Tandem duplication of mitochondrial DNA in the black-faced spoonbill, *Platalea minor*

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Mitochondrial (mt) heteroplasmy in the control region (CR) of the black-faced spoonbill was investigated using LA-PCR. To avoid amplification of transpositional nuclear genome fragment from mtDNA (numt), PCR product of the almost-complete mitochondrial genome was amplified using primers designed to anneal on the *COIII* gene. Then nested LA-PCR product was amplified between the *cyt b* and *12S rRNA* genes using the almost-complete mitochondrial genome PCR product as a template. Nucleotide sequencing revealed tandem duplication composed of two units. The first contains *cyt b*-1, *tRNA*^{Thr}-1, *tRNA*^{Pro}-1, *ND6*-1, *tRNA*^{Glu}-1 and CR1, and the second consists of *cyt b*-2, *tRNA*^{Thr}-2, *tRNA*^{Pro}-2, *ND6*-2, *tRNA*^{Glu}-2 and CR2, followed by *tRNA*^{Phe} and *12S rRNA*. The duplicated *cyt b*-2 sequence coincided with 499 bp at the 3' end of *cyt b*-1. With the exception of the CR, the other genes in the duplicated sequence were identical to the original corresponding gene. Even though both CR1 and CR2 contain functional blocks, such as a poly-C site, a goose hairpin and a TAS structure in Domain I, the 3' end of CR1 was followed by a 112 bp sequence (non-coding region) that was not found in CR2 or in sequence homology analysis of similar genes. Meanwhile, CR2 ended in a complicated repeat sequence. The 5' flanking region in the Domain I (Region A) and the 3' flanking region in the Domain I (Region B) of the two CRs evolve in quite different manners: Region A was highly variable between CR1 and CR2 in the same individuals, while Region B was almost identical between them, which indicates concerted evolution.

Key words: black-faced spoonbill, concerted evolution, mitochondrial gene order, *Platalea minor*, tandem duplication

INTRODUCTION

Control region (CR) of the mitochondrial DNA (mtDNA) is characterized as being the most variable region of mtDNA (Brown et al., 1986; Fauron and Wolstenholme, 1976; Chang and Clayton, 1985), despite its functional significance involved in the initiation of duplication and transcription (Desjardins and Morais, 1990, 1991). Examination of the mutations in this region provides useful information of inter- and intra-species phylogeography (Moore, 1995; Zhang and Hewitt, 1996; Sorenson and

Quinn, 1998).

The avian mitochondrial genome encodes the same set of genes as other vertebrate mtDNAs, and is organized in a very similar fashion. However, they are arranged in a unique gene order. The contiguous *tRNA*^{Glu} and *ND6* genes are located immediately adjacent to the control region of the molecule, just ahead of the contiguous *tRNA*^{Pro}, *tRNA*^{Thr}, and *cyt b* genes (Desjardins and Morais, 1990; Quinn and Wilson, 1993). The avian CR is also divided into a conservative central domain (Domain II) with flanking variable left and right domains (Domains I and III). The high similarity of Domain II observed among members of the avian species is represented by the conservative motives in C, D, and F boxes (Brown et al.,

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1986), while Domains I and III contain numerous substitutions, indels, and repeat sequences (Wenink et al., 1994; Berg et al., 1995).

PCR techniques have greatly enhanced molecular phylogenetic analysis using mtDNA; however, these techniques have also revealed a high degree of heteroplasmy in mtDNA. The transposition of mtDNA sequence to the nuclear genome (numt) has been documented in a wide variety of taxa from fungi to vertebrates, and mounting evidence suggests that it is a common, if not ubiquitous, phenomenon (Zhang and Hewitt, 1996). Quinn and White (1987) documented the first example of a numt in birds. Numts present interesting opportunities for both the study of molecular evolution and phylogenetic analysis (Zhang and Hewitt, 1996; Quinn, 1997; Sorenson and Quinn, 1998). Arctander (1995) and Sorenson and Fleischer (1996) amplified nuclear sequences from avian blood samples and identified six different numts derived from the mtDNA control region in diving ducks (*Aythya*), which are highly similar to the mtDNA sequences of ducks.

Another potential source of apparent heteroplasmy arises from the duplication of a section of mtDNA. Duplicated gene segments, including the CR, have been reported in many taxa, such as ticks (Black and Roehrdanz, 1998; Campbell and Barker, 1999; Shao et al., 2005), an insect (Ogoh and Ohmiya, 2004), sea cucumbers (Arndt and Smith, 1998), fish (Lee et al., 2001), frogs (Sano et al., 2005), lizards (Kumazawa and Endo, 2004; Amer and Kumazawa, 2005), snakes (Kumazawa et al., 1996), and turtles (Parham et al., 2006).

With respect to mtDNA in avian species, falcons and a primary group of songbirds have a unique gene order consisting of *cyt b*, *tRNA^{Thr}*, CR, *tRNA^{Pro}*, *ND6*, *tRNA^{Glu}*, *nc* (non-coding sequence, which has similarity with the CR), *tRNA^{Phe}*, and *12S rRNA* (Mindell et al., 1998). Parrots of the genus *Amazona* have duplicated mitochondrial CRs, resulting in a gene order of *cyt b*, *tRNA^{Thr}*, *p-ND6* (partial *ND6*), *p-tRNA^{Glu}* (partial *tRNA^{Glu}*), CR1, *tRNA^{Pro}*, *ND6*, *tRNA^{Glu}*, CR2, *tRNA^{Phe}*, and *12S rRNA* (Eberhard et al., 2001). These CRs contain the typical conserved sequence features.

Albatrosses from the genus *Thalassarche* (Abbott et al., 2005) also have an altered mtDNA gene order resulting from a duplicated sequence. The duplicated section begins with *d-cyt b* (degenerate partial copy of *cyt b*) + *p-cyt b* (partial copy of *cyt b*), followed by *tRNA^{Thr}-2*, *tRNA^{Pro}-2*, *ND6-2*, *tRNA^{Glu}-2*, and ends with the CR. The *d-cyt b* is a 120 bp fragment with 70% similarity to *cyt b*, and *p-cyt b* is a 39 bp fragment that is identical to a functional copy of the *cyt b* 5' flanking sequence. The total length of CR1 in albatross is 973 bp, whereas CR2 is estimated to be up to 2300 bp, due to the approximately 1300 bp repeat sequence in Domain III. The degree of similarity between the conserved elements in CR1 and CR2 is

thought to be of functional importance. Sequence variations between Region A (110 bp) and B (217 bp) in both CR1 and CR2 are quite dissimilar; Region A sequences in CR1 and CR2 evolved independently, while Region B sequences in both CRs appear to have evolved in concert.

The complete mtDNA genome of the black-faced spoonbill has been reported (GenBank Accession No. NC010962; Lee et al., 2007). We attempted to perform a polymorphic analysis using the CR region of this species, and found that the sequence outputs obtained by direct sequencing contained two peaks which look like heterozygotes. The mtDNA genome recorded in GenBank of this species contained no duplications as the common bird gene order. However, the sequencing of the genome in GenBank was performed using the shotgun method, whereas ours was obtained by direct sequencing of PCR products.

This study describes an investigation into the source of mitochondrial heteroplasmy in the black-faced spoonbill (Hancock et al., 1992; Matheu and Hoyo, 1992). We hypothesized that the most probable cause was a combination of numt (Sorenson and Fleischer, 1996; Kidd and Friesen, 1998; Ruokonen et al., 2000) and secondary heteroplasmy, which gives rise to two types of mtDNA in the cells of an individual (Grzybowski, 2000). A third possibility that we investigated was duplication of the CR region, which has been reported to occur in several avian taxa (Mindell et al., 1998; Abbott et al., 2005; Gibb et al., 2007; Tatarenkov and Avise, 2007).

MATERIALS AND METHODS

Muscle samples from two individuals (Lab. No. 08kh03 and 08kh04) found dead at the Beppu river mouth in Aira Town, Kagoshima Prefecture, were obtained from the Aira Animal Hospital and stored in 99% ethanol. Approximately 2 mg of sliced tissue was placed in 285 µl of cell lysis solution (Puregen Inc., USA) with 15 µl of 20 mg/ml proteinase K and incubated for 2 hours at 55°C on a rotator. Nucleic acids were extracted using the Puregen Tissue Kit (Puregen Inc., USA).

To avoid amplification of the numt fragment, the almost-complete mitochondrial PCR product was amplified by the LA-PCR method with a primer set (LCO3.kh and HCO3.kh) designed to anneal to the *COIII* gene (Fig. 1). Then nested LA-PCR product was amplified between the *cyt b* and *12S rRNA* genes using the almost-complete mitochondrial genome PCR product as a template with TaKaRa LA *Taq* polymerase (TaKaRa Bio Inc., Otsu, Japan) using a Lcytb1.kh and 12sR2.kh primer set, of which, the latter was designed based on 12sR2 (Abbott et al., 2005) sequences, and on the mtDNA sequence submitted by Lee et al. (2007; Accession No. NC_010962). Duplication was examined using an Lcon9.kh and Hcon1.kh primer set with TaKaRa Ex *Taq* HS polymerase (TaKaRa Bio Inc. Otsu, Japan). Five specific internal

primer sets (Lcytb1.kh and Hnon-c.kh, LND6.kh and Hcyt b4.kh, Lnon-c.kh and H454.gr, Lcon1.kh and Hcon2.kh, LND6.kh and 12sR2.kh) were designed (Table 1) and used for sequencing.

PCR products were enzymatically purified (PCR Product

Fig. 1. Schematic representation of the mitochondrial (mt) genome of the black-faced spoonbill, *Platalea minor*. PCR products of the almost complete mt genome and sequence determined region (*cyt b* to *12S rRNA*) are shown as arrows.

Pre-Sequencing Kit; USB Corp., USA), and direct sequencing was performed with a Dye Terminator Cycle Sequencing Kit (Beckman Coulter Inc., USA) following the manufacturer's protocol. Cycle sequencing consisted of 30 cycles of 96°C for 20 sec, 50°C for 20 sec, and 60°C for 150 sec. Capillary sequencing was conducted with a CEQ 2000 XL DNA Sequence Analysis System (Beckman Coulter Inc., USA), and the detected waves were analyzed using CEQ Software, Sequence Analysis version 2 (Beckman Coulter Inc., USA). Alignment of sequence data and construction of a neighbor-joining tree were performed using MEGA 4 (Tamura et al., 2007). Genetic distances were calculated using Kimura-2 parameter method (Kimura, 1980) with bootstrap of 1000 replications.

RESULTS

Gene order LA-PCR products between *cyt b* and *12S rRNA* which were amplified using the almost-complete mitochondrial PCR product as a template (Fig. 1) were determined using five PCR fragments (A, B, C, D and E) which were obtained by amplification using internal primers (Fig. 2). Our results (Accession No. AB519130 to AB519131) show that the gene order of mtDNA in the black-faced spoonbill contains a tandem duplication composed of two units. The first is composed of the sequences for *cyt b-1*, *tRNA^{Thr}-1*, *tRNA^{Pro}-1*, *ND6-1*,

Table 1. Primer list used in this study

primer	gene	Sequence 5'-3'	Source
Lcytb1.kh	<i>cyt b</i> ¹	GCTATACACTATACCGCAGACAC	This study
cbF1.kh	<i>cyt b</i> ^{1,2}	TTGAATCGGCAGCCAACCCGTAG	(Abbott and Double 2003)
Hcytb4.kh	<i>cyt b</i> ^{1,2}	GATGAAGGGGTGCTCTACGG	This study
LND6.kh	<i>ND6</i> ^{1,2}	CCTGCACCATAACCCATAACACGTCAATCC	This study
HND6.kh	<i>ND6</i> ^{1,2}	GGATTGACGTGTTATAGGGTATGGTGCAGG	This study
Lcon1.kh	<i>CR</i> ^{1,2}	GCCCCATTATATTGCAATAATGCTAGG	This study
Lcon4.kh	<i>CR</i> ¹	ACAATAATATGTAAGGTGACAATAATCC	This study
Lcon5.kh	<i>CR</i> ²	ACAATAACATGTAGGGTGACAACAATCT	This study
Lcon6.kh	<i>CR</i> ^{1,2}	ATGAATGCTCGCCTGGCACCCCTTGCCACTC	This study
Lcon9.kh	<i>CR</i> ^{1,2}	GGGTATGGGGAATCATCTTGACACTG	This study
Lcon8.kh	<i>CR</i> ^{1,2}	TTCACATGTCATCAGCACTGGAATTAC	This study
Hcon1.kh	<i>CR</i> ^{1,2}	GGGGTCCGTATAGGGGTTATTTATTTTCG	This study
H454.gr	<i>CR</i> ^{1,2}	GCCCTGACATAGGAACCAGAG	Baba et al. 2001
Hcon2.kh	<i>CR</i> ²	GGGACGTTTGTGCACGTGCATGTGAC	This study
Lnon-c.kh	non coding region	CTCGAACATCCTCCAAACACCACGACCATC	This study
Hnon-c.kh	non coding region	TTGATGGGGATGGATGTGCGATGGGTAAGG	This study
12sR2.kh	<i>12S rRNA</i>	GCTATAACTAAGTCAAGTTTACACTTATTG	(Abbott et al. 2005)
Lco3-1.kh	<i>COIII</i>	TCGGGTCCTCCTTCCTATCAGTCTGCCTCTTAC	This study
Hco3-1.kh	<i>COIII</i>	TGTTACGGTAACACCTGATGCTAGGAGGATGGC	This study

*¹ and ² in gene indicate whether the primer can amplify both duplicated gene or one of them.

Fig. 2. Schematic representation of mitochondrial gene order and the position of primers for the black-faced spoonbill. Open arrowheads indicate primers used for amplification of fragments. Shaded arrowheads indicate inner-primers used for sequencing. Black arrowheads primers, Lcon4.kh and Lcon5.kh are used to distinguish CR1 and CR2, respectively. Line connecting Lcon9.kh and Hcon1.kh shows a PCR product to indicate the tandem duplication of this area.

tRNA^{Glu}-1, and CR1, and the second of *cyt b-2*, *tRNA^{Thr}-2*, *tRNA^{Pro}-2*, *ND6-2*, *tRNA^{Glu}-2*, and CR2, followed by *tRNA^{Phe}* and *12S rRNA*.

The presence of the tandem repeat of these genes were also determined by PCR using Lcon9.kh annealed at middle of CR region and Hcon1.kh annealed near 3' end of CR region. It resulted in 2300 bp of PCR fragment containing 5' flanking region of CR1, *cyt b-2*, *tRNA^{Thr}-2*, *tRNA^{Pro}-2*, *ND6-2*, *tRNA^{Glu}-2*, and 3' end of CR2 (Fig. 2), instead of almost-complete mitochondrial PCR product.

Comparison between these tandem-duplicated genes Sequence analysis determined that the duplicated *cyt b-2* sequence coincided with 499 bp at the 5' end of *cyt b-1*. The sequences for *tRNA^{Thr}-2*, *tRNA^{Pro}-2*, and *tRNA^{Glu}-2* were also identical to those for *tRNA^{Thr}-1*, *tRNA^{Pro}-1*, and *tRNA^{Glu}-1*, respectively, and the *ND6-2* sequence matched the *ND6-1* sequence.

On the other hand, sequencing of the duplicated CRs of this species revealed complicated structures. The 1007 bp at the 3' end of both of the CRs had a high similarity and was readily aligned. Domain I of both CR1 and CR2 was 364 bp in length and had a poly-C site and a TAS element (Ramirez et al., 1993). The region between the 47th bp and 211st bp at the 3' end of Domain I contained one indel and 18 substitutions, which enabled us to discriminate CR1 from CR2. The remaining sequences in Domain I were highly similar, with the exception of two substitutions between CR1 and CR2.

Domain II is the most conserved domain in the control region, and contains the F box, D box, C box, and BSB (Bird Similarity Box). The Domain II sequences of both CR1 and CR2 had a length of 463 bp and contained these elements. In Domain III, the CSB-1 (Conserved-Sequence Block-1; Sbisá et al., 1997) was detected in both CR1 and CR2, and the 181 bp at the beginning of Domain III was identical between the two CRs. The 3' end of CR1 is followed by a 112 bp non-coding region, which could not be aligned to CR2, and could not be matched with a similar mtDNA sequence by a BLAST search. The 3' end of CR2 contains a relatively long and complicated repeat sequence, beginning at 251 bp from the start of Domain III. This repeat sequence contains two CA-rich repeats measuring 11 bp and 22 bp in length.

Dissimilarity of Domain I in CR1 and CR2 To examine the variation arising from substitutions in CR1 and CR2, an additional set of primers was designed that was able to distinguish CR1 and CR2 (Lcon4.kh and Lcon5.kh; Fig. 2). MtDNA from the two individuals from Kagoshima and from fourteen fallen feathers collected in 2007 from Imazu estuary in Fukuoka city (AB519132–AB519141) were analyzed using the primer sets Lcon4.kh and H454.gr for the partial CR1 region, and Lcon5.kh and H454.gr for the CR2 region. Subsequently, five haplotypes were defined and aligned as shown in Fig. 3.

CR region sequences by a direct sequencing of PCR

Fig. 3. Alignment of duplicated sequences in the mitochondrial control regions of the black-faced spoonbill. Two control regions (CR1 and CR2) from two individuals are marked to indicate the Poly-C region, Termination Associated Sequence (TAS), F-, D-, B-, and Bird similarity (BSB) boxes, and the Conserved Sequence Block-1 (CSB-1).

Fig. 4. Neighbor-joining trees constructed using Region A + B (285 bp), Region A (146 bp) and Region B (139 bp) in CR1 and CR2 of 5 individuals.

product amplified with Lcon1.kh which anneals both CR1 and CR2 had many double peaks, especially in Domain I. When CR1 and CR2 were amplified separately using Lcon4.kh annealed at 3' end of CR1 and Lcon5.kh annealed at that of CR2, they did not show any double peaks in their outputs. Compared with CR1 sequence and CR2 sequence, every double peak was interpreted clearly, suggesting that our observation of double peaks, or heteroplasmy of mtDNA must be duplication of CRs.

When CR1 and CR2 were aligned separately, there were clear substitution differences in the first 146 bp of Domain I (Region A) and the final 139 bp of Domain I (Region B). In Region A, most of the substitutions in CR1 and CR2 were different from each other. By contrast, the substitutions in Region B were almost identical between CR1 and CR2 in the same individuals.

Three neighbor-joining (NJ) trees constructed using Region A, Region B and Regions A + B (Fig. 4) revealed a different topology. The NJ trees for Region A and Regions A + B were divided into 2 clades: CR1 and CR2. The bootstrap value dividing CR1 and CR2 clades in the tree for Region A (99%) was higher than that of the tree for Region A + B (82%). On the other hand, in the NJ tree for Region B, CR1 and CR2 from the same individuals were closely clustered.

DISCUSSION

Avian gene order In this study, tandem duplication of

the region between *cyt b* and the CR was found in the mtDNA of the black-faced spoonbill. The gene order of the tandem duplication of this species begins with *cyt b*-1, *tRNA^{Thr}*-1, *tRNA^{Pro}*-1, *ND6*-1, *tRNA^{Glu}*-1, and CR1, and is repeated in *cyt b*-2, *tRNA^{Thr}*-2, *tRNA^{Pro}*-2, *ND6*-2, *tRNA^{Glu}*-2, and CR2. This gene order is similar to that of the genus *Thalassarche* (Abbott et al., 2005), with the exception of *cyt b*-2, which is composed of *d-cyt b* (a 120 bp degenerated copy of *cyt b*) and *p-cyt b* (a copy of the last 39 bp of *cyt b*) in the latter.

Gibb et al. (2007) proposed 4 types of avian gene order: Standard avian (chicken), duplicated *tRNA^{Thr}*-CR (albatross), duplicated CR (*Amazona* parrots), and remnant CR2 (falcon), and indicated that the albatross gene order represents the initial reorganization of avian gene order resulting from gene duplication (Fig. 5). The black-faced spoonbill has longer tandem duplication than any other avian species detected until now, suggesting that the black-faced spoonbill may exhibit an initial type of tandem duplication of mtDNA. Even the black-faced spoonbill has the longest tandem duplication than that of albatross, then parrots and falcon, it is not necessary to indicate evolutionary order of Aves, because recent phylogeny of Aves by Hackett et al. (2008) are different from it, suggesting that mtDNA tandem duplication around CR may have occurred independently in several taxa.

Comparison of CR1 and CR2 Ruokonen and Kvist (2002) extensively described the structural and evolution-

Fig. 5. Schematic representation of mitochondrial gene order in chicken (standard), black-faced spoonbill, albatross, parrot and falcon.

ary characteristics of the CRs of 68 avian species. A poly-C sequence was detected near the 5' end of Domain I in the black-faced spoonbill (Fig. 3), which is a conserved feature in many birds, including Struthioniformes, Galliformes, Falconiformes, and Sphenisciformes (Haring et al., 2001; Ritchie and Lambert, 2000). The conserved palindrome motifs, or TAS (Termination-associated sequence) which consisted of 5'-TACAT-3' (Saccone et al., 1991) and 5'-ATGTA-3' palindrome motif (Desjardins and Morais, 1990) were identified in this species (Fig. 3).

CSB1 (Sbisa et al., 1997), which regulates duplication of the H-strand (Walberg and Clayton, 1981), was identified at the beginning of Domain III, as were other conserved blocks of the F, E, D, and C boxes (Brown et al., 1986), and a BSB (Bird Similarity Box; Ruokonen and Kvist, 2002) in Domain II. Because these features were preserved in both CRs, they may play an important functional role. In addition, CR2 contained a long complicated repeat at the 5' end of Domain III (Piganeau et al., 2004; Mjelle et al., 2008). At this position, the repeat structure might signal the termination of transcription.

In Domain I, Region A was highly variable between CR1 and CR2 in the same individual, while Region B in CR1 and CR2 was similar. The simultaneous genetic variation observed in Region B is termed "concerted evolution" (Li, 1997), as typified by five species of the albatross mitochondrial genome (Abbott et al., 2005). According to Li (1997) concerted evolution was first suggested as co-evolution by Edelman and Gally (1970) in the rDNA from *Xenopus* (Brown et al., 1972). Concerted

evolution is now commonly used in a high interspecific homogeneity of the multigene families (Zimmer et al., 1980). Mechanism of concerted evolution of tandemly-repeated gene such as rDNA is explained in Liao (2000).

The discovery of these tandem repeats in mtDNA in several species in the same genus (Eberhard et al., 2001; Abbott et al., 2005) indicates that we should further investigate the species in genus *Plataea* or family Threskiornithidae (Orbis group).

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