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Mitochondrial 16S and 12S rRNA/tRNA-Val Gene Analysis in Tiger Barbs (*Puntius tetrazona*)

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Abstract: Sequence analysis of the mitochondrial 16S rRNA and 12S rRNA genes have been used for molecular taxonomy in many fishes. Three varieties (normal, green and yellow) of tiger barb were sampled to identify the genetic polymorphism using the mtDNA study. In this study, a 620 and 730 bp hyper variable region of the partial mitochondrial 16S rRNA and 12S rRNA/tRNA-Val gene have been analyzed. Sequence analysis of these genes were applied to indicate the arrangement patterns between these varieties and the sequence was found to be usually A+ T rich. The nucleotide composition indicates that the green variety, in contrast with that of normal and yellow varieties. The analysis of the gene sequences revealed a degree of similarity between normal, green and yellow with the percent in range of 15 to 73. The phylogenetic tree was constructed with these sequences. Normal and yellow variety was closer than the green variety with the genetic distance in range of 0.17 to 0.35.

Key words: Tiger barb, mitochondrial, polymorphism, 16S rRNA, 12S rRNA/tRNA-Val

Introduction

Tiger barbs (*Puntius tetrazona*) which is natives to South East Asia is one of the popular ornamental fish species cultured and is a significant component of the aquarium fish industry in Malaysia. The fish has 4 vertical black bands and a reddish body and hence the name tiger barb. Tiger barb is an active fish and this behavior coupled with their brightly coloured body, is a much desired aquarium fish all over the world. The various forms of bands and colour of tiger barbs bring an interest to study its genetic evolution and variability.

The mitochondrial genome of vertebrates is a single, small, double stranded; circular DNA molecules contained in mitochondria and up to several thousand copies of the mitochondrial genomes are found per cell. In animals the mitochondrial genome is approximately 16,500 bp long and possesses 13 genes coding for proteins, two genes coding for ribosomal RNA's, 22 genes coding for transfer RNA's and one major non-coding region, the d-loop, which contains the origin of replication (Meyer, 1993).

The mtDNA analysis has many features, which makes it useful for population genetic study. The mtDNA has been completely sequenced in many different organisms belonging to different phyla or taxa. It is do not depend on the availability of hybridization probe and very useful for the study of population structure within species. Knowledge of the base sequence of an entire mtDNA genome would allow comparisons with other known mtDNA genomes through the analysis of gene order. The basis of non-shared mtDNA sequences will be consistently separated the species although the analysis has revealed extensive homology between them.

It has revealed extensive structuring of population of many fish species however in some fish it has not successfully identified. Because of its high rate of evolution, mitochondrial DNA is extremely useful molecule for high-resolution analysis of evolutionary processes (Brown *et al*., 1979). It has been used in the phylogenetic analysis of insects (Misof *et al*., 2000), amphibians (Morita, 1999) and fishes (Richards and Moore, 1996). The sequence of ribosomal RNA molecules has been widely used for phylogenetic studies and sequences differences in hyper variable regions reflect strain variations.

Mitochondrial DNA has become a popular tool in phylogenetic systematic and population biology. Animal mitochondrial have a number of properties that makes them attractive to work with. They are independent units and can thus be easily extracted from the cell and separated from genomic DNA (Avise *et al*., 1987). The gene order is highly conserved within phyla (Moritz *et al*., 1987; Meyer, 1993). Work using mitochondrial genes has been done to study interrelationships of populations, families and even orders (Moritz *et al*., 1987; Lamb *et al*., 1994). There are several studies that have found phylogenetic relationships above the family level, difficult to resolve with the use of ribosomal RNA genes (Orti and Meyer, 1997). It is seem ideal for phylogenetic studies at lower taxonomic levels, such as for resolving interrelationships of genera and species (Hillis and Dixon, 1991). The objective of this study was to use polymerase chain reaction (PCR) based on mitochondrial DNA analysis to examine the genetic relationship among three variety of tiger barb (normal, green and yellow). The comparison of mtDNA gene arrangement between the varieties was used to develop the dendogram.

Materials and Methods

DNA extraction and gene amplification: Fish were collected from the Peninsular of Malaysia and preserved in 95% ethanol until used. Total DNA of tiger barb from three varieties (normal, green and yellow) was extracted from the muscle tissue by Pure-Gene Tissue Kit II (BST TechLab).

Universal L2S10 (5'-CGC CTG TTT ATC AAA AAC AT-3') and H3080 (5'-CCG GTC TGA ACT CAG ATC AC GT-3') primers were used to amplify a partial region of 16S rRNA gene (about 620 bp) (Palumbi *et al*., 1991). The second portion was obtained from the 12S rRNA/tRNA-Val (about 730 bp) was amplified using the following universal primer pair: OMT16SF ('5 – TGC CAG CCA CCG CGG TTA TAC CT –3') and tRNA02 (5' – GGA TGT CTT CTC GGT GTA AG – 3') (Saiki *et al*., 1988). PCR reactions included 1X PCR buffer, 5 mM MgCl, 0.2 mM dNTPs (Biotools), 0.03 μM primer and 0.5U taq (Biotools) in a 15 μl reaction. Amplification was carried out for 35 cycles using denaturing temperature at 95°C for 1 min followed by annealing temperature at 55°C for 1 min and extension temperature at 72°C for 2 min. The amplification products were electrophoresed at a 2% (w/v) agarose gel to ensure the successful of mtDNA isolation.

PCR purification: The PCR products were purified to remove unincorporated primers and nucleotides by the commercial enzyme kit (PCR product Pre- Sequencing Kit); the PCR product was mixed with the mixture of 0.7 μl SAP, 0.7 μl EXON I, 1.6 μl deionized water and incubated at 37°C for 15 min followed by 80°C for 15 minutes.

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Sequencing analysis: The sequence amplification was performed with the Big Dye Terminator Cycler Sequencing Kit (ABI, Perkin Elmer). The purified products (2 µl) were mixed with 1 µl reaction mixture, 0.5 µl primer (for each reaction the forward and reverse primer were prepared) and 6.5 µl distilled water. The reaction mixture was performed in a Gene Amp PCR System 2400 at 96°C for 30, 15 second at 50°C and 4 min at 60°C for 25 cycles. Purification of sequence reaction of PCR product was done with the mixture of 3M sodium acetate and 95% ethanol (2:30). The mixture was mixed with the PCR product and incubated at room temperature for 15 minutes. The sample was centrifuged at 15,000 rpm for 20 min; the supernatant was removed and the pellet was mixed with a 200 µl 70% ethanol and centrifuge again. The pellet was air dried to remove the ethanol. The formamide (12 µl) was added into the sample, incubate at room temperature, pipetting up and down, incubated at 95°C for 2 min and hold on ice. Labeled fragments were analyzed using Automated Sequencer of ABI Prism 310 Genetic analyzer (Perkin-Elmer).

Data analysis: Sequence alignment was done using the Clustal W program with default settings (Higgins and Sharp, 1988). Juke-Cantor distances were calculated and the phylogenetic tree was constructed using UPGMA method. Bootstrapping was done for 1000 replicates. Phylogenetic tree was also constructed using Kimura 2 parameter distances (Kimura, 1980) and the results were identical when transition plus transversions or only transversions were used for the analysis.

Results and Discussion

A 620 and 730 bp fragment of mitochondrial 16S rRNA and 12S rRNA/tRNA-Val was amplified using universal primers (Fig. 1) and was further sequenced. These primers successfully sequence the 16S rRNA and 12S rRNA/tRNA-Val gene fragments and nucleotide sequence of around 500 and 539 bp were obtained. The nucleotide base composition of the sequenced rRNA segments showed that like most other fish mitochondrial rRNA gene (Oohara and Okazaki, 1996). The A+T content were between 42-60% for all varieties studied. Similar observation has been made in 16S rRNA gene of Atlantic herring, *Clupea harengus* (Kornfield and Bogdanowicz, 1987). Alternatively, a high A+T content could be imposing constrain on the sequence.

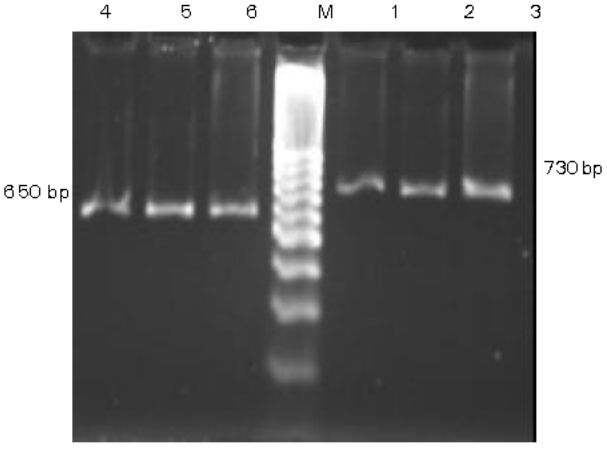

Fig. 1: The amplification of partial 16S rRNA and 12S rRNA/tRNA-Val gene in three varieties of tiger barb. The DNA extracted from the samples were used as templates and successfully amplified. Lane 1 and 4, normal variety; lane 2 and 5, green variety; lane 3 and 6, yellow variety; lane 1 to 3 represent the amplification of partial 16S rRNA gene (650 bp) while Lane 4 to 6 were amplified 12S rRNA/tRNA-Val gene (730 bp). Lane M are molecular weight marker (100 bp Plus).

Table 1: Genetic distance values among tiger barb variety for 16 and 12S rRNA Gene

	Normal	Green	Yellow
16SrRNA			
Normal	0.0000		
Green	0.0718	0.0000	
Yellow	0.2839	0.3589	0.0000
12SrRNA			
Normal	0.0000		
Green	0.0341	0.0000	
Yellow	0.2589	0.2700	0.0000

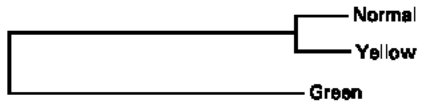

Fig. 2: Phylogenetic tree based on genetic distances from analysis of the aligned partial mitochondrial 16S rRNA gene sequences of tiger barb. Distances were estimated by Kimura's two parameter model.

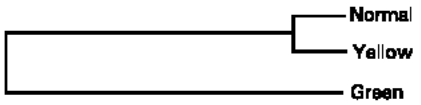

Fig. 3: Phylogenetic tree based on genetic distances from analyses of the aligned partial mitochondrial 12S rRNA/tRNA-Val gene sequences of tiger barb. Distances were estimated by Kimura's two parameter model.

In this study we address the relationship among three variety of tiger barb (normal, green and yellow) to assess mtDNA variation. The tiger barb complete sequence has not been determined yet. The comparison of mtDNA gene arrangement between different fishes orders belonging to the same class shows that the gene organization is conserved between the same fish class.

The distance matrix analysis for the tiger barb variety used in this study (Table 1). Phylogenetic tree constructed from the 16S rRNA and 12S rRNA/tRNA-Val sequence data were shows in Fig. 2 and 3. The sequences were aligned in Clustal W and the tree was constructed in PHYLIP using the neighbour joining method. Normal and yellow variety was closer than the green variety for the analysis of gene 16S and 12S/tRNA-Val with the genetic distance in range of 0.03 to 0.35. The average for percent of similarity between normal and green was 5 and 73% for normal and yellow while 69% for green and yellow. Such DNA sequences are variable enough for investigation of phylogenetic relationships, because optimal similarities of DNA sequences for phylogenetic studies are between 70 and 100% (Hillis and Dixon, 1991).

Sequencing work was orientated toward phylogenetic and evolutionary studies, where it was sufficient to sequence the DNA regions from only one individual representing each species under study. In this technique PCR is used to provide sufficient amplified mtDNA to support sequencing in analysis using either one of the PCR primer. The amplified DNA can be reamplified using one primer in excess (Gyllensten and Ehrlich, 1988). This provides a single stranded template, which improves the quality of the sequencing results. Analysis of this and hyper variable regions may permit an investigation of genetic relatedness of fish population at the sub specific levels. The analysis will be valuable in studies involving molecular taxonomy, particularly for those species that are difficult to identify using morphologic characteristic and in epidemiological research. Therefore mtDNA sequences are considered to be a good molecular marker for the reconstruction of fish phylogenies.

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