



Designing Primers for Amplification of Coi, Cyt-B, Nd2 and Fib7 in Swiftlet (*Aerodramus* spp.)

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ABSTRACT

Background: Edible bird's nests (EBN) are highly nutritious and valued food for their numerous health benefits. Consequently, their high economic value and increasing global consumption demand for EBN have contributed to a rise in counterfeited food issue. Currently, the common method for identifying the origin of edible bird nests is the DNA barcoding PCR-based technique because of the limitations of morphological identification methods; however, the primers currently used for PCR face specific challenges in amplifying DNA from edible bird nests. Thus, newly designed primers are necessary.

Methods: This study aims to redesign primers from the DNA barcode sequences of the mitochondrial genes cytochrome-c oxidase subunit I (COI), NADH dehydrogenase subunit II (ND2), cytochrome b (Cyt-b) and the nuclear gene β -fibrinogen intron 7 (Fib7) of Swiftlet belonging to the *Aerodramus* genus.

Result: Four primer pairs AeroCOIf/AeroCOIr, AeroND2f/AeroND2r, AeroCytbf/AeroCytbr Cyt-b gene and AeroFib7f/AeroFib7r were designed successfully amplifying DNA from *Aerodramus* spp. The resulting amplicon sizes were 157 bp for COI, 150 bp for ND2, 130 bp for Cyt-b and 173 bp for Fib7 with the optimal annealing temperature of 60°C. For the result of the primers sensitivity investigation, the AeroCOIf/AeroCOIr, AeroND2f/AeroND2r, AeroCytbf/AeroCytbr and AeroFib7f/AeroFib7r primers was able to detect DNA concentration at 15×10^{-4} ng/ μ L, 15 ng/ μ L, 15×10^{-3} ng/ μ L and 15 ng/ μ L, respectively. These results demonstrate that these new primers could effectively amplify DNA from EBN belonging to *Aerodramus* spp.

Key words: *Aerodramus* genus, Cytochrome B, Cytochrome-C oxidase subunit I, DNA barcode sequences.

INTRODUCTION

Swiftlets, morphologically, are small insect-eating birds that use their salivary glands to produce saliva to build nests. Nonetheless, edible bird nests (EBN) largely originate from only two species of swiftlets, *Aerodramus fuciphagus* and *Aerodramus maximus* (Daud *et al.*, 2021), which belong to the *Aerodramus* genus. These species are primarily found in Southeast Asia countries such as Vietnam, Thailand, Malaysia, Philippines and Indonesia (Liu *et al.*, 2020) because these areas have appropriate environmental conditions for the growth of them: humidity of about 90%, temperature of 28 ~ 30°C (Yifeng *et al.*, 2018). Nowadays, EBN is used as a health supplement for boosting the immune system (Careena *et al.*, 2018), addressing undernutrition, increasing metabolic activity and promoting healthier skin. Moreover, it also has neuroprotective, antiviral, anticancer and bone-strengthening properties among other benefits (Quek *et al.*, 2015; Hou *et al.*, 2017). Owing to these great benefits for human health, EBN has become a precious and expensive bioproduct. As a result, the production of counterfeit products using lower-quality materials, including white fungus, agar agar, collagen hydrolysate, sterculia gum, pork rinds, egg white, amylum and red gulfweed, can occur throughout any stage of the supply chain, from production to the perpetration of fraud in commerce that affects consumers benefits (Marccone, 2005). In fact, a traditional identification method for EBN authentication such as morphological identification met

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challenging because the homologous shape between genuine EBN and fake EBN. Therefore, developing a novel method for verifying commercial EBN products is definitely significant based on a promising solution as genetic approach (Lin *et al.*, 2009).

Currently, among DNA-based techniques used for food traceability, DNA barcoding is a cost-effective and reliable method for solving this problem (Kress *et al.*, 2015; Willette *et al.*, 2017; Guan *et al.*, 2018; Li *et al.*, 2022) because DNA carries all the genetic information of an organism and can be evaluated through commonly used genes in the

mitochondrial DNA (mtDNA) (Yang and Rannala, 2017). In DNA barcoding method, DNA segments from specific gene regions is used as a molecular marker to identify tissue samples of the species (Long *et al.*, 2020; Melo *et al.*, 2021). It is an ideal tool for research, analysis and molecular determination, especially for degraded DNA samples. Specifically, cytochrome-c oxidase subunit I (COI), NADH dehydrogenase subunit II (ND2) and cytochrome-b (Cyt-b) are genetic markers in mtDNA due to their characteristics such as short length, rapid evolution and extensive databases (Junaid *et al.*, 2017; Quek *et al.*, 2018; Wang *et al.*, 2019; Sajali *et al.*, 2021; Utami *et al.*, 2022). These advantages make them a popular choice in studies aimed at determining the origin of specimens or discovering new species, particularly avian species (Hebert *et al.*, 2003; Yang and Rannala, 2017). In addition, β -fibrinogen intron 7 (Fib7), a nuclear gene (nDNA), was also utilized to identify and classify EBN produced by closely related species (Dai *et al.*, 2015; Lv *et al.*, 2021) because of the highly conserved features of this region. However, the available primer pairs have limitations, such as difficulty in amplifying degraded DNA, low sensitivity and specificity and being time-consuming and costly for DNA sequencing (Lin *et al.*, 2009; Orbayinah *et al.*, 2019). Therefore, in order to overcome the limitations designing new primer pairs is more useful in identifying the origin of EBN. In this study, mtDNA and nuclear DNA, including COI, ND2, Cyt-b and Fib7 genes, were selected to design new primer pairs for the efficient amplification of short DNA barcode sequences from the bird nests of *Aerodramus* spp. Specifically, these new primer pairs can be more suitable for degraded DNA and are specialized for EBN. Moreover, they can offer higher sensitivity and also reduce the time and cost of sequencing, thus contributing to the development of a kit for identifying EBN of uncertain origin.

MATERIALS AND METHODS

Samples collection

The DNA samples extracted from bird nests were provided by the Molecular Biology Laboratory, Institute of Food and Biotechnology Technology, Can Tho University, originating from different swiftlet house farms in the Mekong Delta (09/2022-10/2023).

Gene sequences collection

The COI gene sequences from 3 genus of swiftlet (*Aerodramus* spp.; *Collocalia* spp.; *Apus* spp.) were collected from the genbank database available on the National Center for Biotechnology Information (NCBI) website (<http://www.ncbi.nlm.nih.gov/>). Similarly, the partial ND2, Cyt-b, Fib7 sequences also were obtained from NCBI.

Multiple sequences alignment

Alignment analysis was used to compare the assembled gene sequences to get primers that are specific for *Aerodramus* genus which are not matched with other genus of swiftlet. BioEdit program was used for multiple sequences alignment in order to select the variative region of *Aerodramus* spp. compared to *Collocalia* spp. and *Apus* spp.

Primer design

The results of multiple sequences alignment were then used for designing primer accessed via the Primer3 version 4.1.0 website (<https://primer3.ut.ee/>). The start position and the end position of the chosen gene region were entered into the Included Region and then clicked Pick Primers. The program will provide several potential primer candidates with their particular features. The chosen primer candidates must contain more than 50% GC content of forward and reverse primer. In addition, the melting temperature difference between the two primers was limited to be within 4°C and the length of each primer's length ranges from 15 bases to 25 bases.

PCR reaction

In this study, four primer pairs, AeroCOIf/AeroCOIr, AeroND2f/AeroND2r, AeroCytbf/AeroCytbr and AeroFib7f/AeroFib7r, were used to amplify four gene regions, COI, ND2, Cytb and Fib7 in bird species belonging to the *Aerodramus* spp. (Table 1).

The components involved in the PCR reaction were presented in Table 2. The PCR conditions in COI, ND2, Cytb and Fib7 gene were followed by an initial denaturation of 95°C for 3 mins, 35 cycles of [95°C for 15 s, 60°C for 30 s and 72°C for 1 min] and a final extension of 72°C for 7 mins. The results of the PCR reaction were electrophoresed with 2% agarose gel stained with SafeView and visualized using gel documentation.

Table 1: Sequences information of four primers used in this study.

Primer name	Primer sequence (5'-3' direction)	Product length (bp)
AeroCOIf	CGAGCAGAACTTGGACAACC	157
AeroCOIr	GCGCACCGATTATGAGGG	
AeroND2f	ATGCCTGATCTACCGGACAA	150
AeroND2r	CGGTTGTAGTGGGGATCCT	
AeroCytbf	CCTCACCCCTCCTCCATGAAT	130
AeroCytbr	GAGCGGGGTGAGTATGATGA	
AeroFib7f	GGACCTACTAAGTGCTTCTGGA	173
AeroFib7r	GTCTGTGTCAATTCCTTGGCA	

RESULTS AND DISCUSSION

Multiple sequences alignment

From NCBI, 6 COI partial gene sequences of swiftlets belonging to the *Aerodramus* genus were downloaded from the NCBI database with accession numbers KY350188.1, KY350193.1, KY350191.1, KY350190.1, JQ174502.1 and JQ173912.1. In addition, 2 COI partial sequences of *Collocalia* spp. (JQ174497.1, JQ174500.1) and 2 sequences of *Apus* spp. (KU722438.1, KY242302.1) were also selected for multiple sequence alignment to find the highly conserved region in *Aerodramus* spp. and distinct from 2 other genera (*Collocalia* spp. and *Apus* spp.) in order to enable the design of a primer pair that amplify a short COI sequence from *Aerodramus* spp. The results showed that there were several variative regions between *Aerodramus* spp. and 2 other genera, specifically the position is from 52 bp to 91 bp and the position is from 201 bp to 230 bp potentially to design primers.

For ND2 gene region, 7 ND2 partial sequences of *Aerodramus* spp. (EU085333.1, EU085332.1, KJ671363.1, KJ671358.1, KJ671355.1, KJ671344.1, KJ671340.1), 2 sequences of *Collocalia* spp. (KF818946.1, KF818945.1) and 2 sequences of *Apus* spp. (KR905656.1, KR905653.1) were obtained for multiple sequence alignment. The region from 231 bp to 383 bp was selected for primer design using Primer3 tool with forward primer was located from 231 bp to 290 bp and reverse primer was from 345 bp to 383 bp.

Cyt-b gene

9 sequences belonging to the genus *Aerodramus* spp (MG653625.1, KJ671364.1, KJ671365.1, KJ671366.1, KJ671367.1, KJ671369.1, KJ671370.1, KJ671371.1,

KJ671372.1, KJ671373.1, KJ671374.1, KJ671375.1, KJ671379.1, KJ671380.1, KJ671384.1, JN709906.1, JN709907.1, JN709922.1, JN709923.1), 3 sequences from the genus *Collocalia* spp (JQ353853.1, MG322679.1, MG653638.1) and 2 sequences from the genus *Apus* spp (JQ353923.1, JQ353899.1) were compared for sequence alignment. The gene segment ranging from position 489 to 625 bp was selected for primer design.

Fib7 gene

7 sequences from the genus *Aerodramus* spp (AY513077.1, AY513075.1, AY513078.1, JQ520052.1, AY513098.1, AY513101.1, JQ520047.1), 2 sequences from the genus *Collocalia* spp (JQ520050.1, JQ520049.1) and 2 sequences from the genus *Apus* spp (JQ520068.1, JQ520069.1) were compared to identify suitable sequence regions for designing forward and reverse primers. The gene segment from position 565 to 749 bp was chosen for designing PCR amplification primers targeting the Fib7 gene sequence of the *Aerodramus* spp swiftlets. The forward primer was designed based on the sequence region from position 565 to 583 bp, while the reverse primer was designed based on the sequence region from position 724 to 749 bp.

Primer design

The result of designing the COI primer pair provided by the Primer3 software (Table 3) most values meet the requirements for primer design, but in terms of primer efficiency, the self-dimer formation energy of the reverse primer (-9.89 kcal.mol⁻¹) does not satisfy the criterion of being greater than -9 kcal.mol⁻¹. However, this value deviates only slightly from the requirement, so the primer pair is still used for specificity testing using the Primer Blast tool.

Table 2: The table of PCR reaction component concentrations.

Order number	Components	Reaction concentration	Volume per reaction
1	My taq mix 2X	1X	12.5 µl
2	DNA template	12 ng	4 µl
3	Primer (F)	10 µM	0.75 µl
4	Primer (R)	10 µM	0.75 µl
5	BiH ₂ O		7.0 µl
Total			25 µl

Table 3: The properties of primer pairs provided by Primer3 tool.

Primer name	Product size (bp)	T _m (°C)	ΔG of Hairpin-loop structure (kcal.mol ⁻¹)	ΔG of Self-dimer structure (kcal.mol ⁻¹)	ΔG of Hetero-dimer structure (kcal.mol ⁻¹)
AeroCOIf	157	59.13	-0.19	-3.9	-3.61
AeroCOIr		58.36	-0.33	-9.89	
AeroND2f	150	58.50	0.16	-9.75	-8.02
AeroND2r		59.09	-0.45	-10.76	
AeroCytbf	130	58.48	0.73	-5.38	-12.06
AeroCytbr		58.96	0.57	-3.61	
AeroFib7f	173	58.90	-0.2	-3.54	-5.09
AeroFib7r		58.77	0.63	-3.14	

Similarly, for the primer design results of the ND2, Cyt-b and Fib7 genes, primer pairs were selected to assess their specificity.

The results of the specificity test using the Blast software for all four primer pairs showed the ability to specifically bind with swiftlets belonging to *Aerodramus* spp.

The optimization of the annealing temperature

The annealing temperature of the primer pair amplifying the COI gene: The results of the COI primer pair were shown in Fig 1. PCR product bands were successfully amplified at all tested temperatures, resulting in a 157 bp size. The primer pair consistently produced bright bands, but there were differences in band intensities across the tested temperature levels. The lowest intensity was observed at 55°C, gradually increasing with higher annealing temperatures. However, at 60°C, the band intensity was higher than at 61°C. Additionally, at an annealing temperature of 60°C, the brightest band was obtained without any non-specific products or primer dimers. This temperature was chosen for the next experiment.

The annealing temperature of the primer pair amplifying the ND2 gene: the results of ND2 primers are shown in Fig 2 where PCR product bands at all tested annealing temperatures were amplified correctly at the expected size of 150 bp. All annealing temperatures produced bright bands without any non-specific products or primer-dimers. Among the tested temperatures, 60°C and 61°C exhibited better results compared to the other temperatures. but 60°C was chosen for further investigation due to its smaller deviation from the average melting temperature provided by the software.

The annealing temperature of the primer pair amplifying the Cyt-b gene: The results of gradient annealing temperature survey for Cyt-b primer pairs were shown in Fig 3, the PCR product bands at all tested annealing temperatures were amplified at the correct size of 130 bp. There were no non-specific products or primer-dimers. Specifically, at 60°C the band intensity is brightest. Therefore, the optimal annealing temperature for amplifying the Cytb gene using this primer pair is determined to be 60°C and it was chosen for further investigation.



Fig 1: The PCR annealing temperature's reaction of COI primers.

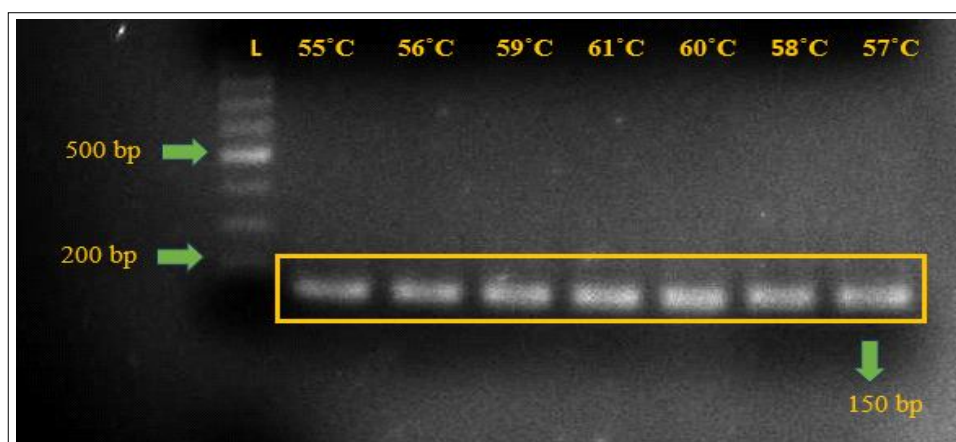


Fig 2: The PCR annealing temperature's reaction of ND2 primers.

The annealing temperature of the primer pair amplifying the Fib7 gene: the results were shown in Fig 4, where all amplified product bands have a size of 173 bp. No non-specific products are observed, but primer-dimers are present. At 60°C, the band intensity is relatively higher compared to 61°C and it represents the highest intensity among the tested annealing temperatures. Therefore, the

optimal annealing temperature for the AeroFib7f/AeroFib7r primer pair is determined to be 60°C and it was selected for further investigation.

The sensitivity of primer pairs

The sensitivity analysis results of the COI primer pair: the results of the sensitivity assessment of the COI primer pair

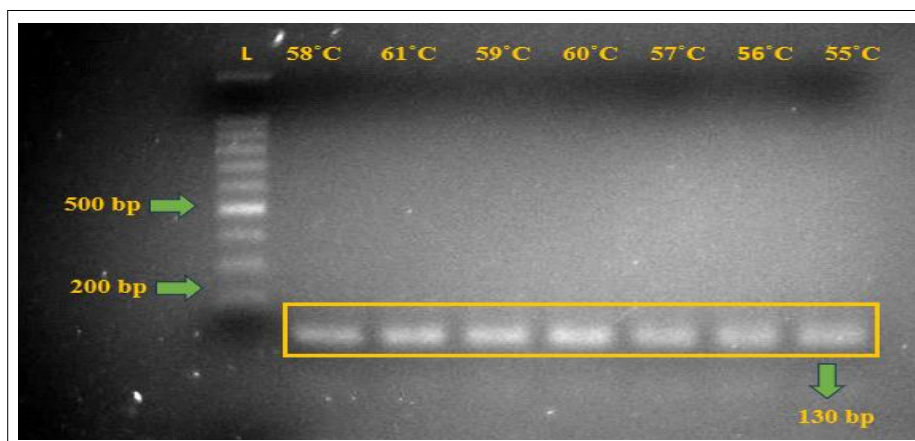


Fig 3: The PCR annealing temperature's reaction of Cyt-b primers.

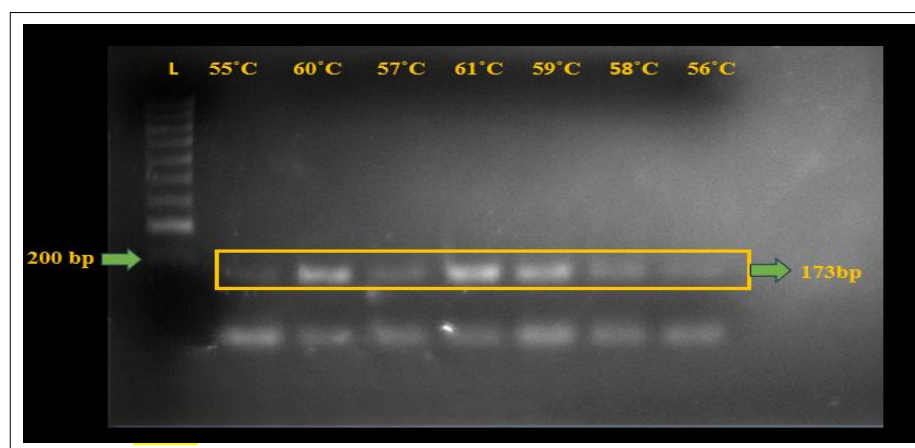


Fig 4: The PCR annealing temperature's reaction of Fib7 primer.

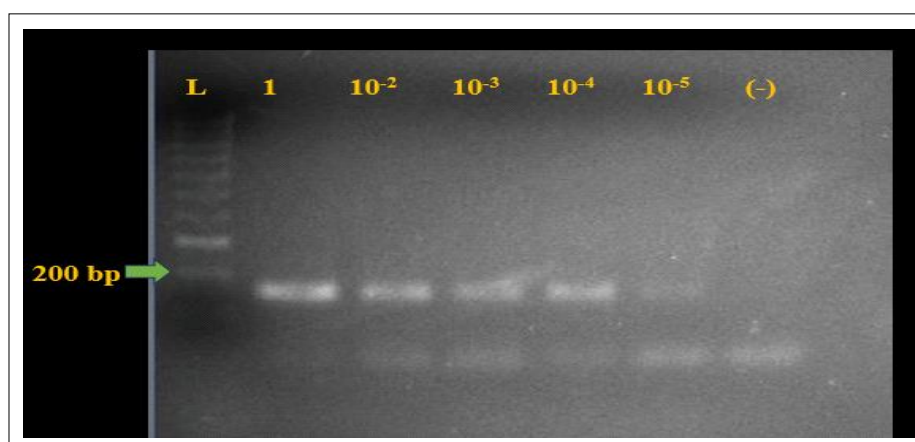


Fig 5: The PCR dilution concentration of COI primers.

in Fig 5 showed that as the DNA concentration decreased from 100 times to 10000 times, the intensity of the band also decreased but was still visible. At a DNA concentration lower than 15×10^{-5} ng/ μ L, the band of the amplified product became fainter and almost invisible. Therefore, the sensitivity of the reaction was determined to be 15×10^{-4} ng/ μ L.

The sensitivity analysis results of the ND2 primer pair: from Fig 6 the result of the sensitivity of ND2 primer pair, it can be observed that at the first dilution factor of DNA sample, no product band appeared, only the product was observed at the original DNA concentration with a size of 150 bp. The extended smeared bands indicate that the DNA concentration is too low compared to the sensitivity of the primers, causing the primer to fail to bind to the target sequence and amplify the product. Therefore, the sensitivity of the reaction is 15 ng/ μ L.

The sensitivity analysis results of the Cyt-b primer pairs: from Fig 7 (A), it can be seen that at the initial dilution factor 100 times, the Cytb primer pair was still able to amplify a clear and bright product band at 130 bp and the intensity of the band is not significantly different from that of the original DNA concentration. However, at the dilution factor of 1000 times, the product band becomes fainter, but still observable and at subsequent dilution factors, no product

band is detected. Therefore, the sensitivity of the reaction is 15×10^{-3} ng/ μ L.

The sensitivity analysis results of the Fib7 primer pair: From Fig 8 (B), it can be observed that at the original DNA concentration, a clear and bright product band is visible at 173 bp. At the first dilution factor, no product band is detected, but at the lower dilution of 1000 times and 10000 times, faint and indistinct bands can be observed. This could be due to the low initial DNA concentration, resulting in extremely low DNA amounts at dilutions of 100, 1000 and 10,000 times, with minimal differences, so the amount of DNA obtained during pipetting at different concentrations is random and lower concentrations tend to yield more DNA, resulting in the appearance of bands. This possibility is supported by the absence of bands in the negative control sample containing BiH₂O, indicating no contamination between the dilutions. Hence, the sensitivity of the reaction is 15 ng/ μ L.

The results from multiple sequence alignment of the COI gene sequence align with Hebert *et al.* (2003), suggesting that the COI mitochondrial gene exhibits high mutation rates and significant variation among avian species. Hence, the COI gene region is often suitable for identifying closely related species, such as swiftlet species belonging to the genera *Aerodramus* spp., *Collocalia* spp.

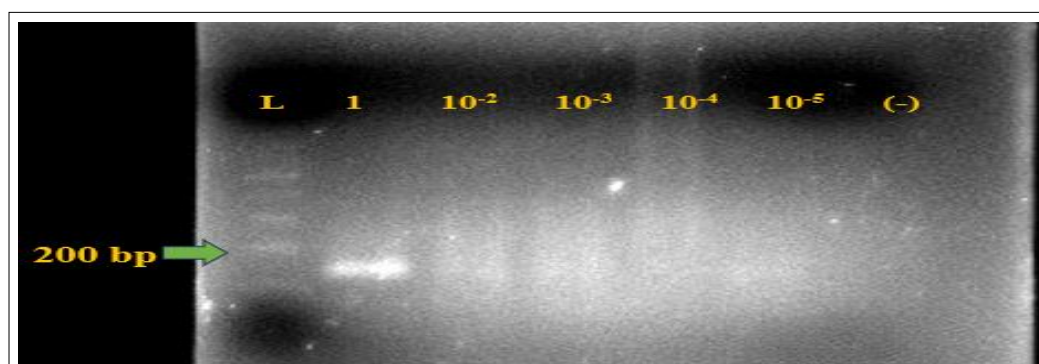


Fig 6: The PCR dilution concentration of ND2 primers.

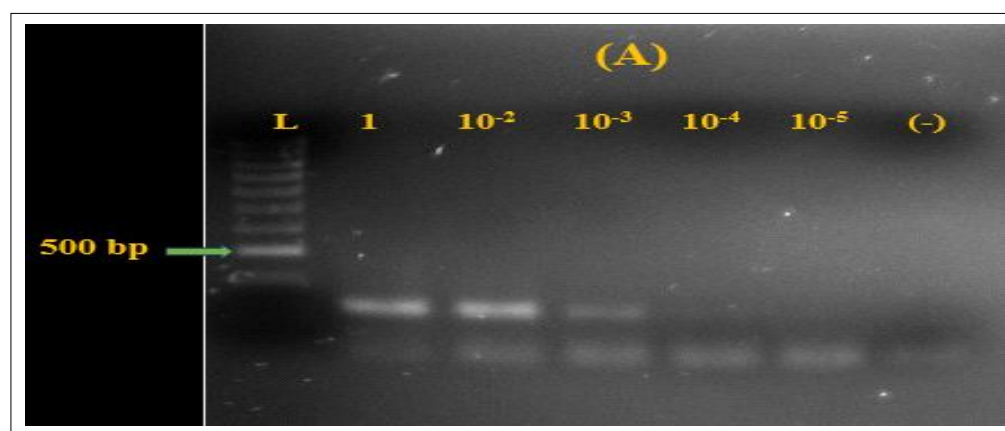


Fig 7: The PCR dilution concentration of Cyt-b primers.

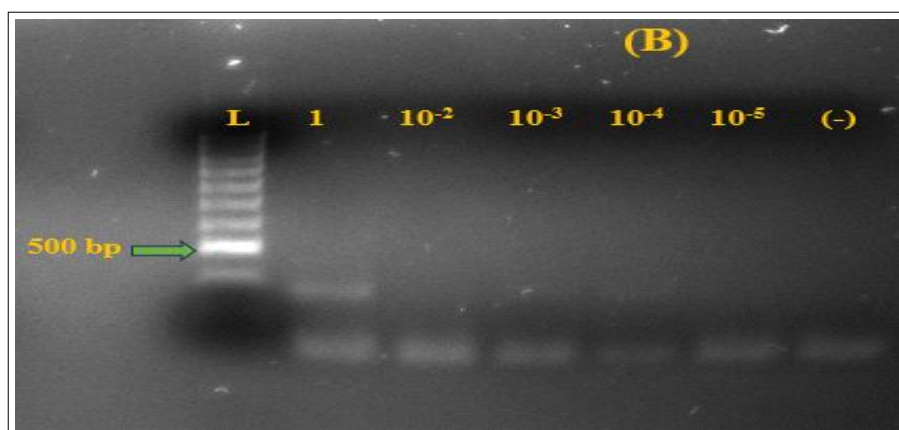


Fig 8: The PCR dilution concentration of Fib7 primers.

and *Apus* spp., due to several appropriately varied sequence regions suitable for primer design. Moreover, selecting a shorter DNA sequence, approximately 100-300 bp, for primer design targeting easily degraded DNA would be more suitable and effective compared to longer sequences (Dai *et al.*, 2015). Similarly, the comparison of the ND2 gene sequences reveals the presence of conserved segments characteristic of higher-order species, along with specific variant positions that could be useful for species differentiation. This outcome aligns with previous analyses showing that the ND2 mitochondrial gene exhibits the highest nucleotide variability, accounting for 9.7% when compared to other genes in swiftlet samples (Quek *et al.*, 2018). Therefore, the mitochondrial ND2 gene sequence appears as a useful molecular marker capable of distinguishing closely related swiftlet species. The chosen sequence size for amplification falls within the range of 100-300 bp (from position 230 to 385 bp), is suitable for amplifying easily degraded DNA, such as from bird's nests and is relatively shorter than the sequence chosen in a previous study differentiating the origins of bird nests in Thailand, where the selected region length was 356 bp (Lv *et al.*, 2021).

The primer pairs designed for the amplification of COI, ND2 and Cyt-B genes exhibited complete specificity towards the *Aerodramus* genus. The primer pair targeting the Fib7 gene exhibits potential matches with some swiftlet species closely related within the Apodidae family. These species include *Collocalia linchi*, *Collocalia esculenta*, *Collocalia affinis*, *Raphidura leucopygialis* and *Hydrochous gigas*, as indicated by NCBI Blast results, but this Fib7 primers did not match the DNA sequence of other organisms. Therefore, this lack of specificity does not compromise the study's primary objective, which is to detect DNA from swiftlets capable of producing edible bird nests, notably associated with two prominent species, *Aerodramus fuciphagus* and *Aerodramus maximus*. Therefore, despite this limitation, this primer pair continues to be utilized in the experiments.

In the sensitivity analysis experiment, the sensitivity of mtDNA was definitely higher than the sensitivity of nDNA.

This was also suitable with characteristics between two gene regions when the mtDNA existence in multiple copies in cells. Furthermore, nDNA analysis requires higher amount of template than mtDNA leading to less sensitivity of analysis (Orbayinah *et al.*, 2019).

CONCLUSION

This study redesigned four primer pairs that successfully amplified regions of four genes from edible bird nest's DNA with amplicon sizes of 157 bp for COI, 150 bp for ND2, 130 bp for Cyt-b and 173 bp for Fib7. The optimal annealing temperature of four primer pairs was 60°C. The sensitivity of the primer pairs amplifying the COI, ND2, Cytb and Fib7 genes had detection limits of DNA concentration at 15×10^{-4} ng/μL, 15 ng/μL, 15×10^{-3} ng/μL and 15 ng/μL, respectively. Based on the study's findings, the newly designed primers demonstrate high sensitivity and the ability to amplify short PCR products, making them well-suited for the application of PCR techniques in identifying market-available edible bird's nest products because these products frequently contain low concentrations and low-quality DNA due to processing.

ACKNOWLEDGEMENT

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RECOMMENDATION

These designed primers should be tested on market-available swiftlet nest samples.

Conflict of interest

We declare that they have no conflict of interest.

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