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Published in:
Molecular Phylogenetics and Evolution

DOI:
10.1016/j.ympev.2010.06.010

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2010

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Download date: 06-04-2022
A novel mitochondrial gene order in shorebirds (Scolopacidae, Charadriiformes)

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A R T I C L E   I N F O

Article history:
Received 25 March 2010
Revised 13 June 2010
Accepted 15 June 2010
Available online 19 June 2010

Keywords:
mtDNA
Control region
Gene duplication
Concerted evolution
Philomachus pugnax
Sandpiper
Aves

A B S T R A C T

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 found. 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: tRNAPro and tRNAGlu. 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, 1980, 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 phylogenetic and population genetic studies (Avise, 2000; Bensch and Här lid, 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 heteroplasmy, 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. Karlionova and P. Pinchuk) and in breeding areas in Sweden (by K.A. Thuman), Finland (by D.B. Lank) and in 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) 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. (1993); 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 16S/r for ruff 1481191 (caught on 4 March 2005 in The Netherlands, and resighted in Russia on 15 May 2005, Sur- gut, 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 | 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. |

<table>
<thead>
<tr>
<th>Region</th>
<th>Primers</th>
<th>SEQUENCE</th>
<th>Application</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome b</td>
<td>b86</td>
<td>5'-TCAATNGGGAGCCACCCTAGATAACACCC-3'</td>
<td>Long template-1</td>
<td>O. Haddrath (unpublished)</td>
</tr>
<tr>
<td></td>
<td>b3</td>
<td>5'-GGAGCACCCCTTTTACTACGGCTC-3'</td>
<td>Long template-2</td>
<td>T. Burke (unpublished)</td>
</tr>
<tr>
<td></td>
<td>b71</td>
<td>5'-GGAGAGATCTACTGGAGCGCCTC-3'</td>
<td>Long template-3</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>b5</td>
<td>5'-TCCACCGCTTACTTCACATAAGA-3'</td>
<td>Backwards into CR; no microsatellite</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>cytend</td>
<td>5'-CAGACGGTTGTAACAGCAGCCACCTGACACC-3'</td>
<td>Backwards into Domain II-BSB</td>
<td>This study</td>
</tr>
<tr>
<td>tRNA$^{199}$</td>
<td>PropR</td>
<td>5'-AAACGGAGGAGGACCTGC-3'</td>
<td>Into C-string</td>
<td>O. Haddrath (unpublished)</td>
</tr>
<tr>
<td>NADH6</td>
<td>ND6p2R</td>
<td>5'-TGGTACGCTGACGAGGGCA-3'</td>
<td>Into C-string</td>
<td>This study</td>
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<tr>
<td></td>
<td>ND6p6R</td>
<td>5'-CTCCTGCGAGATCTGGGCT-3'</td>
<td>Into C-string</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>ND6L3</td>
<td>5'-ACTGCTGCAATGGCCCCACAGG-3'</td>
<td>Into C-string</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>ND6HR</td>
<td>5'-TYCNGATGATTTCTGGG-3'</td>
<td>Into C-string</td>
<td>O. Haddrath (unpublished)</td>
</tr>
<tr>
<td>Control region</td>
<td>H451</td>
<td>5'-CTGCTGACTGCTACACCGCAG-3'</td>
<td>Into C-string</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>L141</td>
<td>5'-CTCATATATCTCTACACCTGCG-3'</td>
<td>Into $\psi$-rRNA$^{199}$</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>LH402</td>
<td>5'-TGAACTGACGGCAGCTAGG-3'</td>
<td>Into $\psi$-rRNA$^{199}$</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>L438</td>
<td>5'-TACTGGATACCAACCACAGG-3'</td>
<td>Forward in 12S; into microsatellite</td>
<td>Wenink et al. (1993)</td>
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<td></td>
<td>L716</td>
<td>5'-ACTTGCCCTGCACGGGTTAG-3'</td>
<td>Forward into ND6; no microsatellite</td>
<td>This study</td>
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<td></td>
<td>125S RNA</td>
<td>H1537</td>
<td>5'-TACCGCGGATGGCCACCAAGG-3'</td>
<td>Into microsatellite</td>
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<td></td>
<td>H1827</td>
<td>5'-CCAGCGCTCTCTCTAGAGG-3'</td>
<td>Long template-1</td>
<td>M. Burke (unpublished)</td>
</tr>
<tr>
<td></td>
<td>16S/cR</td>
<td>5'-TTCCTAAAGCTGCGCCCAACC-3'</td>
<td>Long template-2</td>
<td>D. Haddrath (unpublished)</td>
</tr>
<tr>
<td></td>
<td>H1390</td>
<td>5'-TCAAGGGATATACGGGGCGCAGTAC-3'</td>
<td>Long template-3</td>
<td>This study</td>
</tr>
</tbody>
</table>
Results

The complete long template (LT) PCR product between the 3′-end of cytochrome b and the 12S rRNA gene was obtained for two individuals. Based on the published mitochondrial gene order and CR size in shorebirds closely related to ruffs (Buehler and Baker, 2003; Paton et al., 2002), the expected size of the LT PCR product (range from cytochrome b to 12S rRNA gene) was ~3.2 kb. The LT fragment obtained in both individuals was 4.5 kb (primer combination b71 and H1390), and was confirmed with two different primer combinations (Table 1). Clones of products amplified off the LT with internal primers ND6L3 and H402 yielded two different CR sequences in each individual (Fig. 2). The assembly of sequences from nested amplifications off the LT (using primers anchored in cytochrome b, 12S rRNA, tRNAPro and ND6) showed that ruffs have duplicates of the CR and adjacent genes ND6, tRNAPro, and tRNAGlu (Fig. 2, GenBank GQ255993). All sequences aligned with sequences of ruddy turnstones and red knots, and had a very similar base composition and the same general molecular organization 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 heteroplasmacy was observed 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 clones and with the genomic sequence from SIB-258 from the Siberian breeding population. Within individuals, the sequences showed “apparent heteroplasmacy” at nine positions at the 5′-end of Domain I. 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 tRNAPro backwards into CR1 or in 12S backwards in CR2 (see Fig. 2, Table 1) were in perfect alignment.

Domain III of the two CRs was nearly identical under 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 tRNAPro (Fig. 4). The tRNAPro at the 3′-end of CR1 and the tRNAPro 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 turnstones, ending with a 4-nucleotide repeat sequence (microsatellite) of ~40 repeats which was very similar in structure to red knot and ruddy turnstone sequences. 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 (ND61) was 540 nt long between tRNAPro and tRNAGlu. Cloned products of the amplifications of the 3′-end of ND6, obtained with the primers ND6LR3 and H402, were identical. ND62 was followed by a complete tRNAPro and tRNAGlu. Both copies aligned with ND6 of ruddy turnstones (Paton et al., 2002). Sequences of the amplicons using primers anchored in...
cytochrome b and ND6\textsuperscript{1} or in CR\textsuperscript{2} 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 amplicons 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

![Fig. 3](image.png)

**Fig. 3.** Apparent heteroplasmy in the control region of ruffs *Philomachus pugnax* due to gene duplication. The chromatogram starts at the C-string at the 5'-end and continues 55 nt into the CR; the double peaks indicated ‘apparent heteroplasmy’. This sequence is part of a 2500 nt PCR product obtained from amplification off genomic template of ruff 1463006 with a primer anchored in ND6 and the CR primer H451 (see inset). Below are sequences of clones of amplicons off Long Templates of ruff 1481191 and ruff 1479281 showing that the ‘apparent heteroplasmy’ is due to two different control regions (CR\textsuperscript{1} and CR\textsuperscript{2}). Inset: graphical presentation of the gel representing amplification in four individuals of which one showed the larger band that indicated the duplication.

![Fig. 4](image.png)

**Fig. 4.** Schematic representation following Buehler and Baker (2003) of the two control regions (CR) in ruffs *Philomachus pugnax* and comparison with control regions elements in the closely related red knots *Calidris canutus*. Conserved sequence blocks (CSB) and bird similarity box (BSB) are indicated in grey, (*) indicates 1 nt in the conserved sequence blocks where ruffs and red knots were different. The two CRs in ruffs were similar until the TACAT promoter. The dotted box in CR\textsuperscript{1} indicates degenerate sequence (88 nt). The striped box indicates the 4-nt repeat at the end of CR\textsuperscript{2}.
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 orthopwise pairwise divergence was 7.7 ± 0.02%.

4. Discussion

The mitochondrial genome of the ruff between cytochrome b and 12SrRNA 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 amplification 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). CR1 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 125rRNA) 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. 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 amplification 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.

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Acknowledgments

We thank Oliver Haddrath for inspiring ideas and assistance in the laboratory, and the many insightful discussions. Tissue samples of ruffs outside The Netherlands were collected by Natalia Karlianova, David Lank and Michael Soloviev and other volunteers. Katherina Thuman and Fredrik Widemo kindly provided DNA samples from the breeding population in Gotland, Sweden. Joop Jukema and the Frisian Wilsterflappers traditionally catch ruffs on their Frisian stopovers site and provided us with the chance to collect blood samples and colour-mark individuals which were sighted on breeding grounds in Norway and Siberia. Jos Hooijmeijer provided logistical management of the ruff field work and database. YIV was supported by Dutch Science Foundation (NWO) travel grants (R84-606, R87-307). AJB was supported by an operating grant (200-07) from the Natural Sciences and Engineering Research Council of Canada. This study was financed by a start-up grant to TP from the University of Groningen. Sergio Pereira, Erika Tavares, Robert M. Zink, Ritset C. Jansen and Irene Titelmann provided valuable advice and insights.

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