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DNA barcodes highlight two clusters within the little penguin (*Eudyptula minor*): time to reassess species delineation?

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Abstract The use of DNA barcodes (haplotypic variation in a 648 bp segment of the cytochrome *c* oxidase subunit I [COI] gene within the mitochondrial genome, starting from base 58 at the 5′ end of the gene) as part of a species description is an accepted part of modern taxonomy. The evidence COI provides is compelling since a sequence of DNA is biological data obtained from living material. Early in the use of COI, it became apparent that it might highlight potential cryptic species and inform the debate around their status. The little penguin (*Eudyptula minor*) has been the subject of such debate. DNA barcodes from 53 little penguins were assessed to determine the specific status of this species across its range. Analysis of these data indicates distinct Australian and New Zealand haplotypes that may be indicative of separation at the species level. The specific status for the 2 populations is also supported by behavioural evidence and geographic isolation.

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Keywords *Eudyptula minor*; DNA barcode; COI; haplotype

INTRODUCTION

The use of DNA sequences as part of a species description is an accepted part of modern taxonomy (Rubinoff 2006). For a number of years the Consortium for the Barcode of Life (CBOL) has promoted the use of a 648 bp segment of the cytochrome *c* oxidase subunit I (COI) gene, starting from base 58 at the 5′ end of the gene, to provide a standardised comparator with which to distinguish species (Hebert & Gregory 2005. COI is a component of a membrane-bound enzyme found in the mitochondrial cristae that is involved in cellular respiration. Therefore, COI is under a significant level of structural and functional constraint (Waugh 2007). However, the nucleotides of the COI gene show sufficient variation (usually in the non-coding third position) to allow for differentiation between species (Hebert *et al*. 2003). Moreover, insertions and deletions are rare (Blaxter 2003). In general, the longer 2 species have diverged from each other over evolutionary time, the greater the number of nucleotide differences between their sequences. Thus, sequence distance approximates evolutionary distance. Intraspecific variation in this gene is generally <10% of that observed between congeneric species. This particular segment of the COI gene is commonly referred to as the "DNA barcode" and, for brevity's sake, that term or the abbreviated "barcode" will be used in this paper.

For taxonomic purposes, haplotypic variation in sequences of the COI gene can be used as part of a species description. In addition, this data can be

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Table 1. The 6 subspecies that have been recognised in *Eudyptula minor*. The current checklist of the Birds of New Zealand does not recognise any subspecies.

Subspecies	Distribution
E. m. novaehollandiae	Southern Australia & Tasmania
E. m. iredalei	Northern North Island
E. m. variabilis	Southern North Island & Cook Strait
E. m. albosignata	Eastern South Island
E. m. minor	Western South Island & Stewart Island
E. m. chathamenssis	Chatham Islands

used to provide information to precipitate or assist discussions about species delimitation (Tavares & Baker 2008). The evidence COI provides is particularly compelling since a sequence of DNA is biological data obtained from living material, and is not subject to variations with age or season (Waugh 2007).

DNA barcoding has been particularly useful in identifying otherwise unidentifiable remains following predation or birdstrike on aircraft (Waugh *et al.* 2011; Yang *et al.* 2010; Galbraith *et al.* 2015). Early in the use of COI, it became apparent that COI might be useful for highlighting potential cryptic species and informing the debate around their status (Velona *et al.* 2015; Smith *et al.* 2011; Saitoh *et al.* 2015; Allabadian *et al.* 2013; Johnsen *et al.* 2010; Kerr *et al.* 2007; Penton *et al.* 2004). For example, Kerr *et al.* (2007) identified 15 putative species among the birds of North America that contained 2 distinct barcode clusters, which they suggested represent cryptic species. Thus, the analysis of barcode sequences for the birds of New Zealand is likely to highlight species in which such distinct clusters are found.

While speciation may result in genetic divergence, speciation is not caused by genetic divergence. Ultimately, it is for taxonomists to conclude whether these DNA clusters are real expressions of taxonomic differences or merely extreme intraspecific genetic variation (Waugh 2007). A comprehensive DNA barcode characterisation of New Zealand's avifauna is largely complete and awaits publication (Waugh 2011).

Up to 6 subspecies of the Australasian species, little penguin (*Eudyptula minor*), have been recognised (Kinsky & Falla 1976) based on morphological characteristics such as back colour, flipper pattern and measurements as well as distribution (Table 1). Furthermore, the genus was considered to contain 2 species until 1976 when they were amalgamated (Banks *et al.* 2002; Heather & Robertson 2005).

Baker *et al.* (2006) reported a genetic split between a white-flippered variant found on the Banks Peninsula and birds from other areas around New Zealand. Two mitochondrial DNA clades were identified by Banks *et al.* (2002) who observed divergence between little penguins from Australia and those from Otago and the rest of New Zealand. Tavares & Baker (2008) found intraspecific clusters in DNA barcodes from New Zealand and Australian birds (Tavares & Baker 2008). A recent study, employing a variety of genetic markers other than COI, identified 2 congeneric taxa within *Eudyptula*; one from Australia and one from New Zealand (Grosser *et al.* 2015). Moreover, they suggest that the Australian clade has recently migrated to the Otago region. However, the specific sequences used have not been subject to the same calibration testing as the COI barcode and the degree of variation at these loci that would indicate separation at the species level is not clear.

Eudyptula minor has been the subject of ongoing debate but is currently recognised as a single species without designation of subspecies in the most recent Checklist of the Birds of New Zealand because, "In view of the continuing uncertainty of the taxonomic status of these various populations, including white-flippered birds often classified as *E. albosignata* or *E. minor albosignata*, we have placed all the little penguins in one species *E. minor* and not recognised any subspecies" (Gill *et al.* 2010). The aim of this paper is to cast light, using DNA barcodes, on this discussion.

METHODS

DNA barcode sequences (a 648 bp segment of the cytochrome *c* oxidase subunit I gene, starting from base 58 at the 5′ end of the gene) from 53 specimens of *E. minor* (Table 2) were analysed using Bayesian inference of phylogeny (see Table 1 for accession numbers and details of specimen location). The program employed for this analysis was MrBayes (Huelsenbeck *et al*. 2011), which uses Markov chain Monte Carlo (MCMC) methods to estimate the posterior distribution of model parameters. One million cycles were run before a phylogenetic tree with the highest possible posterior probability was produced. Burn in time was determined by assessment of the number of generations required for the standard deviation of split to reach a value of <0.01. In addition, Kimura-2-parameter (K2P) genetic distance correction analysis was carried out. Software for K2P analysis, available on the Barcode of Life website, was used (BOLDSystems 2015). Both these methods build phylogenetic trees based on genetic distance.

Table 2. Details of *Eudyptula minor* specimens analysed in this study. Specimens designated as the white-flippered morph are indicated.

Accession number	Number of bases	Location	GPS coordinates	Notes
EU525350	914	Wellington	41.15 S, 174.506 E	
EU525351	896	Wellington	41.15 S, 174.506 E	
EU525352	902	Wellington	41.15 S, 174.506 E	
EU525353	908	Wellington	41.15 S, 174.506 E	
EU525354	913	Wellington	41.15 S, 174.506 E	
EU525355	671	Canterbury	44.00 S, 176.50 E	
EU525356	827	No data	No data	
EU525357	719	Banks Peninsula	43.51 S, 173.03 E	White flippered
EU525358	812	Northland	36.36 S, 174.54 E	
EU525359	745	Northland	36.36 S, 174.54 E	
EU525360	841	Canterbury	43.345 S, 172.461 E	White flippered
EU525361	841	Canterbury	43.345 S, 172.461 E	White flippered
EU525362	841	Canterbury	43.345 S, 172.461 E	White flippered
EU525363	789	Canterbury	43.345 S, 172.461 E	White flippered
EU525364	841	Canterbury	43.345 S, 172.461 E	White flippered
EU525365	841	Canterbury	43.345 S, 172.461 E	White flippered
EU525366	833	Canterbury	43.345 S, 172.461 E	White flippered
EU525367	802	Canterbury	43.345 S, 172.461 E	
EU525368	764	Banks Peninsula	43.75 S, 173.13 E	White flippered
EU525369	709	Marlborough	41.748 S, 173.495 E	
EU525370	695	Marlborough	41.748 S, 173.495 E	
EU525371	687	Western Australia	32.182 S, 115.412 E	
EU525372	841	Western Australia	35.035 S, 117.563 E	
EU525373	708	Victoria	No data	
EU525374	673	Western Australia	34.122 S, 122.205 E	
EU525375	731	Great Barrier Island	36.112 S, 175.636 E	
EU525376	692	Canterbury	43.345 S, 172.461 E	White flippered
EU525377	689	Canterbury	43.345 S, 172.461 E	White flippered
EU525378	816	Marlborough	41.748 S, 173.495 E	
EU525379	742	Marlborough	41.748 S, 173.495 E	
EU525380	841	Western Australia	32.182 S, 115.412 E	
EU525381	841	Western Australia	34.122 S, 122.205 E	
EU525382	841	Western Australia	34.122 S, 122.205 E	
EU525383	841	Victoria	38.5072 S, 145.243 E	
EU525384	841	Victoria	38.5072 S, 145.243 E	
EU525385	779	Victoria	38.5072 S, 145.243 E	
EU525386	841	Victoria	38.5072 S, 145.243 E	
EU525387	841	Victoria	38.5072 S, 145.243 E	
EU525388	841	Victoria	38.5072 S, 145.243 E	

Table 2. Continued.

EU525389	841	Victoria	38.5072 S, 145.243 E
EU525390	841	Western Australia	35.035 S, 117.563 E
EU525391	841	Western Australia	35.035 S, 117.563 E
EU525392	840	Western Australia	35.035 S, 117.563 E
EU525393	839	Western Australia	35.035 S, 117.563 E
EU525394	841	Western Australia	35.035 S, 117.563 E
EU525395	841	Western Australia	32.182 S, 115.412 E
EU525396	839	Western Australia	32.182 S, 115.412 E
EU525397	728	Western Australia	32.182 S, 115.412 E
EU525398	737	Northland	36.36 S, 174.54 E
EU525399	811	Northland	36.36 S, 174.54 E
EU525400	787	Northland	36.36 S, 174.54 E
EU525401	668	Northland	36.36 S, 174.54 E
EU525402	841	Northland	36.36 S, 174.54 E
EU525403	766	Northland	36.36 S, 174.54 E

In addition, an alignment of the DNA barcode sequences from these birds was carried out using ClustalX version 0.1 (copyright 2003 Ramu & Co.) to identify sites within the barcode region that were diagnostic of any clusters observed. The DNA sequences (Table 2) referred to in this paper have been previously published by Tavares & Baker (2008).

RESULTS

Both the Bayesian tree and the K2P tree of 53 *E. minor* DNA barcodes showed 2 distinct clades correlating to broad geographical location (Fig. 1). Twenty-one specimens in 1 clade came from Australia (8 from Victoria, 13 from Western Australia). Of the 32 specimens in the second clade, 8 came from Northland, 1 from Great Barrier Island and 5 from Wellington in the North Island, while 4 came from the Marlborough region, 11 from the Canterbury region and 2 from Banks Peninsula in the South Island. One sample had no associated geographical location data, but grouped with the Australian samples.

The divergence between the Australian and New Zealand specimens was nearly 20 times (19.32) that of the divergence found within the New Zealand specimens and more than 30 times (34.07) that observed within the Australian specimens (Table 3).

Analysis of an alignment of New Zealand and Australian birds highlighted 23 diagnostic sites (nucleotide bases) within the barcode region, each of which differed between birds from the 2 geographic locations but did not differ between birds from the same geographic location. There were only 3 other nucleotide positions that differed, which were not diagnostic and rather represent *within* clade variation.

DISCUSSION

The use of DNA barcodes as part of a species description is now common place (Hebert *et al*. 2003; Ward *et al*. 2009; Vences *et al*. 2005; Ajmal Ali *et al*. 2014) and has been used to resolve taxonomic ambiguity in a variety of organisms (Lambert *et al*. 2005; Hebert *et al*. 2004; Smith *et al.* 2006; Blagoev *et al.* 2013). One species whose status has been the subject of discussion over a long period of time is *E. minor*. In this case, DNA barcodes have provided more evidence that Australian and New Zealand members of this genus form 2 distinct clusters. These are worthy of further examination to determine whether they might be regarded as separate species. On its own, divergence at the barcode region of the COI gene may not be sufficient evidence to justify such an investigation. However, when correlated with the geographic separation of the populations involved as well as with other evidence presented in the debate relating to their status (Banks *et al.* 2002; Gill *et al*. 2010; Holdaway *et al.* 2001), further investigation is warranted.

For this species, geographical isolation is more significant than might be assumed in a marine bird species capable of swimming relatively long distances. *Eudyptula minor* tends to remain within 25 km of the shore during its foraging expeditions, precluding regular trans-Tasman excursions (Croxall & Davis 1999; McKenzie 2011). It is

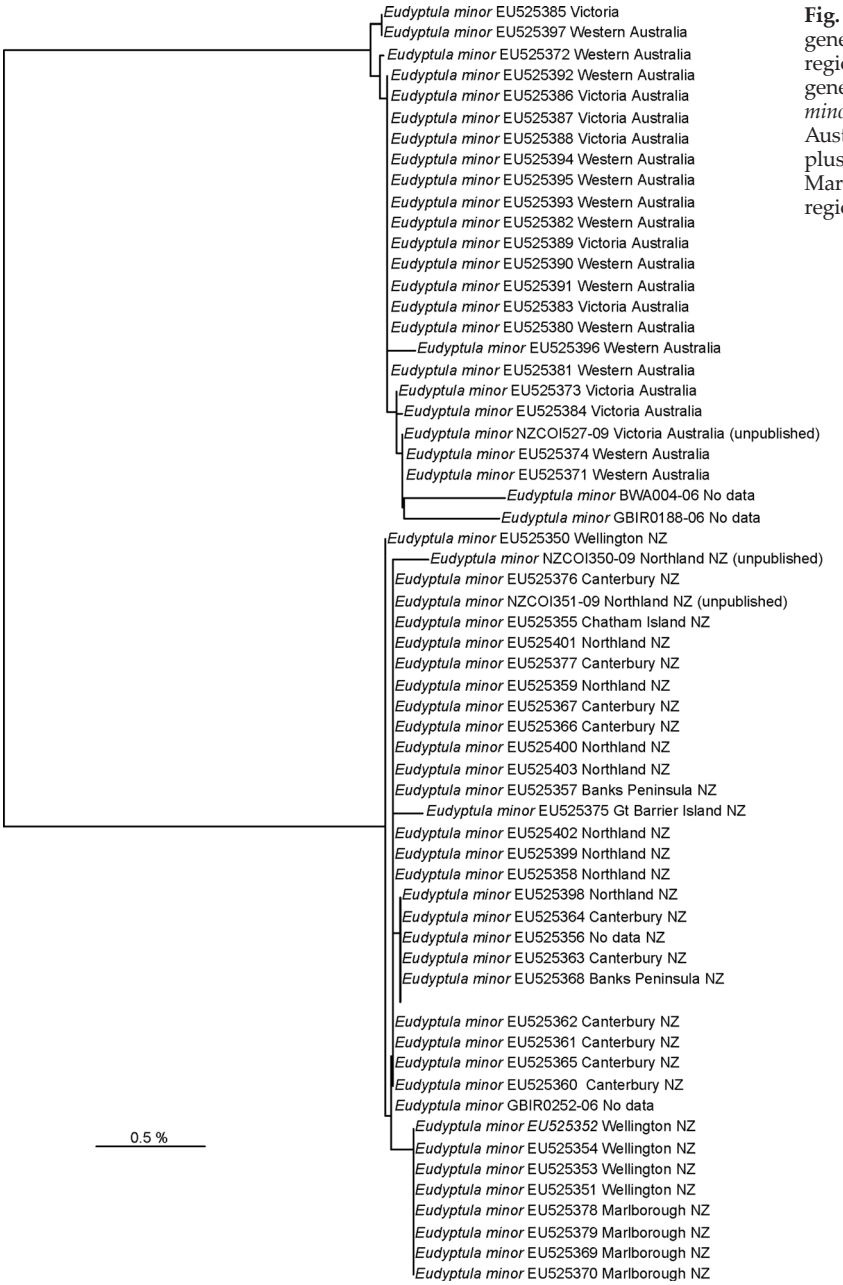

Fig. 1. A Kimura-2-Parameter genetic distance tree of the barcode region of the mitochondrial CO1 gene from specimens of *Eudyptula minor* collected in Western Australia, Victoria, Australia plus the Northland, Wellington, Marlborough and Canterbury regions of New Zealand.

noteworthy that the Australian specimens, although collected from widely separated regions, showed homogeneity at the barcode region. However, their distribution is along a continuous coastline or in the case of the Bass Strait, has numerous stepping stone islands. The same is largely true for New Zealand specimens.

DNA barcodes did not support the variety of subspecies that have been proposed in the past. Contrary to the findings of Baker *et al.* (2006), there was no apparent difference in COI haplotypes between the white-flippered morphotype found in the Banks Peninsula and Canterbury regions and those from other regions (Fig. 1). One sample obtained from Genbank and identified by Baker *et al.* (2006) as *E. albosignata* (a name sometimes given to the white-flippered morphotype) had no geographic data associated with it and is also not

Table 3. Kimura-2-Parameter genetic distance at the DNA barcode locus for 53 specimens of *Eudyptula minor* from Australia (n = 21) and New Zealand (n = 32).

	n	Comparisons	Taxa	Minimum distance (%)	Mean distance (%)	Maximum distance (%)	SE Distance (%)
Within species	53	1596	1	0	1.84	4.21	0.046
Within New Zealand specimens	32	561	1	0	0.097	0.323	0.004
Within Australian specimens	21	253	1	0	0.055	0.292	0.005

significantly different at the barcode region from the other little penguins of New Zealand. Nor were any of the other New Zealand specimens, proposed as subspecies, distinguishable by this means.

Where a debate arises over the status of a particular species, DNA barcoding offers a source of objective evidence that can help resolve issues by augmenting existing taxonomic data. In the case of *E. minor*, an unresolved debate can be assisted by this data, which supports the hypothesis that they comprise 2 species, suggesting that a review of all the evidence might be timely.

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