Morphometric and Molecular Techniques for Sex Identification in Greater Coucal (*Centropus sinensis intermedius*)

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ABSTRACT

Accurate gender recognition in birds is crucial for research and conservation. Nevertheless, distinguishing sexes in weakly dimorphic birds poses challenges for conservationists, hindering efforts to boost endangered populations. Greater coucal exhibits weak Sexual Size Dimorphism (SSD) and lacks established criteria or methodology for morphometric sexing. Therefore, this study aimed to determine the sex of a weakly dimorphic bird by amplifying the CHDZ and CHDW genes, examining seven (7) morphological characters for standardization morphometric analysis using discriminant function analysis (DFA). Freshly plucked feathers from 66 Greater Coucals were used in Touchdown Polymerase Chain Reaction (tPCR), amplified a single Z band in males and both Z and W bands in females. Amplification of the 300-400 bp segment of the CHD1-Z gene and 200-300 bp segment of CHD1-W using the 2550F/2718R primer set gene can be used to distinguish the sex of adult Greater coucal (males 30: females 36). The established function is (D = (-61.74) + (0.29) bill culmen length + (0.50) total head length + (0.60) bill depth) allowed for 100% correct classification where female if D > 0 and male if D < 0. Thus, the formulated function equation, intended for practical application in the field, emphasizes the potential for gaining valuable insights into diverse facets of the biology, behavior, and ecology of monogamous bird species by exploring size differences. This first field-based, cost-effective study of sexual dimorphism in birds provides valuable resources for future coucal management while offering important genetic and morphological data in Malaysia. It also provides the groundwork for more effective conservation and breeding programs, where accurate sex identification is crucial in decision-making.

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INTRODUCTION

Accurate gender recognition is critical in veterinary, medicinal, and ecological studies, and it is useful in enforcing regulations or reviving endangered and protected bird species (Lee *et al.,* 2009; Ghorpade *et al.,* 2012; Kaplan *et al.,* 2022; Withaningsih *et al.,* 2024). However, sexing a bird that is weakly dimorphic in size but monomorphic in plumage has proven difficult for Malaysian conservationists seeking to increase captive bird populations of endangered species. Sexing male and female birds apart when there are no obvious plumage differences is extremely difficult, especially when behavior-based sex determination is unavailable, or there is a slight size difference in dimorphic bird species (Vucicevic *et al.,* 2013; Puan *et al.,* 2018; Stehlíková Sovadinová *et al.,* 2024).

However, it is expensive and lacks instant identification, hindering post-capture behavioral studies (Griffiths *et al.,* 1998; Purwaningrum *et al.,* 2019). Alternative blood or feather analysis methods are costly, time-consuming, and invasive (Cardoni *et al.,* 2009; Dechaume-Moncharmont *et al.,* 2011). Observation-based techniques yield uncertain results (Matta-Camacho *et al.,* 2009; Guerra-Paramo *et al.,* 2015).

Behavioral sexing is unreliable outside the breeding season (Henry *et al.,* 2015; Leys and Grieves, 2023). Consequently, researchers have turned to morphometry, a cost-effective and reliable field-based method for sex recognition in birds (Mischler *et al.,* 2015; Ferrer *et al.,* 2016; Seyer *et al.,* 2020; Nepshinsky *et al.,* 2021; Tobella *et al.,* 2023). The Greater coucal (*Centropus sinensis intermedius*), or But-but Besar, is a large, long-tailed, non-migratory bird found in the Indian Subcontinent and Southeast Asia. It inhabits forests, grasslands, and urban gardens (Wong, 2016). Although the plumage is monomorphic, there is a weak dimorphism in size, with females slightly larger than males (Ali and Ripley, 1971). During the mating season, it forms monogamous pairs, is omnivorous, and slow-moving. While data suggests that this species is classified as Least Concern (Birdlife International (2022) in Malaysia, this is not always the case, as these species face conservation challenges due to their reliance on the forest.

This bird is traditionally used in the Indo-Malayan area to make Crow Pheasant Oil for treating injuries (Payne, 2020), though its effectiveness and impact on populations have not been studied. No reliable criteria have been described for morphometric sexing in Greater coucal. Therefore, this study aimed to identify the sex of weakly dimorphic birds by amplifying the CHDZ and CHDW genes by examining seven (7) morphological characters, providing a standardized morphological evaluation using Discriminant Function Analysis (DFA). This first field-based, cost-effective study of sexual dimorphism birds provides valuable resources for future coucal management and important genetic and morphological data for Malaysia. It also lays the groundwork for more effective conservation and breeding programs, where accurate sex identification is crucial in decision-making (Lee *et al.,* 2008; Liu, 2023).

EXPERIMENTAL

Ethics statement

Ethical review was waived for this study because it does not include animals used for experimentation. Method of data collection, including mist net trapping, feather sampling, and morphological measurements, were conducted to ensure minimal impact on the welfare and well-being of the birds (Evers *et al.,* 2021; Mendoza-Silva, 2023). We have thoroughly reviewed Malaysia's Wildlife Conservation Act 2010 (Act 716). Permissions are required for sampling activities within gazetted areas such as protected areas, wildlife reserves, and national parks. Our sampling locations were outside these gazetted areas, and based on our understanding of the Act, no particular permissions are required for conducting research in these locations.

Data collection

The research was conducted in a variety of habitats, including shrublands, woodlands, paddy fields, and urban forest, at various locations in Northern Peninsular Malaysia, including Kedah (Pendang; 5.9930° N, 100.4773° E, Sungai Chepir; 5.8530° N, 100.4602° E, Mahang; 5.3245° N 100.7548° E and Kulim; 5.3717°N, 100.5533° E), Perak (Sungai Bayor; 5. 2495° N, 100.7784° E), and Perlis (Kampung Ujung Bukit; 6.4721° N, 100.1859° E). Sixty-six (66) adult Greater coucal were sampled from June to September.

To optimize capture while limiting non-target species contacts, sound luring was employed in combination with a mist-netting method (Rahlin and Vilag, 2019). Birds caught in the mist net were carefully removed and identified, with confirmation by an on-site expert through behavioral observation and examination of the cloaca, particularly during the breeding season (Davidson and Yeap, 2010). Linear measurements were taken in millimeters for tarsus, bill culmen length, bill depth, bill width, and total head length using Electronic Digital Caliper (Techno 6"/150mm). The wing and tail length were measured using a centimeters (cm) metal ruler.

Weight was taken in grams (g). To eliminate bias, all measurements were performed by the same individual using the same procedure and measuring instruments (Henry *et al.,* 2015). A nondestructive (Yufei *et al.,* 2015), freshly plucked (Presti *et al.,* 2011) wing feather was collected by plucking the feather near the skin. Wing feathers that are either big primary or secondary feathers were favored over smaller feathers for their consistent source of DNA (Segelbacher, 2002; Vili *et al.,* 2013). Samples were then put in sterile plastic bags and stored at –20°C.

Molecular sex identification

According to the manufacturer's instructions, DNA was extracted from the superior umbilicus region of a wing feather using NucleoSpin® Tissue (50 preps) Macherey-Nagel, Germany. The region on the superior umbilicus of a feather to the inferior umbilicus (tip of the feather) was used in the analysis due to the presence of the blood clot (approximately 1 cm from the basal tip of the calamus, but just before the after shaft) (Horvath *et al.,* 2005). The final reaction PCR amplification mixture was 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 /2718R (Fridolfsson and Ellegren, 1999) for the modified touchdown PCR cycling condition was as follows: 94°C for 2 min, then 10 cycles of 94°C for 30 s, 54°C (annealing temperature was reduced 1°C per cycle) for 15 s and 72°C for 30 s, followed by 30 cycles of 94°C for 30 s, 44°C for 30 s and 72°C for 30 s, and finally 72°C for 5 min (Cakmak *et al.,* 2017). The PCR product was run on 2 % agarose gel stained with FloroSafe DNA Stain (1st BASE) for 1 hour at 70 V.

Statistical analyses

Body measurements were reported as means and standard errors. Discriminant Function Analysis (DFA) with enter independents was applied to the measurements. All the assumptions of discriminant function analysis were met, including the homogeneity of covariances (Box's M test) (Hahs-Vaughn, 2016), the Shapiro-Wilk normality test (Shapiro and Wilk, 1965) for males and females separately in each of the

two groups, the Independence Samples T Test (Levene, 1960), Welch's T-Test for unequal population variances (Welch, 1947).

We confirmed no multicollinearity of the selected measurements (r < 0.50 for all pairwise correlations). The cut-off point for classifying cases between male and female birds was developed based on functions at group centroids (Sikora and Dubiec, 2007). To validate the model, the jack-knife procedure was used to assess the percentage of correctly sexed individuals by discriminant function analysis (Dechaume-Moncharmont *et al.*, 2011). All tests were two-tailed; the accepted significance level was P < 0.05. The statistical analyses were performed using IBM SPSS Statistics 27.

RESULTS AND DISCUSSION

Morphological differences between sexes

DNA analysis effectively established distinctive male and female banding patterns in sixty-six (66) (30 male: 36 female) Greater coucal. The amplified product of the CHD gene was identified as homogametic (ZZ) (single band) and heterogametic (ZW) (double bands). CHD1-Z amplified products were 300-400 bp in size, whereas CHD1-W amplified products were 200-300 bp. Figure 1 shows the electrophoresis gel of PCR product for the CHD gene of Greater coucals. The findings were consistent across three replications. The observed gap between Z and W fragments in this analysis employing 2550F/2718R ranges matched Fridolfsson and Ellergen. (1999), who reported that the fragment difference ranged from 150 to 250 bp. This primer set was designed to amplify both gene copies in the region conserved between CHD-Z and CHD-W genes.
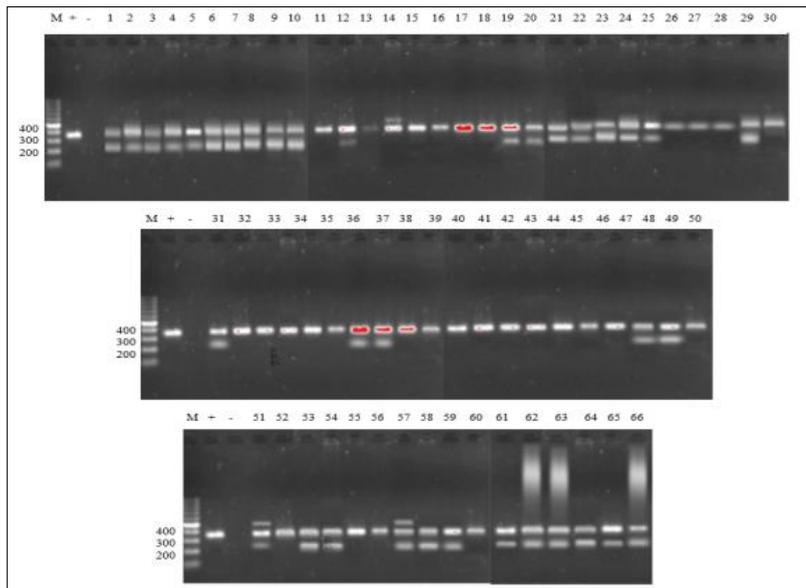

Fig.1: Electrophoresis gel showing PCR product for CHD gene of Greater coucals. M: GeneRuler™100 bp DNA ladder; (+) positive control; (-) negative control; male Z lanes display single band of 300-400 bp; female lanes display double bands with amplified W of 200-300 bp.

Following validation in the Independent Sample t-test (Table 1), the significant differences were only indicated in the mean for tarsus, bill culmen length, and bill depth. The total head length variable did not pass the homogeneity of variance on Levene's Test with $p < 0.05$. Thus, the bottom row of equal variances, which was not assumed, was utilized. Welch's t-test result noted a significant difference in the mean for total head length.

The discriminant analysis result demonstrated four statistically significant predictors distinguishing males from females. However, due to the violation of the covariance equality assumption of Box's M test, only the three best predictors were used in the equation. Three statistically significant PVs demonstrated higher F values, thus contributing to the group membership discrimination. The discriminant function equation was written mathematically as follows:

$$D = (-61.74) + (0.29) \text{ bill culmen length} + (0.50) \text{ total head length} + (0.60) \text{ bill depth}$$

Based on centroids derived from DFA (cut score: 0), a bird with a score closer to -2.34 ($D < 0$) is likely male, while a score near 1.95 ($D > 0$) indicates female. This equation function allowed for 100% correct classification by original grouped cases and cross-validated grouped cases (leave-one-out cross-validation).

Table 1. Morphological characteristics for male and female Greater coucal

Variables (mm)	Males (*n*=30) Mean ± SD (range)	Females (*n*=36) Mean ± SD (range)	*t*	*p*	Dimorphism (%)
Wing	209.14 ± 3.70 (203.00-215.60)	208.70 ± 7.71 (196.00-224.30)	0.34	0.75	0.19
Tail	246.50 ± 8.63 (231.70-263.30)	249.60 ± 10.08 (231.00-269.30)	-1.31	0.19	1.25
Tarsus	65.80 ± 0.87 (64.16-67.52)	64.48 ± 0.98 (62.50-66.46)	5.75	0.00*	2.03
Bill culmen length	39.41 ± 1.34 (37.05-41.92)	42.56 ± 1.32 (40.22-44.81)	-9.61	0.00*	7.69
Total head length	73.98 ± 0.77 (72.66-75.54)	77.64 ± 1.49 (75.20-80.81)	-12.84	0.00*	4.83
Bill depth	19.83 ± 1.01 (18.22-21.63)	22.60 ± 0.95 (21.06-24.47)	-11.46	0.00*	13.06
Bill width	24.63 ± 0.84 (23.06-26.22)	24.91 ± 1.05 (23.10-26.67)	-1.17	0.25	1.13

*Significance difference

Greater coucal is known as monogamous, with limited mating competition, monomorphic in plumage and dimorphic in size, with females slightly larger and males contributing more to incubation and offspring care (Andersson, 1995). Female-biased sexual size dimorphism (SSD) was observed in this bird (Ali and Ripley, 1971), but the morphometric data was not published in detail. Significantly, this finding emphasized dimorphic traits in females, contradicting the prevailing thought that male birds tend to be statistically larger than females (Darwin, 1871).

Most studies have stressed the importance of these variables due to their stable structures using a range of bird species with classification success of 99-100%, 96.6%, and 88-99%, respectively (Mawhinney and Diamond, 1999; Chochi *et al.,* 2002; Almeida *et al.,* 2020; Pay *et al.,* 2021; Redondo-Gómez *et al.,* 2021; Reyes *et al.,* 2022; Hughes *et al.,* 2023; Suastegui *et al.,* 2023; Leys and Rhodes, 2024; Meissner *et*

al., 2024). Measuring a single variable in the field is considered the most practical approach to reducing handling time and minimizing bird stress (Dechaume-Moncharmont *et al.,* 2011; Saez-Gomez *et al.,* 2017). However, when morphometric overlap occurs (Demongin, 2013), the sexing accuracy is reduced.

Hence, a combination of variables is recommended, as in Little Stints (*Calidris Minuta*) (Mazur and Remisiewicz, 2016). Using wing length as a predictor variable, the equation D1 = -47.496+0.484 (wing) effectively identified the sex of 78.8% of birds. Meanwhile, when the combination of wing and tarsus length was used in the equation D2 = −50.428+0.421(wing)+0.420 (tarsus), the function correctly classified 82.7% of the birds. It was more accurate than the single predictor variable used. A combination of head-bill length and wing length was beneficial in adult Carrion crows (*Corvus coroner*) and Rooks (*Corvus frugilegus*), where it increased the accuracy of sexing (Fletcher and Foster, 2010). Monogamous and monomorphic coucal species share similar ecological roles, providing biparental care, with males generally contributing more to incubation and offspring care than females (Andersson, 1995).

While SSD is more common in species with polygamous mating systems, it may also be seen in monogamous species with varying degrees. No detailed study has been done on the relationship of the significance morphology in female Greater coucal. However, the functional significance of the size of the total head length, including bill culmen length and bill depth, might be due to its ecological and behavioral roles. The function of sexual selection (intrasexual and intersexual) has been included as the possible cause of SSD. A large female was seen competing for mates by prominently perching and performing advertising behavior. Females can compete for better quality mates since males are more active in nesting duties (Payne, 2020).

As a result, it is suggested that the SSD evolved in the female of this bird due to intrasexual selection (female-female competition), which involves access to high-quality territories, nesting sites, and mates (Rosvall, 2011). Furthermore, SSD could be caused by niche partitioning. In this ecological strategy, this bird species exists with other species in the shared ecosystem to limit competition by using alternative resources or habitats (Ainley and Emison, 1972). Hence, the larger bill morphology (bill depth and culmen length) was seen in females. In addition, parental care is another potential factor that might contribute to the larger bill morphology observed in females. Typically, biparental care for developing offspring is the most prevalent in socially monogamous birds (Cockburn, 2006).

However, there is a tendency for SSD to be reduced or minimized in species where both parents contribute significantly to offspring care. The level of involvement in caring for the offspring in Greater coucal is uncertain as limited is known about parental care. It varies based on ecological conditions and food availability (Safari and Goymann, 2020). Male contributions to offspring care are greater than female contributions (Darwin, 1871). This also supports the observation that the nests are primarily constructed by males, are normally domed-shaped, and made of leaves, twigs, and other materials in Greater coucal (Natrajan, 1997). Therefore, the lack of pronounced size differences may be associated with shared responsibilities in parental care (Andersson, 1995; Goymann *et al.,* 2015).

CONCLUSION

In summary, mild SSD was found where females were larger than males in Greater coucal. This study provided the first field-based, cost-effective method for differentiating the sex of this bird. The established function equation, outlined as $D = (-61.74) + (0.29)$ bill culmen length $+ (0.50)$ total head length $+ (0.60)$ bill depth) allowed for 100% correct classification. A defined cut score between two centroids determines that a bird is categorized as female if $D > 0$ (1.95) and as male if $D < 0$ (-2.34).

Significantly, this study provides the groundwork for more effective conservation and breeding programs, where accurate sex identification is crucial in decision-making. This study recommends exercising caution when employing the function to data from different sources due to the potential risk of misclassification. It is advisable to consider adjustments for each study since different studies may employ different measuring routines.

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AUTHOR'S CONTRIBUTION

Jessey Angat conducted the research and authored and revised the article. Farida Zuraina Mohd Yusof conceived the primary research idea, provided the theoretical framework, designed the study, supervised the research progress, led the review and revisions, and approved the article for submission.

CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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