



RESEARCH ARTICLE

REVISED Eggshell membrane for DNA sexing of the endangered Maleo (*Macrocephalon maleo*) [version 4; peer review: 2 approved]

Pramana Yuda , Andie Wijaya Saputra

Teknobiologi, Universitas Atma Jaya Yogyakarta, Kab. Sleman, DI Yogyakarta, 55281, Indonesia

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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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1. **Sena Adi Subrata**, Universitas Gadjah Mada, Yogyakarta, Indonesia
2. **Jessica Worthington Wilmer** , Queensland Museum, South Brisbane, Australia

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Corresponding author: Pramana Yuda (pramana.yuda@uajy.ac.id)

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REVISED Amendments from Version 3

We edit a few of the sentences in "Introduction" as suggested by the reviewer.

Any further responses from the reviewers can be found at the end of the article

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 Megapodiidae) are a galliform clade, centered in Australasia¹⁶, that are known for their unique super-precocial behavior¹⁷. Megapodes are also renowned as the only bird species that use environmental heat rather than body temperature (brood) to incubate their eggs¹⁸. 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 form¹⁹. 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 blood²⁰ or fresh tissue²¹.

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 ratio²². 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 chicks²³.

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. As in all non-ratite birds, determination of sex in Maleo are based on heteromorphic Z and W chromosomes. Female birds are heterogametic sex ZW, meanwhile males are homogametic sex ZZ^{24,25}. Maleo are endemic to Sulawesi, Indonesia^{16,26}. 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 species²⁷. Among the major threats are the over-exploitation of eggs and loss of connectivity between forest and nesting grounds²⁸. 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 non-invasive DNA samples from the eggshell membrane left in the soil or brought to the soil surface by the hatched Maleo. The DNA of the chicks can be obtained from the extraembryonic membranes that remain attached to eggshell membranes of post-hatched eggs. The extraembryonic membranes consist of the amnion, allantois and chorion, and form to support the life and growth of the bird embryo²⁹. During the embryonic development stage, the allantois sac expands, causing the inner shell membrane and chorion being combined and forming a chorioallantois membrane (CAM), which contains lots of blood capillaries^{29,30}.

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. Samples were egg-shell membranes from maleo chicks that hatched less than 24 hours earlier based on records of staff from semi-natural hatcheries. The samples were collected from the surface and inside the substrate (soil and sand) where the eggs are laid. 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.

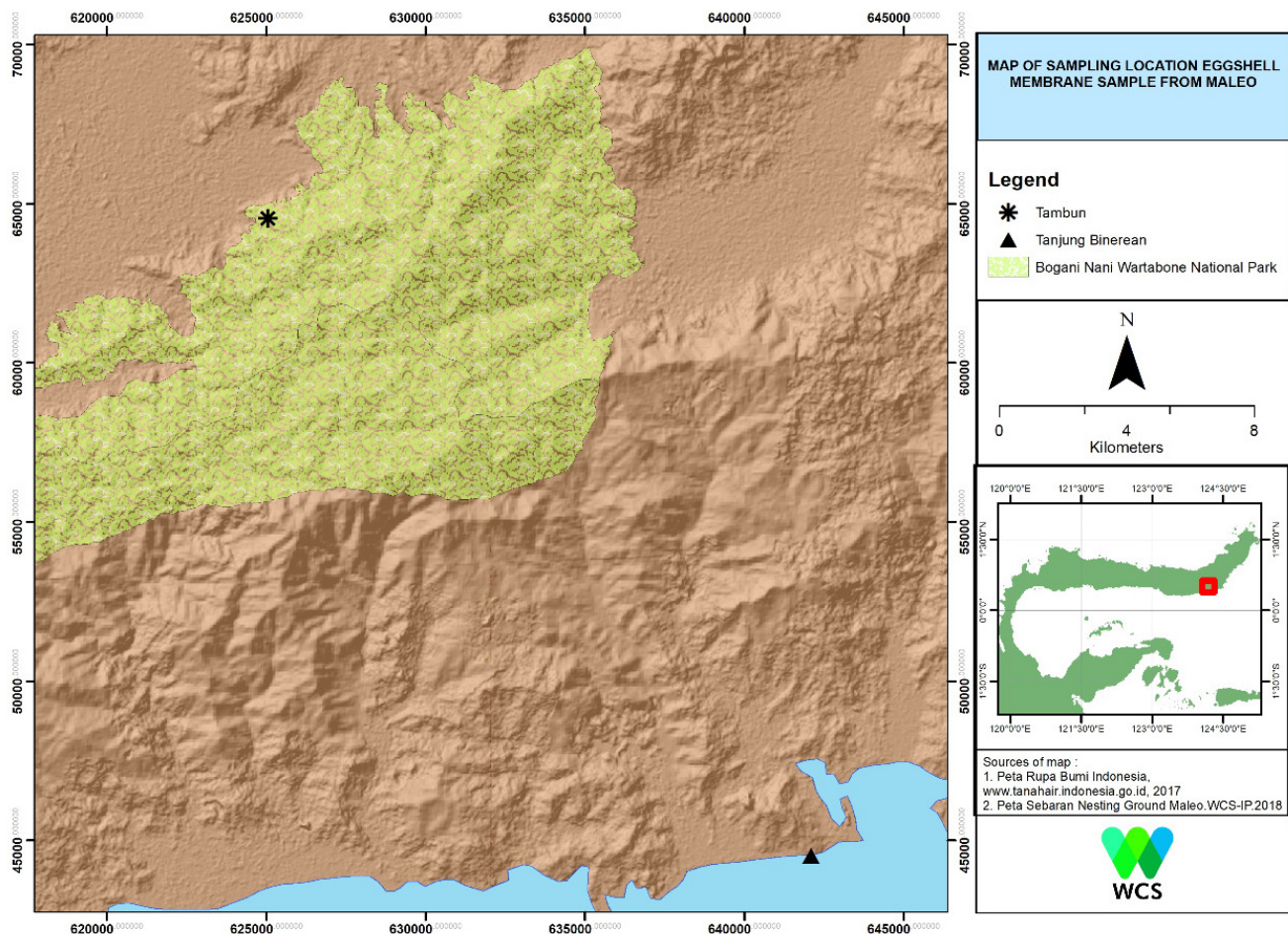


Figure 1. Sampling sites of eggshell membrane of Maleo (*Macrochepalon maleo*) in North Sulawesi, Indonesia.

DNA extraction

We used two different DNA extraction methods: the alkaline lysis method and gSYNC™ 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 used consisted of dry chorioallantois membrane (CAM), included allantois blood vessels, as DNA materials of the chicks, except five samples from Binerean (MB04, MB09, MB10, MB11 and MB12) with no blood vessels. The membrane (20–25 mg) was grinded using a micro-pestle in a 1.5 mL microcentrifuge tube; next, 180 μ L 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 μ L Tris-HCl (1 M, pH 8.0) was added and the tube was vortexed thoroughly, then centrifuged at 12,000 RPM for 5 min. Lysate was removed to new 1.5 mL microtube and store at freezer until used for PCR.

Meanwhile the protocol for gSYNC™ DNA Extraction Kit (Genaid) followed the provided user manual with little modifications. The eggshell membrane (25 mg) was grinded

using a micro-pestle in a 1.5 mL 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 eluded 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/2718R³¹. PCR used a 10 μ l total volume containing 1 μ l (30 ng/ μ l) diluted 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',³¹), 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 egg-shell membranes from the domestic chicken as a positive control. The positive control consistently identified as a female with the same PCR condition in this research.

Amplification of CHD Genes were resolved on a 2% agarose gel. Electrophoresis was conducted using TAE (0.5x) buffer, stained by ethidium bromide (1%), at 100 V for 30 minutes; and 5 μ l PCR product was mixed with 1 μ l 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 7.0.5.3³² and Chromas versi 2.6.5 (Technelysium Pty Ltd). Sequence similarity was probed using NCBI BLAST³³.

Results

All eggshell membranes were successfully extracted, with mean DNA concentrations around 267.5 ng/ μ l (range 47–510.5 ng/ μ l). The average DNA concentration extracted from eggshell membrane collected from coastal nesting grounds (Tanjung Binerean: 213 \pm 179 ng/ μ l,) was significantly lesser than of that of inland nesting grounds (Tambun: 322 \pm 153 ng/ μ l, 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 (\pm 0.03). Meanwhile the average for Tambun and Tanjung Binerean samples were, respectively, 1.85 (\pm 0.03) and 1.84 (\pm 0.01); **Table 1**). 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 sample (MB09) was not amplified. 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 manchuricum*, *Syrnaticus reevesii*).

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/ μ l)
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

No.	Location	Sample Code	Purity (A260/A280 Ratio)	Concentration (ng/μl)
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

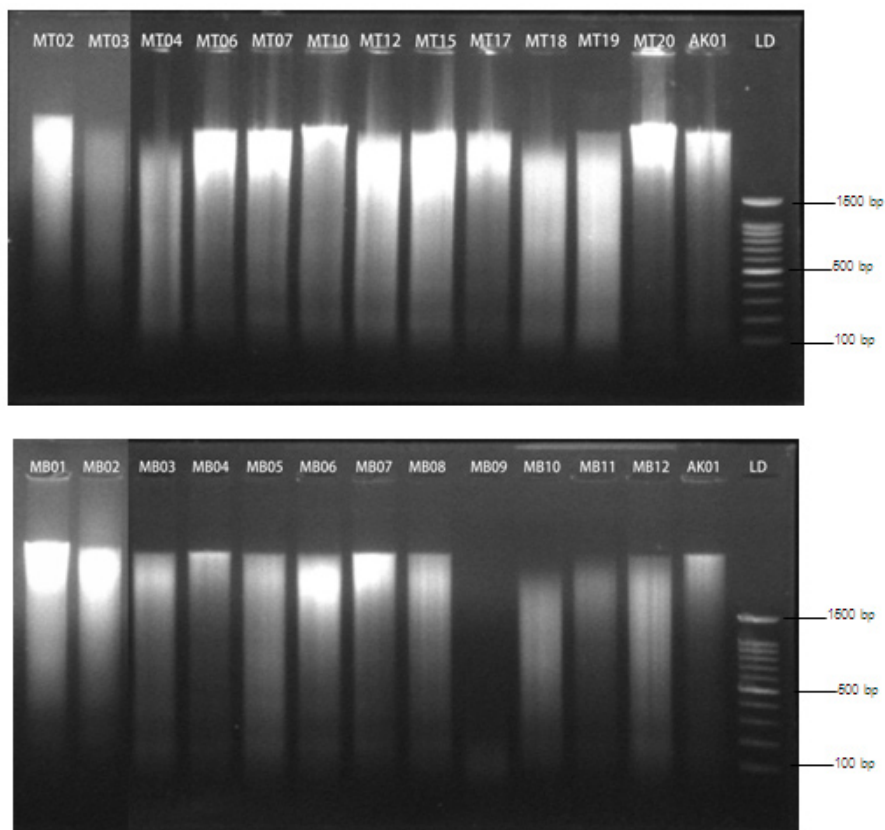


Figure 2. Gel visualizing DNA degeneration of eggshell membrane samples. Above: Tambun; Below: Tanjung Binereaan; LD: 100 bp DNA Ladder (SMOBiO); AK01: domestic chicken eggshell membrane.

In total 9 samples were identified as females and 15 were males (Table 2). Four out of five samples with no allantois blood vessels were identified as females. 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. Care should be taken using eggs to identify the sex of

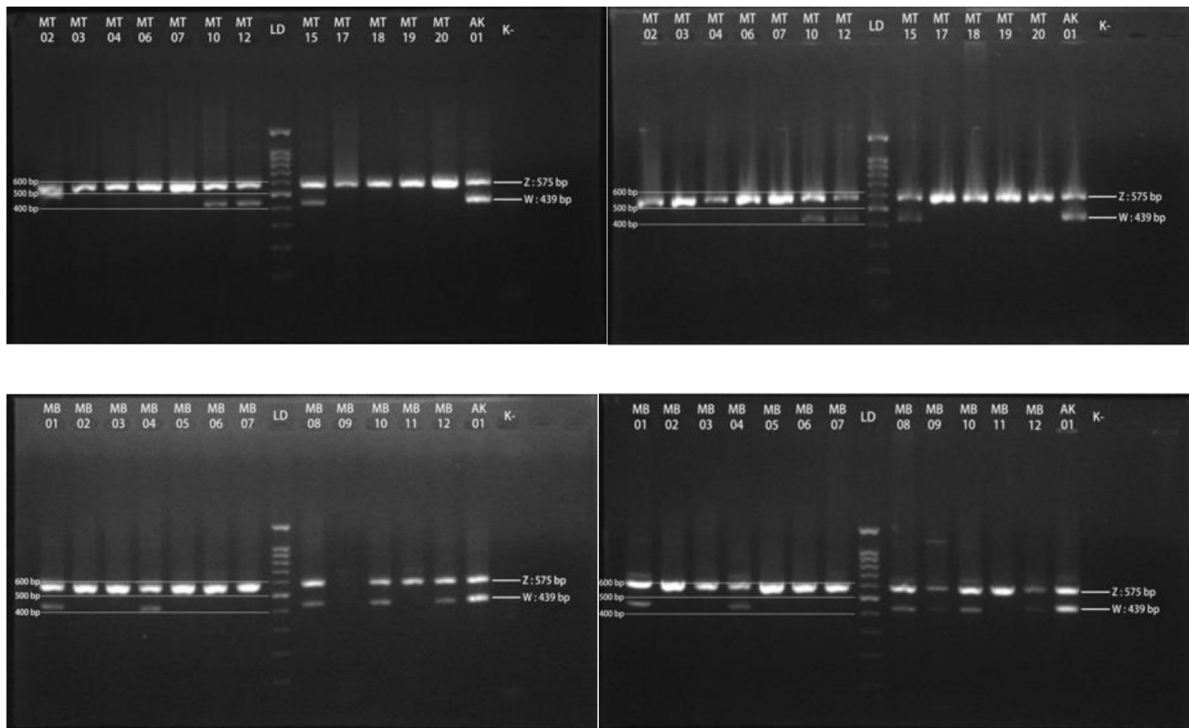


Figure 3. Gel visualizing of CHD-genes amplification of Maleo using extracted DNA (left) and Lysate (right) for molecular sexing of Tambun (above) and Tanjung Binerean (below) samples. AK1, positive control, female chicken; K, negative control, no template.

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

the chicks. The use of freshly laid eggs resulted in inaccurate estimates of the primary ratio, due to contaminants from the DNA of the hen³⁴. In the other hand, using DNA isolated from CAM were reliable to sex the newly hatched Denizli chicken. The results of DNA sexing of chicks in all samples matched with that determined by morphological appearance of gonads³⁵.

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/μl) 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. The quantity of the DNA sample (MB09; 47 ng/μl) might be the cause of the failure, since its quality was relatively good (1.88). 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 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 open 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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Version 3

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Jessica Worthington Wilmer 

Biodiversity and Geosciences Program, Queensland Museum, South Brisbane, Qld, Australia

This revision has incorporated many of the suggestions from my previous review and I now approve the manuscript.

I just have a few very minor language/writing suggestions that should be made to improve the English and information provided.

Introduction:

1. "Megapodes are also known as the only bird species that are not incubating their eggs, but make use environmental heat to incubate their eggs".
Please rewrite this sentence as follows:
"Megapodes are also renowned as the only bird species that use environmental heat rather than body temperature (brood) to incubate their eggs".
2. "The DNA of the chicks can be obtained from the extraembryonic membranes of fertilized egg, which was attached on the hatched eggshell membrane. The extraembryonic membranes were consisted of amnion, allantois and chorion, and formed to support the life and growth of the bird embryo".
Please rewrite these 2 sentences as follows:
""The DNA of the chicks can be obtained from the extraembryonic membranes that remain attached to eggshell membranes of post-hatched eggs. The extraembryonic membranes consist of the amnion, allantois and chorion, and form to support the life and growth of the bird embryo".

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Population genetics, molecular systematics, landscape genetics, evolutionary biology, conservation biology, DNA sexing (megapodes).

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.

Version 2

Reviewer Report 18 November 2020

<https://doi.org/10.5256/f1000research.29940.r74177>

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Jessica Worthington Wilmer 

Biodiversity and Geosciences Program, Queensland Museum, South Brisbane, Qld, Australia

This paper examines the capacity to genetically sex Maleo (*Macrocephalon maleo*) chicks from DNA extracted from the membranes of hatched eggs. This is interesting work and potentially very important for understanding hatching sex ratios of Maleo and for future conservation work of this endangered megapode.

The paper has already gone through one round of review and is generally well presented, however, there are a couple of fundamental issues that need to be highlighted and then addressed by the authors.

1) Egg-Chick membrane anatomy and biology:

- It states in the title and throughout the paper that eggshell membranes are being used but the authors must be careful in implying that the eggshell membranes themselves are the source of chick DNA. The shell membranes are produced by the hen when the shell is formed in the oviduct and, therefore, will always be female. Thus, eggshell membranes on their own without the inclusion embryonic tissue will not be useful for DNA sexing of hatched chicks. So, the source of the chick DNA extracted must be from one of the extraembryonic membranes or some other chick tissue left behind in the shell at hatching. This fundamental egg-biology information must be included in the introduction.
- The issue becomes clearer in the methods section where the authors detail the tissue used for DNA extraction "The eggshell membrane (20-25mg; *mostly with dry allantois blood vessel*) was grinded..." This is the critical information as these blood vessels comprise the extraembryonic arterial system and therefore are the source of chick DNA in this study. The question then is did the authors specifically target these sections of the eggshell membranes because of the blood vessels (with reference to the fundamental egg-chick biology)? If so, then this should be clearly stated as what needs to be done when dealing with hatched eggs.
- "Mostly" is not an accurate scientific term. Please provide detail as to how many eggshell membranes included allantois blood vessels out of the total number (24) of samples. This is

important because of the 9 females that were detected. How many of the samples that didn't include allantois blood vessels were sexed as being female? Those membranes could still include other extraembryonic tissues however, there needs to be a way for the authors to demonstrate with confidence that it is the chick that was sexed as female and not because they have amplified the sex chromosomes of the hen. (see Arnold et al, 2003¹).

2) Maleo and Megapodes

- The introduction needs to include the fundamental biological information that megapodes are the only avian species/group that are not brood incubators (Frith 1956²). All megapodes employ environmental heat to incubate their eggs. Temperature dependent sex biased mortality has been shown to occur in the Australian Brush Turkey (Eiby et al. 2008³) and it may well be that further in depth studies of Maleo may reveal a similar mechanistic interaction between their environmental incubation temperature and hatching sex ratios. The current observed skew in hatching ratio is interesting although the low sample numbers may mean it is not biologically / statistically significant.
- It would also be good to include some basic information regarding sex determination in birds and that in megapodes, as in all other non-ratite birds, sex is determined by heteromorphic Z and W chromosomes (Belter and Deboer, 1984⁴) and, unlike in mammals, females are the heterogametic sex ZW and Males ZZ.

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

Partly

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?

Yes

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

Not applicable

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

No source data required

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Population genetics, molecular systematics, landscape genetics, evolutionary biology, conservation biology, DNA sexing (megapodes).

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.

Reviewer Report 20 October 2020

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Sena Adi Subrata

Department of Forest Resource Conservation, Faculty of Forestry, Universitas Gadjah Mada, Yogyakarta, Indonesia

The authors have provided substantial correction. I agree with all revisions. I suggest a minor improvement in Figure 3 : characters representing length of ladder should be clear enough. At present, it is hard to read the characters.

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?

Yes

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

Yes

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

Yes

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

Yes

Are the conclusions drawn adequately supported by the results?

Yes

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.

Version 1

Reviewer Report 08 September 2020

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Sena Adi Subrata

Department of Forest Resource Conservation, Faculty of Forestry, Universitas Gadjah Mada, Yogyakarta, Indonesia

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, Universitas Atma Jaya Yogyakarta, Kab. Sleman, Indonesia

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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