JURNAL BIOLOGI UDAYANA

P-ISSN: 1410-5292 E-ISSN: 2599-2856

Volume 29 | Nomor 2 | Desember 2025

DOI: https://doi.org/10.24843/JBIOUNUD.2025.v29.i02.p04

PCR reaction testing by using microsatellite DNA markers on Bali Myna samples (*Leucopsar rothschildi* Stressemann, 1912)

Pengujian reaksi PCR menggunakan penanda DNA mikrosatelit pada sampel jalak bali (*Leucopsar rothschildi* Stressemann, 1912)

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Diterima
28 Desember 2024

Disetujui
30 Desember 2025

ABSTRACT

Genetic research requires a good quality and quantity of DNA template and appropriate conditions to obtain optimal PCR results. This study aimed to evaluate and compare PCR reaction conditions and compositions in Bali Myna (*Leucopsar rothschildi* Stressemann, 1912) samples in order to generate PCR products. Samples were obtained from calamus feathers and buccal swabs of captive Bali Mynas at Taman Nasional Bali Barat and the Friends of National Parks Foundation, Nusa Penida, Bali. The DNA extraction was carried out using Chelex® 5% and GeneJET Genomic DNA Purification Kit Thermo Fisher K0721. This study used microsatellite markers with five pairs primers (primer 1 Lr031337, primer 2 Lr052255, primer 3 Lr104861, primer 4 Lr123908, primer 5 Lr045800). The results indicated that calamus samples performed better than buccal swab samples. DNA extracted using the GeneJET Genomic DNA Purification Kit (Thermo Fisher K0721) yielded better results than DNA extracted using Chelex® 5%, as indicated by successful DNA amplification. PCR amplification using primer 3 was successfully achieved with a reaction mixture consisting of 10 µl (1×) mastermix, 1 µM forward primer, 1 µM reverse primer, and DNA template concentrations of 0.5 ng/µl, 1 ng/µl, 2.5 ng/µl, and 3.5 ng/µl. The PCR was performed at an annealing temperature of 55°C for 35 amplification cycles, producing a PCR product of 250 bp.

Keywords: Bali Myna, DNA isolation, non-invasive methods, PCR reaction

INTISARI

Penelitian genetika memerlukan DNA dengan kualitas dan kuantitas yang memadai serta kondisi PCR yang optimal. Penelitian ini bertujuan untuk membandingkan kondisi dan komposisi reaksi PCR pada sampel jalak bali (*Leucopsar rothschildi* Stressemann, 1912) untuk menghasilkan produk PCR. Sampel berupa bulu kalamus dan usap bukal jalak bali yang diperoleh dari penangkaran di Taman Nasional Bali Barat dan Friends of National Parks Foundation, Nusa Penida. Ekstraksi DNA dilakukan menggunakan Chelex® 5% dan GeneJET Genomic DNA Purification Kit Thermo Fisher K0721. Penanda mikrosatelit yang digunakan terdiri atas lima pasang primer (Lr031337, Lr052255, Lr104861, Lr123908, dan Lr045800). Hasil penelitian menunjukkan bahwa sampel kalamus memberikan kualitas DNA yang lebih baik dibandingkan usap bukal. Ekstraksi DNA menggunakan GeneJET Genomic DNA Purification Kit Thermo Fisher K0721 menghasilkan DNA yang lebih baik dibandingkan Chelex® 5% yang ditunjukkan oleh keberhasilan amplifikasi DNA. Kondisi PCR optimal diperoleh menggunakan primer Lr104861 dengan komposisi reaksi berupa 10 µl mastermix (1×), primer forward dan reverse masing-masing 1 µM, serta DNA dengan konsentrasi 0,5 ng/µl, 1 ng/µl, 2,5 ng/µl dan 3,5 ng/µl

pada suhu annealing 55°C dan 35 siklus amplifikasi. Produk PCR yang dihasilkan berukuran 250 bp.

Kata kunci: Isolasi DNA, Jalak bali, Metode non-invasive, Reaksi PCR

INTRODUCTION

Bali Myna, also known as the Curik Bali (*Leucopsar rothschildi* Stressemann, 1912), is endemic to the island of Bali. The species is classified as critically endangered on the IUCN Red List and faces numerous threats in its natural habitat, including illegal poaching and habitat destruction (Sutomo, 2021; Squires *et al*., 2023). Conservation efforts have been carried out through captive breeding programs. Taman Nasional Bali Barat (TNBB) and the Friends of National Parks Foundation (FNPF) Nusa Penida have successfully bred the Bali Myna in captivity and reintroduced individuals into the wild. However, genetic diversity remains a critical concern and may pose a potential threat to the long-term survival of the species. Genetic diversity has an important role in conservation, it can reflect the genetic structure of a species, forms of adaptation and evolution, also can be used in preparing steps to save populations and considerations for determining long-term conservation strategies. Low genetic diversity poses a serious risk to the future sustainability of a species or population. Genetic research requires a good quality and quantity of DNA template and appropriate conditions of PCR reaction to obtain optimal PCR results. The success of a PCR reaction is determined by the concentration and quality of DNA, the annealing temperature of the primers used, the concentration of $MgCl_2$, the polymerase enzyme, the concentration and quality of the primers, total of PCR cycles, deoxynucleoside triphosphate (dNTP) and other factors such as buffer solutions (Gelfand & White, 1990; Setyawati & Zubaidah, 2021).

A study on genetic diversity of Bali Myna was conducted by Ogata *et al* (2020) using microsatellite DNA markers and mitochondrial *cytochrome c oxidase subunit II* gene. The results show that Bali Myna at the Preservation and Research Center Yokohama Japan has low genetic variation. The low genetic variation in its population in Japan is thought to reflect the low genetic variation in Bali Myna population in Bali as the founder. All the samples used in genetic diversity come from blood and muscle tissue. Non-destructive DNA samples taken from plucked feathers and buccal swabs also can provide genetic material. Non-invasive DNA samples can be taken from egg shells, shed feathers and feces. Non-invasive and non-destructive DNA sampling techniques are becoming more important in genetic research because it will minimize stress to organisms and useful for studying endangered species such as birds (Yufei *et al*, 2015). The research regarding the genetics of Bali Myna has not been widely conducted. Therefore, it is necessary to carry out research regarding the source of tissue in DNA extraction, DNA extraction methods and PCR reaction conditions on Bali Myna samples.

MATERIALS AND METHOD

Time and location of research

Sampling was taken in Bali Myna captivity at Taman Nasional Bali Barat (TNBB) and at the Friends of the National Parks Foundation (FNPF), Nusa Penida. Analysis of the sample was carried out at the Veterinary Biomedical and Molecular Biology Laboratory, Faculty of Veterinary Medicine, Udayana University, Bali. This research was conducted from August 2023 to March 2024.

Materials and tools

The materials used in this research are DNA samples (calamus and buccal swab), Chelex® 5%, GeneJET Genomic DNA Purification Kit Thermo Fisher K0721, 5 pairs of primer (primer 1 Lr031337, primer 2 Lr052255, primer 3 Lr104861, primer 4 Lr123908, primer 5 Lr045800), GoTaq® green master mix, agarose, loading dye 1x, ladder and ethidium bromide. Furthermore, the tools used were vortex, centrifuge, micropipettes, thermo-shaker, Qubit 3.0 fluorometer, nanodrop spectrophotometer, thermocycler PCR, electrophoresis chamber, tray and combs, and UV-transilluminator.

Method

Sample Collection

Samples were taken from 10 individuals of Bali Myna at the Taman Nasional Bali Barat (TNBB) and four individuals of Bali Myna at Friends of National Parks Foundation, Nusa Penida, Bali. The source of the sample comes from calamus feathers and buccal swabs. The calamus were taken from a fresh plucked feather. Two feathers were taken from each individual. Buccal swab was done using a sterile cotton bud and stored in a tube containing a lysis buffer. All sampling procedures were assisted by staff of TNBB and FNPF. All the procedures of sample collection permitted by the Secretariat of Scientific Authority for Biodiversity, National Research and Innovation Agency (BRIN) and Direktorat Konservasi Keanekaragaman Hayati Spesies dan Genetik (KKH-SG).

DNA Extraction

DNA extraction carried out using Chelex® 5%. The feathers were soaked in 3% bleach for 30 seconds and rinsed quickly with sterile water. The calamus was cut by a sterile scissor then ground by mortar and transferred into a micro tube (Pharmawati & Yuni, 2020). As much as 250 µl Chelex® 5% was added to the sample then incubated at 100°C for 15 minutes. Through the incubation, the sample was vortexed 2 times for 15 seconds and cooled to room temperature. Furthermore, the sample was centrifuged for 30 seconds at 14.000 rpm. The supernatant was then transferred into a new sterile tube and stored at 4°C or -20°C for long-term storage. On buccal swab samples, the tube containing cotton bud in lysis buffer was vortexed, then the cotton bud was removed and the tube was centrifuged for 10 minutes at 14.000 rpm. Ethanol was thrown away, then the pellet was air dried. Afterwards, DNA extraction takes the same methods as the feather extraction step.

Apart from Chelex® 5%, DNA extraction was also carried out using GeneJET Genomic DNA Purification Kit Thermo Fisher K0721. The feathers were soaked in 3% bleach and rinsed quickly with distilled water. The calamus of the feather was cut using a sterile scissor then was ground by mortar and transferred into an Eppendorf tube. As much as 400 µl lysis solution and 20 µl Protein K were added to the sample then vortexed and incubated at 96°C for 1 hour. Furthermore, 200 µl of 96% ethanol were added and the sample was vortexed then transferred into a new tube. The sample was then centrifuged at 8.000 rpm for 1 minute, then the supernatant was thrown away. 500 µl of Wash Buffer I was added to the sample then centrifuged at 8.000 rpm for 1 minute, supernatant was thrown away. 500 µl of Wash Buffer II was added to the sample then centrifuged at 12.000 rpm for 3 minutes. 200 µl of Elution buffer was added then kept at room temperature for 2 minutes. Afterwards, the sample was

centrifuged 2 x 2 minutes at 12.000 rpm and the sample can be used immediately or stored in the freezer.

The concentration and purity of DNA was measured by Qubit 3.0 Fluorometer Invitrogen by Thermo Fisher Scientific and Nanodrop Spectrophotometer. The ratio of absorbance at λ260 nm λ280 nm was calculated to determine the purity of DNA.

Amplification PCR

DNA sample of Bali Myna was amplified using 5 pairs of microsatellite primers based on Ogata *et al* (2020) (Table 1). PCR reactions were carried out using GoTaq® Master Mix containing dNTPs, MgCl2 and buffer. The PCR reaction was divided into 5 different final volumes: 1) The final volume of the mixture was 10 µl consisting of 5 µl GoTaq® Master Mix, 0.5 µl of each forward and reverse primers, 3 µl aquabidest and 1 µl DNA template. 2) The final volume of the mixture was 10 µl consisting of 5 µl GoTaq® Master Mix, 0.5 µl of each forward and reverse primers, 2 µl aquabidest and 2 µl DNA template. 3) The final volume of the mixture was 10 µl consisting of 5 µl GoTaq® Master Mix, 1 µl of each forward and reverse primers and 3 µl DNA template. 4) The final volume of the mixture was 10 µl consisting of 5 µl GoTaq® Master Mix, 0.5 µl of each forward and reverse primers and 4 µl DNA template. 5) The final volume of the mixture was 20 µl consisting of 10 µl GoTaq® Master Mix, 2 µl of each forward and reverse primers and 6 µl DNA template.

Table 1. Microsatellite Primers of Bali Myna

Locus	Primer
Lr031337	F- TTAAAACAGTGTTGAGAAGC R- TACATAGAGACACCACAACC
Lr052255	F- GTTTTCATTAATTTCTGTGAAGG R- ATTGGACTTAACTGAGAAGC
Lr104861	F- TTGTGGTGATGTTTAATAGC R- TCCTCACTACTGCATTAGG
Lr123908	F- AAAGCTGAAGAATCTTTAGG R- GAGTATGCTGTTCAGTTCC
Lr045800	F- GATGTCCTTTCACATCTCC R- CGAGTTACTCAGAAACTGC

Initial denaturation at 95°C for 2 minutes followed by denaturation at 98°C for 20 seconds. The annealing temperature was divided into several conditions 45°C, 50°C, 55°C and 60°C for 15 seconds and extension at 72°C for 30 seconds. The amplification cycle was repeated 35 times then ended with a final extension at 72°C for 5 minutes.

Electrophoresis

PCR products were visualized using 2% agarose gel. Electrophoresis was carried out at 100 Volts for 40 minutes. Moreover, electrophoresis was also carried out using 20% SDS-PAGE (SDS-Polyacrylamide Gel Electrophoresis) and 12% SDS-PAGE (SDS-Polyacrylamide Gel Electrophoresis) at 100 Volts

for 1.5 hours. The gel was then stained using ethidium bromide and was observed with an UV-transilluminator.

Data analysis

Data were analyzed descriptively by comparing DNA concentration, purity, and PCR amplification success among sample sources, extraction methods, and PCR conditions. PCR success was evaluated based on the presence and clarity of DNA bands observed on agarose gel electrophoresis.

RESULTS

Four samples consisting of two calamus samples (BD1 and JC2) and two buccal swab samples (BA1 and JE1) were then extracted DNA using Chelex® 5%. DNA concentration was tested by Qubit 3.0 Fluorometer. DNA concentrations were 0.088 ng/µl (BD1), 0.086 ng/µl (JC2), 0.146 ng/µl (BA1) and 0.162 ng/µl (JE1). As many as 28 samples from calamus were extracted using GeneJET Genomic DNA Purification Kit Thermo Fisher K0721. DNA concentration and purity were tested using a nanodrop spectrophotometer. DNA concentration and purity of each sample shown in Table 2.

Table 2. Concentration and purity of calamus sample extracted using GeneJET Genomic DNA Purification Kit Thermo Fisher K0721

Population	Code of samples	DNA concentration (ng/ µl)	DNA purify (A260/280)
Taman Nasional Bali Barat (TNBB)	JA1	0.5	0.5
	JA2	2.5	1
	BA1	0.5	1
	BA2	3.5	1.167
	JUV1	1	2
	JUV2	-	-
	JB1	-	-
	JB2	1	2
	BB1	0.5	1
	BB2	0.5	1
	JC1	-	-
	JC2	-	-
	BC1	0.5	1
	BC2	0.5	1
	JD1	0.5	0.5
	JD2	-	-
	BD1	-	-
	BD2	0.5	0.5
	JE1	0.5	1
	JE2	1	1

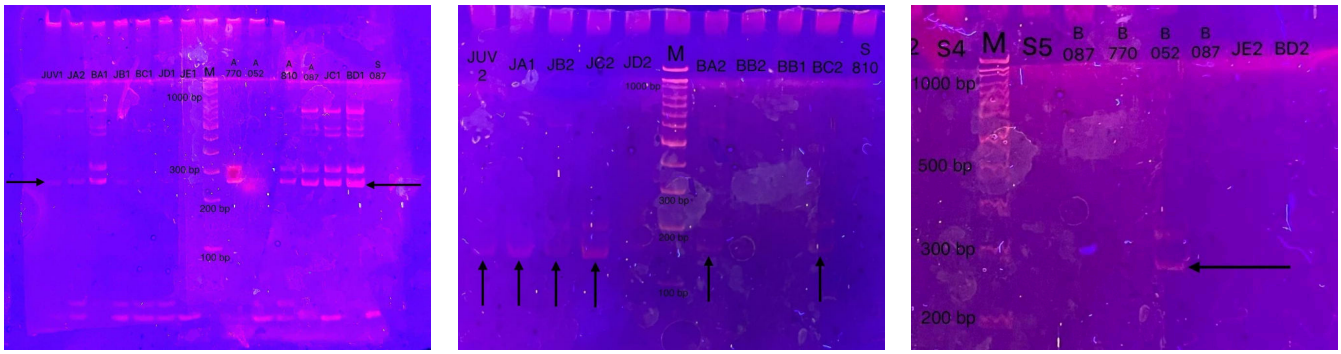
Population	Code of samples	DNA concentration (ng/ µl)	DNA purify (A260/280)
Friends of National Parks Foundation (FNPF), Nusa Penida	A770	0.5	0.5
	B770	0.5	1
	A052	-	-
	B052	0.5	1
	A087	1	1
	B087	0.5	1
	A810	-	-
	B810	0.5	0.5

DNA samples extracted using Chelex® 5% were subsequently subjected to PCR amplification using five pairs of primers with three different reaction compositions: (a) a total reaction volume of 10 µl consisting of 5 µl mastermix, 0.5 µl of each forward and reverse primer, 1 µl of DNA template, and 3 µl of distilled water; (b) a total reaction volume of 10 µl consisting of 5 µl mastermix, 0.5 µl of each forward and reverse primer, 2 µl of DNA template, and 2 µl of distilled water; and (c) a total reaction volume of 10 µl consisting of 5 µl mastermix, 0.5 µl of each forward and reverse primer, and 4 µl of DNA template. PCR products were visualized using 2% agarose. Electrophoresis was done at 100 Volt for 40 minutes. The results showed that no amplification products were detected, as no bands were visible upon PCR products visualization. The optimization steps conducted throughout the study are presented in Table 3.

Table 3. Optimization on the samples

Optimization		Concentrations	Temperature	Optimal conditions	Result
Samples source	Calamus	-	-	Calamus	A total of 18 calamus samples from 12 individuals of Bali Myna could be amplified.
	Buccal swab				
Extraction methods	Chelex	-	-	GeneJET kit	18 samples of DNA extracted using the GeneJET kit could be amplified
	GeneJET kit				
PCR component	DNA	0.5 ng/µl, 1 ng/µl, 2.5 ng/µl dan 3.5 ng/µ	-	0.5 ng/µl, 1 ng/µl, 2.5 ng/µl dan 3.5 ng/µ	Bands can be seen but DNA concentration is still low
	Primer	1 µM	-	1 µM	1 µM of primer can produce clear bands
	Annealing temperatures	-	45°C, 50°C, 55°C, 60°C	55°C	On 55°C produce clear bands, on 45°C and 50°C there are some samples that produce thin bands

A total of 30 samples, consisting of 28 calamus samples and 2 buccal swab samples, were extracted using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, K0721). The final PCR reaction volume for all samples was 20 µl. Each PCR reaction contained 10 µl of 1× mastermix, 2 µl of reverse primer (1 µM), and 2 µl of forward primer (1 µM). The resulting DNA concentrations were 0.5 ng/µl, 1 ng/µl, 2.5 ng/µl and 3.5 ng/µl so that in a DNA volume of 6 µl the total concentrations used were 3 ng, 6 ng, 15 ng and 21 ng. The visualization of PCR products is presented in Figure 1.



(a) (b) (c)

Figure 1. Visualization of PCR products on 30 DNA samples. (a) JUV1, JA2, BA1, JB1, BC1, JD1, JE1, JC1 and BD1 are calamus sample from TNBB; A770, A052, A810 and A087 are calamus samples from FNPF and S087 is buccal swabs sample. b) JUV2, JA1, JB2, JC2, JD2, BA2, BB2, BB1 and BC2 are calamus samples from TNBB and S810 is buccal swabs sample. c) JE2 and BD2 are calamus samples from TNBB and B870, B077, B810 and B052 are calamus samples from FNPF.

DISCUSSION

DNA samples from calamus showed better results compared to samples from buccal swabs. All DNA samples from buccal swabs could not be amplified. These results differ from those reported by Yannic *et al*. (2011), who found that buccal swab samples from ivory gulls (*Pagophila eburnea*) exhibited higher PCR amplification success (100%) than feather-derived samples (83–100%). However, in the study of Turcu *et al* (2023) on sex determination using four bird species (adult and juvenile individuals), pigeons (*Columba livia domestica*), parakeets (*Psittacula krameri*), lovebirds (*Agapornis* spp.) and scarlet-chested parrots (*Neophema splendida*) showed that samples from feathers (96.77%) produced a higher PCR success rate compared to samples from oral swabs (93.55%) in adult birds. In juvenile individuals, oral swab samples (94.29%) produced a higher PCR success rate than samples from fur (72.09%). The amount of DNA obtained from buccal swabs can be affected by some conditions such as the number of bacteria in the mouth area and the time of sampling (Phillips *et al*., 2012). Other factors that affect DNA concentration include sampling techniques, such as the scraping method, and the duration of sample storage (van Wieren-de Wijer *et al*., 2009).

DNA extraction using GeneJET Genomic DNA Purification Kit Thermo Fisher K0721 in both samples (calamus and buccal-swab sample) was also better than the DNA extraction using Chelex® 5%. The results showed PCR product bands were invisible during the DNA visualization process on all samples extracted using Chelex® 5%. DNA extraction using a kit can minimize human error that causes contamination (Hajibabaei *et al*, 2005; Ariyanti & Sianturi, 2019) and the quality of the DNA obtained is relatively pure (Ariyanti &

Sianturi, 2019). DNA extraction using Chelex® 5% has the disadvantage, DNA and RNA produced are relatively small, and the heating process can damage the double-stranded structure of the DNA produced (denaturation). Furthermore, molecules of DNA and RNA will be less stable for long-term storage (Phillips *et al.*, 2012; Walsh *et al.*, 1991; Marwayana, 2015).

Calamus samples of Bali Myna using primer 3 Lr104861 (244-252 bp) and electrophoresis with 12% SDS showed that only the 250 bp allele appeared. The PCR reaction containing 0.5 µl of each primer and 1 µl of DNA template failed to produce detectable amplification products. Similarly, PCR amplification was not observed when the reaction mixture consisted of 0.5 µl of each primer and 4 µl of DNA template. The PCR reaction containing 1 µl of each primer and 3 µl of DNA template produced thin amplification bands. The PCR reaction, composed of 10 µL of master mix, 2 µL of forward primer, 2 µL of reverse primer, and 6 µL of DNA template, produced clear bands corresponding to the target size of 250 bp. Of the 14 Bali Mynas used in this study, 12 individuals (8 from TNBB and 4 from FNPF, Nusa Penida) produced clear PCR bands, whereas 2 individuals from TNBB failed to amplify. This may due to degraded or damaged DNA templates or the presence of inhibitor (Dietrich *et al*., 2013).

The PCR reactions that successfully produced amplification products in this study used a reverse primer at 1 µM, a forward primer at 1 µM, and DNA template concentrations of 3 ng, 6 ng, 15 ng, and 21 ng. The purity of DNA from this study is 0.5, 1.0, 1.167 and 2.0. DNA molecules are pure if a ratio ranging from 1.8-2.0. Samples with purity values below 1.8 indicate the presence of contaminants in the DNA isolate or an insufficient DNA concentration (Lucena-Aguilar *et al*., 2016). The PCR reactions that yielded successful amplification consisted of 2 µL reverse primer, 2 µL forward primer, and 6 µL DNA template. The results of this study indicate that higher primer and DNA template volumes improve PCR product quality. Annealing temperature of 55°C produced clearer bands compared to annealing temperatures of 45°C, 50°C and 60°C. Annealing temperature affects the process of attaching primers to DNA templates. The annealing temperature depends on the type of primer used and the size of the fragment being amplified (Asy'ari & Noer, 2005).

DNA concentration of the non-amplified samples was 0.5 ng/µl and 1 ng/µl so in a DNA volume of 6µl the total concentration was 3 ng and 6 ng. There was no difference between higher and lower DNA concentrations in producing PCR products. Samples with a DNA concentration of 1 ng/µl can't produce PCR products, but samples with a concentration of 0.5 ng/µl can produce PCR products. Low sample quality and inadequate primer quality or concentration can lead to unsuccessful PCR amplification. Studies on conventional PCR assays have demonstrated that varying primer concentrations affects sensitivity and the quality of amplification products. Mardiana *et al*. (2023) compared PCR amplification at 5 µM and 10 µM primer concentrations and found that the higher concentration produced clearer and stronger bands for the target gene than the lower concentration, demonstrating that primer concentration affects product yield and visibility. At concentrations greater than the optimal concentration, primers can form dimers and interfere with target-specific PCR (Park *et al*., 2010). In this study, all DNA samples from buccal swabs could not be amplified because the quality and quantity of DNA from buccal swabs were very low.

CONCLUSION

DNA samples obtained from the calamus were more suitable as a DNA source than those obtained from buccal swabs. In addition, DNA extraction using

the GeneJET Genomic DNA Purification Kit (Thermo Fisher K0721) produced better results than extraction using Chelex® 5%. Optimal PCR amplification using primer 3 (Lr104861) was achieved with a reaction mixture consisting of 10 µl (1×) mastermix, 1 µM forward primer, 1 µM reverse primer, and DNA template concentrations of 0.5 ng/µl, 1 ng/µl, 2.5 ng/µl, and 3.5 ng/µl. The PCR was performed at an annealing temperature of 55°C for 35 amplification cycles. The resulting PCR product had a size of 250 bp. Furthermore, optimization of the DNA extraction process is necessary to obtain DNA with higher concentrations and improved quality.

ACKNOWLEDGEMENT

We thank Sekretariat Kewenangan Ilmiah Keanekaragaman Hayati, National Research and Innovation Agency (BRIN) and Direktorat Konservasi Keanekaragaman Hayati Spesies dan Genetik (KKH-SG) for research permit. We also extend our gratitude to the staff of Taman Nasional Bali Barat (TNBB) and Friends of National Parks Foundation (FNPF), Nusa Penida for their valuable assistance during sample collection. We sincerely thank Prof. Dr. drh. I Gusti Ngurah Kade Mahardika and the staff of the Veterinary Biomedical and Molecular Biology Laboratory, Faculty of Veterinary Medicine, Udayana University, for their support and assistance throughout this research.

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