CHAPTER 1
INTRODUCTION

The Asian Arowana (*Scleropages formosus*) is an ornamental and primary freshwater fishes that is strictly intolerant of saltwater. It's commonly known as the Dragonfish and belongs to the order *Osteoglossiformes* (Nelson, 1994).The Asian arowana has obtained a special status in the world particularly in some Asian countries such as Japan and China as a very popular but very expensive aquarium fish (Goh & Chua, 1999). The name of Dragon fish comes from their resemblance to Chinese dragon and known as lucky fish by many people (Hu et al., 2009).

This fish is listed as an endangered species in the 2006 IUCN Red List (Kottelat et al., 1996) and also is classified as threatened a species with danger of extinction in the CITES list since 1980 (Joseph et al., 1986), therefore farmers are required to attain permission when culturing it (Dawes et al., 1999). According to the World Conservation Union Red list 2007, it is estimated 2,491 freshwater fishes (43%) or 1,074 species fall into the "threatened'' class. Furthermore, this fish consists of geographically isolated strains distributed at different locations around South East Asia that were probably connected to freshwater habitats during the Pleistocene glacial ages (Stearn et al., 1979). Generally, there are three main colour varieties for Asian arowana including Green, Golden and Red along with distinct sub-varieties (Goh & Chua, 1999).

The keeping of ornamental fish is the most widespread animal related hobby in the world that their attraction and value in the market depend on the diversity of species, great variety of colour, shape, behaviour and origin (Tang, 2004). In Malaysia, the ornamental fish trade is almost exclusively made up of freshwater fish species.

Morphological features have been commonly used to distinguish fish. The features include the body shape, relative size of the body parts, size and count of the scales, the relative position, number and type of fin rays, and the body color pattern (Strauss & Bond, 1990). However, species identification solely based on the morphological features can be misleading due to presence of intraspecific variation and subtle differences observed in many closely related species (Teletchea, 2009). Moreover, the identification at early developmental stage can be more difficult and challenging for this fish since it constantly undergoes phenotypic changes in its different life stages and develops its own distinct features at a later life stage. Thus, DNA based identification method is seen to offer greater utility to screen and identify the fish, as it can be performed on any developmental stage using any body part or organic material from the fish (Teletchea, 2009).

Due to environmental destruction, Asian arowana is currently uncommon in the Malaysia while they were widely distributed previously. In fisheries, the conservation of genetic resources is a great task in long-term management because, there might be a need of recovering the lost or decreased genetic variation and they might be possessing special genes or important genetic information (Tang, 2004).

The lack of molecular and genetic information about Asian arowana has hindered the biological study of this fish. Mitochondrial DNA markers are expected to be a useful tool for the understanding the fish's molecular biology (Yue et al., 2006). To date, Mitochondrial DNA sequences have shown some success in phylogenetics studies of many organisms; due to its lack combination (Hayashi et al., 1985; Clayton, 1992); mainly maternal inheritance nature (Kondo et al., 1990; Gyllestein et al., 1991); compact gene packing, with little noncoding intergenic nucleotides and some nucleotide overlapping between genes encoded in opposite strands (Cantatore et al., 1987); Multicopy status in cell, and faster evolution rate which allows variation sufficient to delineate closely related species compared to the nuclear genes (Brown et al., 1982; Drake et al., 1998).

In this study, mitochondrial conserved markers such as Cytochrome c oxidase subunit I (COI) and Cytochrome b (Cyt b) are used for molecular identification of Asian arowana. DNA barcoding is one of the molecular identification tools which is based on short, standardized genes that are able to provide accurate species identification (Hebert et al., 2003). Cytochrome c oxidase I (COI) gene, under the Fish Barcode Initiative, FISH-BOL has been used extensively for species identification and discovery of cryptic species (Ward et al., 2005). DNA barcodes comprise approximately of 600-bp sequences of mitochondrial DNA that have become eminent in the world of genetic applications for global biodiversity assessment (Rubinoff et al., 2006). The efficiency of DNA barcoding depends on the degree of sequence divergence among species and species-level identification and is relatively straightforward when the average genetic distance among individuals within a species does not exceed the average genetic distance between sister species (Hubert et al., 2008). In addition, the mitochondrial cytochrome c oxidase subunit I gene (COI) provide valuable information in species

identification to complete taxonomic information and validation of systemic position and phylogeny (Machordom et al., 2003; Smith et al., 2004; Donald et al., 2005).

The most commonly used mtDNA marker is Cytochrome b (Cyt b) in phylogenic studies (Briolay et al., 1998). This mtDNA marker is considered to be variable enough to explain doubts on the population level, and conserve enough to clarify deeper phylogenetics relationships. The wide application of cytochrome b has made a status as universal metric, in the sense that studies can be easily compared (Mayer, 1994).

Main objectives of this study are;

1. To be able to DNA barcode the different Arowana strains using Cytochrome c oxidase subunit 1(COI)
2. To infer the phylogenetic analysis of arowana strains using Cytochrome b and Cytochrome c oxidase I (COI)

The hypothesis:

- Different Arowana starins will have their unique DNA barcodes
- The Malaysian golden arowana and Indonesian golden arowana can be differentiated by its cytochrome b genes

CHAPTER 2

LITERATURE REVIEW

2.1 Asian Arowana

2.1.1 Classification of Asian Arowana

Asian Arowana is a primitive fish from the Jurassic era (Bonde, 1979), belonging to the order *Osteoglossiformes* and is one of the ancestral teleost clades with bony tongue being one of their primitive features. The *Osteoglossiformes* order is composed of two suborders, *Osteoglossoidei* and *Notopteroidei.* The *Osteoglossoidei* suborder consists two families including *Osteoglossidae* and *Pantodontidae. Osteloglossidae* family is further divided into two subfamilies, *Osteoglossinae* and *Heterotidinae*. The family *Pantodontidae* has only one species, *Pantodon buchholzi* or butterfly fish (Nelson, 1994).

The subfamily *Osteoglossinae* is divided into two genera, *Osteoglossum* and *Scleopages.* There are two species of *Osteoglossum* in South America including *Osteoglossum bicirrhosum* and *Osteoglossum ferreirai* which are referred to as the silver arowana and black arowana respectively. There are three species in *Scleopages* genus. *Scleropages jardinii* in the Gulf Saratoga or Nothern Spotted Barramundi and *Scleropages leichardti* are found in Australia while *Scleropages formosus*, the Asian arowana is distributed in the Southeast Asian regions (Dawes et.al., 1999). The Subfamily *Heterotidinae* consists of *Arapaima gigas* found in South America and *Heterotis niloticus niloticus* distributed in Niger and the upper Nile (Dawes et al., 1999). In 1844, first time it was described by Muller and Schlegel for the genus *Osteoglossum* and coined by Nelson in 1994 the present name *Scleropages formosus*

based on morphological data and supported by Kumazawa and Nishida in 2000 based on the molecular phylogenetic data.

Kingdom	Animalia
Phylum	Chorodata
Class	Actinopterygii
Subclass	Neopterygii
Order	Osteoglossiformes
Family	Osteoglossidae
Genus	Scleropages
Species	formosus

Due to high demand and over exploitation of natural populations, the Convention of International Trade in Endangered Species of Wild Fauna and Flora (CITES) has classified arowana as a highly endangered species under its Appendix I list (Joseph et al., 1986). In addition, several reproductive characteristics such as low fecundity, oral brooding habit and open-water spawning makes this species vulnerable to overexploitation (Tang, 2004).This kind of fish is now bred regularly in Singapore and sold with CITES' permission.

2.1.2 Distribution of Asian Arowana

The Asian arowana (*Scleropages formosus*) is commonly known as the Dragonfish, Asia Bonytongue, kelisa or baju rantai (Tang et al., 2004) widely distributed in Peninsular Malaysia, Sumatra, Thailand, Cambodia and Kalimantan (Tang et al., 2004). In Malaysia, this fish can be found in certain rivers which are located in northern and southern regions such as Penang, Johor, Pahang and Terengganu (Tang, 2004). This fish can also be found in some lake in northern regions such as Kenyir Lake, Bukit Merah Lake (Suleiman, 1999).

2.1.3 Habitat of Arowana

In nature, the arowana can be found in herbaceous swamps or marshland, lakes, rivers and mining pools. This fish prefers to live in still or slow-flowing waters which are turbid or weedy (Dawes et al., 1999).

2.1.4 Morphology

This arowana exhibites a knife shaped compressed body while the abdomen is keeled. The gap of the mouth is very large and the lower jaw sticks out. The dorsal fin base is shorter than the anal fin base and the anal fin base is longer than the head length (Suleiman, 1999). This fish is able to grow up to around 90 cm .The distinction of arowana sexes is not easy when they are young. In general, males are longer than females in size and look slimmer while females have more rounded bodies (Dawes et al., 1999).

2.1.5 Strain of Asian Arowana

There are four commercial verities of Asian arowana (Pouyaud et al., 2003) including Malaysian Gold Arowana, Green Arowana, Indonesian Gold Arowana, Indonesian Red Arowana (Pouyaud et al., 2003)

2.1.5.1 Golden Arowana

Golden Arowana or Blue Base Gold arowana is the most popular and can fetch high price in the market (Tang, 2004). Malaysian Golden arowana is native to Bukit Merah Lake, Perak (Suleiman, 1999). The scales may have different colors such as gold; silver or blue (Figure 2.1).

2.1.5.2 Green arowana

This variety of Arowana is the most common and is widespread throughout Southeast Asia which is found in Malaysia, Thailand, Vietnam and Myanmar (Kottelat et al., 1993). In Malaysia this variety is distributed in Terengganu, Pahang and Johor (Ng & Tan, 1999; Suleiman, 1999). This fish has olive scales and a prominent lateral line. Young fish has yellow fins while the fins of the adult are dark green in color (Figure A1).

2.1.5.3 Indonesian Gold Arowana

Indonesian Gold arowana is found in Sumatra island of Indonesia (Dawes et al., 1999). The scales are copper-gold in color. Scales above the lateral line, dorsal fin and

upper half of its tail are dark green (Figure A2).The lower half of its tail fin, dorsal fin and anal fin have purplish-red to brownish-red color (Dawes et al., 1999).

2.1.5.4 Indonesian Red Arowana

Indonesian Red arowana is the most well known variety which is found in Indonesia's Kalimantan province (Dawes et al., 1999). This variety (Figure A3) can be divided into the first red class and the second red class and it is difficult to differentiate the first class from the second class when the fish is young (Tang, 2004).

In addition, Goh and Chua (1999) reported that there are four naturally occurring varieties found in different geographical locations: Cross back golden or Blue Malayan Bonytongue (native to Bukit Merah and Pahang, Peninsular Malaysia), Super red (native to West Kalimantan, Indonesia), Green (native to Peninsular Malaysia, Indonesia, Myammar, Thailand) and Red-tail Gold (Figure A4) (native to Pekan Baru and Jambi, Indonesia).In this study, Silver Arowana *(Osteoglossum bicirrhosum)* was also used as a single species which is the one of the most popular ornamental fish in South America (Figure A5).

Figure 2.1 Golden Arowana, taken from: Breeding Malaysian, *Scleropages formosus* in Concrete Tanks (Mohamad Zaini Suleiman, 2003)

2.2 Mitochondrion DNA (mtDNA)

Animal mtDNA is small and circular which has been widely used as one of the many molecular tools and is extremely useful for species identification of major vertebrate phyla (Murray et al., 1995; Branicki et al., 2003; Ward et al., 2005). Mitochondrion is responsible for transferring energy from food to a form that cells are able to use. However, most DNA is packaged in the nucleus, while mitochondria also have a small amount of its own DNA. Mitochondrial DNA can be also defined as a single type of circular double-stranded DNA including the light (L) strand and heavy (H) strand. Light strand is rich in cytosine while heavy (H) strand is rich in guanines making approximately 16 kbp length of mitochondrial genome a GC rich region (Brown et al., 2005).

During zygote formation, a sperm cell contributes its nuclear genome to the egg cell but not for its mitochondrial genome. Therefore, mitochondrial genome is known as maternally inherited and both males and females receive their mitochondrial DNA from their mother (Strachan & Read, 1999). Because of this, mitochondria are rather reasonable markers to be used in detecting the correlation of the fish using maternal inheritance.

Most of animal mitochondrial DNA contains 37 genes (Boore, 1999) that are all essential for normal mitochondrial function. Among these genes, 13 genes provide instructions for making enzymes contributed in oxidative phosphorylation process. The rest of genes provide instructions for making molecules called transfer RNA (tRNA) and ribosomal RNA (rRNA), which are chemical cousins of DNA. These types of RNA help assemble protein building blocks (amino acids) in functioning proteins (Boore,

1999).The initial sites for mtDNA replication and mtRNA transcription are located at one non-coding control region called D-loop (Boore, 1999). Mitochondrial DNA possesses several advantages over nuclear DNA. According to Drake's observation, threat of DNA mutation is inversely related to the size of the genome. Hence, nuclear DNA undergoes relatively slow mutation compared with mtDNA and, for this reason, would require a much longer nucleotide sequence which is necessary with mtDNA in order to provide a barcode capable of differentiating species. Each mitochondrion contains several complete sets of mitochondrial genes and, therefore, when sample tissue is limited, the mitochondrion offers a relatively abundant source of DNA (Drake, et al., 1998).

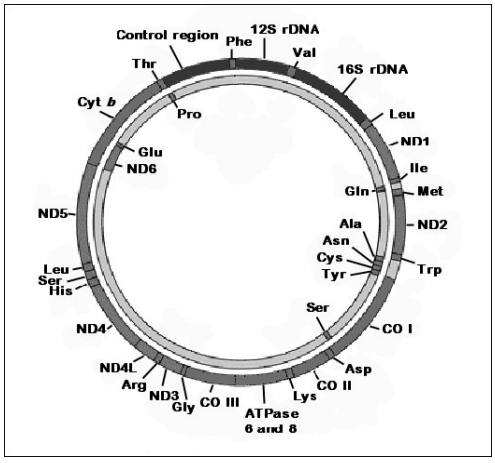


Figure 2.2 Schematic representation of the circular molecule of the "conserved" vertebrate mitochondrial genome. Genes outside and inside the circle are transcribed in the H and L strands, respectively. Protein-coding genes are represented as follows: Cyt *b* - cytochrome *b*; CO I, CO II and CO III - subunits I, II and III of the cytochrome oxidase; ND1-6 - subunits 1 to 6 of the NADH reductase. tRNA are represented by their three-letter amino acid abbreviations. Picture taken from review article (Sérgio Luiz Pereira, Genetics and Molecular Biology, 23, 4, 745-752, 2000).

2.3 Mitochondrion DNA markers

Molecular genetic markers have become a well-established and valuable tool for many applications in population genetics, conservation biology and evolutionary studies as well as for mapping projects (Jarne & Lagoda, 1996). Vertebrate mitochondrial DNA comprises of 6 different markers including, protein coding regions such as cytochrome b, cytochrome c oxidase subunits (COI, II, III), ND1-6 (subunits 1- 6 of the NADH reductase), non protein coding regions involving D-loop control region, 12s rDNA and 16s rDNA (Sérgio, 2000). Among these markers, cytochrome b, cytochrome c oxidase subunits I, II, III and ND1-6 are considered as conserved markers (Figure 2.2). These markers have their own application in different studies (Sérgio, 2000), For example D-loop control region is frequently used in population studies due to the high variability in its nucleotide sequence (Brown et al., 1996; Gissi et al., 1998; Ursing & Arnason, 1998), while protein-coding genes such as cytochrome b are generally used for phylogenetic relationship studies at various levels within population (Sérgio, 2000).

2.3.1 Cytochrome c oxidase subunit I (COI)

Cytochrome c oxidase subunit 1 gene known as single short sequence of mtDNA which is able to code a large transmembrane protein found in the mitochondrion which is highly conserved among species. Cytochrome c oxidase protein works as the terminal electron acceptor in the respiratory chain for reduction of oxygen to water (Waugh, 2007). A segment near the 5-terminus of COI has been selected as a barcode region for some of the animal groups (Hebert et al., 2003).

Hebert and co-workers have achieved the successes of DNA barcoding in a series of publications. What is new and debatable is the idea of using just a small portion of a single gene to identify species from a wide taxonomic range, including animals such as birds, fish and insects (Hebert et al., 2004; Ward et al., 2005; Hajibabaei et al., 2006). In order to obtain DNA barcodes from all species on the planet, DNA barcoding has formed one site as Consortium for the Barcode of Life. Therefore, developments in sequencing technology mean that sequences can be obtained rapidly and inexpensively, so that this barcoding technique may seem conceivable and worthwhile.

Some individuals of North American birds have been identified at species level based on COI that has had a success with rate ranging from 98 to 100% (Hebert et al., 2004). Two studies convincingly indicate the efficacy of DNA barcoding to recover biologically important species. First of all, within a single morphologically identified skipper butterfly species, DNA barcoding separated 10 cryptic species (Hebert et al., 2004). Second, morphologically indistinguishable parasitoid flies (Tachinidae) were shown to be comprised of groups of separate host-specific cryptic species (Smith et al., 2006). Recently, DNA barcoding has discriminated the freshwater fish species from the well-known Canadian fauna, nearly 200 fish were considered as species with high economic value like salmons and sturgeons (Hubert et al., 2008).

In general, fishes constitute a highly diverse group of vertebrates that exhibit deep phenotypic changes during development. Freshwater fishes show more population differentiation than marine species even though marine species can show significant differentiation (Ward et al., 1994).

In case of phylogenetic relationships based on COI, 28 Indian carangid fish species were identified (Persis et al., 2008). Sequence analysis of COI gene very clearly indicated that all the 28 fish species fell into five distinct groups, which are genetically distant from each other and exhibited identical phylogenetic reservation. All the COI gene sequences from 28 fishes provided sufficient phylogenetic information and evolutionary relationship to distinguish the carangid species unambiguously (Persis et al., 2008)

2.3.2 Cytrochrome b (Cyt b)

Cytochrome b (cyt b) has been used as a powerful molecular marker in many fields of molecular studies. It has been used frequently in numerous studies of phylogenetic relationships within mammals, and it is the gene for which most sequence information from different mammalian species is available (Irwin et al., 1991; Jhons & Avise, 1998; Mayer, 1994). These gene codes cytochome b proteins contributed into the electron transport in the respiratory chain of mitochondria and it consists eight transmembrane helices linked by intramembrane or extramembrane domains (Esposti et al., 1993). The sequence variability of cytochrome b makes it most useful for the comparison of species in the same genus or same family. It is probably the best-known mitochondrial gene with regards to structure and function of its protein product (Esposti et al., 1993). Cytochrome b contains both slowly and rapidly evolving codon positions, as well as more conservative, more variable regions and domains overall. Therefore, this gene has been used for a diversity of systematic questions for deep phylogeny (Meyer et al., 1990; Irwin et al., 1999; Cantatore et al., 1994; Lydeard et al., 1997; Kumazawa & Nishida, 2000).

The identification of sturgeon products have been carried out based on cytochrome b gene for 22 sturgeon species (Ludwing et al., 2002). MtDNA sequence information has also proved important in the identification of various tuna species (Thunnus) given the differences in the conservation status and market value of various tuna species (Wen-Feng et al., 2005).

Recently, Studies of mitochondrial cytochrome b gene from representative individuals from 26 harvested fish taxa from Ontario, Canada showed that Interspecific and intraspecific sequence comparisons using phylogenetic analysis provide a precise statistical metrics for species identification of this kind of freshwater fish (Kyle & Wilson, 2006).

2.4 Studies of *Scleropages formosus*

Although Asian arowana is known as one the ornamental fish with a great worth, a few scientific papers have been published about this fish. And yet, there is no study regarding to Malaysian Golden Arowana. The previous studies on Asian arowana relate to classical studies such as taxonomy and physiology of its species (Scott & Fuller, 1976).

In 2000, Kumazawa and Nishida have done their research on Molecular Phylogeny of *Osteoglossoids* from Nagoya University of Japan. They determined the complete sequences of two mitochondrial protein genes, Cyt b and ND2, from 12 *osteoglossiform* species. The results obtained for phylogeny analysis showed that the *osteoglossiforms* diverged from a basal position of the teleostean lineage, that *heterotidines* (the Nile arowana and the pirarucu) form a sister group of *osteoglossines* (arowanas in South America, Australasia, and Southeast Asia), and that the Asian arowana is more closely related to Australasian arowanas rather than to South American ones. However, molecular distances between the Asian and Australasian arowana are much larger than expected from the fact that they are classified within the same genus.

The random amplified polymorphic DNA marker which has been converted to sequence tagged site (STS) marker has been identified and shown to be strain specific in Asian arowana (Yue et al., 2003). Another study claimes that the different colored Asian arowana strains (green, silver, super red and red-tailed golden) can be distinguished genetically based on the molecular data of mitochondrial cytochrome b partial sequence (~300bp) apart from the morphometric and meristic data, in which the authors concluded that each of the colored strain is of different species (Pouyard et al.,

2003). It has captured the attention of both the fish taxonomist and conservation biologists carefully, and well-planned conservation strategy has to be implemented in which it takes into account the different but very closely related species.

Research done on Genetic Structure and Biogeography of Asian Arowana (*Scleropages formosus*) by Tang, Sivananthan, Pillay and Muniandy from University of Malaya shows that arowana consists of a monophyletic groups of mtDNA with three different lineages which represents three different colors, red, green and gold. The red arowana is the out-group but phylogeny was not fully resolved for the gold strains. In contrast, according to phylogenetic tree based on microsatellite data shows that the Asian arowana is a monophyletic group with two lineages while the green arowana is the outgroup and has a closer relationship with Indonesian. However, the mtDNA tree constructed is not associated with the geographical structure but it is better to separate distinct species based on different color varieties.

CHAPTER 3
METHODOLOGY

3.1 Samples

In this study, Scales samples of six varieties of *Scleropages formosus* were used and collected from different sites around Malaysia (Table 3.1). All the scale samples were kept in 95% ethanol and stored in -20°C.

Table 3.1 species, color designation, status, domestication source and geographical origin of the scales samples of *Scleropages formosus* and *Osteoglossum bicirrhosum* collected for this study

Species	Strain	Status	NO of indivitual for cytb	NO of indivitual for COI	sites
Scleropages formosus	**Golden base Malaysian golden**	Domesticated (native to Malaysia)	**7**	**8**	Private breeding farm, Perak
	Malaysian Green	Domesticated (native to Malaysia)	**7**	**8**	KT Nyiur Aquaculture Farm Enterprice ,Perak
	Indonesian Red	Domesticated (native to Kalimantan, Indonesia)	**8**	**10**	Semanggol and Kuala Kangsar Perak, Malacca
	High back gold hybrid (male: Malaysian golden + female: Indonesian golden)	Domesticated	**7**	**8**	Semanggol, Perak
	Blue base Malaysian golden	Wild (native to Malaysia)	**3**	**3**	Bukit Merah, Perak
	Indonesian red tailed golden	Domesticated (native to Indonesia)	**-**	**3**	Malacca
Osteoglossum bicirrhosum	**Silver**	Domesticated (native to South America)	**-**	**8**	Negeri Sembilan
			Total=32	**Total= 48**	

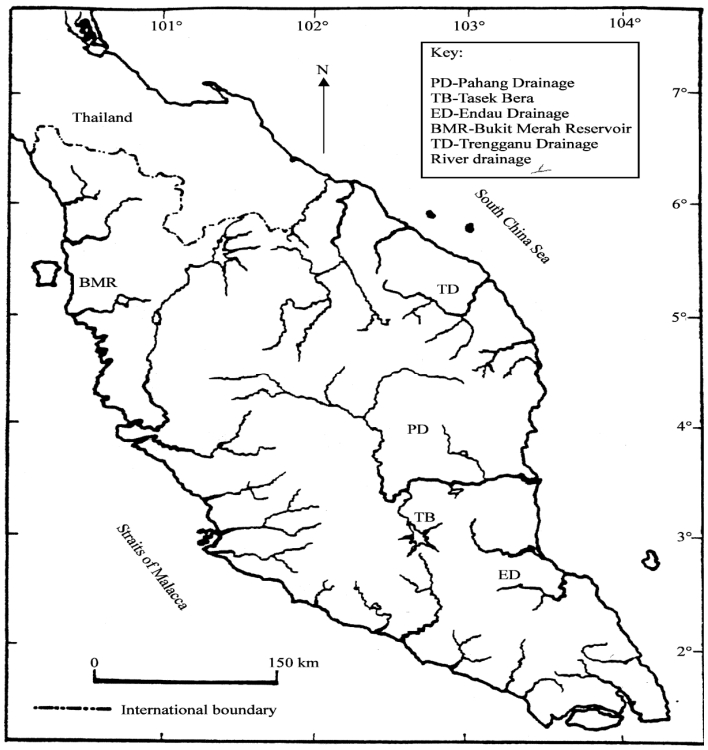


Figure 3.1 Major river drainages and major habitats of Arowana *(scleropages formosus)* in peninsular Malaysia. Figure taken from Rahman S.et al, 2008.

3.2 DNA extraction

The genomic DNA extraction was performed using a GF-1 Tissue Extraction Kit (Vivantis, Malaysia) from scales samples according to the instructions with slight modifications. First of all, 20mg of scale sample was cut into small pieces using a clean scalpel and were placed into a 1.5 ml micro centrifuge tube. Followed by, 250µl of TL Buffer and 20µl of Proteinase K solution (20mg/ml) were added to the sample and vortexed to obtain a homogenous mixture. Then, 12µl of Lysis Enhancer was added and mixed well quickly. Incubation for 1-3 hour was carried out at 65℃ for the tube in a rotating waterbath and mixed occasionally to ensure an overall digestion of the sample. Then, another 20µl of RNase A was added to the sample and mixed. The tube was incubated at 37℃ for 10 minutes.

Next, 600µl of TE Buffer was added to the sample and vortexed and incubated for another 10 minutes at 65°C. After incubation, 200µl of absolute ethanol was added to the sample and mixed well (prevents uneven precipitation of nucleic acid). Then, approximately 600µl of the mixture solution was transferred gently into a column assembled in a clean collection tube and centrifuged at 5000 ×g for 1 minute. The remainder of the original solution was kept and the flow-through was discarded. The column was then washed with 750µl of Wash buffer and centrifuged again at 5000 ×g for 1 minute. The flow-through was discarded. This washing step was repeated once again. Next, the column was centrifuged at 10000 ×g for 1 minute to remove all traces of ethanol.

Finally, DNA elution was carried out by placing the column into a clean micro centrifuge tube, followed by the addition of 200µl of preheated Elution Buffer directly onto the column membrane and incubated under the room temperature for 2 minutes. Next, the solution was centrifuged at 5000 ×g for 1 minute to elute the DNA. The extracted DNA product was then stored at -20°C.

3.3 DNA quantification

The presence of DNA extracted was detected by using both gel electrophoresis and spectrophotometery. Agarose gel electrophoresis confirmed the presence of DNA extracted from the scale samples. For electrophoresis, 3µl of DNA was properly mixed with 3µl of loading dye (Vivantis, Malaysia) and the loaded into 1% agarose gel. After gel electrophoresis and staining, the gel was viewed by UV illumination for bands. The concentration of the DNA extracted was determined by spectrophotometric measurement of UV absorbance using spectrophotometer (Eppendorf, Germany). A ratio of the OD260nm/280nm is an indicator of DNA purity. Experimentally, the ratio of 260 nm/280 nm of a pure DNA solution is between 1.8 to 2.0 which was recorded at 1.8 in this experiment.

3.4 PCR amplification of COI

The partial sequence of mitochondrial cytochrome c oxidase subunit I (COI) was amplified via the Polymerase Chain Reaction (PCR) using a pair of primers, upstream primer **FishF1**: 5'TCAACCAACCACAAAGACATTGGCAC3' and downstream primer **FishR1**: 5'TAGACTTCTGGGTGGCCAAAGAATCA3' (Ward, et.al, 2005). PCR cocktail contained a total volume of 20µl, containing 2.0µl of MgCl2 (25mM), 6µl of GoTaq®Flexi Buffer 5X (green buffer) , 0.5µl of each primer (10µM), 0.5 µl of each dNTP (10mM), 0.6µl of Taq DNA polymerase , and 2µl of template DNA and finally 6.4µl of distilled water. The mixture was triplicate for each DNA sample to obtain totally 60µl of PCR product. Therefore, there is more product of DNA for DNA purification step. The PCR tubes were then placed into the PCR thermal cycle machine (Multigene) in 35 cycles (Table 3.2).

Table 3.2 Temperature and time condition for COI amplification

Step	Temperature	Time
Pre- denaturation	**96^0C**	**5 minutes**
Denaturation	**94^0C**	**45 seconds**
Annealing	**45 ^{0}C**	**45 seconds**
Extension	**72^0C**	**30 minutes**
Final extension	**72^0C**	**10 minutes**

3.5 PCR amplification of Cytochrome b

The partial sequence of mitochondrial cytochrome b gene was amplified via the Polymerase Chain Reaction (PCR) using a pair of primers, upstream primer **LF5267**: 5' AATGACTTGAAGAACCACCGT 3' and downstream primer **H15891**: 5' GTTTGATCCCGTTTCGTGTA 3' (Briolay, Galtier, Brito, & Bouvet, 1998). PCR cocktail for Cyt b amplification was same as COI amplification cocktail while PCR thermal cycles was different. The PCR tubes were then placed into the PCR thermal cycle machine (Multigene) in 40 cycles (Table 3.3).

Table 3.3 Temperature and time condition for Cyt-b amplification

Step	**Temperature**	**Time**
Pre-denaturation	**96^0C**	**5 minutes**
Denaturation	**94^0C**	**45 seconds**
Annealing	**35.6^0C**	**45 seconds**
Extension	**72^0C**	**30 minutes**
Final extension	**72^0C**	**10 minutes**

3.6 Gel electrophoresis

DNA Gel electrophoresis is generally only used after amplification of DNA via PCR. In this case, Agarose gel 1 % was used to analyze the PCR product. The gel was made by dissolving 0.4 mg of agarose powder into 40 ml of 1 X TBE solution (Tris-borate-EDTA: 90mM Tris, 90mM borate and 1mM EDTA) and was shaken thoroughly until all the powder properly dissolved in the solution. Then, the gel was baked for 1 minute in a microwave and poured into a gel rack having a gel cassette and "comb" on it. After 30 minute, the gel becomes solid and the comb was removed and gel was placed in a tank containing buffer medium (1 X TBE). Then, the PCR product was loaded into each well. As well as, 2.5µl of 100bp ladder was loaded into one well and allowed to run using 70 V and 140 A for about 60 minute.

3.7 Staining

Upon finishing the electrophoresis, the gel was removed from the cassette and immersed in ethidium bromide solution (working concentration 0.5µg/ml; 10 mg/ml stock) for 15 minute. Finally, the gel was viewed by UV light using AlphaImager (Alpha Innotech; CA, USA) and then the phonograph of the gel was taken. Thereafter, the expected band was cut and purified.

3.8 DNA Purification

DNA gel extraction was done by using Gel extraction Kit (Axygen). The gel containing the expected DNA band was cut with a sharp and clean scalpel under ultraviolet illumination. After cutting the expected DNA band, the gel was excised and

transfer into a 1.5 ml microcentrifuge tube. After that the gel was sliced into smaller pieces and placed in a tube. Then, it was followed by centrifugation for 30 second at 12,000 × g. A 300 µl of Buffer DE-A was added in order to Resuspend the gel in Buffer. The mixture was vortexed until it becomes homogenous solution. Then, the solution was heated at 75°C until the gel is completely dissolved. In next step, 150µl of Buffer DE-B was added to the tube and mixed together.

The mixture then was transferred into a 2.0 ml microcentrifuge tube having miniprep column on it. Then, the tube was centrifuged at 12,000 × g for 1 minute. The filtrate from the 2.0 ml microfuge tube was discarded and after that, 500µl of Buffer W1 was added on miniprep column and Centrifuged at 12,000×g for 30 sec. After centrifugation, the filtrate was discarded again and the tube was added with 500µl of Buffer W2 (95% ethanol added before using) through the miniprep column. Then, the tube was centrifuged at 12,000×g for 30 second. After discarding the filtrate from the tube, 700µl of Buffer W2 was added one more time to the miniprep column and centrifuged at 12,000 × g for 1 minute. Buffer W2 was used to make sure the complete removal of salt, eliminating potential problems in subsequent enzymatic reactions, such as ligation and sequencing reaction. After that, the flow through was discarded and centrifuged at 12,000 × g for 1 minute for drying the miniprep column.

Finally, the miniprep column was added to a new 1.5 ml micocentifuge tube and 30µl of eluent (65^0C preheated) was added into the miniprep column to elute DNA. The tube was incubated overnight at room temperature. After incubation, the tube was centrifuged for the last time at 12,000×g for 1 minute.

3.9 Testing DNA Purification

In order to confirm the occurrence of DNA in purified sample, the purified sample must be test. Therefore, The gel was made by dissolving 0.4 mg of agarose powder into 40 ml of 1 X TBE solution (Tris-borate-EDTA: 90mM Tris, 90mM borate and 1mM EDTA). After making the gel, the gel was placed into the tank containing buffer medium (1 X TBE). Then, 3µl of purified sample was mixed with 3 µl of loading dye (bromophenol blue) and loaded into the gel. As well as, 2.5µl of 100bp was loaded into one well. Then, the gel was run by voltage 80v and current 200 AMP for 40 minute. Then, the gel was stained by ethidium bromide (working concentration 0.5µg/ml;10 mg/ml stock) for 15 minute and lastly, the picture of the gel showed the occurrence of DNA in purified sample.

3.10 DNA Sequencing

After testing DNA purification, the purified DNA were sequenced in both forward and reverse directions on an ABI 3730XL Automated Sequencer at First BASE Laboratory.

3.11 Data analysis

The total of 624bp of COI and 564bp of cyt b sequences obtained from DNA sequencing were used to analysis of molecular variance, characteristics of sequences and population and phylogenetics studies. The DNA sequences were aligned using the Clustal W (Thompson et al., 1997) with default parametes. After checking of the aligned sequence for presence of premature stop codon, MEGA 4.0.2. (Tamura et al., 2007) was used to determine the base composition, variable and parsimony informative sites. The nucleotide diversity, number of transition and transversion between strains and species, the haplotype diversity were determined using DNAsp (Rozas et al., 2006). The Neighbor Joining (NJ) tree based on both COI and cyt b haplotypes were constructed (Kimura 2 P substitution model; 1,000 bootstraps pseudoreplications) using MEGA 4.0.2 (Tamura et al*.,* 2007) with *Arapaima gigas* extracted from Genbank as outgroup for COI gene tree apart from the sequences obtained for *Osteoglossum bicirrhosum* in this study. In contrast, sequences of *Scleropages jardinii, Scleropages leichardti, Osteoglossum bicirrhosum*, *Osteoglossum ferreirai, Arapaima gigas and Heterotis niloticus* were used as outgroup for Cyt b extracted from NCBI Genbank as used in Kumazawa and Nishida, 2000 study. Appendix B shows a list of all outgroups used in this study along with their accession numbers. The AMOVA (Analysis of Molecular Variance) were conducted using the Arlequin version 3.11 (Excoffier et al., 2007) to estimate the variation within and among strains and the fixation index values between the populations to observe population differentiation. The genealogical relationship among the haplotypes was represented in a network tree using the program TCS version 1.21 (Clement et al., 2000).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 DNA extraction

DNA had been extracted from the scale samples of individuals using genomic extraction method. The DNA concentration was determined using spectrophotometery and also confirmed by gel running (1% agarose gel).

4.2 PCR optimization

The PCR protocol for COI and Cyt b underwent optimization before amplification. Annealing temperature is the most important factor for good PCR amplification was changed by gradient format to find the optimum temperature and create a clearer band. Finally, optimum temperature was found at 35.6° C for Cyt b gene and 45° C for COI gene (Figure 4.1 and 4.2).

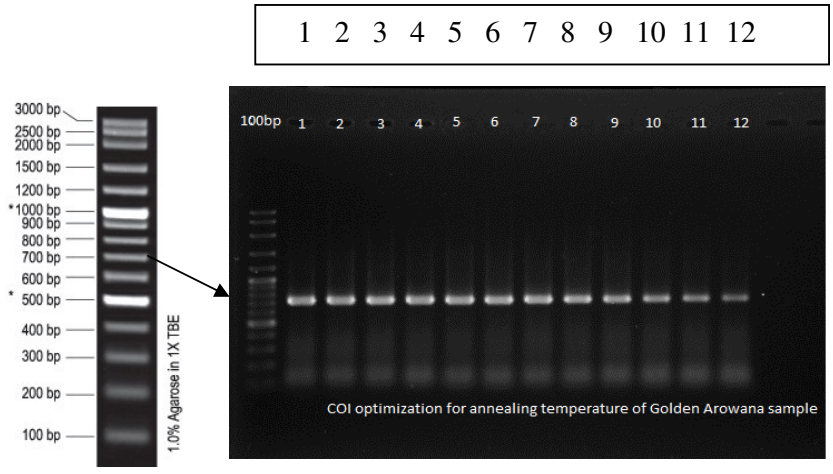


Figure 4.1 PCR optimization of COI gene on 1% agarose gel ; **1:45.0 ° C**; 2:45.9 ° C; 3:46.6 ° C; 4:48.6 ° C; 5:50.8 ° C; 6:53.0 ° C; 7:54.0 ° C; 8:55.9 ° C; 9:57.6 ° C; 10:59.1 ° C; 11:59.5 ° C; 12:60 ° C.

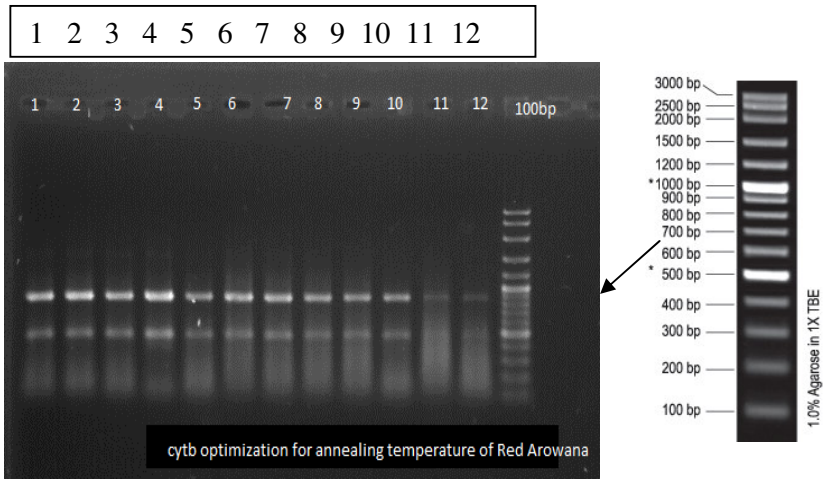


Figure 4.2 PCR optimization of cyt b gene on 1% agarose gel ; 1:35.0 °C; **2:35.6 ° C**; 3:36.6 °C; 4:38.6 °C; 5:40.8 °C; 6:43.0 °C; 7:44.0 °C; 8:45.9 °C; 9:47.6 °C; 10:49.1 °C; 11:49.5 °C; 12:50 °C.

4.3 PCR amplification

After amplification, the required bands were observed on 1% agarose gel (Figure 4.3, 4.4) and then excised manually for DNA purification.

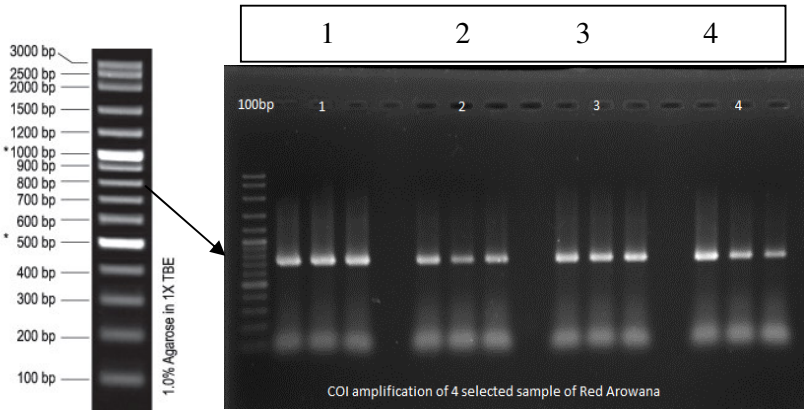


Figure 4.3 PCR amplification of COI from 4 samples of Red Arowana

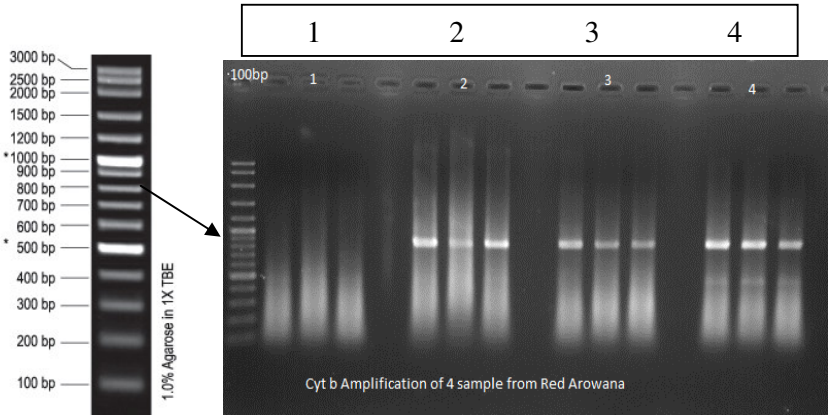


Figure 4.4 PCR amplification of Cyt b from 4 samples of Red Arowana

Note:

1) Each sample was run in triplicates (20µl of the PCR product was loaded into each well of the gel)
2) Marker: 100bp DNA Ladder (Vivantis)
3) Run on 1% agarose gel at 75 volt for 60 minutes

4.4 DNA purification

The expected bands that have been excised are purified to clean the DNA template from any chemicals such as PCR mixture and EtBr that might be stuck on the template. In order to have good DNA sequencing, the DNA template must be clean. If it's not clean, the base can't be detected accurately and noisy sequences will result.

4.5 Testing DNA purification

The purified DNA must be checked and used agarose gel electrophoresis. Figure 4.5 and 4.6 show the bands and confirmation of the DNA purified samples.

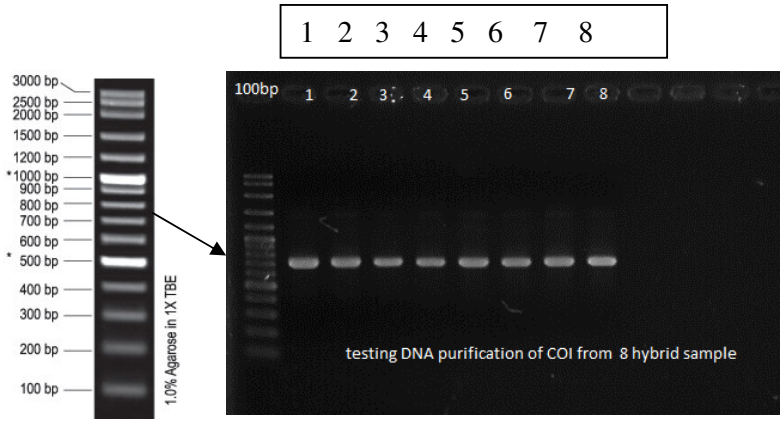


Figure 4.5 DNA purification result of COI from 8 samples from High back gold hybrid (male: Malaysian golden + female Indonesian golden

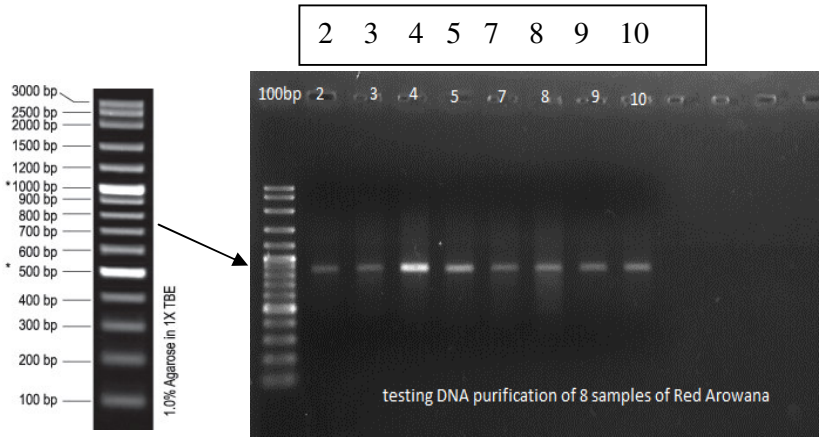


Figure 4.6 DNA purification result of Cyt b from 8 samples from Red Arowana

Note:

1) Marker: 100bp DNA Ladder (Vivantis)
2) Run on 1% agarose gel at 80 volt for 40 minutes

4.6 DNA sequencing

DNA sequencing involves determining the exact order of bases that form a DNA segment. Each base in the nucleotide sequence was detected and used laser by automatic sequencing machine. The result of the chromatogram diagram showed one narrow peak for each base and also confirmed that the sequence quality is good to analyze.

4.7 Data analysis

4.7.1 BLAST Analysis

The sequences obtained from this study were analyzed and used the NCBI BLAST program to confirm that the primers amplified the targeted conserved regions. The result of BLAST Analysis obtained from NCBI could confirm all partial sequences of COI and Cyt b genes used in this study.

4.7.2 Sequences Alignment and characterisation

After alignment using the ClustalW (Thompson et al, 1997) with default parameters, the sequences were then trimmed at the beginning and end till 624bp for COI and 564bp for cyt b to exclude any unsure sequences. Among the 624bp of COI, 459 sites were conserved, 165 sites were variable without any insertion or deletions. In case of 564bp of cyt b, 362 sites were conserved whereas 202 sites were variable.

A total of forty eight and thirty two individuals were used for 5' end partial sequence analysis of COI and cytochrome b genes respectively to produce a total of eighty sequences with no presence of stop codon or gaps resulting from indels. It was inferred that the absence of a stop codon, and unambiguity ruled out the possibility of numts included in the data analysis.

4.7.3 Base composition

MEGA 4.0.2. (Tamura et al., 2007) was also used to analyze base composition (Table 4.1, 4.2). The compositional skew was calculated using the following formula proposed by Perna and Kocher: GC skew = (G-C) / (G+C) and AT skew = (A-T) / (A+T) where C, G, A, and T are the frequencies of the four bases at four-fold degenerate third codon position.

Cytochrome c oxidase I (COI) base composition pattern of forty samples of Asian arowana and eight samples of silver arowana along with outgroups and some sequences taken from GENEBANK were observed (Table 4.1). Overall, the highest value base among all bases is C base averaging at 29.30%. The second highest value is T base, averaging at 28.4%, followed by A base averages at 24.8%.The lowest content is in G base averages 17.5%. At third codon position, A base is showing the highest range, averaging at 33.3%, followed by C base, averaging at 33.2%, T base averages at 26%. The lowest average is G base, at 7.5%. The GC skew was obtained 0.56% whereas AT skew was 0.16%.

These data indicated that the GC content of asian arowana COI gene is higher than another species *Osteoglossoidei*, silver arowana and the frequency of G is a lowest among the four bases. In contrast, AT content of silver arowana is higher than asian arowana. The pattern of codon usage in COI gene was also identified. The most frequently amino acid derived from all COI sequences were leucine (15.87%), followed by alanine (10.00%) and glycine (9.13%).

Cytochrom b base composition patterns of 32 samples of Asian arowana along with outgroups and some sequences taken from GENEBANK were also observed (Table 4.2). In generall, the highest value base among all bases is C base averaging at 33.5%. The second highest value is A base, averaging at 25.9%, followed by T base averages at 24.4%. The lowest content is in G base averages 16.2%. For the third codon, A base is showing the highest range, averaging at 53.1%, followed by C base, averaging at 34.3%, T base averages at 8.0%. The lowest average is G base, at 4.2%. The GC skew was obtained 0.88% and also AT skew was 0.67%. The most frequently amino acid from all Cyt-b sequences were leucine (12.96%), followed by alanine (8.93%) and phenylalanine (8.35%).

On the whole, the base composition, amino acid and codon usage of the COI and Cytb genes are very similar to the corresponding gene sequences reported by Yue et al., 2006. The data of this part of research support that the GC content of Asian arowana mitochondrial genome is higher than another *Osteoglossoidei* species such silver and the same order was described before by Yue et al., 2006. As well as, the frequency of G is the lowest among the four bases in Asian arowna mitochondrial genomes and has been approved in fish mitochondrial genome by Mayer, 1993. In harmony with others' data, COI was the most conserved gene (Noguchi et al., 2000 ; Mayer, 1993).

Table 4.1 Base composition of the codon from 51 sequences of COI with the length of 624 base pairs

	Codon position															
	overall				1st codon				2nd codon				3rd codon			
Samples	T(U)	C	A	G	T-1	C-1	A-1	G-1	T-2	C-2	A-2	G-2	T-3	C -3	A-3	G-3
AR1	27.9	30.0	24.4	17.8	17	26.9	25.5	30.8	42	28.4	15.4	14.4	25	34.6	32.2	8.2
AR2	27.7	30.1	24.5	17.6	17	26.9	25.5	30.8	42	28.4	15.4	14.4	25	35.1	32.7	7.7
AR3	27.7	30.1	24.5	17.6	17	26.9	25.5	30.8	42	28.4	15.4	14.4	25	35.1	32.7	7.7
AR4	27.7	30.1	24.5	17.6	17	26.9	25.5	30.8	42	28.4	15.4	14.4	25	35.1	32.7	7.7
AR5	27.7	30.1	24.5	17.6	17	26.9	25.5	30.8	42	28.4	15.4	14.4	25	35.1	32.7	7.7
AR7	27.7	30.1	24.5	17.6	17	26.9	25.5	30.8	42	28.4	15.4	14.4	25	35.1	32.7	7.7
AR8	27.7	30.1	24.5	17.6	17	26.9	25.5	30.8	42	28.4	15.4	14.4	25	35.1	32.7	7.7
AR9	27.7	30.1	24.5	17.6	17	26.9	25.5	30.8	42	28.4	15.4	14.4	25	35.1	32.7	7.7
AR10	27.7	30.1	24.5	17.6	17	26.9	25.5	30.8	42	28.4	15.4	14.4	25	35.1	32.7	7.7
AR11	27.7	30.1	24.5	17.6	17	26.9	25.5	30.8	42	28.4	15.4	14.4	25	35.1	32.7	7.7
AGO1	27.7	30.1	24.5	17.6	17	26.9	25.5	30.8	42	28.4	15.4	14.4	25	35.1	32.7	7.7
AGO2	27.7	30.1	24.5	17.6	17	26.9	25.5	30.8	42	28.4	15.4	14.4	25	35.1	32.7	7.7
AGO3	27.7	30.1	24.5	17.6	17	26.9	25.5	30.8	42	28.4	15.4	14.4	25	35.1	32.7	7.7
AGO4	27.7	30.1	24.5	17.6	17	26.9	25.5	30.8	42	28.4	15.4	14.4	25	35.1	32.7	7.7
AGO5	27.7	30.1	24.5	17.6	17	26.9	25.5	30.8	42	28.4	15.4	14.4	25	35.1	32.7	7.7
AGO6	27.7	30.1	24.5	17.6	17	26.9	25.5	30.8	42	28.4	15.4	14.4	25	35.1	32.7	7.7
AGO7	27.7	30.1	24.4	17.8	17	26.9	25.0	31.3	42	28.4	15.4	14.4	25	35.1	32.7	7.7
AGO8	27.7	30.1	24.4	17.8	17	26.9	25.0	31.3	42	28.4	15.4	14.4	25	35.1	32.7	7.7
AGR1	27.4	30.4	24.8	17.3	16	27.4	25.5	30.8	42	28.4	15.4	14.4	24	35.6	33.7	6.7
AGR2	27.4	30.4	24.8	17.3	16	27.4	25.5	30.8	42	28.4	15.4	14.4	24	35.6	33.7	6.7
AGR3	27.4	30.4	24.8	17.3	17	26.9	25.5	30.8	42	28.4	15.4	14.4	24	36.1	33.7	6.7
AGR4	27.4	30.4	24.8	17.3	17	26.9	25.5	30.8	42	28.4	15.4	14.4	24	36.1	33.7	6.7
AGR5	27.7	30.1	24.4	17.8	17	26.9	25.5	30.8	42	28.4	15.4	14.4	25	35.1	32.2	8.2
AGR6	27.4	30.4	24.8	17.3	17	26.9	25.5	30.8	42	28.4	15.4	14.4	24	36.1	33.7	6.7
AGR7	27.4	30.4	24.8	17.3	17	26.9	25.5	30.8	42	28.4	15.4	14.4	24	36.1	33.7	6.7
AGR8	27.4	30.4	24.8	17.3	17	26.9	25.5	30.8	42	28.4	15.4	14.4	24	36.1	33.7	6.7
AH1	27.7	30.1	24.2	17.9	17	26.9	25.5	30.8	42	28.4	15.4	14.4	25	35.1	31.7	8.7
AH2	27.7	30.1	24.5	17.6	17	26.9	25.5	30.8	42	28.4	15.4	14.4	25	35.1	32.7	7.7
AH3	27.7	30.1	24.5	17.6	17	26.9	25.5	30.8	42	28.4	15.4	14.4	25	35.1	32.7	7.7
AH4	27.7	30.1	24.5	17.6	17	26.9	25.5	30.8	42	28.4	15.4	14.4	25	35.1	32.7	7.7
AH5	27.7	30.1	24.5	17.6	17	26.9	25.5	30.8	42	28.4	15.4	14.4	25	35.1	32.7	7.7
AH6	27.7	30.1	24.5	17.6	17	26.9	25.5	30.8	42	28.4	15.4	14.4	25	35.1	32.7	7.7
AH7	27.7	30.1	24.5	17.6	17	26.9	25.5	30.8	42	28.4	15.4	14.4	25	35.1	32.7	7.7
AH8	27.7	30.1	24.5	17.6	17	26.9	25.5	30.8	42	28.4	15.4	14.4	25	35.1	32.7	7.7
ABBG1	27.7	30.1	24.5	17.6	17	26.9	25.5	30.8	42	28.4	15.4	14.4	25	35.1	32.7	7.7
ABBG2	27.7	30.1	24.5	17.6	17	26.9	25.5	30.8	42	28.4	15.4	14.4	25	35.1	32.7	7.7
ABBG3	27.7	30.1	24.5	17.6	17	26.9	25.5	30.8	42	28.4	15.4	14.4	25	35.1	32.7	7.7

Samples	overall				1st codon				2nd codon				3rd codon			
	T(U)	C	A	G	T-1	C-1	A-1	G-1	T-2	C-2	A-2	G-2	T-3	C -3	A-3	G-3
AS1	**31.4**	**26.0**	**25.6**	**17.0**	**20**	**24.5**	**26.4**	**29.3**	**42**	**28.4**	**15.4**	**14.4**	**33**	**25.0**	**35.1**	**7.2**
AS2	**31.4**	**26.0**	**25.6**	**17.0**	**20**	**24.5**	**26.4**	**29.3**	**42**	**28.4**	**15.4**	**14.4**	**33**	**25.0**	**35.1**	**7.2**
AS3	**31.4**	**26.0**	**25.6**	**17.0**	**20**	**24.5**	**26.4**	**29.3**	**42**	**28.4**	**15.4**	**14.4**	**33**	**25.0**	**35.1**	**7.2**
AS4	**31.4**	**26.0**	**25.8**	**16.8**	**20**	**24.5**	**26.9**	**28.8**	**42**	**28.4**	**15.4**	**14.4**	**33**	**25.0**	**35.1**	**7.2**
AS5	**31.4**	**25.8**	**25.8**	**17.0**	**20**	**24.5**	**26.4**	**29.3**	**42**	**28.4**	**15.4**	**14.4**	**33**	**24.5**	**35.6**	**7.2**
AS6	**31.4**	**26.0**	**25.6**	**17.0**	**20**	**24.5**	**26.4**	**29.3**	**42**	**28.4**	**15.4**	**14.4**	**33**	**25.0**	**35.1**	**7.2**
AS8	**31.4**	**26.0**	**25.6**	**17.0**	**20**	**24.5**	**26.4**	**29.3**	**42**	**28.4**	**15.4**	**14.4**	**33**	**25.0**	**35.1**	**7.2**
AS 10	**31.4**	**26.0**	**25.6**	**17.0**	**20**	**24.5**	**26.4**	**29.3**	**42**	**28.4**	**15.4**	**14.4**	**33**	**25.0**	**35.1**	**7.2**
ARTG1	27.7	30.1	24.5	17.6	17	26.9	25.5	30.8	42	28.4	15.4	14.4	25	35.1	32.7	7.7
ARTG2	27.7	30.1	24	17.8	17	26.9	25.5	30.8	42	28.4	15.4	14.4	25	35.1	32.2	8.2
ARTG3	27.7	30.1	24.4	17.8	17	26.9	25.5	30.8	42	28.4	15.4	14.4	25	35.1	32.2	8.2
ASIAN AROWANA (GENBANK /BOL)	27.7	30.1	24.5	17.6	17	26.9	25.5	30.8	42	28.4	15.4	14.4	25	35.1	32.7	7.7
OSTEOGLOSSUM BICIRRHOSUM OUTGROUP	31.4	26.0	25.6	17.0	20	24.5	26.4	29.3	42	28.4	15.4	14.4	33	25.0	35.1	7.2
ARAPAIMA GIGAS OUTGROUP	32.4	23.6	27.9	16.2	21	23.1	26.4	29.8	41	28.8	15.4	14.4	35	18.8	41.8	4.3
Avg.	**28.4**	**29.3**	**24.8**	**17.5**	**17**	**26.5**	**25.6**	**30.5**	**42**	**28.4**	**15.4**	**14.4**	**26**	**33.2**	**33.3**	**7.5**

AR= Red Arowana; AGO =Golden Arowana; AGR=Green Arowana; AH= Hybrid Arowana (Gold Malaysia/Indonesia); ABBG= Blue Base Gold Arowana; **AS= Silver Arowana**; ARTG= RED Tail Gold Arowana

Table 4.2 Base composition of the codon from 44 sequences of Cyt b with the length of 564 base pairs

	Codon position															
	overall				1st codon				2nd codon				3rd codon			
Samples	T(U)	C	A	G	T-1	C-1	A-1	G-1	T-2	C-2	A-2	G-2	T-3	C -3	A-3	G-3
AGR1	24.1	33.9	26.2	15.8	26	23.4	23.9	27.1	39	23.9	19.7	17.0	7	54.3	35.1	3.2
AGR2	24.1	33.9	26.1	16.0	26	23.4	23.4	27.7	39	23.9	19.7	17.0	7	54.3	35.1	3.2
AGR3	23.9	34.0	25.9	16.1	26	23.4	23.4	27.7	39	23.9	19.7	17.0	7	54.8	34.6	3.7
AGR 4	23.9	34.0	25.9	16.1	26	23.4	23.4	27.7	39	23.9	19.7	17.0	7	54.8	34.6	3.7
AGR 5	23.9	33.9	25.5	16.7	26	23.4	23.4	27.7	39	23.9	19.7	17.0	7	54.3	33.5	5.3
AGR 6	23.9	34.0	25.9	16.1	26	23.4	23.4	27.7	39	23.9	19.7	17.0	7	54.8	34.6	3.7
AGR 7	23.9	34.0	25.9	16.1	26	23.4	23.4	27.7	39	23.9	19.7	17.0	7	54.8	34.6	3.7
AGO 1	23.9	33.9	25.9	16.3	26	23.4	23.9	27.1	39	23.9	19.7	17.0	7	54.3	34.0	4.8
AGO 2	23.9	33.9	25.9	16.3	26	23.4	23.9	27.1	39	23.9	19.7	17.0	7	54.3	34.0	4.8
AGO3	23.9	33.9	25.7	16.5	26	23.4	23.4	27.7	39	23.9	19.7	17.0	7	54.3	34.0	4.8
AGO 4	23.9	33.9	25.9	16.3	26	23.4	23.9	27.1	39	23.9	19.7	17.0	7	54.3	34.0	4.8
ABBG1	23.9	33.9	25.7	16.5	26	23.4	23.4	27.7	39	23.9	19.7	17.0	7	54.3	34.0	4.8
ABBG2	23.9	33.9	25.7	16.5	26	23.4	23.4	27.7	39	23.9	19.7	17.0	7	54.3	34.0	4.8
ABBG3	23.9	33.9	25.9	16.3	26	23.4	23.4	27.7	39	23.9	19.7	17.0	7	54.3	34.6	4.3
AGO 5	23.9	33.9	25.9	16.3	26	23.4	23.9	27.1	39	23.9	19.7	17.0	7	54.3	34.0	4.8
AGO6	23.9	33.9	25.9	16.3	26	23.4	23.9	27.1	39	23.9	19.7	17.0	7	54.3	34.0	4.8
AGO 8	23.9	33.9	25.7	16.5	26	23.4	23.4	27.7	39	23.9	19.7	17.0	7	54.3	34.0	4.8
AR 2	23.9	33.9	25.5	16.7	26	23.4	23.4	27.7	39	23.9	19.7	17.0	7	54.3	33.5	5.3
AR 3	23.9	33.9	25.9	16.3	26	23.4	23.4	27.7	39	23.9	19.7	17.0	7	54.3	34.6	4.3
AR 4	23.9	33.9	25.7	16.5	26	23.4	23.4	27.7	39	23.9	19.7	17.0	7	54.3	34.0	4.8
AR 5	23.9	33.9	25.9	16.3	26	23.4	23.4	27.7	39	23.9	19.7	17.0	7	54.3	34.6	4.3
AR 7	23.9	33.9	25.9	16.3	26	23.4	23.9	27.1	39	23.9	19.7	17.0	7	54.3	34.0	4.8
AR8	23.9	33.9	25.7	16.5	26	23.4	23.4	27.7	39	23.9	19.7	17.0	7	54.3	34.0	4.8
AR9	23.9	33.9	25.9	16.3	26	23.4	23.4	27.7	39	23.9	19.7	17.0	7	54.3	34.6	4.3
AR 10	23.9	33.9	25.9	16.3	26	23.4	23.4	27.7	39	23.9	19.7	17.0	7	54.3	34.6	4.3
AH2	23.9	33.9	25.9	16.3	26	23.4	23.4	27.7	39	23.9	19.7	17.0	7	54.3	34.6	4.3
AH3	23.9	33.9	26.2	16.0	26	23.4	23.9	27.1	39	23.9	19.7	17.0	7	54.3	35.1	3.7
AH4	23.9	33.9	25.9	16.3	26	23.4	23.4	27.7	39	23.9	19.7	17.0	7	54.3	34.6	4.3
AH6	23.9	33.9	25.7	16.5	26	23.4	23.4	27.7	39	23.9	19.7	17.0	7	54.3	34.0	4.8
AH7	23.9	33.9	25.9	16.3	26	23.4	23.4	27.7	39	23.9	19.7	17.0	7	54.3	34.6	4.3
AH8	23.9	33.9	25.9	16.3	26	23.4	23.4	27.7	39	23.9	19.7	17.0	7	54.3	34.6	4.3
AH10	23.9	33.9	25.9	16.3	26	23.4	23.4	27.7	39	23.9	19.7	17.0	7	54.3	34.6	4.3

Samples	overall				1st codon				2nd codon				3rd codon			
	T(U)	C	A	G	T-1	C-1	A-1	G-1	T-2	C- 2	A-2	G-2	T-3	C -3	A-3	G-3
MSIAN GREEN AROWANA 1 (mu et al)	23.9	34.0	25.9	16.1	26	23.4	23.4	27.7	39	24.5	19.1	17.0	7	54.3	35.1	3.7
MSIAN GOLD AROWANA 1 (mu et al)	23.9	34.0	25.7	16.3	26	23.4	23.4	27.7	39	24.5	19.1	17.0	7	54.3	34.6	4.3
MSIAN RED AROWANA 1 (mu et al)	23.9	34.0	25.7	16.3	26	23.4	23.4	27.7	39	24.5	19.1	17.0	7	54.3	34.6	4.3
ASIAN RED AROWANA (yue et al)	23.9	33.9	25.7	16.5	26	23.4	23.4	27.7	39	23.9	19.7	17.0	7	54.3	34.0	4.8
INDON RED AROWANA 1 (kumazawa et al)	23.9	33.9	25.9	16.3	26	23.4	23.4	27.7	39	23.9	19.7	17.0	7	54.3	34.6	4.3
INDON RED AROWANA 2 (kumazawa et al)	24.1	33.7	25.9	16.3	26	23.4	23.4	27.7	39	23.9	19.7	17.0	7	53.7	34.6	4.3
AUSTRALIAN SPOTTED BARRAMUNDI	26.2	32.4	25.7	15.6	26	22.3	26.6	25.0	39	23.9	19.1	17.6	13	51.1	31.4	4.3
AUSTRALIAN NOTHERN BARRAMUNDI	24.3	34.2	24.3	17.2	24	23.9	23.4	28.2	39	23.9	20.2	16.5	9	54.8	29.3	6.9
BLACK AROWANA	28.0	30.9	25.5	15.6	26	24.5	22.9	26.6	40	23.9	19.1	16.5	18	44.1	34.6	3.7
SILVER AROWANA	27.3	31.2	26.6	14.9	27	23.9	23.4	26.1	40	23.9	19.7	16.5	15	45.7	36.7	2.1
PIRACUCU	30.9	27.8	26.6	14.7	27	21.3	24.5	27.1	40	23.9	20.2	16.0	26	38.3	35.1	1.1
NILE AROWANA	28.7	29.8	27.0	14.5	26	23.4	25.5	25.0	39	25.0	18.6	17.6	21	41.0	36.7	1.1
Avg.	**24.4**	**33.5**	**25.9**	**16.2**	**26**	**23.4**	**23.6**	**27.4**	**39**	**24.0**	**19.6**	**17.0**	**8**	**53.1**	**34.3**	**4.2**

AR= Red Arowana; AGO= Golden Arowana; AGR=Green Arowana; AH= Hybrid Arowana (Gold Malaysia/Indonesia); ABBG= Blue Base Gold Arowana

4.7.4 AMOVA

The AMOVA analysis for both COI and cytochrome b shows (Table 4.3) that the variation among the populations is very much lower than within the population.

Table 4.3 Result of Analysis of Molecular Variance (AMOVA)

Source of variation	Percentage of Variation	
	COI	Cyt b
Among population	38.08%	23.88%
Within population	61.92%	76.12%

4.7.5 Nucleotide diversity

In order to measure the degree of polymorphism within populations, nucleotide diversity or Pi value was calculated and used DNAsp software. Table 4.5 shows the nucleotide diversity of 48 sequences from COI of different strains of Asian Arowana including Green, RED, Gold ,Hybrid , blue Base Gold, Red Tail along with one species from south America, Silver Arowana and also table 8 shows Nucleotide diversity of Cyt b from 32 samples of Asian Arowana strains. Among all sequences of COI, Green Arowana has the highest Pi value, 0.00412 while the lowest Pi value is 0.000 derived from Blue Base Golden. In case of cyt b sequences Green Arowana has the highest nucleotide diversity when compared to other sequences used. Generally, the nucleotide diversity of each population is low, indicating that the divergence of COI and cytochrome b's genealogy is low.

Table 4.4 Nucleotide diversity of COI sequences among Arowna species

Strain/species	NO of Individuals	Nucleotide diversity (standard deviation)
Green	8	0.00412 (0.00196)
Red	10	0.00079 (0.00057)
Golden	8	0.00068 (0.00027)
Hybrid	8	0.00079 (0.00057)
Blue Base Golden	3	0.000 (0.000)
Red Tail Golden	3	0.00214 (0.0071)
Silver	8	0.00080 (0.00038)
Total	48	

Table 4.5 Nucleotide diversity of Cyt b sequences among Asian Arowana strains

Strain	NO of indivituals	Nucleotide diversity (standard deviation)
Green	7	0.00675 (0.00307)
Red	8	0.00190 (0.00046)
Golden	7	0.00084 (0.00030)
Hybrid	7	0.00220 (0.00066)
Blue Base Golden	3	0.00118 (0.00056)
Total	32	

4.7.6 Haplotype analysis

In order to differentiate between species and strains, haplotypes were constructed based on COI and Cytb data using DnaSP software. In DNA barcoding, sharing of the same hoplotypes between different species is not desirable unless it indicates that both of distinct species belong to similar species. In this study, 12 haplotypes has been generated from 48 sample based on COI for DNA barcoding and as well as 10 haplotypes has been generated from 32 sample for Cyt b (Table 4.6 and 4.7). Table 4.8 and 4.9 indicate the distribution of COI and Cytb hapoloypes across all strain and species used in this study. All sequences of COI and Cytb haplotypes respectively from strains of Asian arowana and silver arowana species are shown in Appendix C.

Table 4.6 Variable sites for COI hoplotypes. Dots indicate identity to the sequences of Hap1

	position														
haplotypes	**3**	**6**	**24**	**45**	**51**	**133**	**237**	**244**	**268**	**294**	**312**	**480**	**582**	**621**	**414**
Hap1	**C**	**T**	**A**	**G**	**A**	**A**	**T**	**T**	**G**	**G**	**A**	**T**	**A**	**C**	**C**
Hap2	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**A**	**.**	**C**	**.**	**.**	**.**
Hap3	**.**	**.**	**.**	**.**	**.**	**G**	**.**	**.**	**.**	**A**	**.**	**C**	**.**	**.**	**.**
Hap4	**.**	**.**	**.**	**.**	**.**	**.**	**C**	**C**	**.**	**A**	**.**	**.**	**.**	**.**	**T**
Hap5	**.**	**.**	**.**	**.**	**.**	**.**	**C**	**.**	**.**	**A**	**.**	**.**	**.**	**.**	**.**
Hap6	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**C**	**.**	**.**	**.**
Hap7	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**G**	**C**	**.**	**.**	**.**
Hap8	**T**	**C**	**G**	**A**	**.**	**.**	**.**	**C**	**.**	**A**	**.**	**C**	**.**	**.**	**T**
Hap9	**T**	**C**	**G**	**A**	**.**	**.**	**.**	**C**	**A**	**A**	**.**	**C**	**.**	**.**	**T**
Hap10	**T**	**C**	**G**	**A**	**.**	**.**	**.**	**C**	**.**	**A**	**.**	**C**	**.**	**A**	**T**
Hap11	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**A**	**.**	**C**	**G**	**.**	**.**
Hap12	**.**	**.**	**.**	**.**	**G**	**.**	**.**	**.**	**.**	**A**	**.**	**C**	**.**	**.**	**.**

Table 4.7 Variable sites for Cyt b haplotypes. Dots indicate identity to the sequences of Hap1

	position														
haplotypes	**67**	**69**	**93**	**126**	**204**	**258**	**285**	**294**	**306**	**348**	**351**	**385**	**429**	**444**	**486**
Hap1	**A**	**A**	**A**	**G**	**C**	**C**	**T**	**A**	**C**	**A**	**G**	**G**	**A**	**T**	**A**
Hap2	**G**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**
Hap3	**G**	**G**	**.**	**.**	**.**	**.**	**C**	**.**	**.**	**.**	**.**	**.**	**.**	**.**	**.**
Hap4	**G**	**G**	**G**	**A**	**T**	**.**	**C**	**G**	**G**	**G**	**A**	**.**	**.**	**C**	**G**
Hap5	**G**	**G**	**G**	**A**	**.**	**T**	**C**	**G**	**G**	**.**	**A**	**A**	**G**	**C**	**.**
Hap6	**G**	**G**	**G**	**A**	**.**	**T**	**C**	**G**	**G**	**.**	**A**	**.**	**G**	**C**	**.**
Hap7	**G**	**G**	**G**	**.**	**.**	**T**	**C**	**G**	**G**	**.**	**A**	**.**	**G**	**C**	**.**
Hap8	**G**	**G**	**G**	**G**	**.**	**T**	**C**	**G**	**G**	**.**	**A**	**.**	**.**	**C**	**.**
Hap9	**.**	**G**	**G**	**G**	**.**	**T**	**C**	**.**	**G**	**.**	**A**	**.**	**.**	**C**	**.**
Hap10	**G**	**G**	**G**	**G**	**.**	**T**	**C**	**.**	**G**	**.**	**A**	**.**	**G**	**C**	**.**

Table 4.8 COI haplotype distribution table across all the strains of Asian arowana and silver arowana

Haplotype																
Strains / species	1	2	3	4	5	6	7	8	9	10	11	12	Total (# of individuals)	No. of haplotype	Haplotype diversity (standard deviation)	Nucleotide diversity (standard deviation)
Green				2	5	1							8	3	0.607 (0.164)	0.00412 (0.00196)
Red	1	9											10	2	0.250 (0.180)	0.00079 (0.00057)
Golden		6	2										8	2	0.249 (0.169)	0.00068 (0.00027)
Hybrid		7					1						8	2	0.250 (0.180)	0.00079 (0.00057)
Blue based golden		3											3	1	0.000 (0.000)	0.000 (0.000)
Red tailed gold		1									1	1	3	3	1.000 (0.272)	0.00214 (0.00071)
Silver								6	1	1			8	3	0.406 (0.200)	0.00080 (0.00038)
Total	1	30	2	2	5	1	1	6	1	1	1	1	48			

Table 4.9 Cytochrome b haplotype distribution table across all the strains of Asian arowana

Haplotype														
Strains	1	2	3	4	5	6	7	8	9	10	Total	No. of haplotype	Haplotype diversity (standard deviation)	Nucleotide diversity (standard deviation)
Green	1	1	4	1							7	4	0.714 (0.181)	0.00675 (0.00307)
Red					1	2	1	4			8	4	0.750 (0.130)	0.00190 (0.00046)
Golden					5	2					7	2	0.476 (0.171)	0.00084 (0.00030)
Hybrid						1		4	1	1	7	4	0.714 (0.181)	0.00220 (0.00066)
Blue based golden						1		2			3	2	0.667 (0.314)	0.00118 (0.00056)
Total	1	1	4	1	6	6	1	10	1	1	32			

4.7.7 Population analysis

As shown in Table 4.8, Nine haplotypes are identified based on COI data including 5 haplotypes (COI Hap 1, 6, 7, 11, 12) unique to one individual, 3 haplotypes (COI Hap 3, 4, 5) represented by multiple individuals but unique to a specific strain, and 1 major haplotype (Hap 2) that spanned across all strains except for the green strain. The COI gene sequences of silver arowana yielded 3 separate haplotypes containing 2 haplotypes (Hap 9, Hap 10) unique to one individual and 1 major haplotype (Hap 8) represented by six individuals. Haplotypes for Asian arowanas were characterized by 13 polymorphic sites with 10 parsimony informative sites and 3 singletons. The polymorphic sites resulted in 12 transitions and one transversion (isoleucine to valine) in the amino acid sequence. Haplotype for silver arowna were characterized by 2 polymorphic sites which were both singletons and resulted in 2 transversions (phenylalanine to leucine, valine to methionine). Comparing all the haplotypes from both Asian and silver arowanas, 127 polymorphic sites were identified which resulted 122 parsimony informative and 5 singleton sites with only 6 transversions in the amino acid sequence. The COI haplotype diversity of red tailed golden was the highest followed by the green and the silver due to the equal distribution of samples in each of three haplotypes. The nucleotide diversity was the highest in green followed by the red tailed golden.

In table 4.9, cytochrome b gene sequences obtained for the Asian arowana samples, 10 haplotypes were identified including 6 haplotypes (cytochrome b Hap 1, 2, 4, 7, 9, 10) represented by one individual and 1 haplotype (cytochrome b Hap 3) represented by multiple individuals of the green strain and lastly 3 haplotypes (cytochrome b Hap 5, 6, 8) spanned across more than one strain: Cytochrome b Hap 5: red and gold strains; Cytochrome b Hap 6: red, gold, hybrid and blue based gold strains;

Cytochrome b Hap 8: red, hybrid and blue based gold strains. These haplotypes were characterized by 15 polymorphic sites with 12 parsimonious informative sites and 3 singletons, resulting in 13 transitions and 2 transversions (Tyrosine to alanine, valine to methionine). By involving the publicly available cytochrome b sequences of Asian arowanas in the Genbank, it was shown that the red arowana from Yue et al. (2006) belonged to Hap 5 and one of the Indonesian red arowanas from Kumazawa & Nishida (2000) grouped under Hap 8 while the other formed its own unique haplotype. For the Malaysian arowana of the 3 strains submitted by Mu et al. (2004), each formed one unique haplotype. The haplotype and nucleotide diversity were almost equal among all strains except for gold (Table 4.9).

4.7.7.1 Population pairwise and exact test of differentiation P values

In this study, the population pair-wise and exact test of differentiation P values for COI showed that there was no significant difference among the colored Asian arowana strains except between green and other strains. The silver arowana, which is of a different species and genus clearly indicated significant divergence in its *F*st values compared to the other Asian arowana strains (Table 4.10). The population pair-wise and exact test of differentiation P values based on cytochrome b showed that there was significant difference between the green and all other strains and gold strain with all other strains except red and gold in the non-differentiation exact test (Table 4.11).

Table 4.10 Population pairwise Fst from haplotype frequencies (below diagonal) and non-differentiation exact P value (above diagonal) for COI. The grey shaded cells indicate significant difference

POP	RED	GOLD	GREEN	HYBRID	BLUE BASED	RED TAILED	SILVER
RED		**0.18359**	**0.00005**	**0.70309**	**1.00000**	**0.11556**	**0.00000**
GOLD	**0.01299**		**0.00025**	**0.46633**	**1.00000**	**0.10903**	**0.00013**
GREEN	**0.57143**	**0.48214**		**0.00057**	**0.01084**	**0.04955**	**0.00041**
HYBRID	**-0.0667**	**0.01299**	**0.57143**		**1.00000**	**0.14939**	**0.00041**
BLUE BASE	**-0.18421**	**-0.04348**	**0.57007**	**-0.17483**		**0.39029**	**0.00654**
RED TAILED	**0.36877**	**0.15493**	**0.25834**	**0.28994**	**0.25000**		**0.05666**
SILVER	**0.68022**	**0.55357**	**0.46429**	**0.64286**	**0.66416**	**0.36122**	

Table 4.11 Population pairwise Fst from haplotype frequencies (below diagonal) and non-differentiation exact P value (above diagonal) for cytochrome b. The grey shaded cells indicate significant difference

POP	GREEN	RED	GOLD	HYBRID	BLUE BASE
GREEN		**0.00542**	**0.00298**	**0.01424**	0.06477
RED	**0.26724**		0.05390	1.00000	1.00000
GOLD	**0.40476**	**0.26388**		**0.00494**	**0.03290**
HYBRID	**0.28571**	-0.07933	**0.37943**		1.00000
BLUE BASE	**0.30236**	-0.22560	**0.40310**	-0.21471	

4.7.8 Phylogenetics analysis

Phylogenetic trees were constructed (Figure 4.7, 4.8) through MEGA 4 version based on Kimura 2 parameter.The phylogenetic trees for COI and cytochrome b genes had similar topologies using neighbor joining, and maximum parsimony. With 10 and 12 parsimony informative sites for COI and cytochrome b gene sequences respectively, the phylogenetic analysis recognized all the strains of *Scleropages formosus* as single monophyletic clade, which the same goes to the silver arowana in the COI gene tree. The outgroup sequences obtained from Kumazawa and Nishida (2000) study exhibited the same pattern, as reported previously in the cytochrome b gene tree. The depth of divergence of the silver arowana in the COI gene tree was only slightly shallower compared to the *Arapaima gigas*. Both trees showed that the Asian arowana collected in this study can be divided into two lineages: green and red/gold, with exception of COI Hap 6 of the green strain in the COI gene tree. It was also observed that the haplotype which consists of mixed combinations of red, gold, hybrid and its sub-varieties were not highly resolved as indicated by the low bootstrap values and shallow coalescence in both trees. Another interesting observation was that in the cytochrome b gene tree, the Malaysian arowana consists of three different strains obtained from GENBANK forming a monophyletic clade which are separated from its corresponding strains of the data collected in this study. In this clade, the red individuals were shown to form a sub-clade with the green, instead of the gold, which contradicted the result obtained from this study's data.

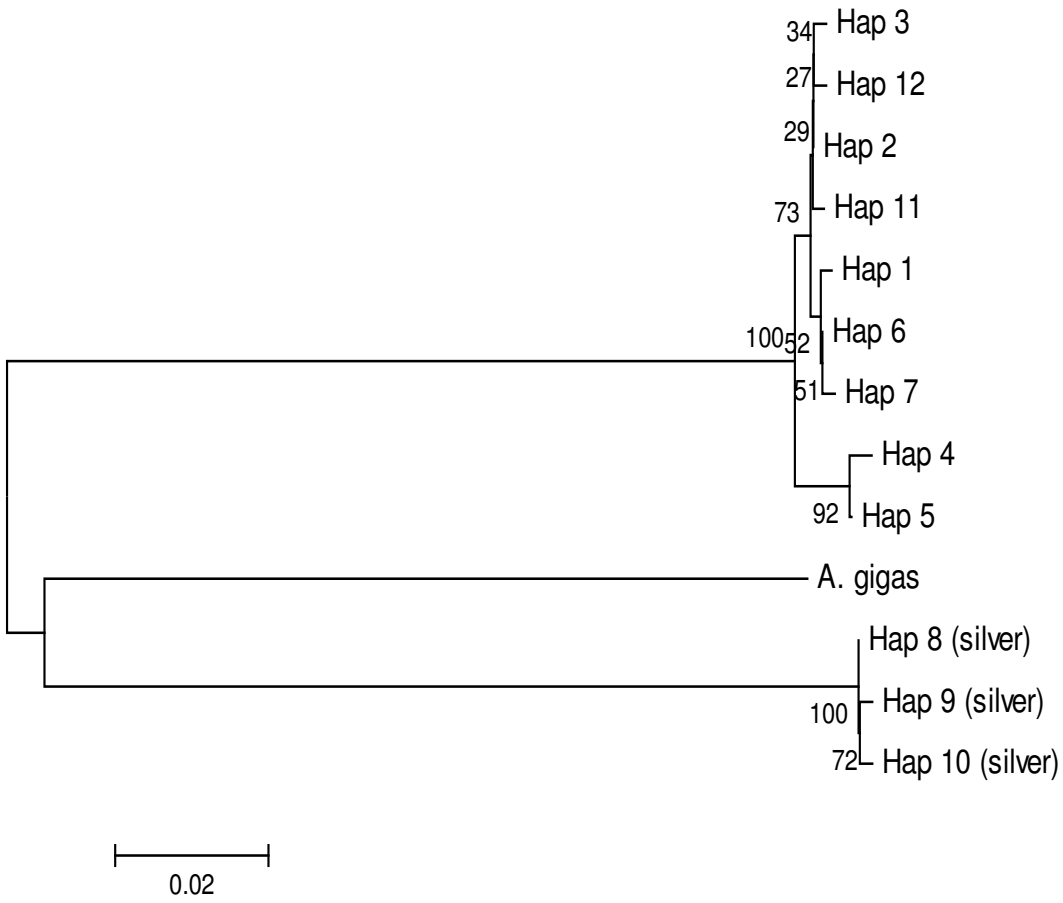


Figure 4.7 Neigbor joining (NJ) phylogenetic tree of haplotypes of Asian and silver arowana inferred from mtDNA sequences of COI

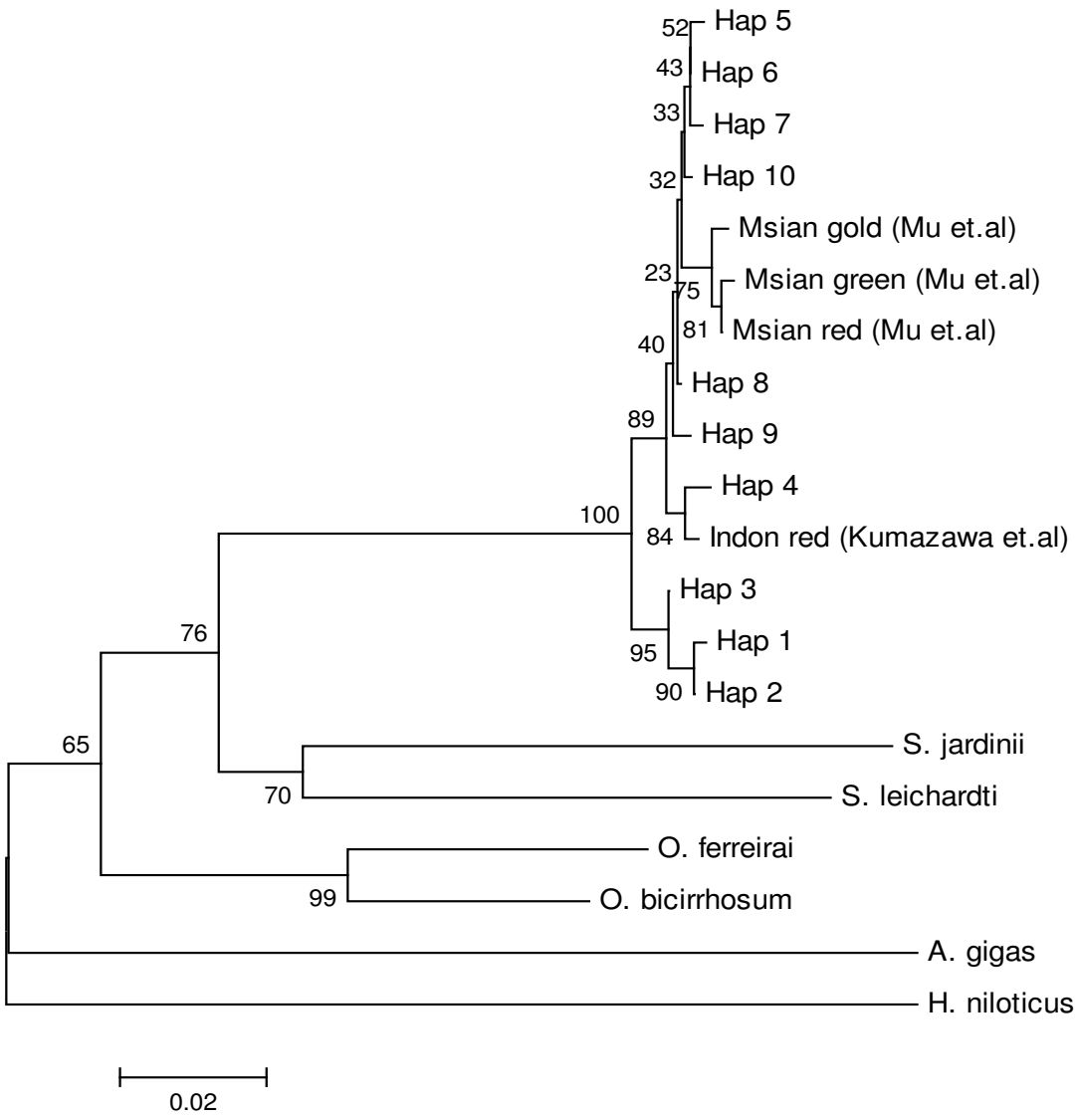
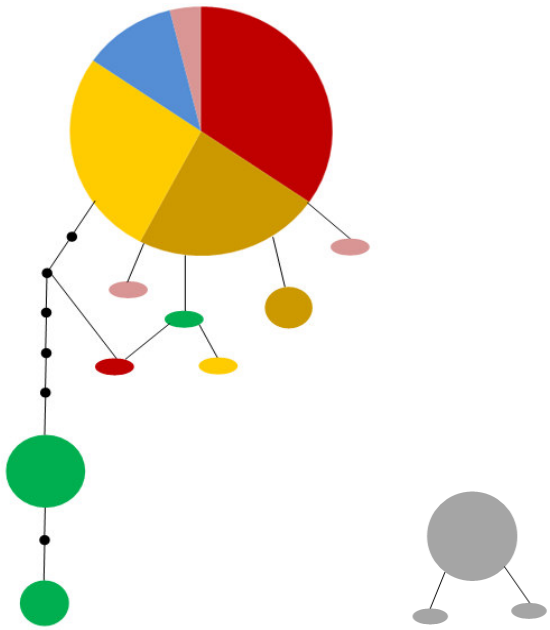


Figure 4.8 Neigbor joining (NJ) phylogenetic tree of haplotypes of Asian arowana inferred from mtDNA sequences of cytochrome b

4.7.9 Parsimony network

(a)



(b)

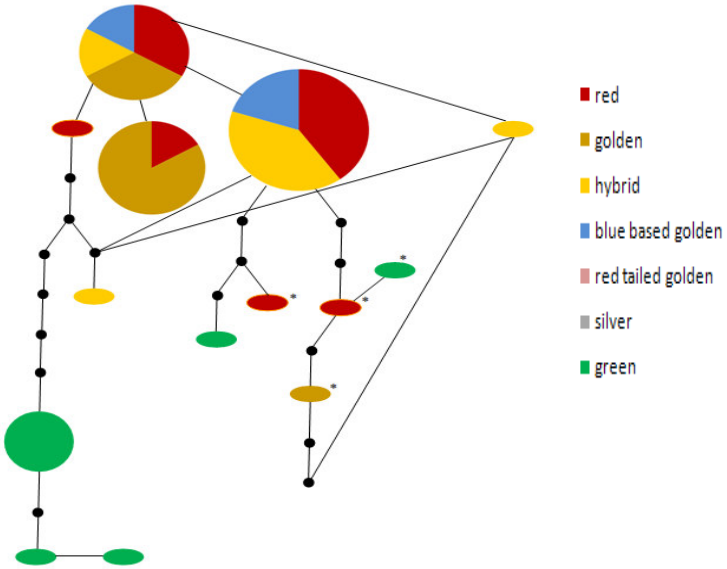


Figure 4.9 Parsimony network for *Scleropages formosus* – red, golden, green, red tailed golden, blue based golden and hybrid and *Osteoglossum bicirrhosum* – silver. (**a:** COI genealogical tree**, b:** cytochrome b genealogical tree). The sizes of the circles are proportional to the number of individuals constituting the haplotype. Oval – haplotype unique to one individual, circle – shared haplotype with more than one individuals of one or more strains. Black dots represents missing haplotype and each line represents one nucleotide change. * In the figure: haplotypes were generated from the sequence obtained from NCBI Genbank database.

The different geographical distributions of red and red tailed gold from Indonesia, blue base and golden base gold from Malaysia in which the breed purity has been maintained in the hatchery for commercial purposes were not reflected in the genetic partitioning, as the Indonesian red and red tailed golden, Malaysian golden base gold, wild blue base gold largely shared a common ancestral haplotype in COI network study in Figure 4.9 (a). In Figure 4.9 (b), Cyt b network study also showed the same haplotype distribution where the red, gold and blue base gold have shared common ancestral haplotypes.

As discussed, DNA barcoding was not able to differentiate stains of Asian arowana due to its lack of haplotype diversity derived from COI data. It was also reflected in shallow coalescence in the phylogenetic trees (Figure 4.7, 4.8) as well as insignificant pairwise population F_{st} values and exact test of sample non-differentiation index (Table 4.10, 4.11) except for the gold population in which it showed significant difference compared to other strains for Cyt b gene (Table 4.11).

The result for this part clearly contradicted the result from the previous study in which the different strain can be distinguished through different haplotypes that formed distinct clades on the cytb gene tree (Pouyard et al., 2003). The green strain on the other hand showed significant differentiation from other strains for both mtDNA and possesses higher haplotype numbers that are all exclusive to itself (Table 4.8, 4.9).

In the genealogy studies for mtDNA genes, high differentiation was clearly shown although the differentiation is not high enough for these exclusive haplotypes to belong to a separate entity as what has been observed in closely related *Scleropages. jardinii, Scleropages leichardti* and other outgroups. The distribution of the green strains on both gene trees raised the possibility that there was genetic structure within the green strain captive stock due to founder individuals which are taken from different wild populations and different geographical locations around Malaysia, as previously reported (Rahman et al., 2008). Although the sample size for the data collection for each of the strains, especially the red tailed golden and blue base gold strain (n = 3) is small, increasing the number of samples will increase the number of haplotypes in each colored strain and reveal more possible shared haplotypes among the strains, but it will not change the general ancestral haplotype that is commonly shared among various colored strains. This was corroborated as the same observation was achieved when more

sequences (publicly available in GENBANK) were added into the study. It was hypothesized that each strain of arowana diverged quite recently that it was not reflected in the COI and cytochrome b mtDNA, as the different strains might not have sufficient amount of time for these mitochondrial genes to exhibit patterns consistent to the lineage sorting. The authors agreed that the divergence of each strain could be reflected in the highly variable mitochondrial regions such as D-loop and the control regions.

CHAPTER 5

CONCLUSION

In conclusion, this study showed that there was genetic differentiation especially in the green strains compared to other strains, but it did not show clear genetic structure among red and gold strains and their sub-varieties to support emergence of new species within *Scleropages formosus*. The result where the green strains formed a paraphyletic group in both the gene trees also corroborated this conclusion. It was inferred that the samples collected by Pouyard et al. (2003) were mostly from highly domesticated stocks, that it failed to capture the largely common haplotype shared among all the strains.

Our results were incongruent with the taxonomic divisions done by the previous study (Pouyard et al., 2003). Our genetic relatedness of the different strains also contradicted with the previous genetic relationship study conducted using microsatellite data which stated that the genetic distance between red and green is less than between red and gold (Yue et al., 2002). From this study, it was shown that both COI and cytocrome b can be good molecular identification markers for *Scleropages formosus* as compared to other closely related species. Nevertheless, these markers would not be suitable to distinguish the different strains of this species except for the green strains. Therefore, more research should be done using more markers such as D-loop, ND2 and ATPase to obtain more concrete evidence of the relationship among individuals of *Scleropages formosus.*

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APPENDIX A

Figur A1: Malaysian Green Arowana,

http://arowana168.co.uk/wp-content/uploads/2009/06/green-arowana-500x238.jpg

Figure A2: Indonesian Gold Arowana

http://wb3.itrademarket.com/pdimage/64/1231364_img_6565.jpg

Figure A3: Indonesian Red Arowana

http://arowana168.co.uk/wp-content/uploads/2009/06/G1R-500x180.jpg

Figure A4: Indonesian red- tail Gold Arowana

http://arowana168.co.uk/wp-content/uploads/2009/06/red-tail-golden-arowana-500x294.jpg

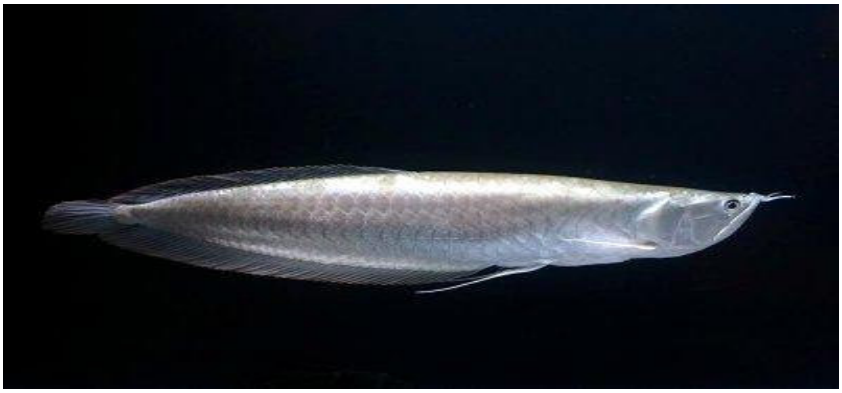

Figure A5: Silver Arowana

http://www.aqua-fish.net/imgs/fish/silver-arowana-profile.jpg

APPENDIX B

List of out groups used in this study

Scientific name	Accesses number	Database	URL
***1.Arapaima gigas* (COI)**	**EF523611**	Nucleotide Collection (nr/nt)	http://www.ncbi.nlm.nih.gov/nuccore/146221455?report=GenBank
2.Scleropages jardinii	**AB035236**	Nucleotide Collection (nr/nt)	http://www.ncbi.nlm.nih.gov/nuccore/11862847?report=GenBank
3.Scleropages leichardti	**AB035237**	Nucleotide Collection (nr/nt)	http://www.ncbi.nlm.nih.gov/nuccore/11862849?report=GenBank
4.Osteoglossum bicirrhosum	**AB035238**	Nucleotide Collection (nr/nt)	http://www.ncbi.nlm.nih.gov/nuccore/11862851?report=GenBank
5.Osteoglossum ferreirai	**AB035239.1**	Nucleotide Collection (nr/nt)	http://www.ncbi.nlm.nih.gov/nuccore/11862853?report=GenBank
***6.Arapaima gigas* (cytb)**	**AB035241.1**	Nucleotide Collection (nr/nt)	http://www.ncbi.nlm.nih.gov/nuccore/11862857?report=GenBank
7.Heterotis niloticus	**AB035240.1**	Nucleotide Collection (nr/nt)	http://www.ncbi.nlm.nih.gov/nuccore/11862855?report=GenBank

APPENDIX C

COI sequences of 12 hoplotypes from Arowana's strains used in this study. Identity with the reference sequences is indicated by dots.

```
Hap 1   G T C G G T A C T G C C C T C A G C C T C C T A A T C C G C
Hap 2   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 3   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 4   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 5   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 6   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 7   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 8   . . T . . C . . C . . T . . T . . . . . T . . G . . T . . .
Hap 9   . . T . . C . . C . . T . . T . . . . . T . . G . . T . . .
Hap 10  . . T . . C . . C . . T . . T . . . . . T . . G . . T . . .
Hap 11  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 12  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

Hap 1   G C A G A A C T A A G C C A G C C T G G A G C C C T C C T T
Hap 2   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 3   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 4   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 5   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 6   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 7   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 8   . . . . . . . . C . . . . . A . . . . . T . . T . G . . . .
Hap 9   . . . . . . . . C . . . . . A . . . . . T . . T . G . . . .
Hap 10  . . . . . . . . C . . . . . A . . . . . T . . T . G . . . .
Hap 11  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 12  . . . . . . . . . . . . . . . . . . . . G . . . . . . . . .

Hap 1   G G T G A C G A C C A A A T C T A T A A T G T T A T C G T A
Hap 2   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 3   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 4   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 5   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 6   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 7   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 8   . . . . . T . . T . . . . . T . . . . . . . C . . . . . C
Hap 9   . . . . . T . . T . . . . . T . . . . . . . C . . . . . C
Hap 10  . . . . . T . . T . . . . . T . . . . . . . C . . . . . C
Hap 11  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 12  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

Hap 1   A C A G C A C A C G C T T T C G T A A T A A T T T T C T T C
Hap 2   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 3   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 4   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 5   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 6   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 7   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 8   . . . . . . . . T . . . . . T . . . . . G . . . . . . . . .
Hap 9   . . . . . . . . T . . . . . T . . . . . G . . . . . . . . .
Hap 10  . . . . . . . . T . . . . . T . . . . . G . . . . . . . . .
Hap 11  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
```

Hap 12	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Hap 1	A	T	G	G	T	C	A	T	G	C	C	A	A	T	T	A	T	A	A	T	T	G	G	G	G	G	C	T	T	T
Hap 2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 3	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 8	.	.	A	.	.	A	.	.	A	.	.	.	.	.	.	.	.	.	.	.	C	.	.	C	.	.	A	.	.	.
Hap 9	.	.	A	.	.	A	.	.	A	.	.	.	.	.	.	.	.	.	.	.	C	.	.	C	.	.	A	.	.	.
Hap 10	.	.	A	.	.	A	.	.	A	.	.	.	.	.	.	.	.	.	.	.	C	.	.	C	.	.	A	.	.	.
Hap 11	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 12	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Hap 1	G	G	C	A	A	C	T	G	A	C	T	A	G	T	C	C	C	A	C	T	T	A	T	G	A	T	C	G	G	A
Hap 2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 8	.	.	T	.	.	.	.	.	.	.	.	T	A	.	T	.	.	.	.	.	G	.	.	A	.	.	.	.	.	T
Hap 9	.	.	T	.	.	.	.	.	.	.	.	T	A	.	T	.	.	.	.	.	G	.	.	A	.	.	.	.	.	T
Hap 10	.	.	T	.	.	.	.	.	.	.	.	T	A	.	T	.	.	.	.	.	G	.	.	A	.	.	.	.	.	T
Hap 11	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 12	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Hap 1	G	C	C	C	C	A	G	A	C	A	T	A	G	C	A	T	T	C	C	C	G	C	G	A	A	T	A	A	A	C
Hap 2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.
Hap 9	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.
Hap10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.
Hap 11	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 12	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Hap 1	A	A	C	A	T	A	A	G	C	T	T	C	T	G	G	C	T	T	C	T	A	C	C	C	C	C	T	T	C	C
Hap 2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.
Hap 5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.
Hap 6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 8	.	.	T	.	.	G	.	.	T	.	.	T	.	.	A	.	.	A	.	.	G	.	.	G	.	.	.	.	.	A
Hap 9	.	.	T	.	.	G	.	.	T	.	.	T	.	.	A	.	.	A	.	.	G	.	.	G	.	.	.	.	.	A
Hap 10	.	.	T	.	.	G	.	.	T	.	.	T	.	.	A	.	.	A	.	.	G	.	.	G	.	.	.	.	.	A
Hap 11	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 12	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Hap 1	T	T	C	T	T	A	C	T	C	C	T	C	T	T	G	G	C	C	T	C	T	T	C	C	G	G	A	G	T	A
Hap 2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

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Hap 4     . . . C . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 5     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 6     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 7     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 8     . . . C . C . . T . . T . . A . . . . . A . . T . . G . . .
Hap 9     . . . C . C . . T . . T . . A . . . . . A . . T . . G A . .
Hap 10    . . . C . C . . T . . T . . A . . . . . A . . T . . G . . .
Hap 11    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 12    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

Hap 1     G A A G C A G G G G C T G G A A C A G G A T G G A C G G T T
Hap 2     . . . . . . . . . . . . . . . . . . . . . . . . . . A . . . . . .
Hap 3     . . . . . . . . . . . . . . . . . . . . . . . . . . A . . . . . .
Hap 4     . . . . . . . . . . . . . . . . . . . . . . . . . . A . . . . . .
Hap 5     . . . . . . . . . . . . . . . . . . . . . . . . . . A . . . . . .
Hap 6     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 7     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 8     . . . . . T . . T . . A . . . . . . . . . . . . A . . T . . A
Hap 9     . . . . . T . . T . . A . . . . . . . . . . . . A . . T . . A
Hap 10    . . . . . T . . T . . A . . . . . . . . . . . . A . . T . . A
Hap 11    . . . . . . . . . . . . . . . . . . . . . . . . . A . . . . . .
Hap 12    . . . . . . . . . . . . . . . . . . . . . . . . . A . . . . . .

Hap 1     T A C C C C C C T C T A G C G G G T A A T C T G G C C C A T
Hap 2     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 3     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 4     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . C
Hap 5     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . C
Hap 6     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 7     . . . . . . . . . . . G . . . . . . . . . . . . . . . . . . . .
Hap 8     . . . . . A . . . T . . . . A . . A . . C . . A . . A . . . .
Hap 9     . . . . . A . . . T . . . . A . . A . . C . . A . . A . . . .
Hap 10    . . . . . A . . . T . . . . A . . A . . C . . A . . A . . . .
Hap 11    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 12    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

Hap 1     G C C G G A G C A T C C G T A G A T C T A A C T A T T T T T
Hap 2     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 3     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 4     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 5     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 6     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 7     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 8     . . A . . . . . . . . . . C . . C T . . . . . . . C . . .
Hap 9     . . A . . . . . . . . . . C . . C T . . . . . . . C . . .
Hap 10    . . A . . . . . . . . . . C . . C T . . . . . . . C . . .
Hap 11    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 12    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

Hap 1     T C T T T A C A C C T G G C T G G A G T C T C A T C C A T C
Hap 2     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 3     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 4     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 5     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 6     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 7     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 8     . . A C . . . . T . . . . . A . . T . . T . . . . . T . . T
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Hap 9	.	.	A	C	.	.	.	.	T	.	.	.	.	.	A	.	.	T	.	.	T	.	.	.	.	.	T	.	.	T
Hap 10	.	.	A	C	.	.	.	.	T	.	.	.	.	.	A	.	.	T	.	.	T	.	.	.	.	.	T	.	.	T
Hap 11	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 12	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Hap 1	C	T	C	G	G	G	G	C	A	A	T	T	A	A	C	T	T	T	A	T	T	A	C	C	A	C	T	A	T	T
Hap 2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 4	.	.	.	.	.	A	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.
Hap 5	.	.	.	.	.	A	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 8	T	.	A	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	T	.	.	A	.	.	C
Hap 9	T	.	A	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	T	.	.	A	.	.	C
Hap 10	T	.	A	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	T	.	.	A	.	.	C
Hap 11	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 12	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Hap 1	A	T	T	A	A	C	A	T	A	A	A	A	C	C	C	C	C	A	G	C	C	A	T	T	A	C	C	C	A	A
Hap 2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 8	.	.	C	.	.	T	.	.	G	.	.	G	.	.	.	.	.	T	.	.	.	.	.	C	.	.	T	.	.	.
Hap 9	.	.	C	.	.	T	.	.	G	.	.	G	.	.	.	.	.	T	.	.	.	.	.	C	.	.	T	.	.	.
Hap 10	.	.	C	.	.	T	.	.	G	.	.	G	.	.	.	.	.	T	.	.	.	.	.	C	.	.	T	.	.	.
Hap 11	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 12	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Hap 1	T	A	C	C	A	A	A	C	A	C	C	T	C	T	A	T	T	T	G	T	C	T	G	A	T	C	T	G	T	T
Hap 2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C
Hap 3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C
Hap 4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C
Hap 7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C
Hap 8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	A	.	.	.	.	.	.	.	C	.	.	C
Hap 9	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	A	.	.	.	.	.	.	.	C	.	.	C
Hap 10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	A	.	.	.	.	.	.	.	C	.	.	C
Hap 11	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C
Hap 12	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C

Hap 1	C	T	A	G	T	A	A	C	C	G	C	T	G	T	C	C	T	T	C	T	A	C	T	T	C	T	C	T	C	C
Hap 2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 8	.	.	.	.	.	.	.	.	T	.	.	A	.	.	.	.	.	.	.	.	T	.	.	C	.	.	T	.	.	T
Hap 9	.	.	.	.	.	.	.	.	T	.	.	A	.	.	.	.	.	.	.	.	T	.	.	C	.	.	T	.	.	T
Hap 10	.	.	.	.	.	.	.	.	T	.	.	A	.	.	.	.	.	.	.	.	T	.	.	C	.	.	T	.	.	T
Hap 11	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap 12	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

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Hap 1   C T C C C A G T C T T A G C T G C A G G A A T C A C T A T A
Hap 2   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 3   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 4   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 5   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 6   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 7   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 8   . . T . . . . . . . . . . . . . . . . . T . . T . . A . . .
Hap 9   . . T . . . . . . . . . . . . . . . . . T . . T . . A . . .
Hap 10  . . T . . . . . . . . . . . . . . . . . T . . T . . A . . .
Hap 11  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 12  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

Hap 1   C T T C T A A C A G A T C G C A A C C T T A A C A C C A C A
Hap 2   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 3   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 4   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 5   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 6   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 7   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 8   . . . T . . . . . . . C . . T . . . T . A . . . . . T . . T
Hap 9   . . . T . . . . . . . C . . T . . . T . A . . . . . T . . T
Hap 10  . . . T . . . . . . . C . . T . . . T . A . . . . . T . . T
Hap 11  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 12  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

Hap 1   T T C T T T G A C C C A G C T G G G G G A G G G G A T C C A
Hap 2   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 3   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 4   . . . . . . . . . . . . . . . . . . . . . . . . . A . . . . .
Hap 5   . . . . . . . . . . . . . . . . . . . . . . . . . A . . . . .
Hap 6   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 7   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 8   . . . . . C . . . . . . . . . . . . . . . . . . . . C . . .
Hap 9   . . . . . C . . . . . . . . . . . . . . . . . . . . C . . .
Hap 10  . . . . . C . . . . . . . . . . . . . . . . . . . . C . . .
Hap 11  . . . . . . . . . . . G . . . . . . . . . . . . . . . . . .
Hap 12  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

Hap 1   A T C C T A T A T C A A C A C C T A T T C T G A
Hap 2   . . . . . . . . . . . . . . . . . . . . . . . .
Hap 3   . . . . . . . . . . . . . . . . . . . . . . . .
Hap 4   . . . . . . . . . . . . . . . . . . . . . . . .
Hap 5   . . . . . . . . . . . . . . . . . . . . . . . .
Hap 6   . . . . . . . . . . . . . . . . . . . . . . . .
Hap 7   . . . . . . . . . . . . . . . . . . . . . . . .
Hap 8   . . . . . . . . . . . . . . . . . T . . . . . .
Hap 9   . . . . . . . . . . . . . . . . . T . . . . . .
Hap 10  . . . . . . . . . . . . . . . . . T . . . . A . . .
Hap 11  . . . . . . . . . . . . . . . . . . . . . . . .
Hap 12  . . . . . . . . . . . . . . . . . . . . . . . .
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APPENDIX C

1) Cyt b sequences of 10 hoplotypes from Arowana's strain used in this study. Identity with the reference sequences is indicated by dots.

```
Hap 1    A T G G C C A G C C T C C G A A A A A C C C A C C C A A T C
Hap 2    . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 3    . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 4    . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 5    . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 6    . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 7    . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 8    . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 9    . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 10   . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

Hap 1    G C C A A A A T C A T A A A C G A C G C C C T T G T T G A C
Hap 2    . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 3    . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 4    . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 5    . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 6    . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 7    . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 8    . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 9    . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 10   . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

Hap 1    C T C C C A A C A C C A T C C A A C A T T T C C G C C T G A
Hap 2    . . . . . . G . . . . . . . . . . . . . . . . . . . . . .
Hap 3    . . . . . . G . G . . . . . . . . . . . . . . . . . . . .
Hap 4    . . . . . . G . G . . . . . . . . . . . . . . . . . . . .
Hap 5    . . . . . . G . G . . . . . . . . . . . . . . . . . . . .
Hap 6    . . . . . . G . G . . . . . . . . . . . . . . . . . . . .
Hap 7    . . . . . . G . G . . . . . . . . . . . . . . . . . . . .
Hap 8    . . . . . . G . G . . . . . . . . . . . . . . . . . . . .
Hap 9    . . . . . . . . G . . . . . . . . . . . . . . . . . . . .
Hap 10   . . . . . . G . G . . . . . . . . . . . . . . . . . . . .

Hap 1    T G A A A C T T C G G C T C C C T T C T A G G C C T C T G C
Hap 2    . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 3    . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 4    . . G . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 5    . . G . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 6    . . G . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 7    . . G . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 8    . . G . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 9    . . G . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 10   . . G . . . . . . . . . . . . . . . . . . . . . . . . . .

Hap 1    C T G G G G G C C C A A A T T C T A A C A G G C C T C T T C
Hap 2    . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
Hap 3    . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
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Hap 4  . . . . . A . . . . . .
Hap 5  . . . . . A . . . . . .
Hap 6  . . . . . A . . . . . .
Hap 7  . . . . . .
Hap 8  . . . . . A . . . . . .
Hap 9  . . . . . A . . . . . .
Hap 10 . . . . . A . . . . . .

Hap 1  C T A G C C A T A C A C T A C A C C T C T G A C A T C T C A
Hap 2  . . . . . .
Hap 3  . . . . . .
Hap 4  . . . . . .
Hap 5  . . . . . .
Hap 6  . . . . . .
Hap 7  . . . . . .
Hap 8  . . . . . .
Hap 9  . . . . . .
Hap 10 . . . . . .

Hap 1  A C T G C C T T C T C C T C C G T A G C C C A C A T C T G C
Hap 2  . . . . . .
Hap 3  . . . . . .
Hap 4  . . . . . T . . . . . .
Hap 5  . . . . . .
Hap 6  . . . . . .
Hap 7  . . . . . .
Hap 8  . . . . . .
Hap 9  . . . . . .
Hap 10 . . . . . .

Hap 1  C G A G A C G T C A A C T A C G G A T G A T T C A T C C G C
Hap 2  . . . . . .
Hap 3  . . . . . .
Hap 4  . . . . . .
Hap 5  . . . . . .
Hap 6  . . . . . .
Hap 7  . . . . . .
Hap 8  . . . . . .
Hap 9  . . . . . .
Hap 10 . . . . . .

Hap 1  A A C C T A C A C G C A A A C G G C G C T T C A T T C T T C
Hap 2  . . . . . .
Hap 3  . . . . . .
Hap 4  . . . . . .
Hap 5  . . . . . T . . . . . .
Hap 6  . . . . . T . . . . . .
Hap 7  . . . . . T . . . . . .
Hap 8  . . . . . T . . . . . .
Hap 9  . . . . . T . . . . . .
Hap 10 . . . . . T . . . . . .

Hap 1  T T C A T C T G C A T C T A T C T T C A C G T A G C A C G A
Hap 2  . . . . . .
Hap 3  . . . . . C . . . . . .
Hap 4  . . . . . C . . . . . G . . . . . .
Hap 5  . . . . . C . . . . . G . . . . . .
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Hap 6  . . . . . C . . . . . G . . . . . .
Hap 7  . . . . . C . . . . . G . . . . . .
Hap 8  . . . . . C . . . . . G . . . . . .
Hap 9  . . . . . C . . . . . .
Hap 10 . . . . . C . . . . . .

Hap 1  G G C C T C T A C T A C G G C T C C T A C C T A T A C A A A
Hap 2  . . . . . .
Hap 3  . . . . . .
Hap 4  . . . . . G . . . . . .
Hap 5  . . . . . G . . . . . .
Hap 6  . . . . . G . . . . . .
Hap 7  . . . . . G . . . . . .
Hap 8  . . . . . G . . . . . .
Hap 9  . . . . . G . . . . . .
Hap 10 . . . . . G . . . . . .

Hap 1  G A A A C A T G A A A C A T C G G A G T G G T C C T G C T A
Hap 2  . . . . . .
Hap 3  . . . . . .
Hap 4  . . . . . G . . A . . . . . .
Hap 5  . . . . . A . . . . . .
Hap 6  . . . . . A . . . . . .
Hap 7  . . . . . A . . . . . .
Hap 8  . . . . . A . . . . . .
Hap 9  . . . . . A . . . . . .
Hap 10 . . . . . A . . . . . .

Hap 1  C T C C T A G T A A T A A T A A C A G C C T T C G T A G G A
Hap 2  . . . . . .
Hap 3  . . . . . .
Hap 4  . . . . . .
Hap 5  . . . . . A . . . . .
Hap 6  . . . . . .
Hap 7  . . . . . .
Hap 8  . . . . . .
Hap 9  . . . . . .
Hap 10 . . . . . .

Hap 1  T A C G T C C T C C C A T G A G G A C A A A T A T C A T T T
Hap 2  . . . . . .
Hap 3  . . . . . .
Hap 4  . . . . . .
Hap 5  . . . . . .
Hap 6  . . . . . .
Hap 7  . . . . . .
Hap 8  . . . . . .
Hap 9  . . . . . .
Hap 10 . . . . . .

Hap 1  T G A G G C G C A A C A G T C A T C A C T A A T C T C C T A
Hap 2  . . . . . .
Hap 3  . . . . . .
Hap 4  . . . . . C . . . . . .
Hap 5  . . . . . G . . . . . C . . . . . .
Hap 6  . . . . . G . . . . . C . . . . . .
Hap 7  . . . . . G . . . . . C . . . . . .
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Hap 8  . . . . . C . . . . . .
Hap 9  . . . . . C . . . . . .
Hap 10 . . . . . G . . . . . C . . . . . .

Hap 1  T C A G C A G T C C C C T A C A T C G G A G A C A T A C T A
Hap 2  . . . . . .
Hap 3  . . . . . .
Hap 4  . . . . . .
Hap 5  . . . . . .
Hap 6  . . . . . .
Hap 7  . . . . . .
Hap 8  . . . . . .
Hap 9  . . . . . .
Hap 10 . . . . . .

Hap 1  G T C C A A T G A A T C T G G G G C G G C T T C T C A G T A
Hap 2  . . . . . .
Hap 3  . . . . . .
Hap 4  . . . . . G . . . . . .
Hap 5  . . . . . .
Hap 6  . . . . . .
Hap 7  . . . . . .
Hap 8  . . . . . .
Hap 9  . . . . . .
Hap 10 . . . . . .

Hap 1  G A C A A C G C C A C A C T A A C C C G A T T C T T C G C C
Hap 2  . . . . . .
Hap 3  . . . . . .
Hap 4  . . . . . .
Hap 5  . . . . . .
Hap 6  . . . . . .
Hap 7  . . . . . .
Hap 8  . . . . . .
Hap 9  . . . . . .
Hap 10 . . . . . .

Hap 1  T T C C A C T T C C T A T T C C C A T T T A T A
Hap 2  . . . . . .
Hap 3  . . . . . .
Hap 4  . . . . . .
Hap 5  . . . . . .
Hap 6  . . . . . .
Hap 7  . . . . . .
Hap 8  . . . . . .
Hap 9  . . . . . .
Hap 10 . . . . . .
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