Assessment of genetic and morphological differentiation among populations of Diederik Cuckoo (*Chrysococcyx caprius)*

Rae M. Smith[1,2], Desiré L. Dalton[1,3], Monica Mwale[1,4], Lisa Jane Nupen[1,6], Chantelle Pretorius[1], Jamie Bojko[3], Kim Labuschagne[1], Isa-Rita M. Russo[5], Samuel T. Osinubi[6]

[1]South African National Biodiversity Institute, P.O. Box 754, Pretoria, 0001, South Africa

[2]Department of Agriculture and Animal Health, College of Agriculture and Environmental Sciences, University of South Africa, Johannesburg, South Africa

[3]School of Health and Life Sciences, Teesside University, Middlesbrough, TS1 3BX, United Kingdom

[4]National Institute for Theoretical and Computational Sciences (NITheCS), KwaZulu-Natal, South Africa

[5]Organisms and Environment Division, Cardiff University, School of Biosciences, Sir Martin Evans Building, Museum Avenue, Cardiff, CF10 3AX, UK

[6]FitzPatrick Institute of African Ornithology, University of Cape Town, South Africa

*Corresponding author: DL Dalton, d.dalton@tees.ac.uk, +44 75 777 29448

ORCID:

Rae Smith: 0000-0002-1379-3949

Desire Dalton: 0000-0001-5975-6425

Monica Mwale: 0000-0003-2180-8917

Lisa Nupen: 0000-0003-1030-6057

Chantelle Pretorius: 0000-0003-1396-8312

Jamie Bojko: 0000-0001-5972-0844

Isa-Rita Russo: 0000-0001-9504-3633

Samuel Osinubi: 0000-0002-5143-6985

Abstract

Diederik Cuckoo (*Chrysococcyx caprius*) is a widely distributed species that occurs south of the Sahara Desert and migrates seasonally between breeding and non-breeding sites. It is currently unknown if the species consists of a single panmictic population or if it is genetically structured. To investigate this, we analysed sequence variation in three mitochondrial and two nuclear gene regions in combination with morphological measurements in specimens from four localities. Phylogenetic relationships were estimated using maximum likelihood methods and included specimens of Klaas's Cuckoo (*Chrysoccyx klaas*), Red-chested Cuckoo (*Cuculus solitarius*) and African Cuckoo (*Cuculus gularis*) to characterise levels of genetic differentiation. Haplotype networks and analysis of molecular variance were used to characterize the spatial distribution of genetic diversity. Lastly, a principal component analysis was performed to investigate morphological variation among localities based on selected characters. Molecular analysis identified two mitochondrial lineages that were syntopic (i.e., samples collected from the same locality in South Africa either grouped with clade one or two). The magnitude of divergence between- vs. within-clades was low (0.4-1%), but significant (F_{ST}: 0.84-0.88). Lack of apparent phylogeographic structure provides support for the absence of physical barriers to gene flow. Further, morphological analyses did not show any pattern of variation among lineages. The emergence and persistence of shallow mitochondrial divergence among sympatric lineages in Diederik Cuckoo may possibly be attributed to maternal divergence in host selection of these brood parasites.

Keywords: Phylogeny, genetic diversity, population structure, mtDNA, nuclear DNA

Introduction

The subfamily Cuculinae includes 54 species that are distributed across Europe, Africa, Asia and Australasia (Pratt and Beehler 2014). Thus far, only a few of these species such as Common Cuckoo (*Cuculus canorus*) and some of the Australian Cuckoos have been the subject of intensive study. Cuckoos vary in appearance but are often slender, long-tailed, zygodactyl birds (Aragón et al. 1999). The bills are stout, generally with a hooked tip. They vary considerably in size, ranging from 15 - 63 cm, with little sexual dimorphism in size. However, in some cases, depending on the genus, males or females can be larger. Old World Cuckoos are parasitic breeders that lay their eggs in the nests of other birds with some being reported to be host generalists while others are specialists (Johnsgard 1997). Host species for several cuckoos are not known, however in general, hosts are smaller than the cuckoo itself and include

insectivorous species of moderate size such as babblers and shrikes or of small size such as warblers, chats, pipits, weavers, and sunbirds (Davies 2010). The Common Cuckoo parasitizes over 100 species across its range, however in any single locality only a few species are parasitized (Marchetti et al. 1998). Several young cuckoos eject the host eggs or host young out of the nest. The Asian Koel (*Eudynamys scolopacea*) and Channel-billed Cuckoo (*Scythrops novaehollandiae*) are examples of cuckoos that do not eject due to the large size of the host species and may be raised alongside host young (Payne 2005). In these cases, cuckoos may outcompete host chicks for food leading to their death. In species where cuckoos are raised with host chicks, more than one cuckoo egg will be laid in each nest (Davies 2010). Depending on species, cuckoo eggs may closely match host eggs in colour and size or may not match at all.

The genus *Chrysococcyx* includes 15 species, four of which occur in Africa and the remainder occur in southeast Asia and Australasia. Several African species show seasonal movements between breeding and nonbreeding grounds within Africa known as intra-African migration (Moreau 1972). The Diederik Cuckoo is a widespread species, occurring from western to eastern Africa and most of southern Africa (Fry et al. 1988). Information on the migratory behaviour including timing and duration of migration between breeding and non-breeding grounds of this species is limited and is mainly based on species occurrence data. It has been reported that Diederik Cuckoos were almost absent in southern Ghana during September and October (Macdonald 1980). Diederik Cuckoos migrate to southern Africa from central or eastern Africa (September to October) but may arrive a month later depending on the onset of rain (Macdonald 1980, Hockey et al. 1989). They spend approximately six months in southern Africa and depart in April, breeding between November and April. Diederik Cuckoos regularly migrate seasonally, however, it is unknown if individuals from different populations in Africa, migrate to the same breeding or non-breeding areas. Satellite telemetry has revealed that the African Cuckoo (*Cuculus gularis*) breeds in central Nigeria and then migrates to Cameroon and western Central African Republic at the beginning of the dry season in central Nigeria. Migratory routes were variable between birds, but individuals demonstrated high fidelity for non-breeding grounds (Iwajomo et al. 2017). However, intra-African migration is a diverse and complex process which can vary considerably between species. In addition, intra-African migration may be severely impacted by habitat loss. The tropics are expected to lose extensive amounts of biodiversity due to anthropogenic-induced climate change (Alroy 2017). It has been reported that half of the known migratory birds are still flying historical migratory routes when

compared to 30 years ago (Kirby et al. 2008). Although, there are several data deficiencies in the species' assessment (IUCN 2016), the Diederik Cuckoo is currently listed as 'Least Concern' due to their large geographic range, across a wide range of habitats (forest-edge, semi-desert, and woodland habitats) in nearly all temperate and tropical biomes within Africa (Rowan 1983, Fry et al. 1988) (Figure 1). Our study aims to investigate for the first-time genetic differentiation and morphological variation among populations of Diederik Cuckoo from southern, western and eastern Africa using three mitochondrial (mtDNA) markers (Cytochrome c oxidase subunit I (COI) and the rRNA genes (12S, 16S)), two nuclear genes (β-fibrinogen exons 5 and 6 (FIB5) and recombination activating 1 (RAG1) gene), and five morphological characters.

Materials and methods

Study sites and sample collection

Trapping was conducted using varying numbers and arrays of mist nets, with species-specific playback, during morning (06h00 – 10h00) and evening (15h00 – 18h00) sessions. Between December 2015 and January 2019, 54 Diederik Cuckoos were trapped as part of a larger study on intra-African migratory birds. Sampling localities for Diederik Cuckoos were: West Africa (Jos, Nigeria, n = 9 and Damongo, Ghana, n = 3), East Africa (Entebbe, Uganda, n = 3) and Southern Africa (Limpopo Province, South Africa, n = 39). Additionally, blood samples were also collected from three closely related African cuckoo species (Klaas's Cuckoo *Chrysococcyx klaas* (n = 4); Red-Chested Cuckoo *Cuculus solitaries* (n = 7) and African Cuckoo *Cuculus gularis* (n = 1)). Generally, samples were collected during the breeding season across the sub-regions. Trapped birds were ringed using individually coded aluminium rings that followed the ringing scheme in each country. The aluminium rings ensured individuality of samples as well as the identification of individual birds if later re-trapped. Biometrics and ring numbers were uploaded to the South African Bird Ringing Unit (SAFRING) online database. Blood samples were collected using the brachial venipuncture method with 27-gauge needles and 100 µl capillary tubes, and the blood samples were stored in lysis buffer (Seutin et al. 1991). All collected biological materials were stored at the Biobank of the South African National Biodiversity Institute (SANBI). The project was registered and approved as P14/23 by the Research and Ethics Committee (RESC) of SANBI. Permission to do research in terms of Section 20 of the Animal Diseases Act, 1984 (Act No. 35 of 1984) was issued by the Department of Agriculture, Land Reform and Rural Development (DALRRD). A dispensation on Section 20 approval in terms of the Animal Diseases Act, 1984 (Act No. 35 of 1984) was

also issued by DALRRD to store the samples collected for the project at the SANBI Wildlife and Conservation Biobank. The lead bird ringer on the project was licensed in accordance with the South African Bird Ringing Unit (SAFRING), and collection permits (ZA/LP/93056; ZA/LP/WMD/1257; CPM 36408) were secured from the Limpopo Provincial Department of Economic Development, Environment and Tourism during each sampling year. Blood samples were collected under South African Veterinary Council (SAVC) authorisation (AL17/15903). Additionally, support for sampling was obtained from relevant national and local authorities in Nigeria, Ghana and Uganda.

Morphological data collection

All trapped individuals were weighed and measured (mass (g), wing, tail, tarsus and head length (mm)) before blood samples were collected, and immediately released after sampling. Mass (g) was measured either using a spring balance (bird is weighed in the bird bag and the bird bag is weighed afterwards to determine the mass of the bird) or a digital scale (small plastic container was tared before each measurement, then the bird was placed in the container and weighed). Wing length (mm) was measured using a wing rule, placing the bend of the wing against the top of the rule, flattening the wings and feathers so that the measure is maximised, and taking the reading from the tip of the longest wing feather (the primaries). Tail length (mm) was measured using a flat rule and taking the measurement from the base of the tail to the tip of the longest tail feather. Head length (mm) was measured using a digital caliper, and the measurement was taken from the back of the skull to the front of the skull. This measure excludes the length of the culmen from the total head length. Tarsus length (mm) was measured using a digital caliper, and the measurement was taken from the notch on the metatarsus (where it meets the tibiotarsus) to the top of the bone above the bent toes. Morphological measurements have been included in SAFRING.

Molecular methods

Genomic DNA was successfully extracted from 56 out of 66 specimens of four species of cuckoos from two genera: 1) Diederik Cuckoo (*Chrysoccyx caprius*), 2) Klaas's Cuckoo (*Chrysoccyx klaas*), 3) Red-chested Cuckoo (*Cuculus solitaries*) and 4) African Cuckoo (*Cuculus gularis*) (Table 1). DNA was extracted from blood samples using the Quick-DNA Miniprep Plus Kit (Zymo Research) following the manufacturer's instructions. The extracted DNA was quantified using a Nanodrop™ 1000 Spectrophotometer (Thermo Fisher Scientific) and samples were stored at -20ºC until further laboratory work. Portions of three mitochondrial

DNA genes and two nuclear genes were targeted from the cuckoo DNA samples. Mitochondrial gene regions included: Cytochrome oxidase 1 (COI: BirdF1, 5'-TTCTCCAACCACAAAGACATTGGCAC-3' and BirdR1, 5'-ACGTGGGAGATAATTCCAAATCCTG-3'; Hebert et al. 2004), the 16S ribosomal RNA gene (16S: 16S-f, ,5'-CGCCTGTTTAACAAAAACAT-3', and 16S-r, 5'-CCGGTCTGAACTCAGATCACGT-3'; Miya and Nishida 1996) and the 12S ribosomal RNA gene (12S: 12S-f , 5'- TGACTGCAGAGGGTGACGGGCGGTGTGT -3' and 12S-r, 5'-AAAAAGCTTCAAACTGGGATTAGATACCCC -3'; Kocher et al. 1989). We targeted two nuclear gene sites, namely the β-fibrinogen gene, intron 5 (FIB5) using the primers FIB5 (5'-CGCCATACAGAGTATACTGTGACAT-3') and FIB6 (5'-GCCATCCTGGCGATTCTGAA-3'; Marini and Hackett 2002). Secondly, we targeted the Recombination activating gene 1 (RAG1) using the RAG-1-F1 (5'-GATTCTGTCACAACTGTTGGAGT-3'), and RAG-1-R2 primers (5'-TCCCACTTCTGTGTTAGTGGA-3'; Gardner et al. 2010). Amplification of the various gene regions was conducted using *Taq* DNA Polymerase 2x Master Mix RED (Ampliqon) at a final concentration of 1.5 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.05 M Tris/HCl pH 8.5, $(NH_4)_2SO_4$ and 0.1 units μl^{-1} Taq DNA polymerase. Additionally, 0.1 µM of the forward and reverse primers and 2-4 µl of DNA template (50 ng/µl) was added and the mixture was made up to final reaction volume of 15 µl with ddH_2O. Targeted gene regions were amplified in a SimpliAmp thermocycler (Thermo Scientific, California, USA). The PCR protocol consisted of an initial cycle of 5 min at 95ºC followed by 35 cycles of 95ºC for 30 s; 50-60ºC for 30 s and 72ºC for 30 s, with a final extension step of 72ºC for 10 min. Amplification was confirmed by agarose gel electrophoresis on a 2% gel (140 volts (V) for 30 min). The PCR products were purified using 5 U of Exonuclease I (Thermo Fisher Scientific) and 1 U FastAP™ Thermosensitive Alkaline Phosphatase (Thermo Fisher Scientific) incubated at 37ºC for 15 min, followed by 85ºC for 15 min. The cycle sequencing reactions were prepared using the BigDye™ Terminator kit 3.1 (Applied Biosystems) as per manufacturer's instructions. The amplification conditions were as follows: 4 min of initial denaturation at 94°C, followed by 40 cycles of 85°C for 10 s, at 53°C for 10 s, and 60°C for 2 min 30 s. Cycle sequenced products were purified using BigDye™ Xterminator Sequencing clean-up Kit (Thermo Fisher Scientific) as per manufacturer's instructions. Briefly, samples were added to 45 µl SAM™ solution along with 10 µl of the BigDye Xterminator™ solution and were shaken at 2000 rpm for 30 min at room temperature. Sequences were visualised on the ABI PRISM 3500 Genetic Analyser (Thermo Fisher Scientific).

Phylogenetic and sequence comparison analyses

All sequences have been deposited in GenBank (Accession no's OQ067723-OQ067764, OQ068504-OQ068540, OQ147134-OQ147180 and OQ158800-OQ158828). Forward and reverse sequences were edited and aligned to create a consensus sequence in Geneious R10.2 (Biomatters inc.) using the default settings (Kearse et al. 2012). Not all gene regions were successfully sequenced for every individual (Table 1). All sequences were manually trimmed and checked for ambiguous peaks. To test for nuclear mitochondrial pseudogenes (numts), we searched for stop codons, insertions and/or deletions and double peaks in the COI region, and insertions and/or deletions and double peaks in 12S rRNA and 16S rRNA. Further, phylogenetic trees of individual gene regions were assessed for long branches and/or deep divergence, outside the clade of other sequences of the same species which may identify sequence errors, incorrect sequence assembly and/or numts (Sangster and Luksenburg 2021). Sequence polymorphic sites in nuclear genes corresponding to heterozygous individuals were coded with the International Union of Pure and Applied Chemistry (IUPAC) ambiguity codes. Phylogenetic relationships were reconstructed using the maximum likelihood (ML) method, separately for the nuclear and mtDNA genes. Reference sequences were obtained from the National Centre for Biotechnology Information (NCBI) GenBank. Published reference sequences used for each gene region is indicated in Table 1. The quality of the forward and reverse sequence reads was assessed using Geneious® v. 10.0.9, before being assembled into consensus sequences for alignments. We determined the best fitting substitution model in MEGA7 (Kumar et al. 2016). Phylogenetic relationships were reconstructed by the ML method based the Kimura 2-parameter (K2) model for 12S, the K2 model + gamma (G) for 16S and the K2 model for the concatenated mitochondrial dataset. The Tamura 3-parameter (T92) model was used for RAG1. Branch support values were estimated using non-parametric bootstrap with 1,000 replicates. To estimate phylogeny for FIB5, we used a hierarchical Bayesian inference (BI) approach as implemented in MrBayes 2.1 with the phased haplotype data only (see below) and *C. klaas* haplotypes treated as outgroup. Each run consisted of four simultaneous Markov chain Monte Carlo (MCMC) chains with a length of 1 million generations. Trees were sampled every 1000 generations with the first 20% discarded as burn-in with MCMC, to approximate the posterior probabilities of the trees. The T92 + G model was subsequently used to generate the ML tree.

Inter- and intraspecific p-distances between subspecies and lineages were calculated using ML genetic distance in MEGA7. Population genetic diversity measures for the mtDNA and nDNA

sequence datasets for number of haplotypes (H), haplotype diversity (Hd), polymorphic sites (S), nucleotide diversity (π), and levels of gene-flow were all calculated using DNASP 6.12.03 (Rozas et al. 2003). Haplotype reconstruction for both gene regions were done using the median joining network (Bandelt et al. 1999) using POPART (Leigh and Bryant 2015). The nuclear DNA sequence was only analysed after phasing the data using the algorithms provided by Stephens et al. (2001) and Wang and Xu (2003) for heterozygous sites (polymorphic nucleotide positions) in DNASP. The pairwise number of differences which can reflect patterns of population dynamics were estimated through mismatch distributions in DNASP and the test of neutrality (Tajima's D and Fu's F statistic) in Arlequin 3.5.2.2 (Schneider et al. 2000). Analysis of Molecular Variance (AMOVA) was used as implemented in Arlequin 3.5.2.2 to estimate hierarchical partitioning of genetic variation by testing hypotheses about genetic variation and geographic differentiation among the localities of the Diederik Cuckoo (South Africa versus East and West Africa using 10,000 permutations. To test for genetic and population differentiation (for populations with more than five individuals only) and among the clades of the Diederik Cuckoo, F_{ST} pairwise comparisons were computed using Arlequin with 1,000 permutations.

Morphometric analysis

Principal component analyses (PCA) were generated using ggplot2 (Wickham 2016) in R v.4.1.3 to explore morphometric measures. The sample set included 39 (75%) males, 11 (21%) females and 2 samples were of unknown sex (4%). Morphometric measures (Tarsus length, wing length, head length, tail length, and mass) were compared between the samples collected from various localities (Ghana, Nigeria, South Africa and Uganda), the sex (male and female) of the animals, and the two 'clades' identified via our phylogenetic analysis and haplotype mapping. To determine whether there were any significant differences in the means of morphological characters between locations, sexes, and clades not evident from the PCA analyses, we conducted a Kruskal-Wallis test in Microsoft Excel for Windows XP Professional. This non-parametric test was chosen as head length and mass were not normally distributed.

Results

Mitochondrial analysis

Phylogeny, diversity and genetic distance

Here, we identified double peaks and long branches for COI which were attributed to numts (Sangster and Luksenburg 2021), thus this region was excluded from further analysis as their

presence may confound results. Numts and sequence errors were not identified for 12S or 16S. The ML tree identified monophyly of *Chrysococcyx caprius* (99% bootstrap support) with *Chrysococcyx klaas* as a sister taxon (Figure 2). Within *C. caprius* two divergent lineages (well supported monophyletic clades) were identified for both 12S and 16S (Figure 2, Supplementary Figures 1 and 2) with high bootstrap support (100%). The first clade included birds from South Africa (Limpopo), Ghana, Uganda and Nigeria whereas the second clade only consisted of individuals from South Africa (Limpopo). Moderate to high Hd (0.4 to 0.75) and low π (0.0011 to 0.0047) was detected (Table 2). The 12S rRNA and 16S rRNA genes showed an absence of differentiation between localities (Fst = 0.048 to 0.082, $p > 0.05$), however significant differentiation for both gene regions were identified between clades (Fst = 0.84 to 0.88, $p < 0.05$) (Table 2). The distribution of haplotypes among localities for the concatenated dataset were associated with the two clades identified by the phylogenetic analysis. In clade 1, haplotypes H1 and H3 were shared amongst localities whereas H4 consisted of only South African individuals. Clade 2 consisted of haplotypes H2 and H5 and were only detected in birds from South Africa (Limpopo, Figure 2). For the 12S rRNA dataset, clade 1 had three haplotypes that included individuals from all four localities (Supplementary Figure 2). The most common haplotype (H4) was shared among all localities, while the other two were private being restricted to Nigeria and South Africa (Limpopo). The 16S rRNA network (Clade 1) had two shared widespread haplotypes and only one private haplotype (H3) restricted to South Africa (Limpopo). Clade 2 was more restricted, with all haplotypes in both 12S rRNA and 16S rRNA networks being detected in individuals from South Africa (Limpopo) only, suggesting some genetic sub-structure (Supplementary Figure 1). However, this clade was only represented by one haplotype in the 16S rRNA dataset while three haplotypes were identified in the 12S rRNA dataset. Tajima's D (Clade 1: -0.67 and 0.94, Clade 2: -1.23 and 0.00) and Fu's Fs neutrality tests (Clade 1: -0.63 and 0.87, Clade 2: -0.92 and 0.00) were positive and negative respectively for each clade (Table 2A) and the mismatch distribution analysis (Supplementary Figure 3A) was bimodal for both mtDNA gene regions.

Pairwise interspecific genetic distance between *Chrysococcyx klaas* and *C. caprius* varied from 2.6-6.9%, depending on the mtDNA gene region analysed (Table 4A and B) whereas genetic distance between species of *Chrysococcyx* and *Cuculus* varied between 3.9-14.5%. Pairwise intraspecific genetic distance between sampling localities (South Africa, Nigeria, Uganda and Ghana) of *C. caprius* varied from 0-0.4%. The pairwise genetic distance between clades 1 and 2 was 0.4% for 12SrRNA and 1% for 16S rRNA (Table 4A and B).

Nuclear analysis

Phylogeny and diversity

A total of 55 sequences from four geographic populations were analysed for the nuclear DNA sequence dataset (Table 1). The resulting ML trees for the two nuclear gene regions, FIB5 and RAG1, were similar in that there was no clustering by locality (Supplementary Figures 4 and 5). For each of the nuclear trees generated, there was no clustering by sampling locality. However, as with the mtDNA trees *Chrysococcyx klaas* was placed as closely related to *Chrysococcyx caprius*. Similar to mtDNA analysis, nuclear DNA haplotype diversity was high (0.72 and 0.86). The FIB5 dataset identified 21 haplotypes connected in a star-like network with no more than eight inferred mutational changes to connect any pair of haplotypes. Six haplotypes were shared and fifteen were restricted to localities (Supplementary Figure 4). In RAG1 one haplotype (H2) was widespread being shared among all localities (Supplementary Figure 5). There was evidence of genetic substructure based on private haplotypes (H1, H3, H4, H5 and H6) identified in the South African (Limpopo) samples and in a single sample from Nigeria (H7). Tajima's D and Fu's Fs neutrality tests varied per locality and overall was positive and negative (0.60, -1.00 and -0.83, -0.08, respectively; Table 2B). As with the mtDNA dataset, the mismatch distribution analysis (Supplementary Figure 3B) was bimodal.

Phylogenetic analysis reconstructed similar topology, irrespective of the data set used (mtDNA or nuclear DNA; Figure 2, Supplementary Figure 5A), with a basal split between *Cuculus* species and a branch consisting of the two *Chrysococcyx* species (*C. klaas* and *C. caprius*). The arrangement of *Cuculus* species differed between data sets, most likely due to the absence of all related sub-family Cuculinae species reference sequences for each region to verify phylogenetic relationships.

Morphometric analysis

The principal component analysis used five morphometric measures and did not identify differences between locality (Figure 3A), sex (Figure 3B) and clade (Figure 3C). The sum of all the eigenvalues gave a total variance of 5 (PC1 = 1.73, PC2 = 1.19, PC3 = 0.97, PC4 = 0.74 and PC5 = 0.37). The first and second principal components contained 34.67% and 23.74% of the variation. In all cases, the first and second principal components accounted for 58.41% of the variance and this percentage was considered large enough to explain the whole dataset. Other PC combinations do not show any discernible differences (Supplementary Figure 6).

With regards to locality (Figure 3A), there was little morphological variation with sample overlap among all localities; however, some variation is visible between Nigerian and South African birds along PC1. Some individual South African birds showed longer tarsus and wing length and higher mass, whereas certain Nigerian birds displayed shorter tarsus and wing length and lower mass. Tail and head length were similar among individuals for all locations. There was no clear cluster separation when distinguishing samples by sex (Figure 3B). Lastly, individuals from Clade 1 were highly scattered, with little morphological differentiation between the clades (Figure 3C). The Kruskal-Wallis test indicated statistical significant difference in tarsus length between Ghana and South Africa ($p = 0.019$) and between Nigeria and South Africa ($p = 0.003$) as well as statistical significance in wing length between Nigeria and South Africa ($p = 0.008$) and Uganda and South Africa ($p = 0.045$).

Discussion

Our study provides new insights into the population structure of Diederik Cuckoo, using samples located in the northern and southern distribution of their range and a combination of genetic markers. Nuclear phylogenetic trees were shallow and network analysis for both FIB5 and RAG1 gene regions identified private and shared haplotypes that did not correlate either with geography or lineages identified by mtDNA markers. AMOVA showed non-significant genetic variation among populations, indicating that most genetic variation exists within populations (Table 3). A shallow phylogenetic tree and star-like haplotype network may suggest a recent population expansion (Richards et al. 1998) leading to homogeneity across populations. Although lack of differentiation was observed for nuclear markers, two sympatric lineages in South Africa were identified based on mtDNA analysis. This finding may be attributed to yet undetermined selective pressures acting on populations of Diederik Cuckoo.

Morphological characters and lack of phylogeographic structure between geographic regions

Lack of morphological differences were detected between sexes of Diederik Cuckoo. It has been previously reported that in several Old-World Cuckoos, instead of selection on males to become larger, both sexes are slightly smaller with the evolution of brood parasitism (Krüger et al. 2007). Here, there was little morphological variation with sample overlap among all localities, however, tarsus and wing length were significantly shorter in birds from Nigeria, Uganda and Ghana compared to South Africa. Larger-bodied birds have been reported at higher latitude, whereas wing length may be influenced by external factor such as adaptation to local environment. Shorter wings may be selected due to greater requirements for manoeuvrability

at breeding sites with high predation rates (James 1970, Alatalo et al. 1984). Here, lack of phylogeographic structure was observed between populations located in the northern and southern distribution which may be attributed to high levels of contemporary gene flow and dispersal, or populations may have only recently been isolated and still share substantial amounts of ancestral variation. Limited data is available describing the migratory behaviour of Diederik Cuckoo. Intra-African migration varies greatly between species (Cox et al. 2011, Cox et al., 2013) and certain species are altitudinal migrants while other species are long-distance migrants moving across the continent. It is likely that Diederik Cuckoo are long-distance migrants, which is supported by the identification of vagrants. Using Ethiopia as a passage (Figure 1), Diederik Cuckoo has been reported in Cyprus, Israel and Saudi Arabia (BirdLife International, 2023). It is currently unknown if extralimital records of birds in Saudi Arabia are vagrant individuals or uncommon summer breeders. Slatkin (1987) reported that even sporadic long-distance migration may be sufficient to prevent substantial genetic differentiation via genetic drift. Presence of morphological differentiation in the absence of phylogenetic structure may be attributed to morphological differences being ecophenotypic and not based entirely on genetic differences (James 1983) or may be due to rapid evolution of genes responsible for morphological differences over a time scale that is too short for mtDNA to detect geographic differentiation (Ball et al. 1988).

Cuckoo molecular divergence

Phylogenetic analysis of mtDNA sequences identified the existence of two lineages (Clades 1 and 2, Figures 2A and B) within *Chrysococcyx caprius*. Clade 1 includes birds that are distributed across the continent (Uganda, Nigeria, South Africa and Ghana) whereas Clade 2 is limited to birds collected from South Africa. In a single location in South Africa (Limpopo), the two sympatric lineages were detected. Divergent but sympatric mtDNA lineages within a single species is considered rare; however, it has been previously described in various groups including birds (Quinn 1992, Spottiswoode et al. 2011, Hogner et al. 2012, Block et al. 2015), mammals (Hoelzer et al. 1994) and invertebrates (Xiao et al. 2012, Giska et al. 2015). In birds, divergent mtDNA lineages (approximately 2% sequence divergence) have been identified in the Savannah Sparrow (*Passerculus sandwichensis*), a widespread North American Songbird (Zink et al. 2005), and in a Madagascan bird species, the spectacled tetraka (*Xanthomixis zosterops*), where sympatric mtDNA haplotypes (approximately 5% sequence divergence) has been described (Block et al. 2015). In both cases, an absence of phylogenetic groupings was observed in the nuclear genome (Zink et al. 2005, Block et al. 2015), similar to what has been

identified in our study. Benham and Cheviron (2019) further investigated patterns of divergent, sympatric mtDNA lineages in the Savannah Sparrow and identified that divergence most likely arose from a single large and panmictic population rather than due to a pattern of isolation followed by secondary contact and admixture. In the study presented here, pairwise genetic distance was low and was estimated to be between 0.4 to 1.0% between divergent mtDNA lineages, whereas within-lineages genetic distance varied from 0-0.1%. Genetic distance identified in this study was lower than reported for other avian species with range-wide sympatry of two divergent mtDNA lineages. For example, Hogner et al (2012) identified two mtDNA haplogroups in Common Redstart (*Phoenicurus phoenicurus*) with an estimated divergence of 5%. However, genetic distances based on analysis of mtDNA is reported to be highly variable between avian sister-species (0.78% to 11.77%; Tavares and Baker, 2008) making taxonomic delineation complicated. Thus, in this study due to lack of morphological differences between clades, low genetic distance in mitochondrial genes and absence of genetic variation in nuclear genes, we currently do not support the classification of lineages as separate species or sub-species.

Here we hypothesise that genetic divergence of populations within a brood parasitic species might (1) reflect geographic divergence as in any avian species or (2) be a consequence of specialization on and adaptation to different host species. It is unlikely that geographic barriers are driving genetic divergence within *Chrysococcyx caprius* as genetic structure was not associated with geographic locality. It may additionally be unlikely that sympatric individuals in South Africa might represent different breeding populations that are temporarily in contact during part of the annual cycle. Migrant Diederik Cuckoo arrive in South Africa between September and October and approximately a month later courtship displays, and egg-laying occurs after which the males take up territories and call continually until February (Reed 1968). In this study, sampling in South Africa occurred during and directly after breeding between November and January. However, aggregation in South Africa of different populations of Diederik Cuckoo could arise due to high food availability that attract individuals from multiple breeding locations, thus it cannot be excluded that the divergent lineage may represent birds arriving in South Africa from an unknown locality. In regard to the second hypothesis, divergence could be at the population level and contribute to speciation. For example, the African Indigobirds (*Vidua* spp.) are host-specific brood parasites that have developed behavioural mechanisms (e.g. mimic mouth markings and song of their hosts) that has led to reproductive isolation and differentiation in allele frequencies in both nuclear and

mitochondrial DNA (Sorenson et al. 2003). Alternatively, divergence could be present only among female lineages, for example female host-specific races via mimicry of host egg coloration has been documented in Common Cuckoo and Cuckoo Finch (Fossøy et al. 2016, Gibbs et al. 2000; Spottiswoode et al. 2022).

Cuckoos are obligate brood-parasites that lay their eggs in the nest of other bird species. In order to avoid rejection by the host species, cuckoos display a strong selection for egg mimicry (Dawkins and Krebs, 1979). In general, female cuckoos are reported to show high fidelity for a single host species, whereas males will mate with females irrespective of their preferred host species. However, a further study conducted by Fuisz and de Kort (2007) suggested that in Common Cuckoos (*Cuculus canorus*), non-random mating may occur since females prefer the call of male cuckoos from their own habitat. In support of this finding, Fossøy et al. (2011) and (2016) identified significant genetic differentiation at both nuclear (microsatellites and W-linked CHD-W gene) and mtDNA markers in sympatric populations of Common Cuckoos with different host preferences resulting in the separation of immaculate blue eggs from brown speckled eggs. Furthermore, Spottiswoode (2010) reported that eggs found in the nests of different host species differed significantly in shell thickness (eggshell strength) among gentes of *Chrysococcyx caprius*. In Diederik's Cuckoo, approximately 24 authenticated host species have been described in southern Africa; however, only six species are generally parasitised: Cape Sparrow (*Passer melanurus*), Cape Weaver (*Ploceus capensis*), Southern Masked Weaver (*P. velatus*), Spotted-backed Weaver (*P. cucullatus*), Yellow Weaver (P. *subaureus*), and the Southern Red Bishop (*Euplectes orix*; Payne 1967, Reed 1968, Jensen and Vernon 1970, Rowan 1983). The Red Bishop is the most commonly targeted (Payne 1967, Jensen and Jensen 1969) as this species' breeding season is similar to the Diederik Cuckoos (Craig 1982, Ferguson 1994). The breeding season of Weaver and Sparrow hosts occur a few weeks prior to the migration of Diederik Cuckoo to southern Africa (Reed 1968, Rowan 1983, Maclean 1993). There is the potential that divergent lineages observed in this study may be associated with variation in host species selection. However, further analysis would be required in order to confirm this hypothesis. Genetic samples from Diederik Cuckoo collected from host species should be conducted in order to determine if divergent lineages are specific to host species. Although nuclear sequencing showed a lack of differentiation, further analysis such as sequencing of the W-linked gene (CHD-W) or next generation sequencing with a larger sample set, may determine the likelihood of divergent lineages being associated with either female

fidelity or non-random mating. To support this line of enquiry, analysis of variation in male calls should be conducted since no significant morphological features could be distinguished.

Acknowledgements

This research was supported by the International Foundation for Science, Stockholm, Sweden (IFS; J/4611-3), with additional support from the African Bird Club (ABC; Expedition Award) and the British Ecological Society (BES; EA17/1146).

Conflict of interests

The authors report that they have no conflict of interests.

References

Alatalo RV, Gustafsson L, Lundberg A (1984) Why do young passerine birds have shorter wings than older birds? Ibis, 126: 410-415

Alroy J (2017) Effects of habitat disturbance on tropical forest biodiversity. Proc Natl Acad Sci USA 114: 6056–6061

Aragón S, Moller AP, Soler JJ, Soler M (1999) Molecular phylogeny of cuckoos supports a polyphyletic origin of brood parasitism. J Evol Biol 12: 495-506

Bandelt HJ, Forster P, Röhl A (1999) Median-joining networks for inferring intraspecific phylogenies. Mol Biol Evol 16: 37-48

Ball Jr RM, Freeman S, James FC, Bermingham E, Avise JC (1988) Phylogeographic population structure of red-winged blackbirds assessed by mitochondrial DNA. Proceedings of the National Academy of Sciences, 85: 1558-62

Benham PM, Cheviron ZA (2019) Divergent mitochondrial lineages arose within a large, panmictic population of the Savannah sparrow (*Passerculus sandwichensis*). Mol Ecol 28: 1765-83

BirdLife International (2023) Species factsheet: *Chrysococcyx caprius*. Downloaded from http://www.birdlife.org on 16/03/2023

Block NL, Goodman SM, Hackett SJ, Bates JM, Raherilalao MJ (2015) Potential merger of ancient lineages in a passerine bird discovered based on evidence from host-specific ectoparasites. Ecol Evol 5: 3743-55

Craig AJ (1982) Breeding success of a Red Bishop colony. Ostrich 53: 182-8

504 Cox DT, Brandt MJ, Mcgregor R, Ottosson U, Stevens MC, Cresswell W (2011) Patterns of
505 seasonal and yearly mass variation in West African tropical savannah birds. Ibis, 153:
506 672-83
507 Cox DT, Brandt MJ, McGregor R, Ottosson U, Stevens MC, Cresswell W (2013) The
508 seasonality of breeding in savannah birds of West Africa assessed from brood patch and
509 juvenile occurrence. Journal of Ornithology, 154: 671-83
510 Davies N (2010) Cuckoos, cowbirds and other cheats. A&C Black
511 Dawkins R, Krebs JR (1979) Arms races between and within species. Proceedings of the Royal
512 Society of London. Series B. Biological Sciences, 205: 489-511
513 Ferguson JW (1994) The importance of low host densities for successful parasitism of diederik
514 cuckoos on red bishop birds. Afr Zool 29: 70-3
515 Fossøy F, Antonov A, Moksnes A, Røskaft E, Vikan JR, Møller AP, Shykoff JA, Stokke BG
516 (2011) Genetic differentiation among sympatric cuckoo host races: males matter.
517 Proceedings of the Royal Society B: Biological Sciences 278: 1639-45
518 Fossøy F, Sorenson MD, Liang W, Ekrem T, Moksnes A, Møller AP, Rutila J, Røskaft E,
519 Takasu F, Yang C, Stokke BG (2016) Ancient origin and maternal inheritance of blue
520 cuckoo eggs. Nature communications 7: 1-6
521 Fry CH, Keith S, Urban EK (eds.) (1988) The Birds of Africa. Vol. 3. London, UK: Academic
522 Press.
523 Fuisz TI, De Kort SR (2007) Habitat-dependent call divergence in the common cuckoo: is it a
524 potential signal for assortative mating? Proceedings of the Royal Society B: Biological
525 Sciences 274: 2093-7
526 Gardner JL, Trueman JW, Ebert D, Joseph L, Magrath RD (2010) Phylogeny and evolution of
527 the Meliphagoidea, the largest radiation of Australasian songbirds. Mol Phylogenet Evol
528 55: 1087-102
529 Gibbs HL, Sorenson MD, Marchetti K, de L Brooke M, Davies NB, Nakamura H (2000)
530 Genetic evidence for female host-specific races of the common cuckoo. Nature 14: 183-
531 186
532 Giska I, Sechi P, Babik W (2015) Deeply divergent sympatric mitochondrial lineages of the
533 earthworm *Lumbricus rubellus* are not reproductively isolated. BMC Evol Biol 15: 1-3
534 Hebert PD, Stoeckle MY, Zemlak TS, Francis CM (2004) Identification of birds through DNA
535 barcodes. PLoS biology 2: e312
536 Hockey PAR, Underhill LG, Neatherway M, Ryan PG (1989) Atlas of the Birds of the
537 Southwestern Cape. Cape Bird Club, Cape Town

538 Hogner S, Laskemoen T, Lifjeld JT, Porkert J, Kleven O, Albayrak T, Kabasakal B, Johnsen
539 A (2012) Deep sympatric mitochondrial divergence without reproductive isolation in the
540 common redstart *Phoenicurus phoenicurus*. Ecol Evol 2: 2974-88
541 Hoelzer GA, Dittus WP, Ashley MV, Melnick DJ (1994) The local distribution of highly
542 divergent mitochondrial DNA haplotypes in toque macaques *Macaca sinica* at
543 Polonnaruwa, Sri Lanka. Mol Ecol 3: 451-8
544 IUCN (2016) *Chrysococcyx caprius*. The IUCN Red List of Threatened Species 2016:
545 e.T22684026A93011786
546 Iwajomo SB, Willemoes M, Ottosson U, Strandberg R, Thorup K (2017) Data from: Intra-
547 African movements of the African cuckoo *Cuculus gularis* as revealed by satellite
548 telemetry. Movebank Digital Repository, doi:10.5441/001/1.b800b7c3
549 James FC (1970) Geographic Size Variation in Birds and Its Relationship to Climate. Ecology
550 51: 365-390
551 James FC (1983) Environmental component of morphological differentiation in birds. Science,
552 221: 184-6
553 Jensen RA, Jensen MK (1969) On the breeding biology of southern African cuckoos. Ostrich
554 40: 163-81
555 Jensen RA, Vernon CJ (1970) On the biology of the didric cuckoo in southern Africa. Ostrich
556 41: 237-46
557 Johnsgard PA (1997) The avian brood parasites: deception at the nest. Oxford University Press
558 Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A,
559 Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Drummond A (2012) Geneious
560 Basic: An integrated and extendable desktop software platform for the organization and
561 analysis of sequence data. Bioinformatics 28: 1647–1649
562 Kirby JS, Stattersfield AJ, Butchart SHM, Evans MI, Grimmett RFA, Jones VR, O'sullivan J,
563 Tucker GM, Newton I (2008) Key conservation issues for migratory land- and waterbird
564 species on the world's major flyways. Bird Conserv Int 18: S49–S73
565 Kocher TD, Thomas WK, Meyer A, Edwards SV, Pääbo S, Villablanca FX, Wilson AC (1989)
566 Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing
567 with conserved primers. Proc Natl Acad Sci USA 86: 196-200
568 Krüger O, Brooke MD (2007) Brood parasitism in birds. Reproductive biology and phylogeny
569 of Aves. NHSP, Enfield. 2: 327-66
570 Kumar S, Stecher G, Tamura K (2016) MEGA7: molecular evolutionary genetics analysis
571 version 7.0 for bigger datasets. Mol Biol Evol 33: 1870-4

572 Leigh JW, Bryant D (2015) POPART: full-feature software for haplotype network
573 construction. Methods Ecol Evol 6: 1110-6
574 Macdonald MA (1980) Observations on the diederik cuckoo in southern Ghana. Ostrich 51:
575 75-9
576 Maclean GL (1993) Roberts' Birds of Southern Africa. Cape Town, South Africa: Trustees of
577 John Voelcker Bird Book Fund
578 Marchetti K, Nakamura H, Gibbs HL (1998) Host-race formation in the common cuckoo.
579 Science 282: 471-472
580 Marini MÂ, Hackett SJ (2002) A multifaceted approach to the characterization of an
581 intergeneric hybrid manakin (Pipridae) from Brazil. Auk 119: 1114-20
582 Miya M, Nishida M (1996) Molecular phylogenetic perspective on the evolution of the deep-
583 sea fish genus Cyclothone (Stomiiformes: Gonostomatidae) Ichthyol Res 43: 375-98
584 Moreau RE (1972) Palaearctic-African bird migration systems. agris.fao.org
585 Payne RB (1967) Interspecific communication signals in parasitic birds. American Naturalist
586 101: 363–375
587 Payne RB (2005) The cuckoos. Oxford: Oxford University Press
588 Pratt TK, Beehler BM (2014) Birds of New Guinea. In Birds of New Guinea. Princeton
589 University Press.
590 Quinn TW (1992) The genetic legacy of Mother Goose–phylogeographic patterns of lesser
591 snow goose Chen *caerulescens caerulescens* maternal lineages. Mol Ecol 1: 105-17
592 Reed RA (1968) Studies of the diederik cuckoo *Chrysococcyx caprius* in the Transvaal. Ibis,
593 110: 321–331
594 Richards MB, Macaulay VA, Bandelt H-J, Sykes BC (1998) Phylogeography of mitochondrial
595 DNA in western Europe. Ann Hum Genet 62, 241-260
596 Rozas J, Sánchez-DelBarrio JC, Messeguer X, Rozas R (2003) DnaSP, DNA polymorphism
597 analyses by the coalescent and other methods. Bioinformatics. 19: 2496-7
598 Rowan MK (1983) The doves, parrots, louries, and cuckoos of Southern Africa Croom Helm,
599 London (UK)
600 Sangster G, Luksenburg JA (2021) Sharp increase of problematic mitogenomes of birds:
601 Causes, consequences, and remedies. Genome Biol Evol 13: evab210
602 Schneider S, Roessli D, Excoffier L (2000) Arlequin: a software for population genetics data
603 analysis. User manual ver. 2: 2496-7
604 Seutin G, White BBN, Boag PPT (1991) Preservation of avian blood and tissue samples for
605 DNA analyses. Can J Zool 69: 82–90

606 Slatkin M (1987) Gene flow and the geographic structure of natural populations. Science, 236:
607 787-92
608 Sorenson MD (2003) Avian mtDNA primers. Boston University: Boston, MA, USA.
609 Spottiswoode CN (2010) The evolution of host-specific variation in cuckoo eggshell strength.
610 Journal of Evolutionary Biology 23: 1792-9
611 Spottiswoode CN, Stryjewski KF, Quader S, Colebrook-Robjent JF, Sorenson MD (2011)
612 Ancient host specificity within a single species of brood parasitic bird. Proc Natl Acad
613 Sci USA 108: 17738-42
614 Spottiswoode CN, Tong W, Jamie GA, Stryjewski KF, DaCosta JM, Kuras ER, Green A,
615 Hamama S, Taylor IG, Moya C, Sorenson MD (2022) Genetic architecture facilitates
616 then constrains adaptation in a host–parasite coevolutionary arms race. Proc Natl Acad
617 Sci USA 119: e2121752119
618 Stephens M, Smith NJ, Donnelly P (2001) A new statistical method for haplotype
619 reconstruction from population data. Am J Hum Genet 68: 978-89
620 Tavares ES, Baker AJ (2008) Single mitochondrial gene barcodes reliably identify sister-
621 species in diverse clades of birds. BMC Evolutionary Biology 8: 1-4
622 Wang L, Xu Y (2003) Haplotype inference by maximum parsimony. Bioinformatics 19: 1773-
623 80
624 Wickham H (2016) Programming with ggplot2. In ggplot2 (pp 241-253) Springer, Cham
625 Xiao JH, Wang NX, Murphy RW, Cook J, Jia LY, Huang DW (2012) Wolbachia infection and
626 dramatic intraspecific mitochondrial DNA divergence in a fig wasp. Evol Int J Org Evol
627 66: 1907-16
628 Zink RM, Rising JD, Mockford S, Horn AG, Wright JM, Leonard M, Westberg MC (2005)
629 Mitochondrial DNA variation, species limits, and rapid evolution of plumage coloration
630 and size in the Savannah Sparrow. Condor 107: 21-8
631
632
633
634
635
636
637
638
639

Tables

Table 1. The cuckoo samples included in our genetic-based analyses, with the unique sample and ring numbers (or Genbank accession numbers), gene regions successfully sequenced, and sampling localities for each individual. Reference sequences obtained from National Centre for Biotechnology Information (NCBI) GenBank are also shown.

Common name	Latin name	Sex	Sample number	12S	16S	FIB5	RAG1	Measure ments	Localities	Ring number	Co-ordinates	
African cuckoo	*Cuculus gularis*	Female	N06	No	Yes	Yes	No	---	Ghana	D69104	N 09° 05′ 17 9″	W 01° 48′ 33 8″
Diederik Cuckoo	*Chrysococcyx caprius*	Male	CK030	Yes	Yes	Yes	Yes	Yes	Ghana	T000601	N 09° 05′ 17 9″	W 01° 48′ 33 8″
Diederik Cuckoo	*Chrysococcyx caprius*	Male	N05	No	No	Yes	Yes	Yes	Ghana	4A58729	N 09° 05′ 17 9″	W 01° 48′ 33 8″
Diederik Cuckoo	*Chrysococcyx caprius*	Male	CK025	Yes	Yes	Yes	Yes	Yes	Nigeria	4A58728	N 09° 52′ 18 9″	E 08° 58′ 42 8″
Diederik Cuckoo	*Chrysococcyx caprius*	Male	CK026	Yes	Yes	Yes	Yes	Yes	Nigeria	4A58726	N 09° 52′ 41 0″	E 08° 58′ 29 4″
Diederik Cuckoo	*Chrysococcyx caprius*	Unknown	CK027	Yes	Yes	Yes	Yes	Yes	Nigeria	4A58730	N 09° 51′ 07 3″	E 08° 58′ 52 0″
Diederik Cuckoo	*Chrysococcyx caprius*	Male	CK028	Yes	Yes	Yes	Yes	Yes	Nigeria	E46117	N 09° 51′ 07 3″	E 08° 58′ 52 0″
Diederik Cuckoo	*Chrysococcyx caprius*	Male	CK029	No	No	Yes	Yes	Yes	Nigeria	4A58729	N 09° 52′ 18 0″	E 08° 58′ 16 6″
Diederik Cuckoo	*Chrysococcyx caprius*	Male	CK031	No	Yes	Yes	Yes	Yes	Nigeria	4A58727	N 09° 52′ 35 1″	E 08° 58′ 48 4″
Diederik Cuckoo	*Chrysococcyx caprius*	Female	N07	Yes	No	Yes	Yes	Yes	Nigeria	4A58742	N 09° 52′ 35 1″	E 08° 58′ 48 4″
Diederik Cuckoo	*Chrysococcyx caprius*	Male	N08	No	Yes	Yes	Yes	Yes	Nigeria	4A58745	N 09° 52′ 35 1″	E 08° 58′ 48 4″
Diederik Cuckoo	*Chrysococcyx caprius*	Male	CK001	Yes	Yes	No	No	Yes	South Africa	4A58708	S 22° 18′ 50 5″	E 29° 21′ 29 0″
Diederik Cuckoo	*Chrysococcyx caprius*	Male	CK002	Yes	Yes	Yes	Yes	Yes	South Africa	4A58709	S 22° 18′ 50 5″	E 29° 21′ 29 0″
Diederik Cuckoo	*Chrysococcyx caprius*	Male	CK003	Yes	Yes	Yes	Yes	Yes	South Africa	4A58710	S 22° 19′ 13 5″	E 29° 21′ 29 3″
Diederik Cuckoo	*Chrysococcyx caprius*	Male	CK004	Yes	Yes	Yes	No	Yes	South Africa	4A58711	S 22° 16′ 02 2″	E 29° 19′ 49 3″
Diederik Cuckoo	*Chrysococcyx caprius*	Male	CK005	Yes	Yes	Yes	Yes	Yes	South Africa	4A58712	S 22° 18′ 06 4″	E 29° 21′ 51 8″
Diederik Cuckoo	*Chrysococcyx caprius*	Female	CK006	Yes	Yes	Yes	Yes	Yes	South Africa	4A58713	S 22° 18′ 06 4″	E 29° 21′ 51 8″
Diederik Cuckoo	*Chrysococcyx caprius*	Male	CK007	Yes	Yes	Yes	Yes	Yes	South Africa	4A58714	S 22° 20′ 17 1″	E 29° 20′ 03 0″
Diederik Cuckoo	*Chrysococcyx caprius*	Male	CK008	Yes	Yes	Yes	Yes	Yes	South Africa	4A58715	S 22° 30′ 14 7″	E 29° 21′ 15 3″
Diederik Cuckoo	*Chrysococcyx caprius*	Male	CK009	Yes	Yes	No	Yes	Yes	South Africa	4A58716	S 22° 44′ 17 6″	E 28° 46′ 40 5″
Diederik Cuckoo	*Chrysococcyx caprius*	Male	CK010	Yes	Yes	Yes	Yes	Yes	South Africa	4A58717	S 24° 10′ 20 9″	E 29° 01′ 05 8″
Diederik Cuckoo	*Chrysococcyx caprius*	Male	CK011	Yes	Yes	Yes	Yes	Yes	South Africa	4A58718	S 24° 10′ 13 2″	E 29° 01′ 10 4″
Diederik Cuckoo	*Chrysococcyx caprius*	Male	CK012	Yes	Yes	Yes	Yes	Yes	South Africa	4A58719	S 24° 10′ 09 4″	E 29° 01′ 14 9″
Diederik Cuckoo	*Chrysococcyx caprius*	Male	CK013	Yes	Yes	Yes	Yes	Yes	South Africa	4A58721	S 24° 10′ 08 5″	E 29° 01′ 19 9″
Diederik Cuckoo	*Chrysococcyx caprius*	Male	CK014	Yes	Yes	Yes	Yes	Yes	South Africa	4A58722	S 24° 09′ 39 0″	E 29° 02′ 29 3″
Diederik Cuckoo	*Chrysococcyx caprius*	Male	CK015	Yes	Yes	Yes	Yes	Yes	South Africa	4A58724	S 24° 10′ 23 6″	E 29° 01′ 22 8″
Diederik Cuckoo	*Chrysococcyx caprius*	Male	CK016	No	No	Yes	Yes	Yes	South Africa	4A58725	S 24° 10′ 52 4″	E 29° 01′ 56 4″
Diederik Cuckoo	*Chrysococcyx caprius*	Male	CK020	Yes	Yes	Yes	Yes	Yes	South Africa	4A58735	S 22° 23′ 16 9″	E 29° 20′ 07 7″
Diederik Cuckoo	*Chrysococcyx caprius*	Male	CK021	No	Yes	Yes	Yes	Yes	South Africa	4A58736	S 22° 20′ 39 5″	E 29° 19′ 45 2″
Diederik Cuckoo	*Chrysococcyx caprius*	Male	CK024	No	Yes	Yes	Yes	Yes	South Africa	4A58740	S 24° 10′ 42 3″	E 29° 01′ 44 8″
Diederik Cuckoo	*Chrysococcyx caprius*	Female	N11	No	No	Yes	No	Yes	South Africa	4A58755	S 22° 19′ 15 9″	E 29° 21′ 28 5″
Diederik Cuckoo	*Chrysococcyx caprius*	Male	N12	Yes	Yes	No	Yes	Yes	South Africa	4A58759	S 22° 19′ 15 9″	E 29° 21′ 28 5″
Diederik Cuckoo	*Chrysococcyx caprius*	Male	N13	No	Yes	No	Yes	Yes	South Africa	4A58724	S 24° 10′ 20 9″	E 29° 01′ 05 8″
Diederik Cuckoo	*Chrysococcyx caprius*	Male	N14	No	Yes	No	Yes	Yes	South Africa	4A58751	S 24° 10′ 20 9″	E 29° 01′ 05 8″

Diederik Cuckoo	*Chrysococcyx caprius*	Male	N15	Yes	Yes	Yes	No	Yes	South Africa	4A58762	S 22° 43′ 13 2″	E 28° 46′ 09 8″
Diederik Cuckoo	*Chrysococcyx caprius*	Female	N16	No	Yes	Yes	Yes	Yes	South Africa	4A58760	S 22° 19′ 15 9″	E 29° 21′ 28 5″
Diederik Cuckoo	*Chrysococcyx caprius*	Male	N18	No	Yes	Yes	Yes	Yes	South Africa	---	---	---
Diederik Cuckoo	*Chrysococcyx caprius*	Male	N20	Yes	Yes	No	Yes	Yes	South Africa	4A58753	S 22° 19′ 15 9″	E 29° 21′ 28 5″
Diederik Cuckoo	*Chrysococcyx caprius*	Male	N21	Yes	Yes	Yes	Yes	Yes	South Africa	4A58750	S 22° 19′ 15 9″	E 29° 21′ 28 5″
Diederik Cuckoo	*Chrysococcyx caprius*	Male	N23	Yes	Yes	Yes	Yes	Yes	South Africa	4A58747	S 24° 10′ 20 9″	E 29° 01′ 05 8″
Diederik Cuckoo	*Chrysococcyx caprius*	Female	N24	Yes	No	Yes	No	Yes	South Africa	4A58768	S 22° 43′ 13 2″	E 28° 46′ 09 8″
Diederik Cuckoo	*Chrysococcyx caprius*	Unknown	N26	No	Yes	No	Yes	Yes	South Africa	---	---	---
Diederik Cuckoo	*Chrysococcyx caprius*	Female	N27	No	No	Yes	Yes	Yes	South Africa	4A58769	S 22° 43′ 13 2″	E 28° 46′ 09 8″
Diederik Cuckoo	*Chrysococcyx caprius*	Female	N30	No	Yes	Yes	No	Yes	South Africa	---	---	---
Diederik Cuckoo	*Chrysococcyx caprius*	Female	N31	Yes	No	Yes	No	Yes	South Africa	4A58767	S 24° 10′ 20 9″	E 29° 01′ 05 8″
Diederik Cuckoo	*Chrysococcyx caprius*	Male	N32	Yes	No	Yes	No	Yes	South Africa	4A58771	S 22° 43′ 13 2″	E 28° 46′ 09 8″
Diederik Cuckoo	*Chrysococcyx caprius*	Male	N02	Yes	No	Yes	No	Yes	Uganda	AB6992	N 0°03'07 0"	E 32°32'06 6"
Diederik Cuckoo	*Chrysococcyx caprius*	Male	N03	Yes	Yes	No	Yes	Yes	Uganda	AB6993	N 0°03'07 0"	E 32°32'06 6"
Diederik Cuckoo	*Chrysococcyx caprius*	Male	N04	No	No	Yes	Yes	Yes	Uganda	AB6994	N 0°03'07 0"	E 32°32'06 6"
Klaas's Cuckoo	*Chrysococcyx Klaas*	Male	CK022	Yes	No	Yes	Yes	---	South Africa	4A58737	S 22° 19′ 15 9″	E 29° 21′ 28 5″
Klaas's Cuckoo	*Chrysococcyx Klaas*	Male	CK023	No	No	Yes	Yes	---	South Africa	4A58738	S 22° 16′ 02 2″	E 29° 19′ 49 3″
Klaas's Cuckoo	*Chrysococcyx Klaas*	Male	N10	No	Yes	Yes	Yes	---	South Africa	CC98403	S 22° 16′ 02 2″	E 29° 19′ 49 3″
Red-chested cuckoo	*Cuculus solitaries*	Unknown	CK017	Yes	Yes	Yes	No	---	South Africa	4A58707	S 22° 43′ 13 2″	E 28° 46′ 09 8″
Red-chested cuckoo	*Cuculus solitaries*	Male	CK018	Yes	Yes	Yes	Yes	---	South Africa	4A58720	S 24° 10′ 20 9″	E 29° 01′ 05 8″
Red-chested cuckoo	*Cuculus solitaries*	Unknown	CK019	Yes	Yes	Yes	Yes	---	South Africa	4A58723	S 24° 09′ 52 2″	E 29° 02′ 59 2″
Red-chested cuckoo	*Cuculus solitaries*	Unknown	N29/N28	Yes	No	Yes	No	---	South Africa	4A58763	S 24° 09′ 52 2″	E 29° 02′ 59 2″
Common cuckoo	*Cuculus canorus*	Unknown	LN734268 1	Yes					GenBank			
Common cuckoo	*Cuculus canorus*	Unknown	MN06786 7 1		Yes				GenBank			
Common cuckoo	*Cuculus canorus*	Unknown	EU739243 1			Yes			GenBank			
Common cuckoo	*Cuculus canorus*		XM00955 7568 1				Yes		GenBank			
Lesser cuckoo	*Cuculus poliocephalus*		KT378620 1		Yes				GenBank			
Indian Cuckoo	*Cuculus micropterus*		MZ048030 1		Yes				GenBank			
Black-billed cuckoo	*Coccyzus erythropthalmus*		AY274015 1	Yes					GenBank			

645

646

647

648

649

650

651

652

653

Table 2. Genetic diversity indices for the different populations of *Chrysococcyx caprius* based on (A) 12S ribosomal RNA subunits (369 bp) and 16S alignments (501 bp; separated by comma respectively) and (B) β-fibrinogen exons 5 and 6 (FIB5, 536 bp) and recombination activating 1 (RAG1, 916 bp; separated by comma respectively). Pairwise comparisons (F_{ST}) were only calculated between South Africa and Nigeria. Values in **bold** are significant.

A

Locality/ clades	N	No. of haplotypes (H)	Haplotype diversity (Hd)	No. of polymorphic sites (S)	Nucleotide diversity (π)	Tajima's D	Fu's F	F_{ST}
South Africa	24, 29	5, 4	0.66, 0.75	6, 5	0.0047, 0.0039	0.27, 1.13	0.60, 2.02	0.082, 0.048
Ghana	1, 1	1, 1	-	-	-	-	-	-
Nigeria	5, 6	2, 2	0.40, 0.60	1, 1	0.0011, 0.0013	-0.81, 1.45	0.09, 0.80	0.082, 0.048
Uganda	2, 1	1, 1	-	0, -	0, -	-	-	-
Clade 1	24,30	3, 3	0.36, 0.62	2, 2	0.0010, 0.0016	-0.67, 0.94	-0.63, 0.87	**0.88, 0.84**
Clade 2	7,7	3, 1	0.52, 0.00	1, 0	0.0016, 0.0000	-1.23, 0.00	-0.92, -	
Overall	31, 37	6, 4	0.60, 0.72	7, 5	0.0041, 0.0035	-1.13,	0.17, 0.44	-

B

Locality	N	No. of haplotypes (H)	Haplotype diversity (Hd)	No. of polymorphic sites (S)	Nucleotide diversity (π)	Tajima's D	Fu's F
South Africa	54, 22	18, 6	0.80, 0.41	16, 11	0.0068, 0.0584	-0.42, -1.78	**-6.80**, -1.11
Ghana	4, 1	4, 1	1, 1	10, -	0.0124, -	0.08, -	-0.40, -
Nigeria	16, 7	7, 2	0.79, 0.29	1, 2	0.0100, 0.0238	0.56, -1.24	0.62, 0.86
Uganda	4, 0	2, -	0.50, -	7, -	0.0079, -	-0.82, 0	3.25, -
Overall	78, 30	26, 8	0.86, 0.72	54, 5	0.0020, 0.0043	0.60, -1.00	-0.83, -0.08

Table 3. Analysis of molecular variance (AMOVA) results (P<0 05) for (A) all populations for the 12S and 16S gene dataset (separated by comma respectively), (B) the populations of *C. caprius* for the FIB and RAG1 data (separated by comma respectively). Values in **bold** are significant.

A

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation (ns)	Fixation Indices
Among groups	1, 1	1.81, 1.53	0.33, 0.33	44.70, 41.6	F_{CT}: 0.44, 0.416
Among populations within groups	2, 2	0.08, 0.50	-0.33, -0.34	-44.70, -43.2	F_{SC}: -0.81, -0.739
Within populations	28, 33	20.92, 26.67	0.75, 0.81	100.0, 101.5	F_{ST}: 0.00, -0.015
Total	31, 36	22.81, 8.70	0.75, 0.80		

B

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation (ns)	Fixation Indices
Among groups	2, 1	7.81, 0.32	0.18, 0.27	10.04, 48.72	F_{CT}: 0.10, 0.487
Among populations within groups	1, 1	1.28, 0.04	-0.07, -0.33	-3.50, -59.69	F_{SC}: -0.42, -1.164
Within populations	74, 27	127.35, 16.44	1.72, 0.61	93, 110.97	F_{ST}: 0.06, -1.110
Total	77, 29	136.44, 16.80	1.83, 0.54		

Table 4. Pairwise genetic distance between members of the genera *Chrysococcyx*, *Coccyzus* and *Cuculus* for mt DNA sequences (A) 12S ribosomal RNA subunits (rRNA; 369 bp), (B) 16S rRNA (501 bp) and nuclear DNA sequences, (C) recombination activating 1 (RAG1; 916 bp) and (D) β-fibrinogen exons 5 and 6 (FIB5, 536 bp).

A

	1	2	3	4	5	6
[1] *Chrysococcyx caprius* (Clade 1)						
[2] *Chrysococcyx caprius* (Clade 2)	0.004					
[3] *Chrysococcyx caprius*	N/A	N/A				
[4] *Coccyzus erythropthalmus*	0.037	0.036	0.041			
[5] *Cuculus canorus canorus*	0.041	0.041	0.046	0.008		
[6] *Cuculus solitarius*	0.039	0.042	0.044	0.011	0.003	
[7] *Chrysococcyx klaas*	0.026	0.027	0.029	0.054	0.056	0.056

B

	1	2	3	4	5	6	7	8
[1] *Chrysococcyx caprius* (Clade 1)								
[2] *Chrysococcyx caprius* (Clade 2)	0.010							
[3] *Chrysococcyx caprius*	N/A	N/A						
[4] *Cuculus solitarius*	0.139	0.145	0.140					
[5] *Cuculus gularis*	0.143	0.141	0.143	0.018				
[6] *Cuculus poliocephalus*	0.144	0.142	0.144	0.036	0.030			
[7] *Cuculus canorus*	0.143	0.141	0.142	0.021	0.009	0.027		
[8] *Cuculus micropterus*	0.143	0.141	0.143	0.020	0.015	0.027	0.006	
[9] *Chrysococcyx klaas*	0.069	0.068	0.069	0.121	0.118	0.126	0.117	0.118

C

	1	2	3	4	5	6
[1] *Chrysococcyx caprius* (Clade 1)						
[2] *Chrysococcyx caprius* (Clade 2)	0.000					
[3] *Chrysococcyx caprius*	N/A	N/A				
[4] *Cuculus solitarius*	0.029	0.029	0.029			
[5] *Cuculus gularis*	N/A	N/A	N/A	N/A		
[6] *Chrysococcyx klaas*	0.018	0.018	0.018	0.018	N/A	
[7] *Cuculus canorus*	0.032	0.032	0.032	0.004	N/A	0.040

D

	1	2	3	4	5	6
[1] *Chrysococcyx caprius* (Clade 1)						
[2] *Chrysococcyx caprius* (Clade 2)	0.000					
[3] *Chrysococcyx caprius*	N/A	N/A				
[4] *Cuculus solitarius*	0.049	0.049	0.049			
[5] *Cuculus gularis*	0.055	0.055	0.055	0.012		
[6] *Chrysococcyx klaas*	0.012	0.012	0.012	0.052	0.058	
[7] *Cuculus canorus*	0.053	0.053	0.053	0.016	0.021	0.056

Figures

Figure 1. Map showing the distribution of Diederik's Cuckoo (*Chrysococcyx caprius*) in Africa (BirdLife International, 2023). Light green indicates native breeding sites, dark green indicates native resident sites and purple indicates the migration passage

Figure 2. (A) Maximum likelihood phylogenetic tree indicating distribution of haplotypes based on Diederik Cuckoo concatenated ribosomal RNA (12S and 16S) and the Kimura 2-parameter (K2) model. Numbers below branches indicate bootstrap values (B) median-joining network.

Figure 3. Scatter plot principal component analysis (PCA) representing the components calculated for the morphometric measurements given for each of the Diederik cuckoo samples. Every spot represents a bird, and every colour represents a (A) locality (GHA = Ghana, NIG = Nigeria, RSA = South Africa and UGA = Uganda; ellipses are a visual grouping sign), (B) sex (male, female and unknown sex) and (C) clade (clade 1, clade 2 and unknown samples).

Supplementary Material

Supplementary Figure 1. (A) Maximum likelihood tree of Diederik Cuckoo ribosomal RNA (16S) based on the Kimura 2-parameter (K2) + gamma (G) model. Numbers below branches indicate bootstrap values. (B) Maximum likelihood tree indicating distribution of haplotypes and (C) median-joining network.

Supplementary Figure 2. (A) Maximum likelihood phylogenetic tree based on Diederik Cuckoo ribosomal RNA (12S) and the Kimura 2-parameter (K2) model. Numbers below branches indicate bootstrap values (B) Maximum likelihood tree indicating distribution of haplotypes and (C) median-joining network.

Supplementary Figure 3. Mismatch distribution for the Diederik Cuckoo showing observed and expected pairwise difference frequencies for concatenated datasets (A) mitochondrial and (B) nuclear DNA.

Supplementary Figure 4. Maximum likelihood tree of Diederik Cuckoo based on the nuclear datasets for (A) β-fibrinogen exons 5 and 6 (FIB5) and (B) Median-joining network of FIB5.

Supplementary Figure 5. Maximum likelihood tree of Diederik Cuckoo based on the nuclear datasets for (A) recombination activating 1 (RAG1) and (B) Median-joining network of RAG1.

Supplementary Figure 6. Scatter plot principal component analysis (PCA) representing the components calculated for the morphometric measurements given for each of the Diederik cuckoo samples.