The Auk 126(2):397–408, 2009

Printed in USA.

RANGE-WIDE PHYLOGEOGRAPHY OF THE LITTLE PENGUIN (*EUDYPTULA MINOR*): EVIDENCE OF LONG-DISTANCE DISPERSAL

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ABSTRACT.—The Little Penguin (*Eudyptula minor*), a colonial-nesting seabird that is widespread in New Zealand and southern Australia, has high dispersal potential but exhibits regional variation in morphology, coloration, and breeding phenology. We present a distribution-wide survey of mitochondrial DNA variation in the Little Penguin to document phylogeographic relationships and genetic structuring and to test for concordance with intraspecific taxonomy. Phylogeographic structuring was absent among Australian colonies (27 localities, 94 individuals), but the distribution of haplotypes among colonies was significantly nonrandom ($\varphi_{ST} = 0.110$, $P < 0.01$). The Australian individuals exhibited close phylogenetic relationships with a subset of New Zealand birds (4 localities, 22 individuals), whereas the remaining New Zealand birds (20 localities, 106 individuals) were phylogenetically distinct, with ≥7% sequence divergence, and exhibited greater levels of genetic variation and geographic structuring ($\varphi_{ST} = 0.774$, $P < 0.05$). These patterns are consistent with earlier suggestions of an origin in New Zealand followed by recent colonization of Australia and back-dispersal to New Zealand. Extinction and re-establishment processes may have been important factors in the development of genetic structuring across a range of spatiotemporal scales. The genetic data are consistent with suggestions that a single subspecies exists in Australia, but not with the subspecies distributions within New Zealand that have been suggested on the basis of morphology and coloration. *Received 26 March 2008, accepted 3 January 2009.*

Key words: Australia, colonization, *Eudyptula minor*, Little Penguin, long-distance dispersal, mitochondrial DNA, seabird, subspecies, Tasman Sea.

Filogeografía de *Eudyptula minor* en su Área de Distribución: Evidencia de Dispersión de Distancias Grandes

RESUMEN.—El pingüino *Eudyptula minor* es un ave marina que anida en colonias y que se distribuye ampliamente en Nueva Zelanda y en el sur de Australia. Esta especie presenta un alto potencial de dispersión pero presenta variación regional en su morfología, coloración y fenología reproductiva. Presentamos un muestreo de la variación del ADN mitocondrial que abarca toda el área de distribución de esta especie para documentar sus relaciones filogeográficas y estructuración genética y también para probar la concordancia con la taxonomía intraespecífica. No existió estructuración filogeográfica entre las colonias australianas (27 localidades, 94 individuos), pero la distribución de los haplotipos entre las colonias fue significativamente diferente a la esperada por azar ($\varphi_{ST} = 0.110$, $P < 0.01$). Los individuos australianos exhibieron relaciones filogenéticas cercanas con un subgrupo de aves de Nueva Zelanda (4 localidades, 22 individuos). Las demás aves de Nueva Zelanda (20 localidades, 106 individuos) fueron filogenéticamente diferentes (≥7% de divergencia en sus secuencias) y exhibieron un mayor grado de variación genética y estructuración geográfica ($\varphi_{ST} = 0.774$, $P < 0.05$). Estos patrones son consistentes con estudios anteriores que sugirieron un origen en Nueva Zelanda seguido por una colonización más reciente hacia Australia, con un nuevo evento de dispersión hacia Nueva Zelanda. Los procesos de extinción y restablecimiento pueden haber sido factores importantes en el desarrollo de la estructuración genética a través de un amplio rango de escalas de espacio y tiempo. Los datos genéticos son consistentes con la sugerencia de que existe una única subespecie en Australia, pero no con la distribución de las subespecies que ha sido sugerida en Nueva Zelanda con base en la morfología y la coloración.

SUBSPECIES DESCRIPTIONS BASED on regional intraspecific variation in coloration or morphology are common in birds, apparently because of demotions from full species status based on adoption of the biological species concept (Mayr 1970, Zink 2004). However, very few studies of genetic variation, and of mitochondrial DNA (mtDNA) phylogeography in particular, have revealed relationships that are concordant with subspecific designations (Ball and Avise 1992, Zink et al. 2000, Zink 2004). According to

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The Auk, Vol. 126, Number 2, pages 397–408. ISSN 0004-8038, electronic ISSN 1938-4254. DOI: 10.1525/auk.2009.08055

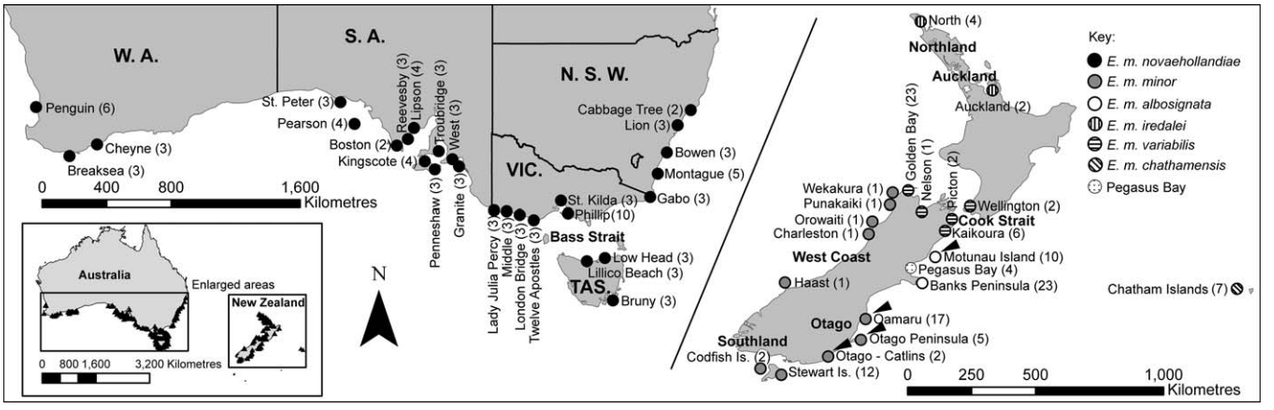

FIG. 1. Distribution of *Eudyptula minor* (Marchant and Higgins 1990, Dann 1994, Robinson et al. 1996, Brothers et al. 2001, Stevenson and Woehler 2007, Heber et al. 2008), distribution of the six subspecies defined by Kinsky and Falla (1976), and sampling localities. Arrows indicate New Zealand colonies that contain representatives of the "Australia and southeast New Zealand" (ASENZ) clade. Numbers in parentheses represent numbers of individuals sampled. Names in bold refer to geographic features, states (Australia; W. A. = Western Australia, S. A. = South Australia, N. S. W. = New South Wales, VIC. = Victoria, TAS. = Tasmania), and regions (New Zealand).

the most recent review, only 3% of avian subspecies tested were monophyletic and, on average, 1.9 independently evolving groups were inferred from mtDNA variation within species, in contrast to the average 5.5 designated subspecies based on phenotypic characters (Zink 2004). However, the reviewed studies typically assessed only phylogenetic relationships among mtDNA lineages, which are likely to be discordant with subspecific taxonomy if features defining the intraspecific groups have arisen recently, effective population sizes are very large, or there is occasional gene flow between groups (Avise 2000). Regardless, under these conditions one could still expect mtDNA variation to be significantly partitioned according to intraspecific designations, but this is rarely tested, perhaps because of inadequate sample sizes.

The Little Penguin (*Eudyptula minor*) is a flightless marine bird that uses land mainly for breeding and for its annual molt. The distribution of colonies is irregular throughout southern Australia, New Zealand, and associated islands, and colony size varies from a few breeding pairs to tens of thousands (Marchant and Higgins 1990). Little Penguins begin breeding at the age of 2 to 3 years (Dann and Cullen 1990), usually lay a clutch of two eggs (Stahel and Gales 1987), and breeding birds live to an average of 7 years (Reilly and Cullen 1979), though several individuals older than 20 years have been recorded in the wild (Stahel and Gales 1987, Dann et al. 2005, C. Challies pers. comm.). As in many seabirds, natal philopatry is considered high in the Little Penguin (Stahel and Gales 1987, Marchant and Higgins 1990). However, banding and genetic studies show that dispersal occurs, albeit infrequently, at least in southeastern Australia (Dann 1992, Overeem et al. 2008, Priddel et al. 2008). During their first year, juveniles may travel distances of several hundred kilometers (Dann et al. 1992, Priddel et al. 2008), and fledglings have been observed migrating to breed at non-natal colonies (Reilly and Cullen 1982, Dann et al. 1995, Priddel et al. 2008).

Despite the high dispersal potential of the Little Penguin, regional variation in coloration and size exists throughout New Zealand and Australia. Kinsky and Falla (1976) used morphometric measurements and plumage coloration to define six geographically discrete subspecies—five in New Zealand and one in Australia (Fig. 1.). Banks et al. (2002) investigated the division of these six subspecies using morphometric, vocalization, and mtDNA characters. Monophyletic mtDNA relationships were observed for only four of the six subspecies, but these were based on only two individuals of each (Banks et al. 2002). Interestingly, two deeply divergent mtDNA lineages were observed, one restricted to New Zealand, and the other shared between Australia and the Otago Peninsula (southeast New Zealand; Fig. 1), a pattern supported by later increased sampling of Australian individuals (Overeem et al. 2008). However, in these mtDNA studies and that of Banks et al. (2008), sampling was insufficient to rigorously assess the monophyly of individual subspecies or the partitioning of genetic variation among subspecies.

Here, we present a distribution-wide survey of mtDNA variation in *E. minor.* We incorporate analysis of 222 individuals sampled from 50 localities (>30 individuals of each subspecies, with the exceptions of *E. m. iredalei* and *E. m. chathamensis*), so that we can assess the null hypothesis that phylogenetic relationships and partitioning of genetic variation reflect the geographic distribution of subspecies designated by Kinsky and Falla (1976) on the basis of morphology and coloration. Our broad sampling in Australia also enabled us to test for genetic structuring throughout this part of the species' range for the first time, and to more rigorously assess the hypothesis of Banks et al. (2002) that Australian *E. minor* were initially derived from New Zealand and then represented a source for back-colonization. We also investigated the origins of a specimen of *E. minor* that was found alive on an island off Namibia (southwest Africa). This study helps to alleviate the knowledge gap concerning levels and mechanisms of population differentiation in penguins highlighted by Friesen et al. (2007).

METHODS

Sample Collection and DNA Extraction

Samples were collected throughout the breeding range of *E. minor*, which occurs along the southern coast of Australia from Fremantle on the west coast (–32°03′S, 115°44′E) to Port Stephens

on the east coast (–32°41′S, 152°04′E), including Tasmania and associated islands, and around New Zealand and its offshore islands, such as the Chathams (–44°00′S, –176°35′W; Fig. 1). One sample was obtained from Namibia in April 2005 (Ichaboe Island, –26°16′S, 14°57′E; J. Kemper pers. comm.), ~11,000 km from the nearest known colony. Samples from colonies in Australia consisted of a small volume of blood taken from the upper surface of the foot of live birds and preserved in Longmire's buffer (Longmire et al. 1991) or ethanol, or from tissues in the case of fox-killed individuals (see Overeem et al. 2008). Samples from New Zealand and Namibia were blood or tissue collected from already deceased birds or feathers collected during molting. Molted feathers were collected from known colony residents (C. Challies pers. comm.) to avoid sampling prebreeding vagrant molters. A set of dead, beach-washed individuals from Pegasus Bay (north of Banks Peninsula) was also analyzed. DNA was extracted using a modified protein precipitation method of Crandall et al. (1999), either from tissue samples or 100 μL of preserved blood.

Sequencing

A 655-base-pair (bp) fragment of the mitochondrial control region was amplified from each individual as indicated by Overeem et al. (2008), following Banks et al. (2002) and Roeder et al. (2002). Sequences from GenBank that were also analyzed were AF468945–AF468956 (Banks et al. 2002); AY194081–AY194086 and DQ017223–DQ017249 (Banks et al. 2008); AF362763 (Slack et al. 2003); and EU043384–EU043403 (Overeem et al. 2008). The Humboldt Penguin (*Spheniscus humboldti*; AY882541; J. Schlosser unpubl. data) was employed as the outgroup, representing the closest related penguin species for which control-region sequence was available (Bertelli and Giannini 2005, Baker et al. 2006, Ksepka et al. 2006). In total, sequences from 157 individuals are newly reported here, and 65 sequences were previously available.

Data Analysis

Tree and network building.—All sequences were aligned manually against those published by Banks et al. (2002). A heuristic tree search under maximum parsimony using tree-bisection reconnection branch-swapping and 100 replicates of random sequence addition was performed using PAUP*, version 4.0b10 (Swofford 2002), with "maxtrees" set to 1,000. Bootstrap support was estimated from analysis of 500 pseudoreplicate data sets. Gaps were treated as missing data. A maximum-likelihood heuristic search was also conducted using PAUP*, under the HKY + I + Γ model (Hasegawa et al. 1985) selected from a hierarchical likelihood-ratio test (hLRT) of 56 candidate models using MODELTEST, version 3.7 (Posada and Crandall 1998). The Hasegawa-Kishino-Yano (HKY) model of nucleotide substitution accounts for a higher occurrence of transitions versus transversions, and we also accommodated rate heterogeneity among sites (Γ), including a proportion of sites assumed to be invariant (I). Bayesian analysis was also conducted using the HKY + I + Γ model in MRBAYES, version 3.1 (Ronquist and Huelsenbeck 2003; nst = 2, rates = invgamma) with four Monte Carlo Markov chains heated according to temp = 0.1 and run for 2,110,000 generations, with sampling every 100 generations. Attainment of asymptotes for LnL, substitution model parameters, and the average standard deviation of split frequencies between duplicate runs were monitored, and preceding generations (100,000) were discarded as burn-in before the calculation of a consensus topology and bipartition posterior probabilities (Huelsenbeck et al. 2002). Network analysis was performed to explore relationships among closely related haplotypes, where assumptions of strictly bifurcating relationships and extinction of ancestral haplotypes may be violated (Posada and Crandall 2001). A haplotype network was constructed using TCS, version 1.21 (Clement et al. 2000).

Tests of geographic variation.—During tests of geographic variation, individuals sampled from some proximate locations were grouped together, such as those within the New Zealand regions of Banks Peninsula, Northland, Oamaru, Chatham Islands, and Otago Peninsula. This was to make sample sizes larger. The samples obtained from Namibia, Southland, and Pegasus Bay were not analyzed further, given their imprecise origin. Analysis of molecular variance (AMOVA; Excoffier et al. 1992) was performed using ARLEQUIN, version 2.0.1.1 (Schneider et al. 2000), incorporating pairwise distance among haplotypes measured according to the Kimura (1980) two-parameter model, the nucleotide-substitution model offered by ARLEQUIN that is most similar to HKY. AMOVA was performed either at the level of colonies alone or with the allocation of colonies to groups based on their location within the inferred ranges of subspecies, countries, or major phylogeographic clades. A Mantel test was also performed, to assess correlation of genetic differentiation and subspecies designation based on a colony pairwise F_{ST} matrix and a binary subspecies difference matrix. Spatial analysis of molecular variance (SAMOVA; Dupanloup et al. 2002) was conducted to assign populations to groups that are maximally differentiated genetically. This approach is somewhat akin to the STRUCTURE program of Pritchard et al. (2000) but is not based on Hardy-Weinberg equilibrium and, hence, can be applied to haploid data such as mtDNA. SAMOVA maximizes the genetic covariance due to differences between groups of populations, under the constraint that the groups must be genetically homogeneous. The algorithm operates such that groups of populations are usually geographically contiguous (Dupanloup et al. 2002).

We assessed the divergence time and migration rates between the New Zealand population and the Australian population using IM, version 3.2 (Hey and Nielsen 2004). IM assesses six demographic parameters of a two-population isolation-with-migration model (Hey and Nielsen 2004), with each scaled by the neutral mutation rate (u). The ancestral population of effective population size N_A ($\theta_A = 2N_A u$) splits into two present-day populations of effective population sizes N_1 ($\theta_1 = 2N_1 u$, New Zealand) and N_2 ($\theta_2 = 2N_2 u$, Australia). Time since divergence t ($t = tu$) of populations is estimated, along with rates of subsequent migration, m_1 ($m_1 = m_1/u$, from N_2 to N_1) and m_2 ($m_2 = m_2/u$, from N_1 to N_2). Maxima of prior distributions were set such that the tails of posterior distributions fell close to zero within these ranges where possible (100 for θ_1 and θ_2, 60 for θ_A, 4 for t, and 5 for m_1 and m_2), and analyses were repeated several times, and with different priors, when this was not the case. Parameters were estimated using the HKY model of mutation implementing a four-chain Metropolis-coupled Markov chain–Monte Carlo procedure with a two-step increment model of chain heating (g1 = 0.02, g2 = 2.00) and a burn-in of 100,000 steps. The time since the most recent common ancestor (TMRCA) of certain haplotypes was also assessed using IM and the molecular-clock calibration of Lambert et al. (2002): 1.43×10^{-6} mutations site^{-1} year^{-1}.

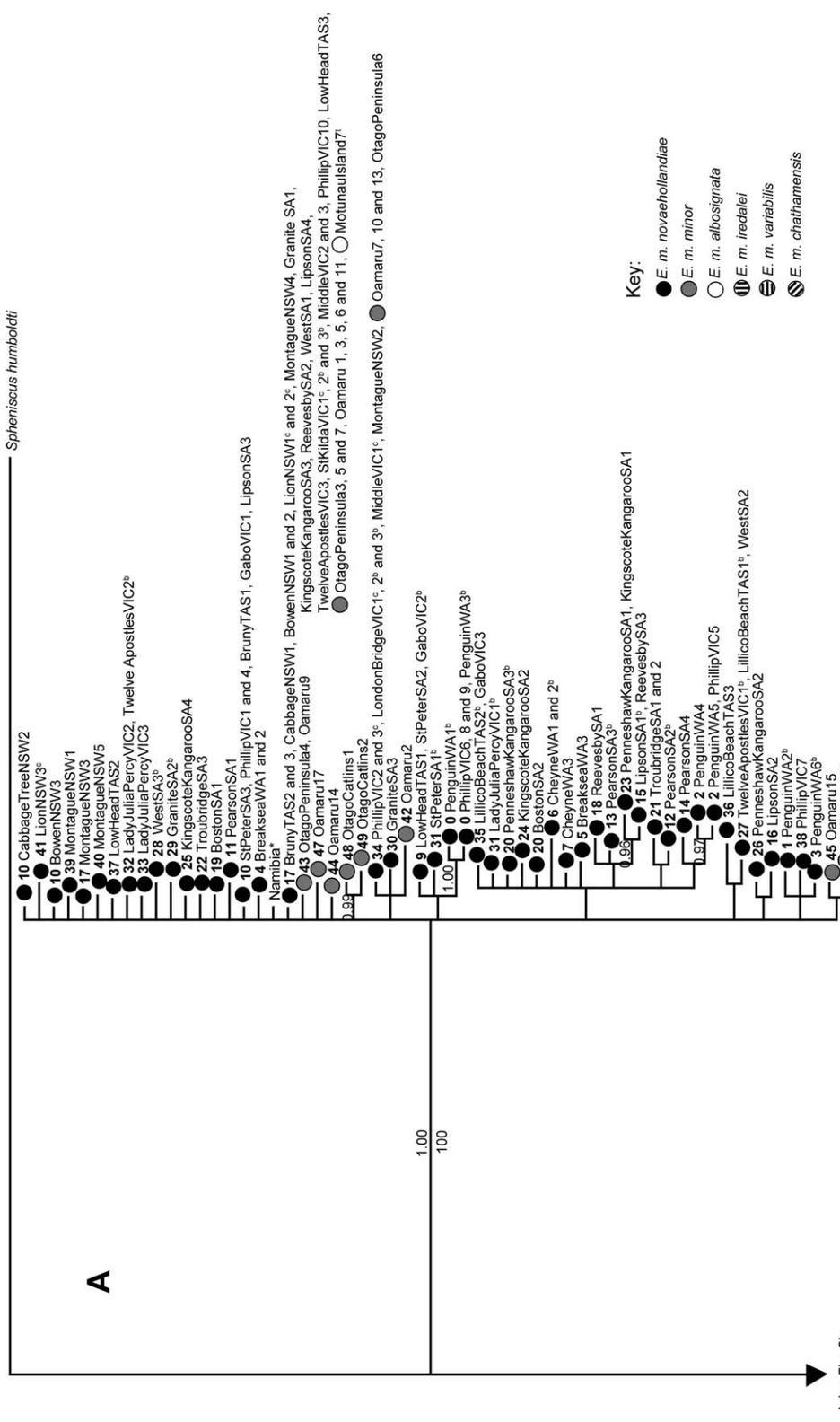

FIG. 2A. Bayesian majority-rule consensus topology of *Eudyptula minor* control-region sequences obtained from within the ranges of subspecies classified by Kinsky and Falla (1976), showing (A) the outgroup and the ASENZ clade and (B) the NZO clade (see Fig. 1). Numbers given before sample names correspond to the haplotype number from the haplotype networks (Fig. 3; NZO network not shown). Closely related haplotypes may differ by ambiguous bases only. Numbers above the branches are Bayesian posterior probabilities when >0.95. Numbers below the branches are maximum-parsimony bootstrap values when >75%. Asterisk represents samples of unknown origin that were not included in further analysis. Superscript letters represent breeding status, as follows: b = breeding; c = chick (i.e., born at locality), and t = translocated from locality (i.e., born at locality). Unmarked individuals are of unknown breeding status. *Figure 2 is continued on the following page.*

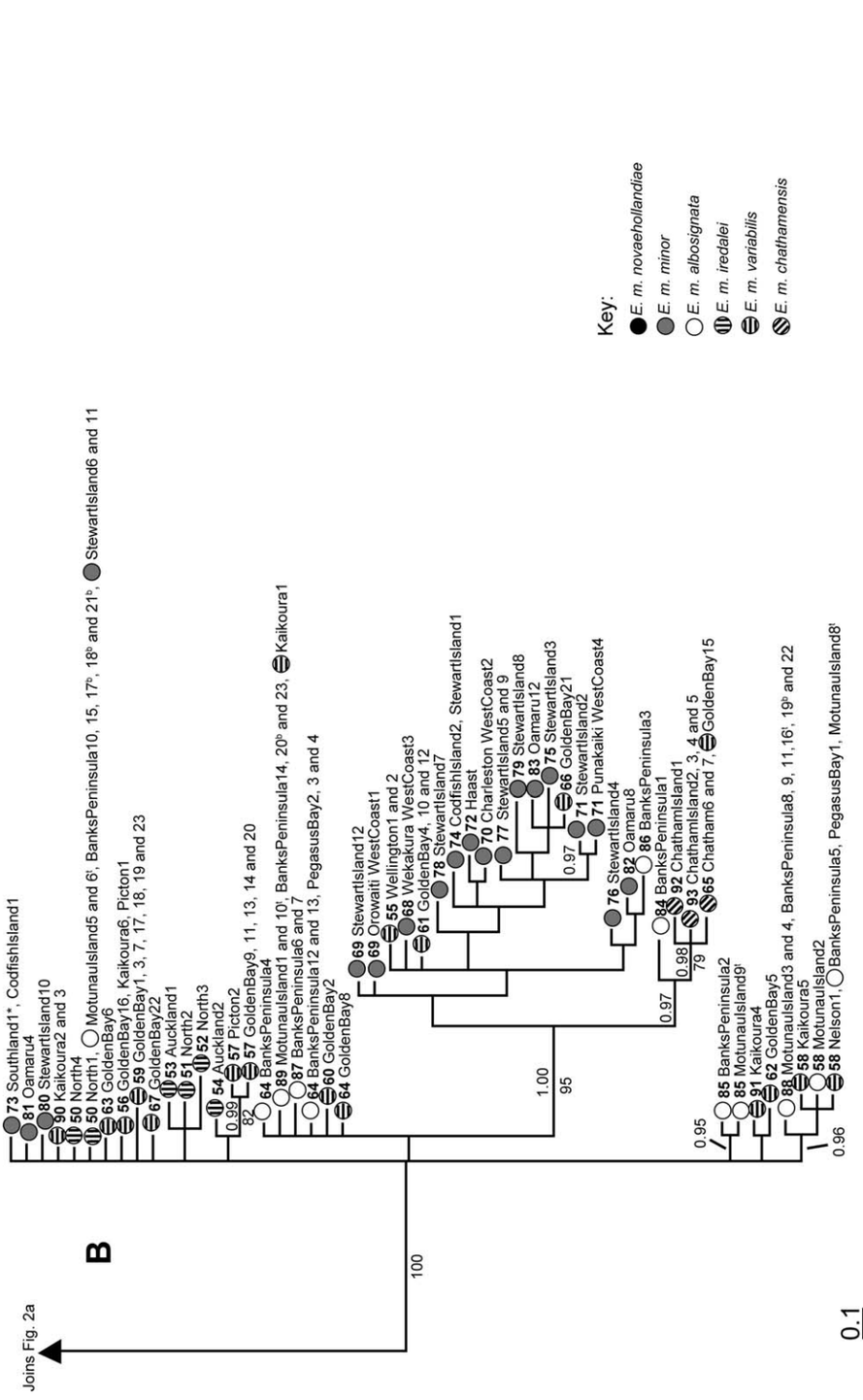

FIG. 2B. *Continued*. Bayesian majority-rule consensus topology of *Eudyptula minor* control-region sequences obtained from within the ranges of subspecies classified by Kinsky and Falla (1976), showing (A) the outgroup and the ASENZ clade and (B) the NZO clade (see Fig. 1).

Results

In all, 222 individual sequences of ≤429 bp each were aligned, consisting of 111 haplotypes, with *S. humboldti* as the outgroup. All samples sequenced during the present study have been entered into GenBank under accession numbers FJ529997–FJ530153. Tree topologies derived from maximum parsimony, maximum likelihood, and Bayesian inference were similar, and the Bayesian majority-rule phylogram is shown in Figure 2. Phylogenetic relationships were inconsistent with the suggested geographic distributions of subspecies, with paraphyletic relationships observed for each taxon (Fig. 2). *Eudyptula minor* is separated into two deeply divergent clades, separated by a minimum of 30 and a maximum of 58 substitutions, representing 7.0–13.5% sequence divergence (uncorrected distance). One clade includes all Australian individuals ($n = 93$), the Namibian individual, plus 22 New Zealand individuals from four localities on the east coast of the South Island: Catlins, Otago Peninsula, Oamaru, and Motunau Island (Fig. 2). Therefore, we have called this clade "ASENZ" (Australia and southeast New Zealand). The other clade includes individuals only from New Zealand ($n = 104$), and we have called this the "NZO" clade (New Zealand only; Fig. 2). Allocation of haplotypes to these two clades received high posterior probability or bootstrap support (ASENZ: 1.00/100%, NZO: 0.91/100%; Fig. 2). Interspecific variation between *S. humboldti* and *E. minor* consisted of 62–78 nucleotide differences (including length mutations) representing 14.5–18.0% sequence divergence (uncorrected distance), which is only slightly higher than the largest divergence observed between the two *E. minor* clades. Intraclade sequence divergence was lower for the ASENZ clade (maximum: 10 substitutions) than for the NZO clade (maximum: 19 substitutions).

AMOVA performed on the entire data set showed significant genetic variation among colonies within countries ($\phi_{SC} = 0.756$, $P < 0.05$), among colonies ($\phi_{ST} = 0.946$, $P < 0.05$), and between countries ($\phi_{CT} = 0.777$, $P < 0.05$). Partitioning of variation shows more clearly that variation was markedly higher between countries (Australia vs. New Zealand, 77.69%) than among colonies within countries (16.87%) or within colonies (5.44%). AMOVA performed with colonies assigned to groups representing subspecies also revealed significant structuring (among subspecies, $\phi_{CT} = 0.781$, $P < 0.05$). Given the phylogenetic topology and branch lengths observed, we assessed whether comparisons against a single subspecies, *E. m. novaehollandiae*, were driving this subspecific structuring. Pairwise subspecies AMOVA were significant only for comparisons against *E. m. novaehollandiae* ($\phi_{CT} = 0.569–0.998$, $P < 0.05$ following sequential Bonferroni correction)—except that involving *E. m. chathamensis* ($\phi_{CT} = 0.997$, $P = 0.038$), which likely reflects the presence of only a single *E. m. chathamensis* colony available for permutation. Likewise, the Mantel test produced a nonsignificant correlation coefficient of intercolony F_{ST} and subspecies difference or identity ($r = 0.033$, $P > 0.05$). Therefore, in most cases, genetic structuring was discordant with the subspecies boundaries designated by Kinsky and Falla (1976).

Bayesian coalescent analysis of 9.1×10^6 iterations under the isolation-with-migration model estimated the θ_1 parameter of New Zealand as ~54 and the θ_2 of Australia as ~44. The migration posterior for m_1 (migration into New Zealand) peaked at 0.1325, and that for m_2 (migration into Australia) peaked at 0.0025, the latter representing the lowest-sized bin into which m_2 could fall, including inferences of zero migration. Posterior distributions obtained for ancestral θ and the divergence time parameter were not bell-shaped. Hence, we assessed the sensitivity of our migration posteriors to changes in priors for these two parameters (θ_A = 40–80, t = 3–5), but the results were convergent. Although we could not calculate a divergence time estimate for the Australian and New Zealand populations given the shape of the posterior, we were able to estimate the TMRCA of the main New Zealand haplotypes within the ASENZ clade ($t = 1.1475$; 95% highest posterior density [HPD]: 0.2625–2.7975), which translates into ≥0.18 million years using the most rapid calibration of Lambert et al. (2002). The results obtained with IM with just one locus provide an approximate indication of the parameters explored, given that a multilocus data set is required to obtain a more accurate result (Hey and Nielsen 2004); however, these migration posteriors indicate highest posterior probability for non-zero movement into New Zealand, but highest posterior probability for negligible movement into Australia. These results are supported by inspection of haplotype relationships; the most abundant Australian haplotypes are observed within New Zealand (haplotypes 17 and 34), and, with one possible exception (haplotype 43), all the other ASENZ haplotypes from New Zealand appear to be derived from these haplotypes, within one to three mutational steps (Fig. 3).

ASENZ clade and variation in Australia.—The ASENZ clade contains 58 haplotypes among 116 individuals. Divergence within this clade is shallower than that within the NZO clade (maximum 10-bp difference between haplotypes), and there is low topological support for the majority of nodes (Fig. 2). Both of the Catlins (Otago) individuals group together with high Bayesian posterior probability ($pP = 0.99$). The remaining New Zealand representatives of the ASENZ clade comprise 14 of the 17 individuals from Oamaru, 1 of 10 Motunau Island individuals, and all 5 Otago Peninsula individuals. Therefore, the ASENZ clade is found within the distribution of three of the six subspecies defined by Kinsky and Falla (1976): Australia (*E. m. novaehollandiae*), Otago (*E. m. minor*), and Motunau Island (*E. m. albosignata*). Some Australian samples strongly supported as sister lineages are geographically proximate (i.e., Kangaroo, Lipson, and Reevesby Islands, South Australia; Bayesian $pP = 0.96$). However, samples from Phillip Island (Victoria) and Penguin Island (Western Australia) share haplotypes even though they are ~4,000 km apart.

AMOVA performed on the individuals within the ASENZ clade (partitioned into their colonies) showed a significant ϕ_{ST} (0.109, $P < 0.01$); however, 89.11% of the variation was within, rather than among, colonies. SAMOVA performed on the ASENZ clade revealed the greatest ϕ_{CT} when colonies were assigned to two groups ($\phi_{CT} = 0.368$, $P < 0.05$; Fig. 4). These groupings were Cheyne Island (Western Australia) against all other ASENZ localities. As the number of groups (K) was increased, only single colonies from Australia were assigned to the additional groups ($K = 3$, Lillico Beach, Tasmania; $K = 4$, London Bridge, Victoria; $K = 5$, Troubridge Island, South Australia; and $K = 6$, Pearson Island, South Australia), where only one subspecies has been defined, rather than segregating the Australian samples from the southeast New Zealand samples. The first New Zealand colony to form a separate grouping was Catlins (Otago), but this did not occur until $K = 8$, with a lower ϕ_{CT} of 0.306 ($P < 0.01$).

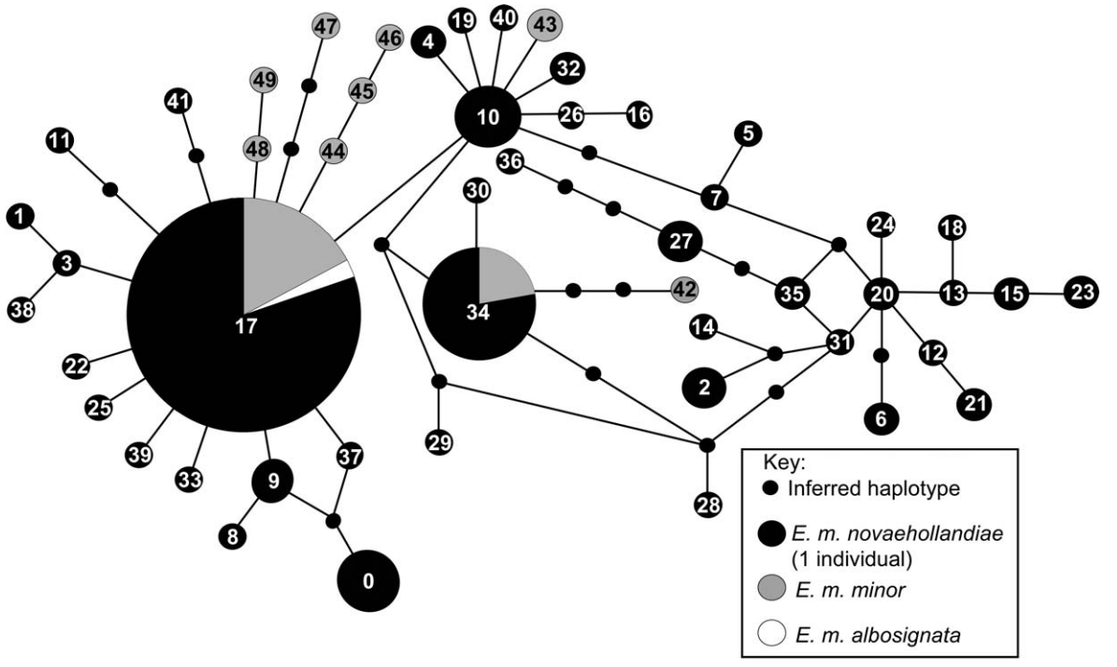

Fig. 3. Australia and southeast New Zealand (ASENZ) clade haplotype network. Shading of circles reflects samples collected within the ranges of the subspecies designated by Kinsky and Falla (1976). Size of circles is proportional to the number of individuals with that haplotype, with circles in the key representing a single individual. Numbers within haplotype circles represent the haplotype number and correspond to the ASENZ clade in Figure 2.

AMOVA performed on only Australian colonies showed low but significant structuring ($\phi_{ST} = 0.110$, $P < 0.01$), with 89.03% of the structuring within, rather than among, colonies. SAMOVA restricted to the Australian colonies revealed the same groupings and trends of ϕ_{CT} with K as for the ASENZ clade (maximum $\phi_{CT} = 0.336$, $P < 0.05$, $K = 2$; single colonies allocated to the new groups with increase in K; Fig. 4). The combined AMOVA and SAMOVA results indicate the presence of genetic structuring that cannot be defined above the level of individual colonies.

NZO clade.—The NZO clade contained 106 individuals from 20 of the 22 New Zealand colonies analyzed, including those from associated islands. The NZO clade does not include any individuals from two of the southeast New Zealand locations sampled (Otago Peninsula and Catlins), and only 3 of 17 individuals from the Oamaru locality. The beach-washed individuals collected from Pegasus Bay shared haplotypes with individuals from the surrounding localities (previously proposed subspecies ranges of *E. m. albosignata* and *E. m. variabilis*; Fig. 2). One NZO subclade received high topological support (1.00/95%), consisting of individuals from around the South Island but also including Wellington from the North Island and from the Chatham Islands (Fig. 2). Within this subclade, separated by at least four mutations (0.93–1.09% uncorrected divergence), is another strongly supported grouping (0.98/79%) of all seven Chatham Islands individuals (*E. m. chathamensis*) and one Golden Bay individual (sampled from within the proposed range of *E. m. variabilis*).

AMOVA clearly showed greater variation among New Zealand than among Australian individuals ($\phi_{ST} = 0.774$, $P < 0.05$) because of the presence of both NZO and ASENZ clades. The partitioning of variation was higher among (77.43%) rather than within (22.57%) the colonies. Variation for the NZO clade ($\phi_{ST} = 0.402$, $P < 0.05$) was higher than that for the ASENZ clade and the Australian samples, with 40.24% and 59.76% variation among and within colonies, respectively. SAMOVA ϕ_{CT} values were also higher for the NZO clade than for the ASENZ clade or the Australian individuals, which indicates the presence of stronger structuring in New Zealand (Fig. 4). Three groupings ($K = 3$) achieved $\phi_{CT} = 0.514$ ($P < 0.01$), which was not appreciably smaller than the ϕ_{CT} value achieved at larger values of K (Fig. 4). Those three groupings corresponded to (1) Chatham Islands; (2) Wellington, West Coast, Southland (Stewart and Codfish Islands), and Oamaru; and (3) Northland, Auckland, Nelson–southern Cook Strait, Kaikoura, Motunau Island, Banks Peninsula, and Pegasus Bay (refer to Fig. 1 for regions).

Namibian individual.—The specimen found off Namibia falls within the ASENZ clade (Fig. 2), which confirms it as *E. minor*. The Namibian Little Penguin specimen did not share a haplotype with any other sampled individual, but it differed from an Australian sequence at only one nucleotide position.

Discussion

Genetics of subspecies.—Phylogeographic mtDNA relationships were paraphyletic with respect to the distributions of each of six subspecies defined by Kinsky and Falla (1976) on the basis of morphology and coloration. Although monophyletic relationships were apparent for at least three of the six subspecies from previous studies (Banks et al. 2002, Banks et al. 2008, Overeem et al. 2008), the

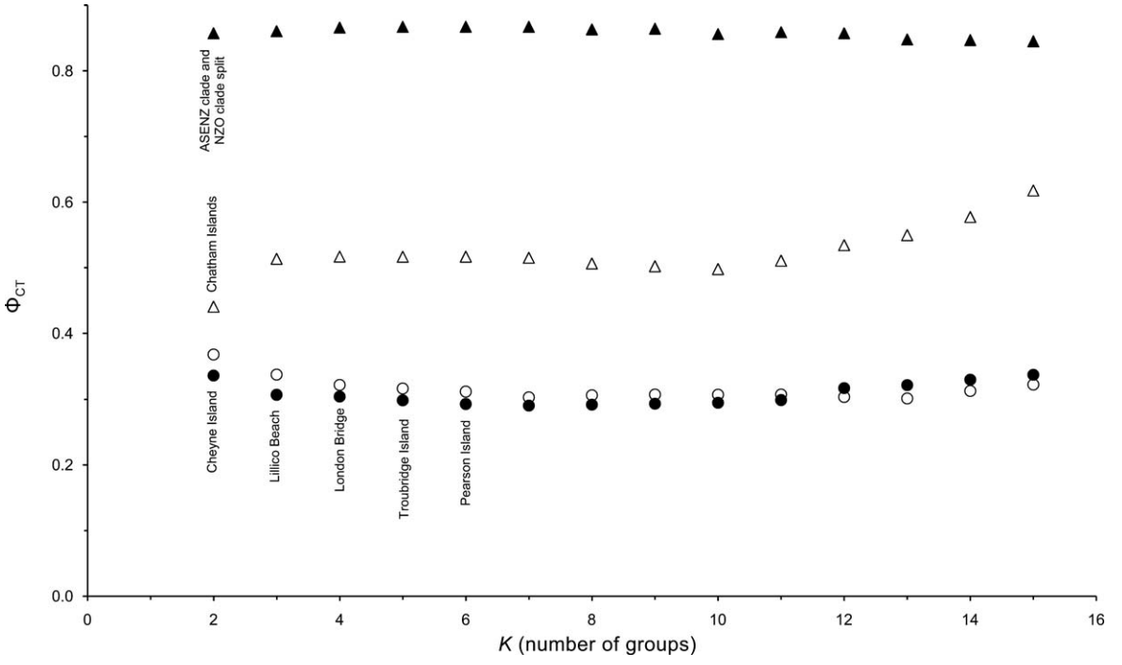

FIG. 4. Spatial analysis of molecular variance. The maximum observed ϕ_{CT} for each predefined number of groups (*K*) is displayed. Analyses were performed on different data sets (closed circles = Australia only, open circles = ASENZ clade, open triangles = New Zealand-only clade, and closed triangles = entire data set). Text adjacent to data points describes the allocation of colonies to groups as the number of groups (*K*) was increased. For example, at *K* = 2, both the ASENZ and Australia data sets exhibited maximum ϕ_{CT} when Cheyne was allocated to one group and all other colonies were allocated to the other group. At *K* = 3, Lillico Beach was removed as a new group, and so forth until *K* = 7, where removed colonies begin to group together or differ between data sets.

increased sampling employed during our study has revealed paraphyletic relationships for all six subspecies (Fig. 2), as is commonly observed in avian studies (Zink 2004). The closest phylogeographic concordance with intraspecific taxonomy was observed for the subspecies *E. m. chathamensis*: all individuals from the Chatham Islands clustered within a small, well-supported clade. However, this clade also included one Golden Bay individual that shared a haplotype with two Chatham Island individuals, indicating either a recent colonization of the Chatham Islands from mainland New Zealand and incomplete lineage sorting, or recent gene flow in the opposite direction. The Chatham Islands most recently emerged only 1–3 mya (Trewick et al. 2007), which is consistent with recent colonization, rather than long-term persistence at the Chatham Islands and recent back-colonization to the mainland.

Although phylogeographic relationships were discordant with intraspecific taxonomy, the partitioning of genetic variation among subspecies was significant ($\phi_{CT} = 0.781$, $P < 0.05$). However, this variation was not partitioned with respect to the majority of subspecies boundaries, with only *E. m. novaehollandiae* appearing to be distinct from other putative subspecies—with the exception of *E. m. chathamensis*, for which only one colony was available for permutation; all other pairwise subspecific ϕ_{CT} values were nonsignificant. In addition, groups of populations recovered by SAMOVA revealed little resemblance to the distribution of subspecies, with the best concordance observed for *E. m. chathamensis*, because the Chatham Islands were distinguished as a separate group.

While we found little concordance of genetic structure with the suggested distributions of subspecies, it is important to remember that this could simply reflect errors in the original measurement or interpretation of morphological characters and color variation, rather than representing a characteristic of genetic variation and subspecific classifications (but see Zink 2004). A more detailed study after that of Kinsky and Falla (1976) questioned Kinsky and Falla's recognition of some subspecies and their geographic distributions, preferring instead the recognition of clinal variation and mixtures of morphotypes at individual locations (Meredith and Sin 1988). This led to the abandonment of subspecies recognition within *E. minor* by Turbott (1990). Consequently, the accuracy of the taxonomic framework against which genetic relationships and variation are assessed will influence the degree of concordance observed (e.g., McKay 2008).

Genetic structuring in Australia.—Although some samples strongly supported as sister lineages were geographically proximate, others were not. Consequently, phylogeographic structuring was absent in Australia. SAMOVA, which aims to define geographically adjacent and genetically homogeneous populations (Dupanloup et al. 2002), returned a maximum ϕ_{CT} for two groups, one of which represented only the Cheyne Island locality. This is consistent with expectations for SAMOVA of data sets that lack broad geographic partitioning of variation. The removal of only single colonies to new groups as *K* was increased (SAMOVA; Fig. 4) and the detection of genetic structuring among colonies

via AMOVA may reflect the development of significantly different haplotype frequencies during establishment of colonies. Colony extinction and formation appear to occur frequently in Little Penguins. Examples of colonies that have become extinct include Port Fairy (in Victoria; Dann and Norman 2006), Cape Direction, Marion Bay, North Clifton, and Pigeon Holes (in Tasmania; Stevenson and Woehler 2007), and seven colonies in Otago, New Zealand (Dann 1994). The best example of a colony that has formed recently is that located at the St. Kilda breakwater, built for the 1956 Melbourne Olympic Games, and Little Penguins are proposed to have established a colony there before 1974 (Cullen et al. 1996). The formation of such colonies and accompanying founder effect may result in genetic differentiation from colonies both nearby and farther away, therefore providing structure that is not broadly geographically partitioned.

Ecologically comparable species.—There is a lack of phylogeographic studies on Spheniscidae species (Friesen et al. 2007), particularly those inhabiting continental areas, and also of seabird species that breed in Australia and New Zealand, but our results exhibit similarities to previous studies. Phylogeographic analysis of the Antarctic Adélie Penguin (*Pygoscelis adeliae*) uncovered two deeply divergent mtDNA lineages (Ritchie et al. 2004). Like our ASENZ–NZO structure, these lineages of Adélie Penguin also overlap in one geographic area, the Ross Sea, where colonies contain individuals from both lineages. It is hypothesized that, as suggested for Little Penguins, this zone of overlap in the distribution of the two clades represents secondary admixture (Ritchie et al. 2004). Both lineages of Adélie Penguin are more diverse than those of the Little Penguin, which is consistent with the larger population sizes—and possibly the longer colony-persistence time—in the Antarctic species.

The Short-tailed Shearwater (*Ardenna tenuirostris*, formerly *Puffinus tenuirostris*; Christidis and Boles 2008) is similar to the Little Penguin in several aspects of its ecology. It is a burrowing, colonial-nesting seabird of southeastern Australia that often nests sympatrically with Little Penguins. Short-tailed Shearwaters have also established new colonies in the past 100 years, and both sexes incubate and tend the young (Marchant and Higgins 1990). A lack of population genetic structuring despite high philopatry in Short-tailed Shearwaters was explained in terms of a recent bottleneck (10,000 years ago), followed by a range expansion involving large numbers of individuals (Austin et al. 1994). This could parallel the lack of broad geographic structuring in Little Penguins throughout southern Australia and local extinction–recolonization dynamics.

Long-distance dispersal of Little Penguins.—The Little Penguin is split into two distinct and well-supported lineages, ASENZ and NZO, with all sampled individuals from two of the Otago localities (Otago Peninsula and Catlins), most of the individuals sampled from Oamaru, and 1 of 10 individuals from Motunau Island, grouped with all the Australian individuals. The NZO clade shows deeper genetic divergence than the ASENZ clade. Banks et al. (2002) suggested that the similar patterns they observed may reflect an origin in New Zealand, with colonization of Australia and then a return to southern New Zealand. Banks et al. (2002) suggested that the colonization of southern New Zealand from Australia occurred after a Pleistocene cooling period during which the previous southern New Zealand residents were displaced to the north. However, Banks et al. (2002) had no genetic data from south of the Catlins to support this theory. Although our results are broadly consistent with the multiple trans-Tasman dispersal hypothesis of Banks et al. (2002)—in particular, our coalescent analysis showing recent dispersal into New Zealand from Australia—our samples from Stewart and Codfish islands, and their positioning within the NZO clade rather than the ASENZ clade, reject northward movement of the species distribution as a mechanism for the lack of the NZO clade in southeast New Zealand. Alternatively, regional extinction of NZO may have been anthropogenic, mediated via movement of Polynesians into southern New Zealand around 1280 C.E., as Boessenkool et al. (2009) suggested in the case of the Waitaha Penguin's (*Megadyptes waitaha*) extinction. However, a minimum estimated TMRCA of 180,000 years ago is observed for a subset of haplotypes that would appear to date back to the colonization of southern New Zealand from Australia (haplotypes 17, 44–49; Fig. 4), which argues against Polynesian-mediated extinction of the Little Penguin in southeast New Zealand, given that Polynesian colonization of New Zealand is dated only to 730 years ago (Higham et al. 1999, Wilmshurst et al. 2008). Our molecular estimate was based on the lower 95% HPD of the Bayesian TMRCA distribution, and one of the most rapid mtDNA control-region calibrations obtained for birds (Lambert et al. 2002), so that we are unlikely to have overestimated the arrival of ASENZ in New Zealand. Otherwise, extinction of NZO representatives from southeast New Zealand may have occurred after the colonization by ASENZ individuals, which suggests that the ASENZ individuals are competitively superior in this region. Future studies comparing the reproductive success of these clades where they share a colony (e.g., Oamaru, Motunau Island) may resolve the question of whether one clade is displacing the other. There is no evidence of a geographic barrier that accounts for the localized presence of the ASENZ clade within New Zealand, and it may be slowly expanding its range.

There is independent evidence to support the hypothesis of multiple trans-Tasman dispersals in Little Penguins. First, this species can swim long distances (Dann et al. 1992, Priddel et al. 2008), and it is not uncommon for the species to be found on the shores of other Southern Hemisphere continents. Several Little Penguins have been observed on Magdalena Island in the Magellan Strait, Chile (one found in a nest over two seasons; A. Jaramillo pers. comm.). Several photographs confirm their identity, and the Little Penguin has been added to the American Ornithologists' Union's South American classification list (Remsen et al. 2009) as a vagrant bird species, given that there were numerous sightings in Chile over 10 years (Valverde and Oyarzo 1996, Brito 2000, Wilson et al. 2000). The penguin specimen that was found off Namibia was confirmed as a Little Penguin by morphological measurements, photographic evidence, and now genetic evidence, although, because of the lack of phylogeographic structuring, we could not narrow down a source beyond the geographic limits of the ASENZ clade.

Other penguin species also disperse long distances; endemic New Zealand and sub-Antarctic taxa occasionally visit the shores of Australia (Woehler 1992). A Northern Rockhopper Penguin (*Eudyptes moseleyi*) has recently been confirmed via mtDNA sequence analysis as having dispersed 6,000 km from its natal colony, crossing the subtropical convergence (de Dinechin et al.

2007). Oceanic dispersal is also implicated for the colonization of newly formed islands (Amsterdam and St. Paul, 0.25 mya) by Northern Rockhopper Penguins originating 6,000 km away (de Dinechin et al. 2009). Although these observations suggest that spheniscids are capable of long-distance dispersal, we should note that "assisted dispersal" (i.e., translocation) via ship cannot be discounted. For example, vagrant Magellanic Penguins (*S. magellanicus*) in New Zealand readily took food from humans, which suggests that their travel may have been assisted (Robertson et al. 1972, Darby 1991). However, we reject anthropogenic dispersal for *E. minor* in the majority, given the depth of the divergence between the NZO and ASENZ clades, and the age of common ancestry estimated for those ASENZ haplotypes observed from New Zealand.

Acknowledgments

Blood samples in Australia were collected under authorization numbers A27/2004 (Deakin University), S11771 (New South Wales), 10002899, 10003981 (Victoria), FA05228, 7339/05 (Tasmania), 10/2003, Q24796, 137 (South Australia), and SF004716, CE000772 (Western Australia). Tissue and feather samples in New Zealand were collected under permit numbers OT-18599-DOA, NO-19323-DOA, and WE-19821-DOA. We thank J. Kemper of African Penguin Conservation Project, C. and J. Challies, P. Collins (Conservation and Land Management), B. Cannell (University of Western Australia), N. Klomp (Charles Sturt University), and Museum Victoria for donations of samples. Many thanks to P. Agnew from Oamaru penguin colony and Department of Conservation rangers D. Houston, M. Ogle, and J. Geritzlehner (among others), A. Spencer, P. McClelland, L. Bridson, B. Beaven, A. Leseberg, M. Aviss, M. Morrissey, B. Lummis, and A. Walker, who kindly donated samples. Thanks to R. Overeem for training in penguin handling and blood collection techniques and for donation of samples. Many thanks to all the Wildlife Officers, colony managers, and penguin enthusiasts who allowed us access to colonies and provided information and logistic support. Thanks to J. Banks for additional information on his samples, J. Monk and A. Rattray for assistance with map production, G. Quinn and C. Styan for supervision and comments on drafts of the manuscript, and C. Austin for assistance and supervision in the earlier stages of this project. Comments by K. T. Scribner and two anonymous reviewers were gratefully received and improved the manuscript. Funding was supplied by an Australian Research Council (Linkage) Grant (LP0453481) to Deakin University (C. Burridge, C. Austin, and P. Dann) with Phillip Island Nature Park. Thanks to other penguin researchers for valuable information and assistance, especially A. Wiebkin and R. Overeem. A huge thanks to the numerous volunteers who performed field work, especially S. Peucker.

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Associate Editor: K. T. Scribner