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ANALYSIS OF THE CHD GENE FOR SEX DETERMINATION OF PROTECTED BIRD SPECIES

Vučićević, M.[1], Stevanović, J.[1], Simeunović, P.[1], Vučićević, I.[1], Đelić, N.[1], Stanimirović, Z.[1]

Summary: Considering that more than 50% of bird species are monomorphic, the sex identification based on phenotypic characteristics is almost impossible. Sex determination in birds is a very important part of program for the protection and conservation of endangered bird species that are under state protection. Implementation of molecular methods enables reliable, rapid and economical determination of sex in most bird species. The aim of this study was determination of sex in some bird species inhabited in Serbia that are under the state protection. DNA was isolated from feathers, so that sampling did not harm the physical integrity of individuals. Amplification of high-conserving chromo-helicase-DNA binding regions was performed with primer set 2550F/2718R. Determination was successful in 10 bird species. This simple procedure can be important for genetic resources conservation programs.

Key words: CHD gene; protected bird species; sex determination;

Introduction

Hunting is a branch of the economy with potentially great importance for Serbia. In our country there are 321 hunting grounds with a total area of nearly 9 million acres [1]. There are 25 bird species grown in the hunting areas in Serbia. According to the Law On Nature Protection [2] there are 21 species of hunting game birds under protection in our country.

Bird populations may be limited by a variety of factors, including food supply, territorial space, nest sites, predation and parasites. There are two particularly crucial moments in avian life cycles where one or more of these factors can limit population densities: climate conditions and breeding periods [3]. Particularly during breeding periods it is important to have a proper male to female ratio. Many avian species are sexually monomorphic. Reproduction is possible by keeping males and females together in avian breeding. Recently it has become apparent that despite the difficulties, sex identification of birds is an essential part of *ex situ* conservation breeding programs for endangered species including hunting ones, and the sex of individuals is an integral component of information required for research concerning ecology, behavior, genetics, and conservation biology [4].

More than 50% of bird species are monomorphic [5], making their sexing based on external morphology impossible. One of the biggest difficulties of sexing avian species stems from the absence of external sex organs in birds. Moreover, even in dimorphic species sex determination is problematic in chicks [6]. Among protected species there is a need for sex determination both in monomorphic and in nestlings of dimorphic birds.

Traditional methods of avian sex determination are based on the observation and study of sex-specific behaviour and the comparison of different morphological entities such as weight and tail length, size and plumage colouration and head plus bill lenght [7] [8] [9] [10]. Cloacoscopia was a highly implied method [11], but requires well-trained stuff. Even experts can misidentify sex of the monomorphic birds. Besides, with exception of ducks and swans, in most of the birds cloaca is morphologically identical in males and females.

Surgical methods (laparoscopy and laparotomy), which enable direct observation of gonads, although successful in most cases, are aggressive [12] and even lethal to the birds [13]. Ultrasonography may also be used in sex identification in many bird species [14], but can be very difficult due to the presence of air sacks [15].

Cytogenetical sex identification is based on differences in morphology of sex chromosomes. In birds, unlike in mammals, females are the heterogametic sex, i.e. have 2 different sex chromosomes (ZW), while males are the homogametic sex (ZZ). However there are many difficulties involved in this method as blood cells do not give satisfactory results and bird cells have a large number of chromosomes, from 40 up to 126 depending on the species [16]. Recently, simpler, cheaper and more effective techniques have been developed, so cytological sex identification is only very rarely used [17].

DNA based techniques are more reliable than the others. Most of the DNA techniques are based on polymerase chain reaction (PCR) method. In the first decades of use, DNA research and techniques were labour intensive, expensive, insecure and slow. However, with the development of the new methods, DNA research became simpler, cheaper, faster and safe. The invention of polymerase chain reaction (PCR) revolutionised the genetic investigations. Certainly PCR has become one of the most important tools for molecular biologists [18]. Also, samples can be as

[1] Miloš Vučićević, teaching assistant, Jevrosima Stevanović, PhD, docent, Predrag Simeunović, teaching assistant, Ivana Vučićević, teaching assistant, Ninoslav Đelić, PhD, professor, Zoran Stanimirović, PhD, professor, Faculty of Veterinary Medicine, Belgrade
Corresponding author: Miloš Vučićević, Faculty of Veterinary Medicine, Blvd. Oslobodjenja 18, 11000 Belgrade, Serbia; E-mail: milosvucicevic@vet.bg.ac.rs; Phone +38162222012

small as a single feather or a small portion of feces and complete determination can be completed in around 4 hours without affecting the reliability of the test. Isolation of genomic DNA from feathers helps reduce handling-stress, eliminates unnecessary bleeding, and minimizes the possibility of infection without compromising the accuracy and reliability of the results.

Chromosome examination has developed over time from the cytogenetic to a molecular level. Most reliable sex determination results are obtained by analysis of sex-specific Chromo Helicase DNA-binding gene (CHD gene) polymorphisms. Owing to the differences in size of CHD W and CHD Z genes, PCR amplification using CHD specific primers produces a single band in male birds and two bands in female.

The aim of this work was to test one universal and non-agressive sexing method for endangered species, particularly those species protected under the Law of the Republic of Serbia [19].

Material and Method

Sampling and DNA extraction

In this study we sexed 30 individuals from 10 avian species: *Podiceps cristatus, Platalea leucorodia, Ciconia ciconia, Anser fabalis, Cygnus olor, Haliaetus albicilla, Falco subbuteo, Aquila heliaca, Buteo buteo* and *Corvus frugilegus.* All these species are protected under the Law of the Republic of Serbia. One thoracic feather was plucked from each bird.

DNA was isolated from the feathers using the KAPA Express Extract kit (KAPA Biosystems, cat No KK7103). 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^{o}C was prolonged to 20 min. 50 µL of the obtained DNA isolate was added to 200 µL of TE buffer. 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) [20].

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

The recommended thermal protocol of KAPA2G Robust HotStart ReadyMix was used: 3 min of initial denaturation at 95^{o}C, followed by 45 cycles of denaturation (15 sec at 95^{o}C), primer annealing (15 sec at 52^{o}C), extension (15 sec at 72^{o}C) and a final extension step at 72^{o}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. A commercial O'RangeRuler™ 50bp DNA Ladder (Fermentas) was used as size marker in order to judge whether Z- and W-bands were obtained.

Results and Discussion

Protocol proved to work well with all tested samples (Figure 1). Gender was determined in 10 bird species of the orders: Podicipediformes, Ciconiformes, Anseriformes, Falconiformes and Passeriformes. There are no previously published data for two species (*Anser fabalis* and *Buteo buteo*). In Platalea and also Accipiteridae and Anatidae species previous attempts gave ambiguous results [21] [22]. All species tested in this study are important for preservation of biodiversity of the particular geographical area they populate and protected under the Law of the Republic of Serbia. Under conditions listed in Material and Methods section, primer set 2550F/2718R amplified distinct bands in 2% agarose gel. In samples originating from female birds we were able to visualize two amplified bands in agarose gel, sized around 400 and 650bp, and in male birds only one band is visualized at approximately 650bp (CHD-Z) which is in accordance with previously published data for related species [23].

The design of primers 2550F/2718R is such that W – fragment is the smaller one, enabling sex determination in birds even if only one fragment is visualized due to the size differences between the bands [24]. In our samples, this occurred in *Falco subbuteo* and had been previously described in Accipitridae, Anatidae, Falconidae, Gruidae, and Scolopacidae [25].

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 programmes are aimed at preservation of various species through intensive breeding of birds, and imply that the sex of individuals is accurately identified.

Certain bird species inhabit the whole northern hemisphere and global conservation status of most of these species is not critical [26]. However, at regional, national and local level, many hunting bird populations are decreasing and

threatened. The most important factors affecting the population size of individual species of game birds are logging,

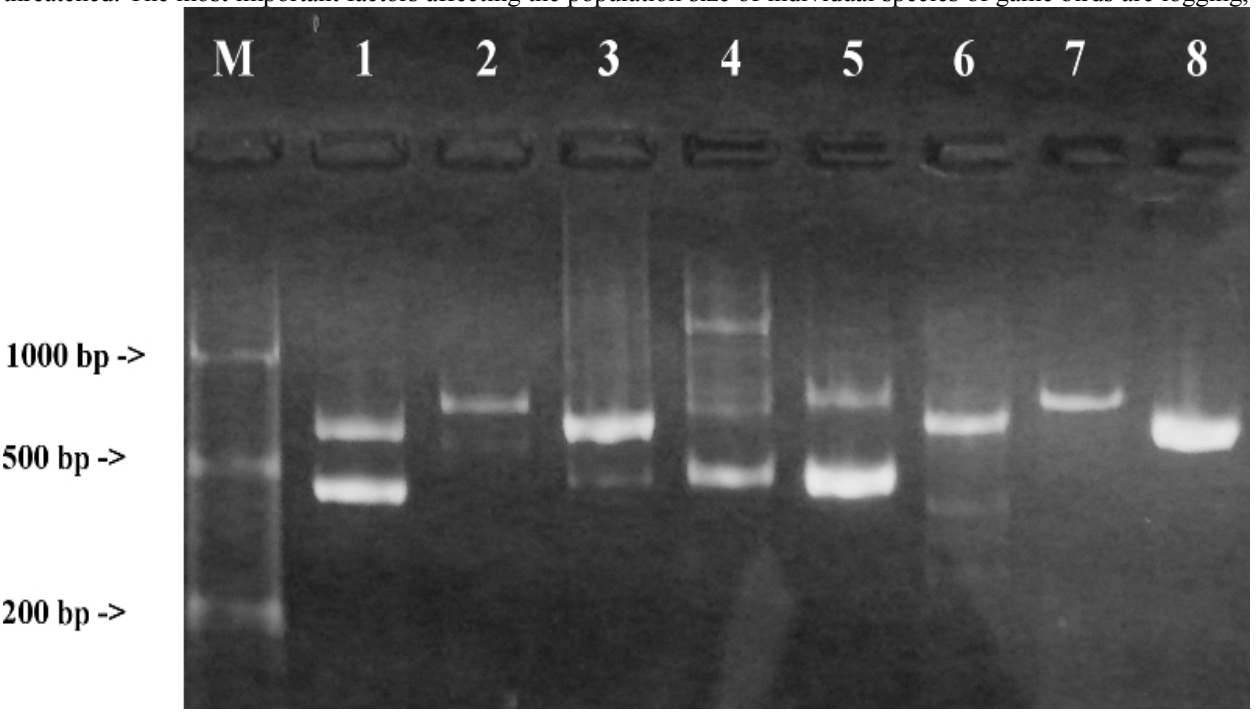

Figure. 1 Ethidium bromide stained agarose gel showing sex determination in different protected bird species with 2550F/2718R set of primers
M – Ladder, 1 – *Corvus frugilegus* (♀), 2 – *Buteo buteo* (♂), 3 – *Aquila heliaca* (♀), 4 – *Falco subbuteo* (♀), 5 – *Haliaeetus albicilla* (♀), 6 – *Cygnus olor* (♂), 7 – *Anser fabalis* (♂), 8 – *Ciconia ciconia* (♂), M – Ladder

disturbing of birds in the nest, use of chemicals in agriculture and forestry, illegal hunting, forest fires, degradation of habitat, predation, environmental pollution and climate changes [27].
There are several initiatives on preservation of biodiversity in Europe or Balkan area that include the Republic of Serbia aimed at, among other things, the preservation and reintroduction of wild populations of many endangered bird species populating this area [28] [29].
Although the legislation in Serbia lists species protected, there is no status classification, but more importantly penalties for killing protected birds are far from the level of European countries. This proves that there is a lot of work to be done in order to avoid further decline in wild bird populations. Indeed, protection of game birds in Serbia is of great importance for wildlife conservation of broader scale the more so that Serbia represents one of six biodiversity centres [30] [31].

Conclusion

To conclude, the method for determining sex in birds presented in this study is a 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 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 potential to be expanded to cover most bird species [32], including protected and endangered, which should be a subject of further research.

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

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