No evidence of genetic structure in a sky island endemic: implications for population persistence under a shrinking thermal niche

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Abstract

Mountain habitats physically isolated from one another ("sky islands") represent a unique system for studying dispersal in seemingly isolated populations. The Cape Fold Belt of southwest South Africa forms a sky island archipelago of high-altitude mountain fynbos of which the Cape Rockjumper Chaetops frenatus is an avian-endemic. Continued contraction of habitat due to increasing temperatures may be causing further isolation of C. frenatus populations beyond their dispersal capacities, resulting in currently declining populations in warmer areas of their habitat. In this study, we sequenced two mitochondrial loci and one nuclear locus of 73 C. frenatus samples from 13 localities representing 8 mountain ranges. We found (1) low overall genetic diversity, (2) no evidence for geographically-based genetic structuring, and (3) no evidence for inbreeding within localities. While this may indicate birds are effectively dispersing, it may also indicate strong selective pressure is being placed on their specific genotype. Haplotype networks suggested that C. frenatus may have experienced a bottleneck or founder effect in their recent genetic past —- a result supported by a significantly negative Tajima's D value. As the first avian genetic study to arise from a range-restricted species of the Cape Fold Belt sky islands, our results show no evidence that C. frenatus are unable to disperse across inhospitable lowland habitat, and thus may not experience isolation due to climate change. We thus potentially found further support that selective pressure in species with highly specialized habitat niches may have a stronger effect than dispersal limitations.

Introduction

The glacial cycles of the Pleistocene recurrently brought formerly isolated populations into contact with one another during cooler periods that increased low-elevation distributions (Hewitt 1996, Hewitt 2000, Avise 2009). Following the last glaciation period, cooler-adapted species either shifted ranges to higher latitude or else became relicts in mountaintop refugia ("sky islands"; McCormack, Huang, Knowles, Gillespie, & Clague, 2009); sky islands are analogous to ocean archipelagos, with individual mountain populations isolated due to low-elevation habitat that acts as a barrier to dispersal. Predicted increases in temperatures due to climate change will likely result in increased isolation of these high-altitude species as lowland habitat expands and alpine habitat shifts upslope (Chamberlain et al., 2012; Kupfer & Cairns, 1996; Scridel et al., 2018; Sekercioglu, Schneider, Fay, & Loarie, 2008). Depending on the distance between populations and their ability to disperse, this isolation can lead to a feedback cycle of increased inbreeding depression among populations descending into an "extinction vortex" (Frankham, 2015).

The inhibited gene flow and localized genetic drift resulting from decreased dispersal in sky islands means alpine species often develop genetic differentiation among isolated populations (McCormack et al., 2009). This isolation can be seen in geographically-based genetic structuring (e.g. Bech, Boissier, Drovetski, & Novoa, 2009; Jackson, Gergel, & Martin, 2015; Lonsinger, Schweizer, Pollinger, Wayne, & Roemer, 2015). By examining genetic structuring in isolated populations of species, areas of low connectivity may be identified (Allendorf & Luikart, 2009). Especially for species at risk, understanding the genetic diversity underlying the spatial structure of populations is important for establishing the appropriate scale and subunits for conservation management (Laikre, 2010). While it has been suggested that species in isolated populations would evolve more efficient dispersal ability (Travis et al., 2013), effective dispersal distance (i.e. that which results in the disperser reproducing and adding to the gene pool) is constrained by population growth rate and distance between habitat patches (Baguette & Schtickzelle, 2006; Van Dyck & Baguette, 2005). However, even if populations are not separated by geographic barriers, they may have low genetic diversity due to strong selective pressure if they occupy highly specialized habitat niches (Orsini, Vanoverbeke, Swillen, Mergeay, & De Meester, 2013).

As would be expected, genetic structuring in sky island endemics has been seen across nearly all taxa, including plants (e.g. Gizaw et al., 2013; Lexer et al., 2013), arthropods (e.g. Knowles & Richards, 2005; Masta, 2000), amphibians (e.g. Vörös et al., 2017), birds (e.g. Bech et al., 2009; Jackson et al., 2015), and mammals (e.g. Browne & Ferree, 2007; Lonsinger et al., 2015). The sky islands of South Africa's Cape Fold Belt have been overlooked for such studies among birds, despite the possibility of elevation-based species diversification (Verboom, Bergh, Haiden, Hoffmann, & Britton, 2015), and presence of high levels of endemism (proportional to geographic size) within the Fynbos biome across taxa (Cowling, 1992; Picker & Samways, 1996; Sharratt, Picker, & Samways, 2000; Wishart & Day, 2002). Here, we focused on the Cape Rockjumper *Chaetops frenatus* , an avian sky endemic restricted to the mountain fynbos of southern African sky islands. The contemporary geographical distribution of mountain fynbos results from recent historical shifts in precipitation regimes; it expanded ˜96–37 KYA ("KYA": thousand years ago) and then contracted resulting in the current isolated patches of alpine fynbos across the Cape Fold Belt (Quick et al., 2016). Population declines correlated with warming habitat (Milne, Cunningham, Lee, & Smit, 2015), as well as a small population size, led to *C. frenatus* being placed as Near Threatened on the IUCN Red List of Endangered Species in 2017 (IUCN, 2017). The possible continued contraction of mountain fynbos habitat due to the expansion of warmer lowland habitat from climate change may be one reason for the decline and isolation of*C. frenatus* populations in warmer areas of their habitat.

In this study, we aimed to determine genetic structuring among sky island populations of *C. frenatus* using a combination of mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) to test if*C. frenatus* are experiencing geographically-based genetic isolation. These three markers were chosen as they remain relevant in studies on avian population structure (e.g. Liu et al., 2016; Dantas et al., 2019; Stervander, Ryan, Melo, & Hansson, 2019), as well as general avian taxonomy (Ericson, Klopfstein, Irestedt, Nguyen, & Nylander, 2014). We hypothesized that unsuitable lowland habitat between the geographically distinct mountain ranges would act as a barrier to effective dispersal between populations, resulting in genetic structuring aligned with the topography of the Cape Fold Belt. If we find geographic genetic structuring, suggesting some populations of*C. frenatus* are genetically isolated from one another, this may indicate key areas in need of conservation. Alternatively, if selective pressure is at work due to the highly specialized habitat occupied by*C. frenatus* , we may find low overall genetic diversity and no genetic structuring (Orsini et al., 2013). As both sister-species within family Chaetopidae represent small relict groups at the far end of early radiation of African passerines of high taxonomic interest (Fjeldså & Bowie, 2008), we chose the Drakensberg Rockjumper *C. aurantius* as our outgroup. We also chose *Picathartes gymnocephalus* as an additional outgroup because the exact taxonomy and phylogenetic placement between the sister families Picathartidae and Chaetopidae, and within Chaetopidae itself, remains unresolved within the oscines (Ericson, Klopfstein, Irestedt, Nguyen, & Nylander, 2014).

Methods

Study area and sample collection

The study area encompassed much of the Western and Eastern Cape provinces of South Africa, spanning the species distribution range for the *C. frenatus* . During 2016 and 2017, we collected 73 blood samples of *C. frenatus* from 13 localities representing eight mountain ranges of the Cape Fold Belt (see Figure 1 for localities as well as samples per mountain range); these eight ranges represent areas with >10 % recorded occurrence rate for *C. frenatus*on the South African Bird Atlas Project ("SABAP") 2 (2007 - present; http://sabap2.adu.org.za), and were sampled as extensively as was possible. For two additional localities we attempted to sample *C. frenatus* based on records from SABAP 1 (1987–1991) as well as from earlier records of SABAP 2 (<2015), but were unable to locate birds at these localities (e.g. Tsitsikamma and Lady Slipper; Figure 1).

Although generally contiguous, many of the mountain ranges in the Cape Fold Belt are interspersed by >20 km of lowland habitat (Lee & Barnard, 2016). This lowland habitat is generally warmer than habitat occupied by *C. frenatus* , and is often also either drier (e.g. Karoo) or densely vegetated (e.g. Albany thicket), thus representing a potential dispersal barrier for the *C. frenatus*(McCormack et al., 2009). Sample localities ranged from 32° to 34° S latitude and 18° to 25° E longitude, spanning from ˜100 metres above sea level ("masl") near the southwest tip of South Africa to ˜1,800 masl in the northern Cederberg mountains (Figure 1). Despite the low elevation occurrence of *C. frenatus*near the southwestern tip of South Africa, this area does in fact consist of the short, Ericaceae- dominated mountain fynbos (Cowling & Heijnis, 2001). We additionally collected 15 blood samples of *C. aurantius* from two localities at 30° S latitude and 27° to 28° E longitude in the Lesotho Highlands, spanning from ˜2,600 masl to ˜2,800 masl (Figure 1). Localities for *C. aurantius* were also selected based on occurrence data from SABAP 2.

We caught wild-living individual *C. frenatus* and *C. aurantius* using snap traps baited with tenebrionoid spp. Upon capture, we recorded a GPS point, and fitted birds with unique numbered aluminium rings (SAFRING). Blood samples (˜50 µL) were collected by brachial vein puncture, whereby a small pin-prick was made with a 26-gauge needle and the resulting blood droplet collected into a capillary tube and preserved in ˜90 % alcohol. The entire sampling process took <5 min of handling time and occurred adjacent to, or within, birds' territories for release immediately after sampling. Blood samples were stored at 4 °C until further use for DNA extraction.

DNA extraction and primer selection

Genomic DNA was extracted from blood samples in a dedicated lab following standard salt extraction techniques (Bruford, Hanotte, Brookfield, & Burke, 1992) using Proteinase K (QIAGEN, Germany; 10 mg mL^{-1}), modified by use of lysis and elution buffers (buffers ATL and AE respectively; QIAGEN, Germany).

Primers were prepared by Integrated DNA Technologies, USA. Two primer sets were selected from Zuccon and Ericson (2012) for mtDNA markers: "ND2" which encodes the NADH dehydrogenase subunit 2 and "cytb" that encodes the cytochrome b. For the selected nDNA marker, the recombination activating gene 1 — - "RAG1", we designed a ˜550 bp primer online (www.ncbi.nlm.nih.gov/tools/primer-blast/) using sequences of *C. frenatus* and our additional outgroup *P. gymnocephalus*(representing the sister family Picathartidae) available from GenBank? (www.ncbi.nlm.nih.gov/genbank/; Date: 04-03-2019; Table S1).

DNA amplification and sequencing

For amplification of mtDNA, PCR reaction occurred in a final volume of 27 µL containing approximately 100 ng of genomic DNA, 0.4 µM of each primer, and 12.5 µL of a mixture dNTPs, $MgCl_2$, Taq polymerase and stabilizers (cytb: iTaq? Universal SYBR Green Supermix® by Bio-Rad Laboratories Inc.; ND2: TopTaq PCR MasterMix ?QIAGEN). For amplification of the nDNA the PCR final volume was 33 µL and contained approximately 200 ng of genomic DNA, 0.6 µM of each primer, and 12.5 µL of a mixture of dNTPs, $MgCl_2$, Taq polymerase and stabilizers (iTaq? Universal SYBR Green Supermix® by Bio-Rad Laboratories Inc.).

Mitochondrial DNA PCR protocol was as follows: initial denaturing step of 94 °C for 5 min, 40 cycles of denaturing at 94 °C (30 s), annealing at 58 °C (ND2) or 60 °C (cytb; 30 s), and extension at 72°C (40 s), final

extension at 72 °C (5 min). Nuclear DNA PCR protocol was as follows: initial denaturing at 94 °C (5 min), 40 cycles of denaturing at 94 °C (30 s), annealing at 51.5 °C (40 s), and extension at 72 °C (40 s), final extension at 72 °C (5 min). Sequence data for *P. gymnocephalus* were retrieved from GenBank for ND2 (Fuchs, Fjeldså, Bowie, Voelker, & Pasquet, 2006), cytb (Ericson & Johansson, 2003), and RAG1 (Barker, Barrowclough, & Groth, 2002); see Table S1 for accession numbers). Sequencing of the three loci was outsourced to Macrogen Europe (Amsterdam, the Netherlands); sequences were only produced in the forward direction. We visually checked each sequence individually for correct base-calling of individual nucleotides.

Phylogenetic analysis

Sequences were aligned in MEGA (v 7.0.62; Kumar, Stecher, & Tamura, 2016) with the ClustalW method. We tested for saturation in each codon of each marker using DAMBE v. 7 (Xia, 2018), and found no evidence of saturation in any of the codon positions. We estimated uncorrected p-distances both among groups/species (*C. frenatus* , *C. aurantius* , and *P . gymnocephalus*) and within groups/species (*C. frenatus* and *C. aurantius*) in MEGA to investigate the degree of divergence and examine overall genetic variation. Population structure was examined using minimum-spanning networks (Bandelt, Forster, & Röhl, 1999) for both cytb and ND2 in PopART (Leigh & Bryant, 2015; http://popart.otago.ac.nz).

Genetic differentiation between populations was examined using an Analysis of Molecular Variance (AMOVA; Excoffier, Smouse, & Quattro, 1992) in ARLEQUIN v. 3.5 (Excoffier & Lischer, 2010). *Chaetops frenatus* and *C. aurantius* sequences were split into eight and two groups respectively based on localities where samples were obtained (see Figure 1). Fixation indices were used to estimate the proportion of genetic variability found within populations (F_{ST}; i.e. within each mountain range), among populations within groups (F_{SC}; i.e. among mountain ranges) and between groups (F_{CT}; i.e. between *C. frenatus* and *C. aurantius*). Due to the lack of nucleotide differences between individuals and populations of *C. frenatus* and *C. aurantius* at the RAG 1 locus, we did not perform an AMOVA on RAG1.

Sequence data were also used to detect signal of population expansion, within each species (*C. frenatus* and *C. aurantius*) as estimated with *Tajima's D* (ARLEQUIN v3.5). When $D = 0$ indicates no evidence of selection, $D > 1$ indicates population contraction, and $D < 0$ indicates a post-bottleneck expansion (Tajima, 1989).

We first used jModelTest v. 2.1.10 (Darriba, Taboada, Doallo, & Posada, 2012) for each marker to determine the evolutionary model that best fit the dataset using Bayesian Information Criterion adjusted for low sample size. Results of jModelTest suggested best fits were two rate categories for each locus, with cytb using uniformly distributed proportions of invariable sites (HKY + I; Hasegawa, Kishino, & Yano, 1985), ND2 using four-category gamma distributed invariable sites (HKY + Γ + I; Hasegawa et al., 1985), and RAG1 using equal rates (K80; Kimura, 1980). Phylogenetic inference trees were created online in CIPRES Science Gateway v. 3.3 (Miller, Pfeiffer, & Schwartz, 2010) with data partitions based on each genetic marker (cytb, ND2, and RAG1), and priors set according to results of jModelTest. As we did not have sequences for the same individuals of *P . gymnocephalus*across all three markers, we used available sequences for RAG1 in our consensus trees. For Bayesian inference of phylogeny we used the program MrBayes v. 3.2.6 on XSEDE with BEAGLE (Miller et al., 2010) to obtain Markov Chain Monte Carlo (MCMC) approximations of posterior trees; MCMC was run for 20,000,000 generations, sampling trees every 1,000 generations, with the first 2,500,000 generations (2,500 trees) discarded as burn-in. For maximum likelihood (ML) inference, we used Garli v. 2.0.1 on XSEDE (Bazinet, Zwickl, & Cummings, 2014) to obtain approximations of posterior trees based on 100 bootstraps; we ran this analysis twice to ensure the independent ML searches produced the same tree topology. We determined best fit phylogeny of Bayesian vs. ML trees based on greater number of node posterior probabilities of >95 % or >70 % (Bayesian and ML respectively; see Bates et al., 2013).

Ethics and Permissions

Data collection received ethics clearance from Nelson Mandela University: Research Ethics Committee (Animal; A15-SCI-ZOO-007) as well as from the Ethics Committee of the Department of Zoology & Entomology, Rhodes University (RU-DZE-2017-10-028). Capture permits have been received from both Western Cape

Province: Cape Nature (AAA041-00565), and Eastern Cape Province: Department of Economic Development and Environmental Affairs and Tourism (CRO55/17CR and CRO56/17CR). Birds were ringed with permission from SAFRING (Ringer no: 17059), and blood sampling technique was confirmed by Dr. Tarryn Fick (BVSc, Newton Park Animal Hospital, Port Elizabeth, SA).

Results

Sequence variation

In total we produced 74 cytb sequences (380 bp; *C. frenatus* N = 59, *C. aurantius* N = 15), 85 ND2 sequences (505 bp; *C. frenatus* N = 70, *C. aurantius* N = 15), and 55 RAG1 sequences (455 bp; *C. frenatus* N = 45, *C. aurantius* N = 10). For the cytb marker we found more genetic variation within *C. aurantius*(N = 5 haplotypes) compared to *C. frenatus* (N = 4 haplotypes). For the ND2 marker we found five haplotypes in *C. aurantius* , and 15 haplotypes for *C. frenatus* (see below for details). Uncorrected p -distances within *C. frenatus* varied from 0.0–0.002, within *C. aurantius* varied from 0.0–0.003, and between *C. frenatus* and *C. aurantius* varied from 0.0–0.043 (Table 2).

Tajima's D for both the cytb and ND2 markers in *C. frenatus* were significantly negative (cytb: *Tajima's D* = -1.70,p = 0.020; ND2: *Tajima's D* = -1.77, p = 0.023), while significantly negative for cytb but not significant for ND2 in*C. aurantius* (cytb: *Tajima's D* = -0.78, p = 0.028; ND2: *Tajima's D* = 1.08, p = 0.893).

Population genetic structure

We found no evidence for genetic differentiation among *C. frenatus* localities, with low fixation indices showing strong evidence for interbreeding among the various populations (cytb: F_{ST} = 0.035, DF = 6; ND2: F_{ST} = 0.057, DF = 6). Despite the proximity of *C. aurantius* sample populations to one another, we found little evidence for interbreeding among them, with higher fixation indices than those for *C. frenatus* (cytb: F_{ST} = 0.277, DF = 1; ND2: F_{ST} = 0.470, DF = 1). The phylogenetic networks did not show any clear pattern of genetic structure among the sky islands (Figure 2).

The haplotype network for cytb showed little variation or pattern, while we found a "starburst" pattern for *C. frenatus* in the ND2 network (Figure 2). When visualized by frequency per locality, ND2 showed little evidence for spatial genetic structure (see inset Figure 3 below). The low number of haplotypes within *C. frenatus* at the cytb locus (N = 4) resulted in a haplotype network which did not have the variability to visualize frequency by locality

Overall, most of genetic variation was within populations of *C. frenatus* (cytb: 96.48 %, ND2: 94.26 %; see Table 3 for summary of AMOVA), with similar variation partitioned between species*C. frenatus* and *C. aurantius* (cytb: 93.86 %; ND2: 93.77 %). We also found that while some variation existed within individual populations of both *C. frenatus* and *C. aurantius* (cytb: 5.59 %; ND2: 5.35 %), there was little variation among populations (cytb: 0.55 %; ND2: 0.88 %). High fixation indices for both cytb and ND2 showed there was little evidence for interbreeding between*C. frenatus* and *C. aurantius* (cytb: F_{CT} = 0.939, DF = 1; ND2: F_{CT} = 0.938, DF = 1).

Our consensus tree based on ML inference resulted in stronger predictive weight of node probabilities compared to the Bayesian tree (see Appendix Figure A1 for Bayesian inference tree), and while we found no obvious structuring within *C. frenatus* , we did find strong support for two clear clades within family Chaetopidae being *C. frenatus* and*C. aurantius* (Figure 3).

Discussion

We found no evidence for genetic structure among populations of*C. frenatus* . The results of population substructure among populations ($F_{ST} < 0.05$) indicated only a slight to moderate genetic differentiation among the populations, similar to two other studies on genetic structuring in sky island endemic birds (Bech et al., 2009; Sittenthaler et al., 2018). Instead, our study seems to provide support for the hypothesis that sky island species with specialized niche requirements may experience selective pressure which limits their

genetic diversity [see Orsini et al., (2013)]. We also found evidence for interbreeding among individuals from different mountain ranges, suggesting that birds may be able to effectively disperse between mountain ranges. This suggests *C. frenatus* is not currently experiencing inbreeding depression. However, the lack of structure may also indicate the chosen loci are under positive selective pressure and so are similar due to mutations relevant for alpine species. The shape of our mtDNA haplotype networks suggest *C. frenatus* experienced a bottleneck or founder effect in their recent genetic past. *Tajima's D* also showed support for a past bottleneck event in *C. frenatus* at both mtDNA markers. In addition, overall low genetic diversity within *C. frenatus*indicates the potential for negative effects from a reduced effective population size.

Population variation, structure, and dispersal

Our finding that within *C. frenatus* most genetic variation was from within populations, as opposed to among populations, suggests some genetic structuring, and our haplotype frequency network implies this is not geographically based (Figure 3). The genetic composition of *C. frenatus* , along with the lack of geographic distribution of genetic populations, suggest that although mountain populations of *C. frenatus* are separated from one another by unsuitable lowland habitat, the actual degree of separation does not hamper effective dispersal. The question then remains as to how *C. frenatus* are managing to effectively disperse across large tracts of unsuitable habitat, presenting an interesting avenue for future research. While in some sky island species low occurrence rates in lowland habitat implied low dispersal, as in Mexican Jays *Aphelocoma ultramarine*(McCormack, Bowen, & Smith, 2008), according to SABAPs 1 and 2 (1987–1991, and 2007–present, respectively) there is currently a complete lack of observations for *C. frenatus* in the lowland habitat separating the mountain ranges of the Cape Fold Belt (http://sabap2.adu.org.za; Harrison et al. 1997).

We also found evidence that while populations may be interbreeding, *C. frenatus* have low overall genetic diversity among populations for both cytb and ND2 markers (i.e. genetic variation <5 %). While we found no variation for our nDNA marker, we decided against the addition of a different marker; the little variation we found for both faster-evolving mtDNA markers suggests the addition of a second (slower-evolving) nDNA marker, or a faster-evolving nDNA marker (i.e. microsattelites) was unlikely to provide additional insight,. Moreover, our choice of nDNA marker (RAG1) remains relevant for determining population structure in birds (e.g. Dantas et al., 2019; Stervander, Ryan, Melo, & Hansson, 2019). Low genetic variation among populations may be from low diversity within the species, or close inbreeding within an otherwise diverse population (Ceballos, Joshi, Clark, Ramsay, & Wilson, 2018); since the results of the AMOVA suggest *C. frenatus*populations are interbreeding, there may be low diversity within the species itself. Low diversity can arise from a recent demographic expansion (e.g. bottleneck), here possibly tracking the mountain Fynbos expansion ˜37 KYA. A bottleneck may have extirpated many individuals, reducing overall genetic variability. Because such a population expansion would be recent in evolutionary terms, there has not been time enough to accumulate measurable mutations. Alternatively, as the loci analyzed are protein-coding, natural selection may be purging all but a few advantageous alleles (e.g. strong stabilising selection due to habitat specialisation; Orsini et al., 2013), as *Tajima's D* 's for the mtDNA loci were negative, indicating a higher frequency of rare alleles than would be expected under neutrality. Although *C. frenatus* had a greater number of ND2 haplotypes than *C. aurantius* (N = 15 and 5, respectively), the number is not proportionally greater considering the difference in distribution coverage (˜750 km and ˜20 km respectively).

Bottleneck or founder effect

The haplotype network for ND2 showed the predicted mtDNA starburst pattern for a population that had experienced a bottleneck or founder effect, i.e. a star-like shape where core haplotype is shared by most populations and then many less-common peripheral haplotypes (Ferreri, Qu, & Han, 2011; inset Figure 3). This result was upheld by *Tajima's D* , where a negative result indicated a population expansion after a bottleneck event (Ramakrishnan, Hadly, & Mountain, 2005); for this study we were unable to determine whether this pattern was due to a bottleneck versus a founder effect. Either instance may be due to changes in habitat distribution during the last Pleistocene glacial period —- a possible extrapolation of the Pleistocene

refugia hypothesis (Grubb, 1982). The Pleistocene refugia hypothesis (sometimes referred to as Holocene Refugia) suggests that repeatedly expanding glaciers toward the end of the Pleistocene epoch resulted in a large proportion of speciation events, especially among sister taxa (Avise, 2009; Avise & Walker, 1998). While the Pleistocene refugia hypothesis has previously undergone criticism stating many of these divergence events preceded the Pleistocene (Knapp & Mallet, 2003; Whitten, 1979), there remains a large number of studies across taxa where speciation or isolation events correlated with major changes in climate ˜37 KYA (e.g. Foltz, Nguyen, Kiger, & Mah, 2008; Johnson & Cicero, 2004; Quick et al., 2016; Ribera & Vogler, 2004; Vörös et al., 2017).

However, Africa did not have glaciers in the Pleistocene, and so changes in distribution of *C. frenatus* were likely due not to glaciers specifically, but to shifts in vegetation structure from changing weather patterns during the last glacial period. As the fynbos experienced a major shift wherein ericaceous fynbos contracted >96 KYA, expanded ˜96–37 KYA, and again retreated ˜37 KYA (Quick et al., 2016), we suggest two possibilities in relation to this hypothesis: (1) *C. frenatus* may have been confined to one mountain range when fynbos retreated >96 KYA, then radiated out from this bottleneck population when fynbos expanded ˜96–37 KYA; or (2) *C. frenatus* was extirpated (or not initially present) in the Cape Fold Belt, but the expansion of ericaceous Fynbos ˜96–37 KYA, along with shrinking surrounding Afromontane forests, leading to colonisation by a founder population of the sister *C. aurantius*lineage. In the second scenario, speciation may have been promoted by geographic isolation resulting in the current differences between the two species. However, the bottleneck or founder population of *C. frenatus* may also have originated much earlier during the Miocene, as Cape Fold uplift during this time period has been suggested as the main source of speciation among fynbos plant communities (Cowling, Procheş, & Partridge, 2009; Pirie et al., 2016).

From a purely genetic perspective, our results suggest *C. frenatus* and *C. aurantius* have low species-level diversity based on uncorrected *p* -distance values. Our recorded cytb*p*- distance result for *C. frenatus* -*C. aurantius* (1.5 %) was below most *p* -distance values for species-level distances of 3.3–12.8 % in passerines (Liu et al., 2016; Luo et al., 2014; Martens, Tietze, & Sun, 2005), although (Martens, Tietze, & Päckert, 2011) suggested sister-species can be <3 %. Similarly, our recorded ND2 *p* -distance result for *C. frenatus* -*C. aurantius* (4.3 %) was at the lower range of *p* -distance values for previous species-level distances of 2.0–13.7 % of passerines (Aliabadian et al., 2012; Luo et al., 2014; Zuccon & Ericson, 2012). However, this is not entirely surprising, as (Martens et al., 2011) indicate one should use caution in applying previous species*p* -distance values to apparent sister species.

Edge extinction

The mountain Fynbos of South Africa extends from the coast of the Cape Peninsula (in the very southwest corner of South Africa), to ˜300 km north into the Cederberg mountains, and ˜600 km east ending near Port Elizabeth. Thus, while the Cape Peninsula consists of habitat which is suitable for*C. frenatus* , there have been no records of birds on these mountains in recorded history (Cohen and Frauenecht 1993); indeed, there are also no records from the eastern coastal edges of mountain fynbos habitat (Lee & Barnard, 2016). Species extinction is often preceded by initial habitat fragmentation and increasing distance between suitable patches, resulting in local population extinction on the edges of a species distribution (Ceballos & Ehrlich, 2002; Woodroffe & Ginsberg, 1998). Conceivably *C. frenatus* were once located in this area, but have since been extirpated. This may have been due to human encroachment (or human-associated species such as domestic cats and dogs, or invasive rats) or the area became unsuitable due to too many years between fires. In the first instance, it may be that *C. frenatus* do occasionally recolonize the mountains only to be again driven off by anthropogenic disturbance, and in both instances it may be that once *C. frenatus* edge territory populations become extinct they are unlikely to recolonize [as suggested by Ceballos and Ehrlich (2002)]. Such edge extinction may have occurred as recently as the last three decades as despite seemingly suitable habitat on the Lady Slipper mountain range near Port Elizabeth, Eastern Cape, where birds were recorded by SABAP1 (1987 - 1991), there are no records from the more recent SABAP2 (2007 to present).

Increased variation at the edge of species' distributions has been attributed to reduced dispersal and gene flow (Sittenthaler et al., 2018), fragmentation, isolation, genetic drift, and small population size (Arnaud-

Haond et al., 2006; Bohme, Schneeweiss, Fritz, Schlegel, & Berendonk, 2007), or human conflict (Woodroffe & Ginsberg, 1998). In addition, while samples collected from the furthest east known occurrence of *C. frenatus* in 2017 (i.e. Kleinrivier), none were recorded on return in 2019 (pers. obs. KNO). As in Black Grouse *Tetrao tetrix* (Sittenthaler et al., 2018), the greatest genetic variation for *C. frenatus* was found in edge populations (i.e. Kogelberg; inset Figure 3). In all cases, these edge populations are placed at increased risk of extinction (Ceballos & Ehrlich, 2002; Woodroffe & Ginsberg, 1998).

Conclusions

Genetic theory predicts reduced effective population size will result in a substantial loss of genetic variation, corresponding to a reduction in allele number and heterozygosity at polymorphic loci (Nei, 1978; Varvio, Chakraborty, & Nei, 1986). It thus seems possible that declining *C. frenatus* populations are resulting in an overall loss of genetic diversity, although low diversity may also be due to selective pressure. While we did not detect any geographical genetic structure in *C. frenatus* populations, their overall low genetic diversity suggests further research is needed to conserve remaining populations and dispersal avenues. Moreover, as *C. frenatus* habitat continues to warm, and suitable alpine Fynbos retreats upslope, it seems likely climate change will impact gene flow in the future. Our finding of low genetic variation and no genetic structuring may be complicated by the specialized niche inhabited by *C. frenatus* ; that being said, a lack of genetic diversity may indicate an inability to adapt to changing environments. The signature of a bottleneck/founder effect within *C. frenatus* suggests an interesting link between paleoclimate, the potential for climate refugia, and current species distributions in the Fynbos. While this is the first genetic structuring study on an avian endemic of the Cape Fold Belt sky islands, our study provides insights into processes that may have impacted speciation and evolution within this unique study system.

Data Accessibility

DNA sequences: The data that support the findings are openly available in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) under accession numbers LR723324-LR723539 (see Supplementary Materials Table S1 for individual accession numbers).

Competing Interests

We declare that none of the authors have competing interests.

Author Contributions

KNO, BS, ATKL, and SJC developed the project idea. KNO, BS, and SE designed the research. KNO performed the research, analyzed the data, and wrote the paper. All authors contributed to editing the paper.

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Tables

Table 1 Overview of forward and reverse primers used to isolate segments of three genetic markers (NADH dehydrogenase subunit 2 —- "ND2", cytochrome b —- "cytb", and recombination activating gene 1 —- "RAG 1") from families Chaetopidae and Picathartidae.

gene	primer	direction	sequence 5' – 3'	source
ND2	ND2-F241	forward	ACCGGRCAATGRGAYATYACYCA	Zuccon and Ericson (2012)
ND2	ND2-R811	reverse	CWRCTGGRGCTATBTCYTGTTT	Zuccon and Ericson (2012)
cytb	cytb-bird-F179	forward	GCATCTACCTACACATYGGCCGAG	Zuccon and Ericson (2012)
cytb	cytb-bird-R655	reverse	TTGGCTGGTGTGAAYTTTTCTGGGTC	Zuccon and Ericson (2012)
RAG1	pica_RAG1F	forward	TGAACTGGAGGCTATAATGC	This study
RAG1	pica_RAG1R	reverse	TTTCATTCCCATGTGCTACA	This study

Table 2 Uncorrected p -distances based on data of *Chaetops frenatus* and *C. aurantius* , for two mtDNA markers (Cytochrome b "cytb", and NADH dehydrogenase subunit 2 "ND2") and one nDNA marker (recombination activation gene 1 "RAG1").

source of p-distance variation	cytb	ND2	RAG1
within species			
C. frenatus	0.001 ± 0.001	0.002 ± 0.001	0.0 ± 0.0
C. aurantius	0.003 ± 0.002	0.003 ± 0.001	0.0 ± 0.0
between species			
C. frenatus - *C. aurantius*	0.015 ± 0.006	0.043 ± 0.008	0.0 ± 0.0

Table 3 Analysis of molecular variace (AMOVA) based on data for 8 south-west South African mountain ranges of *Chaetops frenatus* , and 2 central South African mountain ranges of *C. aurantius* , for two mtDNA markers (Cytochrome b "cytb", and NADH dehydrogenase subunit 2 "ND2"). F-statistics are provided for genetic differentiation between groups (F_{CT}), among populations within groups (F_{SC}), and among populations (F_{ST}). All values significant at $p< 0.001$.

source of variation	locus	DF	variance components	% variation	F-statistic
between two genetic groups (*C. frenatus* and *C. aurantius*)					

source of variation	locus	DF	variance components	% variation	F-statistic
between groups	cytb	1	5.75	93.86	F_{CT} 0.939
	ND2	1	10.29	93.77	F_{CT} 0.938
among populations	cytb	7	0.03	0.55	F_{ST} 0.944
	ND2	7	0.10	0.88	F_{ST} 0.946
within populations	cytb	62	0.34	5.59	F_{SC} 0.090
	ND2	66	0.59	5.35	F_{SC} 0.141
within *C. frenatus* genetic group					
among populations	cytb	6	0.01	3.52	F_{ST} 0.035
	ND2	6	0.04	5.74	F_{ST} 0.057
within populations	cytb	49	0.30	96.48	
	ND2	53	0.59	94.26	
within *C. aurantius* genetic group					
among populations	cytb	1	0.19	27.68	F_{ST} 0.277
	ND2	1	0.50	47.03	F_{ST} 0.470
within populations	cytb	13	0.49	72.32	
	ND2	13	0.56	52.97	

Figure Legends

Figure 1 Map of South Africa showing locations of successful (circles; n = 8) and unsuccessful (triangles; n = 2) sample collection from 10 mountain ranges across the distribution of *Chaetops frenatus*(shaded grey area outlined in dark red), and from two sites (squares; n = 2) within the distribution of the *C.* aurantius (shaded grey area outlined in orange). Numbers in parentheses indicate sample number collected from each range. Samples were collected from Oct 2016 to Oct 2017 in the Western and Eastern Cape provinces of South Africa.

Figure 2 Minimum-spanning haplotype networks for mitochondrial loci cytb and ND2. Each circle represents a haplotype with its size proportional to haplotype frequency, each branch illustrates one nucleotide change and each hash-mark indicates an additional nucleotide difference. Circles are colour-coded according to sampling locality. Distributions of *Chaetops frenatus* and *C. aurantius* are depicted by outlined shaded areas in red and orange respectively.

Figure 3 Consensus tree based on maximum likelihood inference for three genetic markers (two mtDNA: cytb and ND2; one nDNA: RAG1), from*Chaetops frenatus* (n = 71), with outgroups *C. aurantius* (n = 15) and *Picathartes gymnocephalus* (n = 2). Bootstrap scores of >50 are indicated on each branch. Inset: ND2 haplotype frequencies showing relative frequency per locality of each haplotype (*C. frenatus* haplotypes n = 15, "A" through "N"; *C. frenatus* haplotypes n = 5, "O" through "S"). Localities are indicated on inset map of South Africa with species distributions shaded in grey and outlined in red (*C. frenatus*) or orange (*C. aurantius*).

Figure A1 Consensus tree based on Bayesian inference for three genetic markers (two mtDNA: cytb and ND2; one nDNA: RAG1), from *Chaetops frenatus* (n = 71), with outgroups *C. aurantius* (n = 15) and*Picathartes gymnocephalus* (n = 2). Bootstrap scores of >50 are indicated on each branch.

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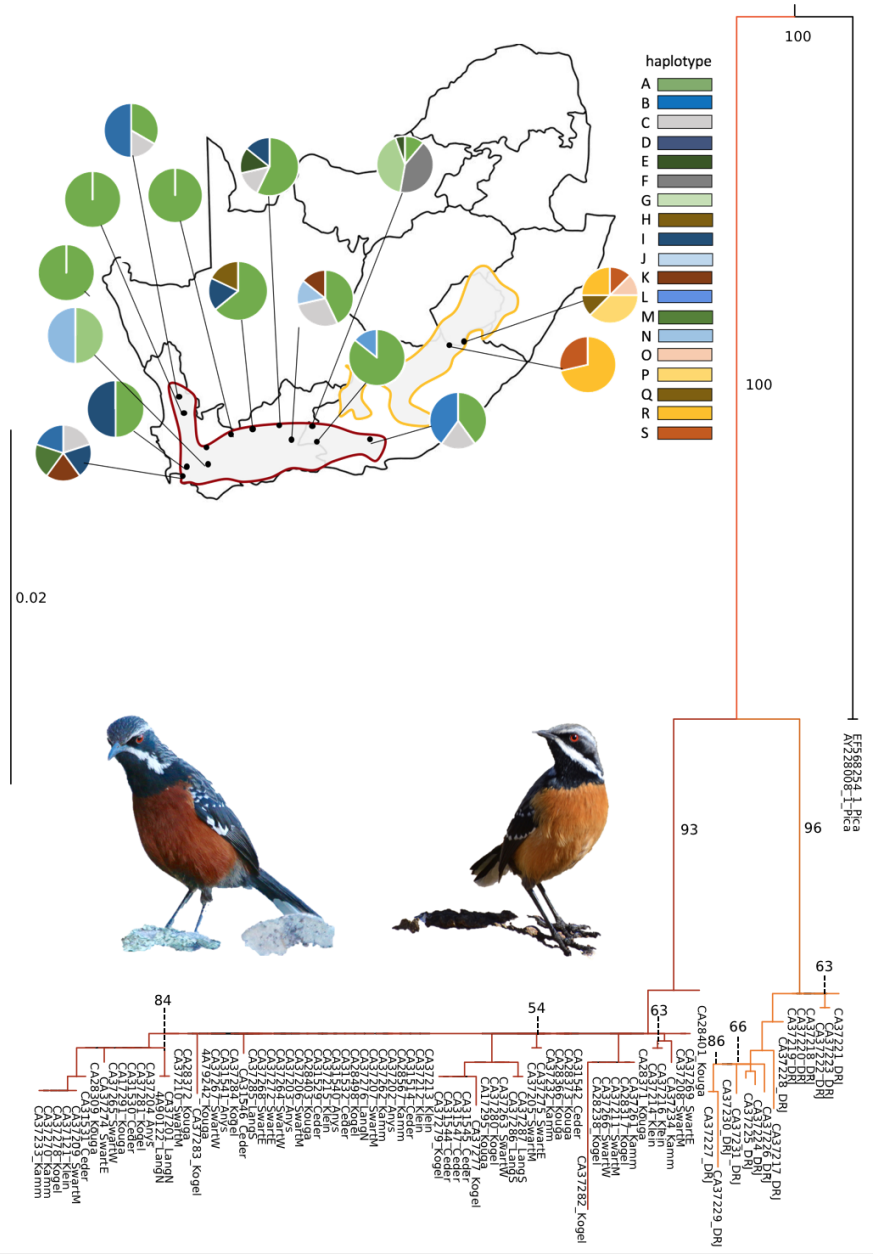

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