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INTRA- AND INTERSPECIFIC SEQUENCE VARIATION IN A PORTION OF THE MITOCHONDRIAL ND6 GENE IN CUCKOOS[1]

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Abstract. A 474 base pair segment of the NADH dehydrogenase subunit 6 (ND6) mitochondrial gene was sequenced for four cuckoo species (family Cuculidae). To assess the potential of this little-studied gene for intra- and interspecific genetic analyses, we examined sequence variation between (1) individuals from sympatric populations of the Common Cuckoo (*Cuculus canorus canorus*) collected in Great Britain, (2) two subspecies of Common Cuckoo (*C. c. canorus* and *C. c. telephonus*) collected at the geographical extremes of this species' range, and (3) four representative Cuculids: the Common Cuckoo, the Rusty-breasted Cuckoo (*Cacomantis sepulcralis*), the Fan-tailed Cuckoo (*Cacomantis flabelliformis*), and Klaas's

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Cuckoo (*Chrysococcyx klaas*). Although we found little genetic divergence at the intraspecific level, the substantial amount of interspecific sequence variation indicated that ND6 may be most useful in resolving evolutionary relationships between cuckoo species rather than within species.

Key words: mitochondrial DNA, ND6, cuckoo, Cuculus canorus, *sequence variation.*

Despite the fact that the entire DNA sequence for the mitochondrion is known for a bird (Desjardins and Morais 1990), most studies analyzing mtDNA sequence variation in birds focus on a narrow set of regions such as the control region (Edwards 1993) and, most often, cytochrome *b* (Edwards et al. 1991). However, little information is available on levels of variation in other mtDNA genes in birds; such data are needed to assess whether they are useful for studies of variation at the intra- and/or interspecific level.

One such gene which may be useful for constructing phylogenies of closely related species or for intraspecific phylogeographic analysis is the NADH dehydrogenase subunit 6 (ND6) gene. Desjardins and Morais (1990) suggested that it may be one of the most rapidly evolving genes in the avian mitochondrial genome because of the relatively high rate of amino acid substitution in Chicken (*Gallus gallus*) ND6 when compared with the same gene in other vertebrates. Analysis of ND6 sequence has since been applied to the resolution of Alcine and Gruid phylogenies (Moum et al. 1994, Wood and Krajewski 1996). Nevertheless, information on ND6 variation at the intra- and interspecific level in other avian assemblages is lacking.

In this study, we designed primers that allowed us to measure variation in a portion of the ND6 gene both within and between species of brood parasitic cuckoos (family Cuculidae). At the intraspecific level, we focused on assessing genetic variation in sympatric British populations of the Common Cuckoo (*Cuculus canorus canorus*) because of the possible evolution of genetically distinct host races within populations of this species (Brooke and Davies 1988, Gibbs et al. 1996). We also examined intraspecific variation between allopatric populations of Common Cuckoos by comparing the above sequences with those obtained from another subspecies, *C. c. telephonus* from Japan. Lastly, to obtain estimates of interspecific variation at the generic and intergeneric level, we sequenced ND6 in three other cuckoo species: the Fan-tailed Cuckoo (*Cacomantis flabelliformis*), Klaas's Cuckoo (*Chrysococcyx klaas*), and the Rusty-breasted Cuckoo (*Cacomantis sepulcralis*).

METHODS

We used the following strategy to develop primers for assaying ND6 variation in cuckoos: First, as described by Gibbs et al. (1996), we used primers ND6C-L (Edwards 1993), a light-strand primer located 23 base pairs from the 5′ end of ND6, and GSH-12s (H. Gelter, unpubl. data), a heavy stranded primer located in 12sRNA, to amplify a region consisting of ND6, Glu-tRNA, the control region, and Phe-tRNA from a British Common Cuckoo using purified mtDNA as the template. Next, we cloned this product and used a primer walking approach to obtain sequence from the rest of the ND6 gene and the adjacent Glu-tRNA. We then used this sequence information to design two additional primers: CUGLU-H (5′-GAGGAGGCCAAGCAGGAATA-3′), a heavy strand primer located at the 5′ end of the Glu-tRNA, and an internal heavy strand primer, CUND2-H (5′-CGGCTGATCCTTTTCCCGAG-3′).

To obtain ND6 sequence from a sample, a double-stranded amplification was first performed using equimolar (0.5 uM) quantities of ND6C-L and CUGLU-H in a reaction mixture consisting of 100 mM Tris-HCl (pH 8.3), 0.5 M KCl, 20 mM $MgCl_2$, 0.01% gelatin, 200 uM dNTP, 2.5 units Taq Polymerase and double-distilled H_20 to a final volume of 50 ul. Thirty amplification cycles were run using the following parameters: (1) 60 sec denaturation at 94°C, (2) 60 sec annealing at 55°C, and (3) 90 sec extension at 72°C. The 514 bp product was then purified from a low-melt agarose gel and subjected to asymmetrical amplification to generate single-stranded product for sequencing (Gyllensten and Erlich 1988). Conditions were identical to the double-stranded amplification except that CUGLU-H was diluted to 1/100 relative to ND6C-L and 35 cycles were performed. Single-stranded products were purified via centrifugation at 7,000 rpm for 8 min through Ultra-free MC (Millipore) filters and resuspended in 23 ul ddH_20.

Chain-termination sequencing (Sanger et al. 1977) using ^{35}S-dATP and Sequenase (USB) and either CUGLU-H or CUND2-H as primers was done for all samples according to the manufacturer's specifications. Products were run out on 8% polyacrylamide gels, exposed for 48 hr on Kodak X-/OMAT AR film and scored manually. Sequences were aligned on ESEE (Cabot 1990) using chicken ND6 as a reference (Desjardins and Morais 1990).

The sequences used to survey inter- and intraspecific variation were generated from genomic DNA isolated from blood samples belonging to four cuckoo species. To examine intraspecific variation in sympatric populations, blood was collected from 27 unrelated Common Cuckoo chicks found in nests of its three most common hosts in Britain (Brooke and Davies 1987; see Gibbs et al. 1996 for sampling locations): the Reed Warbler (*Acrocephalus scirpaceus,* 14 samples), the Meadow Pipit (*Anthus pratensis,* 9 samples) and the Dunnock (*Prunella modularis,* 4 samples). To assess variation between the two Common Cuckoo subspecies, we compared the above samples with sequences from 12 unrelated adults of *C. canorus telephonus* from Saitama, Japan. Finally, for interspecific comparisons, we sequenced ND6 from a Klaas's Cuckoo, a Fan-tailed Cuckoo, and a Rusty-breasted Cuckoo. DNA extraction techniques are detailed in Gibbs et al. (1994).

RESULTS

The L-strand consensus sequence of a 474 bp portion of ND6 for Common Cuckoos from Britain was identical to that generated from the cloned product (see Methods), which was amplified from purified mtDNA. Sequences for all species have been deposited in Genbank under accession numbers U81974–U81978.

TABLE 1. Pairwise substitutions between cuckoo species for a portion of the ND6 gene. Numbers of transitions are shown above the diagonal, whereas the numbers of transversions are shown below the diagonal. Species codes: CCC = *Cuculus canorus canorus*, CCT = *C. c. telephonus*, CFL = *Cacomantis flabelliformis*, CKL = *Chrysococcyx klaas*, CSE = *Cacomantis sepulcralis*, GGA = *Gallus gallus*.

	CCC	CCT	CFL	CKL	CSE	GGA
CCC	—	0	8	8	12	60
CCT	2	—	8	8	12	60
CFL	45	45	—	6	14	60
CKL	48	48	45	—	13	58
CSE	47	46	54	67	—	63
GGA	55	55	47	59	54	—

Within-population comparisons revealed no nucleotide substitutions between the British Cuckoos except for one chick, found in a Dunnock nest, which had a T-C transition at bp 253. Similarly, no differences were detected between the twelve unrelated adult cuckoos from Japan.

A comparison between the two Common Cuckoo subspecies found a small degree of divergence; of 474 base-pairs, only two sites (0.42 %) differed (Table 1). However, both were transitions, with one a conservative substitution, changing the amino acid from histidine to tyrosine.

At the interspecific level, more sequence divergence is apparent (Table 1). Among the four cuckoo species sampled, a total of 111 variable sites (23.4%) were present. Twenty-eight variable sites were at the first position, 10 at the second position, and 73 at the third position. A comparison of the Common Cuckoo and chicken sequences found 108 variable sites (22.8%). Pairwise comparisons found an average of 12.9% divergence between the cuckoo species, with the least divergence (10.8%) occurring between the Klaas's Cuckoo and the Fan-tailed Cuckoo, and the greatest divergence (16.9%) occurring between the Klaas's Cuckoo and the Rusty-breasted Cuckoo (Table 1). The extent of the divergence between the three cuckoo sequences and that of the chicken (mean = 23.7%, range 22.6–24.7), combined with a low average transition: transversion ratio (0.86:1), indicates saturation (Moritz et al. 1987).

Using the Common Cuckoo consensus sequence, we found a general bias towards purines on the light (sense) strand (52.0%) and a strong bias towards adenine and cytosine for all three codon positions; these two base pairs constituted an average of 81.3% of all sites (average for the three cuckoo species: 83.7% first position, 75.3% second position, 84.8% third position). A similar bias also was found in the ND6 gene of Lesser Snow Goose (*Anser caerulescens*) (Quinn and Wilson 1993). The base composition here differs between codon positions, with the first and second positions being richest in adenine, while the third position contains more thymine. The distribution of substitutions revealed some relatively conserved sites at 67–102 bp from the 5′ end and at 424–451 bp. Also, a marked transition : transversion bias (5.4:1) was seen between the sequences for the three cuckoos, a figure consistent with substitution patterns seen between closely-related taxa (Moritz et al. 1987).

DISCUSSION

Our study is the second, after Wood and Krajewski (1996), to assess intraspecific genetic variation in sympatric and allopatric bird populations using the ND6 gene, and we find extremely low variability within a focal population of British Common Cuckoos. This result, combined with previous work on British Common Cuckoo population genetic structure by Gibbs et al. (1996), adds support to the idea that host races as defined by mimetic egg types have either evolved extremely rapidly or that gene flow between host races occurs because female cuckoos periodically parasitize the "wrong" host (Gibbs et al. 1996). The minimal sequence divergence between the two Common Cuckoo subspecies (0.4%) concords with values obtained for crane subspecies (Wood and Krajewski 1996).

Previous attempts to assess interspecific variability in the ND6 gene in birds found 7.9% sequence divergence between two congeneric murres (*Uria* spp.) (Moum and Johansen 1992) and 4.9–6.4% divergence for three congeneric cranes (*Grus* spp.) (Wood and Krajewski 1996). Using sequences from the Genbank database, we compared representatives of 7 Alcine genera (*Alle alle, Cepphus columba, Brachyramphus brevirostris, Fratercula corniculata, Aethia cristatella,* and *Uria aalge*) and found an average of 13.8% sequence divergence (range 11.2%–15.8%) over the same portion of ND6 assayed in this study. This is close to the average value of 12.9% divergence among all cuckoo species found in this study. A surprising result was that a 14.3% sequence divergence was present between two sample congeners, *Cacomantis sepulcralis* and *C. flabelliformis*, whereas *C. flabelliformis* showed only an 11.2% divergence compared to *Cuculus canorus*. This result supports previous suggestions (Slater et al. 1994) that *Cacomantis flabelliformis* may be more appropriately classified as a species within the genus *Cuculus*. However, further detailed molecular work is needed to better assess the evolutionary affinities of these species.

At the interspecific level, the ND6 gene may prove to be a phylogenetically informative marker in the cuckoos. Although we had too few species to generate phylogenetic trees and thus identify the frequency of informative sites, the observed average value for interspecific variation (12.9%) suggests that a number of such sites are likely present. Furthermore, the fact that we could amplify this gene from several different species within the family indicates that the primers used are relatively conserved. As with Moum and Johansen (1992), we believe that this gene is attractive to phylogeneticists studying closely related species because of its relatively small size and its high rate of evolution. Rates of sequence evolution differ among bird assemblages, but our results suggest that ND6 is potentially useful for studies at the interspecific level.

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MORPHOLOGICAL AND VOCAL VARIATION AMONG SUBSPECIES OF THE BLACK-FACED SHEATHBILL[1]

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Abstract. The Black-faced Sheathbill (*Chionis minor*) is a sedentary and polytypic species. Four allopatric subspecies are known, each breeding on one archipelago in the Southern Indian Ocean. To evaluate the degree of isolation of these four subspecies, morphometrics and vocalizations of adult birds of Iles Kerguelen and Crozet were compared with those of the other localities (Prince Edward and Heard Islands). Two groups were distinguished (Prince Edward-Crozet and Kerguelen-Heard) on geographic and morphological criteria. In the eastern group (Kerguelen-Heard), corresponding to higher latitudes, sheathbills were larger and heavier, following Bergmann's Rule. The sheathbills from Iles Kerguelen also had a lower-pitched voice than those from Iles Crozet, consistent with their larger body size. Moreover, the birds from the southernmost locality (Heard Island) had a shorter culmen, consistent with Allen's Rule, but longer tarsi and deeper sheaths. Within the western group (Prince Edward-Crozet), and at Iles Kerguelen, there also was variability on a microgeographical scale. Differences

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