



Original Article

# Phylogeography, Population Structure, and Species Delimitation in Rockhopper Penguins (*Eudyptes chrysocome* and *Eudyptes moseleyi*)

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## Abstract

Rockhopper penguins are delimited as 2 species, the northern rockhopper (*Eudyptes moseleyi*) and the southern rockhopper (*Eudyptes chrysocome*), with the latter comprising 2 subspecies, the western rockhopper (*Eudyptes chrysocome chrysocome*) and the eastern rockhopper (*Eudyptes chrysocome filholi*). We conducted a phylogeographic study using multilocus data from 114 individuals sampled across 12 colonies from the entire range of the northern/southern rockhopper

complex to assess potential population structure, gene flow, and species limits. Bayesian and likelihood methods with nuclear and mitochondrial DNA, including model testing and heuristic approaches, support *E. moseleyi* and *E. chrysocome* as distinct species lineages with a divergence time of 0.97 Ma. However, these analyses also indicated the presence of gene flow between these species. Among southern rockhopper subspecies, we found evidence of significant gene flow and heuristic approaches to species delimitation based on the genealogical diversity index failed to delimit them as species. The best-supported population models for the southern rockhoppers were those where *E. c. chrysocome* and *E. c. filholi* were combined into a single lineage or 2 lineages with bidirectional gene flow. Additionally, we found that *E. c. filholi* has the highest effective population size while *E. c. chrysocome* showed similar effective population size to that of the endangered *E. moseleyi*. We suggest that the current taxonomic definitions within rockhopper penguins be upheld and that *E. chrysocome* populations, all found south of the subtropical front, should be treated as a single taxon with distinct management units for *E. c. chrysocome* and *E. c. filholi*.

**Subject areas:** Population structure and phylogeography, Conservation genetics and biodiversity

**Keywords:** Antarctic Circumpolar Current, conservation genetics, migration, Southern Ocean, speciation, subtropical front

From a terrestrial perspective, the ocean appears homogeneous and with few obvious barriers to gene flow. Many marine species exhibit a considerable capacity for dispersal both as adults and juveniles and are seemingly unimpeded in their movements compared with their terrestrial counterparts. However, barriers to dispersal are present in the open ocean. These barriers include both physical and chemical properties (including depth, temperature, salinity, and current) or ecological barriers such as competition (Boessenkool et al. 2009), and for some taxa these barriers may lead to isolation and divergence (Teske et al. 2011; Bowen et al. 2016; Dornburg et al. 2016; Reid et al. 2016; Munro et al. 2017).

The opening of the Drake Passage and later the Tasmanian Gateway connected the oceans surrounding Antarctica to form the Southern Ocean during the late Eocene (Scher et al. 2006). Demarcated from the Atlantic and Indo-Pacific by the subtropical front (STF), a convergence of warm northern waters and cold sub-Antarctic waters, the Southern Ocean represents a biogeographic province with high endemism for vertebrate and invertebrate animals (Allcock et al. 2012). The dominant oceanographic feature of the Southern Ocean is the Antarctic Circumpolar Current (ACC). No longer impeded by any intervening landmass since the late Eocene, winds push the ~25 000 km eastward ACC clockwise around the Antarctic continent (Rintoul 2010). While the STF tends to act as a barrier between the Southern Ocean and the warmer Atlantic and Indo-Pacific and the Antarctic polar front (APF), a barrier between sub-Antarctic waters and colder waters surrounding the Antarctic continent, the ACC acts to facilitate dispersal within the Southern Ocean. These oceanographic features are major determinants of biological diversity in the Southern Ocean.

Penguins (Order: Sphenisciformes) are emblematic of the Southern Ocean and many taxa are of conservation concern with 10 out of 18 species globally threatened (Trathan et al. 2015; BirdLife International 2017a, 2017b). With their capacity to travel long distances (Thiebot et al. 2012; Ratcliffe et al. 2014) penguins would seem likely to have sufficient gene flow among breeding populations to hamper divergence and for several species this seems to be the case (Freer et al. 2015; Clucas et al. 2016, 2018; Cristofari et al. 2016, 2018) but perhaps less so for others (Levy et al. 2016; Vianna et al. 2017; Clucas et al. 2018). Elucidating lineage diversity within the penguins and patterns of gene flow among populations within species is important for understanding population dynamics and coordinating management strategies.

The northern/southern rockhopper penguin complex (*Eudyptes moseleyi* and *Eudyptes chrysocome*) breeds primarily on the sub-Antarctic islands, islands of the South Atlantic, and the tip of South America with a range that crosses the STF. Given the rockhopper's precarious conservation status, a comprehensive investigation of the taxonomy of rockhopper penguins that includes a broad sampling within each region was a key recommendation of an international action plan for basic research and conservation to investigate and address population changes in rockhoppers (BirdLife International 2017a, 2017b). Dickinson and Rensen (2013) recognized 3 taxa of rockhopper penguins: the northern rockhopper penguin (*E. moseleyi*) and 2 subspecies (*chrysocome* and *filholi*) within the southern rockhopper penguin (*E. chrysocome*). The northern rockhopper penguin (*E. moseleyi*) is distributed in the South Atlantic and South Indian Oceans primarily along the the STF (40°S) and breeding colonies in the Tristan da Cunha archipelago and Gough Island in the South Atlantic and St. Paul Island and Amsterdam islands in the Southern Indian Ocean. The southern rockhopper penguin (*E. chrysocome*) is distributed throughout the sub-Antarctic Southern Ocean primarily south of the STF (39°–45°S) and north of the APF (50°–60°S) with breeding colonies in the Falkland (Malvinas) Islands and islands surrounding the southern tip of South America (western rockhopper penguin, *Eudyptes chrysocome chrysocome*) and Prince Edward Islands, Iles Crozet, Iles Kerguelen, and Heard Island in the Southern Indian Ocean, and Macquarie, Campbell, Auckland, and the Antipodes islands south of New Zealand (eastern rockhopper penguin, *Eudyptes chrysocome filholi*).

Species delimitation plays an essential role in determining evolutionarily significant units in conservation biology (de Queiroz 2007; Hey et al. 2012; Carstens et al. 2013; Rannala 2015). Jouventin et al. (2006) delimited 2 rockhopper species (*E. chrysocome* and *E. moseleyi*) on the basis of plumage, calls, distribution, and mtDNA. Banks et al. (2006) argued that all 3 rockhopper taxa should be delimited as species on the basis of reciprocal monophyly in 3 mtDNA loci (16S, COI, and cytb). Frugone et al. (2018) also argued for 3 rockhopper species on the basis of the Automatic Barcoding Gap Discovery (ABGD; Puillandre et al. 2012) and the Generalized Mixed Yule Coalescent (GMYC; Pons et al. 2006; Fujisawa et al. 2013) methods from mtDNA sequences. However, several lines of evidence suggest historical and ongoing gene flow among rockhopper penguin colonies and potentially even across named taxa. Geolocation

sensor tagging of *Eudyptes* penguins in breeding colonies in the South Atlantic (Ratcliffe et al. 2014) and Indian Oceans (Thiebot et al. 2012) and South America (Oehler et al. 2018) have documented a prodigious ability for ocean travel of thousands of kilometers. Breeding records have also revealed that penguins, like many marine species, exhibit considerable capacity for dispersal (Tennyson et al. 1989; de Dinechin et al. 2007; Demongin et al. 2010) and interspecific pairings with vagrants in *Eudyptes* penguins have been recorded (White et al. 2002; Morrison et al. 2014). Also, despite being morphologically distinct and allopatric, the macaroni penguin (*Eudyptes chrysolophus*) and royal penguin (*Eudyptes schlegeli*) are not reciprocally monophyletic even for mtDNA (Frugone et al. 2018) suggesting that some local morphs of *Eudyptes* penguins may not form evolutionarily distinct lineages. These patterns are commonly observed among other penguin species. For example, for some widely distributed species, such as the king (*Aptenodytes patagonicus*), emperor (*Aptenodytes forsteri*), chinstrap (*Pygoscelis antarcticus*), and Adélie penguins (*Pygoscelis adeliae*), there is little to no evidence of population genetic structure, even when applying large scale multilocus methods such as RADseq (Clucas et al. 2016, 2018; Cristofari et al. 2016, 2018). Therefore, breeding populations connected by gene flow may also be common in groups such as rockhoppers. We adopted a multilocus genetic approach to determine the degree to which rockhopper penguin colonies, subspecies, and species are connected by gene flow and to elucidate the number of potential species within the group. While significant insight has been gained by prior systematic work from both genes and morphology in rockhoppers, none have employed more than a 2 nuclear loci, sampled from more than 10 colonies, and few have adopted explicit model based approaches that test for isolation and migration among either named taxa or among breeding colonies within taxa (Banks et al. 2006; Jouventin et al. 2006; Ksepka et al. 2006; de Dinechin et al. 2007; de Dinechin et al. 2009; Gavryushkina et al. 2017; Frugone et al. 2018). Here, we analyze an extensive sampling including 12 colonies throughout the entire distribution of the rockhopper penguin complex.

## Materials and Methods

### Sampling and Laboratory Methods

Blood samples were collected from adult rockhoppers on their breeding sites and stored in either lysis buffer or ethanol as part of multiple ongoing research projects across the rockhopper's distribution. Details of sampling techniques specific to each location are detailed in the [Supplementary Material](#) section. A list of individual samples and their collecting locations are available in [Supplementary Table 1](#).

Whole genomic DNA was extracted using a standard phenol-chloroform protocol followed by ethanol precipitation. The mitochondrial encoded NADH dehydrogenase subunit 2 (ND2) was amplified for all ingroup and outgroup samples across all taxa using primers L5216 and H6313 from Sorenson et al. (1999). We also amplified conserved autosomal nuclear intron loci including introns from a damage-specific DNA binding protein (08352), myelin basic protein (22187), cathepsin B precursor (26896), acetylserotonin O-methyltransferase-like protein (26928), and 2 anonymous loci (20454 and 27189) described in Backström et al. (2008). For each locus the number assigned refers to the last 5 digits of the Ensembl transcript ID. In cases where primers derived from the literature failed to amplify all samples we constructed *Eudyptes*-specific

internal primers based on sequence alignments from *Eudyptes* and outgroup sequences.

Common sequence tags, either CS1 (5'-ACACTG ACGACATGGTTCTACA) in the forward direction or CS2 (5'-TACGGTAGCAGAGACTTGGTCT) in the reverse direction, were added to all the oligonucleotide sequences in this study. Oligonucleotide tags are commonly used to facilitate the addition of adapters and barcodes to amplicons for next-generation sequencing (Moonsamy et al. 2013). In our study the addition of common 5' tags allowed for CS1 and CS2 to be utilized as sequencing primers for all amplicons across all loci. A complete list of the oligonucleotide sequences used as PCR primers in this study and their properties may be found in [Supplementary Table 2](#).

Polymerase chain reaction (PCR) was performed in 10  $\mu$ L reactions with Fidelitaq™ Master Mix (Affymetrix/USB) and forward and reverse primers (0.5  $\mu$ M each; Integrated DNA Technology) using a thermal profile of 94 °C for 4 min followed by 30 cycles of 1 min at 94 °C, 1 min at 50 °C, and 2 min at 72 °C, and then a final extension cycle for 10 min at 72 °C. The same thermal profile was utilized for all PCRs across all loci. Primers and excess dNTPs were inactivated with ExoSAP-IT® (Affymetrix/USB) following the manufacturer's instructions. Sequencing reactions were performed on ExoSAP-IT® treated PCR amplicons using BigDye® v. 3.1 Cycle Sequencing Kit (Life Technologies). Unincorporated BigDye® terminators were removed from sequencing reactions using the BigDye Xterminator® kit (Life Technologies) and cleaned sequencing products were separated on a 3130 Genetic Analyzer (Life Technologies). PCR amplicons were sequenced in both directions with primers based on the common sequence tags incorporated into all amplicons (CS1 and CS2).

## Population and Phylogenetic Analyses

### Summary Statistics

Complementary reads from at least 2 sequencing runs (one from each direction) were assembled and edited using Geneious v8.1.4 (Biomatters) available from <http://www.geneious.com> (Kearse et al. 2012). Chromatograms were inspected individually and every variation was checked for authenticity. Alignments of sequence assemblies for each locus were done using the Geneious alignment and edited by trimming the ends such that all alignments spanned the same lengths. The phase of nuclear alleles was determined using the PHASE v2.1 (Stephens et al. 2001) algorithms implemented in DnaSP v5 (Librado et al. 2009). All sequences were deposited in NCBI GenBank ([Supplementary Table 3](#)).

We calculated population genetic metrics of nucleotide and haplotype diversity, population mutation rate (Watterson's estimator,  $\Theta_G$ ),  $F_{ST}$ , and Tajima's D and tested for selection using the Hudson-Kreitman-Aguade (HKA) test (Hudson et al. 1987) in DnaSP v5. A 95% confidence interval for pairwise  $F_{ST}$  among geographic localities for the 6 nuclear intron loci combined was obtained in HIERFSTAT using the bootstrapping method (Goudet 2005). We looked for evidence of recombination distinguished from recurrent mutation using the  $\Phi_w$ -statistic (Bruen et al. 2006) implemented in the program SplitsTree v4.10 (Huson et al. 2006). For ND2, we constructed a haplotype network using the integer neighbor joining method in PopArt (<http://popart.otago.ac.nz>; Leigh et al. 2015). We selected the best-fit model of nucleotide substitution for ND2 using Akaike information criteria (AIC) in the program jModeltest2 v2.1.7 (Darrriba et al. 2012).

## Phylogenetic Inference

We used MrBayes v3.2.6 (Huelsenbeck et al. 2001) implemented in Geneious v8.1.4 to generate a Bayesian tree for the ND2 locus with the following settings: the best substitution model inferred by JModelTest2, a Markov chain Monte Carlo (MCMC) chain of 2 000 000 steps with 4 heated chains with a temperature of 0.2, a burnin of 200 000, molecular clock priors with uniform branch lengths, and *E. chrysolophus* as an outgroup.

We constructed a species tree using the combined information across 6 nuclear introns and ND2 in a Bayesian analysis as implemented in \*BEAST2 v2.4.2 (Drummond et al. 2012). We used the best-supported model reported by JModelTest2 that was also an available model in \*BEAST2. A strict molecular clock approach was used for each locus. Priors for substitution rates were estimated based on lognormal distributions. The prior distribution for ND2 was based on a mean of 0.0105 substitutions/site/lineage/Ma and a standard deviation of 0.75 (5–95% quantile range of 0.00231–0.0272). This range is consistent with mtDNA substitution rates reported in birds (Fleischer et al. 1998; Pereira et al. 2006a; Weir et al. 2008; Eo et al. 2010). For the intron loci we based our prior substitution rate distributions on a mean 1/10th that of the ND2 rate or 0.00105 substitutions/site/lineage/Ma and a standard deviation of 0.75 (5–95% quantile range of 0.000231–0.00272).

We justified the substitution prior distribution for the nuclear loci on the basis of previous estimations of nuclear substitution rates in vertebrates relative to those of mtDNA. In birds, substitution rates at nuclear introns are approximately 5–14 times slower than mtDNA (Johnson et al. 2000a, 2000b; Prychitko et al. 2000; Armstrong et al. 2001; Allen et al. 2003). In mammals, single copy nuclear genes were found to have neutral substitution rates approximately 5–20 times slower than mtDNA (Brown et al. 1979; Brown et al. 1982; Pesole et al. 1999; Yu et al. 2004). Amphibians also tend to fall within this range with synonymous substitution rates being 16 times slower for single copy nuclear genes compared to mitochondrial genes (Crawford 2003). We incorporated uncertainty in the nuDNA neutral substitution rate by using prior distributions between approximately 4 and 45 times less (5–95% quantile range) than the mean mtDNA rate and overlapping with the mtDNA distribution.

The species tree was constrained to be monophyletic for *E. c. chrysome*, *E. c. filholi*, and *E. moseleyi* with another crested penguin, the macaroni penguin (*E. chrysolophus*), as an outgroup. MCMCs were run for 300 million generations, sampling every 10 000 generations and discarding the first 100 million as a burnin. Three independent MCMCs, each starting with a different random seed, were run under these conditions and log files and species tree files from each run were combined into a single log file and a single species tree file using the logCombiner and TreeAnnotator software included with BEAST v2.4.2. Convergence was assessed in the program Tracer v1.6 (Rambaut et al. 2013).

## Species Delimitation

To assess the degree to which population structure and admixture occur across colonies, we used Structure v2.3.4 (Pritchard et al. 2000) and the approach of Evanno et al. (2005). Structure analysis was run on the phased haplotypes for the 6 introns. Runs were conducted in batch mode in Structure for 100 000 replicates with a burnin period of 20 000 and 20 replicate runs for each value of  $K$  ( $K$  from 1 to  $n_{\text{colonies}} + 3$ ) using a sequential random seed. Results from Structure were analyzed in Structure Harvester (Earl et al. 2011) and

the individual and population files representing the  $K$  value with the largest  $\Delta K$  were each used as input files in 2 runs of Clump v1.1.2 (Jakobsson et al. 2007) to generate population and individual files for creating a graphical display of Structure results in Distruct (Rosenberg 2003). We repeated this process in separate analyses for all rockhopper penguin colonies across both species, *E. chrysome* colonies including both subspecies, only *E. moseleyi* colonies, only colonies of the *E. c. chrysome* subspecies and only colonies of the *E. chrysome filholi* subspecies.

We assessed population-scaled mutation rates ( $\theta$ ) for each ingroup taxon (*E. c. chrysome*, *E. c. filholi*, *E. moseleyi*) and pairwise, bidirectional migration between each extant taxon and among ancestral populations using IMA2p (Sethuraman et al. 2016). IMA2p was run on the BigGreen computing cluster at Marshall University utilizing 18 computing nodes and 12 cores per node. MCMCs were run for 10 million generations (sampling every 100 generations and discarding the first 6 million as burn-in) using 648 heated chains (3 heated chains per core across 12 cores on each of 18 nodes) with geometric swapping and heating terms of 0.99 and 0.3. Starting priors for population size, migration rates and splitting times were determined based on recommendations in the IMA2 manual. The highest geometric mean (GM) of the Watterson's estimators ( $\approx 2.12$ ) across all loci obtained in DnaSP was set as  $x$  and the population size prior set at  $5x$ , the migration rate prior set at  $x/2$  and the splitting prior set at  $2x$ . In initial runs values for each prior were rounded up to the next highest integer (population size prior = 11, migration rate prior = 1, splitting time prior = 5). Based on preliminary runs using these priors, we found some estimates for migration rates and population size exhibited highest posterior densities (HPDs) exceeding the limits imposed by the priors so we adjusted these initial values for the final analysis (population size prior = 22, migration rate prior = 1, splitting time prior = 18). For ND2, our basis for setting the substitution rate was the same as used in the \*BEAST2 analysis. We used an ND2 substitution rate of 0.0105 substitutions/site/lineage/Ma (0.5 $x$  a typical mtDNA pairwise divergence rate of 0.021 reported for birds) and converted this to a per locus rate by multiplying by the number of sites for each locus. We did the same for each nuDNA sequence but used a substitution rate 1/10th of that used for ND2. For each locus we included a range of site-specific substitution rates from 0.1 $x$  to 10 $x$  as a substitution rate distribution prior. To report  $N_e$ , migration rates and splitting times in demographically relevant values we used a generation time of 9.4 years (Guinard et al. 1998; de Dinechin et al. 2009) and the geometric mean of the scalars across all 7 loci (geometric mean across 1 mtDNA and 6 nuDNA = 0.82034). Examination of autocorrelation values, estimated sample sizes (ESSs), and posterior probability histograms for each estimated population parameter were used to assess MCMC convergence.

We assessed the fit of 5 demographic models designed to determine lineage limits (i.e., 1 vs. 2 lineages) and to test the presence of gene flow between lineages using PHRAPL (Jackson et al. 2017b). Analyses were performed independently at different taxonomic levels. First, to assess differences between the southern and northern rockhopper penguin species, we considered *E. chrysome* as a single taxon that includes both subspecies *chrysome* and *filholi*, and *E. moseleyi* as a separate taxon. Second, we also tested demographic models within *E. chrysome* species considering each subspecies a separate taxon (*chrysome* and *filholi*). Third, to test demographic models within each of the *E. chrysome* subspecies, we considered clusters of colonies separated geographically as independent groups. For example, in the *chrysome* subspecies, models were tested considering populations from South America and the

Falkland (Malvinas) Islands as independent groups. In a similar way, in a different analysis for the *filholi* subspecies, populations from Crozet and New Zealand were considered independent groups. In each analysis, the demographic models were characterized as follows: groups are part of the same lineage (model 1), 2 groups without migration (model 2), 2 groups with symmetric migration (model 3), 2 groups with asymmetric migration from population 1 to population 2 (model 4), 2 groups with asymmetric migration from population 2 to population 1 (model 5). A second set of 81 models was tested including scenarios of complete isolation, isolation with migration, and migration. In this analysis, 3 lineages were considered being the 2 *E. chrysocome* subspecies and *E. moseleyi* independent groups. The set of models included all possible topologies for isolation and isolation with migration models and, to reduce model space, migration was forced to be symmetric (see details of model design in Morales et al. 2017).

Model selection was performed in PHRAPL using gene trees from 6 nuclear loci and 1 mitochondrial locus as input. Maximum likelihood gene trees were estimated for each locus using the rapid hill-climbing mode in RAxML v7.2.8 and the GTR substitution model and estimating gamma and proportion invariant sites (Stamatakis 2014). Gene trees were subsampled at random with replacement 200 times, sampling 4 alleles per lineage in each replicate. Simulation of 100 000 gene trees was conducted using a grid of parameter values for divergence time ( $t$ ) and migration ( $m$ ) designed to encompass the range of potential values of these lineages. These values were  $t = 0.30, 0.58, 1.11, 2.12, 4.07, 7.81$  and  $m = 0.10, 0.22, 0.46, 1.00, 2.15, 4.64$ . The relative scaling of the effective population size of each locus was considered during gene tree simulation (e.g., diploid nuclear locus = 1, mtDNA locus = 0.25). The log likelihood (lnL) and AIC of each model were calculated based on the proportion of matches between simulated and empirical trees. Akaike weights (wAIC) are used to compare models and calculate metrics analogous to model probabilities that go from 0 (low support) to 1 (high support).

Finally, we also conducted a separate species delimitation analysis using the Bayesian Phylogenetics and Phylogeography (BPP) package (Yang et al. 2010, 2014; Yang 2015) using the 6 nuclear intron sequences and 5 randomly selected individuals from each of our 12 rockhopper colonies and for 2 individual *E. chrysolophus* samples. We used 2 approaches in this analysis. First, we conducted a model testing approach for species delimitation against a known phylogenetic tree (the A10 analysis in BPP). We used the species delimitation algorithm 1 with  $\alpha = 6$  and  $m = 4$ . We repeated this analysis for each of 4 different combinations of prior distributions ( $\theta = 0.005, \tau = 0.003$ ;  $\theta = 0.01, \tau = 0.003$ ;  $\theta = 0.005, \tau = 0.001$ ;  $\theta = 0.01, \tau = 0.001$ ) for the population scaled mutation rate ( $\theta$ ) and divergence ( $\tau$ ) in 3 replicate runs for each combination of priors.

However, given that this approach has been found to over split species and may therefore be more indicative of population structure than species limits (McKay et al. 2013; Jackson et al. 2017a; Sukumaran et al. 2017) we also conducted a heuristic species delimitation approach based on estimates of posterior density of the genealogical diversity index (GDI) using BPP (Leaché et al. 2019). BPP uses the multispecies coalescent (MSC) to estimate  $\tau$  and  $\theta$  from a sequence alignment, which is in turn used to calculate GDI as follows:

$$\text{GDI} = 1 - e^{-2\tau/\theta_A}$$

$2\tau/\theta_A$  is the population divergence time in coalescent units for population A ( $2N_A$  generations equals one coalescent time unit where  $N_A$  is the effective population size of population A) and  $1 - e^{-2\tau/\theta_A}$  reflects the probability that 2 sequences in population A coalesce prior, going backwards in time, to the divergence between species A and a sister species B. The probability of this sorted gene tree increases with increasing population divergence. From a meta-analysis of GDI variation across pairs of populations, ranging from populations with low divergence and high gene flow to strongly delimited reproductively isolated pairs of species, a heuristic rule was developed such that  $\text{GDI} < 0.2$  was consistent with within population diversity characterized by comparatively short population divergence and high gene flow and  $\text{GDI} > 0.7$  represented isolated species lineages with deep divergence and low gene flow while  $\text{GDI}$  between 0.2 and 0.7 suggests potential but weakly divergent species lineages (Pinho et al. 2010; Jackson et al. 2017a).

We conducted 3 heuristic BPP species delimitation analyses for 3 different phylogenetic trees using the method outlined in Leaché et al. (2019). First was an analysis using all putative rockhopper species as tips and *E. chrysolophus* as an outgroup. The second was a tree that collapsed *E. c. chrysocome* and *E. c. filholi* as a single branch and the third collapsed all rockhopper penguins onto a single branch. This allowed for GDI comparisons for each branch in the tree, between *E. c. chrysocome* and *E. c. filholi*, between southern rockhoppers and *E. moseleyi*, and between all rockhoppers and *E. chrysolophus*. For each tree, we combined the results from 3 independent runs of the A00 analysis, which estimates  $\theta$  and  $\tau$  from the sequence alignment for a given tree and set of putative species. For a guide tree, we used the tree topology supported by both mtDNA (Figure 2) and the combined mtDNA and nuDNA dataset (Figure 3). Each run incorporated a burnin of 12 000 and an mcmc sample of 120 000 with a sample frequency of 5. As in the model testing A10 analysis, we repeated the analysis for each of the same 4 different combinations of prior distributions for  $\theta$  and  $\tau$ .

## Results

We produced a total of 3513 bp of sequence data across 114 individual rockhopper samples from 12 colonies including 724 bp of partial ND2 sequence and 2789 bp from 6 nuclear introns. Excluding sites with gaps in the alignment we were able to use 3457 bp in our analyses. No locus showed evidence of either recombination by the  $\Phi_w$ -statistic (Bruen et al. 2006) or evidence of selection or deviations from neutrality by the HKA test (Hudson et al. 1987) or Tajima's D statistic (Tajima 1989). Phased haplotypes for the 6 nuclear intron loci ranged from 13 to 54 (median 19.5) with an average intron haplotype diversity of 0.82 and an average number of differences among intron haplotypes within a locus of 2.45. The ND2 locus exhibited 24 haplotypes with a haplotype diversity of 0.86 and an average number of differences between haplotypes of 7.1. See Table 1 for a summary of descriptive statistics for each locus.

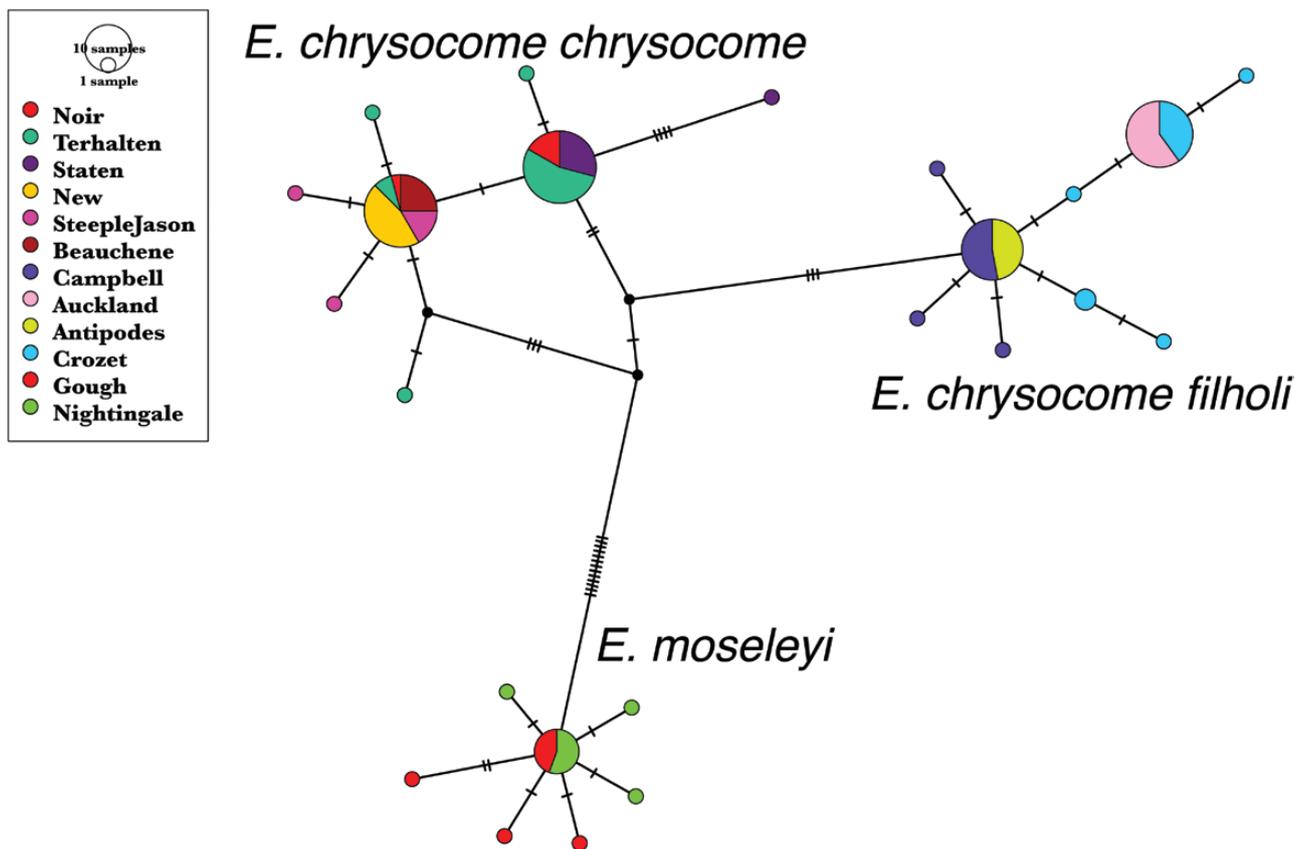
A haplotype network for ND2 based on the integer neighbor joining technique (Figure 1) and a MrBayes gene tree for ND2 (Figure 2) both showed strong mitochondrial differentiation between the 3 rockhopper taxa, *E. c. chrysocome*, *E. c. filholi*, and *E. moseleyi* but little differentiation among colonies or clusters of colonies within each taxon. Posterior probabilities associated with each rockhopper taxon in the MrBayes analysis were all 1 (Figure 2).

\*BEAST2 analysis revealed a topology consistent with both the ND2 haplotype network and ND2 MrBayes analysis with

**Table 1.** Summary table for genetic loci from DnaSP

Locus	Chromosome	<i>n</i>	Sites	Sites <sub>Net</sub>	<i>S</i>	Hap	<i>H<sub>d</sub></i>	<i>P<sub>i</sub></i>	<i>K</i>	$\Theta_c$	Tajima's D
08352	5	108	499	495	25	54	0.942	0.005	2.648	8.489 (4.202)	-1.015
20454	1	108	399	398	16	18	0.900	0.006	2.480	6.756 (2.689)	-0.199
22187	2	108	521	514	14	21	0.794	0.005	2.440	4.578 (2.353)	0.092
26896	3	109	466	433	22	38	0.880	0.009	3.913	8.527 (3.692)	0.161
26928	1	109	496	486	13	16	0.688	0.004	1.936	4.488 (2.181)	-0.276
27189	1	110	408	408	10	13	0.704	0.003	1.287	4.517 (1.843)	-0.711
ND2	Mitochondrial	114	724	723	44	24	0.858	0.010	7.104	11.463 (8.288)	-0.443
All			3513	3457	144	184	0.824	0.006	3.115	6.974 (3.607)	-0.342

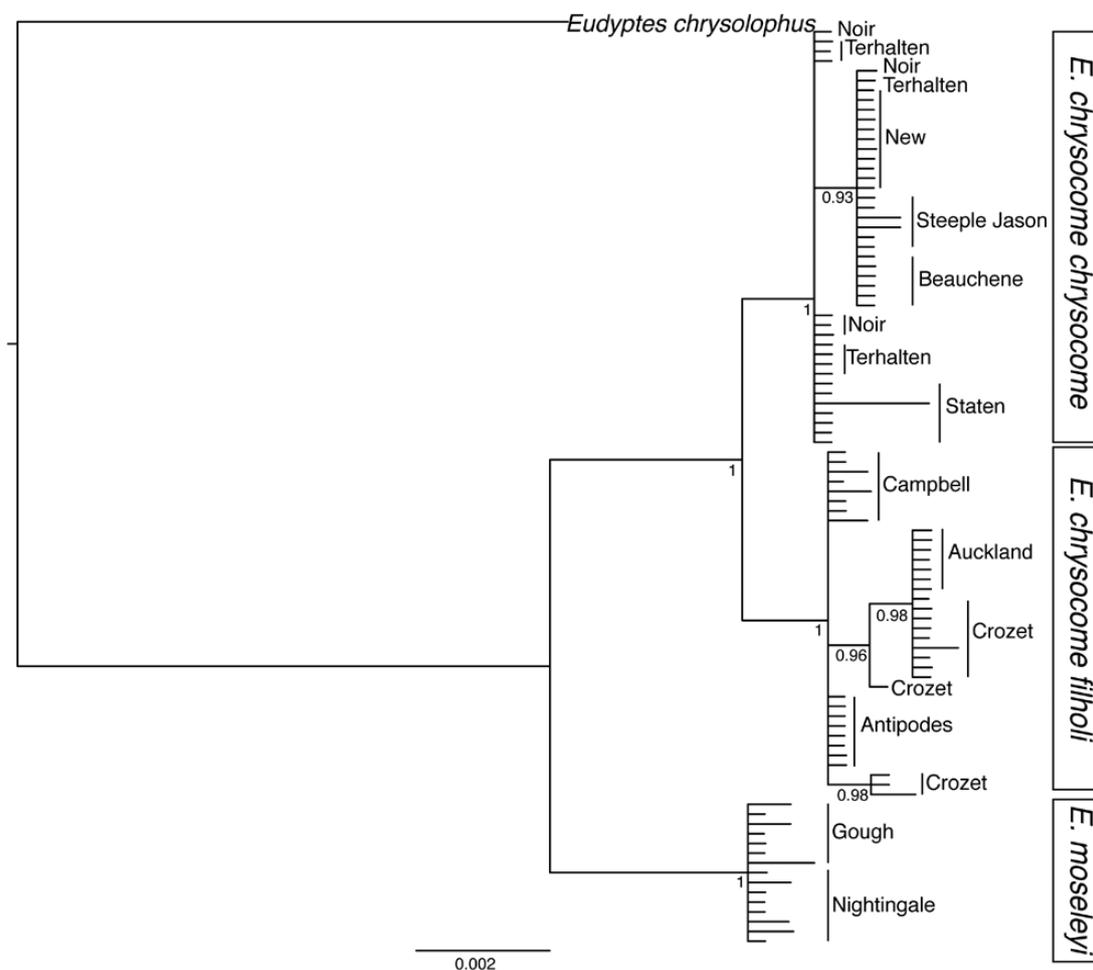
Chromosome number refers to the chicken (*Gallus gallus*) chromosome number to which each nuclear locus was mapped in Backström et al. (2008). *n*, number of individuals with sequence data; Sites<sub>Net</sub>, sequence length (sites) and the number of sites used in the analysis excluding gaps; *S*, number of segregating sites; Hap, number of haplotypes; *H<sub>d</sub>*, the haplotype diversity; *P<sub>i</sub>*, per site nucleotide diversity; *k*, the average number of differences between haplotypes. The population mutation rate ( $\Theta_c$ ) normalized for the net number of sites at a locus and expressed as per 1000 bases with the raw value from DnaSP in parentheses. Permutation tests were conducted on Tajima's D and no statistically significant deviation from 0 was observed in Tajima's D for any locus. The "All" row contains summed values for the number of sites, the number of net sites, the number of segregating sites and the number of haplotypes, and mean values for the remainder of the variables.



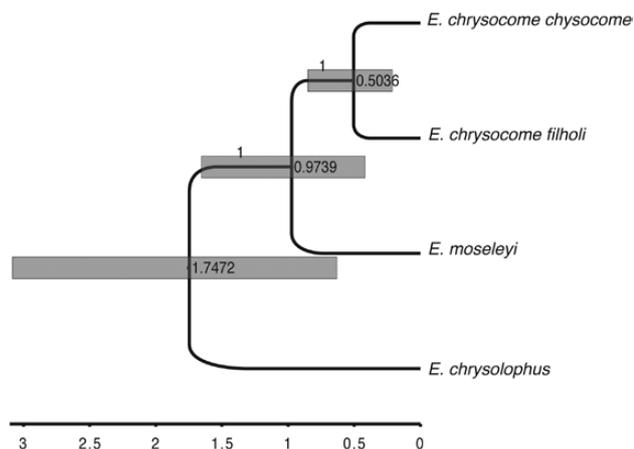
**Figure 1.** ND2 integer neighbor joining haplotype network. ND2 integer neighbor joining network generated in PopArt with reticulation tolerance set to the default value of 0.5. Individual hatch marks on the lines connecting haplotypes indicate mutations.

*E. c. chrysocome* and *E. c. filholi* as sister taxa and a more deeply divergent *E. moseleyi* (Figure 3). Divergence times between the 3 rockhopper taxa and *E. chrysolophus* was estimated at 1.75 Ma (0.63–3.09 95% HPD). The split between *E. moseleyi* and the 2 recognized *E. chrysocome* subspecies was estimated to have occurred 0.97 Ma (0.42–1.66 95% HPD) and the split between *E. c. chrysocome* and *E. c. filholi* estimated to be 0.50 Ma (0.21–0.85 95% HPD). All branches had a posterior probability of 1 and parameters estimated in \*BEAST2 showed strong convergence and ESS values ranging from 469 to 70 357 across the 3 combined independent runs.

Colonies were grouped into 5 geographic groups on the basis of the ND2 haplotype network, ND2 MrBayes tree and geographic proximity; South America (Noir, Terhalten, Staten), the Falkland (Malvinas) Islands (New Island, Steeple Jason, Beauchene), New Zealand (Campbell, Auckland, Antipodes), Crozet, and Tristan da Cunha (Gough, Nightingale). All pairwise comparisons were strongly differentiated for mtDNA. Average  $F_{ST}$  values for nuclear intron loci between the *E. moseleyi* sequences collected in the Tristan da Cunha group and southern rockhopper groups ranged from 0.26 to 0.32 and for the ND2 locus between 0.94 and 0.97.



**Figure 2.** ND2 MrBayes tree. ND2 MrBayes 3.2.6 tree implemented in Geneious 8.1.4 with the HKY+I substitution model, MCMC chain length 2 000 000, 4 heated chains (temp 0.2), 200 000 burnin, subsampling frequency of 1000, and a molecular clock prior with uniform branch lengths. We used the most likely model available in the Geneious implementation of MrBayes as determined by the JModelTest2 AIC criterion (3216.1 HKY+I).



**Figure 3.** \*BEAST species tree. \*BEAST species tree with all rockhopper penguin taxa (*Eudyptes chrysocome chrysocome*, *Eudyptes chrysocome filholi*, *Eudyptes moseleyi*) and *Eudyptes chrysolophus* as an outgroup. Node ages and 95% highest posterior density (HPD) are reported in Ma and branch labels represent posterior probabilities. Scale is in Ma.

Among *E. chrysocome* groups, average  $F_{ST}$  values for nuclear intron loci ranged from 0.01 to 0.18 and for ND2 from 0.15 to 0.9

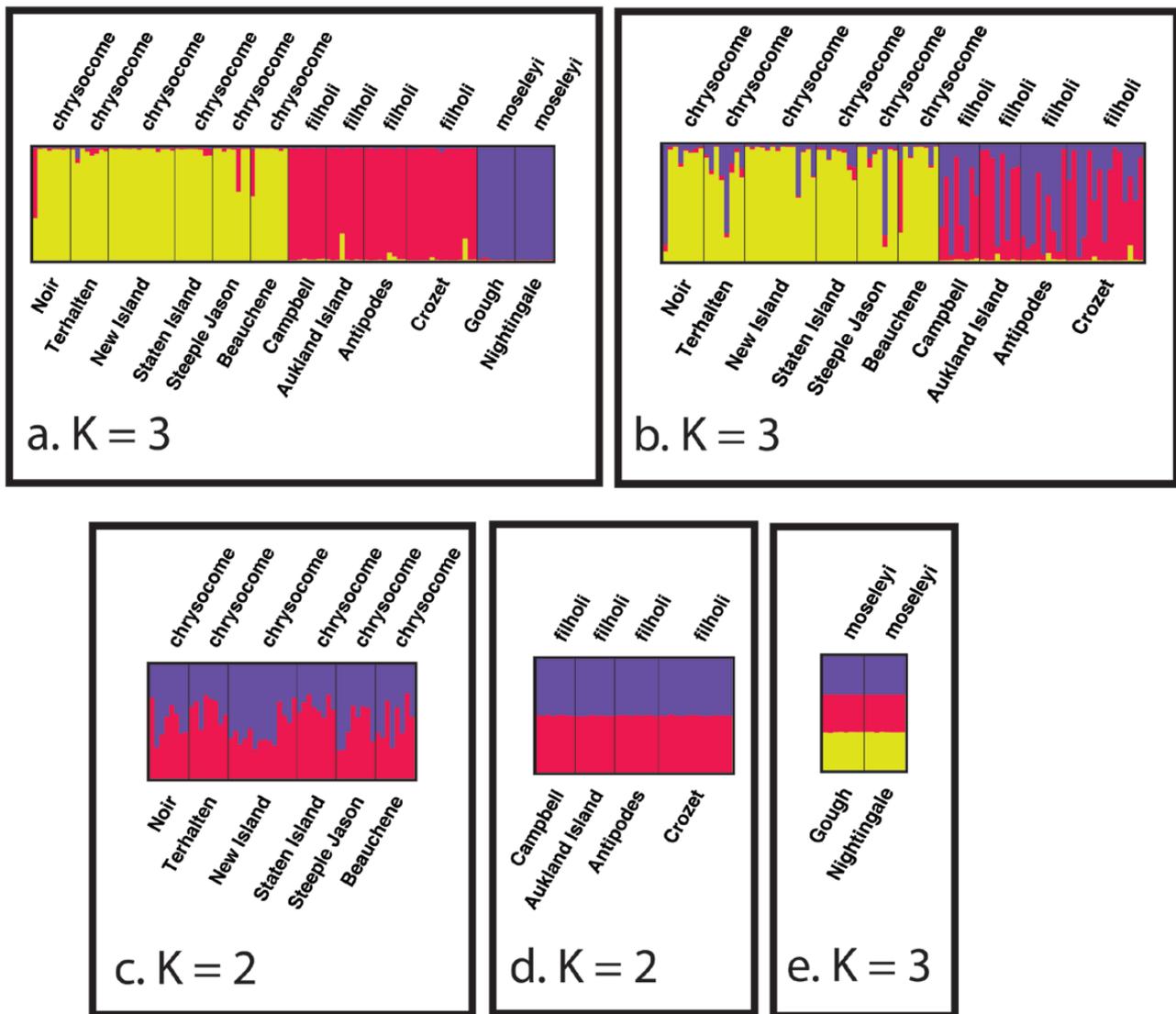
(Table 2). In the combined 6 intron  $F_{ST}$  analysis in HIERFSTAT no 95% confidence intervals included zero for any pairwise comparison between geographic groups (Table 2). While every comparison of intron  $F_{ST}$  among geographic groups was statistically significant based on a 95% confidence interval only those comparisons between *E. moseleyi* and *E. chrysocome* colonies were consistently well above 0.15, a threshold often considered as reflecting meaningful biological differentiation (Frankham et al. 2010).

A hierarchical Structure analysis of the 6 nuclear intron loci showed population structure with limited admixture across the recognized subspecies/species but significant genetic admixture among colonies within each currently recognized taxon (Figure 4). Structure Harvester analysis of the Structure output using the  $\Delta K$  Evanno et al. (2005) method revealed that clusters of  $K = 3$  were the most likely clusters for analyses across all rockhopper taxa (Figure 4a) and within *E. chrysocome* (Figure 4b). Near complete admixture was exhibited in those Structure analyses confined to colonies within *E. c. chrysocome* (6 colonies,  $K = 2$ , Figure 4c), *E. c. filholi* (4 colonies,  $K = 2$ , Figure 4d), and *E. moseleyi* (2 colonies,  $K = 3$ , Figure 4e). Structure analysis showed strong admixture even among geographically widely separated colonies between Crozet in the Indian Ocean and colonies in sub-Antarctic waters around New Zealand in the Pacific Ocean (Campbell, Auckland, Antipodes, Figure 4d).

**Table 2.** Mean pairwise  $F_{ST}$  values  $\pm$  1 SD for 6 nuclear intron loci and ND2 and 95% confidence intervals for pairwise  $F_{ST}$  values for 6 nuclear intron loci

	South America	Falklands	New Zealand	Crozet	Tristan da Cunha
South America		0.06 $\pm$ 0.06 (0.59)	0.11 $\pm$ 0.08 (0.84)	0.16 $\pm$ 0.08 (0.84)	0.32 $\pm$ 0.18 (0.95)
Falklands	0.01–0.05		0.14 $\pm$ 0.11 (0.9)	0.18 $\pm$ 0.12 (0.89)	0.31 $\pm$ 0.12 (0.97)
New Zealand	0.03–0.10	0.03–0.14		0.01 $\pm$ 0.01 (0.15)	0.29 $\pm$ 0.22 (0.94)
Crozet	0.08–0.13	0.06–0.17	0.01–0.05		0.26 $\pm$ 0.22 (0.94)
Tristan da Cunha	0.16–0.25	0.13–0.26	0.09–0.22	0.07–0.21	

Mean pairwise  $F_{ST}$  values  $\pm$  1 SD for 6 nuclear intron loci and pairwise  $F_{ST}$  values for ND2 (in parentheses) as calculated in DnaSP are shown above the diagonal. 95% confidence intervals for  $F_{ST}$  based on all 6 nuclear intron loci combined generated by bootstrapping in HIERFSTAT are shown below the diagonal. Colonies from South America and the Falklands (Malvinas) represent *Eudyptes chrysocome chrysocome*, colonies from New Zealand and Crozet represent *Eudyptes chrysocome filholi* and colonies from Tristan da Cunha represent *Eudyptes moseleyi*.



**Figure 4.** Hierarchical STRUCTURE analysis. Hierarchical Structure analysis for population structure and admixture for all 3 rockhopper penguin taxa on histogram a (*Eudyptes chrysocome chrysocome*, *Eudyptes chrysocome filholi*, *Eudyptes moseleyi*), secondary structure and admixture within each recognized species on histogram b (*Eudyptes chrysocome*) and histogram e (*Eudyptes moseleyi*), and tertiary structure and admixture within the recognized *E. chrysocome* subspecies on histogram c (*E. c. chrysocome*) and histogram d (*E. c. filholi*). Each histogram is constructed from the most likely number of clusters ( $K$ ) according to the  $\Delta K$  criterion as described in Evanno et al. (2005).

Individuals in the Structure analysis between the 3 recognized rockhopper taxa tended to cluster appropriately with other individuals in their taxon, however, there were exceptions where  $>0.05$

of the genetic data for an individual was assigned to a different taxon (Figure 4a). Only one individual *E. c. chrysocome* and one individual *E. c. filholi* were associated with  $>0.05$  admixture with

*E. moseleyi* (0.097, 0.052, respectively). However, a more significant admixture was evident between *E. c. chrysocome* and *E. c. filholi*. Within *E. c. chrysocome*, 7 individuals had >0.05 of their ancestry assigned to *E. c. filholi* (ancestry proportion 0.052–0.589). Within *E. c. filholi*, 3 individuals exhibited admixture with *E. c. chrysocome* at a probability of >0.05 (0.064–0.255, Figure 4a). These data suggest a greater amount of gene flow from *E. c. filholi* to *E. c. chrysocome* than the reverse and very little gene flow between either *E. chrysocome* subspecies and *E. moseleyi* in either direction.

Parameters estimated in the IMA2p analysis showed convergence, low autocorrelation values (<0.02) and high ESS values (11 770–43 757) for all parameters except for the ancestral  $\theta$  for all 3 taxa and the time to the most recent common ancestor for all 3 taxa. These last 2 parameters failed to adequately converge despite long, heated MCMC chains. Estimates for  $\theta$  and migration rates were within at most 2% when compared across the first set of genealogies and the second set of genealogies. IMA2p returns migration rate parameters ( $m$ ) in units of migrations per mutation or  $m = M/u$ , where  $M$  is a migration rate per gene copy and  $u$  is a geometric mean of mutation rates across loci. Significant migration rate parameters, reported forward in time, were estimated from *E. c. filholi* to *E. c. chrysocome* (0.87, likelihood ratio test 36.35,  $P < 0.001$ , Table 3, Figure 5) and from *E. moseleyi* to *E. c. filholi* (0.19, likelihood ratio test 34.94,  $P < 0.001$ , Table 3, Figure 5). Only the migration of alleles from *E. c. filholi* to *E. c. chrysocome* was greater than 1 allele per year as measured by the population scaled mutation rate which is the number of gene copies in the population ( $2N$ ) times the migration rate parameter (highest point in posterior density  $2N_0m_{0.5} = 1.19$ , 95% HPD 0.48–2.17, Table 3, Figure 5). The most likely order for the 3 taxa in terms of population size was *E. c. filholi* > *E. c. chrysocome* > *E. moseleyi* (highest smoothed point in posterior density for population size estimates in demographic units 121 127; 76 413; and 61 064 respectively, Table 3, Figure 6). The split time in years from IMA2p for *E. c. chrysocome* and *E. c. filholi* was estimated by a high point of 482 469 years in a smoothed posterior distribution (mean 568 882 years, 95% HPD 236 102–975 204) while failure to reach convergence left the estimate for the split time between *E. chrysocome* and *E. moseleyi* unreliable.

When a set of 81 models for all 3 taxonomic groups was explored using PHRAPL (including all possible scenarios of isolation and isolation with migration), none of the models achieved a wAIC higher than 0.05. However, models of isolation with migration tended toward higher support compared with models of isolation (Supplementary Figure 1). Because our comparatively limited dataset did not have the power to compare all models for 3 taxa in

a single analysis we divided our analysis in to 4 separate analyses each with 2 taxa (*E. chrysocome* and *E. moseleyi*, *E. c. chrysocome* and *E. c. filholi*, South American *E. c. chrysocome* colonies and Falkland/Maldives *E. c. chrysocome* colonies, and the Crozet *E. c. filholi* colony and New Zealand *E. c. filholi* colonies).

In the first PHRAPL analysis with a set of 5 models, model 4, a model of isolation with asymmetric gene flow from *E. chrysocome* to *E. moseleyi*, achieved the highest support (wAIC = 0.93; Figure 7a, Supplementary Table 4). In the second analysis including *E. c. chrysocome* and *E. c. filholi*, model 1 achieved the highest support meaning that both taxa are acting as a single population lineage (wAIC = 0.73). However, to explore whether there is any signal of divergence and/or isolation, model 1 was removed from the set of models, and the wAIC were recalculated including only those models that considered 2 independent lineages (i.e., models 2–5). Model 3 achieved the highest support in this analysis, demonstrating that there has been significant gene flow in both directions for *E. c. chrysocome* and *E. c. filholi* subspecies (wAIC = 0.55; Figure 7b, Supplementary Table 4).

To look for population structure within *E. c. chrysocome* and *E. c. filholi* each subspecies was divided into 2 geographic groups, South American colonies versus Falkland (Maldives) Islands colonies for *E. c. chrysocome* and Crozet versus New Zealand colonies for *E. c. filholi*. In both analyses, model 1 achieved the highest support indicating that geographic groups are acting as a single lineage (wAIC = 0.82 and 0.95, respectively). We also reanalyzed these models within each subspecies leaving out model 1. In both analyses, a model with symmetrical gene flow received the highest support (model 3, wAIC = 0.55 and 0.73, respectively) showing that populations of *E. c. chrysocome* from South American and Falkland (Maldives) Islands colonies are genetically connected (Figure 7c, Supplementary Table 4), as are *E. c. filholi* populations from Crozet and New Zealand (Figure 7d, Supplementary Table 4).

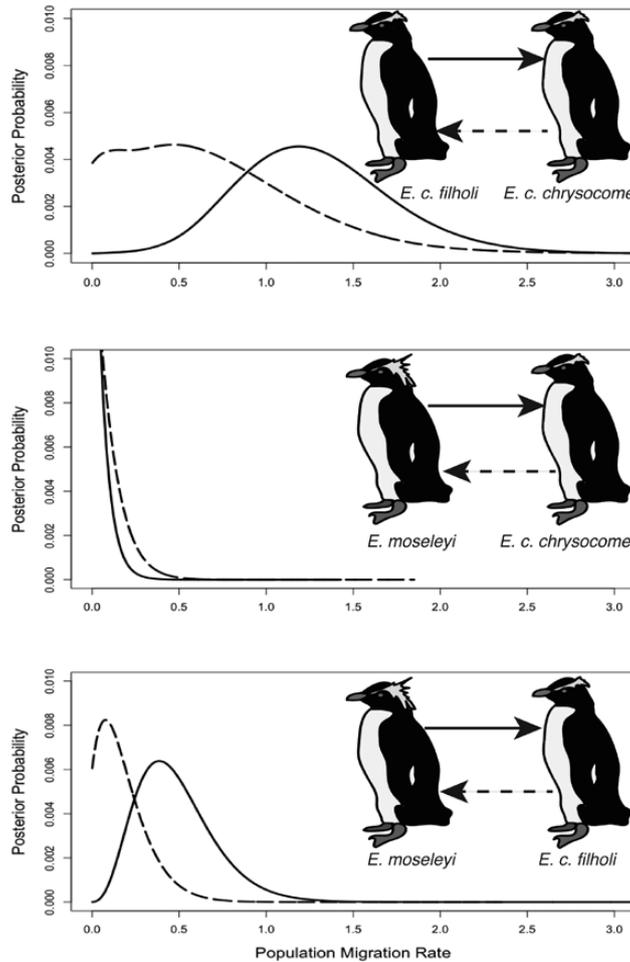
The model testing approach in BPP strongly supported splitting all putative rockhopper species ( $P = 1.0$ ) in every analysis. However, given a widely documented tendency for BPP to over split species (McKay et al. 2013; Jackson et al. 2017a; Sukumaran et al. 2017) we favored the heuristic approach of Leaché et al. (2019) based on suggested GDI cutoff values (Hey et al. 2012; Jackson et al. 2017a). Across all 4 combinations of priors for  $\theta$  and  $\tau$  only *E. moseleyi* consistently exceeded the 0.2 GDI threshold for a potential species lineage and no taxon exceeded the 0.7 GDI threshold for a strongly divergent species lineage (Figure 8). *Eudyptes chrysolophus* had a peak GDI posterior density exceeding the 0.2 GDI threshold for one of the analyses (Figure 8a) and was near this threshold in another (Figure 8c).

**Table 3.** IMA2p population scaled mutation rate ( $\Theta$ ) per locus, effective population size ( $N_e$ ), and migration rates reported forward in time with population scaled migration rates in parentheses

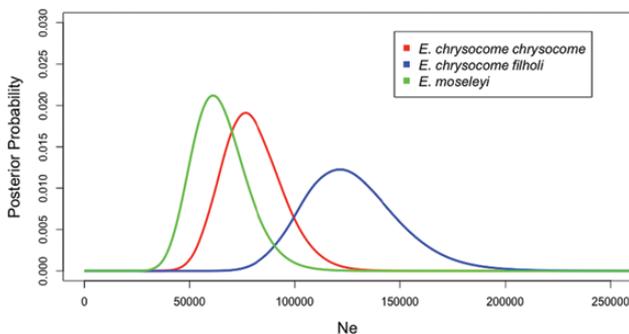
Species/subspecies	Population-scaled mutation rates ( $\Theta$ )	$N_e$ (individuals)	Forward in time migration to <i>chrysocome</i>	Forward in time migration to <i>filholi</i>	Forward in time migration to <i>moseleyi</i>
<i>Eudyptes chrysocome chrysocome</i>	2.52	76 413	NA	0.05 (0.47)	0.01 (0.001)
<i>Eudyptes chrysocome filholi</i>	4.00	121 127	0.87 (1.19) <sup>a</sup>	NA	0.07 (0.08)
<i>Eudyptes moseleyi</i>	2.01	61 064	0.01 (0.001)	0.19 (0.39) <sup>a</sup>	NA

Reported from highest points in posterior distributions.

<sup>a</sup>Migration rates significantly different from zero based on the likelihood ratio test in IMA2p.



**Figure 5.** IMA2p migration rates. Pairwise population mutation rates ( $2Nm$ ) from IMA2p. Posterior probabilities are normalized to 1.0 across all comparisons and the direction of migration is expressed forward in time, from one taxon to another, for each pair of taxa. Direction is specified for each pair of taxa by solid versus dashed lines.



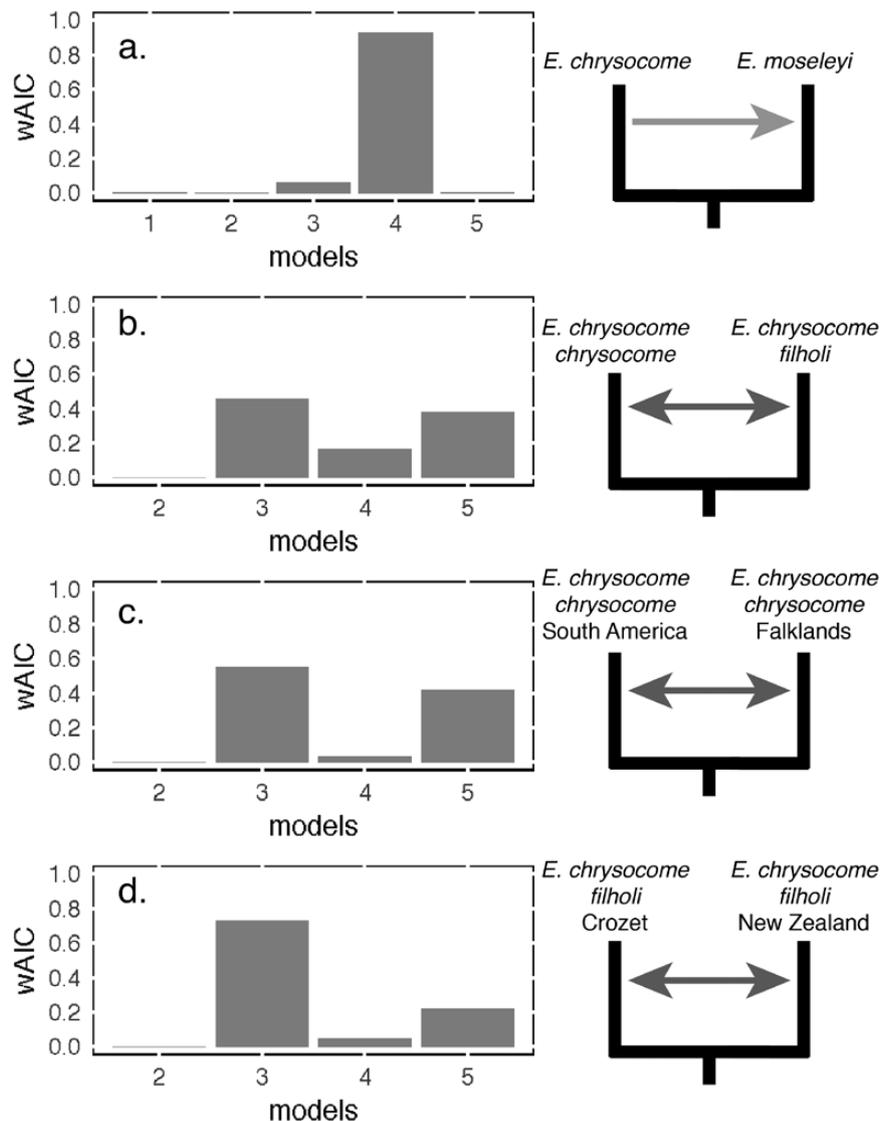
**Figure 6.** IMA2p effective population size. Effective population size ( $N_e$ ) distributions in demographic units estimated by IMA2p for each Rockhopper Penguin taxon. Posterior probabilities were normalized to 1.0 for each taxon.

## Discussion

### *Eudyptes moseleyi* and *E. chrysocome* Are Different Species

Many species concepts have been proposed over nearly a century of investigation of the species problem. However, all these seemingly disparate concepts may be united under a single unified concept that defines species as independently evolving metapopulation

lineages where evolutionary independence is proportional to a reduction in gene flow (de Queiroz 2007) and that is the concept we are applying here. Delimiting species under the unified concept is thus a matter of uncovering evidence of lineage separation. Understanding the balance between divergence, isolation, and gene flow is therefore critical in delimiting species boundaries (Ender 1973; Losos et al. 2009; Jackson et al. 2017a). Populations distributed across environments with few barriers coupled with a high capacity for dispersal should often result in a single, widely distributed species. However, for rockhoppers, the STF is a porous but sufficient oceanographic barrier to have promoted species-level divergence. Previous studies support species-level delimitation between *E. moseleyi* and *E. chrysocome* based on mtDNA (Banks et al. 2006; Jouventin et al. 2006; de Dinechin et al. 2009; Frugone et al. 2018) and fixed phenotypic differences including a more exaggerated crest and low frequency, less complex nuptial vocalizations in *E. moseleyi* (Jouventin 1982; Jouventin et al. 2006). However, caution is warranted when considering single locus mtDNA approaches to species delimitation in isolation or even morphological data when those data are based on a limited geographic sampling or any single rapidly evolving character (Zink et al. 2019). GMYC (Pons et al. 2006; Fujisawa et al. 2013), for example, is known to over split species lineages (Carstens et al. 2013).

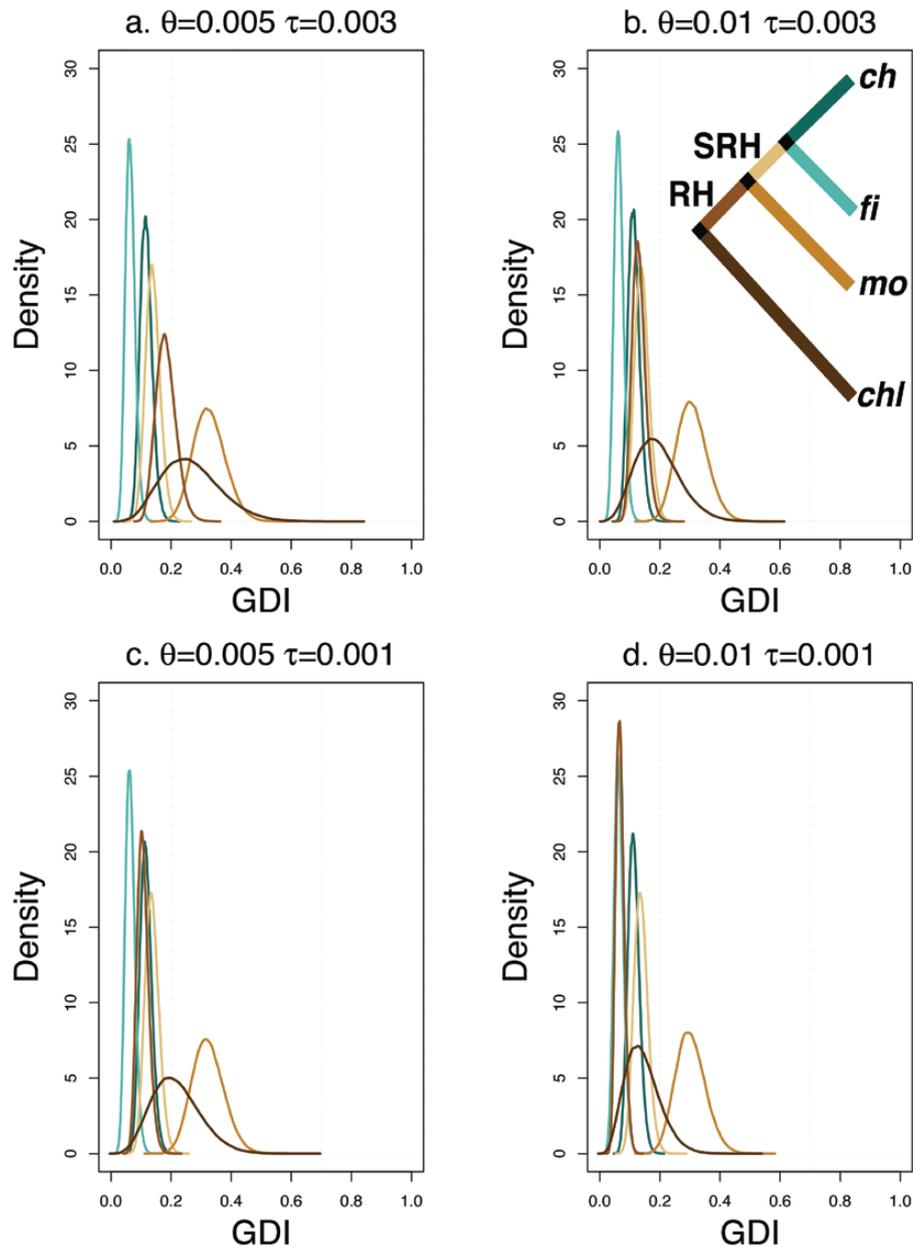


**Figure 7.** PHRAPL model testing analysis. PHRAPL analyses for 4 pairwise comparisons: species level comparisons between *Eudyptes chrysocome* and *Eudyptes moseleyi* (a), subspecies level comparisons within *E. chrysocome* (b), population level comparisons within *Eudyptes chrysocome chrysocome* (c), and population level comparisons within *Eudyptes chrysocome filholi* (d). Five models tested denoted on the x axis of each graph are as follows: a single population lineage (model 1), 2 lineages without migration (model 2), 2 lineages with symmetric migration between pop1 and pop2 (model 3), 2 lineages with asymmetric migration from pop1 to pop2 (model 4), and 2 lineages with asymmetric migration from pop2 to pop1 (model 5). Akaike weights (wAIC) are on the y axis and each graph is accompanied by a tree showing the direction of migration between the 2 lineages in the analysis with the lineage on the left of each tree corresponding to pop1 and the lineage on the right of each tree corresponding to pop2. Analyses b–d all had model 1 with the highest wAIC support in initial analyses and reflect subsequent analyses excluding model 1.

In our study, both PHRAPL and BPP analyses of multilocus introns delimits *E. chrysocome* and *E. moseleyi* as 2 independent lineages and thus 2 species under a unified species concept, and for PHRAPL, species with asymmetric gene flow. A model testing approach in BPP strongly supports *E. moseleyi* as an independent species lineage but a heuristic approach based on GDI provided relatively weak support for species limits in *E. moseleyi*. Given that species delimitation model testing in BPP, like GMYC, is prone to over split lineages this approach may be more indicative of genetic structure than species limits per se and should be considered with some caution (Carstens et al. 2013; McKay et al. 2013; Jackson et al. 2017a; Sukumaran et al. 2017). However, in the more conservative heuristic GDI approach in BPP, only *E. moseleyi* was consistently above the suggested bound for a putative species (GDI > 0.2) but

not above that suggestive of a strongly delimited species (GDI > 0.7, Leaché et al. 2019). Species delimitation among branches in the heuristic BPP analysis was not reciprocal. For example, while the GDI for the *E. moseleyi* branch was above 0.2, the GDI for the sister, southern rockhopper branch was less than 0.2, suggesting that one branch was in the zone of a putative species but the other was not. Leaché et al. (2019) also found a similar result in analyses of various datasets and attributed the discrepancy to differences in  $\theta$  between different lineages. We also found that the outgroup (*E. chrysolophus*) was poorly delimited as a species lineage in the heuristic BPP analysis and we attribute this to the limited sample size for this taxon in our analysis (only 2 individual samples or 4 phased haplotypes).

The results from this and previous studies are congruent in regards to species delimitation of *E. chrysocome* and *E. moseleyi*.



**Figure 8.** Heuristic BPP delimitation based on GDI. Posterior density of the genealogical diversity index (GDI) from 4 BPP analyses using 4 different combinations of  $\theta$  and  $\tau$ . Colors correspond to different branches on the guide tree shown in the upper right corner; *Eudyptes chrysolophus* (chl, dark brown), all rockhoppers (RH, brown), *Eudyptes moseleyi* (mo, gold), southern rockhoppers (SRH, beige), *Eudyptes chrysocome filholi* (fi, light blue), and *Eudyptes chrysocome chrysocome* (ch, dark green). Dashed lines at 0.2 and 0.7 GDI indicate bounds for putative species with <0.2 consistent with single population lineages, >0.7 consistent with well delimited species, and 0.2–0.7 indicating potential species.

This includes strongly supported reciprocally monophyletic mtDNA clades (this study; Banks et al. 2006; Jouventin et al. 2006; de Dinechin et al. 2009; Frugone et al. 2018), explicit species delimitation tests based on distance and branch rates for mtDNA (Frugone et al. 2018), fixed morphological and behavioral differences (Jouventin et al. 1982, 2006), consistently high  $F_{ST}$  ( $F_{ST} > 0.15$ ) for both mtDNA and multilocus nuDNA comparisons, strong support for a 2 lineage model with asymmetric gene flow in PHRAPL, and a GDI consistently greater than 0.2 across multiple priors in a heuristic BPP approach (this study). Any one of these results considered in isolation may make a weak case but when considered together are strong support for 2 species lineages in *E. chrysocome* and *E. moseleyi*.

Our multilocus estimate of divergence time between the more southerly distributed sub-Antarctic *E. c. chrysocome* and *E. c. filholi* and the more northerly distributed *E. moseleyi* was 0.97 Ma. This is congruent with a divergence estimate of 0.90 Ma from de Dinechin et al. (2009) in an MDIV analysis (Nielsen et al. 2001) of multiple mtDNA sequences and with the divergence estimate of 0.77 Ma derived from the Bayesian total evidence penguin phylogeny from Gavryushkina et al. (2017). Divergence time between *E. c. chrysocome* and *E. c. filholi* was comparable for both \*BEAST2 and IMA2p analyses with peak posterior estimates of 0.50 and 0.48 Ma, respectively. Frugone et al. (2018) derived much older divergence times between these taxa than either our study or those of De Dinechin et al. (2009) and Gavryushkina et al. (2017) with the split

between *E. chrysocome* and *E. moseleyi* at approximately 3.06 Ma and a split between *E. c. chrysocome* and *E. c. filholi* at 2.26 Ma. While the estimates in Frugone et al. were unlike ours in that they included fossil calibration, they were solely based only on the mtDNA hypervariable region rather than a multilocus dataset and this may be contributing to an overestimate of divergence times.

Molecular clock-based estimates of divergence times are fraught with assumptions (Bromham et al. 2003; Lovette 2004; Welch et al. 2005; Pereira et al. 2006b) and while they provide useful benchmarks in the timing of species divergence we would urge caution in their interpretation. However, the estimated split of *E. chrysocome* and *E. moseleyi* in our study is broadly contemporaneous with that of some other seabirds with a taxonomic division at the STF, including the northern (*Macronectes halli*) and southern giant petrels (*Macronectes giganteus*; 0.7 Ma; Techow et al. 2010) and the spectacled (*Procellaria conspicillata*) and white-chinned petrels (*Procellaria aequinoctialis*; 0.9 Ma; Techow et al. 2009). Genetic divergence between *E. moseleyi* and *E. chrysocome* is comparable to that found in other sister taxa spanning the STF including *Diomedea dabbenena* on Tristan de Cunha and other taxa in the wandering albatross complex (Burg et al. 2004) and the extinct *Megadyptes waitaha* and the yellow-eyed penguin (*Megadyptes antipodes*; Boessenkool et al. 2009). These comparisons suggest common climatic events during the Pleistocene may be promoting divergence in Southern Ocean seabirds. However, more studies explicitly estimating divergence times from calibrated, multilocus datasets across taxa are needed.

While *E. chrysocome* and *E. moseleyi* likely represent species lineages, both PHRAPL and IMA2p analysis support a comparatively small, but still significant, amount of gene flow between *E. chrysocome* and *E. moseleyi* and in the case of the IMA2p analysis between *E. moseleyi* and *E. c. filholi* in particular. These taxa breed in closer proximity to one another in the Indian Ocean but *E. moseleyi* has a greater tolerance for southward migration crossing the STF than *E. c. filholi* does for northern migrations (Thiebot et al. 2012). At least one occurrence of an *E. moseleyi* individual, genetically assigned to Gough Island 6000 km away in the Atlantic Ocean, was recorded during the breeding season in an *E. chrysolophus* colony on Kerguelen Island (approximately 30 km from the nearest *E. c. filholi* colony; de Dinechin et al. 2007). Even more remarkably, Moors and Merton (1984) recorded *E. moseleyi* in January at the Cook Strait in New Zealand.

Interspecific breeding pairs have been observed between resident and vagrant individuals for many species within *Eudyptes* penguins (Morrison et al. 2014). Crofts and Robson (2015) reported a successful pairing between *E. c. chrysocome* and *E. moseleyi* in an *E. c. chrysocome* colony on East Falkland (Malvinas) and suggest that hybridization may be on the rise with increasing sightings of *E. moseleyi* adults during the breeding season. While *E. chrysocome* and *E. moseleyi* diverged nearly 1 million years ago and have accumulated differences in genetics, morphology and behavior they have apparently not maintained strict reproductive incompatibility.

### ***Eudyptes c. chrysocome* and *E. c. filholi* Do Not Represent Different Species**

While our data support *E. moseleyi* and *E. chrysocome* as independent species lineages, albeit with limited gene flow, *E. c. chrysocome* and *E. c. filholi* exhibit evidence of significant gene flow and as such likely do not represent evolutionarily independent lineages. The single lineage model was the best-supported model in PHRAPL when considering *E. c. filholi* and *E. c. chrysocome*. When

a single lineage model was omitted however, a model of symmetric gene flow between the 2 subspecies received the highest support in PHRAPL. However, consistent with the results from IMA2p, support for the symmetric gene flow model was closely followed by a model of asymmetrical gene flow from *E. c. filholi* to *E. c. chrysocome*. Also, 7 *E. c. chrysocome* individuals were found to have admixture with *E. c. filholi* in the Structure analysis that included all 3 putative rockhopper taxa (>0.05 admixture with *E. c. filholi* and ranging from 0.052 to 0.589, Figure 4a).

In a heuristic BPP approach, no analysis regardless of our priors produced GDI >0.2 for either *E. c. filholi* to *E. c. chrysocome* and GDI posterior densities were consistent across all analyses and similar between the 2 taxa. We know of no evidence for separate species lineages in *E. c. filholi* and *E. c. chrysocome* outside of those suggested on the basis of mtDNA. There appear to be few if any fixed morphological, ecological, or behavioral differences between *E. c. filholi* and *E. c. chrysocome* outside of characters related to the coloration of the patch of skin at the base of the bill (Jouventin 1982) and any other phenotypic diversity that does exist among these taxa could likely be clinal. Outside of our model based BPP analysis, an approach known to over split lineages, there are no multilocus analyses that suggest separate species lineages in southern rockhoppers. Frugone et al. (2018) however suggested that *E. c. filholi* and *E. c. chrysocome* are well delimited species lineages but this was based only on the mtDNA hypervariable region and ABGD and GMYC analyses, the latter of which, like model approaches in BPP, tends to over split lineages (Carstens et al. 2013).

However, we did uncover evidence of population structure between *E. c. chrysocome* and *E. c. filholi*, even if there was not enough independence between these 2 groups to warrant elevating the subspecies to species status. Interestingly, again the pattern resembles that in spectacled and white-chinned petrels (Techow et al. 2009). The species-level divergence time between a northerly distributed spectacled petrel and a southern white-chinned petrel (0.90 Ma) is similar to that for *E. moseleyi* and *E. chrysocome* (0.97 Ma) and divergence times between eastern and western groups within the southerly distributed white-chinned petrel (0.35 Ma) closely resembles the divergence time between subspecies within *E. chrysocome* (0.48 Ma, IMA2p; 0.5 Ma, \*BEAST; this study). This again suggests similar drivers of diversification for both species complexes occurring during the mid to late Pleistocene.

Genetic data in our study was congruent with observational data indicating a far-ranging capacity for dispersal in *Eudyptes* penguins (Moors et al. 1984; Tennyson et al. 1989; de Dinechin et al. 2007; Demongin et al. 2010) and willingness for *Eudyptes* penguins to form interspecific pairs with vagrants (Morrison et al. 2014; Crofts et al. 2015). Structure, IMA2p, PHRAPL, and a heuristic approach in BPP all suggest migration between the 2 recognized subspecies within *E. chrysocome*. We found evidence of gene flow between *E. chrysocome* subspecies with significantly greater gene flow from *E. c. filholi* populations to *E. c. chrysocome* populations in an IMA2p analysis. The direction of gene flow in this analysis is consistent with the fact that *E. c. filholi* has a much larger effective population size compared with *E. c. chrysocome*. It is unclear however as to whether alleles from *E. c. filholi* to *E. c. chrysocome* populations in South America and the Falkland (Malvinas) Islands are being introduced from the Indian Ocean or from the South Pacific (the latter corresponding to the easterly flow of the ACC). Tennyson and Miskelly (1989) recorded 2 adult penguins identified as *E. c. chrysocome* on North East Island, Snares Islands, New Zealand in December 1985 and November 1986 indicating that *E. chrysocome* likely has

substantial capacity for circumpolar dispersal. Also, Oehler et al. (2018) found significant winter dispersal of *E. c. chrysocome* on Noir Island in Chile including male dispersal >3000 km west into the Pacific.

### No Population Structure among Breeding Colonies within Subspecies

While we uncovered evidence for population structure between *E. c. chrysocome* and *E. c. filholi*, we found strong evidence of genetic admixture among colonies within both *E. c. chrysocome* and *E. c. filholi* based on a MrBayes ND2 tree, ND2 haplotype network, multilocus nuclear intron  $F_{ST}$ , Structure and PHRAPL. Colonies showed significant sharing of mitochondrial and nuclear haplotypes within each taxon even when colonies were widely separated, as in the case of the Campbell, Antipodes and Auckland colonies and those on the island of Crozet (separated by approximately 7800 km, Auckland Island—Crozet). PHRAPL analyses within *E. c. chrysocome* and *E. c. filholi* revealed different geographic clusters of colonies within each subspecies were acting as single lineages, or perhaps lineages with symmetric gene flow, even when the geographic clusters were widely separated.

These results are consistent with those of other penguin species showing high genetic admixture among breeding colonies (Freer et al. 2015; Clucas et al. 2016, 2018; Cristofari et al. 2016). Structured populations and ongoing diversification in penguins, as observed in gentoo penguins (*Pygoscelis papua*), appears to be the exception rather than the rule and limited to penguins with a comparatively small at-sea range confined to coastal shelves or cases where distributions are intersected by oceanographic fronts. Divergence within gentoo penguins is similar to that between northern and southern rockhoppers in that it too is mediated by an oceanic front, in the case of gentoo penguins the more southerly APF acts as a barrier and the STF is the barrier in the case of rockhoppers (Levy et al. 2016; Vianna et al. 2017; Clucas et al. 2018).

### Effective and Census Population Sizes

*Eudyptes chrysocome filholi* was estimated to have a greater  $N_e$  (121 127) compared with both *E. c. chrysocome* (76 413) and *E. moseleyi* (61 064, Table 3, Figure 6). Census population sizes for breeding adults ( $N_b$ ) compiled by Bird Life International estimates approximately 1 694 890 individuals for *E. c. chrysocome*, 856 378 individuals for *E. c. filholi*, and 480 600 for *E. moseleyi* (BirdLife International 2017a, 2017b) resulting in  $N_e/N_b$  ratios of 0.05, 0.14, and 0.13 for *E. c. chrysocome*, *E. c. filholi*, and *E. moseleyi*, respectively. These values are similar to, or in the case of *E. c. chrysocome* considerably lower than,  $N_e/N_b$  ratios for other wildlife species (Frankham 1995; Palstra et al. 2008). A number of underlying causes may be responsible for the differences in the  $N_e/N_b$  ratios among these taxa and their comparatively low values, especially for *E. c. chrysocome*. Long-term skews in sex ratios may be in part responsible for these comparatively low  $N_e/N_b$  ratios. Donald (2007) showed a correlation between male-biased sex ratios in birds and IUCN status (Endangered and Critically Endangered) with relatively more males in species under greater threat. This suggests population declines may be affecting male and females differently and in turn negatively affecting  $N_e$ . Ainley and DeMaster (1980) found in Adélie penguins that females breed at a younger age and within any mature age class there is a greater proportion of females breeding than males, however, because breeding is risky there is a higher mortality in females resulting in a male-skewed sex ratio in older age classes.

Pichegru and Parsons (2014) also found that female African penguins (*Spheniscus demersus*) experience higher mortality resulting in male-biased sex ratios. In a colony with high sea lion predation rates, breeding female *E. c. filholi* penguins had lower survival rates than males in a year of nutritional stress when males greatly reduced their provisioning rates (Morrison et al. 2016a, 2016b). Further studies on both nestling and adult sex-ratios in *Eudyptes* penguins would be useful in determining if male-skewed sex ratios may have contributed to a lower  $N_e$ , particularly in threatened populations. Other factors may also contribute to low  $N_e/N_b$  ratios. Lescroel et al. (2009) found that Adélie penguins at Cape Crozier, Ross Island varied significantly in reproductive success with strong selection imposed at the first bout of reproduction. Additionally, population monitoring of Adélie, chinstrap and gentoo penguins over a 38-year period at Signy Island, South Orkney Islands revealed significant population fluctuations (Dunn et al. 2016). All of these factors including biased sex ratio, variation in individual reproductive success, and long-term population fluctuations may be contributing to comparatively low  $N_e/N_b$  ratios in rockhopper penguins.

### Conclusions

Penguin populations fluctuate with the productivity of the oceans and as such act as sentinels of changes in marine ecosystems (Boersma 2008). Understanding their population history is crucial in placing their current conservation plight in context. We hope these findings will inform conservation and management decisions regarding rockhopper penguins. While clearly *E. chrysocome* and *E. moseleyi* should be delimited as separate species, there is little evidence in our results to justify elevating the currently recognized southern rockhopper penguin subspecies (*E. c. chrysocome* and *E. c. filholi*) to species status. Unless additional data were to warrant otherwise, *E. chrysocome* should be viewed as a single widespread species. However, given the evidence of significant population structure between eastern and western regions, *E. chrysocome* should be managed within distinct geographical units corresponding to the currently recognized subspecies.

### Supplementary Material

Supplementary data are available in *Journal of Heredity* online.

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## Data Availability

We have deposited the primary data underlying these analyses as follows:

Sampling locations, sequence alignments, input parameter files for \*BEAST and IMA2p, raw output from IMA2p: Dryad (doi: 10.5061/dryad.6c8d45m).

DNA sequences: Genbank accession numbers (Supplementary Table 3).

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