A peer-reviewed version of this preprint was published in PeerJ on 13 June 2018.

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Haworth ES, Cunningham MJ, Calf Tjorve KM. 2018. Population diversity and relatedness in Sugarbirds (Promeropidae: *Promerops* spp.) PeerJ 6:e5000 https://doi.org/10.7717/peerj.5000

Population diversity and relatedness in Sugarbirds (Promeropidae: *Promerops* spp.)

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Sugarbirds are a family of two socially-monogamous passerine species endemic to southern Africa. Cape and Gurney's Sugarbird (*Promerops cafer* and *P. gurneyi*) differ in abundance, dispersion across their range and in the degree of sexual dimorphism in tail length, factors that affect breeding systems and potentially genetic diversity. According to recent data, *P. gurneyi* are in decline and revision of the species' IUCN conservation status to a threatened category may be warranted. It is therefore necessary to understand genetic diversity and risk of inbreeding in this species. We used six polymorphic microsatellite markers and one mitochondrial gene (ND2) to compare genetic diversity in *P. cafer* from Cape Town and *P. gurneyi* from Golden Gate Highlands National Park, sites at the core of each species distribution. We describe novel universal avian primers which amplify the entire ND2 coding sequence across a broad range of bird orders. We observed high mitochondrial and microsatellite diversity in both sugarbird populations, with no detectable inbreeding and large effective population sizes.

Introduction

The ability to analyse genetic markers at a population level has markedly increased our ecological insight and capacity for conservation planning at the intraspecific population level. By contrasting patterns of genetic diversity at rapidly mutating nuclear encoded loci, such as microsatellites, combined with rapidly mutating mitochondrial (mtDNA) loci, inferences can be made regarding historical gene-flow (Johnson, Toepfer & Dunn, 2003), historical range expansion (Brito, 2007), and sex-biased dispersal (Melnick & Hoelzer, 1992). The use of previously identified microsatellites is advantageous as they can be studied using PCR-based fragment scoring methods (Oliveira *et al.,* 2006), which require minimal costs. Additionally, microsatellites are generally highly polymorphic due to mutational instability (Ellegren, 2004), and thus serve as effective population genetic markers.

Sugarbirds (Promeropidae) are a family of two socially-monogamous nectivorous passerine species endemic to Southern Africa, with the Cape Sugarbird (*Promerops cafer*) occurring in the fynbos biome of south-western South Africa and Gurney's Sugarbird (*Promerops gurneyi*) occurring in the grasslands of eastern South Africa, Swaziland and Zimbabwe (Figure 1). Sugarbird occurrence and abundance is closely tied with that of shrubs in the family Proteaceae. The distribution of *P. gurneyi* is fragmented in comparison to that of *P. cafer*, owing to the sparse occurrence of the silver sugarbush (*Protea roupelliae*), a fire sensitive species that is Gurney's Sugarbird's preferred source of food, shelter and nesting sites (de Swardt, 1991). By contrast, *P. cafer* occurs in a region with a much greater diversity, abundance and more even dispersion of Proteaceae, many of which are used by this species. *Promerops gurneyi* is currently listed as a species of 'Least Concern' both globally and within southern Africa (Taylor & Wanless, 2015; BirdLife International, 2018). However, recent data presented by Lee *et al.* (2017) suggests that populations of *P. gurneyi* are in serious decline. The modification of grassland habitat in South Africa, through transformation for agriculture and changes in fire and grazing regimes, has also been implicated in the decline of the Yellow-breasted Pipit (Pietersen *et al.,* 2017), which shares most of its distribution with *P. gurneyi*.

Sugarbirds exhibit fierce territoriality toward other nectivorous birds, including the Malachite and Orange-breasted Sunbirds, and toward other Sugarbirds with which they compete for renewable nectar resources and mating opportunities (Daniels, 1987). Given that *Protea* utilized

by *P. gurneyi* are sparser and less diverse than those utilized by *P. cafer* (Calf, Downs & Cherry, 2003), and given that territory size and quality have effects on breeding success, *P. cafer* may be substantially less energetically constrained than *P. gurneyi* in terms of foraging and reproduction (Calf, Downs & Cherry, 2001). Thus, we expect that the abundance and distribution of *Proteaceae*, such as *Protea roupelliae,* could have significant effects on the breeding system, population size, and seasonal movements of *P. gurneyi*.

Extremely high rates of extra-pair paternity (EPP) (>70%) have been observed in *P. cafer* (Henderson, 1999; McFarlane *et al.,* 2009), and is thought to be associated with their extreme sexual dimorphism in tail length, with long-tailed males being more likely to succeed in extra-pair copulations than short-tailed males. Foerster *et al.,* (2003) showed that high rates of EPP promotes increased offspring heterozygosity and fitness in the Blue Tit (*Parus caeruleus*), which may also be the case for *P. cafer,* and is consistent with high genetic diversity observed in previous studies (Feldheim, Mcfarlane & Bowie*,* 2006). In contrast, the breeding system of *P. gurneyi* is poorly understood, and rates of EPP have not yet been measured. However, given the low levels of sexual dimorphism in *P. gurneyi*, we assume that rates of EPP may be lower. Alternatively, male reproductive success in *P. gurneyi* may be more heavily influenced by other factors, such as resource availability (O'Brien & Dawson, 2011).

Given this disparity in occurrence, abundance and sexual selection we predict that *P. gurneyi* populations may show lower genetic diversity than *P. cafer*, and greater risk of inbreeding within habitat patches. In this study we aimed to characterize and compare genetic diversity, relatedness and genetic drift in populations of *P. gurneyi* and *P. cafer*. We also investigated whether inbreeding poses a risk to the persistence of a population of *P. gurneyi* in Golden Gate Highlands National Park, within the Maloti-Drakensberg bioregion of Southern Africa. We compared this with a population of *P. cafer* from Helderberg Nature Reserve, in the south-western Cape. These populations are at the centre of each species distribution and were the sites of previous ecological studies, that provided the samples analysed here. We used six polymorphic microsatellite markers (Feldheim, Mcfarlane & Bowie, 2006) and designed universal avian primers for the entire mitochondrial NADH dehydrogenase II (ND2) gene to compare levels of heterozygosity, inbreeding, and effective population size between these populations. Our results should serve as a useful basis for future phylogeographic and conservation assessments of *P. gurneyi*.

Methods and materials

Sample collection and DNA extraction

Blood samples were obtained by brachial vein puncture (one droplet, approximately 20 ul per bird) from a population of *P. gurneyi* in Golden Gate Highlands National park, Free-State, in 1998, and from one individual at Sani Pass, KwaZulu-Natal, in 2004. Samples were similarly obtained from a population of *P. cafer* in Helderberg, Western-Cape, in 1999, where sampling was targeted around nests. All samples were collected during prior ecological studies (Calf, Downs & Cherry, 2001; 2003) under permits from the relevant provincial conservation agency (Free State Department of Environmental Affairs and Tourism and Cape Nature Conservation Permits respectively to Kathleen Calf and Gordon Scholtz, 1998-1999) with mist netting of birds licensed (KMCT) under the South African national bird ringing scheme (http://safring.adu.org.za). Samples were stored in 1mL of a modified PBS solution (phosphate buffered saline blood storage buffer: 3 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4, 0.14 M NaCl, 6 mM EDTA, 0.2% NaN_3 (w/v)) which was kept on ice after sampling and stored long-term at -20ºC. This buffer includes EDTA to prevent enzymatic digestion of nucleic acids, while the Sodium Azide, NaN_3, is an antimicrobial.

Samples from other species, used to determine the taxonomic range of ND2 primer amplification, were available from previous studies in our laboratory. These included mountain pipit, *Anthus hoeschi*, blue crane, *Anthropoides paradiseus*, Angolan cave chat, *Cossypha ansorgei*, Cape parrot, *Poicephalus robustus*, Namaqua sandgrouse, *Pterocles namaqua*, and eastern clapper lark, *Mirafra fasciolata*. Tissue samples of chicken, *Gallus gallus domesticus* and Ostrich, *Struthio camelus*, were obtained from supermarket meat, and marsh owl, *Asio capensis*, from a roadkilled individual sampled from a carcass found beside the R50 road at Delmas (Long, Lat: 028.681, -26.141).

We extracted genomic DNA by salting out. Digests were prepared by adding 1 mg blood to 500 µL DNA lysis buffer (100 mM NaCl, 50 mM Tris.HCl, 100 mM EDTA, 1% SDS w/v) supplemented with 0.2 mg proteinase-K, before incubation at 57ºC overnight. Following digestion, a volume of 20 µL RNase A (25 mg/mL) was added, before incubation at 37ºC for 60 minutes. Proteins and other cellular contents were precipitated by addition of 180 µL 5 M NaCl (final concentration 1.3 M) followed by agitation and centrifugation. DNA was then extracted by

combining the supernatant with ice-cold isopropyl alcohol (1:1), washed with 70% ethanol, and resuspended in ½ x TE buffer (5 mM tris, 0.5 mM EDTA). We confirmed successful extraction using agarose gel electrophoresis (1% agarose, 1 x TAE buffer, 100V, 15 min), assessed DNA quantity and quality using a NanoDrop™ spectrophotometer, and prepared working stocks of 50 ng/µL.

Microsatellite genotyping

We used multiplex PCRs to amplify the six microsatellite loci developed by Feldheim, Mcfarlane & Bowie (2006). Reactions contained 5 µL Platinum® PCR Multiplex Mix (Applied Biosystems), 25 ng template DNA, 0.015 M of each primer (Table 1), made up to a final volume of 10 µL using ultra-pure H_2O. Reactions were amplified in an Applied Biosystems™ 2720 thermocycler under the following conditions: long denaturation at 95ºC for 10 min followed by 33 cycles of denaturation at 95ºC for 30 sec, annealing at 56ºC for 1 min 30 sec, and extension at 72ºC for 30 sec, with a final extension step at 60ºC for 30 min.

We used agarose gel electrophoresis to confirm successful PCR amplification (2% agarose, 1 x TAE buffer, 100V, 15 min), and conducted fragment analysis on an ABI3500xl Genetic Analyzer with a Liz-500 size standard (Applied Biosystems) at the DNA Sanger Sequencing Facility, University of Pretoria. We analysed electropherogram results using GeneMarker® v1.95 (SoftGenetics) and designed separate genotyping panels for either species. We checked the consistency of fragment assignment by replicate amplification and scoring of 20% of samples.

ND2 primer design

Sorenson *et al.* (1999) developed a range of universal avian mitochondrial primers, however, most of these make use of degenerate sites which reduce their utility for sequencing. We used the Primer3 v.2.3.7 (Untergasser *et al.,* 2012) plugin in Geneious® vR10.2.2 to design universal avian ND2 primers against *Taeniopygia guttata* (NC_007897.1) with comparative alignment across 16 other bird species (Table 2). Primer L3977 is within the Methionine tRNA gene (positions 3958 – 3977 in *T. guttata*). Primer H5191 is within the Asparagine tRNA gene (positions complementing 5211 - 5191 in *T. guttata*). Giving an expected product length of 1254 bp (1213 bp target sequence, including 23 bp of the Methionine tRNA, the complete 1041 bp of ND2, 70 bp Tryptophan tRNA gene, 69 bp complementary to the Alanine tRNA and 10 bp non-genic nucleotides). We authenticated our primers by PCR amplification across a "Noah's ark" of

birds and used NCBI BLAST to confirm successful gene-targeting for a subset of individuals following Sanger sequencing. By designing primers in the conserved tRNAs flanking ND2, we were able to amplify the entire ND2 coding sequence across a broad range of species using a single protocol.

Mitochondrial gene sequencing

Mitochondrial genes were amplified in 10 µL PCR reactions comprised of 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM forward and reverse primer (Table 1), 0.5 U/µL Supertherm® Taq polymerase, and 2 µL of template DNA, made up to final volume with ultra-pure H_2O. Reactions were placed into an Applied Biosystems™ 2720 thermocycler for amplification under the following conditions: long denaturation at 94ºC for 5 min, followed by 33 cycles of denaturation at 94ºC for 30 sec, annealing at 56ºC for 30 sec, and extension at 72ºC for 45 sec, followed by 2 cycles of denaturation at 94ºC for 30 sec and extension at 72ºC for 1 min 30 sec. Successful amplification was confirmed using agarose gel electrophoresis (2% agarose, 100V, 15 min), and DNA precipitation was performed to remove unincorporated dNTPs and primers in preparation for sequencing.

Cycle-sequencing reactions were each comprised of 1 µL BigDye® Terminator v3.1 Ready Reaction Mix, 1 µL BigDye® Sequencing Buffer, 3.2 µM forward primer (Table 1), and 2 µL template DNA, made up to a final volume of 10 µL with ultra-pure H_2O. Reactions were placed into an Applied Biosystems™ 2720 thermocycler for amplification under the following conditions: long denaturation at 96ºC for 3 min, followed by 30 cycles of denaturation at 96ºC, and annealing and extension at 60ºC for 4 min. Following DNA precipitation, cycle-sequencing products were sequenced on either ABI3730xl or ABI3500xl DNA Analysers at the Sanger Sequencing Facility, University of Pretoria. The process was then repeated using the reverse primer to confirm sequences and increase coverage.

Data analysis

We used Cervus 3.0.7 (Kalinowski, Taper & Marshall, 2007) to measure allele frequencies at the 6 microsatellite loci, Genepop v4.6 (Rousset, 2008) to test for deviations from Hardy-Weinberg and linkage disequilibrium among loci, and Micro-checker v2.2.3 (Van Oosterhout *et al.,* 2004) to identify and correct for null-alleles. We conducted parentage analysis using Cervus 3.0.7 (Kalinowski, Taper & Marshall, 2007) in order to identify parent-offspring trios. We conducted

parent-pair simulations for 10000 offspring under the conservative assumption that 25% of potential fathers had been sampled after trail runs with different parameters, allowing 2 mismatches to account for the Z-linked locus (Pro86) as well as any null-alleles or misscoring.

We used Coancestry V1.0.1.7 (Wang, 2011) to calculate pairwise relatedness estimates (Queller and Goodnight, 1989) and inbreeding coefficients (Lynch and Ritland, 1999) for both sugarbird populations, and used R-Studio V1.1.383 (Ross *et al.,* 1996) to graph the frequency distribution of pairwise relatedness estimates. We then estimated the effective size of both populations using the linkage-disequilibrium method (Waples & Do, 2010) implemented in NeEstimator V2.01 (Do *et al.,* 2014) with Jack-knifing across loci. Given that *P. cafer* exhibits high rates of EPP and given that rates of EPP have not yet been measured in *P. gurneyi*, we estimated effective population sizes assuming both random and monogamous mating.

Sampling of *P. cafer* was targeted around nests, and thus our *P. cafer* dataset included a higher proportion of close relatives (parent-offspring, siblings, and half-sibling). In order to investigate whether sampling methods may affect our estimates of effective population size, we used Friends and Family (de Jager *et al.,* 2017) to produce a reduced *P. cafer* dataset with a lower overall mean relatedness. This analysis identified groups of potentially related individuals (relatedness > 0.25), and randomly removed individuals from each group until sample size was equal for both datasets.

We used MEGA 7.0.26 (Kumar, Stecher & Tamura, 2016) to align and trim our mitochondrial sequence data and to calculate nucleotide diversity. We then converted our sequence data to NEXUS format using PGDSpider v2.1.1.2 (Lischer & Excoffier, 2012), and used Popart v1.7 (Bandelt, Forster & Röhl, 1999) to construct a median-joining ND2 haplotype network. In addition to the sequence data that we produced, complete ND2 coding sequences of both *P. cafer* (accession number DQ125990) and *P. gurneyi* (accession number GU16832.1) were downloaded from GenBank and included in our analyses.

Results

Microsatellite diversity

All 6 microsatellite loci were highly polymorphic in both *P. cafer* and *P. gurneyi* (Table 2). There were no significant deviations from Hardy-Weinberg equilibrium within loci but there was

a general tendency towards a slight deficiency of heterozygotes across loci (10 of 12 comparisons). Numbers of alleles and heterozygosity were slightly higher in *P. cafer* than *P. gurneyi*. We suspected null-alleles at the marker Pro66 due to excess homozygosity and failed amplification of this locus in one sample, and so we used Micro-checker to obtain corrected genotypes at this locus.

Additionally, 1-2 base pair shifts were observed at several Pro66 alleles (4bp repeat motif), these scored consistently across replicate PCRs and parent-offspring comparisons and were considered as separate alleles in analyses. We also observed a slight overlap in allele size ranges between markers Pro25 and Pro86 in the *P. cafer* panel, both of which are labelled with the same fluorophore. Fortunately, most individuals with alleles in the overlapping region were identified as females, which are hemizygous for the Z-linked Pro86, and so manual scoring was possible.

ND2 primer authentication

As shown in Table 3, the region to which the forward primer L3977 binds is highly conserved across all 17-species included in our alignment, with only one variable site. The region to which H5191 binds is less conserved, with 6 variable sites across 17 species, however, all 11 of the bird species included in the "Noah's ark" produced clean bands following PCR amplification with the avian ND2 primers L3977 and H5191 (Figure 2). Of the 5-additional species selected for sanger sequencing (*A. hoeschi, A. paradiseus, C. ansorgei, S, camelus, and P. robustus*), each sample yielded over 1100bp of high quality sequence data (GenBank accession numbers; MG972851, MG972852, MG972853, MG972854, and MG97285).

Mitochondrial diversity

We obtained 1150bp, including the complete ND2 coding sequence, from 10 *P. cafer* (GenBank accession numbers; MG972856- MG972865) and 15 *P. gurneyi* (GenBank accession numbers; MG972866- MG972879). These were combined for phylogenetic analysis with the single existing ND2 sequences of each species from GenBank, each of which was a singleton haplotype. MtDNA diversity matches the pattern from microsatellites, with higher nucleotide and haplotype diversity observed in *P. cafer* (π =0.0024; H=0.84) compared to *P. gurneyi* (π =0.0012; H=0.76), despite a smaller sample of the former. We observed 8 haplotypes in *P. cafer* and 6 haplotypes in *P. gurneyi* (Figure 3).

Inbreeding and relatedness

Mean relatedness and inbreeding coefficients were low for both species. Bell-shaped curves were obtained for both relatedness frequency distributions (Figure 4), with mean relatedness being slightly lower in *P. gurneyi* (mean = -0.022; variance = 0.040) than in *P. cafer* (mean = -0.016; variance = 0.030) with wide variance. Mean inbreeding coefficients were slightly higher for *P. gurneyi* (mean F = 0.028; variance = 0.026) than for *P. cafer* (mean F = 0.024; variance = 0.013). We also used Cervus to confirm four parent-offspring pairs in our *P. gurneyi* dataset and six parent-offspring pairs in our *P. cafer* dataset, although we suspect that the proportion of second order relatives is significantly higher in our *P. cafer* dataset due to sampling of some half-sibling fledgelings

Effective population size

The effective population size of *P. cafer* in Helderberg, Western-Cape, was estimated to be 99 individuals (66-182; 95% CI) assuming random mating and 198 individuals (132-356; 95% CI) assuming monogamous mating. The effective population size of *P. gurneyi* in Golden Gate Highlands National Park, Free-State, was estimated to be 133 individuals (55-983; 95% CI) assuming random mating and 223 individuals (111-1627; 95% CI) assuming monogamous mating. The reduced *P. cafer* dataset, filtered for relatives, yielded somewhat higher estimates of effective population size, of 157 individuals (87-582: 95% CI) assuming random mating, and 316 individuals (175-1144: 95% CI) assuming monogamous mating.

Discussion

Both sugarbird species exhibit high levels of microsatellite and mitochondrial diversity, with relatively large local effective population sizes and no detectable inbreeding. This is consistent with previous findings in *P. cafer* (Feldheim, Mcfarlane & Bowie, 2006) and other African nectivorous birds, such as the orange-breasted sunbird (Chan, Van Vuuren & Cherry 2011) and the Ruwenzori double-collared sunbird (Bowie, Sellas & Feldheim, 2010).

High diversity in *P. gurneyi* was unexpected, given their fragmented occurrence and the sparse distribution of suitable habitat. Seasonal migration of *P. gurneyi* has been inferred bird monitoring projects, such as the South African Bird Atlas Project 2 – (SABAP2) citizen science project. These movements include altitudinal movements within regions, and movement from the inland escarpment to the south-east coast (Hockey, Dean & Ryan, 2004: de Swardt, 1991;

SABAP2, 2017). One possibility is that diversity in *P. gurneyi* is maintained through unrecognised gene-flow among regional populations, which are separated by large areas of unsuitable habitat. Alternatively, high levels of diversity may reflect a historically large metapopulation of *P. gurneyi* within this particular region. Our study site, Golden Gate Highlands National Park is one of several large protected areas within the Maloti-Drakensberg Bioregion, the largest untransformed area within the distribution of *P. gurneyi*. Stands of *Protea roupelliae*, a key feature of this species habitat, are dispersed across this bioregion, with intervening *Protea caffra* shrubs and other nectar providing plants providing connectivity among patches. SABAP2 results suggest that the Maloti-Drakensberg area is the stronghold of this species, with the possibility of more extensive connections to the south-eastern coastal area. The single sample from Sani Pass, 130 km SE of Golden Gate, in the Ukhahlamba-Drakensberg World Heritage area, shared a mitochondrial haplotype with the Golden Gate population but also carried several unique microsatellite alleles suggesting the possibility of population divergence across this region but not long-term isolation.

Thus our samples from the north-eastern periphery of a large and well connected regional population may not reflect diversity in other isolated regions further North in Mpumalanga and Limpopo provinces of South Africa, and the eastern highlands of Manicaland province, Zimbabwe. We expect that the inclusion of samples from these regions would yield additional mitochondrial haplotypes and private microsatellite alleles. Irrespective of the contribution of phylogeographic structure to this diversity, it is unlikely that inbreeding poses an immediate risk to the persistence of *P. gurneyi* in the Golden Gate Highlands National Park.

Differences in genetic diversity

All measures of diversity were higher in *P. cafer* than in *P. gurneyi*. Several factors could contribute to this disparity. Firstly, our sample of *P. cafer* was somewhat larger than that of *P. gurneyi*. This is unlikely to be a substantial contributor to interspecific differences in diversity as substantial samples (> 48 individuals) were analysed from each species, each from a single population at the centre of the species range. Some of our measures, such as heterozygosity and nucleotide diversity, are robust to variation in sample size. Although the proportion of close relatives was low in both datasets, this was higher in *P. cafer*, which would tend to reduce observed diversity in that species, contrary to the observed differences. Additionally, these

differences in diversity remained after randomly subsampling *P. cafer* to compare equal sized samples.

Secondly, there may potentially be some degree of ascertainment bias – we used microsatellite markers which were developed in *P. cafer* and selected for high allelic diversity (Feldheim, Mcfarlane & Bowie, 2006), therefore, there may be lower allelic diversity at these markers in *P. gurneyi* due to increased mutational stability (Huang *et al.,* 2016). All microsatellite markers showed high diversity in both species, with extensive overlap in allele size ranges and a low and similar frequency of null alleles. Ascertainment bias would not affect differences in mtDNA diversity.

Diversity may be influenced by the promiscuous breeding system in *P. cafer*, which shows a high level of extra-pair paternity despite long-term social monogamy (Henderson, 1998; McFarlane *et al.,* 2009). *Promerops gurneyi* also shows long-term social monogamy, however the frequency of extra-pair paternity may be lower in this species, where territories are more dispersed and reduced sexual dimorphism implies lower levels of sexual selection than in the extremely dimorphic *P. cafer*. We believe that the best explanation for higher diversity in *P. cafer* may higher abundance and higher connectivity across the species' distribution, in the Protea rich fynbos biome.

Inbreeding and relatedness

Our panel only included 6 microsatellite markers, but all were highly polymorphic and effective in assigning parentage. The frequency distribution of relatedness estimates yielded bell-shaped curves for both populations (Figure 4), suggesting that our sampling was not biased by related individuals. Mean relatedness was low for both populations, and slightly higher for *P. cafer* (-0.016) compared to *P. gurneyi* (-0.022). This may be a reflect more first and second order relatives in our *P. cafer* dataset (six parent-offspring pairs and several sibling pairs and half siblings) compared to that of *P. gurneyi* (four parent-offspring pairs).

Mean inbreeding coefficients were low in both populations, with little variation in the degree of inbreeding among individuals. We detected slightly higher rates of inbreeding in *P. gurneyi* (mean F = 0.028) compared to *P. cafer* (mean F = 0.024), which may be attributed to higher overall diversity in *P. cafer.* In total, we conclude that it is highly unlikely that inbreeding poses

298 a threat to the persistence of either *P. gurneyi* in the Maloti-Drakensberg or *P. cafer* in the South-
299 western Cape. However, it remains to be seen whether the same can be said of the several
300 smaller disjunct regional populations of *P. gurneyi*.

301 **Effective population size**

302 Sugarbirds form socially-monogamous breeding pairs which often re-unite in subsequent
303 breeding seasons, however, while *P. cafer* exhibits high rates of EPP (>70%), the breeding
304 system of *P. gurneyi* remains poorly understood. We predict that the mating system of
305 Sugarbirds is not random, nor is it purely monogamous. The linkage disequilibrium method
306 implemented in NeEstimator V2.01 requires specification of either a random or monogamous
307 mating system, we estimated the effective size of both populations assuming either random or
308 monogamous mating.

309 The effective size estimated for each population was considerably larger than our sample size,
310 suggesting that both populations were derived from relatively large and stable historical
311 populations. The effective population size of *P. cafer* was estimated as 99 individuals (66-182;
312 95% CI) under the assumption of random mating, and 198 individuals (132-356; 95% CI) under
313 the assumption of monogamous mating. The effective population size of *P. gurneyi* was
314 estimated as 133 individuals (55-983; 95% CI) under the assumption of random mating, and 223
315 individuals (111-1627; 95% CI) under the assumption of monogamous mating. Estimates of
316 effective size based in linkage are upwardly biased by sample size, with confidence intervals
317 strongly influenced by the number of loci analysed. Estimates of effective population size were
318 larger for *P. gurneyi* than for *P. cafer*, perhaps due to the slightly higher proportion of relatives
319 in the *P. cafer* dataset. There are wide and broadly overlapping confidence intervals around all
320 these estimates. These figures may be understood as indices of local abundance and relatedness,
321 with values larger than sample size suggesting that additional sampling is required to attain
322 stable estimates of long-term effective population size.

323 In contrast to sampling of *P. gurneyi*, sampling of *P. cafer* was targeted around nests, and
324 therefore contained a higher proportion of relatives**.** Filtering the *P. cafer* dataset for relatives
325 yielded a much higher estimate of effective population size, with 95% upper confidence intervals
326 comparable to those estimated for *P. gurneyi*. This demonstrates that slight differences in
327 sampling strategies can affect interspecific comparisons of effective population size, specifically

when using methods based on Linkage-Disequilibrium. In future it may be necessary to use coalescent-based Bayesian approaches which are independent of sample size.

Conclusions

We observed unexpectedly high microsatellite and mitochondrial diversity in *P. gurneyi* with no detectable inbreeding. It remains unclear whether differences in genetic diversity between *P. gurneyi* and *P. cafer* reflect differences in breeding systems or connectivity among regional populations. In future studies we hope to determine whether phylogeographic structure exists between the disjunct regional populations of *P. gurneyi* in southern Africa, however, sample collection is made difficult by the sparse distribution of *P. roupelliae* and the unpredictable seasonal movements of this species in relation to weather and flowering feed trees. We also intend to increase our panel of nuclear markers by including universal avian microsatellites (Dawson *et al.,* 2013) and to extend our comparative assessment to similarly fragmented declining grassland species such as the Drakensberg Rockjumper, the Sentinel Rock Thrush, and the Ground Woodpecker.

Acknowledgements

We would like to acknowledge Craig Symes and Bradley Gibbons for their assistance with sample collection, and Paulette Bloomer, Arrie Klopper, Claire Lenahan, Kate Henderson and Dawie de Swardt for fruitful discussions that have informed this article.

References

Altchul S, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic alignment search tool. *Journal of Molecular Biology* 215: 403-410. DOI:10.1016/S0022-2836(05)80360-2.

Bandelt H, Forster P, Röhl A. 1999. Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution* 16: 37–48. DOI:10.1093/oxfordjournals.molbev.a026036

BirdLife International. 2018. Species factsheet: *Promerops gurneyi*. *Available at* http://www.birdlife.org (accessed 22 February 2018).

Bowie RCK, Sellas AB, Feldheim KA. 2010. Development of ten highly polymorphic microsatellite markers for Sunbirds (Aves: Nectariniidae) with broad cross-amplification utility in at least eight species. *Conservation Genetics* 11: 1159–1162.

Brito PH. 2007. Contrasting patterns of mitochondrial and microsatellite genetic structure among Western European populations of tawny owls (*Strix aluco*). *Molecular Ecology* 16: 3423–3437.

Calf KM, Downs CT, Cherry MI. 2001. Territoriality and breeding success in Gurney's sugarbird, *Promerops gurneyi*. *African Zoology* 36: 189–195.

362 Calf KM, Downs CT, Cherry MI. 2003. Foraging and territorial behaviour of male Cape and
363 Gurney's Sugarbirds (*Promerops cafer* and *P. gurneyi*). *African Zoology* 38: 297–304.
364 Chan C, Van Vuuren BJ, Cherry MI. 2011. Fynbos fires may contribute to the maintenance of
365 high genetic diversity in orange-breasted sunbirds (*Anthobaphes violacea*). *South African*
366 *Journal of Wildlife Research* 41: 87–94.
367 Daniels CL. 1987. The feeding ecology of nectarivorous birds in the Natal Drakensberg. MSc
368 Thesis, University of Natal.
369 Dawson DA, Ball AD, Spurgin LG, Martín-Gálvez D, Stewart IRK, Horsburgh GJ, Potter J,
370 Molina-Morales M, Bicknell AWJ, Preston SAJ, Ekblom R, Slate J, Burke T. 2013.
371 High-utility conserved avian microsatellite markers enable parentage and population
372 studies across a wide range of species. *BMC Genomics* 14: 176. DOI: 10.1186/1471-
373 2164-14-176
374 de Swardt DH. 1991. The seasonal movements of Gurney's Sugarbird (*Promerops gurneyi*) in
375 the Lydenburg area, Transvaal. *Ostrich* 62: 40–44
376 de Jager D, Swarts P, Harper C, Bloomer P. 2017. Friends and Family: A software program for
377 identification of unrelated individuals from molecular marker data. *Molecular Ecology*
378 *Resources* 17: e225-e233. DOI: 10.1111/1755-0998.12691.
379 Do C, Waples RS, Peel D, Macbeth GM, Tillett BJ, Ovenden JR. 2014. NeEstimator v2: Re-
380 implementation of software for the estimation of contemporary effective population size
381 (Ne) from genetic data. *Molecular Ecology Resources* 14: 209–214. DOI: 10.1111/1755-
382 0998.12157
383 Ellegren H. 2004. Microsatellites: simple sequences with complex evolution. *Nature Reviews* 5:
384 435–445.
385 Feldheim KA, Mcfarlane ML, Bowie RCK. 2006. Isolation of highly polymorphic autosomal
386 microsatellite loci and a sex-linked locus from sugarbirds. *Molecular Ecology Notes* 6:
387 1019–1021.
388 Foerster K, Delhey K, Johnsen A, Lifjeld JT, Kempenaers B. 2003. Females increase offspring
389 heterozygosity and fitness through extra-pair matings. *Nature* 425: 714–717.
390 Henderson C. 1999. Provisioning, paternity and reproductive success in the Cape sugarbird,
391 *Promerops cafer*. MSc Thesis, University of Stellenbosch.
392 Hockey PAR, Dean WRJ, Ryan PG. 2005. *Roberts – Birds of Southern Africa, VIIth* ed. Cape
393 town: The trustees of the John Voelcker Bird Book Fund.
394 Huang J, Li WJ, Jian ZY, Yue BS, Yan YF. 2016. Genome-wide distribution and organization of
395 microsatellites in six species of birds. *Biochemical Systematics and Ecology* 67: 95–102.
396 Johnson JA, Toepfer EJ, Dunn PO. 2003. Contrasting patterns of mitochondrial and
397 microsatellite population structure in fragmented populations of greater prairie-chickens.
398 *Molecular Ecology* 12: 3335–3347.
399 Kalinowski ST, Taper ML, Marshall TC. 2007. Revising how the computer program CERVUS
400 accommodates genotyping error increases success in paternity assignment. *Molecular*
401 *Ecology* 16: 1099–1106.
402 Kumar S, Stecher G, Tamura K. 2016. MEGA7: Molecular evolutionary genetics analysis
403 version 7.0 for bigger datasets. *Molecular Biology and Evolution*. 33: 1870–1874.
404 Lee ATK, Altwegg R, Barnard P. 2017. Estimating conservation metrics from atlas data: the case
405 of southern African endemic birds. *Bird Conservation International* 27: 323-336. DOI:
406 10.1017/S0959270916000307.
407 Lischer H, Excoffier L. 2012. PGDSpider: an automated data conversion tool for connecting

408 population genetics and genomics programs. *Bioinformatics* 28: 298-299.
409 Lynch M, Ritland K. 1999. Estimation of pairwise relatedness with molecular markers. *Genetics*
410 152: 1753-1766.
411 McFarlane ML, Evans MR, Feldheim KA, Préault M, Bowie RCK, Cherry MI. 2009. Long tails
412 matter in sugarbirds-positively for extrapair but negatively for within-pair fertilization
413 success. *Behaviour Ecology* 21: 26-32.
414 Melnick DJ, Hoelzer GA. 1992. Differences in male and female macaque dispersal lead to
415 contrasting distributions of nuclear and mitochondrial DNA variation. *International*
416 *Joural of Primatology* 13: 379–393.
417 O'Brien EL, Dawson RD. 2011. Plumage color and food availability affect male reproductive
418 success in a socially monogamous bird. *Behaviour Ecology* 22: 66-72.
419 Oliveira EJ, Pádua JG, Zucchi MI, Vencovsky R, Lúcia M. 2006. Origin, evolution and genome
420 distribution of microsatellites. *Genetetics and Molecular Biology* 29: 294-307.
421 Pietersen DW, Little IT, Jansen R, Mckechnie AE. 2017. Predicting the distribution of the
422 Vulnerable Yellow-breasted Pipit (*Anthus chloris*) using Species Distribution Modelling.
423 *Emu - Australian Ornithology*. DOI: 10.1080/01584197.2017.1372689
424 Queller DC, Goodnight KF. 1989. Estimating relatedness using genetic markers. *Evolution* 43:
425 258-275. DOI: 10.1111/j.1558-5646.1989.tb04226.x
426 Ross I, Robert G, Ihaka R, Gentleman R. 1996. R: A Language for Data Analysis and Graphics.
427 *Journal of Computational and Graphical Statistics* 5: 299-314.
428 Rousset F. 2008. GENEPOP'007: A complete re-implementation of the GENEPOP software for
429 Windows and Linux. *Molecular Ecolology Resources* 8: 103-106.
430 SABAP2. 2017. South African Bird Atlas Project 2. *Available at http://www.sabap2.adu.org.za*
431 (Accessed 22 February 2018)
432 Sorenson MD, Ast JC, Dimcheff DE, Yuri T, Mindell DP. 1999. Primers for a PCR-Based
433 Approach to Mitochondrial Genome Sequencing in Birds and Other Vertebrates.
434 *Molecular Phylogenetics and Evolution* 12: 105-114.
435 Taylor MR, Peacock F, Wanless RM. 2015. *The 2015 Eskom Red Data Book of Birds of*
436 *Southern Africa, Lesotho and Swaziland*. BirdLife South Africa, Johannesburg.
437 Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG. 2012.
438 Primer3 - new capabilities and inferences. *Nucleic Acids Research* 40: e115. DOI:
439 10.1093/nar/qks596.
440 Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P. 2004. MICRO-CHECKER: Software
441 for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology*
442 *Notes* 4: 535–538.
443 Wang J. 2011. Coancestry: A program for simulating, estimating and analysing relatedness and
444 inbreeding coefficients. *Molecular Ecology Resources* 11: 141–145.
445 Waples RS, Do C. 2010. Linkage disequilibrium estimates of contemporary Ne using highly
446 variable genetic markers: A largely untapped resource for applied conservation and
447 evolution. *Evolutionary Applications* 3: 244–262.
448

Figure 1(on next page)

Sugarbird species distributions and sampling sites

Distribution of Cape (red) and Gurney's (green) Sugarbirds. Large circles show sampling sites at Cape Town (lower left) and Golden Gate Highlands National Park (mid right), respectively. The small circle indicates the single sample of *P. gurneyi* from Sani Pass.

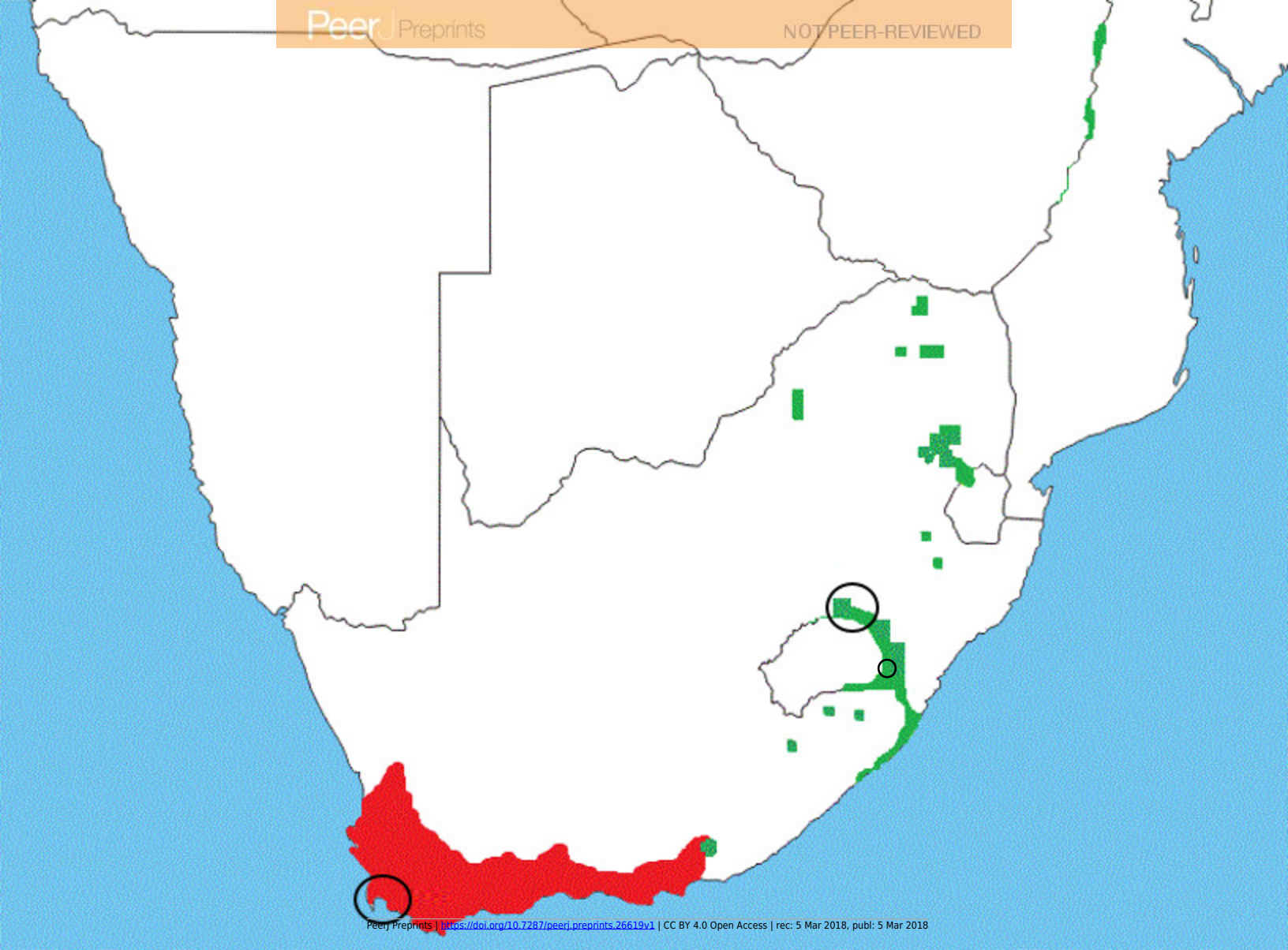

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Figure 2(on next page)

PCR results from L3997 - H5190 universal avian ND2 primers

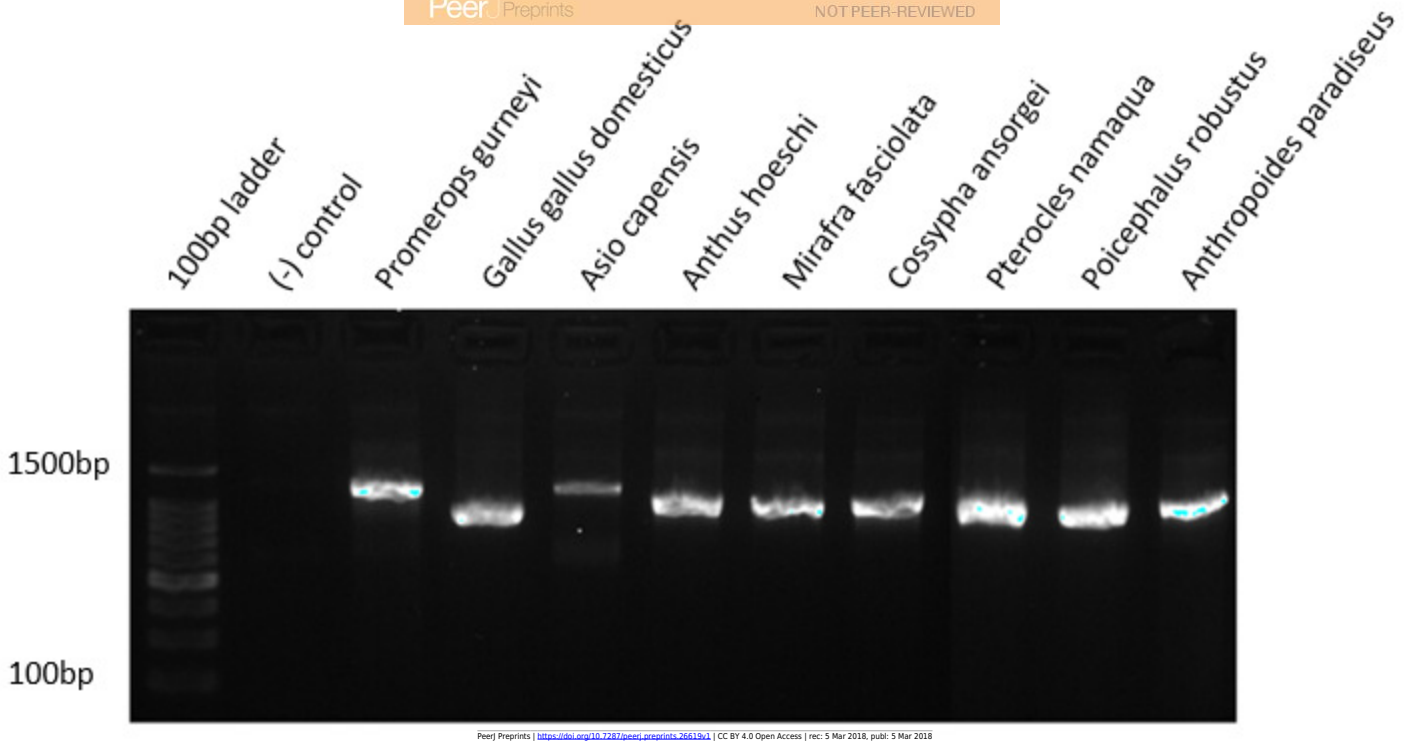

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Figure 3(on next page)

ND2 haplotype networks for Cape and Gurney's Sugarbirds

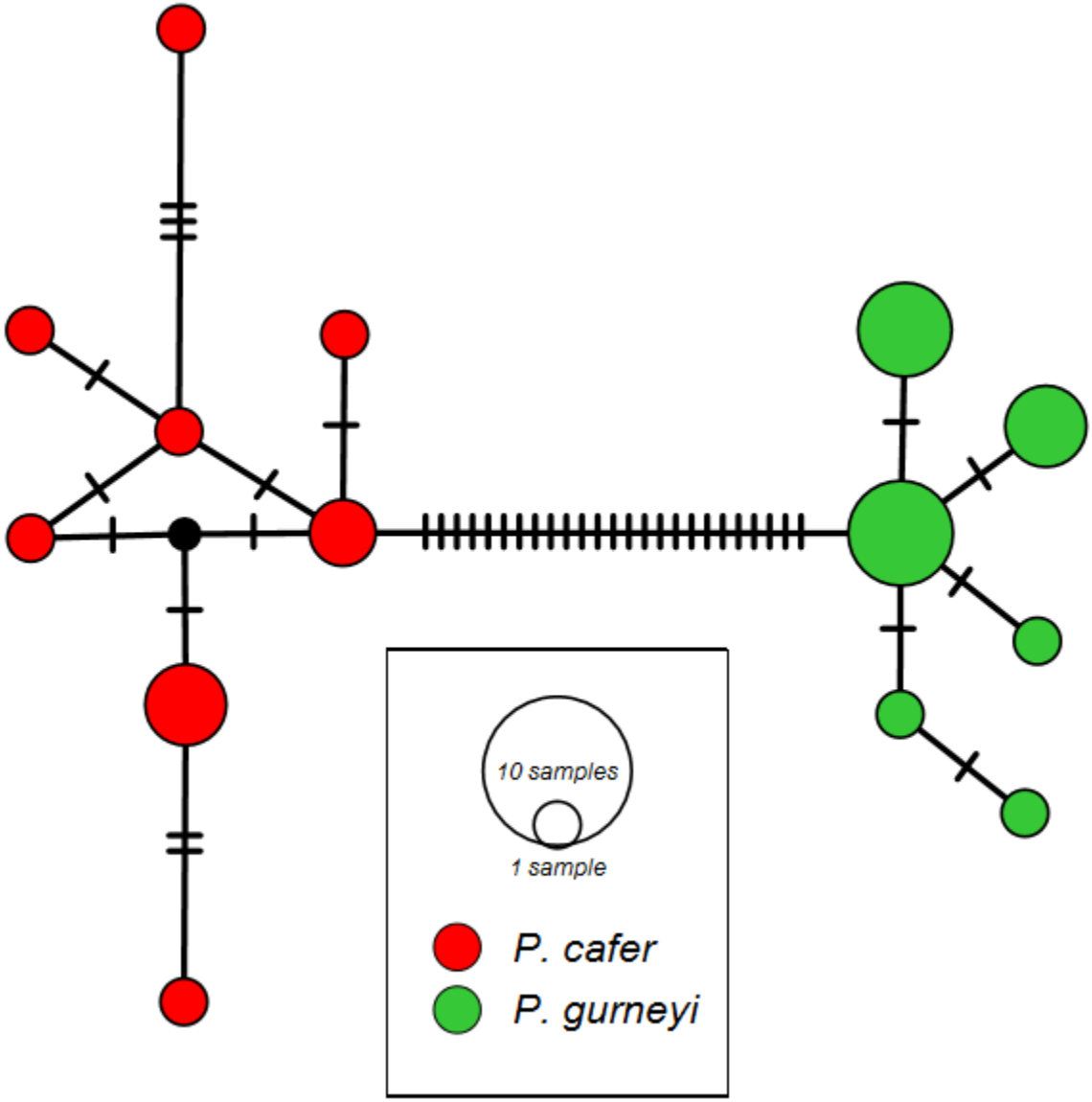

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Figure 4(on next page)

Distribution of pairwise relatedness estimates in Cape and Gurney's Sugarbirds

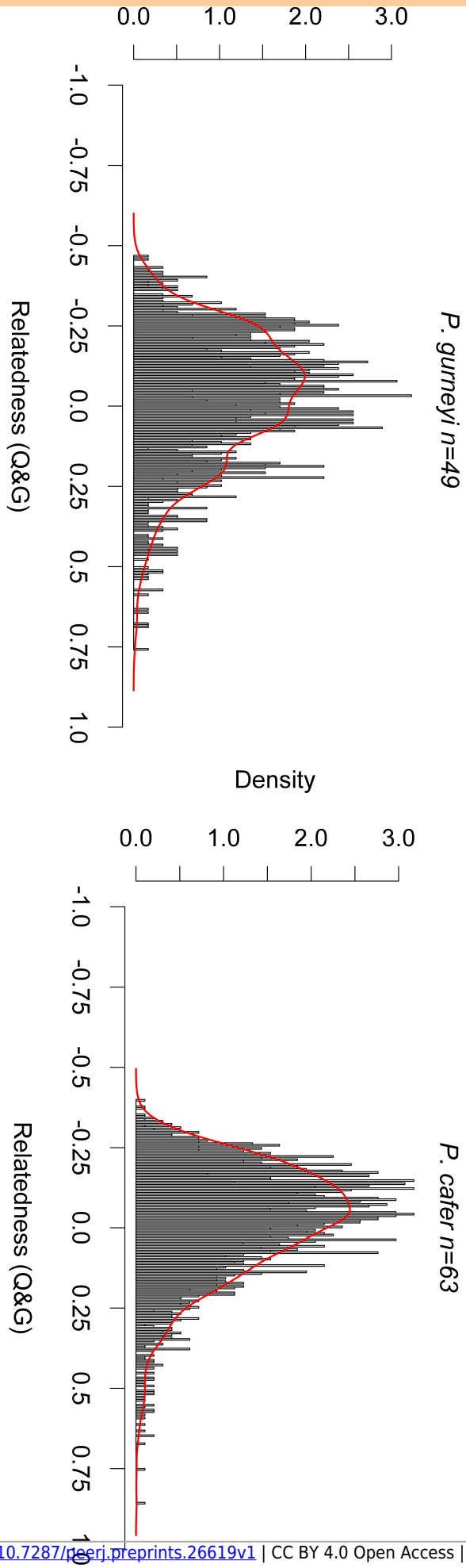
0.0
1.0
2.0
3.0
-1.0
-0.75
-0.5
-0.25
0.0
0.25
0.5
0.75
1.0
Relatedness (Q&G)
P. gurneyi n=49
Density
0.0
1.0
2.0
3.0
-1.0
-0.75
-0.5
-0.25
0.0
0.25
0.5
0.75
1.0
Relatedness (Q&G)
P. cafer n=63

Table 1(on next page)

Microsatellite and mitochondrial primer sequences

Locus	Repeat type	Primer sequence 5' to 3'	Reference
Pro24	Hexa	**F**: TCGTCATCTTGCAACCAAAA **(FAM)**	(Feldheim *et al* . 2006)
		R: TCAGCAGCAAACATGAAACC	
Pro25	Tetra	**F**: CGAGAGCCAGGATTCATTTTCC **(VIC)**	(Feldheim *et al* . 2006)
		R: AGCCAGAATTTGTCCTGTCTG	
Pro66	Tetra	**F**: GCTTGATTAAGGTGCCGAAA **(NED)**	(Feldheim *et al* . 2006)
		R: GCAGGACACAGAGCACTCAA	
Pro86	Penta	**F**: CAGACCTTGGAACAGGCTTC **(VIC)**	(Feldheim *et al* . 2006)
		R: GGCTCCCTCAATTCCTTCTC	
Pro19	Tetra	**F**: TGGAACAGTCCACTTCATGC **(NED)**	(Feldheim *et al* . 2006)
		R: CAACTTTCCTAGCAAAAGGCAC	
Pro90	Tetra	**F**: TTGGAGGGAAGAAGATCTGGG **(PET)**	(Feldheim *et al* . 2006)
		R: CATTCCTTGCCCATTCTGCTG	
ND2		**F**(L3977) : GGCCCATACCCCGAAAATGA	(This study)
		R(H5191) : GGATCGAAGCCCATCTGCCTA	

Table 2 (on next page)

Genetic diversity in Cape and Gurney's Sugarbirds

Diversity statistics in P. gurneyi and P. cafer. Statistics for microsatellite loci are Number of alleles (N_A), Sample size (n), Observed heterozygosity (H_o), Expected heterozygosity (H_e), Polymorphic Information Content (PIC), Locus inbreeding coefficient - potentially representing observed homozygote excess due to null alleles (F(null)) and Allele size ranges. Mitochondrial statistics also include nucleotide diversity (pi) and gene-diversity (H, equivalent to H_e).

P. gurneyi

Locus	N_A	n	H_O	H_e	PIC	F(null)	Size range (bp)
Pro24	14	49	0.82	0.84	0.81	0.010	181-262
Pro25	9	49	0.80	0.81	0.78	0.003	201-235
Pro86	9	24	0.83	0.84	0.80	-0.006	302-352
Pro19	10	49	0.78	0.85	0.82	0.042	166-203
Pro66	11	31	0.71	0.88	0.86	0.096	319-355
Pro90	12	49	0.90	0.87	0.85	-0.019	204-241
Mean	10.5	-	0.81	0.85	0.82		

Locus	N_A	n	π	H	Size (bp)
ND2	6	15	0.0012	0.76	1041

P. cafer

Locus	N_A	n	H_O	H_e	PIC	F(null)	Size range (bp)
Pro24	19	63	0.86	0.92	0.90	0.029	187-324
Pro25	16	63	0.84	0.83	0.81	0.012	185-278
Pro86	16	37	0.81	0.89	0.87	0.040	273-352
Pro19	8	63	0.76	0.79	0.75	0.006	154-186
Pro66	20	61	0.84	0.92	0.91	0.046	275-340
Pro90	15	63	0.87	0.88	0.87	0.003	180-245
Mean	15.2	-	0.83	0.87	0.85		

Locus	N_A	n	π	H	Size (bp)
ND2	8	11	0.0024	0.84	1041

Table 3(on next page)

ND2 primer site alignment across Avian orders

Alignment of ND2 primer sequences across 17 bird species spanning several orders. Genbank accession numbers for sequences used in primer design are shown at left. L3977 in tRNA-Met matches perfectly to all species excepting a 1bp mid-primer A-G transition in Aythya americana. H5191 matches perfectly to Taeniopygia guttata (the design reference) with 1-3 mid-primer mismatches in all other species.

Accession number	Speceis name	L3977 G G C C C A T A C C C C G A A A A T G A	H5191 G G A T C G A A G C C C A T C T G C C T A
NC_007897.1	*Taeniopygia guttata*	. . . . .	. . . . .
NC_000880.1	*Vidua chalybeate*	. . . . .	. . . . . T . . .
NC_010774.1	*Syrmaticus humiae*	. . . . .	. . . . . C . . . . .
NC_010771.1	*Syrmaticus ellioti*	. . . . .	. . . . . C . . . . .
NC_010770.1	*Syrmaticus reevesii*	. . . . .	. . . . . C . . . . .
NC_010767.1	*Syrmaticus soemmerringi ijimae*	. . . . .	. . . . . C . . . . . A . .
NC_010781.1	*Lophura ignita*	. . . . .	. . . . . C . . . . .
NC_010778.1	*Phasianus versicolor*	. . . . .	. . . . . C . . . . .
NC_007238.1	*Gallus varius*	. . . . .	. . . . . T . . . . .
NC_007240.1	*Gallus sonneratii*	. . . . .	. . . . . A . .
NC_007329.1	*Gallus lafayetii*	. . . . .	. . . . . A . .
NC_003408.1	*Coturnix japonica*	. . . . .	. . . . . A . .
NC_010195.1	*Meleagris gallopavo*	. . . . .	. . . . . T . C . . . . .
NC_000877.1	*Aythya americana*	. . . . . G	. . . . . C . . . . A . .
NC_029846.1	*Falco naumanni*	. . . . .	. . . . . C . . . . . A . . A . .
NC_000878.1	*Falco peregrinus*	. . . . .	. . . . . C . . . . . A . .
NC_000879.1	*Smithornis sharpei*	. . . . .	. . . . . A . .

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