Available online at www.derpharmachemica.com

Scholars Research Library

Der Pharma Chemica, 2015, 7(10):223-228
(http://derpharmachemica.com/archive.html)

ISSN 0975-413X
CODEN (USA): PCHHAX

Mitochondrial DNA genetic diversity of arowana fish local variants on the D-loop hypervariable region: Study in the southern region of Papua-Indonesia

Yohanis Ngili,[1] Daniel Lantang,[2] Richardo Ubyaan,[3] and Epiphani Imelda Yosephin Palit[4]

[1]*Biochemistry Research Group, Department of Chemistry, University of Cenderawasih, Jayapura-Indonesia*
[2]*Microbiology Research Group, Department of Biology, University of Cenderawasih, Indonesia*
[3]*Chemistry Study Program, University of Cenderawasih, Indonesia*
[4]*Biostatistics Research Group, Department of Mathematics, University of Cenderawasih, Indonesia*

ABSTRACT

The southern region of the provinces of Papua that includes Merauke and Diegul Boven regency, has the potential of natural resources in the areas of inland waters. One of the many fish species that bring financial benefits are arowana fish. This fish is mostly found in the southern region of Papua and the price is quite expensive for a particular variant. Traditional fishing patterns and local knowledge-based society should continue to be pursued in order to improve the productivity of arowana fish. The existence of arowana fish in Papua are common in the southern region due to the large flow of creeks and major rivers in the region. The existence of arowana fish are only found in the southern region of Papua affect the genetic diversity of the fish. The purpose of this study is to analyze and characterize the pattern of nucleotide mutations biomolecular Arowana fish on the hypervariable region. Primers designed for amplification of 1 kb arowana fish along the D-loop region of mitochondrial. Based on the results obtained by analysis of mitochondrial DNA nucleotide sequence arowana fish in the D-loop region through the direct sequencing of samples arowana fish. Number of dominant mutations in Papua arowana fish as many 9 positions on the D-loop region of mtDNA fish. Nucleotide sequence analysis of the variability of the sample arowana fish in southern waters of Papua provide identifying information mutations were highly variable in both positions, quantity, and type of mutation that occurs. The study results that have been obtained are describe variants arowana fish that are in the local waters of Papua, in the southern region of Papua; has successfully illustrates the genetic map of the fish through the design of an efficient and comprehensive primer on the overall mitochondrial arowana fish. From total mutations, mutations are unique ie transversion substitution mutation at position n + 2, changes into a cytosine nucleotides adenine, A → c. Overall output of the data obtained from this study is very useful for the development of biotechnology in particular fisheries primer design methods and techniques of fish reproducible DNA isolation.

Keywords: Diversity, Genetic, DNA, Arowana Fish, Southern Papua

---

INTRODUCTION

Almost all animals have the mitochondrial genome consisting of 37 genes, which consists of 13 protein-coding genes (*protein-coding genes*), 2 ribosomal RNA (rRNA) and 22 transfer RNA (tRNA), which is required for the translation of proteins encoded by mtDNA (Boore, 1999). They also have a major gene control regions that initiate mtDNA replication and transcription of mitochondrial gene expression. General mitochondrial genome mutate 5-10 times faster than the DNA in the core gene (chromosome) [1-3].

Asian arowana (dragonfish; *Scleropages formosus*, *Osteoglossidae*) is one type of fish that live in freshwater habitats. This fish is one of the most expensive types of ornamental fish in the world and is listed in the *Convention on International Trades in Endangered Species of Wild Fauna and Flora* (CITES) as a species of "*highly endangered*". This arowana fish has three varieties of Asian arowana basic colors: green, gold, and red with some subvarian are almost extinct [4-5].

Based on taxonomic studies, the order consists of two subordo *Osteoglossiformes* namely *Osteoglossoidei* and *Notopteroidei*. *Orteoglossoidei* suborder have two families: *Osteoglossidae* and *Pantodontidae*. *Osteoglossidae* family consists of 7 species: *Scleropages formosus* (southeast Asia region), *S. jardini* (Northern Australia and Papua New Guinea), *S. leichardti* (eastern Australia), *Osteoglossum bicirrhosum* (South America), *O. ferreirai* (South America), *Arapaima gigas* (South America) and *Heterotis niloticus* (West Africa). *Pantodontidae* family has only one species, butterfly fish, *Pantodon buchholzi* (west Africa). Among the eight species *Osteoglossoidae*, mitochondrial genomes have been successfully sequenced the only two species, namely *O. bicirrhosum* and *P. buchholzi*. While the suborder *Notopteroidei* have 3 families with 56 species, there are only a full mtDNA sequences to a single species, the golden-eyed (*Hiodon alosoides*, *Hiodontoidae*) [6-8]

Although the Asian Arowana fish is one of the most expensive fish in the world, but still relatively few scientific publications that publish on genomic studies to analyze gene variants in the mitochondrial genome arowana fish. Most publications on arowana fish only related to classical studies relating to taxonomy, and physiology of a species of arowana. Specific objectives of this study are profiles Describing genetic mutations and variants of Papua local arowana fish in areas that do not code for genes (non-coding region) in the mitochondrial genome and the nucleotide mutation analysis. Characterizing genetic diversity variant arowana fish that is in the southern region of Papua and examine the pattern of DNA polymorphism and genetic distance and makes kinship analysis on the data GenBank arowana fish.

MATERIALS AND METHODS

The study was conducted in the southern region of the province of Papua, which is an area of diverse variants Arowana fish ecosystem. The area where research on Muting District and Ulilin District in regency of Merauke and Waropko District which is the regency of Boven Diegul. While the genetic diversity analyzes performed in the Laboratory of Biochemistry, Faculty of Mathematics and Natural Sciences, University of Cenderawasih.

DNA extraction of arowana fish

The method used in DNA extraction method is phenolchloroform, with steps as follows: Part of the body of the fish to be extracted is a piece of fin fish weighing 5-10 mg. Then inserted into the tube which has been filled with 500 µl NTES urea solution, is then inserted into the tube and added 15 µL/ml protein kinase. The next process, stirred with a vortex and incubated at 55 °C for 1 h. Subsequently, the mixture is stirred with a vortex and added with a solution of phenolchloroform 1000 µL. Then centrifuged at 1000 rpm for 10 min. Formed supernatant was transferred into a new tube and then added 1000 µL solution of 90% ethanol and 10 µL of sodium acetate. After the mixture was centrifuged at a speed of 10,000 rpm for 10 min to form a white precipitate. Separated from the mixture between the DNA and the solution. DNA that has been separated dried at room temperature, then, DNA coupled with 50-100 µL DNA rehydration solution [1, 6, 7, 10].

Testing the quality and quantity of DNA and DNA amplification of fish

1% agarose gel is placed in a solution of 1X TBE buffer (0.001 M EDTA triborate), then the DNA template as many 3 µL mixed with 3 µL of *loading dye*. The mixture is then taken as many as 3 µL and put in 1% agarose gel. Electrophoresis process carried out at a voltage of 100 V for 20 min. Results of electrophoresis, seen by using transilluminator. Dry mixing 1 unit taq (Promega), 2 µL *DNA template*, 2 µL primer and 21 µL of distilled water with a total PCR volume of 25 µL [10-11].

Electrophoresis and sequencing data analysis

Results of the amplification process is viewed by using 1% agarose gel. In each process used DNA electrophoresis of PCR results as many 9 µL and 3 µL marker as many base pairs with a range of 100 bp to 1500 bp. Electrophoresis results were observed using UV Transilluminator and documented with Polaroid film cameras. Sequencing process, carried out also by calculating the heterozygote and genetic distance [9]. Heterozygote is a fusion of different alleles at the same locus, calculated using equation. While the genetic distance, calculated based on the Polaroid translated into data based on the presence or absence of DNA bands. Genetic diversity seen by analyzing the genetic distance based on UPGMA program and Bayesian Inference [12].

RESULTS AND DISCUSSION

Arowana fish is a freshwater fish that is quite popular and is one of the pride of the Indonesian state fish because the price is quite expensive and are used as ornamental fish. It can be said that until now only arowana fish that can compete with the popular ornamental fish developed abroad to become the best ornamental fish. Arowana fish popularity due to bodies beautiful shape, style pool gallant but graceful, and color that attracts attention. That excess arowana fish from freshwater fish of other species.

Based on the data, when viewed from the arowana fish export growth overall from the year 2005-2014, it is known that the demand for arowana fish from overseas markets is very high and an increasing trend from year to year. Arowana fish export sales growth from year to year increase of nearly 40 percent. During this time the foreign market Arowana fish generally come from countries in Asia such as Japan, China, Taiwan, Hong Kong, Korea, and Singapore, if the market arowana has penetrated the European market, the arowana fish export value will increase. Arowana fish export value is also sufficient to provide a role in the country's foreign exchange earnings.

Arowana fish is one type of fish that live in freshwater habitats and is very intolerant of sea water (intolerant of saltwater). Arowana fish has three varieties of basic colors of asian arowana: green, gold, and red with some subvarian endangered [2, 4, 14-18]. Green Arowana fish is a fish species are generally found in almost the entire southeast Asian region. Varieties of other endangered and separate geographical areas are "super red", which is only found in the upper part of the Kapuas River (Lake protected Sentarum, West of Kalimantan) [8, 11, 13]. Other arowana fish that is golden (cross back golden) or blue (blue Malayan), abundant in the Lake Hills area of Pahang and red (Malay Peninsula, Malaysia). Arowana fish tail red-golden color comes from Pekanbaru region and Jambi [5, 11].

PCR amplification results along 1 kb

Preparation of template DNA for PCR begins with taking DNA samples of fish on the local fish fins and the the tail. The number of samples each place (Merauke and Boven Diegul) of 6 variants of fish. Cell lysis is then performed to obtain mitochondrial DNA using standard procedures without prior purification. Extract DNA lysis is a template for the Polymerase Chain Reaction (PCR). In Figure 1 is shown 1 kb fragment of the D-loop region obtained through amplification of human mtDNA in vitro using primer pair ARfor and ARrev. DNA detection results showed that the samples gave results of amplification measuring approximately 1 kb fragment indicated the presence of one band which is located between the band 1000 bp and 1500 bp band (Fig. 1). These results are as expected, ie, amplifying the D-loop region with a length of about 1 kb.

Stages PCR conditions used for DNA amplification arowana fish is as follows: Phase I (initial denaturation, at 95 °C for 3 min) and followed by a phase II: denaturation, at a temperature of 94 °C, for 1 min; annealing process at a temperature of 50 °C for 1 min, and the elongation/polymerization at a temperature of 72 °C for 1 min. In the second stage of the process was repeated for 30 cycles. The final stages of this amplification process is the final extension at 72 °C for 10 min.

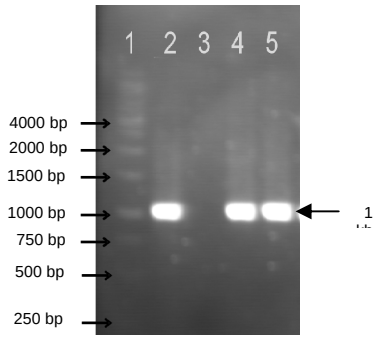

Fig 1. PCR fragments of 1 kb Arowana fish on the D-loop region. Amplification using primers ARfor and ARrev. line 1: 1 kb ladder marker, line 2: control (+), line 3 is the control (-), and line 4-5 is a sample (1 kb) Arowana fish representing Merauke arowana fish and Diegul Boven arowana fish

On the results showed the DNA fragment at position 1 kb for samples which show that amplification occurs in mtDNA with primer template ARfor and ARrev. Positive control function in the PCR process is to determine the course of the PCR process. The emergence of the tape on the positive control to prove that the PCR process is going well and the band that emerged in the sample are bands of fragment D-loop region. This is reinforced by its participation in the process of PCR negative control. Negative control in the PCR process aims to determine the

possibility of contaminants in the PCR reaction mixture. No emergence of the tape on the negative control proves that the reaction mixture contained no contaminants that could interfere with the PCR process. 1 kb fragment obtained tape were shown in electrophoresis results showed that the fins and tail of fish, mitochondrial DNA is found quite a lot. Suborganel main function of mitochondria is producing energy in the form of ATP in the cells of fish. Cell regeneration process requires energy obtained from food oxidation process in mitochondria, therefore, the blood cells are found pretty much the mitochondria. In large amounts, found in the mitochondria of cells that have a high metabolic activity such as sperm tail which actively divide and epidermis under arowana fish skin.

Results of direct sequencing analysis

Analysis of the 1 kb region of mtDNA performed on the D-loop region and as a standard used in GenBank sequence arowana fish. To determine the nucleotide sequence of the mutated analyzed using Seqman and MegAlign DNASTAR program by placing parallel sample nucleotide position (*align*) with the nucleotide sequence of CRS. In this program can also be seen form the circled electropherogram display shows the difference between the sample with a nucleotide sequence of the nucleotide sequence comparison (Fig. 2).

Based on the analysis of mutations in the D-loop region of mtDNA to 12 Arowana fish samples from Papua: The Merauke regency and Digul Boven regency, found some of the amount, type, and position of mutations in the D-loop region of mtDNA.

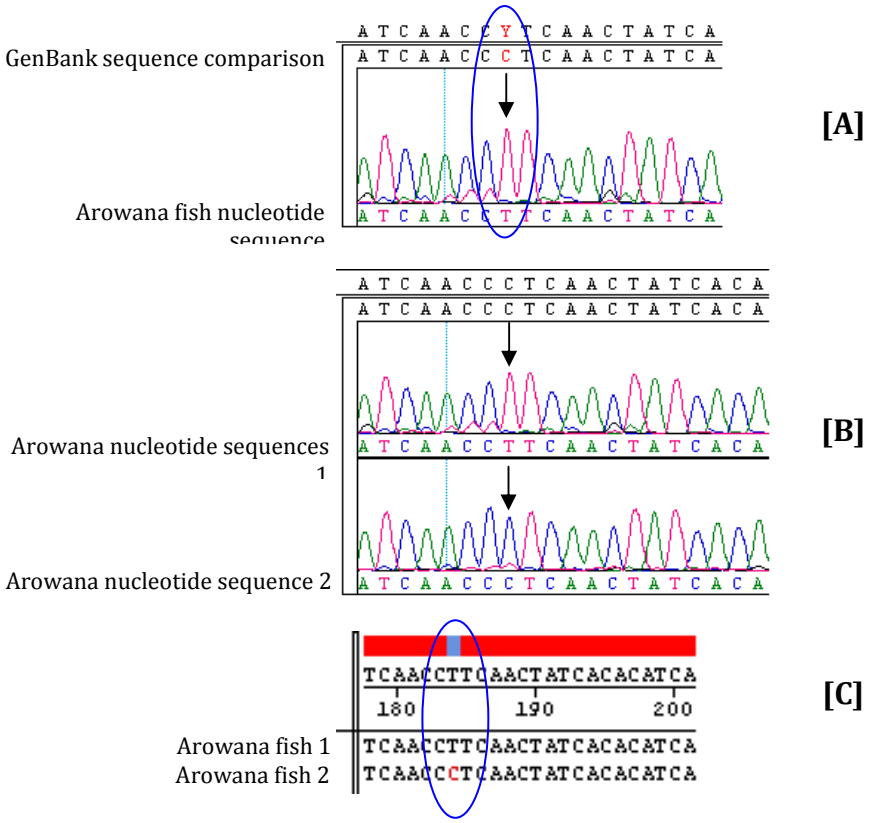

Fig. 2. At [A] is the result of mutation analysis of sequences Arowana fish 1 (Merauke) in the D-loop region through mutation electropherogram display using DNASTAR Seqman Program. In the figure appear in a certain position there is a difference between sequence mutations comparison with the GenBank sequences arowana fish 1, the base turned Thymine into Cytosine (C → T). While [B] is the result of a comparative analysis of mutations in these positions. In the picture above looks at this position in the nucleotide sequence 1 Arowana fish is Thymine bases (T) which is indicated by a red spectral peaks, while in Papua 2 shows the base Cytosine (C) the blue spectrum, together with a comparative sequence GenBank. In Figure [C], the result of alignment nucleotide sequence at a specific position using DNASTAR MegAlign program. In the picture above looks at the position of nucleotide sequences in fish Arowana 1 is Thymine (T), while Fish Arowana 2 shows the base Cytosine (C)

In Table 1 shown that mutations occurred in all the samples vary in the position, type and amount. The type of mutation that occurred among mutation transition and transversion mutations. Results of the analysis of the genetic

pattern of the overall sample as specified in the table shows the types of mutation nucleotides contained on arowana fish populations Papua, Merauke regency and Diegul Boven regency.

Table 1. The number and type of mutation variants of the D-loop Papua Arowana fish originating from Merauke regency and Boven Diegul regency

Nucleotide positions in the D-loop	Reference sequence	Arowana fish in Southern Papua												nucleotide changes
		Arowana fish at Merauke						Arowana fish at Boven Diegul						
		Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11	Sample 12	
n	T	.	.	c	.	.	.	.	.	.	.	.	.	T→c
n+1	C	t	t	t	t	t	t	.	t	t	t	t	t	C→t
n+2	A	.	c	c	.	.	.	.	.	.	c	.	.	A→c
n+3	T	.	c	c	.	.	.	c	.	.	c	.	.	T→c
n+4	T	.	.	.	.	.	.	.	.	c	.	.	.	T→c
n+5	T	.	c	c	.	.	.	.	.	.	c	.	.	T→c
n+6	C	t	.	.	t	.	t	.	.	.	.	.	t	C→t
n+7	C	t	.	.	t	t	t	.	t	t	.	t	t	C→t
n+8	A	g	.	.	g	.	g	.	.	.	.	.	g	A→g
n+9	C	.	.	.	.	.	.	t	.	.	.	.	.	C→t
n+10	C	t	.	.	t	t	t	.	t	t	.	t	t	C→t
n+11	G	a	.	.	a	a	a	.	a	a	.	a	a	G→a
n+12	A	g	.	.	g	.	g	.	.	.	.	.	g	A→g
n+13	T	.	.	.	.	c	.	.	c	c	.	c	.	T→c
n+14	A	g	g	g	g	g	g	g	g	g	g	g	g	A→g
n+15	T	c	.	.	c	c	c	.	c	c	.	c	c	T→c
n+16	T	.	c	.	.	.	.	.	.	.	c	.	.	T→c
n+17	A	g	.	.	g	g	g	g	g	g	.	g	g	A→g
n+18	T	.	c	.	.	.	.	c	.	.	c	.	.	T→c
n+19	G	.	.	.	.	.	.	a	.	.	.	.	.	G→a
n+20	T	.	.	.	.	.	.	c	.	.	.	.	.	T→c
n+21	A	g	.	.	g	g	g	.	g	.	.	g	g	A→g
n+22	A	g	g	g	g	g	g	g	g	g	g	g	g	A→g

Table 2. Analysis of the patterns of mutations in Papua arowana fish populations: from Merauke and Boven Diegul regency

	Arowana fish at Merauke regency						Arowana fish at Boven Diegul regency					
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11	Sample 12
Number of variants per sample arowana[a]	12	8	7	12	10	12	8	10	10	8	10	12
Percentage variants[b] (in percent)	52	35	30	52	43	52	35	43	43	35	43	52
Number of variants that no mutation	11	15	16	11	13	11	15	13	13	15	13	11
Most variants of the D-loop[c]	1	3	1	1	3	1	2	3	1	3	3	1
Comparison with the mutation variant that is not mutated[d]	1,09	0,33	0,43	1,09	0,77	1,09	0,53	0,77	0,77	0,53	0,77	1,09
Most mutations identity[e]	6A→g	4T→c	3T→c	6A→g	4A→g	6A→g	3A→g 4T→c	4A→g	3A→g 3T→c 3C→t	4T→c	4A→g	6A→g
The type of substitution mutation that occurred[f]	transition	transition	transition	transition	transition	transition	transition	transition	transition	transition	transition	transition

[a] *The number of mutations per sample arowana [number of nucleotide mutations in all samples in all positions mtDNA D-loop arowana fish].*
[b] *Percent number of variants per sample fish (calculated per variant total arowana fish, ie per 23)*
[c] *Comparison of variants of the D-loop*
[d] *The calculation result if more than one, the mutated variant more than homology with comparators and vice versa*
[e] *Results of the analysis of the number and type of mutations that occur in a sample of arowana fish regardless of the position of the mutation*
[f] *Arowana fish types of nucleotide mutations that occurred only viewed by the highest number of mutations*

From the data presented in Table 1 is shown that mutations that occur in Papua arowana fish originating from the waters of Merauke and Boven Diegul highly variable (hypervariable). Mutations that occur do not show a specific pattern of the DNA comparison. The results of further analysis and comparison test on the D-loop region of the comparable data showed new mutations in the arowana fish in both places in the southern part of Papua (Table 2).

Total number of nucleotide mutations in the D-loop region of mtDNA along the 1 kb of the CRS are 23 variants of the mutation. Of total mutations, mutations are unique ie transversion substitution mutation at position n + 2, changes into a cytosine nucleotides adenine, A → c.

CONCLUSION

From the stages of research that has been done, it can be concluded that the arowana fish in the southern region of the province of Papua, which is a regional ecosystem arowana fish, there are various variants. The region where the arowana fish habitat is quite numerous in Muting District and Ulilin District in the Regency of Merauke and District Waropko which is the regency of Boven Diegul. Primers designed for amplification of 1 kb arowana fish along the D-loop region of mitochondrial amplify about 600 bp in the forward and 600 bp reverse sequence. Based on the results obtained by analysis of the nucleotide sequence of arowana mitochondrial DNA D-loop region through the direct sequencing of samples arowana southern of Papua. Number of dominant mutations in Papua arowana fish as many as 9 positions on the D-loop region of mtDNA fish. From total mutations, mutations are unique ie transversion substitution mutation at position n + 2, changes into a cytosine nucleotides adenine, A → c. Nucleotide sequence analysis of the variability of the sample arowana fish in southern waters of Papua provide identifying information mutations were highly variable in both positions, quantity, and type of mutation that occurs.

Acknowledgements
The authors gratefully acknowledge the financial support by the Directorate General of Higher Education of Indonesia for Research Grant of the Master Plan for the Acceleration and Expansion of Indonesian Economic Development (MP3EI) to YN (Grant No. 0094/E5.1/PE/2015-Batch 1)

REFERENCES

[1] Boore, J.L. *Nucleic Acids Res*, (**1999**) 27: 1767-1780.
[2] Broughton, R.E., Milam, J.E., and Roe, B.A. *Genome Res*, (**2001**) 11: 1958-1967.
[3] Briolay, J., Galtier, N., Brito, R.M., and Bouvet, Y. *Mol. Phyl. Evol*, (**1998**) 9: 100-108.
[4] Hilton, E.J. *Zool.J.Linn. Soc*., (**2003**) 137: 1-100
[5] Kumazawa, Y., and Nishida, M. *Mol Biol Evol*, (**2000**) 17: 1869-1878
[6] Inoue, J.G., Miya, M., Tsukamoto, K., and Nishida, M. *Mol. Phyl. Evol*. (**2001**). 20(2): 275-285.
[7] Machida, R.J., Miya, M.U., Nishida, M., and Nishida, S. *Mar Biotechnol*, (**2002**) 4: 406-417
[8] Natalia, Y., Hashim, R., Ali, A., and Chong, A. *Aquaculture*, (**2004**) 233: 305-320.
[9] Yue, G.H., Liew, W.C., and Orban, L. *BMC Genomics*, (**2006**) 7: 242
[10] Yue, G.H., and Orban, L. *Mar Biotechnol*, (**2001**) 3: 199-204
[11] Pouyaud, L., Sudarto, and Teugels G.G. *Cybium*, (**2003**) 27(4): 287-305.
[12] Huelsenbeck, J.P., and Ronquist, F. *Bioinformatics*, (**2001**) 17: 754-755
[13] Roberts, T.R. *Mem.Calif. Acad, Sci*, (**1989**) 14: 1-210.
[14] Yue, G.H., Li, Y., Lim, L.C., and Orban, L. *Aquaculture*. (**2004**). 237: 89-102.
[15] Meyer, A. Elsevier Science Publishers, (**1993**) 1-38
[16] Nelson, J.S. 3rd ed, 600 p. New York. (**1994**) J. Wiley & Sons.
[17] Voris, H.K. *J. Biogeogr*., (**2000**) 27: 1153-11667.
[18] Yue, G.H., Chen, F., and Orban, L. *Mol. Ecol*, (**2000**) 9: 1007-1009.