Rapid sexing of selected *Galliformes* by polymerase chain reaction

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ABSTRACT: Vent sexing of one-day-old chicks in commercial hatcheries has long been common practice and can be highly accurate. However, there are circumstances when this technique is not applicable such as smaller breeds, non-domestic birds, or where is the necessity of precise sexing. In this study we present a simple and reliable method for fast gender determination in selected Galliformes for which phenotypic determination of sex is difficult until maturity. Four species were tested: two commercial species – chicken *(Gallus gallus)* and turkey *(Meleagris gallopavo)*, and two game birds – common pheasant *(Phasianus colchicus)* and wood grouse *(Tetraro urogallus).* DNA was tested with universal single-pair primers polymerase chain reaction (PCR) detecting W chromosome specific sequence yielding a single band of length specific for each species. The method was developed with regards to time consumption and cost-effectiveness giving results in less than two hours. The method may also be used for early sexing in commercial chicken and turkey flocks as well as sexing of smaller game birds flocks or for research laboratories when rapid sexing of selected Galliformes cells is required.

Keywords: sex determination; fowl; game fowl; polymerase chain reaction

Sexing one-day-old chicks can be usually accomplished by one of the two methods: (1) vent sexing or (2) feather sexing. Each method deals with some difficulties making its use in small flocks unsuitable. Vent sexing requires highly trained specialists to visually differentiate male from female genitalia. The difficulty in visually sexing Galliformes is due to the small size of external genitalia at hatching (1–2 mm in length and height), to subtle differences between male and female genital morphology and, also, to the genitalia location at the ventral part of the cloaca which is hidden under the dorsal lip. In addition, chickens' genitalia differ in shape within each sex (over fifteen different shapes are to be considered) (Nakayama, 1993). Therefore, relatively few people have experience with determining the sex of birds because of the difficult nature of the process and most of them are employed by large commercial hatcheries.

Feather sexing is based on differences in feather characteristics (length or colour) at hatching. This technique can be of major interest in the case of specific crosses between, for instance, a rapid-feathering males and slow-feathering females as sex specific phenotypes are expressed at hatching. However, most breeds of chickens do not have these feather sexing characteristics and feathering of both sexes appears identical (Stromberg, 1977). Conversely, the genetic sex of many game birds cannot be determined at hatching. Chickens are then raised all together until secondary sex characteristics become recognizable. In pre-cited situations

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Supported by the Czech Science Foundation (Grant No. 523/09/P590) and by the Ministry of Education, Youth and Sports of the Czech Republic (Project No. ME10104).

and considering the limitations of both venting and feather sexing, the most convenient method of sexing chickens in small flocks of chickens or game birds remains, up to date, to wait until one or the other sex clearly exhibits its secondary sex characteristics.

In addition to pre-cited techniques, molecular sexing of one-day-old chicks is a relatively simple and highly accurate technique which has been already extensively described (Trefil et al., 1999, 2002; Clinton et al., 2001; Granevitze et al., 2007; Rosenthal et al., 2010). It is usually based on W chromosome specific chromobox-helicase-DNA-binding gene (CHD-W) sequence, the first gene being isolated from W chromosome (Ellegren, 1996). This gene is well conserved between avian species and it has been shown that a single set of PCR primers can be used to sex chicks in most birds (Griffiths et al., 1996). These primers simultaneously amplify the homologous parts of CHD-W gene and of its homologue, CHD-Z gene, localized on Z chromosome (Griffiths and Korn, 1997). Once amplified, these genes can be revealed as two-band differing by their length on agarose gel. However, some species of birds yield two bands of similar length which makes sex differentiation difficult (Griffiths et al., 1998).

In the present study, we describe a simple, fast, one-band-yield, no DNA isolation alternative to the procedure described by Trefil et al. (2002) for molecular sexing of selected Galliformes. We also demonstrated its application in the chicken, turkey, pheasant and wood grouse.

MATERIAL AND METHODS

A specific W chromosome sequence was selected by comparative analysis between chicken W (NW_001488744.1) and Z chromosome with regards to coding regions located on W chromosome. Predicted sequences were tested using BLAST sequence analysis tool (Altschul et al., 1990) for their possible homology with the rest of the known chicken genome. Ten candidate primer pairs were drafted out and tested.

Tissue samples were collected from our experimental breeds (*Gallus gallus, Meleagris gallopavo, Phasianus colchicus*) and from the grouse conservation station (*Tetraro urogallus*, South Bohemia). A total of 59 adult birds (30 *Gallus gallus,* 15 *Meleagris gallopavo,* 10 *Phasianus colchicus,* and 4 *Tetraro urogallus*) along with a total of 89-day-old chicks of known sex (30 *Gallus gallus,* 45 *Meleagris gallopavo,* 10 *Phasianus colchicus,* and 4 *Tetraro urogallus*) were used. For each bird, at first a 10 µl blood sample was collected and then suspended in 90 µl citrate buffer solution to prevent coagulation. Along with blood, two feathers per bird were also collected and the lower part of their calamus (about 2 mm long at the distal end of the quill) isolated and placed into a 2 ml plastic tube. DNA from blood, further used as control, was also isolated using Dneasy kit (Qiagen GmbH, Hilden, Germany) according to the manufacture protocol. The initial preparation for PCR consisted in 10× Taq polymerase buffer, 10 nmol dNTP, 10 pmol of pre-tested primers and 0.5 U CombiTaq polymerase (all Top-Bio, s.r.o., Prague, Czech Republic). Bi-distilled water was added up to a final volume of 20 µl per tube. The whole lower calami were then added to each tube and the final preparations subjected to PCR.

Control PCRs were run in parallel using primers for the cytochrome b (CYTB) gene from mitochondrion (forward – 5’ GCCCCTCAGAATGATATTTG; reverse – 5’ CCATCCAACATCTCAGCATG) and then subjected to the same conditions as described above. PCR reaction was carried out using a thermal cycler (Biometra, Göttingen, Germany) adjusted at an initial step of 10 min at 94°C followed by 30 cycles of 30 s at 94°C, 20 s at 54°C, and 40 s at 72°C. The resulting products were visualized on a 1% agarose gel stained with SYBR green (Sigma-Aldrich, St. Louis, USA) and analyzed with a Kodak photoimaging system. Amplified bands were cut, purified using Qiagen purification kit (Qiagen GmbH, Hilden, Germany) and sequenced (Macrogen Corp., Amsterdam, the Netherlands). The final sequences were aligned using a CLC sequence viewer software (CLC bio, Aarhus, Denmark).

RESULTS AND DISCUSSION

Out of ten tested primer pairs only one pair showed required characteristics. Based on sequencing data, forward primer was further slightly modified to give stronger amplification in wider annealing range. Using primer pair (forward – 5’ GGGTGTAACATGAGAAGAAC; reverse – 5’ GCACAGATGGAGACAAAAGC) we could successfully determine the female sex of all the samples evaluated (Figure 1A). However, there

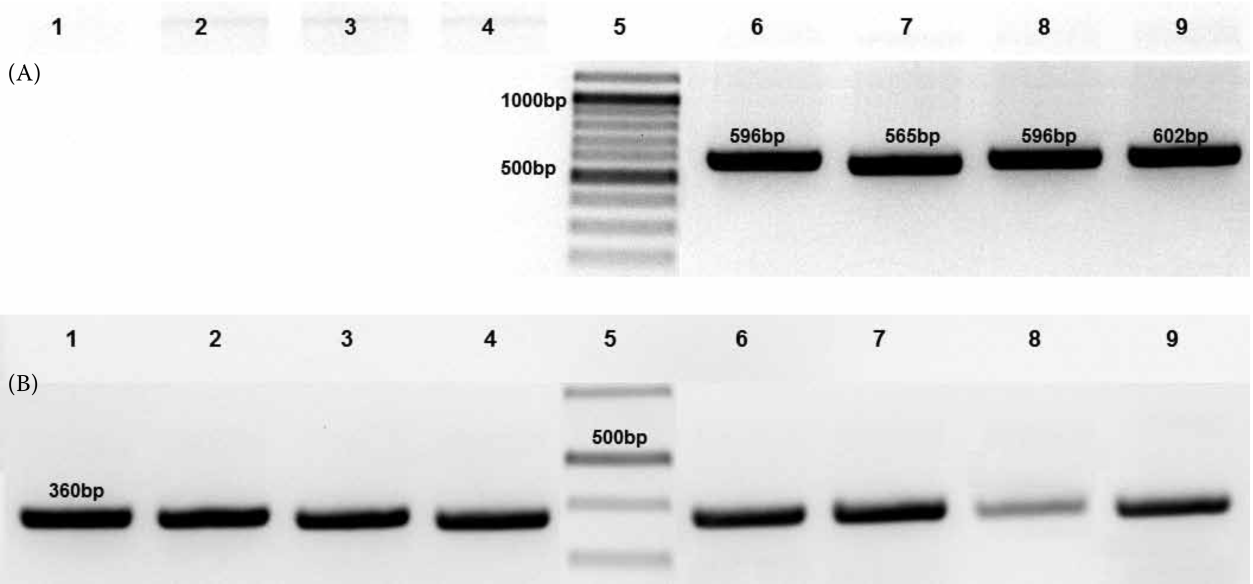

Figure 1. Comparison between sexes among the four studied species (A) and parallel control reaction for CYTB (B). From left to right: chicken, turkey, pheasant, grouse. Lines 1–4 = males, lines 6–9 = females

were subtle differences between the amplified sequences (Table 1). Sequencing and alignment of the obtained sequences show very high homology between all four species with noticeable long deletion of 33 base pairs in turkey fragments (Figure 2).

Interestingly, our method which does not require an initial step consisting in DNA isolation/purification can be performed directly from unprepared tissues. Thus, the quantity of DNA present in the epithelial cells located at the distal end of feathers appears sufficient to initiate PCR reaction. We have also tested adding the whole blood (1 µl of 10 × diluted blood in citrate buffer, data not shown) into reaction similarly to Rosenthal (2010) as an alternative to calami epithelia. Because hemoglobin interferes with polymerases (Al-Soud and Radstrom, 2001) specific blood inhibitors resistant to polymerase and buffer (usually with BSA) needed to be used (e.g. Phire Hot Start DNA Polymerase, Finnzymes Oy, Vantaa, Finland) and the reaction is still more prone to give false results.

The female genotype was revealed as a single band while the male genotype yielded no band. This is, indeed, a major difference to the previously described method (Griffiths et al., 1996) which, in our study, was successfully tested in 4 different species. However, this method is known to give false negative results when two amplified sequences are of a similar length. To avoid false negative results in our test, parallel control PCR reaction using the universal primers for CYTB gene from mitochondrion was performed. The primers used yielded a 360 bp long band regardless of sex or species thus confirming they underwent the appropriate reaction (Figure 1B). It therefore appears that the procedure used in the present study is of practical interest in most species of birds, providing a simple, reliable, and quick approach to determine sex in one-day-old chicks.

In addition to pre-cited applications, sex determination by molecular techniques can also be of higher interest when working with embryo cells from avian species. For example, a freshly ovipos-

Table 1. Detected fragments homology (%) and length (bp) of amplified fragments

Species	1	2	3	4	Length
Gallus gallus (1)	–	89	92	92	596
Meleagris gallopavo (2)	89	–	90	90	565
Phasianus colchicus (3)	92	90	–	93	596
Tetraro urogallus (4)	92	90	93	–	602

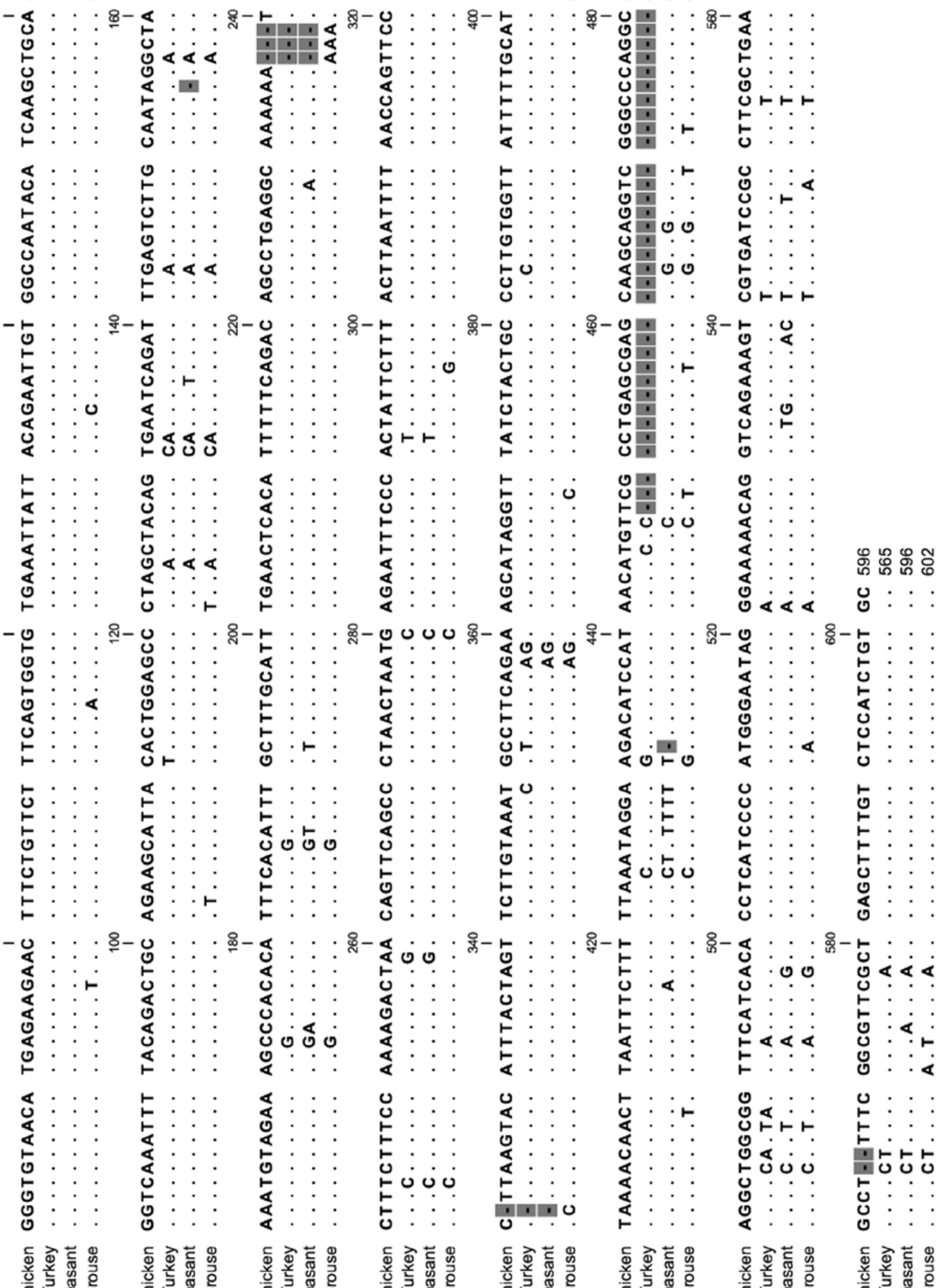

Figure 2. Alignment of four sequenced PCR fragments shows very high homology between four experimental species except for 33 bp deletion in turkey sample (position 444–476). The matching bases are displayed as dots. Deletions are displayed as hyphens with gray background

ited chicken egg already contains about 30 000 to 60 000 blastodermal cells (BCs) that have pluripotent characteristics. They are often manipulated with the aim of developing germ-line chimaeras and also transgenic birds. Primordial germ cells (PGCs), which are precursors of the germ cells, appear during the early embryonic stages in the germinal crescent through which they colonize the developing gonad (Ginsburg and Eyal-Giladi, 1986) after being vehicled into the bloodstream (Stage 10–12 according to Hamburger and Hamilton, 1951). These cells can be isolated, optionally genetically modified (van de Lavoir et al., 2006), and then transferred and transported into the bloodstream of another embryo, thus contributing to the production of a chimeric germline (Yasuda et al., 1992; Tajima et al., 1998). Currently, both BCs and PGCs are intensively studied as a potential tool for the preservation of endangered species. Based on the present study, fresh or frozen cells with properly determined gender could become rapidly accessible for the construction of chimeric birds with reproductive organs producing gametes of any selected species. This technique was recently tested to develop Houbara bustard chicken chimeras from PGCs (Wernery et al., 2010).

In conclusion, the technique described in the present study appears as reliable but also faster and cheaper than the sexing procedures previously published. It can therefore be of practical interest in any of pre-cited Galliformes at hatching, covering a broad range of applications including smaller flocks of game birds, research laboratories, as well as biotechnical manipulations of blastodermal or primordial germ cells for the production of chimeras.

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Received: 2011–10–26
Accepted after corrections: 2011–12–08

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