RESEARCH ARTICLE

Eggshell membrane for DNA sexing of the endangered Maleo (*Macrocephalon maleo*) [version 1; peer review: 1 approved with reservations]

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Abstract

Background: Noninvasive DNA sampling has been applied across many avian genetic studies for a variety of purposes including conservation and management of endangered birds. However, its application in megapodes is still lacking. The previous genetic studies on megapodes used either blood or fresh tissue. Here we present the first demonstration of the use of eggshell membrane for research on endangered Maleo (*Macrocephalon maleo*).

Methods: We used 24 post-hatched eggshell membranes collected from two different sites, Tambun and Tanjung Binerean, in North Sulawesi, 12 samples in each. Two different DNA extraction methods: alkaline lysis method and gSYNC™ DNA Extraction Kit were applied. To determine the sex of Maleo, we utilized PCR-based DNA sexing using CHD genes, with the primer set 2550F/2718R.

Results: We successfully extracted all samples; the mean sample concentration was 267.5 ng/μl (range 47–510.5 ng/μl) and samples were of high purity (A260/280 ratio 1.85±0.03). All samples were used to successfully identified sexes, 9 females and 15 males.

Conclusions: Our research clearly illustrates that eggshell membranes can be used for DNA sexing and open the possibility to build noninvasive DNA collections over large spatial scales for population study of endangered birds.

Keywords

eggshell membrane, endangered birds, DNA sexing, , Maleo

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Saputra AW: Data Curation, Investigation, Project Administration, Writing – Original Draft Preparation

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Introduction

Studies on molecular ecology have a great impact on our knowledge on ecology and evolution of animals, i.e. the phylogenetic relationships and systematics of organisms, population genetics, mating systems, micro-evolutionary processes and host-parasite interactions¹⁻⁴. Oftentimes a necessary prerequisite for answering evolutionary or ecological questions is access to a good DNA sample. Birds' blood contains nucleated red blood cells with abundant DNA, making it a preferred source of DNA⁵. However, obtaining blood requires the capture of a bird, which can provoke an increased level of stress and might result in unusual behavior or nest desertion. For example, blood sampling has been reported to reduce annual survival in Cliff Swallows (*Petrochelidon pyrrhonota*)⁶, although the effects of blood collection in free-living adult and immature birds is not thought to have major negative effects on adult survival, reproductive success, body condition, or behavior⁷.

As an alternative to invasive sampling, researchers have adopted noninvasive sampling methods such as DNA capture from molted feathers^{8,9}, feces^{10,11}, and egg shell membrane^{12,13}. In addition the application of moderately invasive sampling such as buccal swabs^{14,15} has also increased.

Megapodes (family Megapodidae) are a galliform clade, centered in Australasia¹⁶, that are known for their unique super-precocial behavior¹⁷. Their ground-living habits, large body size and large egg size make them particularly vulnerable to human persecution, habitat destruction and habitat loss: 11 out of 21 species are now considered endangered or threatened in some form¹⁸. Given this precarious conservation situation, the application of noninvasive DNA sampling techniques is crucial for megapode birds. Yet previous genetic studies on this family have used either blood¹⁹ or fresh tissue²⁰.

Previously, all megapodes were assumed to be monogamous. The mating system is considered to correlate with sexual selection, with sexually dimorphic birds are non-monogamous and monomorphic birds are monogamous. The evolution of non-monogamous systems in birds was believed to be an adaptive solution to an unbalanced sex ratio²¹. The sex-ratio in Maleo (*Macrocephalon maleo*) is unknown, but based on previous assumptions, it is expected that the Maleo has an evenly balanced sex ratio. Even though Maleo are slightly sexual dimorphic, the available population data only report total population size and never mention sex ratio. A study on the correlation of incubation temperature and sex ratio of chicks has been carried out in the Australian brush-turkey (*Alectura lathami*), which revealed that at average temperature the hatched chicks in the proportion of 1:1 of male and female chicks²².

The purpose of our study was to determine whether the eggshell membrane of the endangered Maleo, a monotypic genus within the megapodes, could be successfully extracted and amplified for DNA sexing. Adult male and female Maleo are morphologically slightly different, but the chicks are not. To determine the sex in Maleo chicks, vent sexing has been conducted. Base

on cloaca size and shape, a one-day-old male Maleo chick cloaca is bigger (3.96 ± 0.11 cm) and rounded, than the female cloaca (3.20 ± 0.10 cm), which is more oval in shape. The concentration of estrogen in female birds was also higher²³. Until recently, no molecular technique has been applied for sex determination of Maleo.

Maleo are endemic to Sulawesi, Indonesia^{24,25}. The bird is a burrow-nesting megapode that incubates its eggs in communal nesting sites on beaches (coastal nesting grounds) and in soil heated by volcanic activity mostly at inland localities. Due to its small, severely fragmented population and continued rapid decline, the International Union for Conservation of Nature has classified Maleo as an endangered species²⁶. Among the major threats are the over-exploitation of eggs and loss of connectivity between forest and nesting grounds²⁷. To minimize these threats at some nesting grounds, conservation programs are currently removing eggs and hatching them in safer, semi-natural hatcheries, built close to the nesting grounds. These facilities provide an opportunity to collect noninvasive DNA samples from the egg-shell membrane left in the soil or brought to the soil surface by the hatched Maleo.

Methods

Study sites and genetic sampling

Post-hatched egg-shell membranes were collected from semi-natural hatcheries of Maleo at two different nesting grounds: an inland geothermal heated nesting ground at Tambun (Bogani Nani Wartabone National Parks and a sun-heated sand beach nesting ground at Tanjung Binerean, North Sulawesi, Indonesia (Figure 1). All samples were collected from 4th April until 1st May 2018. To prevent post-sampling contamination, each sample was placed separately in a zip-lock plastic bag and stored in silica gel for delivery to laboratory. The samples were stored at -40°C until DNA extraction were conducted.

DNA extraction

We used two different DNA extraction methods: the alkaline lysis method and gSYNCTM DNA Extraction Kit (Genaid). For the alkaline lysis method, we followed the recommended procedure for rapid preparation of mouse tails or nail lysates suitable for amplification using DNA polymerase from hyperthermophilic archaeon *Pyrococcus kodakaraensis* (KOD FX Neo 1103; TOYOBO Co. Ltd.). The eggshell membrane (20–25 mg; mostly with dry allantois blood vessel) was grinded using a micro-pestle in a 1.5 mL microcentrifuge tube; next, 180 µL NaOH (50 mM) was added, the suspension mixed thoroughly by vortexing and then incubated at 90°C in water-bath for 10–29 min. Following this, 20 µL Tris-HCl (1 M, pH 8.0) was added and the tube was vortexed thoroughly, then centrifuged at 12,000 RPM for 5 min. Supernatant was removed to new 1.5 mL microtube and store at freezer until used for PCR.

Meanwhile the protocol for gSYNCTM DNA Extraction Kit (Genaid) followed the provided user manual with little modifications. The eggshell membrane (25 mg; mostly with dry allantois blood vessel) was grinded using a micro-pestle in a 1.5 mL

Figure 1. Sampling sites of eggshell membrane of Maleo (*Macrochepalon maleo*) in North Sulawesi, Indonesia.

microcentrifuge tube; 300 μ l GST Buffer (Tris, SDS) and 30 μ l Proteinase K (10 mg/ml) was added to the sample mixture, mixed thoroughly by vortexing and incubated at 60°C in water-bath overnight or until the tissue was lysed completely. Next, 200 μ l GSB Buffer was added to the sample mixture, mixed thoroughly by pulse-vortexing and incubated at 70°C for 10 min. After this, 200 μ l ethanol (100%) was added to the sample mixture, which was mixed thoroughly by pulse-vortexing and brief spinning of the tube to remove drops from the inside of the lid. Next, a GS Column was placed in a Collection Tube and the mixture (including any precipitate) was carefully transferred to the GS Column, which was centrifuged at 14,000 RPM for 1 min then the GS Column was placed in a new Collection Tube. Following this, 400 μ l W1 Buffer was added to the GS Column and centrifuged at 14,000 RPM for 1 min then flow-through was discarded. Next, 750 μ l Wash Buffer was added to the GS Column, centrifuged at full speed for 1 min, then the flow-through was discarded, the tube centrifuged at 14,000 RPM for an additional 3 min to dry the column, 50 μ l of preheated Elution Buffer (pH 7.5–9.0) added to the membrane of the GS Column. The GS Column was then left to stand for 3 min, following a final centrifugation at full speed for 2 min to elute the DNA.

The eluted DNA (1 μ l) was quantified using NanoVue Plus™ (Biochrom, Harvard Bioscience, Inc), at A260 nm. The 260/280 nm absorbance ratio was also measured to give an indication of purity of the DNA. Pure DNA has expected ratios of 1.7–1.9.

DNA sexing

To determine the sex of Maleo, we applied PCR based DNA sexing by using CHD genes, with the primer set 2550F/2718R²⁸. PCR used a 10 μ l total volume containing template DNA (genomic DNA or lysate), 1.2 μ l sterile dH₂O, 5 μ l 2x PCR buffer KOD FX Neo, 2 μ l dNTPs (2 mM), (TOYOBO Co. Ltd.), 0.3 μ l Primer 2550F (10 μ M; 5'-GTT ACT GAT TCG TCT ACG AGA-3'), and 0.3 μ l Primer 2718R (10 μ M; 5'-ATT GAA ATG ATC CAG TGC TTG-3',²⁸), and 0.2 U KOD polymerase enzyme. PCR was carried out in a Veriti™ 96-well thermal cycler (Applied Biosystems™). For genomic DNA templates, the following profile was used: 1 cycle at 94°C for 2 min followed by 35 cycles of 98°C for 10 sec, 53°C for 30 sec and 68°C for 45 sec; and a final extension at 68°C for 7 minutes. For lysate as DNA template, the PCR profiles was the same for DNA genome, except that it was run for more cycles (40x). We

employed eggshell membrane from female domestic chicken as positive control, and tube without sample as negative one.

Amplification of CHD Genes were resolved on a 2% agarose gel. Electrophoresis was conducted using TAE (0.5×) buffer, stained by ethidium bromide (1%), at 100 V for 30 minutes; and 5 µl PCR product was mixed with 1 µl loading dye. After finish, the gel was visualized and analyzed on Gel Logic 200 Imaging System and Kodak Molecular Imaging Software. To confirm that the amplified fragments were the CHD genes, the PCR products of one male and one female sample, respectively, were sequenced. The gels were cut on upper and lower bands for female samples and the single band for male sample, then purified for sequencing. The sequence reactions were carried for both direction in sequencing services laboratory provided by 1st BASE Laboratories (Apical Scientific Sdn Bhd, Malaysia). The sequences were check and edited manually on Bioedit version 7.0.5.3 (Hall, 1999) and Chromas versi 2.6.5 (Technelysium Pty Ltd). Sequence similarity was probed using **NCBI BLAST** (Zhang *et al.*, 2000).

Statistical analysis

Mann-Whitney U-test (at a significance level of 95%) was performed to assess the DNA concentration differences which were collected on Tanjung Binerean and Tambun. Analysis was performed using SPSS v17.

Results

All eggshell membranes were successfully extracted, with mean DNA concentrations around 267.5 ng/µl (range 47–510.5 ng/µl). The average DNA concentration extracted from eggshell membrane collected from coastal nesting grounds (Tanjung Binerean: 213±179 ng/µl,) was significantly lesser than of that of inland nesting grounds (Tambun: 322±153 ng/µl, $p=0.004$; Data Supp.1). These results demonstrate that all samples were adequate for further PCR based analysis.

The 260/280 nm absorbance ratio of all samples ranged from 1.81 to 1.89, with an average of 1.85 (± 0.03). Meanwhile the average for Tambun and Tanjung Binerean samples were, respectively, 1.85 (± 0.03) and 1.84 (± 0.01); **Table 1**.

Table 1. Concentrations and purity of DNA extracted from eggshell membrane of Maleo (*Macrocephalon maleo*).

No.	Location	Sample Code	Purity (A260/A280 Ratio)	Concentration (ng/µl)
1	Tambun	MT03	1.848	127.5
2	Tambun	MT02	1.821	204.0
3	Tambun	MT17	1.879	280.0
4	Tambun	MT18	1.845	302.5
5	Tambun	MT10	1.822	308.0
6	Tambun	MT04	1.881	308.5
7	Tambun	MT12	1.829	342.0
8	Tambun	MT06	1.869	349.5
9	Tambun	MT07	1.898	364.5
10	Tambun	MT19	1.832	381.0
11	Tambun	MT20	1.846	383.0
12	Tambun	MT15	1.887	510.5
Average (Tambun)			1.855	321.75
13	Tanjung Binerean	MB09	1.880	47.0
14	Tanjung Binerean	MB11	1.859	72.5
15	Tanjung Binerean	MB10	1.813	155.0
16	Tanjung Binerean	MB08	1.818	174.4
17	Tanjung Binerean	MB04	1.839	183.0
18	Tanjung Binerean	MB07	1.844	206.5
19	Tanjung Binerean	MB12	1.858	209.0
20	Tanjung Binerean	MB05	1.841	232.0
21	Tanjung Binerean	MB02	1.821	249.5
22	Tanjung Binerean	MB03	1.852	250.0
23	Tanjung Binerean	MB06	1.816	286.0
24	Tanjung Binerean	MB01	1.893	495.0
Average (Tanjung Binerean)			1.845	213.3
Average (all samples)			1.850	267.5

This result suggested good purity of DNA extracted from eggshell samples. However, gel visualization of extracted DNA showed smears in all samples (Figure 2), pointing to some DNA degradation.

Sex determination

Out of 24 samples in which extracted DNA was used as a template, one did not amplify. Meanwhile all samples based on lysate were successfully amplified. There was complete agreement in gender determination across all Maleo samples that were run with different DNA template (Figure 3). Females showed two bands (545 bp and 395 bp), whereas males exhibited one band (545 bp). Three sequences of CHD1 genes have been deposited in GenBank (accession numbers MT074328, MT074329 and MT074330). Sequence similarity searches on the upper band revealed a match with CHD-Z genes of other bird species (i.e. *Anser cygnoides*, *Anser reevesii*, *Anas penelope*). Meanwhile the lower band matched CHD-W submissions of other birds (i.e. *Gallus gallus*, *Crossoptilon mantchuricum*, *Syrnaticus reevesii*).

In total 9 samples were identified as females and 15 were males (Table 2). Based on this limited sample, the sex ration of Maleo's chicks in Tambun and Tanjung Binerean is biased towards

males. The sex ratio males to females was 1.6, and significantly different from the hypothetical sex ratio 1:1 for Maleo.

Discussion

This study has demonstrated the first successful DNA isolation from eggshell membranes of a megapode bird. Our success rate (100%) compares favorably to that of previous avian eggshell membrane studies of Black-tailed Godwits (*Limosa limosa*), which also successfully extracted DNA from all 47 eggshell membranes¹³. The freshness of the samples might be one of the determinant factors of DNA extraction success. Our samples were relatively fresh, extracted 5–15 days after collection and kept in the freezers until extraction, with no concomitant extraction failure and high purity (1.85 ± 0.03) and concentration (267.5 ng/ μ l) of DNA.

PCR amplification of CHD genes succeeded in 96% of the eggshell membrane samples, with only one eggshell membrane isolate out of 24 failing to amplify. However, using lysate as DNA template for PCR resulted in 100% amplifications across 24 samples. This result show that eggshell membrane isolates yielded DNA with little amplification problems. Compared to blood DNA isolates, eggshell membrane DNA isolates of Black-tailed Godwits (*Limosa limosa*) also yielded fewer amplification problems¹³. One eggshell membrane DNA isolate out of 21

Figure 2. Gel visualizing DNA degeneration of eggshell membrane samples. Above: Tambun; Below: Tanjung Binerean; LD: 100 bp DNA Ladder (SMOBiO); AK01: domestic chicken eggshell membrane.

Figure 3. Gel visualizing of CHD-genes amplification of Maleo using extracted DNA (left) and Lysate (right) for molecular sexing of Tambun (above) and Tanjung Binerean (below) samples. AK1, positive control, female chicken; K, negative control, no template.

Table 2. Identified sex of Maleo (*Macrocephalon maleo*) based on molecular sexing with different templates.

No.	Sample Code	Template	
		Extracted DNA	Lysate
1	MT02	Male	Male
2	MT03	Male	Male
3	MT04	Male	Male
4	MT06	Male	Male
5	MT07	Male	Male
6	MT10	Female	Female
7	MT12	Female	Female
8	MT15	Female	Female
9	MT17	Male	Male
10	MT18	Male	Male
11	MT19	Male	Male

No.	Sample Code	Template	
		Extracted DNA	Lysate
12	MT20	Male	Male
13	MB01	Female	Female
14	MB02	Male	Male
15	MB03	Male	Male
16	MB04	Female	Female
17	MB05	Male	Male
18	MB06	Male	Male
19	MB07	Male	Male
20	MB08	Female	Female
21	MB09	-	Female
22	MB10	Female	Female
23	MB11	Male	Male
24	MB12	Female	Female

and 3 samples of Black-tailed Godwit (*Limosa limosa*) did not amplify for 2 and 5 of the 11 microsatellite loci. The amplification success rates was 99.1%¹³. Meanwhile the success rate of eggshell membrane of Sage Grouse (*Centrocercus urophasianus*) for DNA sexing was only 55.6%¹².

This study demonstrates that hatched eggshell membrane provides useful noninvasive DNA material as an alternative to invasive sampling in sex determination studies of Maleo. Information of the sex of the hatched eggs are important to understand demographic issues, such as the demographic consequences of offspring sex ratio bias or whether there is any sex-specific mortality or dispersal. Furthermore, this information is very important for translocation programs of endangered species, including Maleo.

This study provides additional evidence that noninvasive DNA samples yield reliable results and eliminating the need for capture and invasive sampling. Collection of post-hatched eggshell membrane of Maleo, and other megapodes does not require specific skills. This noninvasive DNA sampling also opens the possibility to build participation of local community or local conservation area staff on DNA collections over large spatial scales. Furthermore, the collected samples provide sufficient samples required for population and other ecological and evolutionary study of endangered bird species.

Data availability

Underlying data

NCBI GenBank: Macrocephalon maleo isolate MT10-FTB_UAJY_01 chromo-helicase DNA binding protein (CHDZ) gene, partial cds. Accession number [MT074328](#).

NCBI GenBank: Macrocephalon maleo isolate MB01-FTB_UAJY_02 chromo-helicase DNA binding protein (CHDZ) gene, partial cds. Accession number [MT074329](#).

NCBI GenBank: Macrocephalon maleo isolate MT10-FTB_UAJY_03 chromo-helicase DNA binding protein (CHDZ) gene, partial cds. Accession number [MT074330](#).

Mendeley Data: Eggshell membrane for DNA sexing of the endangered Maleo (Macrocephalon maleo). <http://doi.org/10.17632/mjp5n9pcj3>.

This project contains the following underlying data:

- Electrophoresis photos. (Folder containing photos of DNA extraction and PCR sexing gels.)
- Sequences. (Folder containing raw sequencing files.)

Data hosted with Mendeley Data are available under the terms of the [Creative Commons Attribution 4.0 International license](#) (CC-BY 4.0).

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Open Peer Review

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In general, the article provided a new finding that never been reported, and discussed possible contribution of the finding to the species conservation. However, there was unclear information particularly in the methods section that should be resolved before the article can be published.

Here is my suggestion:

- In the introduction section, a correction is needed on the family name of the bird.
- In methods section, particularly genetic sampling, a further explanation is required regarding sample collection: how to collect the membranes ? How old is the samples ? Furthermore, the author should refer supernatant as lysate. Volume and DNA concentration should be explicitly mentioned when describing PCR condition. Method of sex determination of positive control (egg shell membrane of chicken) should be described. Report of statistical test should be removed because it was meaningless without argument in the discussion.
- The author should mention and analyze a failed sample in the result and discussion sections. Most likely it correlated with DNA quality and quantity. When comparing sex ratio, it should be done in the same way.
- In the discussion section, particularly when discussion freshness of a sample, the author should provide data on the old of the sample. In the Figure 3, a scale indicating length of the amplicon/ladder should is required.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

No

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Wildlife molecular ecologist

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 28 Sep 2020

Pramana Yuda

Thank you for reviewing our article and some valuable recommendations for improvement. We have incorporated the correction and recommendations in our revised article.

- Family name was corrected
- In method section we added description of sample age, collection technique, the volume and concentration of the DNA, and the more description on positive control for sex determination. We also removed the statistical test.
- Failed sample (MB09) in sex determination was added in 'Sex determination' section and discussion.
- Length of amplicon was added in Figure 3
- The description on age and freshness of samples were added in method and discussion sections.

Competing Interests: No competing interests were disclosed.

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