

Genetic evidence for three species of rockhopper penguins, *Eudyptes chrysocome*

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Abstract The taxonomy of rockhopper penguins, *Eudyptes chrysocome* (Forster 1781), is contentious. Some authorities recognise three subspecies based on morphological differences and geographical separation of breeding populations while others suggest that morphological differences support classifying rockhopper penguins as two distinct species. The taxonomy of rockhopper penguins is of more than academic interest as breeding colonies worldwide have declined markedly in size since the 1930s and rockhopper penguins are currently listed as vulnerable by the IUCN. We compared the genetic distances between three mitochondrial gene regions from the three putative rockhopper penguin subspecies with the distances between various penguin sister species to clarify the taxonomy and systematics of rockhopper penguins. Genetic distances between the rockhopper penguin taxa, relative to other closely related penguin species, support reclassifying the three rockhopper penguin subspecies as species. Reclassification of rockhopper

penguins as three species could result in their conservation status being upgraded from vulnerable to endangered.

Introduction

Taxonomy is a crucial tool for understanding biodiversity and setting priorities for the management of species in danger of extinction (McNeely 2002). The conservation status of species is dependent on several factors including population size and distribution (e.g. The IUCN Red List 2004). If the taxonomy of species is incorrect, population size and distribution is not known accurately and therefore conservation management priorities and strategies cannot be assigned correctly.

Rockhopper penguins, *Eudyptes chrysocome* (Forster 1781) have a circumpolar distribution, breeding on small subantarctic and southern temperate islands (Marchant and Higgins 1990). There is disagreement about the taxonomy of rockhopper penguins. Currently, most authorities recognise three subspecies of rockhopper penguins that differ morphologically (del Hoyo et al. 1992; Marchant and Higgins 1990). The southern rockhopper, *Eudyptes c. chrysocome* breeds on the Falkland Islands and the islands around Cape Horn, South America. The eastern rockhopper penguin, *Eudyptes chrysocome filholi* Hutton 1879, breeds on Marion Island and other islands in the eastern sector of the southern Indian Ocean, Macquarie Island and the New Zealand subantarctic islands. The northern rockhopper penguin, *Eudyptes c. moseleyi*

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Mathews and Iredale 1921, breeds on Tristan da Cunha and Gough Islands in the South Atlantic, and Amsterdam and St Paul Islands in the middle of the southern Indian Ocean (del Hoyo et al. 1992). The three subspecies are distinguished by differences in the length of the tassels of the crests, the size and colour of the fleshy margin of the gape, colour pattern on the underside of the flipper and differences in the size of the superciliary stripe in front of the eye. Additionally, the northern rockhopper penguin is larger than the other two subspecies (Marchant and Higgins 1990; Williams 1995). Behavioural characteristics have also been found to vary between northern rockhoppers and their conspecifics (Jouventin 1982).

It has also been suggested that *E. chrysocome* should be reclassified as two species consisting of the southern rockhopper, corresponding to *E. c. chrysocome* and *E. c. filholi*, and the northern rockhopper, corresponding to *E. c. moseleyi* (Cooper et al. 1990; Jouventin 1982). Penguins appear to be morphologically conservative (Fordyce and Jones 1990) and it is likely that the marine lifestyle of penguins somewhat constrains the development of unique morphological characters. Analysis of DNA is shedding new light on penguin relationships (Baker et al. 2006) and sequences for mitochondrial genes have been used to identify other cryptic penguin populations (Banks et al. 2002).

The distributions of the ectoparasitic chewing lice of rockhopper penguins also suggest that the three rockhopper penguin populations may not be as connected as is generally thought. Chewing lice have limited mobility away from their hosts and are thought to rely on close contact between hosts to transfer to new host taxa (Marshall 1981). Penguins are parasitised by 15 species of lice in two genera, *Austrogoniodes* and the monotypic *Nesiotinus*. Within rockhopper penguins, the host-specific louse *Austrogoniodes keleri* Clay 1967 is present only on southern rockhoppers; the multi-host *Austrogoniodes concii* (von Kéler 1952) parasitises only northern rockhoppers and *Austrogoniodes hamiltoni* Harrison 1937 parasitises only eastern rockhoppers (Price et al. 2003).

Worldwide, rockhopper penguin populations have decreased markedly. For example, a survey conducted in 2000/2001 estimated that populations of southern rockhopper penguins in the Falkland Islands have declined to 20% of the number present in 1932/33 (Pütz et al. 2003). Likewise, eastern rockhopper penguins breeding on Campbell Island have decreased from an estimated 1.6 million breeding birds in 1942 to 103,000 birds in 1985 (Cunningham and Moors 1994). It has been suggested that competition with fishing fleets for prey, oil exploitation (Bingham 2002), introduced

predators, human collection of eggs, hunting of adults for fishing bait (Birdlife International 2000) and/or increasing sea temperatures (Cunningham and Moors 1994) (Guinard et al. 1998) are responsible for the decline. In 2000, the IUCN-World Conservation Union reclassified the species as vulnerable (from lower risk) due to this population decline. The IUCN Red List (IUCN 2004) also notes that the status of rockhopper penguins may warrant upgrading to endangered (facing a very high risk of extinction in the wild) if the subspecies were reclassified as full species. Given the magnitude, speed and extent of the population declines, clarification of the alpha taxonomy of *E. chrysocome* is urgently required.

Methods

Blood samples were collected from wild rockhopper penguins (Fig. 1; Table 1) as described in Chérel et al. (2005). DNA was extracted from blood samples using QIAGEN DNeasy kits following the manufacturer's protocol for extraction from animal tissues. Portions of three mitochondrial genes, the small ribosomal subunit (12S) cytochrome *b* (cytb) and cytochrome oxidase subunit 1 (COI) were amplified using polymerase chain reactions (PCR). Polymerase chain reactions were carried out with an Eppendorf Mastercycler thermal cycler and consisted of 2.5 μ L of Hotmaster buffer (Eppendorf), 1.2 μ L of dNTPs (8 mM), 2.5 μ L of each primer (2.5 μ M), 0.125 μ L of Hotmaster *Taq* (5 units/ μ L, Eppendorf), 0.8 μ L of DNA and 15.375 μ L of water. A negative control was incorporated in each amplification round using water rather than DNA. Primers are specified in Table 2. Sequencing was carried out on an ABI 3730 capillary sequencer.

We combined the portions of our sequences (GenBank accession numbers DQ525741–DQ525800) that were homologous with the sequences of Baker et al. (2006) (GenBank accession numbers DQ137169–DQ137204, DQ137209–DQ137226) and aligned them manually in MacClade (Maddison and Maddison 1992). Phylogenetic trees were estimated using maximum parsimony (MP) and neighbour joining (NJ) as implemented in Paup*4.0b10 (Swofford 2002).

We also conducted a mixed model Bayesian analysis using Mr Bayes 3.1 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) with three partitions corresponding to the three gene regions. Models for the Bayesian partitions were general time-reversible (GTR) (Tavaré 1986) plus proportion invariable plus gamma (Rodríguez et al. 1990; Yang et al. 1994) for 12S and cytb regions, and Hasegawa-Kishino-Yano 85

Fig. 1 Collection sites (arrows) of the rockhopper penguin samples

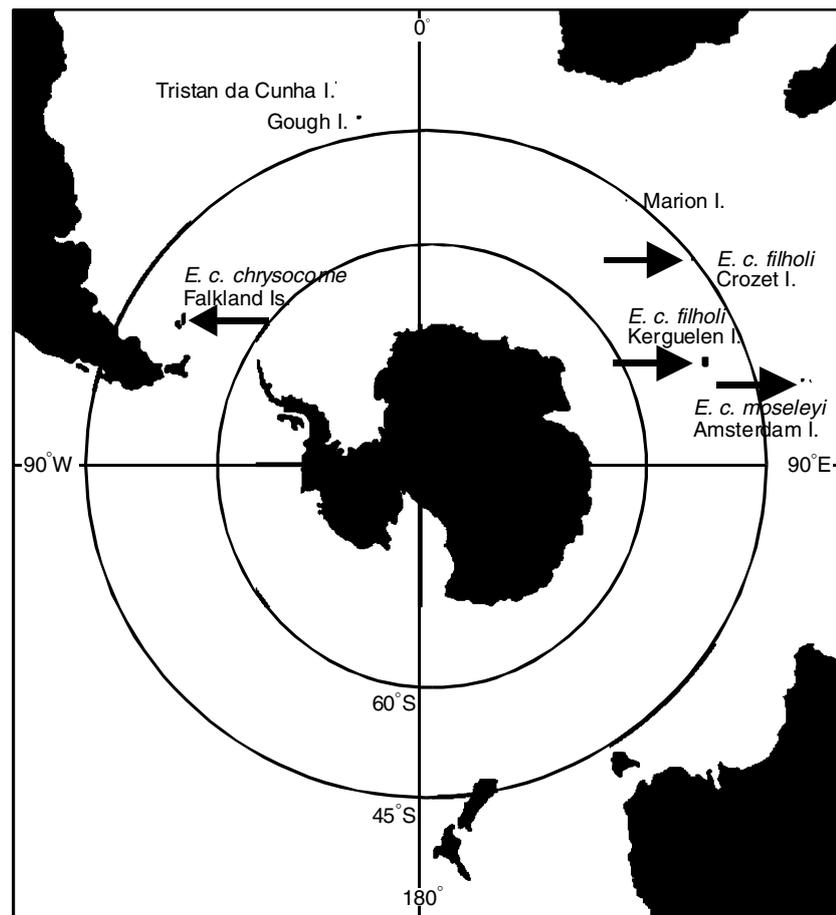


Table 1 Collection location and number of *Eudyptes chrysocome* sequenced

Taxon	Collection location	Number of individuals sequenced
<i>Eudyptes c. chrysocome</i> southern rockhopper	New I, Falkland Islands group	5
<i>Eudyptes c. filholi</i> eastern rockhopper	Crozet I	5
	Kerguelen I	5
<i>Eudyptes c. moseleyi</i> northern rockhopper	Amsterdam I	5

Table 2 Primers used to sequence portions of the small ribosomal subunit (12S) cytochrome *b* (*cytb*) and cytochrome oxidase subunit 1 (COI). R = A or G, Y = C or T

Gene	Primer name	Primer sequence	Reference
12S	12SA1	ACTGGGATTAGATACCCCACTAT	Modified from Kocher et al. (1989)
	12SR1	GAGGGTGACGGGCGGTATGTACG	Modified from Kocher et al. (1989)
COI	LCOI5508	ACTYATCCGCGCAGAACTTGGCC	This study
	HCOIrev	RACTTCTGGGTGACCAAAAAATC	This study
<i>cytb</i>	<i>cytbF</i>	ACAGGCATTATCCTCCTRCTCAC	This study
	<i>cytbR</i>	AGAGGATTAGGAGGATGGTGAAG	This study

(HKY) (Hasegawa et al. 1985) plus gamma (Yang et al. 1994) for COI. Models were selected using MrModelTest Test (Nylander 2004). Mr Bayes estimated the model parameters from the data using one cold and three heated Markov chains. The Monte

Carlo Markov chain length was 2,000,000 generations and we sampled the chain every 100 generations. We discarded the first 5,000 samples as burnin and thus estimated our phylogeny and posterior probabilities from a consensus of the last 15,000 sampled trees.

Genetic distances for individual genes within the three subspecies were compared with genetic distances between the three subspecies. The rockhopper penguin inter-subspecific genetic distances were also compared with the genetic distances between other sister penguin species as listed in Figs. 3–5 (Genbank accession numbers DQ137187, DQ137188, DQ137190–DQ137193, DQ137196, DQ137197, DQ137199–DQ137204, DQ137170, DQ137171, DQ137173–DQ137182, DQ137185, DQ137186, DQ137210, DQ137211, DQ137213–DQ137220, DQ137223, DQ137225, DQ137226). Sister species for comparison were selected from the phylogeny of Baker et al. (2006).

Rockhopper penguin sequences were pooled according to subspecies to test the partitioning of genetic variance between the three subspecies using AMOVA (Excoffier et al. 1992) as implemented in Arlequin 3.01 (Excoffier et al. 2005).

Results

We analysed 1441 nucleotides (nt) of three mitochondrial genes (12S = 394nt, COI = 379nt and cytb = 668nt). The genetic distances between the three clades were much greater than the distances within clades (Tables 3, 4). An MP analysis of the three genes concatenated found a single most parsimonious tree with three distinct clades that clearly corresponded to the three putative subspecies (Fig. 2).

NJ and the Bayesian analysis (results not shown) also found the same relationships for the rockhoppers as MP. Monophyly of each of the three rockhopper clades was strongly supported by Bayesian posterior probabilities of 1.00 for the *E. c. chrysocome* and *E. c. moseleyi* clades and 0.88 for *E. c. filholi*.

Uncorrected pairwise genetic distances between the three rockhopper subspecies were similar to the genetic distances between other penguin sister species (Figs. 3, 4, 5).

The AMOVA analysis found that 92% of the molecular variation was due to differences between the three subspecies. The Φ_{ST} value (0.922, $df = 2$) was highly significant ($P < 0.001$).

Discussion

Phylogenetic trees with limited or shallow phylogeographic structure are indicative of species that have occupied ranges free of impediments to gene flow;

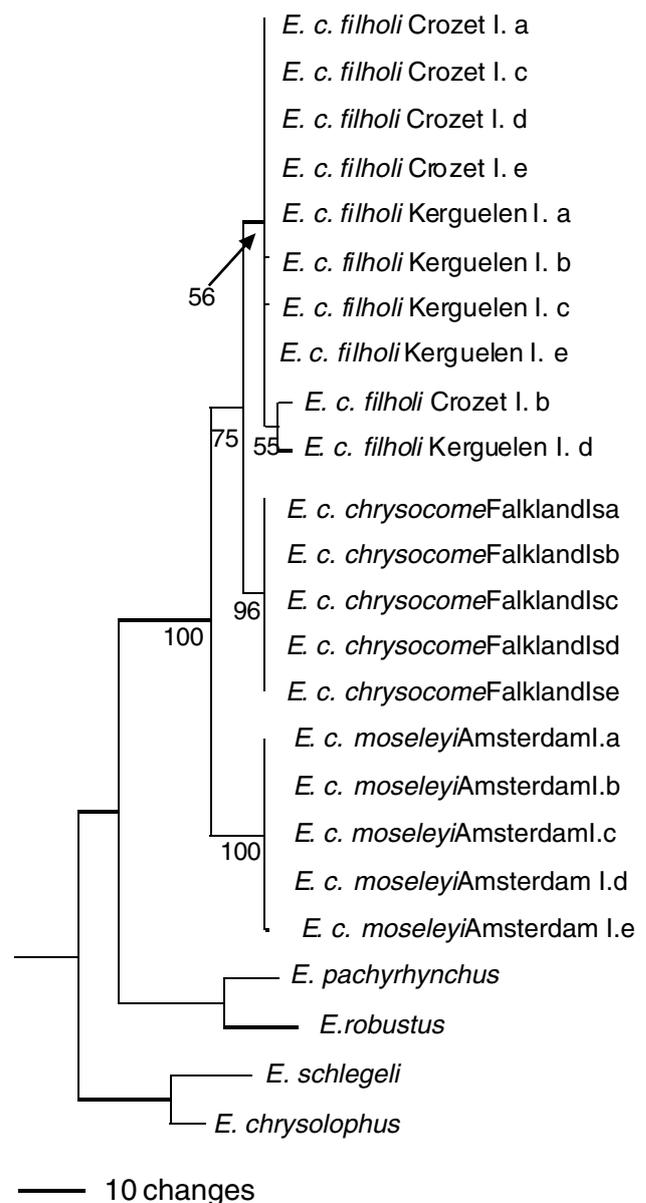


Fig. 2 Single most parsimonious tree for the three rockhopper penguin subspecies (tree length = 986; consistency index (CI) excluding uninformative characters = 0.50; homoplasy index (HI) excluding uninformative characters = 0.4988). The letters *a–e* differentiate individuals. Other penguin taxa are trimmed from the phylogeny for clarity. Numbers below the branches indicate non-parametric bootstrap support values >50 (of 100 replicates) for the rockhopper penguin nodes. Bootstrap values for the other *Eudyptes* penguin nodes are not shown

conversely, geographically separated populations that form monophyletic groups are suggestive of long-term barriers to gene flow (Avice 1994). The three monophyletic clades we found from the MP, NJ and Bayesian analyses of the rockhopper penguins sequences (Fig. 2) suggest there have been barriers to the exchange of mitochondrial genes.

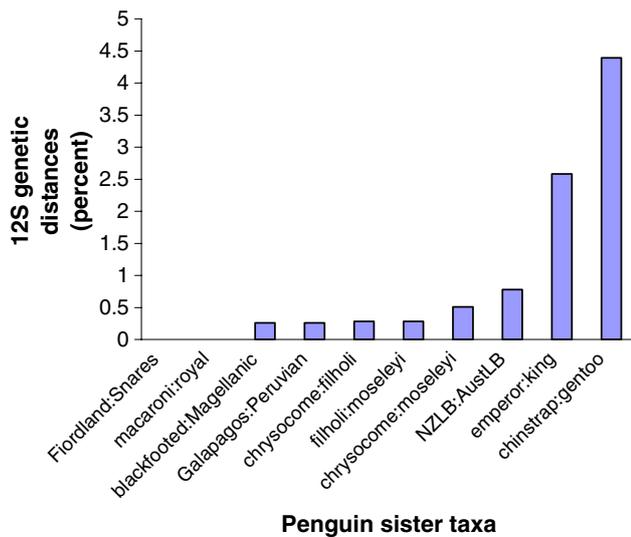


Fig. 3 Comparison of uncorrected pairwise distances for various penguin sister taxa for the small ribosomal subunit (*12S*) region. *NZLB* New Zealand little blue penguin, *Eudyptula minor* and *AustLB* Australian little blue, *E. minor* as discussed in Banks et al. (2002) and Baker et al. (2006)

The level of variation between the three rockhopper subspecies compared with the levels of variation between other penguin sister taxa (Figs. 3–5), support elevating the three subspecies to species. Reclassification of the three subspecies as full species may result in the conservation status of rockhopper penguins being changed from vulnerable to endangered by the IUCN due to the smaller population size and limited distributions of the new species. Although the genetic dis-

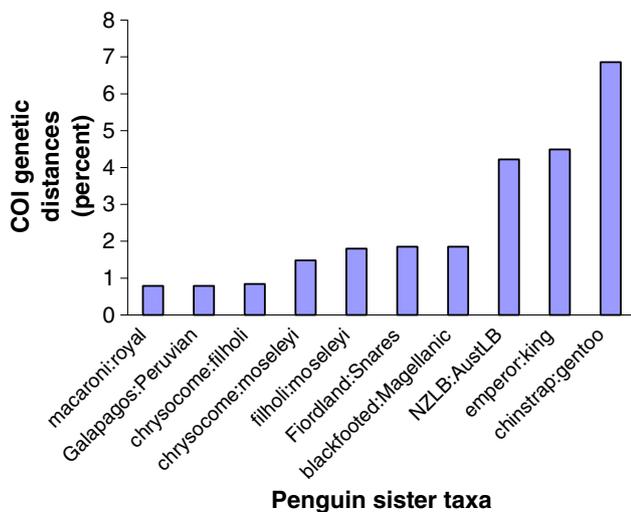


Fig. 4 Comparison of uncorrected pairwise distances for various penguin sister taxa for the cytochrome oxidase subunit 1 (*COI*) region. *NZLB* New Zealand little blue penguin, *Eudyptula minor* and *AustLB* Australian little blue, *E. minor* as discussed in Banks et al. (2002) and Baker et al. (2006)

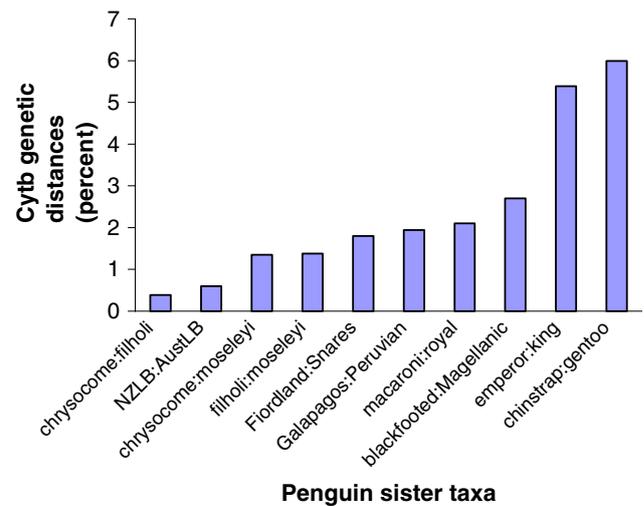


Fig. 5 Comparison of uncorrected pairwise distances for various penguin sister taxa for the cytochrome *b* (*cytb*) region. *NZLB* New Zealand little blue penguin, *Eudyptula minor* and *AustLB* Australian little blue, *E. minor* as discussed in Banks et al. (2002) and Baker et al. (2006)

Table 3 Rockhopper intra-subspecific genetic distances for the three mitochondrial genes sequenced

Gene region	Southern (%)	Eastern (%)	Northern (%)
12S	0	0.05	0
COI	0	0.1	0.08
cytb	0	0.2	0

Abbreviations: *12S* small ribosomal subunit; *COI* cytochrome oxidase subunit 1; *cytb* cytochrome *b*

Table 4 Rockhopper inter-subspecific genetic distances for the three mitochondrial genes sequenced

Gene region	Southern: eastern (%)	Southern: northern (%)	Eastern: northern (%)
12S	0.3	0.5	0.3
COI	0.8	1.5	1.8
cytb	0.4	1.4	1.4

Abbreviations: *12S* small ribosomal subunit; *COI* cytochrome oxidase subunit 1; *cytb* cytochrome *b*

tances show that eastern and southern rockhoppers are more closely related to each other than either is to northern, our results do not support classifying rockhopper penguins as two species.

There are many definitions of species and although many definitions are similar (Hey 2001), there is no clear definition of species (Agapow et al. 2004). In this study, we use a phylogenetic species concept (PSC) (Cracraft 1983) that interprets the monophyly of the three rockhopper clades as suggesting the three

populations should be classified as species. It has been argued that phylogenetic species are the most useful for conservation for reasons such as the size of populations are estimated more accurately (Cracraft 1997, for a fuller list of reasons). Others have suggested that the PSC inflates the number of species, and therefore the number of endangered species, by reducing the size of populations and ranges, leading to apathetic attempts to manage endangered species (Agapow et al. 2004). We would argue that, given the marked decline of rockhopper penguin populations and the relatively high levels of genetic diversity between the populations, reclassifying the three rockhopper subspecies as species is appropriate. Reclassification is also supported by morphological differences and the allopatric distribution of rockhopper penguins. Reclassification of the rockhopper subspecies may provide impetus to developing management strategies to reverse the population declines.

The close relationship we found between eastern and southern rockhoppers is also supported by shared morphological characters as the two subspecies share shorter plumes (in comparison with northern rockhoppers), a small area of black plumage at the tip of the underside of the flipper and a thin grey leading edge on the underside of the flipper (Marchant and Higgins 1990; Williams 1995). Morphological characters also support the genetic distinctiveness of the northern rockhopper with its broad superciliary stripe, very long crests reaching past the junction of the black and white plumage on the throat, large area of black plumage on the tip of the underside of the flipper and a thick black leading edge on the flipper (Marchant and Higgins 1990; Williams 1995). The distinctiveness of northern rockhoppers within the rockhopper group is also supported by differences in the date of maximal egg laying and call characteristics (Jouventin 1982).

Currently, there are no obvious barriers to gene flow between the rockhopper penguin subspecies. Oceanographic fronts have been suggested as a barrier to dispersal for other species. For example, there are genetic differences between populations of Patagonian toothfish on either side of the Antarctic Polar Front (AF), a region where northward moving Antarctic surface waters sink below subantarctic waters (Shaw et al. 2004). Northern rockhoppers breed north of the Subtropical Front (STF), which is marked loosely in the Indian Ocean by the southern limit of water warmer than 12°C at 200-m depth (Park et al. 1993). Other rockhopper penguin breeding colonies lie further south, south of the Subantarctic Front (SAF) (del Hoyo et al. 1992). In the Indian Ocean, the main biogeographical barrier for fish is the SAF (Koubbi 1993). North of Kerguelen and

Crozet Islands, the STF, SAF and AF converge abruptly separating warm subtropical water to the north from cold, oxygen-rich water from the sub Antarctic (Park et al. 1993) and it seems likely that this frontal system is a more probable barrier to the exchange of mitochondrial genes than mere distance. Northern rockhoppers breeding on Tristan da Cunha and Gough Islands in the South Atlantic Ocean and on Amsterdam and St Paul Islands in the southern Indian Ocean are separated by approximately 7,500 km and yet Amsterdam (northern rockhopper) and Crozet Islands (eastern rockhopper) that lie north and south of the STF/SAF, respectively are separated by only 2,300 km. Rockhoppers have also been recorded travelling large distances during the non-breeding season. For example, both eastern and southern rockhoppers have been recorded on the Snares Islands, which are at least 900 km from the closest rockhopper breeding grounds (Miskelly et al. 2001).

The basal position of northern rockhoppers suggests that the rockhopper lineage was derived from a speciation event following a long-distance colonisation event when the common ancestor of the rockhopper, Fiordland and Snares crested penguins crossed the STF. Given that the majority of *Eudyptes* species breed south of the STF, it seems most likely that the colonisation was from south to north of the STF. Although Fiordland and Snares crested penguins straddle the STF today, the STF has varied historically, at times lying north (Knox 1990) of the present day distributions of Fiordland and Snares crested penguins (Marchant and Higgins 1990). Divergence between the northern rockhopper, and the southern and eastern populations could have followed a dispersal event by the ancestral rockhopper penguin south of the STF.

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