Molecular sexing of the Black Stork *Ciconia nigra*: sex ratios in the Portuguese population

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Abstract
We sexed 125 chicks of black stork ringed between 2003 and 2005 in Portugal. We used a combination of molecular methods which effectively determine sex in this species. Analysis was done mainly using feather samples, applying a non invasive approach to a threatened population. Sex ratios and female proportion within broods were calculated. An excess of females was observed during the years studied, and globally, the deviation from parity was statistically significant. This result needs to be confirmed and related to changes in habitat quality. The sex ratio of black stork populations should be monitored in the long term as they may be an important source of information for monitoring ecological stress. This is the first assessment of sex in a wild population in Portugal.

Key words: Black Stork, *Ciconia nigra*, sex ratio, Portugal

Received 6 February 2007; accepted 4 April 2007

INTRODUCTION

Unlike many other bird species, it is not possible to reliably determine the sex of Black Storks *Ciconia nigra* by external morphological observation. Cramp (1977) described the sexes in this species as "alike", although other authors have pointed out discrete differences such as adult males being on average larger (Del Hoyo et al. 1992). Molecular determination of sex in birds is now a common procedure with a wide range of applications from sexing chicks and forensics to bird aviary management. It allows sexing from a small sample of tissue using a non invasive procedure for the wild animals. However, the same techniques do not apply for all species. Molecular sexing has been described for *Ciconia boyciana* (Itoh et al 1997) and other storks (Tomasulo et al 2002) but to date there has been no published data on molecular methods of determination of sex in black stork. Sexing of animals yields important information for ecological and population studies, individual life history, and long term monitoring of ringed birds. It also allows the calculation of sex ratios in a population which is subjected to environmental variation or change and adaptation of the species. Sex ratio adjustments have recently been described for some species depending on habitat quality (Stauss et al 2005) and maternal condition (Gilbert et al 2005).

Among black storks breeding in Europe, different populations exist; one of the smallest, 405-483 pairs (Cano Alonso et al, in press), breeds in the Iberian Peninsula. The Iberian population is geographically isolated (Cramp 1977, Birdlife International 2004) and partly resident (Bernis 1974, Parkes & Torés Sanchez 2003), although most birds migrate to winter in Africa (Cramp 1977). It is thought that black storks show breeding site fidelity, so this population might be isolated in reproductive terms. Mainly because of its small population size, the species is considered threatened in Portugal and Spain, with a recent attribution of the IUCN Red List category of Vulnerable (Cano Alonso & Hernandez Garcia 2004, Almeida et al 2005). Part of the Iberian population, around 100 pairs (Rosa et al 2001), breeds in Portugal, choosing undisturbed areas of rocky habitat or forest (Franco & Pacheco, pers. comm.). Since the 1990s an annual monitoring of nests has been performed in Portugal, including ringing of chicks. In 2003, other studies were started, involving satellite tracking of some individuals (Franco 2006) and a genetic study of the population which indicates low genetic variability (data not presented, Fernandes et al in prep).

Knowledge of the sex of an individual is important in population studies because many life history characters including survival, fecundity, and dispersal may differ between sexes. Sex ratio is also of interest to evolutionary biologists as the target itself of natural selection (Lessells et al 1996).

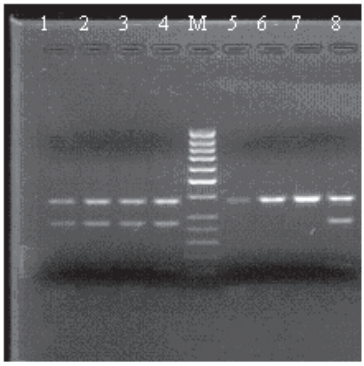
We describe an effective combination of methods for sexing black stork and present results obtained for individuals ringed in Portugal from 2003 to 2005. Sex ratios of the population in Portugal were calculated.

MATERIALS & METHODS

Bird sampling

Material was collected in Portugal in 2003-2005 (Table 1). During nest control, all chicks from each brood were sampled corresponding to a total number of 137 samples. In certain cases the same nests were controlled during two consecutive years. Around 6-10 growing feathers were plucked from the chicks, which were between 33 and 52 days of age (n = 123). Feathers were stored in paper envelopes in a dry and dark place. Moreover, to guarantee a high DNA content in one sample per brood, approximately 200µl of blood was collected from one of the chicks in each brood (n = 34) and later stored at -20ºC.

Figure 1. Molecular sexing of Black Stork. Results of *Asp*700I digestion of PCR products amplified with P2/ P8. Samples with two bands (378bp and 280bp) are females (1, 2, 3, 4 and 8) and with one band (378bp) are males (5, 6 and 7). M = 100bp marker (Fermentas).



Two control samples from black storks of known sex were used, to verify results of molecular sexing. One tissue sample was obtained from a dead animal whose sex was determined by internal morphological observation and the other was composed of feathers from a zoo animal.

Sex ratio

We used the total of broods in which all chicks were sexed to calculate the sex ratio within each brood. Sex ratio was also calculated for each year and for the global sample using the total number of males and females. We used chi-square statistics to test for independence between years and significant deviations from 1:1 ratio. We performed an ANOVA to test for independence of sex ratio in each brood over the years using Program R - 2.1.1 (RDCT 2005).

Molecular analysis

DNA was extracted from blood using the Puregene blood kit (Gentra) or from feathers using the Nucleospin tissue DNA extraction kit (Macherey-Nagel) with some modifications as follows: we used a higher concentration of proteinase K per sample (20-25µl for a solution of 20mg/ml). Digestion was performed overnight at 55ºC. The lysis buffer volume was adjusted to the size and number of the feathers (350-600µl). We also used a higher quantity of cold (-70ºC) ethanol (350-500µl). For feather samples 30µl of DTT was previously added, freshly prepared, following the recommendation of Sorenson (pers.comm; Sefc et al. 2003). DNA was finally recovered in 50-80µl of elution buffer and stored at 4ºC for immediate use.

We amplified all samples with primers P2 (5´-TCTGCATCGCTAAATCCTTT-3´) and P8 (5´-CTCCCAAGGATGAGRAAYTG-3´) described by Griffiths et al. (1998) and previously used for bird species. Template DNA (1.5-5µl) from blood and (1,5-10µl) from feathers was carried out in a 23µl reaction volume containing 0.9mM dNTPs, 1.6 pmol of each primer, 2.5ul of PCR buffer $(NH_4)_2(SO)_4$, 2.7 mM $MgCl_2$, 0,1mg/ml BSA and 1U of Taq polymerase (MBI Fermentas). When amplification failed, ImmoMix ready mixture for PCR (Bioline) was used and up to 10µl of DNA was added. Amplification was performed in an iCycler BioRad thermocycler. PCR reactions consisted of a touchdown profile with a first step of 4 min denaturation at 94ºC, 5 cycles of 30s denaturation at 94 ºC, 30s annealing at 55ºC and 30 s extension at 72ºC, followed by 35 cycles of 30s denaturation at 94 ºC, 20s annealing at 50ºC and 20 s extension at 72ºC and a final extension step at 72ºC for 5 min.

After PCR amplification we added an enzyme digestion step (Sacchi et al 2004). The products of PCR were submitted to restriction enzyme digestion with *Asp700* or *HaeIII* (Roche). The reaction mix was performed in a total volume of 15µl and contained 0.5 units of enzyme, 12 µl of PCR product and 1.5 µl of 10x enzyme buffer. The digest was incubated at 37ºC for 3 hours.

To detect whether contamination of samples with exogenous DNA or PCR products had occurred, tubes without samples were

Table 1. Sampling of Black Stork *Ciconia nigra* in Portugal and number of chicks of each sex determined by molecular analysis. Female proportions within broods indicated as an average, standard deviation indicated between brackets. Sex ratio expressed in terms of percentage of females.

Year	Number of nests visited	Number of animals sampled	No. of Males	No. of females	Average proportion of females within broods	Sex ratio
2003	19	49	17	29	0.54 (0.36)	0.63
2004	21	53	21	30	0.58 (0.28)	0.59
2005	12	35	11	17	0.65 (0.33)	0.61

included in the DNA extraction and PCR amplification procedure as negative controls. DNA extractions, pre-PCR and post-PCR pipetting were carried out in different rooms and aerosol-resistant filter pipette tips were used to avoid contamination.

The PCR products and the products of digestion were separated by 2% agarose gel electrophoresis in 0.5x TBE at 70 V, and stained with ethidium bromide.

Results and discussion

In Black Stork, female samples present three bands of 378 bp, 280 bp and 107 bp, although the third one may be present as a very weak signal in the gel, visible only by direct observation under UV transillumination. Male samples present a single band of 378 bp (Figure 1).

A total of 125 birds were successfully sexed. From the individuals ringed and sampled in Portugal, 12 failed to be amplified and sexed, most probably because of the low amounts of DNA present in the sample. The use of feather samples with this method proved to be easy and reliable, making it applicable to other monitoring programs in storks and other bird species.

Table 1 summarizes results of sex determination for each year in Portugal. Among the Portuguese animals ringed and sexed between 2003 and 2005, a significant number is expected to be later controlled in the wild (17% of the black storks ringed in the last 30 years in Portugal were later recaptured; data from Portuguese Ringing Centre). Knowledge of their gender is basic information and may be relevant for further studies. Four of these animals were satellite tracked, so knowledge of their sex can be further related with dispersion distances, territory movements, migration timings and other aspects of their biology.

Concerning brood sex proportion, we calculated the average proportion of females in each year (see table 1) and noticed an apparent excess of female production. As an example, we can refer to four broods sampled in 2003 (three with 3 chicks and one with 4 chicks) which were exclusively composed of female chicks. An ANOVA test was performed with year as a factor but no differences were found (F value =0.3439, $P > 0.05$).

We did calculations of sex ratio per year and an excess of females was demonstrated for all the years (Table 1). We first tested if there were differences for the number of females and males among years and chi-square was not significant ($\chi^2 = 0.181$; $P > 0.05$). Then we pooled the data from three years and tested for deviation from a 1:1 sex ratio. Chi-square was then significant ($\chi^2 = 5,83$; $P < 0.01$) showing an overproduction of females. These results are preliminary and need to be related with other factors. Sex ratio may be determined by mothers and can be related to habitat quality (Stauss et al 2005, Sasvari & Nishiumi 2005). Black storks in the Iberian Peninsula can be subjected to strong environmental variations caused by droughts or wet springs and differences in food availability.

Another preliminary study on a different black stork region in Spain, sexing 38 individuals, found an overall sex ratio tending to 1:1 (Fernandez-Garcia & Lanzarot in press). Known sex ratios of other bird species have shown non significant deviations from an expected 1:1 ratio (e.g. Rudnick pers.comm., Laaksonen et al 2004, Lessells et al 1996). However, by comparison, our sex ratios for the black stork of 0.59 to 0.63 are higher than others. For instance, Montagu's harrier and Eurasian kestrel presented lower sex ratios than black stork, varying from 0.510 to 0.614 and 0.47 to 0.59 respectively (Leroux & Bretagnolle 1996; Laaksonen et al 2004). On the other hand, a significant bias in the 1:1 sex ratio was found in wood stork, in Brasil and the United States, one favoring females and hypothetically related to low quality sites, and another with a higher proportion of males in sites with a low level of predation (Seccomandi et al 2003).

These results are indicative of a sex ratio deviation towards females and form a basis for future comparisons. A wider sample over the years will be necessary to confirm significant variations. Records of environmental variations such as temperature and rainfall will also be useful to relate to these results. The sex ratio of the black stork population should be monitored in the long term as it may be an important source of information for monitoring ecological stress. We believe this molecular approach is an effective way of routinely sexing chicks using a non invasive approach. Molecular sexing is a reliable method of obtaining data on sex ratios of wild populations and that information can be related to other ecological factors.

ACKNOWLEDGEMENTS

This study was financially supported by Junta de Extremadura (Spain). We would like to thank the BTVS from ICN and the Riga Zoo for tissue samples. We also thank all the collaborators involved in field work and sample collection, in particular, Victor Pizarro and Juan Carlos Aronja.

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