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Evaluation of methods for molecular sex-typing of three heron species from different DNA sources

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Abstract: Genetic markers are a useful tool for bird population monitoring, especially when combined with ringing data, and particularly so in vulnerable species. This study compared the effectiveness of two DNA extraction methods: a standard protocol and a commercially available kit. The molecular sex-typing was performed using PCR-based methods with a 2550F/2718R set of primers in three species of herons: Great Egret, Purple Heron, and Grey Heron. Genomic DNA was isolated from feathers, eggshells, and eggshell swabs from 26 individuals. Overall, better DNA yields and purity were obtained by using the standard protocol isolation method. The highest DNA yield was obtained from the pin feathers compared to the contour feathers and eggshells, both of which had lower yields. Eggshell swabs indicated possible contamination with parental/sibling DNA. Our evaluation demonstrates that the optimization of laboratory procedures is beneficial, particularly when different types of noninvasive tissue samples are available.

Key words: Ardeidae, *CHD*, sex determination, polymerase chain reaction

1. Introduction

Bird ringing has been used for over a century to study bird migration and biology, and even today it is one of the essential tools in bird studies (Jouventin et al., 1994; Baillie et al., 2007; Klaassen et al., 2014). The value of bird ringing data can increase if the data for the ringed individuals are coupled with sex information. Noninvasive genetic analyses can provide information about the birds' sex, population demography, and behavioral ecology (Horváth et al., 2005; Schmaltz et al., 2006; Hogan et al., 2008; Rudnick et al., 2009). Use of molecular markers for sex can help with tracking and predicting population status and growth in birds. The chromo helicase DNA-binding (*CHD*) gene, which plays an important role in the control of transcription elongation and chromatin remodeling, is the accepted molecular marker used for avian sex determination (Ellegren, 1996; Griffiths et al., 1996; Griffiths and Korn, 1997). In birds, females are heterogametic (ZW) and males are homogametic (ZZ). Therefore, use of primers based on the *CHD* gene and sex-typing applying polymerase chain reaction (PCR) can be performed to detect sex-specific fragments in samples (Griffiths et al., 1998; Fridolfsson and Ellegren, 1999).

Colonial waterbirds, such as herons, are a group of birds whose survival depends on the extent and quality of wetland habitats (Kushlan, 2008). To study their migration and wintering, regular monitoring and marking of breeding populations were carried out during the past 10 years in Croatia (Kralj and Barisic, 2013). Both the Great Egret and the Purple Heron are endangered species in Croatia and are listed on the Croatian Red List, with total populations below 180 and 140 breeding pairs, respectively (Tutiš et al., 2013). The Grey Heron is not threatened since its breeding population in Croatia exceeds 3000 pairs. Samples for this study were collected from two colonies, at the Jelas fishpond and Nature Park Kopački Rit at Ćošak Šume. The abovementioned heron species do not have pronounced sexual dimorphism; the male's skeletal elements are only 2%–4% larger than the female's, with the least variation occurring in the Great Egret (Del Hoyo et al., 2010). Therefore, only molecular methods may be reliable for sex-typing of these species.

The aim of this work was to evaluate methods for DNA extraction and molecular sex identification by using noninvasive sampling in three heron species.

2. Materials and methods

2.1. Sampling

Sampling was performed in the continental part of Croatia from colonies in Ćošak Šume (Kopački Rit Nature Park,

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45°38.15′N, 18°50.76′E) and at the Jelas fishpond (near the Sava River, 45°08.27′N, 17°48.08′E) during regular monitoring and ringing in 2015. We sampled three species: the Great Egret (*Ardea alba*), the Purple Heron (*Ardea purpurea*), and the Grey Heron (*Ardea cinerea*) (Table). We collected noninvasive samples of contour (molted) feathers and eggshells; these were harvested around nests of the Great Egret and the Grey Heron. Only contour (molted) feathers with transparent calamus and intact barbs on the vane were collected. The Purple Heron and the Great Egret were sampled during ringing; from each nestling of the Great Egret and Purple Heron, two pin (blood) feathers were plucked (Table). The samples were stored in paper envelopes at room temperature and kept separately to avoid cross-contamination.

2.2. Isolation of genomic DNA

Isolation of DNA from each sample type was performed twice; the DNA was placed in two separate tubes and then used in two different extraction protocols. In detail, the tips of the pin feathers (two from each nestling) were cut into pieces of 3 to 5 mm long. The calamus of the contour feather (molted) was cut in half and placed into two separate tubes. From the eggshell, a piece of vascularized membrane was excised and cut into smaller fragments. From the same eggshells, the external surface was swabbed as described by Schmaltz et al. (2006) by using cotton tips. To avoid cross-contamination with nestling DNA, swabs were taken only from eggshells that did not have blood smears (which could have come from the mother) on the surface. A similar amount of each sample was used for both DNA extraction protocols.

We used two methods to extract DNA from noninvasive samples (Table). Genomic DNA was isolated using the DNeasy Blood and Tissue Kit (QIAGEN, Germany) and a standard protocol for DNA isolation according to De Volo et al. (2008). For DNA isolation with the DNeasy Blood and Tissue Kit, manufacturer instructions were followed. De Volo et al.'s protocol was modified as follows: samples were incubated in 360 µL of extraction buffer containing 10 mM Tris-Cl (pH 8.0), 10 mM EDTA, 100 mM NaCl, 2% sodium dodecyl sulfate (SDS, Carl Roth GmbH, Germany), 40 mM dithiothreitol (DTT, Carl Roth GmbH), and 2 mg/mL proteinase K (Carl Roth GmbH) in final concentrations. Furthermore, after overnight incubation at 56 °C, keratin, vascularized membranes, and cotton tips were precipitated with 300 µL of 3 M sodium acetate (Carl Roth GmbH). Samples were vortexed, incubated on ice, and centrifuged at 4 °C and 16,000 × *g* for 10 min. Genomic DNA in the supernatant was precipitated at –20 °C for 30 min with the addition of 1:1 isopropanol (v/v). Samples were centrifuged again, and the pellet was washed with 70% ethanol. After centrifugation, DNA was air-dried and dissolved in 40 µL of nuclease-free water (Promega, USA). DNA quantity (concentration) and quality (A_{260}/A_{280}) were quantified using a NanoPhotometer (Implen GmBH, Germany).

2.3. The *CHD*-based molecular sexing protocol

Sex-specific fragments of the *CHD* gene were amplified with primer pair 2550F/2718R (Fridolfsson and Ellegren, 1999) in a total volume of 20 µL. The PCR reaction contained 10 µL of Takara Emerald Mix (Clontech, USA), 2 µM of each primer, and 30–50 ng of genomic DNA. The

Table. The origin of samples and DNA concentration used in the study of three heron species.

Species	No. of individuals sampled	Collected material (age)	Sampling site	DNA concentration ng/µL (range)	
				Standard protocol	QIAGEN kit
Great Egret, *Ardea alba*	4	Pin feather (nestlings)	Jelas fishpond	652–1728	65–1608
Great Egret, *Ardea alba*	5	Contour (molted) feather (adults)	Jelas fishpond	3–58	3–23
Purple Heron, *Ardea purpurea*	7	Pin feather (nestlings)	Jelas fishpond	762–2728	83–1595
Grey Heron, *Aredea cinerea*	10	Eggshell	Nature Park Kopački Rit	50–473	24–99.5
	10	Eggshell swabs	Nature Park Kopački Rit	23–30	5–20
Total sample size	26				

thermal cycling protocol consisted of initial denaturation at 95 °C for 5 min followed by 40 cycles at 95 °C for 30 s, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min, with a final elongation step at 72 °C for 7 min. PCR products were visualized on 2% agarose gels in Tris-acetate-EDTA buffer and visualized using SYBR Safe DNA gel stain (Invitrogen, USA).

3. Results

DNA was extracted from 26 samples (Table) obtained during regular heron colony monitoring in Croatia. The pin and contour feathers, as well as eggshells, provided enough DNA for molecular sex-typing. Isolation of DNA with the standard protocol provided more genomic DNA from pin feathers (413–2780 ng/μL) and contour feathers (3–58 ng/μL), eggshells (50–473 ng/μL), and eggshell swabs (23–30 ng/μL) when compared to isolation with the commercial kit (Table). We were unable to extract DNA from one contour feather of Great Egret using the commercial kit as opposed to isolation with the standard protocol. When the two isolation protocols were compared, results showed that the highest DNA yield was obtained from pin feathers and the lowest yield from contour feathers (Table). Isolation procedure did not influence DNA quality (ratio A_{260}/A_{280}) in feathers (contour and pin) or eggshells. All samples had relatively good DNA quality (1.8–2.0) except for swabs and one eggshell, and these two samples had a lower DNA quality (0.610–1.639).

The primer set 2550F and 2718R was efficient in heron sex identification for both extraction protocols (Figure 1). In subsequent sex-typing of ringed birds, we used the modified standard protocol for DNA extraction. Results of molecular sexing based on DNA obtained from pin feathers of Grey Egret and Purple Heron nestlings are presented in Figure 2. PCR bands were clearly visible in all tested samples, showing a size of 450 bp for the W-chromosome and 600 bp for the Z-chromosome. All the Purple Heron nestlings were males, whereas nestlings of the Great Egret were females.

For the Grey Heron, amplification of the *CHD* gene was successful only for three eggshells; it indicated two males and one female. Of three swab samples taken from the aforementioned eggshells, only one sample was successfully amplified, showing the *CHD-Z* fragment indicating a male. Amplification failed for the other seven collected samples even though they did not have a lower DNA concentration (19–23 ng/μL) than those with successful amplification (23–50 ng/μL).

4. Discussion

We optimized the procedure for DNA isolation based on the quantity and quality of isolated DNA from contour and pin feathers as well as eggshells of three species of the heron family: the Great Egret, Grey Heron, and Purple Heron. We showed that DNA yield obtained with the modified standard protocol was higher compared to the commercially available kit. In the study by Harvey et al. (2006), the same commercial kit was used for DNA extraction from the feathers of the Black-capped Chickadee (*Poecile atricapilla*). Obtained DNA yield (1.16 ± 0.72 ng/μL) was lower compared to our results (6–15 ng/μL), while in the study by Horvath et al. (2006), the authors obtained a higher DNA yield (92.19 ± 76.8 ng/μL) from the molted feathers of Spanish Imperial Eagles. De Volo et al. (2008) isolated DNA by using the standard protocol from molted feathers of free-ranging Goshawks. Their results showed that yield differed between feather types (tail, secondary, primary feather, alula, and covert), with the highest yield

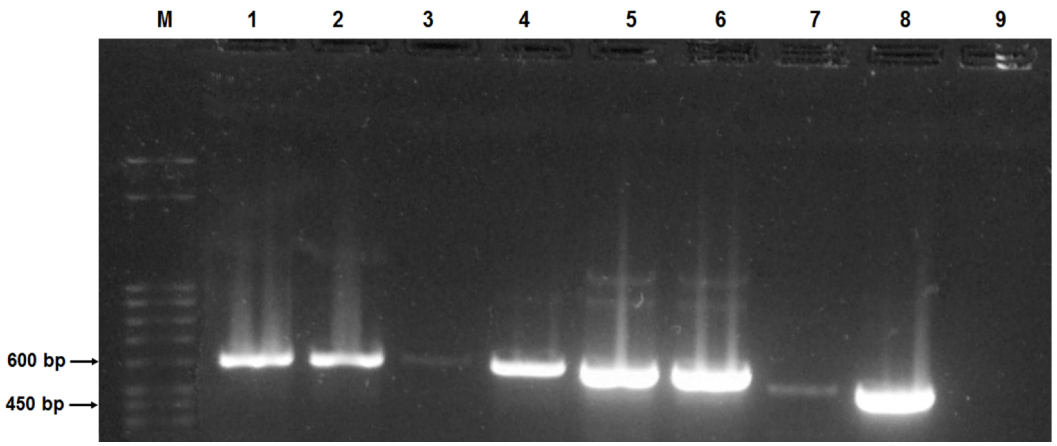

Figure 1. DNA extraction with two different protocols from different noninvasive samples. Lines 1, 3, 5, and 7: DNA extraction with commercial kit; Lines 2, 4, 6, and 8: DNA extracted with modified standard protocol. Lines 1–2: eggshells (Grey Heron); lines 3–4: eggshell swabs (Grey Heron); lines 5–6: pin feathers (Purple Heron); lines 7–8: contour feathers (Great Egret); 9: negative control; M: molecular marker.

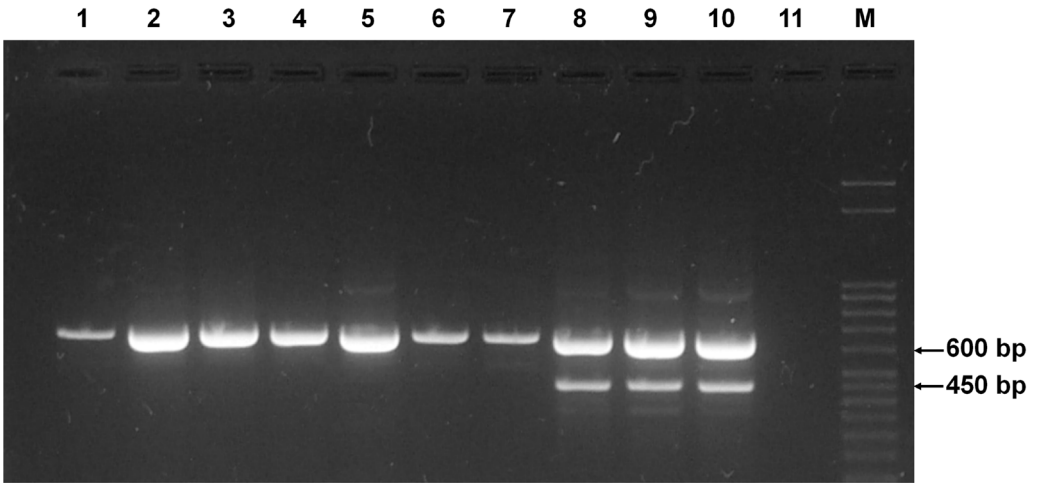

Figure 2. Heron sex-typing with 2550F/2718R primers. Lines 1–7: Purple Heron nestlings, males (600 bp for Z chromosome); 8–10: Great Egret nestlings, females (600 bp for Z chromosome and 450 bp for W chromosome); 11: negative control; M: molecular marker.

obtained from tail feathers (mean value of 24.6 ng/μL). Hogan et al. (2007) also used the standard protocol for DNA extraction, but the authors did not report yields and the quality of the DNA was assessed by PCR amplification. The variations in DNA yield between different studies may be the result of differing size and type, as well as the condition of the feathers.

Our results showed that the eggshells provided less DNA compared to pin feathers, but this DNA had better purity, possibly indicating presence of proteins in the sample. Bush et al. (2005) reported that DNA yield from eggshells was 39 ng/μL, while in the study by Trimbos et al. (2009), DNA yield was in a range from 32.7 to 543.68 ng/μL when the same commercial kit was used. This could be explained by differences in the size of the vascularized membrane initially used for DNA isolation, the number of blood vessels present in the membrane, and the degree of sample degradation due to environmental exposure. In the study by Martín-Gálvez et al. (2011), the authors reported lower DNA yield from eggshell swabs (mean ± SE: 8.38 ± 0.61 ng/μL) compared to our isolation with the standard protocol (21.5 ± 5.7 ng/μL), but they used Chelex-based DNA isolation. Our results showed that DNA yield from contour feathers was lower in comparison with that from eggshells (Table). However, it is important to note that the amount of DNA obtained from contour feathers was sufficient for sex determination. Previous studies have shown that the condition of feathers was a critical factor in determining successful PCR amplification regardless of the feather type (down, semiplume, and contour). DNA degradation is influenced by unfavorable weather conditions and therefore affects DNA quality and concentration (Bello et al., 2001; Horváth et al., 2005; De Volo et al., 2008; Hogan et al., 2008).

Many bird species, including herons, are sexually monomorphic. Therefore, reliable sex determination in the field is very difficult and requires extensive experience with visual sex identification (Morinha et al., 2012). In young birds, sex is almost impossible to determine without using molecular methods (Dubiec and Zagalska-Neubauer, 2006; Wang et al., 2011). The findings of our study are congruent with other studies; they indicate that molted feathers are a good source of DNA for molecular sex identification and should thus be collected during ringing events (Bello et al., 2001; Horváth et al., 2005; Harvey et al., 2006). While the DNA obtained from feathers is usually of lower quality and concentration than DNA obtained from blood (Horváth et al., 2005; Hogan et al., 2008), the quality of feather DNA is sufficient for reliable sex identification. Furthermore, feather samples are easier to collect and feather collection is less stressful for birds than blood collection. When ringing efforts and research are focused on vulnerable populations, as in our study, it is better to collect freshly plucked pin feathers from nestlings than molted feathers and eggshells. This approach enables researchers to link an individual's sex with the tag serial number. This association of the bird's sex with its identity can be very useful for future monitoring efforts that aim to assess population status and predict population growth.

The findings of the present study indicated that eggshells can also serve as adequate sources of DNA. However, it is important to be aware of the possibility of cross-contamination with parental and sibling DNA when eggshells are used (Strausberger and Ashley, 2001; Schmaltz et al., 2006). Both females and males of the Grey Heron take part in the incubation of eggs (Hancock and Kushlan, 1984). During incubation, female and/or male cells may adhere to the eggshell surface and lead to the

detection of parental DNA on the surface of the eggshell (Schmaltz et al., 2006; Trimbos et al., 2009). In the present study, we detected contamination in only one eggshell, but to determine the exact source of DNA (sibling, paternal, or maternal) found on the external surface of the eggshell, additional genotyping analysis should be performed.

The primers used in this study, 2550F and 2718R (Fridolfsson and Ellegren, 1999), proved to be successful in amplification of the *CHD* gene in the studied heron species. The size of the PCR fragments was in concordance with previous studies reporting avian *CHD* loci (Ellegren, 1996). We determined that all examined nestlings of the Great Egret were females and all nestlings of the Purple Heron were males (Figure 2). It is important to note that the sample sizes for this study were selected to facilitate evaluation of DNA extraction procedures, and not for the assessment of sex ratios in these populations. Due to the small sample sizes, the sex biases observed in our samples are unlikely to be representative of the respective populations.

In conclusion, our study showed that the standard isolation protocol was more effective (i.e. DNA concentration was higher) than the commercial kit. The results also suggest that there is a need for preliminary testing of different isolation protocols and techniques at the start of molecular sex-typing: the suitability of the isolation protocols may be taxon-specific. The data collected in this study covered only two nests in the colony by type. The sample sizes were too small to estimate population sex ratios, which could significantly differ in adult populations.

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