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Simple Multiplex PCR for Rapid Diagnosis of Sex of Ducks and Duck Embryos

Santosh Haunshi,* S.C. Saxena, A. Pattanayak[1], S. Bandyopadhaya[2], A.K. Tyagi[3], K. M. Bujarbaruah[4]

Molecular Genetics Laboratory
Division of Poultry Science
ICAR Research Complex for N.E.H. Region
Umroi Road, Umiam-793 103, India

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Abstract

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A simple and rapid multiplex polymerase chain reaction (PCR) was developed for quick diagnosis of sex of duck and duck embryos. W chromosome specific DNA sequence was selected and primers were designed to amplify 335 bp fragment from female sex while 16S ribosomal sequence was selected to design primers to amplify 468 bp PCR products both in male and female sex as an internal control. Nucleotide sequences of W chromosome specific DNA fragments of Khaki Campbell and Indian Runner breeds of duck were found to be identical both in size and sequences. The study concluded that the protocol was successful in precisely identifying the gender of ducklings belonging to both Indian Runner and Khaki Campbell breeds of ducks and duck embryos.

Key words: Multiplex PCR, duck, embryo, sex diagnosis.

Introduction

Normally, sexing in ducks is carried out by vent sexing at day old stage. However, at embryonic level there are few methods available for identification of sex in ducks. Ogawa *et al.* (1997) have developed a PCR based sexing protocol using a set of primers without internal control to amplify the fragment of a non-repetitive DNA sequence from the EE0.6 of W chromosome of chicken for sexing of 14 different species of birds including domestic duck. Using same set of primers and other internal control primers Itoh *et al.* (2001) devised multiplex PCR protocol for detection of gender in ducks and other species of birds. However, the PCR protocol described by Ogawa *et al.* (1997) is without internal control and is very lengthy, whereas, that described by Itoh *et al.* (2001) is also very lengthy, and primers used in their study have as many as 23 cross dimers. Hence, there is a need for simple protocol

*Corresponding author: Tel: +91-364-2570305,
Fax: +91-364-2570362, E-mail: santosh575@rediffmail.com
[1]Division of Plant Biotechnology
[2]Division of Veterinary Parasitology
[3]Division of Animal Health
[4]ICAR, New Delhi

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for rapid detection of gender of ducks and their embryos for investigations involving sex differentiation and determination and also in generation of germ line chimeras and transgenic studies. Therefore, the present study was carried out with the objective of developing a simple, rapid and one step procedure for gender identification of ducks and duck embryos through multiplex PCR protocol with internal control primers.

Materials and Methods

Blood samples from 10 Khaki Campbell and eight Indian Runner breeds of ducks (*Anas platyrhynchos*) were collected aseptically in a sterile syringe from brachial vein. The sex of these breeds of duck was determined from adult morphology. Fertile eggs of ducks were incubated and eggs were taken out from incubator between 5 and 8d after setting. The embryos along with their associated membranes were placed in petri dishes and amniotic fluid and tissue samples of embryos were collected in microcentrifuge tubes. About 50 µl of amniotic fluid was aspirated to a microcentrifuge tube and cells were pelleted at 12000xg for 1 min and then pelleted cells were used for DNA extraction after discarding the supernatant.

The genomic DNA was extracted from whole blood using conventional PCI method (Sambrook and Russel, 2001). Non repetitive W chromosome specific DNA sequence of duck (Ogawa *et al.,* 1997) was retrieved from the Genbank database and used to design primers to amplify 335 bp single fragment of W chromosome specific sequence from nucleotide position of 19 to 353 in female ducks only.

ShD - F primer 5'-
TAGTAGCCGCAGATAGGAAATG-3'

ShD - R primer 5'-
ACAAATGCCAATGAAATAGGTT -3'

Primers were also designed to generate a 468 bp fragment of the 16S ribosomal gene (Ramirez *et al.,* 1993) from nucleotide position of 579 to 1046 in both male and female sex to serve as internal control.

16S R - F primer 5'
ACGAGAAGACCCTGTGGAAC 3'

16S R – R primer 5'
GGGAAGGCGTGCTTGTAGTA 3'

PCR reactions were carried out in a volume of 25 µl reaction mixture consisting of 12.5 µl Qiagen multiplex PCR master mix with HotStar Taq DNA polymerase, 1X Q-solution (Qiagen - USA), 150 ng of genomic DNA, 1.5 µM of ShD – F & R primers and 0.5 µM of 16S R - F & R primers. Various PCR variables were assessed to optimize reactions and final assay conditions were standardized at 94 C for 1 min followed by 30 cycles of 94 C for 30 s, 53 C for 30 s and 72 C for 30 s., with final extension step of 72 C for 7 min. PCR reactions for sexing were carried out in programmable Thermal Cycler (Applied Biosystems, Foster city, USA). After amplification PCR products were analyzed on 1.75% agarose gel in 1X TAE buffer and bands were visualized under UV light after ethidium bromide staining (Sambrook and Russel, 2001). The size of the PCR products was determined with the help of Kodak 1D software. As many as 35 samples were tested for identification of gender of ducks and their embryos.

The PCR products of W chromosome specific sequence from Khaki Campbell and Indian Runner ducks were purified using sample exonuclease shrimp alkaline phosphatase digestion method. Direct sequencing of purified PCR products was carried out using Big Dye terminator V3.1 (ABI) chemistry in ABI prism 3100 genetic analyzer to ascertain the specificity of primers and to compare the nucleotide sequence between Khaki Campbell and Indian runner breeds of ducks. Sequencing of PCR products was carried out at Labindia Sequencing facility, Gurgaon (India).

Results and Discussion

Initially, primers specific to W chromosome and 16S ribosomal gene were tested individually using DNA samples from adult male and female ducks of known sex. W chromosome specific primers could amplify a single 335 bp fragment only in female ducks whereas, 16S ribosomal gene primers generated a 468 bp PCR product both in male and female ducks. It was established by testing that ShD - F & R primers were unique to female sex chromosome and, therefore, are useful for identifying sex of ducks. While 16S R - F & R primers were identified as suitable primers to serve as internal control since they could amplify the portion of ribosomal gene sequence both in male and female ducks. So, in any gender identification test in ducks with these set of primers if one gets only one band of 468 bp the sample could be identified as male if both 335 bp and 468 bp PCR products were observed then it is established that the sample is from female ducks (Fig. 1). Multiplex PCR to detect gender of ducks was efficient over a wide range of the template DNA concentration from 15 - 300 ng and that PCR cycles as few as 30 were ideal for efficient multiplex PCR.

Subsequently, the multiplex PCR protocol was tested to identify the gender of embryos. It was found that multiplex PCR protocol was successful in determining the gender of embryos as well (Fig. 2). This particular protocol was also successful in identifying the gender of embryos using genomic DNA extracted from cells of amniotic fluid by Wizard genomic DNA purification kit (Fig. 3). Clinton *et al.* (2001) have also demonstrated the detection of gender of chicken embryos by multiplex PCR using DNA extracted from amniotic fluid cells. The results of sex diagnosis of embryos were same whether DNA extracted from soft tissues or amniotic fluid cells were used for multiplex PCR. The PCR protocol for sex identification described in the present study requires less time as compared to the protocol without internal control primers developed by Ogawa *et al.* (1997) and to the one developed by Itoh *et al.* (2001) in adult ducks with internal control primers.

The W chromosome specific primers used in this study were also tested in

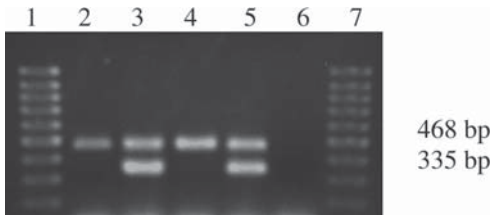

Fig. 1. Multiplex PCR for sexing of Khaki Campbell and Indian Runner breeds of ducks. Lane 1 & 7 are molecular size (MS) markers, lane 2 & 3 are male & female Khaki Campbell ducks, lane 4 & 5 are male & female Indian Runner ducks and lane 6 is negative control.

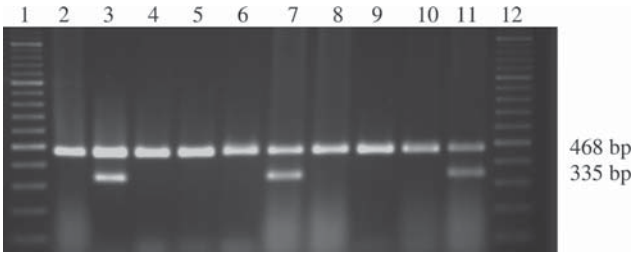

Fig. 2. Multiplex PCR for sexing embryos using DNA samples from various embryos. Lanes 1 & 12 are MS markers and lanes 2 to 11 are DNA samples from embryos.

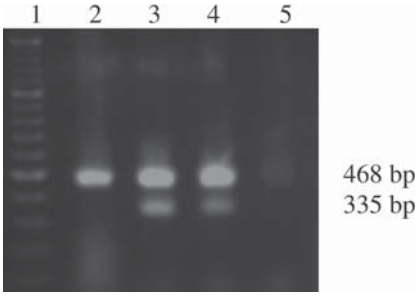

Fig. 3. Multiplex PCR for gender identification of embryos using DNA from amniotic fluid cells. Lanes 1 is MS marker, Lane 2 to 4 are amniotic DNA extracted using Wizard Genomic DNA purification kit and lane 5 is negative control.

chickens; however, they failed to amplify the DNA fragment from either of the sex, so it appears that they are specific to duck species and thus also serve as a means to identify the species of ducks simultaneously. Sequencing of W chromosome specific PCR products revealed that the nucleotide sequence from both Khaki Campbell and Indian Runner breeds of ducks were identical both in size and composition. Finally it is concluded that multiplex PCR protocol described in the present study is simple and rapid which is useful for quick detection of gender of ducks and duck embryos using DNA extracted either from whole blood samples, embryonic tissues or amniotic fluid cells.

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