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Molecular characterization of ornamental fish (*Poeciliidae*) using mitochondrial DNA 12S rRNA and 16S rRNA genes

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ABSTRACT

Mitochondrial DNA (mtDNA) ribosomal genes have been used as molecular markers for fish species identification in many studies. In this study, two mtDNA ribosomal genes namely 12S rRNA and 16S rRNA have been utilized to characterize ornamental fish species from Poeciliidae family. Five ornamental fish species namely Poecilia sphenops, Poecilia reticulata, Limia vittata, Xiphophorus hellerii and Xiphophorus maculatus were used. PCR amplification was performed and DNA sequencing was carried out in order to study the genetic relationship of fish species. More polymorphisms were seen in 16S rRNA as compared to 12S rRNA. Phylogenetic tree results showed two clusters with one cluster consisting of Limia vittata, Xiphophorus hellerii and Xiphophorus maculatus and the second cluster consisting of Poecilia sphenops and Poecilia reticulata.

Keywords: Mitochondrial DNA, Poeciliidae, phylogenetic tree

INTRODUCTION

Identification and classification of animal species is a key prerequisite for many biological studies. Frezal and Leblois (2008), state that the identification of species depends on the knowledge and experiences of taxonomists [1]. Species identification strictly on the basis of morphological characters alone is quite unreliable, because of considerable geographical and ecological variability [2,3].Study by Herbert et al. (2003), indicate four main significant limitations for species identification based on morphological characters [4]. First, species recognition using phenotypic plasticity and genetic variability in the characters can lead to incorrect identifications. Second, this method overlooks morphologically puzzling taxa, which are common in many groups. Third, many individuals cannot be identified since the morphological keys are often effective only for a particular life stage or gender and fourth, misdiagnoses of the species still can occur.

Recently, a number of new methods have been developed and utilized for fish species identification. According to Bossier (1999), morphological features are more suitable for identification of fresh fish but in situation of processed fish this method is not suitable since they do not retain enough morphological characteristics for identification purpose [5]. Traditional method based on separation and characterization of specific protein using electrophoretic techniques such as isoelectric focusing (IEF) [6] and capillary electrophoresis (CE) [7] are proved to be reliable and easy to be used in food identification, but not suitable for heat-treated products as thermal treatment as in canning, smoking or drying leads to irreversible loss of solubility [6, 8]. Application of techniques based on the analysis of

nucleic acids such as mitochondrial DNA or nuclear DNA offer an advantage over protein-based techniques since they are not dependent on tissue source, age of the individual or/and sample damage [9, 10].

Kochzius (2009) and Teletchea (2009)suggested that mitochondrial DNA (mtDNA) genes are promising markers for fish species identification when compared to nuclear genes due to special features of mtDNA [11, 12]. Mitochondrial DNA occupy high copy number in each cell as well as small in size 15-20 Kb which made mtDNA successful to recover from limited or degraded samples [13]. The features of maternal inheritance pattern without recombination [14] and rapid mutation rate made mtDNA suitable as a tool for studying phylogeny and genealogy of taxa through matrilineage [15]. All these mtDNA characteristics make it useful for analysis of processed samples [16]. Several mtDNA markers such as cytochrome B, cytochrome oxidase I, hypervariable region and ribosomal genes (12S rRNA and 16S rRNA) have been used in species identification.

Study by Ludwig *et al.* (2004) has showed that the application of small subunit of ribosomal RNA gene as a standard method for identifying microbial organisms [17].The mitochondrial ribosomal genes including 12S rRNA and 16S rRNA and nuclear ribosomal genes such as 28S rRNA, 5.8S rRNA and 18S rRNA are widely used as genetic markers for phylogenetic analyses [18]. According to Hillis and Dixon (1991), ribosomal RNA sequences have been used to infer phylogenies across a very broad spectrum, from studies among the lineages of life to relationships among closely related species and populations [19]. It has been reported that mtDNA 12S rRNA and 16S rRNA genes have been used extensively as molecular markers to categorize mammals, birds, shrimp and other species [10]. According to Kitano et al. (2007), several conversed region that found in mtDNA 12S rRNA and 16S rRNAloci and the characteristic of high copy number occupied by mtDNA has made these region as a choice for species identification [20].

Multiple DNA based approaches have been developed for species identification, including DNA hybridization, restriction enzyme digestion, random PCR amplification, species-specific PCR primer use, and DNA sequencing [10]. The application of DNA sequencing has provided a new insight into identification of animal species. DNA sequence-based identification utilizes the refined Sanger sequencing method which is still the ‘‘gold standard’’ but requires samples that contain DNA of only one specimen [11] Currently, DNA sequencing analysis is the most used method for molecular species identification [21].

The ornamental fish is popular as aquarium fish in Malaysia due to their attractive of physical appearance, size and color. The definition of ornamental fish is referring to an aquatic animal that kept in the aquarium or a garden pool and not suitable for fishing. The Ornamental species include fishes, invertebrates such as coral, crustaceans (e.g., crabs, hermit crabs, shrimps), mollusks (e.g., snails, clams, scallops), and also live rock [22]. In Malaysia, more than 550 varieties of ornamental fish belonging to 250 species are cultured and 95% of them are commercially exported. Many of ornamental fish belong to freshwater habitat. The ornamental fish species belong to nine families namely *Cyprinids, Cobitids, Cypinodontidss, Anabantidis, Poecilids, Characins, Cichlids, Osteoglossid and Callchthyids. Poecilids* family becomes the second highest ornamental fish exported after aquatic plant [23].

Poecilids also known as *Poeciliidae* belong to a single family of freshwater fish known as the *Poeciliidae*[24]. The family *Poeciliidae* is a widespread and diverse group of small-sized fishes that includes 22–29 genera and more than 200 species [25]. *Poeciliidae* is one of four groups of *Cyprinodontiform* order fishes that evolved internal fertilization [26]. *Poeciliidae* family consists of guppies, mollies, platies, and swordtails. All *Poeciliidae* family shares a similar body shape with a distinct upturned mouth, and in most cases the males are substantially smaller than the females [24]. Guppies and mollies belong mostly to the genus *Poecilia* while the swordtails and platies belong to the genus *Xiphophorus*.[24], has reported that in *Poeciliidae* family, hybridized species occurred between guppy-molly [24]. Hence, it is important to study genetic affinity of the ornamental fish especially *Poeciliidae* family since there are less study on assessment of genetic background of *Poeciliidae* family through molecular DNA marker especially mtDNA ribosomal genes to distinguish hybridized species from wild type species. This is the first study on genetic background of ornamental fish from *Poeciliidae* family conducted using mtDNA ribosomal genes 12S rRNA and 16S rRNA as molecular markers to investigate the molecular relationship of *Poeciliida*e family.

MATERIALS AND METHODS

Sample Collection

In this study, two individuals from each of the five species of *Poeciliidae* family were selected to confirm the DNA sequence. *Poeciliidae* family consist of three distinct genera of fish species (Table 1). The scientific name for each of the fish species was referred to the Department of Fisheries Malaysia.

Genomic DNA extraction

Genomic DNA was extracted from ethanol-preserved muscle tissue from fish species using QIAamp tissue kit following provided manual (Qiagen, Valencia CA). The integrity of the extracted DNA was assessed by agarose gel electrophoresis. The presence of high molecular weight (HMW) DNA was observed under UV light using Image. The extracted DNA was quantified using spectrophotometer prior storage at −20 °C. The reading for extracted genomic DNA of fish species was recorded for further use in PCR amplification.

Table 1: List of *Poeciliidae* family fish species

Genus Name	Common Name	Scientific Name	Picture
Limia	Cuban Limia fish	*Limiavittata*	
Poecilia	Guppy fish	*Poecilia reticulata*	
	Black Molly fish	*Poecilia sphenops*	
Xiphophorus	Platy fish	*Xiphophorusmaculatus*	
	Swordtail fish	*Xiphophoru shellerii*	

PCR amplification of 12S rRNA, 16S rRNA

In this study, published universal primers were used to amplify partial sequence of 12S rRNA (L1067F: 5'-AAA CTG GGA TTA GAT ACC CCA CTAT-3' and H1478R: 5'-GAG GGT GAC GGG CGG GCG GTG TGT-3', and 16S rRNA (L2510F: 5'-CGC CTG TTT ATC AAA AAC AT-3', and H3080R: 5'-CCG GTC TGA ACT CAG ATC ACG T-3') genes [27]. These primers generate PCR products of 350 bp and 550 bp for 12S rRNA and 16S rRNA respectively.

The PCR mixture was amplified in 20 uL consisting of 1 uL of 10 pmol of each primer (Sigma, USA), 0.32 uL of 10 mM dNTPs (Bioline, USA Inc), 1X PCR buffer (Bioline, USA Inc), 2 uL of 25 mM MgCI2 (Bioline, USA Inc) and 1 U of Taq DNA Polymerase (Bioline, USA Inc). The reaction conditions are 95 °C for 3 minutes followed by 30 cycles of 95 °C for 30 seconds; 61 °C (12S rRNA)/58 °C (16S rRNA) for 1 minute; 72 °C for 1 minute and a final extension at 72 °C for 5 minutes. All PCR amplicons were checked by electrophoresis on 1% agarose gel and then purified using PCR purification kit (Qiagen, USA). The purified PCR product was adjusted to 20-30 ng/uL of concentration using water for further use in sequencing.

Direct sequencing

A total of 10 µl sequencing reaction was prepared consisting of purified PCR product, 3.3 pmol of primers, and 1:8 ABI BigDye® Terminator versions 3.1. PCR cycle sequencing was performed on GeneAmp PCR System 9700 (Applied Biosystems). The following thermal cycle condition was used: initial denaturation at 96°C for 1 minute, followed by 25 cycles of denaturation at 96°C for 10 seconds, annealing at 50°C for 5 seconds, extension at 60°C for 4 minutes, and final hold at 4°C. The cycle sequencing reaction then was purified by ethanol precipitation prior to sequencing on ABI the 3130*xl* Genetic Analyzer

Analysis of the Sequences

The polymorphisms reported in this study were analyzed using MEGA 4 and BioEdit ver. 4.0 software. The presence of polymorphism such as transition, transversion, insertion and deletion were recorded. The Neighbor-Joining tree was constructed using Kimura 2-parameter distance model via Mega 4 software. Kimura-2-parameter distance model corrects for multiple hits, taking into account transitional and transversional substitution rates, while assuming that the four nucleotide frequencies are the same and that rates of substitution do not vary among sites [28].

RESULTS AND DISCUSSION

In this study, mitochondrial DNA 12S rRNA and 16 S rRNA genes have been used to characterize five ornamental fish species of *Poecilids* family namely *Poecilia reticulata, Limia vittata, Poecilia sphenops, Xiphophorus maculatus* and *Xiphophorus hellerii*. A 350 bp and 550 bp of PCR products was amplified byPCR amplification of mtDNA12S rRNA and 16S rRNA gene respectively. Based on sequence analysis of partial amplification of mtDNA 12S rRNA and 16S rRNA the highest percentage of GC content for both regions were found in *Limia vittata* with 52.07% and 48.80% respectively (data not showed). *Poecilia reticulata* was found to have the lowest GC contents with 49.15% for partial mtDNA 12S rRNA and 45.44% for mtDNA16S rRNA (data not showed).

The percentage of similarities between each species was analyzed using BioEdit software (Table 2). The highest percentage of similarities of two species was seen between *Limia vittata* and *Xiphophorus hellerii* in both mtDNA 12S rRNA and 16S rRNA genes with 98.59% and 98.64% respectively (Table 2). All fish species showed more than 90% of sequence similarities between two fish species for both mtDNA 12S rRNA and 16S rRNA genes (Table 2).

Table 2: The pairwise comparison of fish species showing percentage of similarities

Species	12S rRNA (%)	16S rRNA (%)
Poeciliareticulata and *Poeciliasphenops*	95.93	95.08
Poeciliareticulata and *Limiavittata*	92.60	91.23
Poeciliareticulata and *Xiphophorusmaculatus*	91.83	91.39
Poeciliareticulata and *Xiphophorushellerii*	91.29	90.20
Poeciliasphenops and *Poeciliareticulata*	95.93	95.08
Poeciliasphenops and *Limiavittata*	93.57	93.25
Poeciliasphenops and *Xiphophorusmaculatus*	93.76	92.61
Poeciliasphenops and *Xiphophorushellerii*	92.25	92.07
Limiavittata and *Poeciliareticulata*	92.60	91.23
Limiavittata and *Poeciliasphenops*	93.57	93.25
Limiavittata and *Xiphophorusmaculatus*	97.61	97.81
Limiavittata and *Xiphophorushellerii*	98.59	98.64
Xiphophorusmaculatus and *Poeciliareticulata*	91.83	91.39
Xiphophorusmaculatus and *Poeciliasphenops*	93.76	92.61
Xiphophorusmaculatus and *Limiavittata*	97.61	97.81
Xiphophorusmaculatus and *Xiphophorushellerii*	96.24	97.46
Xiphophorushellerii and *Poeciliareticulata*	91.29	90.20
Xiphophorushellerii and *Poeciliasphenops*	92.25	92.07
Xiphophorushellerii and *Limiavittata*	98.59	98.64
Xiphophorushellerii and *Xiphophorusmaculatus*	96.24	97.46

The lowest sequence similarities for mtDNA 12S rRNA and 16S rRNA was observed between *Xiphophorus hellerii* and *Xiphophorus maculatus* (91.29%) and between *Poecilia reticulata* and *Xiphophorus hellerii* (90.20%) respectively (Table 2).

The sequence analysis results showed that the highest total polymorphisms in both mtDNA 12S rRNA and 16S rRNA, was seen in *Poelicia reticulata*. No transversions polymorphism was found in *Limia vittata* and *Xiphophorus hellerii* for mtDNA 12S rRNA and 16S rRNA. Interestingly, one insertion was found in fish species *Poecilia sphenops* for mtDNA 12S rRNA (Figure 1) and for mtDNA 16S rRNA it was observed in *Xiphophorus maculatus*(Figure 2). Transitions T ↔ C is the common polymorphism observed in both mtDNA genes. *Limiavittata* and *Xiphophorus hellerii* were found to have less polymorphisms compared with other fish species in both mtDNA 12S rRNA and 16S rRNA genes (Table 3). The only transversion G ↔ C was seen in *Poeciliareticulata* in mtDNA analysis of 16S rRNA. A total of 194 of polymorphisms were observed in mtDNA 16S rRNA compared to 100 polymorphisms in mtDNA 12S rRNA (Table 3).

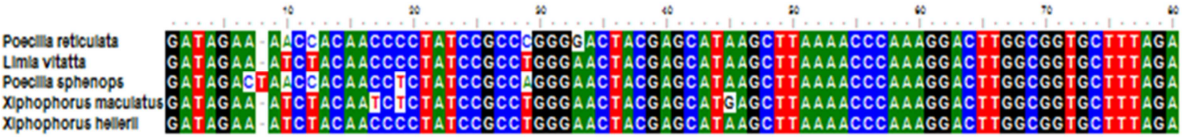

Figure 1: Multiple ClustalW alignment of mtDNA 12S rRNA showed insertion T was observed in *Poecilia sphenops*

Figure 2: Multiple ClustalW alignment of mtDNA 16S rRNA showed insertion C was observed in *Xiphophorus maculatus*

Table 3: Total polymorphism observed in mtDNA 12S rRNA and 16S rRNA genes

Fish Species	Total Polymorphism	
	12S rRNA	16S rRNA
Poecilia reticulata	24	50
Limia vittata	1	3
Poecilia sphenops	20	36
Xiphophorus maculatus	4	5
Xiphophorus hellerii	1	3

Phylogenetic tree is the mathematical structure which models the evolutionary history of a group of sequences. In phylogenetic tree the ancestor is located in the tree of trunk; organisms that have arisen from it are placed at the end of the tree branches. The distance of one group from other group indicates the degree of relationship. There are two types of phylogenetic tree, unrooted and rooted tree. An unrooted tree illustrated the relatedness of the nodes without creating assumptions regarding ancestry; meanwhile rooted tree is directed trees corresponding to the most recent ancestor.

In this study, an unrooted neighbor-joining phylogenetic tree was constructed from partial sequence of mtDNA 12S rRNA and 16S rRNA using MEGA software with 1,000 replicates. Two main clusters were observed from neighbor-joining of mtDNA 12S rRNA (Figure 3) and 16S rRNA (Figure 4). Similar pattern of neighbor-joining tree was observed in both mtDNA 12S

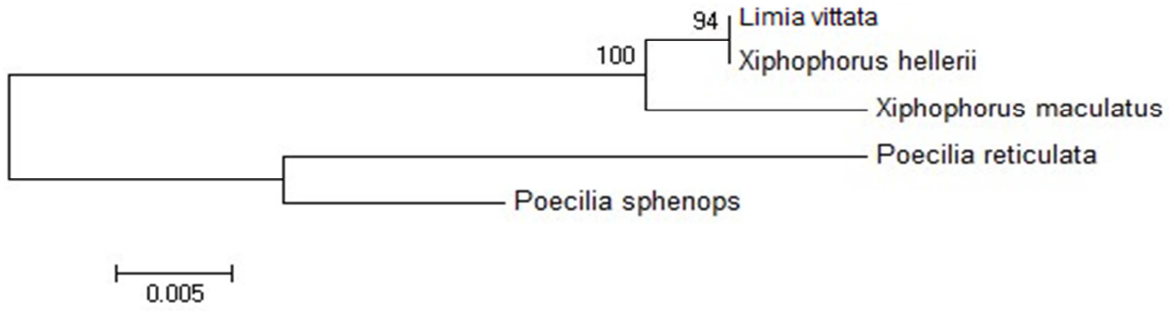

Figure 3: A Neighbor-joining phylogenetic tree of mtDNA 12S rRNA gene with 1000 replicates

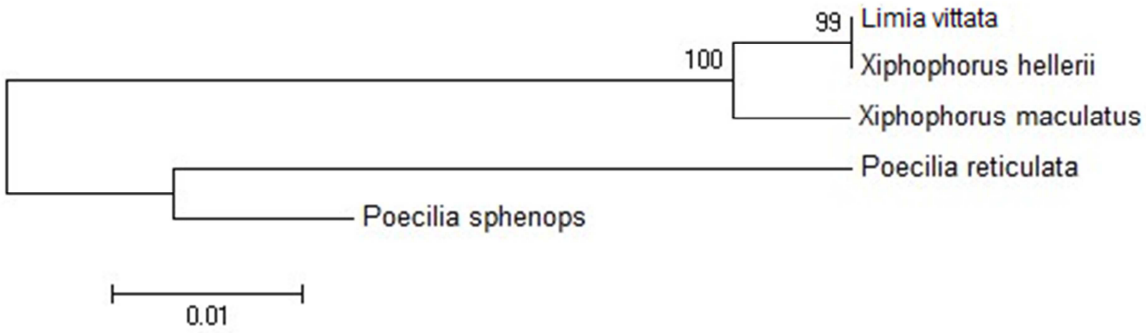

Figure 4: A Neighbor-joining phylogenetic tree of mtDNA 16S rRNA gene with 1000 replicates

rRNA and 16S rRNA (Figure 3 and Figure 4). Neighbor-joining tree of mtDNA 12S rRNA and 16S rRNA showed that *Xiphophorus maculatus* was found to be closely related to *Limia vittata* and *Xiphophorus hellerii* with 100% bootstrap value (Figure 3 and Figure 4). Though, *Xiphophorus maculatus* and *Xiphophorus hellerii* was from the same genus, phylogenetic tree showed that these two species was genetically differ since they occupy different branch in the NJ tree separate branch. This study also indicated that *Limia vittata* and *Xiphophorus helleri* are sister species and share common ancestor with *Xiphophoruxs maculatus* (Figure 3 and Figure 4). A bootstrap value of 99% and 94% was observed between *Limia vittata* and *Xiphophorus hellerii* in Figure 3 and Figure 4 respectively, suggest that these two species were genetically closely related.Based on phylogenetic tree, *Poecilia reticulata* and *Poecilia sphenops* was genetically distant with *Limiavittata*, *Xiphophorus maculatus* and *Xiphophorus hellerii* since they were clustered in different branch (Figure 3 and Figure 4).

CONCLUSION

The present study amplified partial sequence of mtDNA 12S rRNA and 16S rRNA from ornamental fish species namely *Poecilia sphenops, Poecilia reticulata, Limia vittata, Xiphophorus maculatus and Xiphophorus hellerii*. The polymorphisms observed in fish species were useful for intra-species comparison. This study also revealed that some fish from *Poeciliidae* family such as *Poeciliareticulata* and *Poecilia sphenops* were genetically distant from other species (*Limiavittata, Xiphophorus maculatus* and *Xiphophorus hellerii*). Interestingly, both result from neighbor-joining of mtDNA 12S rRNA and 16S rRNA showed similar finding. In future, further study using more

fish species is required to confirm the genetic relationship. In summary, both mtDNA 12S rRNA and 16S rRNA genes are suitable to be used as a molecular markers for fish species characterization.

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