GENDER IDENTIFICATION IN EMU (DROMAIUS NOVAEHOLLANDIAE) - A REVIEW

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ABSTRACT

Distinct phenotypic sexual dimorphism servers as one of the key factors that can enable one to categorize the individual to a particular sex. However in case of ratite bird emu (Dromaius novaehollandiae) this phenotypic distinctness between males and females is less evident and careful observation of adult birds would help to identify the sexes. Other methods of sex identification such as the sexing by feather pattern observation, vent sexing, surgical sexing and karyotyping do not appear to yield confirmatory results. The identification of a sequence of DNA on sex chromosomes and then its use as a marker for sex diagnosis in such species where sexing of the birds is difficult can solve the problem, as the pairing of the birds could be done at the chick stage. Such confirmatory methods of sexing would be highly beneficial for the emu farmers and breeders.

Key words: Ratite, Sex chromosomes, Sexual dimorphism.

Ratites are flightless birds, with a raft-like breastbone devoid of a keel. In these birds, breast muscles are vestigial to non-existent. Having evolved from flighted ancestors, ratites share numerous characteristics with other birds. Ratites are precocial birds that produce young capable of moving and foraging for feed within the first 48 h after hatching (Angel, 1996).

Gender identification is extremely important for breeding management in avian species. In many species of monomorphic birds sex determination on the basis of phenotypic study or external morphology is difficult, especially at the young stage. Similar difficulties are encountered by the farmers while determining sex in Emu (Dromaius novaehollandiae) as no sexual dimorphism occurs in these birds during immature stage.

Various methods of gender identification have developed over the years in ratites as well as in other avian species. Recently developed molecular methods for the assignment of sex in avian species have facilitated studies in both ecology and conservation. The breeding programmes of ratites would benefit from the accurate identification of male and female birds. The current review focuses on the literature for gender identification methods that have been used in ratite as well as some non ratite birds which can also be applicable for the ratite birds.

Vocalisation: The voice change in emu begins when the birds are 10 to 14 months of age. At this time both sexes will be heard making grunting sounds. Soon after the female goes through a physical change whereas the male does not, her throat sac begins to develop. During this time the sounds she makes will vary from grunting to a guttural drumming. By the time she is sexually mature, the female would have “found her voice” and it will be easily distinguished from that of a male grunt (http://www.aae-emu.org). Emu can be sexed, after they reach sexual maturity at 18 to 24 months of age, by the difference in the type of calls made by the opposite sexes during the breeding season.

The mature emus have visual characteristics that can assist in sex assignment as the female emus usually have a pronounced air sac at the base of the throat. They also make a booming and drumming sound unlike the males which make pig like grunting sound and have no pronounced air sac (Robinson, 1996 and Reddy, 2005).

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Phenotypic and behavioural difference between the sexes: Feather pattern in male chicks is bullseye pattern and females have an irregular feather pattern (http://www.aea-emu.org). Reddy (2005) stated that the adult emu females are larger than the males, especially during breeding season when the males may be fast. The female is the dominant member of the pair and the brooding behavior has been observed in case of males.

Vent sexing: Gender identification can be done through cloacal examination by studying the presence of phallus or clitoris. In emu it is difficult to tell the difference between the sexes from 3 to 14 months because the growth rate of the sexual organs is non-existent, often causing mistakes in gender identification. The sexual organs enlarge at puberty and then again at the onset of breeding season. In ostrich sex determination carried out by the use of a proctoscope in addition to digital examination for the presence of phallus on the ventral floor of proctodeum, revealed that it is difficult to sex the ostriches, as the penis of the male is still tiny and easily confused with the clitoris of the female (Samour et al., 1984). Visual examination for the presence of a phallus with a sulcus and visible blood vessels has been used for distinguishing males from the females in juvenile ostriches (Gandini and Keffen, 1985). Stewart (1989) noted that digital examination and proctoscopy made vent sexing was much easier in 1-3 months old ostrich birds.

Sex assignment in emu can be done at sixteen months of age or older by a vent sexing method called “three man take down”, wherein two handlers grab a leg each and turn the bird upside-down, while the third person using a gloved finger, inverts the vent exposing the phallus or the clitoris thus visually confirming the sex of the bird (Brackett, 1996).

Ogawa et al. (1998) tested the signs of genetic differentiation between homomorphic Z and W chromosomes on ratites, by using two marker genes (Z-linked ZOV3 and the gene for the iron-responsive element- binding protein) and one marker sequence of a part of a presumptive pseudogene (W-linked EE0.6 of the chicken). Their homologues, maintaining 71–92 per cent identities to the chicken counterparts, were found in both the emu (Dromaius novaehollandiae) and the ostrich (Struthio camelus). Their locations were visualized on chromosome preparations by fluorescence in situ hybridization. In the case of the emu, these three marker sequences were localized on both members of the fifth pair of a
female, thus revealing no sign yet of genetic differentiation between the Z and the W. The finding was the same with regard to both members of the fourth pair of male ostriches. In the female ostrich, however, the sequence of the gene for the iron responsive element-binding protein was missing from one of the pairs, thus revealing the differentiation by a small deletion of W from Z. Ellegren (2001) studied that it is difficult to count all the chromosomes in avian karyotype as most of them are microchromosomes.

**DNA based techniques for sex identification:**

The developments in DNA technology have enabled DNA typing as the most reliable method for sex identification in mammals and birds. The invention of Polymerase Chain Reaction (PCR) by Kary Mullis in the mid 1980s has revolutionized the field of molecular biology. Over the years scientists have come up with various variants of PCR such as multiplex PCR, reverse transcriptase PCR, real time PCR, nested PCR and Random amplified polymorphic DNA (RAPD) which have contributed immensely to the research in the field of molecular biology. Polymerase chain reaction can be used as an effective tool for gender identification of the monomorphic birds such as the Emu. Molecular methods for sex diagnosis require isolation of DNA from body tissues, the feather and blood have been found most favorable tissues for extraction of DNA.

Bello and Sanchez (2001) developed a rapid and accurate protocol for high quality DNA isolation from feather. The technique included a lysis step of feather quill, which differed in temperature and time depending on the feather size. Purification of genomic DNA was performed with phenol: chloroform: isooamyl alcohol extraction and ethanol precipitation. Malago et al. (2002) devised a technique for large scale DNA extraction in ostrich from feather bulb in which feather bulbs were submerged in wells from a 96 well PCR plate containing 20 µl of 0.2 N NaOH. The plate was covered with a plastic lid, placed in a PCR machine with the hot lid disabled and the machine set for 75°C, 20 min. The neutralisation was then carried by adding 180 µl of a 0.04 M Tris-HCl (pH 7.5) solution.

A technique of DNA isolation from the feather of kiwi involving the DNA isolated from the pulp of 1-4 feathers by incubation at 55°C overnight with 200µl of SET buffer (100 mM NaCl, 10 mM EDTA, 50 mM Tris-Cl pH 8.0), 100µg Proteinase K, 0.5 per cent SDS, and 20 mM dithiothreitol. The mixture was then extracted with an equal volume of phenol: chloroform and DNA was precipitated with 0.5 volumes of 7.5 M ammonium acetate and 3 volumes of ethanol (Huynen et al., 2002). Honkatukia et al., (2003) also opined that in avian species blood or feathers could be the source for DNA extraction. Feather sexing can be referred to as tissue sexing. He reported that small freshly-plucked feathers were a highly effective way of collecting the proper amount of tissue cells needed for performing the analysis. DNA isolation from feathers was difficult because only the feather quill contained tissue from which DNA could be extracted.

The isolation of genomic DNA in lesser Rhea was attempted from the calamus of feathers and best results were obtained by using a saline, non-phenolic method of DNA extraction; the DNA was repurified using Chelex 100 resin (Fraire et al., 2006). DNA extraction by using feather reduces the stress of the bird and avoids excessive blood loss (Cerit and Avanus, 2007 and Jeyakumar, 2013).

**Phenol:** chloroform method of DNA extraction has been used for isolating DNA from whole blood. The DNA was precipitated from the aqueous phase using 3M sodium acetate and 8 to 10 ml of isopropyl alchohol (Jaferien, 2008).

**Polymerase Chain Reaction (PCR):** Saitoh et al. (1991) first reported DNA typing in avian species. A family of repetitive sequences, designated the EcoRI family, and was found in the DNA of the chicken W chromosome by hybridization with the W chromosome-specific Xhol family probe. It was found that 70 per cent to 90 per cent of the DNA in the chicken W chromosome was shown to be occupied by repetitive sequences. Petitte and Keglemeyer (1992) developed a PCR assay using primers specific to the Xhol repetitive element. This assay was used by them to identify the sex of early chicken embryos. The PCR amplification of W-chromosome markers performed by using specific primers was carried out by isolating DNA from 5-7 day-old embryos by proteinase-K digestion and results were obtained within a day using as little as one ng of template DNA (Clinton, 1994). W linked
sequence was also used as a marker for sex assignment in Tongariro forest kiwis (Grant, 2001). The EE0.6 sequence is found to be conserved in all species of birds examined both in Carinatae and Ratitae. A counterpart sequence of EE0.6 was found to be present on the Z chromosome. The cloning and comparison of W and Z-linked EE0.6 sequences has been done in 12 different species. Four forward and three reverse primers were selected to amplify parts of the EE0.6 sequence by polymerase chain reaction (PCR). By choosing a suitable combination of primers for EE0.6 and a set of primers for a Z/W-common sequence, as an internal control, the sex of 36 species belonging to 16 different orders of Carinatae could be determined clearly by PCR. The sex of two other species representing different orders could be distinguished by Southern blot hybridization using ET15 as a probe. For the two Ratitae species, emu and ostrich, EE0.6 sequences on W and Z chromosomes could not be distinguished either by PCR or Southern blotting (Itoh et al., 2001).

Huynen et al. (2002) designed w1 and k7 primers based on the sequence of the sex specific marker kW1. The primers were used to sex all extant species of ratites. All species sexed produced a DNA fragment of about 350 bp as well as shadow bands slightly smaller than 350 bp. An additional PCR product of about 150 bp was only observed in case of females. Further a technique of sex assignment in all the Kiwi species was developed by designing new sex specific w5 and w7 primers that bind to a different site of the W specific locus, kW1 (Gen Bank accession number: AF 308932) (Huynen et al. 2003). Fraire and Mortella (2006) carried out sex determination in lesser rhea (Rhea pennata pennata) using w1 and k7 primers. PCR products were separated by horizontal gel electrophoresis and sex was assigned on the basis of difference in banding pattern observed in 1.5 per cent agarose. When the amplification of emu DNA using w1 and k7 primers, designed for kW1 locus, the amplification products were separated on 2 per cent agarose gels stained with ethidium bromide, after separation a single sex specific band of approximately 150 bp was observed only in female subjects (Costantini et al., 2008).

Multiplex PCR: Multiplex PCR is a variant of PCR in which two or more loci are simultaneously amplified in the same reaction. Relative concentration of the primers at the various loci, the concentration of PCR buffer, cycling temperatures and the balance between magnesium chloride and dNTPs are important for successful PCR (Henegariu et al., 1997). Griffiths et al. (1998) described a DNA based sex identification test based on two conserved CHD (chromo- helicase- DNA- binding) genes, CHD-Z and CHD-W, which are located on avian sex chromosomes of all the birds with possible exception of ratites. Test employs PCR with single set of primers, P8 and P2. Primers amplify homologous sections of both genes and incorporate introns whose lengths usually differ. When examined on gel the females show a second, distinctive CHD-W band and males show single CHD-Z band. This test was not successful in sexing the ratite bird ostrich.

A fast, accurate and inexpensive multiplex PCR procedure for large scale sex typing of ostriches using DNA extracted from feathers. They used female specific primers SS1 and SS2 and one of the standard internal control primer pairs OSM5 and OSM7, LO14, VIAS-OS4 and VIAS-OS14 that amplified a microsatellite region. They diagnosed the females by observing a female specific band and males by absence of the female specific band, besides the control primers produced bands in both males and females (Malago et al., 2002). Malago et al. (2005) devised a fast, cheap and accurate PCR multiplex reaction for large scale sex typing in ostrich. They developed a duplex PCR in which they used W chromosome specific OSFES or SS primers along with an internal control primer VIAS-OS14. Similarly triplex PCR involving all the three primers was also developed.

Random Amplified Polymorphic DNA (RAPD): DNA fragments produced by PCR using chosen short (usually 10-mer) primers are called RAPD markers, such markers could be used in sex identification. If the selected RAPD marker is on the W chromosome it would be amplified only in females and provide a female specific marker (Welsh and McClelland, 1990; Williams et al., 1990).

The length of primer determines the length of target size, when the primer length decreases primers could encounter a great number of target sites and this could increase the chance of amplifying a sex specific locus (Griffiths and Tiwari, 1993). RAPD assay using two hundred different 10 mer
primers of arbitrary sequence reported the identification of W-linked marker in the ostrich (Struthio camelus) as one primer (D10) generated a female specific band. Sex specificity of the primer was confirmed by testing the 21 birds individually. Cloning and sequencing of the candidate DNA fragment was done to design longer primers in order to optimize a PCR for sex diagnosis (Bello and Sanchez, 1999).

The main drawback of RAPD-PCR technique is that the primers used in the PCR reaction usually fail to amplify the same DNA sequence in different species, because most of the W chromosome is noncoding DNA that evolves and changes the sequence of its DNA reasonably quickly. As a consequence, the primers used in the PCR usually fail to find or amplify the same DNA sequence in a different species and thus sex identification is limited to a few closely related species (Griffiths, 2000). W linked DNA markers in ostrich was identified by the use of RAPD analysis, four female specific Sequence Characterised Amplified Regions (SCARS) were identified for sex identification immature ostriches (Hinckley et al., 2005). The RAPD assay involves low annealing temperature, usually 35- 40°C, which reduces the specificity of reaction and hence leads to repeatability of the results (Dubiec and Neubauer, 2006).

The reliability of RAPD markers can be questioned as such markers had low reproducibility, sensitivity to reaction conditions and also competition between different DNA fragments caused more weekly amplified bands, which disappeared in the presence of bright polymorphic bands. However, these disadvantages were outweighed by technical simplicity and low cost of RAPD markers (Cerit and Avanus, 2007).

**Minisatellite:** Minisatellites are also called variable number of tandem repeats (VNTRs). Minisatellites contain a number of tandem repeat sequences and their numbers differ between minisatellite alleles. Human minisatellite probe 33.15 has been used for sex identification in 33 species belonging to 13 genera of South American parrots (Miyaki et al., 1997). The RAPD and minisatellite methods are species-specific, laborious and time consuming (Lessells and Mateman, 1998).

**Microsatellite:** Microsatellites are short tandem repeats that are short regions in the genome (Russel, 2002). If the repeating units are two, three, or four base pairs long, they are called short tandem repeats (STRs) or microsatellites. Nesje and Roed (2000) reported microsatellite loci NVHfp 102 and fp 49 for sex identification in peregrine, gyrfalcon, merlin, kestrel, and hobby. They found that the amplification of locus NVH fp102 and locus NVH fp49 easily identified the female falcons and peregrines respectively.

**Amplified Fragment Length Polymorphism (AFLP):** AFLP has been used as a method for sex identification in ostrich (Struthio camelus) and shag (Phalacrorax aristotelis), however RAPD has been reported to be a simpler method in comparison to AFLP for sex identification, as in case of the latter there is use of acrylamide gels, radioactive markers or high-pressure liquid chromatography (HPLC) purified primers. Consequently, the safety requirements, cost and preparation time are high in AFLP (Griffiths and Orr, 1999). However, AFLP is more repeatable than RAPD and also it allows producing a greater number of bands per assay, which gives a higher chance of detection of sex-specific fragments. However, due to the complexity of the test and its expensiveness, it has been used for sex identification extremely rarely (Dubiec and Neubauer, 2006).

**Restriction Fragment Length Polymorphism (RFLP):** RFLP technique has been used as complementary method in minisatellite (Miyaki et al., 1997) and DNA probes like DQSG10 (Quinn et al., 1990), pv47-2 (Millar et al., 1992), pMg1 (Millar et al., 1996) for sex identification in geese, brown skua, and purple swanphne respectively.

Kloet (2001) described the identification of a female (W-chromosome) specific randomly amplified polymorphic (RAPD) 1.3 kb DNA fragment (ESEXW) in the emu (Dromaius novaehollandiae). Southern blot experiments and sequence analysis revealed that a related (96 per cent similarity) sequence exists on the emu Z- chromosome (ESEXZ). The sequences of ESEXW and ESEXZ were further subjected to PCR and restriction digestion, with EF9 and ER10 primers and BglII enzyme respectively, for the development of a two-primer CAPS (cleaved amplified polymorphic sequence) assay for reliable sex identification of the emu.
Chromo-Helicase DNA (CHD) genes: The CHD gene encoding chromo-helicase DNA binding protein 1 was the first gene discovered on the avian W chromosome (CHDW) (Griffiths and Tiwari, 1995). This CHD gene is present in both sexes and is named as CHD-Z and CHD-W on Z chromosome and W chromosomes, respectively (Griffiths and Korn, 1997). In most avian species the length of the CHD gene is slightly longer in the W chromosome as compared to the Z due to the presence of additional DNA bases in intron region. However in some avian species for example some terns, pukeko, most owls and hawks intron size of CHDW and CHDZ genes show very similar sizes (Griffiths et al., 1998 and Kahn et al., 1998).

CHD has been reported as an improved basis for DNA sexing by PCR, since CHD-Z and CHD-W were sexually dimorphic genes conserved in most avian species (Fridolfsson and Ellegren, 1999). Multiple primers P2 – P8 and 1237L - 1237H, have been designed to screen the intron size difference in CHD genes. Since the intron size of CHDW and CHDZ genes were identical in ostrich and emu (ratites), both females and males exhibited a single band by 1237L - 1237H (Kahn et al., 1998) and P2 – P8 (Griffiths et al., 1998) primer pairs. Griffiths (2000) mentioned the problem which existed with CHD gene was that if a PCR was designed to only amplify CHD1-W, a female would yield a single band and a male would provide nothing. The lack of a band could also mean that the PCR reaction had failed and could easily result in mis-sexing. Malago et al. (2002) reported the P2 and P8 primers did not amplify CHD genes in ratites.

DNA Sequencing in Ratite Sex Determination: The sex specific fragments produced by PCR primers can be subjected to DNA sequencing. The sequence data obtained can be used to study the homology with other sequences present on sex chromosomes of ratites and design sex specific primers. Dideoxy sequencing of sex specific RAPD PCR product revealed a DNA sequence data for a locus that is W chromosome linked in the kiwi, ostrich, cassowary, rhea, and emu. This sex specific sequence was named as kW1, at the amino acid level, this sequence was found to have a significant homology to X-linked genes in platyfish and Caenorhabditis elegans (Huynen et al., 2002).

Fraire and Mortella (2006) used the w1 and k7 primers for DNA sexing in lesser rhea (Rhea pennata pennata). They sequenced the 150 bp fragment, which amplified only in females. The sequence of 150 bp fragment was found to have 86 per cent homology with the 350 bp fragment, which amplified from the Z chromosome. The difference between the two fragments was found to be due to internal deletion of approximately 200 bp.

CONCLUSION
The sexing of the emu birds has been attempted through various techniques, though the method of phenotypic sexing (i.e. bulls eye feather pattern of male chicks, vocalisation in adult birds or brooding behaviour of adult males) appear to be simple methods of sex determination but they need experience and minute observation. Similarly surgical sexing though confirmatory but its involved with stress for the chicks which may endanger their lives. Karyotyping is time consuming as well as non confirmatory method of sex determination in the emu birds as the ZW chromosomes exhibit homomorphism and show similar banding pattern when stained in G or R banding. Therefore, DNA sexing appears to be the most confirmatory method of sex determination in the ratite birds as a maker which if located on a particular sex chromosome (Z or W) would amplify in that sex only when such marker sequence would be subjected to polymerase chain reaction. Such sex specific markers can also be targeted through other techniques in molecular biology as Fluorescence in situ hybridization. However, the development of a reliable but and effective method for sex assignment of the ratites will immensely benefit the emu breeders and farmers as they would be able to pair these monogamous birds at an early age and thus farming of these birds would become relatively easier and thus effective managemental decisions can be taken at the earliest.

REFERENCE


