GENETIC CHARACTERISATION OF MALAYSIAN EDIBLE NEST SWIFTLET (*Aerodramus fuciphagus* Thunberg, 1812) USING MICROSATELLITE AND MOLECULAR SEXING MARKERS

By

MUHAMMAD AMIN BIN OSMAN

Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Master of Science

August 2019

FP 2019 79

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

GENETIC CHARACTERISATION OF MALAYSIAN EDIBLE NEST SWIFTLET (*Aerodramus fuciphagus* Thunberg, 1812) USING MICROSATELLITE AND MOLECULAR SEXING MARKERS

By

MUHAMMAD AMIN OSMAN

August 2019

Chairman : Sumita Sugnaseelan, PhD
Faculty : Agriculture

The White-nest swiftlet (*Aerodramus fuciphagus*) is a swiftlet species which produce edible bird nest (EBN) from pure saliva, that is considered of high value in the international market. Due to increased demand and high value of the nests, over-harvesting had occurred in many locations, believed to be their natural habitats including in Malaysia. However, the introduction of swiftlet ranching or farming has increased the overall population of this species, leading to exclusion of this swiftlet species from the list of protected animals under the legislation Wildlife Conservation Act 2010 (Act 716), Malaysia. Exploitation and rapid population expansion of this species will trigger unpredictable changes in its genetic structure and reproduction over time. It is with these in mind that the present study was conducted. The main objectives of the study were to determine the genetic structure and correctly differentiate the sex of *A. fuciphagus* in Peninsular Malaysia. These main objectives were accomplished using microsatellite and molecular sexing markers.

In the study analysing the genetic structure of *A. fuciphagus*, 90 *A. fuciphagus* samples (blood, embryos, feathers and muscle tissues) were randomly collected from three different populations (N = 30 per population): i) Northern Region (NR), that was Perak, ii) Eastern Region (ER), that comprised of Kelantan and Terengganu, and iii) Southern Region (SR) which was Johor. DNA was extracted from the samples and were assessed for 12 species-specific and cross-species microsatellite loci. PCRs were accomplished by using touchdown programmes, and the PCR products were separated using Polyacrylamide Gel Electrophoresis (PAGE). The results showed that eight microsatellite loci were polymorphic (11 to 15 alleles per locus). Observed heterozygosity values for all populations were low to moderate, ranging from 0.28 (SR) to 0.44 (ER) and lower than the expected heterozygosity values. All loci showed significant deviation from Hardy-Weinberg equilibrium (HWE)

($p<0.05$). The mean of inbreeding coefficients (FIS) ranged from 0.465 (ER) to 0.636 (SR), indicating that inbreeding was present in the sampled populations. The genetic distance between populations showed that SR and NR tended to be the most distantly related. The mean FST value was 0.059. Analysis of molecular variation (AMOVA) indicated that a greater proportion of the genetic diversity was within the populations (92.9%) than among populations (7.06%). The structure analysis showed that the populations could be grouped into two clusters; SR was in a cluster of its own, while ER and NR were clustered together.

In the molecular sexing study, seven primer sets for sexing birds were screened in *A. fuciphagus* using a total of 13 feather samples that were randomly selected from Perak. From the screening analysis, only one primer set (P8/WZ/W) was successfully differentiated the sex of *A. fuciphagus*. PCR amplification produced a single 255 bp DNA fragment for males which was derived from *CHD-Z* (CHD gene region in the sex chromosome Z), while for the females it produced two fragments (144 and 255 bp). The 144 bp fragment was from *CHD-W*. Results from sequencing showed that there were no variations in the base sequences of the *CHD-W* and *CHD-Z* amplified fragments within the same sexes, except for one male sample (A23) where at position 166, a base substitution occurred (from G to A). Phylogenetic analysis of *CHD-W* showed that four (Apodiformes, Pelecaniformes, Gruiformes and Accipitriformes) out of the five orders investigated had 100% similarity among birds from the same order. Whilst phylogenetic analysis of *CHD-Z* did not show 100% similarity among birds from the same order. The results suggest that for the CHD gene, evolution occurred at a higher rate in males compared to females.

In conclusion, this study suggests that there was sufficient genetic variation among the EBN swiftlets in Malaysia. However, the risk of inbreeding should be a concern. Therefore, it is important to ensure a healthy population size for the industry to grow as this will significantly decrease the effects of genetic drifts in the founder populations, as well as maintain the genetic diversity and adaptive ability of the *A. fuciphagus* populations. In addition, the accomplishment to correctly identify the sex of *A. fuciphagus* using molecular sexing marker is beneficial for further studies on sex ratio and breeding management in *A. fuciphagus*.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENCIRIAN GENETIK BURUNG WALIT DI MALAYSIA (*Aerodramus fuciphagus Thunberg, 1812*) MENGGUNAKAN PENANDA MIKROSATELIT DAN PENJANTINAAN MOLEKULAR

Oleh

MUHAMMAD AMIN OSMAN

Ogos 2019

Pengerusi : Sumita Sugnaseelan, PhD
Fakulti : Pertanian

Burung walit sarang putih (*Aerodramus fuciphagus*) adalah spesies burung walit yang menghasilkan sarang yang boleh dimakan daripada air liur, di mana sarangnya bernilai tinggi di pasaran antarabangsa. Oleh kerana peningkatan permintaan dan nilai tinggi sarang burung, penuaian sarang burung secara berlebihan berlaku di kebanyakan tempat yang dipercayai habitat semulajadinya, termasuk di Malaysia. Walau bagaimanapun, pengenalan terhadap penternakan burung walit telah sedikit sebanyak mempengaruhi penambahan dalam bilangan populasi keseluruhan spesies ini, yang akhirnya membawa kepada pengecualian spesies burung walit ini daripada senarai haiwan terlindung dibawah undang-undang Akta Pemuliharaan Hidupan Liar (Akta 716), Malaysia. Eksploitasi dan perkembangan pesat populasi spesies ini akan menyebabkan perubahan yang tidak menentu dalam struktur genetik dan reproduksi dalam suatu jangka masa. Dengan mempertimbangkan perkara ini, maka kajian ini dijalankan. Objektif utama kajian ini adalah untuk mengenalpasti struktur genetik dan menentukan dengan tepat jantina *A. fuciphagus* di Semenanjung Malaysia. Objektif utama ini tercapai melalui penanda mikrosatelit dan penjantinaan molekular.

Dalam kajian penilaian struktur genetik *A. fuciphagus*, 90 sampel *A. fuciphagus* (darah, embrio, bulu dan sampel tisu otot) diambil secara rawak daripada tiga populasi berbeza (N = 30 setiap populasi): i) Kawasan Utara (NR), iaitu Perak, ii) Kawasan Timur (ER), yang merangkumi Kelantan dan Terengganu, dan iii) Kawasan Selatan (SR), iaitu Johor. DNA diekstrak daripada sampel (keseluruhan berjumlah 90) dan dianalisa untuk 12 lokus mikrosatelit yang spesies-spesifik dan spesies-silang. Keseluruhan PCR dicapai dengan menggunakan beberapa program *touchdown*, dan produk PCR diasingkan melalui kaedah *Polyacrylamide Gel Electrophoresis* (PAGE). Keputusan menunjukkan lapan lokus mikrosatelit adalah polimorfik (11 hingga 15 allele

pada setiap lokus). Nilai heterozigositi yang diperhatikan untuk semua populasi berkisar antara rendah ke sederhana, iaitu dari 0.28 (SR) hingga 0.44 (ER), dan lebih kecil daripada nilai heterozigositi yang dijangka. Semua lokus menunjukkan sisihan ketara ($p<0.05$) daripada keseimbangan Hardy-Weinberg (HWE). Min koefisien pembiakbakaan dalam (F_{IS}) berjulat antara 0.465 (ER) hingga 0.636 (SR); ini menunjukkan pembiakbakaan dalam wujud di populasi yang disampel. Jarak genetik di antara populasi memperlihatkan SR dan NR sebagai paling jauh hubungannya. Purata nilai F_{ST} adalah 0.059. Analisis variasi molekular (AMOVA) menunjuk bahawa kepelbagaian genetik lebih menjurus kepada kepelbagaian di dalam populasi (92.9%) daripada di antara populasi (7.06%). Analisis struktur menunjukkan bahawa keseluruhan populasi boleh dikumpulkan kepada dua kluster; SR membentuk satu kluster sendiri sementara ER dan NR dikumpul dalam satu cluster lagi.

Dalam kajian penjantinaan molekular, tujuh set primer untuk penjantinaan burung telah disaring pada *A. fuciphagus* dengan menggunakan 13 sampel bulu yang telah dipilih secara rawak dari Perak. Daripada analisis saringan, hanya satu set primer (P8/WZ/W) yang berjaya membezakan jantina *A. fuciphagus*. Amplifikasi PCR menghasilkan satu fragmen DNA 255 bp bagi jantan yang berasal daripada *CHD-Z* (kawasan CHD gen di kromosom jantina Z), manakala bagi betina ia menghasilkan dua fragmen (144 dan 255 bp). Fragment 144 bp berasal daripada *CHD-W*. Keputusan daripada *sequencing* menunjukkan tiada perubahan pada jujuran bes di fragment teramplifikasi *CHD-W* dan *CHD-Z* di antara sesama jantina, kecuali pada satu sampel jantan (A23) dimana pada posisi 166, penukaran bes telah berlaku (daripada G ke A). Analisis filogenetik pada *CHD-W* menunjukkan empat (Apodiformes, Pelecaniformes, Gruiformes and Accipitriformes) daripada lima order yang dikaji mempunyai 100% persamaan antara burung dalam order yang sama. Manakala, analisis filogenetik pada *CHD-Z* tidak menunjukkan persamaan 100% antara burung dalam order yang sama. Keputusan ini mencadangkan bahawa bagi gen CHD, evolusi berlaku pada kadar yang lebih cepat pada jantan (*CHD-Z*) berbanding betina (*CHD-W*).

Kesimpulannya, kajian ini mencadangkan terdapatnya kehadiran variasi genetik yang mencukupi pada burung walit di Malaysia. Walau bagaimanapun, risiko pembiakbakaan dalam harus diberi perhatian sewajarnya. Oleh yang demikian, amatlah penting untuk memastikan saiz populasi yang sihat untuk pertumbuhan industri walit kerana ianya akan mengurangkan kesan hanyutan genetik dalam populasi pengasas serta memastikan kepelbagaian genetik dan kebolehan beradaptasi populasi *A. fuciphagus*. Tambahan pula, kejayaan dalam memastikan jantina *A. fuciphagus* dengan tepat menggunakan penanda molekular jantina adalah berfaedah untuk kajian akan datang untuk penentuan kadar seks dan pengurusan pembiakbakaan *A. fuciphagus*.

ACKNOWLEDGEMENTS

First and foremost, my honest appreciation goes to the All Mighty God for giving me the strength and grace to complete this study.

I am most grateful to my father and my mother for their sacrifices and supports through all of my life with their love and money. I am also thankful to my siblings who supported me directly or indirectly in encouraging me to finish my study.

I am also indebted to my wonderful supervisor, Dr. Sumita Sugnaseelan who not only gave me support, but also cared for my well-being. I also like to express my sincere thanks to my supervisory committee members, Dr. Nurul Izza Ab Ghani and Prof. Jothi Malar Panandam for their encouragements, advices and suggestions that helped a lot in completing my study.

I wish to express my sincere gratitude to everyone who helped me or contributed to the success of my work; special thanks to the staff of Genetic Laboratory, Miss Kamariah Jamhari, for her assistance. Thank you.

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

Sumita Sugnaseelan, PhD
Senior Lecturer
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

Nurul Izza Ab Ghani, PhD
Senior Lecturer
Faculty of Science
Universiti Putra Malaysia
(Member)

Jothi Malar P.V. Panandam, PhD
Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Member)

_____
ZALILAH MOHD SHARIFF, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 08 October 2020

TABLES OF CONTENTS

		Page
ABSTRACT		i
ABSTRAK		iii
ACKNOWLEDGEMENTS		v
APPROVAL		vi
DECLARATION		viii
LIST OF TABLES		xiii
LIST OF FIGURES		xv
LIST OF ABBREVIATIONS		xvii
CHAPTER		
1	**INTRODUCTION**	1
	1.1 Research Problem	2
	1.2 Research Hypothesis	2
	1.3 Objectives	2
2	**LITERATURE REVIEW**	3
	2.1 Swifts and Swiftlets	3
	2.2 Swiftlet Industry	5
	2.3 Swiftlet Farming	6
	2.3.1 Cave and birdhouses	7
	2.3.2 Edible nest swiftlet	8
	2.4 Genetic Diversity	9
	2.5 Genetic Characterisation	9
	2.6 Microsatellites	12
	2.6.1 Distribution and sequence	13
	2.6.2 Advantages, disadvantages and applications	14
	2.6.3 Mutation mechanism	15
	2.6.4 Genotyping of microsatellite loci	17
	2.7 Genetic Studies in Swiftlet	18
	2.8 Studies of Birds' Diversity using Microsatellite Markers	20
	2.9 Molecular Sexing	21
3	**MATERIALS AND METHODS**	24
	3.1 Experimental Materials	24
	3.2 Sample Collection	27
	3.3 DNA Extraction	27
	3.4 DNA Quality and Quantification	28
	3.5 Microsatellite Primers	30
	3.6 Polymerase Chain Reaction (PCR) Optimization	34
	3.7 DNA Pooling	34

3.8 PCR Amplification of Microsatellites 34
3.9 Microsatellite Genotyping Techniques 37
3.10 Microsatellite Sequencing 38
3.10.1 PCR product purification 38
3.11 Molecular Sexing 39
3.11.1 Experimental material 39
3.11.2 Sample collection 39
3.11.3 Primers for molecular sexing 39
3.11.4 PCR amplification 41
3.11.5 Agarose gel electrophoresis 42
3.11.6 Sequencing 42
3.12 Statistical Analysis 43
3.12.1 Genetic variation and relationship within swiftlet populations in Peninsular Malaysia 43
3.12.1.1 Heterozygosity 43
3.12.1.2 Hardy-Weinberg Equilibrium (HWE) 43
3.12.1.3 Inbreeding coefficient 44
3.12.2 Genetic variation and relationship between swiftlet populations in Peninsular Malaysia 44
3.12.2.1 F-statistics and gene flow 44
3.12.2.2 Analysis of Molecular Variance (AMOVA) 45
3.12.2.3 Estimation of genetic admixture 45
3.12.2.4 Genetic distance and phylogenetic analysis 45
3.12.3 Sexing markers analysis 46

4 RESULTS 47
4.1 DNA Extraction and PCR Optimization 47
4.2 DNA Pooling 49
4.3 Allele Frequency Distributions 50
4.4 Microsatellite Loci Analysis 52
4.5 Genetic Diversity within Populations 54
4.5.1 Observed and effective number of alleles 54
4.5.2 Heterozygosity 56
4.5.3 Hardy-Weinberg Equilibrium (HWE) 59
4.5.4 Estimation of inbreeding 59
4.5.5 Analysis of molecular variance (AMOVA) 60
4.6 Genetic Variation and Relationship between Populations 61
4.6.1 F-statistics and gene flow 61

	4.6.2	Estimation of genetic admixture in the three populations investigated	62
	4.6.3	Genetic distance and phylogenetic analyses	66
4.7	Molecular Sexing Analysis		67
	4.7.1	Post-mortem findings	67
	4.7.2	PCR product amplification	68
	4.7.3	Gel purification and sequencing	71
	4.7.4	PCR product nucleotide sequence alignment	71
	4.7.5	Comparison of sex evolution between *A. fuciphagus* and other avian species	72
5	**DISCUSSION**		78
5.1	Polymorphism Screening using DNA Pooling		78
5.2	Genetic Diversity at the Microsatellite Loci		78
5.3	Genetic Diversity within Populations		79
5.4	Genetic Variation and Relationship between the Populations		84
5.5	Molecular Sexing		87
6	**CONCLUSION**		91
REFERENCES			93
APPENDICES			106
BIODATA OF STUDENT			116

LIST OF TABLES

Table		Page
2.1	Subfamilies, tribes and genera of the Apodidae	4
2.2	Relationship between swifts and hummingbirds	4
2.3	Perfect, imperfect and composite microsatellites repeat	13
2.4	Advantages and disadvantages of PAGE	17
3.1	The investigated colonies in the study	26
3.2	Information on species-specific microsatellite loci for *A. fuciphagus*	31
3.3	Information of microsatellite loci primers for swallow species	32
3.4	Composition of the PCR reaction used in this study	35
3.5	PCR protocol used for amplification of microsatellite loci Aef4 and Aef112	35
3.6	PCR protocol used for amplification of microsatellite loci Aef24, Aef109, Aef115 and Aef133	36
3.7	PCR protocol for amplification of microsatellite loci Aef27, Aef104, TaBi1, TaBi4, TaBi6, TaBi8, TaBi10, TaBi12, TaBi21, TaBi25, TaBi34, Tal4, Tle4, Tle8, Tle14, Tle15, Tle16, Tle17, Tle18, Tle19, Tle20 and Tle21	37
3.8	Chemicals and reagents used for the preparation of 8% polyacrylamide gel	37
3.9	Information on *A. fuciphagus* individuals used for molecular sexing	39
3.10	Seven primers tested for identifying the sex of *A. fuciphagus*	40
3.11	PCR protocol used for five molecular sexing primers (P8/P2/P0, 1237L/1272H, 1237L/1272H/GW2, P8/P2 and P8/M5)	41
3.12	PCR protocol used for two sexing primer pairs (P8/WZ/W and 2550F/2718R)	41
4.1	Size of products from PCR amplifications of DNA pools for the twelve microsatellite loci	50
4.2	Alleles at high frequencies in different populations	52
4.3	Observed (Na) and effective (Ne) number of alleles, Shannon information index (I) and allele size ranges for the microsatellite loci investigated	53
4.4	Observed (Na) and effective (Ne) number of	55

	alleles for the eight polymorphic loci in the three populations	
4.5	Private alleles in the three populations	56
4.6	Observed (Ho) and expected (He) heterozygosities at the eight polymorphic microsatellite loci for the three populations	58
4.7	Probability values of Chi-square test for Hardy-Weinberg equilibrium for the eight polymorphic microsatellite loci in the three populations	59
4.8	Inbreeding coefficient (F_{IS}) for the eight polymorphic microsatellite loci in the three populations	60
4.9	Analysis of Molecular Variance (AMOVA) for the three populations	61
4.10	Gene differentiation (F_{ST}) and gene flow (N_M) among the three populations	62
4.11	F-statistics and gene flow at the eight polymorphic microsatellite loci across the populations	62
4.12	Posterior probability values for K in clusteringassignment	63
4.13	Membership of each population in each of the two clusters inferred	65
4.14	Genetic distances among three populations	66
4.15	Percentage of similarity between males (CHD-Z) of *A. fuciphagus*	72
4.16	Percentage of nucleotide similarity between male *A. fuciphagus* with other birds of the same Apodiformes order or Aves class	73
4.17	Percentage of nucleotide similarity between females *A. fuciphagus* with other birds of the same Apodiformes order or Aves class	74

LIST OF FIGURES

Figure		Page
2.1	A nest patch on the inward-inclining wall (<90° to the horizontal line)	7
2.2	Examples of microsatellite and repeat units	13
2.3	Slippage during DNA replication	16
2.4	Unequal crossing over between homologous chromosomes	16
3.1	Locations of the sampled colonies	25
4.1	DNA quality as displayed by AGE of ten swiftlets samples	47
4.2	Polyacrylamide gel showing PCR amplicons from optimized PCR composition and protocol at microsatellite locus Aef27 for six swiftlet samples	48
4.3	Polyacrylamide gel showing PCR amplicons from optimized PCR composition and protocol at microsatellite locus Aef115 for six swiftlet samples	48
4.4	Polyacrylamide gel showing PCR amplicons of three pooled DNA samples for two microsatellite loci	49
4.5	Allele frequencies at Aef112, Aef27 and Aef133 loci in Eastern (ER), Southern (SR) and Northern (NR) regions	51
4.6	Clustering assignment of 90 animals representing the three populations	63
4.7	Evanno method to detect the real K	64
4.8	Individual proportion of membership (q) of three populations of edible nest swiftlets	65
4.9	Consensus tree generated from 1000 bootstrap values using NJ based on Nei's DA genetic distances	66
4.10	Consensus tree generated from 1000 bootstrap values using UPGMA based on Nei's DA genetic distances	67
4.11	Male *A. fuciphagus* (M1, M2 and M3) determined by post-mortem	68
4.12	Agarose gel showing PCR amplicons from an optimized PCR composition and protocol by P8/WZ/W sexing primer set for eight swiftlet samples	69
4.13	Agarose gel showing PCR amplicons from and optimized PCR composition and protocol by P8/WZ/W sexing primer set for five swiftlet samples	69
4.14	Agarose gel showing PCR amplicons from	70

	optimized PCR compositions and protocols by two sexing primer sets for eight swiftlet samples	
4.15	Agarose gel showing PCR amplicons from optimized PCR compositions and protocols by two sexing primer sets for five swiftlet samples	70
4.16	Agarose gel showing PCR amplicons from optimized PCR compositions and protocols by 1237L/1272H sexing primer set for eight swiftlet samples	71
4.17	Neighbour-joining tree based on DA genetic distances showing the genetic relationship between male *A. fuciphagus* and male birds of the same Apodiformes order	75
4.18	Neighbour-joining tree based on DA genetic distances showing the genetic relationship between male *A. fuciphagus* and male birds of the same and different order (Apodiformes, Accipitriformes, Columbiformes, Galliformes and Passeriformes)	76
4.19	Neighbour-joining tree based on DA genetic distances showing the genetic relationship between female *A. fuciphagus* and female birds of the same and different order (Apodiformes, Passeriformes, Pelecaniformes, Accipitriformes and Gruiformes)	77

LIST OF ABBREVIATIONS

AFLP	Amplified fragment length polymorphism
AMOVA	analysis of molecular variance
bp	base pair
CE	capillary electrophoresis
df	degree of freedom
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphates
DVS	Department of Veterinary Services Malaysia
EDTA	ethylediaminetetraacetric acid
EtBr	ethidium bromide
F_{IS}	inbreeding coefficient
F_{IT}	deficiency or excess of average heterozygotes in a group of populations
F_{ST}	degree of gene differentiation among populations
GBS	genotyping-by-sequencing
He	expected heterozygosity
Ho	observed heterozygosity
HWE	Hardy- Weinberg equilibrium
IAM	Infinite Allele Model
MAGE	MetaPhor agarose gel electrophoresis
ml	millilitre
mM	millimole
MNA	mean number of alleles
MNE	mean number of effective alleles
mtDNA	mitochondrial DNA
Na	observed number of alleles
Ne	effective number of alleles
Ng	nanogram
NGS	next-generation sequencing
NJ	neighbour-joining
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
RAPD	randomly amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
rpm	rotations per minute
SNP	single nucleotide polymorphism
SSR	simple sequence repeat
STR	short tandem repeat
TBE	Tris/Borate/EDTA
UPGMA	unweighted pair group method with arithmetic mean
UV	ultraviolet

CHAPTER 1

INTRODUCTION

Edible nest swiftlets consist of several species of swiftlets that produce an edible bird nest with a high commercial value (Lim, 2011). History indicates that edible bird nest (EBN) consumption began around 1500 years ago in China during the Tang dynasty (Lim & Cranbrook, 2002). The increased wealth in the Asian region and the steady increase in the price of EBN products have made the EBN the 'Caviar of the East'. Due to the increase in demand and high value of the nests, over-harvesting had occurred in many locations, believed to be their natural habitats, including in Malaysia. Analysis of reported studies showed a decrease of the EBN swiftlet population for Black-nest species (*Aerodramus maximus*) at the Great Cave of Niah from approximately 300,000 in 1999 (Hobbs, 2004) to 150,000 in 2008 (Anwarali *et al*., 2008). However, the introduction of swiftlet ranching or farming has increased the overall population of these several species, leading to exclusion of one of the high value swiftlet species, White-nest swiftlet (*Aerodramus fuciphagus* Thunberg, 1812) from the list of protected animals under the Wildlife Conservation Act 2010 (Act 716), Malaysia (Kamarudin & Abd Aziz, 2011).

Among the several edible nest swiftlet species, *A. fuciphagus* is one of the species which produce the EBN of high value in the international market due to the fact that the nests are made from pure saliva (Kang & Lee, 1991) with high nutritional properties that include crude protein (62.0%), carbohydrate (27.3%), inorganic ash (2.1%) and 0.14% lipid (Marcone, 2005). Thus, it results in rapid exploitation and population expansion of this species. The sources of rapid expansion of the birds were reported to come from natural caves initially, but the recent sources may have come from other established man-made colonies (Aowphol *et al*., 2008). Consequently, this will trigger unpredictable changes in the genetic structure of the species over time.

The EBN swiftlet is a non-sexually dimorphic animal, due to the absence of distinct morphological characteristics between male and female birds. This makes it difficult when trying to determine the sex of the birds. By utilising the PCR and molecular method for sexing, it is possible to determine the sex of birds accurately. In many studies involving evolution and ecology such as sex ratio evaluation, demography, conservation and sexual selection, the ability to identify the individuals' sex is crucial. However, only a few sexing primers can successfully differentiate the sex of birds, depending on the species of investigating birds, such in the case reported on hummingbird (Hagadorn *et al*., 2016).

1.1 Research Problem

Commercialized species require sustainable breeding potential for growth and survival of the industry, and this relates to the importance of genetic variation. *A. fuciphagus* is a very important commercial species in Malaysia. However, there is a lack of available genetic information on this species, especially on its population genetic structure in Malaysia. In addition, as *A. fuciphagus* is a non-sexually dimorphic bird, identifying molecular sexing markers to differentiate the sexes will not only aid in the evaluation of genetic variation between the sexes but, more importantly, will be a vital tool for monitoring of the *A. fuciphagus* breeding population.

1.2 Research Hypothesis

The introduction of EBN swiftlet ranching and growth of the swiftlet industry had triggered a rapid population expansion of this species, which may lead to increased genetic variation and evolutionary potential. Evaluating genetic structure among and within the populations would enable the industry to adopt and implement appropriate strategies to ensure the continuity of production and growth of the EBN industry. Meanwhile, identification of suitable molecular sexing markers for swiftlet may also enable sustainable breeding management of this species through the monitoring of the breeding populations of this species.

1.3 Objectives

The general objectives of this study were to provide information on the genetic structure of *A. fuciphagus* in Peninsular Malaysia using microsatellite, and correctly identify the sex of *A. fuciphagus* using molecular sexing markers. This may serve as a baseline for monitoring the effects of swiftlet ranching and EBN harvesting on the genetic variability of swiftlet populations, and to evaluate genetic variation between sex, as well as monitor the breeding population of this species respectively.

The specific objectives were:

1. To identify and evaluate polymorphic microsatellite loci for *A. fuciphagus*.
2. To evaluate the genetic structure among and within populations of *A. fuciphagus* in Peninsular Malaysia.
3. To estimates the genetic distances and identify cluster relationships among *A. fuciphagus* populations.
4. To identify molecular sexing markers for *A. fuciphagus* and compare the amplified nucleotide sequences with those of other bird species.

CHAPTER 2

LITERATURE REVIEW

2.1 Swifts and Swiftlets

Swifts, generally classified as insectivorous birds, are distributed worldwide and share the same feature of having large salivary glands that are critical for the production of saliva in nest building (except in Cypseloidinae and *Hirundapus*) (Chantler & Driessens, 2000). Swiftlets (tribe Collocaliini) are small-sized swifts (Cranbrook *et al*., 2013) that inhabit mainly the limestone caves and are found in the Indian Ocean, Pacific Islands, Australia and South-East Asia (Thomassen *et al*., 2003).

Traditional classification had divided swifts into two main families; the tree swifts (Hemiprocnidae) and typical swifts (Apodidae), and within Apodidae, Cypseloidinae is the sister group to the sub-family Apodinae (Brooke, 1970; Chantler & Driessens, 2000). Brooke (1970) further subdivided Apodinae into three tribes (Apodini, Collocaliini and Chaeturini) (Table 2.1), and this has been supported by published molecular studies by Price *et al*. (2004), Price *et al*. (2005) and Thomassen *et al*. (2005). Swifts and hummingbirds have also been confirmed to be closely related (Table 2.2).

Table 2.1: Subfamilies, tribes and genera of the Apodidae. (Modified from: Brooke, 1970; Chantler & Driessens, 2000)

Family	Subfamily	Tribe	Genus
Apodidae	Cypseloidinae	Cypseloidini	*Cypseloides*
			Streptoprocne
	Apodinae	Collocaliini	*Collocalia*
			Aerodramus
			Hydrochous
		Chaeturini	*Mearnsia*
			Zoonavena
			Telacanthura
			Rhaphidura
			Neafrapus
			Hirundapus
			Chaetura
		Apodini	*Aeronautes*
			Tachornis
			Panyptila
			Cypsiurus
			Tachymarptis
			Apus

Table 2.2: Relationship between swifts and hummingbirds. (Source: Chantler & Driessens, 2000)

Superorder	Order	Family	Subfamily
Apodimorphae	Trochiliformes	Trochilidae	Phaethornithinae
			Trochilinae (Hummingbirds)
	Apodiformes	Apodidae	Cypseloidinae
		Hemiprocnidae	Apodinae (Swifts)

Taxonomy and phylogeny of swiftlets (Apodidae: Collocaliini) are controversial topics. Mayr (1937) reported, “their classification presents the most difficult problem in taxonomy of birds”. The controversy arises mainly because there is a high similarity in the morphology among the species, where the individuals were dull grey in colour and possess similar structural character development. All swiftlets were originally placed into a single genus, *Collocalia*, and for over a hundred years, this classification was used. The discovery of echolocation in

swiftlets by Novick (1959) was the main reason for the revision of swiftlet taxonomy. Brooke (1970) split the echolocating species into the genus *Aerodramus*, and the non-echolocating species into the genus *Collocalia* and *Hydrocous* (consist of a single species, *Hydrochus gigas*). This has been supported by the swiftlet phylogenetic classification works, conducted by Thomassen *et al.* (2005) and Price *et al.* (2005).

Currently, there appear to be approximately 24 species of swiftlets recorded in the world (Ibrahim *et. al*., 2009) and five of these species can be found or located in Peninsular Malaysia and Borneo Island; *Aerodramus fuciphagus*, *Aerodramus maximus, Hydrochus gigas*, *Collocalia esculent* and *Cypsiurus balasiensis*. Of these five species, *A. fuciphagus* and *A. maximus* are the species of interest presently due to the high market value of their nests, believed to have both aphrodisiac and medicinal values.

2.2 Swiftlet Industry

The swiftlet industry is unique in a way that the main product is the EBN. According to Idris *et al*. (2014), the value of the EBN industry in the world is estimated to be more than 10 billion Ringgit Malaysia. The high value of the industry is mainly based on the beneficial properties of EBN through scientific and non-scientific reports. Marcone (2005) reported that EBNs from Malaysia and Indonesia were composed of 62.0% crude protein, 27.3% carbohydrate, 7.5% moisture, 2.1% inorganic ash and 0.14% lipid. EBN is also rich in minerals such as calcium, magnesium, potassium and sodium (Norhayati *et al*., 2010). However, Norhayati *et al*. (2010) reported higher level of minerals in four different brands of processed EBN compared with unprocessed EBN samples, which may be of health concern as excessive intake of minerals can be harmful to health (Nerbass *et al*., 2015; Reid *et al*., 2015; Ndanuko *et al*., 2017). Kathan & Weeks (1969), showed that the beneficial effects of EBN was due to the major component in glycoprotein called sialic acid, which accounts for 8.6%. The nutrient content of EBN may be affected by the location of breeding sites and/or seasonal variations (Norhayati *et al*., 2010). Nutrients in the nests have been claimed to be beneficial for reducing the risk of cardiometabolic disease associated with estrogen deficiency such as diabetes and cardiovascular (Hou *et al*., 2015b). EBN also may possess anti-inflammatory (Vimala *et al*., 2012) and antioxidant properties (Hou *et al*., 2015a). Furthermore, EBN suggested to be effective for the improvement of bone loss (Matsukawa *et al*., 2011) and it may be a potential anti-degenerative agent for treating osteoarthritis (Chua *et al*., 2013).

In Malaysia, the EBN industry has been pioneered by Chinese entrepreneurs and commercialized in the late 1980s. As the second biggest producer of EBN after Indonesia, Malaysia aims to reach 40 percent sales growth from the current 20 percent by the year 2020 (Siti-Nabiha *et al*., 2013). Hong Kong (50%), China (8%), Taiwan (4%) and Macau (3%) are the biggest importer of EBNs from Malaysia (Babji *et al*., 2015). By the end of 2006, there were

approximately 36,000 units of swiftlet farms in Malaysia, with a reported average annual growth rate of 35% per year over the last 5 years (Hameed, 2007).

In August 2011, China banned the importation of EBNs from Malaysia (DVS, 2017) which had a great impact on the swiftlet industry as a whole. According to the Malaysian Federation of Bird's Nest Merchants Association, the annual production of birds' nests in Malaysia has reached 1 billion ringgit (290 million U.S dollars). However, the prices have dropped to at least 20% due to the ban by China (Babji *et al*., 2015). Despite the lifting of the ban in 2013, EBN processing facilities needed to be inspected for standards compliance by the Certification and Accreditation Administration of the People's Republic of China before these processing facilities are allowed to export their EBN products to China. In 2013, only eight companies from Malaysia were approved. To help the industry, led by Department of Veterinary Services Malaysia (DVS), efforts have been made to develop the industry's downstream capabilities such as producing value-added products. Various products of ready-to-drink health tonics and cosmetics have been in the market across the region to promote the benefits of EBN to domestic consumers (Thorburn, 2015).

2.3 Swiftlet Farming

Swiftlet farming began at the caves which are the natural habitat of swiftlets. In Malaysia, the first farming record was reported in Niah Cave in Sarawak as early as in 1878 (DVS, 2010). Traditionally, the skilful nest collector will harvest the swiftlet nest located high in the cave's ceiling by climbing the ladder made from bamboo (Siti-Nabiha *et al*., 2013). The nature high risk of farming from the cave and the realization of the lucrative bird nest market have triggered entrepreneurs to seek an alternative to the traditional way, which is through swiftlet farming in man-made buildings that are more humane, economic, sustaining and environmental friendly (DVS, 2010). The edible nest swiftlet started to inhabit man-made buildings recorded as in the late 1940s in Penang, Perak and also in Kuala Lumpur (Anuar, 2011). Ever since then, swiftlet farming has started to move from the caves to man-made buildings in urban areas.

Nowadays, swiftlet farming is converting an abandoned building to be a house for swiftlets (Siti-Nabiha *et al*., 2013). This has gained momentum after the Asian Economic Crisis 1997-1998 when many buildings were left empty after a lot of small to medium scale businesses closed down (Hameed, 2007). Hence, some of the landlords decided to convert their untenanted properties into swiftlet houses.

2.3.1 Cave and birdhouses

As mentioned earlier, the natural habitat of swiftlet is in caves, specifically limestone caves (Ibrahim *et al.*, 2009). The two species (*A. fuciphagus* and *A. maximus*) that produced edible nest constructed their nests deep inside the caves. Viruhpintu *et al.* (2002), reported that *A. fuciphagus* and *A. maximus* avoided interspecific competition for nesting space by using different areas of the cave wall. *A. fuciphagus* nested in groups ranging from ten to more than 1000 nests in one area of the caves. The nest site selection by *A. fuciphagus* is also not random; they choose the inward-inclining wall (angle of lower than 90° to the horizontal line) as shown in Figure 2.1 to construct their nests. The sites characterized as smooth and concave with supporter or the protruding "U-shaped rock" are generally selected by the birds to attach their nests.

Figure 2.1: A nest patch on the inward-inclining wall (< 90° to the horizontal line). (Source: Viruhpintu *et al.*, 2002)

To encourage the birds nesting away from caves, buildings were created or modified to imitate the cave-like atmosphere which is comfortable for the birds (Anuar, 2011), and swiftlet song playbacks were used to attract the birds to colonized the buildings (Aowphol *et al*., 2008; Lim, 2011). The two major factors to be considered in constructing swiftlet houses are location and environment (Idris *et al.*, 2014).

The most strategic location to build the house was at the highly populated area of swiftlet (Idris *et al*., 2014), assuming that the swiftlet chicks will search for new homes due to the crowding in their natal houses. The houses must also be built near to feeding sites. Swiftlets feed on flying insects, so the location of the houses must be near to places with abundant insects. The study conducted by Manchi & Sankaran (2010) showed that edible nest swiftlets were more active in forested areas. Petkliang *et al*. (2017), also reported similar findings in Thailand with the highest swiftlet foraging intensity occurred over water bodies, forest and open paddy fields where high numbers of prey insects (Hymenoptera, Diptera and Hemiptera) were available.

The air temperature, relative humidity, air velocity and light intensity within the birdhouses must also be taken into account when considering the suitability of the birdhouse environment (Ibrahim *et al*., 2009). According to Ibrahim *et al*. (2009), the recommended air temperature ranges between 26°C and 35°C. Low air temperature will reduce the production of swiftlet nest while high air temperature will trigger an uncomfortable environment for the swiftlet and at the same time damage the swiftlets' eggs. They also recommended relative humidity of between 75 and 90%, because high relative humidity encourages fungi to thrive within the nesting plank. Swiftlets refuse to build their nests on planks with fungus growth. On the other hand, lower relative humidity will dry the swiftlet nests, causing cracks in the nests. For the orientation, the south-north orientation should be considered when constructing the swiftlet entrance to the house as it will help to maintain the internal temperature and light intensity by avoiding direct exposure to sunlight. Lastly, air ventilation is important in providing the air movement to the building. The air movement will reduce the air temperature and relative humidity in the building. However, both air temperature and relative humidity must be within a suitable range.

2.3.2 Edible nest swiftlet

Edible nest swiftlets mainly inhabit the Indo-Pacific region, with the highest diversity in South-East Asia (Cranbrook *et al*., 2013). The two main species that have been contributed towards the production of EBN traded worldwide are the White-nest swiftlet (*A. fuciphagus)* and black-nest swiftlet *(A. maximus).* The nests of *A. fuciphagus* are made from pure saliva, while the nests of *A. maximus* may include some of the swiftlets' feathers (Kang & Lee, 1991). The edible nest swiftlets can be categorized as small birds with a total body length of around 127 mm. Usually, it is impossible to distinguish the *A. maximus* from *A. fuciphagus* during flight. However, if looked closely, *A. maximus* has a more robust build, the rump appears to be darker compared with the normal greyish colour of *A. fuciphagus*, and its wing appears to be longer (Kang & Lee, 1991).

The construction of the nest by both *A. maximus* and *A. fuciphagus* occurs at night, and they spend approximately 25 to 60 min a day for nest construction (Kang & Lee, 1991). However, *A. maximus* peak building period is reported to occur between 2200 and 0400 hours, and between 0500 and 0600 hours

(Kang & Lee, 1991). For *A. fuciphagus*, peak building activity occurs before the pre-emergence period (0500 – 0600 hours) and immediately after they return to their respective houses between 1800 and 2000 hours (Ramji *et al*., 2013). According to Kang & Lee (1991), *A. maximus* lays an egg per clutch compared to *A. fuciphagus* which lays two eggs per clutch. Both species incubate their eggs for approximately 25 days (Kang & Lee, 1991). The nestling of *A. maximus* fledges after about 46 days, while *A. fuciphagus* fledges after 40 days.

The White-nest swiftlet (*A. fuciphagus*) has received more attention than the Black-nest swiftlet (*A. maximus*) due to the high value of its nest in the international market. White-nests occupy almost 99.0% of total production in Malaysia (Hameed, 2007). *A. fuciphagus* is known to be permanent monogamous, and breeders of this species tend to have a high nest-site fidelity (Viruhpintu *et al*., 2002). *A. fuciphagus* usually has a long annual breeding season, approximately around nine months beginning in August or September each year until the following year in April (Lim & Cranbrook, 2002). According to Lim (2011), swiftlets construct nests and lay eggs as soon as environmental and essential physiological factors permit to maximize their annual breeding success. Generally, this species takes approximately 92 to 120 days for a complete breeding cycle, with three bouts of breeding per year (Lim & Cranbrook, 2002). However, fecundity between April and July is the lowest (Lim, 2011).

2.4 Genetic Diversity

Genetic diversity is the variation present in alleles and genotypes of a particular group (population, species or group of species) under study (Frankham *et al*., 2002). Genetic diversity is a broad term encompassing the variations occurring among the various loci chosen for screening from the total genetic makeup of a single species or a group of species (Bhandari *et al*., 2017). The major concern in conservation biology is to maintain the genetic diversity within a population. The International Union for Conservation of Nature (IUCN) includes genetic diversity as one of three types of biodiversity that deserves attention in conservation practice, together with the ecosystem and species diversities (McNeely *et. al.,* 1990). Low genetic diversity increases the risk of extinction in populations in the face of environmental changes as it lacks evolution potential. Loss of genetic diversity also is one indication that the population is undergoing inbreeding, and inbreeding leads to reductions in reproductive fitness (Frankham *et al.,* 2002). Many factors contribute to genetic diversity in populations that include mutation, migration, selection, genetic drift and non-random mating (Frankham *et al.*, 2002; Li *et al*., 2005; Tillmar, 2010).

2.5 Genetic Characterisation

Characterisation is the act of describing individuals. It includes all aspects, either with reference to the phenotypes or the genotypes of the individuals. In genetics, characterisation refers to the detection of variation resulting from

either the differences in deoxyribonucleic acid (DNA) sequences at specific or nonspecific locations in the genome, specific genes or modifying factors (de Vicente *et al*., 2005). Genetic characterisation can be referred to as the description of attributes following Mendelian inheritance or involving a specific DNA sequence. Genetic characterisation helps in enhancing the power of detecting diversity. This is because the molecular method used in genetic characterization is capable to disclose the variation in genotypes, which is the highest level of variation showed by individual DNA sequences without being influenced by an environmental factor (de Vicente *et al*., 2005).

Genetic or molecular markers are defined as specific genes or DNA sequences that may or may not produce detectable traits and have a known or unknown location on a chromosome, and can be used to study population genetics. Toro *et al.* (2009), defined them as DNA locations presenting different detectable variants. They play very important roles in the estimation of the genetic diversity within and among populations. They also help in understanding the origin, migration and level of admixture in animal populations. This information is a useful guide in making decisions and choices for the conservation activities. Genetic markers are also used for the identification of threatened breeds, prioritize conservation decisions among the breeds and development of plans to improve these breeds (Hanotte & Jianlin, 2005). There are many types of genetic or molecular markers used for the assessment of genetic diversity (Brown, 1996; Allan & Max, 2010) and some of them are described briefly in the following paragraphs.

Restriction fragment length polymorphism (RFLP): This technique is the first DNA-based genetic marker developed (Botstein *et al*., 1980), involving the digestion of particular DNA molecules with particular restriction enzymes resulting in many reproducible fragments of certain lengths (Weising *et al*., 2005). The differences in the pattern of restriction fragments between individuals arise as a result of mutations (base substitutions, deletions or insertions) within DNA sequences. Agarose gel electrophoresis (AGE) had been used to separate these fragments before subjected to Southern blotting onto a membrane and hybridization with a labeled probe (locus-specific probes or multilocus probes). However, nowadays RFLP usually referred to as PCR based RFLP or PCR-RFLP. This method is a modification of RFLP by exploiting the use of PCR to amplify targeted DNA sequence, followed by the digestion of PCR products with specific restriction enzymes and separation of the digestion fragments by AGE. PCR-RFLP requires only a small amount of DNA, shorter methodology and not requiring probes as compared with RFLP (Weising *et al*., 2005).

RFLP has played an important role in the human genome project as well as provided information on many genetic diseases (Emadi *et al*., 2010). RFLP analysis is useful to determine the individual that may be at risk for certain disease or a carrier of the mutated gene. The location of a specific gene for disease on a chromosome can be achieved by comparing the DNA from a set of family members who suffered from certain diseases and compared it to the

RFLP allele which showed the same inheritance pattern for the condition. RFLP is one of the first methods used in DNA fingerprinting, profiling or testing (Firas & Abdulkareem, 2015). However, RFLPs suffered drawbacks as some of the restriction enzymes are expensive, and the technique is not suitable for high throughput analysis because the technique is not suitable for the simultaneous analysis of a large number of different single nucleotide polymorphism (SNP). This is due to the requirement for specific primer pair and restriction enzyme for each SNP (Rasmussen, 2012).

Amplified fragment length polymorphism (AFLP): It is developed in 1993 by combining principles of both RFLP and PCR (Vos *et al*., 1995). It involved digestion of the DNA with two different restriction enzymes, followed by ligation of oligonucleotide adaptors (with defined sequence) to these fragments. These restriction fragments with adaptors will create target sites for primer annealing in fragment amplification by the PCR technique.

AFLP has many advantages such as high sensitivity, high reproducibility and high level of polymorphism compare to those of other markers (randomly amplified polymorphic DNA (RAPD), RFLP and microsatellites) (Brumlop & Finckh, 2010). AFLP was highly efficient in genomic fingerprinting. The AFLP fingerprints are used for species identifications (Heubl, 2010; Osman *et al*., 2011) or in phylogenetic analysis involving different species (Osman *et al*., 2011; Todd *et al*., 2011). In the case of the absence of sequence information, AFLP can also be used to determine the intraspecific variation in species (Osman *et al*., 2011; Todd *et al*., 2011). In addition, AFLP also can be applied in linkage mapping (Mahanil *et al*., 2012; Niedziela *et al*., 2012), marker assisted selection (MAS) and quantitative trait locus (QTL) mapping (Emara & Kim, 2003; Ontivero *et al*., 2011; Xiao *et al*., 2012). However, the drawback is that the markers are dominant bi-allelic, that is, the dominant homozygous and heterozygous genotypes are indistinguishable (Jehan & Lankhanpaul, 2006).

Mitochondrial DNA (mtDNA) genes: MtDNA is a small circular molecule range around 15 kb to 20 kb (Avise, 1994). Within the coding region, cytochrome b is one of the genes that most widely used in phylogenetic work, especially in vertebrates (Meyer, 1994). In the non-coding region, the displacement loop (D-loop), is widely used in evolution study because it has the highest rate of nucleotide substitution in mtDNA (Cecconi *et al*., 1995). The polymorphism of mtDNA markers has been widely used to identify maternal lineages because the transmission of mtDNA only comes from the female germline (Cortés *et al*., 2008; Achili *et al*., 2009). The mtDNA analyses are more suitable to study hybridization, introgression and incomplete lineage sorting (Firas & Abdulkareem, 2015). Mitochondrial DNA also has been used in forensic science due to the advantage of its high copy number and maternal lineages. The sex chromosome markers and mtDNA were used effectively to identify individual identity or ancestry and proved to be useful in cases of missing persons when maternal relatives separated by several generations (Adrian & Jennifer, 2014).

Single nucleotide polymorphism (SNPs): SNPs are variation of sequence (alleles) at single base pair (bp) positions in genomic DNA, exist in normal individuals in some population(s) with the least frequent allele have a frequency of 1% or greater (Jehan & Lakhanpaul, 2006). In non-model organisms, SNPs often observed about once in every 300 bp (Seeb *et al.*, 2011). SNP markers are identified from DNA sequences of targeted regions within the genome. SNP markers are usually less mutable compared to the other markers, making it a good choice for studying complex genetic traits. It is also a powerful marker that can provide information about population history (Gibbs *et al*., 2009; Lewis *et al*., 2011). SNPs have played a very important role in farm animal research, particularly concerning population genetic structure, genetic differentiation, origin and evolution (Yang *et al*., 2013). SNPs are also important tools in crop and livestock breeding programs (Firas & Abdulkareem, 2015). The advantages of SNPs include high polymorphism and reproducibility. The disadvantages are the high initial cost involved and that detection requires some prior knowledge of the sequence.

Microsatellites or simple sequence repeats (SSR): This is the genetic marker used in the present population genetics study to determine the genetic characterization of *A. fuciphagus*. For that reason, it is described in detail in Section 2.6.

2.6 Microsatellites

Microsatellites, also known as SSRs or short tandem repeats (STRs) are repetitive DNA regions composed of small motifs of 1 to 6 nucleotides repeated in tandem (Toth *et al*., 2000). Tautz & Renz (1984), in a study involving five phylogenetically distinct eukaryotes including humans, *Drosophila*, yeast, sea urchin and *Stylonychia* had proved the abundance of microsatellites. Microsatellites can be categorized based on repeat motif sizes such as shown in Figure 2.2. Meanwhile, when categorizes according to the type of repeat sequence, microsatellites can be classified as perfect, imperfect, interrupted or composite (Oliveira *et al.,* 2006). Details of it can be seen in Table 2.3.

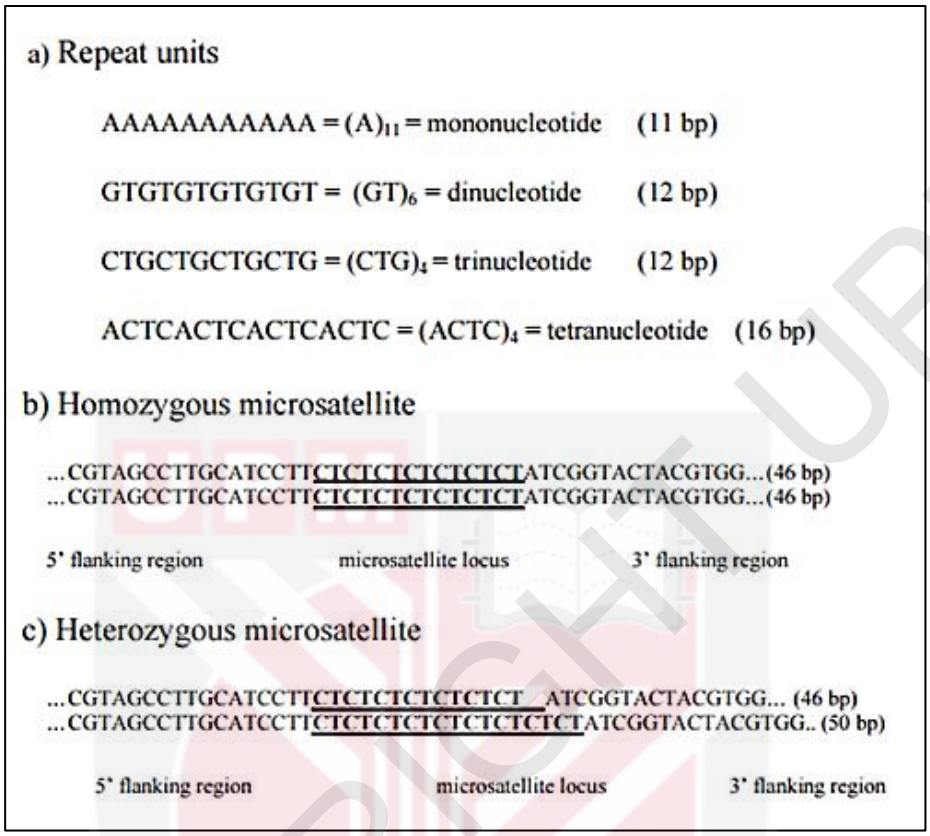

Figure 2.2: Examples of microsatellite and repeat units. (Source: Mburu & Hanotte, 2005)

Table 2.3: Perfect, imperfect and composite microsatellites repeat

Repeat Type	Example
Perfect	… TC TC TC TC TC TC TC TC TC ...
Imperfect	… TC TC TC TC T**A** TC TC TC TC...
Imperfect	… TC TC TC **TG AT** TC TC TC TC...
Composite	… TC TC TC TC **GA GA GA GA GA** ...

2.6.1 Distribution and sequence

Microsatellites are found in both prokaryotic and eukaryotic genomes, found anywhere in genomes, both in protein-coding and noncoding regions (Field &

Wills, 1998; Toth *et al.*, 2000; Varshney *et al*., 2005). It appears that SSRs in noncoding regions are more abundant compared to in exons (Hancock 1995). In eukaryotic organisms, microsatellites generally occur in noncoding regions (Hancock 1995; Li *et al*., 2005). Generally, poly C/G sequences are less abundant than poly A/T repeats. Intergenic regions and introns of numerous taxa including vertebrates are dominant with dinucleotide repeats while in exons in all taxa shows trinucleotide repeats as the most abundant. AC/TG repeats are the most common dinucleotide repeats compared to AG and AT repeats in most of the taxa investigated (Toth *et al*., 2000). In molecular genetic studies, the most common choices are the sequences of di-, tri- and tetranucleotide repeats (Selkoe & Toonen, 2006).

2.6.2 Advantages, disadvantages and applications

There are many advantages of microsatellite markers such as locus specific in nature, highly polymorphic and hypervariable due to high mutation rate, relatively abundance and random distribution throughout the genome, being co-dominant markers where heterozygotes can be distinguished from homozygotes, low quantity of DNA required for detection and ability to be amplified by using Polymerase Chain Reaction (PCR) (Bravo *et al.,* 2006; Pokhriyal *et al*., 2012). In contrast, there are also some limitations or disadvantages associated with microsatellites: a) presence of null allele, alleles that consistently failed to amplify in the annealing primer site, leading to inaccurate estimations of allele frequencies and segregation rates (Hoshino *et al*., 2012), b) homoplasy, i.e. two alleles that are identical by size but not by descent (different evolutionary history) or sequence (Anmarkrud *et al*., 2008; Pokhriyal *et al.,* 2012), and c) presence of stutter bands, the products of slippage by the DNA polymerase during the PCRs, making it difficult to score alleles (Walsh *et al.*, 1996).

Microsatellites have been used in many different areas of research: a) in forensic, widely used for individual identification and relatedness test (Azam *et al*., 2012; Pokhriyal *et al*., 2012), b) in population genetic studies for understanding of the population structures and differences, genetic drift, genetic bottleneck, and also estimating genetic distance and relationships (Sodhi *et al.*, 2008; Bray *et al.*, 2009), c) in conservation biology for decision making on conservation of endangered population (Padilla *et al.*, 2009; Amigues *et al*., 2011), d) in linkage analysis and Quantitative Trait Loci (QTL) mapping to enable use of marker-assisted selection (Raadsma *et al*., 2009; Hu *et al*., 2010), and e) in biomedical diagnosis as markers to detect some diseases due to the mutations of certain microsatellite alleles in coding regions of the DNA that can contribute to a lot of medical disorders (Pokhriyal *et al.,* 2012).

Taking into consideration of the advantages and disadvantages of the various molecular markers, microsatellites were selected as the most appropriate

genetic marker to be used in the present population genetics study, that is to determine the genetic characterization of *A. fuciphagus*.

2.6.3 Mutation mechanism

Microsatellites have high mutation rates, estimated at 10^{-2} to 10^{-6} per locus per generation depending on species or organism (Ellegren, 2000). There are two mechanisms proposed as to explain the mutation mechanisms of microsatellites: a) replication slippage, a theory that assumes slipped-strand mispairing during DNA replication is the main cause of microsatellite mutations (Levinson & Gutman, 1987), and b) unequal crossing over during meiosis (Hoshino *et al*., 2012).

In the first theory, the result of misalignment between a newly synthesized DNA strand and its complementary template strand gives rise to mutations as illustrated in Figure 2.3. The two strands dissociate and reanneal incorrectly, forming a loop but are stable due to the repetitive nature of the sequence. A loop formed on the nascent strand will result in mutation by repeated expansion, while a loop on the template strand will result in a reduction of repeat length (Jentzsch *et al*., 2008). The second theory, as illustrated in Figure 2.4 is not affecting microsatellite mutation much because it involves an exchange between homologous chromosome repeated units. However, this mechanism may be responsible for microsatellite multistep mutations (Grover & Sharma, 2011).

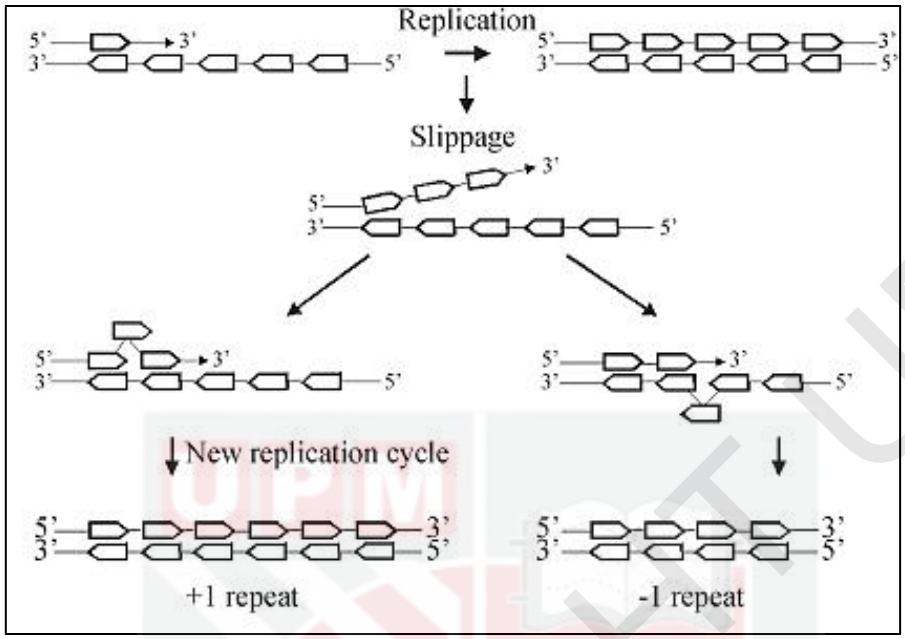

Figure 2.3: Slippage during DNA replication. Assume that there were five repeats motif for the original DNA molecule, symbolize by a box. Slippage leads to the formation of new alleles with either six or four repeat motifs, depending on strand containing the polymerase error. (Source: Oliveira *et al*., 2006)

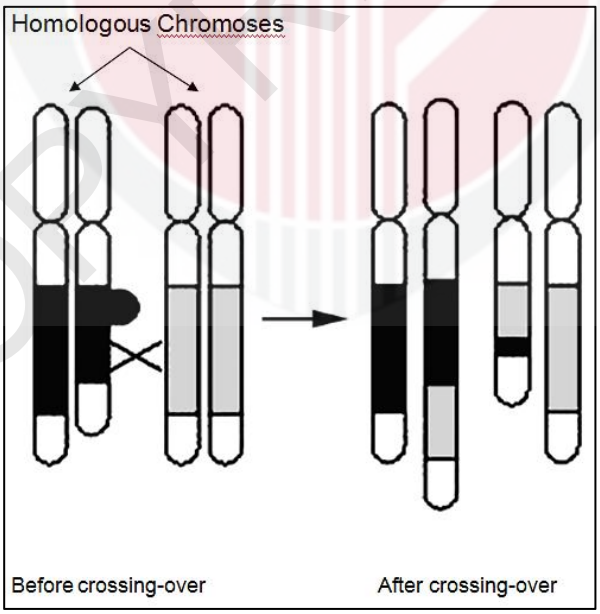

Figure 2.4: Unequal crossing over between homologous chromosomes. Black and grey regions correspond to microsatellite repeat sequences. (Source: Oliveira *et al*., 2006)

2.6.4 Genotyping of microsatellite loci

There are many genotyping methods currently used for microsatellite fragment analysis to determine the length of polymorphism. Those methods include MetaPhor agarose gel electrophoresis (MAGE), polyacrylamide gel electrophoresis (PAGE), capillary electrophoresis (CE) and DNA sequencing.

MAGE is a mixture of metaphor agarose and other chemicals, which according to Lonza Rockland, Inc. (USA), gives twice the resolution capability of the finest sieving agarose product. It is used to separate the DNA fragments of 20 to 800 bp. MAGE is still popularly used in Malaysia, although many studies elsewhere currently started to apply the use of capillary electrophoresis. MAGE is a method proven to be easy and quick for screening large numbers of samples for small length polymorphism (Ochsenreither *et al*., 2006). On the contrary, in the comparative study of the three electrophoresis separation methods by Sánchez-Pérez *et al*. (2006), they reported a lower accuracy of MAGE compared to PAGE and CE. This also supported by Wang *et al*. (2009) that found a lower resolution of MAGE than the PAGE, but they reported both methods (PAGE and MAGE) have difficulty in scoring the allele size.

PAGE is a traditional method used for DNA molecules separation (Bishop *et al*., 1967; Maniatis *et al*., 1975). Currently, PAGE has also been used for the separation of PCR amplified fragments of DNA. The fragments of DNA in polyacrylamide are usually visualized using silver staining or ethidium bromide (EtBr) (Wang *et al*., 2003). The advantages and disadvantages of using polyacrylamide gels are shown in Table 2.4.

Table 2.4: Advantages and disadvantages of PAGE. (Source: Barril & Nates, 2012)

Advantages	Disadvantages
Stable chemically cross-linked gel	Polyacrylamide is a neurotoxin (when unpolymerized)
Produce sharp bands	Tedious and time consuming to prepare the gels
Provide a good separation for low molecular weight fragments	Gel cannot be reused

CE is the current method of choice for genotyping microsatellites. Capillary sequencers are used to provide automated allele size estimation. The use of fluorescently labelled primers in combination with the multiplex system in CE produces high detection sensitivity in amplified DNA fragments (Mansfield *et al*., 1996; Wenz *et al*., 1998; Ramachandran *et al*., 2003). CE is fast due to its ability to run more than three markers in a single capillary and gives high

throughput analysis (Ochsenreither *et al*., 2006). However, CE requires highly sophisticated instruments and fluorescently labelled primers, making the other methods cheaper than CE (Wang *et al*., 2009).

A new CE system has been developed that uses disposable microchannel cartridges containing a sieving-gel matrix with EtBr and does not require labelled primers (QIAxcel, Qiagen, Germany). Although this system has a relatively lower resolution compared to other CE systems, it is cheaper and more convenient to use especially for mapping and large-scale population analysis (Wang *et al*., 2009).

The advent in technologies called next-generation sequencing (NGS) had enabled high throughput genotyping in genetic studies such as population genetics by exploiting some protocols [e.g. RAD (Baird *et al*., 2008), 2bRAD (Wang *et al*., 2012) and ddRAD (Peterson *et al*., 2012)]. These methods can be collectively called genotyping-by-sequencing (GBS). The main advantage of GBS in a study involving population genetics is the ability to generate high quantities of data for improved statistical power. The use of GBS also can resolve some issues involving microsatellite-based population studies such as estimation of allele size sequence length due to the ability of GBS to consider the actual allele size. Additionally, it also eliminated the time-consuming method of genotyping using capillary or gel electrophoresis runs. The disadvantages of GBS usually related to the cost of NGS technologies. However, many researches had been done to develop a rapid and cost-effective method for microsatellite GBS such as reported by Vartia *et al*. (2016) using individual combinatorial barcoding.

2.7 Genetic Studies in Swiftlet

The studies related to genetic characteristics of swiftlets are still lacking. Researches that had been conducted usually only focusing on the genetic relationship of swiftlets and only a few studies explaining and describing the genetic variations of swiftlets. Various genetic markers had been used such as mtDNA genes, nuclear markers and SSR. The following are a summary of these studies.

Aowphol *et al.* (2008), reported sufficient gene flow among 10 colonies of swiftlets from Thailand to prevent genetic isolation. Results from microsatellite loci showed a high genetic variation within and between colonies of *A. fuciphagus* suggest a genetically viable future for this species in Thailand.

Hendra *et al*. (2014), studied the population structure and genetic variation of EBN swiftlet (*C. fuciphaga*) in Riau, Indonesia using three microsatellite markers. The results showed high genetic variability (0.855) and low genetic variation between populations indicate that no isolation occurs. However, the

inbreeding coefficiency (FIS) showed a positive value, indicating inbreeding occurs on the swiftlet population in Riau.

Interestingly in a study by Lin *et al*. (2009), they successfully used the sequence of cytochrome b gene in mtDNA for genetic identification of EBN sample, and further compared the sequence with the sequences of swiftlets in GenBank. This study has arisen a promising method to identify the authenticity of EBN products.

Goh *et al*. (2013), reported on a novel DNA sequence at the mitochondrial control region (D-loop) of *A. fuciphagus*. The primer walking technique had been used to isolate the control region. They suggested that DNA marker derived from the control region is a promising marker to resolve the problem regarding the low-level phylogenetic relationships among closely related swiftlets as well as helping in understanding the genetic structure of *A. fuciphagus* populations.

Cranbrook *et al*. (2013), studied on the origins of house farm swiftlet by morphometric and genetic evidence using mtDNA *Cyt-b* gene, and suggested that original pioneers of swiftlet in Malaysia were drawn from at least 3 wild sources of 2 species Northern grey-rumped swiftlets (*A. inexpectatus germane*) in Penang and Southern grey-rumped swiftlets (*A. inexpectatus* subsp.) in Kuala Terengganu, and Thunberg's swiftlet (*A. fuciphagus fuciphagus)* in Johor.

Goh *et al*. (2018), focused on mitochondrial genetics by using mitochondrial cytochrome-*b* (mtDNA Cyt-b) with samples of swiftlets collected from birdhouses (Peninsular and Bornean Malaysia) and wild grey-rumped swiftlets, *A. inexpectatus,* collected from island of Mantanani Besar, Sabah and Seringgis, Terengganu. Genbank data from birdhouse swiftlets in Thailand and Vietnam were also included to extend the comparisons. The reanalysis of three previous studies of genetic diversity in birdhouse swiftlets coupled with the new supplementary data of mtDNA Cyt-b showed that the multiple maternal lineages observed within ranched swiftlets of Malaysia were also detected widely across the Southeast Asian region. Furthermore, they also stated that ranched swiftlets exhibited behavioural characters that may be considered as evidence of domestication. Ranched swiftlets prefer to seek buildings as nest sites compare to the natural caves as evidence in Peninsular Malaysia. The interaction between birds seeking for buildings as nest sites and human designing suitable habitats for the birds may have led to the rapid selection of new genotype. This might be suited to the definition of domestication in which the man has manipulated and changed the way of life by engulfing animals within human societies.

The above-mentioned studies gave brief information regarding the genetic relationships and variations of the swiftlets. However, more thorough studies are required to obtain a clearer picture of the genetic structures of this species,

especially in Malaysia. Studies conducted on genetic variations also are not much reliable as the studies used small sample sizes for some colonies and the number of markers used also varies between each study.

2.8 Studies of Birds' Diversity using Microsatellite Markers

The content of this section is based on recent publications on genetic diversity studies in birds. This review includes swiftlet closely related species, endangered birds and birds of different distribution patterns. This review aims to observe different patterns of genetic diversity and also the reliability of microsatellite markers to evaluate genetic diversity.

Dor *et al*. (2012), studied the patterns of genetic differentiation and gene flow between two barn swallow subspecies, European (*Hirundo rustica rustica*) and East Mediterranean (*Hirundo rustica transitiva*). The barn swallow (*Hirundo rustica*) has a wide geographic distribution encompasses considerable geographic differences in term of morphology and behaviour. The totals of eight microsatellite loci were investigated for this study. Results showed that low but significant genetic variations occur among the subspecies, and considerable gene flow among these populations despite the existence of differences in morphological and behavioural traits. They suggested that the divergence in signal due to the mate choice results in these subspecies undergoing initial differentiation.

Billerman *et al*. (2011), documented the natural long-distance colonization event by six pairs of barn swallow (*Hirundo rustica*) migrating from South America to Buenos Aires Province, Argentina in 1980, and has grown to thousands of pairs. To test for evidence of a genetic founder event, eight microsatellite loci were investigated. The results showed no founder effect occurrence as evidenced by no significant differences in heterozygosity or allelic diversity, and there was no population variation compared to the large North American populations. They suggested that this migration event may be related to the increase in the South American population by ongoing immigration from North America and not associated with a strong demographic bottleneck.

Malpica & Ornelas (2014), studied the broad-tailed hummingbird (*Selasphorus platycercus*) which exhibited both long-distance migratory and sedentary behaviour. 152 samples from 18 populations were taken from the USA and Mexico. Eight species-specific microsatellite markers were used in the study. Results showed the mean number of alleles (MNA) per locus ranged from 1.37 to 6.87. They also saw a lack of genetic diversity between the populations of sedentary and migratory *Selasphorus platycercus* and gene flow estimates were greater than 1 (3.4 to 15.3).

Ross & Bouzet (2014), used 12 microsatellite primers to investigate genetic diversity patterns for 176 lark sparrows (*Chondestes grammacus*) across Western, Central and Eastern USA with a total of 6 populations. The mean for observed heterozygosity (Ho) across all populations was 0.603. Allelic richness per locus did not vary widely across populations ranged from 6.13 to 10.63. FIS were positive for three populations (range from 0.057 to 0.094). In general, the results indicated clinal variation among populations of long-distance migratory birds, which may reflect an initial evolutionary divergence, secondary contact zones and local adaptation of populations towards continuous variable environments.

Abalaka *et al*. (2015), studied the genetic diversity of rock fire finch (*Lagonosticta sanguinodorsalis*), a range-restricted bird species by collecting and analysing 161 blood samples from three populations in Nigeria. 11 microsatellite loci were investigated and results showed that the number of alleles per locus ranged from 2 to 18. The FIS values within populations were low but significant (0.024 - 0.054), indicating some deviation from Hardy-Weinberg equilibrium (HWE). Although the distance between populations is short, there was some population structure observed, indicating that the populations are somewhat isolated. They suggested that it may be due to behaviour of the birds, who exhibit high site fidelity (prefer high altitude location) and do not move far from their natal place.

Studied on endangered species was conducted by Nims *et al*. (2008) on Galapagos penguin (*Spheniscus mendiculus*) using five microsatellite markers. 116 adults' individuals from 5 subpopulations across the Galapagos archipelagos were selected. The total number of alleles per locus ranged from 2 to 4 with a total of 15 alleles reported for five microsatellite loci investigated. Results showed a low level of genetic variation throughout the populations and the seemingly high level of gene flow between subpopulations.

From all the reviews, it shows that microsatellite markers are powerful tools to investigate genetic diversity in many species of birds. Furthermore, it helps to explain and prove the behavioural aspect and relationships of the birds investigated.

2.9 Molecular Sexing

The individual's sex information is crucial in numerous studies of evolutionary biology, ecology, conservation and breeding (Fridolfsson & Ellegren, 1999). The difficulty to identify the sex of sexually monomorphic bird species has made the molecular sexing method for birds attractive and important. Molecular sexing helps in accurately and rapidly identifying sex. Female birds are heterogametic (ZW), while males are homogametic (ZZ). The first W-linked gene was discovered (Griffiths & Tiwari, 1995), identified later as a gene encoding for a chromo-helicase-DNA binding protein (*CHD*), is found to be

present in most of the birds (Ellegren, 1996; Griffiths *et al*., 1996) and is related to the human *CHD1* (Woodage *et al*., 1997). A related Z-linked gene *CHD-Z* has also been reported (Griffiths & Korn, 1997). Griffiths *et al*. (1996), proved that this is a remarkably conserved gene that a single set of PCR primers is already enough to determine the sex of birds in Aves class, except for ratites. This primer simultaneously amplifies the homologous parts of *CHD-W* and *CHD-Z*. The two *CHD* products were of the same size but could be differentiated using restriction enzyme which selectively cut the *CHD-Z* DNA fragment; generating two electrophoretic bands for the females and only one for the males. To overcome the problem of same PCR product sizes for *CHD-Z* and *CHD-W*, Griffiths *et al.* (1998) proposed a protocol for sexing of birds using two PCR primers which anneal at the conserved exonic region and amplified across an intron, which is noncoding and less conserved, varied in length between *CHD-W* and *CHD-Z*.

Fridolfsson & Ellegren (1999), however, claimed that the primers suggested by Griffiths *et al*. (1998) were not always successful for differentiating *CHD-W* and *CHD-Z* fragments by using standard agarose electrophoresis, and therefore, proposed another set of primers which consistently amplified different sizes of DNA fragments for *CHD-W* and *CHD-Z* in the vast majority of bird species. This particular pair of primers (2550F and 2718R) was designed based on the constant size differences between *CHD1W* and *CHD1Z* introns. By exploiting the use of a highly conserved pair of primer flanking at the intron, male characterized by showing a single fragment (*CHD1Z*), while female displayed either one (*CHD1W*) or two fragments (*CHD1W* and *CHD1Z*). From the vast majority of species successfully amplified the fragment, *CHD1Z* fragments ranged between 600 and 650 bp in size while *CHD1W* fragments between 400 and 450 bp. Almost similar method was designed by Kahn *et al*. (1998) which based on two highly conserved regions that flank the intervening introns that are common to both Z-linked and W-linked *CHD* genes. From it, a pair of primers was synthesized (1237L and 1272H). The sizes of amplification products for this pair of primers for each species differ over a range of 210 to 285 bp and the size differences between the W and Z-specific copies of this amplification also vary among species. Thus, some of the species showed very small size differences such as those found in Great Horned Owl (*Bubo virginiasus*) and Red-tailed Hawk *(Buteo jamaicensis*). Furthermore, the size differences in some species cannot be resolved by standard AGE and needed the use of a non-denaturing PAGE method.

The most widely used molecular sexing method for birds usually involved the exploitation of variation in size between introns of the *CHD* gene on the Z and W sex chromosomes (Kahn *et al*., 1998; Fridolfsson & Ellegren, 1999). Heterogametic (ZW) females are expected to have two different sized introns while homogametic (ZZ) males should only have one intron size. However, in some species, the introns of *CHD-Z* appeared to be polymorphic and heterozygote males (e.g. ZZ') also produced introns with two different sized which make it difficult for sex identification (Dawson *et al*., 2001; Lee *et al*., 2002; Robertson & Gemmell, 2006). Due to this concern, another molecular sexing technique to improve the reliability in birds sexing had been introduced

by amplifying an additional W chromosome-specific DNA fragment of a different size than what had been produced by the pre-existing sexing primer pairs to independently confirm the existence of the W chromosome. Therefore, Shizuka & Lyon (2008) developed a reverse primer (GWR2) designed to sit within the intron of the W chromosome and amplify a small DNA fragment that served as a W-specific marker. This primer combines with the primer pair (1237L and 1272H) developed by Kahn *et al*. (1998) were used to amplify the genes of the individual birds. It was essentially a multiplex reaction of two primer pairs, the 1237L/1272H that amplified the entire *CHD1* intron of both Z and W chromosomes and 1237L/GWR2 pair that amplified a short fragment when the W chromosome was present.

Over the years, abundant molecular sexing methods had been designed to accurately sexing the birds. However, the differences in species of birds being investigated usually resulted in only a few sexing methods that can successfully differentiate the sex of birds. Hagadorn *et al*. (2016) had evaluated 11 primer pairs from the *CHD* region on sex chromosomes in birds and only obtained two primer sets (P8/P2 and 1237L/1272H) that successfully amplified the DNA and able to determine the sex in hummingbirds. This method of sex determination is important and can be applied to identify sex more accurately and can be used as important data for particular studies.

CHAPTER 3

MATERIALS AND METHODS

3.1 Experimental Materials

Swiftlets from three regions of Peninsular Malaysia were investigated in the present study for specific objectives one, two and three. The three regions were the northern region (NR), eastern region (ER) and southern region (SR) (Figure 3.1). All of the samples used in this study came from man-made birdhouses. The swiftlets of a single birdhouse were considered as a colony.

Five colonies in the state of Perak were sampled for the northern region. They were from Beruas, Lekir, Taiping, Gerik and Sungkai. Six samples were randomly collected from each colony. Samples for the eastern region were from four colonies which were located at Kuala Terengganu (Terengganu), Kota Bharu, Bachok and Tanah Merah (Kelantan). Seven to eight samples were randomly collected from each colony. The southern region was represented by five colonies from the state of Johor; they were from Segamat, Mersing, Kluang, Johor Bahru and Muar. Six samples were randomly collected from each colony. The detailed information on the colonies investigated in this study is provided in Table 3.1.

For specific objective four, samples were collected from the Perak population (northern region). The samples were feathers from 13 *A. fuciphagus* individuals, including those from three confirmed males (by post-mortem of carcass).

The approval for handling and sampling of the swiftlets was obtained from the Institutional Animal Care and Use Committee (IACUC), Faculty of Veterinary Medicine, UPM with the reference number UPM/IACUC/AUP-R044/2014.

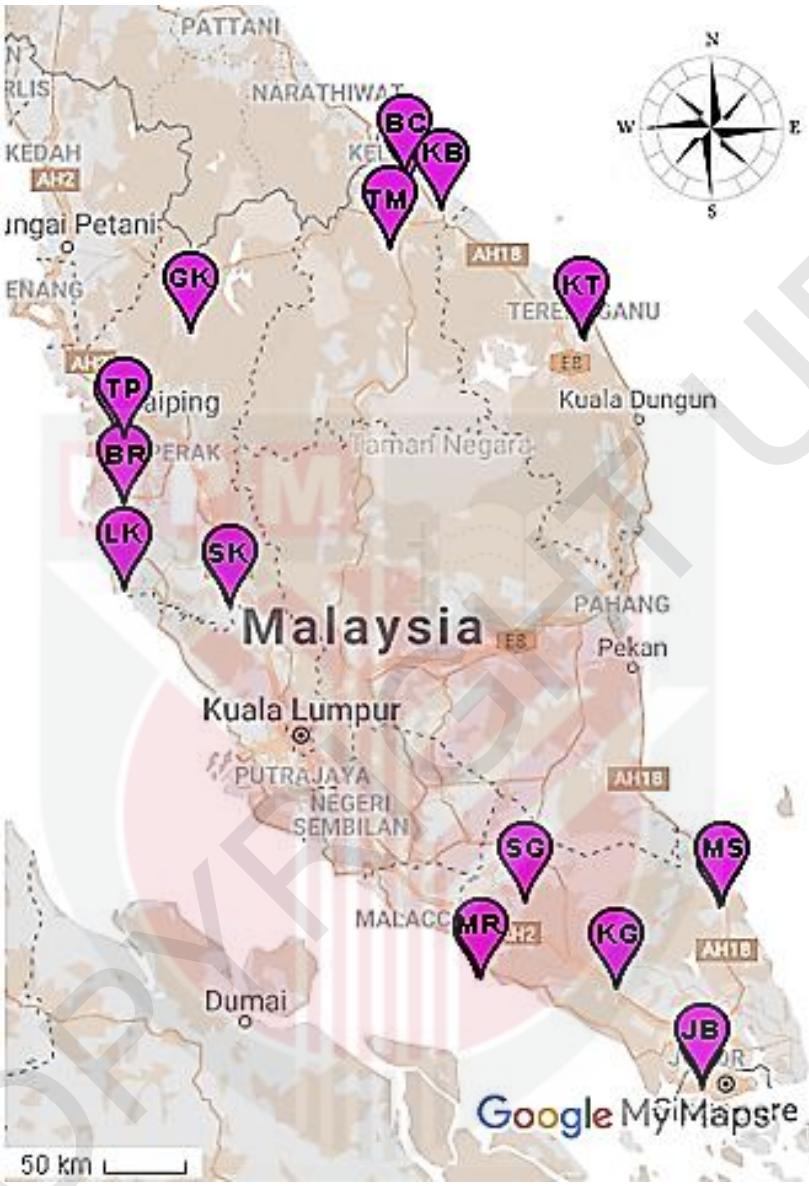

Figure 3.1: Locations of the sampled colonies
Northern region; NR (TP: Taiping, GK: Gerik, BR: Beruas, LK: Lekir, SK: Sungkai), Eastern region; ER (BC: Bachok, KB: Kota Bharu, TM: Tanah Merah, KT: Kuala Terengganu) and Southern region; SR (MR: Muar, SG: Segamat, KG: Kluang, MS: Mersing, JB: Johor Bahru). (Source: Google Map)

Table 3.1: The investigated colonies in the study

Colonies	Sample Size	Geographic Coordinates		Location	Region
SK	6	N 4°00'00.8"	E 101°18'36.3"	Sungkai, Perak	Northern
LK	6	N 4°04'05.5"	E 100°47'19.4"	Lekir, Perak	Northern
BR	6	N 4°30'02.9"	E 100°46'53.4"	Beruas, Perak	Northern
TP	6	N 4°50'59.0"	E 100°43'59.0"	Taiping, Perak	Northern
GK	6	N 5°25'42.4"	E101°07'46.9"	Gerik, Perak	Northern
KB	8	N 6°07'00.4"	E 102°16'39.7"	Kota Bharu, Kelantan	Eastern
BC	8	N 6°04'10.5"	E 102°23'49.9"	Bachok, Kelantan	Eastern
TM	7	N 5°48'32.0"	E 102°08'49.5"	Tanah Merah, Kelantan	Eastern
KT	7	N 5°19'46.6"	E 103°08'13.3"	Kuala Terengganu, Terengganu	Eastern
SG	6	N 2°35'44.5"	E 102°53'49.0"	Segamat, Johor	Southern
MS	6	N 2°28'19.7"	E 103°49'10.1"	Mersing, Johor	Southern
KG	6	N 2°00'40.1"	E 103°17'01.4"	Kluang, Johor	Southern
JB	6	N 1°31'19.4"	E 103°47'55.8"	Johor Bahru, Johor	Southern
MR	6	N 2°03'10.4"	E 102°35'03.7"	Muar, Johor	Southern
Total	90				

N: North, E: East

3.2 Sample Collection

For the study on population genetics (specific objectives one, two and three), blood, feathers, embryos or muscle tissue samples from edible nest swiftlets were collected as the source of nuclear DNA. Samples, of one of the types, were randomly collected from six to eight different individuals for each colony. Feathers (primary feathers) and muscle tissue samples were obtained from recently dead or injured chicks that had dropped from their nests at their breeding colonies. These samples were maintained frozen by storing in individual sealed envelopes and vials, respectively, in the freezer at low temperature (-20°C). Bloods were collected from living birds by authorized veterinary officers. The mist netted bird was placed dorsally on a stable surface and the brachial vein area was prepped by medical-use alcohol swab. Then, 26G, 1/2-inch needle and 1 ml syringe were used to withdraw 0.1 ml of blood from the brachial vein area. Pressure was applied by using an alcohol swab on the punctured site to avoid hematoma. Finally, two to three drops of blood were dropped onto FTA® Classic-Cards. The cards with the blood samples were placed in individual plastic bags, sealed (to avoid contamination) and stored in freezers (-20°C) until further analysis. The embryos (eggs) were collected from the breeding colonies with each embryo originating from a different nest (to avoid a close relationship between samples). All samples were stored in -20°C freezer in the laboratory. Samples were labelled according to the locations and type of samples. The identification numbers and sample types used in this study are given in Appendix A (1 - 3).

For the molecular sexing study (specific objective four), the initial plan was to collect swiftlet carcasses, identify the sex of the dead birds through post-mortem, and then screened the male and female samples using the molecular markers for sexing. Thirteen swiftlet carcasses (from dead or injured chicks that had dropped from their nests at breeding colonies) were collected from Perak and a post-mortem was conducted on these carcasses by a veterinarian. It was hoped that these carcasses would represent samples from male and female birds. Of the 13 carcasses, only three could be successfully confirmed as those of male birds based on the identification of the testes. Hence, feather samples from the three carcasses identified as males (M1, M2, M3) were used as control males. Meanwhile, the 10 feather samples (A11, A12, A13, A14, A15, A16, A21, A23, A25 and A26) of unidentified sex were used as tested individuals.

3.3 DNA Extraction

DNA was extracted from feathers, embryos, muscle tissues and blood by using the DNeasy® Blood & Tissue kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. This method comprises four steps: cell lysis, binding of DNA to a silica membrane, washing to remove non-DNA cell contents and DNA elution with a low salt buffer. The detailed protocol is given below.

Fifteen milligrams of a tissue sample were cut into small pieces and placed in a 1.5 ml microcentrifuge tube. For the feather sample, 5 – 10 mm of the calamus tip from a primary feather was placed in a 1.5 ml microcentrifuge tube. For dried blood spots on FTA cards, three 3 mm diameter discs were cut from one FTA card with a single-hole paper punch, and the blood card punches were placed in a 1.5 ml microcentrifuge tube. Then, for all sample types, 180 µl of buffer ATL was pipetted into each microcentrifuge tube. After that, 20 µl proteinase K was added to the sample, mixed well by vortexing for 15 s, and incubated at 56°C for 2 hours. Then, 200 µl of buffer AL and ethanol were added to the sample simultaneously and mixed well by vortexing for 15 s. Subsequently, the mixture was poured carefully to the QIAamp Spin Column (contain silica gel-membrane causing the DNA to specifically bind to it while contaminants pass through) that was placed in a 2 ml polypropylene collection tube without wetting the rim. The cap was closed, and the mixture was centrifuged at 8000 rotations per minute (rpm) for 1 min. The collection tube containing the flow-through was discarded and the QIAamp Spin Column was then transferred to another clean 2 ml collection tube. Then, 500 µl of wash buffer AW1 was added to the QIAamp Spin Column and centrifuged at 8000 rpm for 1 min. Again, the QIAamp Spin Column was transferred to another clean 2 ml collection tube, and the tube containing the filtrate was discarded. The QIAamp Spin Column cap was opened carefully and 500 µl of wash buffer AW2 was added, and the tube was centrifuged at 14,000 rpm for 3 min (to dry the membrane since subsequent reaction may be interfered by residual ethanol). The collection tube containing filtrate was discarded again and QIAamp Spin Column was transferred to a clean 1.5 ml microcentrifuge tube. The QIAamp Spin Column was carefully opened and 200 µl of Buffer AE was added directly to the membrane to elute the DNA. The column was left for 1 min to incubate at room temperature to increase the DNA yield. The tube was then centrifuged at 8000 rpm for 1 min. The eluted DNA in the 1.5 ml microcentrifuge tube was placed at –20°C for long term storage while the QIAamp Spin Column was discarded.

3.4 DNA Quality and Quantification

The DNA quantity and quality were checked using spectrophotometry and agarose gel electrophoresis. The details of the steps are given below.

In spectrophotometry, the DNA sample is exposed to ultraviolet (UV) lights at different wavelengths. Nucleic acid absorbs light of wavelength 260 nm, and proteins absorb light of wavelength 280 nm. The component called photo-detector in the spectrophotometer measures the light that passes through the sample, and the absorbance (A) or optical density (OD) for a particular wavelength is given. The absorbance or OD of 1 at the wavelength of 260 (A260 = 1) for double stranded DNA corresponds to DNA concentration of 50 µg/ml or 50 ng/µl. The purity of a nucleic acid sample is determined by the ratio of the absorbance at 260 and 280 nm (A260/280). For pure DNA, A260/A280 is within the range from 1.8 to 2.0.

First, five hundred microliters of water were placed in a 10 mm path length standard glass cuvette and served as a blank to calibrate the spectrophotometry at 260 nm and 280 nm. Then, the extracted DNA was diluted by adding 5 µl of the DNA with 495 µl water, and the content of the cuvette was mixed well. The absorbance values of A260 and A280 were measured and the A260/280 ratio was calculated. The readings were taken for three replicates from each sample, and the average was determined. The formula below was used to determine the DNA concentration of the sample:

DNA concentration (ng/µl) = A260 x 50 ng/µl x dilution factor

where, in the present case the dilution factor was 100. Finally, the extracted DNA stock was used to prepare the working stock with a standardized DNA concentration of 25 ng/µl by diluting the required volume of extracted DNA stock in distilled water, making a total volume of 50 µl.

Gel electrophoresis is a method to separate a mixture of DNA or RNA fragments according to the molecular weight by using electrical current. In DNA quality check, a low concentration of agarose gels (0.4 - 0.8%) was applied for electrophoresing of whole genomic DNA. Smaller fragments such as amplified microsatellite markers normally used 1.2 - 2% agarose gels (Weising *et al*., 2005).

Agarose gel (0.8%) was prepared by first suspending 0.8 grams of low melting agarose gel (Sigma) in 100 ml of 1x Tris Borate Ethylediaminetetraacetric acid EDTA buffer (TBE) in a 500 ml conical flask. The top of the flask was covered with parafilm with a small hole punctured to allow small escape of steam. The flask was swirled gently to mix the content and boiled in a microwave oven for 50 s, and after that, the steps of swirling and boiling were repeated for 20 s until the agarose was completely dissolved and became clear (a total time of 2 to 4 min). Then, the flask was slightly cooled under running tap water and placed inside the fume cabinet and added with 3 µl of EtBr (0.4 mg/ml) and mixed. The mixture was gently poured into the gel caster with an inserted comb to build wells in hardened agarose gel. Any small air bubbles were removed while the mixture was still liquefied. The gel was allowed to solidify for 30 min. After that, the comb was wet with some 1X TBE buffer and carefully removed.

The gel was placed into the electrophoresis chamber filled with pre-cooled 1X TBE buffer such that it was submerged in the buffer solution. Then, 1 µl of 6X gel loading dye was mixed to 5 µl of each DNA sample before loaded into each well of the gel. The loading dye acts to increase the density of the DNA so it sinks into the wells, and also provides a visual marker as to how far the DNA has travelled in the gel. The DNA quality was checked with reference to the lambda Hind III as a standard ladder (Research Biolabs, UK) loaded into the first well of the gel. The electrophoresis chamber was connected to an electric power supply by negative and positive sockets. The electrophoresis was then run at 75 volts for one hour (usually the dye would have travelled 1/2 – 3/4 of the way down the gel). The presence of electric current was confirmed by the

formation of air bubbles on both sides of the electrophoresis chamber. DNA migrates from the negative charge to the positive charge due to the phosphate group in the DNA. Finally, the gel was transferred from the electrophoresis chamber into the gel documentation system (Vilber Lourme, France) and visualized under UV light, and the DNA banding patterns were documented by photography.

3.5 Microsatellite Primers

A total of 28 microsatellite loci were investigated in this study. Eight of the primer pairs were species-specific, designed for *A. fuciphagus* (Aowphol *et al*., 2008), while the other 20 primer pairs were designed for swallow species (three species in the genus *Tachycineta*: Makarewich *et al*., 2009). Information on these loci are provided in Table 3.2 and Table 3.3.

Table 3.2: Information on species-specific microsatellite loci for *A. fuciphagus*.

Locus	Primer Sequence (5'- 3')	Repeat Motif	Allele Size Range (bp)
Aef4	F: M-AGA ACA TTT CCC CAA CCA CTT R: AAT GTT GGC AAT GTG CCT TA	(TATC)14	238 - 266
Aef24	F: M-TGG AAG TCT TGT ATG ATG GAC AG R: CCA TAG TTG CAG GGA TAG TCT G	(TAGA)15	226 - 262
Aef27	F: M-CCA TAA CCT AAA TCC CCC TAC C R: CAG CTG GTG TGC TGA GAA AA	(GATA)16	210 - 222
Aef104	F: M-GGA GAA TCT GGG AGA GCT GA R: GTG TCT TTC TGG TTC CAT CTT TAT GCA G	(TATC)11 (TGCC)10	162 - 194
Aef109	F: M-TGC CTC TAT ATG CAC ACA TGC R: TTT TTA CCA TTT CAT TGC CTT TT	(TAGA)12	168 - 192
Aef112	F: M-TTT TTG CCC TCA CAG TGT CC R: CAG ACC TCC TTG ATG TCC TGA	(TATC)13	226 - 242
Aef115	F: M-CAC ACA CTA TTT TTG GGC AGA R: AAG GTG CTT GGC ATT AGT GAA	(TAGA)12	214 - 234
Aef133	F: M-GTC CAG TGC CTA CAA TGC TG R: AAT CCG GAT AAC ATC TCC TCT T	(TATC)17	212 - 228

M: Fluorescently labelled M13 tail

Table 3.3: Information on microsatellite loci primers for swallow species.

Locus	Primer Sequence (5'- 3')	Repeat Motif	Allele Size Range (bp)
TaBi1	F: GCTGAATTTGGACAACTCACCCTC R: CTCCCAATCAGAAGCAGAAGCAC	(TATC)13	297 - 358
TaBi4	F: GAAGTTCCCATAGCTGGCTCTAAGAC R: TAGTGGTGAAAGACAGCTTGCTGG	(TATC)12	257 - 300
TaBi6	F: GAACTGTTATCTGCTCTCCTGCCC R: GCTTCAAACTGGTAGGTTTGTGGAG	(GTT)16	269 - 328
TaBi8	F: GCCAACATCCTTCTCTGTAAATCCC R: AGCAGATGTAACTGTTGCCCTTTTG	(GTn)19	320 - 344
TaBi10	F: GCCGTTCCTAAACTGTGACTCCTG R: GGCACAATGAGCATCATAAACGC	(CAA)12	341 - 385
TaBi12	F: CATGGGATTTTTACTTCCAGGG R: GGGACTCTCATGTCTTTGGTGATC	(CTT)55	301 - 506
TaBi21	F: CACATATTACACAGTCTCGCACC R: CAGACTTTGCTATTACAGTGCCAC	(CTn)24(GnTn)7	311 - 433
TaBi25	F: CACTGCGTACCTAAAATCTCTGG R: CTGAAGTCTAGCACTGGAAGTCTG	(GTT)13	122 - 180
TaBi34	F: GGAAGCACGAGATGGTATTCAC R: CCCACCAGAATCCTCCACAG	(CnAn)21	180 - 220
Tal4	F: TCCGCTACAGGAACTGAATAGG R: GACTATGCAAAGATTAGATCCCACC	(TATC)13(TA)2 (TC)(TA)6	230 - 257

Table 3.3: Continued

Locus	Primer Sequence (5'- 3')	Repeat Motif	Allele Size Range (bp)
Tle4	F: GACACGGGAGCCAAATGTC R: CTATGGGGATAGTTTTCTGCTG	(GAA)22 (CAA)3	251 - 299
Tle8	F: TTAGCACTGCCTAAACGATAGTCTAG R: TGCAGCCAGGCAACGATG	(GnTn)16	231 - 250
Tle14	F: AATGAGACCACACGCCAAAGTC R: CTTAATTGTGAGAGCCAACCATCG	(GA)16	205 - 234
Tle15	F: AGAGAGGGAATGGGGTTGC R: GAGAGTTGATTGTCTAGGTCTAGGC	(AnCn)6(G) (AnCn)13	248 - 276
Tle16	F: GTCCTATTCCTTCTAGGGATTGAG R: GTATTTTTGCTGCCATACGCTC	(AnCn)25	253 - 264
Tle17	F: CTTCATGAGTCTCAGACACACCTC R: GATGACTACTACAGGAATATGGGTTC	(GnTn)9(G) (GnTn)10	228 - 242
Tle18	F: GACGGATAGTGAATGTAGAAGACAG R: GCATGCACATTTTCCTGTAGC	(AnC)7	154 - 221
Tle19	F: CACTGCTGTGATGTCTTTCAGG R: CTCTTAAGCTTCTGAACTGTCAGG	(CnAn)16	153 - 173
Tle20	F: CCTCTAATATAACAACATACCCAGTGC R: GAAGTACCTTGACCCAGGCAC	(CnAn)18	377 - 400
Tle21	F: CTAAGTTAAGTCGGTTTTGCGTG R: GCACCTTACGGTAGTGTGGC	(TG)10(TC)2 (TG)4	166 - 177

3.6 Polymerase Chain Reaction (PCR) Optimization

PCR optimization was carried out to improve the efficiency of PCR and decrease genotyping errors. Some factors can affect optimal PCR amplification, these include concentrations of reagents, annealing temperature and the PCR protocol. The PCR mixture and protocol were optimized for each of the 28 microsatellite primer pairs by varying the concentration of $MgCl_2$ (1.2 – 2 mM) and the annealing temperature (52 – 64°C). Three different touchdown PCR protocols were developed as shown in Tables 3.5 – 3.7. DNA samples from three individuals (A27, A28 and A29) at concentrations of 25 ng/µl per reaction were used for this purpose.

3.7 DNA Pooling

DNA pooling in PCR is a procedure that combines several individual samples into one PCR reaction. It is a practical method to save cost and time. In the present study, it was used to identify the polymorphic microsatellite loci. Pooled DNA samples were prepared with each pool containing equal concentrations of DNA from 9 samples of the same region. Three DNA pools were prepared, one for each region and samples from all the colonies in the region were included in the pools. Pool 1 contained the DNA samples from Northern region (A11, A12, A24, A25, A30, A31, A32, A42, A52), pool 2 contained the DNA samples from Eastern region (TM2, TM3, KB2, KB3, KT1, KT2, BC8, BC9, BC10) and pool 3 contained the DNA samples from Southern region (J11, J12, J21, J22, J42, J49, J56, J57, J61). Then the three DNA pools were PCR amplified for the 12 microsatellite loci, which showed successfully amplification during the PCR optimisation process as described in section 3.6.

3.8 PCR Amplification of Microsatellites

PCR was carried out in a total volume of 25 µl. Table 3.4 shows the composition of the PCR reactions. For amplification of two of the loci (Aef27 and Aef109), 1.2 mM MgCl2 per reaction was used, while for the other loci, 1.5 mM MgCl2 was used. PCRs were accomplished by using touchdown protocols as shown in Tables 3.5 – 3.7. Taq polymerase and deoxyribonucleotide triphosphates (dNTP) used were from Promega, USA.

Table 3.4: Composition of the PCR reaction used in this study

Reagent	Stock Concentration	Desired amount	1 sample (µl)
PCR Buffer	5 X	1 X	5
$MgCl_2$	25 mM	1.2 or 1.5 mM	1.2 or 1.5
dNTPs	10 mM	0.2 mM	0.5
Forward-primer	10 µM	1 µM	2.5
Reverse-primer	10 µM	1 µM	2.5
Taq polymerase	5 unit	1 unit	0.2
DNA	25 ng/µl	50 ng	2
Pure water			10.5 - 10.8
Total			25

Table 3.5: PCR protocol used for amplification of microsatellite loci Aef4 and Aef112

Stage	Temperature (°C)	Time	Cycle
Initial denaturation	95	5 min	
Denaturation	95	30 s	38 cycles
Annealing	64	30 s	
Extension	72	45 s	
Final extension	72	10 min	

Table 3.6: PCR protocol used for amplification of microsatellite loci Aef24, Aef109, Aef115 and Aef133

Stage	Temperature (°C)	Time	Cycle
Initial denaturation	95	5 min	
Denaturation	95	30 s	3 cycles
Annealing	64	30 s	
Extension	72	45 s	
Denaturation	95	30 s	3 cycles
Annealing	63	30 s	
Extension	72	45 s	
Denaturation	95	30 s	3 cycles
Annealing	62	30 s	
Extension	72	45 s	
Denaturation	95	30 s	6 cycles
Annealing	61 - 56	30 s	
Extension	72	45 s	
Denaturation	95	30 s	20 cycles
Annealing	55	30 s	
Extension	72	45 s	
Final extension	72	10 min	

Table 3.7: PCR protocol used for amplification of microsatellite loci Aef27, Aef104, TaBi1, TaBi4, TaBi6, TaBi8, TaBi10, TaBi12, TaBi21, TaBi25, TaBi34, Tal4, Tle4, Tle8, Tle14, Tle15, Tle16, Tle17, Tle18, Tle19, Tle20 and Tle21

Stage	Temperature (°C)	Time	Cycle
Initial denaturation	95	5 min	
Denaturation	95	30 s	38 cycles
Annealing	64 - 56	45 s	
Extension	72	45 s	
Final extension	72	10 min	

3.9 Microsatellite Genotyping Technique

The PCR amplicons were genotyped using the PAGE method. First, the glass plates with spacers in gel caster were assembled after cleaning it beforehand. After that, the 8% polyacrylamide gel solution was prepared as shown in Table 3.8.

Table 3.8: Chemicals and reagents used for the preparation of 8% polyacrylamide gel

Stock Solution	Desired Concentration/ Percentage (%)	1 gel with 1.0 mm thickness (ml)
30% Acrylamide/ Bis	8.0	1.6
1X TBE Buffer	30.0	1.8
10% APS	0.05	0.03
TEMED	0.1	0.006
Water		2.6

The gel solutions were prepared first without adding APS and TEMED into the solutions. It was stored at 4°C for 15 min and allowed to warm to room temperature. After 15 min, APS and TEMED were added to the gel solutions and swirled gently before transferred into the glass cassettes using a pipette. Thereafter, a well-forming comb was inserted without trapping air under the teeth. Polymerization was allowed to occur at room temperature for 90 min to ensure maximum reproducibility in gel pore size. Then, the comb was removed, and the wells were rinsed completely with water. The gel was then transferred to electrophoresis chambers and pre-cooled 1X TBE Buffer was added between chambers so that buffer filled the wells. Five µl of each sample was loaded into each well. Then, the lid was put on the electrophoresis tank and connected to the power supply. Electrophoresis was then conducted at 35 volts for three hours. The PCR amplicon sizes were estimated with reference to the 25 bp DNA standard ladder (Research Biolabs, UK) loaded into the first and last wells of the gel (usually first and tenth wells) and visualized under UV light after staining with EtBr with the aid of the gel documentation system (Vilber Lourme, France).

3.10 Microsatellite Sequencing

The PCR product from each different polymorphic locus that showed a single DNA fragment after genotyping was purified using Geneall®Expin™ Combo GP (Geneall Biotechnology, Korea). The procedures were as followed:

3.10.1 PCR product purification

Each 20 µl of chosen PCR product was pipetted into a 1.5 ml microcentrifuge tube. Then, 100 µl of Buffer PB was added into the microcentrifuge tube (five volumes of the Buffer PB to 1 volume of the sample) and mixed. The mixture was transferred to a Spin/Vacuum (SV) column (provided in the kit) and centrifuged at 14,000 rpm for 30 s. The filtrate was discarded, and the SV column was re-inserted to the same collection tube. Then, 700 µl of Buffer NW was added to the SV column and centrifuged at 14,000 rpm for 30 s. The filtrate was discarded and the SV column was re-inserted into the same collection tube. The SV column was centrifuged again at 14,000 rpm for 1 min to remove the residual wash buffer. After that, the SV column was transferred to a new 1.5 ml microcentrifuge tube. Then, 50 µl of Buffer EB was dispensed directly onto the centre of the SV column membrane for optimal elution of DNA and left for 1 min at room temperature. Finally, the SV column was centrifuged at 14,000 rpm for 1 min. The purified DNA was kept at -20°C until sent out for sequencing. Subsequently, the purified products were sent to the sequencing service provider (First Base Laboratories Sdn Bhd, Selangor) for sequencing. All sequences were aligned using CLUSTALW of the Bio edit software and microsatellite repeat motifs were determined manually through visual observation.

3.11 Molecular Sexing

3.11.1 Experimental material

Thirteen *A. fuciphagus* individuals including three males (determined by post-mortem) from Northern Region (Perak) swiftlet houses were randomly selected to be used in the study as shown in Table 3.9. All DNA samples were extracted using DNeasy® Blood & Tissue kit (Qiagen, Hilden, Germany).

Table 3.9: Information on *A. fuciphagus* individuals used for molecular sexing

Sample ID	Sex	Sample Type
M1	Male	Feather
M2	Male	Feather
M3	Male	Feather
A11	Unknown	Feather
A12	Unknown	Feather
A13	Unknown	Feather
A14	Unknown	Feather
A15	Unknown	Feather
A16	Unknown	Feather
A21	Unknown	Feather
A23	Unknown	Feather
A25	Unknown	Feather
A26	Unknown	Feather

M1, M2, M3 = control (the sex was determined by post-mortem)

3.11.2 Sample collection

All samples were taken from feathers including for the three carcasses of males (after the gender of the birds had been confirmed by post-mortem). Details are as described in Section 3.2.

3.11.3 Primers for molecular sexing

A total of seven primer pairs for molecular sexing were used in this study. Details of these primers are shown in Table 3.10. All primers were developed for the chromo-helicase-DNA binding gene (*CHD*) region in the sex chromosome of birds. Primers P8/P2/P0, P8/WZ/W and 1237L/1272H/GW2 used the multiplex PCR method.

Table 3.10: Seven primers tested for identifying the sex of *A. fuciphagus*.

Forward Primer	Forward Primer Sequence	Reverse Primer	Reverse Primer Sequence
P8[a]	5'-CTCCCAAGGATGAGRAAYTG-3'	P2[a]	5'-TCTGCATCGCTAAATCCTTT-3'
P0[a]	5'-ATTGAGTTGGAACCAGAICA-3'.		
1237L[b]	5'-GAGAAACTGTGCAAAACAG-3'	1272H[b]	5'-TCCAGAATATCTTCTGCTCC-3'
P8[c]	5'-CTCCCAAGGATGAGRAAYTG-3'	WZ[c]	5'-CCCTTCACTTC CATTAAAGC-3'
		W[c]	5'-ACCCAACCCAAAAGTACAAG-3'
1237L[d]	5'-GAGAAACTGTGCAAAACAG-3'	1272H[d]	5'-TCCAGAATATCTTCTGCTCC-3'
		GW2[d]	5'-CCTGTAAAAACCACCCAACC-3'
2550F[e]	5'-GTTACTGATTCGTCTACGAGA-3'	2718R[e]	5'-ATTGAAATGATCCAGTGCTTG-3'
P8[f]	5'-CTCCCAAGGATGAGRAAYTG-3'	P2[f]	5'-TCTGCATCGCTAAATCCTTT-3'
P8[g]	5'-CTCCCAAGGATGAGRAAYTG-3'	M5[g]	5'-YTYMCTTCAYTTCCATTAAAGC-3'

[a] Original primer design from Han *et al*. (2009), [b] Original primer design from Kahn *et al*. (1998), [c] Original primer design from Wang *et al.* (2011), [d] Original primer design from Shizuka and Lyon (2008), [e] Original primer design from Fridolfsson and Ellegren (1999), [f] Original primer design from Griffiths *et al*. (1998) and [g] Original primer design from Bantock *et al*. (2008).

3.11.4 PCR amplification

PCR was carried out in a total volume of 25 µl. The composition of the PCR reaction was as stated in Table 3.4 with 1.5 mM of MgCl2 concentration. PCR amplifications were accomplished by using the protocols shown in Table 3.11 and Table 3.12.

Table 3.11: PCR protocol used for five molecular sexing primers (P8/P2/P0, 1237L/1272H, 1237L/1272H/GW2, P8/P2 and P8/M5).

Stage	Temperature (°C)	Time	Cycle
Initial denaturation	95	5 min	
Denaturation	95	30 s	
Annealing	48[1]/56[2]	1 min	35 cycles
Extension	72	1 min	
Final extension	72	5 min	

[1] = Annealing temperature for primer P8/P2/P0, P8/P2 and P8/P5, [2] = Annealing temperature for primer 1237L/1272H and 1237L/1272H/GW2.

Table 3.12: PCR protocol for two sexing primer pairs (P8/WZ/W and 2550F/ 2718R)

Stage	Temperature (°C)	Time	Cycle
Initial denaturation	95	5 min	
Denaturation	95	30 s	
Annealing	60 - 50	30 s	10 cycles
Extension	72	45 s	
Denaturation	95	30 s	
Annealing	50	30 s	30 cycles
Extension	72	45 s	
Final extension	72	5 min	

3.11.5 Agarose gel electrophoresis

The procedure was similar with that described in section 3.4. However, agarose gel was prepared to 3% concentration and electrophoresis was run at 80 volts for one hour before the gel was visualized under UV light to observe the PCR amplicon bands. 100 bp ladder was used as a marker for size reference.

3.11.6 Sequencing

PCR products that showed variations in genotypic patterns were chosen for further investigation. The PCR products were purified and sent for sequencing to First Base Laboratories Sdn Bhd, Selangor. One male control (sex determine by post-mortem) together with two expected males and two expected female samples (identified as such from banding patterns) were selected for sequencing (a total of five samples). The two expected male and one male control samples displayed only a single band pattern on electrophoresis, and therefore, the PCR products were subjected to the same purification method as described in 3.10.1. Since the two expected female samples showed two band patterns on electrophoresis, the DNA bands of interest were visualized with the help of an UV transilluminator and excised from the agarose gel using a sterile scalpel. Each gel piece excised was transferred into a 1.5 ml microcentrifuge tube and weighed.

The selected samples were purified using Geneall®Expin™ Combo GP (Geneall Biotechnology, Korea). Five microlitres of Buffer GB were added to one milligram of gel. Then, the gel was incubated at 50ºC and alternately was vortexed for 2 to 3 min until it had completely melted (5 – 10 min). The colour of the mixture was checked after the sliced gel had dissolved completely; it should be yellow (similar to the colour of Buffer GB). If the colour of the mixture became brown or purple, 10 µL of 3 M sodium acetate, pH 5.0 must be added to adjust the pH, but this pH adjustment is not needed if the reason for the change in colour was due to the loading dyes (e.g. bromophenol blue, xylene cyanol). The mixture was transferred to a Spin/Vacuum (SV) column (provided in the kit) and was centrifuged for one minute at 14,000 rpm. If the mixture volume was larger than 700 µL, then the mixture was divided into several batches until all of the mixture has been filtered through the SV column. The filtrate was discarded and the SV column was reinserted into the same collection tube. A volume of 500 µl of Buffer GB was applied to the SV column and the tube was centrifuged at 14,000 rpm for 30 s. The filtrate was discarded, and the SV column was reinserted into the same collection tube. The following steps were the same as the procedure described in 3.10.1. The purified DNAs were kept in the -20°C freezer until sequencing.

3.12 Statistical Analysis

3.12.1 Genetic variation and relationship within swiftlet populations in Peninsular Malaysia

The means of observed and effective number of alleles and Shannon information index per locus per population that serve to evaluate the genetic variation within a population were estimated using the Popgene software (Yeh *et al.,* 1999). The allele and genotype frequencies at each locus for each population were calculated to determine genetic variability within and between populations using the Popgene software.

3.12.1.1 Heterozygosity

The measurement of genetic variation within a population is called heterozygosity. The Ho is defined as the percentage of loci that is heterozygous per individual or the number of individuals heterozygous per particular locus (Ojango *et al*., 2011). Ho at each locus for each breed or population was estimated using the POPGENE software. Ho is simply calculated as follows:

Ho = n (Ai Aj) / N
where n (Ai Aj)= Number of individuals with genotype Ai Aj
N = Total number of individuals in the sample

Individual breed or population mean heterozygosity was estimated by summing the heterozygosities at each locus for the breed or populations and averaging this value over all loci.

Expected heterozygosity (He) or gene diversity is defined as the estimated heterozygous individuals for any randomly chosen locus in a random mating population. It is calculated from individual allele frequencies (Ojango *et al*., 2011). He at each locus for each breed was estimated using the POPGENE software. It is calculated as follows:

$$He = 1 - \sum pi^2$$

where pi is the frequency of i-th allele for locus

3.12.1.2 Hardy-Weinberg Equilibrium (HWE)

HWE occurred in a population when the genotype frequencies remain constant from one generation to next. This occurs in large, random mating populations with the absence of selection, non-random mating, migration and mutation (Wigginton *et al*., 2005). The deviation from HWE expectation for each locus in a population was tested using the Chi-square test of the Popgene software.

$X^2 = \sum \frac{(O-E)^2}{E}$

Where O = Observed heterozygosity
E = Expected heterozygosity

3.12.1.3 Inbreeding coefficient

The probability of an individual is identical by descent (IBD) in two homologous alleles are called inbreeding coefficient (F_{IS}). It can measure the genetic inbreeding that occurs within a population, and therefore, estimating the deviation from HWE. F_{IS} was calculated in all populations by comparing the expected with H_o as follows:

$$F_{IS} = 1 - (H_O / He)$$

where

H_O = observed heterozygosity
He = expected heterozygosity

3.12.2 Genetic variation and relationship between swiftlet populations in Peninsular Malaysia

3.12.2.1 F-statistics and gene flow

F-statistics (Weir & Cockerham, 1984) are a measure of heterozygotes deficit relative to expected HWE proportions in the specified populations (Allendorf & Luikart, 2007). It describes the distribution of genetic variation within a species or population using a series of coefficients: F_{IS}, F_{ST}, and F_{IT}. F_{IS} is a measure of inbreeding within the population and has been explained in Section 3.11.1.3. F_{ST}, on the other hand is a measure of divergence in allele frequency or differentiation of genetic among populations. This value indicates the amount of gene exchange among the populations. F_{IT} is a measure of overall departure from HWE in the entire population resulting from both inbreeding within population (F_{IS}) and allele frequency divergence among subpopulations (F_{ST}). It is capable of measuring the degree of reduction in heterozygosity of individuals from the entire population. F-statistics for each locus and overall, including population pairwise F_{ST} and F_{IT} values were calculated using the formulas below with the aid of the Popgene software.

$$F_{ST} = 1 - (H_S / H_T)$$
$$F_{IT} = 1 - (H_O / H_T)$$

where

H_O = observed heterozygotes averaged overall populations
H_S = expected heterozygotes averaged overall populations
H_T = expected HWE proportion of heterozygotes using the allele frequencies averaged overall population

The gene flow (Nm) was calculated from the mean F_{ST} values for the 8 microsatellite loci using Popgene software

$$Nm = (1 - F_{ST})/ 4 F_{ST}$$

3.12.2.2 Analysis of molecular variance (AMOVA)

The distribution of genetic diversity within and between populations is generally calculated through an analysis of molecular variance (AMOVA). In this study, AMOVA was performed using ARLEQUIN 3.1 software (Excoffier *et al*., 2005).

3.12.2.3 Estimation of genetic admixture

To estimate the genetic admixture in the swiftlet populations, structure analysis was performed.

Structure analysis

The Structure software version 2.3.4 (Pritchard *et al.,* 2010) was used to analyse the genetic structure of the studied populations. This program uses genotype data by implementing a model-based clustering method for inferring population structure. It is also capable of identifying admixture individuals by assigning individuals to the inferred populations. The analyses were performed utilizing the admixture model with correlated allele frequencies. To decide on the appropriate number of clusters (K), two to five inferred clusters were ran with each inferred cluster were ran for three independent runs. All analyses used a burn-in period of 10,000 iterations for data collection. The phenomenon where the larger K's in a state of little changes when the real K is reached together with the increase in variance among run was not observed in this study. When K was increased, the LnP(D) increased continuously, as shown later in Table 4.12 (section 4.6.2). Therefore, the Evanno method, which is considered as a formal method to detect the real K, was used. The values of LnP(D) for each K were plotted and the delta K(ΔK) statistics, which is based on the second-order rate of change in LnP(D) between successive K values (Evanno *et al*., 2005) were estimated.

3.12.2.4 Genetic distance and phylogenetic analysis

In a study of animal diversity, phylogenetic analyses using clustering methods are commonly used. Genetic distances can measure the differences of genes between populations and further determined the genetic relationship among these populations (Ojango *et al*., 2011). In this study, genetic distances between the three populations based on the eight loci were estimated using Nei's standard genetic distances (Nei, 1972).

Phylogenetic trees were constructed based on Nei's DA genetic distances (1983). This genetic distance method is based on allele frequency procedure that assumes the Infinite Allele Model (IAM) holds true. Both the Neighbour-joining (NJ) (Saitou & Nei, 1987) and unweighted pair group method with

arithmetic mean (UPGMA) (Sneath & Sokal, 1973) methods, which represent the two most commonly used methods for constructing the trees were used. The robustness of tree topologies was evaluated with a bootstrap test of 1000 resampling across loci. These analyses were conducted using MEGA 7 (Kumar *et al*, 2016).

3.12.3 Sexing markers analysis

To determine the validity of the sexing primers for identifying the sex of *A. fuciphagus*, two expected male samples (A16 and A23) and two expected female samples (A15 and A21), together with a control male sample (M3) that had the sex confirmed through post-mortem were sent for sequencing. The nucleotide sequences of expected males and females of *A. fuciphagus* were aligned and compared individually using BLASTN, (National Centre of Biotechnology Institute, NCBI) with a control male sequence. The similarity in the nucleotide sequences among the expected males and females, and the control were compared, and the percentage of identity was estimated using Clustal Omega software (http://www.ebi.ac.uk/Tools/msa/clustalo) to confirm the sex of the *A. fuciphagus* and the CHD-W and CHD-Z genes.

After confirmation, the nucleotide sequences of males and females *A. fuciphagus* were aligned and compared individually using BLASTN, NCBI with homologous nucleotide sequences of fourteen other bird species from the same Apodiformes order (*Apus apus* and *Calypte costae*) and Aves class to understand the evolution of CHD gene. Phylogenetic trees using the NJ method (Saitou & Nei, 1987) for male and female *A. fuciphagus* with fourteen other bird species from the same Apodiformes order and other bird species of the same Aves class were constructed using MEGA 7 (Kumar *et al*, 2016). In addition, a phylogenetic tree was constructed for the male *A. fuciphagus* with birds from the Apodiformes order (*Apus apus and Calypte costae*), using information available in the GenBank, to determine the genetic relationship between the birds in the same order.

CHAPTER 4

RESULTS

4.1 DNA Extraction and PCR Optimization

DNA was successfully extracted from tissue and blood samples of 90 birds. The DNA quality was tested using AGE and the result for some samples is presented in Figure 4.1. The DNA was generally of good quality, although some of the samples showed signs of DNA degradation or some contamination (i.e. the presence of smear on the gel). The concentration and purity ranges for the DNA samples are presented in Appendix B (1 – 3).

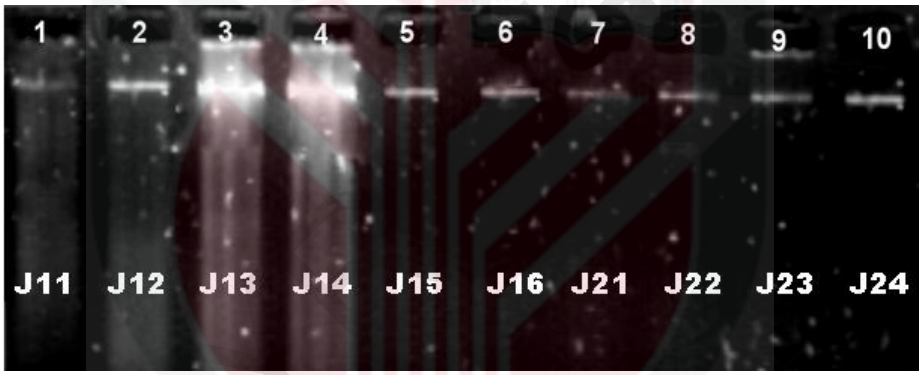

Figure 4.1: DNA quality as displayed by AGE of ten swiftlets samples.

The PCR amplification was optimized by varying the annealing temperature and $MgCl_2$ concentrations. Of the 28 microsatellite loci investigated using DNA samples from three individuals (A27, A28, A29) as described in Section 3.6, only 12 loci showed successful amplification. Figures 4.2 and 4.3 show the examples of digital images of the electrophoresis gels with the PCR products for two successfully amplified loci generated using optimized mixtures and protocols for six samples.

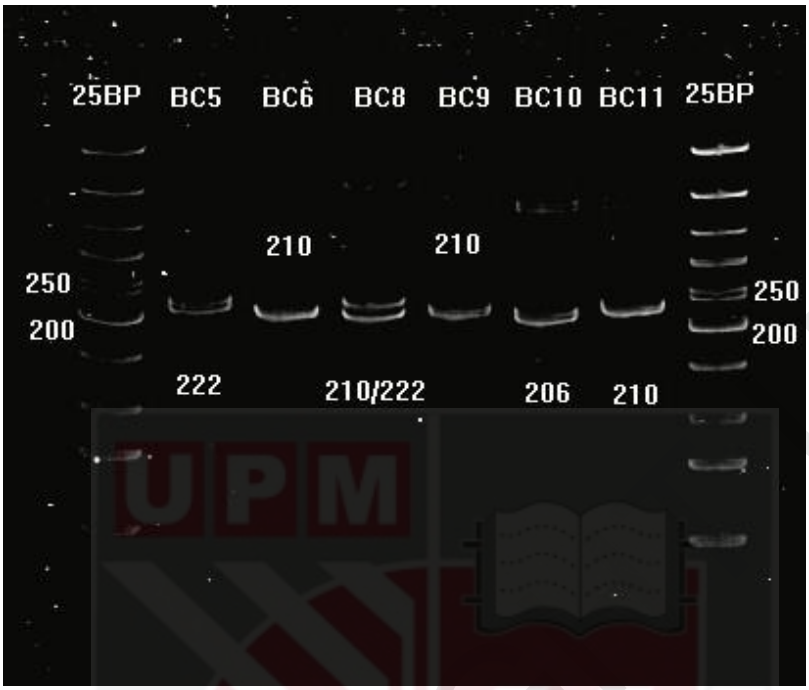

Figure 4.2: Polyacrylamide gel showing PCR amplicons from optimized PCR composition and protocol at microsatellite locus Aef27 for six swiftlet samples. 25BP; 25 bp DNA ladder.

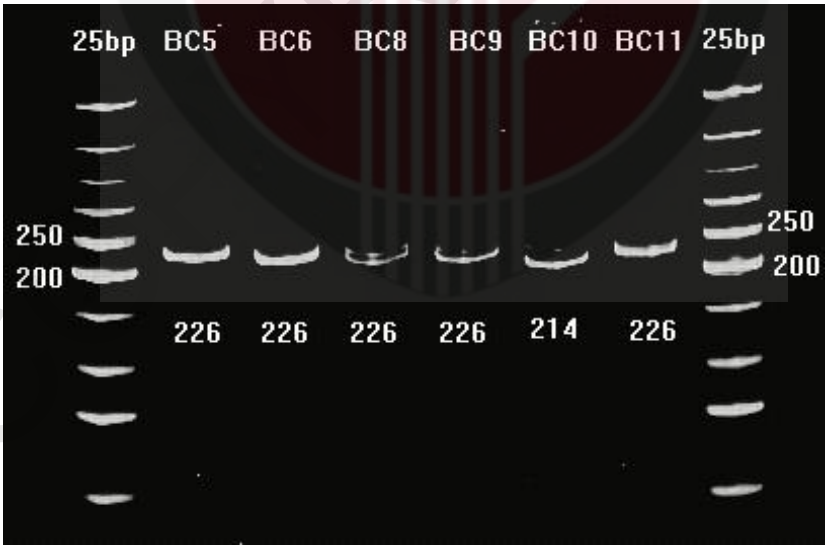

Figure 4.3: Polyacrylamide gel showing PCR amplicons from optimized PCR composition and protocol at microsatellite locus Aef115 for six swiftlet samples. 25BP; 25 bp DNA ladder.

4.2 DNA Pooling

To reduce cost, time and labour, a DNA pooling procedure was adopted to identify the polymorphic loci from 12 loci that showed successful amplification previously. Figure 4.4 shows the digital image of the electrophoresis gel for PCR products for pooled DNA samples for two microsatellite loci (Aef104 and Aef27). All the DNA pools with nine samples, each showed more than one band for eight microsatellite loci. These served as indicators of polymorphism at these loci. The DNA pools from the other four microsatellite loci showed only a single band, indicating the monomorphic nature of these loci. The loci that showed monomorphism were excluded from further analysis. Table 4.1 shows the results of PCR amplification of DNA pools (a total of 27 samples for three pools) for the loci investigated.

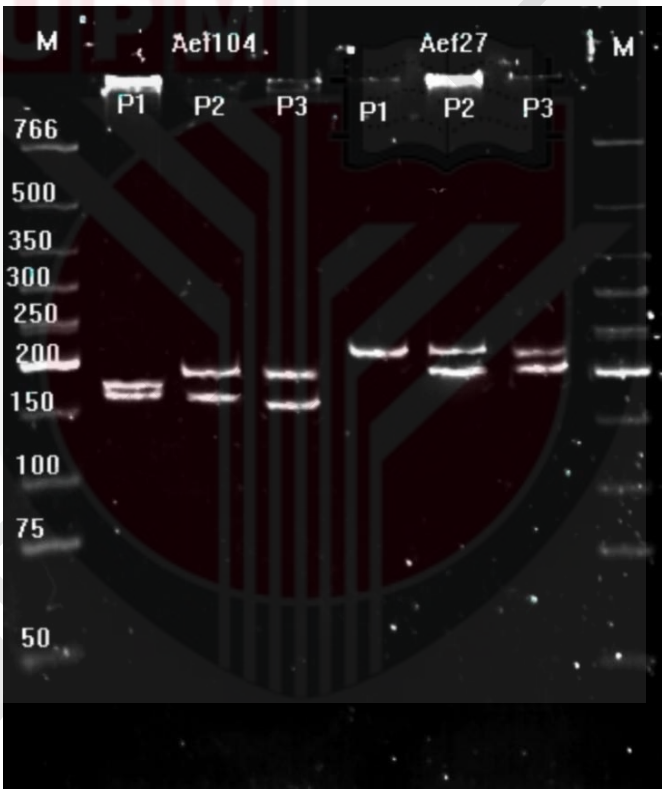

Figure 4.3: Polyacrylamide gel showing PCR amplicons of three pooled DNA samples for two microsatellite loci. P1 – 3: DNA pools, each pool contained nine DNA samples. M: 25 bp DNA ladder.

Table 4.1: Size of products from PCR amplifications of DNA pools for the twelve microsatellite loci

Loci	DNA Amplicon Size (bp)			Allele Size Range	Remarks
	Pool 1	Pool 2	Pool 3		
Aef4	250	242/266	238	238-266	Polymorphic
Aef24	262	226	258	226-262	Polymorphic
Aef27	222	210/222	210/222	210-222	Polymorphic
Aef104	166/182	166/194	162/194	162-194	Polymorphic
Aef109	168/184	180/192	180	168-192	Polymorphic
Aef112	226	238	230/242	226-242	Polymorphic
Aef115	234	214	218	214-234	Polymorphic
Aef133	224	212	228	212-228	Polymorphic
Tle19	169	169	169	169-169	Monomorphic
TaBi25	102	102	102	102-102	Monomorphic
TaBi34	192	192	192	192-192	Monomorphic
Tle17	282	282	282	282-282	Monomorphic

Pool 1 - 3: DNA pools, each pool contained nine DNA samples.

4.3 Allele Frequency Distributions

The genetic variability within and between populations were determined by calculating the frequency of allele at each locus for each of the three populations. The allele frequencies at the eight polymorphic loci for each population are presented in Appendix C. As shown in Figure 4.5, Northern and Southern regions (NR and SR respectively) showed relatively different allele frequencies for the three selected loci. For example, at locus Aef27, the 222 bp allele was presented in low frequency (0.07) in the SR, while it was presented with high frequencies in the NR (0.32) and Eastern region (ER; 0.23). In the same manner at locus Aef112, the 230 bp allele was presented at high frequencies in the SR (0.50) and ER (0.2), while it was presented with low frequency in the NR (0.03).

Table 4.2 shows 12 alleles that showed high frequencies in at least one out of three populations investigated (frequency ≥ 0.2). ER showed a high frequency for all selected alleles, while some of the alleles were presented in low frequencies for either in the SR or NR. For example, at locus Aef112, the 230 bp allele was presented at low frequency in the NR (0.03), but the frequencies

were high in the SR (0.5) and ER (0.2). Meanwhile, at locus Aef133, the 236 bp allele was presented with high frequencies in the ER (0.22) and NR (0.23), but was absent in the SR.

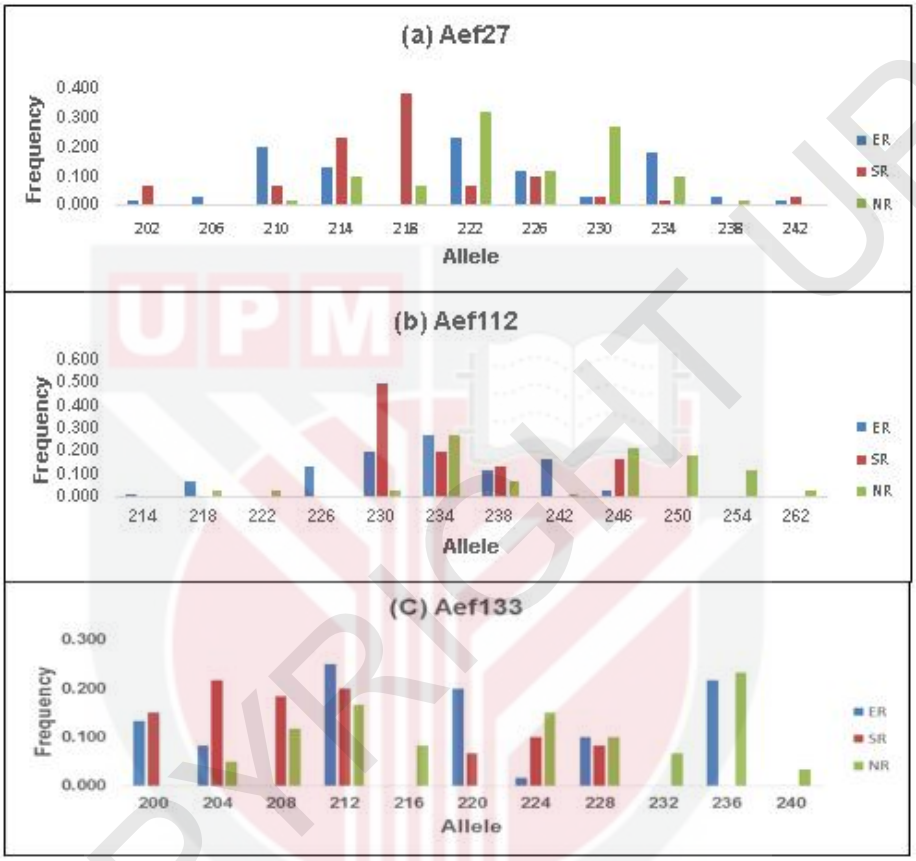

Figure 4.4: Allele frequencies at Aef112, Aef27 and Aef133 loci in Eastern (ER), Southern (SR) and Northern region (NR).

Table 4.2: Alleles at high frequencies in different populations

Locus	Allele Size	Allele Frequency		
		ER	SR	NR
Aef4	254	0.20	0.20	0.15
Aef24	250	0.12	0.33	0.25
Aef27	222	0.23	0.07	0.32
Aef104	162	0.23	0.25	0.07
Aef109	164	0.28	0.10	0.20
Aef109	168	0.23	0.02	0.22
Aef112	230	0.20	0.50	0.03
Aef112	234	0.27	0.20	0.27
Aef115	226	0.20	0.12	0.25
Aef115	230	0.30	0.40	0.13
Aef133	212	0.25	0.20	0.17
Aef133	236	0.22	0.00	0.23

ER = Eastern Region, SR = Southern Region, NR = Northern Region.

4.4 Microsatellite Loci Analysis

In order to assess how informative, the microsatellite loci, the observed number of alleles (Na), Shannon information index and allele size ranges for each microsatellite loci were investigated using pooled population data from all three populations (a total of 90 samples). The results are presented in Table 4.3. A total of 102 alleles were detected across the populations, with an MNA 13. The most polymorphic locus was Aef24 with fifteen alleles, and the least polymorphic loci were Aef27 and Aef133 with eleven alleles each. The mean number of effective alleles (MNE) ranged from 6.2 at Aef112 to 9.2 at Aef4; the mean was 7.7 alleles per locus. The MNE values were less than the MNA values across all populations.

The Shannon information index ranged from 2.05 for Aef112 to 2.34 for Aef4; the mean was 2.2. The observed allele size ranges for all eight loci were mostly similar to the reported allele size ranges from the literatures.

Table 4.3: Observed (Na) and effective (Ne) number of alleles, Shannon information index (I) and allele size ranges for the microsatellite loci investigated.

No.	Locus	Na	Ne	I	Allele Size Ranges (bp)	
					Observed	Reported in Literature
1	Aef4	13	9.2	2.34	226-278	251-283
2	Aef24	15	7.7	2.26	210-278	227-267
3	Aef27	11	7.5	2.13	202-242	215-247
4	Aef104	14	8.7	2.32	154-210	176-216
5	Aef109	14	7.8	2.21	128-196	143-210
6	Aef112	12	6.2	2.05	214-262	224-248
7	Aef115	12	6.5	2.10	214-258	215-243
8	Aef133	11	8.1	2.20	200-240	204-232
	Mean	13	7.7	2.20		
	Std Dev	1.5	1.0	0.10		

4.5 Genetic Diversity within Populations.

4.5.1 Observed and effective number of alleles

Table 4.4 shows the observed and effective number of alleles for each of the investigated microsatellite loci at the population level. The results showed that the SR had the lowest total number of alleles (TNA = 57), while the NR showed the highest TNA (89). SR had the smallest MNA (7.1) as well, while the NR had the highest MNA (11.1). As for MNE, the SR had the lowest value (4.9), followed by the ER (5.6) and the NR had the highest value (7.1). In general, the MNE values were less than the MNA values for all populations.

The results showed that two loci Aef27 and Aef109 had the highest number of alleles (Na) for both the ER (10) and SR (9). NR had Aef24 and Aef104 loci as the highest Na (13). Locus Aef27 had the minimum Na in the NR (8), while it showed the highest Na in both ER and SR. The minimum Na was observed at locus Aef133 for the ER (7) and locus Aef112 at SR (4).

Table 4.5 shows the private alleles detected across the three populations. In general, there were 29 private alleles detected at eight loci. The number of private alleles per population ranged from one (SR) to 22 (NR). All the private alleles showed low frequencies, which ranged from 0.017 to 0.183.

Table 4.4: Observed (Na) and effective (Ne) number of alleles for the eight polymorphic loci in the three populations.

No.	Locus	ER		SR		NR	
		Na	Ne	Na	Ne	Na	Ne
1	Aef4	9	6.7	7	6.5	12	8.8
2	Aef24	8	5.8	7	4.5	13	7.8
3	Aef27	10	6.1	9	4.4	8	4.8
4	Aef104	8	4.3	6	3.6	13	8.0
5	Aef109	10	5.1	9	6.5	12	6.6
6	Aef112	8	5.7	4	3.0	10	5.7
7	Aef115	8	5.4	8	4.4	12	7.7
8	Aef133	7	5.4	7	6.1	9	7.0
	Total	68		57		89	
	Mean	8.5	5.6	7.1	4.9	11.1	7.1
	Std Dev.	1.1	0.7	1.6	1.3	1.9	1.3

ER = Eastern Region, SR = Southern Region, NR = Northern Region

Table 4.5: Private alleles in the three populations.

No.	Population	Locus	Private Allele	Frequency
1	ER	Aef4	238	0.033
2	ER	Aef27	206	0.033
3	ER	Aef109	128	0.017
4	ER	Aef112	214	0.017
5	ER	Aef112	226	0.133
6	SR	Aef104	198	0.033
7	NR	Aef4	226	0.033
8	NR	Aef4	230	0.050
9	NR	Aef24	210	0.083
10	NR	Aef24	218	0.033
11	NR	Aef24	230	0.017
12	NR	Aef24	238	0.033
13	NR	Aef24	274	0.150
14	NR	Aef24	278	0.017
15	NR	Aef104	202	0.033
16	NR	Aef104	210	0.017
17	NR	Aef109	136	0.017
18	NR	Aef109	196	0.017
19	NR	Aef112	222	0.033
20	NR	Aef112	250	0.183
21	NR	Aef112	254	0.117
22	NR	Aef112	262	0.033
23	NR	Aef115	238	0.033
24	NR	Aef115	250	0.100
25	NR	Aef115	254	0.017
26	NR	Aef115	258	0.033
27	NR	Aef133	216	0.083
28	NR	Aef133	232	0.067
29	NR	Aef133	240	0.033

ER = Eastern Region, SR = Southern Region, NR = Northern Region

4.5.2 Heterozygosity

Estimates of observed (H_o) and expected (H_e) heterozygosities for the eight loci according to the three populations are presented in Table 4.6. The results

showed that SR had the lowest mean H_o and H_e (0.28 and 0.79, respectively) compared to ER (H_o: 0.44, H_e: 0.83) and NR (H_o: 0.35, H_e: 0.87). The mean values of H_o were lower than the mean values of H_e for all populations.

The results showed that the locus Aef112 showed an absent of heterozygosity observed at SR and lowest heterozygosity in NR (0.13). ER had a different locus with the lowest H_o (Aef104: 0.17). Out of the eight loci, none showed heterozygosity values of 0.50 and above in all three populations. Most of the loci showed large differences between H_o and H_e in all populations.

Table 4.6: Observed (H_o) and expected (H_e) heterozygosities at eight polymorphic microsatellite loci for the three populations

No.	Locus	ER		SR		NR	
		H_o	H_e	H_o	H_e	H_o	H_e
1	Aef4	0.7667	0.8650	0.7667	0.8599	0.2000	0.9017
2	Aef24	0.3000	0.8429	0.0333	0.7904	0.4000	0.8859
3	Aef27	0.3333	0.8508	0.0333	0.7859	0.4667	0.8034
4	Aef104	0.1667	0.7819	0.3333	0.7305	0.5333	0.8898
5	Aef109	0.3667	0.8175	0.7000	0.8599	0.3667	0.8638
6	Aef112	0.3667	0.8376	0.0000	0.6757	0.1333	0.8395
7	Aef115	0.6333	0.8299	0.2333	0.7853	0.3667	0.8853
8	Aef133	0.5677	0.8294	0.1667	0.8497	0.3333	0.8712
	Mean	0.4375	0.8319	0.2833	0.7922	0.3500	0.8676
	Std Dev.	0.1988	0.0249	0.3003	0.0654	0.1309	0.0322

ER = Eastern Region, SR = Southern Region, NR = Northern Region

4.5.3 Hardy-Weinberg Equilibrium (HWE)

Table 4.7 shows the probability values of the Chi-square test for HWE for the eight loci in the three populations. Of the 24 HWE tests (eight loci in three populations), a total of 21 tests gave significant ($p < 0.05$) deviation from HWE. The deviations from HWE were found for all eight loci in the NR, seven loci in the ER and six loci in the SR.

Of the eight loci, six loci showed significant deviation from HWE ($p < 0.05$) for all the populations. These loci were Aef24, Aef27, Aef104, Aef112, Aef115 and Aef133.

Table 4.7: Probability values of Chi-square test for Hardy-Weinberg equilibrium for the eight polymorphic microsatellite loci in the three populations.

Locus	P value for HWE test		
	ER	SR	NR
Aef4	0.43	0.19	0.00
Aef24	0.00	0.00	0.00
Aef27	0.00	0.00	0.02
Aef104	0.00	0.00	0.00
Aef109	0.00	0.13	0.00
Aef112	0.00	0.00	0.00
Aef115	0.00	0.00	0.00
Aef133	0.00	0.00	0.00

ER = Eastern Region, SR = Southern Region, NR = Northern Region

4.5.4 Estimation of inbreeding

Table 4.8 shows the F_{IS} for the eight loci in the three populations. All populations had positive F_{IS} estimates, ranging from 0.465 in ER to 0.636 in SR. All of the loci showed positive F_{IS} values in all populations and most of it showed a very significant deficit of heterozygotes in all populations.

Table 4.8: Inbreeding coefficient (F_{IS}) for the eight polymorphic microsatellite loci in the three populations.

Locus	F_{IS}		
	ER	SR	NR
Aef4	0.0986	0.0933	0.7744
Aef24	0.6381	0.9571	0.5408
Aef27	0.6016	0.9569	0.4093
Aef104	0.7832	0.536	0.3905
Aef109	0.5439	0.1721	0.5683
Aef112	0.555	1.0000	0.8385
Aef115	0.224	0.6978	0.5788
Aef133	0.3052	0.8005	0.6109
Mean	0.4652	0.6363	0.5897

ER = Eastern Region, SR = Southern Region, NR = Northern Region

4.5.5 Analysis of molecular variance (AMOVA)

AMOVA was carried out using the Arlequin software and the results are presented in Table 4.9. AMOVA analysis permitted the partitioning of the genetic variability between different sources of variation. AMOVA showed significant ($p < 0.01$) variation among the populations as well as within the populations. The analysis indicated that 7.06% of the genetic variability in the analysed populations was due to differences among the populations and that the largest part of the variability (92.94%) was due to differences within the populations.

Table 4.9: Analysis of Molecular Variance (AMOVA) for the three populations.

Source of variation	df.	Sum of Square	Variation Components	Percentage of Variation	P value
Among populations	2	36.94	0.25	7.06	0.00
Within populations	177	588.03	3.32	92.94	0.00
Total	179	624.97	3.57		

4.6 Genetic Variation and Relationship between Populations

4.6.1 F-statistics and gene flow

Table 4.10 shows gene differentiation (F_{ST}) values and gene flow (N_M) among the populations. The highest F_{ST} value was found between SR and NR (0.052), while the lowest was found between ER and NR (0.034).

The highest F_{ST} value between SR and NR indicated that there was less migration between these populations and that they were more isolated from one another compared to ER and NR population that showed a low gene differentiation (the lowest F_{ST} value). As for the gene flow (N_M), the highest value was observed between ER and NR (7.18), while the lowest value was found between SR and NR (4.53).

The highest gene flow observed between ER and NR indicated that there were fewer genetic differences between these populations due to the high exchange of genes between them. Table 4.11 shows F-statistics and gene flow for each locus based on pool data from all three populations. The overall F_{ST} among populations was 5.9%.

Table 4.10: Gene differentiation (F_{ST}) and gene flow (N_M) among the three populations.

Population 1	Population 2	F_{ST}	N_M
ER	SR	0.049	4.833
ER	NR	0.034	7.184
SR	NR	0.052	4.528

ER = Eastern Region, SR = Southern Region, NR = Northern Region

Table 4.11: F-statistics and gene flow at the eight polymorphic microsatellite loci across the populations.

Locus	F_{IS}	F_{IT}	F_{ST}	N_M
Aef4	0.329	0.352	0.034	7.072
Aef24	0.704	0.719	0.051	4.658
Aef27	0.653	0.679	0.076	3.022
Aef104	0.563	0.611	0.110	2.024
Aef109	0.426	0.452	0.044	5.406
Aef112	0.784	0.801	0.079	2.903
Aef115	0.498	0.514	0.031	7.702
Aef133	0.575	0.594	0.046	5.137
Mean	0.563	0.589	0.059	3.980

F_{IS} = the deficiency or excess of average heterozygotes, F_{IT} = the deficiency or excess of average heterozygotes in a group of populations, F_{ST} = the degree of gene differentiation among populations in terms of allele frequencies.

4.6.2 Estimation of genetic admixture in the three populations investigated.

One of the methods used to estimate the genetic admixture in the populations was structure analysis. To identify genetic admixture, the structure software was used to assign studied individuals to the inferred populations so that the genetic structure of the populations in a study can be analysed. Analyses were performed using the admixture model with correlated allele frequencies. Figure 4.6 shows the clustering assignment of the 90 animals representing the three populations with three independent runs, each performed on two to five inferred clusters. All analyses used a burn-in and iterations of 10,000. The value of K = 2 (Figure 4.6) was chosen as this showed the highest delta K (Table 4.12), as suggested by Evanno *et al*. (2005; Figure 4.7).

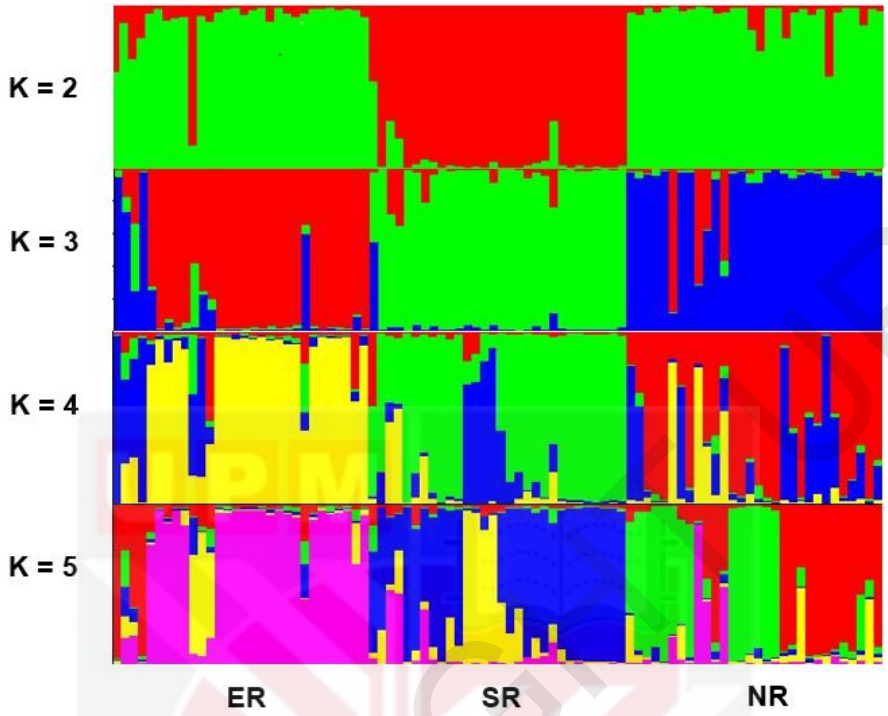

Figure 4.5: Clustering assignment of 90 animals representing the three populations. K = number of clusters. When K (the inferred number of cluster) was two, ER and NR were group into one cluster, while SR was clearly separated into a different cluster. For K=3, the three population were inferred to three clusters.

Table 4.12: Posterior probability values for K in clustering assignment.

K	LnP(D)
2	-3062.6
3	-2941.0
4	-2864.9
5	-2799.3

K= The most probable number of populations, LnP(D) = log-likelihood

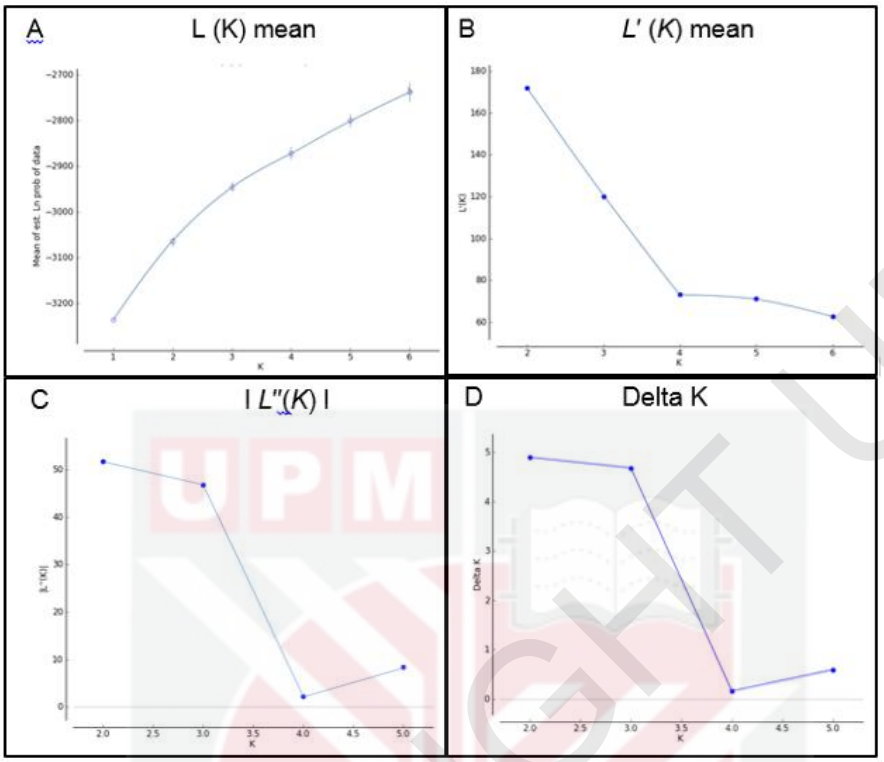

Figure 4.6: Evanno method to detect the real K
(A) L (K); Mean likelihood for each K value over 20 runs. (B) Rate of change of the likelihood distribution (mean) calculated as $L'(K) = L(K) – L(K –1)$. (C) Absolute values of the second order rate of change of the likelihood distribution (mean) calculated according to the formula: $| L''(K) | = | L'(K + 1) - L'(K) |$. (D) ΔK; delta K which calculated as ΔK = mean of $| L''(K) |$ / standard deviation of L(K).

Figure 4.8 displays the two clusters as the two vertices in a triangular distribution corresponding to the animals from the three populations. Cluster 1 represented the SR while Cluster 2 represented ER and NR.

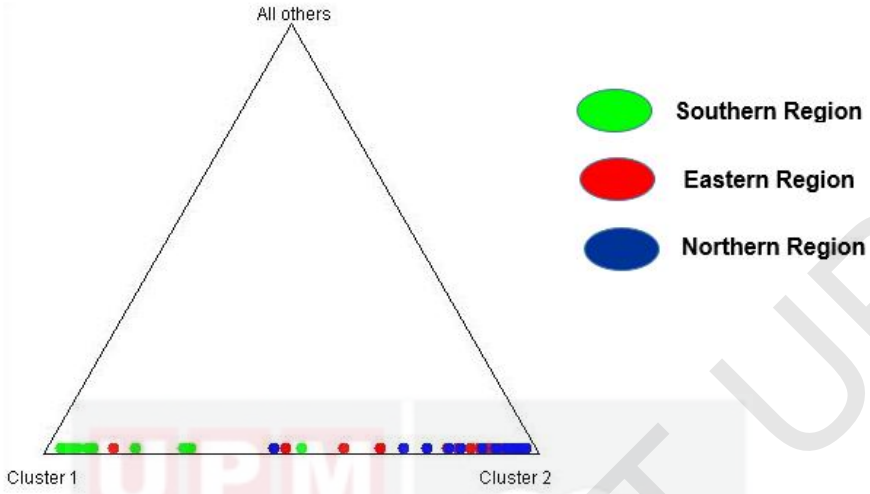

Figure 4.7: Individual proportion of membership (*q*) of three populations of edible nest swiftlets. Each individual is represented by a coloured point (dot). The colour corresponds to the prior population labels. Each of the represents assign to one of the two clusters. Individual who are in one of the corners are assigned exclusively to the cluster concerned. Any points between two vertices exhibit affinities with the two clusters represented by the vertices.

Table 4.13 shows the average proportions of memberships (q) to the two clusters. SR was grouped in cluster 1 (q = 0.94), while ER and NR were grouped in cluster 2 with $q \geq 0.80$.

Table 4.13: Membership of each population in each of the two clusters inferred.

Given population	Proportion of inferred clusters (q)		Number of individuals
	1	2	
ER	0.12	0.88	30
SR	0.94	0.06	30
NR	0.07	0.93	30

ER = Eastern Region, SR = Southern Region, NR = Northern Region

4.6.3 Genetic distance and phylogenetic analyses

Pairwise genetic distances (GD) between the three populations based on 8 investigated microsatellite loci were estimated using Nei's standard genetic distances (1972). The genetic distances among the populations are presented in Table 4.14. The NR and SR were most distantly related (GD = 0.319) and ER was found to be more similar to NR (GD = 0.262) compared to SR (GD = 0.291).

Table 4.14: Genetic distances among three populations

Population	Population	
	Southern Region	Northern Region
Eastern Region	0.291	0.262
Southern Region	****	0.319

Phylogenetic trees were constructed based on Nei's DA genetic distances (1983). Both the NJ and UPGMA methods were used. The robustness of the tree topologies was evaluated with a bootstrap test of 1000 resampling across loci. These processes were conducted using the PopTree2 program.

Figure 4.9 shows an NJ tree based on Nei's DA genetic distances and revealed two clusters. The first cluster consisted of SR, and the second cluster consisted of the ER and the NR.

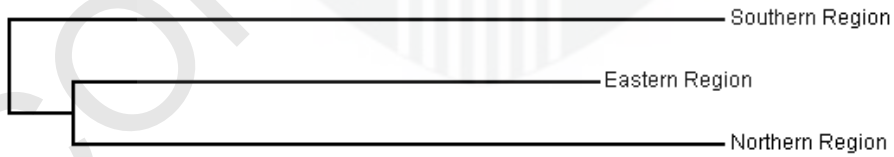

Figure 4.8: Consensus tree generated from 1000 bootstrap values using NJ based on Nei's DA genetic distances.

The UPGMA tree based on Nei's DA genetic distances revealed two clusters (Figure 4.10). ER and NR were grouped as one cluster, while the SR as another cluster.

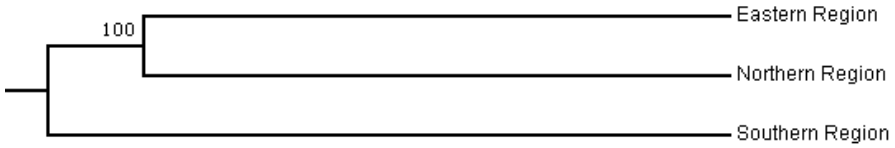

Figure 4.9: Consensus tree generated from 1000 bootstrap values using UPGMA based on Nei's DA genetic distances.

Both phylogenetic analyses revealed the presence of two clusters, ER and NR as one cluster and the SR as another cluster, supporting the result of structure analysis. Bootstrap values for the trees were high for both NJ and UPGMA trees (1000), indicating reliable topology of the phylogeny constructed using Nei's DA genetic distances.

4.7 Molecular Sexing Analysis

4.7.1 Post-mortem findings

In an attempt to obtain samples of male and female swiftlets, post-mortem was conducted on 13 *A. fuciphagus* carcasses to positively confirm the sex of each individual via identification of the sex gonads. From the 13 individual carcasses dissected, the sex gonads could be identified only in three of the carcasses. Based on the distinct testes identified, these three carcasses were confirmed as male *A. fuciphagus* (Figure 4.11) and were used as control (M1, M2 and M3) in the subsequent investigation for molecular sexing.

Figure 4.10: Male *A. fuciphagus* (M1, M2 and M3) determined by post-mortem

4.7.2 PCR product amplification

The results of PCR amplification on thirteen individuals of *A. fuciphagus* for the six different molecular sexing primers developed from the CHD gene region were observed, as shown in the digital images of gels in Figures 4.12 - 4.16. The digital image for sexing primer set 2550F/2718R is not shown as it failed to amplify. From all of the primer sets tested, only one primer set (P8/WZ/W) showed a positive result and was able to differentiate the sex of all thirteen individual *A. fuciphagus* samples. From the results observed in this particular primer set, shown in Figures 4.12 and 4.13, male individuals yielded only a single 255 bp band derived from *CHD-Z*, while female individuals showed an additional 144 bp band derived from *CHD-W*. From thirteen individuals investigated using this particular marker, only two individuals were detected as female. This primer set had been selected for further analyses.

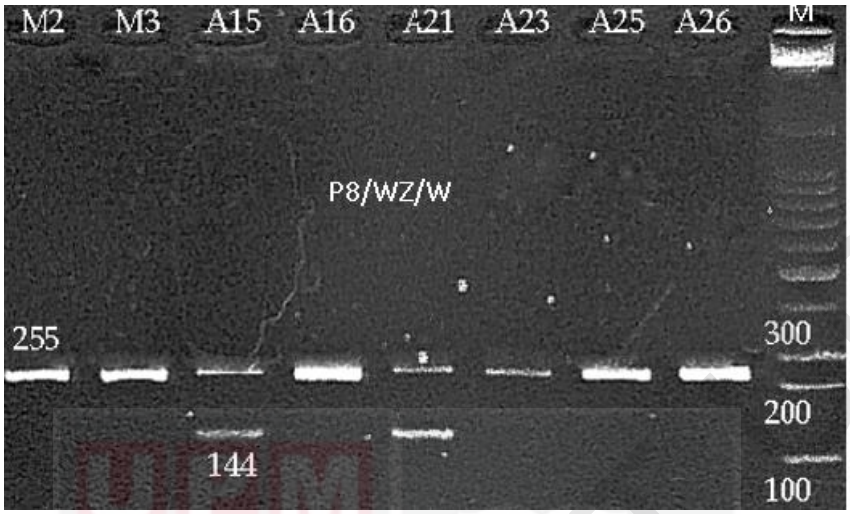

Figure 4.12: Agarose gel showing PCR amplicons from an optimized PCR composition and protocol by P8/WZ/W sexing primer set for eight swiftlet samples (M2, M3: control male 2 and 3; A15, A21: possible female; A16, A23, A25 and A26: possible male; M: 100 bp DNA ladder).

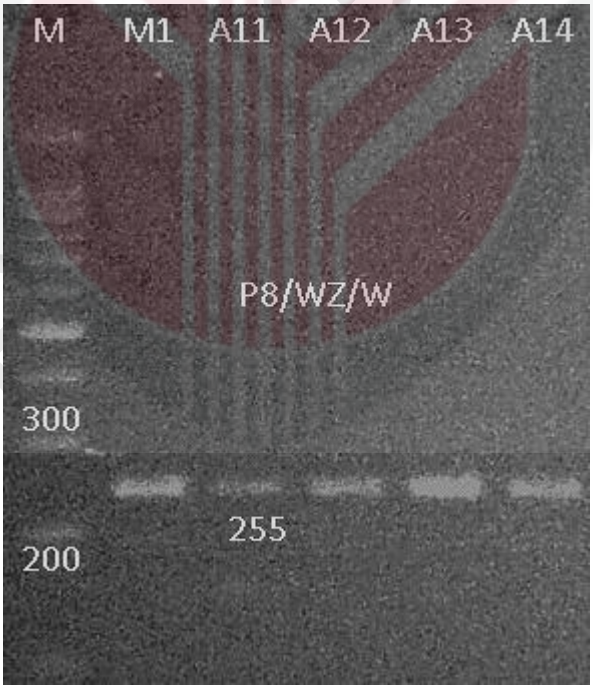

Figure 4.13: Agarose gel showing PCR amplicons from an optimized PCR composition and protocol by P8/WZ/W sexing primer set for five swiftlet samples (M1: control male 1; A11, A12, A13, A14: possible male; M: 100 bp DNA ladder).

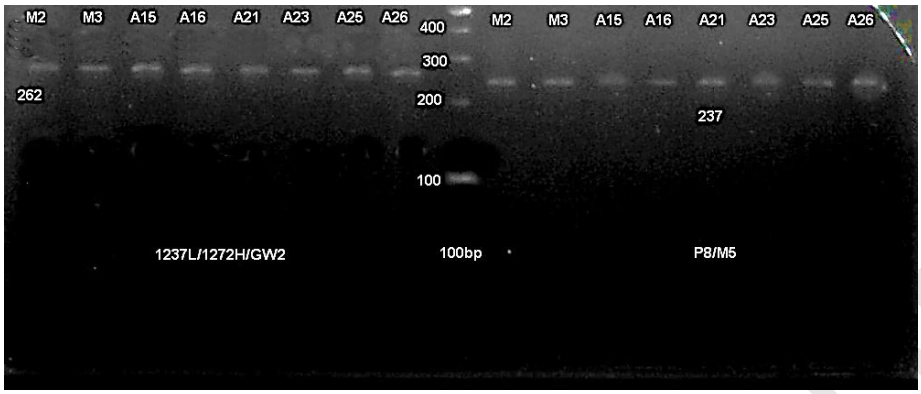

Figure 4.14: Agarose gel showing PCR amplicons from optimized PCR compositions and protocols by two sexing primer sets for eight swiftlet samples (M2, M3: control male 2 and 3; A15, A21: possible female; A16, A23, A25 and A26: possible male; 100bp: 100 bp DNA ladder; 1237L/1272H/GW2 and P8/M5: sex primer).

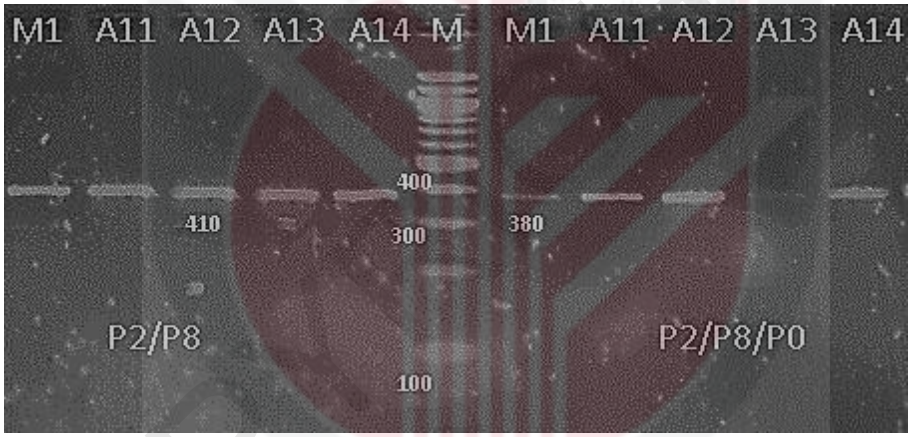

Figure 4.15: Agarose gel showing PCR amplicons from optimized PCR compositions and protocols by two sexing primer sets for five swiftlet samples (M1: control male 1; A11, A12, A13, A14: possible male; M: 100 bp DNA ladder; P2/P8 and P2/P8/P0: sex primer).

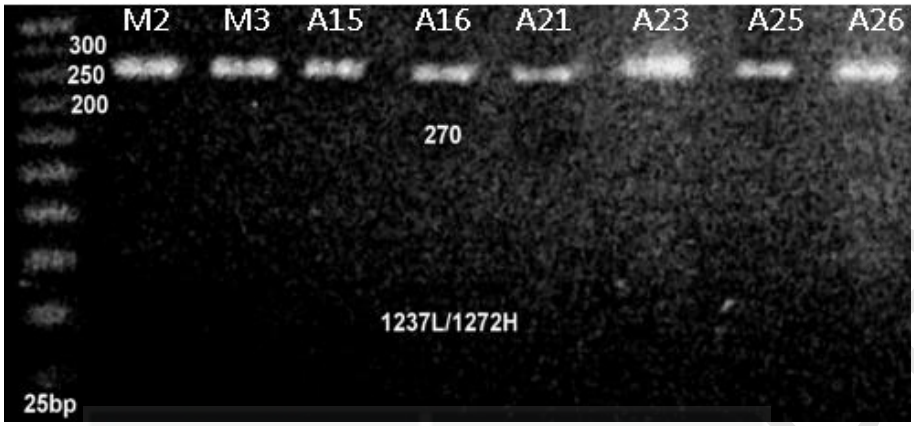

Figure 4.16: Agarose gel showing PCR amplicons from an optimized PCR composition and protocol by 1237L/1272H sexing primer set for eight swiftlet samples (M2, M3: control male 2 and 3; A15, A21: possible female; A16, A23, A25 and A26: possible male; 25bp: 25 bp DNA ladder).

4.7.3 Gel purification and sequencing

The purification of the PCR products for the P8/WZ/W primer set had been done from either PCR product (for male individuals) or the gels (for female individuals). A total of five samples had been selected consist of two possible females (A15 and A21), two possible males (A16 and A23) and one control, M3 (male sample determined by post-mortem). The results of sequences confirmed that A15 and A21 as female due to the present of CHD-W (CHD gene region in the sex chromosome W) and CHD-Z (CHD gene region in the sex chromosome Z) genes. A16, A23 and M3 had been confirmed as male due to the presence of the CHD-Z gene only. The sequence region was 255 bp for *CHD-Z* and 144 bp for *CHD-W*.

4.7.4 PCR product nucleotide sequence alignment

Nucleotide sequence alignment of the partial sequence of the amplified CHD gene showed similarity within both *CHD-Z* and *CHD-W* regions of *A. fuciphagus* are shown in Appendix D (1 – 2). Percentages of similarity within the same *CHD-Z* region are presented in Table 4.15. One male sample (A23) showed variation in base sequence from the other two male samples (M3 and A16) with 99% similarity, while M3 and A16 showed 100% similarity between each other. There was no variation in base sequence within the same sex for female samples (A15 and A21).

Table 4.15: Percentage of similarity between males (*CHD-Z*) of *A. fuciphagus*

Animal ID		Percentage (%)
A16	A23	99
A16	M3	100
A23	M3	99

4.7.5 Comparison of sex evolution between *A. fuciphagus* and other avian species.

The sex evolution of *A. fuciphagus* in the present study with respect to other avian species was investigated based on the percentage of similarity within the CHD gene loci of *A. fuciphagus* compared to 14 other avian species. These 14 avian species were chosen because they were either members of the same Apodiformes order or Aves class.

The nucleotide sequences for *A. fuciphagus* (M3 for *CHD-Z* and A15 for *CHD-W*) were aligned and compared with both the *CHD-Z* and *CHD-W* regions of other birds. Male *A. fuciphagus* showed the percentage of similarity ranged from 74 to 98% with other male birds (*CHD-Z*) of the Apodiformes order or Aves class (Table 4.16). Female (*CHD-W*) *A. fuciphagus*, on the other hand, showed the percentage of similarity with other female birds of the Apodiformes order or Aves class ranging from 83 to 100% (Table 4.17).

Table 4.16: Percentage of nucleotide similarity between male *A. fuciphagus* with other birds of the same Apodiformes order or Aves class.

Reference Male	Species and Common Name	Percentage (%)	Order
A. fuciphagus	*Apus apus* (Common swift)	98	Apodiformes
A. fuciphagus	*Calypte costae* (Costa's hummingbird)	80	Apodiformes
A. fuciphagus	*Pernis ptilorhynchus* (Crested honey buzzard)	82	Accipitriformes
A. fuciphagus	*Accipiter gularis* (Japanese sparrow hawk)	81	Accipitriformes
A. fuciphagus	*Accipiter soloensis* (Chinese sparrow hawk)	81	Accipitriformes
A. fuciphagus	*Circaetus gallicus* (Short-toed snake eagle)	81	Accipitriformes
A. fuciphagus	*Sylvia undata* (Dartford warbler)	76	Passeriformes
A. fuciphagus	*Pyrrhocorax pyrrhocorax* (Red-billed chough)	77	Passeriformes
A. fuciphagus	*Erythura gouldiae* (Gouldian finch)	76	Passeriformes
A. fuciphagus	*Erithacus rubecula* (European robin)	75	Passeriformes
A. fuciphagus	*Coturnix coturnix* (Common quail)	74	Galliformes
A. fuciphagus	*Coturnix chinensis* (King quail)	75	Galliformes
A. fuciphagus	*Columba livia* (Rock dove)	81	Columbiformes
A. fuciphagus	*Columba pulchricollis* (Ashy wood pigeon)	80	Columbiformes

Table 4.17: Percentage of nucleotide similarity between female *A. fuciphagus* with other birds of the same Apodiformes order or Aves class.

Reference Female	Species and Common Name	Percentage (%)	Order
A. fuciphagus	*Apus apus* (Common swift)	100	Apodiformes
A. fuciphagus	*Gyps tenuirostris* (Slender-billed vulture)	91	Accipitriformes
A. fuciphagus	*Gyps bengalensis* (White-rumped vulture)	91	Accipitriformes
A. fuciphagus	*Accipiter virgatus* (Besra)	91	Accipitriformes
A. fuciphagus	*Lanius cristatus* (Brown shrike)	87	Passeriformes
A. fuciphagus	*Pyrrhocorax* (Red-billed chough)	87	Passeriformes
A. fuciphagus	*Sylvia undata* (Dartford warbler)	86	Passeriformes
A. fuciphagus	*Erithacus rubecula* (European robin)	83	Passeriformes
A. fuciphagus	*Egretta garzetta* (Little egret)	83	Pelecaniformes
A. fuciphagus	*Ixobrychus cinnamomeus* (Cinnamon bittern)	83	Pelecaniformes
A. fuciphagus	*Nycticorax nycticorax* (Black-crown night heron)	83	Pelecaniformes
A. fuciphagus	*Ardea alba* (Great egret)	83	Pelecaniformes
A. fuciphagus	*Rallus aquaticus* (Water rail)	87	Gruiformes
A. fuciphagus	*Porzana porzana* (Spotted crake)	87	Gruiformes

The NJ tree based on Nei's DA genetic distances illustrates the genetic relationship between male *A. fuciphagus* and male avian of the same Apodiformes order (Figure 4.17). The tree separates into two different clusters, with the first cluster consisting of *Apus apus* (common swift) and *A. fuciphagus*, while *Calypte costae* (Costa's hummingbird) was presented in the other cluster. This indicated that even though *Calypte costae* was in the same Apodiformes order as *A. fuciphagus*, it had a low percentage of nucleotide similarity (80%). However, *Apus apus* that was also in the same order showed a high percentage of nucleotide similarity (98%) with *A. fuciphagus*. This may be the reason for the separation of Apodiformes order into two clusters in males (*CHD-Z*).

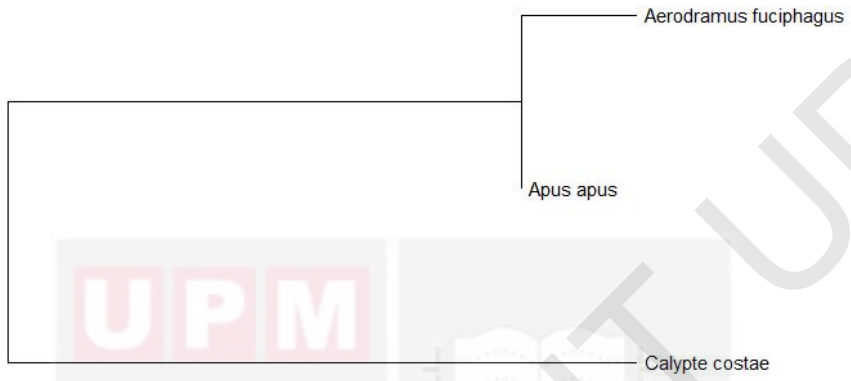

Figure 4.17: Neighbour-joining tree based on DA genetic distances showing the genetic relationship between male *A. fuciphagus* and male birds of the same Apodiformes order.

Figure 4.18 shows the NJ tree based on Nei's DA genetic distances illustrating the genetic relationship between male *A. fuciphagus* and male avian of the same Aves class. The total of five different orders (Apodiformes, Galliformes, Columbiformes, Acciptriformes and Passeriformes) had been selected and revealed four different clusters. The first cluster consisted of Galliformes, Columbiformes, Accipitriformes and Apodiformes order, the second cluster consisted of Apodiformes order, while the third cluster contained Passeriformes order. Interestingly, the phylogenetic tree separated Apodiformes into two different clusters with the *A. fuciphagus* and *Apus apus* in one cluster and *Calypte costae* was in the other cluster together with birds from Galliformes, Columbiformes and Accipitriformes order.

This indicated that *Calypte costae* nucleotide sequence at the CHD-Z gene was closer to birds from Galliformes, Columbiformes and Accipitriformes order rather than to the birds from the same Apodiformes order. Passeriformes order was separated in one cluster of its own, indicating that the nucleotide sequence at the CHD-Z gene for the birds of this order is significantly different from birds of other orders.

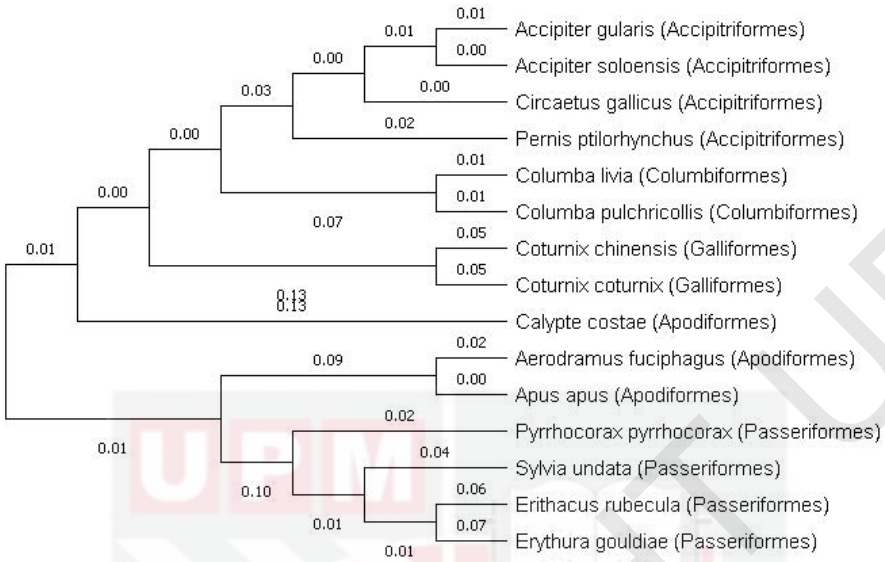

Figure 4.18: Neighbour-joining tree based on DA genetic distances showing the genetic relationship between male *A. fuciphagus* and male birds of the same and different order (Apodiformes, Accipitriformes, Columbiformes, Galliformes and Passeriformes).

Figure 4.19 shows the NJ tree based on Nei's DA genetic distances illustrating the genetic relationship between female *A. fuciphagus* and female avian of the same Aves class. The total of five different orders (Apodiformes, Gruiformes, Pelecaniformes, Acciptriformes and Passeriformes) had been selected and revealed four different clusters. The first cluster consisted of the Passeriformes order, the second cluster consisted of Pelecaniformes order, the third cluster consisted of Apodiformes order, while the fourth cluster consisted of Acciptriformes and Gruiformes order. This result indicated that nucleotide sequences at the CHD-W gene for birds from Acciptriformes and Gruiformes order were closer and can be grouped into the same cluster. Meanwhile, the other three orders (Apodiformes, Passeriformes and Pelecaniformes) were separated as one cluster of their own, indicated that the birds in the same order had a high similarity or the same nucleotide sequence at CHD-W gene.

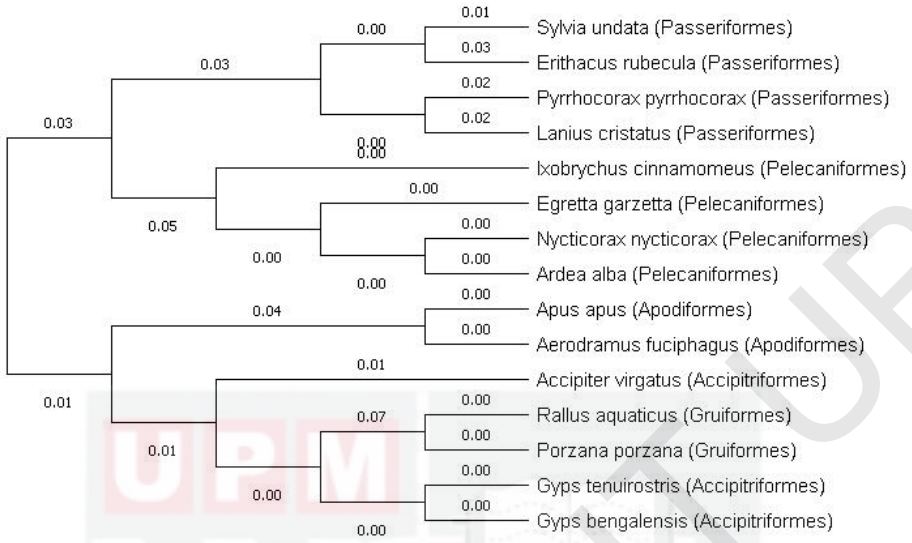

Figure 4.19: Neighbour-joining tree based on DA genetic distances showing the genetic relationship between female *A. fuciphagus* and female birds of the same and different order (Apodiformes, Passeriformes, Pelecaniformes, Accipitriformes and Gruiformes).

CHAPTER 5

DISCUSSION

5.1 Polymorphism Screening using DNA Pooling

In the present study, an initial screening for polymorphism at the microsatellite loci was performed using DNA pools. DNA samples were pooled with nine samples per pool. DNA pooling is an efficient and useful tool in population genetic studies as shown by the results. It saves the cost and time for identifying the polymorphic markers for further investigation. In addition, PCR and electrophoresis using DNA pools allowed rapid estimation of the allele size range, even though the specific allele sizes in some cases could not be accurately estimated. This is useful when there is a need for designing the multiplex system. Screening for polymorphic markers at the microsatellite loci was carried out using PAGE. The advantages of using PAGE as a method for screening are the ability to produce sharp bands and good separation for low molecular weight fragments (Barril & Nates, 2012). Sánchez Pérez *et al*. (2006) reported that PAGE was among the most efficient method and can resolve allelic variation at a fine scale (2 bp).

5.2 Genetic Diversity at the Microsatellite Loci

In the present study, eight microsatellite loci which had been identified as polymorphic and suitable for swiftlet were used. The total number of alleles (TNA) observed in the three populations was 102 (sum for the eight microsatellite loci). It was slightly higher than that reported for the ten colonies of *A. fuciphagus* in Thailand (TNA = 95) where 160 animals were screened for the same eight microsatellite loci (Aowphol *et al*., 2008). Aowphol *et al*. (2008) concluded that the populations studied from the Gulf of Thailand and the Andaman Sea were grouped in a single panmictic population where all individuals are potential partners with the same species origin, Germain's Swiftlet (*A. germani*). Furthermore, it may also be due to the fact that unequal and small sample size had used in the study of ten colonies of *A. fuciphagus* in Thailand ranging from three to 28 samples collected per colony (Aowphol *et al*., 2008), while the samples collected in this study were taken from four to five different colonies for each region with almost the same amount of sample size (n = 6 - 8). It is known that TNA is usually affected by the sample size (Allendorf & Luikart, 2007). In addition, there is a possibility of hybrid species in Malaysia as suggested by Cranbrook *et al*. (2013) that discovered the origin of swiftlet from Peninsular Malaysia is a combined genetic component from the north (*A. inexpectatus* germani, *A. inexpectatus* subsp.) and south (*A. fuciphagus*), which probably increase TNA in Peninsular Malaysia populations.

The number of alleles per locus in the present study ranged from 11 to 15, with a mean of 13 alleles per locus. For the Thailand colonies, the number of alleles per locus ranged from 9 to 18, with a mean of 11.88 (Aowphol *et al*., 2008). In general, the high number of alleles for the individual loci showed that the choice of using microsatellite markers for the present genetic diversity study was a wise decision. It also confirmed that the microsatellite loci screened were highly informative and suitable for genetic diversity evaluation in swiftlet.

5.3 Genetic Diversity within Populations

In order to evaluate the genetic diversity within populations of *A. fuciphagus*, a few analyses were performed using the allelic variation obtained at the eight microsatellite loci. The results were compared with those reported for *A. fuciphagus* from other countries. The MNA is considered as a good indicator of genetic variation. Low MNA indicates low genetic variation. This may be due to genetic isolation, historical bottleneck or founder effect. In contrast, high MNA indicates high allelic diversity, which could be influenced by crossbreeding or an admixture (Ojango *et al*., 2011).

The results of the present study showed that the Southern region exhibited the lowest MNA (7.1) among the three populations, followed by the Eastern region (8.5) and the Northern region (11.1). It may be because the earliest record of *A. fuciphagus* started to build the habitat in man-made buildings occurred during the late 1940's in Penang, Perak and Kuala Lumpur (Anuar, 2011). Othman *et al*. (2008), also reported that higher numbers of birdhouses were recorded on the west coast (n = 5788) compared to the east coast (n = 1627) in Peninsular Malaysia. Therefore, it is expected that population sizes in the Northern region (located on the west coast of Peninsular Malaysia) are far larger than any other regions in Peninsular Malaysia, allowing a bigger gene pool.

The MNA values observed in the present study were higher than those reported from Riau, Indonesia for colonies at Belilas (7.7) and Airmolek (7.0) (Hendra *et al*., 2014). This may be attributed to the differences in sample sizes used in the study. The sample sizes of the *A. fuciphagus* used in Riau (n = 19) were far lower than those used in the present study (n = 90).

When compared with the MNA values reported for 10 colonies (3.63-9.63) from Thailand (Aowphol *et al*., 2008), the values observed in the present study were still slightly higher. However, this may be related to the unequal sample sizes of the colonies investigated in Thailand despite the fact that the total number of the samples were higher (n = 160) compare to the present study (n = 90).

The MNA values observed in the present study for *A. fuciphagus* were higher than reported for both endangered bird and candidate for endangered bird species such that reported by Nims *et al*. (2008) on Galapagos penguin

(*Spheniscus mendiculus*) and Davis *et al*. (2015) on Greater Sage-Grouse (*Centrocercus urophasianus*). The MNA values of endangered species by Nims *et al*. (2008) on Galapagos penguin (*Spheniscus mendiculus*) using five microsatellite primers for 116 adults' individuals from five subpopulations across the Galapagos ranged from 2.4 to 2.8. Another bird species, Greater Sage-Grouse (*Centrocercus urophasianus*), is listed as one of the species being concerned due to the declined in distribution and abundance of it, making it a candidate species protected under U.S Endangered Species Act. Davis *et al*. (2015), reported the MNA values of Greater Sage-Grouse (*Centrocercus urophasianus*) ranged from 4.6 to 7.3 from eight lakes in northeastern California, USA.

The average heterozygosity is the best general measure of genetic variation (Allendrof & Luikart, 2007). High heterozygosity values could be attributed to long-term natural selection for adaptation in heterozygous forms, due to the mixed nature of the breeds or species, or due to historic mixing or gene flow between different populations. On the other hand, low heterozygosity could be attributed to isolation and inbreeding, result in subsequent loss of unexploited genetic potential (Ojango *et al*., 2011).

The results of the present study showed that the Southern region had the lowest H_o (0.28) and H_e (0.79) among the populations studied. The Eastern region had the highest values of H_o (0.44), while, the Northern region had the highest H_e (0.87). The mean values of H_o were lower than the mean values of H_e for all three populations, indicating a heterozygous deficiency.

The *A. fuciphagus* in this present study thus seems to have a moderate amount of genetic variation. The H_o in this present study was, however, lower than that reported for ten colonies in Thailand (0.67 - 0.98; Aowphol *et al.*, 2008) and Riau, Indonesia (0.67; Hendra *et al*., 2014). The larger sample size (n = 160) and the larger swiftlet population size of Thailand would allow a bigger gene pool, thus contributing to the high average heterozygosity observed. In the case of the Riau population, the sample size investigated (n = 19) was smaller than the present study, and only three microsatellite loci were screened. The fact that Indonesia has a bigger population of *A. fuciphagus* than Malaysia may be a key factor for the high average heterozygosity observed. Furthermore, some colonies in Thailand were represented by two (SK1 and SK2) to three (CP1, CP2 and CP3) different birdhouses, while samples from Riau were from two different birdhouses (Airmolek and Belilas). In the present study, the samples were taken from four to five birdhouses per population so that each population were equally represent and minimise the relationship between samples (FAO, 2011).

The lack of heterozygosity in a population shown by the F_{IS}. A large positive value of F_{IS} reflects the existence of a high homozygosity, which reflects inbreeding. A small value indicates that heterozygote genotype is abundant compared to homozygote genotype and a negative value implies an

outbreeding. In the present study, the mean values of F_{IS} were large and positive values were found for all three populations, which indicate inbreeding in all the populations. This result suggests the inbreeding as one of the factors contributing to the low heterozygosity observed in all populations. A high level of inbreeding was observed in Southern region (0.636) followed by Northern region (0.590) and eastern region (0.465). For a bird categorized as a colonial nester (Maisarah & Hafidzi, 2017), it is possible that inbreeding may occur, affecting the heterozygosity of the species.

The F_{IS} values observed in the present study were higher than those reported for two colonies from Riau, Indonesia (0.229; Hendra *et al*., 2014). However, in the study, the populations were only screened using three from eight microsatellites used in the present study, and the sample sizes also were lower (n = 19). Moreover, the history of swiftlet ranching in Indonesia had been started as early as in 1880 at Sedayu, East Java (Lim & Cranbrook, 2002) as compared to Peninsular Malaysia. An important advance in Java due to the discovery of the foster parenting method by successfully transferring the eggs of birdhouses swiftlets to Linchi swiftlet (*Collocalia linchi*) nests (Lau & Melville, 1994) had helped for increasing the harvestable population of *A. fuciphagus* in Indonesia. Furthermore, the sources of eggs in the foster parenting method may come from several different populations which had lessened the inbreeding occurrence in particular birdhouses in Indonesia that practised the foster parenting method. Although there was no mentioning of the foster parenting method being practised in the Riau colonies, the method might has been practised in these colonies due to the widely practised of foster parenting method in Indonesia. All of the factors had contributed to the low inbreeding occurrence in Indonesia populations as compared to the results of the present study in Malaysia.

Inbreeding generally occurs because of small population size (Allendorf & Luikart, 2007), small founder population and selective/non-random mating behaviour of the species. In the present study, to avoid the possibility of getting high F_{IS} due to error in the design of sampling, samples were randomly collected from four to five colonies (birdhouses) from each region. Embryos or eggs were not collected from the same nest to avoid the sample material originating from the same parents.

As mentioned earlier in Chapter 2, *A. fuciphagus* are known as permanent or long-lived monogamous breeders and have been reported to have high nest site fidelity (Viruhpintu *et al*., 2002). This may lead to inbreeding due to the high chance of mating among related individuals or incestuous pairs. Incestuous pair frequently resulted when the birds did not disperse from their natal or breeding territories (Hidalgo *et al*., 2016). Territorial species with continuous partnerships such as in *A. fuciphagus* will lead to limited vacancies for partner choice and dispersal opportunities. This is one of the factors that may contribute to the inbreeding in this present study. Furthermore, there was no record of foster parenting practices in Peninsular Malaysia, indicating that the

development of the colonies may be depended entirely on two major factors, which are birdhouse location and suitable micro-environment (Idris *et al*., 2014).

Birdhouses are generally constructed in an area highly populated by swiftlets (Idris *et al*., 2014). *A. fuciphagus* is known to have a high nest site fidelity as mentioned earlier. Usually, the pairs will occupy the same places or sites every time they build the nests. However, as the number of birds in a colony increases over time, the birdhouse becomes crowded and eventually causes discomfort due to the high competition for breeding and nest building. The swiftlet chicks will then search for new homes nearby. This was supported by the presence of at least one or more swiftlet houses near the birdhouses from which the samples were selected for the present study. It was for this reason that it was ensured that the colonies that represented for each region were not neighbouring birdhouses.

The other factor that determined the selection of locations for birdhouses was feeding areas. In the present study, the birdhouses that represented the populations in the Southern region were generally situated in urban areas, whereas for the populations in the Northern and Eastern regions these were mostly in rural areas. The study conducted by Manchi & Sankaran (2010) showed that EBN swiftlets were more active in forested areas. Furthermore, it has been reported that rural and agricultural areas supported more food sources for birds, especially insects (Lourie & Tompkins, 2000). This may explain the reduction in the production of nests faced by ranchers from the Southern region; there are decreased of feeding areas while the competitions of birdhouses are abundant. This situation would lead to a decrease in population sizes and in turn would increase inbreeding within the colonies. This may explain the high F_{IS} value for the Southern region as compared to the Northern and Eastern regions. That may also explain the result reported by Othman *et al*. (2008) that the number of birdhouses in urban areas had started to decrease, but was gradually increased in the rural areas.

The suitable environment of birdhouses is determined by four main components, which are the air temperature, relative humidity, air velocity and light intensity (Ibrahim *et al*., 2009). The most common problem experienced by the farmers is to maintain the cave-like environment within birdhouses. The birdhouses selected for the present study also had the same problem, especially the air temperature, which was mostly higher than recommended (26°C to 35°C). High air temperature will trigger an uncomfortable environment for the swiftlet and at the same time cause damage to swiftlet eggs. This will affect the performance of the swiftlet colonies and finally cause a decrease in population. Therefore, the small population sizes in some of the colonies sampled, especially in the urban areas, would probably have led to inbreeding in the populations. Furthermore, as the swiftlets are actually wildlife, and ranchers usually only provide the places for the birds to make their nests, there is no pedigree records or breeding programs, making it impossible to identify the genetic relationship between the birds in a particular colony or to control the occurrence of inbreeding.

Hardy–Weinberg equilibrium (HWE) will be achieved in a population if the genotype frequencies remain constant throughout generations (Ojango *et al*., 2011). In the present study, the deviation from HWE was observed in all three populations (eight loci in the Northern region, seven loci in the Eastern region and six loci in the Southern region). The overall number of loci that deviated from HWE was higher compared to that reported for ten colonies of *A. fuciphagus* in Thailand using the same eight microsatellite loci (Aowphol *et al.*, 2008) where only one locus (Aef109) showed significant deviation from HWE. In general, the low genetic diversity observed in the present study in terms of heterozygote deficiency and deviation from HWE could be attributed to many reasons, but the most probable reasons are inbreeding, small population sizes and assortative mating.

It is known that related individuals probably will be sharing the common alleles by descent. In the present study, inbreeding occurrence due to the high chances of mating among related individuals in a colony of *A. fuciphagus* may contribute to the increase in the frequencies of homozygous for the loci concern and decrease in heterozygotes frequencies, leading to the deviations from the expected HWE frequencies. The chance of some alleles becoming fixed will also increase together with a chance of some alleles to be lost permanently; reducing the TNA.

Another explanation for the observed low genetic diversity may be the small population sizes that occurred due to genetic drift, founder events and Wahlund effect. Genetic drift causes random changes in allele frequencies from generation to generation because of sampling (Allendorf & Luikart, 2007). It occurs in all populations with finite size, but small populations suffer the strongest effect. In small populations, random changes in the frequencies of alleles are possible when sampling of gametes and fertilization to create zygotes occur. The allele frequencies will become unpredictable in direction and amount where they may result in loss of some alleles (including beneficial ones) and the fixation, or significant increase in other alleles. This has resulted in a deviation from the HWE. The smaller the sampled population, the larger is the deviation. In a small population, a loss of genetic diversity over time across the entire genome due to genetic drift is possible.

Founder effects arise when a small number of individuals of a species from a large population move to a new habitat or location, establishing a new population from a sample of genes found in the original population (Allendorf & Luikart, 2007). The founder population will be subjected to sampling effects which will cause a loss of genetic diversity in the new population, the extent of which is determined by the size of the founder population. Founder effects include loss of alleles and change in allele frequencies, leading to deviation from HWE. However, HWE can be achieved in later generations, if the population size increases and random mating is practiced. In *A. fuciphagus*, migration of a small group of individuals from the large population of a birdhouse may be due to competition for nest sites or attraction to swiftlet song playbacks from a new man-made birdhouse.

The presence of null alleles may also be a contributing factor to low genetic variability. Null alleles are alleles that consistently fail to be amplified by PCR due to the lack of primer annealing sequence (Dakin & Avise, 2004). Due to this, heterozygotes for null alleles appear to be as homozygotes during allele screening using electrophoresis. The presence of null alleles results in an excess of homozygotes relative to the HWE proportions. In the present study, null alleles are unlikely, but not impossible, to be the cause of the low genetic variability in the populations and the observed heterozygous deficiency within populations. The microsatellite loci used for screening genetic diversity in the present study have been chosen based on their use and results reported in a previous study by Aowphol *et al*. (2008) which showed high genetic variability for each locus. Loci suspected to have null alleles would have produced high homozygosity or non amplifications in some samples (those homozygous for the null alleles) and, therefore, would not have been recommended for use.

AMOVA partitions the genetic variation among the populations. In the present study, AMOVA revealed that most of the variation was found within the population (92.9%). This suggests that total genetic variation is corresponded to within the population in *A. fuciphagus*. A similar pattern of variance partitioning has been observed in another study of swiftlets (Hendra *et al*., 2014) where 97.2% of the variation was within the population or a colony.

5.4 Genetic Variation and Relationship between the Populations

The genetic variation between populations of *A. fuciphagus* was evaluated by estimation of F-statistics, gene flow, genetic admixture and genetic distance, as well as by phylogenetic analysis.

F-statistics (F_{IS}, F_{ST}, and F_{IT}) are measures of the deficit of heterozygotes relative to expected HWE proportions in the specified populations (Allendorf & Luikart, 2007). For a large, randomly mating population, it is expected that the H_o would be equal to the H_e, and F_{IS} would be equal or close to zero. In this case, F_{IT} would be approximately equal to F_{ST}. However, when F_{IS} is negative, which implies no inbreeding, F_{ST} would generally exceed F_{IT}. On the other hand, when F_{IS} is positive, implying inbreeding in the population, F_{IT} would exceed F_{ST}.

In the present study F-statistics for each locus and the overall data, including breed pairwise F_{ST} values, were calculated. F_{IS} was positive (0.564) and F_{IT} (0.589) exceeded F_{ST} (0.059), indicating inbreeding in all populations. The level of genetic differentiation among populations measured in terms of F_{ST} was moderate (5.9%). This means that 5.9% of the total genetic variation corresponded to between populations, and 94.1% of the total genetic variation corresponded to within the population. This result supports the AMOVA result for confirming the fact that the total variation corresponded to within population in *A. fuciphagus*. This could be attributed to the fact that *A. fuciphagus* is a

colonial nesting bird (Maisarah & Hafidzi, 2017) and nest site fidelity behaviour (Viruhpintu *et al*., 2002).

The population differentiation indicated a relatively moderate gene flow between the three populations (N_M = 3.98). The highest gene flow (7.18%) was between Eastern region and Northern region populations. The gene flow observed between Eastern region and Southern region (4.83), and Northern region with Southern region (4.53) showed moderate gene flow. This reflects on the genetic differences between Southern Region towards Eastern region and Northern region, supporting the findings based on the F-statistics. The high gene flow observed between Eastern region and Northern region indicates the genetic closeness between these two populations. This could be attributed to the geographical distance between the Eastern region and Northern region, which is relatively closer compared to the distance between the Southern region from both the Eastern region and the Northern region. The source of birds for rapid expansion of the *A. fuciphagus* ranching initially comes from natural caves. However, in recent times birds that colonize new birdhouses are from other established birdhouses or colonies. As mentioned earlier in Section 5.3, the locations usually selected to build new birdhouses are the highly populated swiftlet areas, assuming that the swiftlet chicks in the established birdhouses will search for new homes when their homes become crowded and there is lack of spaces for nest building. The migration that occurs may contribute to the gene flow observed in this study. Furthermore, the transfer of eggs from established birdhouses to new birdhouses had been practiced by some swiftlet ranchers in Malaysia (Cranbrook *et al*. 2013). The egg transfers are more likely to be to the new birdhouses at a shorter geographical distance from established birdhouses to ensure hatchability and survivability.

The genetic admixture in the three different populations investigated was estimated using structure analysis. The results of the structure analysis showed that the studied populations were split into two clusters: Southern region in one cluster, whereas Northern region and Eastern region in the other cluster. These results are concordant with the F-statistics and gene flow results. This result also agreed with the findings of Cranbrook *et al*. (2013) and Goh *et al*. (2018) on the taxa clustering by using genetic approaches (mtDNA). Cranbrook *et al*. (2013), discovered that the origin of swiftlet in Peninsular Malaysia had combined genetic components from Germain's Swiftlet (*A. inexpectatus germani*) from the north and Thunberg's Swiftlet (*A. fuciphagus*) from the south. Goh *et al*. (2018), on the other hand, separated birdhouses swiftlets into two different clades with Clade 1 representing the majority of birdhouses swiftlets across Peninsular Malaysia and Borneo, and Clade 2 as the less abundant swiftlets, predominantly found in Mentakab and Bentong, Pahang, Peninsular Malaysia. The results of the present study and those of Goh *et al*. (2018) and Cranbrook *et al*. (2013) have justified the original conclusion by Chasen (1935) by summarizing the genetic evidence that on ecological grounds, there are two types of *Aerodramus* swiftlets making white-nests that can be separated based on habit. The first species is the grey-rumped swiftlet (*A. inexpectatus*) which frequently occupied islands, maritime cliffs and coastal stacks, from the Andaman to the islands of Vietnam, while the second species is the brown-

rumped swiftlet (*A. vestitus*), occupying the inland caves and grottoes in Sumatra and Borneo as well as both inland and coastal nesting sites in Java and Nusa Tenggara Barat, Indonesia (Chantler, 2005). However, Goh *et al*. (2018) could not confidently identified birdhouse swiftlets into either of the two wild white-nest species based only on their mitochondrial genetic evidence. This is also due to the undisputed historical evidence that swiftlet ranching in birdhouses began in Java, Indonesia, where *A. fuciphagus* is the only wild white-nest species. It may be that, contrary to the conclusions of this present study as well as both Cranbrook *et al*. (2013) and Goh *et al*. (2018), that all birdhouse populations have descended from these two common origins (*A. inexpectatus* and *A. vestitus*). The results from the present study can only conclude that the population of the Northern region and Eastern region are closely related in terms of genetic similarity that may be due to the high gene flow between them. There are opportunities for genomic research using NGS, which may confirm the origin of birdhouse swiftlets, especially in Malaysia.

Genetic distances between the three populations based on the eight microsatellite loci were estimated using Nei's standard genetic distances (DS) (Nei, 1972). A phylogenetic tree was constructed based on Nei's DA genetic distance (Nei *et al*., 1983), an allele frequency-based procedure that assumes that IAM holds true. Both the NJ method and the UPGMA method were used.
The phylogenetic trees constructed from the NJ method and UPGMA method, based on Nei's DA genetic distances were both consistent with the historical information on the original pioneers drawn from at least two species; Grey-rumped swiftlet (*A. inexpectatus germani*) and Thunberg's Swiftlet (*A. fuciphagus*). The phylogenetic trees further confirmed the clusters identified by the structure analysis. Generally, the accuracy of the phylogenetic tree is confirmed by bootstrap values; nodes with high bootstrap values (above 0.70) are considered significant, whereas nodes with low values (below 0.50) are considered not significant. The tree topologies generated in the present study were confirmed by the relatively high bootstrap value (1) in the UPGMA method.

Finally, two limitations need to be acknowledged and addressed regarding the present study. The first limitation concerns the number of microsatellite markers investigated in the present study, which is only eight polymorphic microsatellite loci compared to the recommended optimum number of loci which ranged from over 30 (Takezaki & Nei, 1996) to approximately hundreds (Pollack *et al*., 1998) to obtain accurate evolutionary inference. For genetic characterisation of animal genetic resources, the Food and Agriculture Organisation of the United Nations (FAO) recommends the use of 30 microsatellite markers (FAO, 2011). The International Society for Animal Genetics (ISAG)–FAO Advisory Group on Animal Genetic Diversity has proposed panels of 30 microsatellite markers for nine major livestock species for this purpose (FAO, 2011). However, the only avian species for which a panel is recommended is the chicken. There is a lack of reported microsatellite markers for swiftlets. Time constraint and cost to design new primers were issues of concern in the present study. This is a common problem faced in molecular characterisation of wild animal populations. Koskinen *et al.* (2004), summarized the number of microsatellite markers used in studies involving wild

animal species and reported a median of 6 microsatellite markers. This was based on 89 population genetic studies reported in the journals Evolution (vol. 53-54), Heredity (vol. 82-85) and Molecular Ecology (vol. 8-9). This suggests that despite the recommended theoretical guidelines, practically the assessments of even 30 microsatellite loci are difficult to achieve due to various limiting factors such as already mentioned. In the present study, the proposed method of identifying microsatellite markers through cross-species amplification of primer sets previously developed for swallows had proved to be not too successful in *A. fuciphagus.* Only four out of the 20 markers investigated were amplified successfully and all four of these markers were monomorphic, which could not be used for further analysis. The second limitation of concern is the sample sizes, only 30 samples were collected from each population. This choice of sample size is related to the fact that the samples collected were from birdhouses; permission was needed from the swiftlet ranchers to enter and collect the samples. The concern of majority of the ranchers was disturbance caused by the people entering the birdhouses may affect the colony performance, population and health. This had restricted the number of birdhouses that could be visited to collect the samples. Furthermore, the birdhouses are regularly cleaned by the ranchers, making it difficult to obtain any swiftlet carcasses or feathers on the floor of the houses during our visits for sample collection.

5.5 Molecular Sexing

Thirteen carcasses were collected from Perak to provide samples for molecular sexing in the present study. Attempt was made to determine the sex of the dead birds *via* post-mortem identification of the sex gonads. However, of the 13 carcasses, only three could be successfully identified as male birds based on the presence of identifiable testes. Feather samples of these three carcasses were used as a control in this study (M1, M2, M3). The reason for a failure to determine the sex of the other carcasses may be due to the various stages of decomposition of the collected carcasses. The carcasses were collected during the visits of the research team to the birdhouses. Alternatively, the carcasses may be of juvenile individuals in which the sex organs may not yet be fully developed.

Feather samples collected from 10 unknown sex of dead chicks or chicks that had dropped from their nests at the breeding colonies were used as candidate samples to screen for the sexing markers. Whereas the three confirmed male samples were served as controls. Hence, the electrophoresis banding patterns for the 10 candidate samples (unknown sex) were compared to those of the controls. Samples showing similar patterns as the confirmed males were identified as male individuals and samples showing banding patterns that differed from the controls were assumed to be female individuals. DNA for all samples were from feathers, so as to standardise the methodology. Hence, shows that even though the source of the DNA would not matter or affect the result.

The first W-linked gene discovered (Griffiths & Tiwari, 1995), identified later as a gene encoding for a chromo-helicase-DNA binding protein (*CHD*), is present in most of the birds (Ellegren 1996; Griffiths *et al*., 1996) and reported to be a highly conserved gene. A related Z-linked gene *CHD-Z* was later reported (Griffiths & Korn 1997). The discovery of the highly conserved gene (*CHD*) had triggered the development of various molecular sexing methods, and one of the most frequently used is PCR-based amplification of the gene for *CHD*. This technique has contributed to different types of primers successfully developed according to the ability to identify the sex of birds for specific or nonspecific species (Kahn *et al*., 1998; Fridolfsson & Ellegren, 1999; Chang *et al*., 2008; Wang & Zhang 2009).

In this study, seven avian sexing marker sets based on the *CHD* region or the sex chromosome in birds had been evaluated and results showed that only one sexing marker set (P8/WZ/W) could successfully differentiate males from females. This may attribute to the fact that differences of species of birds being investigated usually resulted in only a few sexing methods that can successfully differentiate the sex of birds. This had been reported by Hagadorn *et al*. (2016) while evaluating eleven sexing marker sets from the *CHD* region on sex chromosomes in birds and only obtained two primer sets (P8/P2 and 1237L/1272H) resulted in reliable DNA amplification and reliably determined sex of hummingbirds. Similar results had been reported for the most commonly used primers for PCR-based amplification of CHD gene (P2/P8 primer pair) that proved to be not useful for sex identification in some species (Cheng *et al*., 2006; Huynen *et al*., 2006; Jarvi & Farias, 2006).

The results of unsuccessful sexing marker sets may also attribute to the fact that some of the sexing markers set used in this study had very small size differences between the W and Z copies of the intron, making the size differences could not be resolved on standard agarose gels. This likely to be the reason for most of the sexing marker sets used only showed a single band for both female and male individuals. Higher-resolution gels such as polyacrylamide gel may allow sex identification from this sexing marker set. Unfortunately, the reasons that other methods had not used because it is costly and time consuming with uncertain results. Furthermore, the objective of the study was to determine the sexing marker sets that can successfully identify the sex of *A. fuciphagus* and it is achieved even with only one set of primers.

The sexing primer set P8/WZ/W that had successfully identified the sex of *A. fuciphagus* in this study was designed based on conserved regions inside the amplified P2/P8 primer pair sequences. These PCR primer sets had been used for sex identification of Chinese egret and nine other ardeid species (Wang *et al*., 2011). The results from this study showed that the control males (M1, M2 and M3) showed the presence of the CHD-Z (255 bp) gene, while female samples showed the presence of the extra band, *CHD-W* (144 bp). The size of the bands showed was not much different from the results showed for Chinese egret and nine other ardeid species for both *CHD-Z* (250 bp) and *CHD-W* (140bp). Wang *et al*. (2011), stated that there are some advantages in this

method as compared with P2/P8 primer-based technique. First of all, is the fact that only one PCR required, and results are readily analysed by AGE without the need for a tedious PAGE method. Moreover, the lengths of PCR products with this primer set were shorter (140/250 bp, compared to 380 bp for the P2/P8 primer set in ardeid species), which means that even degraded DNA samples can be used, making it possible to use non-destructive materials such as shed feathers in sex identification. Furthermore, since feathers provide less DNA source material (Harvey *et al*., 2006), if this sexing marker set can still successfully identify the sex for *A. fuciphagus* by using feather sample, there will be less problem with other DNA sources such as blood or tissue that have a higher DNA source material compare to feather. The results in this study also showed that the *CHD-W* fragment was smaller in size (144 bp) compared to *CHD-Z* (255 bp), which can avoid females being mistyped as males. This is because of the PCR product from the W allele is smaller than that from Z, it may be amplified preferentially, especially when amplification is weak (Dawson *et al*., 2001).

To further understand the markers for identifying sexing of *A. fuciphagus*, PCR amplicons from candidate male and female samples together with a control sample were sent for sequencing. The results showed no variation among the individuals of a particular sex, that is, among males (*CHD-Z*) and among females (*CHD-W*), except for one male sample (A23). The sample for this male exhibited a base substitution from G (Guanine) to A (Arginine) at base position 166, suggesting point mutations had occurred in the CHD-Z gene. The implications of such mutation can either be negligible or in some cases a serious effect. Changes to the codon due to base substitution may encode different amino acids and cause a change in the protein produced. In some serious cases, the base substitution may change the codon to a 'stop' codon and causes an incomplete protein. However, in a case where changes of codon encode the same amino acid, there will be no changes to protein produced. This is called a silent mutation. In this study, it seems that base substitution at the CHD-Z gene may be a silent mutation as the effect is negligible.

The phylogenetic analysis compared the sex evolutionary pattern of these sexing markers with fourteen other bird species from either the same Apodiformes order or the same Aves class for both male (*CHD-Z*) and female (*CHD-W*). Those bird species were selected based on compatibility and high similarity in the CHD gene from the genome database. The results of the phylogenetic analysis showed a higher percentage of similarity for both female (100%) and male (98%) *A. fuciphagus* with *Apus apus* (common swift). It is expected as both of the species are closely related as both are in the same Apodiformes order. However, interestingly the result of similarity between *A. fuciphagus* and *Calypte costae* (Costa's hummingbird) showed a low similarity for males (80%) while both are also in the same Apodiformes order. Results from the phylogenetic tree also showed that *Calypte costae* was totally in a different cluster than *A. fuciphagus* and *Apus apus* even though they are all from the same Apodiformes order. Phylogenetic tree results also showed that four (Apodiformes, Pelecaniformes, Gruiformes and Acciptriformes) out of five

orders investigated in *CHD-W* (female) had 100% similarity between birds from the same order and none of this similarity had been observed in *CHD-Z* (male). This result suggests that evolution occurred at a higher rate in males (*CHD-Z*) as compared to females (*CHD-W*) and may also explain why *Calypte costae* which is in the same Apodiformes order with *A. fuciphagus* showed a low percentage of similarity in male. Interestingly, Vicoso *et al*. (2013) in analysing the genome and transcriptome of emu (*Dromaius novaechollandiae*) had confirmed that most of the genes on the sex chromosome are shared between both Z and W. Surprisingly, however, levels of gene expression are generally sex-biased, and the male-bias increases after gonad formation. This expression bias suggests that emu sex chromosomes have become masculinized, even without ZW differentiation. Similarly, Mank *et al*. (2007) on predicting a fast-Z effect by focusing on chicken and zebra finch (have female heterogamety) suggested that evolution proceeds more quickly on the Z chromosome, where hemizygous exposure of beneficial non-dominant mutations increase the rate of fixation.

CHAPTER 6

CONCLUSION

The white nest swiftlet (*A. fuciphagus*) has been received more attention nowadays due to the ability of this species to produce EBN of high value in the international market as most of its nests are created from its pure saliva containing glycoprotein with high nutritional values. Hence, increasing the demand for these EBNs. Consequently, over harvesting had occurred in many locations in their natural habitats (limestone caves), leading to the decrease in *A. fuciphagus* populations at some of the natural habitats in Malaysia such as the Great Cave of Niah. Therefore, the introduction of swiftlet ranching had become important to significantly increase the overall population of this species. The increase in man-made houses has been contributed to the rapid expansion of this bird population, especially by established colonies in abandoned buildings. However, unpredictable changes in the genetic structure of this species over time may be triggered because of the exploitation and rapid population expansion. Thus, this study was conducted to evaluate the genetic variability within and among the populations of *A. fuciphagus* in Peninsular Malaysia using microsatellite markers.

Three populations were investigated in the present study. These included four to five colonies for each population. DNA was extracted from blood, tissue, feather or embryo (egg) samples that randomly collected from six to eight animals or carcasses from each colony. The eight microsatellite loci that were polymorphic and suitable for swiftlet species were used for further population genetics investigation. The PCR products were genotyped using 8% PAGE.

In conclusion, the results showed that the eight polymorphic microsatellite loci used in the present study were highly informative and suitable for genetic diversity evaluation in swiftlets. There were sufficient genetic variations present among the studied populations of *A. fuciphagus* in Peninsular Malaysia. There appeared to be sufficient gene flow among populations to prevent genetic isolation. Populations of *A. fuciphagus* from Peninsular Malaysia can be divided into two different clusters: the first cluster consisted of only the Southern region and the second cluster consisted of Northern and Eastern regions.

However, inbreeding was present. Hence, it suggests that genetic diversity may be lost in this species if no appropriate approaches are taken such as good animal husbandry practice for edible bird nest swiftlet as suggested by DVS, or enrich swiftlet rancher knowledge on sustainable swiftlet ranching. These may help to increase genetic diversity in swiftlet populations and at the same time avoids the risk of inbreeding.

In addition, the difficulty for determining bird sex, especially in the absence of sexual dimorphism and a sexually immature individual has made the molecular sexing method as an important method. In this study, the molecular sexing method involving the use of several sexing markers proved to be a good method for differentiating the sex of *A. fuciphagus*. The results showed that a P8/WZ/W sexing marker set can successfully differentiate the gender of *A. fuciphagus.* The comparison of the sex evolutionary pattern of P8/WZ/W marker set with fourteen other bird species from the same Apodiformes order and Aves class had showed that sexing genes of *A. fuciphagus* are closely related to *Apus apus*. This suggests that evolution had occurred at a higher rate in males (*CHD-Z*) as compared to females (*CHD-W*). Hence, this molecular sexing can be utilized to facilitate further studies on sex evolution, sex ratio and breeding management of *A. fuciphagus*.

REFERENCES

Abalaka, J., Noraine, S. H., Ottoson, U., Bloomer, P. & Hansson, B. (2015). Genetic diversity and population structure of the range restricted rock firefinch *Lagonosticta sanguinodorsalis*. *Conservation Genetic*, 16, 411-418.

Achilli, A., Bonfiglio, S., Olivieri, A., Malusa, A., Pala, M., Kashani, B. H., Perego, U. A., Ajmone-Marsan, P., Liotta, L., Semino, O., Bandelt, H. J., Ferretti, L., & Torroni, A. (2009). The multifaceted origin of taurine cattle reflected by the mitochondrial genome. *PLoS ONE*, 4(6), e5753.

Adrian, L. & Jennifer, E. L. (2014). Forensic DNA profiling: State of the art. *Journal of Research and Reports in Forensic Medical Science*, 4, 25–36.

Allan, G. J. & Max, T. L. (2010). Molecular Genetic Techniques and Markers for Ecological Research. *Nature Education Knowledge*, 3(10), 2.

Allendorf, F. W. & Luikart, G. (2007). *Conservation and the Genetics of Populations*. Blackwell Publishing, Malden, Massachusetts, USA.

Amigues, Y., Boitard, S., Bertrand, C., SanCristobal, M. & Rocha, D. (2011). Genetic characterization of the Blonde d'Aquitaine cattle breed using microsatellite markers and relationship with three other French cattle populations. *Journal of Animal Breed and Genetic*, 128, 201–208.

Anmarkrud, J. A., Kleven, O., Bachmann, L. & Lifjeld. L. T. (2008). Microsatellite evolution: mutations, sequence variation, and homoplasy in the hypervariable avian microsatellite locus HrU10. *Biomedical Central Evolutionary Biology*, 8,138.

Anuar, M. (2011). A brief look at the edible-nest swiftlet industry. *In the Proceedings of the 2011 International Conference & Training on Swiftlet Ranching*, 1-11.

Anwarali Khan, F. A., Bujang, M. F., Kassim, M. A., Yap, S. Y., Ketol, B., Marni, W., Sait, I., Laman, C. J., Aimran, A. A., Mawek, Z., Tajuddin, A. A. M. & Abdullah, M. T. (2008). Biodiversity assessment in a Sarawak lowland dipterocarp rainforest of Niah National Park. *Journal of Tropical Biology and Conservation*, 4, 23-37.

Aowphol, A., Voris, H. K., Feldheim, K. A., Harnyuttanakorn, P. C. & Thirakhupt, K. (2008). Genetic Homogeneity among colonies of the White-Nest Swiftlet (*Aerodramus fuciphagus*) in Thailand. *Zoological Society of Japan*, 25, 372-380.

Avise, J. C. (1994). *Molecular Markers, Natural History and Evolution*. Chapman & Hall, New York.

Azam, A., Babar, M. E., Firyal, S., Anjum, A. A., Nabeela, A., Muhammad, A. & Tanveer, H. (2012). DNA typing of Pakistani cattle breeds Tharparkar and Red Sindhi by microsatellite markers. *Molecular Biology Reports*., 39, 845-849.

Babji, A. S., Nurfatin, M. H., Etty Syarmila, I. K. & Masitah, M. (2015). Secrets of edible bird nest. *UTAR Agriculture Science Journal*, 1(1), 32-37.

Baird, N. A., Etter, P. D., Atwood, T. S., Currey, M. C., Shiver, A. L., Lewis, Z. A., Selker, E. U., Cresko, W. A. & Johnson, E. A. (2008). Rapid SNP

discovery and genetic mapping using sequenced RAD markers. *PLoS ONE*, 3, e3376.
Bantock, T. M., Prys-Jones, R. P. & Lee, P. L. (2008). New and improved molecular sexing methods for museum bird specimens. *Molecular Ecology Resources*, 8, 519-528.
Barril, P. & Nates, S. (2012). Introduction to agarose and polyacrylamide gel electrophoresis matrices with respect to their detection sensitivities. *Gel Electrophoresis - Principles and Basics*, 3–15.
Bhandari, H., Nishant, B. A., Srivastava, K., Singh, M. & Hemantaranjan, A. (2017). Assessment of genetic diversity in crop plants - an overview. *Advances in Plants and Agriculture Research*, 7(3), 1-8.
Billerman, S. M., Huber, G. H., Winkler, D. W., Safran, R. J. & Lovette, I. J. (2011). Population genetics of a recent trans-continental colonization of South America by breedingBarn Swallows (*Hirundo rustica*). *The Auk*,128, 506–513.
Bishop, D. H. L., Claybrook, J. R. & Spiegelman, S. (1967). Electrophoretic Separation of Viral Nucleic Acids on Polyacrylamide Gels. *Journal of Molecular Biology*, 26, 373-387.
Botstein, D., White, R. L., Skolnick, M. & Davis, R. W. (1980). Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *American Journal of Human Genetics*, 32(3), 314–331.
Bravo, J. P., Hoshino, A. A., Angelici, C. M. L. C. D., Lopes, C. R. & Gimenes, M. A. (2006). Transferability and use of microsatellite markers for the genetic analysis of the germplasm of some Arachis section species of the genus Arachis. *Genetic and Molecular Biology*, 29, 516–524.
Bray, T. C., Chikhi, L., Sheppy, A. J. & Bruford, M. W. (2009). The population genetic effects of ancestry and admixture in a subdivided cattle breed. *Animal Genetics*, 40, 393–400.
Brooke, R. K. (1970). Taxonomic and evolutionary notes on the subfamilies, tribes, genera and subgenera of the swifts (Aves: Apodidae). *Durban Museum Novitates*, 9, 13-24.
Brown, J. K. M. (1996). The choice of molecular marker methods for population genetic studies of plant pathogens. *New Phytologist*, 133,183-195.
Brumlop, S. & Finckh, M. R. (2010). Applications and potentials of marker assisted selection (MAS) in plant breeding. *Final report of the F+E project* "Applications and Potentials of Smart Breeding". On behalf of the Federal Agency for Nature Conservation December 2010.
Cecconi, F., Giorgi, M. & Makiottini, P. (1995). Unique features in the mitochondrial D-loop region of the European seabass *Dicentrarchus labrux*. *Gene*, 160, 149-155.
Chang, H. W., Chou, T. C. & Gu, D. L. (2008). An improved PCR method for gender identification of eagles. *Molecular and Cellular Probes*, 22, 184–188.
Chantler, P. & Driessens, G. (2000). Swift: A Guide to the Swifts and Treeswifts of the World. Second edition. *Pica Press, Robertsbridge*.
Chantler, P. (2005). Family Apodidae (swifts). In: del Hoyo J, Elliott A & Sargatal A (eds.) Handbook of Birds of the World Vol. 5. Barn Owls to Hummingbirds. *Lynx Edicions, Barcelona*, 388–457.
Chasen, F. N. (1935). A handlist of Malaysian birds. *Bulletin of the Raffles Museum*, 11: i-xx, 1-389.

Cheng, Y. H., Kuo, T. F., Lee, D. N. & Weng, C. F. (2006). Sex identification of the Black-faced Spoonbill (*Platea minor*). *Zoological Studies*, 45(1), 104-113.

Chua, K. H., Lee, T. H., Nagandran, K., Yahaya, N. H. M., Lee, C. T., Tjih, E. T. & Aziz, R. A. (2013). Edible bird's nest extract as a chondro-protective agent for human chondrocytes isolated from osteoarthritic knee: in vitro study. *BMC Complementary Alternative Medicine*, 13(19), 1-9.

Corte´s, O., Tupac-Yupanqui, I., Dunner, S., Garcı´a-Atance, M. A., Garcı´a D., Ferna´ndez J. & Can˜ o´n J. (2008). Ancestral matrilineages and mitochondrial DNA diversity of the Lidia cattle breed. *Animal Genetics*, 39, 649–54.

Cranbrook, E., Goh, W. L., Lim, C. K. & Abdul Rahman, M. (2013). The species of white nest swiftlets (Apodidae, Collocaliini) of Malaysia and the origins of house-farm birds: morphometric and genetic evidence. *Forktail*, 29, 78–90.

Dakin, E. E. & Avise, J. C. (2004). Microsatellite null alleles in parentage analysis. *Heredity*, 93(5), 504-509.

Davis, D. M., Reese, K. P., Gardner, S. C. & Bird, K. L. (2015). Genetic structure of Greater Sage-Grouse (*Centrocercus urophasianus*) in a declining, peripheral population. *The Condor: Ornithological Applications*, 117, 530–544.

Dawson, D. A., Darby, S., Hunter, F. M., Krupa, A. P., Jones, I. L. & Burke, T. (2001). A critique of avian CHD-based molecular sexing protocols illustrated by a Z-chromosome polymorphism detected in auklets. *Molecular Ecology Notes*, 1, 201-204.

De Vicente, M. C., Guzmán, F. A., Engels, J. & Ramanatha Rao, V. (2005). *Genetic characterization and its use in decision making for the conservation of crop germplasm*. The Role of Biotechnology, Villa Gualino, Turin, Italy – 5-7 March 2005.

Dor, R., Safran, R. J., Vortman, Y., Lotem, A., McGowan, A., Evans, M. R. & Lovette, I. J. (2012). Population genetics and morphological comparisons of migratory European (Hirundo rustica rustica) and sedentary East–Mediterranean (*Hirundo rustica transitiva*) barn swallows. *Journal of Heredity*, 103, 55–63.

Dubiec, A. & Zalagska-Neubauer, M. (2006). Molecular techniques for sex identification in birds. *Biological Letters,* 43, 3-12.

DVS. (2010). Good animal husbandry practice for edible-nest swiftlets *Aerodramus* species ranching and its premise, Ministry of Agricultre Malaysia, 1-37.

DVS. (2017). Bilateral Agricultural Cooperation Malaysia – China. *Department of Veterinary Services*, Ministry of Agriculture Malaysia, pp. 1-3.

Ellegren, H. (1996). First gene on the avian W chromosome (CHD) provides a tag for universal sexing of non-ratite birds. *Proceedings of the Royal Society of London Series B*, 263, 1635-1641.

Ellegren, H. (2000). Microsatellite mutations in the germline: implications for evolutionary inference. *Trends in Genetics*, 16, 551-558.

Emadi, A., Crim, M. T., Brotman, D. J., Necochea, A. J., Samal, L., Wilson, L. M., Bass, E. B. & Segal, J. B. (2010). Analytic validity of genetic tests to identify factor V Leiden and prothrombin G20210A. *American Journal of Hematology*, 85(4), 264-270.

Emara, M. G. & Kim, H. (2003). Genetic markers and their application in poultry breeding. *Poultry Science*, 82, 952-957.

Evanno, G., Regnaut, S. & Goudet, J. (2005). Detecting the number of clusters of individuals using the software structure: a simulation study. *Molecular Ecology*, 14(8), 2611-2620.

Excoffier, L., Laval, G. & Schneider, S. (2005). Arlequin (version 3.0): an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online,* 1, 47-50.

FAO. (2011). *Molecular genetic characterization of animal genetic resources*. FAO Animal Production and Health Guidelines. No. 9., Rome.

Field, D. & Wills, C. (1998). Abundant microsatellite polymorphism in Saccharomyces cerevisiae, and the different distributions of microsatellites in eight prokaryotes and S. cerevisiae, result from strong mutation pressures and a variety of selective forces. *Proceedings of the National Academy of Sciences USA*, 95, 1647-1652.

Firas, R. S. & Abdulkareem, A. (2015). Molecular markers: An introduction and applications. *European Journal of Molecular Biotechnology*, 9(3), 118-130.

Frankham, R., Briscoe, D. A. & Ballou, J. D. (2002). *Introduction to conservation genetics*. Cambridge University Press, England.

Frankham, R. (2005). Genetics and extinction. *Biology Conservation*, 126, 131-140.

Fridolfsson, A. K. & Ellegren, H. (1999). A simple and universal method for molecular sexing of non-ratite birds. *Journal of Avian Biology,* 30, 116-121.

Gibson-Hill, C. A. (1949). An annotated checklist of the birds of Malaya. *Bull Raffles Museum*, 20, 1-299.

Gibbs, R. A., Taylor, J. F. & Van Tassell, C. P. (2009). Genome-wide survey of SNP variation uncovers the genetic structure of cattle breeds. *Science*, 324, 528–532.

Goh, W. L., Lim, C. K. & Rahman, M. A. (2013). *Isolation of mitochondrial control region for white-nest swiftlets (Aerodramus fuciphagus) using primer walking techniques. Pertanika Journal of Tropical Agricultural Science*, 36(2), 115-122.

Goh, W. L., Siew, W. S., Davies, S. E. W., Ball, S., Khoo, G., Lim, C. K. & Rahman, M. A. (2018). Genetic diversity among white nest swiftlets of the genus Aerodramus (Aves: Apodidae: Collocaliini) of house-farms in Malaysia. *Raffles Bulletin of Zoology*, 66, 350-360.

Griffiths, R. & Tiwari, B. (1995). Sex of the last wild Spix's macaw. *Nature*, 375, 454.

Griffiths, R., Daan, S. & Dijkstra, C. (1996). Sex identification in birds using two CHD genes. *Proceedings of the Biological Science*, 263, 1251-1256.

Griffiths, R. & Korn, R. M. (1997). A CHD1 gene is Z chromosome linked in the chicken Gallus domesticus. *Gene*, 197, 225-229.

Griffiths, R., Double, M. C., Orr, K. & Dawson, R. J. G. (1998). A DNA test to sex most birds. *Molecular Ecology,* 7, 1071-1075.

Grover, A., & Sharma, P. C. (2011). Is spatial occurrence of microsatellites in the genome a determinant of their function and dynamics contributing to genome evolution? *Current Science*, 100, 859–869.

Guichoux, E., Lagache, L., Wagner, S., Le´ger, P. & Petit R. J. (2011). Two highly validated multiplex (12-plex and 8-plex) for species delimitation

and parentage analysis in oaks (*Quercus spp*.). *Molecular Ecology Resources*, 11, 578–585.

Hagadorn, K. A., Tell, L. A., Drazenovich, T. L. & Ernest, H. B. (2016). Molecular sex identification markers for five North American Hummingbird species. *Conservation Genetics Resources*, 8(4), 427-430.

Haghani, A., Mehrbod, P., Safi, N., Aminuddin, N. A., Bahadoran, A., Omar, A. R. & Ideris, A. (2016). In vitro and in vivo mechanism of immunomodulatory and antiviral activity of edible bird's nest (EBN) against influenza A virus (IAV) infection. *Journal of Ethnopharmacology*, 185, 327-340.

Hameed, S. M. (2007). The 2007 Malaysian swiftlet farming industry report. *SMI Association of Penang*, Penang, Malaysia.

Han, J., Kim, J. H., Kim, S., Park, S. R. & Na, K. J. (2009). A simple and improved DNA test for avian sex determination. *Auk*, 126, 779-783.

Hancock, J. M. (1995). The contribution of slippage-like processes to genome evolution. *Journal of Molecular Evolution*, 41, 1038–1047.

Hanotte, O. & Jianlin, H. (2005). *Genetic characterization of livestock populations and its use in conservation decision-making*. The role of biotechnology, Villa Gualino, Turin, Italy -5 – 7 March 2005.

Harvey, M. G., Bonter, D. N. & Stenzler, L. M. (2006). A comparison of plucked feathers versus blood samples as DNA sources for molecular sexing. *J. Field Ornithology*, 77, 136-140.

Hendra, Yuda, P. & Zahida, F. (2014). *Genetika Populasi Collocalia fuciphaga di Riau Menggunakan Mikrosatelit*. Fakultas Teknobiologi, Universitas Atma Jaya Yogyakarta.

Heubl, G. (2010). New aspects of DNA-based authentication of Chinese medicinal plants by molecular biological techniques. *Planta Medica*, 76, 1963-1974.

Hidalgo, A. N. H., Hall, M. L., Kingma, S. A., Sunnucks, P. & Peters, A. (2016). Incest avoidance, extrapair paternity, and territory quality drive divorce in a year-round territorial bird. *Behavioural. Ecology*, 27, 1808– 1819.

Hobbs, J. J. (2004). Problems in the harvest of edible birds' nests in Sarawak and Sabah, Malaysian Borneo*. Biodiversity and Conservation*, 13, 2209-2226.

Hoshino, A. A., Bravo, J. P., Macedo, N. P. & Morelli, K. A. (2012). Microsatellites as tools for genetic diversity analysis. *Genetic Diversity Microorganisms*, 6, 149-170.

Hou, Z., Imam, M. U., Ismail, M., Azmi, N. H., Ismail, N., Ideris, A. & Mahmud, R. (2015a): Lactoferrin and ovotransferrin contribute toward antioxidative effects of edible bird's nest against hydrogen peroxide-induced oxidative stress in human SH-SY5Y cells. *Bioscience, Biotechnology, and Biochemistry*. 79(10), 1570-1578.

Hou, Z., Imam, M. U., Ismail, M., Ooi, D. J., Ideris, A. & Aahmud, R. (2015b). Nutrigenomic effects of edible bird's nest on insulin signaling in ovariectomized rats. *Drug Design, Development and Therapy*, 9, 4115-4125.

Hu, F., Liu, J. F., Zeng, Z. B., Ding, X. D., Yin, C. C., Gong, Y. Z. & Zhang, Q. (2010). QTL Identification Using Combined Linkage and Linkage Disequilibrium Mapping for Milk Production Traits on BTA6 in Chinese Holstein Population. Asian-Aust. *Journal of Animal Science*, 23(10), 1261-1267.

Huynen, L., Miles, J. & Lambert, D. (2006). Unusual electrophoretic mobility of a DNA fragment of the universal 'non-ratite' sexing marker CHD allows sexing of New Zealand's endangered kiwi ratite *Apteryx* spp. *IBIS*, 148(1), 167-168.

Ibrahim, S. H., Teo, W. C. & Baharun A. (2009). A study on suitable habitat for swiftlet farming. *UNIMAS E-Journal of Civil Eng*ineering, 1, 1-7.

Idris, A., Abdullah, A. A. & Abd Rehman, M. (2014). An overview of the study of the right habitat and suitable environmental factors that influence the success of edible bird nest production in Malaysia. *Asian Journal of Agricultural Research*, 8, 1-16.

Jarvi, S. I. & Farias, M. E. M. (2006). Molecular sexing and sources of CHD1-Z/W sequence variation in Hawaiian birds. *Molecular Ecology Notes*, 6, 1003-1005.

Jehan, T. & Lakhanpaul, S. (2006) Single nucleotide polymorphism (SNP) - methods and applications in plant genetics: a review. *Indian Journal of Biotechnology*, 5, 435–459.

Jentzsch, V. I. M., Bagshaw, A., Buschiazzo, E., Merkel, A. & Gemmell, J. E. (2008). Evolution of Microsatellite DNA. *Encyclopedia of Life Sciences*, John Wiley & Sons Ltd., Chichester, United Kingdom.

Kahn, N. W., St. John, J. & Quinn, T. W. (1998). Chromosome-specific intron size differences in the avian CHD gene provide an efficient method for sex identification in birds. *Auk*, 115, 1074-1078.

Kamarudin, M. I. & Abd Aziz, J. (2011). Managing God-given wealth: the need for inclusivity for sustainable EBN industry. *Paper presented at International Conference of Swiftlet Ranching*, University Zainal Abidin Terengganu, Malaysia.

Kang, N. & Lee, P. G. (1991). The Edible-nest Swiftlet *Aerodramus* spp. *Nat. Malaysiana*, 16, 44-51.

Kathan, R. H. & Weeks, D. I. (1969). Structure studies of *Collocalia mucoid*. I. Carbohydrate and amino acid composition. *Archives of Biochemistry and Biophysics*, 134, 572-576.

Koskinen, M. T., Hirvonen, H., Landry, P. A. & Primmer, C.R. (2004). The benefits of increasing the number of microsatellites utilized in genetic population studies: an empirical perspective. *Hereditas*, 141, 61-67.

Kumar, S., Stecher, G. & Tamura, K. (2016). MEGA 7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Molecular Biology and Evolution*, 33(7), 1870-1874.

Lau, A. S. M. & Melville, D. S. (1994). *International Trade in Swiftlet Nests with Special Reference to Hong Kong*. Traffic International, Cambridge.

Lee, P. L. M., Brain, P. F., Forman, D. W., Bradbury, R. B. & Griffiths, R. (2002). Sex and death: CHD1Z associated with high mortality in moorhens. *Evolution*, 56, 2548-2553.

Levinson, G. & Gutman, G. A. (1987). Slipped & and mispairing: a major mechanism for DNA sequence evolution, *Molecular Biology Evolution*, 4, 203-221.

Lewis, J., Zafiris, A., Christos, D., Dimitrios, L., Peristera, P. & Petros, D. (2011) Tracing cattle breeds with principal components analysis ancestry informative SNPs. *PLoS ONE*, 6, e18007.

Li, M. H., Sternbauer, K., Haahr, P. T. & Kantanen, J. (2005). Genetic components in contemporary Faroe Islands Cattle as revealed by microsatellite analysis. *Journal of Animal Breed Genetic*, 122, 309-317.

Lim, C. K. & Cranbrook, E. (2002). Swiftlet of Borneo: Builder of Edible Nest. *National History of Publication* (Borneo), Kota Kinabalu. pp.171.

Lim, C. K. (2011). Opportunity and Sustainability of Swiftlet Farming in Malaysia. In the proceedings of the 2011 International Conference & Training on Swiftlet Ranching (ICOTOS 2011), Universiti Sultan Zainal Abidin, Kuala Lumpur, Malaysia, 1-16.

Lin Jie-Ru, Zhou Hua, Lai Xiao-Ping, Hou You, Xian Xiao-Min, Chen JianNan, Wang Pei-Xun, Zhou Lian & Dong Yan (2009). Genetic identification of edible birds' nest based on mitochondrial DNA sequences. *Food Research International*, 42, 1053-1061.

Lourie, S. A. & Tompkins, D. M. (2000). The diets of Malaysian swiftlets. *International Journal of Avian Science*, 142, 596-602.

Mahanil, S., Ramming, D., Cadle-Davidson, M., Owens, C., Garris, A., Myles, S. & Cadle-Davidson, L. (2012). Development of marker sets useful in the early selection of Ren4 powdery mildew resistance and seedlessness for table and raisin grape breeding. *Theoretical and Applied Genetics*, 124, 23-33.

Maisarah, B. & Hafidzi, M. N. (2017). Ranging behaviour of edible nest swiftlet (Aerodramus sp.) in Kuala Langat district, Selangor, Malaysia. *Malaysian Applied Biology*, 46(2), 59-66.

Makarewich, C. A., Stenzler, L. M., Ferretti, V., Winkler, D. W. & Lovette, I. J. (2009) Isolation and characterization of microsatellite markers from three species of swallows in the genus Tachycineta: *T. albilinea*, *T. bicolor* and *T. leucorrhoa*. *Molecular Ecology Resources*, 9, 631-635.

Malpica, A. & Ornelas, J. F. (2014). Postglacial northward expansion and genetic differentiation between migratory and sedentary populations of the broad-tailed hummingbird (*Selasphorus platycercus). Molecular Ecology,* 23, 435-452.

Manchi, S. & Sankaran, R. (2010). Foraging habits and habitat use by edible-nest and glossy swiftlets in the Andaman Islands, India. *The Wilson Journal of Ornithology*, 122, 259-272.

Maniatis, T., Jeffrey, A. & Kleid, D. (1975). Nucleotide Sequence of the Rightward Operator of Phage. *Proceedings of National Academy of Science, USA*, 72, 1184-1188.

Mank, J. E., Axelsson, E. & Ellegren, H. (2007). Fast-X on the Z: Rapid evolution of sex-linked genes in birds. *Genome Res*ource, 17, 618-624.

Mansfield, E. S., Vainer, M., Enad, S., Barker, D. L., Harris, D., Rappaport, E. & Fortina, P. (1996). Sensitivity, Reproducibility and Accuracy in Short Tandem Repeat Genotyping Using Capillary Array Electrophoresis. *Genome Resource*, 6, 893-903.

Marcone, M. F. (2005). Characterization of the edible bird's nest the "Caviar of the East". *Food Research International*, 38, 1125-1134.

Markoulatos, P., Siafakas, N. & Moncany, M. (2002). Multiplex polymerase chain reaction: a practical approach. *Journal of Clinical Laboratory Analysis*, 16, 47-51.

Matsukawa, N., Matsumoto, M., Bukawa, W., Chiji, H., Nakayama, K., Hara, H. & Tsukahara, T. (2011). Improvement of bone strength and dermal thickness due to dietary edible bird's nest extract in ovariectomized rats. *Bioscience, Biotechnology and Biochemistry*, 75, 590-592.

Mayr, E. (1937). Birds collected during Whitney South Sea Expedition. XXXIII. American Museum Novitates, 828, 7-13.

Mburu, D. & Hanotte, O. (2005): *A practical approach to microsatellite genotyping with special reference to livestock population genetics*. ILRI Biodiversity project. A manual prepared for the IAEA/ILRI training course on molecular characterisation of small ruminant genetic resources of Asia, October-December 2005, ILRI, Nairobi, Kenya.

McNeely, J. A., Miller, K. R., Reid, W. V., Mittermeier, R. A. & Werner, T. B. (1990). *Conserving the world's biological diversity*. International Union for the Conservation of Nature and Natural Resources, World Resources Institute, Conservation International, WWF-US and World Bank.

Meyer, A. (1994). Shortcomings of the cytochrome b gene as a molecular marker. Trends. *Ecology Evolution*, 9, 278–280.

Morinha, F., Cabral, J. A. & Bastos, E. (2012). Molecular sexing of birds: a comparative review of polymerase chain reaction (PCR)-based methods. *Theriogenology,* 78, 703-714.

Ndanuko, R. N., Tapsell, L. C., Charlton, K. E., Neale, E. P., O'Donnell, K. M. & Batterham, M. J. (2017). Relationship between sodium and potassium intake and blood pressure in a sample of overweight adults. *Nutrition*, 33, 285-290.

Nei, M. (1972). Genetic distance between populations. *American Naturalist*, 106, 283-292.

Nei, M., Tajima, F. & Tateno, Y. (1983). Accuracy of estimated phylogenetic trees from molecular data. *Journal of Molecular Evolution*, 19(2), 153-170.

Nerbass, F. B., Pecoits-Filho, R., McIntyre, N. J., McIntyre, C. W. & Taal, M. W. (2015). High sodium intake is associated with important risk factors in a large cohort of chronic kidney disease patients. *European Journal of Clinical Nutrition*, 69, 786–790.

Niedziela, A., Bednarek, P. T., Cichy, H., Budzianowski, G., Kilian, A. & Aniol, A. (2012). Aluminum tolerance association mapping in triticale. *BMC Genomics*, 13, 67.

Nims, B. D., Vargas, F. H., Merkel, J. & Parker, P. G. (2008). Low genetic diversity and lack of population structure in the endangered Galapagos penguin (*Spheniscus mendiculus*). *Conservation Genetics*, 9, 1413-1420.

Norhayati, M. K., Azman, O. & Wan Nazaimoon, W.M. (2010). Preliminary study of the nutritional content of Malaysian edible bird's nest. *Malaysian Journal of Nutrition*, 16(3), 389-396.

Novick, A. (1959). "Acoustic Orientation in the Cave Swiftlet (Collocalia brevirostris unicolor). *Biological Bulletin of the Marine Laboratory of Woods Hole* ,117, 497–503.

Ochsenreither, S., Kuhls, K., Schaar, M., Presber, W. & Schonian, G. (2006). Multilocus microsatellite typing as a new tool for discrimination of Leishmania infantum MON-1 strains. *Journal of Clinical Microbiology,* 44, 495–503.

Ojango, J. M., Mpofu, N., Marshall, K. & Andersson-Eklund, L. (2011). Quantitative methods to improve the understanding and utilisation of animal genetic resources. In *Animal Genetics Training Resource*, *version* 3, ed. Ojango, J. M., Malmfors, B. & Okeyo, A. M. (Eds). International Livestock Research Institute, Nairobi, Kenya, and Swedish University of Agricultural Sciences, Uppsala, Sweden.

Oliveira, E. J., Pádua, J. G., Zucchi, M. I., Vencovsky, R., Vieira, M. L. C. (2006). Origin, evolution and genome distribution of microsatellites. *Genetics and Molecular Biology,* 29(2), 294-307.

Ontivero, M., Zamora, G. M., Salazar, S., Ricci, J. C. & Castagnaro, A. P. (2011). Isolation of strawberry gene fragment encoding an actin depolymerizing factor-like protein from genotypes resistant to Colletotrichum acutatum. *Genome*, 54, 1041-1044.

Osman, M. A., Dhawan, S. S., Bahl, J. R., Darokar, M. P. & Khanuja, S. P. (2011). AFLP marking and polymorphism among progenies of Gymnema sylvestre: an important medicinal plant of India. *Natural Product Communications*, 6, 1679-1682.

Othman, A., Yatim, S. H., Norazlinda, A. R. & Azimat, N. A. (2008). Distribution of Edible-nest swiftlet (*Aerodramus fuciphagus*) bird house in Peninsular Malaysia. *Journal of Wildlife and Parks*, 25, 8-11.

Päckert, M., Martens, J., Wink, M., Feigl, A. & Tietze, D. T. (2012). Molecular phylogeny of Old World swifts (Aves: Apodiformes, Apodidae, Apus and Tachymarptis) based on mitochondrial and nuclear markers. *Molecular Phylogenetics and Evolution*. 63, 606–616.

Padilla, J.A., Sansinforiano, E., Parejo, J. C., Rabasco, A. & Martínez-Trancón, M. (2009). Inference of admixture in the endangered Blanca Cacereña bovine breed by microsatellite analyses. *Livestock Science*, 122, 314-322.

Peterson, B. K., Weber, J. N., Kay, E. H., Fisher, H. S. & Hoekstra, H. E. (2012). Double digest RADseq: and inexpensive method for de novo SNP discovery and genotyping in model and non-model species. *PloS ONE*, 7, e0037135.

Petkliang, N., Gale, G. A., Brunton, D. H. & Bumrungsri, S. (2017). Wetland, forest and open paddy land are the key foraging habitats for Germain's swiftlet (*Aerodramus inexpectatus germani*) in Southern Thailand. *Tropical Conservation Science*, 10, 1-12.

Pokhriyal, B., Thorat, K., Lamiye, D. A., Joshi, Y. M., Kadam, V. J. & Dubey, R. (2012). Microsatellite Markers – A novel tool in molecular genetics. *International Journal of Research in Pharmacy and Chemistry*, 2(2), 2231-2781.

Pollock, D. D., Bergman, A., Feldman, M. W. & Goldstein, D. B. (1996). Microsatellite behavior with range constraints: parameter estimation and improved distances for use in phylogenetic reconstruction. *Theoretical Population Biology*, 53, 256-271.

Price, J. J., Johson, K. P. & Clayton, D. H. (2004). The evolution of echolocation in swiftlets. *Journal of Avian Biology*, 35,135-143.

Price, J. J., Johnson, K. P., Bush, S. E. & Clayton, D. H. (2005). Phylogenetic relationships of the Papuan Swiftlet and implications for the evolution of avian echolocation. *International journal of avian science*, 1471, 790-796.

Pritchard, J. K., Wen, W. & Falush, D. (2010). *Documentation for Structure Software: Version* 2.3, p. 33. University of Chicago, Department of Human Genetics, Chicago, Illinois, USA.

Raadsma, H. W., Thomson, P. C., Zenger, K. R., Cavanagh, C., Lam, M. K., Jonas, E., Jones, M., Attard, G., Palmer, D. & Nicholas, F. W. (2009) Mapping quantitative trait loci (QTL) in sheep. I. A new male framework linkage map and qtl for growth rate and body weight. *Genetics Selection Evolution*, 41, 34.

Ramachandran, A., Zhang, M., Goad, D., Olah, G., Malayer, J. R. & Rassi, Z. E. (2003). Capillary electrophoresis and fluorescence studies on molecular beacon-based variable length oligonucleotide target discrimination. *Electrophoresis,* 24, 70-77.

Ramji, M. F. S., Lim, C. K. & Rahman, M. A. (2013). Roosting and nest-building behaviour of the White-nest Swiftlet *Aerodramus fuciphagus* (Thunberg) (Aves: Apodidae) in farmed colonies. *The Raffles Bulletin of Zoology*, 29, 225–235.

Rasmussen, H. B. (2012). Restriction Fragment Length Polymorphism Analysis of PCR-Amplified Fragments (PCR-RFLP) and Gel Electrophoresis - Valuable Tool for Genotyping and Genetic Fingerprinting. In S. Magdeldin (Ed.), *Gel Electrophoresis - Principles and Basics*, Intech, 315-334.

Reid, I. R., Bristow, S. M. & Bolland, M. J. (2015). Cardiovascular complications of calcium supplements. *Journal of Cellular Biochemistry*,116, 494–501.

Robertson, B. C. & Gemmell, N. J. (2006). PCR-based sexing in conservation biology: wrong answers from an accurate methodology? *Conservation Genetics*, 7, 267-271.

Ross, J. D. & Bouzat, J. L. (2014). Genetic and morphometric diversity in the Lark Sparrow (*Chondestes grammacus*) suggest discontinuous clinal variation across major breeding regions associated with previously characterized subspecies, *The Auk: Ornithological Advances*, 131, 298-313.

Saitou, N. & Nei, M. (1987). The neighbour-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, 4, 406-425.

Sa´nchez-Pe´rez, R., Ballester, J., Dicenta, F., Aru´s, P. & Martı´nezGo´mez, P. (2006) Comparison of SSR polymorphisms using automated capillary sequencers, and polyacrylamide and agarose gel electrophoresis: Implications for the assessment of genetic diversity and relatedness in almond. *Scientia Horticulture*,108, 310–316.

Sankaran, R. (2001). The status and conservation of the edible nest swiftlets (*Collocalia fuciphaga*) in the Andaman and Nicobar Islands. *Biological Conservation* 97, 283-294.

Seeb, J. E., Carvalho, G., Hauser, L., Naish, K., Roberts, S. & Seeb L. W. (2011). Single-nucleotide polymorphism (SNP) discovery and applications of SNP genotyping in non-model organisms. *Molecular Ecology Resource*, 11, 1–8.

Selkoe, K.A. & Toonen, R. J. (2006). Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. *Ecology Letter*, 9, 615–629.

Shizuka, D. & Lyon, B. E. (2008). Improving the reliability of molecular sexing of birds using a W-specific marker. *Molecular Ecology Resource*, 8, 1249-1253.

Siti-Nabiha, A. K., Dayana, J. & Hasan, A. (2013). "Farming the “white gold” in a world heritage city", *Emerald Emerging Markets Case Studies*, 3(2), 1-10.

Sneath, P. H. A. & Sokal, R. R. (1973). *Numerical Taxonomy. The Principal and Practice of Numerical Classification*. W. H. Freeman, San Francisco, USA.

Sodhi, M., Mukesh, M., Ahlawat, S. P., Sobti, R. C., Gahlot, G. C., Mehta, S. C., Prakash, B. & Mishra, B. P. (2008). Genetic diversity and structure of two prominent zebu cattle breeds adapted to the arid region of India inferred from microsatellite polymorphism. *Biochemical Genetics*, 46(3–4), 124– 36.

Takezaki, N. & Nei, M. (1996). Genetic distances and reconstruction of phylogenetic trees from microsatellite DNA. *Genetics*, 144, 389-399.

Tautz, D., & Renz, M. (1984). Simple sequences are ubiquitous repetitive components of eukaryotic genomes. *Nucleic Acids Res*ource, 12, 4127-4138.

Tillmar, A. (2010). Populations and Statistics in Forensic Genetics. *Linkoping University Medical Dissertations No. 1175, Department of Clinical and Experimental Medicine, Faculty of Health Sciences*.

Thomassen, H. A., Wiersema, A. T., de Bakker, M. A. G., de Knijff, P., Hetebrij, E. & Povel, G. D. E. (2003). A new phylogeny of swiftlets (Aves: Apodidae) based on cytochrome-b DNA. *Molecular Phylogenetic Evolution*, 29, 86-93.

Thomassen, H. A., Tex, R.-J., Bakker, M. A. G. & Povel, G. D. E. (2005). Phylogenetic relationships amongst swifts and swiftlets: a multi locus approach. *Molecular Phylogenetic Evolution,* 37, 264-277.

Thorburn, C. (2015). The edible nest swiftlet industry in southeast Asia: Capitalism meets commensalism. *Human Ecology*, 43, 179-184.

Todd, J., Wu, Y. Q., Wang, Z. & Samuels, T. (2011). Genetic diversity in tetraploid switch grass revealed by AFLP marker polymorphisms. *Genetics and Molecular Research*, 10, 2976-2986.

Toro, M., Ferna´ndez, J. & Caballero, A. (2009). Molecular characterization of breeds and its use in conservation. *Livestock Science*, 120,174–95.

Toth, G., Gáspari, Z. & Jurka, J. (2000). Microsatellites in different eukaryotic genomes: survey and analysis. *Genome Research,* 967–981.

Varshney, R. K., Graner, A. & Sorrells, M. E. (2005). Genic microsatellite markers in plants: Features and applications. *Trends in Biotechnology*, 23, 48-55.

Vartia, S., Villanueva-Cañas, J. L., Finarelli, J., Farrell, E. D., Collins, P. C., Hughes, G. M., Carlsson, J. E. L., Gauthier, D. T., McGinnity, P., Cross, T. F., FitzGerald, R. D., Mirimin, L., Crispie, F, Cotter, P. D. & Carlsson, J. (2016). A novel method of microsatellite genotyping-by- sequencing using individual combinatorial barcoding. *Royal Society Open Science*, 3(1), 150565.

Vicoso, B., Kaiser, V. B. & Bachtrog, D. (2013). Sex-biased gene expression at homomorphic sex chromosomes in emus and its implication for sex chromosome evolution. *Proceedings of the National Academy of Sciences USA*, 110, 6453-6458.

Vimala, B., Hussain, H. & Nazaimoon, W. M. W. (2012). Effects of edible bird's nest on tumour necrosis factor-alpha secretion, nitric oxide production and cell viability of lipopolysaccharide-stimulated RAW 264.7 macrophages. *Food & Agricultural Immunology*, 23, 303-314.

Viruhpintu, S., Thirakhupt, K., Pradatsundarasar, A. & Poonswad, P. (2002). Nest- site Characteristic of the Edible-nest Swiftlet *Aerodramus fuchipagus* (Thunberg, 1812) at Si-Ha Islands, Phattalung Province, Thailand. *The Natural History Journal of Chulalongkorn University*, 2(2): 31-35.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M. & Zabeau, M. (1995). AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research*, 23, 4407– 4414.

Walsh, P. S., Fildes, N.J. & Reynolds, R. (1996). Sequence analysis and characterization of stutter products at the tetranucleotide repeat locus vWA. *Nucleic Acids Resource*, 24, 2807-2812.

Wang, D., Shi, J., Carlson, S. R., Cregan, P. B., Ward, R. W. & Diers, B. W. (2003). A low-cost, high-throughput polyacrylamide gel electrophoresis system for genotyping with microsatellite DNA markers. *Crop Science*, 43, 1828-1832.

Wang, N. & Zhang, Z. W. (2009). The novel primers for sex identification in the brown eared-pheasant and their application to other species. *Molecular Ecology Resources*, 9, 186–188.

Wang, X. W., Rinehart, A. T., Wadl, P. A., Spiers, J. M., Hadziabdic, D., Windham, M. T. & Trigiano, R. N. (2009). A new electrophoresis technique to separate microsatellite alleles African. *Journal of Biotechnology*, 8, 2432-2436.

Wang, Z., Zhou, X., Lin, Q., Fang, W. & Chen, X. (2011). New primers for sex identification in the Chinese Egret and other ardeid species. *Molecular Ecology Resource*, 11, 176-179.

Wang, S., Meyer, E., McKay, J. K. & Matz, M. V. (2012). 2b-RAD: a simple and flexible method for genome-wide genotyping. *Nature Methods*, 9, 808-810.

Weir, B. S. & Cockerham, C. C. (1984). Estimating F-statistics for the analysis of population structure. *Evolution*, 38, 1358–1370.

Weising, K., Nybom, H., Wolff, K. & Kahl, G. (2005). DNA fingerprinting in plants: Principles, methods, and applications. *CRC Press, Boca Raton, FL*.

Wenz, H. M., Robertson, J. M., Menchen, S., Oaks, F., Demorest, D. M., Scheibler, D., Rosenblum, B. B., Wike, C., Gilbert, D. A. & Efcavitch, J. W. (1998). High-precision genotyping by denaturing capillary electrophoresis. *Genome Resource*, 3, 69–80.

Wigginton, J. E., Cutler, D. J. & Abecasis, G. R. (2005). A note on exact tests of Hardy-Weinberg equilibrium. *American Journal of Human Genetic*, 76, 887–893.

Woodage, T., Basrai, M. A., Baxevanis, A. D., Hieter, P. & Collins, F. S. (1997). Characterization of the CHD family of proteins. Proc. Natl. Acad. Sci. USA, 94, 11472-11477.

Xiao, L., Zhao, Z., Du, D., Yao, Y., Xu, L. & Tang, G. (2012). Genetic characterization and fine mapping of a yellow-seeded gene in Dahuang (a Brassica rapa landrace). *Theoretical and Applied Genetics*, 124, 903-909.

Yang, W., Kang, X., Yang, Q., Lin, Y. & Fang, M. (2013). Review on the development of genotyping methods for assessing farm animal diversity. *Journal of Animal Science and Biotechnology*, 4(1), 2.

Yeh, F. C., Boyle, T. & Yang, R. (1999). POPGENE version 1.31. Microsoft window-based Freeware for population genetic analysis (http://www. ualberta.ca/ ~fyeh/popgene.html).

APPENDICES

APPENDIX A1

The identification numbers and sample types of the swiftlets sample used in this study

(Eastern Region)

No.	ID	Sample type
1	KT1	blood
2	KT2	blood
3	KT4	blood
4	KT5	blood
5	KB1	blood
6	KB2	feather
7	KB3	feather
8	KB4	blood
9	KB5	blood
10	KB6	tissue
11	KB7	tissue
12	KB8	blood
13	TM1	blood
14	TM2	blood
15	TM3	blood
16	TM4	blood
17	TM5	blood
18	TM6	blood
19	TM7	blood
20	KT3	blood
21	KT6	tissue
22	KT7	tissue
23	BC3	feather
24	BC4	feather
25	BC5	tissue
26	BC6	tissue
27	BC8	tissue
28	BC9	feather
29	BC10	feather
30	BC11	feather

APPENDIX A2

The identification numbers and sample types of the swiftlets sample used in this study

(Southern Region)

No.	ID	Sample type
1	J11	feather
2	J12	feather
3	J13	feather
4	J14	feather
5	J21	embryo
6	J23	embryo
7	J24	feather
8	J27	feather
9	J49	embryo
10	J52	embryo
11	J55	embryo
12	J58	embryo
13	J62	feather
14	J64	feather
15	J65	feather
16	J66	feather
17	J15	feather
18	J16	tissue
19	J22	embryo
20	J25	feather
21	J41	feather
22	J42	embryo
23	J43	embryo
24	J44	embryo
25	J46	embryo
26	J51	embryo
27	J56	embryo
28	J57	embryo
29	J61	feather
30	J63	feather

APPENDIX A3

The identification numbers and sample types of the swiftlets sample used in this study

(Northern Region)

No.	ID	Sample type
1	A11	feather
2	A12	feather
3	A13	feather
4	A14	feather
5	A15	feather
6	A16	feather
7	A27	feather
8	A28	feather
9	A29	feather
10	A24	feather
11	A25	feather
12	A26	feather
13	A33	tissue
14	A34	tissue
15	A35	blood
16	A30	feather
17	A31	feather
18	A32	tissue
19	A41	feather
20	A42	feather
21	A43	feather
22	A44	feather
23	A45	feather
24	A46	feather
25	A51	feather
26	A52	feather
27	A53	feather
28	A54	feather
29	A55	feather
30	A56	feather

APPENDIX B1

Absorbance reading and concentration of DNA for 30 samples of *A. fuciphagus* from Southern Region.

No.	Sample ID			DNA Quality	DNA concentration (ng/µL)
		A260	A280	A260/A280	
1	J11	0.018	0.014	1.63	51.0
2	J12	0.013	0.009	1.58	57.2
3	J13	0.018	0.012	1.57	80.5
4	J14	0.020	0.014	1.70	72.2
5	J15	0.003	0.002	1.45	45.0
6	J16	0.003	0.002	1.45	35.0
7	J21	0.020	0.022	0.77	41.7
8	J22	0.005	0.003	1.92	20.0
9	J23	0.016	0.015	1.17	46.7
10	J24	0.006	0.004	1.61	25.0
11	J25	0.005	0.006	0.74	25.0
12	J27	0.018	0.012	1.49	77.6
13	J41	0.002	0.003	0.66	23.0
14	J42	0.004	0.006	0.49	23.0
15	J43	0.002	0.003	0.66	30.0
16	J44	0.003	0.005	0.49	19.0
17	J46	0.003	0.002	1.45	15.0
18	J49	0.009	0.006	1.45	45.0
19	J51	0.001	0.003	0.35	21.0
20	J52	0.013	0.017	0.74	64.2
21	J55	0.005	0.005	0.98	25.0
22	J56	0.001	0.002	0.21	15.0
23	J57	0.002	0.001	0.26	25.0
24	J58	0.009	0.005	1.92	40.0
25	J61	0.003	0.002	1.45	25.0
26	J62	0.009	0.003	2.81	45.0
27	J63	0.002	0.001	0.23	20.0
28	J64	0.015	0.011	1.56	61.0
29	J65	0.011	0.006	1.82	56.8
30	J66	0.007	0.004	1.92	30.0

APPENDIX B2

Absorbance reading and concentration of DNA for 30 samples of *A. fuciphagus* from Northern Region.

No.	Sample ID			DNA Quality	DNA concentration (ng/µL)
		A260	A280	A260/A280	
1	A11	0.023	0.015	1.65	86.0
2	A12	0.018	0.017	1.12	81.5
3	A13	0.011	0.006	2.22	35.0
4	A14	0.012	0.009	1.43	44.3
5	A15	0.014	0.008	1.64	67.9
6	A16	0.006	0.014	0.71	64.7
7	A27	0.004	0.002	1.92	20.0
8	A28	0.014	0.012	1.28	47.3
9	A29	0.008	0.004	1.92	40.0
10	A24	0.014	0.012	1.41	46.5
11	A25	0.026	0.014	1.49	41.3
12	A26	0.008	0.005	1.69	35.0
13	A30	0.011	0.010	1.14	47.5
14	A31	0.006	0.003	1.92	30.0
15	A32	0.012	0.009	1.39	42.9
16	A33	0.006	0.004	1.55	20.0
17	A34	0.007	0.005	1.26	25.5
18	A35	0.005	0.007	0.71	63.3
19	A41	0.005	0.003	1.79	55.9
20	A42	0.012	0.008	1.41	66.9
21	A43	0.007	0.003	2.33	33.5
22	A44	0.005	0.007	0.71	64.2
23	A45	0.007	0.004	1.51	45.0
24	A46	0.008	0.005	1.43	48.1
25	A51	0.010	0.007	1.33	36.7
26	A52	0.007	0.006	1.02	59.5
27	A53	0.009	0.007	1.45	61.6
28	A54	0.010	0.008	1.44	59.1
29	A55	0.009	0.007	1.25	42.2
30	A56	0.018	0.012	1.62	45.0

APPENDIX B3

Absorbance reading and concentration of DNA for 30 samples of *A. fuciphagus* from Eastern Region.

No.	Sample ID			DNA Quality	DNA concentration (ng/µL)
		A260	A280	A260/A280	
1	KT1	0.006	0.004	1.70	30.0
2	KT2	0.006	0.003	2.16	30.5
3	KT3	0.015	0.011	1.56	52.5
4	KT4	0.011	0.006	1.82	55.0
5	KT5	0.007	0.004	1.92	60.0
6	KT6	0.005	0.005	0.98	35.0
7	KT7	0.003	0.002	1.45	25.0
8	KB1	0.012	0.007	1.69	61.6
9	KB2	0.009	0.004	2.14	25.0
10	KB3	0.028	0.017	1.67	32.2
11	KB4	0.013	0.009	1.58	45.0
12	KB5	0.020	0.014	1.70	50.0
13	KB6	0.016	0.015	1.17	35.0
14	KB7	0.003	0.003	0.98	45.0
15	KB8	0.005	0.003	1.92	43.0
16	TM1	0.010	0.003	3.15	50.9
17	TM2	0.007	0.003	2.16	32.5
18	TM3	0.011	0.006	1.90	52.5
19	TM4	0.005	0.002	2.50	44.0
20	TM5	0.012	0.005	2.40	60.0
21	TM6	0.007	0.003	2.33	35.0
22	TM7	0.011	0.006	1.85	37.5
23	BC3	0.007	0.005	1.33	50.9
24	BC4	0.015	0.011	1.65	93.0
25	BC5	0.007	0.004	1.92	33.6
26	BC6	0.005	0.005	0.92	29.5
27	BC8	0.006	0.003	1.92	32.5
28	BC9	0.006	0.004	1.61	29.2
29	BC10	0.003	0.002	1.45	22.5
30	BC11	0.008	0.005	1.65	32.5

APPENDIX C

Allele frequencies at the eight loci in Eastern, Southern and Northern region

Locus	Allele No.	Allele Size	Allele frequency		
			ER	SR	NR
Aef4	1	226	0.000	0.000	0.033
	2	230	0.000	0.000	0.050
	3	238	0.033	0.000	0.000
	4	242	0.067	0.100	0.117
	5	246	0.017	0.000	0.067
	6	250	0.067	0.150	0.100
	7	254	0.200	0.200	0.150
	8	258	0.183	0.000	0.067
	9	262	0.167	0.083	0.117
	10	266	0.167	0.000	0.183
	11	270	0.100	0.117	0.083
	12	274	0.000	0.183	0.017
	13	278	0.000	0.167	0.017
Aef24	1	210	0.000	0.000	0.083
	2	218	0.000	0.000	0.033
	3	226	0.017	0.017	0.000
	4	230	0.000	0.000	0.017
	5	238	0.000	0.000	0.033
	6	242	0.067	0.000	0.100
	7	246	0.150	0.133	0.000
	8	250	0.117	0.333	0.250
	9	254	0.233	0.183	0.050
	10	258	0.167	0.233	0.017
	11	262	0.217	0.033	0.083
	12	266	0.033	0.000	0.100
	13	270	0.000	0.067	0.067
	14	274	0.000	0.000	0.150
	15	278	0.000	0.000	0.017
Aef27	1	202	0.017	0.067	0.000
	2	206	0.033	0.000	0.000
	3	210	0.200	0.067	0.017
	4	214	0.133	0.233	0.100
	5	218	0.000	0.383	0.067
	6	222	0.233	0.067	0.317
	7	226	0.117	0.100	0.117
	8	230	0.033	0.033	0.267
	9	234	0.183	0.017	0.100
	10	238	0.033	0.000	0.017
Aef104	1	154	0.167	0.000	0.017
	2	158	0.367	0.000	0.033
	3	162	0.233	0.250	0.067
	4	166	0.050	0.000	0.183
	5	170	0.000	0.050	0.167
	6	174	0.000	0.433	0.033
	7	178	0.017	0.000	0.033
	8	182	0.083	0.100	0.183
	9	186	0.017	0.000	0.067
	10	190	0.000	0.133	0.117
	11	194	0.067	0.000	0.050
	12	198	0.000	0.033	0.000
	13	202	0.000	0.000	0.033
	14	210	0.000	0.000	0.017

ER = Eastern Region, SR = Southern Region, NR = Northern Region

APPENDIX C (CONTINUED)

Locus	Allele No.	Allele Size	Allele frequency		
			ER	SR	NR
Aef109	1	128	0.017	0.000	0.000
	2	132	0.017	0.017	0.000
	3	136	0.000	0.000	0.017
	4	152	0.017	0.050	0.017
	5	156	0.167	0.133	0.100
	6	164	0.283	0.100	0.200
	7	168	0.233	0.017	0.217
	8	172	0.167	0.233	0.083
	9	176	0.017	0.000	0.200
	10	180	0.017	0.167	0.050
	11	184	0.067	0.167	0.033
	12	188	0.000	0.000	0.033
	13	192	0.000	0.117	0.033
	14	196	0.000	0.000	0.017
Aef112	1	214	0.017	0.000	0.000
	2	218	0.067	0.000	0.033
	3	222	0.000	0.000	0.033
	4	226	0.133	0.000	0.000
	5	230	0.200	0.500	0.033
	6	234	0.267	0.200	0.267
	7	238	0.117	0.133	0.067
	8	242	0.167	0.000	0.017
	9	246	0.033	0.167	0.217
	10	250	0.000	0.000	0.183
	11	254	0.000	0.000	0.117
	12	262	0.000	0.000	0.033
Aef115	1	214	0.033	0.050	0.083
	2	218	0.067	0.050	0.100
	3	222	0.033	0.167	0.117
	4	226	0.200	0.117	0.250
	5	230	0.300	0.400	0.133
	6	234	0.150	0.133	0.033
	7	238	0.000	0.000	0.033
	8	242	0.083	0.050	0.050
	9	246	0.133	0.033	0.050
	10	250	0.000	0.000	0.100
	11	254	0.000	0.000	0.017
	12	258	0.000	0.000	0.033
Aef133	1	200	0.133	0.150	0.000
	2	204	0.083	0.217	0.050
	3	208	0.000	0.183	0.117
	4	212	0.250	0.200	0.167
	5	216	0.000	0.000	0.083
	6	220	0.200	0.067	0.000
	7	224	0.017	0.100	0.150
	8	228	0.100	0.083	0.100
	9	232	0.000	0.000	0.067
	10	236	0.217	0.000	0.233
	11	240	0.000	0.000	0.033

ER = Eastern Region, SR = Southern Region, NR = Northern Region

APPENDIX D1

Partial nucleotide sequence of CHD gene for *A. fuciphagus* in female sample A15.

A15 (ZW)

CTCCCAAGGAGGAGAAACCGTGCAAAACAGGTGTCATCAGATTCTGAGTGA
TTTGTAGTACTTTCATGTTGCTTGATGGTCTTTGTTTGGTTTGAGTTGTTGGT
TTTGGATTTATGATTGTTTTCTTTTTCCTTGTGTGAACACATAGTTTTGACAG
GTTATGGTAAACATTTACTTAAATGTTTGTAATCACATAGCTTTATACTTACTT
CTTCTGAAATTCCAGATGAGCTTTAATGGAAGTGAAGGG

250bp

A15 (W)

CTCCCAAGGATGAGGAACTGTGCAAAACAGGTATCTGGGTTTTGATCAACTA
ATTTGATTTAATTGTTGTTGTTTAATTGCTTTTTTTCATTGCTGCTGTTTTGGC
TTGTACTTTTGGGTTGGGT

125bp

Partial nucleotide sequence of CHD gene for *A. fuciphagus* in female sample A21.

A21 (ZW)

CTCCCAAGGATGAGAAAACGTGCAAAACAGGTGTCATTTGATTCTGAGTGAT
TTGTAGTACTTTCATGTTGCTGATGGTTTTGTTTGGTTTGAGTTGTTGGTTTT
GGATTTATGATTGTATTTCTTTTTCCTTGTGCTGAACACATAGTTTTGACAGG
TTAGGTAAAGATTTACTTAAATTTTGTAATCACATAGCTTTATACTACTTCTTC
TGAAATTCCAGATGAGCTTTAATGGAAGTGAAGGG

247bp

A21 (W)

CTCCCAAGGATGAGGAACTGTGCAAAACAGGTATCTGGGTTTTGATCAACTA
ATTTGATTTAATTGTTGTTGTTTAATTGCTTTTTTTCATTGCTGCTGTTTTGGC
TTGTACTTTTGGGTTGGGT

125bp

APPENDIX D2

Partial nucleotide sequence of CHD gene for *A. fuciphagus* in male sample.

A16 (ZW)

CTCCCAAGGATGAGGAACTGTGCAAAACAGGTGTCACTTGATTCTGACTGA
TTTGTAGTACTTTCATGTTGCTGATGGTTTTGTTTGGTTTGAGTTGTTGGTTT
TGGATTTATGATTGTTTTCTTTTTCCTTGTGTGAACACATAGTTTTGACAGGT
TAGGTAAAAATTTACTTAAATTTTGTAATCACATAGCTTTATACTACTTCTTCT
GAAATTCCAGATGAGCTTTAATGGAAGTGAAGGG

245bp

A23 (ZW)

CTCCCAAGGATGAGGAACTGTGCAAAACAGGTGTCACTTGATTCTGACTGA
TTTGTAGTACTTTCATGTTGCTGATGGTTTTGTTTGGTTTGAGTTGTTGGTTT
TGGATTTATGATTGTTTTCTTTTTCCTTGTGTGAACACATAGTTTTGACAGGT
TAGGTAAAGATTTACTTAAATTTTGTAATCACATAGCTTTATACTACTTCTTCT
GAAATTCCAGATGAGCTTTAATGGAAGTGAAGGG

245bp

M3 (ZW)

CTCCCAAGGATGAGGAACTGTGCAAAACAGGTGTCACTTGATTCTGACTGA
TTTGTAGTACTTTCATGTTGCTGATGGTTTTGTTTGGTTTGAGTTGTTGGTTT
TGGATTTATGATTGTTTTCTTTTTCCTTGTGTGAACACATAGTTTTGACAGGT
TAGGTAAAGATTTACTTAAATTTTGTAATCACATAGCTTTATACTACTTCTTCT
GAAATTCCAGATGAGCTTTAATGGAAGTGAAGGG

245bp

BIODATA OF STUDENT

The student was born in Kuala Lumpur, Malaysia on 13 July 1989. He had his secondary education at SBPI Gopeng, Perak. He continued his studies at the Universiti Putra Malaysia and obtained his first degree in BSc of Animal Science in 2012. He then enrolled as a Master student at Department of Animal Science, Faculty of Agriculture, Universiti Putra Malaysia in February 2014.