UNIVERSITI PUTRA MALAYSIA

MOLECULAR CHARACTERIZATION OF MALAYSIAN SWIFTLET SPECIES AND DEVELOPMENT OF PCR-ELISA METHOD FOR RAPID IDENTIFICATION OF EDIBLE BIRD'S NEST

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By

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

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MOLECULAR CHARACTERIZATION OF MALAYSIAN SWIFTLET SPECIES AND DEVELOPMENT OF PCR-ELISA METHOD FOR RAPID IDENTIFICATION OF EDIBLE BIRD'S NEST

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Chair : Abdul Rahman Bin Omar, DVM, PhD
Faculty : Institute of Bioscience

Edible birds' nest (EBN) is a precious functional food that has been used for several hundred years by Chinese communities around the world. EBN is mainly comprised of a type of secretion from the salivary gland of 4 swiftlet species in the *Aerodramus* genus. In Malaysia, EBNs are obtained from 2 *Aerodramus* species; *Aerodramus fuciphagus* and *Aerodramus maximus*. Due to the limited supply and high price of genuine EBN, counterfeit EBN products are often found in the market. To differentiate the genuine nests from adulterants, one of the suggested methods is to look at the molecular level of EBN swiftlet species to identify unique sequence in each species. Therefore, the general objective of this study was to conduct a molecular characterization of Malaysian swiftlet species and develop a PCR-ELISA method for rapid identification of EBN. The specific objectives were (i) to characterize Malaysian swiftlet species based on sequencing and phylogenetic analysis of cyt-b and ND2 genes, (ii) to design and optimize primers, probes and PCR-ELISA protocols for the detection of EBN, and (iii) to evaluate the performance of the developed PCR-ELISA using commercially purchased EBN and adulterants. For phylogenetic analysis of Malaysian EBN swiftlet species, DNA extracted from EBN and swiftlet pectoral samples were sequenced at the cyt-b and ND2 gene regions to serve as phylogenetic markers. The information obtained were used to confirm the identity for each species by referring the results to the NCBI website, construct the phylogenetic tree of Malaysian swiftlet species and used for probe design for the development of PCR-ELISA method. From the phylogenetic analysis, it was observed that *Apus affinis* and *Aerodramus* species have a genetic distance of 11.1-14.7% and 18.6-20.0%, respectively, for cyt-b and ND2 gene. Amongst the *Aerodramus* species, both *A. fuciphagus* and *A. maximus* have a genetic distance of 7.5-9.5% for cyt-b gene and 8.9-11.5% for ND2 gene, indicating that both species were more closely related to one another than *A. affinis.* Amongst the white nest swiftlets, they were further separated into either natural cave nests or nests from swiftlet ranch, with genetic distance of 1.7-2.1% for cyt-b gene and 2.0-2.5% for ND2 gene respectively. Not only that, the sequenced data also allowed the successful identification of unique gene regions displaying SNPs as potential molecular markers to differentiate between Malaysian EBN swiftlet species.

By identifying DNA sequences containing at least 2 unique SNPs between species of *A. fuciphagus, A. maximus* and *A. affinis* as potential probe regions, specific probes and primers targeting species-specific regions on cyt-b and ND2 were successful identified. For the development of PCR-ELISA, several factors were also successfully optimized. It was found that optimized results were obtained when 10 × digoxigenin-labelled PCR products were hybridized with 7.5 pmol/well biotin-labelled probe concentration, at 37^{o}C for 1 hr. *A. affinis* gave lower O.D. readings compared to both *Aerodramus* species and thus, the cut-off value for detection of EBN authenticity is set at 1.5. The performance of developed PCR-ELISA was evaluated using commercial EBN samples and adulterants. When tested against a commercially purchase fake nest and three common adulterants; the probe does not recognize the adulterants. This shows the specificity of the probe towards the *Aerodramus* species but not the adulterants, allowing detection of authentic EBN in the sample. Finally, when the performance of PCR-ELISA was evaluated through the spiking test, EBN samples that were spiked with higher concentrations of adulterants have O.D. readings that tend to drop in accordance to the nests concentration. Overall, Malaysian swiftlet species were successfully characterized based on sequencing and phylogenetic analysis of cyt-b and ND2 genes. The primers and probe for PCR-ELISA as well as PCR-ELISA protocols were successfully developed and optimized. The performance of the developed PCR-ELISA was also found to be specific to EBN when the assay was tested using commercially purchased edible birds' nests and adulterants. In conclusion, this study had successfully achieved all the objectives of the study and with the successful development of PCR-ELISA for detecting genuine EBN from samples that were spiked with adulterants, the EBN industry will be able to increase or regain back the confidence level of the consumers and increase the sales of EBN.

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

PENCIRIAN MOLEKULAR SPESIES BURUNG WALIT MALAYSIA DAN PEMBANGUNAN KAEDAH PCR-ELISA UNTUK PENGENALAN CEPAT SARANG BURUNG YANG BOLEH DIMAKAN

Oleh

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Sarang burung yang boleh dimakan (EBN) merupakan makanan fungsian yang berharga dan digunakan selama beberapa ratus tahun oleh masyarakat Cina di seluruh dunia. EBN terdiri daripada sejenis rembesan dari kelenjar air liur beberapa spesies dalam genus *Aerodramus*, seperti *Aerodramus fuciphagus* dan *Aerodramus maximus*. Oleh kerana bekalan yang terhad dan harga tinggi untuk EBN tulen, produk palsu EBN sering dijumpai di pasaran. Untuk membezakan sarang yang tulen dari barang noda, salah satu kaedah yang dicadangkan adalah melalui pendekatan molekular burung walit yang menghasilkan EBN dan mengenalpasti jujukan unik di setiap spesies. Oleh itu, objektif umum kajian ini adalah untuk menjalankan pencirian molekular spesies burung walit Malaysia dan menghasilkan kaedah PCR-ELISA untuk identifikasi cepat EBN. Objektif khusus kajian ini adalah untuk (i) mencirikan spesies burung walit Malaysia berdasarkan penjujukan dan analisis filogenetik gen cyt-b dan ND2, (ii) reka bentuk dan optimumkan primer, proba dan protokol PCR-ELISA untuk pengesanan EBN, dan (iii) menilai prestasi PCR-ELISA terorak menggunakan dagangan komersial EBN dan barang noda. Untuk analisis filogenetik spesies EBN burung walit Malaysia, DNA yang diekstrak daripada EBN dan sampel pektoral burung walit dijujuk pada gen cyt-b dan ND2 sebagai penanda filogenetik. Maklumat yang diperolehi telah digunakan untuk pengesahan identiti setiap spesies dengan merujuk pada keputusan di laman web NCBI, untuk konstruk pokok filogenetik spesies burung walit Malaysia dan untuk reka bentuk proba bagi pembangunan kaedah PCR-ELISA. Berdasarkan analisis filogenetik, ia didapati bahawa *Apus affinis* dan spesies *Aerodramus* mempunyai jarak genetik 11.1-14.7% dan 18.6-20.0%, masing-masing, untuk gen cyt-b dan ND2. Di antara spesies *Aerodramus*, kedua-dua *A. fuciphagus* dan *A. maximus* mempunyai jarak genetik 7.5-9.5% untuk gen cyt-b dan 8.9-11.5% untuk gen ND2, dan ini menunjukkan bahawa kedua-dua spesies ini berkait rapat antara satu sama lain daripada *A. affinis*. Di antara burung walit bersarang putih, species burung ini dibahagikan kepada sarang burung walit dari gua semulajadi atau sarang burung walit dari ladang ternakan burung walit, dengan jarak genetik 1.7-2.1% untuk gen cyt-b dan 2.0-2.5% untuk gen ND2 masing-masing. Bukan itu sahaja, data jujukan juga membolehkan pengenalan gen unik yang mempunyai SNPs sebagai penanda molekular berpotensi untuk membezakan spesies EBN burung walit Malaysia.

Dengan mengenal pasti jujukan DNA yang mengandungi sekurang-kurangnya 2 SNPs unik antara spesies *A. fuciphagus*, *A. maximus* dan *A. affinis* sebagai rantau proba berpotensi, proba dan primer spesifik yang mensasarkan rantau spesies-spesifik cyt-b dan ND2 telah berjaya dikenalpasti. Untuk pembangunan PCR-ELISA, beberapa faktor telah berjaya dioptimumkan. Ia didapati bahawa keputusan optimum diperolehi apabila 10 × produk PCR yang berlabel digoxigenin menghibrid pada kepekatan 7.5 pmol/perigi proba berlabel biotin, pada 37^{o}C selama 1 jam. *A. affinis* memberikan bacaan O.D. yang lebih rendah berbanding dengan kedua-dua spesies *Aerodramus*, oleh itu nilai pemisahan untuk mengenal pasti ketulenan EBN ditetapkan pada 1.5. Prestasi PCR-ELISA juga dinilai menggunakan dagangan komersial EBN dan barang noda. Apabila diuji pada satu dagangan komersial EBN dan tiga barang noda, proba tersebut tidak dapat mengesan barang noda tersebut. Ini menunjukkan kespesifikan proba terhadap spesies *Aerodramus* tapi bukan pada barang noda, dan membolehlan pengesahan EBN tulen dalam sampel. Akhirnya, apabila prestasi PCR-ELISA dinilai melalui ujian spika, sampel EBN yang dicampuraduk dengan kepekatan tinggi barang noda memberi bacaan O.D. yang jatuh mengikut kepekatan sarang burung walit. Secara keseluruhan, spesies burung walit Malaysia telah berjaya dicirikan berdasarkan penjujukan dan analisis filogenetik gen cyt-b dan ND2. Primer dan proba PCR-ELISA, termasuk protokol PCR-ELISA juga berjaya direka bentuk dan dioptimumkan. Prestasi PCR-ELISA terorak juga spesifik pada EBN apabila diuji menggunakan dagangan komersial EBN dan barang noda. Kesimpulannya, kajian ini telah berjaya mencapai semua objektif kajian ini dan dengan PCR-ELISA terorak untuk mengesan tetulenan EBN dari sampel yang dicampuraduk dengan barang noda, industri EBN akan dapat meningkatkan atau mendapat balik keyakinan konsumer dan meningkatkan jualan EBN.

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I certify that a Thesis Examination Committee has met on 14 January 2016 to conduct the final examination of Sue Mei Jean on her thesis entitled "Molecular Characterization of Malaysian Swiftlet Species and Development of PCR-ELISA Method for Rapid Identification of Edible Bird's Nest" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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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:

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

β-fib7	β-fibrinogen intron 7
2DE	2D electrophoresis
ABTS	2,2'- azino-di-3-ethylbenzthiazoline sulfonate
BLAST	Basic local alignment search tool
cyt-b	Cytochrome-b
DIG-dUTP	Digoxigenin-11-dUTP
CE-GC	Capillary electrophoresis- gas chromatography
CTAB	Cetyltrimethylammonium bromide
EBN	Edible bird's nest
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
FTIR	Fourier transform infrared spectroscopy
GAPDH	Intron 11 of glyceraldehyde 3-phosphate dehydrogenase
GC	Gas chromatography
GMP	Good manufacturing practice
GS-MS	Gas chromatography-mass spectrometry
HKY + I	Hasegawa-Kishino-Yano, with invariant sites
HPAE-PAD	High performance anion exchange chromatography with pulsed amperometric detection
HPTLC	High-performance thin layer chromatography
ML	Maximum likelihood
mtDNA	Mitochondrial DNA
myo	Myoglobin intron 2
NCBI	National Center for Biotechnology Information
ND2	NADH dehydrogenase subunit-2
NJ	Neighbour joining
O.D.	Optical density
PCR	Polymerase chain reaction
PCR-ELISA	Polymerase chain reaction-enzyme linked immunosorbent assay
pI	Isoelectric point
qPCR	Real-time PCR
rRNA	Ribosomal RNAs
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscope
SNP	Single nucleotide polymorphism
TAE	Tris-acetate-EDTA
tRNA	Transfer RNA
VNTR	Variations in the number of tandem repeats

CHAPTER 1

INTRODUCTION

Edible birds' nest (EBN) is a precious functional food that has been used for several hundred years by Chinese communities around the world (Lin *et al.*, 2009). EBN is mainly comprised of a type of secretion from the salivary gland of 4 swiftlet species in the *Aerodramus* genus, such as *Aerodramus fuciphagus* and *Aerodramus maximus* (Hamzah *et al.*, 2013a). Due to the limited supply and high price of genuine EBN, counterfeit EBN products are often found in the market (Yang *et al.*, 2014). Various fake materials including *Tremella* fungus, karaya gum, red seaweed are added to increase the net weight of the birds' nest (Ma & Liu, 2012). Nests of other non-edible species of *Aerodramus* are also added to make up for the loss weight of EBN during the cleaning process (Lau & Melville, 1994).

A number of detection methods have been introduced to differentiate the adulterants from genuine EBNs. Most of the detection methods relied on sophisticated but costly and time-consuming equipments, such as microscopy, infrared spectrometry and protein electrophoresis to differentiate adulterants such as *Tremella* fungus, karaya gum and red seaweed. However, current available methods cannot differentiate the counterfeit EBN of other non-edible birds nest from the genuine product due to their identical morphology and similar physiochemical properties (Marcone, 2005; Wu *et al.*, 2010; Zhang *et al.*, 2012a). To differentiate genuine nests from non-edible birds' nests, one of the suggested methods is to look at the molecular level of edible birds' nest swiftlet species to identify unique sequence or region in each species (Lee *et al.*, 2003; Tukiran *et al.*, 2015).

In order to conduct a molecular analysis, DNA must first be extracted from the samples. However, DNA extraction for EBN samples have always been a challenge due to DNA losses, degradation and extraction interference (Atashpaz *et al.*, 2010; Wu *et al.*, 2010). For molecular studies on EBN, an optimal DNA extraction method to improve DNA yield and quality is crucial. Not only that, an assay with high sensitivity and specificity is also required for the detection of EBN. PCR-ELISA is a rapid and powerful immunodetection assay that has been used mainly for detection and differentiation of target genes (Mousavi *et al.*, 2006; Di Pinto *et al.*, 2012). Due to its high sensitivity and specificity, various studies on the use of PCR-ELISA as a detection and diagnostic method were proven successful, particularly in the medical sector (Cosan *et al.*, 2011; Raji *et al.*, 2011; Tahk *et al.*, 2011).

To develop the PCR-ELISA assay, unique regions need to be identified to serve as probes for the genetic identification of EBN. Whilst identifying the unique marker between edible and non-edible birds' nest species, similar molecular information can also be used for phylogenetic analysis. As there is limited publication on the phylogeny characterization of Malaysian EBN swiftlet species, the phylogenetic study provides valuable information on the species origin and diversification of the EBN swiftlet species in Malaysia.

Hence, the first hypothesis of this study is that the phylogenetic characterization can be applied for grouping and differentiation of Malaysian EBN swiftlet species and EBN samples. The second hypothesis of this study is that the newly developed PCR-ELISA method based on cytochrome-b (cyt-b) and/or NADH dehydrogenase subunit-2 (ND2) genes will allow the detection of EBN. The general objective of this study is to conduct a molecular characterization of Malaysian EBN swiftlet species and develop a PCR-ELISA method for rapid identification of edible birds' nest. This can be achieved through three specific objectives listed below:-

(i) to characterize Malaysian EBN swiftlet species based on sequencing and phylogenetic analysis of cytochrome-b (cyt-b) and NADH dehydrogenase subunit-2 (ND2) genes,
(ii) to design and optimize primers, probes and PCR-ELISA protocols for the detection of EBN,
(iii) to evaluate the performance of the developed PCR-ELISA using commercially purchased nests and adulterants.

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