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UNIVERSITÀ DEGLI STUDI DI TORINO

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Short title: Sexing Hooded Crows.

Summary

In this paper we propose some criteria for sexing Hooded Crow (*Corvus cornix*) belonging to different age groups in NW Italy. The morphometric data were validated by a feather and tissue molecular sexing technique which offers a valid and reliable method to sex birds without ambiguity. Our results suggest that tarsus and head-bill length provide the best biometric criteria for sex determination in the Hooded Crow because it is a measurement that does not change with age. Nevertheless, in those cases where tarsus and head-bill length gives an ambiguous sex determination, wing and third-primary lengths can be used as additional criteria, but, in these cases, it is to determine the age of the birds. In order to determine sex, we also propose linear discriminant analyses based on the tarsus and head-bill length and on the head-bill length alone. Furthermore, biometric data analysis was supported by DNA analysis.

Introduction

A wide range of behavioural, ecological and evolutionary studies involves the determination of sex ratio in natural populations. In addition, the sex ratio is important in conservation and wildlife management programmes. In the field, however, assessing sex of bird species that are sexually monomorphic in plumage, such as the Hooded Crow *Corvus cornix*, is difficult. For this species, effective population management in NW Italy would benefit from knowledge of the population sex ratio and sex-specific resource use. Numerous studies on the Hooded Crow have suggested that males are significantly larger than females (external measurements) (Stresemann 1920, Cramp & Perrins 1994, Acquarone *et al* 2002). In recent years, DNA-based tests have been developed for the detection of sex in some species of birds. In particular, such a test has the potential to increase the confidence in biometric data for sexing. In this regard, a general approach is based on the amplification by polymerase chain reaction (PCR) of the conserved chromo-helicase-DNA-binding (CHD) genes located on the sex chromosomes of all non-Ratite birds (Ellegren 1996, Griffiths *et al* 1996 and 1998, Sacchi *et al* 2004). This PCR test employs a single set of primers that amplifies homologous fragments of both the CHD-W gene, unique to females (the heterogametic sex with W and Z chromsosomes), and the CHD-Z gene which occurs in both sexes. The amplification products of the W and Z CHD genes differ in length, thus allowing birds to be sexed. This paper aims to describe the external measurements of the Hooded Crow and to provide simple criteria that can be used to facilitate sexing in ringing studies. Biometric data was supported by gonadal examination and DNA analysis for sexing all samples.

Material and Methods

Study site and data collection

Material for the study was carcasses of Hooded Crows, culled during a management program. Some Crows were caught with Larsen traps placed in five localities in the Cuneo plain (district of Caramagna Piemonte 44°47'N 7° 44'E, Savigliano 44°39'N 7°38'E, Murello 44°45'N 7°36'E, Sant'Antonio Baligio 44°33'N 7°39'E, Racconigi 44°46'N 7°41'E), while others were shot down in five localities (District of Murello, Savigliano, Moretta 44°45'N 7°32'E, Cavallermaggiore 44°43'N 7°41E, Cavallerleone 44°44'N 7°40'E). In these areas we estimated crow density to be 20.3 individuals per km^2 (with 95% confidence interval [16.3-25.3]) using line transect method (Buckland *et al* 1993) and Distance software. In total 159 individuals were examined in the period March 2005 - July 2007. Small feathers were collected for the PCR sexing method. Feathers were put in plastic bags and stored at -20 °C until analysis.

Morphometric measurements

Wing length was measured to the nearest 0.5 mm, as the distance between the carpal joint and the tip of the longest primary feather (wing closed), using a ruler with a stop at the zero mark (the '*maximum length*' method Stresemann 1920, Ticehurst 1938). The third primary was also measured, to the nearest 0.5 mm, using a ruler with a pin at the zero mark and by inserting the pin at the base between the third and second primary. Wings with lost, broken or heavily worn primaries were not measured. Tarsus length was measured to the nearest 0.1 mm, using a pair of callipers and with the toes at right angles to the tarsus (Svensson 1992).

Head-bill length was measured to the nearest 0.1 mm, using a pair of callipers, from the back of the head to the tip of the bill.

Age and sex determination

Age was determined by examination of plumage (Svensson 1992) and each sex was grouped into three age classes: first calendar-year (1 cy, Euring code = 3), second calendar-year (2 cy, Euring code = 5), and older than second calendar-year (>2 cy, Euring code = 6). The sex of all 159 birds was established either by gonad examination or, when this was not possible, by DNA analysis. To validate the PCR test for sex identification in crows, PCR analysis was carried out on individuals of known sex. Tissue and feather samples from ten individuals, six females and four males, were used. Subsequently the test was used to identify the sex of all individuals of unknown sex. Muscle tissue or the basal tips of the calamus from two or three feathers, were place in a 1.5 ml microcentrifuge tube. Genomic DNA was extracted using the NucleoSpin Tissue Kit (Macherey-Nagel) according to the manufacturer's instructions. A region of the CHD gene was amplified by PCR using the P2550F and 2718R primers (Roche Diagnostics) proposed by Fridolfsson and Ellegren (1999). PCR reactions were performed using 5 μl of DNA solution in 10 μl of final volume containing PCR Buffer 1X (QIAGEN), 0.2 mM of each deoxynucleotide triphosphate, 0.5 μM primers and 0.005 U/μl HotStarTaq (QIAGEN). Cycling conditions consisted of an initial denaturation step at 95 ℃ for 15 min followed by 10 cycles at 94 ℃ for 30 s, a touch–down from 60 ℃ to 50 ℃ of 1 ℃/cycle of annealing temperature for 30 s and at 72 ℃ for 45 s. Then 30 additional cycles were ran at 94 ℃ for 30 s, at 50 ℃ for 30 s and at 72 ℃ for 30 s. A final extension step at 72 ℃ for 5 min was carried out for all reactions. PCR was performed in a Gene-Amp PCR System 2400 thermal cycler (Applera Biosystems). Negative controls were included to verify that samples had not been contaminated with exogenous DNA or PCR products. This is important as the human CHD-1 gene can be a contaminant.

Statistical analysis

Statistical analyses of morphological data were conducted in R and SAS software.
Tarsus, wing, third primary, head-bill length differences between sexes within age classes were analysed by t-tests.
We employed two-way ANOVAs to compare sex and age differences in tarsus and head-bill length as well as interactions between both factors.
Finally, a linear discriminant analysis was carried out on a randomly selected sample of birds of known sex (training set) and the resulting linear discriminant function was then used to predict the sex of the remaining birds of known sex (test set).

Results

Molecular sex determination

The PCR molecular sexing test correctly identified the sex of all 10 individuals of known sex. Then, 66 crows were analyzed with this PCR molecular sexing test and for all samples it was possible to identify sex without ambiguity. The test with the primer set P2550F-2718R gave rapid and clearly interpretable results, amplifying a single 700 base pair (bp) fragment in males (the product of the CHD-Z gene) and two fragments (500bp and 700bp) in females (products of the CHD-W and CHD-Z genes) (Figure 1).

Sex and age differences in biometrics

Table 1, 2, 3 and 4 show the statistical estimates for, respectively, tarsus, wing, third primary and bill-head length of males and females within each of the three age groups (1 cy, 2 cy and >2 cy) and t-test performed for each group. In particular, independent from age, considering tarsus length:

- birds <52.5 mm can be categorised as females;
- birds >61.1 mm can be categorised as males;

considering head-bill length:

- birds < 90.2 are females,
- birds > 96.1 are males

Nevertheless, in those cases where tarsus and head-bill length alone gives an ambiguous sex determination, wing and third-primary lengths can also be used, as suggest their highly significant relationship with sex shown in this study. It is, however, important to determine the age of the bird through examination of wing plumage before using these last two parameters.
In particular, after age determination, considering wing length:

- birds of 1 cy with >308 mm are males and < 290 mm are females;
- birds of 2 cy with >320 mm are males and < 295 mm are females;
- birds of >2 cy with >320 mm are males and < 301 mm are females.

In particular, after age determination, considering third primary length:

- birds of 1 cy with >238 mm are males and < 226 mm are females;
- birds of 2 cy with >245 mm are males;
- birds of >2 cy with >245 mm are males and < 229 mm are females.

Figure 2 gives box plots for the measurement of tarsus within males and females and within six suitable categories. The analysis of the figure (a) confirms that birds with tarsus > 61.0 are males, while not considering the outliers of the data for males, would lead us to conclude that birds with tarsus < 55.0 are females. Part (b) highlights how the measurement of the tarsus is already determined in first year and maintained with age
Figure 3 gives box plots for the measurement of head-bill length within males and females and within six suitable categories. It is clear that even the bill-head length is already determined in the first year and maintained with age in females, whereas in males measure is determined a little later (in the 2Y).
Table 5 and Table 6 show the output of two-way ANOVAs for unbalanced data performed with SAS software on overall measurement of tarsus and, respectively, head-bill length.
Statistical analyses highlight that, with reference to morphometric variables, males are on average larger than females and, in particular, there are no significant interactions between sex and age factors on tarsus length and on head-bill length.
In order to determine the best measurements for sexing Hooded Crows, a forward stepwise linear discriminant analysis was performed on a randomly selected sample of birds of known sex (training set) and the effectiveness of the discriminant analysis was assessed in terms of the proportion of individuals of known-sex that were classified correctly using the remaining birds of known sex (test set).
In particular, inear discriminant analysis incorporating head-bill and tarsus length for the training set (consisting of 48 individuals sexed by DNA and by gonad examination), yielded a model which correctly predicted sex in 87.5% of the test set (of size 62), with 32 of the 34 males and 21 of the 28 females being correctly classified.

This model provides the following linear function

$$LD = 0.911997 \times HB + 0.225644 \times T,$$

where HB is the head-bill length (mm) and T is the tarsus length (mm), and a threshold for males of 97.92: so, any individual with a LD value greater than or equal to the threshold is categorized as a male.

In addition, we note that a linear discriminant analysis incorporating only head-bill length produced a model which correctly predicted sex in 85.5% of the test set, with 31 of the 34 males and 22 of the 28 females being correctly classified.

Such a model provides the following rule: any individual with a head-bill lenght (mm) greater than or equal to the HB threshold given by 93.1 is categorized as a male.

Discussion

Being able to assign sex accurately is important for studies comparing ecological and behavioural sex differences. Because many species show only limited sexual dimorphism in plumage traits, we tried to develop sexing tools involving biometrics issues for Hooded Crow.

Setting out from definite and certain sexing data, obtained from gonad analysis and matched with molecular techniques, we could elaborate biometric data, in particular about wing, third-primary and tarsus length. Our results suggest that tarsus length is the best biometric way for sex determination in the Hooded Crow, because it is a measurement that does not change with age. This finding is in accordance with previous studies carried out on different species of crows (Tella *et al* 1995). As a matter of fact, box plots (Figure 2) already give us an idea of how important the difference in tarsus length between males and females is, regardless of age classes. This idea is confirmed by t-tests (Table 1) and ANOVAs performed on overall measurement of tarsus (Table 5). In particular, it is statistically evident that sex influence the length of tarsus ($p<0.0001$), while the age and the interaction sex*age having no effect on it.

Another biometric way for sex determination is based on the head-bill length: this measurement appears not to change with age in females and determined slightly more late (in 2Y) in males. Box plots (figure 3) shows the difference in head-bill length between males and females and the difference between males in age 1Y and males of the other two age groups. But it is statistically evident (Table 6) that sex influence the length of head-bill ($p<0.0001$), while the age and the interaction sex*age having no effect on it. The difference between males in age 1Y and males of the other two age groups is significant (Tukey multiple comparisons of means 95%: $p=0.0007157$ for 1Y vs 2Y; $p = 0.0000647$ for 1Y vs >2Y)

Nevertheless, if tarsus length and head-bill length give an ambiguous sex determination, then wing and third-primary lengths can also be used. In such a case, it becomes necessary to determinate the age. In fact, ANOVAs performed on overall measurement of wing ($p<0.0001$ for sex, $p<0.0001$ for age) and of third primary ($p<0.0001$ for sex, $p=0.0008$ for age) show that both sex and age have an effect on these measures. This is due to the fact that wing and third primary change with age.

We hypothesize that, if the measure of the tarsus in both sexes does not change with age, being determined at an early age (1Y), and if the measure of the skull, including the bill, does not change with age, as determined in females in the first year and in males only a little later (2Y), the skeleton of Hooded Crow inreaches the final size in the early periods and does not grow with the attainment of adulthood.

In addition, linear discriminant analyses based on the tarsus and head-bill length and on the head-bill length alone, presented in this study, allow the sex identification of more than 87% and, respectively, 85% of birds.
Finally the study confirms the usefulness of DNA techniques for improving the ability to sex accurately bird species. Furthermore, when the necropsy analysis is not possible, molecular methods represent a reliable tool to test biometric features for determining sex in Hooded Crows.

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Table 1 Comparison of the mean and minimum-maximum tarsus (mm) of males and females Hooded Crown. The three age groups are first calendar-year (1 cy), second calendar-year (2 cy), and older than second calendar-year (>2 cy) Statistics presented are the 95% confidence interval for the mean (CI), the t-test statistic and the probability value (p).

Age	Number of individuals	Sex	Mean	Min-Max	CI for mean	t-test
1 cy	29	M	59.21	52.5-65.0	[58.25; 60.17]	3.9001
	22	F	56.79	52.7-61.1	[56.06; 57.52]	(p=0.0002928)
2 cy	26	M	59.64	55.2-63.3	[58.94; 60.34]	7.1707
	44	F	56.04	51.3-60.4	[55.38; 56.70]	(p=6.982e-10)
>2 cy	17	M	59.75	56.0-63.8	[58.75; 60.75]	4.9505
	5	F	55.10	54.1-57.4	[53.45; 56.75]	(p=7.702e-05)
	72	M	59.50	52.5-65.0	[58.99; 59.99]	9.5017
	71	F	56.21	51.3-61.1	[55.73; 56.69]	(p < 2.2e-16)

Table 2. Comparison of the mean and minimum-maximum wing (mm) of males and females Hooded Crown. The three age groups are first calendar-year (1 cy), second calendar-year (2 cy), and older than second calendar-year (>2 cy) Statistics presented are the 95% confidence interval for the mean (CI), the t-test statistic and the probability value (p).

Age	Number of individuals	Sex	Mean	Min-Max	CI for mean	t-test
1 cy	19	M	309.26	290-317	306.31-312.22	5.0317
	16	F	294.81	267-308	289.16-300.46	(p=1.680e-05)
2 cy	21	M	313.95	295-329	309.79-318.11	5.4930
	25	F	303.00	285-320	299.22-306.78	(p=0.0002091)
>2 cy	27	M	325.15	301-340.5	321.60-328.70	6.5622
	16	F	308.06	291-320	304.41-311.71	(P=6.867e-08)
	67	M	317.13	290-340.5	314.52-319.74	7.9508
	57	F	302.12	267-320	299.41-304.83	(p=1.058e-12)

Table 3. Comparison of the mean and minimum-maximum third primary (mm) of males and females Hooded Crown. The three age groups are first calendar-year (1 cy), second calendar-year (2 cy), and older than second calendar-year (>2 cy) Statistics presented are the 95% confidence interval for the mean (CI), the t-test statistic and the probability value (p).

Age	Number of individuals	Sex	Mean	Min-Max	CI for mean	t-test
1 cy	19	M	233.64	226-241	231.72-235.54	3.1808
	15	F	224.27	197-238	218.27-230.27	(p=0.005482)
2 cy	20	M	237.72	213-249	233.27-242.17	2.6845
	25	F	230.12	213-245	226.24-234.00	(p=0.01027)
>2 cy	27	M	244.96	229-257	242.20-247.72	4.5697
	15	F	234.57	224-245.5	230.56-238.58	(p=4.612e-05)
	66	M	239.51	213-257	237.40-241.62	5.8063
	55	F	229.74	197-245.5	227.06-232.42	(p=5.419e-08)

Table 4 Comparison of the mean and minimum-maximum bill-head length (mm) of males and females Hooded Crown. The three age groups are first calendar-year (1 cy), second calendar-year (2 cy), and older than second calendar-year (>2 cy) Statistics presented are the 95% confidence interval for the mean (CI), the t-test statistic and the probability value (p).

Age	Number of individuals	Sex	Mean	Min-Max	CI for mean	t-test
1 cy	32	M	94.68	90.2-101.4	[93.76; 95.60]	8.5601
	31	F	90.13	86.4-93.6	[89.54; 90.72]	(p=7.104e-12)
2 cy	21	M	97.84	90.6-114.0	[95.81; 99.86]	6.1701
	36	F	91.48	86.8-96.1	[90.77; 92.19]	(p=1.797e-06)
>2 cy	17	M	98.09	93.3-105.7	[96.35; 99.82]	4.8894
	8	F	91,09	84.2-95.2	[88.37; 93.80]	(p=6.137e-05)
	70	M	96.46	90.2-114.0	[95.56; 97.35]	10.4756
	75	F	90.88	84.2-96.1	[90.39; 91.37]	(p<2.2e-16)

Table 5. Output of SAS program: two-way ANOVAs for tarsus length of male (sex 1) and female (sex 2) Hooded Crowns. The three age groups are first calendar-year (age 1), second calendar-year (age 2), and older than second calendar-year (age 3).

Parameter	Estimate		Standard error	t value	Pr > |t|
Interc.	55.10000000	B	0.92240233	59.74	<.0001
sex 1	4.65294118	B	1.04931836	4.43	<.0001
sex 2	0.00000000	B	.	.	.
age 1	1.69545455	B	1.02185890	1.66	0.0994
age 2	0.94545455	B	0.97340168	0.97	0.3331
age 3	0.00000000	B	.	.	.
sex*age 1 1	-2.23839572	B	1.20047188	-1.86	0.0644
sex*age 1 2	-1.05608803	B	1.16677941	-0.91	0.3670
sex*age 1 3	0.00000000	B	.	.	.
sex*age 2 1	0.00000000	B	.	.	.
sex*age 2 2	0.00000000	B	.	.	.
sex*age 2 3	0.00000000	B	.	.	.

Table 6. Output of SAS program: two-way ANOVAs for bill-head length of male (sex 1) and female (sex 2) Hooded Crowns. The three age groups are first calendar-year (age 1), second calendar-year (age 2), and older than second calendar-year (age 3).

Parameter	Estimate		Standard error	t value	Pr > |t|
Interc.	91.08750000	B	0.98792252	92.20	<.0001
sex 1	7.00073529	B	1.19803203	5.84	<.0001
sex 2	0.00000000	B	.	.	.
age 1	-0.95685484	B	1.10808823	-0.86	0.3893
age 2	0.39027778	B	1.09218944	0.36	0.7214
age 3	0.00000000	B	.	.	.
sex*age 1 1	-2.45013046	B	1.38965718	-1.76	0.0801
sex*age 1 2	-0.64041783	B	1.42266428	-0.45	0.6533
sex*age 1 3	0.00000000	B	.	.	.
sex*age 2 1	0.00000000	B	.	.	.
sex*age 2 2	0.00000000	B	.	.	.
sex*age 2 3	0.00000000	B	.	.	.

Figure 1. PCR fragment analysis

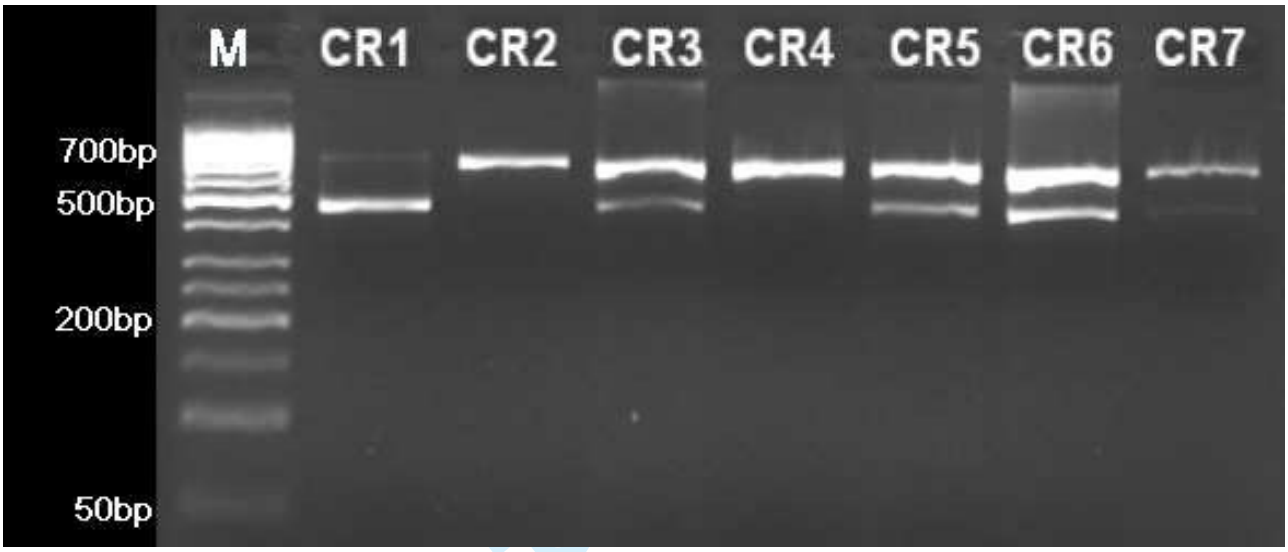

Figure 2 Box plot for the measurement of tarsus (a) in males (M) and females (M) and (b) in six categories. The three age groups are first calendar-year (age1), second calendar-year (age2), and older than second calendar-year (age>2).

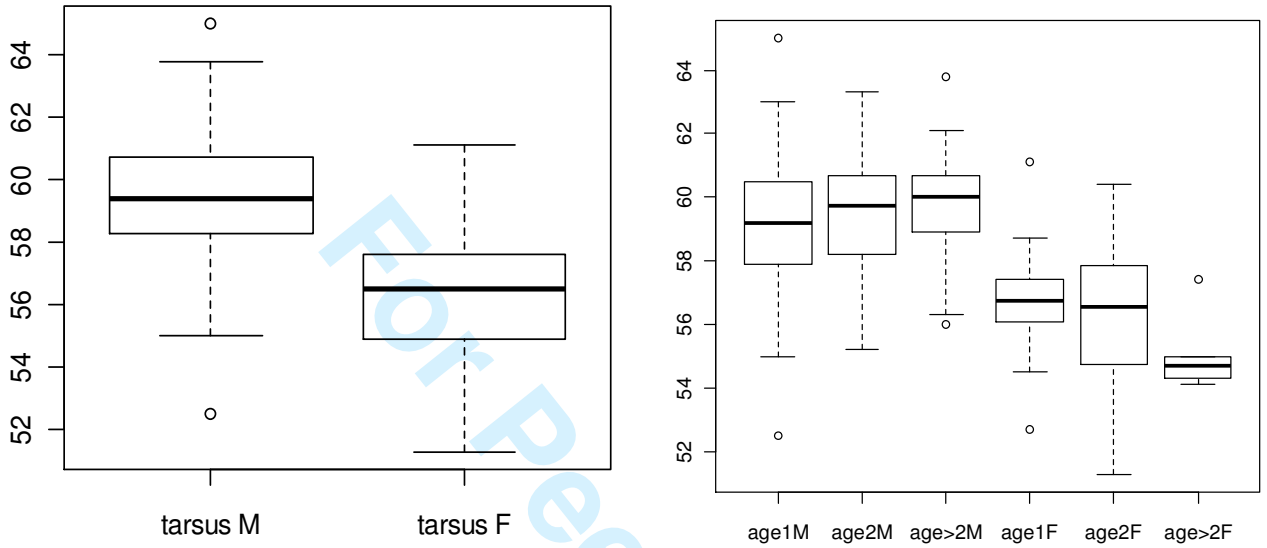

Figure 3 Box plot for the measurement of BH (a) in males (M) and females (M) in six categories. The three age groups are first calendar-year (age1), second calendar-year (age2), and older than second calendar-year (age>2).