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Cloning and expression of prolactin gene in laying pigeon

MIN ZHANG[1], AI-TING ZHANG[2], SHA ZHU[3], LIAN-LONG LIU[4] and X T ZOU[5]*

Zhejiang University, Hangzhou 310 058 China

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ABSTRACT

In order to understand the phylogenetic diversity and expression character of prolactin (PRL) molecule, the cDNA of PRL was isolated and cloned from female American silver king pigeon (*Columbia livia*) pituitary gland by a degenerate primer and nested reverse transcription polymerase chain reaction (RT-PCR) based strategy. The expected expression of mRNA in different tissues and status was quantified by real-time quantitative PCR (FQ-PCR). The cloned cDNA contains 658bp nucleotides, encoded 219 amino acids and deduced amino acid sequences showed high similarities and identity with the corresponding sequences of chicken, budgerigar, India pealfowl, comb duck, duck, goose, turkey, ring-necked pheasant, Java sparrow, ostrich, ring-necked pheasant and quail. *PRL* was widely expressed in tissues analyzed, and highest level of PRL mRNA expression was observed in the pitutary whereas the lowest in the kidney. Comparable high level of PRL mRNA expression was observed in oviduct and ovary. PRL mRNA expression increased significantly in incubation, compared to laying.

Key words: Clone, Gene expression, Pigeon, Prolactin

Certain birds like *Columbia* species incubate and foster the neonatal by nature. Pigeon is a kind of economic animal in many countries, especially in China. The American king pigeons are raised for egg, meat, and even the feces can be used as feedstuff for other livestock. The reproduction period of king pigeon is about 45 days, and each cycle can be divided into courtship, incubation and feeding of the squabs. The interval of egg laying can be shortened to 10–14 days, when the egg is taken away immediately after laying. During this stage pigeon just show incubation behaviors then come into the next laying. However, the egg production is limited by the incubation behaviors.

It is reported that, increased concentration of prolactin (PRL) plays a role in cessation of egg laying and incubation during the active period of lay (Maney *et al.* 1999, Curlewis 1992). PRL was originally described as a distinct anterior pituitary hormone with 'lactogenic' activity (Stricker and Grueter 1928), and the factor was purified and given the name prolactin shortly thereafter. PRL affects more processes than all other pituitary hormones combined. Among these are the control of mammary gland development, initiation and maintenance of lactation, immune modulation, osmoregulation, control of hypothalamic releasing-inhibiting factors, behavioral modification and nesting behavior regulation (Freeman *et al.* 2000). Cloning of PRL cDNA in avian species was conducted in chicken (Hanks *et al.* 1989, Watahiki *et al.* 1989), turkey (Kang *et al.* 2004), Japanese quail (AB162003), domestic duck (Kansaku *et al.* 2005), comb duck (AM180924), goose (Liu *et al.* 2008) and budgerigar (AB362879). However, it is not known if the genomic structure and the regulatory mechanisms for pigeon PRL gene expression are shared in different avian species. And the studies about expression difference between laying and incubation of pigeon are rare.

MATERIALS AND METHODS

Animals and samples: Laying pigeons were female-female paired and the egg was picked out immediately after laying, the pigeons do not have the status of feeding of the squabs. So the reproduction periods including laying and incubation, without broodness. Samples for DNA clone and analyze tissue distribution were collected from 720-day old adult female American king pigeons. Birds (6) were killed by cervical dislocation, and tissues (pituitary, kidney, liver, heart, pancreas, spleen, oviduct, and ovary) were removed and immediately snap-frozen in liquid nitrogen and stored at –80°C until use. For analyze expression difference of PRL mRNA, 6 birds from each period (incubation and laying) were selected in random and then killed by cervical

Present address: [1]Ph.D. (20517028@zju.edu.cn), Nutrition and Safety Center of Wilmar Biotechnology Research and Development Center Co., Ltd., Shanghai 200 137, China; [5] Professor (xtzou@zju.edu.cn), [2,,3,4] Feed Science Institute, Zijingang Campus.

dislocation, pituitary and ovary were collected. All samples were immediately snap-frozen in liquid nitrogen, and then stored at –80°C until use.

Total RNA extraction: Total RNA from pigeon were isolated using Trizol total RNA isolation kit. The isolated RNA was analysed using a Spectra Max 190 spectrophotometer to determine purity and concentration (OD_{260}/OD_{280}). All RNA samples were stored at –80°C until used for first strand complementary DNA (cDNA) synthesis.

Reverse transcription (RT) and polymerase chain reaction (PCR): Two micrograms of total RNA was reverse transcribed by incubation at 42°C for 1 h in a 20µl mixture consisting of 10 U avian myeloblastosis virus reverse transcriptase, 20 U RNase inhibitor, 2.4 mM Oligo (dT10) primer, 1 mM each dNTP, 50 mM Tris–HCl (pH 8.3), 10 mM $MgCl_2$, RNA samples were denatured at 80° for 5 min and placed on ice for 5 min together with the Oligo (dT10) primer and dNTP before reverse transcription (RT).The RT reaction was terminated by heating at 95° for 5 min and quickly cooled on ice.

Two microliters of RT reaction mix was used for PCR in a final volume of 50 µl containing 0.4µl Taq DNA polymerase, 5µl 10×PCR buffer, 1µl each dNTP, 1.5 µl $MgCl_2$, 1µl specific primers for target genes. And PCR conditions were: 94°C 2 min, 55°C 45s, and 72°C 1 min. The nucleotide sequences of primers for Cloning *PRL* were designed according to *Anser anser* (EU647469), *Coturnix coturnix* (AB162003), *Meleagris gallopavo* (U05952), *Numida meleagris* (AB451557), *Pavo cristatus* (AB451556) and *Phasianus colchicus* (AB451555), using Primer Premier 5.0 software (Table 1) ÿand were synthesized commercially.

We ligated 2µl PCR products directly to the 1µl PUCm-T vector (BBI) in a 10 µl reaction using 2µl T4' RNA ligase(TaKaRa) at 22µ overnight. Two microliters of this ligation reaction was then transferred to the competent cells of TOPO10 (Omega), then spread onto LB agar plates containing 50 µg/ml of ampicillin and X-gal. The plates were inversely placed in a 37°C incubator for at least 18 h and then shifted to 4°C for 2–3 h to allow proper color development. White colonies were utilized for colony PCR. The positive colonies were amplified in 50 ml LB solution with 50 µg/ml of ampicillin. The amplified plasmids with PCR inserts were send sequencing by Shanghai Shengong. Finally, the obtained sequence was analyzed by BLAST and related software.

Table 1. Nucleotide sequences of primers used for cDNA isolation and quantitative RT-PCR of PRL in relation to GADPH housekeeping gene AF036934

Primers	Primer sequences	Size(bp)
DP-F1	PRL-F1: 5'- ATGAGCAACAVA GGGGCTTCA -3'	About 690
DP-F2	PRL-R1: 5'- CTTCAYTGAAA GGTTTGTTGCTG -3'	
DP-R1	PRL-F2: 5'- GCAATTGCTATCAT GGATTAGGC -3'	
DP-R2	PRL-R2: 5'- CATGGATTA GGCGGCACTTC -3'	
qRT-PCR-F	PRL-F: 5- CGGGTTCATACT GGTGAGATTGGA -3	112
qRT-PCR-R	PRL-R: 5- GCAGCAGGTTGTAAAA GGCAAAGAGT -3	
GADPH -F	5'-GCAGGAACATCCCGAAGAAGC -3'	191
GADPH -R	5' –CCCGGAAGTGACCATGAGTAG- 3'	

F, Forward; R, reverse; DP, degenerate primers; qRT-PCR, real-time quantitative RT-PCR.

Sequence analysis: The cDNA sequence and the deduced amino acid sequence were compared with the sequences in the GeneBank database using BLAST program available for the NCBI Internet website. Analyses of amino acid sequences and structures were performed by MegAlign program. The phylogenetic tree was constructed based on their amino acid sequences by ClustalW method (Higgins *et al.* 1996).

Quantitative analysis of mRNA expression: Total RNA was isolated from pigeons collected with Trizol reagent, quantified, and then made a reverse transcription (RT) as described above. The SYBR Green RT-PCR assay was conducted to determine mRNA expression by iQTM5 . The real-time PCR amplifications were carried out in a total volume of 25 µl, containing 12.5 µl of 2 × SYBR Green Supermix, 1µl of diluted cDNA and lµl of each primer. Gene-specific primers were designed by Primer Express 2.0 and Beacon designer software, and amplified a product of 112 bp from RLR cDNA template. A fragment of 191bp GADPH cDNA was amplified as internal standard gene to calibrate cDNA template (Table 1), the primers were designed according to pigeon (gene bank number: GU968532).

The PCR temperature profile was 95°C for 5 min followed by 45 cycles of 95°C for 5s, 60°C for 30 s, and 72°C for 30s. To confirm that only one PCR product was amplified and detected, dissociation curve analysis of amplification products was performed at the end of each PCR reaction. After the PCR program, data from three replicate samples were analyzed with the SDS 2.0 software . The comparative C_T method was used to analyze the expression level. The ΔC_T (differences in the C_T between the target and internal control) for each sample was subtracted from that of the calibrator, and then the expression level was calculated by $2^{-\Delta CT}$.

Statistical analysis: Data were analyzed by using ANOVA-Oneway t-test SPASS software, version 16.0. Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

cDNA fragments were obtained by nested RT-PCR from pigeon pitutary total RNA primed with degenerated P1 and P2 primers. The identity of cDNA fragments were 658 bp, contained 615bp open reading frame, encoded 219 amino

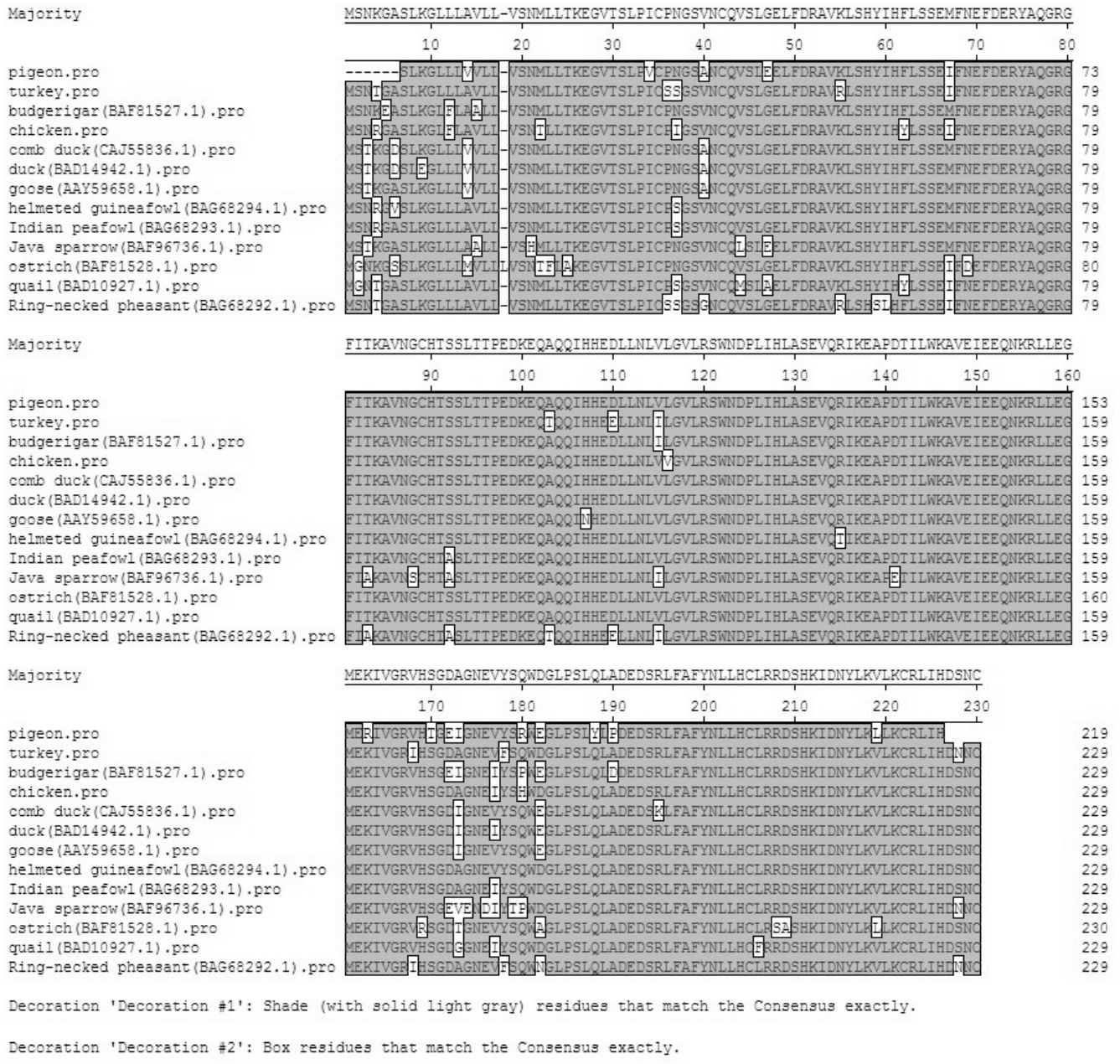


Fig.1. Multiple alignment of the amino acid sequences of PRL in Clustal W Lasergene

acids, and confirmed as PRL gene by nucleotide sequencing, and submitted to Genbank with access number, GU968532.

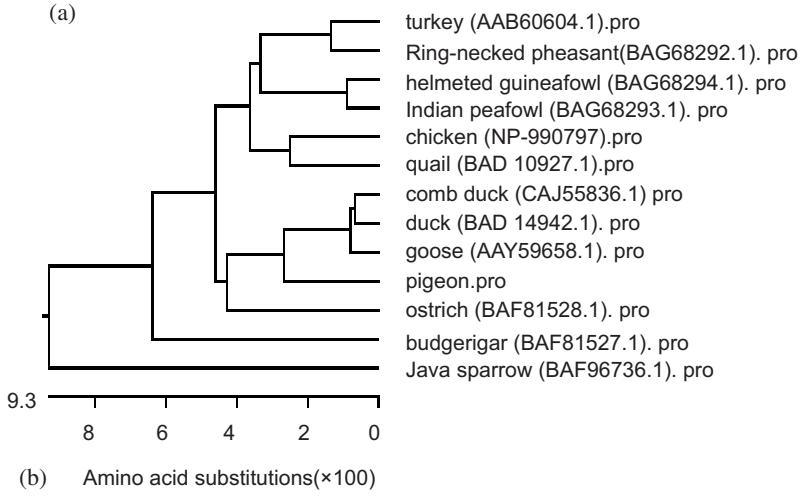
The deduced amino acid sequences of American king pigeon PRL showed high similarities and identities with other avian (Fig.2). The identities of *PRL* deduced amino acid sequence with the corresponding sequences of turkey (AAB60604.1), budgerigar (BAF81527.1), chicken (Genbank accession no. NP_990797), comb duck (CAJ55836), duck(BAD14942.1), domestic goose (AAY59658), helmeted guineafowl (BAG68294.1), Indian peafowl (BAG68293), Java sparrow (BAF96736.1), ostrich (BAF81528.1), quail (BAD10927), and Ring-necked pheasant (BAG68292.1) were 90.4%, 93.2%, 91.3%, 95.0%, 92.7%, 92.2%, 89.0%, 91.3%, 91.8%, and 88.6%, respectively. Phylogenetic analysis indicates that pigeon PRL is more closely related to the duck and goose orthologs compared to that of other species. This work successfully cloned the American king pigeon PRL gene segments by degenerate primers. And the ORF encoded a PRL peptide of 219 amino acids. The pigeon PRL shared high amino acid sequence identity with other avian species. It shared a greater than 90% sequence identity with other commonly studied

Percent identity

Di-Brgence

	1	2	3	4	5	6	7	8	9	10	11	12	13		
1		90.4	93.2	91.3	95.0	94.5	95.0	92.7	92.2	89.0	91.3	91.8	88.6	1	pigeon. pro
2	10.3		91.7	93.0	92.1	91.7	92.6	94.8	94.8	89.1	90.0	93.4	97.4	2	turkey.pro
3	7.2	8.8		93.9	94.3	94.8	93.8	93.9	94.8	92.6	90.4	92.6	89.5	3	budgerigar (BAF81527.1).pro
4	9.2	7.3	6.4		93.0	93.4	93.4	95.6	96.5	89.5	91.7	95.2	90.4	4	chicken. pro
5	5.2	8.3	5.9	7.3		98.7	98.7	95.6	95.2	90.4	92.6	93.0	90.4	5	comb duck (CAJ55836.1) pro
6	5.7	8.8	5.4	6.9	1.3		98.3	95.2	95.6	90.8	92.1	93.4	90.0	6	duck (BAD14942.1). pro
7	5.2	7.8	5.4	6.9	1.3	1.8		95.6	95.6	90.8	92.6	93.4	90.8	7	goose (AAY59658.1).pro
8	7.7	5.4	6.4	4.5	4.5	5.0	4.5		98.3	90.4	92.6	95.2	92.1	8	helmeted guineafowl(BAG68294.1). pro
9	8.2	5.4	5.4	3.6	5.0	4.5	4.5	1.8		92.1	92.1	96.1	93.0	9	Indian peafowl(BAG 68293.1). pro
10	11.9	11.8	7.8	11.3	10.3	9.8	9.8	10.3	8.3		86.5	90.0	88.2	10	Java sdarrow (BAF96736.1). pro
11	9.2	10.8	10.3	8.8	7.8	8.3	7.8	7.8	8.3	15.0		91.7	87.8	11	ostrich (BAF81528.1). pro
12	8.7	6.9	7.8	5.0	7.3	6.9	6.9	5.0	4.0	10.8	8.8		90.8	12	quail (BAD 10927.1).pro
13	12.4	2.7	11.3	10.3	10.3	10.8	9.8	8.3	7.3	12.9	13.4	9.8		13	Ring-necked pheasant (BAG68292.1). pro
	1	2	3	4	5	6	7	8	9	10	11	12	13		

(a)



(b) Amino acid substitutions(×100)

Fig. 2. Multiple alignment of the amino acid sequences sequence distance (a) and phylogenetic trees (b) of PRL in Clustal W Lasergene. Scale bar indicates the substitutionrate per residue.

birds, such as chicken, duck, goose, turkey, and quails. The high identities indicated the close evolutionary relationship of pigeon with goose and duck.

The PRL gene was expressed in all tissues examined in female pigeons (Figure 3). Pituitary was found to contain the highest amount of mRNA ($P < 0.05$) followed by spleen ($P<0.05$), oviduct ($P<0.05$), liver, heart, ovary, pancreas ($P < 0.05$) and kidney. PRL was synthesized and secreted by the anterior pitutary gland and lower levels of PRL were also expressed at extrapituitary sites: the central nervous system, the immune system, the uterus and its associated tissues of conception, in both avian and mammals (Freeman *et al.* 2000). Consistent with our studies that PRL mRNA had a remarkable mRNA expression amount in pitutary and widely presented among tissues, and indicated potential physiological function of PRL in pigeon immunity, production and osmoregulation.

The relative expression of ovary PRL gene mRNA in incubation was significantly increased ($P<0.05$), while the expression of pitutary PRL mRNA was increased numerically. Angelier *et al.* (2007) reported that levels of prolactin changed with age and experience in a long-lived

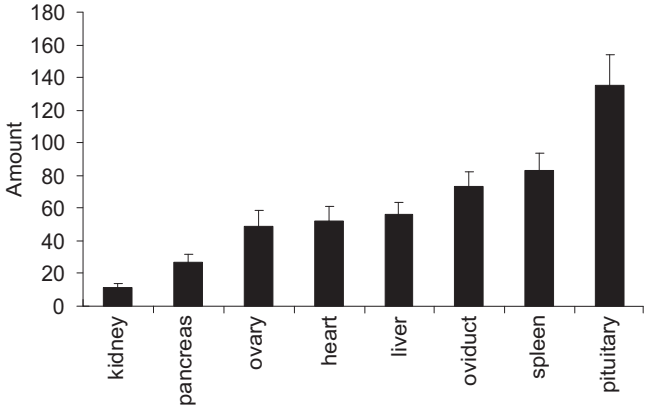


Fig. 3. Comparison of relative amount of PRL mRNA in various tissues of female pigeons. Data (mean±standard error of the mean, n=6) with different superscript letters significantly differ (P<0.05).

bird during the brooding stage. When plasma PRL changed, the mRNA of pigeon *PRL* in pituitary also rise dramatically after laying, and the difference was significant as plasma PRL did in turkey (Freeman *et al.* 2000, Proudman and Opel 1981). However, there was no significant difference between laying and incubation pigeon in pituitary PRL mRNA expression. This probable because of the hormone secretion of pigeon associated more with parental behavior than the initiation and termination of incubation (Wentworth *et al.* 1983), and PRL started to increase during laying, and peaked at the crop milk was produced (Wentworth *et al.* 1983). In southern China, pigeon are reared as a kind of poultry, and have wide market in southeast Asia for its delicious tastes and abundant nutrients. In order to have maximized egg production and minimized laying intervals, laying pigeons were female-female paired and the egg was picked out immediately after laying. The lack of parental behavior reduced the stimulation in pituitary PRL mRNA expression, although the laying pigeon remained the incubation behavior in the laying interval. The significant difference of ovary PRL mRNA expression was observed in different laying period, because that ovary not only serves as a target for endocrine prolactin action but also as a site of local prolactin hormone production (Schw Rzler *et al.* 1997).

In summary, the sequences of PRL genes in birds which have the incubation behaviors were identified. And the pigeon PRL were also widely existent and synthesized, especially in pituitary. The ovary PRL mRNA expression increased significantly in incubation, compared to laying.

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