

SHORT COMMUNICATION

Molecular sexing and sexual difference in carpal spur length of the Gray-headed Lapwing *Vanellus cinereus* (Charadriidae)

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Carpal spurs are projecting bony cores covered with a keratinous outer layer on the forward edge of the bird wing near the carpal joint. Prominent carpal spurs are found in all species of screamers (Anhimidae) and in some species of geese (Anseridae), jacanas (Jacanidae), and plovers (Charadriidae); they are usually well developed in both sexes, however males usually have slightly longer spurs than do females (Rand 1954; Prater et al. 1977). These projections of the carpalia are, anatomically, processes of metacarpal I, the original function of which was as an attachment of the extensor muscles (Rand 1954). There have been few studies focusing on the function of wing spurs, and their biological significance remains to be elucidated.

In the Spur-winged Plover *Vanellus spinosus*, which has one of largest carpal spurs for a bird of its size, the spurs have been considered to function as a form of armature (Cramp et al. 1983), because during their threat display (towards other plovers or intruders) their spurs are brandished demonstratively. In contrast, both female and male of the Northern Jacana, *Jacana spinosa*, are reported to spread their wings and attempt to strike each other with their carpal spurs during courtship (Rand 1954).

The Gray-headed Lapwing, *V. cinereus*, is one of the members of the Charadriiformes that has carpal spurs. Because the nests of this species are constructed on the ground of open land, they are particularly suitable for observation and capture to investi-

gate functional aspects of carpal spurs. Information concerning the species is, however, somewhat contradictory, with some authors finding no conspicuous morphological differences between the sexes (Okugawa et al. 1970; Prater et al. 1977), whereas others have referred to males having longer carpal spurs than females (Kobayashi 1956; Sakane 1957). An initial goal of this study was to investigate the spurs of the Gray-headed Lapwing and to confirm whether sexual dimorphism in spur size occurs in this species or not.

Molecular typing of the Chromo-Helicase-DNA-binding protein (CHD) genes has recently been developed as a reliable sexing system for many avian species (Ellegren 1996; Griffiths et al. 1996; Fridolfsson et al. 1998; Kikuchi & Ishii 2002; Mizuta et al. 2003; Seki 2003). This technique has also proven successful among the Charadriidae for sexing Kentish Plover *Charadrius alexandrinus* (Blomqvist et al. 2002; Szekely et al. 2004). As a means of investigating the extent of sexual dimorphism in the Gray-headed Lapwing, we first attempted to sex (molecularly) wild-caught birds, then compared the external measurements of females and males.

MATERIALS AND METHODS

1) Birds

Adult Gray-headed Lapwings were captured singly at their nests by means of a noose trap (under permission of the Ministry of the Environment, Japan) on the Ogura reclaimed land, Kyoto, Japan (34.9°N, 135.8°E) in spring (April and May) 2003 and 2004. All nests had been constructed in rice fields prior to those fields being flooded. After measuring their ex-

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ternal body features and taking blood samples, each bird was marked with a numbered metal ring provided by the Ministry of the Environment and released immediately. Twenty six individuals from 22 nests were studied.

2) Genomic DNA and PCR

Small blood samples (approximately 50 μ l) were obtained by brachial vein puncture, following Kajita (1999), and stored in 99% ethanol at room temperature until use. Genomic DNA was extracted using a commercial kit (AquaPure Genomic DNA Isolation Kit, Bio Rad). Each PCR reaction was carried out in a 50 μ l mixture containing 0.25 units of DNA polymerase (KOD Dash, TOYOBO), 2.5 mM each of dNTPs, and 10 mM each of the primers 2550F (5'-GTTACTGATTCGCTACGAGA-3') and 2718R (5'-ATTGAAATGATCCAGTGCTTG-3') for amplifying the CHD genes (Fridolfsson & Ellegren 1999) using a thermal cycler (The Mini Cycler PTC-150-16, MJ Research). The thermal profile was as follows: initial denaturing at 94°C for 2 min followed by a touch-down scheme where the annealing temperature was lowered by 1°C per cycle from 60°C to 50°C, and 30 additional cycles including denaturing at 94°C for 30 sec, annealing at 50°C for 30 sec, and extension at 74°C for 30 sec. A final extension step at 74°C for 4.5 min was added. PCR products were electrophoresed on a 3% agarose gel at 100 V for 30 min and revealed with ethidium bromide stain.

3) External morphological features

According to Svensson (1970), we measured the following characters of each individual using calipers and rulers: total length, wing length (unflattened), total head length, total bill length, bill depth (at proximal edge of nostrils), bill width (BW; at proximal edge of nostrils), tarsus length, and tail length. The measurements for carpal spur length (CSL; length of the longer one), breast-band depth, tail-band depth, wattle length (length of the longer one), and wattle width (width of the wider one) are shown in Fig. 1. Each bird was also weighed using a balance.

4) Data analysis

The differences between measurements of males and females were tested statistically using the Wilcoxon two-sample test. Since we performed 14 tests for the external features, the critical value for the Bonferroni multi-test correction was $0.05/14 = 0.00357$. To derive the Fisher's discriminant function

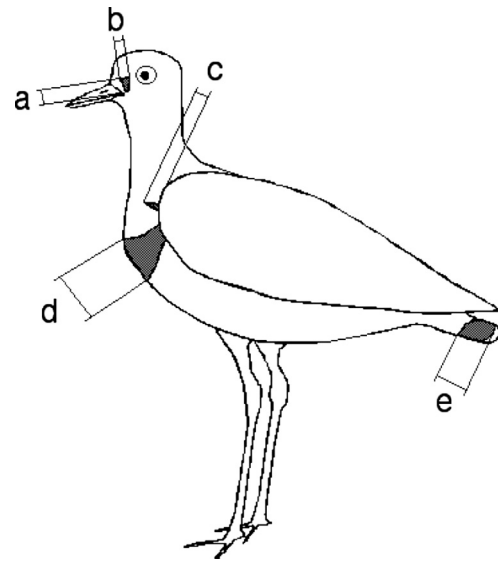


Fig. 1. Measurements. a: wattle length, b: wattle width, c: CSL (carpal spur length), d: breast-band depth, and e: tail-band depth.

for discriminating between the sexes, regression analysis was performed using the VBA Analysis tool of Microsoft Excel 2001.

RESULTS

1) Molecular sexing of the Gray-headed Lapwing

Genomic DNA was successfully extracted from 26 wild caught Gray-headed Lapwings. To determine each individual's gender we performed PCR to amplify a portion of the CHD genes (Fig. 2). All birds showed a PCR product of about 600 bp, which is expected if the template is the CHD-Z gene on the Z chromosome. Some birds showed an additional 450 bp PCR product and others did not, suggesting that the former were females (ZW) and the latter were males (ZZ), because the 450 bp product is expected if the template is the CHD-W gene on the W chromosome. This is consistent with the results for the chicken, *Gallus domesticus*, of known sex, the males of which showed only a 600 bp band whereas the females showed two bands of 600 bp and 450 bp. On this basis, we identified 12 individual Gray-headed Lapwings as females and 14 as males.

2) Comparison of external features between females and males

Following the results of molecular sexing using the CHD genes, we compared the 14 external measure-

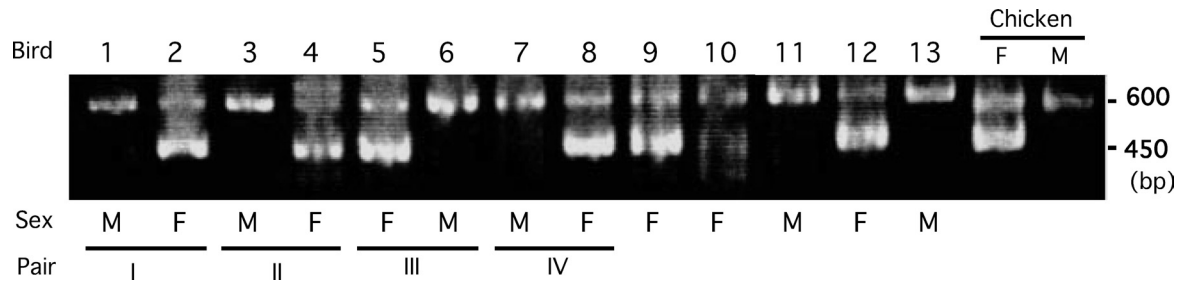


Fig. 2. Molecular sexing of birds using PCR of the Chromo-Helicase-DNA-binding protein (CHD) genes for Gray-headed Lapwings. Pairs are numbered from I to IV. Female and male chickens, *Gallus domesticus*, were used as controls. F: female, M: male.

Table 1. Mean values of morphological measurements of the Gray-headed Lapwing.

Characters	Female	Male	P-value
Total length	336.3±12.1 (12)	341.7±12.1 (14)	0.2917
Wing length	235.5±4.6 (12)	236.9±7.5 (14)	0.6620
Total head length	73.30±1.97 (12)	74.29±1.77 (13)	0.2210
Total bill length	41.37±3.00 (12)	42.02±2.91 (14)	0.4875
Bill depth	10.15±0.39 (12)	10.43±0.56 (14)	0.2617
Bill width	8.50±0.49 (12)	8.79±0.43 (14)	0.1358
Tarsus length	76.26±3.45 (12)	76.90±2.70 (14)	0.3545
Tail length	109.8±2.8 (11)	109.4±3.7 (14)	0.3380
Carpal spur length	3.69±0.54 (12)	5.08±1.35 (14)	0.0006*
Breast-band depth	11.6±3.7 (8)	12.6±4.2 (8)	0.5849
Tail-band depth	28.1±3.2 (8)	29.5±3.6 (8)	0.5905
Wattle length	5.17±0.66 (12)	5.71±1.36 (14)	0.2578
Wattle width	5.03±1.03 (12)	5.62±1.12 (14)	0.2268
Body weight	266.6±19.7 (10)	280.1±15.9 (13)	0.1629

The units of measurements, mean±standard deviation, are g for body weight and mm for all others. The numbers of individuals are in parentheses. P values are from Wilcoxon two-sample tests. Asterisk indicates $P<0.001$.

ments, including CSL, of males and females (Table 1). Mean values of all measurements were found to be larger in males than in females, with only one exception, which was tail length—females had longer tails. Wilcoxon two-sample tests indicated that the gender difference was significant only for CSL ($P=0.0006$, which was significant even after the Bonferroni correction), but not in other characters ($P>0.10$). However, the range of values overlapped considerably, even for CSL, which ranged from 3.0 mm to 4.9 mm for females and from 3.8 mm to 9.0 mm for males. Furthermore, the spur length of the male was not always longer than that of his probable mate; in two pairs the female's spurs were longer than her mate's (Fig. 3a).

By taking CSL as the explanatory variable after standardizing by BW, a discriminant function, $D=4.293(\text{CSL}/\text{BW})-2.117$, was derived. This func-

tion correctly categorized the sex of 81% (21/26) of the birds (Fig. 3b). None of the attempts at improving the reliability were successful; for example, adding other parameters as explanatory variables (data not shown).

DISCUSSION

Among the 14 external features of the Gray-headed Lapwing that were measured, only CSL was shown to differ significantly between the sexes—spurs were longer in males than in females. Sexing, by means of the molecular typing of the CHD genes, was unambiguous. The sexual difference in CSL observed here was not an effect of differences in body mass between males and females, but a result of disproportionate growth of the carpal spurs. Our results are consistent with the previous reports of sexual dimor-

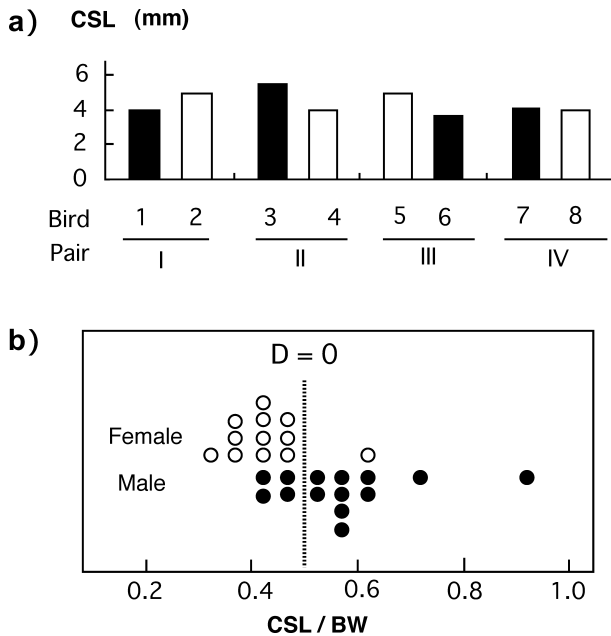


Fig. 3. CSL of Gray-headed Lapwing. (a) CSL (carpal spur length) of individuals in pairs. Birds and pairs are numbered as shown in Fig. 2. White bars indicate females, and black bars males. (b) Distribution of CSL with standardizing by BW (bill width). The broken line is based on the discriminant function, $D=4.293 (\text{CSL}/\text{BW})-2.117$, and indicates the demarcation between females and males ($D=0$). This is almost equivalent to using the value of CSL/BW of 0.5 ($2.117/4.293$) or larger as an indicator of maleness. Open circle: female; closed circle: male.

phism of their carpal spurs (Kobayashi 1956; Sakane 1957), but not with that of larger body size in males (Hayman et al. 1986). It is noteworthy that, in spite of this difference in spur length, the most reliable discriminant function derived from the external measurements failed to sex 19% of the birds. Thus in order to achieve accurate sexing of all individual Gray-headed Lapwings, molecular analysis is necessary.

In Gray-headed Lapwings, the sharing of parental duties is still controversial (Nakamura & Nakamura 1995), although Okugawa et al. (1970) reported that both members of the pair incubate. During the course of this research, one female and one male were identified in each of the four probable mating pairs by CHD typing; thus our results provide further evidence in support of a parental contribution by both sexes, at least as far as incubating the eggs. Our results also confirm the high reliability of this molecular sexing system. Notably, when we compared carpal spur length between the members of a pair, we were unable to find evidence for a simple relationship indi-

cating the importance of carpal spur size for mating success. Gray-headed Lapwings are, however, known to make a threat posture, during which they extend their wings horizontally, when intruders approach their nests. Many such threat displays were made towards us while we were setting the traps in this study (data not shown), and it appeared as if they were attempting to display their carpal spurs to us. These various results taken together indicate that the Gray-headed Lapwing uses its wing spurs not in mating behavior, but as weapons, in much the same way as the Spur-winged Plover (Cramp et al. 1983). Even if this is the case, however, several questions concerning the function of the wing spurs remain unanswered; in particular the reason for the sexual difference in CSL. Accurate molecular sexing, by means of the CHD genes will facilitate further investigation.

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