

ORIGINAL ARTICLE

Complete mitochondrial genome sequence of *Bonasa sewerzowi* (Galliformes: Phasianidae) and phylogenetic analysis

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Abstract *Bonasa sewerzowi*, the smallest and most southerly distributed grouse species in the world, is a bird endemic to China. The population of *B. sewerzowi* had shown a declining trend, which made it to be the endangered species in the China Red Data Book and Category I of nationally protected animals. So far, however, most studies about this species were mainly focused on the morphological and ecological aspects. In order to further study the feature of *B. sewerzowi*, the complete mitochondrial genome (mitogenome) of *B. sewerzowi* was sequenced by Illumina Hiseq 2000 high-throughput sequencing. Then, we focused on comparative genomics of two *Bonasa* species to find their characteristics. Finally, phylogenetic position of *Bonasa* was made based on the mitogenome dataset. Our results revealed that: (1) the mitogenome of *B. sewerzowi*, consisting of 16658bp, displayed typical genome organization and gene order found in other previously determined Galliformes mitogenomes; (2) the structure and composition of mitogenomes were similar between *B. sewerzowi* and *B. bonasia*; (3) the monophyly of *Bonasa* was well supported, which had a closer phylogenetic relationship with *Meleagris gallopavo*.

Key words *Bonasa sewerzowi*, mitogenome, comparative genomics, phylogeny.

1 Introduction

Bonasa is unique to palearctic realm, with major two species in China, namely, the Chinese grouse (*B. sewerzowi*) and the hazel grouse (*B. bonasia*). *B. sewerzowi*, one of the least known grouse in the world (Johnsgard, 1983; Storch, 2000b), is a bird endemic to China, inhabiting the conifer-dominated mountains between 2400 and 4300m elevation. It is the smallest grouse in the world (Johnsgard, 1983; Sun *et al.*, 2005), and the most southerly distributed grouse species in the world (Sun, 2000), with distributing in Gansu, Qinghai, Sichuan and some other places. It is also one of the rarest grouse species in the world (Storch, 2000b). The population of *B. sewerzowi* had shown a declining trend with the threat of hunting and habitat loss or fragmentation, which made it to be the endangered species in the China Red Data Book (Zheng & Wang, 1998). It was list as “Lower Risk (near threatened)” species (IUCN 1996) and threatened species (IUCN 2004), and Category I of nationally protected animals (Sun, 2000). *B. bonasia*, a small and unobtrusive grouse species, inhabits coniferous and mixed forests of the Eurasian boreal forest belt, and also mountainous areas of Central and Eastern Europe

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(Bergmann *et al.*, 1996). It has the narrowest requirements of habitat structure among forest grouse species in the Palearctic Region (Swenson, 1995), and prefers the semi-natural forests containing dense understory (Swenson, 1995; Bergmann *et al.*, 1996). This species is considered to be a keystone species for old-growth forests, an umbrella species for old-growth forest species assemblages and a good indicator for measuring the intensity of forestry (Kajtoch *et al.*, 2012). *B. bonasia* is endangered throughout Europe (Swenson & Danielson, 1991; Storch, 2000a), and listed as the Category II of nationally protected animals in China (Zhao, 2001).

Characterized by the distinct secondary sexual traits and variable mating systems (Wittenberger, 1978), life history parameters, and social organization (Lucchini *et al.*, 2001), grouse is particular interested to ecologists and ethologists, and extensive researches have been made (Mougeot *et al.*, 2007; Storch, 2007). So far, the research of *B. sewerzowi* mainly focused on the aspect of spawning rhythm (Sun *et al.*, 2002), population (Wang *et al.*, 1987; Sun *et al.*, 2003), morphometrics (Sun *et al.*, 2005), ethological and acoustical characters (Scherzinger *et al.*, 2006), nest site selection (Sun *et al.*, 2007), the natural history, behavior and conservation (Sun & Fang, 2010) and the sexual ornamentation (Yang *et al.*, 2013). The study of *B. bonasia* concentrated mainly on the habitat suitability at the landscape scale (Mathys *et al.*, 2006), the habitat selection in a montane forest (Müller *et al.*, 2009) and the occurrence in fragmented forests (Kajtoch *et al.*, 2012). However, the characteristic feature and phylogenetic analysis of *Bonasa* based on the complete mitogenome has rarely reported.

In this study, the mitogenome of *B. sewerzowi* (Galliformes: Phasianidae: Tetraoninae) was sequenced and made a general introduction of its genome organization. Besides, combined the mitogenome of *B. bonasia* (FJ752435) obtained from GenBank database, a detailed analysis of comparative genomics was made to get further information about *Bonasa*. Furthermore, the phylogenetic position of *Bonasa* was made from the level of mitogenome based on 42 species of 24 genera, combining with 2 Anseriformes species as outgroups, in order to provide molecular evidence for phylogenetic relationship of this genus with other Galliformes species.

2 Materials and methods

2.1 Specimen collection

The specimen of *B. sewerzowi* was collected in Lianhuashan, Gansu, China in 2008. The sample was preserved in 100% ethanol and stored at -20°C. The voucher specimen was deposited in Key Laboratory of Zoological Systematics and Evolution, Institute of Zoology, Chinese Academy of Sciences.

2.2 DNA extraction, amplification and sequencing

Total DNA was extracted using traditional phenol-chloroform-isoamyl alcohol method (Dang *et al.*, 2008) and stored at -20°C. According to the position and sequence of primers offered by Sorenson (2003), combining mitogenomes of other Galliformes species were downloaded from GenBank database, the 12 sets of primers were confirmed with minor changes based on the conserved sequences and used to amplify the complete mitogenome (Table 1). Besides, the quality of primer pairs was evaluated by Oligo 6.0. The PCR reaction program was described as follows: 93°C denaturation for 2 min; (92°C for 10 s, 58–53°C for 30 s, 68°C for 10 min) × 20 cycles; (92°C for 10 s, 53°C for 30 s, 68°C for 10 min, and each cycle increases 20 s) × 20 cycles; 68°C for 7 min; 4°C insulation. The PCR amplifications were carried out in a volume of 15 µL, which contained 2.4 µL of 2.5 mmol/L dNTPs, 2.1 µL of each primer (10 µmol/L), 1.5 µL of 10×LA PCR Buffer I (Mg²⁺ Free), 1.5 µL of 25 mmol/L MgCl₂, 1 µL of DNA templates, 0.18 µL of 5 U/µL LA Taq polymerase (Takara, Dalian, China) and 4.22 µL ddH₂O. PCR products with good quality were purified with DNA Gel Purification Kit after separation by electrophoresis in a 1.0% agarose gel. The 12 target fragments were sequenced by Shenzhen Huada Gene Technology Co., LTD using Illumina Hiseq 2000 high-throughput sequencing system. The gaps after assembly were filled by the sanger sequencing using adjacent primers provided by Sorenson (2003) (Table 1).

Table 1. Primers used in this study.

Name	Sequence (5'-3')	Length (bp)
L1263x	AAAGCATGGCACTGAA	16
H2891x	TGRTGGCTGCTTKAAGGCCAC	22
L2260x	CAAGGTAAGYGTACCGGAAGGTG	23
H4644x	TCRAATGGGGCRCGGTTRGTTC	26
L3803x	CTACGTGATCTGAGTTCAGACCG	26
H6681x	GGTAAAGAGTGCCAATRTCTTTRTG	25
L5758x	GGAGGHTGAATRGGHCTAAACCARAC	26
H7122x	ATGGTRGTGATRAAGTTGATDGCYCC	26
L6615x	CCTCTGTAAAAAGGACTACAGCC	23
H8121x	GGGCAGCCRTGRATTCATTC	20
L7525x	GTATGAGCCCACCAATATTYAC	23
H8628x	TCRTAGGTTTCAGTATCATTGRTGDCC	26
L8386x	GCCTCATCCCCATCATAGAAGA	23
H10884x	GGGTCAAATCCRCATTCTAYGG	23
L10635x	CACCACTTCGGATTYGAAGCAGC	23
H12344x	CTATGTGGCTHACRGAYGAGTARGC	25
L11458x	TCHACMCGAACACAYGGCTCHGA	23
H13563x	TGDAGTGCRGCTGTRTRGC	20
L13040x	ATCCRTTGGTCTTAGGARCCA	21
H15049x	GTGTCTGCDGTGTARTGYATKGC	23
L14080x	TCAACYCATGCMTTCTTYAARGC	23
H16064x	CTTCAGTTTTTGGTTTACAAGACC	24
L15725x	AAACCAGAATGRTAYTTYCTATTYGC	26
H1530x	GGTGGCTGGCACAAGATTAC	22
Supplement-1-L1263x	AAAGCATGGCACTGAA	16
Supplement-1-H1859b	TCGATTACAGAACAGGCTCCTCTA	24
Supplement-2-L10635x	CACCACTTCGGATTYGAAGCAGC	23
Supplement-2-L10236b	TTCTGAGCMTTCTTCCAYTCMAG	23
Supplement-2-L11458x	TCHACMCGAACACAYGGCTCHGA	23
Supplement-2-H11837b	ARGGTKGCTTCRAATGCRATRTARAA	26
Supplement-2-H12344x	CTATGTGGCTHACRGAYGAGTARGC	25
Supplement-3-L15413b	GGWGGATTYTCAGTAGACAACCC	23
Supplement-3-L15725x	AAACCAGAATGRTAYTTYCTATTYGC	26
Supplement-3-H1530x	GGTGGCTGGCACAAGATTAC	22

2.3 Sequence assembly, annotation and analysis

The sequence data were assembled using the SOAPdenovo software. The location and secondary structure of tRNAs were identified by the online software tRNAScan-SE 1.21 (Lowe & Eddy, 1997). If necessary, the tRNA secondary structure was manually calibrated based on the characteristics of a typical cloverleaf structure. The positions of remaining tRNA gene (tmS(agy)) unpredicted by tRNAScan-SE 1.21, protein-coding genes (PCGs), rRNA genes and CR were identified by comparing with the mitogenome of *B. bonasia* (FJ752435). Referred to the srRNA secondary structures of *Gallus gallus* and *Anas platyrhynchos* obtained from RNA database (<http://www.rna.cccb.utexas.edu/>), *Pseudopodoces humilis* (Yang *et al.*, 2010) and *Podoces hendersoni* (Ke *et al.*, 2010), the srRNA secondary structure of *B. sewerzowi* was predicted. The lrRNA secondary structure of *B. sewerzowi* was predicted based on the secondary structure of *Xenopus laevis* in RNA database, *Bos taurus* (Burk *et al.*, 2002), *Pseudopodoces humilis* (Yang *et al.*, 2010) and *Podoces hendersoni* (Ke *et al.*, 2010). Statistical analysis, such as nucleotide composition of different regions and codon usage of PCGs, were executed in MEGA4.1 (Kumar *et al.*, 1994).

The 41 Galliformes mitogenomes available in the GenBank database combined with *B. sewerzowi* were used to reconstruct phylogenetic relationship of *Bonasa*, with two Anseriformes species (*Anser anser*, EU932689; *Anseranas semipalmata*, AY309455) as outgroups. All PCGs were translated into amino acids and align with ClustalW, then

retranslated back to nucleotide sequences in MEGA 4.1. The remaining genes were aligned by online software M-Coffee (<http://tcoffee.crg.cat/>). A mitogenome dataset (PCGs+rRNAs+tRNAs+CR) was made. MrModelTest2.2 together with MrMTgui was used to select optimal model for this dataset (GTR+I+G). Maximum parsimony (MP), Maximum likelihood (ML) and Bayesian inference (BI) methods were employed to analyse this dataset. MP analysis was constructed using heuristic search in PAUP*4.0b10 with 1000 bootstrap resamplings. ML analysis was performed using RAxML-7.0.3 with the optimal model including 1000 replications. BI analysis was executed in MrBayes 3.1.2 also with the optimal model. Four Markov chains (one cold chain and three hot chains) were allowed to run 1000000 generations with Markov Chain Monte Carlo (MCMC) method, sampling every 100 generations, and the first 1000 burn-in samples were discarded.

3 Results

3.1 Genome structure and organization in *B. sewerzowii*

The complete mitogenome of *B. sewerzowi* was first sequenced and characterized. The high-throughput sequencing yielded effective assembly data with sequence depths (X) of 6118.62 after filtering out some reads, such as low-quality or adapter-sequence-polluted reads. The annotated mitogenome has been deposited in the GenBank database with the accession number KJ997914. This mitogenome was 16658bp in size and it encoded 37 genes typically found in most Galliformes: 13 PCGs, 2 rRNA genes, 22 tRNA genes (Fig. 1). In addition, a total of 1127bp of non-coding control region (CR) existed in mitogenome with locating between trnE and trnF. The gene order was consistent with other Galliformes

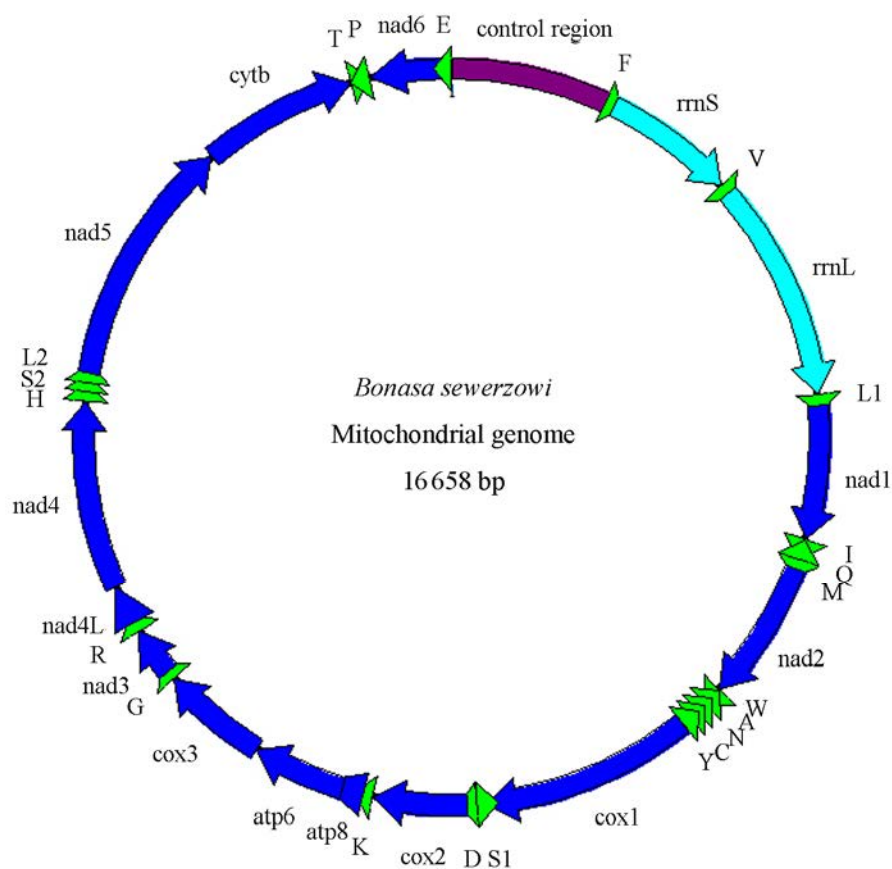


Fig. 1. Gene map of the *B. sewerzowi* mitochondrial genome. Transfer RNA genes are designated by single-letter amino acid codes. L1, L2, S1, and S2 denote trnL (uur), trnL (cun), trnS (ucn) and trnS (agv), respectively.

species (Mindell *et al.*, 1998). Similar to other Galliformes mitogenomes, all genes were coded on the H-strand, except for one PCG gene (*nad6*) and eight tRNA genes (*trnQ*, *trnA*, *trnN*, *trnC*, *trnY*, *trnS*(*ucn*), *trnP* and *trnE*).

3.1.1 Protein-coding genes

All PCGs of *B. sewerzowi* mitogenome started with a typical ATG codon with the exception of *cox1* gene, which used GTG as the initiation codon. There were four types of termination codon in PCGs: one (*cox1*) with AGG, one (*nad6*) with TAG, three (*nad2*, *cox3*, *nad4*) with incomplete termination codon T and the other eight with TAA. The three most abundant codons were CGA, UCA and CUA, in which the third positions select A. Leu was the most abundant amino acid (17.83%), while Cys was the lowest one (0.66%) in PCGs of *B. sewerzowi* mitogenome.

3.1.2 rRNA and tRNA genes

The two genes encoding the large and small ribosomal RNA subunits (*rrnL* and *rrnS*) were located between *trnV* and *trnL* (*uur*), and between *trnF* and *trnV*, respectively. The length of *rrnL* was 1610bp with an A+T content of 57.02%, while *rrnS* was 968bp in size with an A+T content of 52.79%. The predicted secondary structure of *rrnL* and *rrnS* contained 6 domains with 59 stem-loop structures and 3 domains with 46 stem-loop structures, respectively (Figs 2–3).

The length of tRNAs were changed from 66bp to 78bp in *B. sewerzowi* mitogenome. All tRNAs can be folded into the typical cloverleaf secondary structure with exception of *trnS* (*agy*), in which lost the DHU arm. The anticodon arm (5 bp) and the loop (7nts) were both conserved in all tRNAs except for *trnY* and *trnL* (*ucn*), which contained 3–4 matched base pairs in the anticodon arm. There were 30 mismatched base pairs, consisting of 17 G-U pairs, 1 A-G, 1 A-A, 2 U-U, 2 C-C, 2 U-C and 5 A-C mismatches. Amino acid accepting arm had higher mismatches compared with other arms, while DHU arm owned fewer mismatches.

3.1.3 The control region

According to the differences of base variation rate and base content, CR can be divided into 3 domains (I–III) (Randi & Lucchini, 1998). The positions of the 3 domains (I–III) were 1–307, 308–775 and 776–1127 in *B. sewerzowi* mitogenome, respectively (Fig. 4). Domain I contained two parts, A (1–157) and B (158–307), which a similar "goose hairpin" structure (34–58) of *Anas caerulea* (Quinn, 1992) and two conserved regions (extended termination- associated sequence, ETAS), ETAS1 (60–121) and ETAS2 (119–157), existed in part A. There were box F (345–372), E (390–409), D (450–474), C (498–523) and a conserved bird similarity box (737–750) in Domain II. Domain III included a poly(C) sequence (776–788) which was similar to replication initiation of mammalian heavy chain (O_H), and bidirectional promoter of translation (LSP and HSP, 972–988).

3.2 Comparative genomics analysis in two *Bonasa* mitogenomes

To date, only one complete mitogenome sequence from Galliformes, *B. bonasia* (FJ752435), was sequenced and available in GenBank database. The gene arrangement was compact in two *Bonasa* species with similar composition and gene order. The lengths of gene overlap and interval were 1–10bp, which the largest intervals (10bp in *B. sewerzowi*, 9bp in *B. bonasia*, respectively) located at *trnL*(*uur*)-*nad1*, while the biggest overlaps (10bp in two *Bonasa* species) existed between *atp8* and *atp6*. For the mitogenome dataset with 94.46% conserved sites, the P-distance was 0.053 between two *Bonasa* species.

3.2.1 A+T content and nucleotide skew

The nucleotide compositions of two *Bonasa* mitogenomes were C>A>T>G (A=30.2%, T=25.5%, C=30.9% and G=13.4% in *B. sewerzowi*, A=30.1%, T=25.8%, C=30.6% and G=13.5% in *B. bonasia*, respectively), with A+T content slight greater than G+C content. In the four major partitions (PCGs, rRNAs, tRNAs and CR) from two *Bonasa* mitogenomes, the highest A+T content was observed in CR region (Fig. 5). Based on the A+T content of CR region, Domain III showed the largest A+T content, followed by Domain I and Domain II was the lowest. In three other kinds of partitions, A+T content revealed the tendency of tRNAs > rRNAs > PCGs in *B. sewerzowi*, while tRNAs > PCGs > rRNAs

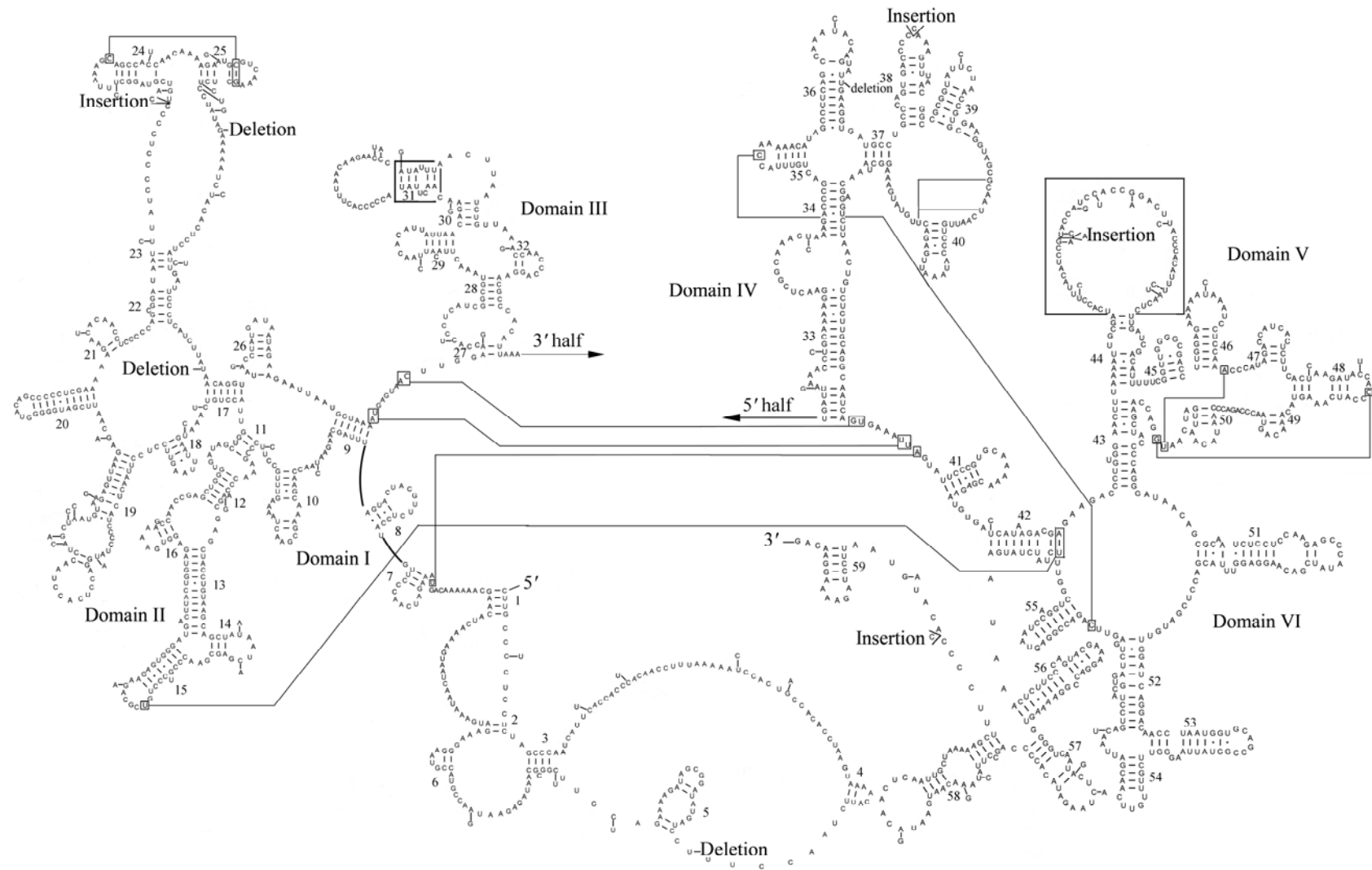


Fig. 2. The lrRNA secondary structure of *Bonasa sewerzowi* mitogenome and comparasion with *B. bonasia*. The different nucleotides in *B. bonasia* was pointed out.

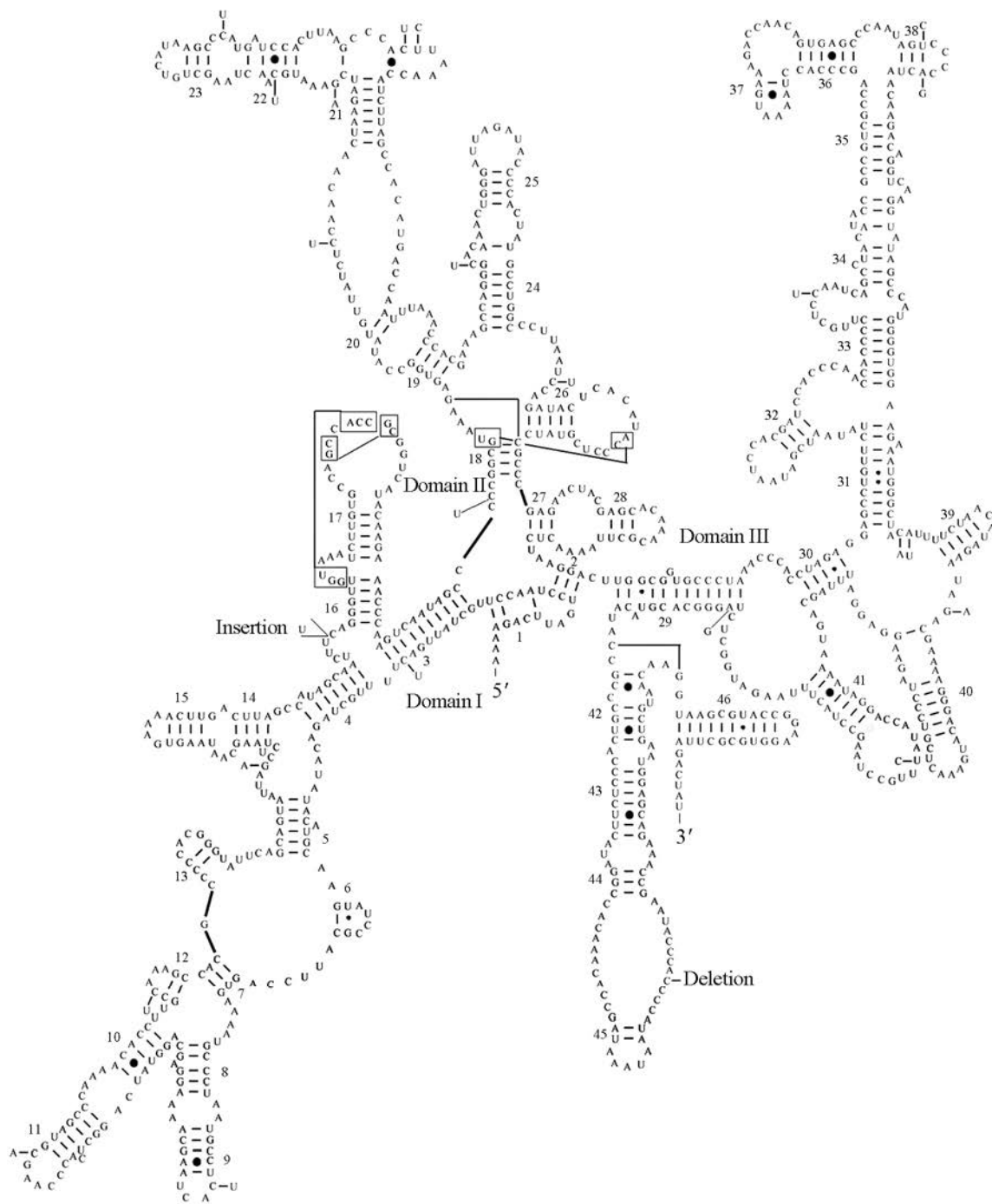


Fig. 3. The srRNA secondary structure of *Bonasa sewerzowi* mitogenome and comparison with *B. bonasia*. The different nucleotides in *B. bonasia* were pointed out.

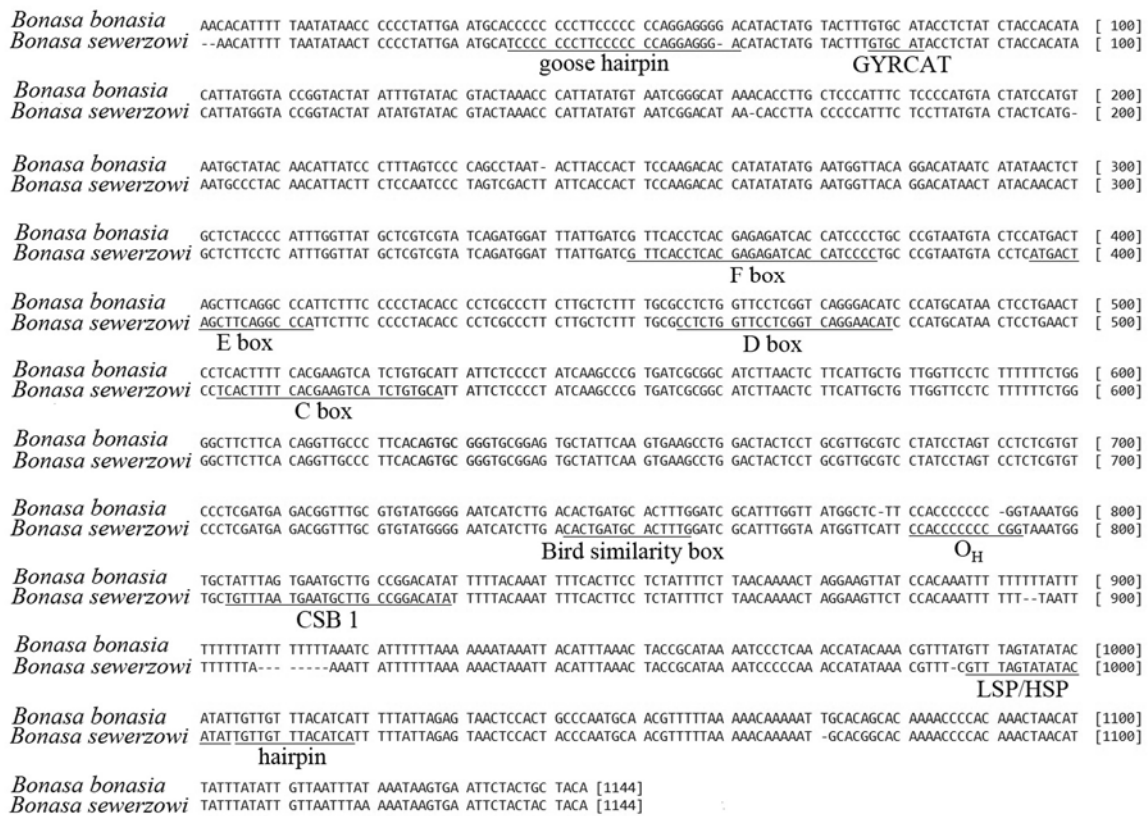


Fig. 4. The structure of CR in *Bonasa sewerzowi* mitochondrial genome and comparison with *B. bonasia*.

existed in *B. bonasia*. In the comparison of A+T content in three codon positions of PCGs, the second codon position contained higher A+T content than the first and third codon positions. Based on the nucleotide compositions of rRNAs, rrnL included larger A+T content than rrnS. The loops of all tRNAs showed higher A+T content than those in stems divided according to tRNA secondary structure model of mitogenome (K-N model) proposed by Kumazawa and Nishida (1993) and the tRNA secondary structure in *B. sewerzowi*.

The distributed trend of AT-skew and GC-skew were similar in two *Bonasa* mitogenomes (Fig. 5). The skew degree of four major partitions as mentioned above showed that rRNAs had higher AT-skew with A skew than others, while PCGs owned larger GC-skew with obvious C skew. According to the skew of CR region, Domain II showed the largest T skew, while Domain I and Domain III contained higher C skew. The three codon positions of PCGs revealed distinct differences, with T/C skew exhibited in the second codon position and obvious A/C skew in the third codon position. The rrnS and rrnL both showed higher A/C skew. The trend of stems in tRNAs owned slight T/G skew, while loops had A/C skew.

3.2.2 Transition and transversion of PCGs

In the comparison of nucleotide substitution in PCGs of two *Bonasa* mitogenomes, sequence alignment of PCGs revealed a 93.87% nucleotide sequence identity between *B. sewerzowi* and *B. bonasia*, only with 615 transition (Ts) and 81 transversion (Tv) mutations ($R=Ts/Tv=7.59$), showing that they were closely related. The third codon position existed more both Ts and Tv compared with the first and third codon positions. Besides, the second codon position contained higher R value than the first and third codon positions. Among 13 PCGs except for atp8, the T-C transition was larger than A-G, while transversion mainly existed in T-A and C-A of all PCGs. There was the highest R value in nad4L and the lowest one in cyt b.

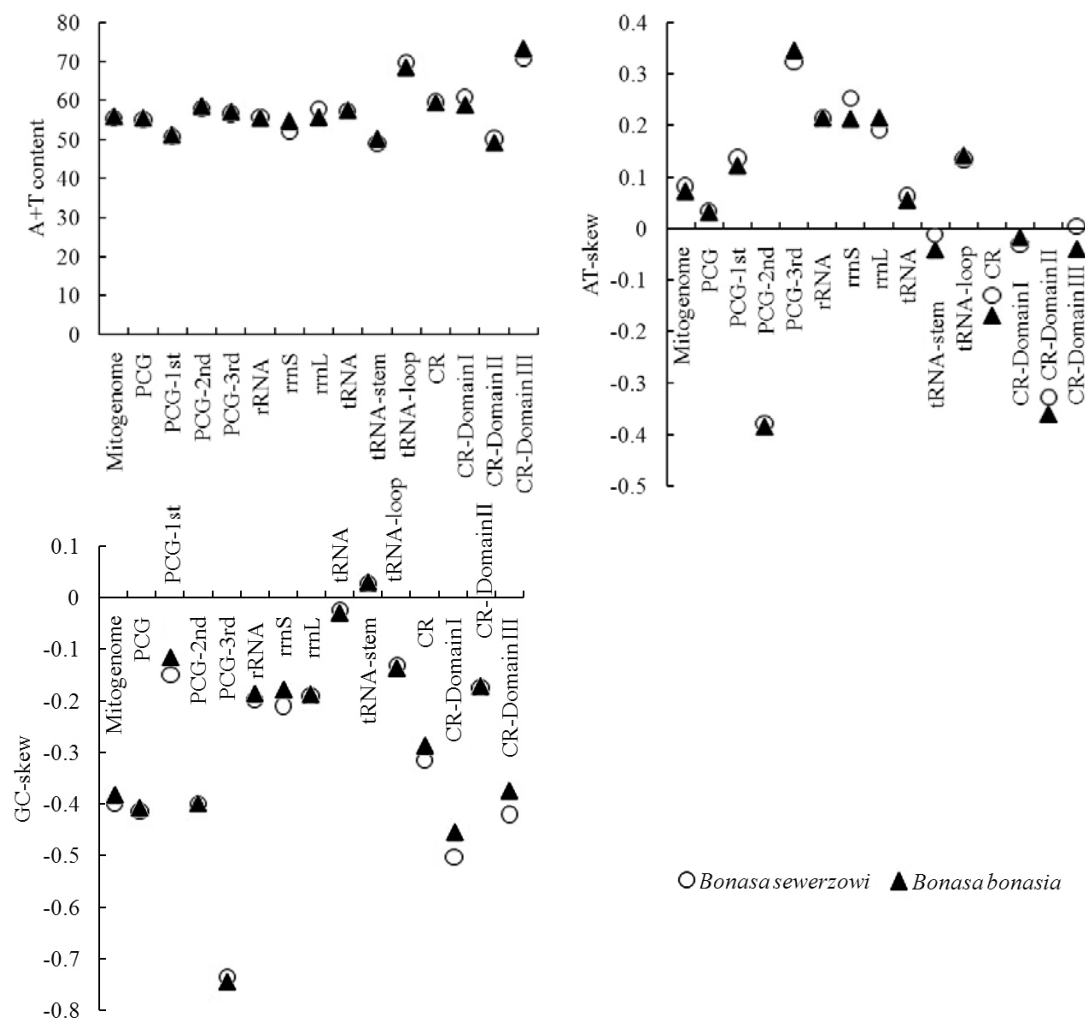


Fig. 5. Nucleotide composition of different partitions from two *Bonasa* mitogenomes. AT-skew, $(A-T)/(A+T)$; GC-skew, $(G-C)/(G+C)$; PCG-1st, the first codon positions of PCGs; PCG-2nd, the second codon positions of PCGs; PCG-3rd, the third codon positions of PCGs.

3.2.3 Comparison of RNA structures

The whole set of 22 tRNAs typical of typical of Galliformes mitogenome was found in *B. sewerzowi*, and the comparison with *B. bonasia* was made. The secondary structure of tRNA in *B. sewerzowi* was similar to that in *B. bonasia* with only 38 different bases. Those nucleotides in the stem of tRNA which was essential in maintaining the secondary structure were more conserved. There was relative conservative sites in trnL (uur), trnQ, trnM and trnY, while trnF and trnE showed larger differences in two *Bonasa* mitogenomes. The stems of rrnS and rrnL secondary structures were conservative in two *Bonasa* mitogenomes (Figs 2–3). The structures of rrnS showed that there were only 24 nucleotide differences with T-C transition the most common format, while rrnL contained 76 nucleotide differences also with T-C the highest one.

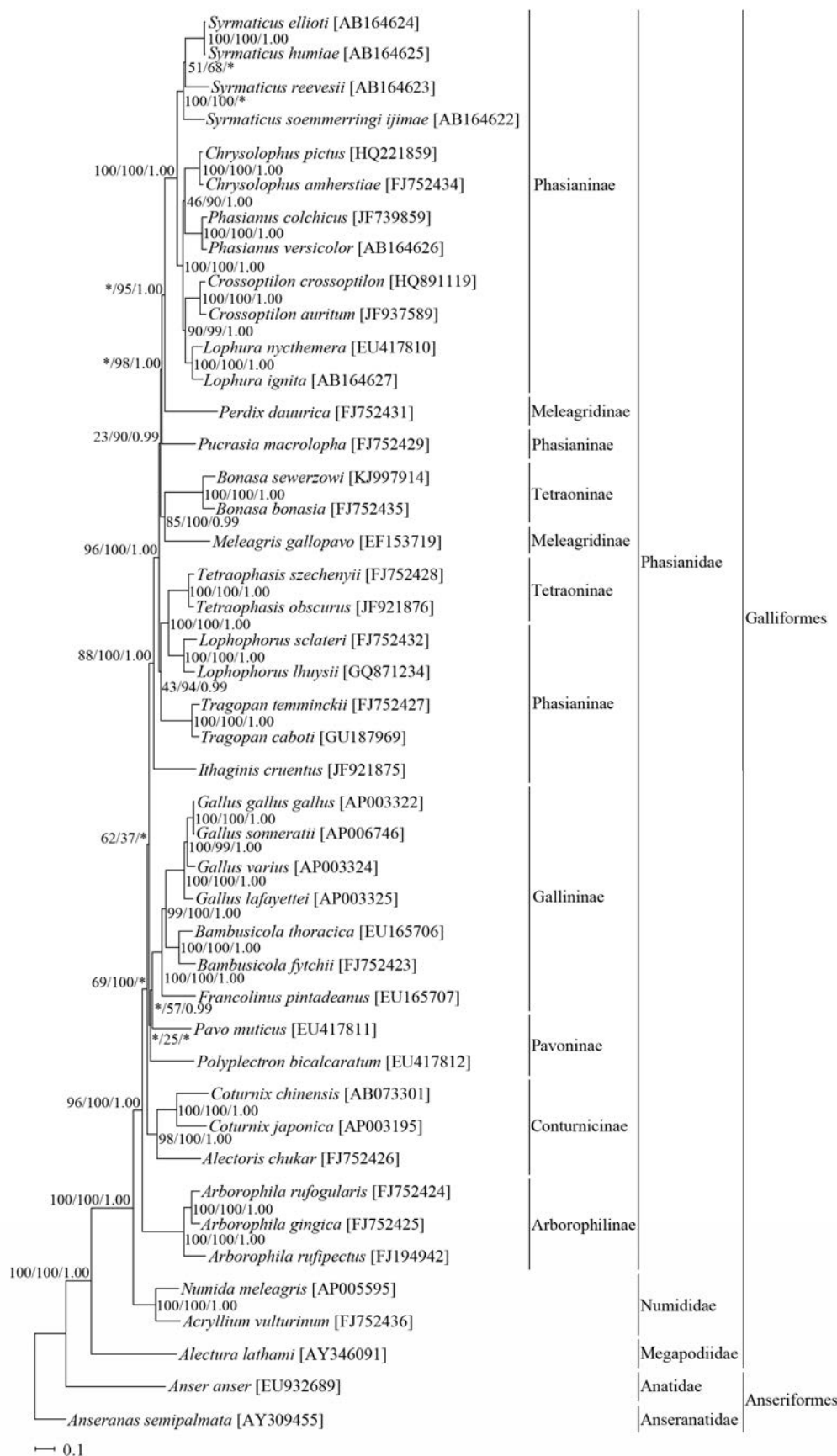


Fig. 6. The phylogenetic relationship of *Bonasa* among Galliformes based on the complete mitogenome. Branch lengths and topologies were obtained from Maximum Likelihood analyses. The numbers were the bootstrap values of MP/ML/BI trees in turn. * indicates that MP or BI tree was inconsistent with ML tree.

3.3 Phylogenetic position of *Bonasa*

When the complete mitogenome dataset was analysed, there were similar topologies among MP, ML and BI trees (Fig. 6). Based on the phylogenetic trees, *Bonasa* was recovered as monophyletic with *B. sewerzowi* clustered with *B. bonasia* (bootstrap value = 100% in MP and ML, posterior probabilities 1.00 in BI, respectively). Within Galliformes, *Bonasa* formed a sister group with *Meleagris gallopavo* with strong supports (bootstrap value = 85% and 100% in MP and ML, posterior probabilities 0.99 in BI, respectively).

4 Discussion

4.1 Characteristics in two *Bonasa* mitogenomes

In the two *Bonasa* mitogenomes, the highest A+T content was observed in the Domain III of CR region, while the lowest one existed in the stems of tRNAs. According to the A+T content in PCGs, the second codon position revealed the highest value rather than the third codon position. AT trends in different partitions of mitogenome may be the results of two transcription-dependent processes that result in different mutation rates on the transcribed and nontranscribed strands of a gene: transcription-coupled repair and deamination (Francino & Ochman, 1997). The third codon position in PCGs revealed A skew and C skew, therefore, PCGs was likely to choose A or C as their third codon position. T skew existed in the second codon position in PCGs indicating the preferential selection of T base in this position. There was also T skew in the Domain II of CR region, which was the most conservative region among 3 domains (Baker & Marshall, 1997).

There were higher Ts than Tv in 13 PCGs, in agreement with the conclusion that nucleotide substitution concentrated mainly on transition during the mitogenome evolution of animals (Irwin *et al.*, 1991). The third codon position contained more Ts and Tv, which indicated the nucleotide variation in PCGs of two *Bonasa* mitogenomes mainly concentrated on the third codon position under the lower selection pressure. The R in all PCGs was larger than 2.0, which indicated the nucleotide mutation was not saturation (Knight & Mindell, 1993).

Loop regions usually had fast evolutionary rate with larger differences among species, while stem regions were relative conservative (Noller, 1984), which may due to the different selection pressures. In comparison of RNA secondary structures of two *Bonasa* mitogenomes, the stem regions being essential in maintaining the secondary structure of RNA were also more conservative than loop regions. There were also a large number of GU mismatches in tRNAs, which also played an important role in maintaining the stability of tRNA secondary structure. The most nucleotide differences in tRNAs of two *Bonasa* mitogenomes were transition.

4.2 Phylogenetic analysis

In the topologies this study reconstructed, the monophyly of *Bonasa* was well supported with the sister relationship between *B. sewerzowi* and *B. bonasia*, which was also supported by many similar morphological features, such as nares covered with black plumes, forehead white and larynx black in male, and larynx brownish yellow in female. Furthermore, *Bonasa* and *Meleagris*, representing two subfamilies Tetraoninae and Meleagridinae respectively, formed a clade and were embedded in Phasianinae birds, indicating a closer phylogenetic relationship, which was similar to previous studies (Shen *et al.*, 2010; Jiang *et al.*, 2014).

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