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Cloning and Comparison of Prolactin Promoter in Galliformes

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To investigate the mechanism regulating transcription of the prolactin (*PRL*) gene in avian species, the *PRL* promoter region in Ceylon junglefowl, Japanese quail, ring-necked pheasant, turkey, Indian peafowl and helmeted guineafowl were cloned and sequenced. In each species, approximately 4,800–5,900 bp were sequenced. The *PRL* promoters of 7 galliformes including red junglefowl were found to have, on average, 91.2% sequence identity over the entire region and 97% sequence identity was observed in the proximal promoter (from the initiation codon (+55) to −130). Moreover, average of sequence identities was 91.6% among 11 avian species (7 galliformes, duck, Java sparrow, budgerigar and ostrich). In the *PRL* proximal promoter, putative Pit-1 binding site and vasoactive intestinal peptide response element were conserved among avian species. These results suggest that that the mechanisms involved in gene expression of *PRL* may be conserved in Galliformes.

Key words: Galliformes, prolactin, promoter

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Introduction

Prolactin (PRL) is mainly produced in the anterior pituitary gland, and is thought to have arisen from a common ancestral protein with growth hormone and placental lactogen by gene duplication at least 500 million years ago (Niall *et al.,* 1971; Miller and Eberhardt, 1983; Nicoll *et al.,* 1986). In avian species, PRL is the key hormone for broody behavior. The onset of incubation behavior is initiated by increasing levels of plasma PRL, and its maintenance at high concentration during the egg incubation period followed by its rapid decline after hatching in many precocial birds (Sharp *et al.,* 1979, 1988; Lea *et al.,* 1981; Lea and Sharp, 1982; El Halawani *et al.,* 1986; Youngren *et al.,* 1991; Kuwayama *et al.,* 1992). Moreover, levels of *PRL* mRNA in the anterior pituitary gland are correlated with levels of plasma PRL during the reproductive cycle (Shimada *et al.,* 1991; Talbot *et al.,* 1991; Wong *et al.,* 1992a; Kansaku *et al.,* 1994). On the other hand, in altricial species, levels of PRL increase at the late stage of incubation behavior and high levels are maintained until the mid stage of brooding (Buntin, 1979; Goldsmith *et al.,* 1981; Wingfield and Goldsmith, 1990; Kikuchi *et al.,* 1999). Unlike mammals where the synthesis and release of PRL is under inhibitory control, in avian species expression of the *PRL* gene is mainly tonically regulated by vasoactive intestinal peptide (VIP) (Macnamee *et al.,* 1986; Talbot *et al.,* 1991; Youngren *et al.,* 1994), Moreover, VIP-stimulated *PRL* expression was associated with cAMP and protein kinase A (Kansaku *et al.,* 1998; Kang *et al.,* 2002).

In chicken, several variations within the *PRL* promoter region were identified by comparison of several breeds (Zadworny *et al.,* 2002). They reported the presence of a 24 bp insertion/deletion at position −358 of the chicken *PRL* gene when White Leghorn was compared with red junglefowl, whereas there is no difference between red junglefowl and silkies. Moreover, allelic frequencies in this position showed large differences between breeds. Since White Leghorn rarely expresses incubation behavior unlike red junglefowl and silkies, it was thought that this 24 bp insertion/deletion might have an effect on the expression of *PRL* mRNA and indeed, Cui *et al.* (2006) reported that this 24 bp insertion/deletion was associated with egg production. In turkey *PRL* promoter, the presence of several genetic variations was also reported (Zadworny *et al.,* 2002), and one of these variants was associated with higher rates of expression of broody behavior. Comparison of promoter activity between these two genetic variants using a reporter assay indicated that there were significant differences in activation of transcription (Kansaku *et al.,* 2008). Although the nature of the difference in the activity of transcription complex is unknown, cis-acting elements such as the Pit-1 binding site in chicken (Ohkubo *et al.,* 2000) and the VIP response element (VRE) in turkey (Kang *et al.,* 2004) are known to activate transcription of *PRL*.

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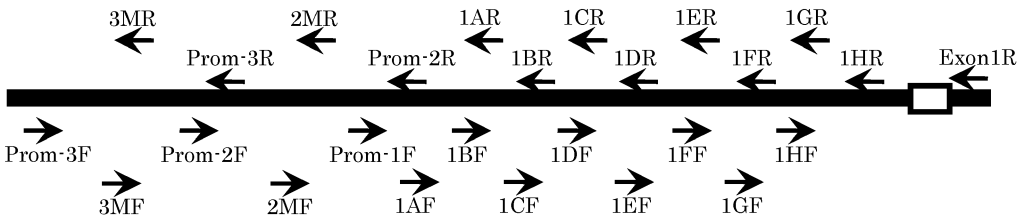
However, it is unknown if the promoter sequences including cis-acting elements are conserved and the mechanisms regulating transcription of *PRL* are common in galliformes. Hence, this study was conducted to clone several additional galliform *PRL* promoters and compare those sequences to investigate if cis-acting elements, such as Pit-1 binding site and VRE, are conserved, and if regulatory mechanisms of affecting gene transcription of *PRL* are common in Galliformes.

Materials and Methods

All experimental procedures for the use and care of the animals in the present study were approved by the Animal Care Committee of Azabu University. Blood samples were collected from Ceylon junglefowl (*Gallus lafayetii*) and helmeted guineafowl (*Numida meleagris*) (provided from Tokyo University of Agriculture, Atsugi, Japan), Japanese quail (*Coturnix japonica*) and turkey (*Meleagris gallopavo*) (provided from Nagoya University, Nagoya, Japan), ring-necked pheasant (*Phasianus colchicus*) (obtained from Chubu-chisha Inc., Hamamatsu, Japan) and Indian peafowl (*Pavo cristatus*) (obtained from local breeder, Sagamihara, Japan). Genomic DNA was extracted from blood samples. In brief, 10 μl of blood sample was mixed with 500 μl of cell lysis solution (0.15 M NaCl, 15 mM Na-Citrate) and centrifuged at 3,000 rpm for 10 min. Precipitates were washed two times with saline (0.835% NaCl, 0.5 mM EDTA). After aspiration of supernatant, precipitates were dispersed with 50 μl of saline, and mixed with 500 μl of Proteinase K solution (0.1% Proteinase K, 10 mM Tris-HCl, 10 mM EDTA, 0.5% SDS) and the mixture was incubated for 12 h at 37℃. After Proteinase K treatment, protein was removed by phenol-chloroform extraction. Thereafter, genomic DNA was precipitated by ethanol precipitation, dried and dissolved in 200 μl of TE buffer. Primers were designed based on nucleotide sequences of *PRL* promoter regions in turkey (Kurima *et al.,* 1995), Japanese quail (GenBank accession number: AB162005), ring-necked pheasant (AB 162004), and red junglefowl (NW_001471637). The *PRL* promoter region of each species was amplified by PCR using primer pairs (Prom-1F - Exon1R, and Prom-3F - Prom-2R), individually (Fig. 1). DNA sequencing was conducted using the primers indicated in Fig. 1 and dye-terminator chemistry on Applied Biosystems Model 310 sequencer by the dideoxy-mediated chain-termination method (Sanger *et al.,* 1977). The cloned sequences were analyzed by using FASTA and Philip softwares. On the basis of these results, phylogenetic trees were constructed using the Neighbor-Joining method (Saitou and Nei, 1987) using MEGA 4.0. Reliability of the trees was evaluated by bootstrap method with 1,000 replications.

Results and Discussion

PCR amplification between primers Prom-1F and Exon 1R and Prom-3F and Prom-2R yielded sequences of approximately 3,000–3,600 bp and 2,000–2,200 bp respectively and the amplicons were sequenced using primers indicated in Figure 1. In total, about 4,800 to 5,900 bp of promoter sequence was obtained from the 6 species (Ceylon junglefowl (4,879 bp); turkey (4,876 bp); pheasant (4,724 bp); Japanese quail (5,001 bp); peafowl (4,913



Prom-3F	5′-CATGCAGAGATCATGCTAAG-3′	Prom-3R	5′-ACAAGACCAGCATTCAGGCA-3′
3MF	5′-CATTAAAGTTCTSTGAGGG-3′	3MR	5′-ATTCAGATGACTGAGCATGC-3′
Prom-2F	5′-AGCCTGAATGCTGGTCTTGT-3′	Prom-2R	5′-GAAGGGAGAAGCAATCAACT-3′
2MF	5′-ACCTTCTGGTTSTGAYGC-3′	2MR	5′-CTTCTCCAAAATGTTCCC-3′
Prom-1F	5′-AAGTACAGGTCTGGGAATCT-3′	1AR	5′-GGTGGAAAGATAGCTGTCTT-3′
1AF	5′-CATAAGTCATATGAGGATCC-3′	1BR	5′-GGGACTTTGGACTCAGTGAT-3′
1BF	5′-AAGAGCACAAACCATGTATG-3′	1CR	5′-GAGGAGATTGAGTGGATCTC-3′
1CF	5′-ATAGGGGTTGGAAGGCACAT-3′	1DR	5′-GACCTAGATGTCCTGGTAGA-3′
1DF	5′-CTGACCCCATTCTCATCCCT-3′	1ER	5′-GTTCTGTACATGTGGAACAGG-3′
1EF	5′-GTCAACCTGTTGTCTACCAG-3′	1FR	5′-GTCGTAATCAGTGGAATCT-3′
1FF	5′-ATAGTTACGAAATAATGGAG-3′	1GR	5′-ATGGAGGCTTCTGGCAAGAC-3′
1GF	5′-CACAGACACATTAGATCAGG-3′	1HR	5′-GAGTATGGCTGGATGTAGAG-3′
1HF	5′-GCTGTATGGAGACAAACACA-3′	Exon1R	5′-CTTGKTGCTCATGGTAGRGA-3′

Fig. 1. **Primer location of galliformes *PRL*.** Open box represents Exon 1. Sequences are given in the 5′ to 3′ direction. Primer sequence was expressed using IUB codes.

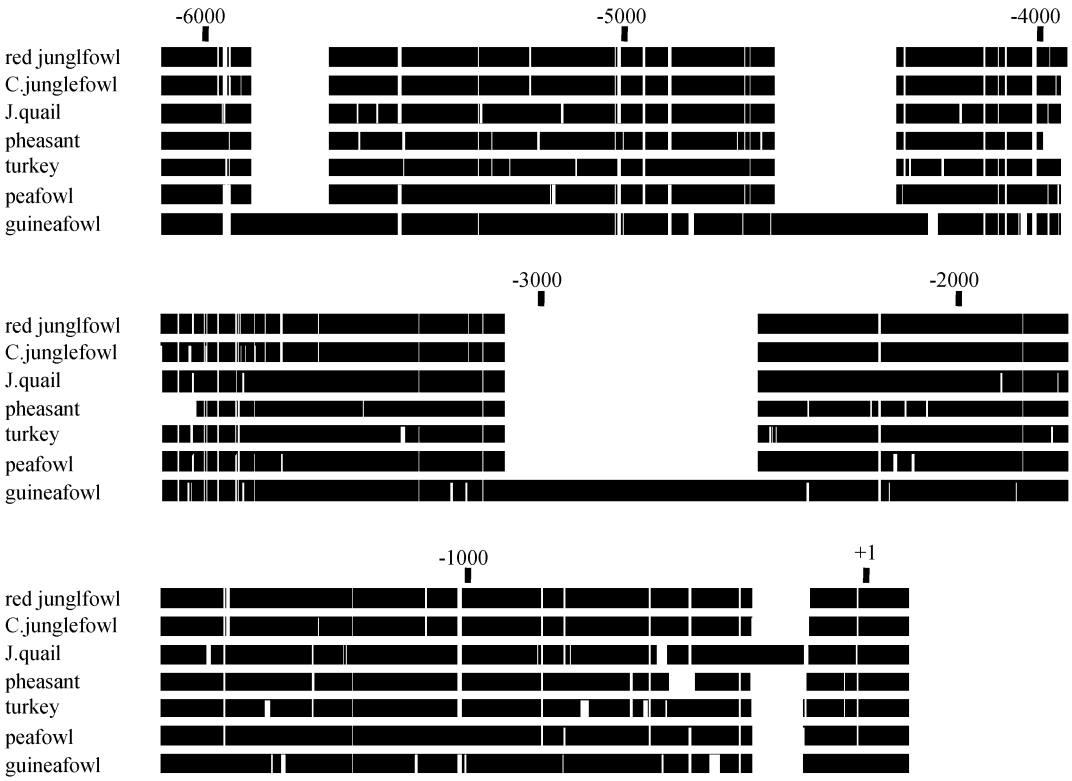


Fig. 2. **Schematic diagram of insertions/deletions at *PRL* promoter region in Galliformes (red junglefowl, Ceylon junglefowl〔C. junglefowl〕, Japanese quail〔J. quail〕, ring necked pheasant〔pheasant〕, turkey, Indian peafowl〔peafowl〕, helmeted guineafowl〔guineafowl〕).** The total length of aligned sequence is approximately 6,300 bp. The presence or absence of sequences compared with other galliformes are indicated black and white, respectively.

bp); and guineafowl (5,925 bp) (GenBank accession number AB162436, AB452964, AB452961, AB452962, AB452960 and AB452963, respectively.)). Then approximately 6,300 bp of sequences were obtained by alignment between the *PRL* promoter sequences in Galliformes. Various insertions/deletions among 7 galliformes *PRL* promoter including red junglefowl (NW_001471637) are schematically presented in Fig. 2. In guineafowl, relatively large specific insertion sequences, which consisted of 615 bp, 275 bp and 187 bp were found at −2289, −4120, and −5419 upstream from the predicted transcription start site, respectively. Following BLAST analysis, 615 bp insertion at −2289 had high similarity to chicken repeat 1 (CR1) transposable element (Kaiser *et al.,* 2007; Kriegs *et al.,* 2007). In this study, the sequence of turkey *PRL* was different from that reported by Kurima *et al.* (1995) in several positions. These genetic variations coincided with previously reported sequence by Zadworny *et al.* (2002). They reported that 1 of the variations at approximately −1300 was associated with higher rate of expression of broody behavior. In other galliformes, this region is well conserved and similar to the sequence by Kurima *et al.* (1995). On the other hand, the 24 bp insertion which is observed at position −358 in White Leghorn chickens was absent from 6 galliformes used in this study, meaning that this 24 bp insertion may be related to the rare expression of incubation behavior in White Leghorn chickens as in previous reports (Zadworny *et al.,* 2002; Cui *et al.,* 2006).

Sequence identity ranged from 86.1 to 98.3% (average 91.2%) over the entire cloned sequences of galliformes *PRL* promoter including red junglefowl (NW_001471637) (Table 1). In contrast, analysis of the mouse (AL591843) and rat (NW_047491) *PRL* promoter up to −5,000 shows fairly low identity (57.5%) in spite of being members of the same subfamily. These results may be related to differences in the evolutionary rate of change of *PRL* among species. On the basis of amino acid sequence, pituitary *PRL* has a slow evolutionary rate in birds, whereas the lineage leading to rodents, primates and some other mammals indicated episodes of rapid evolution (Wallis, 2000; Forsyth and Wallis, 2002). Thus, the *PRL* promoter of Galliformes is unusually highly conserved compared to within mammals suggesting that the basic mechanisms regulating transcription of *PRL* gene may be

Table. 1. **Sequence homology of avian *PRL* promoter region**

	RJF	CJF	J. quail	pheasant	turkey	peafowl	guineafowl	duck	budge.	J. sparrow
RJF		98.3	89.6	92.2	92.5	93.7	89.7			
CJF	99.5		89.3	92.1	92.7	93.4	89.5			
J. quail	97.8	97.3		88.7	89.1	89.9	86.1			
pheasant	95.6	95.1	95.1		94.4	92.6	88.7			
turkey	96.7	96.2	96.2	98.9		92.9	88.8			
peafowl	97.3	96.7	96.2	96.2	97.3		90.1			
guineafowl	98.4	97.8	97.3	96.2	97.3	97.8				
duck	90.7	90.2	91.3	89.6	89.6	90.2	92.4			
budge.	90.7	90.2	90.2	88.5	89.6	90.2	91.4	90.3		
J. sparrow	86.3	86.3	85.8	84.7	85.2	85.8	85.9	86.5	90.3	
ostrich	88.9	88.3	88.3	87.0	88.3	88.9	90.2	84.8	86.6	79.3

Values above and under the diagonal line represent homology of entire sequence from ATG codon (+55) to approximately −5000 upstream and proximal promoter (+55 to −130), respectively.
RJF: red junglefowl, CJF: Ceylon junglefowl, J. quail: Japanese quail, pheasant: ring necked pheasant, peafowl: Indian peafowl, guineafowl: helmeted guineafowl, duck: Peking duck, budge.: budgerigar, J. sparrow: Java sparrow.

common among galliformes and that *PRL,* itself, may have retained functions which are not compensated by the evolution of the cytokine superfamily in aves. In addition, phylogenetic analysis resulted in a mostly similar structure to previous phylogenetic studies (Sibley and Ahlquist, 1990; Pereira and Baker, 2006). However, turkey and pheasant were positioned in close proximity, and generated an independent cluster from other species (Fig. 4A). This result agrees with the phylogenetic studies based on CR1 transposable elements (Kaiser *et al.,* 2007; Kriegs *et al.,* 2007). Thus, turkey and pheasant may be close relationship species.

The sequences within the proximal promoter region (+55 to −130) in Ceylon junglefowl, Japanese quail, pheasant, turkey, peafowl and guineafowl were compared to red junglefowl, duck (Kansaku *et al.,* 2005), Java sparrow (Hiyama *et al.,* in press), budgerigar and ostrich (Kansaku *et al.,* 2008) (Fig. 3). The transcription start site in these galliformes species was predicted to be in the same position as turkey, chicken and duck (Kurima *et al.,* 1995; Ohkubo *et al.,* 2000; Kansaku *et al.,* 2005), since the translation initiation codon (ATG) was observed at similar position in all species used in this study and the sequence similarity was an average of 97.0% within the galliformes, and 91.6% among all species compared (Table 1). In addition, the phylogenetic tree based on the proximal promoter matched with the tree based on the entire cloned promoter sequence within galliformes (Fig. 4B). It is noteworthy that a putative Pit-1 binding site between −76 and −103 in chicken (Ohkubo *et al.,* 2000) and VRE at −40 to −74 in turkey (Kang *et al.,* 2004) were almost completely conserved in these galliformes as in duck, ostrich and budgerigar (Kansaku *et al.,* 2008). Although, in mammals, Pit-1 plays an important role associated with cAMP in the *PRL* gene expression mechanism (Mangalam *et al.,* 1989; Iverson *et al.,* 1990; Howard and Maurer, 1995), the role of Pit-1 in aves is controversial since levels of Pit-1 mRNA in pituitary gland do not correlate to the expression of *PRL* mRNA (Wong *et al.,* 1992b). On the other hand, the VRE core sequence at −53 to −64 has been found to be highly conserved between avian and mammalian species, and mutation of this core sequence resulted in loss of VIP-stimulated PRL secretion in turkey (Kang *et al.,* 2004). Moreover, they found the binding of Pit-1 and other 2 proteins to VRE. Hence, basal expression of *PRL* may be regulated by not only Pit-1 but also DNA binding protein via VRE. Since VRE core sequence is strongly conserved in a wide range of avian species, these regulating mechanism for *PRL* gene expression are most likely common among avian species. In addition, a novel WD motif DNA binding protein (*PRL* regulatory element binding; PREB) was cloned and characterized for up-regulating *PRL* promoter activity in rats (Fliss *et al.,* 1999). It is notable that PREB binds to a site non-identical to the Pit-1 binding element of *PRL* proximal promoter and that the *PREB* gene is also highly conserved across species. However, since there are no reports of common function of VRE between avian and mammalian species or PREB homologous PRL binding protein in aves, further studies are required to elucidate the existence and function of PREB in avian species.

In conclusion, the basic structure of *PRL* promoter is strongly conserved in Galliformes. In particular, within the proximal promoter, several potential sequences for regulating transcription of *PRL* are conserved between widely avian species. These results suggest that the mechanisms involved in gene expression of *PRL* may be conserved in Galliformes.

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red junglefowl  TGAATATGAA TGTGGAAGAG AGGCAATTTG ATGTTTGTAA TTATCGAGGT AAACTCCACG
C. junglefowl   TGAATATGAA TGTGGAAGAG AGGCAATTTG ATGTTTGTAA TTATTGAGGT AAACTCCACG
J. Quail        TGAATATGAA TGTGGAAGAG AGACAATTTG ATGTTTGTAA TTATCGAGGT AAACTCCACG
pheasant        TGAATATGAA TGTGGAAGAG AGGCAATTTG ATGTTTGTAA TTACCGAGGT AAACTCCACA
turkey          TGAATATGAA TGTGGAAGAG AGGCAATTTG ATGTTTGTAA TTACCGAGGT AAACTCCACA
peafowl         TGAATATGAA TGTGGAAGAG AGGCAATTTG ATGTTTGTAA TTATCGAGGT AAACTCCACA
guineafowl      TGAATATGAA TGTGGAAGAG AGGCAATTTG ATGTTTGTAA TTATCGAGGT AAACTCCACG
duck            TGAATATGAA TGTGGAAGAA AGGCAGTTTG ATGTTTGTAA TTATCGAGGT AAACTCCACG
J. sparrow      TGAATATGAA TGTGGAAGAA AGGCAATTTG ATGTTTGTAA TTATGGAGGC AAACTCCACA
budgerigar      TGAATATGAA TGTGGAAGAA AGGCAATTTG ATGTTTGTAA TTATCGAGGC AAACTCCATG
ostrich         ---------- ---------- AGGCAATTTG ATGTTTGTAA TTATCGAGAT AA-CTCCATG

red junglefowl  ACCTGCTGAA TGTATGCAAA AGTGGACCCC GGATGGTGTA TATAAATCTG ACGTG--CAG
C. junglefowl   ACCTGCTGAA TGTATGCAAA AGTGGACCCC GGATGGTGTA TATAAATCTG ACGTG--CAG
J. Quail        ACCTGCTGAA TGTATGCAAA AATGGACCCC GGATGGTGTA TATAAATCTG ATGTG--CAG
pheasant        ACCTGCTGAA TGTATGCAAA C-TGGACCCC GGATGGTGTA TATAAATCTG ACATG--CAG
turkey          ACCTGCTGAA TGTATGCAAA C-TGGACCCC GGATGGTGTA TATAAATCTG ACATG--CAG
peafowl         ACCTGCTGAA TGTATGCAAA AGTGGACCCC GGATGGTGTA TATAAATCTG ACATG--CAG
guineafowl      ACCTGCTGAA TGTATGCAAA AGTGGACCCC GGATGGTGTA TATAAATCTG ACATGTGCAG
duck            ACCTGTTGAA TATATGCAAA A-TGGACCCC GGATGGTGTA TATAAATCTG GTATGTGCAG
J. sparrow      ACCTGCTGAA TGTATGCAAA A-TGGACCCT GCATGGTGTA TATAAGAGCA GTATGTGCAG
budgerigar      ACCTGCTGAA TGTATGCAAA A-TGGACCAT GGATGGTGTA TATAAAAGTG GCATGTGCAG
ostrich         ACCTGCTGAA TGTATTCAAA A-TGGACCCT AGATGGTGTA TATAAACCTG ACACGTTCAG

red junglefowl  AAAGTAAGAG CAGGTATTGA GATTTCTTTC TGGTAGAGCA AGTCATCACA CAGAATCCCT
C. junglefowl   AAAGTAAGAG CAGGTATTGA GATTTCTTTC TGGTAGAGCA AGTCATCACA CAGAATCCCT
J. Quail        AAAGTAAGAG CAGGTATTGA GACTTCTTTC TGGTAGAGCA AGTCATCACA CAGAATCCCT
pheasant        AAAGTAAAAG CAGGTATTGA GACTTCTTTC TGGTAGAGCA AGTCATCACA GAGAATCCCT
turkey          AAAGTAAGAG CAGGTATTGA GACTTCTTTC TGGTAGAGCA AGTCATCACA GAGAATCCCT
peafowl         AAAGTAAGAG CAGGTATTAA GACTTCTTTC TGGTAGAGCG AGTCATCACA CAGAATCCCT
guineafowl      AAAGTAAGAG CAGGTATTGA GACTTCTTTC TGGTAGAGCA AGTCATCACA CAGGATCCCT
duck            AAAATAAAAG CAAGTATTGA GACTTCTTTC TGGCAGAGCA AGTCATCCTA CAGGGTCTCT
J. sparrow      AGAATAGCAG CAAGAATTGA GATTTCTTTC TGGTAAAGAA AGTCATCATC CAGAATCTCT
budgerigar      AGAATAAGAA CAAGTATTGA GACTTCTTTC CGGTAGAGCA AGTCATCATA CAGAATCTCT
ostrich         AAAATAAGAG TAAGTATTTA GACTTCCTTT TGGTAGAGCA AGTCACCACA TAGGATCCCT

red junglefowl  ACCATG
C. junglefowl   ACCATG
J. Quail        ACCATG
pheasant        ATCATG
turkey          ACCATG
peafowl         ACCATG
guineafowl      ACCATG
duck            ACCATG
J. sparrow      ACCATG
budgerigar      ACCATG
ostrich         ACCATG
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Fig. 3. **Comparison of *PRL* promoter at −130 to +55 in avian species.** VRE (Kang *et al.*, 2004) and Putative Pit-1 binding sites (Ohkubo *et al.*, 2000) are indicated by underline and dash underline, respectively. TATA signal, predicted 5′ UTR and ATG codon are showed by double underline, bold, and bold with double underline respectively. C. junglefowl: Ceylon junglefowl; J. quail: Japanese quail; pheasant: ring necked pheasant; peafowl: Indian peafowl; guineafowl: helmeted guineafowl; duck: Peking duck; J. sparrow: Java sparrow.

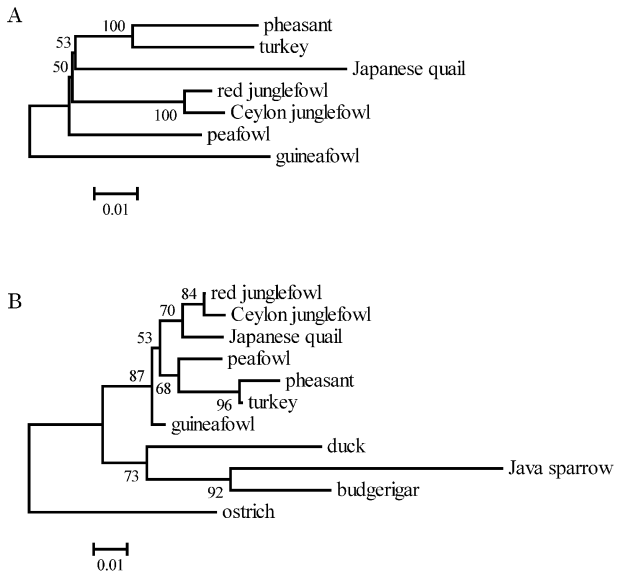

Fig. 4. **Neighbor-Joining tree based on promoter region sequence among 7 galliformes (A) and proximal promoter region sequence among 11 avian species (B).** The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. Pheasant: ring necked pheasant, peafowl: Indian peafowl, guineafowl: helmeted guineafowl, duck: Peking duck.

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