A novel mitochondrial gene order in shorebirds (Scolopacidae, Charadriiformes)

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Although the mitochondrial genome in birds has highly conserved features, with protein genes similar to mammals and amphibians, several variations in gene order around the hypervariable control region have been noted. Here we report a novel gene arrangement around the control region in shorebirds (Charadriiformes). In ruffs Philomachus pugnax, the mitochondrial genome between cytochrome b and 12S rRNA was over 1.5 kb longer than reported for other Charadriiformes and contained a duplication of the control region together with NADH dehydrogenase subunit 6 (ND6) and the adjacent transfer RNAs: tRNA Pro and tRNA Gln. Sequence data from 68 individuals from several stopover and breeding populations show that the duplication is widespread in ruffs. Similar gene re-arrangements have been found independently in unrelated tube-nosed seabirds and spoonbills.

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1. Introduction

Two hundred and fifty million years ago, sometime after the bird-crocodile split but before the radiation of modern birds, a novel gene re-arrangement near the control region (CR) evolved in the avian mitochondrial genome (Desjardins and Morais, 1990, 1991; Quinn and Wilson, 1993). Since then gene duplications and re-arrangements have arisen independently several times among birds (Gibb et al., 2007; Haring et al., 1999; Mindell et al., 1998; Singh et al., 2008). They occur more often around the CR, a site for initiation and termination of DNA replication, than elsewhere in the mitochondrial genome (Fujita et al., 2007). The duplication of the CR often involves changes in the gene order and duplications of the protein-coding NADH dehydrogenase subunit 6 (ND6) and the flanking transfer RNAs (Pereira (2000); Fig. 1).

Sequence variation in the highly polymorphic CR and variations in gene order have both been applied as markers in phylogeographic and population genetic studies (Avise, 2000; Bensch and Härlid, 2000; Gibb et al., 2007; Mindell et al., 1998; Singh et al., 2008). However, gene duplications and re-arrangements complicate the usage of the CR as a marker. Co-amplification of the duplicate copies can result in sequences with “apparent heteroplasmy”. Also CR sequences obtained across individuals, populations or species may not be homologs (i.e., one CR may be preferentially amplified and sequenced for some samples, while the second copy may be obtained for others, resulting in comparisons across non-homologous sequences). In this case observed sequence variation in the CR can be either paralogous (comparisons among gene duplicates) or orthologous (inter-individual comparison). This is a serious issue since it does not present any obvious symptoms such as “apparent heteroplasmy.”

This paper reports on gene duplications and sequence similarity in the mitochondrial (mtDNA) of a basal Charadriiformes species (Baker et al., 2007), the ruff (Philomachus pugnax, Scolopacidae). Ruffs are an interesting case for studies of the relationship between phenotypic and genotypic variation in populations as they appear genetically variable (Segre et al., 1970) though geographically unstructured (Verkuil, 2010). Morphometric variation, however, indicates segregating selective pressures in areas within its vast migratory and breeding range (Karlinova et al., 2007). Sequence variation in the hypervariable CR has been shown to be informative in determining genetic population structure in shorebirds (Buehler and Baker, 2005; Buehler et al., 2006; Wenink et al., 1993, 1994). In ruffs, however, sequences of the CR obtained with primers designed in the conserved regions of the CR and ND6, indicated multiple copies. This hinted at re-arrangements in the gene order around the CR, although other potential sources of duplicates are
the amplification of nuclear mitochondrial pseudogenes (numts) along with the target mtDNA sequence or heteroplasmacy, i.e. the coexistence of multiple copies of mtDNA molecules within individuals.

Duplicates are often degenerate but also can have high sequence similarity, as CR copies may be kept similar by concerted evolution (Tatarenkov and Avise, 2007). In vertebrates, evidence for concerted evolution of duplicate CR regions has been found in fish (Tatarenkov and Avise, 2007), frogs (Kurabayashi et al., 2008), snakes (Jiang et al., 2007), and birds (parrots (Eberhard et al., 2001), albatrosses (Abbott et al., 2005) and warblers (Singh et al., 2008)). We discuss two alternative explanations for the high similarity of the two CRs in ruffs: (1) recent duplication of the CR with enough time for fixation but not enough to accumulate mutations, or (2) concerted evolution (Tatarenkov and Avise, 2007).

2. Methods and material

Blood samples of ruffs (Philomachus pugnax) were collected on spring staging sites in The Netherlands (by J.C.E.W. Hooijmeijer and authors), Belarus (by N. Karlonova and P. Pinchuk) and in breeding areas in Sweden (by K.A. Thuman), Finland (by D.B. Lank) and Siberia, Russia (by M.Y. Soloviev, P.S. Tomkovich et al.). Samples were stored in 98% ethanol at -80 °C. All except the Swedish samples are curated in the collections of the Royal Ontario Museum, Toronto, ON, Canada, and the University of Groningen Shorebird LifeLines Blood Bank, The Netherlands.

DNA was isolated with DNeasy Blood and Tissue Kit (Qiagen) or by standard phenol–chloroform extractions and stored at –20 °C. To screen for genetic variation throughout the migratory and breeding range of ruffs, the 5’-end of the CR was amplified using shorebird primers anchored in ND6 and the conserved blocks in the CR: ND6L3 & H451/H772 (Wenink et al. (1994); see Table 1). However, the sequences obtained varied in size which indicated multiple copies of the CR. Therefore, to explore the gene order around the CR and to exclude the possibility of amplifying nuclear copies, long template (LT) was amplified that spanned the region between cytochrome b and the 12 S ribosomal RNA genes (12S), using Expand Long Template PCR (Roche, Basel, Switzerland). Subsequently the LT amplicon was used as a template to obtain partial sequence of the region. The LT primers used were b86 with H1827 for ruff 1479281 (caught on 20 December 2004 in The Netherlands), b3 with 16ScR for ruff 1481191 (caught on 4 March 2005 in The Netherlands) and resighted in Russia on 15 May 2005, Sur- gút, Khanty/Mansi, 60°10’N, 74°00’E), and b71 with H1390 for both individuals (Table 1). The LT PCR profile was 2 min denaturation at 94 °C, followed by 10 cycles of 92 °C for 30 s, 63 °C for 30 s and 68 °C for 12 min and 25 cycles of 92 °C for 30 s, 63 °C for 30 s

![Table 1](https://example.com/table1.png)

Table 1: Primers used for amplification of long template DNA from which nested amplifications were made to establish the gene order around the mitochondrial control region of ruffs (Philomachus pugnax). For each primer, the location is given and a short explanation of why it was applied.

Fig. 1. Gene arrangements around the control region (CR) in birds: (A) gene order reported as common in Aves (Desjardins and Morais, 1990); (B) first alternative gene order found in Aves (Mindell et al., 1998; Bensch and Härlid, 2000); (C) gene order in parrots (Eberhard et al., 2001), and (D) gene order in albatrosses (Abbott et al., 2005). ψ: locus is degenerate.
and 68 °C for 12 min + 20 s cycle elongation for each successive cy- cle. Final elongation at 68 °C was for 7 min.

The amplified LT mtDNA segments were excised from agarose gels, purified by spinning through filter tips and stored at −20 °C. Nested amplifications were performed with primers anchored in cytochrome b, 12SrRNA, tRNA^{Phe} and ND6 (see Table 1 and Fig. 2 for primer sequence and location). To isolate the 5'-ends of the CRs, PCR products were amplified off LT (ruff 1479281) and geno- mic DNA (ruff 1481191) with internal primers ND6L3 and H402, and cloned into pCR².1 following the manufacturer's instructions (Invitrogen). Off each ligation, 10 positive clones were sequenced with M13 primers. Only sequence variation that was observed in >1 clone was considered real variation. For comparison, additional PCR products using genomic templates were obtained for two indi- viduals, 1463006 (caught on 17 March 2004 in The Netherlands) and SIB-258 (nestling DNA collected on 17 July 2004 on Taymir Peninsula Siberia, 74'09'N, 99'34'E).

A segment of CR that can only be obtained when the alternative gene arrangement was present was amplified in 68 ruffs, using the forward CR primer L141 and the reverse primer PropR anchored in tRNA^{Pro}, and was confirmed with sequences of ruddy turnstones and red knots, and had a very similar base composition and the same general molecular organi- zation in three CR domains as other birds (Pereira et al., 2004). Amplification of Domain I of the CR with primers ND6L3 and H451 off genomic DNA (of ruff 1463006) yielded PCR products of two different sizes: one band of the expected size of about 1 kb and one band of about 2.5 kb. Apparent heteroplasmy was ob- served in the sequences of the 2.5 kb fragment, indicating that both CRs were included in the 2.5 kb fragment (Fig. 3). Sequences of the 2.5 kb fragment aligned perfectly with the LT fragments and with the genomic sequence of SIB-258 from the Siberian breeding popu-lation. Within individuals, the sequences showed "apparent het- eroplasmy" at nine positions at the 5'-end of Domain I (Fig. 3). Domain I in ruffs was similar to the Domain I of red knots (Fig. 4).

Domains II of the two CRs were identical. Sequences of Domain II obtained from the 1 and 2.5 kb amplicons using the primers L438 and H1537 were identical for both LT and genomic templates. Also, sequences from amplifications off LT, either anchored in tRNA^{Pro} backwards into CR² or in 12S backwards in CR² (see Fig. 2, Table 1) were in perfect alignment.

Domain III of the two CRs was nearly identical until the TACAT promoter near the 3'-end. After the TACAT promoter the first CR had 88 nt of apparently random sequence, which ended in a complete functional copy of tRNA^{Pro} (Fig. 4). The tRNA^{Pro} at the 3'-end of CR² and the tRNA^{Pro} downstream of cytochrome b were identical and showed a functional clover leaf folding pattern. The second CR aligned throughout with Domain III of red knots and ruddy turn- stones, ending with a 4-nucleotide repeat sequence (microsatellite) of ~40 repeats which was very similar in structure to red knots and ruddy turnstones. Overall, the two CRs were similar, with the first CR being 1120 nt long and the second 1183 nt, as the latter included the 4-nucleotide repeat at the 3'-end.

Two copies of ND6 were found. The first ND (ND6²) was 540 nt long between tRNA^{Pro} and tRNA^{Glu}. Cloned products of the ampli- cons of the 3'-end of ND6, obtained with the primers ND6LR3 and H402, were identical. ND6² was followed by a complete tRNA^{Glu}. Both copies aligned with ND6 of ruddy turnstones (Paton et al., 2002). Sequences of the amplicons using primers anchored in

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**Fig. 2.** Overview of the gene order between cytochrome b and the 12SrRNA gene in ruffs *Philomachus pugnax*. Amplified fragments (and their relative sizes) used to make the alignment and the primers used in amplifications are given (see also Table 1). Black segments are from nested amplifications off long templates; grey segments indicate sequence from genomic DNA. Dashed parts of PCR products were not sequenced. ‘P’: degenerate sequence block of 88 nt.

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3. Results

The complete long template (LT) PCR product between the 3'-end of cytochrome b and the 5'-end of 12S tRNA was obtained for two individuals. Based on the published mitochondrial gene or-
cytochrome b and ND6\textsuperscript{1} or in CR\textsuperscript{1} and ND6\textsuperscript{2} were in perfect alignment (Fig. 2 and Table 1).

To evaluate paralogous and orthologous sequence similarity in Domain I, sequences of the two cloned individuals were compared. Sequence of 386 nt was obtained, and the 333 nt of Domain I of both CRs were aligned (GenBank GQ 174508, 174509, 1874510, 174511). The average sequence divergence of the orthologous copies of the first domains of the two CRs was 1.5% (2.1% in CR\textsuperscript{1} and 0.9% in CR\textsuperscript{2}). Within individuals, average sequence divergence of Domain I was 1.8% (2.4% in ruff 1479281 and 1.2% in 1481191), most variable sites were located at the 5'-end. Downstream, at position 236, a shared substitution in the two control regions was found within an individual that was not shared by the two control regions in the other individual (Fig. 5).

A large fragment of CR\textsuperscript{1} was successfully amplified from 68 ruffs from a variety of breeding and migratory staging sites. The amplification started at position 124 using forward primer L141 and ended in tRNA\textsubscript{Pro} using reverse primer PropR, indicating that all individuals had the alternative gene arrangement. The average pairwise sequence divergence of the orthologous copies of Domain I and II...
between each pair of the 68 individuals was 5.5 ± 0.01% (28 segregating sites in 504 nt). Within the 209 nt fragment that overlapped with Domain I obtained from the clones from individuals the average orthoparalogous pairwise divergence was 7.7 ± 0.02%.

4. Discussion

The mitochondrial genome of the ruff between cytochrome b and 125SrRNA was over 1.5 kb longer than reported for other Charadriiformes (Buehler and Baker, 2003; Paton et al., 2002). This additional piece included full duplications of the CR, ND6, tRNAPro and tRNAGlu. Since the duplicate sequences were obtained from a 4.5 kb long template, heteroplasmy in individuals can be excluded as the source of sequences variation. Also, the size of the LT ampli-con was larger than most nuclear mitochondrial pseudogenes (numts) reported in birds (Pereira and Baker, 2004). Hence numts are an unlikely source of sequence variation. Moreover, in the ND6 coding genes sequenced from LT, no stop codons or unusual frame shifts were found; hence the LT sequence is unlikely to be a numt. The duplication was present in all 68 samples obtained throughout the species distribution range, indicating that duplicate state of the CRs is not a transient or unstable feature found in a particular individual or deme.

The length of CR² (1183 nt) was comparable to CRs in red knots, ruddy turnstones and blackish oystercatchers Haematopus ater, which are 1168, 1172 and 1239 nt, respectively (Buehler and Baker, 2003; Paton et al., 2002). CR² was shorter because the 4-nt microsatellite-like repeat was absent. Due to the duplications, together with the albatrosses (Abbott et al., 2005) and black-faced spoonbills Platalea minor (Cho et al., 2009), ruffs possess the largest control region area (between cytochrome b and 125SrRNA) reported for Aves.

The re-arrangement in ruffs reflects a gene order that is unique in the order Charadriiformes, in that ruffs have full duplicates of the CR, ND6, tRNAPro and tRNAGlu. Alternative gene orders in birds often involve pseudogenes and a degenerate copy of the CR (Mindle et al., 1998) as in Phylloscopus warblers (Bensch and Hårlid, 2000) and in Falconiformes (Cadahia et al., 2009). Gene orders similar to ruffs, with two almost identical copies of the CR, were previously reported for blackcaps Sylvia atricapilla and reed warblers Acrocephalus scirpaceus (Singh et al., 2008), but in these species no sequences of the region before the first CR is available so it remains unclear whether they had a duplicate ND6 and tRNA genes. In Amazona parrots (Eberhard et al., 2001), osprey Pandion haliaetus and ivory-billed aracari Pteroglossus azara (Gibb et al., 2007), the first tRNAPro, ND6 and tRNAGlu are degenerate or pseudogenes. The black-faced spoonbill has a duplicate of the CR and the adjacent cyt b, ND6, tRNAPro, and tRNAGlu, and similar to ruffs most variation between CRs is found in the 3' end (Cho et al., 2009). In Thalassarche albatrosses (Abbott et al., 2005) the duplicate CR is also flanked by duplicate ND6, tRNAPro, and tRNAHis, the only difference between the gene arrangements in albatrosses and ruffs is that albatrosses had a second copy of tRNAPro. These similarities across taxa must represent convergent evolutionary events in bird genomes (Bensch and Hårlid, 2000).

The observed high level of similarity between the two CRs (especially in Domain II) in ruffs might be a consequence of: (1) recent duplication of the CR, or (2) concerted evolution. In ruffs paralogous sequence divergence was relatively low, being 1.8%. This value is similar to Amazona parrots, where the average sequence divergence of the paralogous CR copies was 1.4% and CRs were considered to be under concerted evolution (Eberhard et al., 2001). However, in our study the comparison between orthologous and paralogous sequence variation is hampered by sample size as paralogous copies were obtained for only two individuals. The supposed mechanisms for concerted evolution are gene conversion (sequences stay homogenized by recombination) or tandem replication (sequences are kept homogenized due to replication slippage) (Kurabayashi et al., 2008; Eberhard et al., 2001). Of these mechanisms only gene conversion allows for unevenness in sequence similarity between Domains as was observed in ruffs. The supposedly functional Domain II is most similar between copies (Fig. 4). In this Domain the observation of a shared substitution between the two CRs within individuals but not between individuals (Fig. 5), suggests concerted evolution (but this phenomenon can also be explained by homoplasies). Sites in Domain I provided a situation opposing concerted evolution as substitutions were shared between orthologs but not between paralogs (Fig. 3).

As the possibility of concerted evolution remains unresolved, we continue to consider the hypothesis of recent fixation of duplicates. Under the scenario of recent fixation, there would not have been enough time to accumulate mutations, so similarity between copies is expected, and, beyond functional constraints, mutation should be randomly distributed. We, however, observed a considerable unevenness in the distribution of substitutions. All variation between CR¹ and CR² was found near the 5' end of Domain I and 3' end of Domain III. Also, the degeneration of the microsatellite repeat at the 3' end of Domain III indicates that the duplication was not recent. Alternatively, the duplication event itself created the observed unevenness in distribution of substitutions, given its position at the 5' and 3' end of the duplicated segment. The probability of fixation of a gene duplication increases with decreasing effective population size (N_e) as predicted by the nearly neutral theory (Ohta, 1973; Zhang, 2003). The ruff, however, has a large historical N_e (>10,000) as determined by genetic methods (Verkuil et al., 2010), and we therefore exclude the possibility that the gene duplication became fixed due to a recent population bottleneck or low effective population size (see also Lynch et al. (2001)).

In conclusion, the sequence similarity in Domain II can be either the consequence of a process of homogenization or a consequence of recent duplication. We did not compare orthologous and paralogous copies of tRNAs, tRNAPro and tRNAGlu, however, the observed sequence similarity between copies of the tRNAs, tRNAPro and tRNAGlu indicates that also these duplicate genes are either recent or homogenized (Jiang et al., 2007).

This paper showed that identical gene re-arrangements can occur independently in unrelated birds (shorebirds, tube-nosed seabirds and spoonbills) (Abbott et al., 2005; Cho et al., 2009). If concerted evolution of paralog copies of the control region would apply to ruffs, then there is no active selection against the duplication of genes in the mitochondrial genome. Maintaining two full control regions allows for gene conversion to act as a repair mechanism to avoid transcription error, which might represent a recombinational force in the animal mtDNA as suggested by Tatarenkov and Avise (2007).
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