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# Molecular identification of species and production origins of edible bird's nest using FINS and SYBR green I based real-time PCR

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ABSTRACT

The increasing demand and consumption of edible bird's nest (EBN) by people worldwide has contributed to the food fraud issue. To ensure the authenticity of EBN in regard to their origin, rapid and accurate analytical methods are very much needed. In this study, forensically informative nucleotide sequencing (FINS) technique based on mitochondrial and nuclear DNA sequences, and phylogenetic analysis was performed to identify the species and production origins of raw and commercial EBNs. The cytochrome *b* (Cyt *b*), NADH dehydrogenase subunit 2 (ND2), 12S ribosomal RNA and beta-fibrinogen intron 7 gene markers used were able to identify and classify EBN produced by *Aerodramus fuciphagus* and *Aerodramus maximus*. It was newly discovered that EBN from man-made houses and natural caves were genetically differentiable using the mitochondrial Cyt *b* and ND2 genes. The phylogenetic results revealed that all EBN samples were well-separated into two groups following their species origin and production origin. A rapid and cost-effective identification alternative of SYBR green I based real-time PCR assay targeting a 177 bp of the mitochondrial Cyt *b* gene was developed and it efficiently differentiated genuine EBN from counterfeits. This FINS and SYBR green I based real-time PCR are highly sensitive, specific and reliable methods for identification of EBN origins and could be useful for preventing fraud substitution and mislabelling of EBN to ensure food safety.



## 1. Introduction

Edible bird's nest (EBN) or "Yan Wo" in Mandarin, is one of the most precious animal-derived foods, particularly prized in Chinese communities around the world. It is traditionally regarded as a functional food for skin rejuvenating and health promoting. EBN has been demonstrated scientifically to inhibit inflammation and influenza virus, proliferate cell, improve bone strength and dermal thickness, reduce tumour production, and treat erectile dysfunction and osteoporosis (Chua et al., 2013; Guo et al., 2006; Kong et al., 1987; Ma, Liu, & Dai, 2012; Matsukawa et al., 2011; Roh et al., 2011; Vimala, Hussain, & Nazaimoon, 2012). EBN is produced from the saliva of swiftlet species, which belongs to the family Apodidae and genus *Aerodramus*. The *Aerodramus fuciphagus* and *Aerodramus maximus*, commonly known as white-nest and black-nest swiftlets, respectively, are the two dominant EBN-producing species in Malaysia (Wu et al., 2010).

EBN produced by these two swiftlet species possessed different nutritional composition and physicochemical properties (Quek, 2017), and therefore vary in economic value. In terms of commercial market value, EBN originated from *A. fuciphagus* is more expensive than that from *A. maximus* (Quek, Chin, Yusof, Tan, & Law, 2015). Due to their high price and increasing consumer demand, fraud substitution of higher-priced EBN with lower-priced EBN commonly occurred in the market, for higher economic gain. Intentional substitution or mislabelling of food is considered a food fraud (Okuma & Hellberg, 2015). Fraudulent is quite rampant as EBN from different swiftlet species share very similar appearance, especially after production processing. Upon soaking and drying processes, morphological characteristics of EBN are greatly altered

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and it becomes difficult to identify EBN following its swiftlet species by visual inspection. Therefore, it is necessary to develop an efficient, specific and sensitive method for species identification of EBN to verify products labelling accuracy, to protect consumers' rights and to ensure fair trade in EBN-related industry. Molecular methods provide the only means for accurate and reliable EBN identification to the species level.

Several molecular methods, such as polymerase chain reaction (PCR), forensically informative nucleotide sequencing (FINS) and real-time PCR, have been developed to identify species origin of food, including seafood, fish, milk, honey, fruit juice and olive oil (Chapela et al., 2002; Giménez, Pistón, Martín, & Atienza, 2010; Han et al., 2012; Keskin & Atar, 2012; Mayer, 2005; Schnell, Fraser, Willerslev, & Gilbert, 2010). Previous studies on EBN identification focused on swiftlet species of *A. fuciphagus* only, using nested PCR and phylogenetic analysis (Lin et al., 2009), SYBR green I based real-time PCR (Wu et al., 2010), and TaqMan based real-time PCR (Guo et al., 2014).

FINS is a DNA-based technique that combines DNA sequencing and phylogenetic analysis, to accurately identify the species origin of unknown samples based on informative nucleotide sequences (Li, Zhang, But, & Shaw, 2011). The FINS comprises four basic steps, namely DNA extraction, selection and amplification of a specific DNA fragments, DNA sequencing, and phylogenetic analysis against a sequence database (Li et al., 2011). FINS is similar to BLAST analysis where it involves sequencing of amplified DNA products (Huang et al., 2014). Although DNA sequencing is the most direct and simple way to obtain sequence information from PCR products, it is also time-consuming and expensive (Lockley & Bardsley, 2000). A SYBR green I based real-time PCR with high specificity and sensitivity may be an alternative to FINS for rapid detection of EBN. This real-time PCR has gained popularity as it is more cost-effective and time saving compared to other fluorescent-based method (Sasmono et al., 2014). The cytochrome *b* (Cyt *b*), NADH dehydrogenase subunit 2 (ND2) and 12S ribosomal RNA (12S) genes of mitochondrial DNA (mDNA), and beta-fibrinogen intron 7 (Fib7) gene of nuclear DNA (nDNA), are usually employed in phylogenetic studies of swiftlets (Thomassen, den Tex, de Bakker, & Povel, 2005; Thomassen et al., 2003). However, only Cyt *b* and Fib7 genes that have been demonstrated as molecular markers in previous EBN identification studies (Guo et al., 2014; Lin et al., 2009; Wu et al., 2010).

In this study, FINS and SYBR green I based real-time PCR were used for genetic identification of EBN origins. FINS can identify unknown samples to the exact species, while real-time PCR allows rapid detection of EBN without performing DNA sequencing step. The mitochondrial Cyt *b*, ND2 and 12S, and nuclear Fib7 genes sequences were employed to identify and classify EBN via species and production origins through phylogenetic analysis by means of FINS technique. The SYBR green I based real-time PCR assay was established for rapid detection of genuine EBN. This is the first study to identify swiftlet species and production origins of EBN using mitochondrial and nuclear DNA molecular markers, which is useful for overcoming EBN fraud and confirming the authenticity of commercial EBN.

## 2. Materials and methods

### 2.1. Edible bird's nest collection

Eleven types of unprocessed EBN samples originated from two swiftlet species, *A. fuciphagus* and *A. maximus*, were collected from different locations in Malaysia from year 2012–2013. The species and production origins of EBN samples were declared by the experienced farmers and harvesters based on morphological characteristics. Two commercial EBN samples, two counterfeit samples and one Hasma sample as negative control were purchased from local markets. The details of EBN samples are summarised in Table 1.

### 2.2. DNA extraction

DNA was extracted from all samples using the DNeasy® *mericon*™ Food Kit (Qiagen, Hilden, Germany) following manufacturer's instructions with minor modifications. The samples were ground into fine powder using pestle and mortar with liquid nitrogen, and screened through a 1 mm sieve. About 25 mg of EBN sample or 5 mg of hasma sample was mixed with 1.3 mL of Food Lysis Buffer and 5 μL of proteinase K, and incubated at 60 °C for a longer incubation time of 1 h. After centrifugation, 700 μL of clear supernatant was mixed with chloroform. A 350 μL of upper aqueous phase recovered from the next centrifugation was mixed with Buffer PB before passed through QIAquick spin column. The column was washed with Buffer AW2 and centrifuged twice for 1 min to dry the silica membrane. The DNA was then eluted with 100 μL of Buffer EB. The extracted DNA was quantified by BioSpectrometer® Kinetic Spectrophotometer (Eppendorf, Hamburg, Germany).

### 2.3. PCR amplification and DNA sequencing

The quality of extracted DNA from all samples was evaluated by PCR. The mitochondrial Cyt *b*, ND2 and 12S, and nuclear Fib7 primers were used for PCR amplification. The Cyt *b* primers used were ND5 (5′- TAG CTA GGA TCT TTC GCC CT -3′) and Thr (5′- TCT TTG GTT TAC AAG ACC AAT GTT -3′) (Desjardins & Morais, 1990). Primers L5215 (5′- TAT CGG GCC CAT ACC CCG AAA AT -3′) (Hackett, 1996) and H6313 (5′- CTC TTA TTT AAG GCT TTG AAG GC -3′) (Johnson & Sorenson, 1998) were used to amplify ND2 gene region, and primers L1267 tRNAPhe (5′- AAA GCA TGG CAC TGA AGH TG -3′) and H2294 tRNAVal (5′- CTT TCA GGT GTA AGC TGA RTG CTT -3′) (Sorenson, Ast, Dimcheff, Yuri, & Mindell, 1999) for 12S gene region. For Fib7, PCR amplification was accomplished with primers Fib-BI7L (5′- TCC CCA GTA GTA TCT GCC ATT AGG GTT -3′) and Fib-BI7U (5′- GGA GAA AAC AGG ACA ATG ACA ATT CAC -3′) (Prychitko & Moore, 1997).

PCR amplification was performed in a total volume of 50 μL, which contained 10 ng of DNA template, 0.4 μM of each primer and 1 × MyTaq™ Mix PCR buffer (Bioline, London, UK). The PCR amplification was conducted in a C1000 Touch™ thermal cycler (Bio-Rad, California, USA). The thermal cycling conditions for mitochondrial genes comprised an initial denaturation step at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 15 s, optimal annealing at 58 °C for 30 s and extension at 72 °C for 1 min, with a final extension step at 72 °C for 7 min. For Fib7 gene, the optimal annealing temperature was set at 50 °C. Three PCR amplification replications were performed to ensure consistency and repeatability of the results obtained.

PCR products for all genes were analysed on a 1.5% (w/v) agarose gel pre-staining with Red-Safe™ DNA dyes (iNtRON Biotechnology, Sungnam, Korea) in 1 × TAE buffer and electrophoresed at 80 V for 60 min. The gel was visualised under UV light using a Gel Doc™ XR imaging system (Bio-Rad, California, USA). Size of the PCR products was estimated by direct comparison with the 100 bp HyperLadder™ DNA ladder (Bioline, London, UK). All PCR products were directly sequenced in both directions with the same primers used for PCR amplification by Sanger dideoxy method using an ABI3730x1 automated DNA sequencer (Applied Biosystems, California, USA).