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SEX DETERMINATION IN GAME BIRDS MANAGEMENT

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Summary: Sex determination in birds is important to ensure good game bird management. Considering that nestlings of almost all bird species are monomorphic the sex identification based on phenotypic characteristics is almost impossible. The timely formation of bird pairs and flocks is the base of good management and for this reason, it's necessary to know the bird sex in hunting area. The aim of this work was to develop a suitable method for rapid sex determination based on molecular markers (CHDZ, CHDW) which could be used in game birds. DNA was isolated from feathers and the amplification of the CHD gene was performed using 2550F/2718R set of PCR primer. Sex was determined using CHD gene in all tested samples of 7 game bird species. Method developed in this study could be used for establishing of sustainable management of hunting bird species and for effectively sex determination in game birds.

Key words: CHD gene; game birds; sex determination; sex ratio

Introduction

Sex determination based upon phenotypic characteristics in adults of monomorphic and in nestlings of dimorphic species is very difficult if not impossible. Sufficient knowledge of the age and sex structure of the annual harvest is essential to the proper management of a game population. Ornithologists generally agree that there are variations in the sex ratio among the wild birds' species [1]. There is a relationship between sex ratio and demography, behaviour and population persistence. Behaviours such as polygyny, extra-pair copulation, mate-guarding and co-operative breeding have frequently been linked to sex ratio, and indeed may have evolved in response to them [2]. The cause of this variation other than expected 1:1 found in most mammalian species has not been explained yet [3] [4]. Skews in sex ratio have implications for ecology, monitoring and conservation [5].

In breeding centers a great number of birds are bred but also traded with, for which purposes sex determination is of extreme importance. 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. This is done in order to maintain physiological behavior of animals and prevent behavioral disorders and pathological behaviors which can frequently lead to exitus in birds. When individuals of one sex are found in excess, they are not able to fight for territory. These animals remain alone and they are called "floating" individuals [6] [7]. These floaters are often young, inexperienced individuals [8]. Unambiguous identification of individual sex is necessary to enforce legislation to protect all endangered bird species.

Unambiguous offsprings' gender determination is of significance in many bird species. Gender determination criteria may be particularly important for species in which males and females differ in size because an undetected bias in sex ratio of a sample may in turn bias the results of investigations into growth, survivorship, foraging behavior and other aspects of a species' biology [9].

In some bird species a certain number of males and females is released into the hunting areas and successful reproduction can be achieved only at that specified ratio. Among monogamous species important are Grey Partridge (*Perdix perdix*), Rock Partridge (*Alectoris graeca*) Common Quail (*Coturnix coturnix*), Hazel Grouse (*Tetrastes bonasia*), Eurasian Woodcock (*Scolopax rusticola*), European Turtle Dove (*Streptopelia turtur*), Domestic Goose (*Anser anser*), Whooper Swan (*Cygnus cygnus*), Eurasian Collared Dove (*Streptopelia decaocto*) and Northern Goshawk (*Accipiter gentilis*) and the most common polygamous species are Common Pheasant (*Phasianus colchicus*), Western Capercaillie (*Tetrao urogallus*) and Great Bustard (*Otis tarda*). Forming couples and flocks is desirable to do as early as possible, therefore it is necessary to determine the sex immediately after hatching. It is equally important not to harm offsprings during the tissue sampling. Besides, in some countries during the hunting season is allowed to hunt only males or only females.

Having all above mentioned in mind, the purpose of this study was to develop a suitable method for rapid sex determination based on molecular markers which could be used in hunting bird species. For molecular methods of sex determination most common samples collected are blood, feathers, bucal swab or stool. Blood sampling implies rough handling and is overall a very stressful event to birds and as such has a high risk level. Sampling of feathers and feces is most desirable because no physical contact with the animal is necessary and it does not violate physical

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and psychological integrity of the animals. It is critically important to minimize handling-induced stress when sexing chicks, fragile individuals, or endangered species [10]. In this study we used the feathers as a sample for sex determination.

Material and Method

Sampling and DNA extraction

The sex identification test involved 35 individuals from 7 avian species: Greylag Goose (*Anser anser*), Bean Goose (*Anser tabalis*), Rook (*Corvus frugilegus*), Common Quail (*Coturnix coturnix*), Grey Partridge (*Perdix perdix*), Eurasian Woodcock (*Scolopax rusticola*) and Common Pheasant (*Phasianus colchicus*). For each mentioned species, sex was determined in 5 animals. One thoracic feather was plucked from each bird and placed into marked envelope.

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 manufacturer's recommendations. 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) [11].

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

Developed method proved to work well for all sampled species (Figure 1). DNA isolated from just one feather was sufficient for amplification sex specific CHD genes and for sex determination. Under conditions listed in Material and Methods section, primer set 2550F/2718R amplified distinct bands in 2% agarose gel. The overall sexing of all bird species done with the primer set 2550F/2718R consistently showed male birds being represented by a single band fragment (CHD-Z) visualised at approximately 650 bp, whereas females are represented by two amplified bands, sized around 400 and 650bp (CHD-Z and CHD-W).

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 [12]. In our samples, this occurred in *Anser anser* and *Phasianus colchicus* and had been previously described in Accipitridae, Anatidae, Falconidae, Gruidae, and Scolopacidae [13].

First methods of sex determination were based upon observation and study of reproductive behavior such as parental behavior as the most reliable one. More reliable methods are based on comparing different morphological entities, such as weight and tail length [14], size and plumage colouration [15], sex specific behavior and head plus bill lenght [16].

The difficulty of sexing avian species stems from the absence of external sex organs in birds. Cloacoscopia was a highly implied method [17], 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 of sex determination are laparoscopy and laparotomy. Both of these methods allow for direct observation of gonads [18]. Laparoscopy requires anaesthesia by a veterinary surgeon. Birds need post-operative intensive care after the surgery. Laparotomy is done by placing an incision on the left side of the abdomen between the last two ribs. The incision must be big enough to allow placing the metal instrument in the abdominal cavity that seperates parts of gastro-intestinal tract and alows visualization of the gonads. The gonads are placed along the spine immediately below the thoracic cavity. In males we find two testicals while in females only one ovarium is found in most cases. A clearer visualisation of gonads is achieved by laparoscopy, utilising fiber optical cables but for this procedure it is also necessary to perform a small incision on the abdomen. Problems that occur with these two methods are atrophycal gonads in non breeding individuals, and small gonads in speices that are morphologicaly small in size as well as in offsprings of all species but also the fact that the examination can be harmful and even lethal to the birds [19].

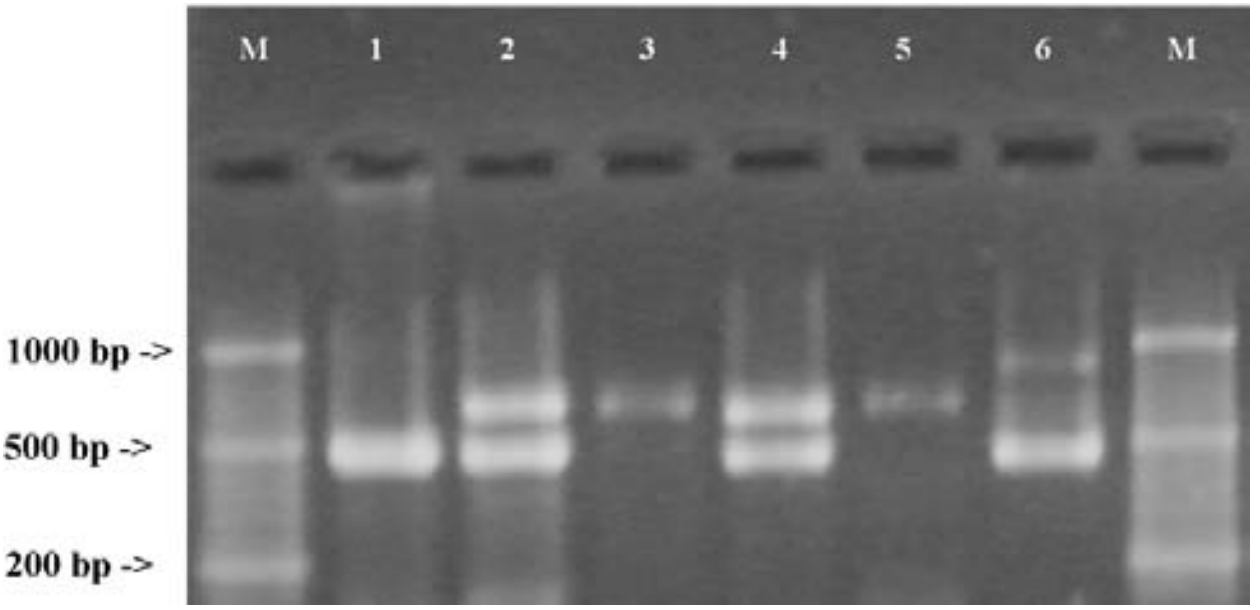

Figure 1: Ethidium bromide stained agarose gels showing sex determination in different game bird species with 2550F/2718R set of primers
M – Ladder, 1 – *Anser anser* (♀), 2 – *Corvus frugilegus* (♀), 3 – *Perdix perdix* (♂), 4 – *Coturnix coturnix* (♀), 5 – *Scolopax rusticola* (♂), 6 – *Phasianus colchicus* (♀), M – Ladder

Cytogenetic could be of use in sex determination of birds. In birds males are homogametic (ZZ) and females are heterogametes (ZW). The method is based on difference in morphology of sex chromosomes. W chromosome has lost most of the genes during the evolution and is therefore reduced in size, while the Z chromosome is very conserved and larger than the W chromosome. However there are many difficulties involved in this method as blood cells give no satisfactory results and bird cells have a large number of chromosomes, from 40 up to 126 depending on the species [20]. Also most of the chromosomes of avian species are microchromosomes and it is difficult to count them accurately [21]. Although handling birds is a stressful application for them, they have to be handled twice for karyotyping; once for picking feathers to obtain newly grown ones and a second time for collection of the growing feather pulps. For these reasons karyotyping is not a preferable method for sex identification in birds.
Over years genetic sex determination has developed from the chromosome level to molecular level. Griffiths and Tiwari [22] discovered the CHD gene on W chromosome in 1995. A very closely related copy of this gene was soon after discovered also in the Z chromosome by Griffiths and Korn [23] in 1997. These two genes have been used for sex identification in a wide range of species. In mammals gender specific sequence is the Sry gene. In birds a structure homologous to the Sry gene does not exist, however highly conserved CHD1W/CHD1Z genes [24], EE0.6 [25], and Wpcki genes [26] can be used. These genes represent excellent markers for sex determination as they form a functional part of DNA and have evolved very slowly so they are highly conserved. CHD is surely the most significant among them because it can be used in almost all bird species, the exception being ratites [27]. Polymerase chain reaction (PCR) is a reliable, economical, fast and not that complicated method for determining sex in birds [28].
Sex determination methods evolved in two different directions. It is improtant to reduce stress level during sampling as much as possible. Equally important is to found a method that is most accurate and most reliable. Primary problem is the subjectivity of traditional methods so the result depends on the observer. Other disadvantages of these methods are uncertainty of results, necessity of violating the physical and psychological integrity of individuals, risk for bird or a person who performs sampling and determination, a slow response time and inability to determine sex at nestlings.
However, developments in molecular genetics have largely overcome this problem [29] [30]. Nowadays, samples can be as small as a single feather and assays can be completed in around 4 hours without affecting the reliability of the test. DNA extraction from feathers helps reduce handling-stress, eliminates unnecessary bleeding, and minimizes the chance of infection without compromising the accuracy and reliability of the results. For this reason, sex identification using molecular methods has proved to be a valuable tool in wildlife conservation in addition to behaviour studies and breeding programs [31].

Conclusions

The balance of the sex ratio in a small population is important in the conservation management of endangered and hunting bird species [32]. For the management and conservation of avian species, for the study of animal ecology, behaviour, population structure and life history, sex identification is necessary.

DNA based techniques are more reliable than traditional. Molecular techniques offer the advantage of a non-invasive sexing method and do not require anaesthesia or rough handling. Amplification of sexual dimorphic genes conserved in most avian species is the main advantage of CHD sequence and CHD gene can serve as an almost universal tag for the determination of sex in birds [33]. Method developed in this study facilitates and accelerates sex determination in game birds and contributes to the conservation management of hunting bird species.

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

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