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Determining the sex of a monomorphic threatened, endemic passerine in the sky islands of southern India using molecular and morphometric methods

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Identifying the sex of an individual is often a basic requirement for many biological studies. This is often critically important for threatened or endangered species that may require different conservation strategies for the two sexes. In many passerine birds, like the threatened, endemic White-bellied Shortwing, *Brachypteryx albiventris*, found only in the Shola forests of the Western Ghats, however, the sexes are often monomorphic and indistinguishable in the field. There has been some conflicting information in the historic incidental records on how the sexes can be identified in this species. We conducted molecular sexing to determine the sexual identity of 99 individuals captured in the field and examined the possibility of using some key morphological and morphometric variables to predict the sex of these individuals. Of all the variables tested, the sexes could be distinguished only by the relatively greater wing and tarsus length of the males. We then examined the sexual identity of 149 individuals that were captured over four years of fieldwork during a long-term study of this species. This study thus provides important baseline data for an ongoing exploration of the ecology and demography of the two sexes of this unique threatened bird.

Keywords: Molecular sexing, monomorphic and threatened species, morphometric variables, Shola forest.

KNOWING the sexual identity of an individual is often a basic requirement for any biological study, whether at the level of a population or an individual. This usually becomes critically important in ecological and behavioural field studies[1]. In population demography, for example, it is essential to know the sex of individuals as there may be sex-specific dispersal or mortality[2]. In some cases, the two sexes may have different methods of foraging[3] and may even experience differential predation pressures[4]. Identifying the sex of an individual is thus clearly necessary for a complete understanding of many natural processes.

Many species of birds are, however, monomorphic and difficult to sex visually, particularly in the field and some even in hand. Some examples are the Hill Mynah, *Gracula religiosa* and the Black-capped Chickadee, *Parus atricapillus*. The White-bellied Shortwing, *Brachypteryx albiventris* in the Western Ghats is one such species. The Shortwing is a small bird (< 25 cm, < 25 g) found only in the dense understorey (within 2 m off the forest floor) of the Shola forests in the high-elevation sky islands of the Western Ghats[5]. Being a highly specialized species adapted to a unique habitat, its biology is likely to have been shaped by various ecological processes, which may have also acted differentially on the two sexes. The Shortwing is also a threatened, endemic species which was thought to be rare, with less than 100 sightings in 150 years, until recently[6]. Like most endemic, understorey bird species, the Shortwing may also be sensitive to habitat disturbance[7]. Hence understanding the impact of various ecological processes, both natural and more recently anthropogenic, is likely to be of critical importance in developing conservation strategies for the species. Such strategies may also need to consider individual life-history traits, which usually differ between the two sexes. Establishing the sexual identity of individuals is thus essential for any field study of this bird. Previous information[8] and our own studies[5,6,9] have, however, clearly established that the sexes of this species cannot be visually distinguished during non-invasive observational studies in the wild.

In some avian species, the sex of captive individuals can be determined by examining the cloacal protrusion[10,11], but this method involves examining the angle of the cloaca. The male cloaca is directed upwards, whereas the female one points backwards. This method is, however, difficult to execute unless adequately trained with sexually dimorphic birds[10]. Cloacal protrusions are also known to enlarge

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and show differences only during the breeding season. Additionally, female passerine birds lose feathers on their abdomens forming a brood patch in the breeding season just before the onset of egg-laying. This patch, characterized by thickened skin with increased venation for more efficient thermoregulation[10], can be used to sex individuals. Both these sexing methods can be utilized for Shortwings, but only seasonally and when the bird can be closely examined in hand.

Sexual dimorphism and variation in morphological characters occur as a distinguishing feature in many species of birds[12]. Ali and Ripley[8] reported the sexes of the White-bellied Shortwing to be identical except in wing length, which was believed to be larger for males (78–83 mm) than the females (73–78 mm). More recently, Rasmussen and Anderton[13] suggested that males have a longer tail length (68–72 mm) than the females (63–68 mm), but surprisingly did not comment on the wing length of the sexes. They also reported a sexual dimorphism in eye colour, with the males exhibiting darker iris colour than the females. There does not, therefore, appear to be any consensus on which morphological features could ideally be used to differentiate the sexes in the Shortwing. One of the motivations for this study was to determine which morphological parameters could best be used to identify the sexes in this species.

In birds, males are homogametic (ZZ) and females heterogametic (ZW); this difference in the chromosome complement has been traditionally used for avian sex determination[14]. More recently, however, molecular methods that exploit the presence of unique W-specific DNA sequences in females have replaced the more traditional chromosomal techniques[14]. Male passerines, for example, have two similar copies of a *CHD1* gene (*CHD1*-Z, Z-linked), whereas females in addition to a single copy of this gene, have one copy of a different-sized allele of the same gene *CHD1*-W, W chromosome-linked[15,16]. This difference has now been used to sex individuals from across different families of birds[14,17].

The principal objectives of our study were, thus to (a) determine the sex of White-bellied Shortwing individuals using standard molecular markers, (b) determine the morphometric characters that could be used to differentiate the sexes in this species, and (c) evaluate which of these techniques can provide us an accurate and convenient method to sex this endemic, threatened bird.

The study was conducted in the montane evergreen Shola forests[18] in the Western Ghats, at elevations ranging from 1400 to 2400 m amsl. The topography is undulating, with patches of forests and grasslands. These forests, with an annual rainfall of about 406 cm, are perhaps one of the wettest regions on earth[19].

The study was conducted in the Grasshills National Park, Periyar Tiger Reserve, Peppara Wildlife Sanctuary, Kodaikanal Reserve Forests, Ooty and Baba Budan Hills in the Western Ghats between 2004 and 2008. Birds were captured using mist-nets placed on natural trails in the forest. These nets, of dimension 12 m × 2 m, were placed at a density of ten nets per hectare in each plot. The nets were kept 'open' to capture birds from the break of dawn (0550–0600 h) for 5 h each sampling day. Each net was visited at least every 30 min in a cyclic manner such that birds do not stay in it for long. Birds were transferred from the net to individual, dark-coloured cotton bags till they were tagged with numbered metal bands. A total of 149 birds were captured during the study.

For every individual captured, bill length and tarsus length were measured with a digital vernier calliper (Series 500, Mitutoyo Corp., USA); flattened chord wing length and tail length with a wing rule (WING15ECON, Avinet Inc., USA), and body mass with weighing scales (30, 100 and 600 g, Pesola AG, USA)[10,11]. The brood patch was scored as present or absent, and the cloacal protrusion classified as that of male or female, whenever possible, following Ralph[11] and De Beer *et al.*[10].

For 33 individuals captured, blood samples were collected following Sutherland *et al.*[20]. About 10–20 μl blood was taken from the ulnar vein using a heparanized micro-haematocrit capillary tube and stored in blood lysis buffer at room temperature in the field and later at –20°C in the laboratory. For 66 individuals, feather samples were collected in separate covers.

We used standard primers P2/P8 (ref. 17) to amplify and examine the length variation of the *CHD* gene to determine the sex of 99 different individuals. DNA was extracted using a DNeasy tissue kit (Qiagen, Germany) following the manufacturer's protocols, except that for the feather samples, 20 μl of 10 mg/ml DTT (dithiothreitol) was added to the lysis buffer to aid the breakdown of the feather shaft[21,22]. All the PCR reactions were carried out with 1× *Taq* buffer B, 2 mM $MgCl_2$, 1.5 U *Taq* DNA polymerase (Bangalore Genei, India), 0.25 mM dNTP mix (Eppendorf, Germany), 0.2 μM of each primer (Sigma–Aldrich Chemicals, India), and about 1–1.5 μl of DNA extract. We conducted a standard 35-cycle PCR with the primers P2/P8 (ref. 17) to amplify the target regions with a denaturation of 30 s at 93–95°C and annealing for 30 s at 52.5°C. All PCR products were checked visually by running 2 μl of the product in 3% agarose gels (Bangalore Genei). We classified all individuals that showed two bands as females and a single band as a male.

The positive results of molecular sexing of feather samples from the 61 individuals were used with morphometric measurements to conduct a discriminant function analysis[23] in order to create a model, if possible, that would explain the relationship between sexual identity and the morphometric variables that were recorded. We used the stepwise procedure to include independent variables in the discriminant model, based on their contribution to the model. At each step, the predictor with the largest '*F* to enter' value that exceeded the entry criteria (default 3.84) was added to the model. The individuals

that had some morphometric data missing were classified as ‘unsexed’. All analyses were conducted using JMP SAS (ref. 24) and SPSS (ref. 25)

Of the 149 Shortwings that we captured, we collected blood samples from 33 individuals and feather samples from 66 individuals. We were able to sex all the 33 former individuals, and 61 of the 66 latter individuals using molecular techniques. The five individuals that could not be sexed had feather samples that were over two years old. Morphometric data were collected from 116 individuals that belonged to a single population from the Grasshills National Park.

The discriminant function was able to group 84.48% of the individuals to the correct sex. All the variance could be explained by a single discriminant function (eigenvalue = 1.02, canonical correlation = 0.711, Wilks’ lambda = 0.494), which contributed significantly to the model that effectively segregated the sexes, than to a random model ($\chi^2 = 36.63$, d$f = 2$, $P = 0.000$; Figure 1). The discriminant model increased our ability to predict the sex of an individual by 52% and was marginally better at discriminating males (86.5%) than females (81%).

Wing length and tarsus length were the two most important variables in determining the sex of the White-bellied Shortwing. In the stepwise procedure of the discriminant analysis, these two variables entered the model and were used for the analysis. All the other variables had lower ‘*F* to enter values’, indicating that the addition of these variables would not have added significantly to the discriminating capability of the model (Figure 1). The structure matrix that examined the correlation of each predictor with the discriminant function, but remained unaffected by collinearity, also revealed the relative importance of different variables (Table 1). This analysis too showed that wing length and tarsus length were indeed the most discriminating variables while body weight, bill length and tail length were of less importance, thus justifying their exclusion in this analysis. The highest ‘*F* to remove’ value for any variable in the model was for wing length (28.077), indicating a relatively higher contribution of this variable to the model.

The standardized canonical discriminant function coefficient allows us to compare variables measured at different scales; it showed that wing length (0.833) was correlated more to the function than was tarsus length (0.526). Males and females were significantly different in their wing length (ANOVA, $F_{1,111} = 114.39$, $P < 0.0001$; Figure 2) and in their tarsus length (ANOVA, $F_{1,111} = 36.91$, $P < 0.0001$).

Of the 116 Shortwings captured at Grasshills, we were able to sex only seven (6%) of the individuals using cloacal protrusion. We did not attempt to sex the birds using this method when the cloaca were not enlarged. Of the seven individuals, sexing information from molecular and morphometric analysis was available for only five, thus successfully confirming their sexual identity.

Table 1. Pooled within-group correlations between discriminating morphometric variables and standardized canonical discriminant functions (structure matrix)

Variable measured	Correlation with discriminant function
Wing length	0.851
Tarsus length	0.554
Weight*	0.409
Bill length*	0.401
Tail length*	0.185

Variables have been ordered by absolute size of correlation within the function. *Variables not used in this analysis.

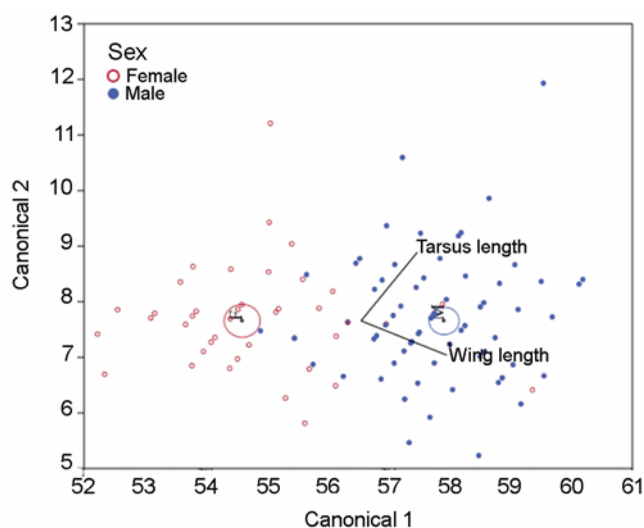

Figure 1. Discriminant function plot of male and female Shortwing’s based on morphometric variables.

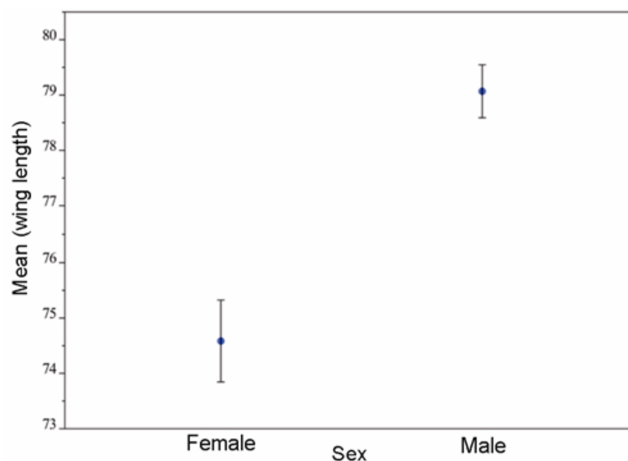

Figure 2. Difference in wing length between male and female Shortwings.

The brood patch was present in only seven (6%) of all the Shortwing individuals captured. Molecular methods confirmed three of these individuals to be females. Application of the morphometric model, however, showed two others of these seven individuals to be males;

molecular analysis could not be conducted on these individuals. The final two individuals could not be sexed by any of these methods.

Our results clearly indicate that sexes of the White-bellied Shortwing can indeed be identified by molecular techniques and further distinguished by specific morphometric traits. Male Shortwings are characterized by greater wing and tarsus length. The morphological characters studied, however, failed to differentiate between the sexes when individuals were examined in hand.

Our findings clearly support the earlier discovery of a greater wing length in male Shortwings, reported by Ali and Ripley[8]. Moreover, we have discovered a role for tarsus length in this sexual dimorphism. We do not, however, find any support for the view of Rasmussen and Anderton[13] that male Shortwings can be distinguished by their greater tail length. It is not possible for us to explain this discrepancy as the source and sample size of measurements by Rasmussen and Anderton[13] have never been clearly specified. It may also be possible that the individuals measured by them were of different ages, or were in different stages of feather moult.

This study has revealed that the sexual identity of individual Shortwings can be established in the field only with great difficulty. The nature of the cloacal protrusion is informative and was able to correctly sex the five individuals in hand, although this is a small sample size and more verification is necessary. It should also be noted that this trait would be of value in the field only during the breeding season of the species. The brood patch, however, was only able to correctly predict the sex of three of the five individuals tested, again a small sample size. Considering the variability and unpredictability in the breeding period (see ref. 9), this method may not be able to provide sufficient information on all individuals in an area or at different locations in different times of the year. We were also not able to distinguish the sexes on the basis of iris colouration, as was suggested earlier by Rasmussen and Anderton[13].

We now believe that wing length could potentially be used to sex individual Shortwings, but there appears to be some overlap in this trait between the sexes and hence an associated degree of error. Even the molecular methods of sexing that we studied were not entirely satisfactory; DNA extraction, for example, failed when the feather samples were extracted two years after collection. These methods, however, appear to be the most successful in sexing individual Shortwings. It must nevertheless be noted that obtaining DNA from birds is usually rather difficult and often involves complex methods to capture individuals successfully.

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