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Sex determination in ratite and non ratite birds by molecular method

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ABSTRACT

In spite of number of methods for sex determination in birds, it is very difficult to distinguish sex especially in ratite birds due to lack of sexual dimorphism. Chromodomain helicase DNA binding 1 gene (CHD 1) is the choice of gene for gender differentiation using PCR based molecular method. In present study, non ratite CHD gene specific primers viz. 1237L/1272H, 2550F/2718R, P2/P8, P2/P3 and ratite bird specific primers viz.W5/ W7 and W1/ K7 were used for gender differentiation in ratite birds. The ratite bird specific primer W5/W7 was the only primer, which determined the sex in emu as well as ostrich successfully, while 1237L/1272H, 2550F/2718R, P2/P8, P2/P3 primers were unable to discriminate sex in emu and ostrich but ratite and non ratite primers can be used to discriminate the sex in non-ratite bird, primarily in chicken. In an alternative approach of PCR-RFLP, the high resolution melting curve (HRM) analysis showed conflicting pattern in both sexes of ratite birds but in chicken HRM analysis showed clear cut differential melting temperature in both sexes, hence HRM can be used for gender differentiation successfully.

Key words: Emu, Gender differentiation, HRM, Non ratite, Ostrich, PCR, Ratite

The ratite birds—flightless, having flat breastbones, lacking a keel for attachment of flight muscles—include ostriches, emus, kiwis etc. Emu and ostrich farming is an emerging enterprise in Indian poultry sector. Emu farming in India was established during the mid 1990s and has been a growing business ever since. India has more than 2,000 emu farms and the number is rapidly increasing. The ostrich farming is also increasing along with emu and poultry farming. With the increasing demands of valuable products of ratite birds in many countries including India, it is important to design breeding plans especially for various economically important traits. An early sex determination is an important key for any breeding plan and it will reduce the maintenance costs and will improve the efficiency of breeding programmes. In many bird species, including ostrich and emu, due to lack of sexual dimorphism, it is very difficult to distinguish between males and females based on their external morphologies, especially at very young age (Cerit and Avanus 2007, Reynolds *et al.* 2008). There are number of approaches for sex determination in ratite and non ratite birds, viz. behavioral observation (Gray and Hamer 2001), gonad examination by laparotomy (Maron and Myers 1984), vent sexing (Bramwell 2003), feather sexing (Cerit and Avanus 2007), cytogenetic approaches (Harris and Walters 1982) etc. These all approaches are time consuming, stress inducing as well as economically inefficient (Morinha *et al.* 2012). To avoid these limitations, the DNA based sex determination is the choice for sexing in ratite as well as in non ratite birds (Griffiths *et al.* 1998). In birds, female is heterogametic (females present Z and W chromosomes) and the male is homogametic sex (males have 2 Z chromosomes). The differences in the nucleotide sequences of Z and W chromosomes in birds permit the precise sex determination (Dubiec and Zagalska-Neubauer 2006). In DNA based method, the chromodomain helicase DNA binding 1 gene (CHD 1) was the first valid gene for sex delineation in non ratite birds (Fridolfsson and Ellegren 1999). In present study, non ratite CHD gene specific primers 1237L/1272H (Kahn *et al.* 1998), 2550F/2718R (Fridolfsson and Ellegren 1999), P2/P8 (Griffiths *et al.* 1998), P2/P3(Griffiths and Tiwari,1995), ratite bird specific primer W5/ W7 (Huynen *et al*. 2003) andW1/ K7 (Huynen *et al*. 2002) were used to determine the sex delineation in ratite (emu and ostrich) and non ratites (chicken).

MATERIALS AND METHODS

Samples collection and DNA extraction: Blood samples from chicken (10) and 24 plucked feather samples from emu (18) and ostrich (6) were collected from breeding farms located around using standard sampling procedure. Genomic DNA from blood sample was isolated with some modification in procedure described by John *et al.* (1991).

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Table 1. CHD gene, kw 1 locus, W specific locus and vector specific primers for determination of sex in chicken, emu and ostrich birds

Primer name	Gene amplified	Primer sequence (5'–3')	Amplicon (bp)	Reference
P2 / P3	CHD	F: TCTGCATCGCTAAATCCTTT R:AGATATTCCGGATCTGATAGTGA	110 (♀ & ♂)	Griffiths and Tiwari (1995)
P2 / P8	CHD	F: TCTGCATCGCTAAATCCTTT R: CTCCCAAGGATGAGRAAYTG	369 (♂) 369, 379 (♀)	Griffiths *et al.* (1998)
2550/ 2718	CHD	F: GTTACTGATTCGTCTACGAGA R: TCCAGAATATCTTCTGCTCC	453 (♂) 453 & 594 (♀)	Fridolfsson and Ellegren, (1999)
1237L/ 1272H	CHD	F: GAGAAACTGTGCAAAACAG R: TCCAGAATATCTTCTGCTCC	239(♂) 250 & 239 (♀)	Kahn *et al*. (1998)
W1/ K7	kw1 locus	F: ACCAGCCTTTAAACAAGCTATTAA R: TCTCTTTTGTTTTAGACACCCT3	350 & 300 (♂) 350, 300 & 150 (♀)	Huynen *et al*. (2002)
W5/W7	W specific locus	F: AATCACCCTTTAAACACAAGCT GTTAAAGCAA R: CCTTTCTCAAATCTCTCTTTTGT TCTAGACAC	350 (♂) 350 &~200 (♀)	Huynen *et al*. (2003)

Table 2. PCR conditions and PCR reactions used for sex determination in chicken, emu and ostrich birds

Primers	PCR conditions					PCR master mix
	Initial denaturation	Denaturation[1]	Annealing[2]	Extension[3]	Final extension	
P2/P8	94°C(5min)	94°C (45 sec)	47°C (45 sec)	72°C(45 sec)	72°C (10min)	AMP Mix (5X)–5.0 µlemPCR additive-2.0 µlem PCR enzyme mix-2.0µl Forward primer (10 Pm)- 1.0µlReverse primer (10 Pm)- 1.0µlNuclease free water-12µl Genomic DNA (30ng/µl)-2.0 µl
P2/P3	94°C (5min)	94°C (30 sec)	55°C (45 sec)	72°C(60 sec)	72°C (10min)	
2550/2718	94°C (5min)	94°C- (30 sec)	55°C (45 sec)	72°C (60 sec)	72°C (10min)	
1272/1237	94°C (5min)	94°C- (30 sec)	55°C (45 sec)	72°C(60 sec)	72°C (10min)	
W1/K7	94°C (5min)	94°C- (40 sec)	51°C (40 sec)	72°C(40 sec)	72°C (10min)	
W5/W7	94°C (5min)	94°C- (30 sec)	50°C- (30 sec)	72°C(30 sec)	72°C (10min)	

Repeat step 1 to 3 for 35 cycles.

Genomic DNA from the apex of feathers was extracted using kit as per manufacturer. Quality and quantity of DNA were checked by spectrophotometer at 260 nm wavelength.

PCR amplification: Six pairs of primers covering CHD gene segment (Table 1) were used to amplify respective fragments from avian, emu and ostrich birds. PCR was performed in Veriti thermal cycler with final volume of 25 µl using em PCR mastermix containing 5X AMP Mix, emPCR additive and emPCR enzyme mix (Table 2). The standardized PCR protocols for each primer is presented in Table 2.

HRM (high resolution melting): HRM analysis was performed with standard protocols for detection of sequence variations in the PCR amplified fragments, through changes in melting temperature (Tm) of DNA duplex. In present study, ABI PRISM 7500 fast real time PCR system with 7500 HRM software version 2.0 was used for HRM curve analysis.

RESULTS AND DISCUSSION

Genomic DNA was isolated from blood samples of chicken and feather samples of emu and ostrich and analysis of PCR products for CHD gene, using 1237L/1272H primer on agarose gel revealed 2 fragments of about 239 bp and 250 bp in female chicken whereas only single fragments of 250 bp in male chicken was observed (Fig. 1). Hence,1237L/ 1272H primers successfully distinguished sex in chicken. Similar result was also reported by Kahn *et al*. (1998) and Jensen *et al*. (2003). However, only single fragments of 250 bp were observed in either sex of emu and ostrich. The present finding indicated the intron size of CHDW and CHDZ genes are identical in both the sexes of ostrich and emu (ratites). Similar result in emu and ostrich was also reported by others (Ellegren 1996, Kahn *et al*. 1998, Cerit and Avanus 2006). Therefore, the 1237L / 1272H primer pair is appeared precise for sex determination in avian (non ratites birds) only whereas it could not discriminate sex in emu and ostrich (ratites birds).

In chicken, 2550F / 2718R primers produced 2 fragments of 453 bp and 594 bp in female chicken while only single fragment of 453 bp in male chicken. In ratite birds, only single fragment of 453 bp was observed in either sex of emu and ostrich (Fig. 1). Similar results were also reported by Fridolfsson and Ellegren (1999) in Greater Flamingo, Hornfeldt *et al*. (2000) in Tengmalm's owl. Present finding revealed that 2550F/ 2718R primers are ratite bird specific so, it could not determine the sex in the ratite birds (emu and ostrich).

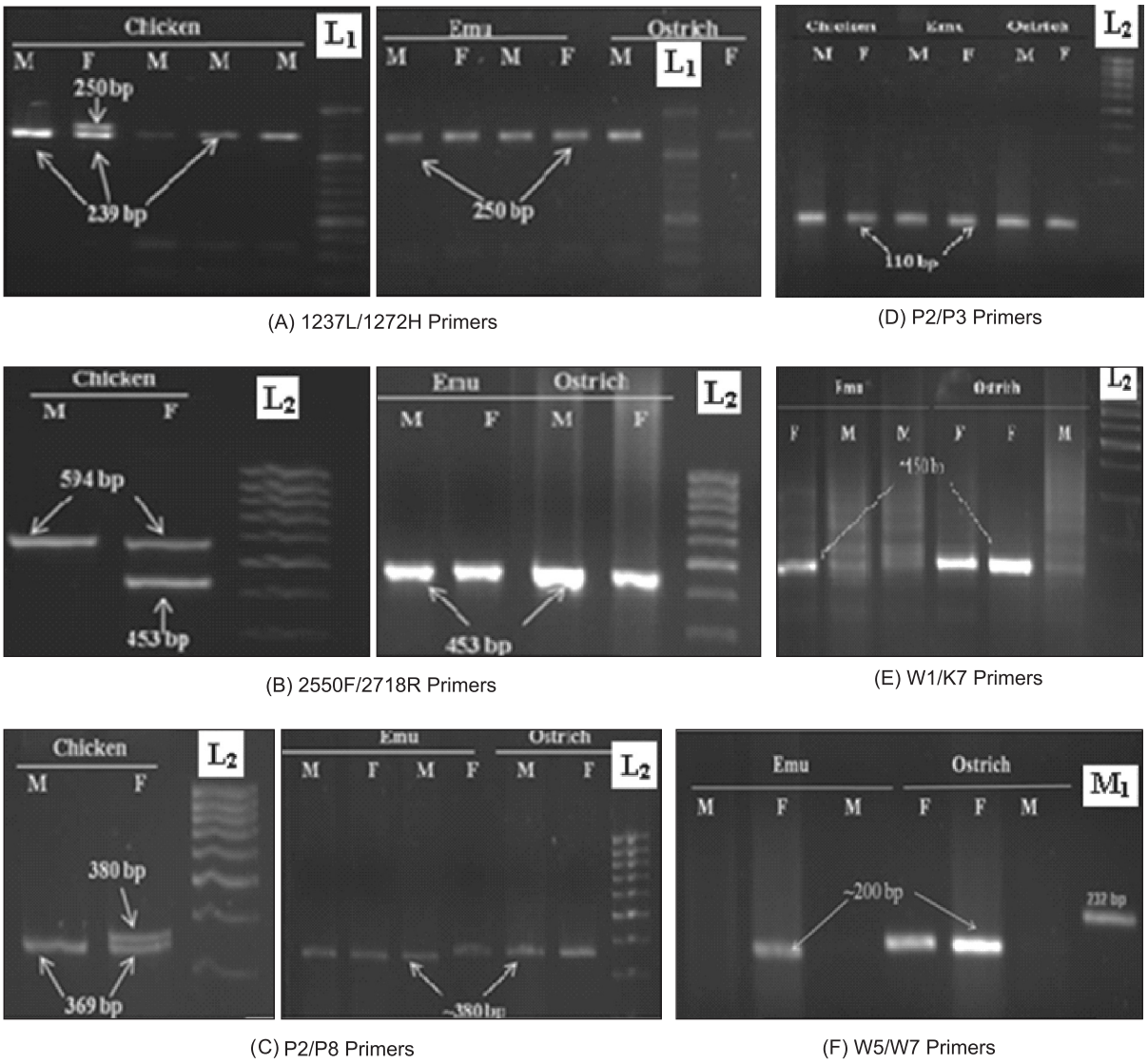
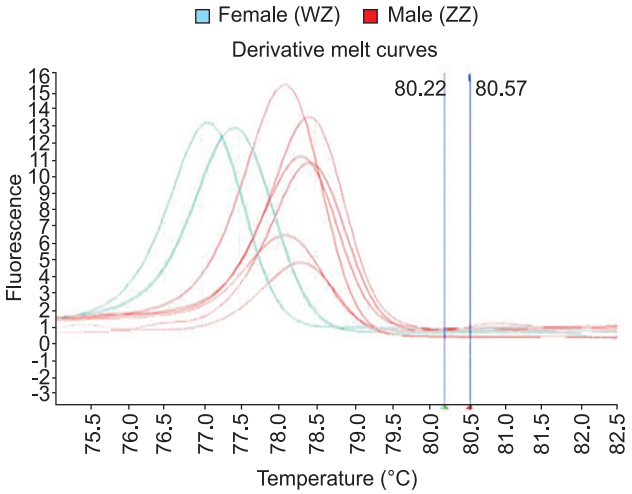


Fig. 1. (A-F). Agarose gel electrophoresis of PCR products generated using primer sets in chicken, emu and ostrich: (A) 1237L/1272H primers; (B) 2550F/2718R primers; (C) P2/P8 primers; (D) P2/P3 primers; (E) W1/K7 primers; (F) W5/W7 primers; where, M, male; F, female, L_1: 20bp DNA ladder, L_2: 100 bp DNA ladder, M_1: 232 bp DNA marker.

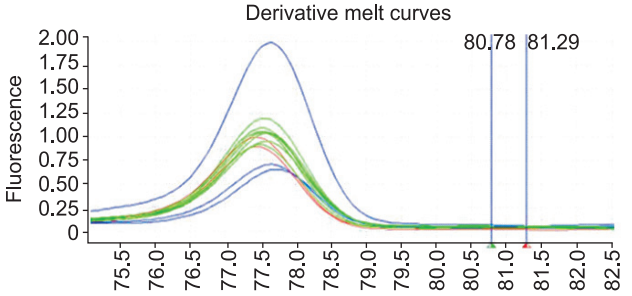
In female chicken, 2 fragments of 369 bp and 380 bp were observed using P2/P8 primers whereas only single fragment of 369 bp in male chicken (Fig.1). Hence, P2/P8 primers successfully discriminated sex in chicken. Similar findings were also reported by Miyaki *et al*. (1998) and Griffiths *et al.* (1998) in non-ratite birds, however, in ratite birds, only single fragment of 380 bp was observed in either sex (Fig. 1). Similar results were also reported by Constantini *et al.* (2008) in juvenile emu and by Cerit and Avanus (2006) in ostrich and emu. Hence, because of specificity of P2/P8 primers to non ratite birds, it could not determine the sex in the ratite birds.

W1/K7 primers produced single fragment of 150 bp in female sex of emu and ostrich whereas multiple faint bands without any specific and compact band was observed in the male of emu and ostrich (Fig. 1). Similar results were also reported by Constantini *et al.* (2008) in emu, Huynen *et al*. (2002) in kiwi. W5/W7 primers produced single fragment of 200 bp in female emus and ostrich; whereas, no amplification was observed in male of emu and ostrich (Fig. 1). All chicken DNA samples also showed no amplification using W5/W7 primer. So these primers failed to discriminate male and female in chicken. Huynen *et al*. (2003) also used W5/W7 primers to discriminate sex in Kiwi and observed 2 fragments of 350 bp and 200 bp in female Kiwi whereas only single fragment of 350 bp in male Kiwi.

P2/P3primers produced single compact fragment of 110 bp in chicken, emu as well as in ostrich (Fig. 1). Initially, PCR-SSCP was performed on PCR products obtained by P2/P3 primer pair; however, it could not obtain differential band patterns on 8% polyacrilamide gel. As PCR-SSCP is time consuming and laborious, limit high throughput applicability (Morinha *et al.* 2012), an alternative high resolution melting curve (HRM) analysis was performed



High resolution derivative melt curve for PCR products amplified by P2/P3 primer in chicken



High resolution derivative melt curve for PCR products amplified by P2/P3 primer in emu and ostrich

Fig. 2. High resolution melting curve analysis in chicken, emu and ostrich.

to differentiate male and female birds. The melting curve analysis of PCR product amplified from emu, ostrich and chicken samples showed differences in the T_m of the PCR amplicons from different species as well as within species but there was conflicting pattern between male and female in emu and ostrich (Fig. 2). However, in chicken the melting temperature of PCR product ranged from 78.08 to 78.43 for male and 77.12 to 77.47 for female which can be used for gender differentiation successfully (Fig. 2).

In the present study, the sex determination of emu as well as ostrich was successfully done by W5/W7 primer only, while 1237L/1272H, 2550F/2718R, P2/P8, P2/P3 primers were unable to discriminate sex in emu and ostrich but these primers can be use in to discriminate the sex in non ratite bird, primarily in chicken. The high resolution melting curve (HRM) analysis was successfully used to determine the sex in non-ratite birds by clear cut differential melting temperature in both sexes but it produced conflicting pattern between male and female sex of emu and ostrich, hence HRM can be used for gender differentiation only for non ratite birds.

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