Acta Ecologica Sinica 32 (2012) 232–239

Contents lists available at SciVerse ScienceDirect

Acta Ecologica Sinica

journal homepage: www.elsevier.com/locate/chnaes

The complete mitochondrial genome and phylogenetic analysis of *Nyctereutes procyonoides*

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ARTICLE INFO

Article history:
Received 18 January 2010
Revised 12 April 2012
Accepted 13 July 2012

Keywords:
Nyctereutes procyonoides
Mitochondrial genome
Canidae
Phylogeny

ABSTRACT

The complete mitochondrial genome sequence of the raccoon dog (*Nyctereutes procyonoides*) was determined by using the long and accurate polymerase chain reaction. The entire mitochondrial genome sequence is 16,713 bp in length contains two ribosomal RNA genes, 13 protein-coding genes, 22 transfer RNA genes and 1 control region. Most mitochondrial genes are encoded on the H strand, except for the ND6 gene and 8 tRNA genes. The base compositions of mitochondrial genomes present clearly A–T skew. All the transfer RNA genes can be folded into the typical cloverleaf-shaped structure except tRNA-Ser (AGY), which lacks the dihydrouridine arm. Protein-coding genes mainly initiate with ATG and terminate with TAA. Some reading frame intervals and overlaps are found in the mitochondrial genome. The control region can be divided into three domains: the extended termination associated sequences (ETASs) domain, the central conserved domain and the conserved sequence blocks (CSBs) domain. Three conserved sequence blocks (CSBs) and one extended termination associated sequences (ETAS-1) is found in the control region. The phylogenetic analysis based on the concatenated data set of 14 genes in the mitochondrial genome of Canidae shows that the raccoon dog has close phylogenetic position with the red fox (*Vulpes vulpes*) and they constitute a clade which has an equil evolutionary position with the clade formed by the genera *Canis* and *Cuon*.

1. Introduction

The raccoon dog (*Nyctereutes procyonoides*) is a middle-sized carnivore originating from eastern Asia [1]. As one of the canine family belonging species, raccoon dog is the only extant species in its genus. It is a native of southeastern Siberia, China, North Vietnam, Korea and Japan, and is also found in northeastern Russia and Mongolia [2,3]. The number of native raccoon dogs has declined due to hunting, fur trade, urbanization and diseases while the non-native populations grow up owing to the widely introducing of this animal to central and northern Europe from the 1920s, thriving in moist forests with abundant undergrowth [4,5]. The wild raccoon dog is also the only Canidae capable of spending 4–5 months of the year in superficial winter sleep in areas with cold winters [1]. For the unique life style and importance as a fur-bearing animal kept on farm, the raccoon dog has been widely studied in physiological adaptations, population ecology, food composition, diet niche, reproduction and diseases [6–11]. There are also some reports in comparative cytogenetics and chromosome painting devoted to discussing the relationship between domestic dog and raccoon dog and the subspecies differentiation of this animal [12–15]. According to their reports, there are at least three different subspecies of raccoon dogs distinguished: *N. p. procyonoides* and *N. p. ussuriensis* living in continental Asia and *N. p. viverrinus* living in Japan. But there is a lack of final delineation of the sub-species in other distribution areas and the phylogenetic position of raccoon dog within Canidae is also under discussion.

As the energy conversion organelle, mitochondria have their own expression system. In most mammals, mitochondrial genome is a closed circular molecule approximately ranging in size from 15 kb to 17 kb [16]. Most mammalian mitochondrial genomes reveal an overall conservation of gene order and a very compact organization of genetic information, which typically includes 13 protein-coding genes, 2 ribosomal RNA (rRNA) genes, 22 transfer RNA (tRNA) genes and 1 non-coding region (control region) [17–20]. Because of their small size, fast evolutionary rate, relatively conserved gene content and organization, maternal inheritance and limited recombination [21–25], many of the mitochondrial genomes are extensively used for studying population structure and phylogenetic relationships at various taxonomic levels [26–28]. Since the human mitochondrial genome was sequenced [29], a large number of mammalian complete mitochondrial genome data have come forth. To date (August, 2011), there are five Canidae species whose mitochondrial genomes have been sequenced, they are *Canis familiaris*, *Canis lupus*, *Canis latrans*,

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http://dx.doi.org/10.1016/j.chnaes.2012.07.003

Vulpes vulpes and *Cuon alpinus* [30–32]. Although some partial mitochondrial genes of raccoon dog have been studied so far, the complete mitochondrial genome sequence has yet to be reported.

In the present study, we sequenced the complete mitochondrial genome of raccoon dog for the first time, examined the genomic structure, gene order, codon usage and base composition. In combination with the complete genome data from GenBank, we also analyzed the phylogenetic relationships within Canidae based on the concatenated data of two ribosomal RNA genes and 12 heavy-strand encoded protein-coding genes. This will help us in further comprehending this species and making a more convictive definition of the phylogenetic relationships within the Canidae.

2. Materials and methods

2.1. Sample collection and DNA extraction

Liver sample was obtained from a female raccoon dog captured from the Dalai Lake Nature Reserve in Inner Mongolia of China. The sample was preserved at −80 °C. Total genomic DNA of the preserved sample was isolated by the standard phenol–chloroform extraction techniques with slight modification and visualized on 1.0% agarose gel [33].

2.2. PCR amplification and sequencing by primer walking

The mitochondrial genome of the raccoon dog was amplified by using the long and accurate polymerase chain reaction (LA-PCR) and Sub-PCR technique according to the manufacture's instruction (TaKaRa, China). Five pairs of LA-PCR primers were designed based on the mitochondrial genome sequences of *C. lupus*, *C. familiaris* and *Vulpes vulpes* (Table 1). PCR cycling was performed in a MJ Model PTC-200 thermal cycler.

LA-PCR was carried out in a 50 μl reaction mixture containing 1 μl template DNA, 5 μl 10 × LA PCR buffer (TaKaRa), 4 μl dNTP Mixture (2.5 mM), 2 μl primer (10 pM) and 1 μl LA-PCR Taq polymerase (5 U/μl). Cycling conditions were 95 °C for 5 min, followed by 35 cycles of 30 s at 95 °C, 40 s annealing at 57 °C, and an extension at 72 °C for 4 min and 30 s, with a final extension at 72 °C for 10 min.

The LA-PCR products were purified from 1% agarose gel with the DNA Agarose Gel Extraction Kit (Omega, USA). Eleven Sub-PCR primer pairs (Table 2) were designed to amplify the LA-PCR products into numerous small fragments, with each fragment sequence overlapping the next by at least 100 bp. PCR cycles were 95 °C for 5 min, followed by 35 cycles of 40 s at 95 °C, 30 s annealing at 57 °C, and an extension at 72 °C for 2 min and 30 s, with a final extension at 72 °C for 10 min. The Sub-PCR products were also purified from 1% agarose gel by using the DNA Agarose Gel Extraction Kit (Omega, USA) and sequenced on ABI 3730 xl by using a primer-walking strategy.

2.3. Sequence analysis

Sequences were assembled by using DNAMAN version 6.0 (Lynnon Biosoft, Quebec, Canada) and the complete sequence was submitted for BLAST searching. Two ribosomal RNA genes and the translation initiation sites of protein-coding genes were determined by comparison of corresponding sequence with reported mitochondrial genome sequences of other Canidae by using Clustal X program version 1.83 [34]. The locations of thirteen protein-coding genes were identified by using software SEQUIN version 9.20. The tRNA genes were identified by tRNA Scan-SE program version 1.21 (http://lowelab.ucsc.edu/tRNAscan-SE/)[35] and their secondary structures were also predicted by this program. Remaining tRNA genes which could not be identified by tRNA Scan-SE were predicted by proposed secondary structures [36] and anti-codons and their secondary structures were predicted manually. Base content and codon usage were calculated by using MEGA version 4.1 [37].

2.4. Phylogenetic analysis

The mitochondrial genome sequences of raccoon dog in this study, together with previously reported mitochondrial genome sequences of 8 Canidae species and 1 outgroup species (brown bear, *Ursus arctos*) from GenBank were used to perform phylogenetic analysis (Table 3). 12S rRNA genes, 16S rRNA genes and 12 heavy-strand encoded protein-coding genes were included for phylogenetic analysis [38–43]. The light-strand encoded ND6 gene was not used because it deviates markedly in nucleotide and amino acid composition from the other protein-coding genes [44,45]. The 14 mitochondrial genes were assembled in a partitioned matrix. The partition homogeneity test (ILD test) [46,47] was implemented in PAUP* version 4b10 with 1000 random addition sequence replicates [48]. Comparisons of gene partitions in a multiple partition and in all possible combinations were performed according to codon positions. All the ILD tests represented non-significant at $\alpha = 0.05$. Then the 14 mitochondrial genes were analyzed as a concatenated data set of 13,454 bp each in length for further analysis. The phylogenetic analysis was performed by using maximum parsimony (MP) [49,50], maximum likelihood (ML) [42,51] and Bayesian (BI) [52–55] inference methods.

MP and ML analyses were performed by using PAUP* version 4b10 [48]. The MP tree was obtained from heuristic searches (by using TBR tree swapping method) of 1000 random addition replicates. Bootstrap values (BP) of MP analysis were obtained by performing 1000 replicates of the same analysis. To select the model of DNA substitution that best fits the data for use in ML and Bayesian searches, a hierarchical likelihood ratio test approach implemented in the program ModelTest version 3.7 was used on the MP topology [56,57]. An iterative approach to search the ML tree was used to circumvent the prohibitive computational time of a large taxon set. The topology obtained in the MP analysis was used to estimate all the parameters of the best model specified by ModelTest. The estimated parameters were then used in a heuristic search for the ML tree. The tree obtained at this step is used to re-estimate the parameters. The new parameters are used in the next heuristic search. The cycle of parameter estimation and tree searching is performed until the estimates have stabilized [40,58]. Bootstrap values of ML tree were obtained by performing 100 replicates.

Table 1
Primers designed for amplifying and sequencing the complete mitochondrial genome of *Nyctereutes procyonoides* in the LA-PCR.

Primer no.	Product lenght (bp)	Upstream primer sequence(5′ → 3′)	Downstream primer sequence(5′ → 3′)
1-F/R	3275	TCCCTCTAGAGGAGCCTGTTC	GGGTATGGGCCCGATAGCTT
2-F/R	3311	GGCGGATAAAAGAGTTACTTTGATAGAG	GCGAATTTAACTTTGACAAAGTCATGT
3-F/R	3337	GAAGAAAGGAAGGAATCGAACC	GCGTAGGGATGATAATTTTTAGCATT
4-F/R	3904	GTATTTGCTGCCTGCGAAGC	TAGTGGTGGGATTGGTTGTGC
5-F/R	3534	GGGTATTGCTCAGTAGCCATAGC	GGTTTGCTGAAGATGGCGGTATAT