NON- INVASIVE SEX DETERMINATION OF GUINEA FOWL KEETS *(NUMIDA MELEAGRIS)* BY POLYMERASE CHAIN REACTION

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Abstract

Early sex determination of birds is crucial for controlled breeding for both production and conservation. Amidst the potential of the guinea fowl *(Numida meleagris)* industry for the economies of African countries and as a rich genetic resource to be conserved, inability to accurately sex guinea fowl keets is a major constraint for breeding. In the present study sex of day-old guinea keets (n=132) and keets of 4 - 8 weeks (n=72) was determined by polymerase chain reaction (PCR) by amplifying a sequence homologous to Chicken *Eco*R1 fragment of 0.6 kb (EE0.6) using Universal Sex Primer 1(USP 1) and Universal Sex Primer 3 (USP 3) together with internal control primers using DNA extracted from feathers. Out of 72 keets, aged 4 - 8 weeks, 38 were identified as males and 34 were identified as females. Out of the 132, day-olds 69 and 63 were identified as female and male keets, respectively, were Results from PCR were confirmed by the presence of respective gonads. The methods described can be used for accurate sex determination of guinea fowl keets from day-old with minimal stress and discomfort to the birds. The method can be used by researchers, breeders, conservationists directly or to develop farmer friendly methods in the future.

Introduction

Accurate sex determination is crucial at every stage of poultry production, breeding and conservation. Gender determination in chicks and young birds is important in the purchase of starter flock, early selection of birds for breed improvement and breeding programs in poultry production. Chick sexing is also important in *in situ* and *ex situ* conservation of animal genetic resources of a given breed (Morinha, Sabral & Bastos 2012; Sulandari & Zein, 2012). Early sex determination is crucial to study behavior, ecology and population structures for effective design of captive breeding programmes for birds (Liu, Zhao & Li 2010; Li *et al.*, 2012; Angat & Yusof, 2015). Amidst the importance of knowing gender of hatchlings early, there exist little or no sexual dimorphism among juveniles in most avian species. Vent sexing and feather sexing are the most common methods used for chick sexing for selective breeding (Kalina et al., 2012). Vent sexing is achieved by examination of external genitalia in the cloacal duct.

Accurate vent sexing in chicks is a difficult practice due to small size of genitalia and its ventral location, minimal differences in male and female external genitalia, and differences in shape of genitalia even within a single gender (Kalina et al., 2012; Liu et al., 2012). This makes vent sexing at hatch a highly specialised task that demands prior understanding of morphological differences between and within sexes for a given

bird species. Feather sexing can be done by visual discrimination based on colour, length or rate of feathering (Cerit & Avanus, 2007b; Kalina et al., 2012). However, it can only be done in breeds where an autosex genotype has been incorporated (Cerit & Avanus, 2007b; Liu *et al*., 2010; Morinha et al., 2012).

Helmeted guinea fowl is a poultry bird indigenous to Africa, that is now raised throughout the world. Guinea fowl derives its name from Coast of Guinea where it is thought to have evolved (Moreki & Radikara, 2013). Its' meat is a delicacy due to its characteristic gamy flavour while its production is an option for diversification of agriculture. However, both guinea fowl production and breeding are limited by several challenges including difficulty in sex determination in day-old chicks, known as guinea keets (Teye & Adam, 2000; Moreki & Radikara, 2013). Teye *et al.,* (2000) reported absence of sexually dimorphic features up to the latter part of life when males can often be identified by large dangling lobed wattles compared to smaller wattles in females. Hence, guinea fowls belong to a category of birds known as monomorphic birds. About half of the world's' bird species are said to be monomorphic with little or no sexual dimorphism particular during early stages of life (Jensen, Pernasetti & Durrant 2003; Liu et al., 2010).

Molecular sexing has been proposed as an alternative for sexing monomorphic birds. Molecular sexing involves utilization of DNA extracted from blood or other tissues, amplification of specific DNA sequences coupled with or without restriction digestion to generate sex specific banding patterns. However, there has been no reported gene identified on Z and W (sex) chromosomes which is unique to male or female gender in birds (Griffiths & Tiwari, 1993; Trukhina & Smirnov, 2014). However, Several genetic markers have been identified for avian gender determination that utilize allelic difference in homologous genes on Z and W chromosomes such as Chromo helicase DNA binding protein gene (CHD)(Griffiths et al., 1998).

Drosophila Nipped-B homolog (NIPBL) (Suh & Kriegs, 2011) and Chicken *Eco*R1 fragment of 0.6 kb sequence (EE0.6) (Itoh et al., 2001). Since their description these markers have been utilized for sex determination in several species of monomorphic birds (Clinton et al., 2001; Cerit & Avanus, 2007a; Harvey et al., 2006 and Kalina et al., 2012). Thanou *et al*.,(2013) applied CHD based markers for accurate sex determination of three of European Phalacrocoracidae species. Liu *et al*. (2010) applied CHD based primers for accurate sex determination of chicks and embyos in Chinese game cocks. Sulandari & Zein (2012) demonstrated that CHD based primers can be utilized to determine sex of 56 Indonesian wild birds. Vucicevic *et al* (2013) successfully applied PCR based on CHD specific primers for sex determination of 50 species of birds after evaluating the method on 58 species.

Itoh *et al.,* (2001) studied the utility of a region homologous to Chicken *Eco*R1 fragment of 0.6 kb sequence (EE0.6) for sex determination of a wide variety of Carinatae birds. EE0.6 sequence present on W chromosome is highly conserved in Carinatae birds. Z chromosome also contain a region homologous to EE0.6. However, the allelic length between the homologous sequence is different with a wide range of variation among species. After sequencing Z and W linked EE0.6 in 12 different species Itoh *et al*., (2001) designed four forward and three reverse primers from these diverse sequences. Different combinations of forward and reverse primers were attempted on 36 different species to identify suitable sex specific primer pairs for selected species.The combination of USP 1 and USP 3 together with internal control primers CPE15F and CPE15R was able to develop differential banding patterns for all Gallifomes tested including guinea fowl, and some birds belonging to other orders including Rock dove, Whale-headed stork and White pelican (Itoh *et al*., 2001).

The present study reports on a method based on Polymerase Chain reaction using Universal Sex Primer 1 (USP 1) and Universal Sex Primer

3 (USP 3) first described by Itoh *et al.* (2001) for accurate and non-invasive sex determination of guinea fowl keets at day 1 using their wing feathers.

Experimental

Animals

A total of 132 day-old keets and 72 keets of ages between 4 and 8 weeks of helmeted guinea fowls *(Numida meleagris)* were used for the study. Day-old keets were obtained after 28 days incubation, period using an artificial incubator, from eggs collected from a breeder flock maintained at the Livestock and Poultry Research Centre (LIPREC) of University of Ghana, Accra, Ghana (n = 154) and egg assemblers (those who are engaged in collection of fertile eggs from farmers and the wild habitats for sale) around Damango (n = 106) and Weichau (n = 82) located in Northern and Upper West regions of Ghana, respectively, between July 2012 and May 2013. Birds aged between 4 and 8 weeks were obtained from the experimental flock maintained at the Livestock and Poultry Research Centre of the University of Ghana. Sex discrimination of birds was not possible using phenotypic features at this stage. After sample collection the two groups of keets (day old keets and keets of age between 4 and 8 weeks) were raised in a deep litter house up to 24 weeks at Animal Research Institute, Accra, Ghana. At the end of the growth period the birds were euthanized and dissected to identify the gonads.

Feather sample collection

Feather samples were collected from young birds (4-8 weeks). Four to six feathers were collected into sterile polythene bags, transported and stored at room temperature for 1-2 weeks. Calami of feathers were cut to 1 mm pieces into sterile microcentrifuge tubes. In addition,n whole feathers from three different sites, including down feathers, wing feathers, and feathers from dorsal strips using sterile tweezers, were collected separately from newly hatched guinea fowl keets within 24 h after 28 days incubation period. Care was taken not to cause pain and trauma during sample collection.

DNA extraction and quatification

DNA was extracted from cut rachis of feathers of young guinea fowls and cut whole feathers from day-old keets by following protocol for tissues using DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA, USA) with 50 µl volume for the last DNA elusion step. DNA extraction was carried out at Biotechnology Laboratory, Animal Research Institute, Accra, Ghana. Extracted DNA was stored at -20 °C. After the preliminary amplification trials DNA samples were quantified by measuring absorbance at 260 and 280 nm using a Carri 6000i Spectrophotometer (Varian Inc. USA).

Primers and PCR conditions

Sex specific region of the long arm of W chromosome homologous to Chicken EE0.6 was amplified using sex s pecific primers USP1 (5'CTATGCCTACCACMTTCCCTATTGC3'), USP 3 (5'AGCTGGAYTTCAG WSCATCTTC T3') and internal control primers, Forward control primer (CPE15F) (AAGCATAG AAACAATGTGGGAC) and Reverse control primer (CPE15R) (5'AACTCTGTCTGGAAG GACTT3'), using a modified protocol described by Itoh *et al.,* (2001). For DNA extracted from cut rachis of young guinea fowls, PCR was performed in a final volume of 20 µl containing 0.2 mM each of dNTPs, 2 µM of each target and control primers, 10 ng of DNA extracted from feather, 0.25 U Taq Polymerase, 1 x Green Go Taq flexi buffer and 1.5 mM magnesium chloride (GoTaq® PCR Core System I, Promega Corporation, Madison, USA).

The cyclic conditions for PCR were as follows: Initial denaturation at 95 °C for 10 min, followed by 35 cycles at 95 °C for 30 secs, annealing at 60 °C for 30 secs, elongation at 72 °C for 60 secs and 72 °C for 5 min for final elongation. For DNA extracted from cut whole feathers plucked from the wing of day-old keets, PCR was performed in a final volume of 20 µl

containing 0.2 mM each of dNTPs, 2 µM of each target and control primers, 5 ng DNA, 0.4 U Taq Polymerase, 1 x Green Go Taq flexi buffer, 1.5 mM magnesium chloride (GoTaq® PCR Core System I, Promega Corporation, Madison, USA). Cycling conditions were similar to that of DNA from cut rachis described previously except for cycle number that had to be increased to 45 for adequate amplification for unambiguous differentiation of sex specific bands. PCR was performed in a Bio-Rad C1000 TM Thermal cycler (Bio-Rad Laboratories, Inc.CA, USA) at Molecular Genetics Laboratory, Department of Animal Science, University of Ghana, Legon.

PCR products were separated on 1.5 per cent agarose gel at 100 V for 30 min by electrophoresis in TBE (1 M Tris base, 1M Boric acid, 0.02 M EDTA) stained with gel red with a 100 bp marker and was visualized and recorded under a Ultra Violet transilluminator (Molecular Imager ® Gel Doc ™ XR + System, Biorad Laboratories, Inc., CA, USA). Genetic sex was determined from appearance of sex specific bands on the gel.

Results

Banding patterns

Using gel electrophoresis, PCR products from feathers from all age groups yielded similar banding patterns, where positive amplification was observed (except for some samples of shows feather types of day old keets) under the conditions specified in this paper. Fig. 1 represents the appearance of bands on the gel for PCR products from 12 birds from DNA extracted from keet wing feathers according to protocols described. Amplicons of female origin produced two bands of sizes 250 bp and 370 bp (F) while those from males produced only single bands of 250 bp (M).

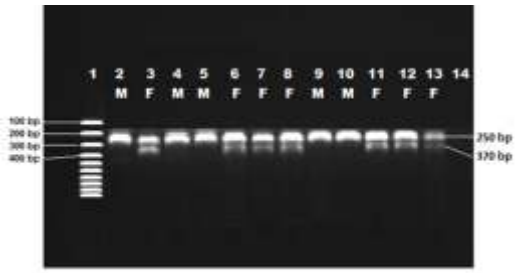

Fig. 1.*Banding patterns observed for males (M) and females (F) upon gel* electrophoresis together with 100 bp size marker (lane 1) and negative control (Lane 14)

Gender determination of young birds

Out of 72 young birds (aged 4 to 8 weeks) used for the study 38 were identified as males and 34 were identified as females by PCR. All the identified males were confirmed as males by presence of testes while all females identified were confirmed as females by the presence of ovaries when euthanized at maturity.

Gender determination of day old keets

Out of the 132 guinea keets 69 samples were identified as females and 63 were males according to PCR with DNA from wing feathers at day 1. After 12 mortalities during growth period observed between 8 and 12 weeks 120 birds remained up to 24 weeks. The flock that reached maturity (24 weeks) included 62 males and 58 females, according to PCR at hatch. All 62 remaining males contained testes while all females identified by PCR contained ovaries when dissected after been euthanized at maturity.

Comparison of results for different feather types for day-old keets

Although the method described and recommended for non-invasive sex determination in

keets in this paper use wing feathers, some preliminary observations are noteworthy. During initial optimization attempts to obtain similar results for different feather types at day old using 2 µl of DNA extract for all three feather types, considerable differences in sensitivity between different feather types under conditions described were observed. Table 1 summarizes the results observed for different feather types at day 1 when 2 µl DNA extract was used.

TABLE 1

Comparison of results obtained for PCR from different sources of DNA from - day old keets

Source of DNA	*No. of keets identified*		*No. of keets unidentified*	*Per cent of keets identified accurately*	*Per cent of keets not identified*
	Females	Males			
Feathers from shank	22	13	97	26.51	73.48
Feathers from dorsal strips	25	21	86	34.84	65.15
Wing feathers	69	63	0	100	0.0

Concentration of DNA extracts

Feather samples from group of birds aged 4 to 8 weeks contained an average DNA concentration of 5 ± 1.56 ng/ µl. Mean DNA concentrations for down feathers, feathers from dorsal strip and wing feathers were 0.24 ±0.18, 0.13 ± 0.07, and 2.24 ± 0.68 ng/ µl, respectively.

Discussion

Avian sexing is important in a wide range of applications in several branches of avian biology including ethology, ecology, genetics and evolution (Griffith et al., 1998; Cerit & Avanus, 2007b; Han et al., 2009). Several methods have been described for avian sex determination including vent sexing, laparoscopy, steroid sexing, feather sexing or karyotyping for monomorphic birds from nestling stage to maturity (Cerit & Avanus, 2007b). Laparoscopy yields most accurate results but may be harmful to the birds (Saino, Ellegren & Moller 1999), hence, is not suitable for-day old-keets and young birds. Vent sexing is simple but may not yield accurate results in chicks (Kalina et al.,2012). Feather sexing is only possible in specific breeder lines in which an auto sex character has been introduced by crossing (Cerit & Avanus, 2007 b; Kalina et al., 2012).

Molecular sexing utilizes PCR to generate differential banding patterns for males and females. So far Chromohelicase DNA binding protein gene (CHD) is the most widely used gene for molecular sexing due to presence of allelic variants that differ in length on sex specific Z and W chromosomes (Griffiths & Tiwari, 1993; Griffith *et al.,* 1998 ;Kalina et al., 2012). Several workers have designed primers for selective amplification of these sequences (Griffith *et al.,* 1998; Horng *et al.,* 2006; Han *et al.,* 2009).Ogawa *et al.,* (1997) described a unique sequence present on W chromosome of chicken conserved among Carinatae species for the purpose of sex determination. Itoh *et al.* (2001) utilized this unique sequence homologous to Chicken *Eco*R1 fragment of 0.6 kb sequence (EE0.6) for sex identification of Carinataes, including guinea fowls and evaluated primers available and PCR conditions to detect W specific sequences. PCR with such primers has been extensively used for sexing birds in a wide range of applications including population studies (Bush *et al.,* 2011). Behavioural studies (Gray, & Hammer 2001; Lewis *et al.,* 2002), were study of mating systems and establishing sex ratios (Wittingham & Dum 2000; Wink *et al.,* 2011), and captive

breeding programs for conservation (Jensen *et al.,* 2012; Kasuga *et al.,* 2012). Malagó *et al.* (2002) reported on adapting PCR with previously described primer combinations for large scale sex typing in Ostriches.

During the present study banding patterns similar to that observed by Itoh *et al.* (2001) for guinea fowls under the conditions specified in this paper, were observed. The forward W specific primer USP1 and reverse W specific primer USP 3 anneal to W chromosome sequences in females to yield an amplified fragment of 370 bp. As W chromosomes is female specific presence of 370 bp band indicates a genetic female. The second band of 250 bp is the amplified product of internal control primers annealing to *spindlin* gene present both on Z and W chromosomes. Incorporation of internal control primers act as a positive control to detect amplification under same conditions. Thus, under conditions described in the present study a sample from female origin will yield two bands of sizes 250 bp and 370 bp while a sample of male origin will only produce a single band of 250 bp amplified from *spindlin* gene (Fig. 1).

In the present study cut rachis from feathers of young guinea fowls and whole feathers from day old keets instead of blood were used. This makes the present approach non-invasive, causing minimal stress to birds compared to collection of blood. This is particularly useful in guinea fowls as they are extremely sensitive to stress. Feathers can be conveniently plucked from young guinea fowls or with sterile tweezers at day-old. The collected feathers can be easily transported at room temperature making this method useful in breeding and study of wild populations. Thus, the present study provides evidence that cut rachis from growers and whole wing feathers provide adequate cell numbers for amplification.

Similar observations were made by Harvey *et al.* (2006) in their attempt to compare use of plucked feathers and blood for molecular sexing in Black-capped Chickadees. Harvey *et al.* (2006) recommended plucked feathers for sex determination particularly in wild birds. Smith et al., (2003) discussed utility of feathers as a source of DNA and proposed the use of feathers in a wide range of application in molecular genetics. Bello, Francino & Sanchez, (2001) described a purification method for genomic DNA with phenol: chloroform: isoamyl alcohol extraction and ethanol precipitation from bird feathers belonging to 120 bird species. Angat & Yusof (2015) applied PCR for non-invasive sex determination of Stripe-throated Bulbul (*Pycnonotus finlaysoni*), Olive-winged Bulbul (*Pycnonotus plmosus*), White-throated kingfisher (*Halycon smyrnensis*) and Collard Scops Owl (*Otus lettia*). Liu *et al*., (2010) applied feathers plucked from breast feathers to develop a PCR based kit for non-invasive sex determination of Chinese game cocks. Use of feathers instead of blood is becoming important in the back drop of emerging animal rights and welfare issues (Cerit & Avanus, 2007a).

Different sensitivities observed for different feather types of day old keets during the preliminary stages of this study suggested possible variations in DNA concentrations in different types of feathers in hatchlings. Results from Spectrophotometry that was available subsequently further explained these observations. The observed differences in DNA concentration and sensitivities may be due to varying number of viable cells in the pulp of rachis due to differences in size or structure between different types of feathers. Majority of feathers from the shank and dorsal side did not produce observable amplification amidst repeated optimization indicating low numbers of viable cells. This suggest that the observed DNA concentrations 0.24 ± 0.18, 0.13 ± 0.07 ng/ µl for these types of feathers could not produce detectable amplification under the specified PCR conditions following specified imaging method. Future research is necessary into approaches to improve DNA yields from these feathers or to further incrase sensitivity of PCR by modifying PCR conditions.

No previous literature available on comparative quantitative or semi-quantitative DNA

yields from the type of feathers used in the current study from hatchlings for meaningful comparisons. Even with wing feather DNA samples 100 per cent sensitivity was only observed after several optimization reactions during preliminary stages of this study that has not been discussed here. However, all feathers plucked from the wings produced detectable bands in day-old keets under conditions specified in this paper. This suggest that 5 ng of DNA can produce reliable results using PCR conditions specified in the methodology. Thus, the use of four to six feathers, plucked from the wing for consistent and reliable results for sex determination in guinea keets under conditions specified. Use of wing feathers and extraction method described may also be useful in other applications of molecular genetics such as parentage testing, genotyping for marker assisted selection and diversity studies etc., in guinea fowls.

Out of 69 female and 63 male keets sexed at hatch, 62 males and 58 females reached maturity with 12 recorded mortalities. Out of the females 48 contained ovulating ovaries while rudimentary non ovulating ovaries were observed in 10 females. This difference in point of lay may be due to breed differences, and is worth investigating in the future in order to obtain a uniform flock for large scale intensive production.

Sex identified by the presence of respective gonads of all surviving guinea fowls dissected at maturity was in agreement with results from PCR. This suggests that sex determination by PCR using wing feather DNA in keets, or cut rachis in other age groups of guinea keets, yields accurate results, and can be reliably used in any application that needs information on sex of guinea keets.

Conclusion

The method described in this paper based on PCR using plucked wing feathers can be used for rapid, reliable and non-invasive sex determination in guinea fowl keets from day old in controlled breeding programmes aimed at improving breeds, breeder stock management and in captive breeding programs for conservation. The current method can also be used as a baseline method to evaluate the different approaches followed by farmers for sex determination of keets, and to develop more farmer friendly methods in the future. Future research is also recommended to evaluate the different feather types as sources DNA for molecular genetic studies of this important bird species.

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