Faculty of Resource Science and Technology

PHYLOGENY OF SELECTED SPECIES IN FAMILY STURNIDAE INFERRED FROM MTDNA CYTOCHROME OXIDASE SUBUNIT 1 GENE

Nurul Ain binti Mohd Khairi (24705)

Bachelor of Science with Honours
(Animal Resource Science and Management)
2012

Declaration

No portion of the work referred to in this dissertation has been submitted in support of an application for another degree of qualification of this or any other university or institution of higher learning.

Nurul Ain binti Mohd Khairi

Animal Resource Science and Management

Faculty of Resource Science and Technology

Universiti Malaysia Sarawak

Table of Contents

Table of Contents ………………………………………………………... I
List of Abbreviations..………………………………………………… II
List of Tables…………………………………………………………... III
List of Figures………………………………………………………….. IV
Abstract………………………………………………………………… V
Acknowledgement……………………………………………………... 1
1.0 Introduction and Objectives ………………………………………. 2-4
    1.1 Problems Statement…………………………………………... 5
    1.2 Objectives…………………………………………………….. 5
2.0 Literature Review …………………………………………………. 6-9
3.0 Materials and Methods……………………………………………….. 10
    3.1 Sampling Collection …………………………………………. 10
    3.2 Species Identification…………………………………………. 11
    3.3 Laboratory Methodology……………………………………….. 11
        3.3.1 DNA Extraction of tissue samples……...…………... 11
        3.3.2 Polymerase Chain Reaction (PCR…..……………….. 11-13
        3.4.3 Purification of PCR Products……………………….. 13
    3.4 Statistical Analysis…………………………………………… 14
4.0 Results………………………………………………………………... 15
    4.1 DNA Extractions………………………………………………... 15
    4.2 Polymerase Chain Reaction…..………………………………….. 16
    4.3 Purification of PCR Products………………………………… 17
    4.4 DNA Sequencing………………………………………………... 18
    4.5 Data Analysis…………………………………………………. 18
        4.5.1 Sequence Analysis…………………………………... 19
        4.5.2 Base Composition…………………………………. 19
        4.5.3 Pairwise Genetic Distance…………………………... 20
        4.5.4 Phylogenetic Tree Analysis…………………………. 21-25
5.0 Discussion………..………...…………………………………………. 26
    5.1 DNA Extraction………………………………………………. 26
    5.2 Polymerase Chain Reaction…………………………………... 26-27
    5.3 Purification of PCR Products………………………………… 27
    5.4 DNA Sequencing………………………………………………... 28
    5.5 Data Analysis…………………………………………………. 28-30
6.0 Conclusion and Recommendations……………………………………... 31
7.0 References…………………………………………………………. 32-34
8.0 Appendices…………………………………………………………. 35-39

List of Abbreviations

DNA	Deoxyribonucleic acid
mtDNA	Mitochondrial deoxyribonucleic acid
COI	Cytochrome oxidase subunit 1
ddH_2O	Deionized distilled water
$MgCl_2$	Magnesium chloride
PCR	Polymerase Chain Reaction
UV	Ultraviolet
kbp	Kilobase pair
bp	base pair
FRST	Faculty of Resource Science and Technology

List of Tables

Table 1 List of species, individual abbreviation and locality

Table 2 COI sequences

Table 3 PCR Mastermix

Table 4 Parameter for PCR process for 30 cycles

Table 5 Percentage of nucleotide frequencies observed in seven samples of starlings and mynas.

Table 6 Pairwise genetic distance among all the individuals

List of Figures

Figure 1 Visualisation of extraction products of tissue samples. L: 1 kb DNA ladder. Lane 1: AT04. Lane 2: AT05. Lane 3: AJ01. Lane 4: AJ02.

Figure 2 Visualisation of PCR products of extraction samples. L: 100bp DNA ladder. Lane 1: 9.11 TM. Lane 2: 11.11 TM. Lane 3: AT01. Lane 4: AT02. Lane 5: AT03. Lane 6: AT04. Lane 7: AT05. Lane 8: AJ01. Lane 9: AJ02. Lane 10: GR01. Lane 11: negative control.

Figure 3 Visualisation of purification of PCR products. L: 100bp DNA ladder. Lane 1: 11.11 TM. Lane 2: AT03. Lane 3: AT04. Lane 4: AT05. Lane 5: AJ01. Lane 6: AJ02. Lane 7: GR01.

Figure 4 Neighbour-Joining (NJ) tree constructed using PAUP 4.0b10 program with bootstrap 50% majority-rule with Kimura-2 parameter model of evolution with 1000 replicates.

Figure 5 50% majority-rule consensus maximum parsimony tree with bootstrap value using PAUP 4.0b10 program with Kimura-2 parameter (1000 replicates).

Figure 6 50% majority-rule consensus maximum likelihood tree with bootstrap value using PAUP 4.0b10 program with Kimura-2 parameter, analysed by model K81uf+I selected by Akaike Information Criterion (AIC) using MODELTEST 3.7.

Figure 7 50% majority-rule consensus Bayesian analysis tree with Bayesian Posterior Probability (BPP) values using MrBayes 3.1.2 program with Markov chain Monte Carlo (MCMC) (10^4 generations).

Phylogeny of Selected Species in family Sturnidae inferred from mtDNA Cytochrome Oxidase Subunit 1 gene

Nurul Ain binti Mohd Khairi

Animal Resource Science and Management
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak

ABSTRACT

Phylogeny of birds in the family Sturnidae (starlings and mynas) has always been an intriguing topic about their relationship and genetic distances among the species within the family. The species were collected within Sarawak. The mitochondrial DNA used in this study is Cytochrome Oxidase subunit 1 (COI). The phylogenetic trees of the family Sturnidae were constructed by using Bayesian analysis, Maximum Likelihood, Maximum Parsimony and Neighbour-Joining methods. The genetic distances were determined by using Kimura two-parameter and Bootstrap analysis in the relationships among the species within the family Sturnidae.

Key words: Sturnidae, COI, phylogenetic

ABSTRAK

Filogeni burung dari famili Sturnidae (perling dan tiong) selalu menjadi subjek yang menarik minat tentang hubungan dan jarak genetik di kalangan spesis dalam famili tersebut. Spesis-spesis tersebut telah didapati dalam Sarawak sahaja. DNA mitokondria yang akan digunakan dalam kajian ini adalah Cytochrome Oxidase Subunit 1 (COI). Pokok-pokok filogenetik famili Sturnidae dibina dengan menggunakan kaedah analisis Bayesian, Maximum Likelihood, Maximum Parsimony dan Neighbour-Joining. Jarak genetik ditentukan dengan menggunakan Kimura two-parameter dan analisis Bootstrap dalam hubungan di kalangan spesis-spesis dalam famili Sturnidae.

Kata kunci: Sturnidae, COI, filogenetik

Acknowledgements

Bismillahirrahmanirrahiim. Praise to Allah the Almighty for giving me the chance to finish this Final Year Project report. I would like to express my gratitude to my mother, Puan Azmani Jaafar for her guidance and moral support. I am also entirely grateful to my supervisor, Prof. Dr. Mustafa Abdul Rahman for his support and advice. Not only that, many thanks to the Head of Zoology Department, Dr. Yuzine Esa; to my mentor, Prof. Dr. Andrew Alek Tuen for giving me the moral support; and also Prof. Dr. Mohd Tajuddin Abdullah for his guidance. Many thanks to Mr Isa Sait, Mr Trevor Allen Nyaseng, Mr Huzal Irwan Husin and Mr Nasron Ahmad for their help in sampling methods and laboratory protocols; and also to the Postgraduate students who are always ready to help us; and my fellow friends who are together with me till the end in helping each other to finish the Final Year Project. Thank you very much.

1.0 Introduction and Objectives

Birds are living organisms which have a beak, a pair of scaly legs, a pair of wings and feathers covering their body. Birds are divided into two groups which are ratite and karinate. Ratite are birds that cannot fly as they have flat sternum and pectoral muscles which are not well developed while karinate are birds that can fly and have one keel sternum with flying muscles attached to it.

Order Passeriformes has 86 families including the Family Sturnidae under Superfamily Muscicapoidea. There are two subfamilies under the Family Sturnidae which are Subfamily Graculinae and Subfamily Sturninae. According to Bockheim and Congdon (2001), the 114-sturnid species make up 29 genera. Most species from Family Sturnidae in Peninsular Malaysia and Borneo are starlings and mynas. The characteristics of mynas and starlings are that they have long strong bills and legs, strong wings for flight and they are also noisy. Most Asian species have evolved to live on grassy areas. Most starlings and mynas live in areas such as houses, barns, gardens and residential areas. Some of the more unique ones can be found in the lowland dipterocarp forest and also in highlands. Based on Forsyth *et al.* (2011) in Rockjumper Birding Tours Malaysia & Borneo Trip Report 2011, they have recorded on some of the species from Family Sturnidae and some of them are migratory birds such as Daurian Starling (*Agropsar sturnus*). The sighted species were Asian Glossy Starling (*Aplonis panayensis*), Hill Myna (*Gracula religiosa*), Javan Myna (*Acridotheres javanicus*), Jungle Myna (*Acrdotheres fuscus*), Common Myna (*Acridotheres tristis*), Crested Myna (*Acridotheres cristatellus*) and a migrant, Daurian Starling (*Agropsar sturninus*).

The species that will be used in this study are Asian Glossy Starling (*Aplonis panayensis*), Hill Myna (*Gracula religiosa*), Common Myna (*Acridotheres tristis*) and Javan Myna or also known as White-vented Myna (*Acridotheres javanicus*). According to Cibois and Cracraft (2004), the phylogenetic hypothesis shows genus *Acridotheres* does not belong together with the Asian clade which comprises of genera *Gracula* and *Aplonis* which means it is not closely related but more closely related to the mynas from Afro-Asian clade. Moreover, genera *Aplonis* and *Gracula* are under Subfamily Graculinae while genus *Acridotheres* is under Subfamily Sturninae (Lovette and Rubenstein, 2007). Based on Lovette *et al.* (2008), Common Myna (*Acridotheres tristis*) and Javan Myna (*Acridotheres javanicus*) have been introduced to many countries including Asia. The outgroup for this phylogenetic study is Asian Paradise Flycatcher (*Terpsiphone paradisi*) from Family Muscicapidae under the same superfamily as Family Sturnidae which is Superfamily Muscicapoidea (Phillips and Phillips, 2011).

The study of systematics has always been evolved from one time to another as new scientists are always finding the truth of the historical relationships of organisms to fit in the Tree of Life like for example, which organisms are closely- or not closely-related species. The word systematics stem from the Latinised Greek word 'Systema' and can be defined as the classification of living organisms into hierarchical series of groups emphasizing their phylogenetic relationship (Guerra-García *et al.*, 2008). Systematics is important in determining the classification of taxa and the names of the species according to their relationship. In order to carefully arrange the taxonomy of species within the family, several methods such as molecular phylogeny is essential to determine the evolutionary relationships of the species.

Phylogeny is defined as historical relationships of organisms and the evolution of the taxa. Phylogeny is important for the study of systematics since the relationships among the species can be obtained via phylogenetic study. By studying the history of the species, the phylogenetic tree can be constructed as the relationship and the connection of the taxa are able to determine. The phylogenetic relationships can be determined by analysing the data obtained by the mtDNA. However, the number of samples for phylogenetic study must be adequate to acquire more precise data. In understanding the phylogeny of the species within the family, we will be able to understand their evolutionary differences. Mitochondrial DNA (mtDNA) is used in this phylogenetic study as it evolves more rapidly than nuclear DNA and resulting higher chances of accurate DNA sequences and differences of closely-related species. Therefore, it is recommended to use the COI as a primer in phylogenetic study of animals including birds.

1.1 Problems Statement

The species selected for this study are Asian Glossy Starling (*Aplonis panayensis*), Hill Myna (*Gracula religiosa*), Javan Myna (*Acridotheres javanicus*) and Common Myna (*Acridotheres tristis*). Asian Glossy Starling and Common Myna are commonly found near the roadside where the sources of food are. According to Lovette and Rubenstein (2007), Asian Glossy Starling and Hill Myna are much more closely-related rather than Common Myna and Javan Myna by using Ultrametric Bayesian likelihood based on mitochondrial protein-coding sequences (4116 nucleotides). As COI is used as the primer gene in this study, the result of phylogenetic analysis might be slightly different compared to Lovette and Rubenstein (2007). However, for the outgroup which is the family Mimidae which is abundance in North, Central and South America and also West Indies, is the most closely-related to family Sturnidae. Therefore, another outgroup has been chosen which is Asian Paradise Flycatcher from family Muscicapidae which is also under Superfamily Muscicapoidea.

1.2 Objectives

1) To construct the phylogenetic tree of selected species from the family Sturnidae in Sarawak using COI gene
2) To investigate the relationship of closely-related species from the family Sturnidae in Sarawak by using COI gene

2.0 Literature Review

Family Sturnidae is under the Superfamily Muscicapoidea which includes the Family Turdinae and Family Mimidae. The starlings are distributed in the Old World (one species has been introduced in the New World) (Zuccon *et al.*, 2006). Starlings and mynas are small to medium-sized, have long wings, strong legs, and a short tail. The family Sturnidae is closely-related to the family Mimidae (mockingbirds) which cannot be found in Malaysia as their distribution is limited more to the west side of the globe. However, family Muscicapidae (flycatchers) which can be found in Malaysia are also closely-related to the family Sturnidae. According to Voelker and Spellman (2004), their results indicate that Turdinae+Sturnidae+Cinclidae clade is sister to the Muscicapinae. Not only that, based on Cibois and Cracraft (2004), their analyses suggest that Muscicapoidea is composed of three main groups which are the Cinclidae, Muscicapidae and Sturnidae. The species that are being studied which are the Asian Glossy Starling (*Aplonis panayensis*), Hill Myna (*Gracula religiosa*), Javan Myna (*Acridotheres javanicus*) and Common Myna (*Acridotheres tristis*) have different subfamily. Based on Lovette and Rubenstein (2007), Asian Glossy Starling and Common Hill Myna are under the same subfamily which is Graculinae while Common Myna and Javan Myna are under subfamily Sturninae. This explains the differences in relationship among the species and the distances of the phylogeny.

Phylogenetic study is important in determining the ancestral traits and the phylogeny of family Sturnidae. Various molecular analyses can be used to build the phylogenetic tree of family Sturnidae which include Bayesian analysis, Maximum Parsimony, Maximum Likelihood and Neighbour-Joining. Monophyly of Passerida is recognized by both parsimony

and maximum-likelihood analyses (Ericson and Johansson, 2003). According to Zuccon *et al.* (2006) which used the maximum parsimony analysis based on four parsimonious trees in family Sturnidae, Asian Glossy Starling (*Aplonis panayensis*) and Hill Myna (*Gracula religiosa)* are very closely-related as they are separated by only one branch while Common Myna (*Acridotheres tristis*) and Javan Myna (*Acridotheres javanicus*) are closely-related to each other but quite far away separated from Asian Glossy Starling and Hill Myna. This result is quite similar to Lovette and Rubenstein (2007) which used the Ultrametric Bayesian likelihood phylogenetic analysis which is differ from Zuccon *et al.* (2006) which used different phylogenetic analysis.

Mitochondrial genomes are targets for characterization, population genetic and phylogenetic studies since they are small, circular, maternally inherited and have high yield of copy-number per cell. Not only that, mtDNA *cox* subunit 1 is the most highly conserved than other two genes coding cytochrome oxidase, therefore it is mostly used for phylogenetic studies (Kandil *et al.,* 2010). Based on Folmer *et al.* (1994), mitochondrial cytochrome c oxidase subunit 1 (COI) appears to be among the conservative protein-coding genes in the mitochondrial genes of animals. However, there is a concerned about nuclear sequences will be amplified instead of the targeted mtDNA sequences (Sorenson and Quinn, 1998). This might be true but recent research has proved that most PCR amplifications of the primers and the DNA were a success. According to Hebert *et al.* (2003), COI divergences among the 13 320 species pairs ranged from low of 0.0% to a high 53.7%. The COI genes in the congeneric species of animals regularly have substantial sequence divergence. In fact, more than 98% of species pairs showed greater than 2% sequence divergence (Hebert *et al.*, 2003).

There are various methods for constructing phylogenetic trees but only four methods are chosen; Neighbour-Joining (NJ), Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian inference. NJ is a method of producing a unique final tree under the principle of minimum evolution (Saitou and Nei, 1987). This method uses distance matrix and does not assume a molecular clock. It shows efficient results of correct tree topology and can be used for any type of evolutionary distance data. NJ also provides the branch lengths of the tree. MP method chooses the best tree that has the smallest number of nucleotide substitutions required for explaining the evolutionary changes of DNA sequences (Saitou and Imanishi, 1989). However, this method does not utilize all the available information such as singular sites that are discarded during the process of tree making (Sourdis and Nei, 1988). This explanation shows that this method is inefficient than other methods but it performs better if the number of nucleotide substitutions is small.

The method of ML uses discrete characters and depends on the specification of data and a probability model to describe the data (Huelsenbeck and Crandall, 1997). The parameter values of the model influences the probability of observed data under the assumed model therefore, the parameter value that maximizes the probability of observed data is chosen. The model choice is based on several model tests such as Hierarchical Likelihood-Ratio tests (hLRTs) and Akaike Information Criterion (AIC). AIC is an unbiased estimator of the expected relative Kullback-Leibler information quantity of distance (K-L) (Posada and Buckley, 2004). This test provides a measure of fit between model and data and includes a penalty for overparametization (Sullivan and Joyce, 2005). The model favoured is the model that has the lowest value of AIC because the amount of information lost is used to approximate the real process of nucleotide substitution (Posada and Buckley, 2004). AIC has

advantage over hLRTs in model selection since AIC removes the need to travel through model space by pairwise comparisons.

Bayesian inference method is based on posterior probability of a tree that uses Bayes' theorem and Markov chain Monte Carlo. Bayes' theorem is used to combine the prior probability of a phylogeny with the likelihood to produce posterior probability distribution on trees (Huelsenbeck *et al.,* 2001). Markov chain Monte Carlo (MCMC) is used to evaluate the posterior distribution of phylogenetic trees (Yang and Rannala, 1997). This method produces best trees similar to those generated by ML method but the posterior probabilities are quite different from the bootstrap proportions. In Bayesian analysis, one of the partition-specific substitution models is selected by using several tests such as hLRTs or AIC which is based on ML estimates (Nylander *et al.,* 2004).

3.0 Materials and Methods

3.1 Sampling Collection

The Asian Glossy Starlings (*Aplonis panayensis*) were captured at East Campus of UNIMAS by mist netting. The Common Myna (*Acridotheres tristis*) and Javan Myna (*Acridotheres javanicus*) were also sampled from the UNIMAS Campus grounds. There were also some voucher specimens of Common Myna available in the Zoology museum so some of the tissues were extracted from three different individuals. Hill Myna (*Gracula religiosa*) is also proved hard to capture thus, a voucher specimen from Zoology Museum was used and some of the tissues were extracted. The tissues are stored in 70% or more ethanol at -20°C.

Table 1: List of species, individual abbreviation and locality

Species	Individual Abbreviation	Locality
Aplonis panayensis	9.11 TM	East Campus, UNIMAS
Aplonis panayensis	11.11 TM	East Campus, UNIMAS
Acridotheres tristis	AT01	West Campus, UNIMAS (Museum specimen)
Acridotheres tristis	AT02	West Campus, UNIMAS (Museum specimen)
Acridotheres tristis	AT03	West Campus, UNIMAS (Museum specimen)
Acridotheres tristis	AT04	East Campus, UNIMAS
Acridotheres tristis	AT05	East Campus, UNIMAS
Acridotheres javanicus	AJ01	East Campus, UNIMAS
Acridotheres javanicus	AJ02	East Campus, UNIMAS
Gracula religiosa	GR01	West Campus, UNIMAS (Museum specimen)

3.2 Species Identification

The specimens were identified by using The Birds of Borneo (Smythies, 1999) and Phillips' Field Guide to the Birds of Borneo, 2nd edition (Phillips and Phillips, 2011).

3.3 Laboratory Methodology

3.3.1 DNA Extraction of tissue samples

The DNA was extracted by using manual CTAB method. CTAB method is very effective for binding the DNA strongly to prevent DNA degradation by nucleases (Ramji, 2007). DNA extractions were done from ten different individuals comprise of four different species which are the Asian Glossy Starling (*Aplonis panayensis*), Common Myna (*Acridotheres tristis*), Javan Myna (*Acridotheres javanicus*) and Hill Myna (*Gracula religiosa*). Then, the extractions underwent gel electrophoresis (0.4 g of Agarose powder, 40 ml of TAE buffer) with 1 kb DNA ladder at 90 volts for 30 minutes. Then, the extractions were visualized under the UV light transilluminator.

3.3.2 Polymerase Chain Reaction (PCR)

Polymerase chain reaction is a process that allows rapid generation of large amounts of DNA sequences that are easier to purify and less damaged (Clark, 2005). This process was conducted to amplify the ten DNA extractions from the tissue samples. The primer used is partial mitochondrial DNA COI-f (forward) and COI-e (reverse). The sequences are as in Table 2.

Table 2: COI sequences

Primer	Sequence
COI-f	5’-CCT GCA GGA GGA GGA GAT CC-3’
COI-e	5’-CCA GAG ATT AGA GGG AAT CAG TG-3’

Table 3: PCR Mastermix

PCR Components	Volume for 1x reaction (µl)
5x Taq buffer	5.0
1.5 mM $MgCl_2$	2.0
0.2 mM dNTPs	0.5
0.4 mM COI-f	1.0
0.4 mM COI-e	1.0
ddH_2O	14.2
Taq polymerase	0.3
DNA template	1.0
Total	**25.0**

Table 4: Parameter of PCR process for 30 cycles

Parameter	Temperature (^{o}C)	Duration (mins)
Initial denaturation	95	3
Denaturation	95	1
Annealing	50.5	1
Extension	72	2
Final extension	72	3
Soaking	4	No time limit

Taq polymerase and DNA template were added lastly into each tube accordingly after equal volume of other mixed PCR components were added into each tube. *Taq* polymerase is used to elongate the new strand of DNA and the optimum temperature for its activity is 70°C. After the PCR was done, the PCR products underwent gel (1%) electrophoresis (0.4 g of Agarose powder, 40 ml of TAE buffer) with 100 bp DNA ladder at 90 volts for 30 minutes. Then, the PCR products were visualized under the UV light transilluminator.

3.3.3 Purification of PCR Product

The PCR products were then purified by using the Promega Wizard™ SV Gel PCR Clean-Up System. The protocol prepared by Promega was followed to purify the products. Then, the products were stored at -20°C before sending to a private laboratory for DNA sequencing. The DNA sequencing is to separate DNA molecules whose lengths differ by just a single nucleotide (Brown, 1994). This is being done by gel electrophoresis of polyacrylamide gel from the negative pole to the positive pole. Polyacrylamide is used to create the gel because

the pores of this gel are smaller than the agarose gel which is crucial in separating the DNA molecules.

3.4 Statistical Analysis

Various programmes for statistical analysis were used such as Chromas Version 1.45 and Clustal X Version 1.81 for visualizing and analysing the DNA sequence analysis result (Thompson *et al*., 1997). The BLAST program (Basic Local Alignment Search Tool) is used to find and compare the nucleotide or protein sequences of our own DNA sequences with the sequences from the databases (NCBI, 2012). The MEGA Version 3.1 (Molecular Evolutionary Genetic Analysis) is used to check the stop codons in the DNA sequences and to determine the sequence analysis of base sites (Kumar *et al.,* 2004). Another program which is PAUP 4.0b10 is used to construct the phylogenetic tree of Family Sturnidae by using three ways which are the Neighbour-Joining (NJ), Maximum Parsimony (MP) and Maximum Likelihood (ML). The Bayesian analysis that is used to construct the phylogenetic tree is by using the MrBayes 3.1.2 programme (Huelsenbeck and Ronquist, 2001).

4.0 Results

4.1 DNA Extraction

DNA from the samples was extracted by using CTAB method (manual). During the water bath step, the time taken for the tissues to completely dissolve was from 2 to 3 hours. The final volume of the extraction products was 50µl by adding ddH_2O. For Hill Myna (*Gracula religiosa*) sample, the DNA pellet that should be in the tube after centrifugal was unavailable. More extractions were done for this sample until the DNA pellet was visible and could proceed to the next step. Similar processes were done for other samples which have no visible bands during visualization of gel electrophoresis.

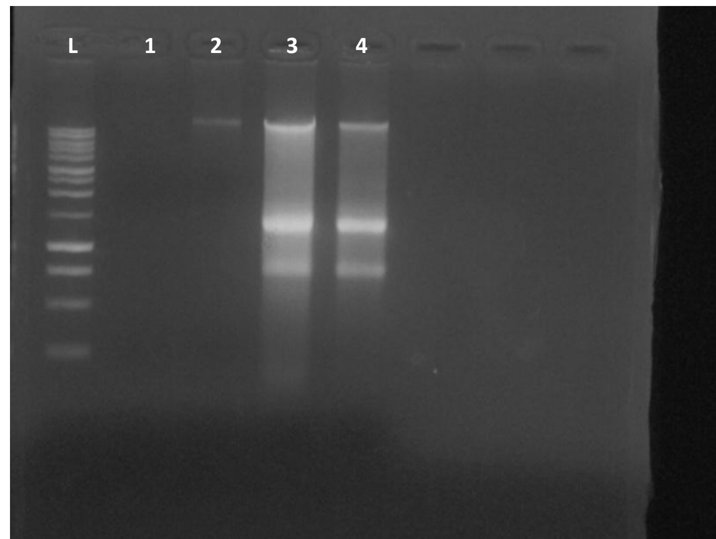

Figure 1: Visualisation of extraction products of tissue samples. L: 1 kb DNA ladder. Lane 1: AT04. Lane 2: AT05. Lane 3: AJ01. Lane 4: AJ02.

4.2 Polymerase Chain Reaction (PCR)

The successful DNA extractions which have clear and visible bands were prepared for the PCR. The PCR mastermix was done including for the negative control and error. The negative control acts as indicator for contamination during PCR mastermix preparation. No band for negative control shows that the PCR mastermix is not contaminated. The error tube is prepared for possibilitie of pipetting error during PCR mastermix preparation. The recipe is as in Table 3. The annealing temperature was adjusted from 50°C to 52°C to get the most suitable temperature for all species. The temperature, 50.5°C, is the best temperature for all species and the visualisation shows clear and visible bands although for Hill Myna (*Gracula religiosa*), the band is faint but visible.

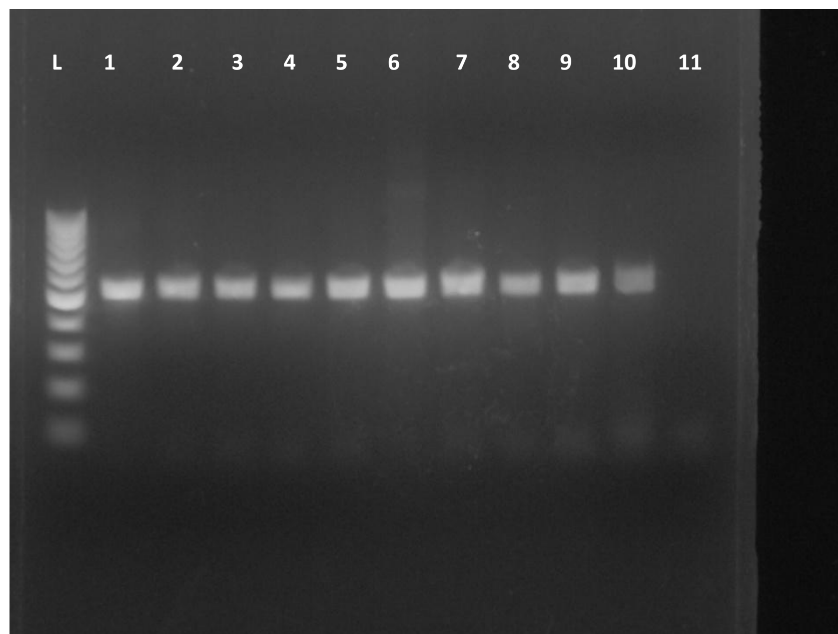

Figure 2: Visualisation of PCR products of extraction samples. L: 100bp DNA ladder. Lane 1: 9.11 TM. Lane 2: 11.11 TM. Lane 3: AT01. Lane 4: AT02. Lane 5: AT03. Lane 6: AT04. Lane 7: AT05. Lane 8: AJ01. Lane 9: AJ02. Lane 10: GR01. Lane 11: negative control.

4.3 Purification of PCR Products

Purification of PCR products is a necessary to remove any impurities such as primer dimer, cellular components or other contaminants that can disturb the DNA sequencing and the chromatograph of DNA sequences will show many noisy backgrounds due to unpurified PCR products. It is important to obtain good DNA for sequencing. Therefore, the PCR products were purified by using Promega Wizard™ SV Gel PCR Clean-Up System kit. Samples 9.11 TM, 11.11 TM, AT03, AT04, AT05, AJ01, AJ02 and GR01 were purified by using the kit. The visualization shows that the bands are situated between 500 and 600 base pairs. Samples that showed bands during visualization were prepared to send to a private laboratory.

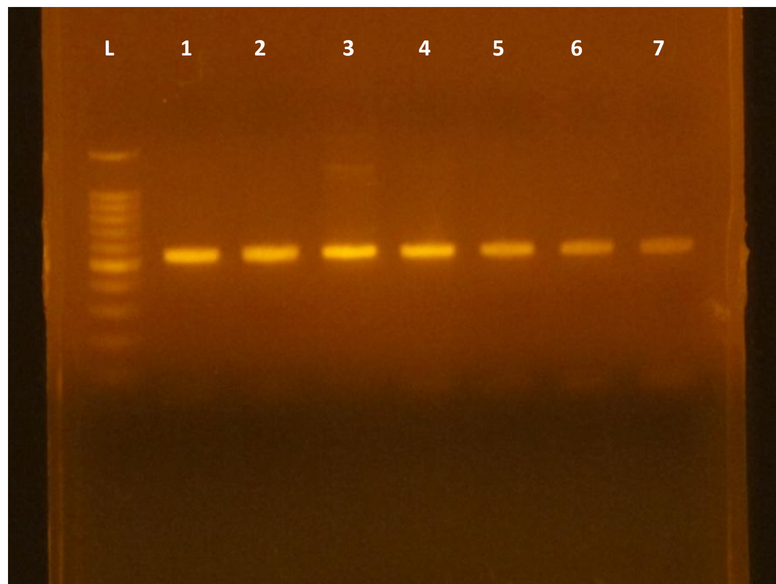

Figure 3: Visualisation of purification of PCR products. L: 100bp DNA ladder. Lane 1: 11.11 TM. Lane 2: AT03. Lane 3: AT04. Lane 4: AT05. Lane 5: AJ01. Lane 6: AJ02. Lane 7: GR01.