Genotypic Characteristics of Red Tilapia & their Relationship with Other *Oreochromis* Spp.

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Abstract - Red tilapia is found in some provinces in the country but there is few genetic information about them. There is little information on the genetic characteristics of red tilapia cultured in the Philippines. Genotypic characteristics is necessary to determine their genetic background to be able to form a founder population with enhanced genetic traits like fast grower, bright coloration, high survival and high fecundity rate that are necessary for commercialization. The study evaluated the genotypic characteristics of red tilapia and to identify the evolutionary divergence of amino acid and base differences. Moreover, the study aimed to determine the percent similarities in nucleotide sequences of red tilapia. Collection of fish samples for DNA extraction were gathered from some provinces of the country. There were six treatments named after the source provinces such as MUNOZ, ZAMBALES, LAGUNA, FAC, BATANGAS AND MUNBA. 20 grams of fish sample taken from the dorsal part of the fish for DNA extraction, CO1 gene amplification, and gel electrophoresis. Data analysis using DNA Baser Sequence Assembler v4x software. To facilitate specimen identification, sequence contigs were compared to sequences available in Genbank of the National Center for Biotechnology Information (NCBI) and in Identification Engine-Barcode of Life Database Systems (ID-BOLD). A NJ tree was made in the Molecular Evolution Genetic Analysis (MEGA) software version 6.0. Sequence analyses accomplished using the MEGA v.6 software and Sequence Analysis Engine of Bold Systems. Genotypic characteristics exists within and between the strains. Results revealed that from 18 samples, ten Red Tilapia strains have identical DNA sequences with *Oreochromis niloticus*, seven have identical DNA sequences with *O. mossambicus* and one has identical DNA sequences with *O. aureus.* In amino acid divergence, Group 1 versus Group 2 have 28 number of base difference with p-distant value of 0.1489. Group 1 vs Group 3 have 40 number of base difference with p-distant value of 0.2128. Group 2 vs Group 3 have 34 number of base difference with p-distant value of 0.1809. On percent similarities, Group 1 vs Group 2 have 93.8 to 94.7 % similarities in nucleotides sequences, Group 1 vs Group 3 have 92.8 % similarities in nucleotides sequences, and Group 2 vs Group 3 have 92.9 % similarities in nucleotides sequences.

Index Terms- Genotypic characteristics, nucleotide sequences, number of base difference, percent similarities

1 Introduction

Tilapia, a native fish in Africa is the common name for almost a hundred species of fish that belongs to family Cichlidae [1]. This fish has been introduced in tropical waters of Asia and America as one of the most important sources of animal protein for human food [2].

The Philippine red tilapia strain was a cross between *O. mossambicus-hornorum* hybrid x *O. niloticus* [3] and this contradicts by [4], who reported that the Philippine red tilapia was composed of *O. mossambicus* x *O. niloticus* hybrid; and this strain exhibited high growth performance and high fecundity. [5], reported that DNA sequences and taxonomic analysis of Florida red and Philippine red tilapia hybrids were identical to those of *O. hornorum* and *O. aureus.* Thus, the Philippine red tilapia hybrid strain, which originated from Israel red Nile tilapia was originally an Egypt red tilapia (*O. niloticus* x *O. aureus*).

Conventional identification of morphological characteristics of red tilapia has limitations because they share common sets of protein profile. It is also difficult to determine red tilapia on species level [6] because it has been crossed with different species of Tilapia before it came to the Philippines.

A molecular tool such as DNA-based barcode is a potential application in aquaculture as this could identify individuals, families, and species. They are also important in the identification of hybrid strains within and between populations. Genetic markers have important implication in aquaculture to maintain genetic variability in the population. Genetic guidelines should also practice by the tilapia fingerling producers to maintain genetic diversity. DNA barcoding is a technique used to identify species of organism by using short DNA sequence from a standard (primer) and a region from the sample to analyze using molecular laboratory protocol [7]. It is also a tool used to assess phylogenetic history and genetic differences in a population [8], [9], [10], and assess genetic variability and inbreeding levels in stocks that leads to the loss of genetic diversity that can have detrimental effect on the performance of the individual, i.e., inbreeding depression [11]. Specimens for identification can be any parts of the fish such as fillets, fins, fragments, juveniles, larvae, eggs, or tissue as long as these were properly preserved.

Red tilapia has become the point of interest to many aquaculturist and researchers worldwide [12]. Essa *et al.* (1998), [13] reported that hybrid red tilapia plays an important role in the development of aquaculture industry because it evolved into much superior
quality of strains than its parents' species that their hybrid gives potential benefit for commercialization.

There is a need to develop genetically improved red tilapia by selective breeding to produce progeny that is better adapted to culture conditions, good conversion of foods into flesh and fast grower in order to meet the growing demand for high quality fingerlings. This study is looking at the biological information of red tilapia using molecular tool. Thus, this study used cytochrome *c* oxidase subunits 1 (CO1) to determine their genetic information to be used on aquaculture.

The purpose of the study was to evaluate the genotypic characteristics of red tilapia and their relationship with other *Oreochromis* spp. To identify the evolutionary divergence of amino acid and base differences and, to determine the percent similarities in nucleotide sequences of red tilapia.

2. MATERIALS AND METHODS

Collection of Samples

DNA genome samples were collected from 18 fish cages. One fish per cage were collected and brought to Molecular Biotechnology and Biology Laboratory in the College of Arts and Sciences, CLSU, Science City of Munoz, Nueva Ecija. Washing, cleaning and cutting of fillet at 20g taken from dorsal parts of fish and placed on tissue vials with cover for DNA extraction.

There are six treatments, with three replicates.

Treatment	Strains
1	MUNOZ
2	ZAMBALES
3	LAGUNA
4	FAC
5	BATANGAS
6	MUNBA

DNA Extraction, CO1 Gene Amplification and Gel Electrophoresis

Genomic DNA was extracted using DNeasy blood and tissue kit (QIAGEN® Group, Hilden Germany) following manufacturer's protocol. Initially, 180µL Buffer ATL and 20µL proteinase K were added to the homogenized tissue before incubating at 56°C. After incubation, 200 µL Buffer AL and 200 µL ethanol (95%) were added to the working solution. Sample was pipetted to the DNEasy Mini Spin column and centrifuged at 8000 rpm for 1 min. Supernatant was discarded and DNEasy Mini Spin column was transferred to a new 2 mL tube. Five hundred microliters of Buffer AW1 was then dispensed to sample and centrifuged for 3 min. Flow-through was again discarded and DNEasy Mini Spin column was finally placed in a new 1.5 mL tube. Buffer AE was directly pipetted to DNEasy Mini Spin column membrane before subjecting to final centrifugation. The final concentration of extracted DNA was determined using NanoDrop 2000c Spectrophotometer (Thermo Scientific, Waltman, MA).

Portion of mitochondrial cytochrome c oxidase I (COI) gene was amplified, using primer sets of [14]. Initially, the mastermix was made containing 5 µL of *Taq* Master Mix (Vivantis Technologies, Shah Alam, Malaysia), 1.5 µL nuclease free water (Promega Corporation, Madison, WI), 1 µL of forward and reverse primers and 2.5 µL DNA template. COI gene amplification was performed in programmable thermal cycler (Flex Cycler, Analytic Jena AG, Germany). Thermal condition consisted of initial denaturation at 94 C for 4 min, followed by 40 cycles of denaturation at 94 C for 30 s, annealing at 60.1 C for 1 min, elongation at 72 C for 30 s with an additional extension step of 7 min at 72 C.

PCR products were visualized on 1% agarose gel. Ten microliters of PCR products were loaded to the gel and electrophoresed at 150 volts for 45 min. Size of amplicons was determined using 100 base pair molecular weight ladder (Hoffman-La Roche Ltd., Basel, Switzerland). Electrophoresed gel was stained using GelRed™ Nucleic Acid Gel Stain (Life Technologies, India) for 30 min and visualized using Alphadigidoc Pro Imaging System (Alphainnotech Corporation, San Leonardo, CA). Sixty microliters of unpurified PCR products were sent to First BASE Laboratories, Malaysia, for bidirectional sequencing using BigDye Terminator v3.1 Cycle Sequencing Kit and ABI PRISM 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA) following manufacturer's protocol.

Data Analysis and Specimen Identification

Bidirectional sequences were assembled using DNA Baser Sequence Assembler v4.x (Heracle Biosoft SRL, Romania) and were aligned using Clustal Omega v 1.1.1 [15]. To facilitate specimen identification, sequence contigs were compared to sequences available in GenBank of the National Center for Biotechnology Information and in Identification Engine-Barcode of Life Database Systems (ID-BOLD) of the International Barcode of Life. Identification through DNA barcoding is accomplished by comparing unknown sequences to known barcodes available in database through different matching algorithms [16]. A Neighbor-Joining (NJ) Tree [17] was made in the Molecular Evolution Genetic Analysis (MEGA) software version 6.0 [18] to compliment the sequence matching result in Basic Local Alignment Search Tool and ID-BOLD. Bootstrap values for each node of the NJ tree were set to 1000 pseudo replications. Genetic distances were computed using Kimura two-parameter model [19], most commonly used genetic model in DNA barcoding studies. Sequence analyses
were all accomplished using MEGA v.6 software and

Sequence Analysis Engine of BOLD Systems.

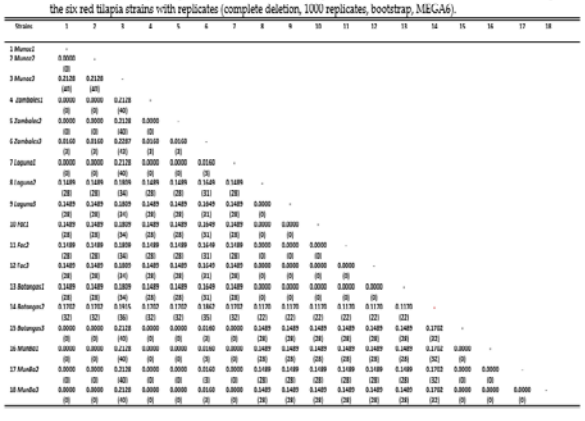
3. RESULTS AND DISCUSSION

Genotypic Characteristics

Generally, with aid of DNA barcoding processes, several biological processes can be inferred which can lead to drawing conclusions about their genotypic characteristics among others. Moreover, assessment of DNA sequences can determine how genetic sequences are closely related to other sequence whose function is actually known from biological or biochemical information [20]. In this study, DNA sequences yielded three distinct groups for three different species: *Oreochromis niloticus*, *O. mossambicus* and *O. aureus*. Species identification revealed that the six red tilapia strains, of three (3) species, one (1) genus and 18 samples in this study composed of mixed population of tilapia species: Group 1 composed of MUNOZ 1, 2; ZAMBALES 1, 2, 3; LAGUNA 1; BATANGAS 3; and MUNBA 1, 2, 3 have identical sequences with *Oreochromis niloticus*. Group 2, composed of LAGUNA 2, 3; FAC 1, 2, 3; BATANGAS 1, 2 have identical sequences with *Oreochromis mossambicus*, and group 3 composed of MUNOZ 3 identical sequences with *Oreochromis aureus*.

Table 3 shows that MUNOZ 3 (group 3 of *Oreochromis aureus*) have p-distance value of 0.2128 and number of base differences is 40 against MUNOZ 1 & 2 (group 1 of *O. niloticus*) which indicate that there is a genetic variability between MUNOZ 1 & 2 against MUNOZ 3. LAGUNA 2, 3 (group 2 of *O. mossambicus*) with p-distance value of 0.1489 and number of base differences is 28 against LAGUNA 1implies that there is also a genetic variation among treatments. BATANGAS 3 (group 1 of *O. niloticus*) had p-distance value of 0.1702 and 32 amino acid base differences against BATANGAS 2 (group 2 of *O. mossambicus*). This implies that there is a genetic variability between BATANGAS 3 and BATANGAS 2.

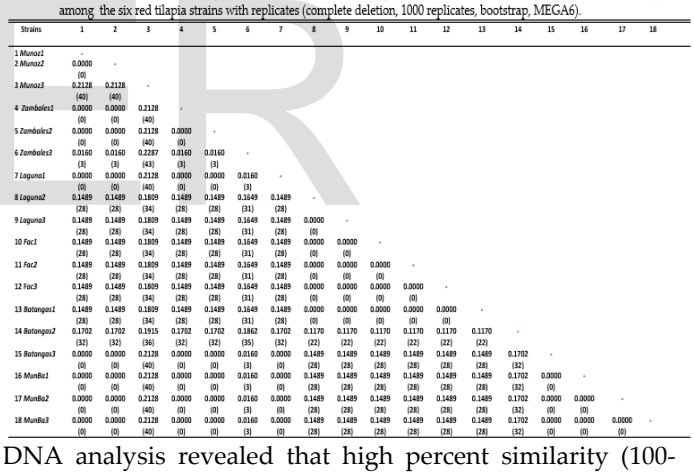
Table 3. Estimates of evolutionary divergence of Amino Acid differences (p-distance model) and number of base differences (in parenthesis) among the six red tilapia strains with replicates (complete deletion, 1000 replicates, bootstrap, MEGA6).



This study reflected that amino acid distances between *O. niloticus* and *O. mossambicus* is 0.1489 and 28 number of base differences. Between *O. mossambicus* and *O. aureus* amino acid distances is 0.1809 with 34 number of base differences. Amino acid distances between *O. niloticus* and *O. aureus* is 0.2128 and number of base difference is 40.

Table 4 of percent similarities in nucleotide sequences, shows that group 1 (MUNOZ 1, 2; ZAMBALES 1, 2, 3; Laguna 1; BATANGAS 3; MUNBA 1, 2, 3) against group 2, had 93.8 to 94.7 % similarities in nucleotide sequences. Group 1 versus group 3 (MUNOZ 3) had 92.8 % similarities in nucleotide sequences. Group 2 (LAGUNA 2, 3; FAC 1, 2, 3; BATANGAS 1, 2) against group 3 (MUNOZ 3) had 92.9 % similarities in nucleotide sequences. These indicate that relationship between group 1 and group 2 is closer than group 1 and group 3. The lower value of similarities in nucleotide sequences, indicate that there is high a genetic variability between the two groups. This indicate that there is a high genetic variability between group 1 and group 3 than group 1 and group 2. Melo *et al*. (2006) [21] reported that low variability within individuals could be linked to disproportion of males and females used in reproduction.

Table 3. Estimates of evolutionary divergence of Amino Acid differences (p-distance model) and number of base differences (in parenthesis) among the six red tilapia strains with replicates (complete deletion, 1000 replicates, bootstrap, MEGA6).



DNA analysis revealed that high percent similarity (100-99.1%) and low number of base differences ranges from 0 – 3 of red tilapia in group 1 (MUNOZ 1, 2; ZAMBALES 1, 2, 3; LAGUNA 1; BATANGAS 3; MUNBA 1, 2, 3) showed that these strains are closely related to *O. niloticus.*

Group 2 (LAGUNA 2, 3; FAC 1, 2, 3, BATANGAS 1,2) with 93.8 – 94.7 percent similarities in nucleotide sequences and number of base differences is 28, indicates that these strains are closely related to *Oreochromis mossambicus*. Group 3 (Munoz 3) with 92.8 percent similarities in nucleotide sequences and high number of base differences 0- 40 indicate that this strain is not closely related to neither *O. niloticus* nor *O. mossambicus* but rather closely related to *Oreochromis aureus*. These results revealed that high genetic variability exists due to genetic differences at the individual level.

CONCLUSION

Genotypic characteristics exists within the strain between. Ten Red Tilapia have identical DNA sequences with *Oreochromis niloticus*. Seven Red Tilapia have identical DNA sequences with *O. mossambicus.* One Red Tilapia has identical DNA sequences with *O. aureus.* Group 1 versus Group 2 have 28 number of base difference with p-distant value of 0.1489. Group 1 vs Group 3 have 40 number of base difference with p-distant value of 0.2128. Group 2 vs Group 3 have 34 number of base difference with p-distant value of 0.1809 Group 1 vs Group 2 have 93.8 to 94.7 % similarities in nucleotides sequences. Group 1 vs Group 3 have 92.8 % similarities in nucleotides sequences. Group 2 vs Group 3 have 92.9 % similarities in nucleotides sequences.

RECOMMENDATIONS

Red tilapia has great potential for large-scale aquaculture industry. It is important to improve the quality of seeds through genetic breeding, nutrition and feeding technologies and production technologies. Improved management of Brood stock by taking extra careful to prevent high level of homozygosity and subsequent deterioration of its genetic quality. Increase stocking density in ponds. And introduce red tilapia in marine cage technology with high stocking density.

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REFERENCES

[1] Trewavas, E. (1983). Tilapine fishes of the genera *Sarotherodon*, *Oreochromis* and *Danakila*. Cornel University Press. Itaca, New York.

[2] El- Sayed, M., Abdel-Fattah. (2006). Tilapia culture: CABI Publishing, 875 Massachusetts Avenue, 7th floor Cambridge, M A 02139 USA.

[3] Eguia, M. R. R. and R. V. Eguia. (1998). Growth of five Asian red tilapia strains in saline environments. Aquaculture, 173: 161-170.

[4] Pruginin, Y., L. Fishelson and A. Koren. (1998). "Intensive Tilapia Farming in Brackishwater from An Israeli Desert Aquifer". Pp. 75-82. In: R.S.V. Pullin, T. Bhukaswan, K. Tonguthai and J. L. Maclean (eds.) The second international Symposium on tilapia in Aquaculture. ICLARM Conference proceedings 15. MCC P.O. Box 1501, Makati, Philippines.

[5] Shirak, A., M. Cohen-Zinder, R. M. Barroso, E. Seroussi, M. Ron and G. Hulata. (2009). DNA Barcoding of Israel endemic and introduced cichlids. Israel Journal in Aquaculture-Bamidgeh 61 (2): 83-88.

[6] Ardura, A., A. R. Linde, J.C., Moriera and E. Garcia-Vazquez. (2010). DNA barcoding for Conservation and management of Amazonian commercial fish. Biological Conservation 143: 1438-1443.

[7] Becker, S., R. Hanner and D. Steinke. (2011). Fishery-barcode of Life (Fish-Bol): Brief status report. Mitochondrial DNA 22: 3-9.

[8] Davidson, W. S. (1998). Analytical methods of food authentication. In P.R. Ashurst, and M. J. Dennis (eds.) Blackie Academic and Professional, pp. 182-203.

[9] Bossier, P. (1999). Authentication of seafood products by DNA patterns. Journal of food Science 64:189-193. Doi: 10. 1111/j. 1365-2621. 1999. Tb 15862.x

[10] Lockley, A. K. and R. G. Bardsley. (2000). DNA based methods for food authentication. Trends in Food Science Technology 11: 67-77.

[11] Fessehaye, Y., J. Komen, M. A. Rezk, R. W. Ponzoni and H. Bovenhius. (2007). Effect of inbreeding on the performance of Nile Tilapia. Aquaculture 272: 247-247.

[12] Wohlfarth, G. and G. Hulata. (1983). Applied genetics of tilapia. ICLARM Studies and Review 6. International Center for Living Aquatic Resources Management, Manila Philippines.

[14] Ward, R.D., T.S. Zemlak, B.H. Innes, P.R. Last andP.D.N. Hebert. 2005. DNA barcoding Australias fish species. Philosophical Transactions of the Royal Society B 360: 1847-1857.

[15] Sievers, F., A. Wilm, D. Dineen, T.J. Gibson, K. Karplus, LI. W. Lopez, R. McWilliam, H. Remmert, M. J. Siding, J.D. Thompson and D.G. Higgins. 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using clustal omega. Molecular System and Biology 7: 539.

[16] Erikson, D.L. and W.J. Kress. 2012. Future Directions. DNA Barcodes: Methods and Protocols. Methods in Molecular Biology. Humana Press. pp. 459-465.

[17] Saito, N. and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4: 406-425.

[18] Tamura, K., G. Stecher, D. Perterson, A. Filipski and S. Kumar. 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6. Molecular Biology and Evolution 30 (12): 2725-2729.

[19] Kimura, M. 1980. "A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences" Journal of Molecular Evolution 16 (2): 111-120.

[20] HALL, BG. 2001. Phylogenetic trees made easy a how-to manual for molecular biologist. Sunderland, Massachusetts: Sinauer Associates.

[21] Melo, Dc., D.A.A. Oliveira, L.P. Ribeiro, C.S.Teixeira, A.B. Sousa, E.G.A. Coelho, D.V. Crepaldi, And E.A. Teixeira. 2006. Caracterização genética de seis plantéis comerciais de tilápia (*Oreochromis*) utilizando marcadores microssatélites. Arq. Bras. Med. Vet. Zootec. 58: 87-93.