Biosaintifika 10 (3) (2018) 629-635

Biosaintifika
Journal of Biology & Biology Education

http://journal.unnes.ac.id/nju/index.php/biosaintifika

Barcoding of Fin Clip Samples Revealed High Exploitation of *Plectropomus leopardus* in Spermonde Archipelago

✉Agus Nuryanto, Hendro Pramono, Kusbiyanto, Muhammad Iqbal Ghifari, Novi Andareswari

DOI: http://dx.doi.org/10.15294/biosaintifika.v10i3.16142

Faculty of Biology, Universitas Jenderal Soedirman, Indonesia

History Article

Received 27 September 2018
Approved 19 November 2018
Published 31 December 2018

Keywords

Grouper; Genetic Distances; Forensic Analysis; Phylogenetic Relationship

Abstract

Spermonde Archipelago in South Sulawesi is among groupers sources in Indonesia for International Marine Live Trade. It was not much known which species are mostly exploited in those areas during the study because only fin clip samples could be obtained. Here we performed DNA forensic analysis to groupers fin clips collected from Spermonde Archipelago using barcode marker to know what species were exploited and which ones was the most exploited species. A total of 110 fin clip samples were obtained during the field trips. Sequencing of the cytochrome c oxidase 1 gene was successful for only 36 samples. Species determination was based on sequences similarity to conspecific sequence available in barcoding of life database (BOLD). BOLD similarity test placed the 36 fin clip samples into four different species, namely *Cromileptes altivelis*, *Epinephelus ongus*, *Plectropomus leopardus*, and *Plectropomus maculatus*. The K-2P taxonomic tree showed clear separation among species which was supported by high K2P genetic distances among species. Most of fin clips were identified as *Plectropomus leopardus* indicates that this species was the most exploited in Spermonde Archipelago. This proved that molecular identification not only can be used to biodiversity study in certain area but also in forensic analysis of a threatened wildlife. This study has important contribution for conservation effort of those groupers species, especially *P. leopradus* and provides new approach for taxonomist and conservation managers to identify samples, especially when only incomplete specimens are available.

How to Cite

Nuryanto, A., Pramono, H., Kusbiyanto, K., Ghifari, M. I., & Andareswari, N. (2018). Barcoding of Fin Clip Samples Revealed High Exploitation of *Plectropomus leopardus* in Spermonde Archipelago. *Biosaintifika: Journal of Biology & Biology Education*, 10(3), 629-635.

✉ Correspondence Author:
Jl. dr. Soeprano 63A Grendeng, Purwokerto 53122, Central Java, Indonesia.
E-mail: anuryanto2003@yahoo.com

p-ISSN 2085-191X
e-ISSN 2338-7610

INTRODUCTION

Grouper is the most popular fish group in international marine live trade. It has high economic values as well as high export demands (Harikrishnan *et al.*, 2010). In Southeast Asia, grouper has very high prices (Pierre *et al.*, 2008). In Spermonde Achipelago, the prices of life individuals reach 250,000 IDR per kilogram, while dead individuals only priced for 50,000 IRD per kg (personal observation). Therefore, grouper has been widely farmed in Southeast Asian countries, including Indonesia. However, grouper farming was unable to meet market demand. This condition makes natural capture is still representing the largest portion of grouper exports in the world (Pierre *et al.*, 2008) with Indonesia that supports a largest portion to global grouper catch (Ottolenghi *et al.*, 2004). Grouper stock in Indonesia become overfished (Scales *et al.*, 2007) and put *Plectropomus leopardus*, *Epinephelus coioides* listed as near threatened, while *Chromileptes altivelis* as vulnerable species (http://www.iucnredlist.org/details/44684/0). In this case, conservation efforts are urgently needed. Fundamental research such as proper identification of target species into species level become a vital step.

In the case of incomplete individual samples were found during the field trips, forensic analysis through DNA barcoding is the only approach suitable to reveal taxonomic status of the samples. The cytochrome c oxidase 1 (CO1) is widely used as barcode marker in such studies (Mabragaña *et al.*, 2011; Radulovici *et al.*, 2010) and it is believed as highly precise marker for species identification (deWaard *et al.*, 2011). It has been reported that CO1 barcode could properly identify fish (von der Heyden *et al.*, 2014; Hubert *et al.*, 2012; Muricy, 2011;), even from fillet samples (Wong and Hanner, 2008); and fish larvae with limited morphological features (Pegg *et al.*, 2006; Nuryanto *et al.*, 2017).

Due to the explanation above, this study was conducted to figure out what species were exploited and which one was the most exploited in Spermonde Archipelago, South Sulawesi. The partial sequences of the COI gene were used as a forensic barcode to reveal taxonomic status of fin clip samples of groupers that region. This research provides valuable information about the application of animal systematics, especially in forensic analysis of wildlife specimen. In addition, information from this study is also important for conservation effort of those groupers species and provides new approach for taxonomist and conservation managers to identify samples, especially when only incomplete specimens are available.

METHODS

Study area, sampling technique, and sample handling

Grouper samples were collected randomly by the fisherman from all around Spermonde Archipelago (Figure 1). Small piece of fin clip tissue samples were cut off randomly from life grouper individuals owned by middlemen. This sampling technique was applied to avoid the individual getting die, otherwise the price become lowered. Tissue samples were collected during the field trips in 2012. The samples were then preserved directly in 96 % ethanol and afterwards they were stored on temperature of 4 °C until the DNA analysis. The DNA processing was performed in 2015.

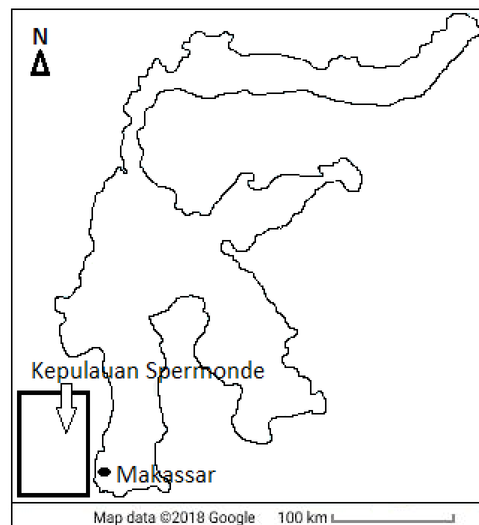

Figure 1. Sulawesi Island with sampling site in Spermonde Archipelago.

DNA extraction and barcode marker amplification

Genomic DNA was isolated using slightly modified Chekex®100 method by Kochzius and Nuryanto (2008). Approximately of 2 mm^2 tissue samples were extracted in 5% of 100 mL chelex, mixed with 0.5 mM dithiotreithol (DTT) and 2 µL proteianse-K. The mixtures were incubated in thermomixer at 56°C for approximately of 6 hours and homogenized manually after each hour incubation. The success of DNA isolations were checked by migrating the extracted DNA in agarose 1%, stained using 1% ethidium bromide and visualized under the UV-light.

The cytochrome c oxidase I (COI) gene fragments of the samples were amplified using

forward primer 5'atctttggtgcatgagcaggaatagt3', instead of using FishF2 and reverse primer FishR2-5'tagacttctgggtggccaaagaatca3' (Ward *et al.*, 2005). The PCR reactions was carried out in a total volume of 50 µl contained 29.8 µl ultrapure water; 1X PCR buffer, $MgCl_2$ 5 mM, 0.01 mM of each primer, 0.05 mM of each dNTP, 1 U Taq polymerase, and 4 µl of template DNA. The thermal cycles were as follow: one cycle at 95 °C for 5 minutes and followed by 35 cycles of 1 minute at 94 °C, 1minute annealing at 54 °C, and 1.5 minutes at 72 °C. The final extension was carried out at 72 °C for 10 minutes. Amplification products were visualized on 1 % Agarose gel over the UV-ligh and photographed.

Sequencing and sequence editing

The big dye terminator sequencing technique was performed in 1st BASE Asia (www.base.asia.com). The resulted sequences were edited manually in BioEdit software ver. 7.0.5. (Hall, 2011). All sequences were translated to amino acid sequences using web-based software called ORFfinder (*https://www.ncbi.nlm.nih.gov/orffinder*) to check that we amplified functional gene, rather than pseudo-gene. Multiple sequences alignment was done using ClustalW (Thompson *et al.*, 1994) in BioEdit software ver.7.0.5 (Hall, 2011). Haplotype's sequences were generated in Arlequin software ver. 3.5 (Excoffier & Lischer, 2010). All haplotypes has been deposited in the genbank with the accession number of KY950370-KY900387.

Data analysis

Taxonomic status of the fin clip samples was defined by comparing the CO1 sequences of the samples to previously published sequences listed in barcode of life database (BOLD) (Ratnasingham & Hebert, 2007) using BIN value of 3% according to species level database. Genetic divergences within and among species were estimated based on Kimura 2 parameters (K2P) substitution model as implemented in Arlequin software version 3.5. (Excoffier & Lischer, 2010). The K2P neighbour-joining (NJ) tree was reconstructed in MEGA software 6.0 versions (Kumar *et al.*, 2008). Topology of the tree was supported by 1000 non-parametric bootstrap replicates.

RESULTS AND DISCUSSION

PCR amplification

A total of 110 fin clip samples were obtained during the field trips. However, amplification and sequencing proccesses was only successful for 36 samples. This minor success of amplification was due to the use of old preserved fin clip samples. The fin clips samples were stored for at least three years until DNA processing since they were collected in 2012, while DNA analysis was performed in 2015. Although theoretically, DNA can still be amplified from ancient material, in fact it is more difficult to amplify DNA from either ancient samples or even from old preserved samples compared to the fresh collected ones. This could be due to that DNA in long preserved tissue will be fragmented. Fragmented DNA sometimes become very difficult to be amplified. According to Hird *et al.* (2006) amplification of highly degraded DNA was very hard, especially if target fragment quite long and the success of amplification rate reduced with the increased of target fragments.

In one hand, our result was different to the result of a study by Sutrisno (2012) and Zimmermann *et al.* (2008) that reported a successful amplification of less than six years preserved moths. In other hand, our result is also similar to Sutrisno (2012) and Zimmermann *et al.* (2008) for older samples. These difference and similarity of our success to Sutrisno (2012) and Zimmermann *et al.* (2008) on PCR amplification of old preserved samples could be due to that we used different animal and tissues types. Here we used fish and fin clips tissue samples while Sutrisno (2012) and Zimmermann *et al.* (2008) used body tissues of moths. This argument is supported by Srinivasan *et al.* (2002) which proved that fixative affect the success of DNA amplification from preserved tissue and leads to the amplification of artifacts amplicons. Moreover, DNA quality of preserved tissues linearly decreased with preservation times and become more fragmented. Fragmented DNA would be very difficult to be amplified. This means the success of DNA amplification from old preserved samples are varied among organismal groups and tissue types. Therefore, it is not surprising if there will be some differences and similarities of the success of PCR amplification from one study to the others.

Homology test and genetic species concept

Homology test of the samples to the sequences available di bold system databases showed that the samples had sequences similarity ranges from 97.24 % to 100% to the sequences of conspecific. These mean that our samples had sequences divergences of less than 3% as the standard divergence value in bold systems. These similarity values mean that all of our samples could be identified accurately into species level.

Among 36 sequences of fin clips, a total of 18 fin clip samples were genetically identified as *Plectropomus leopardus*. Nine samples were identified as *Cromileptes altivelis*, seven fin clips were identified as *Epinephelus ongus*, and the remaining two fin clips were identified as *P. maculatus* (Table 1).

There were no doubt to assign the fin clip samples into those four species due to all samples show sequences similarity of more than 97% either to sequences in bold systems or to genbank databases. This means that sequences divergence between our samples with their conspecific sequences available in boldsystems and genbank databases less than 3%. According to Pegg *et al.* (2006) different fish species can be delineated based on sequence divergence of 1% to 3%. Even if we refer to Pereira *et al.* (2013), maximum genetic divergence within species might reach 8.5% depend on fish group. In other words, genetic similarity among individuals within species can be less than 97%. Therefore, our assignment of the fin clips samples into four species based on 97.24% sequences similarity was very convincing.

The Kimua 2 Parameters (K-2P) neighbour-joining tree topology (Figure 2) supported the result of DNA barcoding. Similar support was also obtained when the trees were constructed using maximum parsimony analysis based on Kimua 2 Parameters. In higher category, the ingroup samples formed a monophyletic group or monophyletic clade compared to the out-group samples (Figure 2). If we look into detail to our samples in Figure 2, the samples were divided into two sub-monophyletic groups (sub-clades) and we call as sub-clade A and B. It can also be found on the tree that each individual sample formed monophyletic groups with their conspecific relatives taken from database. For example, all fin clips identified as *P. leopardus* was put together in a same monophyletic group with *P. leopardus* JN021314 from genbank and referred to as sub sub-clade C. All individual samples which were identified as *P. maculatus* formed a monophyletic clade with *P. maculatus* KR863514 (sub subclade D). The fin clip samples identified as *Epinephelus ongus* were placed together with *E. ongus* KP194568 in one monophyletic group, called as sub sub-clade E. The remaining samples identified as *Cromileptes altivelis* were also combined together with KJ594967 to form another monophyletic group called as sub sub-clade F.

If we go backward in the branching pattern of the tree (Figure 2), we came to genus levels. It is clearly found that samples belong to single genus are placed together in a single monophyletic group (sub-clade A) with rather short branch length. The placement of *E. ongus* and *C. altivelis* into a single monophyletic group was due to that they have closer relationship compared to *P. leopardus* and *P. maculatus* with genetic divergence 0.146 (Table 2). Since the both *E. ongus* and *C. altivelis* belong to the different genera, they are combined because no congenera species are available but by rather long branch length (sub-clade B). This means that both species are combined together in a taxonomic category above genus. This separation is congruence with the result of molecular identification that showed that genus *Plectropomus* consist of *P. leopardus* and *P. maculatus*. The separation of both subclade and sub-subclade were supported by high bootstraps values. These mean that the separation of those groups was statistically reliable and improve the quality of identification result.

A clear discrimination among species and above species level as indicated in the taxonomic tree (Figure2) provides additional scientific data about the reliability of the COI gene as barcode marker in species delimitation, especially in fish. The power of this gene as a species barcode marker because it is changeable enough (Nuryanto *et al.*, 2007; Nuryanto and Kochzius, 2009) due to its high mutation rate which is higher compared to other mitochondrial genome (Hebert *et al.*, 2003). Clear separation of different species and above species category was also reported in fish larvae (Nuryanto *et al.*, 2017), or even among strain within species (Nuryanto *et al.*, 2018).

The K-2P genetic divergences among fin clip samples within species range from 0.000 in *P. maculatus* up to 0.016 in *C. Altivelis* (Table 2). These mean that low genetic divergences were found among individuals from single species. Similar low genetic divergences were also reported

Table 1. Species assigned based on BOLD database, similarity value, and number of individual (N)

Genus	Species in BOLD Database	Similarity Value (%)	N
Cromileptes	Cromileptes altivelis	97.24-99.79	9
Epinephelus	Epinephelus ongus	99.81-100	7
Plectropomus	Plectropomus leopardus	99.81-100	18
	Plectropomus maculatus	100	2
		Total	36

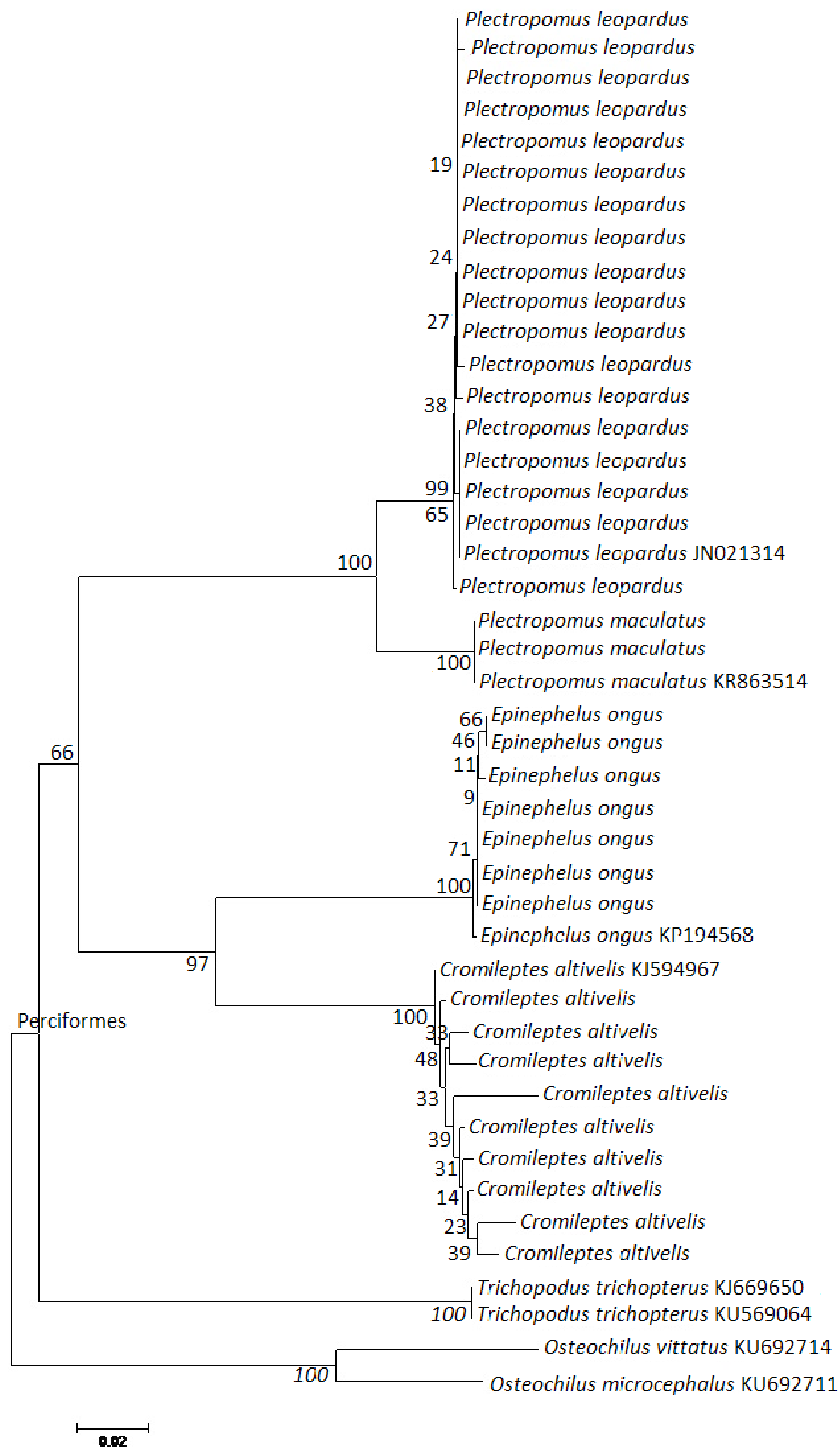

Figure 2. Phylogenetic tree based on 633 bp of the mitochondrial DNA COI gene using genetic distances Kimura 2-parameter; bootstrap analysis with 1000 replicates

by Nuryanto *et al*. (2007) on *Tridacna* spp with the values range from 0.011 up to 0.028. Higher intra-specific genetic divergence was reported by Hubert *et al*. (2012) on other fish group with the value reach up to 0.080. Therefore, the placement of fin clips samples into four different species was

Table 2. Intra- and inter-species K-2P genetic divergence

Species	*C. altivelis*	*E. ongus*	*P. leopardus*	*P.maculatus*
C. altivelis	0.016			
E. ongus	0.146	0.002		
P. leopardus	0.219	0.218	0.002	
P. maculatus	0.213	0.226	0.050	0.000

correct since they have low genetic divergences (less than 0.030).

Inter-species genetic divergences were range from 0.05 between *P. leopardus* and *P. ongus* up to 0.226 between *P. maculatus* and *E. ongus* (Table 2). These observed high values of inter-species genetic divergences are common phenomena. Several studies also reported high genetic divergences among species in various animal groups, such as in fish (Peg *et al.*, 2006; Pereira *et al.*, 2013; Nuryanto *et al.*, 2017; Nuryanto *et al.*, 2018); and Crustacea (Barber *et al.*, 2002).

Molecular identification had also been performed on *Epinephelus* from India (Sachithanandam *et al.*, 2012) and from Israel (Dor, 2012). In general, molecular barcoding was carried out on individuals with clear or definite taxonomic status. Whereas, molecular barcoding for forensic analysis on unidentified tissue samples of Indonesia's groupers was rather rare, especially in regard to the most exploited species. Therefore, the result of this study proved that molecular identification is not only valuable for taxonomic diversity study in certain area, but it can be also addressed as DNA forensic analysis to trace illegal fishing of near threatened and vulnerable species such as *Plectropomus leopardus* and *Cromileptes altivelis,* respectively. This research provides valuable information about the application of animal systematics, especially in wildlife forensic analysis. In addition, information from this study was also important for conservation effort of those groupers species. This study also provides new approach for taxonomist and conservation managers to identify samples, especially when only incomplete specimens are available.

CONCLUSION

Forensic analysis using COI barcode successfully identified fin clips samples into species level. A total of four groupers species was exploited during the sampling and most of them were genetically identified as *P. leopardus*, means that *P. leopardus* was the most exploited grouper in Spermonde Archipelago.

ACKNOWLEDGEMENTS

We would like to thanks to: SPICE Project for the funding during the field trips and to Research and Public Services Institute of Jenderal Soedirman University for the funding during DNA analysis under International Collaborative Research scheme. We would like also to thanks to some persons from Unhas, University of Bremen, and Free University of Brussels (Marc Kochzius) who make the field trips was possible. We were also thanks Anggi Pratama for the help during laboratory works. Our thanks also delivered to Faculty of Biology Jenderal Soedirman University for the supports by allowing the researcher to use the facilities. High appreciation is also delivered to reviewers for their comments and suggestion which make the scientific value of this study become increased.

REFERENCES

Barber, P.H., Palumbi, S.R., Erdmand, M.V., & Moosa, M.K. (2002). Sharp genetic breaks among populations of *Haptosquilla pulchella* (Stomatopoda) indicate limits to larval transport: patterns, causes and consequences. *Molecular Ecology*, *11*(4), 659-674.

deWaard, J.R., Hebert, P.D.N., & Humble, L.M. (2011). A Comprehensive DNA Barcode Library for the Looper Moths (Lepidoptera: Geometridae) of British Columbia, Canada. *PLoS ONE*, *6*(3), 1-6.

Dor, L. (2012). Establishment of genetic markers for breeding the white grouper (*Epinephelus aeneus*). *Master Thesis*. Jerusalem: Faculty of Agriculture, Food, and Environment, the Hebrew University of Jerusalem.

Excoffier, L. & Lischer, H.E.L. (2010). Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources*, *10*, 564-567.

Hall, T.A. (2011). BioEdit: An important software for molecular biology. *GERF Bulletin of Biosciences*, *2*(4)1, 60-61.

Harikrishnan, R., Balasundaram, C., & Heo, M.S. (2010). Molecular studies, disease status and prophylactic measures in grouper aquaculture: Economic importance, diseases and immunol-

ogy. *Aquaculture*, *309*, 1-14.

Hebert, P.D.N., Ratnasingham, R., & deWaard, J.R. (2003). Barcoding animal life: cytochrome c oxidase subunit I divergences among closely related species. *Proceeding Royal Society London B*, *270*, S96-99.

Hird, H., Chisholm, J., Sánchez, A., Hernandez, M., Goodier, R., Scheede, K. Boltz, C., & Popping B. (2006). The effect of heat and pressure processing on the fragmentation of DNA and implications for detection of meat using real-time PCR. *Food Additives and Contaminants*, *23*(07), 645-650.

Hubert, N., Meyer, C.P., Bruggeman, H.J., Guerin, F., Komeno, R.J.L., Espiau, B., Caussee, R., Wiliams, J.T., & Planes, S. (2012). Cryptic diversity in Indo-Pacific coral reef fishes revealed by DNA barcoding provides new support to the Centre-of-Overlap hypothesis. *PloS One*, *7*(3), e28987

Kochzius, M. & Nuryanto, A. (2008). Strong genetic population structure in the boring giant clam *Tridacna crocea* across the Indo-Malay Archipelago: implications related to evolutionary processes and connectivity. *Molecular Ecology*, *17*, 3775-3787.

Kumar, S., Nei, M., Dudley, J., & Tamura, K. (2008). MEGA: A biologist-centric software for evolutionary analysis of DNA and protein sequences. *Briefings in Bioinformatics*, *9*, 299-306.

Mabragaña, E., de Astarloa, J.M.D., Hanner, R., Zhang, J., & Castro, M.G. (2011). DNA barcoding identifies Argentine fishes from marine and brackish waters. *PLoS One*, *6*, e28655.

Muricy, G. (2011). Diversity of Indo-Australian Plakortis (Demospongiae: Plakinidae), with description of four new species. *Journal of the Marine Biological Association of the United Kingdom*, *91*(2), 303-319.

Nuryanto, A., Amalia, G., Khairani, D., Pramono, H., & Bhagawati, D. (2018). Molecular characterization of four giant gourami strains from Java and Sumatra. *Biodiversitas*, *19*(2), 528-539.

Nuryanto, A., Pramono, H., & Sastranegara, M.H. (2017). Molecular identification of fish larvae from East Plawangan of Segara Anakan, Cilacap, Central Java, Indonesia. *Biosaintifika: Journal of Biology and Biological Education*, *9*(1), 33-40.

Nuryanto, A. & Kochzius, M. (2009). Highly restricted gene flow and deep evolutionary lineages in the giant clam *Tridacna maxima*. *Coral Reefs*, *28*, 607-619.

Nuryanto, A., Solihin, D.D., Soedharma, D., & Blohm, D. (2007). Molecular phylogeny of giant clams based on mitochondrial DNA cytochrome c oxidase I gene. *HAYATI Journal of Biosciences*, *14*, 162-166.

Ottolenghi, F. (2004). *Capture-Based Aquaculture: the Fattening of Eels, Groupers, Tunas and Yellowtails*. FAO. Rome.

Pegg, G.G., Sinclair, B., Briskey, L., & Aspden, W.J. (2006). MtDNA barcode identification of fish larvae in the southern Great Barrier Reef, Australia. *Scientia Marina*, *70*(S2), 7-12.

Pereira, L.H.G., Hanner, R., Foresti, F., & Oliverira, C. (2013). Can DNA barcoding accurately discriminate megadiverse Neotropical freshwater fish fauna? *BMC genetics*, *14*(1): 20.

Pierre, S., Gaillard, S., Prevot-D'alvise, N., Aubert, J., Rostaing-Capaillon, R., Leung-Tack, D., & Grillasca, J-P. (2008). Grouper aquaculture: Asian success and Mediterranean trials. *Aquatic Conservation: Marine and Freshwater Ecosystems*, *18*(3), 297-308.

Radulovici, A.E., Archambault, P., & Dufresne, F. (2010). DNA barcodes for marine biodiversity: moving fast forward? *Diversity*, *2*, 450-272.

Ratnasigham, S. & Hebert, P.D.N. (2007). BOLD: The barcode of life data system (www.barcodinglife.org). *Molecular Ecology Notes*, *7*, 355-364.

Sachithanandam, V., Mohan, P.M., Muruganandam, N., Chaaithanya, I.K., Dhivya, P., & Baskaaran, R. (2012). DNA barcoding, phylogenetic study of *Epinephelus* spp. from Andaman coastal region, India. *Indian Journal of Geo-Marine Sciences*, *41*(4), 203-211.

Scales, H., Balmford, A., & Manica, A. (2007). Monitoring the live reef food fish trade: Lessons learned from local and global perspectives. *SPC Live Reef Fish Information Bulletin*, *17*, 36-42.

Srinivasan, M., Sedmak, D., & Jewell, S. (2002). Effect of fixatives and tissue processing on the content and integrity of nucleic acids. *Americana Journal of Pathology, 161*(6), 1961-1971.

Sutrisno, H. (2012). The impact of storage times of museum insect specimens on PCR success: Case study on moth collections in Indonesia. *HAYATI Journal of Biosciences*, *19*(2), 99-104.

Thompson, J.G., Higgins, D.G., & Gibson, T.J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignments through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Research, 22*(22), 4673-4680.

von der Heyden, S., Berger, M., Toonen, R.J., van Herwerden, L., Juinio-Menez, M.A., Ravago-Gotanco, R., Fauvelot, C., & Bernardi, G. (2014). The application of genetics to marine management and consrvation: examples from the Indo-Pacific. *Bulletin of Marine Science*, *90*(1): 123-158.

Ward, R.D., Zemlak, T.S., Innes, B.H., Last, P.R., & Hebert, P.D.N. (2005). DNA barcoding Australia's fish species. *Phil. Trans. R. Soc. B* 360: 1847-1857.

Wong, E.H.K. & Hanner, R.H. (2008). DNA barcoding detects market substitution in North American seafood. *Food Research International*, *41*, 828-837.

Zimmermann, J., Hajibabaei, M., Blackburn, D.C., Hanken, J., Canti, E., Posfai, J., & Evans Jr, T.C. (2008). DNA damage in preserved specimens and tissue samples: a molecular assessment. *Frontiers in Zoology, 5*, 18, doi: 10.1186/1742-9994-5-18.