Characterization of the red knot (*Calidris canutus*) mitochondrial control region

Deborah M. Buehler and Allan J. Baker

Abstract: We sequenced the complete mitochondrial control regions of 11 red knots (*Calidris canutus*). The control region is 1168 bp in length and is flanked by tRNA glutamate (glu) and the gene *ND6* at its 5′ end and tRNA phenylalanine (phe) and the gene *12S* on its 3′ end. The sequence possesses conserved sequence blocks F, E, D, C, CSB-1, and the bird similarity box (BSB), as expected for a mitochondrial copy. Flanking tRNA regions show correct secondary structure, and a relative rate test indicated no significant difference between substitution rates in the sequence we obtained versus the known mitochondrial sequence of turnstones (Charadriiformes: Scolopacidae). These characteristics indicate that the sequence is mitochondrial in origin. To confirm this, we sequenced the control region of a single individual using both purified mitochondrial DNA and genomic DNA. The sequences were identical using both methods. The sequence and methods presented in this paper may now serve as a reference for future studies using knot and other avian control regions. Furthermore, the discovery of five variable sites in 11 knots towards the 3′ end of the control region, and the variability of this region in contrast to the more conserved central domain in the alignment between knots and other Charadriiformes, highlights the importance of this area as a source of variation for future studies in knots and other birds.

Key words: D-loop, *Calidris canutus*, Charadriiformes, Aves, evolution.

Résumé : Les auteurs ont séquencé en entier la région de contrôle du génome mitochondrial chez 11 bécasseaux maubèches, *Calidris canutus*. La région de contrôle mesure 1168 pb et elle est bordée des gènes de l'ARNt glutamate (glu) et *ND6* du côté 5′ et des gènes de l'ARNt phénylalanine (phe) et *12S* du côté 3′. Cette séquence possède les motifs conservés F, E, D, C, CSB-1 ainsi que la BSB (« bird similarity box ») tel qu'attendu pour une copie mitochondriale. Les ARNt bordant cette région montrent la bonne structure secondaire et un test du taux relatif n'indique aucune différence significative du taux de substitution entre la séquence observée et celle rapportée pour les tournepierres. Ces caractéristiques indiquent que la séquence est bien d'origine mitochondriale. Afin de le confirmer, les auteurs ont séquencé la région de contrôle chez un individu à partir de l'ADN mitochondrial purifié et de l'ADN génomique. La séquence obtenue dans les deux cas était identique. Cette séquence et les méthodes employées peuvent maintenant servir de référence pour de futures études des régions de contrôle chez les bécasseaux ou d'autres espèces d'oiseaux. De plus, l'identification de cinq sites variables à proximité de l'extrémité 3′ de la région de contrôle chez ces 11 bécasseaux, de même que la variabilité observée au sein de cette région par opposition au domaine central plus conservé entre les bécasseaux et d'autres charadriiformes, souligne l'importance de cette région en tant que source de variation pour de futures études chez les bécasseaux et d'autres oiseaux.

Mots clés : boucle en D, *Calidris canutus*, charadriiformes, oiseaux, évolution.

[Traduit par la Rédaction]

Introduction

The control region is a non-coding sequence of variable length found in the mitochondrial genome. In vertebrates, this region contains promoters for transcription, the heavy strand replication origin (O_H), and the displacement loop (D-loop; Chang and Clayton 1986). Research based on the distribution of variable nucleotide positions and differential frequencies of the nucleotides shows that the mitochondrial control region is divided into three domains (Saccone et al. 1991). Most of the variability in the control region is concentrated in domains I and III. These domains show variation in length and an elevated number of nucleotide substitutions. Central domain II is conserved compared with flanking domains I and III (Baker and Marshall 1997).

Mitochondrial DNA (mtDNA) has many advantages as a marker for phylogenetic analyses (Moore 1995) and is one of the most frequently used markers in avian molecular systematics because of its maternal inheritance, haploidy, and rapid rate of evolution (Baker and Marshall 1997). For intraspecific investigations, the control region is particularly useful because it evolves 3 to 10 times faster than the average for the whole molecule (Brown et al. 1979) and is thus a

Received 29 December2002. Accepted 1 April 2003. Published on the NRC Research Press Web site at http://genome.nrc.ca on 11 June 2003.

Corresponding Editor: B. Golding.

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 doi: 10.1139/G03-034

highly polymorphic region of mtDNA providing a rich source of variation for determining population structure within a species (Wenink et al. 1993).

One drawback to the use of control region sequences for population analysis is the existence of numts (nuclear mitochondrial DNA segments; Sorenson and Quinn 1998). The streamlined nature of the mitochondrial genome is a product of the gradual transfer of information from the organelle to the nucleus (Perna and Kocher 1996). Through this process copies of mtDNA sequence can be transferred to the nucleus to create nuclear pseudogenes. Because recent nuclear transfers retain high sequence identity with mtDNA, these pseudogenes may be inadvertently amplified during PCR targeting the mitochondrial genome. The existence of numts requires that researchers take precautions against the amplification of nuclear pseudogenes when using mtDNA for systematic or population genetics research.

Red knots (*Calidris canutus*) are long distance migrant shorebirds. They breed in the high arctic tundra and each year embark on migrations of up to 15 000 km to marine staging and wintering areas at different latitudes throughout the world. Their population biology and physiology in relation to their circumpolar migrations have long fascinated researchers. The control region of red knots has been used in population genetics analysis by Baker et al. (1994), who amplified and sequenced 255 bp of domain I for 25 knots from four subspecies. They found very low variability, both in number of haplotypes and in sequence divergence. This study has been cited as an example of a species with very little phylogeographic structure (Avise 2000; Baker and Marshall 1997; Wennerberg et al. 2002). Although precautions were taken by amplifying some sequences from purified mtDNA, the mitochondrial origin of sequences obtained from genomic DNA isolated from blood was never definitively confirmed.

The purpose of this paper was to sequence and characterize the complete control region of red knots to verify that the sequence is of mitochondrial origin. The verification is done through a series of precautions and tests including the amplification of a relatively long fragment for sequencing, the location of all expected structural components, the confirmation of proper secondary structure in surrounding tRNA's and a relative rate test of sequence evolution between the sequence and that of a known mitochondrial sequence in turnstones. To further check that the product amplified from genomic template produces the same sequence as that from purified mitochondrial DNA, we used both methods on the same individual. Additionally, we examined diversity in a sample of 11 complete control regions to locate areas of high variability.

Methods and materials

Red knots were sampled globally using ground traps in breeding areas and cannon nets in wintering and staging areas. A few drops of blood were taken from each bird and stored in 50 mM EDTA and 70% v/v ethanol. For a single individual (MKP311), both total genomic DNA and purified mitochondrial DNA isolated from liver and heart tissue using a caesium chloride (CsCl) gradient method described in Van Wagner and Baker (1990) were used as template. All of the samples used are part of the Royal Ontario Museum collections. DNA was isolated using standard phenol–chloroform extractions (Sambrook et al. 1989).

The following protocol was used to obtain sequence from genomic DNA and purified mtDNA from the same individual (MKP 311) and genomic DNA alone from 10 other knots. A 2000-bp fragment encompassing the control region and the flanking tRNA glutamate (glu), tRNA phenylalanine (phe), and *ND6* regions was amplified. Although numts longer than 2000 bp have been found in the human genome (Tourmen et al. 2002; Woischnik and Moraes 2002), very few numts (2.76%) were found to encompass the entire control region. (Woischnik and Moraes 2002). The initial long fragment was amplified using primers 12SH (5′-GGATAACAATTTCACACAGGGTGAACCTTCCGGTACACTTACC-3′) and LproT (5′-CACGACGTTGTAAAACGACGCTCCCAAAGCTGGTATTTC-3′) (T. Paton, personal communication). Amplification was performed as specified by the manufacturer using an Expand Long Template PCR Kit (Roche, Basel, Switzerland) and the following PCR profile: 94°C for a 2-min denaturation; 10 cycles of 92°C for 30 s, 63°C for 30 s, and 68°C for 12 min; 25 cycles of 92°C for 30 s, 63°C for 30 s, and 68°C for 12 min, increasing the time interval by 20 s/cycle; 68°C for 7 min; and holding at 4°C. Nested amplifications were performed to obtain different sections using primers L98, H401, L438 (Wenink et al. 1993), and H1537 (5′-TGACCGCGGTGGCTGGCACAAG-3′) (O. Haddrath, personal communication) and were carried out in a reaction volume of 25 μL containing 1.0 μL of DNA, 2.5 μL of 10× EH buffer (10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% w/v gelatin, 1.6 mg bovine serum albumin/mL), 0.5 U *Taq* DNA polymerase (Qiagen), 5 mM of each dNTP, and 2.5 pmol of each primer. The amplification protocol consisted of the following: 95°C for a 2-min denaturation; 36 cycles of 95°C for 45 s, 50°C for 45 s, and 72°C for 90 s; 72°C for 5 min; and holding at 4°C. Sequences were obtained using overlapping primers, H401, L98, L438, and H772 (Wenink et al. 1993), and a newly designed primer, KnotMidCRL (5′-GCAACGGGTGAATACAATCTAAGAC-3′). The presence of a cytosine string at the 5′ end of domain I and an imperfect tetranucleotide microsatellite at the 3′ end of domain III made it necessary to obtain sequence from tRNA glu and tRNA phe with primers ND6Lend (5′-AAACTYAACAACCACCCACA-3′) (O. Haddrath, personal communication) and H1537, respectively. All sequences were obtained using the Sanger dideoxy chain-termination method (Sanger et al. 1977) and the Thermo Sequenase Radiolabelled Terminator Cycle Sequencing Kit (United States Biochemical, Cleveland, Ohio) with ^{33}P on a 6% w/v polyacrylamide gel.

Knot sequences were aligned with GenBank control region sequences of dunlins (*Calidris alpina*) (Wenink et al. 1993), ruddy turnstones (*Arenaria interpres*), blackish oystercatchers (*Haematopus ater*) (Paton et al. 2002), and chickens (*Gallus gallus*) (Valverde et al. 1994) using ClustalW (Thompson et al. 1994), and final alignment adjustments were performed manually. The boundary of domains I and II was defined 20 bp in the 5′ direction from the conserved F-box. In the alignment of knot, dunlin, turnstone, oystercatcher, and chicken, a high degree of sequence similarity is seen after this point. Domain III was defined begin-

Table 1. Base composition (%) in the control region of red knots.

Domain	A	C	G	T	Total (bp)
I	30.8	32.6	13.8	22.8	329
II	21.8	25.3	19.7	33.2	467
III	46.2	25	6.7	22	372
Complete D-loop	32.1	27.2	13.9	26.7	1168

ning with the conserved sequence block (CBS-1) (Ruokonen and Kvist 2002).

The proper folding structure of the tRNA phe and tRNA glu sequences was determined by mapping the sequences onto the model structure presented for chicken tRNA folding (Desjardins and Morais 1990). Finally, a relative rate test for sequence evolution was performed using the program Phyltest (Kumar 2001) between the control region sequences of knots and turnstones, with the oystercatcher sequence as an outgroup. This analysis is a two-cluster test of significant difference in branch lengths (number of substitutions per site) between the taxa of interest, using a two-tailed normal deviate test (Takezaki et al. 1995). The turnstone sequence is of confirmed mitochondrial origin and was taken from Paton et al. (2002), who used purified mtDNA from tissue and sequenced from 15-kb-long templates.

Results and discussion

Complete control region sequences were obtained from 11 individuals. Sequence obtained from genomic DNA was identical to that obtained from purified mtDNA in individual MKP311 (GenBank accession No. AY198135). The alignment of the control region of knots with dunlin, turnstone, oystercatcher, and chicken control regions is shown in Fig. 1. The control region is 1168 bp in length, slightly shorter than the turnstone (1172 bp) and oystercatcher (1296 bp) control regions, and is flanked by tRNA glu and the gene *ND6* at its 5′ end, and tRNA phe and the gene *12S* on its 3′ end. The arrangement of genes surrounding the control region matches that of other Charadriiformes and Galliformes, but differs from some Picidae, Cuculidae, and Passeriformes Suboscines (Mindell et al. 1998). Figure 2 shows a schematic representation of the control region of knots and its characteristics.

Base composition in the control region resembles that found in other avian control regions (Baker and Marshall 1997). There is a lack of L-strand guanines in all domains, but it is most pronounced in domain III. Adenines and cytosines are most prevalent in domain I, cytosines and thymines in domain II, and adenines and cytosines in domain III (Table 1).

We found domain I of the control region to be 329 bp in length, and located two conserved elements within this domain. A cytosine string at the 5′ end is widely conserved among birds (Ruokonen and Kvist 2002) and forms a hairpin structure with a string of guanines a short distance downstream in Galliformes and Anseriformes. In knots, however, this string forms a loop. The reason behind the conservation of this cytosine string remains unknown. Termination-associated sequences (TAS), elements involved in the termination of DNA synthesis, are also found in domain I. These elements contain TATAT or TACAT motifs that are conserved to a variable degree (Foran et al. 1988). Only a single TACAT motif was found in knots, as well as in dunlins, turnstones, and oystercatchers (Fig. 1).

Domain II was found to be 467 bp in length and contained five conserved sequence blocks identified as F, E, D, C, and the bird similarity box (BSB) when compared with sequences presented in Ruokonen and Kvist (2002). All Charadriiformes and chicken had identical sequence in the BSB. Dunlin and knot sequences were identical in all boxes, Charadriiformes were identical in box D, and turnstones, dunlins, and knots were identical in boxes C and E. A 3-bp deletion was found in box C for all Charadriiformes when aligned to chicken. A T string was located between the C box and the BSB in knots. This string was interrupted with cytosines to a varying degree in turnstones, oystercatchers, and chickens and was slightly shorter in dunlins (Fig. 1).

Domain III was found to be 372 bp in length and the CSB-1 sequence was located at its 5′ end. The O_H is thought to occur close to this conserved sequence block (Ghivizziani et al. 1994). In addition, knots possessed a tetranucleotide microsatellite repeat at the 3′ end of domain III. This type of repeat has also been found in dunlins, turnstones, and oystercatchers (Charadriiformes), as well as in Adélie penguins (*Pygoscelis adeliae*, Sphenisciformes; Ritchie and Lambert 2000). In knots, it is not a perfect repeat, but instead follows a pattern of $(CAAA)_3(CAA)$. Stutter patterns produced at the far end of this microsatellite on sequencing gels indicate the possibility of heteroplasmy (intra-individual length variation) in this repeat. This type of heteroplasmy was first reported in the order Ciconiiformes (Berg et al. 1995).

Domain III of the chicken control region contains a single bidirectional promoter for the transcription of both light and the heavy strands (LSP/HSP) (L'abbé et al. 1991). This promoter sequence (5′-GTATAATATATATACA-3′) is found towards the end of domain III, just before the 5′ end of the CAAA repeat in the alignment with Charadriiformes. In Adélie penguins, a putative LSP–HSP promoter (5′CATTAATATATATAATAG-3′) also precedes the CAAA repeat (Ritchie and Lambert 2000). In knots, a palindromic motif with a cytosine string is aligned with the chicken transcription promoter sequence. The TATA promoter sequence is also absent from the other Charadriiformes in the alignment. Knots, dunlins, turnstones, and oystercatchers do possess a well-conserved TACAT motif approximately 50 bp upstream from the chicken light and heavy strand promoters (LSP and HSP) in the alignment. When folded, this motif forms a cruciform structure slightly smaller than those formed by the chicken and penguin motifs.

Flanking the knot control region are tRNA's glu and phe. To verify the authenticity of the sequence, we tested the secondary structure of these tRNA regions. To function cor-

Fig. 1. L-strand (5′→3′) control region sequences for four members of Charadriiformes and chicken. Alignment generated by CLUSTAL W (Thompson et al. 1994). Domain I C-string, domain II T-string, and domain III CAAA-microsatellite are bolded and in italic, putative TAS elements and transcription promoters are underlined, and conserved sequence blocks (F, E, D, C, BSB, and CSB-1) are highlighted.

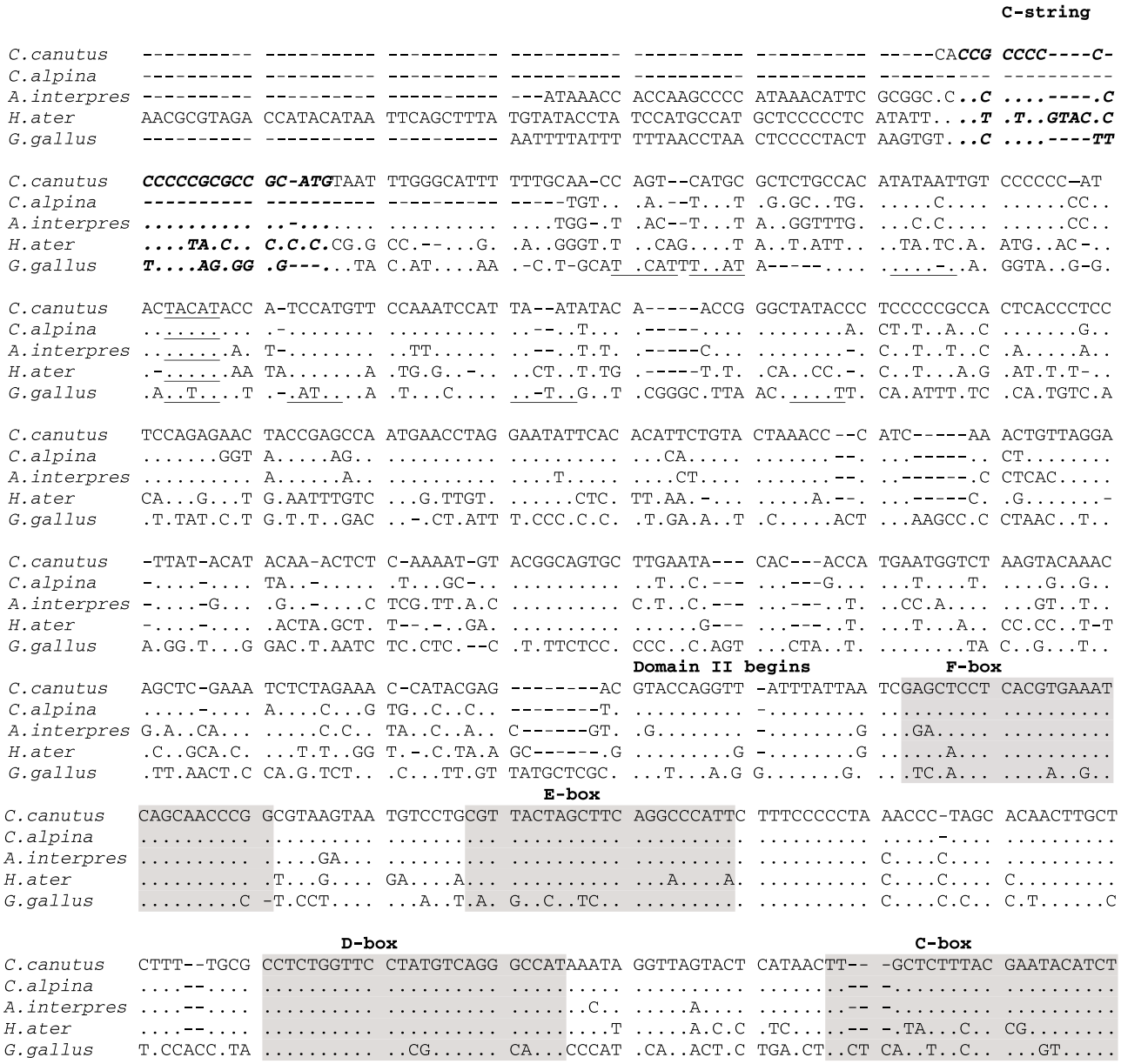


Fig. 1. (*concluded*).

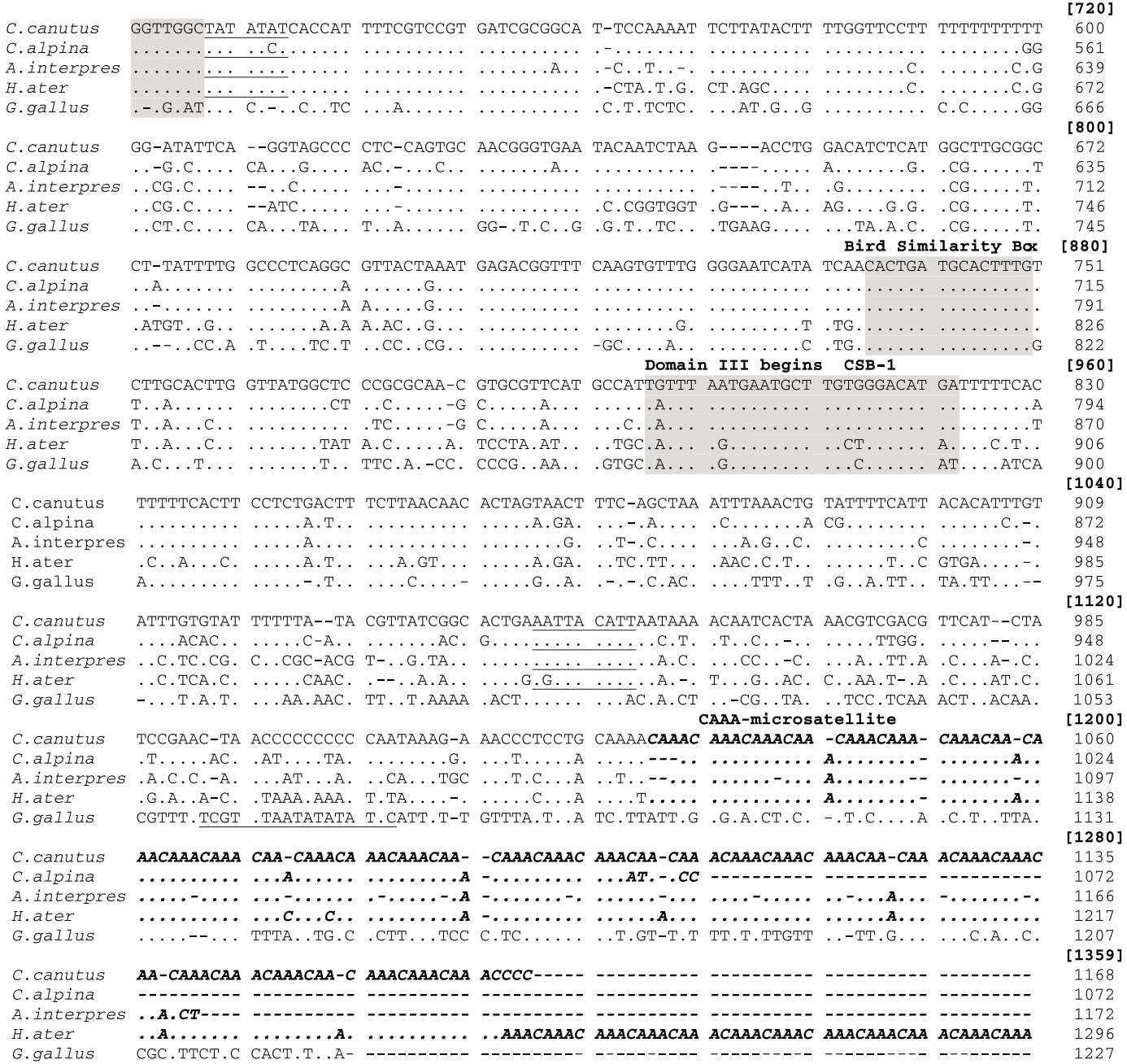


Fig. 2. A schematic representation of the control region of red knots and its characteristics.

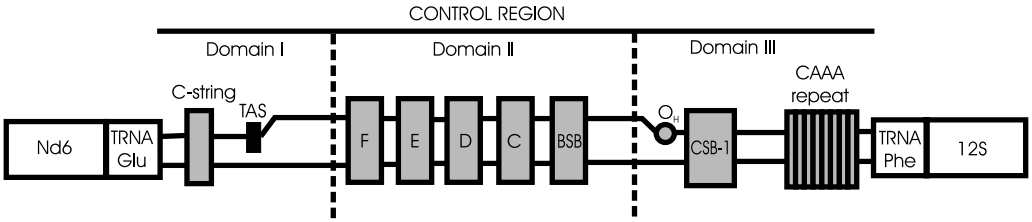
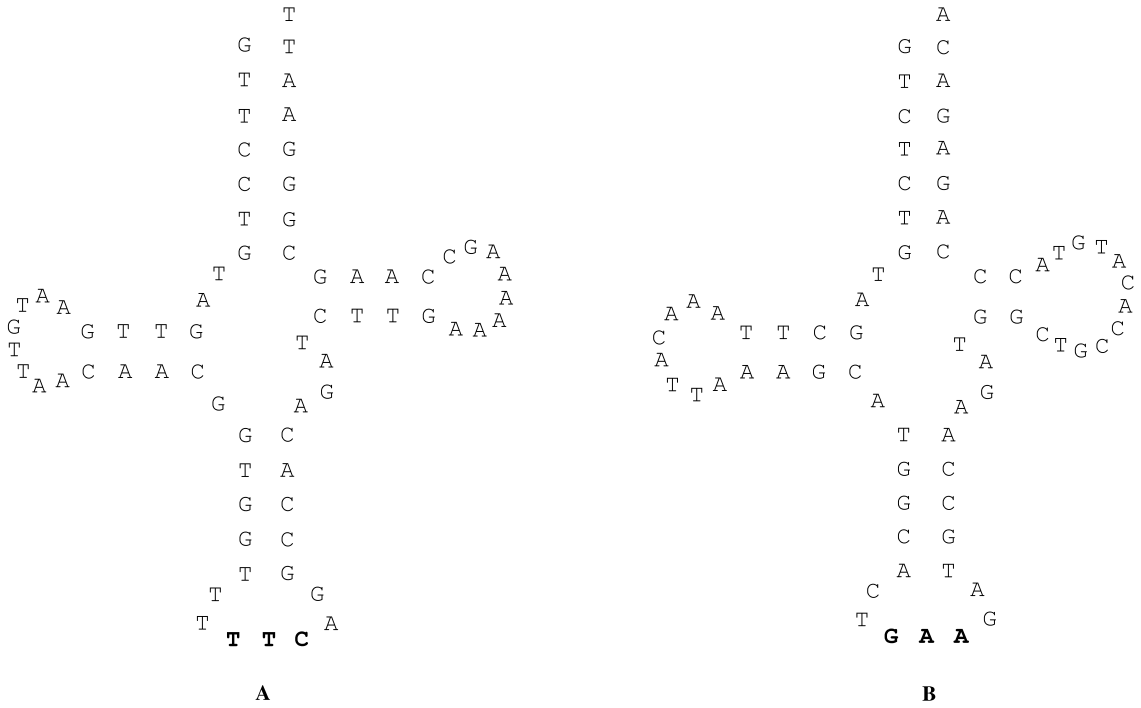


Fig. 3. Cloverleaf folding pattern shown by tRNA glu (A) and tRNA phe (B), which flank the control region of knots.



rectly, these tRNA's must fold into a clover-leaf motif. Figure 3 shows that the tRNA sequences folded correctly as predicted by the tRNA folding model published for the chicken (Desjardins and Morais 1990).

Finally, the relative rate test of sequence evolution found no significant difference in rates of substitution between the control region sequences of knots and turnstones (knot = 3.2×10^{-3} substitutions/site/million years; turnstone = 2.8×10^{-3} substitutions/site/million years; $Z = 0.67$). This finding supports the argument that the sequence is mitochondrial in origin. The absolute rate of mutation in the nucleus is much lower than that in the mitochondria, despite the fact that lack of functionality allows pseudogenes to acquire more mutations over time (Arctander 1995). Thus, if the knot sequence were a nuclear pseudogene then the rate of substitution relative to turnstones would be much lower.

Preliminary screening for genetic variation using the full control region sequence of 11 individuals showed 8 variable sites (1 transversion and 7 transitions) producing 8 haplotypes (Table 2). This is more variation than was found in 25 individuals using only the 5′ end of the control region (Baker et al. 1994). In this study, five of the eight variable sites were located towards the 3′ end of the control region indicating a high level of variability in this region. Similar levels of variability have been found towards the 3′ end of the control region in other bird species (Ruokonen and Kvist 2002). Additionally, in our alignments of the knot control region with that of other Charadriiformes, both the 3′ and 5′ ends showed high levels of variability in contrast to the central domain where sequence blocks F, E, D, and C and especially the BSB showed relative conservation. These characteristics highlight the importance of the 3′ and 5′ ends of the control region as sources of variability and possible markers for future population genetics work in knots and other bird species.

In summary, this study presents the first complete control region sequence of red knots. The control region is 1168 bp in length and contains all of the expected conserved sequence blocks and repeat structures. Furthermore, substitution rate in the sequence did not vary significantly from that of the control region in turnstones, and flanking tRNA regions folded correctly into secondary structure. Finally, sequence obtained from purified mtDNA is identical to sequence obtained using total genomic DNA. These characteristics verify that the sequence presented is mitochondrial in origin and indicate that our primers and protocol of using relatively long template genomic DNA for sequencing can be used to produce mitochondrial copies and not nuclear pseudogenes of the control region. In addition, the sequence itself can serve as a reference for future population genetics studies in knots. Furthermore, this study shows the value of

Table 2. Variable sites found in the control regions (excluding the 3′ tetranucleotide repeat) of a sample of 11 knots representing five subspecies (GenBank accession Nos. AY198136–AY198146).

Individual	Subspecies	Haplotype	Variable sites							
			1	1	2	6	7	8	8	8
			3	9	8	0	8	0	2	9
			8	8	5	6	2	7	7	9
M5315	*C. c.* subsp. *rufa*	1	T	T	T	G	A	T	A	T
REKN5B	*C. c.* subsp. *rufa*	1	–	–	–	–	–	–	–	–
11428006	*C. c.* subsp. *roselaari*	2	–	–	–	–	–	–	–	C
11428176	*C. c.* subsp. *roselaari*	3	A	–	–	–	G	C	–	–
MKP311	*C. c.* subsp. *roselaari*	4	–	–	C	–	–	–	–	C
AJB6050	*C. c.* subsp. *piersmai*	5	–	–	–	A	–	–	G	–
AJB6051	*C. c.* subsp. *piersmai*	1	–	–	–	–	–	–	–	–
80261066	*C. c.* subsp. *islandica*	1	–	–	–	–	–	–	–	–
80261067	*C. c.* subsp. *islandica*	6	–	–	–	–	–	–	–	C
KN12	*C. c.* subsp. *canutus*	7	–	C	–	A	–	–	G	–
KN13	*C. c.* subsp. *canutus*	8	–	–	–	A	–	–	–	C

Note: Variation is represented with respect to the first individual listed and identities are indicated by dashes. Haplotypes are identified by number in the third column. Sites are numbered according to the complete control region sequence presented in Fig. 1.

the 3′ end of the control region as a source of variation and possible markers, setting the stage for a large-scale phylogeographic survey using a larger sample size and sequence from both the 5′ and 3′ ends of the control region in knots, and studies examining the 3′ end of the control region for markers in other birds species.

Acknowledgements

This research was supported by an NSERC PGS A scholarship (PGSA-231177-2000) to D.M.B. and grants from NSERC (200-02) and the Royal Ontario Museum Foundation to A.J.B. We thank Oliver Haddrath for technical support in the lab and stimulating discussion in the analysis. We also thank Dr. David Irwin and Tara Paton for helpful comments and suggestions on the manuscript.

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