Comparison of vent sexing and polymerase chain reaction for reliable sex determination in guinea fowls

K. M. J. AHIAGBE*, B. B. KAYANG, A. NAAZIE, P. K. BOTCHWAY & A. A. AGBOLOSU

(K. M. J. A.: Animal Research Institute, Council for Scientific and Industrial Research, P.O. Box AH 20, Achimota, Accra, Ghana; B. B. K., P. K. B.: Department of Animal Science, University of Ghana, Legon, Ghana; A. N.: Livestock and Poultry Research Centre, University of Ghana, Legon,Ghana.; A. A. A.: Department of Animal Science, University for Developmental studies, Tamale, Ghana)

**Corresponding author's current email address: mariestaa@gmail.com*

ABSTRACT

The guinea fowl is an important poultry species with great economic potential in Africa. It is a monomorphic bird with less conspicuous sexual dimorphism. Inability to sex birds accurately early in their life imposes multiple challenges on breeding, conservation and production of these birds. Several methods have been employed for sexing monomorphic birds each with specific advantages and disadvantages. In the present study, sexes of 215 guinea fowls were determined by Polymerase Chain Reaction (PCR), Vent Sexing and determination of gonads. PCR was used to amplify a sequence homologous to Chicken *Eco*R1 fragment of 0.6 kb (EE0.6) using Universal Sex Primer (USP)1 and USP3 and internal control primers. Vent sexing could only diagnose 48.7 % of males accurately while it was able to diagnose females with an accuracy of 81%. Results from PCR was in complete agreement with sex indicated by gonads. Differences in results between PCR and vent sexing were significant ($p < 0.05$). Vent Sexing alone is not reliable for sexing guinea fowls prior to breeding decisions. Molecular sexing using the method described is recommended for accurate sex determination for breeders and researchers while future research is necessary to develop farmer friendly guidelines for reliable sex determination of guinea fowls.

Original Scientific Paper. Received18 Jul 16; revised 20 Apr 17

Introduction

The helmeted guinea fowl (*Numida meleagris*) is an important poultry species in West Africa. It is native to Africa and derives its name from Guinea Coast where it is believed to have evolved (Crowe, 1978). In Ghana guinea fowl is widely reared in the three Northern regions where small holder farmers make greater financial gains compared to raring chicken (Teye & Gyawu, 2001; FAO, 2014). It is an important source of income and protein and plays important socio-cultural, economic and nutritional roles for its keepers (Dei & Karbo, 2004; Avornyo *et al*., 2013; Dei *et al*., 2014). Due to its West African origin the indigenous population of this species is expected to be rich in adaptive genes representing a high degree of genetic variation.Guinea fowls are hardy, disease resistant (Jacob & Pescatore, 2013) and hence suitable for low input agriculture common in tropical countries.

Demand for guinea fowl meat always exceeds the supply in Ghana (Karbo & Avornyo, 2006). Therefore, development of the guinea fowl industry plays a vital role in strategies to ensure food and nutrition security particularly in the Northern part of the country. However the development of the guinea fowl industry as a

sustainable means of livelihood is limited by several challenges including inability to accurately sex guinea fowls (Teye *et al*., 2000). Moreki & Radikara (2013), described inability to sex guinea fowls as a challenge for commercialization of guinea fowl production in Africa.

The guinea fowl is a monomorphic bird with no sexually dimorphic features expressed in early stages (Awotwi, 1975; Teye *et al.,* 2000). Inability to determine sex of monomorphic birds results in multiple challenges in breeding, breed improvement and several other points in the guinea fowl value chain. Unless the sex of parents is known, guinea fowl keepers and breeders cannot maintain a good breeding stock for selection. Teye *et al*., (2000), reported that the inability to sex the birds early often leaves a flock with none or few females after sexing following methods used by farmers. It becomes difficult for breeders and farmers to introduce the correct sex ratio to ensure high fertility and hatchability. Cerit & Avanus (2007b), reported that difference in care cost of male and female and time spent for reproduction causes considerable financial losses in raring monomorphic birds. Thus, it is important to develop reliable methods for early sexing that can be easily adopted by farmers.

Several methods have been used to sex monomorphic birds with varying degrees of accuracy including examining the cloacal duct, endoscopy, hormone determination, and cell gene analysis (Jones *et al*., 1984; Cerit & Avanus, 2007b). Laparoscopy yields the most reliable results although it can be harmful or lethal to the birds (Saino *et al*., 1999; Cerit & Avanus, 2007a). Faecal steroid sexing is expensive, largely depends on the method used while yielding accurate results only in breeding season with mature birds (Cerit & Avanus, 2007b).

Feather sexing and vent sexing are among the most used methods by breeders for chick sexing. Feather sexing can only be performed in well-established breeds in which an autosex character such as feather colour or rate of feathering has been introduced into the breeds (Kalina *et al.,* 2012). Although this has been achieved in chicken, so far no such breed of guinea fowls with genetically incorporated autosex characters in which feather sexing can be performed accurately has been developed. Vent sexing on the other hand is a method popularised by a Japanese Professor, Kioshi Maui and is the most widely used method for chick sexing in chicken (Cerit & Avanus, 2007b). Teye *et al*., (2000), proposed the use of vent sexing for sexing guinea fowls from the sixth week of age.

Sex in birds is genetically determined. The female is heterogametic (ZW) while the male is homogametic (ZZ). Several assays based on Polymerase Chain Reaction (PCR) have been described to determine the genetic sex of monomorphic birds (Griffiths & Tiwari, 1993; Griffiths *et al.,* 1998; Itoh *et al*., 2001; Huang *et al.,* 2003; Jensen et al., 2003; Horng et al., 2006; Cerit & Avanus, 2007a). These assays are based on variations in alleles or sequences present in Z and W chromosomes including Chromohelicase DNA binding protein (CHD) gene (Griffiths *et al.,* 1998) and *Eco*R1 fragment of 0.6 bp sequence (EE0.6) described first in the long arm of Chicken W chromosome (Ogawa *et al.,* 1997). Itoh *et al*., (2001) cloned EE0.6 sequences from 12 different avian species and proposed four forward and three reverse primers that can be usefull in determination of sex in 36 different species of Carinatae family using PCR with a suitable forward and reverse primer pair. Differential banding patterns were observed in male and female guinea fowls for PCR with Universal Sex Primer (USP)1 and USP3 as sex specific primers together with internal control primers that amplify a common sequence present on both W and Z chromosomes. Ahiagbe *et al.,* (2012) successfully applied these primers for non-invasive sex determination of guinea fowls including keets.

In this study we determined genetic sex of young guinea fowls using primers described by

Itoh *et al.,* (2001) by using a modified protocol with DNA extracted from feathers and compared the genetic sex with sex suggested by vent sexing, using gender determined by the presence of respective gonads as the standard.

Materials and methods

Animals

Two hundred and fifteen 16 weeks old guinea fowls from Ghanaian local breeds whose sex could not be differentiated externally were used for the study. The birds were randomly selected from a grower phase flock maintained at the Livestock and Poultry Research Centre (LIPREC) of University of Ghana.

Vent sexing and samples

Sexes of each of the 215 birds were determined by both vent sexing and PCR at 16 weeks. Each bird was tagged and vent sexed by observing presence of phallus in the cloaca by a trained animal breeder according to the procedure described by Teye *et al.,* (2000). Feather samples were collected into sterile polythene bags and transported for laboratory analysis. After sampling birds were maintained up to maturity. At 24 weeks all surviving birds were euthanized to confirm the sex by presence of ovaries and testes.

The results from vent sexing were compared with the genetic sex determined by PCR and the presence of respective gonads.

DNA extraction from feathers

About 4 mm length of the proximal follicular end of the calamus was cut into pieces with sterilized scissors. DNA was extracted from feather follicles using DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA, USA) from all 215 birds at Biotechnology laboratory of Animal Research Institute, Accra, Ghana. Extracted DNA was stored at -20^{0}C until use.

PCR for sex determination

Primers used were Universal Sex Primer 1 (USP1) (5'CTATGCCTACCACMTTCCCTATTGC3'), Universal Sex Primer 3 (USP3) (5'AGCTGGAYTTCAGWSCATCTTCT3') together with internal control primers namely Forward Control Primer (CPE15F) (AAGCATAGAAACAATGTGGGAC), Reverse Control Primer (CPE15R) (5'AACTCTGTCTGGAAGGACTT3') (Itoh *et al.,* 2001). 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, 20 ng of extracted feather DNA, 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) by modifying the protocol described by Itoh *et al.,* (2001). The PCR conditions used for the thermal cycler (Bio-Rad C1000 ^{TM} Thermal cycler, Bio-Rad Laboratories, Inc. USA) were as follows: Initial denaturation at 95°C for 10 minutes, followed by 35 cycles at 95°C for 30 seconds, annealing at 60°C for 30 seconds, elongation at 72°C for 60 seconds and finally 72^{o}C for 5 minutes for final elongation (Ahiagbe *et al.,* 2012). PCR was performed at Molecular Genetics Laboratory, Department of Animal Science, University of Ghana, Legon, Ghana.

PCR products were separated on 1.5% agarose gel at 100 V for 30 min by electrophoresis in TBE buffer (1 M Tris base, 1M Boric acid, 0.02 M EDTA), stained with gel red and visualized under a UV transilluminator. Size of bands were determined relative to 100 bp size marker (Promega Corporation, Madison, USA). Appearance of sex specific bands was studied to determine genetic sex. PCR was performed in duplicates to veryfy the reproducibility.

Results from vent sexing were later evaluated based on genetic sex as the standard due to its

accuracy reported elsewhere (Huang et al., 2003; Horng et al., 2006; Cerit & Avanus 2007a; Kalina et al., 2012) and confirmed during the current study by the presence of respective gonads.

Statistical analysis

Conformity or deviation of vent sex to/from genetic sex was evaluated by performing Chi-squared test with Yates correction for continuity. Dichotomous Nominal correlation coefficient was also calculated to evaluate the correlation between genetic sex and gonadal sex and genetic sex and vent sex (Naiman *et al.,* 1983). Percent error rate (number of birds wrongly assigned/total number of birds x 100%) was calculated for both PCR and vent sexing.

Results

Genetic sex determined by PCR

When target sex specific genes were amplified using USP1 and USP3 in a single reaction with internal control primers CPE15F, CPE15R with DNA extracted from feathers as template, samples of female origin produced 2 bands of sizes 250 bp and 370 bp upon gel electropho-resis while samples from males resulted only in a single band of 250 bp (Fig. 1). Results for genetic sex for all 215 birds were consistent in all duplicates.

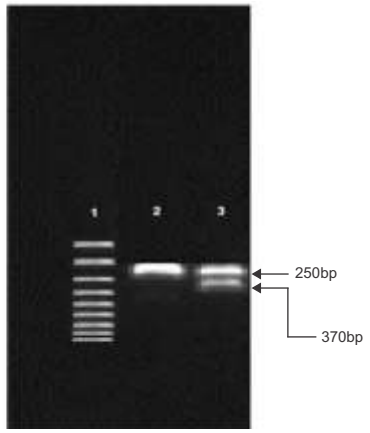

Fig.1 Differential banding patterns of PCR products from male and female Guinea fowls (Lane1, 100 bp size marker; Lane 2: PCR products from a male; lane 3, PCR products from a female)

Population structure according to PCR-based sexing and vent sexing

Fig. 2 represents the population structure according to genetic sex determined by PCR (a) and vent sexing (b). Out of the total of 215 birds sampled PCR detected 115 males (53.5%) and 100 females (47.5%) while vent sexing identified 75 males (35%) and 140 females (65%).

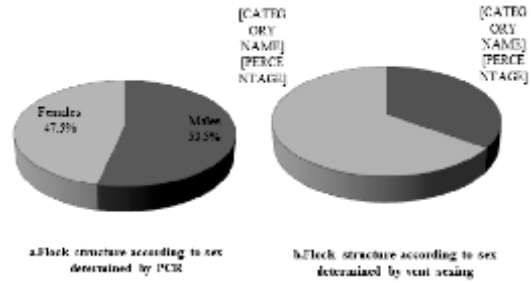

Fig. 2. Flock structure by PCR-based sexing (a) and rent sexing (b)

Comparison of genetic sex with gonadal sex

All birds (115/215) identified as males by PCR were characterized by the presence of testes while the remainder (100/215) identified as females contained ovaries when dissected after being euthanized. Seventy four of the females (74/100) showed developing follicles while the remaining twenty six (26/100) contained small non ovulating ovaries. Due to complete agreement of results from PCR with gonadal sex the genetic sex was directly compared with the results from vent sex.

Comparison of genetic sex (gonadal sex) with vent sexing

Table 1 represents the frequencies of observations based on genetic sex (gonadal sex) and vent sex determinations in the guinea fowl population studied.

TABLE 1

Observed frequencies for sex determined by vent sexing for genetic male and female guinea fowls confirmed by gonads

Genetic sex/ Gonadal Sex	*Vent sex*	*Frequency*	*% Frequency*
Male (115)	Male	56	26.05
	Female	59	27.44
Female (100)	Female	81	37.67
	Male	19	8.84
Total (215)		215	100

Of the 115 males detected by PCR (and confirmed by gonadal sexing), vent sexing correctly identified only 56 (48.7%) and wrongly assigned 59 (51.3%) as females. Similarly, out of the 100 females detected by PCR, sexing by the vent method accurately identified 81 (81%) and wrongly designated 19 (19%) as females. Vent sexing therefore had an overall error rate of 78/215 or 36.3% in this study.

Statistical Analysis

Chi-squared calculated was 29.86 with Yates correction. This suggests that results from vent sexing deviate from results from genetic sex significantly. Dichotomous nominal correlation coefficient between genetic sex and vent sex was 0.27 and was statistically significant ($P < 0.05$).The correlation coefficient between genetic sex and gonadal sex was +1.

Discussion

When target sex specific genes were amplified using USP1 and USP3 in a single reaction with internal control primers CPE15F and CPE15R from DNA extracted from feathers as template, differential banding patterns observed for males and females were similar to earlier reports by Itoh *et al.,* (2001). Ahiagbe *et al.,* (2012) reported similar results in their attempts to apply PCR for non-invasive sex determination of guinea fowl keets.Even before the conformity of methods are compared statistically the flock structures determined and represented in Fig. 2 suggests a considerable deviation of the two methods. Majority of birds were males accor-ding to genetic sex while the majority of birds were females according to vent sexing. There is no data available from previous work that describes population structure of unselected populations in confinement or in wild popula-tions of guinea fowls for comparisons.

Confirmation of genetic sex with gonadal sex

The results from PCR were completely in agreement with the sex identified by presence of their respective gonads. Presence of gonads is the ultimate indicator of sex. This suggests that results from PCR are accurate and hence can be reliably used to identify the sex of guinea fowls. Use of PCR as a diagnostic tool for accurate sex determination has been recommended by previous studies on several species (Huang et al., 2003; Horng et al., 2006; Cerit & Avanus, 2007; Kalina et al., 2012). Thus in this study there on we directly compared genetic sex and vent sexing in guinea fowls.

Comparison of genetic sex and vent sex

In the current study vent sexing identified females with a greater accuracy (81%) than males (48.7%).This may be due to anatomical differences between male and female guinea fowls. The male guinea fowl is characterized by the presence of phallus in the cloaca. However depending on the location, the phallus may not be visible during vent sexing. This may be one of the reasons for observed error rate in males using vent sexing. Females do not possess phallus and this probably makes it easier to be identified by vent sexing. However further investigations into anatomical differences of the cloacal duct of guinea fowls is necessary to better understand the reasons for this differences. Guidelines for vent sexing guinea fowls should be then developed while evaluating accuracy with determination of gonads or PCR prior to disseminating the guidelines to breeders.

Beside these observed differences in error rates, Chi-square calculated was 29.86 with Yates correction factor was significant. Thus there is significant deviation of results expected from gonadal sex and from observed results according to vent sexing, while results from PCR were in complete agreement with gonadal sex. Dichotomous nominal correlation coefficient between gonadal sex and vent sex was 0.27 representing a weaker positive correlation that is statistically significant ($P<0.005$), while a correlation coefficient of +1 between gonadal sex and PCR results indicates that results from two methods are perfectly correlated.

This indicates that although theres a positive correlation between genetic sex and vent sex, vent sexing is not a reliable technique for accurate sexing in the grower phase. As indicated by the results, genetic sex determined by PCR is a reliable method for sex determination in guinea fowls and can be used for sex determination for breeding in any situation that needs accurate information of the gender of parents. As sex is determined by the genetic blue print of an animal, molecular sexing provides a better alternative for sexing monomorphic birds including guinea fowls.

Conclusions and recommendations

Vent sexing alone is not reliable for accurate sex determination of guinea fowls during the grower phase. Until further research identifies sexually dimorphic features, if any, based on scientific evidence verified by gonadal sex or genetic sex, determination of genetic sex by PCR with sex-specific primers is recommended for accurate sex determination for breeders and researchers. However, PCR cannot be directly adopted by farmers at the farm level. Therefore the method described here can be applied to validate indigenous knowledge of farmers on sexing guinea fowls and to develop farmer friendly guidelines for sex determination in guinea fowls. Further research is also necessary to study anatomical differences between male and female guinea fowls in order to develop guidelines for vent sexing guinea fowls to increase accuracy of vent sexing.

Acknowledgement

The research was partly supported by Canadian International Development Agency through Food and Agricultural Budgetory support Scheme of Council for Scientific and Indistrial Research, and Ministry of food and Agriculture of Ghana. Authors are greatful for their financial support. Authors also ackowledge Managemnt and staff of Livestock Poultry Resesearch Centre of University of Ghana and Animal Research Institute for their encouragement and contributions.

REFERENCES

Ahiagbe, K. M. J., Kayang, B. B., Avornyo, F. & Botchway, P. K., (2012) Development of molecular Assay for rapid reliable and non-invasive sex determination of Guinea fowl keets (*Numida meleagris*).*CSIR-Animal Research Institute 2012 Annual Report,* CSIR-ARI, Accra, Ghana, 40-42.

Avornyo, F. K., Salifu, S., Moomen, A. & Agbolosu, A. A. (2013) Effect of dietary protein on the performance of local guinea keets in the Northern Region of Ghana. *Greener Journal of Agricultural Sciences,* **3**(7), 585-591.

Awotwi, E. K. (1975) Some aspects of the reproductive physiology of male and female guinea fowls. *Unpublished MSc. Thesis.* Faculty of Agriculture, Univ. of Ghana, Legon.

Cerit, H. & Avanus, K., (2007a) Sex determination by CHDW and CHDZ genes of avian sex chromosomes in *Nymphicus hollandicus*, *Turkish Journal of Veterinary and Animal Sciences*, **31**(5), 371–374.

Cerit, H. & Avanus, K., (2007b) Sex identification in avian species using DNA typing methods. *World's Poultry Science Journal*, **63**, 91–100.

Crowe, T.M. (1978) The evolution of Guinea fowl (Galliformes, Phasinidae, Numidae). *Annnals of the South African Museum*, **76**, 43– 136.

Dei, H. K., Alenyorege, B., Okai, D. B. & Larbi, A. (2014) Assessment of rural poultry production in northern Ghana. *Ghanaian Journal of Animal Science,* **8**(1), 101-114.

Dei, H. K. & Karbo, N. (2004) *Improving Small holder Guinea fowl Production in Ghana: A training manual*. University for Development Studies, Tamale and Animal Research Institute (CSIR), Nyankpala Station, Tamale, Ghana.

FAO (2014) Poultry Sector Ghana. *FAO Animal Production and Health Livestock Country Reviews*. No. 6. Rome.

Griffiths, R. & Tiwari, B., (1993) The isolation of molecular genetic markers for the identification of sex. *Proceedings of the National Academy of Sciences of the United States of America*, **90**, 83248326.

Griffiths, R., Double, M. C., Orr, K. K. & Dawson, R. J. G. (1998) A DNA to sex most birds. *Molecular ecology*, 7, 1071-1075.

Horng, Y. M., Wu, C. P., Wang, Y. C. & Huang, M. C. (2006) A novel molecular genetic marker for gender determination of Pigeons. *Theriogenology*, **65**, 1759-1768.

Huang, M. C., Lin, W. C., Horng, Y. M., Rouvier, R. & Huang, C. W. (2003) Female-specific DNA sequences in geese. *British poultry science*, **44**, 359364.

Itoh, Y., Suzuki, M., Ogawa, A., Munechika, I., Murata, K. & Mizuno, S. (2001) Identification of the sex of a wide range of Carinatae birds by PCR using primer sets selected from chicken EE0.6 and its related sequences. *The Journal of heredity*, 92, 315321.

Jacob, J. & Pescatore, T. (2013) *Raising guinea fowl*. University of Kentucky College of Agriculture, Food and Environment, Lexington, KY, 40546, USA.

Jensen, T., Pernasetti, F. M. & Durrant, B., (2003) Conditions for rapid sex determination in 47 avian species by PCR of genomic DNA from blood, shell-membrane blood vessels, and feathers. *Zoo Biology,* **22**, 561571.

Jones, D. M., Samour, R. J., Knight, J. A. & Finch, J. M. (1984) Sex determination of monomorphic birds by fibreoptic endoscopy. *Veterinary Research,* **115**, 596-598.

Kalina, J., Mucksová, J., Yan, H. & Trefil, P. (2012) Rapid sexing of selected Galliformes by Polymerase Chain Reaction. *Czech Journal of Animal Science,* **57**(523), 187192.

Karbo, N. & Avornyo, F. K. (2006) State of Guinea fowl Industry in the Northern Region. *A Report commissioned by ActionAid International Ghana*. RUMNET Publishers, Tamale, Ghana.

Moreki, J.C. & Radikara, M.V. (2013) Challenges to Commercialization of Guinea Fowl in Africa, *International Journal of Science and Research,* **2**(11), 436-440.

Naiman, A., Rosenfeld, R. & Zirkel, G., (1983) *Understanding Statistics*, McGraw-Hill, Inc. , New York.

Ogawa, A., Solovei, I., Hutchison, N., Saitoh, Y., Ikeda, J., Macgregor, H., & Mizuno, S. (1997) Molecular characterization and cytological mapping of a non-repetitive DNA sequence region from the W chromosome of chicken and its use as a universal probe for sexing Carinatae birds. *Chromosome Research*, **5**, 93101.

Saino, N., Ellegren, H. & Moller, A. P. (1999) No evidence for adjustment of sex allocation inrelation to paternal ornamentation and paternity in Barn Swallows. *Molecular Ecology* **8**, 399-406.

Teye, G. A. & Gyawu, P. (2001) The benefits of intensive indigenous Guinea fowl production in Ghana. *World Poultry,* **17**(9), 53-54.

Teye, G.A., Gyawu, P., Agbolosu, A., Dei, H.K. & Adjekum, Y.A. (2000) Simple technique for sexing young guinea fowl. *World Poultry*, **16**(7), 22-23.