morphological feature (Teletchea, 2009), and also lack of local taxonomist may stymie morphological-based in fish identification (Hopkins and Freckleton, 2002). However, the conventional method of identification is still carried out even though there are difficulties in some fish groups, both commercial and non-commercial fish groups (Thomsen et al., 2012a), incomplete checklist (Love et al., 2010; Rogers and Ellis, 2000) and leaving databases flawed with errors (Daan, 2001). Currently, the molecular approach is applied in ecology including implementing biodiversity assessment in marine and freshwater ecosystems (de Vargas et al., 2002; Huete-Pérez and Quezada, 2013; Thomsen et al., 2012b).

In this research, the molecular approach has been carried out to assess the marine fish biodiversity in Java and Bali, where the biogeographical similarities of these two islands were clustered into the Sundaland region. This research has been divided into three stages. Initial research focuses on the identification and barcoding of several Indonesian marine fish species that are focused on the accuracy of molecular identification in the Internal Transcribed Spacer (ITS) region and were confirmed by the cytochrome c oxidase subunit I (COI) region. Second, to identify tropical marine fish species, which has not been yet registered their complete mitochondrial DNA

sequences in the GenBank database. In this study, we tried to improve the genetic information deposited in an open source system of the National Center for Biotechnology Information (NCBI). The final stage of this research was the application of environmental DNA metabarcoding approach from seawater samples in Indonesian waters. The steps of the study described in the next sub-chapter.

1.2 Barcoding of Commercial Marine Fish

DNA barcoding, a currently accepted method for identification of terrestrial and aquatic animals and plants, has attracted much attention and numerous advantages. The accuracy of DNA-based identification is nearly 100%, which indicates that this method can prove the identification of specimens under different environmental conditions (Meyer and Paulay, 2005). Identification based on DNA barcode has been accepted globally with various advantages; such as a very simple and useful universal tool that includes all the animals both in the form of fresh and processed products samples (Giusti et al., 2017; Pepe et al., 2007). The barcoding system uses sequences that have a diversity in the single region of mitochondrial DNA, cytochrome c subunit I gene (COI), and then deposited to the GenBank database. The GenBank has become central to deposit diverse taxa from all parts of the world. With the increase of the molecular database, scientists have demonstrated their effectiveness in conducting DNA barcoding from freshwater fish to deep-sea fish (Lakra et al., 2011; Ward et al., 2005). For the specific purposes, beside COI gene region, the ITS fragment is located from small to large subunits of ribosomal RNA and has potentially magnified differences between species

due to the fast rate of evolution (Chow et al., 2009). Recently, ITS region has been adopted for the environmental DNA (eDNA) analysis (Minamoto et al., 2017). In this research, we performed the molecular identification of marine fish species from six sampling sites (Jawa and Bali Island) with COI and ITS gene region to improve the information in the GenBank database, especially on the ITS sequences of Indonesian marine fish species.

1.3 Complete Mitochondrial Genome

Besides the COI and *cyt-b* region, 12S ribosomal RNA is used for identification of species (Jordan et al., 2010). However, there are still many limitations and weaknesses of each fragment. Therefore, some researchers are using other regions of mitochondrial DNA sequence, such as ND1 and D-loop (control region) to conduct the studies (Pourkazemi et al., 1999; Verspoor et al., 1999). Several studies have been carried out for mapping of the complete mitochondrial genome to improve the accuracy in molecular studies.

In vertebrates, the mitochondrial genome is maternally inherited, considered as conservative or lacks recombination, and evolves rapidly. Research on several complete mitochondrial genomes have been able to precisely genes mapping on mammalian (Anderson et al., 1981; Anderson et al., 1982), lampreys (Lee and Kocher, 1995), amphibian (Macey et al., 1997), reptiles (Kumazawa and Endo, 2004; Macey et al., 2004), birds (Quinn and Wilson, 1993) and marsupials (Janke et al., 1994). Additionally, it is found that there is no deviation in the seven species of bony fishes, namely *Crossostoma lacustre* (Tzeng et al., 1992), *Cyprinus carpio* (Chang et al., 1994), *Oncorhynchus mykiss* (Zardoya et al., 1995), *Gadus morhua* (Johansen and

Bakke, 1996), *Protopterus dolloi* (Zardoya and Meyer, 1996), *Polypterus ornatipinnis* (Noack et al., 1996), and *Latimeria menadoensis* (Inoue et al., 2005).

Currently, technology in sequencing is becoming sophisticated and advanced, which is marked by many complete mitochondrial genome sequences are deposited in the GenBank database. Up to the year 2011, based on NCBI Reference Sequences (RefSeq) release-49, it was reported that 16,248 organisms or 18,236,994 sequences had been recorded which increased by 49.7% in the number of organisms and 14.5% in the number of sequences recorded (Pruitt et al., 2011).

The complete mitochondrial genomes are the primary data in genetic information in both plants and animals. This genetic information can be consequently used to further study in genome evolution, genetic structure, phylogenetic relationship, phylogeography, and population genetics (Boore et al., 2004; Ingman and Gyllensten, 2006; Macey et al., 2004). The mitochondrial genome supplies various parts of some protein machinery that is necessary for oxidative phosphorylation by utilizing a series of five multiple-subunit enzymes located within the mitochondrial inner membrane. The mitochondrial DNA of most metazoan species is predominantly inherited maternally (Giles et al., 1980). This clonal inheritance, coupled with a substitution rate in vertebrates is typically 5-10 times more than that of nuclear DNA (Brown et al., 1979). It has made mitochondria an essential source of DNA polymorphism information for the study of genetic population among the broad range of species including fish. In this study, we performed tree Lutjanid species (*Lutjanus vitta*, *Lutjanus fulviflamma*, and *Lutjanus*

carponotatus) complete mitochondrial genome from Indonesian water.

1.4 Tropical marine fish biodiversity assessment through environment DNA (eDNA) approach

Marine ecosystems make a considerable contribution to biodiversity value (Nielsen, 2012). Furthermore, the economic benefit is also generated from marine ecosystems with the presence of aquatic biota that is used in fisheries and numerous products that are also capable of producing many derivative products (Costanza, 1999; FAO, 2016). These are very beneficial for humans as well as for food, medicine, and other industries (Kadam and Prabhasankar, 2010; Lordan et al., 2011; Ngo et al., 2011).

The current health of the marine ecosystem is monitored by estimating fish biodiversity (Bremner et al., 2003). But unfortunately, the presence of these marine fishes is subjected to considerable pressure, and the condition has been decreased due to the excessive human exploitation (Collette et al., 2011; Hutchings, 2000; Jackson et al., 2001; Pauly et al., 2002). The methods commonly used for estimating marine biodiversity is by a bottom trawls and rotenone poisoning, which is very limited to certain areas (Lang and Baldwin, 1996). At present, the rapid development of technology has also influenced the development of the molecular field. Molecular identification at the species level is experiencing rapid growth. However, the traditional method of identification is still carried out even though there are difficulties in both non-commercial and commercial fish groups (Thomsen et al., 2012a) due to an incomplete checklist (Love et al., 2010; Rogers and Ellis, 2000) and leaving databases flawed with errors (Daan, 2001).

Nowadays, the extraction and analysis of genetic materials are obtained directly from the environment by collecting these living particles as an alternative approach to monitoring marine fish (Taberlet et al., 2012). This approach is first carried out on terrestrial sediment samples that can reveal mammalian, bird, and plant ecosystems (Willerslev et al., 2003) which are extinct and still exist today. Furthermore, the approach successfully revealed information on various taxa, habitats, and weather conditions (Anderson-Carpenter et al., 2011; Taberlet et al., 2012; Willerslev et al., 2004). In this research, we have collected marine water samples from the Java and Bali Islands to identify the biodiversity of marine species in this area. The environmental DNA approach for fish biodiversity study mainly depends on the amplification of sequences of the target gene by PCR, here we used the universal MiFish primer set targeting the mitochondrial 12S rRNA gene (163-185 bp) because this gene contains sufficient information to identify fishes up to species level (Miya et al., 2015a). To our comprehension, we are conducting the environmental DNA metabarcoding approach for the first time by using the MiFish primer to study the marine fish biodiversity in the Indonesian waters. From this research, we found that the MiFish primer set effectively amplified sequences and in most cases these sequences able to differentiate up to the species level.

Chapter 2

Tropical marine fish DNA barcoding to improve the sequence information of partial ribosomal RNA region and tRNA-Valine in the NCBI database

2.1 Introduction

The DNA barcoding is currently widely accepted methods for identification of terrestrial and aquatic animals, and plants have been attracted much attention and numerous advantages. The accuracy of DNA-base identification is near 100% accurate, that indicating this method can able to prove in the identification of specimens under different environmental conditions (Meyer and Paulay, 2005). Identification based on DNA barcode has been agreed globally with various advantages possessed it ss very simple and uses as a universal tool that includes all the animals both in the fresh samples and or processed products (Giusti et al., 2017; Pepe et al., 2007). This barcoding system uses sequences that have diversity in a single region of mitochondrial DNA cytochrome c subunit I gene (COI) and deposited to the GenBank database as central bioinformatics. Scientists have demonstrated their effectiveness in conducting DNA barcoding in freshwater fish and deep-sea fish (Lakra et al., 2011; Ward et al., 2005). Beside COI gene, the other regions for DNA barcoding are 12S and 16S rRNA (Cawthorn et al., 2012), cytochrome b (Sevilla et al., 2007), NADH-5 (Johnson and O'Brien, 1997) and control region (Mitchell and Hellberg, 2016). The Internal transcribed spacer (ITS) fragment is another candidate for DNA barcoding, which has some advantages on delimitation indicator by genetic distance measurement in fungi (Del-Prado et al., 2010) and potentially magnified differences

between species due to faster evolution (Chow et al., 2009). The ITS region is located from the small subunit (SSU) to the large subunit (LSU) of ribosomal RNA and or in another word, it is partial 12S and 16S rRNA including tRNA-valine, which has been used in this study. Recently, the shorth fragment in SSU to LSU was adopted for the environmental DNA (eDNA) analysis (Yamamoto et al., 2017).

The goal of this research is to improve the DNA barcode database for the commercial fish species inhabiting in the tropical coastal and offshore waters around the Java and Bali Islands, Indonesia, which would be useful data for their further molecular analysis. The study area has a high biodiversity potential but very vulnerable due to the many pressures that exist. The increasing population in Java Island causes changes in the land conversions, that impact on changes in natural biodiversity structures in both land, estuary and marine ecosystems. The marine ecosystem in Java Island influenced by two oceanic ecosystems, Java sea in the north and the Indian Ocean in the south (Sharp, 1996). The uniqueness of the two ecosystems makes Java island fascinating characteristics. Compare to the other nation in the Indian Ocean, Indonesia has about 3,215 fishes species, and it is the highest in the number of species than other countries (Wafar et al., 2011). From this study, total of 169 commercial fish specimens have been obtained and their DNA sequences deposited in the GenBank database. To be applicable for both typical

barcoding method and recently adopted NGS analysis, we amplified and used both the typical COI region and Internal transcribed spacer (ITS) region.

2.2 Materials and Methods

Fish samples have been collected from the six stations of Java Island and one sampling station in the Bali Island (Figure 1-1). In Java Island, sample has been taken from the Banten 6°0′50.00″S 106°10′21.00″E (Banten Province), Pelabuhanratu 6°59'20,92"S 106º32'29,91E (West Jawa Province), Pekalongan 6º51'32,10"S 109º41'09,52"E (Central Jawa Province), Malang 8º26'05,65"S 112º40'55,31"E, Gresik 6º52'56,65"S 112º12'15.87"E and Banyuwangi 8º12'07,52"S 114º23'07,18"E (East Jawa Province), and Denpasar 8º45'23"S 115º10'05,68"E (Bali Province). The three sampling sites (Malang, Pelabuhan Ratu, and Bali) are representative of the south coast of Jawa, and another site is the representative of the northern coast of Jawa (DJPT, 2011). The samples have been collected from the local traditional fish markets which were in dead condition upon purchasing time, and no specific permission was required for this study.

Figure 2.1. Sampling stations in Java and Bali, Indonesia

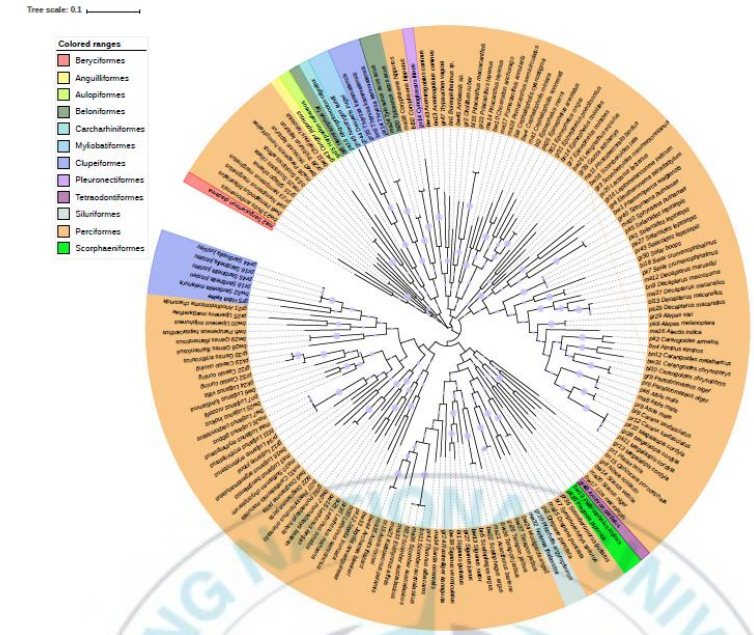
Two sets of universal fish primer targeting the cytochrome c oxidase sub-unit I (COI) region, BCL-BCH (Baldwin et al., 2009; Handy et al., 2011) and ITS primer (Forward F2 5'-CCM YCT AGA GGA GCC TGT YCT RDA A-3'-Reverse R1 5'-CAT GAT GCA AAA GGT AC-3') were used to obtain the partial sequences of each gene, respectively. The ITS gene is targeting around 600 bp sequence. Both the PCR mixture (20µL) contained 11.2 µL ultra-pure water, 1 µL of forward and reversed primer (0.5 µM), 0.2 µL Ex Taq DNA polymerase (TaKaRa, Japan), 2 µL 10X ExTag Buffer, 2 µL dNTPs (1 µM, TaKaRa, Japan), and 2 µL genomic DNA as template. The PCR condition was carried out under the following setting: 95^{o}C for 5 min in initial denaturation, followed by denaturation at 95^{o}C for 30 sec in 40 cycles, 50^{o}C for 30 sec in annealing and 72^{o}C for 45 sec in extension step, and final extension at 72^{o}C

for 5 min. The PCR products were then run in the 1.5% agarose gel electrophoresis and cut the target area from the gel (~600 for both COI and ITS). The gel was purified with the AccuPrep® Gel purification kit (Bioneer, Korea). All sequences were aligned and submitted to the GenBank (Table 2). The pairwise evolutionary distance among the family was determined by Kimura 2-Parameter method. The Maximum Likelihood (ML) tree was constructed, and 1000 bootstrap analysis was carried by Mega 7 (Kumar et al., 2016).

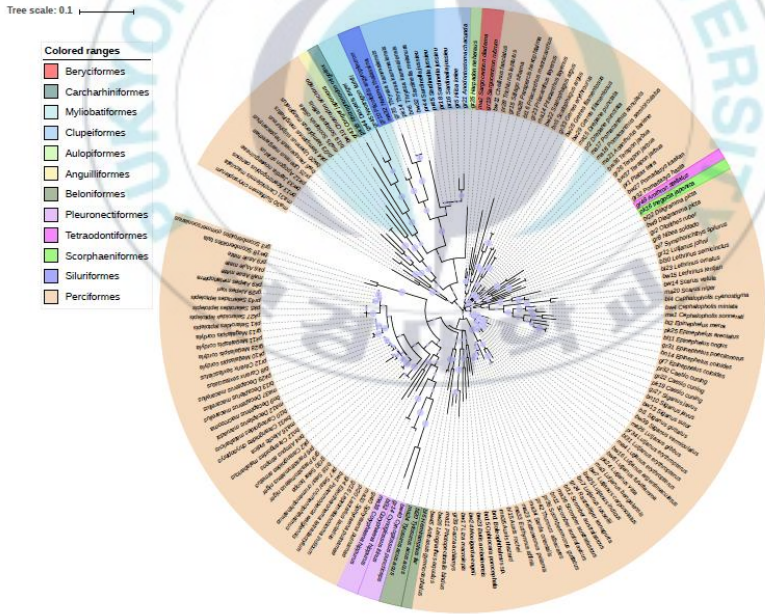
2.3 Results

A total of 169 (COI and ITS) sequences were generated from 169 fish samples representing 136 genera, 50 families, and 12 orders (Table 2.1). The common name, taxonomic assignment, as well as GenBank accession number for all sequences, are shown in Table 2. The new mitochondrial DNA sequences from ITS region were generated for 78 (46.15) of tropical marine commercial fish species from Java and Bali, Indonesia those were registered in the GenBank database. Direct sequencing of the COI gene and ITS gene region produced more than 500bp nucleotide per taxon (607bp for COI and 629bp for ITS region). Un-ambiguity and simplicity were observed among all the sequences, and no stop codon, deletion and insertion were observed in all amplified sequences. The nucleotide frequencies of COI sequences are 29.6%

T(U), 23.9% A, 28.7% C, and 17.8% G than ITS region are 19.4% T(U), 34.7% A, 25% C, and 20.9% G. The average of transitional pairs (si = 62.8) were higher than average of transversion pairs (sv = 37.2), with average ratio is 1.69. The phylogenetic trees were constructed both for COI and ITS sequences of commercial fish species (Figure 2.2), then average K2P distance within taxonomic levels measured only for COI sequences is 0.245.



a) COI



b) ITS

Figure 2.2. The K2P distance Maximum Likelihood tree obtained from the a) COI and b) ITS sequences generated from 169 fish samples. Each color representative of each order. Phylogenetic tree annotation using open source in iTOL (https://itol.embl.de/)

Table 2.1. Species (including Order and Family name) and GenBank accession number of specimens, with gray color shading representing the first entries into the GenBank database for the ITS gene region.

Order	Family	Species	Common Name	Habitat Distribution	GenBank Accession no. for confirmation	GenBank accession no.	
						COI gene	ITS region
Perciformes	Gerreidae	*Gerres erythrourus*	Deep-bodied mojarra	Indo-West Pacific	KF714946	MH085827	MH085661
		Gerres filamentosus	Whipfin silver-biddy	Indo Pacific	KF714948	MH085819	MH085624
		Gerres filamentosus			KF714948	MH085820	MH085625
Perciformes	Serranidae	*Epinephelus merra*	Honeycomb grouper	Indo Pacific	AP005991	MH085799	MH085579
		Epinephelus ongus	White-streaked grouper	Indo-West Pacific	KU668638	MH085802	MH085585
		Epinephelus poecilonotus	Dot-dash grouper	Indo-West Pacific	KU722933	MH085803	MH190799
		Epinephelus coioides	Orange-spotted grouper	Indo-West Pacific	KY849518	MH085801	MH085748
		Epinephelus coioides			KY849518	MH085800	MH085641
		Epinephelus areolatus	Areolate grouper	Indo Pacific	KU668623	MH190817	MH085713
		Cephalopholis miniata	Coral hind	Indo Pacific	KU668646	MH085805	MH085601
		Cephalopholis sonnerati	Tomato hind	Indo Pacific	KU668634	MH085806	MH190804
		Cephalopholis cyanostigma	Bluespotted hind	Western Pacific	KU668647	MH085804	MH085580
Perciformes	Mullidae	*Parupeneus heptacanthus*	Cinnabar goatfish	Indo-West Pacific	KJ202184	MH085847	MH085602
		Upeneus sulphureus	Sulphur goatfish	Indo-West Pacific	KP194654	MH085851	MH085617
		Upeneus margarethae	Margaretha's goatfish	Indian Ocean	KC147801	MH085845	MH085724
Perciformes	Scaridae	*Scarus vetula*	Queen parrotfish	Western Central Atlantic	FJ584083	MH085809	MH085611
		Scarus niger	Dusky parrotfish	Indo Pacific	KP194654	MH085810	MH085681
Perciformes	Haemulidae	*Pomadasys kaakan*	avelin grunter	Indo-West Pacific	HQ676796	MH085852	MH085623
		Pomadasys hasta	Saddle grunt	Indo-West Pacific	KM522836	MH085853	MH190798
		Diagramma picta	Painted sweetlips	Indo-West Pacific	KF009586	MH085904	MH085606
		Diagramma picta			KJ202150	MH085903	MH085594
		Plectorhinchus orientalis	Indian Ocean oriental sweetlips	Indo-West Pacific	HQ676789	MH085905	MH085596
Perciformes	Drepanidae	*Drepane punctata*	Spotted sicklefish	Indo-West Pacific	KM273123	MH085841	MH085677
		Drepane punctata			KM079332	MH085842	MH085717
Perciformes	Mugilidae	*Moolgarda engeli*	Kanda	Indo Pacific	JQ431912	MH085787	MH085620
		Liza macrolepis	Largescale mullet	Indo Pacific	KP128677	MH085900	MH085614
Perciformes	Labridae	*Choerodon anchorago*	Orange-dotted tuskfish	Indo-West Pacific	KF714916	MH085825	MH085607
		Cheilinus fasciatus	Redbreasted wrasse	Indo Pacific	KF809396	MH085789	MH085608
Perciformes	Acanthuridae	*Acanthurus bariene*	Black-spot surgeonfish	Indo-West Pacific	KF009560	MH085850	MH085682
Perciformes	Pomacanthidae	*Pomacanthus annularis*	Bluering angelfish	Indo-West Pacific	FJ583876	MH085785	MH085679
		Pomacanthus semicirculatus	Semicircle angelfish	Indo-West Pacific	FJ583886	MH085786	MH085680
Perciformes	Latidae	*Psammoperca waigiensis*	Waigieu seaperch	Indo-West Pacific	KM079334	MH085775	MH085600
Perciformes	Sphyraenidae	*Sphyraena putnamae*	Sawtooth barracuda	Indo-West Pacific	KC970510	MH085780	MH085664
		Sphyraena putnamae			KC970510	MH085781	MH085673
Perciformes	Polynemidae	*Leptomelanosoma indicum*	Indian threadfin	Indo-West Pacific	MF281369	MH085755	MH085650
		Eleutheronema tetradactylum	Fourfinger threadfin	Indo-West Pacific	JF513842	MH085754	MH085639
Perciformes	Balistidae	*Sufflamen chrysopterum*	Halfmoon triggerfish	Indo-West Pacific	FJ584131	MH085791	MH190805
		Canthidermis maculata	Rough triggerfish	Western Pacific	AP009206	MH085790	MH085689
Perciformes	Terapontidae	*Terapon jarbua*	Jarbua terapon	Indo Pacific	KT231928	MH085844	MH085657
		Terapon jarbua			KP267659	MH190821	MH086630
		Terapon jarbua			KF999839	MH190822	MH087631
Perciformes	Ephippidae	*Platax teira*	Longfin batfish	Indo-West Pacific	KJ668153	MH085838	MH085716
Perciformes	Gobiidae	*Trypauchen vagina*	burrowing goby	Indo Pacific	KJ865406	MH085777	MH085666
		Acentrogobius caninus	Tropical sand goby	Indo-West Pacific	KU692204	MH085762	MH085634
		Acentrogobius caninus			KU692204	MH085763	MH085747

Table 2.1. Continued

Order	Family	Species	Common Name	Habitat Distribution	GenBank Accession no. for confirmation	GenBank accession no.	
						COI	ITS

Perciformes	Gobiidae	*Boleophthalmus boddarti*	Boddart's goggle-eyed goby	Indo-West Pacific	KJ013053	MH085792	MH085737
		Ophiocara porocephala	Northern mud gudgeon	Indo-West Pacific	JN021236	MH085797	MH085749
Perciformes	Sillaginidae	*Sillago sihama*	Silver sillago	Indo-West Pacific	KP112423	MH085788	MH085649
Perciformes	Nemipteridae	*Nemipterus marginatus*	Red filament threadfin bream	Western Pacific	KM522839	MH085795	MH085699
		Nemipterus marginatus			JQ681506	MH085796	MH085723
		Pentapodus bifasciatus	White-shouldered whiptail	Western Central Pacific	KY362916	MH085836	MH085605
Perciformes	Trichiuridae	*Trichiurus lepturus*	Largehead hairtail	Tropical and subtropical	KP112479	MH085794	MH085633
		Trichiurus lepturus			KP112482	MH085793	MH085725
Perciformes	Scombridae	*Scomber australasicus*	Blue mackerel	Indo-West Pacific	KX781882	MH085913	MH085694
		Scomber australasicus			AB102725	MH085914	MH085586
		Scomber australasicus			AB102725	MH085915	MH085745
		Rastrelliger kanagurta	Indian mackerel	Indo-West Pacific	AP012948	MH085911	MH085655
		Sarda orientalis	Striped bonito	Indo Pacific	KX768133	MH085916	MH085692
		Thunnus albacares	Yellowfin tuna	Worldwide	KY984984	MH085917	MH085731
		Euthynnus affinis	Kawakawa	Indo-West Pacific	KX768124	MH085918	MH085691
		Katsuwonus pelamis	Skipjack tuna	Worldwide	KF597042	MH085920	MH085683
		Auxis rochei	Bullet tuna	Atlantic, Indian and Pacific (Western)	KT003827	MH085919	MH085588
		Auxis thazard	Frigate tuna	Atlantic, Indian and Pacific (Western central)	KM055419	MH190813	MH190806
		Scomberomorus guttatus	Indo-Pacific king mackerel	Indo-West Pacific	EU871700	MH190912	MH085729
Perciformes	Lethrinidae	*Lethrinus ornatus*	Ornate emperor	Indo-West Pacific	KM079313	MH085817	MH190797
		Lethrinus lentjan	Pink ear emperor	Indo-West Pacific	KF714957	MH085818	MH085612
		Lethrinus mahsena	Sky emperor	Indian Ocean	JF952782	MH085816	MH085728
		Lethrinus semicinctus	Black blotch emperor	Indo-West Pacific	KU944049	MH085815	MH085597
Perciformes	Siganidae	*Siganus sutor*	Shoemaker spinefoot	Indian Ocean, Indonesia	KT997958	MH085907	MH085610
		Siganus guttatus	Orange-spotted spinefoot	Eastern Indian Ocean and Western Pacific	JN021251	MH085909	MH085578
		Siganus vermiculatus	Vermiculated spinefoot	Indo-West Pacific	KF715018	MH085910	MH085632
		Siganus javus	Streaked spinefoot	Indo Pacific	KT997929	MH085908	MH085658
		Siganus javus			KT997929	MH190823	MH085744
Perciformes	Apogonidae	*Archamia bleekeri*	Gon's cardinalfish	Indo-West Pacific	AB890029	MH085835	MH085635
		Jaydia novaeguinea		Indo Pacific	KX281185	MH085807	MH085628
		Jaydia truncata	Flagfin cardinalfish	Indo Pacific	KY371151	MH085808	MH085707
Perciformes	Priacanthidae	*Priacanthus tayenus*	Purple-spotted bigeye	Indo-West Pacific	KT985639	MH085758	MH085654
		Priacanthus tayenus			KT985639	MH085759	MH085676
		Priacanthus macracanthus	Red bigeye	Western Pacific	JQ691316	MH085757	MH085589
Perciformes	Sciaenidae	*Otolithes ruber*	Tigertooth croaker	Indo-West Pacific	KX778043	MH085760	MH085637
		Nibea soldado	Soldier croaker	Indo-West Pacific	KP722746	MH085761	MH085642
Perciformes	Scatophagidae	*Scatophagus argus*	Spotted scat	Indo Pacific	KU234319	MH085813	MH085618
		Scatophagus argus			KU234319	MH085814	MH085739
Perciformes	Ambassidae	*Ambassis sp.*	Glassy fish	Indo-Pacific	KX144849	MH085822	MH085636
Perciformes	Caesionidae	*Caesio cuning*	Redbelly yellowtail fusilier	Indo-West Pacific	KX866814	MH085863	MH085726
		Caesio cuning			KP194254	MH085864	MH085653
		Caesio cuning			KF809392	MH085865	MH085710
Perciformes	Lutjanidae	*Lutjanus erythropterus*	Crimson snapper	Indo-West Pacific	KP939271	MH085857	MH085727
		Lutjanus erythropterus			KP939271	MH085858	MH085598
		Lutjanus erythropterus			KP939271	MH085859	MH085669
		Lutjanus gibbus	Humpback red snapper	Indo Pacific	MF409615	MH190812	MH085686

Table 2.1. Continued

Order	Family	Species	Common Name	Habitat Distribution	GenBank Accession no. for confirmation	GenBank accession no.	
						COI	ITS
Perciformes	Lutjanidae	*Lutjanus argentimaculatus*	Mangrove red snapper	Indo-West Pacific	JN182927	MH085861	MH085613
		Lutjanus johnii	John's snapper	Indo-West Pacific	KJ013052	MH085860	MH085646
		Lutjanus bengalensis	Bengal snapper	Indo-West Pacific	FJ171339	MH085862	MH085668
		Lutjanus carponotatus	Spanish flag snapper	Indo-West Pacific	KP194641	MH085868	MH085604

		Lutjanus indicus		Indian Ocean	KF830880	MH085869	MH085621
		Lutjanus fulviflamma	Dory snapper	Indo Pacific	MG002617	MH085867	MH085603
		Lutjanus vitta	Brownstripe red snapper	Indo-West Pacific	EU600101	MH085866	MH085712
		Lutjanus russellii	Russell's snapper	Western Pacific	KJ202173	MH085870	MH085741
		Lutjanus notatus	Bluestriped snapper	Western Indian Ocean	JF483844	MH190812	MH085688
		Scolopsis ciliata	Saw-jawed monocle bream	Indo-West Pacific	KY362946	MH085856	MH085685
		Scolopsis affinis	Peters' monocle bream	Western Pacific	KY362936	MH085837	MH085592
		Symphorichthys spilurus	Sailfin snapper	Western Pacific	FJ584135	MH085855	MH085582
Perciformes	Carangidae	*Scomberoides tala*	Barred queenfish	Indo-West Pacific	JX261091	MH085839	MH085615
		Scomberoides commersonnianus	Talang queenfish	Indo-West Pacific	JX261017	MH085840	MH085638
		Selaroides leptolepis	Yellowstripe scad	Indo-West Pacific	KM522839	MH085874	MH085700
		Selaroides leptolepis			KM522839	MH085875	MH190807
		Selaroides leptolepis			KM522839	MH085876	MH085714
		Selaroides leptolepis			KM522839	MH085877	MH085732
		Atule mate	Yellowtail scad	Indo Pacific	KU170601	MH085895	MH085672
		Atule mate			KU170601	MH190815	MH085701
		Atule mate			KU170601	MH085896	MH085719
		Selar crumenophthalmus	Bigeye scad	Indo Pacific, East Africa	KY984985	MH085872	MH085591
		Selar crumenophthalmus			KJ984985	MH085873	MH085702
		Selar boops	Oxeye scad	Pacific Ocean	KU535571	MH085871	MH085660
		Decapterus macarellus	Mackerel scad	Western Atlantic, Global	KY371379	MH085882	MH085695
		Decapterus macarellus			KM986880	MH085883	MH085587
		Decapterus macarellus			KM986880	MH085884	MH085715
		Decapterus maruadsi	Japanese scad	Indo-West Pacific	KX610924	MH085880	MH085675
		Decapterus macrosoma	Shortfin scad	Indo Pacific, Southeast Atlantic	KF841444	MH085881	MH085743
		Alepes vari	Herring scad	Indo-West Pacific	KF714896	MH085897	MH085659
		Alepes melanoptera	Blackfin scad	Indo Pacific	HQ560986	MH085898	MH085704
		Alectis indicus	Indian threadfish	Indo Pacific	NC037050	MH085892	MH085678
		Parastromateus niger	Black pomfret	Indo-West Pacific	KJ192332	MH085885	MH085643
		Parastromateus niger			JX261055	MH190818	MH085718
		Carangoides malabaricus	Malabar trevally	Indo-West Pacific	KJ174514	MH085879	MH085746
		Carangoides malabaricus			FJ237668	MH085899	MH085584
		Carangoides armatus	Longfin trevally	Indo-West Pacific	AP004444	MH085893	MH085698
		Carangoides chrysophrys	Longnose trevally	Indo Pacific	HQ560957	MH085878	MH085626
		Caranx sexfasciatus	Bigeye trevally	Indo Pacific	KT805946	MH085890	MH085583
		Caranx sexfasciatus			KJ202140	MH085891	MH190809
		Megalaspis cordyla	Torpedo scad	Indo-West Pacific	KM522836	MH085886	MH190798
		Megalaspis cordyla			KM522836	MH085887	MH085705
		Megalaspis cordyla			KM522836	MH085888	MH085706
		Megalaspis cordyla			KM522836	MH085889	MH085647
		Atropus atropos	Cleftbelly trevally	Indo-West Pacific	HQ560973	MH085894	MH085738

Table 2.1. Continued

Order	Family	Species	Common Name	Habitat Distribution	GenBank Accession no. for confirmation	GenBank accession no.	
						COI	ITS
Perciformes	Coryphaenidae	*Coryphaena hippurus*	Common dolphinfish	Atlantic, Indian and Pacific	KF814117	MH085770	MH085599
		Coryphaena hippurus			AP009206	MH085771	MH085696
	Pinguipedidae	*Parapercis hexophtalma*	Speckled sandperch	Indo Pacific	MF123971	MH085798	MH085687
	Leiognathidae	*Gazza achlamys*	Smalltoothed ponyfish	Indo-West Pacific	HQ993142	MH085772	MH085663
		Photopectoralis bindus	Orangefin ponyfish	Indo-West Pacific	KY849543	MH085768	MH085674
		Leiognathus equulus	Common ponyfish	Indo-West Pacific	KF714954	MH085773	MH085622
	Eleotridae	*Butis amboinensis*	Olive flathead-gudgeon	Eastern Indian Ocean	KU692386	MH085812	MH085619
	Lactariidae	*Lactarius lactarius*	False trevally	Indo-West Pacific	KU535572	MH085843	MH085722

Clupeiforme r	Clupeidae	*Hilsa kelee*	Kelee shad	Indo-West Pacific	KX786662	MH085830	MH085640
		Sardinella jussieu	Mauritian sardinella	Western Indian Ocean, Vietnam	KY849547	MH085833	MH085833
		Sardinella jussieu			KY849547	MH085832	MH085721
		Sardinella jussieu			KY849547	MH085834	MH085733
		Sardinella jussieu			KY849547	MH085831	MH085734
		Sardinella melanura	Blacktip sardinella	Indo-West Pacific	KX223945	MH085828	MH085627
		Anodontostoma chacunda	Chacunda gizzard shad	Indo-West Pacific	KC466691	MH085829	MH085652
	Eugraulidae	*Thryssa kammalensis*	Kammal thryssa	Indo-West Pacific	KX223961	MH085765	MH085662
		Thryssa kammalensis			KY849558	MH085766	MH190808
		Thryssa kammalensis			KY849558	MH085767	MH085740
Scorphaenif ormes	Platycephalidae	*Platycephalus indicus*	Bartail flathead	Indo-West Pacific	KY463442	MH085821	MH085616
		Inegocia japonica	Japanese flathead	Indo-West Pacific	JX488247	MH085779	MH085708
Pleuronectif ormes	Cynoglossidae	*Cynoglossus itinus*	Speckled tongue sole	Northwest Pacific	KP112240	MH085750	MH085648
Beryciforme s	Holocentridae	*Sargocentron diadema*	Crown squirrelfish	Indo Pacific	JF494418	MH085901	MH085645
		Sargocentron rubrum	Redcoat	Indo-West Pacific	EU600149	MH190810	MH085651
Siluriformes	Ariidae	*Plicofollis argyropleuron*	Longsnouted catfish	Indo-West Pacific	KY849545	MH085823	MH085644
		Netuma thalassina	Giant catfish	Indo-West Pacific	KC569771	MH085824	MH085690
Beloniforme s	Belonidae	*Tylosurus acus*	Agujon needlefish	Western Atlantic	KC970513	MH085782	MH085593
		Tylosurus acus			KC970513	MH085783	MH085684
	Hemiramphidae	*Hemiramphus far*	Black-barred halfbeak	Indo-West Pacific	KF714951	MH085848	MH085581
Anguilliformes	Congridae	*Conger japonicus*	Beach conger	Northwest Pacific	EF607455	MH085764	MH190801
Tetraodontif ormes	Tetraodontidae	*Arothron stellatus*	Stellate puffer	Indo Pacific	KC409389	MH085811	MH190803
Aulopiform es	Synodontidae	*Harpadon nehereus*	Bombay-duck	Indo-West Pacific	JX534239	MH085769	MH085656
Myliobatifo rmes	Dasyatidae	*Dasyatis zugei*	Pale-edged stingray	Indo-West Pacific	KM073022	MH085752	MH190802
		Neotrygon kuhlii	Blue-spotted stingray	Southwest Pacific	KU498012	MH085753	MH085665
Carcharhinif ormes	Carcharhinidae	*Rhizoprionodon oligolinx*	Grey sharpnose shark	Indo-West Pacific	KM973188	MH085756	MH085709

2.4 Discussion

The molecular approach dependent initial fishery assessment is the DNA barcoding for the commercial marine fish species in Java and Bali water, and it should be developed by the regular evaluation of each region and provinces of Indonesia. This research exceedingly useful and improve current biodiversity information regarding the DNA barcoding and species richness exploited by the Indonesian fishery. This study discussed the results and implications for the biodiversity of commercial marine fish species and then offered the recommendations for further research in the surrounding marine ecosystem.

The DNA barcoding is a part of the effort to gather genetic information to expand the database of Indonesian marine fish. This database can be subsequently used for further study in genetic structure, phylogenetic relationship and phylogeography (Boore et al., 2004; Macey et al., 2004). This report is essential not only for better understanding of genomics and phylogenetics of marine fish species, but also for the practice of molecular ecology and biodiversity management strategies of other fisheries resources. The previous study about the DNA barcoding in Indonesia was successfully documented that 1,172 native freshwater fish species belonging to 79 families and among 1,172 fish species, 630 were endemic species (Hubert et al. 2015). Another researcher reported the DNA barcoding of Indonesian fish only from a particular region, e.g., freshwater fish from Jawa and Bali (Dahruddin et al., 2017), freshwater fish of Lake Laut Aceh (Ariyanti, 2012; Muchlisin et al., 2013), Pogar River Sulawesi Island (Arai et al., 1999), and Lake Matano Sulawesi (Roy et al., 2004).

Several researchers also concern on the certain species including Shark (Prehadi et al., 2015; Sembiring et al., 2015), Orange-Spotted grouper (Antoro et al., 2006), Grouper (Jefri et al., 2015), Seahorse (Lourie and Vincent, 2004), Goby fish species (Winterbottom et al., 2014) and some of coral reef fish species. Beside the COI gene marker for DNA barcode, in this study, we also used the ITS region and we obtained better amplification rate

than the COI region. It should also be noted that this research is the first time to result in the ITS region of the commercial marine fish species from Indonesia. Unfortunately, this ITS segment has limited information in the GenBank database. Interestingly, the ITS sequence will be found in the GenBank, those species have the complete mitochondrial DNA sequences, and current study already deposited in the GenBank database of 77 ITS sequences. This result reinforces another study states that ITS database coverage for the marine animal is less than adequate or incomplete than it is for fungi (Croce et al., 2006).

In this study, the ITS primer produced sequences between the 12S rRNA and 16SrRNA gene, including tRNA-Valine. The length of this target sequence is around 600-700bp. Beside for DNA barcoding marker for fungal communities, the ITS region commonly used for characterizing the diversity and composition of genomic information, and this study was able to identify the specimens until species level for marine fishes. This region is a pre-cursor for DNA transcription from 5′ to 3′ (Das and Deb, 2015). The ITS region also sufficient for easy amplification when only small quantities of DNA are obtained, and it also has a very high variation between closely related species and also commonly used in taxonomy and phylogeny analysis; although currently, COI is a region that has been agreed internationally (Lebonah et al.,

2014).

The ITS region used for identification of cryptic species *Tripterygion ripteronotus* from the Mediterranean Sea by comparing of COI region and ITS region of genetic divergence and ITS region have lower genetic divergence than COI region (Carreras-Carbonell et al., 2007). Here, the phylogenetic trees can performed species in the same family mostly clustered in same family clade, only a small portion of species not grouped with similar family members. The Clupeiformes are gathered in one clade based on ITS sequences while using COI sequences produce different results. Species *Thryssa kammalensis* (family Engraulidae) are unrelated from the *Sardinella melanura* and *Hilsa kelee* (family Clupeidae) (Figure 2). These results show that ITS sequence able to distinguish between family better than COI sequence. Another research used the 12S rRNA, tRNA Valine, and 16S rRNA for phylogenetic study of Batoidea (rays and skates) which include in elasmobranchs fish species (Douady et al., 2003). This study also proves that elasmobranch groups (shark and ray) can be distinguished both by COI and ITS sequences. In this study, Shark represented by species of *Rhizoprionodon oligolinx* and Rays represented by *Dasyatis zugei* and *Neotrygon kuhlii*. Heretofore, minimal molecular studies addressing of ITS region for identification or barcoding has been published, as a result, is finite in the GenBank database for the ITS region.

The ITS region contains some sequences which will be a capable candidate of universal primer for detection of tropical marine fish species by short fragment primer. At least two regions from ITS located in 12S rRNA and tRNA valine (Figure 3). Some environment DNA researchers used the specific primer set for identifying certain species (Wilcox et al., 2013), universal primer set which able to detecting several taxa or specific taxa (Miya et al. 2015), and combination primer set (specific and universal) (Thomsen et al., 2012a; Thomsen et al., 2012b)

In this study, we found that several groups of fish are economic importance, and distributed in several families e.g., Carangidae (19 species), Lutjanidae (13 species), Scombridae (9 species), Serranidae (8 species), Clupeidae (4 species), Lethrinidae (4 species), and Singanidae (4 species). In the Scombridae family, tuna is the most dominant exported commodity which distributed to Europe, USA, and Japan, such as skipjack tuna (*Katsuwonus pelamis*), yellow-fin tuna (*Thunnus albacares*), and big-eye tuna (*Thunnus obesus*) (Comitini and Hardjolukito, 1986). The pelagic fish group is thought to have no genetic differentiation due to the extensive geographical distribution, large populations, and far-reaching potential (NesbÖ et al., 2000). Genetic structure of pelagic fish cannot found by several studies (Kumar et al., 2012; Santos et al., 2010). However, other studies in the Indonesian Archipelago showed that although some fish classified as pelagic fish

(including *Katsuwonus pelamis* and *Rastrelinger kanagurta*) showed variations in the regional genetic differentiation (Jackson et al., 2014).

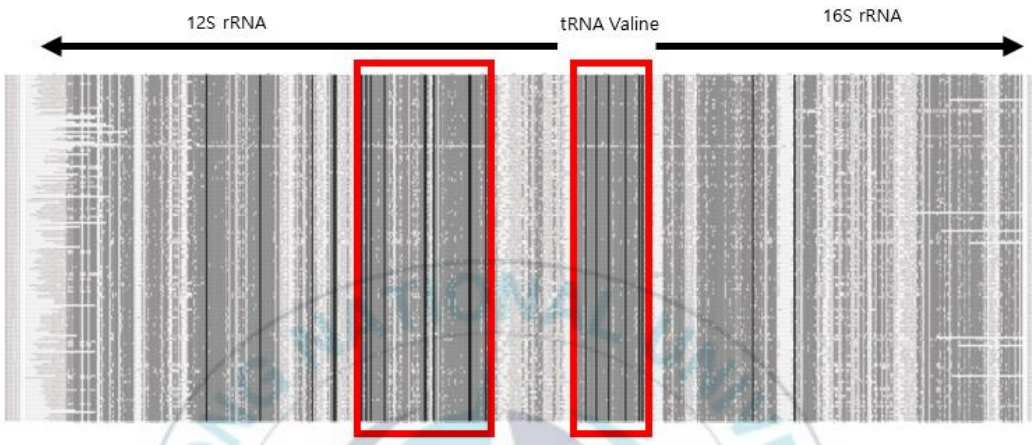


Figure 2.3 Alignment on 169 ITS sequences of tropical marine fish from Indonesia.

Another economical important fish group is Carangidae, which has a size distribution ranging from TL 250 cm to the small size (TL = 16 cm) with diverse body shape from deep and strongly compressed to elongated and fusiform (Randall, 1995). Carangids are an essential source of food for people in Southeast Asia (Mohsin and Ambak, 1996). The groups of marine fishes on average have intraspecific values of K2P genetic distance ranging from 0.24-0.39% (Zhang and Hanner, 2011) which has a lower range when compared with the K2P genetic distance in freshwater fish of 0.3-0.45% (Hubert et al., 2008). This result supported our findings in commercial marine

fish species K2P genetic distance is 0.245. The segment of COI, the Siluriformes fish (Ariidae) had the highest nucleotide composition at 28.8% C followed by 27.5% T(U), 25.2% A and 18.6% G respectively, in an average they have 52.7 % for AT composition (Kartavtsev et al., 2007). In other families mentioned, the A+T percentage ranged between 53-57% and higher than the G+C formation (Cui et al., 2009). Then in the ITS region, nucleotide composition is different from the COI region. In vertebrate, especially for Osteichthyes, the ITS region has an average of 68.0% G+C content, which is higher than the A+T content (Chow et al., 2009).

The Elasmobranchii is one of the major significant from cartilaginous fishes beside another group Holocephalii (Nelson, 1994). It includes rays, shark, and skates, and this is one of the ancient living group, jawed vertebrate diverged from the ancestor of bony vertebrates. In the phylogenetic tree results, Charchanidae (*Rhizoprionodon oligolinx*) and Dasyatidae (*Dasyatis zugei* and *Neotrygon kuhlii*) also diverged in a separate clade from the Actinopterygii. The phylogenetic tree of Elasmobranchii poorly understood, and some of them have commercial and conservation importance. Elasmobranchii has a high compositional nucleotide of 33.6% A, followed by 28.5% C, 20.16% T and 17.6% G on the 12S ribosomal RNA gene, whereas in the COI gene the highest value is 31.8% T followed by 26.01% A, 25.7% C, and 16.3% G, respectively (Pavan-Kumar et al., 2014).

2.5 Conclusion

In conclusion, this study generates the number of COI and ITS sequences and deposited to the GenBank database. We have stored 78 ITS sequences of Indonesian tropical commercial marine fish species, which enriches the database of GenBank of the 12S ribosomal RNA-tRNA Valine-16S ribosomal RNA partial region of the mitochondrial genome. This information will be useful for the molecular identification of fish and the ITS region as well. Further research is essential for completing full mitochondrial genome in the GenBank database for improvement of accuracy in identification based on the different gene markers.

Chapter 3.

Complete mitochondrial genome and characterization of gene arrangement of three snappers to improve the GenBank database for environmental DNA metabarcoding study

3.1 Introduction

The mitochondrial genome is an essential component in almost all eukaryotes for their life. In vertebrates, mitogenomes are around 16-17 kbp in size, compact and the encoded genes are incredibly conserved. The mitochondrial genome in vertebrate typically encoded the canonical 13 protein-coding genes, 22 transfer RNA (tRNAs) genes, two ribosomal RNAs with two non-coding regions, the origin of light strand replication (O_L) and the control region (D-loop). The arrangement of genes order in the mitochondrial genome also conserved and arranged in the same array in the vertebrates for 37 genes and two non-coding regions from hagfish to eutherian mammals (Anderson et al., 1981; Chang et al., 1994; Roe et al., 1985; Tzeng et al., 1992). Nowadays, the mitochondrial DNA sequence is widely using as a useful tool in population genetics and phylogenetic study analysis in vertebrates (Satoh et al., 2016), which will be helpful to enhance the natural stock assessment, its conservation, breeding, culture and production as well as for the proper development strategy of any species.

Therefore, the increasing research in the molecular field shows a sign in improving databases in the GenBank and other open access resources, especially in the genetic information of mitochondrial DNA sequences. The mitogenome information is one of the essential sources of biological data in metazoans (Gissi et al., 2008). The number of databases that are currently

available in GenBank is almost 260,000 (Benson et al., 2012) formally including whole mitochondrial genome. The complete mitochondrial genome was first applied for phylogenetic study for fish at the end of the 20th century (Miya and Nishida, 1999), after that the number of complete mitogenomes deposit in GenBank is increasing sharply, it is also possible for all regions of mitogenome to be used for the identification of animals, such as *Cytochrome b*, 12S ribosomal RNA, and 16S ribosomal RNA. Currently, the most common identification method by using the partial COI region of mitochondrial DNA for official molecular identification has become the standard in animal barcodes, which has been stored in the Barcode of Life project (Ratnasingham and Hebert, 2007).

The use of other regions of mitogenome allows the identification of fast and accurate methods that are very efficient, namely the environmental DNA (eDNA) approach. The platform that has been launched to analyze high-throughput sequencing data is MitoFish pipeline (mitofish.aori.u-tokyo.ac.jp/mifish). Regarding the pipeline, the primary region used here is the part of the 12S ribosomal gene of mitochondrial DNA. However, MitoFish monthly updates on the complete mitogenome and partial genome databases by incorporating RefSeq which have database centers at NCBI, as well as many other database centers such as FishBase (http://www.fishbase.org/), Integrated Taxonomic Information Systems

(https://www.itis.gov/), and the Catalog of Fishes (Fricke et al., 2018). At present, there are more than 1,000 sequences of fish genomes and they have 17,000 fish species database which was confirmed (Iwasaki et al., 2013), even though still more than half of valid fish species database around the world (Froese and Pauly, 2014; Nelson et al., 2016).

Regarding the importance of the whole genome study, we have conducted several studies on the complete mitogenomes of tropical marine fish from Indonesia. Here, we reported the complete mitogenome of *Lutjanus vitta* (GenBank No. MH675887), *Lutjanus fulviflamma* (GenBank No. MH995530), and *Lutjanus carponotatus* (GenBank No. MK092066) to improve the *Lutjanus* mitogenome database. Fishes in the genus *Lutjanus* are usually found in the tropical and subtropical reefs or mangrove forests in the Atlantic, Indian and Pacific Oceans, including the Indo-west Pacific, and the northern Australia (Allen, 1985; Newman et al., 2000), which are primarily inhabitants of shallow coral reef ecosystems (Allen, 1985). Few studies have been conducted on *Lutjanus* fishes about their life cycle, morphology, and molecular characteristics (Chen, 1997; Collins et al., 2001; Li and Chu-Wu, 2007). Among the 73 currently known species in the genus *Lutjanus*, the brown stripe red snapper (*L. vitta*), the dory snapper (*L. fulviflamma*), and the Spanish flag snapper (*L. carponotatus)* are economically important for artisanal and recreational fisheries. These marine fish species are widely

distributed in the western Pacific and Indian Ocean (Iwatsuki et al., 1993; Salini et al., 2006; Williams and Russ, 1997). The *L. vitta* uses the coral reef as their spawning and feeding ground (Freitas et al., 2011), so its abundance and population structure also affected by the changes of the coral reef ecosystem. As shown in its relatives, *L. fulviflamma* shows a seasonal migrating pattern in and out of the coral reefs for its reproduction (Grol et al., 2008; Nagelkerken, 2009). To the understanding the *L. fulviflamma* genetic population structure from a variety of its relatives sharing a habitat, molecular identification would be a useful and accurate tool than the traditional identification methods. The Spanish flag snapper, *L. carponotatus* have potential importance to the commercial fishery (Davis, 1992; Kaunda-Arara and Ntiba, 1997) as valuable sources of food fish, and her sister species from the genus *Lutjanus* are cultured in the Southeast Asia region (Zhang et al., 2004). This full mitochondrial genome of three *Lutjanus* species will provide valuable biological information for the genetic diversity study, establishing relevant protective and developmental measures for the coral reef habitat to ensure the stock protection, modern conservation, reasonable exploitation for sustainable of the resources.

3.2 Materials and Methods

3.2.1 Sample collection and DNA Extraction

The complete mitochondrial genome sequence of *Lutjanus* spp. was determined by the next-generation sequencing (NGS) platform. The *L. vitta* was collected from the coastal water in Pekalongan, Central Java, Indonesia (6º51'45" S 109º4'24" E). The *L. fulviflamma* and *L. carponotatus* were collected from the coastal water in Muncar, Banyuwangi, East Java, Indonesia (8º12'07,52"S 114º23'07,18"E). All specimens are deposited at the Universitas Airlangga, Indonesia. The identification of the sample was made by both the morphological characteristics and the sequence identity in partial COI region to the database (GenBank Accession number for *L. vitta* EU600101; *L. fulviflamma* MG002617; *L. carponotatus* KP194641). The mitochondrial DNA was extracted by the mitochondrial DNA isolation kit ab65321 (Abcam, Cambridge, UK) according to the manufacturer's protocol.

3.2.2 Polymerase Chain Reaction and sequencing of PCR products

The purified mitochondrial DNA was further fragmented into smaller sizes (~350 bp) by Covaris M220 Focused-Ultrasonicator (Covaris Inc., USA). A library for sequencing was constructed by TruSeq® RNA library preparation kit V2 (Illumina, USA) and its quality and quantity was analyzed by 2100 Bioanalyzer (Agilent Technologies, USA). The TruSeq® library performed

end repair, adenylate 3’ ends, adaptor ligation, and last step is PCR enrichment. The end repair conducted at 20ºC for 30 minutes which contained 5.5 µL ultrapure water, 5 µL 10 x T4 DNA ligase buffer (with 10 mM ATP), 2 µL dNTP mix (10 mM), 2.5 µL T4 DNA polymerase (3U/µL), 0.5 µL Klenow DNA polymerase (5 U/ µL), 2.5 µL T4 PNK (10 U/µL), and 47 µL mitochondrial DNA as template. Then, the second step (Adenylate 3’ ends) performed at 37ºC for 30 minutes which contained 16 µL Eluted DNA, 2.5 µL Klenow buffer (10x NED buffer 2), 5 µL dATP (1 mM), 1.5 µL Klenow 3’ to 5’ exo-(5 U/ µL). After completing both processes, DNA purification kit (RBC Cat. YDF300) was used for purification following the manufacturer’s protocol.

The adaptor ligation was performed at room temperature for one hour contained 10.75µL eluted DNA, 1.5µL 10 x T4 DNA ligase buffer, 1.25µL adaptor oligo mix (Illumina index) and T4 DNA ligase (400 U/µL). Then, the last part for TruSeq® library was PCR enrichment which contained 30µL purified index ligation DNA, 10µL 5X clone Phusion buffer (NEB, #F-530), 1 µL of forward and reverse PCR primer (10 pmol), 1.5µL dNTP (10 mM), 0.5 µL Phusion polymerase (NEB, #F-530), and 6µL ultrapure water. The PCR was conducted under the following condition: the initial denaturation step at 98°C for 30 seconds, followed by 15 cycles of denaturation at 98°C for 15 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec.

The final extension at 72°C for 5 minutes was applied for the final steps. The forward primer 1.1 is 5' AAT GAT ACG GCG ACC ACC GAG AT 3', and the reverse primer 1.1 is 5' CAA GCA GAA GAC GGC ATA CGA 3', and all primer was HPLC purified. Then, all DNA result from library preparation was performed for sequencing by Illumina MiSeq sequencer (2 x 300 bp pair ends).

3.2.3 NGS data assembly and complete mitochondrial genome analysis

The raw NGS data was assembled by Geneious ver 11.0.2. The linear gene map of the complete mitochondrial genome was drawn by using OrganellarGenomeDRAW version 1.3.1 (Greiner et al., 2019). The mitogenome structure was determined by sequence comparison with the known full mitochondrial genome of closely related species, including *L. russellii* (Guo et al., 2008). All the tRNAs were predicted by ARWEN (Laslett and Canbäck, 2008) and tRNAscan-SE (Lowe and Eddy, 1997). Finally, the complete mitochondrial DNA sequences were deposited into the GenBank database using Sequin v 15.50 (Benson et al., 2012).

3.2.4 Phylogenetic tree analysis

The phylogenetic tree of *L. vitta, L fulviflamma*, and *L. carponotatus* complete genome were constructed by MEGA7 software with Minimum Evolution (ME) algorithm with 1000 bootstrap replications (Kumar et al.,

2016). The other Lutjanidae species were downloaded from GenBank database, including *Lutjanus russellii* (NC010963), *Lutjanus bengalensis* (NC011275), *Lutjanus kasmira* (NC011578), *Lutjanus rivulatus* (NC009869), *Lutjanus argentimaculatus* (NC016661), *Lutjanus peru* (NC027950), *Lutjanus guttatus* (NC029353), *Lutjanus erythropterus* (NC031331), *Lutjanus sebae* (NC012736), *Lutjanus malabaricus* (NC012736). Furthermore, one species from the different family, *Thunnus albacares* (NC014061), was used as an outgroup taxon.

3.3 Results

3.3.1 Gene organization in the mitochondrial DNA

The *L.vitta* (MH675887), *L. fulviflamma* (MH995530), and *L.carponotatus* (MK0920066) mitochondrial genomes were registered and stored in the GenBank database. This mitogenome was reported for the first time and their sequences and improved the complete genome database at the NCBI. The overall base composition of three snappers has represented in Table 3.1. The comparison of purine and pyrimidine, the C content of three snappers are relatively highest, and G content is the lowest (Miya et al., 2003). This pattern was almost the similar to the Sinipercidae, which has G composition around 16% (Chen et al., 2012). This study found that G contents are 16.4% (*L. vitta*), 16.2% (*L. fulviflamma*), and 16.2% (*L. carponotatus*).

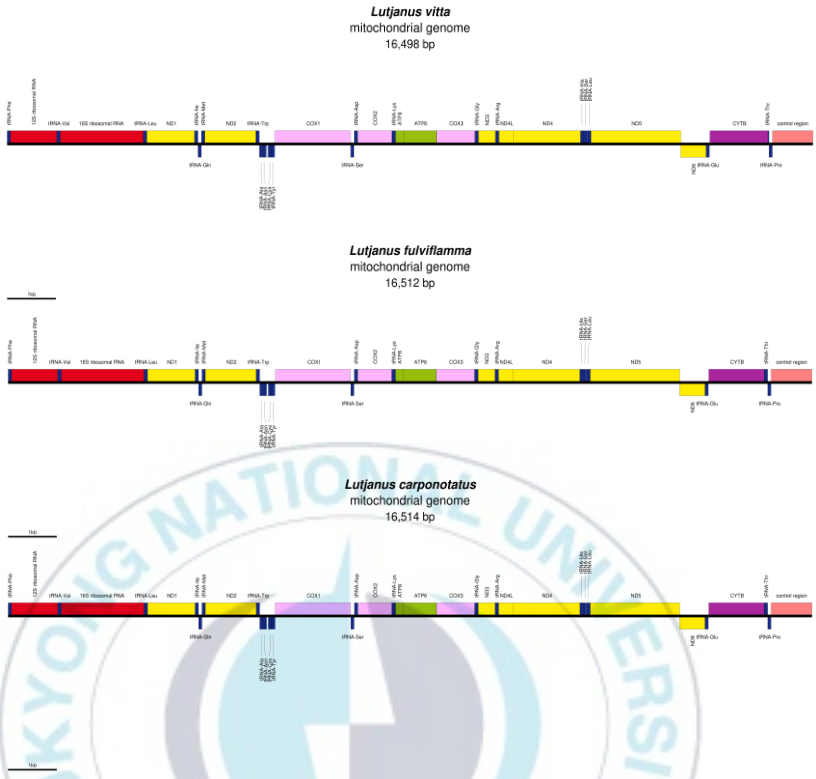


Figure 3.1. Gene map of three Snapper A) *L. vitta*, B) *L. fulviflamma*, and C) *L. carponotatus*

3.3.2 The protein-coding genes

Total 13 protein-coding genes were identified, in which 12 (COX1, COX2, COX3, ATP6, ATP8, ND1, ND2, ND3, ND4L, ND4, ND5, and *Cyt-b*) were encoded on the H-strand, and only ND6 was encoded on the L-strand. At the COX1 gene, the start codon initiated by GTG, but only in the *L. fulviflamma* was initiated by ATC. All other protein-coding genes began with the typical ATG start codons, but in *L. fulviflamma* the ND2 was initiated by the ATA.

The incomplete stop codons were identified in ND2, COX2, COX3, ND3, ND4, *Cyt-b* genes in all three *Lutjanus* spp., besides these six genes in the APT6 gene was identified the incomplete stop codon in *L. fulviflamma* and *L. carponotatus*. Among the 13 PCGs of three Snapper, overlaps of tree reading frames are identified on the similar strand: ND5 and ND6 overlap by four nucleotides, ND4 and ND4L overlap by seven nucleotides, and ATP6 and ATP8 overlap by ten nucleotides (Table 3.1).

3.3.3 Transfer RNAs and ribosomal RNAs

Total 22 tRNA genes ranging from 69-76 bp in length were identified from all three snapper mitogenomes, and tRNA structure was predicted by tRNAscan-SE (Figure 3.2). The shorter tRNA was tRNA-Phe, tRNA-Ser$^{(TGA)}$, tRNA-His, and tRNA-Glu$^{(TTC)}$, while the longest tRNA was tRNA-Lys 76bp in length. Eight tRNAs were located on the L-strand, and the remaining 14 tRNAs were on the H-strand (Figure 3.1). Both the 16S and 12S rRNA genes were found on the H-strand in all three mitochondrial genomes. They were located between the tRNA-Phe and tRNA-Leu and were separated by the tRNA-Val. The size of the 16S rRNA was 1050 bp, 1097 bp, 1097 bp and for the 12S rRNA was 946 bp, 950 bp, and 951 bp in *L. vitta*, *L. fulviflamma* and *L. carponotatus* respectively.

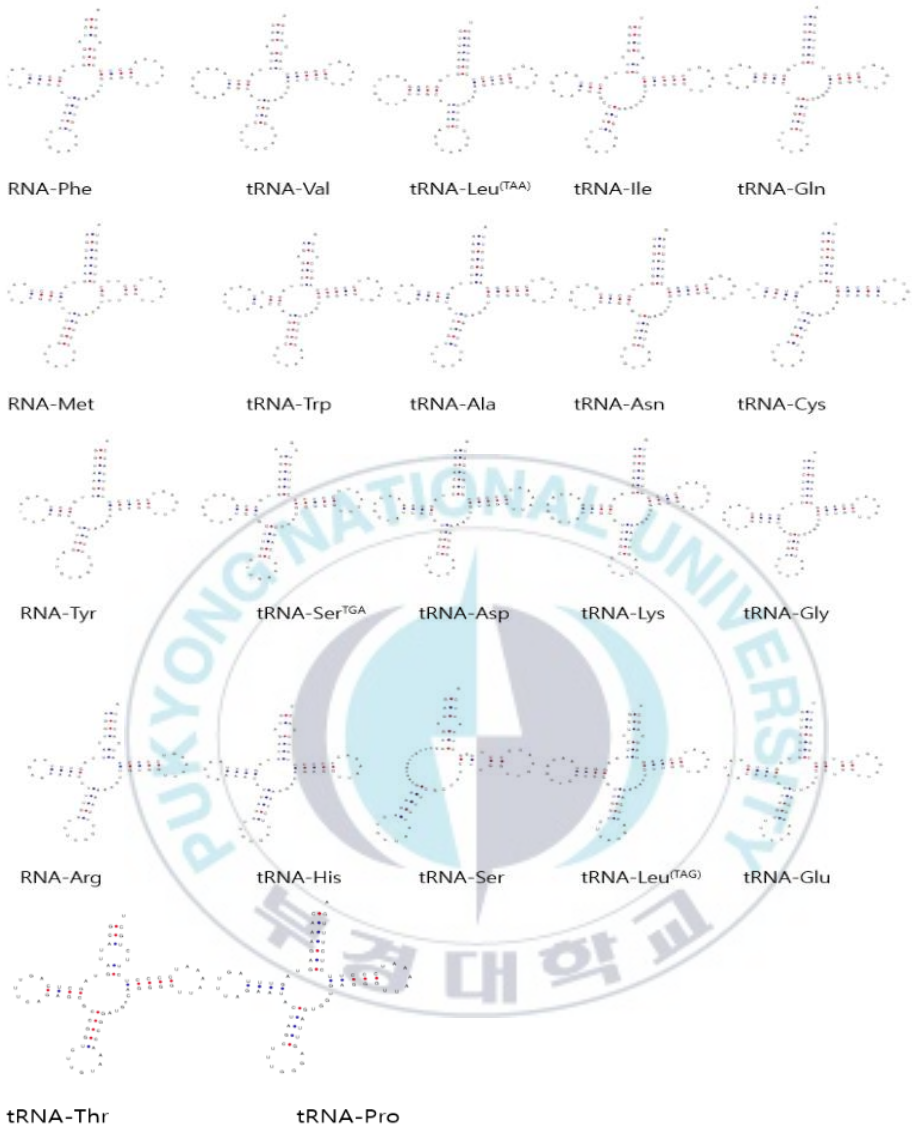


Figure 3.2. Prediction of tRNA structure using tRNAscan-SE

Table 3.1. Summary of Mitochondrial genome of three snappers (Lutjanidae)

Gene	Lutjanids								
	Lutjanus vitta			*Lutjanus fulviflamma*			*Lutjanus carponotatus*		
	Start codon	Stop codon	Position	Start codon	Stop codon	Position	Start codon	Stop codon	Position
tRNAPhe			1 -69			1 -70			1 -69
12S-rRNA			70 -1016			71 -1020			70 -1021
tRNA Val			1017 -1089			1021 -1093			1022 -1094
16S-rRNA			1090 -2786			1094 -2790			1095 -2791
$tRNALeu^{UUA}$			2787 -2860			2791 -2864			2792 -2865
ND1	ATG	TAA	2861 -3835	ATG	TAG	2685 -3839	ATG	TAA	2866 -3840
tRNA Ile			3839 -3908			3843 -3912			3844 -3913
tRNAGln			3908 -3978			3912 -3982			3913 -3983
tRNA Met			3977 -4047			3981 -4051			3982 -4052
ND2	ATG	TA-	4047 -5092	ATA	T--	4051 -5095	ATG	TA-	4052 -5097
tRNATrp			5093 -5164			5097 -5168			5098 -5169
tRNA Ala			5164 -5233			5168 -5237			5169 -5238
tRNAAsn			5235 -5307			5239 -5311			5240 -5312
OL			5308 -5339			5312 -5343			5313 -5344
tRNACys			5340 -5410			5344 -5414			5345 -5416
tRNA Tyr			5410 -5479			5414 -5483			5416 -5486
COX1	GTG	TAA	5472 -7031	ATC	TAG	5891 -7035	GTG	TAG	5488 -7038
$tRNASer^{UAN}$			7034 -7102			7038 -7106			7041 -7109
tRNA Asp			7107 -7178			7111 -7182			7114 -7185
COX2	ATG	T--	7186 -7876	ATG	T--	7190 -7880	ATG	T--	7193 -7883
tRNA Lys			7877 -7952			7881 -7956			7884 -7959
ATP8	ATG	TAA	7953 -8120	ATG	TAA	7957 -8124	ATG	TAA	7960 -8127
ATP6	ATG	TAA	8111 -8794	ATG	TA-	8115 -8797	ATG	TA-	8118 -8800
COX3	ATG	TA-	8794 -9578	ATG	TA-	8798 -9582	ATG	TA-	8801 -9585
tRNAGly			9579 -9650			9583 -9654			9586 -9657
ND3	ATG	T--	9651 -9999	ATA	T--	9655 -10003	ATG	T--	9658 -10006
tRNAArg			9999 -10069			10003 -10073			10006 -10076
ND4L	ATG	TAA	10069 -10365	ATG	TAA	10073 -10369	ATG	TAA	10076 -10372
ND4	ATG	T--	10359 -11739	ATG	T--	10363 -11743	ATG	T--	10366 -11746
tRNA His			11740 -11808			11744 -11812			11747 -11815
$tRNASer^{AGY}$			11809 -11879			11813 -11882			11816 -11884
$tRNALeu^{CUA}$			11882 -11954			11886 -11958			11888 -11960
ND5	ATG	TAA	11955 -13793	ATG	TAA	11959 -13797	ATG	TAA	11961 -13799
ND6	ATG	TAG	13790 -14311	ATG	TAA	13794 -14315	ATG	TAA	13796 -14317
tRNAGlu			14312 -14380			14316 -14384			14318 -14386
Cyt B	ATG	T--	14387 -15533	ATG	T--	14391 -15531	ATG	T - -	14393 -15533
tRNAThr			15528 -15600			15532 -15604			15534 -15606
tRNA Pro			15599 -15668			15603 -15672			15605 -15674
D-Loop			15669 -16759			15673 -16512			15675 -16514

Bold text underlining indicates a gene encoded on L-strand

3.3.4 Non-coding regions

There are two non-coding regions; the origin of light strand replication (*OL*) and the putative control region (*D-Loop*) in the complete mitochondrial genome of all three Lutjanids in this report. A non-coding region, which related with the putative L-strand replication origin is 32 bp, then heavy

strand control region of mitochondrial DNA (D-loop) was identified between the tRNA^{Pro} and tRNA^{Phe}, and it was 830bp, 840bp, and 840 bp length in *L. vitta*, *L. fulviflamma* and *L. carponotatus*, respectively. The OL was located between the tRNA^{Asn} and tRNA^{Cys}, inside the WANCY cluster of tRNAs was 32 bp long in all three snappers, which is similar to those of its relative *Lutjanus russellii* (Figure 3.1).

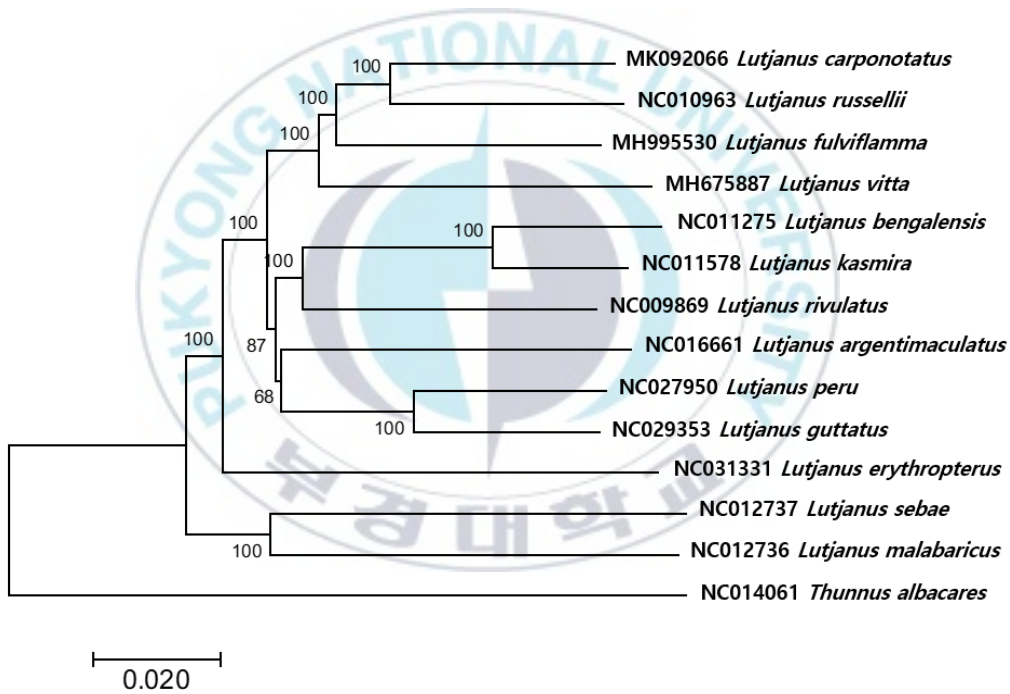


Figure 3.3. Phylogenetic tree of *L. vitta*, *L. fulviflamma*, and *L. carponotatus* within the family Lutjanidae.

3.3.5 Phylogenetic tree analysis

The phylogenetic relationship of *L. vitta*, *L. fulviflamma*, and *L. carponotatus* complete mitogenome within the family Lutjanidae was analyzed and

constructed by MEGA7 software with minimum evolutionary (ME) algorithm (Kumar et al., 2016). The tree topologies performed that *L. carponotatus* was grouped with *L. russelli* (93%), followed *L. fulviflamma* (92%), and *L. vitta* (91%). The phylogenetic tree of three complete mitochondrial genomes in Lutjanidae was constructed by Mega7 using Minimum Evolution (ME) algorithm with 1000 bootstrap replication. The probability of bootstrap at each node and GenBank accession number were shown followed by the scientific name (Figure 3).

3.4 Discussion

The mitogenome of three *Lutjanus* spp. were typically circular molecule and consisted of 13 protein-coding genes (PCGs), 22 tRNA genes, two rRNA genes, and a putative control region. They have similar to the structure of other teleost species (Miya and Nishida, 2000). The heavy strand (H-strand) encoded 23 genes, whereas the light strand (L-strand) encoded the remaining 14 genes. The gene arrangement and order of the 37 genes were utterly identical to those of reported species of Lutjanids such as *Lutjanus russelli* (Guo et al., 2008), *Lutjanus peru, Lutjanus gutattus* (Bayona-Vásquez et al., 2017), and *Lutjanus johnii* (Taillebois et al., 2016).

The tRNAs were clustered in three conserved forms (IQM, WANCY, and HSL) in the mitochondrial genome (Satoh et al., 2016). The IQM (isoleucine,

glutamine, and methionine) cluster was located between ND1 and ND2, the WANCY (tryptophan, alanine, asparagine, cysteine, and tyrosine) cluster was located between ND2 and COX1, and then the HSL (histidine, serine, and leucine) group was located between ND4 and ND5.

This complete mitochondrial genome is the form of baseline for the genetic information, which will be useful for further studies on the population genetics and conservation of coral-reef dependent species in the tropical region. Mitochondrial DNA study is an essential tool in biological evolution. It is due to a relatively fast rate in base substitution (Martin and Palumbi, 1993), maternal inheritance (Birky et al., 1989), and relatively easy of extraction, preservation, and manipulation (Dowling, 1990). For conservation of marine fish resources, mitochondrial DNA has been used in three ways; first, to the analysis of the genetic variation within a population; second, to identify an evolutionarily divergent group of community and also; third, to monitor conservation value of population from phylogenetic or evolutionary views (Moritz, 1994).

The variation of the phylogenetic-based on different genes can be produced (ND4, ND5, COX1, and *cyt-b*) and useful for understanding the evolutionary rate in different species in the different genus. The results suggested that the complete mitochondrial genome could provide reliable information for the molecular-based research including environment DNA (Kelly et al., 2014;

Yamamoto et al., 2017), guts contents analysis (Symondson, 2002), fisheries forensics (Ogden, 2008; Teletchea et al., 2005), and authentication fishery product (Gil, 2007).

3.5 Conclusion

This result is the first complete mitochondrial DNA sequences of three snappers in Lutjanidae, e.g. *L. vitta*, *L. fulviflamma* and *L. carponotatus* from the tropical region of Indonesia. Comparison of the mitogenome and gene arrangement organization, including the phylogenetic relationship study within the family Lutjanidae has been shown here. This genetic information will be used as new tools and have benefits for further understanding of the evolutionary and phylogenetic classification of Lutjanids species. Furthermore, they could be used for the environmental DNA analysis research by supported in the GenBank database for the tropical marine fish species.

Chapter 4.

Diversity studies on marine fish species in Java and Bali by environmental DNA (eDNA) metabarcoding analysis

4.1 Introduction

The existence of coral reef ecosystems, seagrasses, and mangroves in the shallow water region of Indonesia and thousands of small islands spread from eastern Papua to Sumatra, supporting biodiversity in this area (Hutomo and Moosa, 2005). The latest report on the number of marine fish species identified as living in Indonesia is 3,215 species, which is the highest among countries in the Indian Ocean (Wafar et al., 2011). Specifically, in coral reef ecosystems, (Allen and Adrim, 2003) have conducted surveys in 13 locations of observations for 17 years mentioning in total about 2,057 species of marine fish identified. The point out that Indonesia's biodiversity status still does not have accurate data on the number of marine fish species that live in the territory of Indonesia, both in the shallow water region and offshore Indonesia. The Bali Strait region and is one of the essential areas which is the concentration of Bali sardine *Sardinella lemuru* fisheries activities that have significant economic value (Sartimbul et al., 2010), whereas the southern region of Bali (Indian Ocean) is more dominated by tuna fisheries activities (Duggan and Kochen, 2016).

It is well known that the marine ecosystem in East Java and Bali islands as the hotspot of biodiversity, where Pacific and Indian waters meet together forming the excellent fishing grounds several economic importance fish species. Around Bali water, Bali sardine (*Sardinella lemuru*) is the main target

for small-scale fisheries (Pet et al., 1997), then another fisherman with equipment correctly pick out the most economic importance marine species including skipjack (*Katsuwonus pelamis*), yellowfin tuna (*Thunnus albacares*), bigeye tuna (*Thunnus obesus*), albacore (*Thunnus alalunga*), and some neritic tuna species including Indo-pacific king mackerel (*Scomber gutatus*), frigate tuna (*Auxis thazard*), bullet tuna (*Auxis rochei*) and longtail tuna (*Thunnus tonggol*) (Duggan and Kochen, 2016; Ridha et al., 2013). However, over the last several decades, marine ecosystem has suffered significant impacts from both local and global stressors, which including overfishing (Ramenzoni, 2013), pollution (Edinger et al., 1998), tourism (Van der Duim and Caalders, 2002), and global climate change (Hoegh-Guldberg et al., 2007).

But unfortunately, the presence of these marine fishes subjected to considerable pressure, and the situation has decreased due to excessive human exploitation (Pauly et al., 2002). As the result, evidences of declining the fish stocks in those region are being reported (Collette et al., 2011; Hutchings, 2000; Jackson et al., 2001). For example, catch of *Sardinella lemuru* in Bali strait has been dwindling in a significant rate from 108.772 tons in 2006 to 19,663 tons in 2012 (Sartimbul et al., 2018). While for tuna production, in 2004 it reached 400 tons which then experienced a significant decline with a production value of around 100 tons (2006), and until 2009 it still experienced

a downward trend of approximately 60 tons (Nugraha, 2016).

To understand and predict the changes in fish stocks, their continuous surveys along the Bali trait are required. The conventional fish biodiversity surveys are conducted by the direct morphological observation of the specimen caught by a trawl (Lang and Baldwin, 1996). However, those traditional surveys convey several potential problems. One of the major weak points of the trawl surveys is challenging to implement (Demestre et al., 2015) with captured non-selective species (Niamaimandi et al., 2018). Additionally, different survey results often obtained by the different trawling methods (Zimmermann, 2003) and some demersal trawls may destruct the marine ecosystem (Petovic and Markovic, 2013). This species identification is also dependent on the highly trained specialists judged from the anatomic and morphometric characteristic for each species (Teletchea, 2009), which requires considerable observation times and efforts (Thomsen et al., 2012a). We also cannot exclude the potential errors caused by human researchers (Hopkins and Freckleton, 2002), incomplete checklist (Love et al., 2010; Rogers and Ellis, 2000), and leaving databases flawed with errors (Daan, 2001).

Recently, a new method has been introduced to monitor fish biodiversity by analyzing the DNAs extracted directly from the water samples, so-called environmental DNA or eDNA metabarcoding (Stoeckle et al., 2017; Taberlet et al., 2012; Thomsen et al., 2012a). This eDNA method is now widely used

for its short times and relatively low cost for the analysis (Kelly et al., 2014; Shaw et al., 2016; Stoeckle et al., 2017; Yamamoto et al., 2017). The eDNA analysis also has been effective in detecting the endangered species (Laramie et al., 2015; Thomsen et al., 2012b), invasive species (Ardura et al., 2015; Cai et al., 2017; Clusa et al., 2017; Dejean et al., 2012; Klymus et al., 2017; Takahara et al., 2013; Williams et al., 2018), and distribution of a fish species (Yamamoto et al., 2017; Yamamoto et al., 2016).

The eDNA metabarcoding analysis is currently dependent on the PCR amplification by the taxon-specific universal primer set to minimizing biases (Taberlet et al., 2012). Now, the specific primer designed for salmonids (Wilcox et al., 2013), frog (Ficetola et al., 2008), and degenerated fish primers set targeting in *Cyt-b* (Minamoto et al., 2012; Thomsen et al., 2012a). Among them, Mifish primer is one of the most widely used primers set for its high taxon coverage and short amplicon length (~170 bp) with higher detection capacity (Miya et al., 2015b; Yamamoto et al., 2017). Notebly, a web-based bioinformatics tool, the MitoFish pipeline, has been launched recently (http://mitofish.aori.u-tokyo.ac.jp/mifish/), which has changed the paradigm in processing NGS data to be very simple and allows users to analyze fish biodiversity through environment DNA metabarcoding which is user-friendly (Sato et al., 2018).

In this study, we applied eDNA metabarcoding using MiFish pipeline to

estimate the marine fish biodiversity for the first time in Bali Strait. Our objectives were (1) to identify species detected by MiFish metabarcoding without traditional surveillance, and (2) to examine marine fish biodiversity based on eDNA metabarcoding. These approaches will allow us to the identification of local fish genotype (tropical fish) and clarify of eDNA metabarcoding efficiency for regular surveillance in Indonesian marine ecosystem.

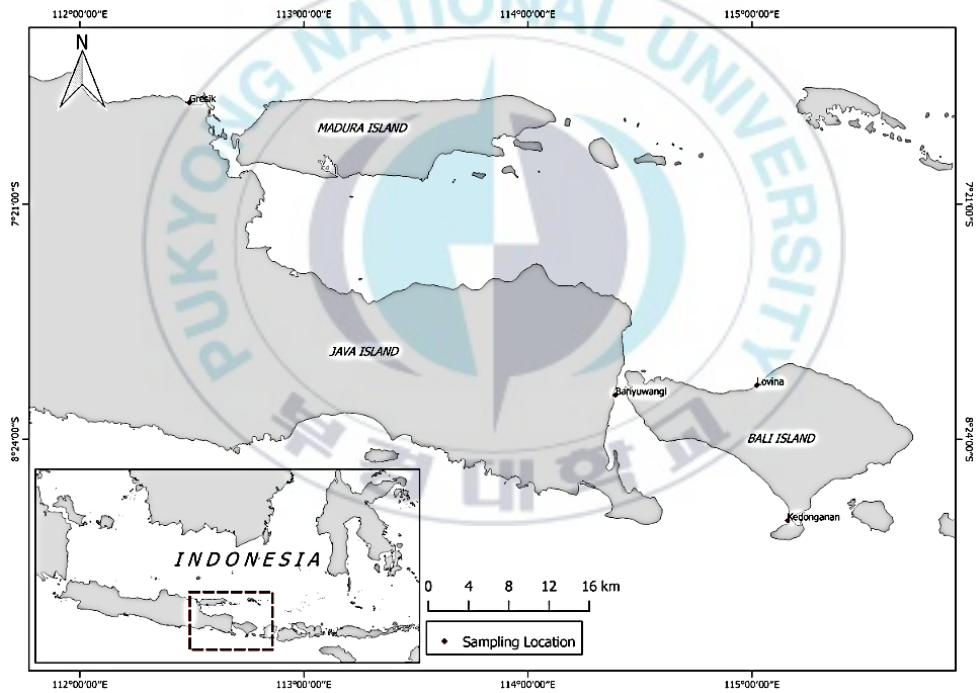


Figure 4.1. Distribution of sampling site in East Java and Bali Island, Indonesia

4.2 Materials and Methods

4.2.1 Sample collection and environmental-DNA extraction

The eDNA water samples were collected from four stations in Java Island (Gresik and Banyuwangi) and Bali Island (Kedunganan and Lovina) on January-June 2018 (Figure 4.1). Five liters of water samples were pooled and immediately stored in ice until brought to the laboratory, at Universitas Airlangga and Udayana University for filtration. Every single filter 0.45 µm pore-sized GN-6 membrane (PALL Life sciences, Mexico) for one liter of the water sample, then the filters were kept and mixed into 2.0 ml tubes with lysis buffer for further analysis. Prevention the cross-contamination in all steps were conducted by washed up with 10% commercial bleach and 70% ethanol for all filtration equipment. The membrane filters DNeasy® Blood, and Tissue Kit (Qiagen, Germany) was used for genomic DNA extraction process, according to the company's guideline. Homogenizing of the membrane filters by TissueLyser II motorized homogenizer (QIAGEN, Hilden, Germany), the quantification of the extracted genomic DNA was measured by using ND-1000 NanoDrop (Thermo Scientific, Waltham, MA, USA). The water temperature, pH value, and TDS were measured by a conductivity meter (CD-4307SD, LUTRON), then salinity was measured by refractometer manual (ATAGO).

4.2.2 Construction of Library and MiSeq sequencing

The MiFish universal primer sets were used to construct the amplicon libraries of partial 12S rRNA marker (Miya et al., 2015a). The total PCR mixture volume was 20 µL, which contained 1.0 µL of MiFish primers (5 pmol each), 2.0 µL dNTPs (2.5mM), 2.0 µL of 10X EX Taq buffer, 0.6 µL DMSO (3%), 0.2 µL of EX Taq Hot Start (TaKaRa Bio Inc. Japan) and 9.20 µL of ultra-pure water. Here, we used 4.0 µL template due to the low genomic DNA concentration that less than 50 ng/µL. The PCR setting condition followed the MiFish primer protocol (Miya et al., 2015b). The gel electrophoresis (1.5% agarose) was performed, and the expected size (250 bp~350 bp) was purified by the AccuPrep® Gel Purification Kit (Bioneer, Republic of Korea). Purified amplicons were pass through the second PCR with the corresponding Nextera XT index (Illumina, San Diego, USA) at the end of each amplicon. The total volume for second PCR mixture was 20 µL which contain 1 µL of a couple of index primers (10 pmol), 0.5 µL dNTPs (10 mM), 4 µL 5X Phusion HF Buffer, 8.3 µL ultrapure water, and 0.2 µL Phusion Hot Start Flex DNA polymerase (New England Biolabs, Hitchen, UK), and including 5 µL amplicons result from the first PCR. The second PCR setting conditions began with 94˚C for 5 min for initial denaturation, followed by 15 cycles of 94˚C for 30 sec for denaturation, 55˚C for 30-sec annealing, and 72˚C for 30 sec for the extension, and an additional 5 min at

72 ˚C for the final extension. The gel electrophoresis and purification were performed similar with the first PCR process, then PCR products with the expected sizes were analyzed by qubit dsDNAHS Assay Kit (Invitrogen, Carlsbad, CA, USA) for quantification of amplicons concentration. The next-generation sequencing was applied using the MiSeq platform (2 X 300 bp).

4.2.3 Bioinformatics analysis of NGS data

Before uploaded NGS raw data to the MiFish pipeline, Phyton27 (an open source software) was used to make the pairing of both reverse and forward sequences with the specific script (Zhang, 2015). In MiFish pipeline, the raw reads by MiSeq sequencing run FASTQC, which will be trimming for the low-quality tail of reads (QV ≤ 20). After that, several steps include assembled paired-end reads and followed by removed N-containing reads, filtered reads by length (~229 bp), run the Usearch (0.99 for clustering of identity, and 10 for minimum read size for filtering), BLASTN based on GenBank database, and then created multi-FASTA files for each samples. The next step is run MAFFT, run the Morphy for each sample, run the Morphy against merged sample, run BLASTN, and finalization of the last process by BLASTN. The entire sequences stipulated to representative genotype by compared to the GenBank database, then the sequences were ascertained as '*species*', '*genus*', and '*unknown* or *unidentified*' level if the sequence identity more than or similar to 99%, 97-98%, and less than 97%, respectively. The

distribution for each species was confirmed by the FishBase (http://www.fishbase.org/) then taxonomic nomenclature was approved under the World Register of Marine Species, WORMS (http://www.marinespecies.org/).

4.2.4 Statistical Analysis in biodiversity indices

The measurements of alpha biodiversity were carried out for the average read number data in each sampling location. Analyses of the index on alpha diversity include the index of Shannon-Wiener (H'), which informs of heterogeneity diversity or total species richness in certain areas (Gray, 2000; Magurran, 1988) and Margalef diversity index (d). The H' index, Margalef index (d), the Pielou's evenness index, and Bray-Curtis similarity analysis was calculated using PRIMER7® software v7 (Clarke and Gorley, 2015).

4.3 Results

4.3.1 Physico-chemical parameters

Water salinity of the sample range from 31 psu in North Java on January-February 2018) to 34 psu started from March in South Bali to June in all station. The lowest salinity in North Java due dilution from Brantas River in the northern part of Java. The temperature of samples ranges from 26^{o}C to 28^{o}C, then pH of the samples ranges from 7.9-8.0 (Table 4.1).

Table 4.1. Geographical coordinate and average value of phisico-chemical parameter measurement.

Month	Sampling Site											
	North Bali (Lovina) 8º09'31''S 115º01'19"E			South Bali (Kedunganan) 8º45'46''S 115º09'35"E			North Java (Gresik) 6º53'43''S 112º29'21"E			South Java (Banyuwangi) 8º12'09''S 114º23'28"E		
	pH	Temp	Salinity	pH	Temp	Salinity	pH	Temp	Salinity	pH	Temp	Salinity
Jan	8.0	26	32	8.0	26	33	7.8	26	31	7.8	26	33
Feb	8.0	26	33	8.0	26	33	7.8	26	31	7.9	26	34
Mar	7.9	27	32	8.0	26	34	7.9	27	32	8.0	26	34
Apr	7.9	27	33	7.9	27	34	8.0	27	33	8.0	26	33
May	8.0	28	34	8.0	27	34	8.0	26	34	8.0	27	34
Jun	7.9	27	34	8.0	28	34	8.0	27	34	8.0	28	34
Average	7.9	26.8	33.0	7.98	26.7	33.7	7.9	26.5	32.5	7.9	26.5	33.7

4.3.2 Analysis of fish haplotype obtained by MiFish pipeline

After clustering and trimming the raw reads (1,948,177) from the MiFish platform, 1,850,348 merged reads (94.98%), and 405 representative haplotypes were assigned from the four sampling stations for six-month monitoring. Total 1,588,953 reads (81%) were assigned into 333 species, and the remaining 162,180 and 99,215 reads were mapped into 27 genera and 25 unidentified genera level, respectively. After the taxonomic assignment, 97,829 reads (5.02%) were discarded which have lower identity between 80-95% identity (Table 4.2). Station-wise average merged reads r (log normalization) shown that north Bali (2.6626) is higher than south Bali (2.2671) followed by South Java and North Java which have similar mean reads is 2.2355 (Figure 4.2).

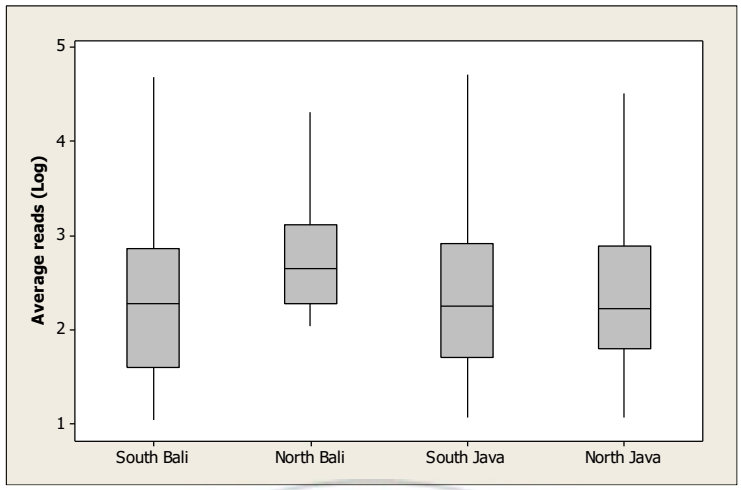


Figure 4.2. Box plot chart of average reads (Log) of eDNA metabarcoding result by MiFish pipeline from four station.

Table 4.2. Summary of taxonomic assignment of MiSeq reads number

	South Bali	North Bali	South Java	North Java
Raw reads	643747	735363	323011	384293
Merged reads	617796	705999	309223	361900
Read number (99-100% identity)	451753	629045	304144	350693
- Haplotypes	242	224	169	94
- Species	224	198	158	85
Read number (95-98% identity)	166043	76954	5079	11207
- Haplotypes	47	57	18	14
- Genera and unidentified genera	44	52	16	11

4.3.3 Marine fish biodiversity in four station

Regarding the environmental DNA metabarcoding analysis using MiFish platform, alpha biodiversity indices in four sampling site have been analyzed by Primer v7. The fluctuation of diversity index during from January to June was observed (Figure 4.3). The North Java region has the lowest average in

Shannon-Wiener index (2.9474) compared to the others, which shows this region to be in the moderate category of alpha diversity index for marine fish species. Wheras the southern and northern part of Bali has the highest average value (3.4426 and 3.4403, respectively), and the South Java (3.011). Another diversity index, Margalef diversity index also showed the same pattern in those regions (Table 4.3). While in general, the distribution of biota in the area is estimated to be evenly distributed with a value of 1 point in all sampling site.

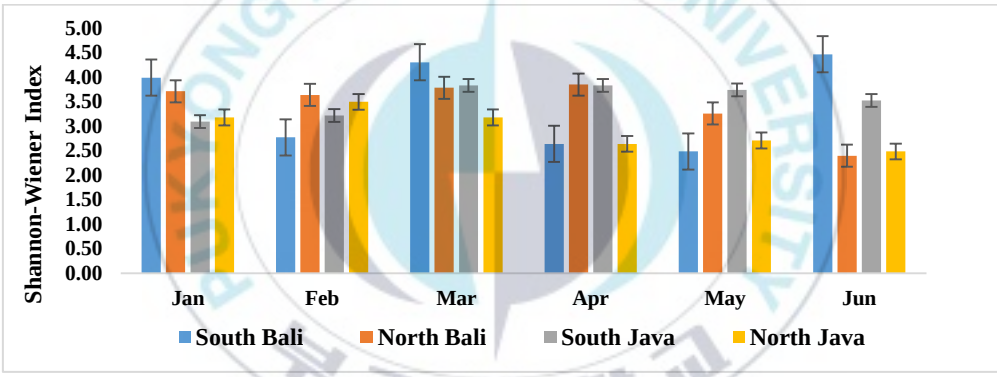


Figure 4.3. Shannon-Wiener index during January-June 2018 in four sampling site.

Table 4.3. Biodiversity Indices in four station of Java and Bali Island

Index	Sampling Sites			
	North Bali	South Bali	North Java	South Java
Shannon-Wiener (H')	3.4403	3.4426	2.9474	3.5385
Margalef (d)	10.3273	12.2959	7.2461	10.8962

The highest number of species were identified in the Order Perciformes (285) followed by Clupeiformes (24), Tetraodontiformes (19), and Scorpaeniformes (14), and remaining order small number of representative haplotypes. The order Perciformes have 43 family includes Carangidae (36), Lutjanidae (28), Serranidae (26), Achanthuridae (16) and Scombridae (16). The highest species numbers within the Perciformes were identified in the genus *Lutjanus* (17), *Epinephelus* (17), *Lethrinus* (12) and *Acanthurus* (8).

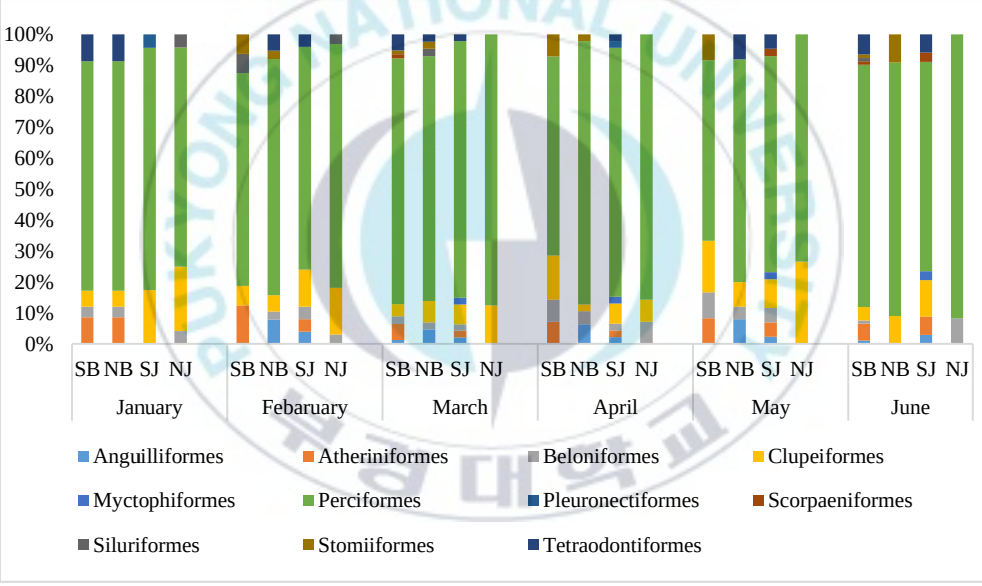


Figure 4.4. Proportion of piscine phyla during January-June 2018 from four sampling site. SB: South Bali; NB: North Bali; SJ: South Java; NJ: North Java

In this study, the Perciformes orders are the most dominant order identified in all study areas, then followed by Clupeiformes and Tetraodontiformes, respectively. The proportion of Perciformes during January-June 2018 shown

dominance in all sampling site (Figure 4.4). Even though between the southern parts of Java and South Bali was separated by the Bali Strait which had a reasonably strong current, this location had almost the same of the oceanography characteristics. However, the southern region of Bali and South Java (Banyuwangi) also shared species identified in this region (Figure 4.5).

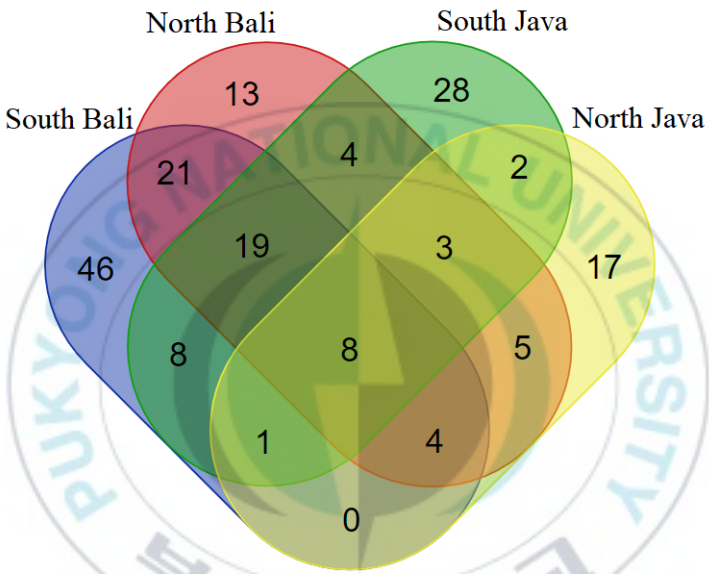


Figure 4.5. Venn diagram of species identified in four sampling site.

4.3.4 Clustering Analysis

Table 4.4. Analysis of Similarity (ANOSIM) pairwise test by Prime v7

Groups	R Statistic	Significance level (%)	Actual Permutations
South Bali, North Bali	0.087	18.2	462
South Bali, South Java	0.757	0.2	462
South Bali, North Java	0.957	0.2	462
North Bali, South Java	0.778	0.2	462
North Bali, North Java	0.656	0.2	462
South Java, North Java	0.913	0.2	462

From the analysis of similarity (ANOSIM) pairwise test by Primer v7 showed that except South Bali-North Bali combination was not significantly different. The ANOSIM showed that the average of R statistical value is 0.723 (Table 4.4). In this study, we have detected pelagic species (12.346) and also coral reef fish associated (87.654%). The proportion of coral reef fishes in all sampling site was dominated than non-coral reef fish species, and 73.33% coral reef fish identified at South Java is higher than other sampling sites. Based on Bray-Curtis Similarity analysis showed that South Java was clustered with both North and South Bali. However, North Bali shared species with North Java (Figure 4.6). In this study, we have identified *Kyphosus vaigiensis*, *Siganus argenteus*, *Spratelloides gracilis*, and *Mulloidichthys flavolineatus* are found exclusively in South Bali. Meanwhile, *Thunnus tongol*, *Liza macrolepis*, *Rhinogobius* sp., and *Platax teira* are found solely

found in North Bali. In North Java, seven species were exclusively found in this region such as *Pseudogobius javanicus*, *Nurchequula flavaxille*, *Stethijulis quinqueradiata*, *Dendrophysa russeli*, *Setipinna taty*, and *Ellochelon vaigiensis*. The *Siganus fuscescens* is the most common fish in all region during January-June 2018 (Figure 4.7).

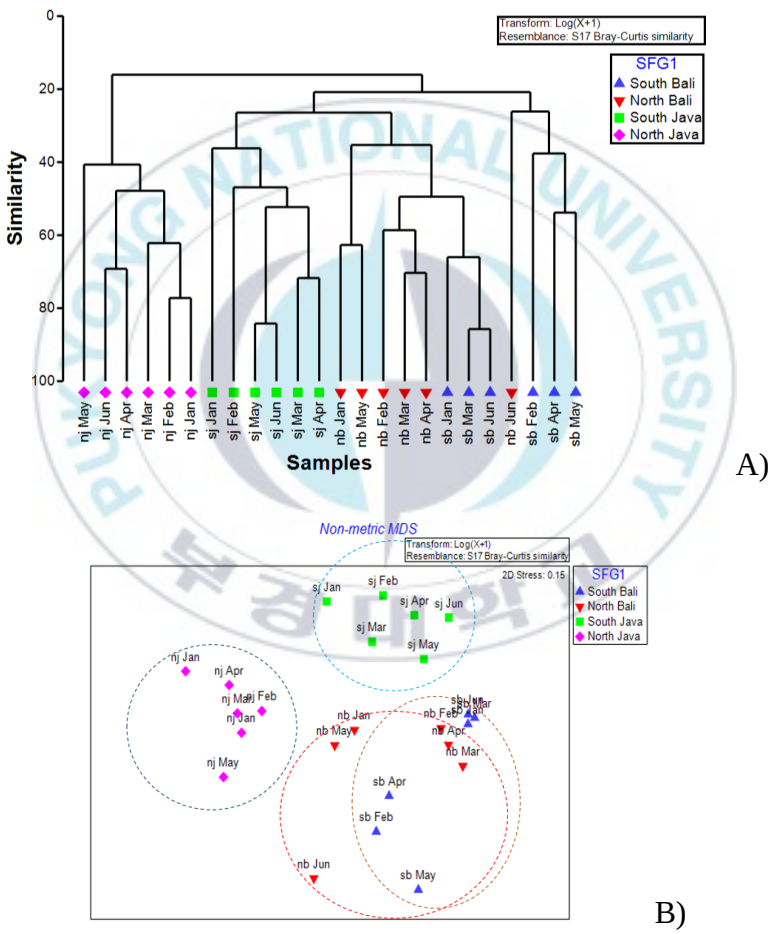


Figure 4.6. Bray-Curtis similarity chart based on four sampling station e-DNA metabarcoding using Primer®v7. A) Dendrogram of similarity B) Non-metric Multidimentional Scaling analysis.

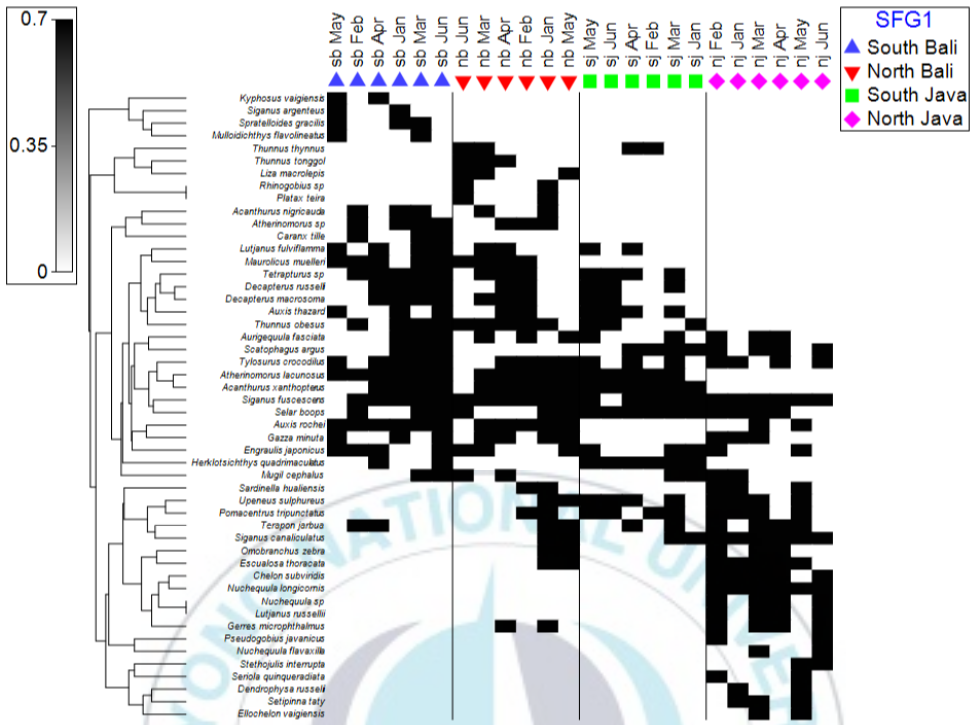


Figure 4.7. Shade plot of eDNA metabarcoding result based on top 50 species in four station

In this study by using Mega v.7.0 program with Maximum Likelihood algorithm, we found the Perciformes showed dominance in this study. There are several order grouping all the members in one cluster, e.g., Anguilliformes and Siluriformes, however, the other orders were split into two groups such as Tetraodontiformes, Clupeiformes, and Beloniformes (Figure 4.8). Here, MiFish primer is not able to distinguish the *Thunnus* spp species (Miya et al., 2015b), and five *Thunnus* species were in one clade group with shallow average genetic distance (0.071). Furthermore, in other Scombridae groups

such as *Sarda* spp., *Euthynnus* spp., *Katsuwonus* sp., *Auxis* spp., and *Acanthocybium* sp., which also faced similar things like *Thunnus* spp.

Besides Scombridae, here we found some species under Carangidae cannot be distinguished within her genus. For instance, the species *Caranx* spp (0.037) and *Carangoides* spp (0.037) failed distinguished within her genera. Likewise, *Pterocaesio* spp (0.011) also difficult to distinguished using MiFish primer. Three species *Pterocaesio digramma*, *Pterocaesio marri*, and *Pterocaesio tessellate* in the same line on the phylogenetic tree. Another species *Achanthurus* spp cannot distinguish between *Ctenochaetus* spp (0.032), and also *Siganus* spp failed distinguished within Siganidae (0.034). Average genetic distance for all 12S short sequence in this research based on MiFish primer is 0.171 (range 0.010-0.391). The low of genetic distance effected to the reposition species in a similar line of the phylogenetic tree.

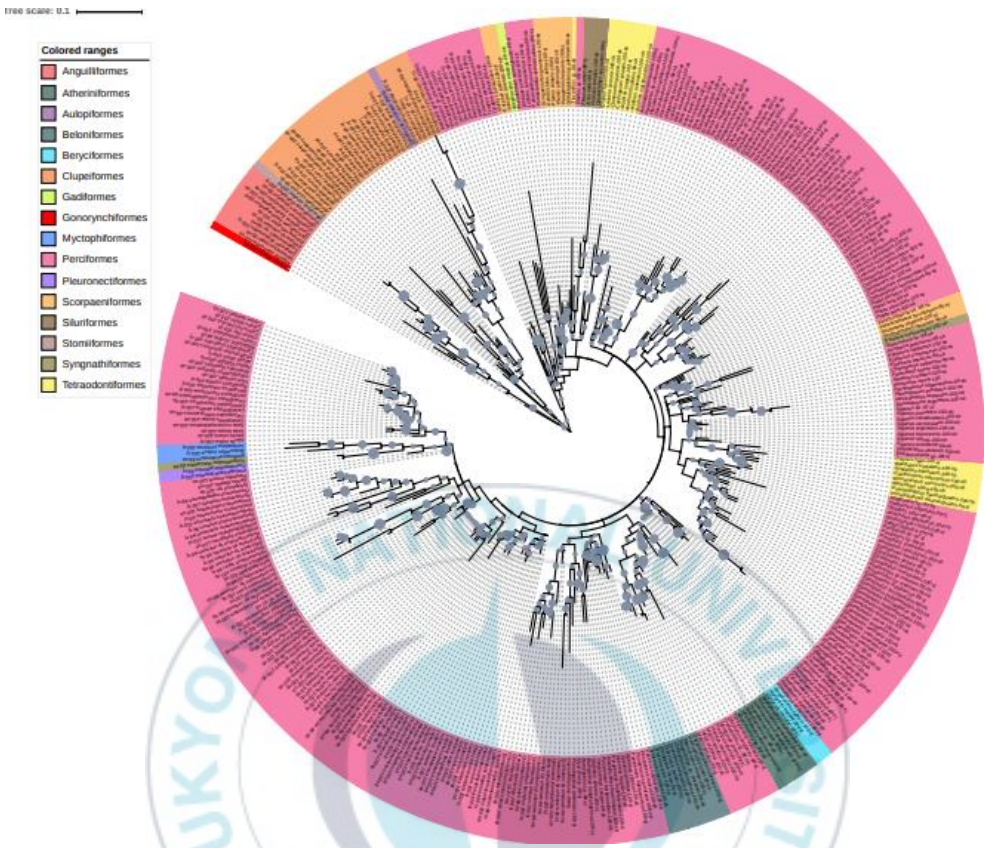


Figure 4.8. Phylogenetic tree of representative haplotypes in the eDNA metabarcoding analysis for the tropical marine fish.

4.4 Discussion

We have demonstrated that environment DNA metabarcoding of marine fish species from the Java and Bali Island by using the MiFish pipeline is an efficient tool for fish biodiversity analysis in Java and Bali Island water. We were able to detect 333 species belonging to 78 families and 16 orders. This result is only about 10.36% of the total fish in the South China Sea (3,215 fish species) and 16.19% of the coral reef fish (2,057 fish species) in Indonesia.

The previous study in Natuna Island and Anambas Island waters has been carried out for 10 days with a relatively complete of equipment and a number of experts in Ichthyology (Ng et al., 2004), while coral-reef fish compilation data in Indonesia has been conveyed for 27 years (1974-2001) by researchers from more than 13 observation locations in Indonesia (Allen and Adrim, 2003). Here, our study shows that environmental DNA metabarcoding can produce data in a relatively short time with a cost-effective compared to conducting traditional surveys to estimate species in waters (Smart et al., 2016; Thomsen et al., 2012a).

Several previous diversity studies in Indonesia, using different methods, such as netting/seining (Unsworth et al., 2007) and Underwater Visual Census, UVC (Abrar, 2017). The UVC method is the most common method in collecting marine fish biodiversity information at the time, and the application of environmental DNA metabarcoding is an initial study in Indonesia. The previous research in the Thousand Islands of Jakarta found about 216 species of coral reef fish in 2011, or 6 more species higher than 2004 (Madduppa et al., 2013). While in the Togean Islands of Sulawesi, about 266 species (Allen and Werner, 2002). This current study was contrasted with the previous researches, which is we were able to identify 333 species within 405 haplotypes.

In the present study, water samples have been collected from the four different

sites, e.g., two locations in Java Island and two locations in Bali Island, which is the representative of varying condition. The measurement of physicochemical parameters shows that distribution is typical in the waters of the Java Sea. Siregar et al. (2015) reported that during January to June 2015, the temperature distribution in the Java Sea ranged from 26-32^{o}C, and this also congruent in sampling locations on Java and Bali (Figure 4.9). Meanwhile, the distribution of salinity in the period January-June 2015 is also still measured in the same way as the results of measurements in the current study, which range from 31-34 PSU (Figure 4.10).

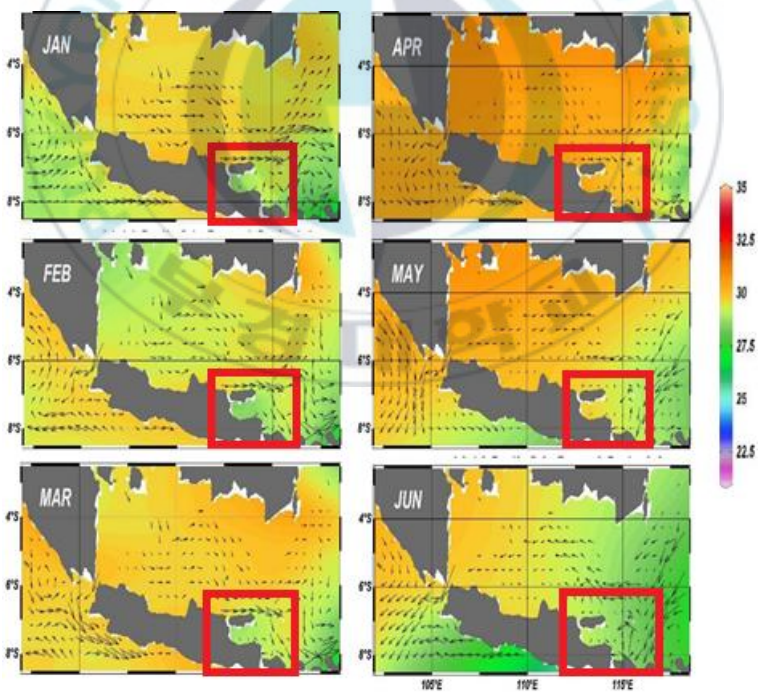


Figure 4.9. Temperature distribution during January – June 2015. The red square is sampling area in east Java and Bali Island. Modification from Siregar et al., 2015

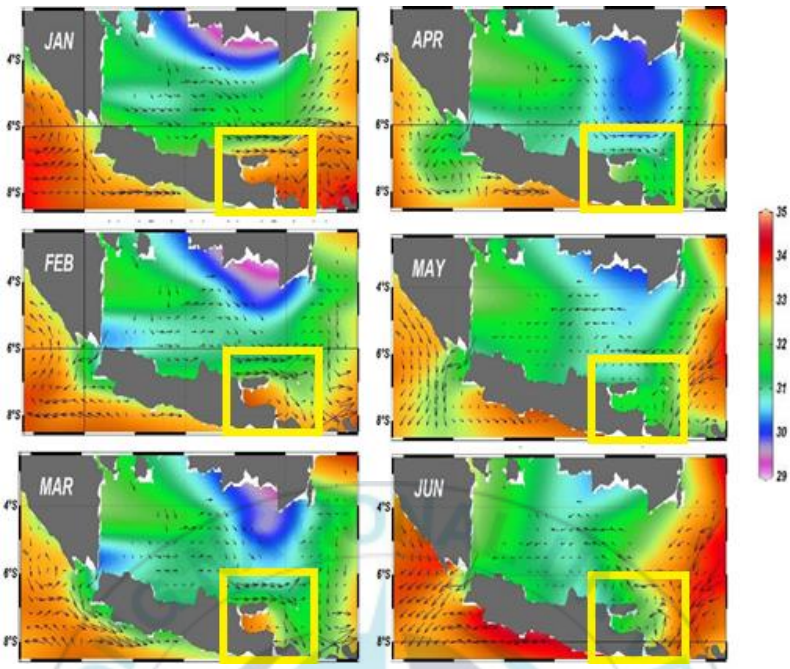


Figure 4.10. Salinity distribution during January – June 2015. The yellow square is sampling area in east Java and Bali Island. Modification from Siregar et al., 2015

In the South Java, samples were collected from the waters of Banyuwangi due to this region was at the eastern end of Java Island and at the same time was very close to the Indian Ocean. The Bali Strait, that separates Java and Bali Island, which is productive waters and many traditional fishing activities operate in this area, especially the Bali sardine (*Sardinella lemuru*) fishery (Purwaningsih, 2015). Several studies in this region are actively monitoring seasonal plankton distribution (Khasanah et al., 2013), abundance of Bali sardine *Sardinella lemuru* (Simbolon et al., 2011; Susilo, 2015), and water quality parameters (Megawati et al., 2014) in the Bali Strait waters.

The northern region of Java Island chosen in this research is Gresik, which is about 22 km far from the Surabaya city (capital of East Java Province). This region is one of the centers of traditional fisheries activities that are quite active in supporting the supply of capture fisheries products in East Java. The Gresik region faces the Java Sea directly with the characteristics of the waters that have low waves (Bird and Ongkosongo, 1980). The Java Sea in the middle of the Indonesian archipelago is a part of the Southeast Asian waters with the western region limited by the Sumatra Island, Kalimantan Island on the north side, and Sulawesi Island on the east side (Wei et al., 2016; Wyrtki, 1961). During the south-east monsoon (June-August), surface current direction comes from the western side to the east side, then during the north-west monsoon (December-February) is vice versa (Siswanto, 2008). It means that the Java Sea in the Java Island shares nutrient and fisheries resources with Bali Island.

Between the two locations in Java Island, species detection by eDNA approach is showing an exciting result, from the South Java, we have found 189 representative haplotypes, 158 species (≥99-100% identity) within 49 families and 12 orders, while from the North Java region found 108 representative haplotype, 85 species (≥99-100% identity) within 40 families and 11 orders. Even though some studies explained that northern Java is facing overfishing problem due to the excess fisheries activities in this region

(Fauzi and Anna, 2010; Squires et al., 2003; Triarso, 2012), but in this study, we identified some pelagic and demersal species are still diversity and require periodically survey to ensure the marine fishes abundance and biomass. The characteristics of the Indian Ocean and the Java Sea also allow for the differences in fisheries resources in the south of this region (Lumban-Gaol et al., 2015; Syamsuddin et al., 2016). Pelagic and demersal fish resources have been detected in this study, providing biodiversity information in the waters of Java and Bali. Artisanal fisheries in the southern region of Java are entirely developed with the use of ornamental fish as a fishery commodity and other species for domestic consumption. The exploitation of coral-reef fish for aquarium fish was growing intensively (Idris et al., 2013). The lack of study on biodiversity of marine fish both in the South and North of Java was occured, primarily through molecular approaches. It should be noted that this study of eDNA metabarcoding is the first time to be carried out with the samples collected from the Indonesian water. However, the results of this study still need to be developed in the broader Java region and even become a routine survey agenda in conducting monitoring through the environmental-DNA metabarcoding approach.

Bali Island has received considerable attention in managing the area with the support of highly developed cultural and tourism activities. Also, Bali is a part of the coral triangle research center, so that several studies on biodiversity

are carried out in this region (Green and Mous, 2008; Hoeksema, 2007). The previous survey obtained data about the diversity of coral reef fish that is fascinating, as many as 977 species of fish have been identified, which consists of 320 genera in 88 families in Bali waters (Allen and Erdmann, 2012).

In this study, Kedunganan is located in the southern region of Bali directly facing the Indian Ocean, while in the northern part of Bali, it has been carried out in the Lovina Beach, which is directly facing the Java Sea. The environmental DNA metabarcoding approach successfully detected 231 representatives of haplotypes, 206 species (99-10% identity) which were belonging to 53 families and 12 orders from the South Bali, while 230 identified haplotypes, 196 species 99-100% identity (56 families and 14 orders) were determined from the North Bali. This research reinforces the findings of the previous study which states that Bali Island has a remarkable characteristic of biodiversity in coral reef fish of Indo-Pacific species (56.4%), western Pacific (25.3), Indian Ocean (3%), and Indonesian endemics (3.3%) (Allen and Erdmann, 2013). This condition is supported by the existence of a suitable seagrass and coral-reef ecosystem (Ginting et al., 2015; Hoeksema and Putra, 2000), so that the abundance and biodiversity of marine fish are still quite high. In Bali, more coral reef fish had been detected than the sampling sites of Java.

For quantification of biodiversity measurement, here, alpha biodiversity used the Shannon-Weiner Diversity Index (H'). The H' value range was 2.94 (North Java) to 3.44 (North Bali). This H' index value is higher than previous studies that have been done using the UVC method; 1.8-2.5 in the Thousand Islands, Jakarta (Dhahiyat et al., 2017; Madduppa et al., 2013), 1.36-3.23 in Kendari, Sulawesi (Adrim et al., 2012), 1.4 in Wakatobi, Sulawesi (Caras and Pasternak, 2009), and 2.183- 2.425 in Karimun Jawa, Central Java (Utomo and Ain, 2013). This biodiversity parameter confirmed that the eDNA metabarcoding could detect not only coral-reef fish (demersal fish), but also can identify pelagic migratory fish species. In the Serranidae group (32 haplotypes, 25 species) which are demersal fish and also coral reef ecosystem associated, but this group can be detected here even though water samples were collected from the surface area. One of the groupers is endangered, namely *Epinephelus akaara* (He et al., 2011). Moreover, the eDNA metabarcoding can also able to detect pelagic fish that may migrate and spawn (Bylemans et al., 2017) in broad range areas e.g., Scombridae family (Beardsley Jr, 1969). In this study, we identified the Order Carangidae (35 haplotypes, 32 species) and Scombridae (20 haplotypes, 18 species). The combination of echo sounding technique and environmental DNA can identify the distribution of Japanese jack mackerel in Maizuru Bay, Japan (Yamamoto et al., 2016).

Moreover, the commercial fish as the target fishery is also very diverse. Based on Bray-Curtis similarity analysis, three station observations cluster in one group except for North Java station, which is different from all stations. This condition clarifies the role of oceanographic parameters in the use of environmental DNA is essential. In this research, we performed sampling from surface water that is quickly displaced by the presence of surface currents generated from the difference in the direction of wind every season. In January, the north-west monsoon season, wind and sea surface currents move towards the north-west, while in June, when the south-east monsoon wind begins to change towards the south-east (Siswanto, 2008). With this change, it is possible for North Java and North Bali to have similarities in terms of fishery resources in the Java Sea.

Meanwhile, in the southern side of Bali, the seasons also affect the movement of seawater that passes through the Bali Strait which effects to the fisheries resources in South Bali and Banyuwangi (South Java) almost similar. Changes in the tides that pass through the Bali Strait allow an exchange of water from the North Bali region to the south of Bali. The Bray-Curtis analysis showed that South Java clustered with two stations in South and North Bali (Figure 4.3).

The results from this current research demonstrated that the Perciformes and Clupeiformes are identified to be quite dominant, and these fish groups are

economically significant that are a mainstay of fisheries activities to the coastal communities in Indonesia. In Perciformes, the grouper (*Epinephelus* spp.), the rabbit fish (*Siganus* spp.), the snapper (*Lutjanus* spp.), scad (*Selar* spp.), and the trevally (*Carangoides* spp) are the most common species caught in the northern region of the Java Sea (Badrudin et al., 2016). Besides the order Perciformes, the Clupeiformes group like the sardine (*Sardinella* spp.), the anchovies (*Stolephorus* spp. and *Engraulis* spp.), and the shad (*Anodontostoma* sp.) are the standard type of fishery catch in this area (Atmadja et al., 2003). Here, the species under the Chaetodontidae family were found in three locations, except for the North Java. The species identified in the family Chatodontidae included *Chaetodon adiergastos*, *Chaetodon auriga*, *Chaetodon kleinii*, *Chaetodon vagabundus*, and *Heniochus varius* (Table 4.6). The species of Chaetodontidae are indicator species for coral reef ecosystem health, which can live only in a healthy and protected coral reef ecosystem (Hourigan et al., 1988; Reese, 1981). So, it can be concluded that in the three sampling locations, there are still excellent coral reef conditions. The existence of coral reef ecosystems in Bali is supported by the awareness of the community in maintaining the ecosystem (Trialfhianty, 2017).

Table 4.5. The list of fish species under the family Chaetodontidae identified in this study

Location	Species Name	% identity	Read numbers
North Bali	*Chaetodon kleinii*	100	817
	Chaetodon vagabundus	100	404
	Heniochus varius	100	125
South Bali	*Chaetodon auriga*	100	12
	Chaetodon kleinii	100	18
North Java	-	-	-
South Java	*Chaetodon adiergastos*	100	37
	Chaetodon kleinii	100	99

The environment DNA metabarcoding is useful for detecting invasive species (Clusa et al., 2017; Williams et al., 2018), migratory species (Goldberg et al., 2015), and biological sewage from fisheries industry and domestic consumption. In this study, we identified three *Sardinella* species (*S. albella*, *S. hualiensis*, and *S. lemuru*), but the *Sardinella hualiensis* not reported from the Indonesian water. The *S. hualiensis* is commonly known as Taiwan sardine, distributed in South China to Hong Kong (http://www.fishbase.se/Country), but this species has been detected in all the study areas. The Taiwan sardine was reportedly identified in the Philippines (Willette et al., 2011; Willette et al., 2014) and the Vietnam waters (Van Quang, 2013). There are two possibilities, firstly, the *S. hualiensis* is migrating to the Java Sea, and their genetic materials are flowing through the Java Sea. Secondly, the DNA of *S. hualiensis* coming from the contamination, that is mixing to the water from the residential areas and or from the fish

processing factories. Some of the sardine canning industry around Banyuwangi are importing raw materials from Taiwan (Ginoga, 2017). Another species, *Siganus woodlandi* has identified in this study, but this species reported from Indonesian yet. The first report of this species is from New Caledonia (Randall and Kulbicki, 2005), which required further research. We were able to identify deep water marine fish and rare marine species. For example, silver light fish, *Maurolicus muelleri* were identified from South Bali, North Bali, and North Java, then *Hypoatherina celebesensis* was identified South Bali. The *M. muelleri*, identified in the central and northwestern tropical waters of the Pacific (Wan and Bian, 2012), carried out vertical migration to feeding activity at night and return to the ocean depth during the day time as part of a strategy to avoid predators (Staby and Aksnes, 2011). The phenomenon of upwelling and down-welling around the Java Sea and the Indian Ocean also observed and contributed to eDNA flow from deep water to the surface. During northwest monsoon, upwelling was found in the Java Sea, and down-welling was shown in the Indian Ocean. During southeast monsoon, upwelling was shown in the Indian ocean, and low intensity of upwelling happen in Java sea (Siswanto, 2008). The Sulawesi silverside *Hypoatherina celebesensis* is a unique species which is a new record was confirmed in 2012 (Sasaki and Kimura, 2012) and has limited distribution in Singapore, Indonesia, and Palau. Report on this eDNA is fascinating, which

allows the discovery of other rare species by increasing the intensity and range in collecting seawater samples (Jerde et al., 2011).

The environemental DNA metabarcoding application is still experiencing difficulties in distinct cryptic species. Previous research by eDNA analysis mentioned due to the short length of MiFish primer, some close related fish like *Sebastes* spp and *Takifugu* spp cannot distinguish by using MiFish universal primer sets (Yamamoto et al., 2017). Here, we got *Cantherhines* spp and *Gerres* spp which are not inhabit in Indonesia water. For instance, the American white spotted filefish, *Cantherhines macrocerus* was identified from North and South Bali, but these species inhabit around Florida, Bermuda to Sao Paulo and Eastern Atlantic (Lubbock and Edwards, 1981), however her sister *Chantherines dumerilii* found in the Indo-Pacific region (Hutchins and Randall, 1982). Also, *Gerres microphthalmus* was identified from North Bali, North Java, and South Java, and this fish has limited distribution around Southern Japan (Iwatsuki et al., 2002). Previous taxonomic study on *Gerres* explained that in the Indo-West Pacific had disclosed several of similar clusters (complexes); *Gerres filamentosus* complex (Iwatsuki et al., 1998). Here, *Gerres oyena* was identified from South Bali, which is a native fish from the Indo-West Pacific (Iwatsuki et al., 1999).

We suppose, with the short sequence of 12S rRNA region of the MiFish primer, eDNA metabarcoding result failed to distinguish closely related

congeners of *Thunnus* (Miya et al., 2015b). Here, beside *Thunnus* spp., another Scombridae includes *Katsuwonus* spp., *Auxis* spp., and *Euthynnus* spp. also failed to distinguish from her group. Some species *Acanthurus* spp. cannot be separated with *Ctenochaetus* spp.; furthermore, *Siganus* spp. has similarly faced the same condition. Secondly, the MiFish pipeline also using their taxonomical system (Table 4.7) based on the MitoFish (Mitochondrial Genome Database of Fish), which is sometimes different from the FishBase and WoRMS (World Register of Marine Species) taxonomical system (Sato et al., 2018).

Increasing the molecular information database is very fundamental, especially in the 12S rRNA segment, which is the target sequence in the MiFish primer. More profound research on universal primers that produce longer sequences will further improve the accuracy of species detection in cryptic species such as the coral-reef fish in the tropical region. Also, the use of the same taxonomy system is expected to be agreed globally.

Table 4.6. Comparison of Taxonomic system within Perciformes in Fishbase, WoRMS, and MitoFish Platform

FishBase		WoRMS		MitoFish	
Order	Family	Order	Family	Order	Family
Perciformes	Acanthuridae	Perciformes	Acanthuridae	Acanthuriformes	Acanthuridae
	Acropomatidae		Acropomatidae	Pempheriformes	Acropomatidae
	Ambassidae		Ambassidae	nd	Ambassidae
	Apogonidae		Apogonidae	Kurtiformes	Apogonidae
	Blenniidae		Blenniidae	Blenniiformes	Blenniidae
	Caesionidae		Caesionidae	nd	Caesionidae
	Carangidae		Carangidae	Carangiformes	Carangidae
	Centrogenyidae		Centrogenyidae	nd	nd
	Chaetodontidae		Chaetodontidae	Chaetodontiformes	Chaetodontidae
	Cirrhitidae		Cirrhitidae	Centrarchifromes	Cirrhitidae
	Drepaneidae		Drepaneidae	nd	nd
	Eleotridae		Eleotridae	Gobiiformes	Eleotridae
	Ephippidae		Ephippidae	Ephippiformes	Ephippidae
	Gempylidae		Gempylidae	Scombriformes	Gempylidae
	Gerreidae		Gerreidae	Gerreiformes	Gerreidae
	Gobiidae		Gobiidae	Gobiiformes	Gobiidae
	Haemulidae		Haemulidae	Lutjaniformes	Haemulidae
	Istiophoridae		Istiophoridae	Pristiophoriformes	Istiophoridae
	Kyphosidae		Kyphosidae	Centrarchiformes	Kyphosidae
	Labridae		Labridae	Labriformes	Labridae
	Leiognathidae		Leiognathidae	Chaetodontiformes	Leiognathidae
	Lethrinidae		Lethrinidae	Spariformes	Lethrinidae
	Lobotidae		Lobotidae	Lobotiformes	Lobotidae
	Lutjanidae		Lutjanidae	Lutjaniformes	Lutjanidae
	Monodactylidae		Monodactylidae	nd	Monodactylidae
	Mullidae		Mullidae	Syngnathiformes	Mullidae
	Pempheridae		Pempheridae	Pempheriformes	Pempheridae
	Pinguipedidae		Pinguipedidae	nd	nd
	Polynemidae		Polynemidae	nd	Polynemidae
	Pomacanthidae		Pomacanthidae	nd	Pomacanthidae
	Priacanthidae		Priacanthidae	Priacanthiformes	Priacanthidae
	Scaridae		Scaridae	nd	nd
	Scatophagidae		Scatophagidae	nd	Scatophagidae
	Sciaenidae		Sciaenidae	nd	Sciaenidae
	Scombridae		Scombridae	Scombriformes	Scombridae
	Serranidae		Serranidae	Perciformes	Serranidae
	Siganidae		Siganidae	nd	Siganidae
	Sillaginidae		Sillaginidae	nd	Sillaginidae
	Sphyraenidae		Sphyraenidae	nd	Sphyraenidae
	Terapontidae		Terapontidae	Centrarchiformes	Terapontidae
Mugiliformes	Mugilidae	Perciformes	Mugilidae	nd	Mugilidae

nd : no data

4.5 Conclusion

The present study provided information regarding the biodiversity of the tropical marine ecosystem using the environmental DNA metabarcoding method from Indonesian water. The environmental DNA analysis in four sampling locations revealed 333 species in 405 representative haplotype (99-100 % identity) and 52 putative species (95-98% identity). The alpha biodiversity in Bali, both in South and North part, have higher than the Java sampling site. The biodiversity Shannon-Wiener Index and Pielou's Evenness Index in north Bali is highest. The Perciformes (72.84%) dominated in this study, followed by Clupeiformes (7.16%) and Tetaraodontiformes (4.69%). The Perciformes most diversity than others order, with 44 families had been identified by eDNA metabarcoding analysis. We performed the marine fish assessment efficiently and cost-effectively by using the eDNA metabarcoding approach from six-months monitoring during January-June 2018. Therefore, proper monitory and regular surveillance should be taken, which may increase the number of marine fish detection in this region. The biodiversity assessment in this region is very crucial to the policy-makers and biologists for sustainable coastal resources management.

Appendix

Appendix 1. Summary of merged reads degenerated by MiFish pipeline were identified on species (99-100% identity)

No.	Species Name	Identity (%)	Reads number station-wise			
			North Bali	South Bali	North Java	South Java
1	*Abudefduf bengalensis*	100	0	0	16	0
2	*Abudefduf sordidus*	100	0	0	0	741
3	*Abudefduf vaigiensis*	100	255	208	0	1437
4	*Acanthocybium solandri*	100	1038	337	0	55
5	*Acanthogobius flavimanus*	100	0	0	0	38
6	*Acanthurus dussumieri*	100	0	213	0	0
7	*Acanthurus lineatus*	100	11615	6541	0	443
8	*Acanthurus nigricauda*	100	1925	1115	0	0
9	*Aeoliscus strigatus*	100	429	0	0	0
10	*Aethaloperca rogaa*	100	0	11	0	0
11	*Alectis ciliaris*	100	553	53	0	0
12	*Alectis indica*	100	0	0	12	0
13	*Alepes djedaba*	100	227	0	61	0
14	*Alepes kleinii*	100	0	0	91	0
15	*Aluterus monoceros*	100	0	25	0	0
16	*Amatitlania nigrofasciata*	100	218	0	0	0
17	*Amblygaster sirm*	100	0	1337	0	122
18	*Amblyglyphidodon aureus*	100	0	0	0	21
19	*Ammodytes personatus*	100	2817	3210	52	1400
20	*Amphiprion ephippium*	100	0	51	0	0
21	*Anguilla marmorata*	100	0	1373	0	0
22	*Anodontostoma chacunda*	100	0	0	0	157
23	*Aphareus rutilans*	100	388	21	0	0
24	*Aplocheilus panchax*	100	132	1787	0	0
25	*Apogon crassiceps*	100	0	0	0	18
26	*Aprion virescens*	100	810	116	0	0
27	*Arctoscopus japonicus*	100	0	0	744	0
28	*Arothron manilensis*	100	0	138	0	0
29	*Arothron mappa*	100	0	0	0	83
30	*Arothron nigropunctatus*	100	113	0	0	0
31	*Atherinomorus aetholepis*	100	606	0	0	0
32	*Atherinomorus cf*	100	0	0	21	0
33	*Atherinomorus forskalii*	100	297	121	0	0
34	*Atherinomorus lacunosus*	100	34417	48125	0	1216
35	*Atherinomorus sp*	100	400	0	0	0
36	*Aurigequula fasciata*	100	2199	911	466	65
37	*Auxis rochei*	100	28461	12099	1357	0
38	*Balistapus undulatus*	100	444	196	0	0
39	*Bathygobius sp*	100	0	0	0	160
40	*Bolinichthys indicus*	100	0	0	0	181
41	*Butis koilomatodon*	100	0	0	144	0
42	*Caesio caerulaurea*	100	565	14	0	1848
43	*Caesio cuning*	100	1493	751	0	592
44	*Calotomus spinidens*	100	113	0	0	0
45	*Cantherhines macrocerus*	100	387	179	0	0
46	*Canthigaster solandri*	100	0	75	16	1121
47	*Carangichthys dinema*	100	0	0	0	32

Appendix 1. Continued

No.	Species Name	Identity (%)	Reads number station-wise			
			North Bali	South Bali	North Java	South Java
48	*Carangichthys oblongus*	100	126	0	0	0
49	*Carangoides chrysophrys*	100	598	20	0	0
50	*Carangoides coeruleopinnatus*	100	115	23	0	0
51	*Carangoides ferdau*	100	608	0	0	0
52	*Carangoides praeustus*	100	0	0	337	0
53	*Caranx bucculentus*	100	227	49	0	0
54	*Caranx heberi*	100	196	27	0	0
55	*Caranx ignobilis*	100	5964	560	0	14
56	*Caranx melampygus*	100	0	277	0	117
57	*Caranx tille*	100	0	211	0	0
58	*Cephalopholis argus*	100	0	917	0	0
59	*Cephalopholis cyanostigma*	100	0	46	0	0
60	*Cephalopholis sexmaculata*	100	0	211	0	0
61	*Cephalopholis sonnerati*	100	574	20	0	0
62	*Chaetodon adiergastos*	100	0	0	0	37
63	*Chaetodon auriga*	100	0	12	0	0
64	*Chaetodon kleinii*	100	817	18	0	99
65	*Chaetodon vagabundus*	100	404	0	0	0
66	*Chanos chanos*	100	0	21	0	0
67	*Cheilopogon cyanopterus*	100	11105	975	0	0
68	*Chelon subviridis*	100	813	28	7693	930
69	*Chelonodon patoca*	100	843	99	0	0
70	*Chirolophis japonicus*	100	0	40	0	0
71	*Cirrhitichthys oxycephalus*	100	0	0	0	80
72	*Congresox talabonoides*	100	0	0	32	26
73	*Ctenochaetus striatus*	100	571	476	0	131
74	*Cypselurus heterurus*	100	4845	1092	0	533
75	*Cypselurus starksi*	100	4572	1623	0	32
76	*Decapterus macarellus*	100	118	0	0	162
77	*Decapterus macrosoma*	100	18048	12709	0	276
78	*Decapterus russelli*	100	49704	26325	0	699
79	*Dendrochirus zebra*	100	0	0	0	46
80	*Dendrophysa russelii*	100	0	0	119	0
81	*Deveximentum indicum*	100	0	12	0	0
82	*Diagramma picta*	100	1103	444	0	0
83	*Diodon hystrix*	100	266	115	0	0
84	*Diodon liturosus*	100	0	0	0	19
85	*Doryrhamphus naia*	100	0	24	0	0
86	*Drepane punctata*	100	355	264	0	0
87	*Echidna nebulosa*	100	172	0	0	0
88	*Elagatis bipinnulata*	100	2392	845	0	0
89	*Encrasicholina devisi*	100	0	0	0	241
90	*Encrasicholina heteroloba*	100	0	0	0	80
91	*Encrasicholina punctifer*	100	0	0	769	2210
92	*Engraulis japonicus*	100	402	35	25384	15151
93	*Epinephelus areolatus*	100	1409	562	0	0
94	*Epinephelus bontoides*	100	123	0	0	51
95	*Epinephelus chlorostigma*	100	163	0	0	0
96	*Epinephelus epistictus*	100	0	34	0	0

Appendix 1. Continued

No.	Species Name	Identity (%)	Reads number station-wise			
			North Bali	South Bali	North Java	South Java
97	*Epinephelus fasciatus*	100	0	90	0	0
98	*Epinephelus maculatus*	100	0	104	0	0
99	*Epinephelus merra*	100	0	315	0	0
100	*Epinephelus ongus*	100	211	0	0	0
101	*Epinephelus polyphekadion*	100	0	37	0	0
102	*Epinephelus quoyanus*	100	124	0	0	0
103	*Epinephelus septemfasciatus*	100	1114	0	302	332
104	*Epinephelus sexfasciatus*	100	0	0	66	0
105	*Erisphex pottii*	100	0	346	0	0
106	*Escualosa thoracata*	100	9192	1313	32991	3128
107	*Eubleekeria splendens*	100	0	784	0	0
108	*Eubleekeria splendens*	100	0	0	0	341
109	*Euthynnus affinis*	100	2210	914	0	46
110	*Fistularia commersonii*	100	139	0	0	0
111	*Gazza minuta*	100	1500	3022	1151	0
112	*Gerres microphthalmus*	100	773	0	4425	221
113	*Gerres oyena*	100	0	20	0	0
114	*Gnathanodon speciosus*	100	2797	459	0	0
115	*Grammatorcynus bilineatus*	100	0	297	0	0
116	*Gymnothorax chilospilus*	100	0	0	0	1087
117	*Gymnothorax flavimarginatus*	100	1106	113	0	128
118	*Gymnothorax richardsonii*	100	1117	336	0	12
119	*Hemigymnus melapterus*	100	0	241	0	0
120	*Hemiramphus lutkei*	100	123	0	0	0
121	*Heniochus varius*	100	125	0	0	0
122	*Herklotsichthys quadrimaculatus*	100	0	11128	0	2669
123	*Hexagrammos stelleri*	100	149	0	225	0
124	*Hilsa kelee*	100	0	0	65	79
125	*Hypoatherina celebesensis*	100	0	101	0	0
126	*Hypoatherina lunata*	100	66044	36698	0	906
127	*Hypoatherina temminckii*	100	0	21	0	0
128	*Inimicus sinensis*	100	170	0	0	0
129	*Istigobius ornatus*	100	0	0	0	16
130	*Katsuwonus pelamis*	100	6539	1144	0	3201
131	*Konosirus punctatus*	100	0	0	23936	115
132	*Kyphosus cinerascens*	100	837	628	0	0
133	*Kyphosus cinerascens*	100	0	0	0	900
134	*Kyphosus vaigiensis*	100	0	8867	0	0
135	*Lactoria cornuta*	100	181	41	0	0
136	*Lampadena luminosa*	100	0	0	0	1014
137	*Lateolabrax japonicus*	100	0	0	0	1039
138	*Lateolabrax maculatus*	100	0	0	0	466
139	*Leptoscarus vaigiensis*	100	493	7255	0	33
140	*Lethrinus atkinsoni*	100	0	143	0	0
141	*Lethrinus cf*	100	486	102	0	0
142	*Lethrinus erythracanthus*	100	1102	163	0	0
143	*Lethrinus genivittatus*	100	1050	154	0	0

Appendix 1. Continued

No.	Species Name	Identity (%)	Reads number station-wise			
			North Bali	South Bali	North Java	South Java
144	*Lethrinus harak*	100	2728	530	0	213
145	*Lethrinus obsoletus*	100	2520	1011	0	16
146	*Lethrinus ravus*	100	0	31	0	0
147	*Lethrinus reticulatus*	100	356	136	0	0
148	*Lethrinus rubrioperculatus*	100	0	365	0	0
149	*Liza macrolepis*	100	10436	0	0	0
150	*Liza macrolepis*	100	0	0	0	51292
151	*Lobotes surinamensis*	100	150	23	0	0
152	*Lobotes surinamensis*	100	0	0	0	0
153	*Lutjanus argentimaculatus*	100	648	541	38	5018
154	*Lutjanus bengalensis*	100	544	0	0	0
155	*Lutjanus bohar*	100	3214	1822	0	57
156	*Lutjanus decussatus*	100	0	1487	0	35
157	*Lutjanus erythropterus*	100	282	55	0	0
158	*Lutjanus fulviflamma*	100	1303	5741	0	52
159	*Lutjanus fulvus*	100	0	137	0	0
160	*Lutjanus gibbus*	100	0	0	0	67
161	*Lutjanus johnii*	100	0	49	0	0
162	*Lutjanus johnii*	100	582	0	0	0
163	*Lutjanus lutjanus*	100	1214	1361	38	136
164	*Lutjanus malabaricus*	100	4591	2289	0	58
165	*Lutjanus rivulatus*	100	0	0	0	38
166	*Lutjanus rufolineatus*	100	542	139	0	0
167	*Lutjanus russellii*	100	0	0	4198	0
168	*Lutjanus sebae*	100	313	0	0	0
169	*Lutjanus vitta*	100	383	70	0	0
170	*Macolor macularis*	100	147	397	0	0
171	*Macolor niger*	100		22	0	0
172	*Maurolicus muelleri*	100	12366	19432	1808	0
173	*Melichthys vidua*	100	392	0	0	0
174	*Micropterus salmoides*	100	0	24	0	0
175	*Monodactylus argenteus*	100	0	1450	0	0
176	*Monotaxis grandoculis*	100	0	12	0	0
177	*Mugil cephalus*	100	439	126	1150	2440
178	*Mulloidichthys flavolineatus*	100	151	2103	0	0
179	*Myripristis berndti*	100	0	0	0	124
180	*Naso lopezi*	100	0	355	0	0
181	*Naso mcdadei*	100	313	474	0	172
182	*Naso thynnoides*	100	37	35	0	0
183	*Naso vlamingii*	100	395	17	0	0
184	*Neopomacentrus cyanomos*	100	0	0	0	1107
185	*Nuchequula flavaxilla*	100	0	0	281	0
186	*Nuchequula longicornis*	100	150	0	1035	0
187	*Nuchequula nuchalis*	100	0	0	0	907
188	*Nuchequula sp*	100	157	0	1025	0
189	*Odontanthias borbonius*	100	0	197	0	0
190	*Odonus niger*	100	0	0	0	1025

Appendix 1. Continued

No.	Species Name	Identity (%)	Reads number station-wise			
			North Bali	South Bali	North Java	South Java
191	*Oedalechilus labiosus*	100	0	0	0	6023
192	*Omobranchus zebra*	100	343	0	792	0
193	*Ophieleotris sp*	100	125	0	0	0
194	*Ophiocara porocephala*	100	150	728	0	0
195	*Ostorhinchus fasciatus*	100	0	0	299	0
196	*Ostorhinchus moluccensis*	100	894	4482	0	0
197	*Ostorhinchus pleuron*	100	0	0	0	29
198	*Oxyporhamphus convexus*	100	837	0	0	321
199	*Paracaesio stonei*	100	0	356	0	0
200	*Paralichthys olivaceus*	100	207	0	82	6134
201	*Paramonacanthus otisensis*	100	476	0	0	0
202	*Parapterois heterura*	100	0	0	0	883
203	*Parexocoetus mento*	100	0	0	0	23
204	*Parupeneus barberinoides*	100	291	0	0	0
205	*Parupeneus heptacanthus*	100	0	0	73	103
206	*Parupeneus indicus*	100	2591	1398	0	131
207	*Parupeneus multifasciatus*	100	0	123	0	0
208	*Pempheris schwenkii*	100	0	297	0	13
209	*Pempheris vanicolensis*	100	117	0	0	65
210	*Petroscirtes breviceps*	100	1323	733	0	32
211	*Pholis nebulosa*	100	0	0	0	40
212	*Pinjalo pinjalo*	100	370	57	0	0
213	*Platax orbicularis*	100	930	249	0	570
214	*Platax pinnatus*	100	0	18	0	0
215	*Platax teira*	100	14088	0	0	194
216	*Plectorhinchus chaetodonoides*	100	0	0	0	19
217	*Plectorhinchus gibbosus*	100	79	0	0	0
218	*Plectroglyphidodon lacrymatus*	100	0	70	0	0
219	*Plectropomus areolatus*	100	158	0	0	0
220	*Plectropomus leopardus*	100	233	0	0	0
221	*Plotosus lineatus*	100	195	476	0	0
222	*Polydactylus plebeius*	100	256	606	0	0
223	*Pomacanthus semicirculatus*	100	1599	0	0	44
224	*Pomacentrus coelestis*	100	0	0	0	194
225	*Pomacentrus tripunctatus*	100	1772	317	6017	1768
226	*Pomadasys kaakan*	100	882	37	307	0
227	*Priacanthus hamrur*	100	0	26	0	0
228	*Pristiapogon exostigma*	100	127	0	0	0
229	*Pristipomoides filamentosus*	100	576	17	0	0
230	*Pristipomoides multidens*	100	742	999	0	34
231	*Pristipomoides typus*	100	0	872	290	16
232	*Protonibea diacanthus*	100	122	0	0	0
233	*Psettodes erumei*	100	414	46	0	0
234	*Pseudaesopia japonica*	100	0	0	0	1419
235	*Pseudobalistes flavimarginatus*	100	1953	2296	0	2280

Appendix 1. Continued

No.	Species Name	Identity (%)	Reads number station-wise			
			North Bali	South Bali	North Java	South Java
236	*Pseudogobius javanicus*	100	927	0	0	0
237	*Pseudopleuronectes yokohamae*	100	0	0	0	737
238	*Pseudorasbora parva*	100	0	89	0	0
239	*Pterocaesio digramma*	100	117	0	0	0
240	*Pterocaesio digramma*	100	0	0	0	708
241	*Pterocaesio marri*	100	0	0	0	72
242	*Pterocaesio tessellata*	100	0	0	0	301
243	*Pterois antennata*	100	0	37	0	0
244	*Pterois volitans*	100	0	0	20	0
245	*Rastrelliger brachysoma*	100	0	20	0	0
246	*Rastrelliger kanagurta*	100	3943	1496	676	418
247	*Rhinecanthus verrucosus*	100	1447	138	0	0
248	*Rhinogobius sp*	100	0	31	0	0
249	*Sarda orientalis*	100	3027	352	0	0
250	*Sardinella albella*	100	0	3456	55	0
251	*Sardinella hualiensis*	100	779	17	1129	136
252	*Sardinella lemuru*	100	392	438	0	0
253	*Sargocentron rubrum*	100	0	0	23	0
254	*Scarus ghobban*	100	116	0	0	0
255	*Scatophagus argus*	100	1271	426	0	1293
256	*Scomber japonicus*	100	658	469	895	31
257	*Scomber scombrus*	100	0	0	18	453
258	*Scomberoides lysan*	100	166	70	0	0
259	*Scomberoides tol*	100	0	54	0	0
260	*Scomberomorus niphonius*	100	127	0	0	0
261	*Scomberomorus sinensis*	100	0	0	0	77
262	*Scorpaena neglecta*	100	0	0	71	0
263	*Scorpaenodes guamensis*	100	0	121	0	227
264	*Sebastes zacentrus*	100	0	0	0	824
265	*Secutor hanedai*	100	265	0	0	0
266	*Secutor megalolepis*	100	231	0	0	0
267	*Selar boops*	100	355	940	0	34797
268	*Selar crumenophthalmus*	100	587	499	73	112
269	*Selaroides leptolepis*	100	460	51	0	0
270	*Seriola rivoliana*	100	0	853	0	36
271	*Setipinna taty*	100	1470	31	268	43
272	*Siganus argenteus*	100	2440	14137	0	0
273	*Siganus canaliculatus*	100	11464	1010	64579	4379
274	*Siganus fuscescens*	100	20542	5824	92201	7265
275	*Siganus punctatus*	100	414	0	0	0
276	*Siganus spinus*	100	190	0	0	0
277	*Siganus vermiculatus*	100	1834	115	0	1391
278	*Sillago japonica*	100	460	0	661	52
279	*Sphyraena jello*	100	926	1169	0	183
280	*Sufflamen fraenatum*	100	0	14	0	0
281	*Taeniamia fucata*	100	0	247	0	87
282	*Terapon jarbua*	100	8056	2200	34736	3313

Appendix 1. Continued

No.	Species Name	Identity (%)	Reads number station-wise			
			North Bali	South Bali	North Java	South Java
283	*Terapon theraps*	100	171	539	0	0
284	*Tetrapturus sp*	100	19709	6433	0	1011
285	*Thryssa baelama*	100	353	1093	0	38
286	*Thryssa setirostris*	100	0	0	31	0
287	*Thunnus maccoyii*	100	183	0	0	0
288	*Thunnus obesus*	100	77342	94408	2130	0
289	*Thunnus thynnus*	100	0	0	564	0
290	*Thunnus tonggol*	100	20	237	0	0
291	*Trachinotus baillonii*	100	1300	330	0	0
292	*Trachinotus blochii*	100	1258	17	0	0
293	*Tribolodon hakonensis*	100	0	0	0	209
294	*Trichopodus trichopterus*	100	249	0	0	0
295	*Tridentiger obscurus*	100	211	0	0	0
296	*Tylosurus acus*	100	171	127	0	0
297	*Tylosurus crocodilus*	100	1264	28236	0	599
298	*Ulua mentalis*	100	0	69	0	0
299	*Upeneus guttatus*	100	0	0	0	95
300	*Upeneus sulphureus*	100	371	282	2772	5772
301	*Upeneus tragula*	100	0	292	0	39
302	*Upeneus vittatus*	100	0	26	0	0
303	*Variola albimarginata*	100	0	16	0	0
304	*Xiphias gladius*	100	721	0	0	0
305	*Ablabys taenianotus*	99	122	0	0	0
306	*Abudefduf sordidus*	99	0	0	0	631
307	*Acanthurus bariene*	99	0	207	0	123
308	*Acanthurus lineatus*	99	0	0	0	529
309	*Acanthurus mata*	99	512	31	0	1049
310	*Acanthurus xanthopterus*	99	2391	1598	0	49156
311	*Acentrogobius pflaumii*	99	0	66	0	192
312	*Acheilognathus intermedia*	99	0	0	31	0
313	*Acheilognathus signifer*	99	603	0	0	0
314	*Acreichthys tomentosus*	99	0	0	17	0
315	*Acropoma japonicum*	99	0	99	0	0
316	*Ammodytes personatus*	99	0	0	0	577
317	*Anodontostoma chacunda*	99	0	0	53	0
318	*Arctoscopus japonicus*	99	109	0	0	0
319	*Arius oetik*	99	0	0	150	170
320	*Arothron hispidus*	99	277	256	0	0
321	*Atherinomorus lacunosus*	99	0	0	0	470
322	*Atherinomorus sp*	99	1117	1002	0	0
323	*Aurigequula fasciata*	99	125	0	0	0
324	*Auxis thazard*	99	2923	613	0	11630
325	*Bathygobius sp*	99	0	0	26	264
326	*Benthosema pterotum*	99	365	0	0	0
327	*Bregmaceros arabicus*	99	173	0	0	0
328	*Caesio caerulaurea*	99	0	0	0	105
329	*Calotomus spinidens*	99	11172	214	0	0

Appendix 1. Continued

No.	Species Name	Identity (%)	Reads number station-wise			
			North Bali	South Bali	North Java	South Java
330	*Caranx ignobilis*	99	0	60	0	0
331	*Caranx tille*	99	140	0	0	0
332	*Centrogenys vaigiensis*	99	350	0	0	0
333	*Cephalopholis sexmaculata*	99	135	0	0	0
334	*Channa striata*	99	0	85	0	0
335	*Chelon subviridis*	99	1453	66	0	0
336	*Clarias sp*	99	174	29	0	0
337	*Clupea pallasii*	99	0	0	0	1062
338	*Ctenochaetus binotatus*	99	0	0	0	50
339	*Culter dabryi*	99	0	0	0	468
340	*Decapterus macarellus*	99	0	16	0	0
341	*Decapterus macrosoma*	99	932	0	0	0
342	*Decapterus russelli*	99	676	0	0	0
343	*Dendrophysa russelii*	99	156	0	0	0
344	*Ditrema viride*	99	0	0	1337	0
345	*Echidna nebulosa*	99	0	0	0	340
346	*Ellochelon vaigiensis*	99	0	0	341	94
347	*Engraulis japonicus*	99	7182	16421	173	0
348	*Engraulis mordax*	99	0	0	77	0
349	*Epinephelus akaara*	99	0	0	148	47
350	*Epinephelus awoara*	99	722	27	0	0
351	*Epinephelus chlorostigma*	99	0	33	0	0
352	*Epinephelus fuscoguttatus*	99	265	155	0	0
353	*Epinephelus malabaricus*	99	173	21	0	90
354	*Epinephelus ongus*	99	0	305	0	0
355	*Epinephelus quoyanus*	99	0	150	0	0
356	*Escualosa thoracata*	99	0	31	0	0
357	*Eubleekeria splendens*	99	0	111	0	0
358	*Gazza rhombea*	99	408	17	90	0
359	*Gerres decacanthus*	99	0	0	38	0
360	*Gymnothorax chilospilus*	99	934	0	0	0
361	*Gymnothorax flavimarginatus*	99	0	0	579	0
362	*Hemibarbus umbrifer*	99	1949	0	0	0
363	*Hemiramphus lutkei*	99	811	497	0	0
364	*Herklotsichthys quadrimaculatus*	99	742	0	0	0
365	*Hexagrammos stelleri*	99	0	0	0	20
366	*Hilsa kelee*	99	293	74	351	0
367	*Hyporhamphus sajori*	99	0	0	0	217
368	*Istiblennius edentulus*	99	0	0	0	1012
369	*Kyphosus cinerascens*	99	3751	0	0	0
370	*Kyphosus pacificus*	99	140	0	0	0
371	*Lepidocybium flavobrunneum*	99	4509	445	0	0
372	*Lethrinus cf*	99	4357	802	331	17
373	*Lethrinus harak*	99	196	0	0	0
374	*Lethrinus lentjan*	99	254	545	0	16
375	*Lutjanus argentimaculatus*	99	194	40	218	0

Appendix 1. Continued

No.	Species Name	Identity (%)	Reads number station-wise			
			North Bali	South Bali	North Java	South Java
376	*Maurolicus japonicus*	99	1857	96	508	180
377	*Maurolicus muelleri*	99	245	0	12	28532
378	*Megalaspis cordyla*	99	0	41	0	0
379	*Mugil cephalus*	99	159	0	2067	0
380	*Muraenesox bagio*	99	2132	0	0	0
381	*Myripristis murdjan*	99	0	0	0	565
382	*Naso unicornis*	99	630	1213	0	28
383	*Naso vlamingii*	99	0	732	0	0
384	*Nematalosa come*	99	9161	4963	0	142
385	*Neopomacentrus azysron*	99	0	0	0	781
386	*Neopomacentrus cyanomos*	99	0	0	0	1431
387	*Nuchequula longicornis*	99	0	0	0	186
388	*Nuchequula nuchalis*	99	0	24	0	0
389	*Odontobutis platycephala*	99	193	0	0	0
390	*Oedalechilus labiosus*	99	0	0	0	479
391	*Omobranchus punctatus*	99	592	380	790	116
392	*Omobranchus zebra*	99	0	0	0	19
393	*Opsariichthys uncirostris*	99	121	0	80	0
394	*Ostorhinchus cookii*	99	672	0	0	4527
395	*Ostorhinchus moluccensis*	99	225	114	0	233
396	*Ostorhinchus taeniophorus*	99	194	0	0	0
397	*Otolithes ruber*	99	0	0	18	0
398	*Oxyurichthys sp*	99	369	0	0	0
399	*Paracaesio stonei*	99	0	18	0	0
400	*Paramonacanthus otisensis*	99	2225	2409	66	212
401	*Platax teira*	99	0	49	263	0
402	*Plectorhinchus gibbosus*	99	0	18	0	0
403	*Plectropomus oligacanthus*	99	622	34	0	0
404	*Pleuronectes pinnifasciatus*	99	0	0	0	18
405	*Plotosus canius*	99	0	0	12	0
406	*Pomacanthus semicirculatus*	99	902	0	0	0
407	*Pomacentrus moluccensis*	99	0	0	0	1229
408	*Pomacentrus nagasakiensis*	99	0	0	0	68
409	*Pomacentrus tripunctatus*	99	283	0	0	0
410	*Pseudaesopia japonica*	99	0	0	109	0
411	*Pseudanthias squamipinnis*	99	0	0	0	659
412	*Pseudobalistes flavimarginatus*	99	0	0	0	1152
413	*Pseudorasbora parva*	99	391	0	0	0
414	*Pterocaesio digramma*	99	0	0	0	627
415	*Rastrelliger kanagurta*	99	3932	0	18	0
416	*Rhinogobius sp*	99	1499	0	0	0
417	*Sarda chiliensis*	99	155	23	0	0
418	*Sardinella albella*	99	0	0	142	0
419	*Sardinella hualiensis*	99	0	152	167	0
420	*Sargocentron rubrum*	99	0	0	0	168

Appendix 1. Continued

No.	Species Name	Identity (%)	Reads number station-wise			
			North Bali	South Bali	North Java	South Java
421	*Scatophagus argus*	99	293	0	873	0
422	*Scomberoides lysan*	99	0	17	0	0
423	*Scomberoides tol*	99	0	0	38	0
424	*Scomberomorus sinensis*	99	2203	1607	0	38
425	*Scorpaena miostoma*	99	0	729	0	0
426	*Scorpaenopsis ramaraoi*	99	152	0	0	0
427	*Selar boops*	99	2922	0	488	0
428	*Seriola quinqueradiata*	99	205	0	10229	0
429	*Setipinna taty*	99	129	0	0	0
430	*Siganus woodlandi*	99	0	19	0	0
431	*Sphyraena jello*	99	0	365	0	0
432	*Spratelloides delicatulus*	99	197	0	0	0
433	*Spratelloides gracilis*	99	2729	5347	0	18
434	*Squalidus japonicus*	99	0	0	70	0
435	*Stethojulis interrupta*	99	0	0	5664	0
436	*Stolephorus brachycephalus*	99	186	707	0	0
437	*Strongylura incisa*	99	0	0	0	44
438	*Syngnathoides biaculeatus*	99	0	12	0	0
439	*Taeniamia fucata*	99	137	0	0	0
440	*Terapon jarbua*	99	459	831	0	0
441	*Thunnus maccoyii*	99	0	170	0	0
442	*Thunnus orientalis*	99	238	795	0	0
443	*Thunnus thynnus*	99	79	256	0	0
444	*Thunnus tonggol*	99	42	0	0	0
445	*Thryssa baelama*	99	798	0	413	178
446	*Trachinotus blochii*	99	535	159	0	0
447	*Tridentiger obscurus*	99	2141	0	0	0
448	*Tylosurus crocodilus*	99	1954	256	8657	630
448	*Upeneus vittatus*	99	156	561	0	2075
449	*Yongeichthys criniger*	99	0	28	0	0

Appendix 2. Summary of merged reads generated by MiFish pipeline were identified on putative species (95-98% identity)

No.	Species name	Identity (%)	Reads number station-wise			
			North Bali	South Bali	North Java	South Java
1	*Acanthurus* sp.	98	0	0	0	12
2	*Atherinomorus* sp.	98	36400	89729	0	31
3	*Barbodes* sp.	98	533	32	0	0
4	*Bathygobius* sp.	98	0	0	0	701
5	*Cantherhines* sp.	98	869	0	0	0
6	*Carangoides* sp.	98	116	0	0	0
7	*Caranx* sp.	98	0	482	0	0
8	*Chelon* sp.	98	0	1792	0	0
9	*Ctenochaetus* sp.	98	0	0	0	40
10	*Decapterus* sp.	98	132	23	0	0
11	*Eleotris* sp.	98	158	253	0	0
12	*Eleutheronema* sp.	98	0	0	139	0
13	*Engraulis* sp.	98	1578	0	0	0
14	*Epinephelus* sp.	98	191	93	0	0
15	*Euthynnus* sp.	98	134	0	0	0
16	*Gazza* sp.	98	0	0	1112	0
17	*Glaucosoma* sp.	98	381	0	0	0
18	*Liza* sp.	98	0	1254	0	0
19	*Lutjanus* sp.	98	633	187	0	102
20	*Moolgarda* sp.	98	136	28	33	1066
21	*Myersina* sp.	98	0	0	0	216
22	*Oedalechilus* sp.	98	1752	0	0	0
23	*Parascorpaena* sp.	98	0	36	0	0
24	*Pempheris* sp.	98	0	83	0	0
25	*Plectorhinchus* sp.	98	243	21	0	0
26	*Scolecenchelys* sp.	98	120	0	0	0
27	*Stolephorus* sp.	98	4676	0	143	0
28	*Thunnus thynnus*	98	52	216	0	0
29	*Triacanthus* sp.	98	0	81	0	0
30	*Acanthurus* sp.	97	0	0	0	624
31	*Caranx* sp.	97	576	0	0	0
32	*Epinephelus* sp.	97	0	19	0	0
33	*Eubleekeria* sp.	97	0	0	171	0
34	*Gazza* sp.	97	0	0	149	0
35	*Herklotsichthys* sp.	97	3442	7229	0	0
36	*Hirundichthys* sp.	97	233	0	0	0
37	*Istiblennius* sp.	97	0	58	0	0
38	*Lates* sp.	97	112	0	0	0
39	*Lethrinus* sp.	97	129	191	0	0
40	*Lutjanus* sp.	97	0	430	0	0
41	*Maurolicus* sp.	97	0	745	0	0
42	*Ostorhinchus* sp.	97	404	0	0	1330
43	*Pempheris* sp.	97	168	19	0	111
44	*Pinjalo* sp.	97	0	62	0	0
45	*Siganus* sp.	97	0	0	22	0
46	*Sphyraena* sp.	97	0	517	0	0
47	*Stolephorus* sp.	97	0	30	118	0
48	*Ambassis* sp. (unidentified)	96	0	47	80	0
49	*Anyperodon* sp. (unidentified)	96	428	49	0	0
50	*Atherinomorus* sp. (unidentified)	96	112	0	0	0

Appendix 2. Continued

No.	Species name	Identity (%)	Reads number station-wise			
			North Bali	South Bali	North Java	South Java
51	*Carangoides* sp. (unidentified)	96	113	100	0	0
52	*Echidna* sp. (unidentified)	96	0	45	0	0
53	*Eubleekeria* sp. (unidentified)	96	8363	58619	0	0
54	*Gerres* sp. (unidentified)	96	1123	290	0	0
55	*Jaydia* sp. (unidentified)	96	526	0	636	0
56	*Lethrinus* sp. (unidentified)	96	1935	1434	0	970
57	*Liza* sp. (unidentified)	96	4785	489	0	0
58	*Nemipterus* sp. (unidentified)	96	0	15	0	0
59	*Neopomacentrus* sp. (unidentified)	96	0	0	0	36
60	*Ostorhinchus* sp. (unidentified)	96	139	0	0	0
61	*Ostorhinchus* sp. (unidentified)	96	217	0	0	0
62	*Parascorpaena* sp. (unidentified)	96	0	0	66	0
63	*Parupeneus* sp. (unidentified)	96	0	23	0	0
64	*Pinjalo* sp. (unidentified)	96	366	0	0	0
65	*Platycephalus* sp. (unidentified)	96	0	0	0	142
66	*Prognichthys* sp. (unidentified)	96	119	0	0	0
67	*Pseudochromis* sp. (unidentified)	96	268	0	0	0
68	*Scartelaos* sp. (unidentified)	96	242	747	8427	171
69	*Scomberoides* sp. (unidentified)	96	181	0	0	0
70	*Sebastapistes* sp. (unidentified)	96	14	0	0	0
71	*Sphyraena* sp. (unidentified)	96	2118	693	107	13
72	*Stolephorus* sp. (unidentified)	96	450	0	0	0
73	*Synodus* sp. (unidentified)	96	122	0	0	0
74	*Taeniamia* sp. (unidentified)	96	0	803	0	0
75	*Arctoscopus* sp. (unidentified)	95	0	0	0	160
76	*Auxis* sp. (unidentified)	95	143	0	0	0
77	*Equulites* sp. (unidentified)	95	1126	108	0	0
78	*Hyporhamphus* sp. (unidentified)	95	124	0	0	0
79	*Myctophum* sp. (unidentified)	95	121	0	0	0
80	*Parapercis* sp. (unidentified)	95	0	0	0	167
81	*Parascorpaena* sp. (unidentified)	95	172	0	0	0
82	*Scartelaos* sp. (unidentified)	95	200	0	0	0
83	*Sphyraena* sp. (unidentified)	95	0	0	0	735
84	*Sphyraena* sp. (unidentified)	95	0	67	0	0
85	*Stolephorus* sp. (unidentified)	95	0	0	27	0
86	*Taeniamia* sp. (unidentified)	95	350	263	0	0
87	*Taenioides* sp. (unidentified)	95	0	0	0	26
88	*Thryssa* sp. (unidentified)	95	0	0	57	0
89	*Thunnus maccoyii*	95	0	312	0	0

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Molecular studies on marine fish diversity in Java and Bali, Indonesia

인도네시아 자바와 발리의 해양 어류 다양성에 관한 분자생물학적 연구

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요약

해양생태계는 유전적 생물다양성에 있어서 담수 및 육지생태계보다 상당한 기여를 하고 있다. 수산업 및 수산관리자원 등 해양생태계의 생물군으로부터 경제적 이익 또한 창출된다. 뿐만 아니라, 해양생태계에서 유래한 다수의 제품들 또한 인간에게 유용한 것(예를 들어, 식품 및 의약품) 및 산업적 소재로 이용된다. 특정 해양생태계의 어류 다양성은 그 생태계 건강성을 나타내는 것으로 정기적인 관찰이 필요하다. 인도네시아는 엄청난 양의 산호초생태계를 자랑하는 해양생물 다양성의 중심지이다.

이전 연구에서 인도네시아의 해양생태계 생물종은 2057 종으로 알려졌었다. 최신의 어류 데이터베이스에서도 3611 종으로 기록되어 인도네시아가 산호초 어업에 있어서 가장 어류자원이 풍부한 나라임을 알 수 있다. 전통적으로, 해양 생물다양성을 추정하기 위해서 바닥 트롤과 로테논 중독 (rotenone poisoning)을 사용하였는데, 이는

특정 지역에 몇 가지 제한이 있습니다. 그러나, 개발도상국이 처한 현실은 지역 분류학자의 부족으로 형태학적 종 규명이 어렵다. 기존의 조사와 지리정보 시스템 기술을 결합한 이전 연구는 어류 분포 및 공간 분석을 이해하기 위해 수행되었다.

현재, 환경 DNA 메타바코딩 분석을 통해 수생생태계의 생물다양성을 모니터링하는 정교한 방법이 개발되어 있다. 해양환경에서 직접 수집한 생물입자의 유전물질 추출 및 분석을 통해 해양어류의 모니터링 및 연구는 시간과 비용을 절약하는 생물다양성 평가에 있어 효과적인 대안이다. 수많은 연구자가 일부 멸종 위기종, 침입종 및 해양어류 분포의 탐지연구를 위해 환경 DNA 메타바코딩을 사용하여 정확도를 입증하였다. 이 연구에서 환경 DNA 메타바코딩은 MiFish 파이프라인 데이터를 이용하여 인도네시아 발리섬의 해양채집 샘플에 의해 수행되었다.

4개 채집정점의 환경DNA 분석은 405개의 haplotype(99-100 % 정확도)에서 333종과 52 개의 추정종(95~98%)을 발견하였다. 발리의 남부와 북부 모두 알파 다양성이 자바섬보다 높았다. 발리 북부의 Shannon-Wiener Index 와 Margalef Index 가 가장 높았다. 이 연구에서 농어목이 295 haplotypes(72.84%)으로 가장 많은 부분을 차지했고, 청어목 29 haplotypes(7.16%), 복어목 19 haplotypes(4.69%) 순으로 나타났다. 농어목이 환경 DNA 메타바코딩을 통해 44 속으로 확인되어 다른 어류목보다 가장 다양성이 높게 나타났다. 발리섬 북부와 남부의 산호초, 해초 및

맹그로브 생태계가 발리섬의 해양생물다양성을 뒷받침하고 있다. Lovina 해변의 북측부분이 가장 생물다양성이 높은 것으로 이번 연구결과를 통해 나타났다. 결론적으로, 발리해협 주변의 환경 DNA 메타바코딩을 통해 그 지역 열대해양어류의 종다양성을 효과적이고 정확도가 높게 확인할 수 있었다. NGS 기반 열대해양어류 연구 특히 산호초어류에서 미확인된 종 연구에서 GenBank 데이터베이스의 개선과 더불어 더 정확한 종 확인이 이루어 질 수 있을 것이다. 따라서 적절한 모니터링 및 정기적인 조사가 이루어진다면, 이 지역에서 확인할 수 있는 해양어류 종 수가 늘어날 것이다. 이 지역의 생물다양성 연구는 연안 자원 유지관리 정책 입안자에게 중대한 자료를 제공할 것이다.