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Identification and Development of Sex Specific DNA Markers in the Ostrich Using Polymerase Chain Reaction

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Abstract: The objective of this study was to identify and develop DNA markers that can be used for sex diagnosis in the ostrich (Struthio camelus) using polymerase chain reaction (PCR) technology. DNA was isolated from 15 male and 15 female, year old, crossbred ostriches. Two bulked DNA samples were prepared by grouping sexes. Random amplified polymorphic DNA (RAPD) analysis was used to screen 1400 arbitrary 10-mer primers for polymorphic markers between the bulks. Potential W-linked markers generated with the female bulked DNA sample were confirmed by individual screening of the 30 samples. Five W-linked RAPD markers, UBC388431, UBC4111300, UBC656,120, UBC793687, and OPL 1 7750 were identified by primers UBC388, UBC411, UBC656, UBC793, and OPL-17. Four RAPD markers were chosen for sequence analysis after further screening using 15 male and 12 female, 14-mo-old, birds sexed at slaughter. The sequence data were used to design pairs of specific primers, 18-24 bp, for PCR amplification of individual genomic DNA from 71 birds of varying age (33 males and 38 females). Primers generated reliable female specific sequence characterized amplified regions (SCARS). Four SCARS, ST793677, ST793665, ST4111245, and ST6561023, were developed, which can be used for sex identification in the ostrich. Cross amplification tests were done using male and female genomic DNA from turkeys, domestic animals (cattle, pigs, sheep, horses, and mink), and human. The PCR primers did not generate the sex specific markers in these species, which indicated that the W-linked markers amplified from ostrich genome may be species specific markers. These results demonstrate that RAPD analysis using bulked DNA samples provides an efficient means for the detection of W-chromosome markers in avian species. The SCARS developed can be used for identifying sex in immature ostriches.

Key words: Sexing, DNA markers, polymerase chain reaction, ostrich

Introduction

Ostrich farming has been dramatically increasing in the past few years. This increase has been driven, in part, by the demands of consumers for low-fat meat, exotic leather, high quality oil and decorating feathers. In some hot and dry areas, such as Southwestern United States, ostrich farming has become more popular because the birds have a remarkable tolerance of heat and require very little water. One problem with ostrich farming is sexing young chicks. After the birds reach sexual maturity at 3-4 years, the sexes may be distinguished phenotypically by plumage and behavior. Cloacal examination was reported to identify 6-wk-old to adult ratite birds including ostriches, since the male has a penis which is visible in the urodaeum, even in young chicks (Samour *et al*., 1984). However, this method is laborious, time-consuming, and inaccurate when a large number of chicks are involved. Cytogenetic approaches, such as karyotype analysis, can sex the birds but is not an efficient way in practice.

Polymerase chain reaction (PCR) (Saiki *et al*., 1985; Mullis and Faloona, 1987) with a thermostable DNA polymerase (Saiki *et al*., 1988) provides a simple, fast and efficient way for genetic diagnostics. Another molecular technique, based upon PCR, known as random amplified polymorphic DNA (RAPD) is performed on a genomic DNA template using single arbitrary 10-mer primers. (Williams *et al*., 1990). It does not require DNA sequence information of target DNA to generate specific binding primers. Each amplified products is derived from a region of the genome that contains two sites present on opposite strands of the genomic DNA with some homology to the short primer. The amplification products generated from one primer often includes 5-10 RAPD loci. With the large number of 10-mer primers available, the probability of detecting DNA markers from specific chromosome regions is high. This technique has been successfully used for the quick construction or saturation of genetic maps, comparative genome studies, and genetic diagnostics in plants (Tanhuanpaa *et al*., 1995; Grattapaglia *et al*., 1996). RAPD together with bulked segregate analysis (Michelmore *et al*., 1991) has proven to speed up the marker screening procedure. We first successfully applied these methods for Y-linked DNA marker identification in domestic animals (Xiong *et al*., 1992, 1993). Although RAPD provides an easy way to screen the genome for DNA markers, sensitivity to changes in

reaction conditions have limited its use as a diagnostic tool. The objective of this study was to develop reliable PCR-based DNA markers that can be used for sex identification in the ostrich with lower costs.

Materials and Methods

DNA Samples:

Bulked samples for RAPD marker screening: Blood samples were collected from 15 male and 15 female, approximately one year old, crossbred ostriches. DNA was isolated from 25ml of whole blood according to established procedures (Sambrook *et al.*, 1989). The isolated DNA was quantified spectrophotometrically and also on the agarose gel prior to dilution to a final working solution of 20 ng/µl. Two bulked samples were prepared by mixing equal volumes of the 15 individual male or female DNA samples.

DNA samples for testing amplification: Twenty seven DNA samples were prepared from the liver tissue of 14 month old ostriches (15 males and 12 females) sexed at slaughter. Fourteen samples of feather root tissue were obtained from 3 to 10 year old ostriches (3 males and 11 females) and DNA was then prepared as previously described. The other male and female DNA samples came from our laboratory stocks from turkeys, cattle, pigs, sheep, horses, and mink. Human DNA samples were obtained commercially (Promega, Madison WI). All of the DNA samples were diluted to a final working concentration of 20 ng/µl.

RAPD amplification and primer screening

RAPD amplification: Arbitrary 10-mer primers were obtained commercially (Operon Technologies, Inc., Alemeda, CA, Kits A to Z, and AA to AS; University of British Columbia, primer numbers 300 to 800). RAPD amplification and primer screening procedure were modifications of the methods of Xiong *et al.* (1993). RAPD reactions were performed in a 15 ml reaction mixture containing 2.5 mM $MgCl_2$, 100 mM each dNTP, 0.4 mM primer, 1 x PCR buffer II and 0.4 unit AmpliTaq DNA polymerase (Perkin Elmer, Foster City, CA) and 40 ng genomic DNA. Amplifications were carried out in Hybaid 96-well thermal cyclers (Hybaid Limited, Middlesex, United Kingdom) programmed an initial 3 min denaturation stage at 92°C, followed by 45 cycles of 1 min at 92°C; 1 min and 45 s at 36°C; and 2 min at 72°C, and followed by a final 7 min extension at 72°C. The amplified products were separated in 2% agarose gels containing 0.5 mg/ml ethidium bromide. DNA gels were documented using a Fotodyne model 3-3000, 312 nm UV transilluminator together with a gel imaging computer system (Fotodyne Inc., Hartland, WI).

Primer screening: Three phases were utilized in screening the products of the primers for W-linked markers. Phase I used the male and female DNA bulks, and screened for polymorphic DNA markers in the female bulk. Phase II used primers that generated female specific markers in phase 1, and individual DNA samples of two males and two females. Phase III was conducted by using the primers that amplify female specific bands in phase II and the DNA samples of the thirty individuals to verify the W-specific markers. Candidate markers were further tested on 27 ostriches sexed at slaughter.

Generation of SCARs

Cloning and sequencing: The female specific RAPD segments were excised and purified from the agarose gel using Geneclean (Bio 101, Inc. La Jolla, CA). The purified fragments were then cloned using the Original TA Cloning Kit (Invitrogen, Corp., San Diego). Positive clones were identified by a PCR-based colony screening protocol (Gussow and Clackson, 1989) with modifications using only one 10-mer primer as insert specific primers and the same RAPD amplification conditions previously described. The Recombinant plasmids were then isolated using the QIAprep Spin Plasmid Kit (QIAGEN, Inc., Chatsworth, CA). Recombinant plasmids were also verified by restriction endonucleases and Southern hybridization using a chemiliminescent detection assay with NEB Phototope™ (New England Biolabs, Inc., Beverly, MA). Sequence analysis was conducted (Genomis Inc., Duluth, GA) and specific longer PCR primers, 18-24 bp, were developed based on the sequence data.

PCR amplification conditions: PCR was conducted in 15 ml reaction mixture containing 2.5 mM $MgCl_2$, 100 mM each dNTP, 8 p moles of each primer 1 x PCR buffer and 0.4 unit AmpliTaq DNA polymerase (Perkin Elmer, Foster City, CA), and 20 ng genomic DNA. Amplifications were done in a Hybaid 96-well thermal cycler (Hybaid Limited, Middlesex, United Kingdom) programmed 3 min at 92°C, followed by 35 cycles of 30 s at 92°C ; 1 min at 55-65°C; and 1 min and 30 s at 72°C, with a final extension step of 7 min at 72°C. Amplified products were resolved electrophoretically in a 2% agarose gel containing 0.5 mg/ml ethidium bromide. Amplitaq Gold DNA polymerase 4 (Perkin Elmer, Foster City, CA) was used to improve PCR performance. We used 0.25 unit Amplitaq Gold per 15 ml reaction mixture. The program for Amplitaq Gold was 9 min at 95°C, followed by 40 cycles of 1 min at 94°C and 1 min at 60°C, with a final step of 10 min at 60°C.

Results and Discussion

Identification of RAPD Markers linked to the W-chromosome: Approximately 1400 primers were screened in this study. Five RAPD markers linked to the W-chromosome were identified: $UBC388_{431}$, $UBC411_{1300}$, $UBC656_{1120}$, $UBC793_{687}$, and $OPL17_{750}$ (Fig. 1). We used a simple nomenclature for the RAPD markers by

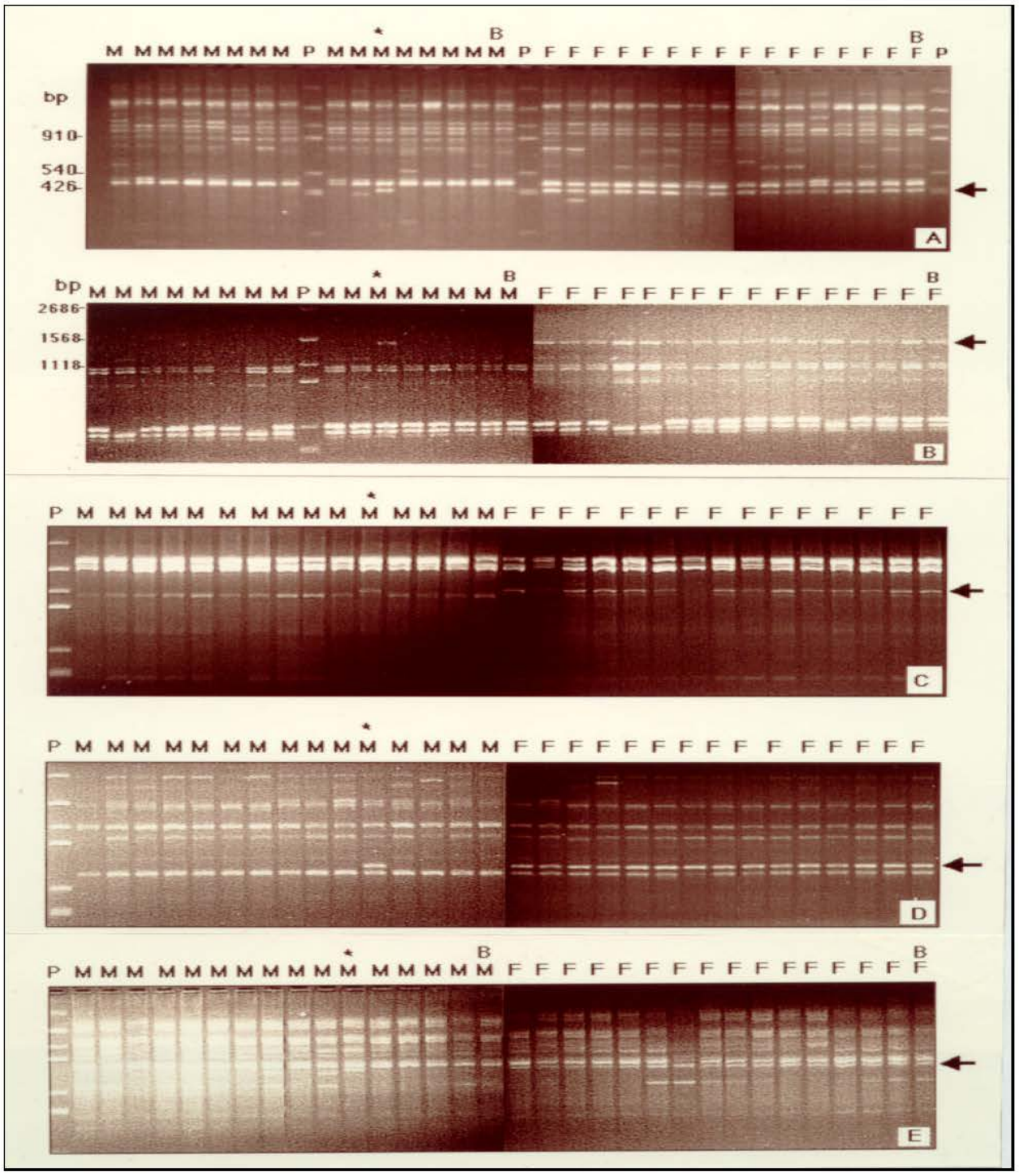

Fig. 1: Ostrich W-linked RAPD markers (arrows) amplified with 10-mer primers.
Lane P, pUC 19 DNA standard; lanes M, males; lanes F, females; B=bulk.
(A) with primer UBC388 (5'CGGTCGCGTC3'), marker size= ~430 bp.
(B) with primer UBC411 (5'GAGGCCCGTT3'), marker size= ~1300 bp.
(C) with primer UBC656 (5'CGTAACCTTG3'), maker size= ~1120 bp.
(D) with primer UBC793 (5'CTCCTCTCTC3'), marker size= ~700 bp.
(E) with primer OPL-17 (5'AGCCTGAGCC3'), marker size= ~750 bp.
*Note: Ostrich incorrectly identified as male at time of sample collection.

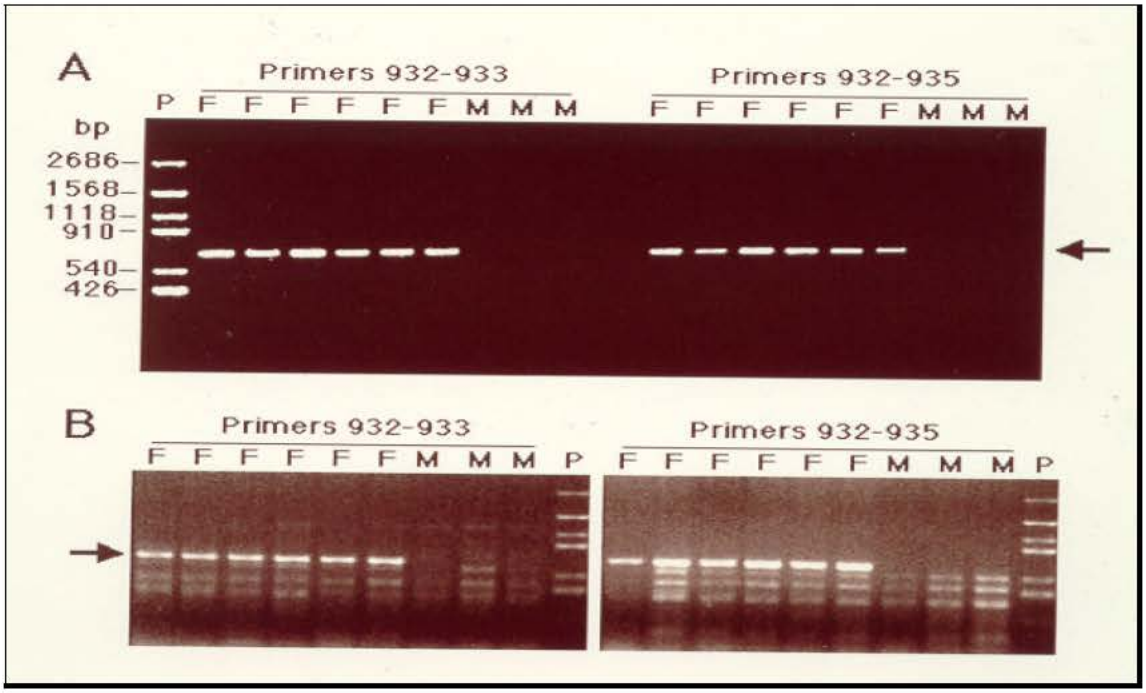

Fig. 2: PCR amplification of the ostrich W-linked markers.

(A) Anneal at 65^0 C. Only the female specific band (arrow) was amplified. Lane P, pUC 19 DNA standard; Lanes F= females; lanes M = males.

(B) Anneal at 550 C. Three non-specific faint bands were generated as well as the female specific band (arrow). These non-specific amplifications may be used as a control for false negative. Lane P, pUC 19 DNA standard; lanes F= females; lane M = males.

combining the primer number and the molecular weight of its products. The stability of RAPD amplifications were tested using different thermal cyclers and switching the sources of Taq polymerase. Marker UBC793$_{687}$ exhibited the highest repeatability. This marker was amplified with primer UBC793 which has the sequence 5'CTCCTCTCTC3'. Primer OPL 17$_{750}$ was the most unstable reproducability under slightly different reaction conditions. Among the 30 samples tested, the five RAPD markers were present in all females and absent in all but one male. This male was only a year old and determined to be a mis-identified female. These five markers were designated W-linked markers since the target chromosome in our screening design was the W-chromosome.

Cloning and sequencing of RAPD markers: The amplified female specific products of the five RAPD markers were cloned. The inserts were identified by a simple colony screening assay, and also verified with restriction enzyme analysis and Southern hybridization (data not published). We found that colony screening using the 10-mer primer as insert specific primers provided an efficient way for positive colony identification (data not shown). The RAPD markers were sequenced with the exception of OPL 17$_{750}$ since it was deemed unstable. About 350 bp sequence data from each end of the inserts were obtained. The terminal 10 base sequences are exactly the same as of the 10-mer primer sequences provided by the vendors. Full length sequences were obtained for markers UBC388$_{431}$, and UBC793$_{687}$.

PCR primer designing and amplification: We used both STSs (sequence-tagged sites) described by. Olsen *et al.* (1989), and SCARs (sequence characterized amplified regions) by Paran and Michelmore (1993) for specific primer design. A SCAR is similar to a STS and defined as a short single-copy DNA sequence identified by PCR amplification using specific oligonucleotide primers. The SCAR primers were designed starting with the RAPD 10-mer primer sequence, and amplified single major bands the same size as the RAPD fragment cloned. The following shows the designed PCR primers, ranging in length from 18 to 24 nucleotides.

Primers Based on RAPD marker UBC793$_{687}$ sequences:
793FI 5'CTCCTCTCTCCATCAATGCTTA-3'
793F2 (-12 bp) 5'TCAATGCTTAGGGCAATGAAAG3'
793RI 3'CGGTCCAACCAGCCCTTA5'(-1O bp)
793R2 3'AACCAGCCCTTACTCTCTCCTC5'
Based on RAPD marker UBC4111300 sequences:
411F (-19 bp) 5'GGGGTGAGCGTGAACATTGAATAG3'
411R 3'GAGGTCCGGTGTATGACAAGTCC5'(-35 bp)
Based on RAPD marker UBC6561120 sequences:

656F (-5 bp) 5'CCTTGCTTACCACCTACTTCTAC3'
656R 3'CGGACCTTTCATCGTCGTAA5'(-92 bp)
Based on RAPD marker UBC388431, sequences:
388F 5'CGGTCGCGTCGTCAGCAATG3'
388R 3'CAAAAATAGAACTGCGCTGGC5'

$UBC388_{431}$ Sequences (see clone 388PI Feb 17 & Feb 24 data)
20 40
CGGTCGCGTCGTCAGCAATGTTCTTCGGGTTACAGTTCCAGTCCCGCGGCCTGCAGG
CGGTCGCGTCGTCAGCAATG (388F)
60 80 100
CTTCACCTACTTTAGACACTTATGCTAAGCAAGAGTCAGACTTATGCTAAGCTGAAG
120 140 160
CTAGGTTGGCTACAATTCCCTTCTCTAGCAAGGATAGATAATAAATGGCCCCAGTGC
180 200 220
AGGGACAGAGAACTTGGAAAAAATGATCAGAGTAAGGTCTGAAAAAAAAAAATAA
240 260 280
CTAAAAGCTTGAAAATACCCAATAAAGGAAGGGGCAGACTGCTTTCTCAATGATCA
300 320 340
GTTGACATTCCAGTGGGGTTTTCTTTTTTCCTTTCTTTATATATATATTTTTAAGCTAA
360 380
TGAATGGTATAACAAAAGTGGCATCTAATGACATCTCAAGCAAAGCTTAAACTGATT
400 420 431
TCCTTTTCTCTGTTTTTATCTTTGACGCGACCG
(388Rnew)CAAAAATAGAACTGCGCTGGC5'
(388R) GAAAATAGAACTGCGCTGGC5'

$UBC793_{687}$Sequences (see clones 793P1 Jan 97 & starI /793 P6 June 96 data)
20 40
CTCCTCTCTCCA**TC**AATGCTTAAGGCAATGAAAGTTTTACTAGAACAAGGAAAGAA
5'CTCCTCTCTCCA**TC**AATGCTTA3' (793FI or 933)
5'CTCCTCTCTCCA**G**AATGCTTA3'(new933)
5'**TC**AATGCTTAAGGCAATGAAAG3'(793F2 or 935)
60 80 100
AGCACTGGCAGCAGTGGGAATAACTAAGCTAGTATTGTAGTTCTTCAGCAAACTCTT
120 140 160
GCTGTCTCAGGAATAATATTAGACACTAAAAATGAATGCTTTGAAGTTATTGAGTGA
180 200 220
CAAAGAAATATTTGAG**CAAT**TAAAACCAGCATAACAGGCAACAGGGTGCAG**CATAT**
240 260 280
ATTGGAAAAATTGACTTTCTTGATATAGTTGTTCTGATTTCTAAAACTTAATTGGGGG
300 320 340
ATTTAGATGCAAAGTTC**CAAT**AAAATAACTTAAATGAAAGATGTATCTGAATCTCCT
360 380
AGAAAATATTGAAACACTTCACTAAAGAAAATTCATAAAAGCACAGCAGAAATACA
400 420 440
CATTTTATACTGACGTA**CATATAT**GTATGGCTATGTGCTGTTAACAAACACAGGGTT
460 480 500
ACTTTCCCAAAGTGAGGAGCCACGATAAAGTCTTGGACTACCCTTGCGAAAGGAGTT
520 540 560
ATGCACGTGCCAAAOTGTTTGCCCTATCAGCAGACACACTGAACTACCACAACAGTG
580 600 620
ATAAGTCTGCTTGTAAAAGCTGCACGCTGCTGCGAATGGGCCAGAACTCCTCCAAGA
640 660
ACAGCAAAAAATTGAATGGGATAATTCAAGTAGAGCCAGGTTGGTCGGGAAT
(793RI or 932) 3'CGGTCCAACCAGCCCTTA5'
680 687 (793R2 or 934) 3'AACCAGCCCTTA
GAGAGAGGAG
CTCTCTCCTC5'

411P2.MI3F.seq

AgATGCTGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTCGGCTT**GAGGC** (RAPD I 0-mer primer 411)
CCGTTATGTGTTTG**GGGGTGAGCGTGAACATTGAATAG**TTGGGAAAAGAAgGGTT
(PCR 24-mer primer 41 IF)
CAAAGAGACCTGAGAAAATGGTACTGGGATGGGAAGATGATTAGGGGGAGATGGG
ATAGTGCCTGCTTCAAGCCCATGCTCTGGTTGCCTGGAACCTGGACCACCTCTAAGT
ACAGAATCCAAGAAAATTAGCCTGGGAGTGGCAGGAGGGATATGATGCATATAAAA
CACTAgGAGAGAGATGTGCTGCTTTGCTGTGGGGATGTGCCAGCGCATGTTGTAgGC
TTCAAGTGGAGCTCCAAAAATATCAGGACCACTTAACAGAgGTTGCGAATCAAGCAG
ATgTTCTCTCCAGAGTAATGGTTTGGTAAAGGAAgCAgTAaCAAATCTCAgCACAAGG
GAAAgCCTTAATCAgAACTTAgTAATATGGCCCACTGGCAAATTAAGACATCNCGAC
CCTACAGACAACAAGACCAAATTCTGTTGACGCATGGACGGGATCTAACTGGGATA
TATTTGGACCTATGACATTATACGANCTTCGCGCCATTCGTATAACAACATCAACGG
GCCGTCCCAACTACCCATGTCTCCACAATAGGNNTTGACTCTCCCCCCAACCCTCTG
TTGATNGGACAACGCGNCCTAGTCGAACATCGGGTAACTCCTTATCTCNTCGTATGG
TNTAGAGATGCGNACNTATCTAACGAGATCACTTGTCATTCCGGGNNNGGNNNNNN
NNNNNNNNNNCCCCCCCTCTGTTTATNTG

4IIP2.MI3R.seq

TAGTAACGGcCGCCAGTGTGCTGGAATTCGGCTT**GAGGCCCGTT**TAATGCAGTAAA
(RAPD I 0-mer primer 41 1)
TTCAGTTCAGCTACCTGAACAGTATGTGGCCTGGAGGTTGGGGCAGGAGGAGCCA
(PCR 23-mer primer 41 IR)
ATTTTTTAACTCAGCTCCAACCTTTCCCTTGCATTCACAATTTCTTGACTTGCAACAC
TAGGTCTGTGAGGGGTTTTTCATCCCACTCAGTCATATTCTCCCCCTTTTCCTTCAGG
AACATCCATAAGTCCTAgTGTGTAGGGCAGGGGCATGGAgAATTCCCATTCCCTCCT
GATGTTTTTTTGAGGTCAACcagAccACTGGACTTTTGGCATTACTCTTGGCCATGCAG
CTGACCTTTGGCTAGTCCTCTAGCATGCTCCCCCTGGTATGGTACATATAGGTCACTA
CTTCTTGATCAACAGGACATCCCACCAATTTATCAgGTGATACCAAAGGGGCAACTG
TGTCCAACTGTGAgAgTTTTGANGTCATGGCATCAGTGACCACAACCTTTTTGTGGTC
CCCCCCCCTTGCCTGTGAACACATCCANCAACCAANCAATGCANTCAATACTCCCTC
CNAATGTCTCATAGGGANATCTTATTGTTGGATCAAACATGCTCCACATATCCTCAT
CACTGATCCAAATCCCCTGTTNCTAGGANACATTACAATTGCTTGGTTCAANCCCCA AAATNTCCTNCCAACTTATN

The underlined sequences represent the sequences of the progenitor RAPD primer. -12,10, 19, 35, 5, 92 bp stand for the number of bases, starting at the first base of the RAPD primer, that were not used as PCR primer sequences. Table 1 lists the SCARs and STSs amplified with these primers.

Markers $ST793_{677}$ and $ST793_{665}$ are only 12 bp different, since primers 793F1 and 793F2 are overlapped. These two markers were the most stable and were tested more than three times on 71 birds of varying age and showed female specific. Markers $ST411_{1245}$ and $ST656_{1023}$ were hardly amplified using Amplitaq DNA polymerase. However, female specific bands were obtained when Amplitaq Gold DNA polymerase was used. Fig. 2 shows the PCR amplification of these female specific markers. Primers $SC793_{687}$ and $SC388_{431}$ can't be reliably used for sex identification either because of the unstable amplification, or no female specific bands amplified.

RAPD has been used for Z-chromosome mapping in the chicken (Levin *et al.*, 1993). In his study eleven W-specific RAPD markers were simultaneously identified. Levin *et al.* concluded that RAPD is an efficient means for screening sex specific markers in birds. More recently, amplified fragment length polymorphism (AFLP) was used to identify W-chromosome markers in the ostrich (Griffiths and Orr, 1999). While AFLP has some advantages over RAPD it is a tedious and expensive procedure compared to RAPD. A similar approach has also been done by Ding *et al.*

Table 1: SCARs and STSs derived from W-linked RAPD markers in the ostriches

SCARs or STSs*	Primers		Polymor-phism	W-linkage
$SC793_{687}$		793FI	Dominant	Yes
	793R2			
$ST793_{677}$		793F1	Dominant	Yes
	793RI			
$ST793_{665}$		793F2	Dominant	Yes
	793R1			
$ST411_{1245}$		411F	Dominant	Yes
	411R			
$ST656_{1023}$		656F	Dominant	Yes
	656R			
$SC388_{431}$		388F	none detected	No
	388R			

(1995).However, once again the method they used is expensive and labor intensive. The RAPD procedure outlined in this paper may be more easily and readably adopted as a tool for identifying simple traits such as sex. In the present study, we used RAPD analysis and bulked DNA samples to detect W-linked DNA markers in the ostrich. Five W-linked RAPD markers were quickly identified. Longer specific primers were then developed. These PCR primers amplified more reliable markers that can be used in sex identification. Early sexing of birds are highly advantageous for ostrich breeders. Such information helps them to manage the ostriches, such as pairing the birds correctly, and selling them earlier. Male and female birds also have different nutritional requirements. Grouping the birds by sex may be essential for the long run of ostrich farming.

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