Preliminary Study on eDNA of the Coelacanth's Habitat Around Deep Sea Conservation Areas of North Sulawesi, Indonesia

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ABSTRACT: Indonesian marine waters have lots of resources, especially rocky fishes including ornamental fishes. However, the utilization levels of these resources are difficult to be assessed due to the limitation of technology and budget. The new technology called Environmental Deoxyribo Nucleic Acid (eDNA) approaches is a promising one that is expected to overcome those issues because of their efficiency in getting results and cost effectiveness. Here, we report the results of our preliminary researches on deep sea eDNA. We collected from 10 stations ranging in 110-200m depths from two areas in North Sulawesi where many coelacanths have been discovered by the Green Eye Project on ecological and distribution studies of Indonesian coelacanths, using Nansen bottle water sampler (1500cc). The collected waters were filtered using Power Water Sterivex DNA Isolation Kits and preserved with the DNAiso Reagent, then were transported to Center for Strategy Research Project, University of the Ryukyus, Okinawa, Japan, where eDNA analyses were conducted. Our preliminary results revealed that the concentrations of eDNA are good, indicating eDNA was successfully extracted. Furthermore, 40 species were detection by using a high through put Illumina MiSeq platform for sequencing analyses, even though we couldn't have detected any coelacanth information.

Key words: deep-sea, environmental DNA, and nansen water sampler

INTRODUCTION

Indonesia as a country has contains of the highest marine biodiversity on the planet laid between 6^0 E to 10^0 S and from 95^0 E to 142^0 E comprises about 18.110 islands with coastlines of about 108.920 km. About 78 % of the Indonesia waters cover territory with shallow seas in the western and eastern parts, the Sunda and Sahul plates separated by the deep Banda Sea is part and center of the Coral Triangle of the earth, which covers only about 2 percent of the global ocean but comprises 76 percent of all known coral species. Although some of the donator from all of the world are giving attention to protect the region's critical biological diversity and marine-dependent livelihoods (Anonymous 2004).

Indonesia has a strategic role as a world maritime shaft in the global supply chain system to connect the Asia-Pacific area with Australia, since it is located between the Asian Continent and the Australia Continent, and also between the Pacific Ocean and the Indian Ocean. With those potentials, God blesses Indonesia with immense marine resources including the biggest marine biodiversity, to control those marine resources abundance; the lots of the budget and times are needed.

A new technology approach can be overcoming of this problem were available can be implemented at around Indonesian marine waters by using the environmental DNA approach. Environmental DNA (eDNA) in aquatic environments refers to genetic material found in the water column. In the case of multicellular organisms, eDNA originates from various sources, such as metabolic waste, damaged tissue or sloughed skin cells (Kelly et al., 2014) (Ficetolla et al., 2008) Ficetola *et al.* [2] was the first study demonstrating the use of eDNA for detecting an aquatic vertebrate species (invasive American bullfrog) from controlled environments and natural wetland, published in 2008. However, this technology is still rare implementation at marine water especially for deep-sea water.

Fig. 1. Map of collection sites of Deep-sea water sampler collected from two areas at Lolak Waters and Manado Bay North of Sulawesi, Indonesia.

Here we would like to predict the coelacanth (*Latimeria menadoensis*) and the others marine fisheries resources existence around the discovered coelacanth sites since years 2006-2015, and also is a potential fishing location by traditional fishermen of the both areas explained above by using the new and sophisticated technology of eDNA. If can be implementation of this technology around Indonesian marine waters, then the research activities of the marine biodiversity abundance will become more efficient and effective in relationship with the budget and times.

METHODOLOGY

Deep-sea water sampling was collected from 10 sites ranging from 110m-200m in depth (table 1.) at front side of the International Coelacanth Research Center and Museum Base at Lolak Waters and Manado Bay North of Sulawesi using Nansen Bottle Sampler (1500 cc) as shown in Fig. 2. The positions were follows the discovered of coelacanth by Green Eye Project on 2007-2015, (Masamitsu I. *et al*.)

The collected waters were filtered using Power Water Sterivex DNA Isolation Kits (Fig.2) and preserved with the DNAiso Reagent and kept in a deep freezer -25^0 C at Faculty of Fisheries and Marine Science, Sam Ratulangi University, until they were transported to Center for Strategy Research Project, University of the Ryukyus, Okinawa, where eDNA analyses were conducted following MiFish protocol at (Miya M. *et al.*2018). Therefore, e-DNA extraction was conducted at Center for Strategy Research Project at University of the Ryukyus Okinawa Japan by using Power Water Sterivex DNA Isolation Kit Samples by followed its protocol as follow (Anonymous, 1993).

Fig. 2. Research activities in the field collections and at Laboratory of Ryukyu University.

We conducted electrophoresis for each part 1st-PCR and 2nd-PCR to amplify the

Intense Signal of MiFish eDNA by Using Universal Primers MiFish-U-F/R, then it was used for MiSeq sequencing process. All the sequence was analyzed for sequence identity at taxonomic assignment processes.

RESULTS AND DISCUSSIONS

The results show us a good quality of DNA concentration of water samples after extraction. The eDNA water sampler was collected from those two areas, Manado Bay and Lolak waters, Sulawesi sea of Indonesia, were each collections site has five stations as shown in Table. 1. And we become more exiting cause the electrophoresis of each part 1st-PCR and 2nd-PCR amplification products show as the sign of the target sequence bp (Fig. 3). As positive control the river samples as exhibited intense signal of MiFish eDNA amplification and negative controls (DW) showed no clear bands. Suggestions that no contamination cleared and feasible to be continued to next of the genome work.

Some number of the species has been analyses at The Center the Strategic research Projects of University of the Ryukyus.

Table 1. Results of collections site, satellite positions, sea`s depth and the number of fish's species was detected at Lolak Waters and Manado Bay North of Sulawesi.

Stations	Positions (Degrees)		Sea's Depth (Meter)	Species (Number)
	Latitude (N)	Longitude (E)		
1	0.9298	124.0351	115	8
2	0.9338	124.0066	150	6
3	0.9339	123.9961	196	14
4	0.9217	124.9889	170	2
5	0.9128	123.9869	110	11
6	1.4699	124.8199	95	3
7	1.4707	124.8179	120	2
8	1.4686	124.8153	135	8
9	1.4659	124.8112	120	6
10	1.4661	124.8156	90	4

As shown in table 1. were 10 sampled sites of two areas are indicated of column one, the second column is satellite positions at latitude of north and longitude of east in degrees, the third column is seas` depth were ranged from 95-196 meters measured by videos eco-sounder with its Global Positioning System (GPS) and the fifth column are the detected fish's species.

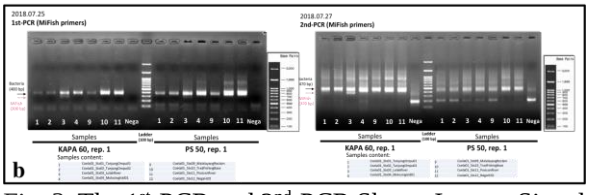


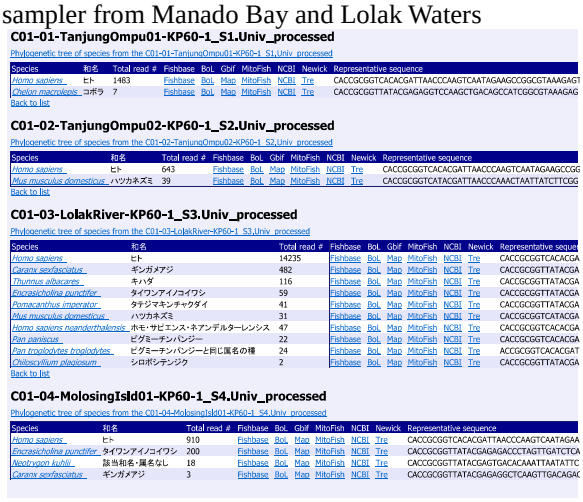
Fig. 3. The 1st-PCR and 2nd-PCR Shows Intense Signal of MiFish eDNA by Using Universal Primers MiFish-U-F/R.

Then finally it can be explained (Table. 2), from all 10 collection sites we found that, the number of fish species have been obtained for taxonomic assignment analysis and the results of species was detection by deposit analysis of eDNA water sampler, based on fish base database, GBIF, Mito fish, and NCBI as shown in Table 4. By using a high through put Illumina MiSeq platform for sequencing analyses, we detected eDNA from 40 fish's species fishes. Even though we couldn't get any information about coelacanth.

Table 2. The results of fishes' detection at Manado Bay and Lolak Waters, Sulawesi Sea of Indonesia by using eDNA

Number	Family	Species Scientific Name	Swimming Layers (M)	Locations
1	Hemiscyllidae	*Chiloscyllium plagiosum*	50	Lolak
2	Dasyatidae	*Neotrygon kuhlii*	170	Lolak
3	Anguillidae	*Anguilla celebesensis*	10	Sariouw,Manado
4		*Anguilla japonica* (※1)	10	Sariouw
5		*Anguilla marmorata*	10	Sariouw, Lolak, Manado
6	Engraulidae	*Encrasicholina punctifer*	35	Lolak,Manado
7	Chanidae	*Chanos Chanos*	50	Lolak
8	Sternoptychidae	*Maurolicus* sp.	400	Lolak, Manado
9	Myctophidae	*Benthosema pterotum*	500	Manado
10		*Diaphus regain*	500	Lolak
11		*Myctophum orientale*	400	Manado
12	Mugilidae	*Chelon affinis*	20	Lolak
13		*Chelon macrolepis* (※1)	10	Manado
14		*Mugil cephalus*	120	Manado
15	Serranidae	*Odontanthias borbonius*	300	Manado
16	Symphysanodontidae	*Symphysanodon katayamai*	183	Lolak, Manado
17	Menidae	*Mene maculate*	200	Manado
18	Carangidae	*Caranx latus* (※2)	140	Manado
19		*Caranx sexfasciatus*	146	Lolak, Manado
20		*Decapterus akaadsi*	170	Manado
21		*Decapterus macarellus*	200	Lolak
22		*Decapterus macrosoma*	214	Manado
23		*Selar crumenophthalmus*	170	Lolak
24	Mullidae	*Upeneus subvittatus*	100	Manado
25	Pomacanthidae	*Apolemichthys trimaculatus*	60	Manado
26		*Pomacanthus imperator*	100	Lolak
27		*Oreochromis niloticus*	5	Lolak
28		*Kuhlia marginata*	5	Lolak
29		*Kali indica*	500	Lolak
30		*Oxyeleotris marmorata*	10	Sariouw
31		*Sicyopterus japonicus* (※1)	5	Sariouw
32		*Sicyopterus lagocephalus*	5	Sariouw
33		*Euthynnus alletteratus*(※2)	150	Sariouw
34		*Katsuwonus pelamis*	260	Lolak
35		*Rastrelliger kanagurta*	90	Lolak, Manado
36		*Thunnus albacares*	250	Lolak, Manado
37		*Thunnus maccoyii*	500	Manado
38		*Paramonacanthus japonicus*	46	Lolak
39		*Lagocephalus gloveri*	450	Manado
40		*Cyclichthys orbicularis*	170	Manado

Table 4. Taxonomic assignment analysis and the results of species detection by Deposit analysis of eDNA water sampler from Manado Bay and Lolak Waters



C01-01-TanjungOmpu01-KP60-1_S1.Univ_processed

Phylogenetic tree of species from the C01-01-TanjungOmpu01-KP60-1_S1.Univ_processed

Species	和名	Total read #	Fishbase	BoL	Gbif	MitoFish	NCBI	Newick	Representative sequence
Homo sapiens	ヒト	1483	Fishbase	BoL	Map	MitoFish	NCBI	Tre	CACCGCGGTCACACGATTAACCCAAGTCAATAGAAGCCGGCGTAAAGAGT
Chelon macrolepis	コボラ	7	Fishbase	BoL	Map	MitoFish	NCBI	Tre	CACCGCGGTTATACGAGAGGTCCAAGCTGACAGCCATCGGCGTAAAGAG

Back to list

C01-02-TanjungOmpu02-KP60-1_S2.Univ_processed

Phylogenetic tree of species from the C01-02-TanjungOmpu02-KP60-1_S2.Univ_processed

Species	和名	Total read #	Fishbase	BoL	Gbif	MitoFish	NCBI	Newick	Representative sequence
Homo sapiens	ヒト	643	Fishbase	BoL	Map	MitoFish	NCBI	Tre	CACCGCGGTCACACGATTAACCCAAGTCAATAGAAGCCGG
Mus musculus domesticus	ハツカネズミ	39	Fishbase	BoL	Map	MitoFish	NCBI	Tre	CACCGCGGTCATACGATTAACCCAAACTAATTATCTTCGG

Back to list

C01-03-LolakRiver-KP60-1_S3.Univ_processed

Phylogenetic tree of species from the C01-03-LolakRiver-KP60-1_S3.Univ_processed

Species	和名	Total read #	Fishbase	BoL	Gbif	MitoFish	NCBI	Newick	Representative sequence
Homo sapiens	ヒト	14235	Fishbase	BoL	Map	MitoFish	NCBI	Tre	CACCGCGGTCACACGA
Caranx sexfasciatus	ギンガメアジ	482	Fishbase	BoL	Map	MitoFish	NCBI	Tre	CACCGCGGTTATACGA
Thunnus albacares	キハダ	116	Fishbase	BoL	Map	MitoFish	NCBI	Tre	CACCGCGGTTATACGA
Encrasicholina punctifer	タイワンアイノコイワシ	59	Fishbase	BoL	Map	MitoFish	NCBI	Tre	CACCGCGGTTATACGA
Pomacanthus imperator	タテジマキンチャクダイ	41	Fishbase	BoL	Map	MitoFish	NCBI	Tre	CACCGCGGTTATACGA
Mus musculus domesticus	ハツカネズミ	31	Fishbase	BoL	Map	MitoFish	NCBI	Tre	CACCGCGGTCATACGA
Homo sapiens neanderthalensis	ホモ・サピエンス・ネアンデルターレンシス	47	Fishbase	BoL	Map	MitoFish	NCBI	Tre	CACCGCGGTCACACGA
Pan paniscus	ピグミーチンパンジー	22	Fishbase	BoL	Map	MitoFish	NCBI	Tre	CACCGCGGTCACACGA
Pan troglodytes troglodytes	ピグミーチンパンジーと同じ属名の種	24	Fishbase	BoL	Map	MitoFish	NCBI	Tre	ACCGCGGTCACACGAT
Chiloscyllium plagiosum	シロボシテンジク	2	Fishbase	BoL	Map	MitoFish	NCBI	Tre	CACCGCGGTTATACGA

Back to list

C01-04-MolosingIsld01-KP60-1_S4.Univ_processed

Phylogenetic tree of species from the C01-04-MolosingIsld01-KP60-1_S4.Univ_processed

Species	和名	Total read #	Fishbase	BoL	Gbif	MitoFish	NCBI	Newick	Representative sequence
Homo sapiens	ヒト	910	Fishbase	BoL	Map	MitoFish	NCBI	Tre	CACCGCGGTCACACGATTAACCCAAGTCAATAGAA
Encrasicholina punctifer	タイワンアイノコイワシ	200	Fishbase	BoL	Map	MitoFish	NCBI	Tre	CACCGCGGTTATACGAGAGACCCTAGTTGATCTCA
Neotrygon kuhlii	該当和名・属名なし	18	Fishbase	BoL	Map	MitoFish	NCBI	Tre	CACCGCGGTTATACGAGTGACACAAATTAATATTC
Caranx sexfasciatus	ギンガメアジ	3	Fishbase	BoL	Map	MitoFish	NCBI	Tre	CACCGCGGTTATACGAGAGGCTCAAGTTGACAGAC

Furthermore, this bioinformatics data was compared to the fish`s distributions and ecological as reported from the Green Eye project 2007-2015 (Masamitsu I. *et al*.) where the site also known as fishing grounds of those target fishes. Here we can have explained that, the results of collections sites, satellite positions, sea`s depth and the number of fish's species was detected at Lolak Waters and Manado Bay North of Sulawesi, explained that the collection site at Lolak Waters relatively higher fisheries resources existence abundance.

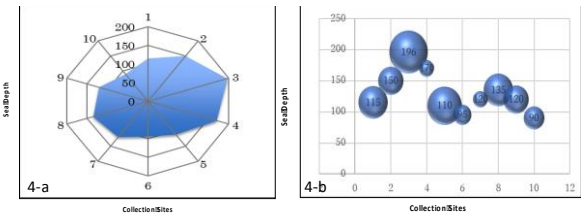


Fig.4. The relationships between the collection sites number and sea`s depth as shown at Fig. 4-a by radar chart and Collections Sites Seas` Depth with balloons pattern analyses (4-b)

As shown at Fig. 4. The relationships between the collection sites number and sea`s depth by radar chart approach and the number of the fishes' species detected was analyzed and perform by using the balloons pattern for easer to understand.

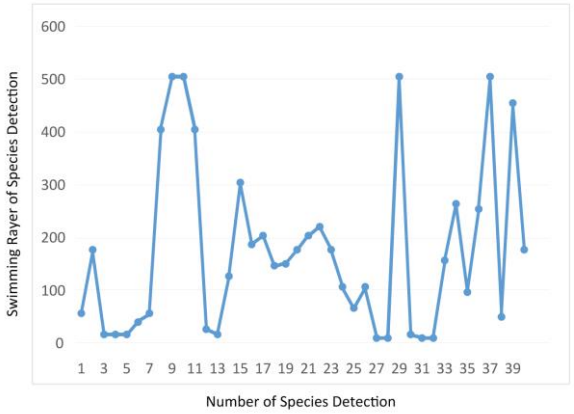


Fig. 5. Relationships between Detected Fishes`Species and Swimming layers of each.

More interesting 40 fishes` species were detected by environmental DNA tools in this study. We found that 8 species are the deep-sea fishes' species or 20 % from the total species detections as shown in Fig. 5. According to the former researches results have been done by Green eye Project team in collaborating among Aquamarine Fukushima, Sam Ratulangi University and Indonesian Institution of Science Republic of Indonesia under umbrella of International Coelacanth Research Center and Marine Museum Indonesia by using the sophisticated equipment of remotely operating vehicles (ROV) during periods surveyed 2006-2016 depths, ranged of 150-225 in meters were encountered swimming layers of the

coelacanth (*Latimeria menadoensis*) around Indonesia seas.

The mostly species were detection are the shallower swimming layers, coral fishes and pelagic species (Fig.6). Only about 7 deep-sea fish species were detected from eDNA water sampler, caused by some reasons the water samples were pickup by Nansen water sampler just limited to the seas` depth ranges around 200 meters.

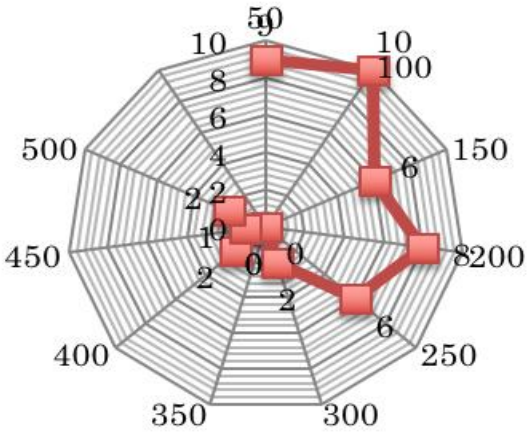


Fig. 6. Relationships between the Sample Sites, Fishes` Swimming Layers and the number of the detected aquatic creatures by spider chart pattern.

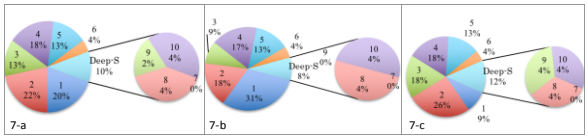


Fig. 7. The relationships between the collections and Fishes` Species Swimming Layers.

Furthermore, we comparing among whole DNA data samples, Lolak sea and Manado Bay in Fig. 7, explained that the percentage of the fish's species were detected by the environmental DNA tools and perform by the lunar eclipse pattern. From this figure show that the mostly the fishes were detected are living at shallower swimming layers. About 90 % of the 40 fish's species are living at shallower sea waters or less than 300 meters in depth and around 10 % of the fish's species are living at deep-sea or more than 300 meters in depth. In Fig. 7-b the fish's species were detected at Lolak waters, in this figure show that only 8 % of the fish's species were detected are at swimming layers categorized of deep-sea and the others of them are more than 90 % are living at shallower and at coral reefs. And Fig. 7-c is at Manado Bay with 12 % at deep-sea were detected and clear based on the PCR results. During our surveyed at the fields by pick up the deep-sea waters at two areas consisted by 10 stations around the discovery of coelacanth and also became the fishing grounds by local fisherman from long years ago, unfortunately we have not yet got any DNA of the coelacanth due to this organism are vey rare in the sea, or because they living in the sea bed cave.

CONCLUSSIONS AND REMARKS

Based on our results analysis to developed universal primer MiFish in a metabarcoding approach to fish eDNA we confirmed that the Lolak Waters are having relatively higher the fisheries resources existence abundance comparing of that on Manado bay. In implementation of the deep-sea environmental DNA research, the un-contamination aspect during fields work is absolutely necessary, therefore for effectiveness and efficiencies research of the marine fisheries resource's existence abundance point of view, the environmental DNA technology approach is suitable to be applied. Finally, this marine environmental DNA technology could be get fruitful in the near future if it could be implemented to Indonesian marine and freshwater due to Indonesia country have very wider territorial in the view of point forecasting the fisheries resources existence abundance.

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