



PCR based sex differentiation in guinea fowl using W chromosome specific sequences

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ABSTRACT

In view of poor sexual bimorphism in guinea fowl, an attempt was made to develop PCR based method for sex differentiation using W chromosome specific sequence. A 370 bp sex specific fragment was amplified in female birds of guinea fowl, chicken, quail and turkey using the EE0.6 specific set of primers i.e. USP1 and USP3, while no amplification was observed in male birds. The 16S rRNA gene specific primers were used to amplify a ~590 bp fragment in both the sexes, which acted as control. A multiplex PCR assay was developed by using sex specific primers and universal 16S rRNA primers for sex differentiation in guinea fowl and other galliformes species. In all the species including guinea fowl, female showed 2 bands i.e. 370 bp female specific and 590 bp common band, while males showed only 590 bp band. These results showed the effectiveness of sex specific primers in sex differentiation in guinea fowl as well as other galliformes species.

Key words: Guinea fowl, Multiplex PCR, Sex identification, W chromosome

Accurate determination of the sex of avian is important for the poultry industry for both economic and welfare reasons. DNA based techniques, developed for sexing in birds were more reliable in sex identification. Mostly these techniques are based on DNA hybridization or polymerase chain reaction (PCR) assays (Dubiec and Zagalska-Neubauer 2006).

The avian W chromosome is small, mostly heterochromatic, filled with large repetitive arrays and shares many features with the mammalian Y chromosome (Ellegren 1996). The W chromosome specific sequences/genes may provide an effective way for sex differentiation using PCR based methods. The Xho I/EcoR I repeats (Itoh *et al.* 2001) and chromo-helicase DNA binding (CHD) gene (Cheng *et al.* 2006 and Cerit and Avanus 2007) have extensively been used for sex identification in avian species. Guinea fowl is one of

many avian species which are considered sexually monomorphic and sex identification based on their external morphology especially in young birds is difficult (Dubiec and Zagalska-Neubauer 2006). The accurate and early (at day-old age) sex identification is must for implementing systematic selection and breeding programmes in guinea fowl. Hence an attempt was made to develop a PCR based method for sex identification in guinea fowl.

MATERIAL AND METHODS

Isolation of genomic DNA: Two adult healthy males and 2 adult healthy females of Pearl variety of guinea fowl as well as chicken, quail and turkey were randomly selected. The 0.5 ml of whole blood was collected from jugular vein of each bird in the tube containing EDTA. The blood was stored at -20°C till further processing. The high molecular weight genomic DNA was isolated using the phenol: chloroform extraction method (Sharma and Appa Rao 2000). The extracted genomic DNA was evaluated for quantity and quality by using spectrophotometer.

Primers: A set of primers, USP1 (5'-CTA TGC CTA CCA CMT TCC TAT TTG C-3') and USP3 (5'-AGC TGG AYT TCA GWS CAT CTT CT-3'), specific to W Chromosome 0.6 kb EcoRI fragment (Ogawa *et al.* 1997) were synthesized. These primers were expected to amplify a 370 bp band in female only. The primers (Forward-5'- CGC CTG TTT ACC AAA ACA T-3', Reverse-5'- CCG GTC TGA ACT CAG

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ATC ACG T-3') specific to partial 16S rRNA gene were used as control and were expected to amplify a ~ 590 bp fragment in both the sexes.

PCR amplification of sex specific band: The PCR reactions were set up in 25 µl reaction volume containing 2.5 µl of 10 X Assay buffer (100 mM Tris- HCl, pH 9.0, 500 mM KCl and 0.1% gelatin), 1.5 mM MgCl₂, 200 mM of dNTPs (dATP, dGTP, dCTP and dTTP), 0.4 mM each of primers (USP1 and USP3), 1 U Taq DNA polymerase (Promega), 50 ng of genomic DNA and autoclaved milliQ water to make up the volume. The amplification was carried out in I-cycler. Protocol for PCR reaction consisted of an initial denaturation at 94° C for 4 min. Followed by 35 cycles of PCR, each cycle consisting of 80 s at 95° C, 90 s at 56° C and 60 s at 72° C, and followed by a final extension step of 5 min at 72° C. After the completion of PCR reaction, the tubes were spun briefly, added 5 ml of gel loading dye to stop the reaction and stored at 4°C till further use. All the PCR products thus amplified were analyzed by electrophoresis for 45–60 min at 7–10 V/cm in a 3% agarose gel stained with ethidium bromide.

Multiplexing of the PCR primers: In view of developing a single tube PCR assay, attempts were made to multiplex the PCR for EE0.6 sequence PCR and 16S rRNA PCR. The PCR reactions were made in 25 ml volume. The PCR reactions were set up in 25 µl reaction volume containing 2.5 µl of 10 X Assay buffer (100 mM Tris- HCl, pH 9.0, 500 mM KCl and 0.1% gelatin), 1.5 mM MgCl₂, 200 mM of dNTPs (dATP, dGTP, dCTP and dTTP), 0.4 mM of EE 0.6 specific primers and 10 pM of 16S rRNA specific primers, 1 U Taq DNA polymerase (Promega), 50 ng of genomic DNA and autoclaved milliQ water to make up the volume. The amplification was carried out in I-cycler. The PCR amplification conditions used for amplification of sex specific band as described above were used for multiplex PCR.

RESULTS AND DISCUSSION

Using the genomic DNA from male as well as female birds of chicken and guinea fowl as template and the EE 0.6 specific set of primers i.e. USP1 and USP3, a 370 bp fragment was amplified in female birds, while no amplification was observed in male birds of guinea fowl as well as of other poultry species i.e. chicken, quail and turkey (Fig. 1A). The *XhoI* and *EcoRI* family repetitive sequences on the chicken W chromosome are useful targets of PCR-based sex identification in different avian species. A unique sequence, EE 0.6 (0.6 kb *EcoRI* fragment) is found on the long arm of the chicken W chromosome and were utilized for sex identification. This sequence is widely conserved on the W chromosome in avian species, however, EE0.6 sequences have their counterpart sequences on the Z chromosome, such as XH0.6RSM in the oriental white stork (*Ciconia boyciana*) (Itoh *et al.* 1997) and variability in W- and Z-linked sequences

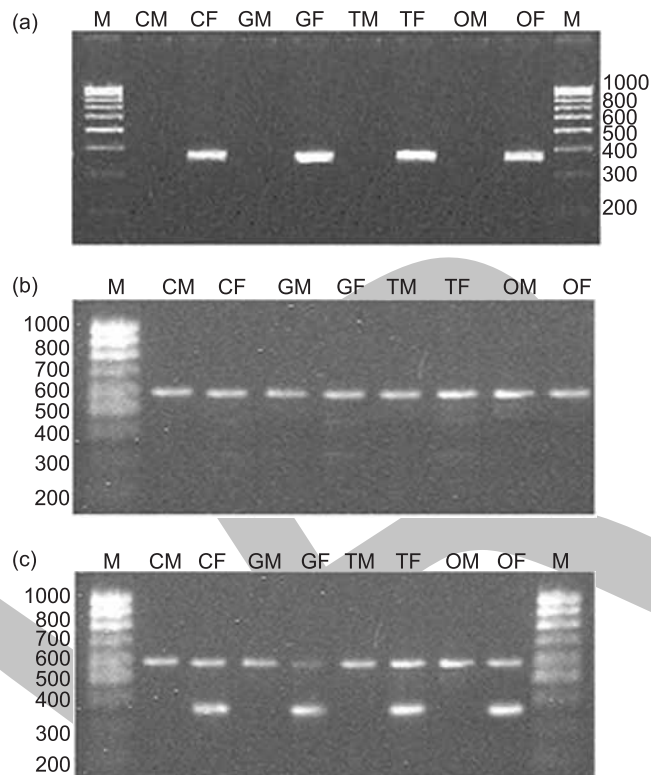


Fig 1. A. Amplification of sex specific band using EE0.6 specific primers i.e. USP1 and USP3. B. Amplification of 16S rRNA fragment (control). C. Amplification of sex specific band using EE0.6 specific primers and 16S rRNA fragment in a multiplex PCR reaction. (M; Molecular size marker-100bp DNA ladder, original form); CM; chicken male; CF; chicken female; GM; guinea fowl male; GF; guinea fowl female; TM; Turkey male; and TF; Turkey female.

among species causes occasional ambiguous results in the sex determination (Griffiths *et al.* 1998). Corté's *et al.* (1999) also found that USP1 and USP3 primers are very effective in sexing of birds from Galliformes order, while in other birds, they could not differentiate the sexes. Itoh *et al.* (2001) also amplified the female specific bands in female of different species under galliformes, however in various species under the order Carinatae, USP1 and USP3 were not successful. Our results also showed the effectiveness of USP1 and USP3 primers in sex differentiation in guinea fowl as well as other galliformes species.

The 16S rRNA gene was used as control. The primers designed to amplify a ~590 bp fragment, amplified this band in both the sexes in all galliformis species (Fig. 1B). We used 16S rRNA gene as control and showed its effectiveness in amplifying the common band in both the sexes. Earlier workers using USP1 and USP3 primers, used suitable combinations of the primers to amplify a W specific band as well as a Z/W common band, as an internal control (Itoh *et al.* 2001) and showed its applicability in Carinatae as well as Galliformes, however the efficiency of 16S rRNA fragment

as control is to be tested in other avian orders.

In view of developing a single tube PCR assay, attempts were made to multiplex the PCR for EE0.6 sequence PCR and 16S rRNA PCR. One female specific band (370 bp) and one common band (590 bp) was observed in all the female birds, while only one band i.e. common observed in all male birds of guinea fowl as well as that of chicken (Fig. 1C). In general, for multiplexing, optimization is must and most critical factor is annealing temperature. In present study, the EE0.6 specific primers and 16S rRNA primers had the similar annealing temperature i.e. 60°C, therefore the PCR conditions used with EE0.6 primers were tested in multiplex PCR amplification and optimum amplification was obtained for 16S rRNA fragment. In all the species including guinea fowl, female showed 2 bands i.e. 370 bp female specific and 590 bp common band, while males showed only 590 bp band. D'Costa and Petitte (1998) and Itoh *et al.* (2001) also used multiplex PCR amplification successfully.

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