Original Article

Comparison of dried blood spot, buccal swab, cloacal swab and feces as DNA sources to identify avian sexes by PCR

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Abstract

Absence of sexual dimorphism makes it difficult to determine bird's sex. A number of both invasive and non-invasive methods as a DNA template of sampling for avian sexing can be used. The aim of this study was to compare the efficacy of different sources of DNA for avian sexing by PCR. Samples were collected from dried blood spots, buccal swabs, cloacal swabs and feces. The highest success rate for sexing determination was from the dried blood spots, accounting for 100%, while those from the buccal swabs, cloacal swabs and feces were 74%, 75.47% and 29.17%, respectively. The non-urate fecal samples gave better results than the urate fecal samples, accounting for 50% and 18.75% success rates, respectively. The difference in the success rates of the method using buccal swabs and that using cloacal swabs was not statistically significant; moreover, both methods are non-invasive. In conclusion, the non-invasive method using buccal or cloacal swabs might be the choice of sources of DNA template for avian sexing by PCR.

Keywords: avian, buccal swab, cloacal swab, feces, sexing

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Introduction

The identification of sex in avian is important for ecology, biology, breeding and conservation. In avian, the neonate and juvenile dimorphism is absent and difficult to determine. In some avian, the adult morphology is similar in both male and female. Sex identification by sex morphology has high risk of being misidentified. A molecular technique for avian sexing is recommended, using intron length polymorphism of chromo-helicase-DNA binding (*CHD*) gene located on Z (*CHD-Z*) and W (*CHD-W*) homologous chromosomes. The gender of avian is determined by the sex chromosome having homogamete (ZZ) in male and heterogamete (ZW) in female (Saitoh et al., 1991; Griffith and Tiwari, 1996; Griffith and Korn, 1997). In 1999, Fridolfsson and Ellegren developed primer 2550F and 2718R for PCR based application of sexing on non-rattie birds. This primer targeted the CHD gene and amplified CHD1W and CHD1Z. A CHD1W fragment is 400-450 bp in size while CHD1Z fragment is between 600-650 bp. In males, only CHD1Z fragment can be amplified while in females amplified CHD1Z and CHD1W fragments were shown.

DNA can be isolated from many kinds of samples such as blood (Ellegren, 1992; Pearce et al., 1997; Fridolfsson and Ellegren, 1999; Quinatana et al., 2008, Bosnjak et al., 2013), feather (Pearce et al., 1997; Bush et al., 2005; Aun and Kumaran, 2010; Bosnjak et al., 2013), egg shell membrane (Pearce et al., 1997; Kimwele et al., 1998), buccal swab (Arima and Ohnishi, 2006; Handel et al., 2006; Bosnjak et al., 2013) and feces (Marrero et al., 2009; Amada, 2012; Bosnjak et al., 2013). Drawing blood is quite an invasive technique and increases stress level in avian. It is also inconvenient to perform in young nestlings. Therefore, the non-invasive techniques using buccal swab, cloacal swab and feces provide important DNA sources for avian ecology. Buccal swab samples have been successfully used to obtain human and nonhuman genomic DNA such as laboratory animals, amphibian and birds (Pidancier et al., 2003; Neuhaus et al., 2004; Handel et al., 2006). In addition, the non-invasive techniques, especially those using feces, could reduce stress of animals during sample collection. However, fecal samples contain inhibitors which probably inhibit the PCR reaction. Cloacal samples for determining sex have not been reported widely in avian. The aim of this study was to compare dried blood spot, buccal swab, cloacal swab and feces as DNA sources to identify avian sexes by PCR.

Materials and Methods

Sample collection: A total 139 samples were collected from fighting cocks and 12 samples from sun conure (*Aratinga solstitialis*) of any age and gender. Among the total of 151 samples, there were 50 buccal swab samples, 53 cloacal swab samples, 24 fecal samples and 24 dried blood spot samples. The buccal epithelial cells were collected by rolling a sterile rayon tipped applicator (Puritan®) inside the mouth and throat for approximately 10 seconds. Each swab was placed in a microfuge tube which contained 0.9% NaCl. The cloacal epithelial cells were harvested by rolling a sterile rayon tipped applicator inside the cloaca approximately 10 seconds and placing it into 0.9% NaCl. The twenty-four fecal samples were separated into two groups, 16 samples with urate and 8 samples without urate. Each one gram of fecal sample was placed into a conical centrifuge tube containing 95% ethanol. All samples were kept at -20°C before genomic DNA extraction. The blood samples were collected and spotted in laboratory filter paper (Whatman grade 1 filter paper, GE healthcare, Buckinghamshire, UK) according to Suriyaphol et al. (2014). Each blood spot in the laboratory filter paper was cut into 2x2 mm^2, fixed by methanol for 20 minutes and air dried for 20 minutes. The research protocol was approved by Chulalongkorn University Animal Committee (approved no. 1631017).

DNA extraction: Genomic DNA was extracted from the buccal and cloacal swab samples by using Presto™ buccal swab g DNA extraction kit (Geneaid Cat. No. GSK100), following the kit protocol. DNA from the fecal samples was isolated by using QIAamp® fast DNA stool mini kit (Qiagen Cat. No. 51640), following the kit protocol.

Polymerase chain reaction: A primer pair 2550F (5'-GTTACTGATTCGTCTACGAGA-3') and 2718R (5'-ATTGAAATGATCCAGTGCTTG-3') was used to amplify CHD1Z gene and CHD1W gene (Fridolfsson and Ellegren, 1999). PCR reaction was performed in a final volume of 25 µl containing 5 picomoles in each primer, DNA template (4-7 mm^2 dried blood spot paper and 50-100 ng DNA from the buccal swab, cloacal swab and fecal samples) and One PCR™plus (Genedirex) using GeneAmp® PCR System 9700 (Applied Biosystems, CA, USA). The thermal profile only for the dried blood spot samples comprised an initial denaturing step at 94°C for 2 min followed by 30 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 45 s, ending with 72°C for 5 min. The DNA from the buccal swab, cloacal swab and fecal samples was measured by a nanodrop 1000 spectrophotometer (Thermo scientific, CA, USA). The PCR protocol was an initial denaturing step a 94°C for 30 s followed by 30 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 45 s, ending with 72°C for 5 min. PCR products were separated by 1% agarose gel with ethidium bromide in 1xTris-acetate-EDTA (TAE) buffer at 120 V for 30 min. One hundred base pair (bp) DNA ladder of 0.5 µg was loaded and used as a marker. The PCR products were visualized by UV light. The primer pair produced one band in males (600-650 bp) and two bands in females (400-450 bp and 600-650 bp).

Statistical analysis: Statistical difference among the experimental groups was analyzed by Anova using program SAS 9.0. Statistical significance was considered at $P<0.05$.

Results

The sex determination from dried blood spot was 100% successful in all samples (n=24). The success rates of PCR amplification for the determination of sex from buccal swab, cloacal swab and fecal samples were 74% (n=50), 75.47% (n=53) and 29.71% (n=24),

respectively. For the two groups of fecal samples, the feces with urate and without urate were able to identify sex with 18.75% (n=16) and 50% (n=8) success rates, respectively (Table 1). After the electrophoresis of PCR products, gender determination was visualized on 1% agarose gel as one band in males (600-650 bp) or two bands in females (400-450 bp and 600-650 bp), which made separation reliable (Fig. 1). Sex determination from the dried blood spot samples showed 9 males and 15 females. The buccal swab samples identified 12 males and 25 females. The cloacal swab samples identified 11 males and 29 females. The fecal samples identified 3 males and 4 females. The quantities of genomic DNA, which was extracted from the buccal swab, cloacal swab and fecal samples, were 28.813±7.222 ng/µl, 71.975±7.620 ng/µl and 73.035±12.404 ng/µl, respectively. The purity of genomic DNA from the buccal swab, cloacal swab and fecal samples was 2.081±0.084, 1.666±0.089 and 2.096±0.145, respectively. Twelve samples of the dried blood spots, buccal swabs, cloacal swabs and feces, each from the same identification number, were analyzed to the sensitivity which had dried blood spot method as the gold standard. The sensitivity of the buccal swab, cloacal swab and fecal samples was 75%, 91.67% and 33.33%, respectively.

Table 1 Success rates of different types of sample (buccal swabs, cloacal swabs, feces and dried blood spots) by PCR amplification

Sample type	Number of success			
	Male (n)	Female (n)	Total (n)	Success rate (%)
Buccal swabs (n=50)	12	25	37	74
Cloacal swabs (n=53)	11	29	40	75.47
Feces (n= 24)	4	3	7	29.17
- Feces with urate (n=16)	-	3	3	18.75
- Feces without urate (n=8)	3	1	4	50
Dried blood spots (n=24)	9	15	24	100

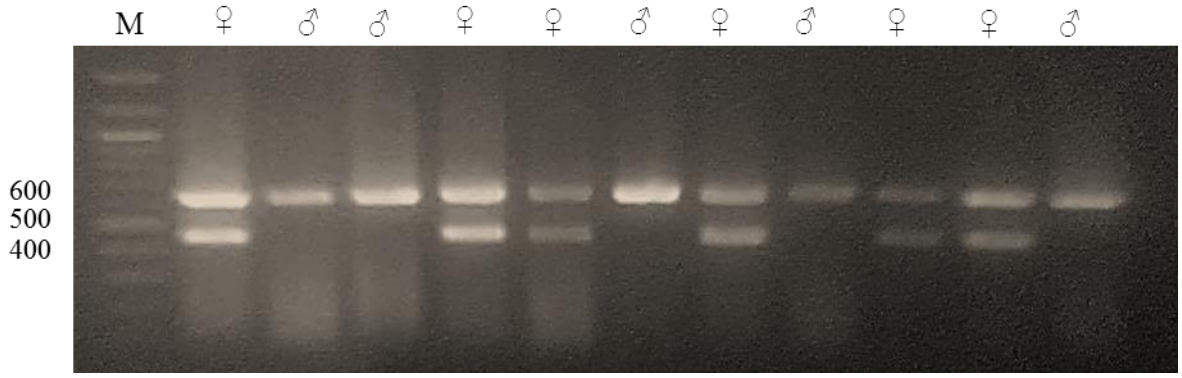

Figure 1 Examples of electrophoresis images of PCR products using primer pair 2550F and 2718R (Fridolfsson & Ellegren, 1999). One DNA band was observed in males (600-650 bp) and two bands in females (400-450 bp and 600-650 bp). The lane M marks the 100 bp DNA ladder.

Discussion

The success rates of avian sex identification by PCR among the four groups were compared. The highest rate was the PCR from dried blood spots (100%). The PCR from dried blood spots on laboratory filter paper with the combination of methanol fixation and boiling procedure was practical, economical and cost-efficient. Methanol fixation was used to prevent diffusion of hemoglobin, the major inhibitor in the blood (McCabe, 1991; Makowski et al., 1995; Suriyaphol et al., 2014). The success rate of amplification by single PCR from Great Cormorants blood was 100% (Arima and Ohnishi, 2006). The determination of bird sexing from dried blood spot was a routine procedure in the laboratory. The results of sexing from the buccal and cloacal swab samples were similar, with the efficacy rates of 74% and 75.47%, respectively. The difference between the buccal and cloacal swab samples was not statistically significant. According to Arima and Ohnishi (2006), the PCR success rates of single PCR, two-step PCR and Amp direct PCR from buccal cell samples were 82.2%, 95.3% and 98.1%, respectively, in 12 wild bird species. The success rates from buccal DNA samples were less reliable than the blood in Great Cormorants. In another study, PCR amplification success rates were 89% in nestling's swifts (*Apus apus*) and 91% in adults. The buccal swab method was practicable for sensitive bird species, especially in nestling birds which are too small for blood sampling (Wellbrock et al., 2012). However, Seki (2003) and Bosnjak et al. (2013) reported that the result of the rate of buccal swab samples was 100%. The success rate of PCR amplification of the cloacal swab sample had not been reported before. In our study, the success rate from the cloacal swab sample was 75.47%, which was not statistically significantly different from that of the buccal swab sample. The cloacal swab method was as non-invasive and efficient as the buccal swab method. The lower success rate of cloacal swab samples compared with blood suggested the contamination of PCR inhibitors in the feces (Baignet et

al., 2005; Arima and Onishi, 2006). Among the four methods, the determination of sex by PCR from fecal samples had the lowest success rate (29.17%). Between the two groups of fecal samples, the ones without urate (18.75%) had higher success rate than those with urate (50%). In our study, the success rate of sex determination from fecal sample was similar to that of Bosnjak et al. (2013), which achieved 25% success rate. In fecal samples, there is a lot of PCR inhibitors such as urea, nutrition, pigments, dead cell, RNA, various microorganisms, gut flora, polysaccharides, chlorophyll, bile salts, hemoglobin, glycolipids and heparin (Monteriro et al., 1997; Oikarinen et al., 2009; Pontriroli et al., 2011; Schrader et al., 2012). Idaghdour et al. (2003) reported that the success rate of sex identification depended on fresh and insect material in the fecal samples. The amplification of DNA was 100% successful in fresh feces, without obvious insect material, and only 17% in older dry feces. Old feces are suspected to have longer exposure to nucleases, causing extensive DNA degradation, making it unsuitable for amplification. In addition, genomic DNA which is extracted from fecal samples is suspected of contamination with genomic DNA from eukaryotic such as protozoa (Nota and Takenaka, 1999). However, the method using fecal samples is non-invasive and fecal samples are quite easy to collect for avian sex identification (Bosnjak et al., 2003). In addition, the quantity and purity of genomic DNA from buccal swab, cloacal swab and fecal samples from commercial test kit in our study were adequate for PCR. Among the three methods, the sensitivity of cloacal swab samples was the highest and that of the fecal samples was the lowest. The sensitivity of fecal sample was quite low because of the contamination of many kinds of PCR inhibitors (Baignet et al., 2005; Arima and Onishi, 2006). According to the results of the present study, it is concluded that avian identification from dried blood spots gives the highest success rate, but it might not be appropriate in nestling birds. Moreover, the success rates of the methods using both buccal and cloacal swab samples are not statistically significant enough to make them efficient samples for the non-invasive method of bird sexing. The fecal samples give the least success rate and sensitivity because there are many kinds of PCR inhibitors. However, fecal sampling is the least invasive method for birds and might, therefore, be the best method for wild bird sample collection.

Acknowledgements

This study was supported by grants from senior project, Faculty of Veterinary Science, Chulalongkorn University. We would like to thank Prof. Dr. Padet Tummaruk, Dr.Kittipong Tachampa for statistical analysis and Prof. Dr. Alongkorn Amornsin for the instruments.

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บทคัดย่อ

การเปรียบเทียบตัวอย่างจากเลือด เซลล์เยื่อบุกระพุ้งแก้ม เซลล์เยื่อบุทวารร่วม และมูลเพื่อแยกเพศในสัตว์ปีกโดยวิธีพีซีอาร์

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การแยกเพศในสัตว์ปีกมีความสำคัญในการเลี้ยงนก เนื่องจากนกสายพันธุ์มีลักษณะภายนอกที่คล้ายคลึงกัน ทั้งในนกเพศผู้และเพศเมีย ทำให้ไม่สามารถแยกเพศได้ งานวิจัยนี้มีวัตถุประสงค์เพื่อเปรียบเทียบความเป็นไปได้และประสิทธิภาพของการเก็บตัวอย่างรูปแบบต่าง ๆ ทั้งจากเยื่อบุกระพุ้งแก้ม เยื่อบุทวารร่วม และอุจจาระ ซึ่งเป็นวิธีที่ค่อนข้างอันตรายน้อย เพื่อมาเปรียบเทียบกับวิธีที่ใช้กันอย่างแพร่หลาย คือ การเก็บตัวอย่างจากเลือด จากการทดลองการแยกเพศ พบว่าตัวอย่างเลือดให้ผลดีที่สุด คือ 100% รองลงมา คือ เยื่อบุทวารร่วม 75.47% เยื่อบุกระพุ้งแก้ม 74% และอุจจาระ 29.17% โดยพบว่าอุจจาระที่ไม่มียูเรตให้ผลดีกว่าอุจจาระที่มียูเรต โดยให้ผล 50% และ 18.75% ตามลำดับ การเก็บตัวอย่างจากเยื่อบุกระพุ้งแก้มและเยื่อบุทวารร่วมนั้นสามารถใช้เป็นแหล่งตัวอย่างที่นำมาแยกเพศได้ โดยไม่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติและยังเป็นวิธีที่อันตรายน้อยต่อตัวสัตว์อีกด้วย ดังนั้น การเก็บตัวอย่างจากเยื่อบุกระพุ้งแก้มและเยื่อบุทวารร่วมอาจเป็นอีกทางเลือกหนึ่งสำหรับการตรวจเพศในสัตว์ปีกได้

คำสำคัญ: แยกเพศ สัตว์ปีก เยื่อบุกระพุ้งแก้ม เยื่อบุทวารร่วม อุจจาระ

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