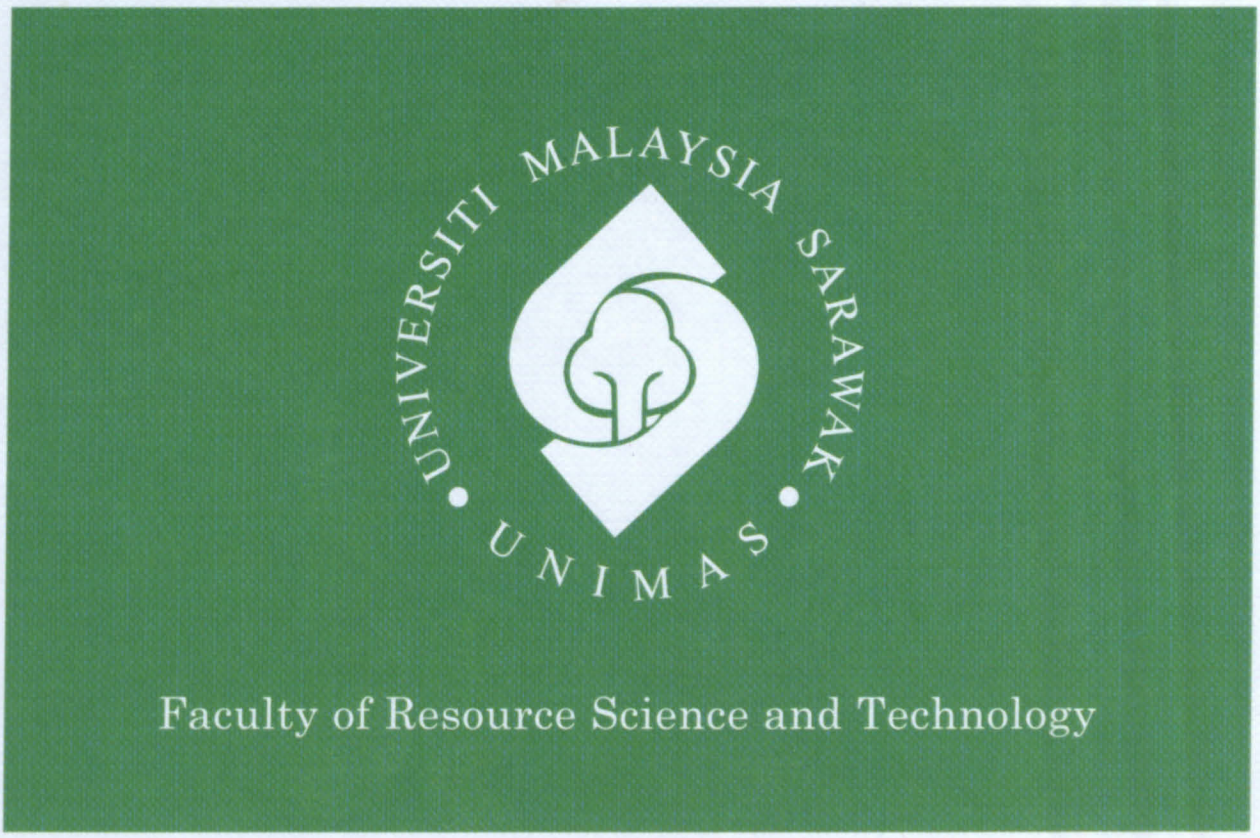

ISOLATION AND CHARACTERIZATION OF PARTIAL FRAGMENT OF MITOCHONDRIAL GENOME (*12S rRNA* – ND2') OF RASBORA *SARAWAKENSIS*

ALICE LAU KUI YAN
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Bachelor of Science with Honours
(Resource Biotechnology)
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Isolation and Characterization of a Partial Fragment of Mitochondrial Genome (*12S rRNA – ND2*) from *Rasbora sarawakensis*

ALICE LAU KUI YAN (46219)

A thesis submitted in partial fulfilment of the requirements for the degree of Bachelor of Science with Honours (Resource Biotechnology)

Supervisor: DR CHUNG HUNG HUI

Bachelor of Science (Honours) Resource Biotechnology
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List of Abbreviations

ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
bp	Base pair
COI	Cytochrome oxidase subunit 1
CSB	Conserved Sequence Blocks
D-loop	Displacement loop
DNA	Deoxyribonucleic Acid
mL	Mililitres
NADH	Nicotinamide adenine dinucleotide
NCBI	National Center for Biotechnology Information
ND 1	NADH dehydrogenase subunit 1
PCR	Polymerase Chain Reaction
spp	species
rRNA	Ribosomal Ribonucleic Acid
T_m	Melting temperature
Taq	*Thermus aquaticus*
tRNA	Transfer Ribonucleic Acid
UV	Ultraviolet
µL	Microlitres

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Isolation and Characterization of Partial Fragment of the Mitochondrial Genome (*12S rRNA – ND2'*) of *Rasbora sarawakensis*

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ABSTRACT

Rasbora sarawakensis is popular ornamental cyprinid fish that native to Borneo. However, the lack of diagnostic morphological characteristic has hindered the identification of this species among *Rasbora* genus. Sequence analysis of mitochondrial genome is a fast and accurate taxonomy method to identify and classify them. In this study, the partial fragment of mitochondrial genome (*12S rRNA – ND2'*) of *R. sarawakensis* was isolated and analysed for the characterization of *R. sarawakensis*. Mitochondrial genome is used instead of nuclear genome because it can trace the direct maternal ancestor of *R. sarawakensis* as mitochondrial genome is maternally inherited. The aim of this study is to isolate and characterize partial fragment of the mitochondrial genome (*12S rRNA – ND2'*) of *R. sarawakensis*. Total genomic DNA from *R. sarawakensis* was isolated through CTAB method. Five pairs of gene specific primers were designed based on the conserved gene sequences from multiple sequence alignment of three *Rasbora* spp for primer walking approach. The size of the five short fragments amplified were around 1000 bp. Trimmed sequencing result and BLASTn analysis shown this fragment (*12S rRNA – ND2'*) have high similarities of 87% with *R. trilineata*. The phylogenetic analysis further strengthen the point that *R. sarawakensis* shared close ancestral relationship with *R. trilineata* in maximum likelihood tree. It is important for further study in biodiversity assessment of *Rasbora* spp in order to regulate international trade of these ornamental fish in the future.

Key words: Mitochondrial genome, *Rasbora sarawakensis*, gradient PCR, phylogenetic tree

ABSTRAK

Rasbora sarawakensis adalah popular ikan cyprinid hiasan yang berasal Borneo. Walaupun begitu, kekurangan ciri morfologi diagnostic telah menghalang pengenalpastian spesies ini dalam Rasbora genus. Analisis sequence genom mitokondria adalah kaedah taksonomi yang cepat dan tepat dalam mengenalpasti dan mengklasifikasikan speisies ini. Dalam kajian ini, sebahagian genom mitokondria (12S rRNA – ND2') dari Rasbora sarawakensis telah diasing dan dianalisis untuk pencirian R. sarawakensis. Genom mitokondria telah diguna dan bukannya genom nuklear kerana genom mitokondria dapat menyurih moyang ibu terus R. sarawakensis disebabkan oleh genom mitokondria adalah diwarisi dari keturunan ibu. Tujuan kajian ini ialah untuk mengasingkan dan mencirikan sebahagian genom mitokondria (12S rRNA – ND2') R. sarawakensis. Keseluruhan genomik dari R. sarawakensis telah diasingkan melalui kaedah CTAB. Lima pasang primer telah direka berdasarkan gen sequence terpelihara melalui "multiple sequence alignment" antara tiga spesies Rasbora untuk kaedah "primer walking". Kelima-lima produck PCR yang digandakan besar mempunyai saiz berhampiran 1000 bp. Keputusan sequencing dan analisis BLASTn atas bahagian genom ini (12S rRNA – ND2') mempunyai kadar homologi sebanyak 87% dengan R. trilineata. Analisis filogenetik mengukuhlan hubungan evolusi dekat R. sarawakensis dengan R. trilineata dalam pokok "maximum likelihood". Hal ini penting bagi kajian lanjut dalam penilaian biodiversiti spesies Rasbora untuk mengawal perdagangan antarabangsa ikan hiasan ini pada masa depan.

Kata kunci: Genom mitokondria, *Rasbora sarawakensis*, gradient PCR, pokok filogenetik

1.0 Introduction

1.1 Background

Rasbora sarawakensis is a species of ray-finned fish in family *Cyprinidae* which is the largest known freshwater fish family. It belongs to subfamily *Rasborinae* which is a diverse group that is probably best known because it include the model organism, the zebrafish, as well as many popular aquarium species (Mayden, 2008). *Rasboras* are perfect ornamental fishes as they have beautiful appearance, elegant and peaceful. According to Department of Fisheries Malaysia (2011), there has increased global demand for ornamental fish exported from Malaysia, especially Singapore, Russia, USA and Europe. Among these ornamental fish, *Rasboras* took up 24% of the market. This situation might poses potential threats to these freshwater fish. Hence, it is necessary to identify and classify the species for the further study in biodiversity assessment of *Rasbora* spp.

Previously, mitochondrial genome had been identified to be a more reliable approach in species classification compared to morphological analysis to delineate species (Dasmahapatra & Mallet, 2006). Mitochondrial genome is the genetic material which is smaller in size than nuclear genome, with about 16,500 base pairs (Taanman, 1999). The mitochondrial genome contains 37 genes which provide instruction for normal mitochondrial functions. Thirteen of these genes encodes 13 protein subunits of enzymes involved in oxidative phosphorylation. The remaining genes encode for 22 transfer RNAs and 2 ribosomal RNAs of mitochondrial ribosome which are necessary in translation of proteins encoded by mitochondrial genome (Taanman, 1999).

Since mitochondrial genome has smaller size, reduced gene content and compact nature, it provide straightforward genome assemblies. It is very commonly used in the study of phylogenetic as the inheritance mode of mitochondrial genome is maternal (Castro *et al.*,

1998). It can trace the direct maternal ancestor of certain organism and provide evidence in phylogenetic relationship. According to Shao *et al.* (2002), only molecular identification can guarantee the identification of fish eggs to species level.

The amount of potential gene arrangements in mitochondrial genome are large enough to make different taxa adopt their own identical state. Thus, comparison of mitochondrial gene arrangement provide significant insights in evolution of organisms in order to identify the species and contribute to biodiversity level (Boore, 1999). Furthermore, this molecules can be used in population genetic studies. In 1984, Avise and Saunders presented the application of mitochondrial DNA in study of populational dynamics of different mitochondrial haplotypes in *Lepomis* species (freshwater sunfish). According to recent studies, DNA barcoding of sequences in mitochondrial gene had been applied in many animals such as birds (Paul *et al.*, 2004), skipper butterflies (Hebert *et al.*, 2004), as well as more than 8,000 species of fishes (Ratnasingham & Hebert, 2007).

However, in Malaysia, there is lack of molecular studies to identify and classify on fish taxonomy and no specific phylogenetic relationship regarding *Rasbora* species has been published. The homogenous appearance of *R. sarawakensis* with some other *Rasbora* spp. increase the difficulty in differentiation of these species. This may cause a confusion on classification of the *Rasbora* spp which lead to poorly regulated international trade in these ornamental fish and eventually poses risks to both biodiversity and economy activities via invasive alien species. Hence, the aim of this research is to construct phylogenetic relationship of *R. sarawakensis* with other *Rasbora* spp. based on the mitochondrial genomic sequences in order to deal with these problems.

1.2 Objectives

The objectives throughout the completion of this project are:

1. To isolate and analyse the DNA sequence of the partial fragment of mitochondrial genome (*12S rRNA – ND2*) of *R. sarawakensis*
2. To understand the phylogenetic relationship of *R. sarawakensis* with other *Rasbora* species

2.0 Literature Review

2.1 Mitochondrial genome

Mitochondrial genome encode for proteins specific for themselves and enable them to replicate by themselves. According to endosymbiosis theory, mitochondria arose from bacteria-like cells that were taken in and assimilated by eukaryotic cells then eventually became organelles in the eukaryotic cells. This theory had been proved through modern sequencing technique (Taanman, 1999). The analysis of rRNA sequences showed mitochondrial genome are different from eukaryotic host cell nuclear genome and it is closely resemble eubacterial genomes (Castro *et al.*, 1998).

DNA in mitochondria was first detected in late 1963 from highly purified yeast by biochemical procedures (Ernster & Schatz, 1981). In 1966, van Bruggen *et al.* had indicated that mitochondrial DNA from higher eukaryotes is circular double-stranded molecule which has different size in different species (Ernster & Schatz, 1981). The parasite *Plasmodium falciparum* has the smallest mitochondrial genome sequenced to date, with 5,967 base pair (Conway *et al.*, 2000) while the largest mitochondrial genome sequenced to date is 366,924 base pair from the plant *Arabidopsis thaliana* (Unseld *et al.*, 2000).

Mitochondrial genome is more commonly used in DNA barcoding than nuclear genome because mitochondrial gene lack of introns and is generally inherited as a haploid from breeding females (Hebert *et al*, 2003). This is because the gamete cells from male have mitochondria mostly located at the tail to generate energy for sperms locomotion and the tail will leave outside the ovum before fertilization take place. As mitochondrial genome is maternally inherited, the recombinant in mitochondrial genome is limited. Hence, compared to nuclear genome with biparental inheritance, the mitochondrial gene can be expected had shared the same underlying genealogy among species (Luo *et al.*, 2011).

Furthermore, mitochondrial genome have higher evolutionary rate, that is, 5 to 10 times than nuclear genome (Castro *et al.*, 1998). This is due to the mitochondrial genome do not have DNA damage repair enzymes to recognize and remove mis-incorporated bases lead to higher rate of single mutations (Paul *et al.*, 2004). The mutation take place in coding sequences and control region caused it evolved rapidly but the spatial arrangement of genes and genome size are constant among species (Castro *et al.*, 1998). The high mutation rate lead to higher degree of variability between individuals (Castro *et al.*, 1998). Besides that, it have higher copy numbers than nuclear genome which can up to 10 copies number in one mitochondria (Phillips *et al.*, 2014). These characteristics of mitochondrial genome have advantage over nuclear genome in routine amplification through polymerase chain reaction as well as used as a molecular marker (Taanman, 1999).

Generally, the circular mitochondrial genome have asymmetric distribution of guanine and cytosine permits the separation of the genome into heavy strand and light strand. The heavy strand encoded the majority of the genes, that are 2 rRNAs, 14 tRNAs and 12 polypeptides. The remaining 8 tRNAs and one polypeptide are coded in light strand (Moraes *et al.*, 2002). Apart from the regulatory region containing promoter and origin of heavy strand replication, the genome have very few non-coding intergenic regions.

Mitochondrial genome have different genetic code with that in nuclear genome. For instant, TGA is a termination codon in nuclear genome but it code for tryptophan in mitochondrial genome. Mitochondrial genome contain displacement loop (D-loop) region which is a three-stranded structure (Taanman, 1999). The majority region in D-loop comprised of Conserved Sequence Blocks (CSB) I, CSBII and CSBIII (Moraes *et al.*, 2002). As these CSBs are located in the D-loop region and the CSBI are located near to the initiation site for heavy strand DNA replication, they are proposed to be involved in mitochondrial DNA replication. There are 2 rRNAs, 22 tRNAs and 13 protein subunits encoded in

mitochondrial genome. The two rRNA species are 12S and 16S respectively. The 22 tRNAs are sufficient to translate all the 13 protein subunits (Moraes *et al.*, 2002).

The 13 protein subunits formed the complex proteins that involved in respiratory chain and ATP synthesis, which are protein complex I, III, IV and V. There are 7 subunits involved in NADH dehydrogenase (protein complex I) formation, ND1, ND2, ND3, ND4, ND4L, ND5 and ND6. The subunit in protein complex III is cytochrome b. There are 3 subunit involved in cytochrome c (protein complex IV) formation, COI, COII and COIII. ATP 6 and ATP 8 are the subunits involved in adenosine triphosphate synthase (protein complex V). The position of gene encoded for these protein subunits in mitochondrial genome are indicated as shown in Figure 2.1.

The alignment of mitochondrial DNA sequences of organism with other species gene sequences is the first step in comparative mitochondrial genomics analysis. The level of conserved sequence across different species can be determined through analysis of nucleotide composition such as order of gene in order to investigate the evolution of the genomes (Wei *et al.*, 2002).

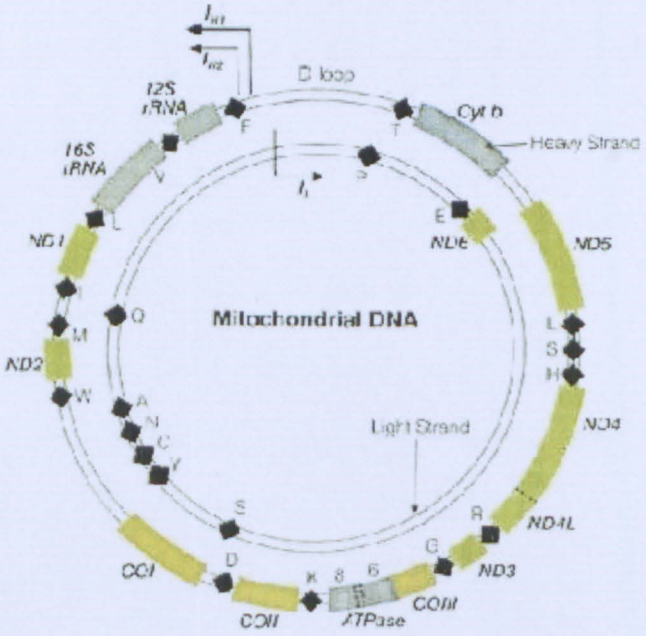

Figure 2.1. General mitochondrial DNA structure with gene and regulatory regions labelled. (Adapted from Kyriakouli *et al.*, 2014).

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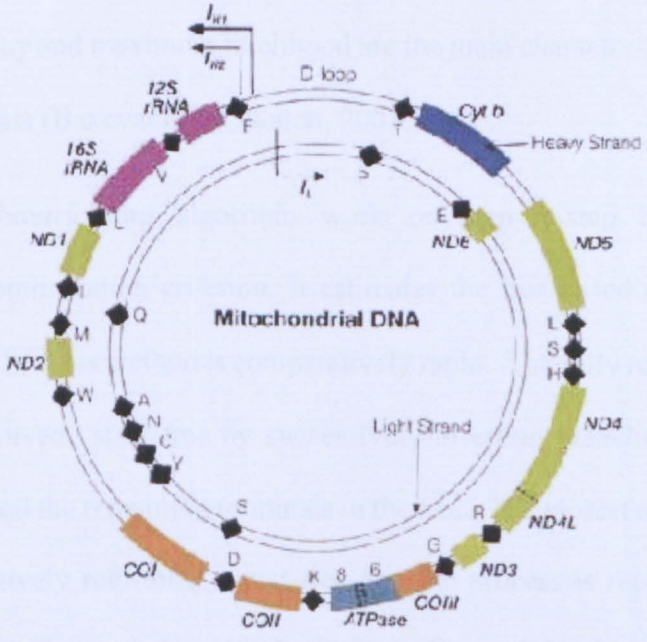

Figure 2.1. General mitochondrial DNA structure with gene and regulatory regions labelled. (Adapted from Kyriakouli *et al.*, 2014).

2.2 Phylogenetic analysis

Phylogenetic tree had been used in evolutionary biology since 1837 by Charles Darwin (Gregory, 2008). Phylogenetic tree is constructed to represent the evolutionary relationship descent of different species from a common ancestor. It shows the relatedness among organisms and is a convenient method to study phylogenetic relationships between species. It provide a rigorous framework to guide biological research that involved evolutionary history (Avise, 2006). DNA sequence alignment had been used to investigate the similarities and differences between species. The sequences may correspond to functional, structural or evolutionary relationship between species.

There has two tree-building method which are distance-based method and character-based method. Distance-based method compute pairwise distances that determined by the tree topology and then discard the actual data. This method only use the fixed distance to build phylogenetic tree (Baxevanis & Ouellett, 2001). On the other hands, character-based method build tree by optimizing the distribution of the actual data patterns for each character. Neighbour-joining is the most commonly applied distance-based method while the maximum parsimony and maximum likelihood are the main character-based method used in phylogenetic analysis (Baxevanis & Ouellett, 2001).

The neighbour-joining algorithm work on step-by-step building procedures, regardless of the optimization criterion. It estimates the tree based on distance matrices (Saitou & Nei, 1997). This method is comparatively rapid. The fully resolved tree is formed from a fully unresolved "star" tree by successively inserting branches between a pair of closest neighbour and the remaining terminals in the tree. The closest neighbour pair is then consolidated, effectively reforming a star tree and the process is repeated until only one terminal is remained (Saitou & Nei, 1997) (Refer to Figure 2.2). It does not permit global

alignment to eliminate negative branches because it does not imply the assumption that the evolution of all lineages happen at the same rate (Baxevanis & Ouellett, 2001).

Maximum parsimony is an optimization criterion that adheres to the principle that the best explanation of the data are provided in a shortest tree, the one with the fewest changes (Huelsenbeck, 1995). It minimize the branch length by reducing the amount of mutation to a minimum level. In maximum parsimony algorithm, two species are related genetically if they tend to share the same nucleotide at similar position (Refer to Figure 2.3). However, this method performs poorly when there is substantial among-site rate heterogeneity (Huelsenbeck, 1995). One approach that can be used to fix this problem is to modify the data set to include only sites that exhibit little or no heterogeneity. This method tend to yield numerous trees that have the same score.

Maximum likelihood method works under evolutionary model which has the highest likelihood of producing the observed data (Baxevanis & Ouellett, 2001). This method is derived for each base position in an alignment. The likelihood is calculated in term of probability of the variation pattern that would be produced at a site, given a particular tree and the overall observed base frequencies (Baxevanis & Ouellett, 2001). The likelihood becomes the sum of the probabilities of each possible reconstruction of substitutions under a particular substitution process (Refer to Figure 2.4). The substitution model should be optimized to fit the observed data. Maximum likelihood tree tend to obtain a single best tree due to their calculation that involved division and decimals, whereas maximum parsimony merely counts discrete steps (Baxevanis & Ouellett, 2001). Hence, maximum likelihood is best optimized for constructing a phylogeny from sequence data input.

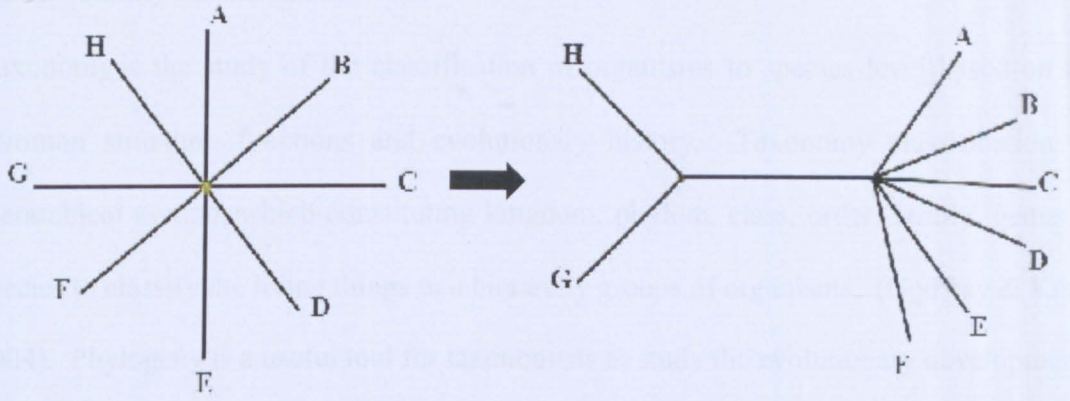

Figure 2.2. The principle of Neighbour-joining method is to find pairs of operational taxonomic units (OTUs) that minimize the total branch length at each stage of clustering of OTUs starting with a starlike tree. (Adopted from Saitou & Nei, 1997).

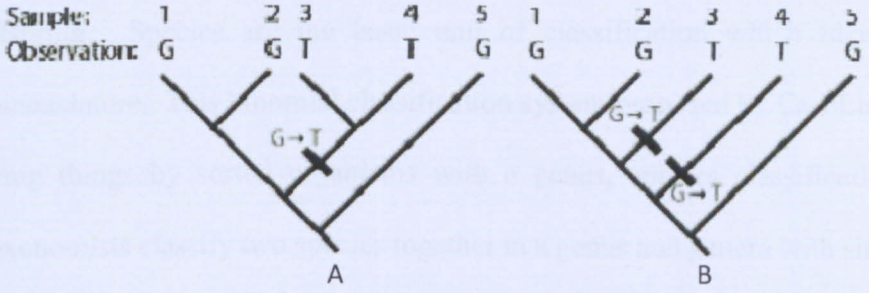

Figure 2.3. The use of maximum parsimony to choose between these two possible trees. In tree A, there has only one change is needed to explain the pattern of nucleotides at a particular sequence position while tree B has two changes in nucleotides. The parsimony method would choose the tree A as it provides a simpler explanation of evolution relationship. (Adopted from Vigilant *et al.,* 1991).

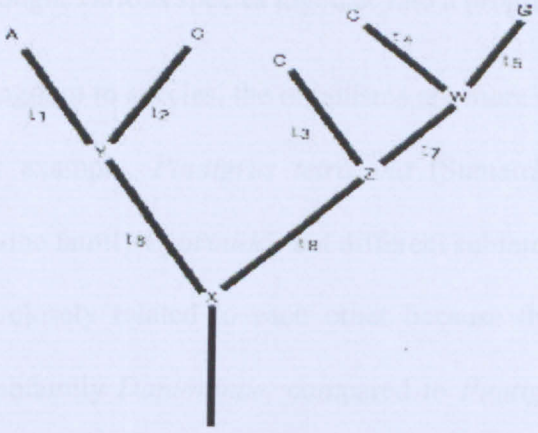

Figure 2.4. In maximum likelihood method, probabilities are considered for every individual nucleotide substitution in a set of sequence alignment. (Adopted from Cawley, 1998).

2.3 Taxonomy classification

Taxonomy is the study of the classification of organisms to species level based on their common structure, functions and evolutionary history. Taxonomy classification is a hierarchical system, which constituting kingdom, phylum, class, order, family, genus and species to classify the living things in a hierarchy groups of organisms. (Godfray & Knapp, 2004). Phylogeny is a useful tool for taxonomists to study the evolutionary development of organisms and relationships between them.

Organisms classed in same species are capable of interbreeding to produce fertile offspring. Species are the basic unit of classification which identified by binomial nomenclature. This binomial classification system proposed by Carl Linnaeus classified all living things by sorted organisms with a genus, species classification (Luketa, 2012). Taxonomists classify two species together in a genus and genera with similar characteristics brought together formed a family. Further, related families are classified in an order and orders are grouped in a class. Related classes are brought together formed a phylum. This classification scheme brought various species together into a progressively larger groups.

Moving from Kingdom to species, the organisms are more closely related (Godfray & Knapp, 2004). For example, *Puntigrus tetrazona* (Sumatrana barb) and *Rasbora sarawakensis* are from same family *Cyprinidae* but different subfamily. *Danio rerio* and *R. sarawakensis* are more closely related to each other because they are in same family *Cyprinidae* and same subfamily *Danioninae,* compared to *Puntigrus tetrazona* which in subfamily *Barbinae*. Systematic operates to identify the species and investigate the evolutionary relationship among species (Godfray & Knapp, 2004).

Taxonomic groups can be used to depict evolutionary relationships of species and construct phylogenetic tree. According to Hodge (2006), Charles Darwin proposed

evolution theory stated that all modern species are arisen from earlier species and all these organisms shared a common ancestry. Combination of Linnaeus's and Charles Darwin's works became the organizing principle and formed foundation of modern taxonomy (Luketa, 2012).

2.4 *Cyprinidae* family

The order *Cypriniformes* are traditionally divided into two superfamilies, *Cyprinioidea* and *Cobitioidea* (Mayden, 2008). The family *Cyprinidae* is the largest of all known freshwater fish families. All members of this family are termed as cyprinids (Refer to Figure 2.5). They are native to North America, Africa and Eurasia, which is the combined continental landmass of Europe and Asia (Zardoya & Doadrio, 1999). According to Nelson (2006), over 2,420 species of cyprinids and about 220 genera had been recognized. The 220 genera is estimated to be more than eight percent of the world's known fishes. According to current molecular and phylogenetic data obtained from fossil evidences, cyprinids may have originated in Asia in the Eocene (Zardoya & Doadrio, 1999). The cyprinids are primary freshwater fishes and they cannot tolerate high salinity environment. Although there are a few species that spend part of their life time in brackish water, for example *Rutilus rutilus,* they cannot reproduce in it especially when pH of water is below 5.5 (Meri *et al.*, 2008).

Cyprinids are characterized by their toothless jaws and palate (Mayden, 2008). They chew their food by using pharyngeal teeth in their throat which the pharyngeal teeth number as an important characteristic in distinguishing species. As a member of superorder Ostareophysi, cyprinids have Weberian organ which is set of bonny ossicles physically connect inner ear to swim bladder and thus amplify the sound waves from greater range of auditory stimuli (Mayden, 2008). Cyprinids usually have thin lips, large scales, absence of