Molecular approach of Vulnerable Short-Toed (*Centropus rectunguis*)

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Abstract. Using touchdown Polymerase Chain Reaction (PCR), this work intends to build a molecular sexing approach in 30 endangered Short-toed Coucals. This approach, which relied primarily on a non-destructive wing feather sample, allowed for the rapid and accurate determination of intron length in CHD1-Z (400 bp) and CHD1- W (260 bp) chromosomes. The umbilicus region of a feather calamus yielded higher quality of DNA with consistent yields in molecular sexing. As a result, this finding contributes to a better knowledge of the species' sex ratio and provide insights into future population studies of *C. retunguis*, a critically endangered bird species.

Keywords: Bird Conservation, CHD1 gene, Non-Destructive Feather Sampling, Umbilicus Region.

1.0 Introduction

Monomorphic birds are difficult to distinguish between sexes, especially for birdwatchers. The Short-toed Coucals, a monotypic species, lacks reliable cues for male and female identification, requiring special conservation attention. Accurate gender identification is critical for wildlife and captive programs such as mating partner selection and sex percentage assessment [1]. Furthermore, it is required for successful ex-situ conservation programs, management techniques, and data gathering in order to save and maintain endangered bird species.

In contrast to non-molecular sexing procedures, requiring bird maturity, they are quick and reliable for sex identification on chicks as early as 5-7 days old [2]. Feathers offer potential for DNA studies in breeding, conservation, and ecology due to their less-invasive nature and reduced handling time, reducing stress for birds [3,4]. Molecular biology techniques have grown in demand due to their improved specificity and quality of results [5].

Avian sex chromosomes have female heterozygous (ZW) and male homozygous (ZZ) sex chromosomes, with molecular sexing techniques depending on the size difference between CHD the gene introns [6]. The use of molecular sexing bird

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research in Malaysia is insignificant, with most studies focusing on monomorphic bird species like Eclectus parrot [7], domesticated chicken [8], White-bellied Sea eagle [9], Spotted Dove, Zebra Dove, Budgerigar and Rock Pigeon [10], Painted storks [11], Stripe-throated Bulbul, Olive-winged Bulbul, White-throated Kingfisher and Collared Scops Owl [12].

In addition to that, recent publications described molecular research on Southeast Asian Barn owl [13], Javan myna [14] and Malaysian White-nest swiftlet [15]. This study aims to develop a molecular sexing method using PCR at the umbilicus region of a wing feather, providing a preliminary sex ratio estimate to address environmental deterioration.

2.0 Literature Review

The Short-toed Coucal is a medium-sized forest-dwelling slender bird in the Cuculidae family, known for its zygodactyl feet and short black tail [16]. The genders are similar based on their plumage, but females display little sexual dimorphism in size for being larger than males. A glimpse of this bird will create confusion with Greater Coucal, but it is actually much smaller in size and has a short black tail (about 38-43 cm). The breeding season is between April to September each year [17]. It is found in the southernmost parts of Thailand, Peninsular Malaysia, Borneo, Brunei, and Indonesia. Their habitat is shrubs in evergreen forests, mainly lowland dipterocarp, riverine forest, and peat swamp.

The population of the Short-toed Coucal has declined by over 50% since 2000 and is listed as a Vulnerable species in the International Union for Conservation of Nature (IUCN) Red List of Threatened Species [18]. Alarmingly, this lowland inhabitant Short-toed Coucal is trending downwards due to the strong logging force leading to the rapid deforestation of lowland forest throughout the Sundaland [19]. Southeast Asia's forests are decreasing faster than anywhere else in the world, with wildlife trade and hunting being the main concerns [20].

Various sexing methods, including surgical procedures and chromosome studies, have been used to determine bird sex. However, these methods are prone to risks, such as laparoscopy, which is invasive and threatening for chicks [2]. Cytogenetics can provide accurate gender recognition, but chromosome examinations are inconvenient and laborious, especially for ostriches due to low size differences [21]. Feathers are being explored as DNA sources for genetic marker-based studies in breeding, conservation, and ecology [22].

Less-invasive feather sexing reduces stress for birds, while blood sampling is practical but laborious. Bird welfare is affected by blood loss and increased metabolic response, which may impair their ability to escape predators or obtain food [23]. Pain can negatively impact data quality due to behavioral, physiological, and neurobiological alterations [24]. Despite these challenges, feathers offer promising solutions for genetic marker-based studies in these fields.

3.0 Methodology

3.1 Feather Samples Collection

With permission from the Department of Wildlife and National Park, Peninsular Malaysia, 30 individual Short-toed coucals were lured into mist nets using birdcall by Ndlovu [25] in Kedah (Pendang; 5.9930° N, 100.4773° E, Guar Chempedak; 5.8530° N, 100.4602° E, Mahang; 5.3245° N 100.7548° E and Kulim; 5.3717°N, 100.5533° E) and Perak (Sungai Bayor; 5. 2495° N, 100.7784° E) and Perlis (Bukit Air Perlis; 6.5454° N, 100.1682° E). The sampling sites were broadened as the low population density recorded in this species.

The sampling took place from April through September of 2019, during the mating season. Mist nets were checked in every 15 to 20 minutes in 15 different sites between 6 am to 6 pm, to ensure that the trapped birds are in excellent state as well as to reduce discomfort. Wing feathers were gathered before rhodamine B was administered to the wing membrane to avoid recaptures [26, 27]. The sexes of each bird were positively validated by expert through gross visual identification of the sex gonads, which assisted in establishing the sex of the bird with amplified product of PCR. Feather samples were put in sterile plastic bags (without contacting within the area of inferior and superior umbilicus) then stored at –20°C.

3.2 DNA Extraction

DNA was extracted from the superior umbilicus region of a wing feather using NucleoSpin® Tissue (50 preps) Macherey-Nagel, Germany according to the manufacturer instructions. The source of DNA on the superior umbilicus region of a feather was used due to the presence of the blood clot (approximately 1cm from the basal tip of the calamus, but just before the aftershaft) (Fig1).

In addition to the standard elution approach, a modification was employed to achieve a high yield and high concentration of eluted DNA by initially performing 50 µL of the elution buffer, then was incubated for 3 min followed by centrifugation step (11 000 x g, 1 min). The procedure was then repeated for another 50 µL followed with the prescribed step. The purity of resultant DNA (ng/µL) extract was qualitatively checked using NanophotometerTM P-Class. Next, the measurement was recorded by referring to the standard of every 1 µL represent DNA concentration in ng/ µL. To measure the intensity of the extracted DNA bands, all samples were electrophoresed in 1% agarose gel.

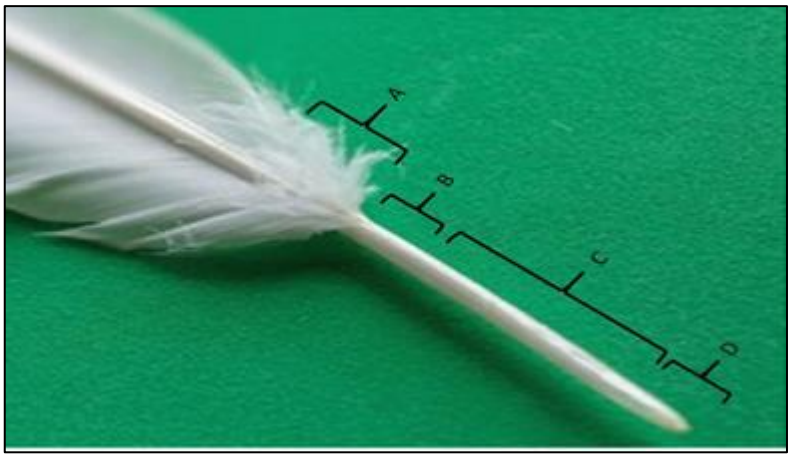

Fig. 1. General view of a typical flight feather: (A) aftershaft, (B) superior umbilicus, (C) Calamus and (D) Inferior umbilicus. Blood clot usually found on the superior umbilicus region.

3.3 PCR Amplifications and DNA Electrophoresis

The final reaction PCR amplification mixture of 50 µL: 25 µL Green Taq Mix (Vazyme), 8 µL DNA template, 2 µL of each forward and reverse primer (1st BASE) and topped up with 13 µL Nuclease-Free water (Sigma-Aldrich). The PCR primers used in this study were 2550F (5'- GTTACTGATTCGTCTACGAGA-3') and 2718R (5'-ATTGAAATGATCCAGTGCTTG-3') [28]. This primer combination amplified a single Z band in males and Z and W bands in females through PCR amplification.

For modified touchdown PCR cycling condition was as follows: 94℃ for 2 min, then 10 cycles of 94℃ for 30 s, 54°C (annealing temperature was reduced 1℃ per cycle) for 15 s and 72℃ for 30 s, followed by 30 cycles of 94℃ for 30 s, 44℃ for 30 s and 72℃ for 30 s, and finally 72℃ for 5 min [29]. Before sexing *C. rectunguis*, preliminary analysis of 2550F/2718R primer pair was conducted using six known sexes (three males: three females) of a polymorphic, Blue-crowned hanging parrot (*Loriculus galgulus)* as control. A positive and negative control were included to prove whether contamination or non-specific amplification of samples exist caused by exogenous DNA or PCR component itself. PCR product were run on 2% agarose gel that was stained with FloroSafe DNA Stain (1st BASE) for 1 hour at 70V.

4.0 Findings

The analysis of 2550F/2718R touchdown PCR-based products from feathers on a 2% agarose gel was practical for sex determination in Short-toed coucal, yielding definite banding patterns in all thirty samples examined (Figure 2, Figure 3 and Figure 4). The band patterns were in accordance with the patterns anticipated by the expert of their previously verified gross visual identification. Based on the amplified product of

the CHD gene, homogametic (ZZ) and heterogametic (ZW) birds were revealed, convincingly stating 10:20 (male: female) of birds under study.

The amplified product size of CHD1-Z was 400 bp, whereas CHD1-W was 260 bp, with a gap of 140 bp between the two genes. In this analysis, the difference was 10 bp smaller than reported by Fridolfsson and Ellergen [28], who estimated a gap between 150 and 250 bp. The findings were consistent across three replications. Faint bands were observed on some of the amplified product of CHD1-W female fragment numbered as 9,11,12,16, and 24. Furthermore, this finding revealed that the purity of the DNA of ratios 260nm/280nm ranged from 1.9 to 2.1.

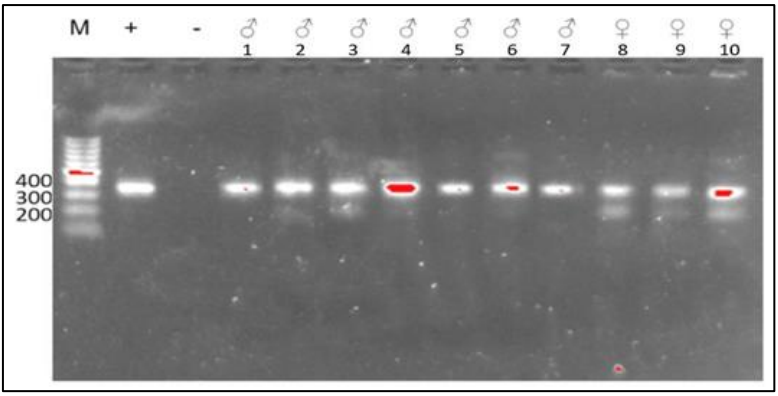

Fig. 2. Electrophoresis gel showing PCR product for CHD gene of Short-toed coucal. M: GeneRuler™100 bp DNA ladder; (+) positive control; (-) negative control; male lanes 1-7(♂) lies between 400 bp; female lanes 8-10 (♀) with amplified W lies between 260 bp.

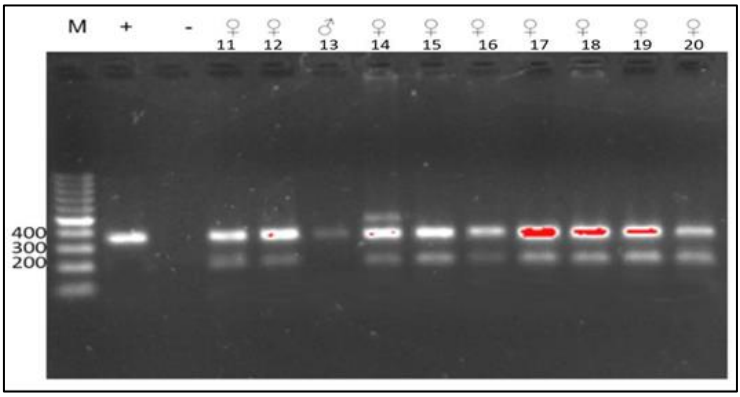

Fig. 3. Electrophoresis gel showing PCR product for CHD gene of Short-toed coucal. M: GeneRuler™100 bp DNA ladder; (+) positive control; (-) negative control; male lanes 13 (♂) lies between 400 bp; female lanes 11,12 and 14-20 (♀) with amplified W lies between 260 bp.

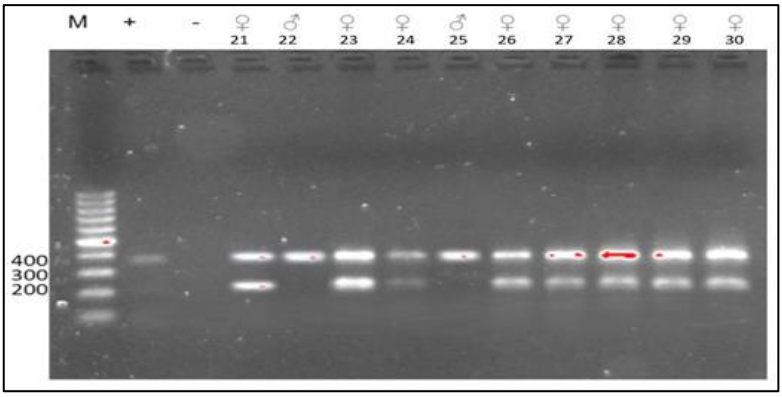

Fig. 4. Electrophoresis gel showing PCR product for CHD gene of Short-toed coucal. M: GeneRuler™100 bp DNA ladder; (+) positive control; (-) negative control; male lanes 22 and 25 (♂) lies between 400 bp; female lanes 21, 23, 24 and 26-30 (♀) with amplified W lies between 260 bp.

5.0 Discussion

When paired with touchdown PCR, the 2550F/2718R primer combination efficiently amplified the CHD gene. Despite advances in PCR- based bird sexing, classical PCR remains common due to its affordable price and simplicity of application [30]. Due to its similarities to *C. sinensis*, determining the status of *C. rectunguis* is difficult. Attempts were made to sex a few Coucals species using the 2550F/2718R primer pair on *Coccyzus americanus* [31], *Centropus bengalensis*, and *Phaenicophaerus curvirostris*, however the combination failed with no clarification. This study concludes that a single primer pair, 2550F/2718R, is universally used in various bird species [32, 33].

Preliminary analysis was conducted on six known sexes of a polymorphic, Blue-crowned hanging parrot to validate its application. Agarose gels are generally considered a viable option due to the significant size difference. The amplified W fragments of female avian birds were misclassified as males, causing a weak band on a second W fragment [25]. The presence of a weak band on a second W fragment on agarose gel numbered as 9,11,12,16, and 24 was most likely caused by primer competition, as primers may complement one CHD gene slightly less than the other during amplification [29].

This finding could help assess sex chromosome structure using DNA sequencing analysis and differentiate physically identical species [34]. A genetic marker for the avian CHD gene could also help determine site-specific management activities for hybrid bird species [35]. The method of sexing birds in *C. rectunguis* is quick and efficient, enabling taxonomic evaluation and genetic data documentation. However, issues with PCR amplification paired with 2550F/2718R, such as misidentification of female birds and loss of heterozygosity, can lead to

misidentification [36]. Success depends on storage time, physical condition, feather type, and size [37].

The study proposes a non-invasive feather sampling method for genetic study of *C. rectunguis*, replacing alternative methods like substitute invasive blood sampling [38], modifications to extraction methods [39], and primer redesigning [40]. The touchdown PCR overcomes low DNA concentration issues and improves specificity by decreasing annealing temperature per cycle [41]. This approach offers faster results with few unspecific products. The procedure for DNA isolation for wing feather were optimized for both higher DNA yield and concentration by specifically performing the two elution steps. The same extraction kit was used for DNA extraction from feather of *Circaetus gallicus* [42] and *Agapornis roseicollis* [43].

Even though DNA concentration (ng/L) was not shown in both mentioned studies, yet amplification success has been observed with minimal unspecific products. Wing calamus was incubated with Proteinase K, overnight by means of increasing DNA yield, which is difference from the protocol that was in this study for *C. rectunguis*. On a pre-lyse sample, the cut pieces of feather were added with Buffer T1 and Proteinase K, incubate for 56°C for only 3 hours. As a result, DNA quality obtained with the two elution steps was optimal compared to the one elution step which was analyzed based on the performance on agarose gel and DNA purity by NanophotometerTM P-Class (data not shown).

Furthermore, contour feather (wing) was used in this study because of its high DNA yield and greater sample size. The preferable application of a larger feather was further supported by the higher success rate of microsatellite amplification in *Ara ararauna, Ara chloropterus* and *Ara macao* [44]. Furthermore, small feathers generate less DNA than all portions of a huge feather combined in *Poecile atricapilla* [45] and *Acridotheres javanicus, Streptopelia chinensis, Lonchura maja* and *Seicercus montis* [12]. Besides that, the condition of the initial sample has an impact on the quality of the extracted nucleic acids. That is why the non-destructive freshly plucked feather was employed over non-invasive molted/shed feather in this study.

Fresh DNA samples in *Egretta eulophotes* are crucial to prevent deterioration or contamination, which can lead to genotyping mistakes. The study found that the umbilicus region was preferred over the calamus tip due to a blood clot, contradicting previous beliefs that DNA was only found at the feather tip [46]. Previous studies have primarily focused on the tip of a feather as a primary source of DNA, with the umbilicus region often overlooked [47]. The applicability of the umbilicus region is limited to large feathers with superior umbilicus [48], causing concerns due to lower yield compared to blood samples.

Feathers as a DNA source have drawbacks such as low concentration and contamination sensitivity as reported in molecular study of *Aquila adalberti*, *Anodorhynchus hyacintinus*, and *Tyto alba javanica* [13]. However, non- destructive sampling methods are crucial to minimize impact on birds and minimize pressure and interference [49]. Non-destructive sample methods are crucial for fragile and threatened species [50], but intrusive blood collection is often used as an alternative DNA source

without considering the welfare of birds. Bled birds increase caution and mortality [23], and invasive blood samples are considered irrelevant in molecular approaches [51, 52].

Nonetheless, most of the paper did not mention detail on the direct consequence from blood sampling that focused on the birds' physiological aspects except for few with minimal impact stated on *Molothus ater* and *Agelarius phoeniceus* [53], *Petrochelidon pyrrhorota* [54], and *Tyryothorus leucotis* [55]. This probably due to quantifying the total impact of blood sampling is difficult to achieve while keeping ethical considerations in mind. Large birds of *Gallus gallus domesticus* and Anatid ducks were extremely reluctant to the physiological aftermath of low blood pressure [56]. Since that, this finding has been generalized and claimed to be relevant in a wide range of bird species [57].

However, the exceptional disagreement occurred since different bird species may react differently depending on their living habitat as well as other essential factors. Feather sampling is more acceptable and risk-free for bird sex identification than blood sampling. Although it has less optimal DNA yield, it offers practical advantages like shorter handling times, no harmful waste, and simple storage [45]. Feather-based research is increasingly used to evaluate ecological and demographic aspects of populations [58, 38].

Feather sampling is a viable option for genetic studies of threatened or fragile chicks, as it allows early DNA analysis with minimal risk [59]. Environmental conditions, type, size, and storage [37] duration is crucial for successful gene amplification. Unfortunately, most researchers disregarded the need of selecting a high-quality feather, hence uncertainty in sexing results. Sexual bias in population was acknowledged as an issue in preserving and managing vulnerable bird species. The sex ratio obtained in this study was 10:20 (10 males 20 females $\neq$ 1:1) of which the number of females was twice that of males.

A pronounced gender imbalance might signal that a population of *C. retunguis* is edging closer to extinction [18]. There were many factors were hypothesized in *C. retunguis* to justify the skewed sex ratio. However, there are several cases when the predicted 1:1 ratio is not demonstrated as indicated by Trivers and Willard [60]. An excess in female adult sex ratio in this study were probably in response to the poor maternal condition [61], sex-biased mortality [62] the availability of food resources [63]. Non-biological factors, such as frequent mist net encounters [64], also contributed to this imbalance. However, the study's findings are inconclusive due to lack of supporting articles.

6.0 Conclusion and Recommendations

Overall, the combination of 2550F/2718R primer pair with touchdown PCR allowed rapid and definite differentiation of the intron length in Z and W chromosomes on thirty individual birds of *C. retunguis* with amplified product size of 400bp for CHD1-Z, whereas 260 bp for CHD1-W. Touchdown PCR performed faster than a conventional PCR condition protocol and produced sharp results with few or no

unspecific products. Therefore, this finding facilitates DNA sequencing analysis by adding to the ability to solve between morphologically similar species in *C. rectunguis* and *C. sinensis*.

As a result, this easy and quick method of sexing birds paves the way for a robust strategy for vital taxonomic evaluation and genetic data documentation in *C. rectunguis*. The umbilicus region of non-destructive feather calamus produced higher quality of DNA quality with consistent yields in molecular sexing. Thus, this finding revealed that the non-destructive approach was more acceptable with minimal risk than intrusive blood sampling. Based on the amplified product of the CHD gene, homogametic (ZZ) and heterogametic (ZW) birds were revealed, convincingly stating 10:20 (male: female) of birds under study.

The preliminary predicted female-biased sex ratio provides an insight on future population study of *C. retunguis by* adding this component into future conservation plan. This, however, warrants additional inquiry into the detailed caused of the sex-biased ratio, as well as its implications for population conservation and the likelihood of reintroduction success. Due to a lack of published data on the possible effects of feather plucking, a thorough investigation should be conducted to determine the long-term consequences of this strategy. Finally, yet importantly, a cooperatively productive discussion can be offered to geneticists and avian ecologists through the integration of discipline in the current and future of *C. retunguis.*

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