

Optimization of Molecular Sex Identification in Ostrich Based on Multiplex PCR

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Abstract

Today, ostrich breeding has been widely developed in Iran and other countries due to the ability of this animal to produce quality meat, leather, and oil. However, one of the main problems in breeding them is sex determination using aggressive techniques with low accuracy. This study aimed to determine the sex of immature ostriches using specific primers in a multiplex PCR reaction. This study considered 20 specimens of unspecified immature and six specimens (three adult males and females) of known-sex African ostriches as controls. SS and OSFES primers were used to amplify part of the female-specific sequence and 18S primer was used as a control in a PCR reaction. The presence of SS and OSFES bands in gel electrophoresis indicated the amplification of the desired parts related to the female sex and the absence of these bands indicates the male sex of the species. In total, out of 20 African ostriches studied, 50% of them belonged to females and 50% of them belonged to males. Later, with the growth of immature individuals, the results of this experiment were confirmed. In this study, it was found that the use of feather samples for DNA extraction and multiplex PCR is a suitable, accurate, and cost-effective method in identifying and determining the sex of young ostrich and leads to more real and reliable results, avoiding stress in birds.

Keywords: Multiplex PCR, Ostrich, SS and OSFES primers, Sex determination

Introduction

The ostrich (*Struthio camelus*) is a large flightless bird in the ratite group that was first introduced by Linnaeus in 1758 (Bertram et al., 2014; Blache et al., 2005; Shanawany et al., 1999). In ancient times, ostriches provided food, clothing, and ornaments for humans (Shanawany et al., 1999). The ostrich breeding in recent years is growing due to the ability of this animal to produce low-fat meat products, suitable leather, high-quality oil, and decorative feathers. Therefore, it can be considered a quality product with high nutritional value in the human diet (Cooper et al., 2005; Poławska et al., 2011; Anon, 1996). Compared to crocodile or elephant skin, ostrich skin is highly flexible, and therefore it is used in the shoe and bag industry. Apart from their importance in breeding, ostrich eggs can also be used in the cooking industry. The volume of an ostrich egg is equal to 24 eggs on average. Eggshells are also used in painting or engraving art objects (Kreibich et al., 1995). The advantage of ostrich farming is that every part of the bird can be used and less than 2% is wasted. The success of ostrich production and their growth, until they reach adulthood is

economically important. However, early sex determination is one of the issues in ostrich breeding. Young male and female ostrich chicks look the same until 12 to 14 months of age (Hallam et al., 1992). Many bird species, including the ostrich (*Struthio camelus*), emu (*Dromaius novaehollandiae*), and pigeons, do not have sexual dimorphism. Therefore, it is difficult to distinguish between males and females based on their external morphology, especially in young birds (Cerit et al., 2007; Jia et al., 2023; Gruszczynska et al., 2019). Early sex determination in this population reduces farm maintenance costs and improves the efficiency of breeding programs (Cerit et al., 2007). Sex determination in ostriches is possible using several phenotypic methods. The traditional methods used are often very time-consuming, expensive, and in some cases aggressive and harmful. Sex determination through cloacal and karyotyping analysis is also impractical and laborious (Dubiec et al., 2006). To avoid these limitations, molecular techniques for determining the sex of birds were developed in the mid-1990s (Griffiths et al., 1996; Ellergren et al., 1997). Modern molecular techniques are non-invasive, accurate, and reliable (Ellegren et

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al., 1997). Mostly, DNA techniques are based on the polymerase chain reaction (PCR) method. PCR-based genotyping requires high-quality genomic DNA and whole blood or feathers of birds can be a source of DNA extraction. In these studies, using PCR and specific primers, fragments of DNA are amplified mainly from the sex chromosome and are identified by agarose gel electrophoresis (Javanmard and Javadmanesh, 2005). Multiplex polymerase chain reaction (PCR) is a type of PCR in which two or more target sequences can be amplified by more than one pair of primers in the same reaction. Multiplex PCR has significant potential for saving time in the laboratory (Markoulatos et al., 2002).

This study aimed to identify and determine the sex of immature ostriches (less than 4 weeks) using specific primers in a multiplex PCR reaction. Reports indicate that, so far, the combination of SS, OSFES, and 18S primers has not been used in multiple replications as well as ostrich sex detection. Therefore, in this study, we will optimize ostrich sex detection for multiple gene loci in a multiplex PCR.

Materials and Methods

In this study, 26 African ostrich breed individuals including six adult species (Three males and three females) and 20 immature chicks (unknown sex) were selected. To extract genomic DNA, samples of feathers isolated from the wings were used. The DNA extraction process from the feathers was performed using the Sina pure DNA kit (Sinaclon, Iran) and then evaluated by 0.8% agarose gel electrophoresis. The primers used in this study consisted of three pairs of SS, OSFES, and 18S primers obtained from previous studies. Table 1 shows the sequence of SS, OSFES, and 18S primers. SS and OSFES primers were used to amplify female-specific sequences and 18S primer was used as a PCR control.

Multiplex PCR reaction

The multiplex polymerase chain reaction was used to determine the sex of ostrich chicks. In this method, more than one target sequence can be amplified by using several pairs of primers in a single reaction. The quality of the DNA pattern in multiplex PCR is determined more effectively than a simplex PCR. To amplify the desired sequences, the reaction mixture including specific primers, master mix, and template DNA was added to 1.5 ml microtubes. Table 2 shows the optimal concentration of materials used in the PCR reaction in a total volume of 25 μ l. After determining the optimal annealing temperature, the temperature and time parameters were determined according to Table 3. Finally, 2 μ l of PCR products along with 1 μ l of loading buffer were inoculated into gel wells and transferred to the electrophoresis tank. After electrophoresis (120 V for 20 min), the bands formed on the gel were photographed.

Results and Discussion

In this study, feather samples were used for genomic DNA. Figure 1 shows the extracted DNA bands on the agarose gel. As can be seen, DNA samples are of appropriate concentration and quantity. Reports showed that DNA extraction from bird feathers, as a non-invasive method, puts less stress on birds, and on the other hand, due to the presence of nuclei in red blood cell (RBC) cells, high amounts of DNA can be extracted from the blood of birds. In a study, Tsuda et al. (2007) used blood samples and ostrich feathers to extract DNA. Their findings showed that the use of feather samples is more appropriate to prevent sampling problems (Tsuda et al., 2007).

Table 1. Primers sequence and product size

primer		Sequence (5'→3')	(bp) Size	References
SS	F	5'TCTACACCTAAGGAGTCCCATATT3'	648	Faghani et al, 2009
	R	5'GGTCTACACCTGTTGAAAATCATT3'		
OSFES	F	5'AGCAGAATTGCTGAGTAAAC3'	432	Alipanah et al., 2016
	R	5'ACAGAGGTTAAAAACCACC3'		
18S	F	5'AGCTCTTTCTCGATTCCGTG3'	256	Huang et al., 2008
	R	5'GGGTAGACACAAGCTGAGCC3'		

Table 2. Reaction mixture for the PCR reaction

Reagents	Volume (µl)
Master mix (2X)	12.5
DNA 100ng/ul	2
Water	7.5
F primer (10mM) (18S+OSEF+SS)	0.25+0.5+0.75
R primer (10mM) (18S+OSEF+SS)	0.25+0.5+0.75
Total	25

Table 3. PCR reaction conditions

	Temperature (°C)	Time (S)	Cycle
Initial denaturation	95	180	1
Denaturation	94	40	33
Annealing	59	40	
Elongation	72	60	
Final elongation	72	300	

Also, the UV absorption spectra for different DNA concentrations showed as can be seen, with increasing DNA concentration, the peak intensity in the region of 260 nm also increases.

Figure 2 showed the PCR products for male and female control samples using 18S, SS, and OSFES primers and the simplex PCR reaction. The presence of SS and OSFES bands in lanes 4 and 5 indicates the amplification of the desired sequences, which is related to the female sex, and the absence of the mentioned bands in lanes 6 and 7 indicates no amplification, which is related to the male sex. In addition, the presence of a 205 bp fragment amplified by 18S primer was observed in all male and female species, representing a successful PCR in all of the reactions.

After confirming the primers in determining the sex of specific ostrich species and achieving the optimal annealing temperature, multiplex PCR was performed using 18S, OSFES, and SS primers to determine the sex of unspecified species. Figure 3 shows the DNA bands resulting from the amplification of the fragments by the respective primers. As can be seen, lanes 1 to 4 have 2 bands related to the amplification of 644 and 407 bp sequences, which are related to the female sex. The absence of bands in lanes 5 to 7 also indicates that the species belong to the male sex. In general, out of

20 ostriches studied, 10 (50%) of them belonged to females and 10 (50%) of were to males. This result was later confirmed with thematuration of ostrich chickens.

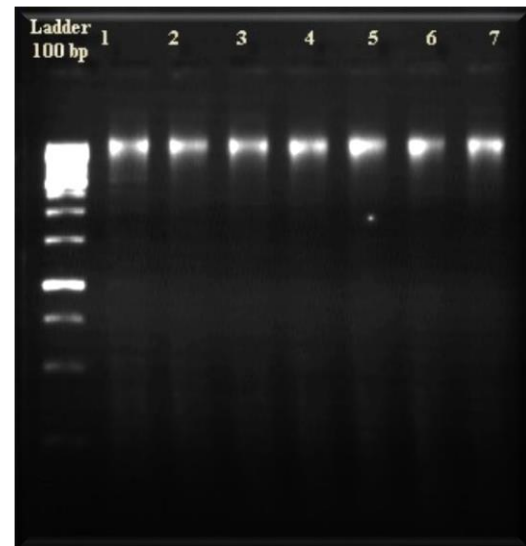


Figure 1. 0.8% agarose gel electrophoresis of genomic DNA samples. Lanes 1-7: DNA extracted from ostrich feathers

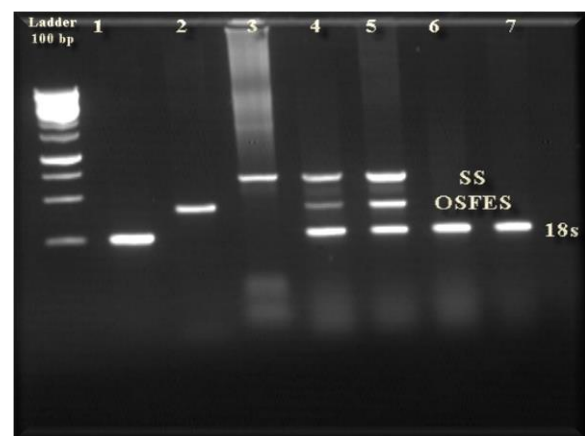


Figure 2. Sex determination of specific ostrich species using PCR. Lane 1: 18S primer amplified fragment, Lane 2: OSFES primer amplified fragment, Lane 3: SS primer amplified fragment. Lanes 4 and 5: Female ostrich-related bands, Lanes 6 and 7: Male ostrich-related bands

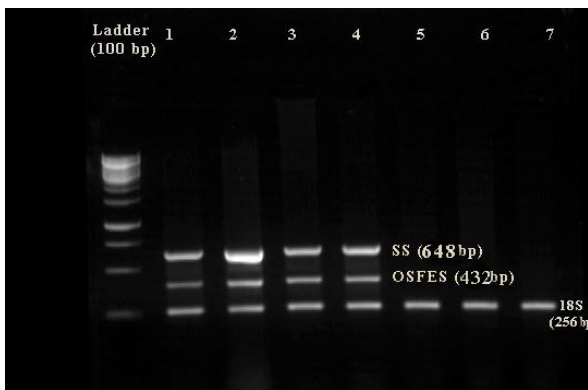


Figure 3. Sex determination of unspecified ostrich species using multiplex PCR reaction. Lanes 1-4: Female ostrich-related bands, Lanes 5-7: Male ostrich-related bands

So far, various studies have been done to determine the sex of ostriches. In one study, Medaglia et al. (2005) determined the sex of ostrich chicks using multiplex PCR reactions. In this research, they used OSFES and SS primers to amplify the desired sequences for the female sex and VIAS-OS primer (amplification of a microsatellite region) as a PCR reaction control (Madaglia et al., 2005). The method developed in this study is inexpensive, accurate, and requires minimal specialized equipment. In addition, they showed that DNA extraction could be done using chicken feathers and sex could be determined in the first days of life. In another study, Billo et al. (1999) used PCR-based methods (RAPD) to determine the sex of young ostriches. They used primer pairs, SS1 and SS2 to determine the sex of the female and primer L014b and L014a as controls in the RAPD assay (Bello et al., 1999).

In birds, sex is determined by two sex chromosomes, so male birds have two identical sex chromosomes (ZZ), and female species have two different chromosomes (ZW). The Z chromosome is present in both sexes, while the W chromosome is present only in females. Thus, in female species (ZW), two DNA bands are observed, the first band corresponding to the large gene on the Z chromosome and the second band to the small gene on the W chromosome, and in males (ZZ), only one band is observed because both chromosomes are the same size. Some of the genes on the W chromosome are protected, and by using these genes, the sex of the bird in all species can be determined using a pair of primers and a PCR reaction. There are currently several independent primer sets for determining the

sex of birds using DNA. Reports demonstrated that the 18S gene is known to be the most stable housekeeping gene and cannot be used to determine mRNA levels due to the lack of a poly-A tail. On the other hand, high expression of the 18S rRNA gene compared to other genes can lead to baseline error in PCR analysis (Bouma et al., 2004). SS primers for the W chromosome also produce a 648 bp fragment in females. OSFES primers also produce a 432-bp DNA fragment that amplifies part of a specific female sequence. In this study, we first used the above primers together with the 18S primer in multiplex PCR. In 2005, Maine et al. used a multiplex PCR to determine the sex of 6-month-old ostriches. The experimental group also included 19 immature birds that were randomly selected. To test the PCR reaction, two primer pairs were used, including primers SS1 and SS2, which amplify a 650 bp fragment in the female, and primers L014a and L014b, which amplify a 280 bp fragment in both sexes. The results showed that out of 19 unspecified species, 10 samples were male and 9 were female ostriches. In addition, the findings also showed that DNA extracted from feathers could help farmers identify the sex of birds at an early age (Mine et al., 2005). In a similar study, Alipanah et al. (2010) used PCR reaction and OSFES and VIAS-SO14 primers to identify and determine the sex of ostrich chicks. Their results showed that the use of PCR and OSFES primer in determining the sex of day-old chicks is easy, low cost, and high accuracy (Alipanah et al., 2010).

Conclusion

In the past, invasive and time-consuming methods such as laparoscopy and morphology were used to determine the gender of birds, but nowadays DNA-based techniques are used, due to the lack of anesthesia and the use of blood or feather samples for DNA extraction. It has resulted in real and reliable results. In this research, multiple PCR reactions and specific primers were used to determine the sex of ostrich chicks. The findings showed that the use of ostrich feathers in DNA extraction, in addition to reducing the stress on the bird, also facilitates sampling. On the other hand, the use of molecular markers and primers specific to the sex of the female is a suitable, accurate, and cost-effective way to identify and determine the sex of immature species.

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Conflict of Interest

None of the authors have any conflicts of interest.

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