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MOLECULAR SEX DETERMINATION OF 20 BIRD SPECIES PROTECTED IN THE REPUBLIC OF SERBIA

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Considering that more than 50% of bird species are monomorphic, the identification of gender based on phenotypic characteristics is extremely difficult. The aim of this study is sex determination in species inhabiting the Republic of Serbia, all under the state protection and declared by IUCN as endangered. DNA was isolated from feathers. Sex determination was based on sex-specific CHD gene amplified by 2550F/2718R primer set. Sexing gave good results in all 91 samples from 20 species including 6 species where molecular sexing has not previously been successful.

Key words: 2550F/2718R, CHD gene, molecular sexing, protected birds

INTRODUCTION

With intensive development of industry and decreased ecological consciousness of human society the number of bird species that are extinct or on the verge of extinction is increasing. Numerous bird protection programs involve intensive breeding of birds, and imply that the sex of individuals is accurately identified (Ito *et al*., 2003). In many bird species it is necessary to have a defined number of individuals and a specific ratio between opposite sexes within the same breeding space. In zoological gardens and breeding centres large numbers of birds are bred and traded, making sex determination extremely important (Lens *et al*., 1998).

Having in mind that adults are monomorphic in a majority of bird species (Griffiths *et al*., 1998), sexing based on their external morphology is impossible. Moreover, even in dimorphic species sex determination is problematic in nestlings (Kahn *et al*., 1998).

Traditional methods of avian sex determination are based on the observation of sex-specific behaviour and comparison of different morphological entities (Tella and Torre, 1993; Baker and Piersma, 1999; Jodice *et al*., 2000; Mendenhall *et al*., 2010). Alternatives are aggressive surgical methods (Griffiths and Phil, 2000) and ultrasonography (Hildebrandt *et al*., 1995), which can be difficult due to the presence of air sacks (Jensen and Durrant, 2006).

Accordingly, a number of recent studies have focused on the development of efficient molecular methods for sex identification, which are gaining undivided attention as an aid in research and conservation of many bird species (Cerit and Avanus, 2007). Most reliable sex determination results are obtained by analysis of sex-specific Chromo Helicase DNA-binding (CHD) gene polymorphisms.

Being functional part of DNA and having evolved very slowly, the CHD gene is highly conserved even among distant species. Ellegren (1996) suggested that, due to its high degree of conservation, the CHD gene can serve as an almost universal tag for the determination of sex in birds, with the exception of ratites. PCR amplification of the CHD gene with the set of primers used in this study produces a single Z-band in male and two bands (Z and W) in female birds. This allows for developing a simple, cost effective and non-agressive method for determining the sex of most bird species including endangered and species protected under the law.

According to BirdLife International (2012) within the territory of Republic of Serbia 35 Important Bird Areas (IBAs) are identified, covering a total area of 766 960 ha. Nine globally threatened and a total of 320 bird species listed on the IUCN red list populate these areas.

The aim of this work was to asses usefulness of a simple, universal and non-agressive sexing method for endangered bird species, and particularly species protected under the Republic of Serbia Law on Nature Protection ("Sluzbeni glasnik Republike Srbije", no. 36/2009, 88/2010 and 91/2010 – corr.) and on the IUCN Red List of threatened species (International Union for Conservation of Nature - IUCN 2011).

MATERIALS AND METHODS

Sampling and DNA extraction

A total of 91 samples of 20 species representing 8 avian orders all protected under the Law of the Republic of Serbia were provided by The Zoological Garden of Belgrade (Table 1). Samples of *Gyps fulvus* were provided by the Bird of Prey Protection Fundation Belgrade. One to ten thoracic feathers were sampled from all species tested in this study by plucking from each bird.

DNA isolation

DNA was isolated from the feathers using the KAPA Express Extract kit (KAPA Biosystems, Cat. No. KK7152). Quills were cut into 2-5 mm long pieces and afterwards, DNA was extracted following the kit protocol. The incubation step of the protocol at 75°C was prolonged to 22 min. 50 μL of the obtained DNA isolate was added to 200 μL of 1xTE buffer (Serva, Cat. No. 39799.01). Ten μL of the obtained dilution of DNA isolate were used in the PCR reaction.

PCR amplification

The following set of primers was used for the amplification of the CHD gene:
2550F (5'-GTTACTGATTCGTCTACGAGA-3') and
2718R (5'-ATTGAAATGATCCAGTGCTTG-3') by Fridolfsson and Ellergen (1999).

Table 1. Birds sampled for sex determination in the study

Order	Species	Status by the The Law on Nature Protection*	Status by IUCN	Previous data	N°**
Podicipediformes	*Podiceps cristatus*	Strictly protected species	Least concerne	Vucicevic *et al*., 2012	3
Ciconiiformes	*Platalea leucorodia*	Strictly protected species	Least concerne	Lee *et al*., 2008, DP***	4
	Ciconia ciconia	Strictly protected species	Least concerne	Cortes *et al*., 1999, DP***	3
Anseriformes	*Anser fabalis*	Protected species	Least concerne	No previous data available	2
	Anser anser	Strictly protected species	Least concerne	No previous data available	4
	Branta canadensis	Protected species	Least concerne	Boutette *et al*., 2002	2
	Cygnus olor	Protected species	Least concerne	Ong and Vellayan, 2008	11
Falconiformes	*Gyps fulvus*	Strictly protected species	Least concerne	Dolonec and Sinko,2009	13
	Haliaeetus albicilla	Strictly protected species	Least concerne	Lee *et al*., 2008	6
	Milvus milvus	Strictly protected species	Near threatened	Vucicevic *et al*., 2012	2
	Neophron percnopterus	Strictly protected species	Endagered	Vucicevic *et al*., 2012	6
	Falco subbuteo	Strictly protected species	Least concerne	Ito *et al*., 2003	4
	Aquila heliaca	Strictly protected species	Vulnerable	Cortes *et al*., 1999, DP***	4
	Buteo buteo	Strictly protected species	Least concerne	No previous data available	3
Galliformes	*Perdix perdix*	Protected species	Least concerne	No previous data available	5
	Coturnix coturnix	Protected species	Least concerne	No previous data available	4
Columbiformes	*Columba livia*	Strictly protected species	Least concerne	Jensen *et al*., 2003	4
Passeriformes	*Corvus corax*	Protected species	Least concerne	Vucicevic *et al*., 2012	3
	Corvus frugilegus	Protected species	Least concerne	Vucicevic *et al*., 2012	5
Charadriiformes	*Scopolax rusticola*	Protected species	Least concerne	No previous data available	3

*Ministry of Environment, Mining and Spatial Planning, Republic of Serbia; **Number of samples; ***Different Primers

The amplification was carried out in 25 μL reaction volume containing 12.5 μL of KAPA2G Robust HotStart ReadyMix (Kapa Biosystems, PN KK7152) and 1.25 μL of each primer from 2550F/2718R primer set and 10 μL DNA sample.

The recommended thermal protocol of KAPA2G Robust HotStart ReadyMix was used: 3 min of initial denaturation at 95°C, followed by 45 cycles of denaturation (15 sec at 95°C), primer annealing (15 sec at 52°C), extension (15 sec at 72°C) and a final extension step at 72°C, which lasted 8 min.

Visualization of PCR products

The PCR products were visualised with UV light after staining the 2% agarose gel with ethidium bromide using commercial O'RangeRuler^{TM} 50bp DNA Ladder (Fermentas).

RESULTS

Sex determination by PCR amplification of the CHD gene was successful in all 91 samples from 20 species used in this study: *Anser anser*, *A. fabalis, Aquila heliaca*, *Branta canadensis*, *Buteo buteo, Ciconia ciconia*, *Columba livia*, *Coturnix coturnix, Corvus corax*, *C. frugilegus*, *Cygnus olor*, *Falco subbuteo*, *Gyps fulvus*, *Haliaeetus albicilla*, *Milvus milvus*, *Neophron percnopterus*, *Perdix perdix, Platalea leucorodia*, *Podiceps cristatus, Scolopax rusticola* (Figure 1). In six of these species (*Anser anser, A. fabalis, Buteo buteo, Perdix perdix, Coturnix coturnix* and *Scopolax rusticola*) molecular sexing was successful for the first time.

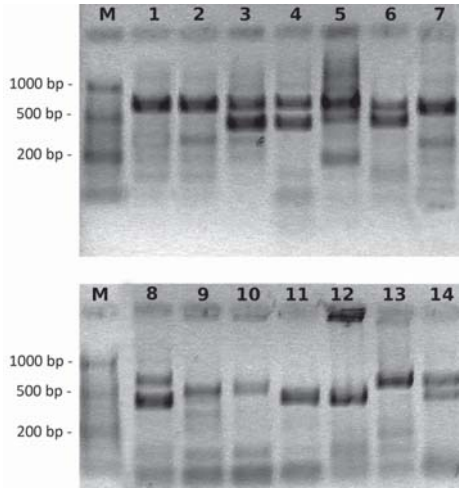

Figure 1. Ethidium bromide stained agarose gels showing sex determination in different avian species using PCR protocol with 2550F/2718R set of primers: M – Ladder, 1 – *Podiceps cristatus* (♂), 2 – *Neophron percnopterus* (♂), 3 – *N. percnopterus* (♀), 4 – *Milvus milvus* (♀), 5 – *Aquila heliaca* (♀), 6 – *Corvus frugiliferus* (♀), 7 – *Gyps fulvus* (♂), 8 – *Aquila haeliaca* (♀), 9 – *Cygnus olor* (♂), 10 – *Anser anser* (♂), 11 – *Cygnus olor* (♀), 12 – *Anser anser* (♀), 13 – *Ciconia ciconia* (♂), 14 – *C. ciconia* (♀), M – Ladder

In samples originating from female birds we were able to visualize two bands on agarose gel, sized around 400 and 650 bp. Alternatively, due to preferential amplification of the shorter PCR product, only one band is visualized at around 400 bp (CHD-W). This was the case in some samples from *Anser anser* and *Cygnus olor* (Figure 1). In male birds only one band is visualized at approximately 650 bp (CHD-Z). These intron sizes are in accordance with previously published data (Ong and Vellayan, 2008).

DISCUSSION

The method used in this study gave good results with species belonging to orders Podicipediformes, Ciconiiformes, Anseriformes, Falconiformes, Galliformes, Columbiformes, Passeriformes, Charadriiformes including six species (*Anser anser, A. fabalis, Buteo buteo, Coturnix coturnix*, *Perdix perdix,* and *Scolopax rusticola*) with no previous data available on molecular sexing. In Platalea and also Accipitridae and Anatidae species previous attempts at molecular sexing gave ambiguous results (Wang *et al*., 2007; Ong and Vellayan, 2008). Also a larger number of samples was included for species where the protocol by Vucicevic *et al*. (2012) was previously employed, confirming the reliability and robustness of the method.

All species tested in this study are important for preservation of biodiversity of the particular geographical area they populate and are protected under the Law of Republic of Serbia. Additionaly, sampling method and DNA extraction from feathers used in this study was employed in order to keep the sexing method in accordance with welfare considerations.

As birds are very sensitive to stress two major aspects are to be taken into regard when determining bird sex, especially when dealing with birds in the wild. One is that stress level to which the birds are exposed needs to be brought to a minimum, so handling and sampling of material need to be as non-aggressive as possible. For molecular methods of sex determination the most common samples collected are blood, feathers or stool. Blood sampling implies rough handling and is overall a very stressful event to birds and as such carries a high risk level. Sampling of feathers and feces is most desirable as it is in consistence with welfare, as no physical contact with the animal is necessary (Jensen *et al*., 2003). The other is that the method needs to be as accurate and reliable as possible. In this study we opted for the methods that would carry the lowest risk and stress level for the bird, while maintaining accuracy of the method and simplicity for the person performing sampling of feathers.

There are several initiatives on preservation of biodiversity in Europe or Balkan area that include Republic of Serbia aimed at, among other things, the preservation and reintroduction of wild populations of many endangered bird species populating this area. *Haliaeetus albicilla* monitoring program in Western Serbia is implemented since 2003 by the Center for Multidisciplinary Studies (CMS) and Birds of Prey Protection Fund (Skoric *et al*., 2007), The White-tailed Sea Eagle action plan (Probst and Gaborik, 2011). Furthermore, Eurasian Spoonbill (*Platalea leucorodia*) colony on Perleska bara is protected, since this

site is within the "Stari Begej – Carska bara" special nature reserve and Ramsar site (Tucakov, 2004).

Although the legislation in Serbia lists the protected species, there is no status classification, but more importantly penalties for killing protected birds are far from the level of European countries (Probst and Gaborik, 2011). This proves that there is a lot of work to be done in order to avoid further decline in wild bird populations. Obviously, protection of wildlife in Serbia has an impact on wildlife in other geographical areas.

To conclude, the method for determining sex in birds presented in this study is reliable, economical, fast, simple and does not include aggressive sampling for DNA extraction, a fact highly important when dealing with bird species that are endangered. The results presented could have an important impact on many programmes for the protection and reintroduction of endangered bird species, thus allowing for preservation and enrichment of biodiversity in the Republic of Serbia. Due to the conservation of the CHD gene this method has the potential to be expanded to cover most bird species, including protected and endangered, which is a subject of further research.

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We declare that the experiment complies with the current laws of Serbia, where it was performed.

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MOLEKULARNA DETERMINACIJA POLA KOD DVADESET VRSTA PTICA ZAŠTIĆENIH U SRBIJI

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SADRŽAJ

Uzimajući u obzir da je više od 50% vrsta ptica monomorfno, identifikacija pola zasnovana na fenotipskim karakteristikama je izuzetno teška. Cilj ovog rada jeste determinacija pola kod vrsta koje naseljavaju Republiku Srbiju a pritom su pod zaštitom države i vode se kao ugrožene od strane IUCN-a. DNK je izolovana iz perja. Determinacija pola se bazirala na polno specifičnom CHD genu, amplifikovanom 2550F/2718R setom prajmera. Određivanje pola je dalo dobre rezultate kod svih 91 uzoraka 20 vrsta uključujući i 6 vrsta gde molekularno određivanje pola prethodno nije bilo uspešno.