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RESEARCH ARTICLE

Contrasting genetic structuring between colonies of the World's smallest penguin, *Eudyptula minor* (Aves: Spheniscidae)

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Abstract The Little Penguin, *Eudyptula minor*, is a seabird that nests in colonies throughout New Zealand and southern Australia. Individuals from different colonies in southeast Australia differ significantly in morphology and ecology, suggesting that some genetic structuring may exist among colonies. In contrast, the marking of individuals with flipper bands has revealed some, albeit infrequent, movement between colonies. To determine the extent of genetic structuring, we tested the null hypothesis of substantial gene flow within southeast Australia by examining patterns of genetic variation across seven colonies separated by up to 1,500 km. Phylogeographic structuring was absent for mitochondrial control region sequences (2–3 individuals per colony). Microsatellite allele frequencies at five loci and mitochondrial haplotype frequencies (50 individuals per colony) were also homogenous among the majority of colonies sampled, although two colonies at the western periphery of the sampling range were distinct from those to the east. The genetic homogeneity among the majority of colonies can be explained by low but consistent contemporary gene flow among them, or a recent founder event in Bass Strait following the last marine transgression. The genetic break towards the western end of the sampling distribution appears best explained by differences in sea surface temperature and, consequentially breeding phenology, the latter hindering genetically effective migration.

Keywords Breeding phenology · Sea surface temperature · Microsatellite · Mitochondrial DNA · Natal philopatry

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Introduction

The Little Penguin (*Eudyptula minor*: Spheniscidae) is the World's smallest penguin, attaining an average size of ~33 cm in height and 1,100–1,200 g in weight (Marchant and Higgins 1990). It occurs throughout coastal waters of New Zealand and southern Australia (Marchant and Higgins 1990). Breeding colonies occur in a range of coastal habitats such as beaches, rocky headlands and offshore islands and vary in size from only a few breeding pairs up to 35,000 individuals (e.g. Gabo Island) at densities of 310 burrows/hectare (Marchant and Higgins 1990; Waas 1990; Dann et al. 1996). Individuals mature at 2–3 years (Dann and Cullen 1990; Dann et al. 1995). The timing of breeding varies geographically, but tends to commence in winter or spring, with 1–2 clutches of two eggs laid per season per breeding pair (Kinsky 1960; Jones 1978; Reilly and Cullen 1982; Gales 1985; Dunlop and Wooller 1986; Fortescue 1995; Perriman and Steen 2000; Johannesen et al. 2003). *Eudyptula minor* attracts considerable community interest and is popular with tourists, particularly the synchronised

'parades' of individuals crossing beaches to their burrows at dusk. Approximately 500,000 tourists visit the Phillip Island colony annually to view the penguins, and similar attractions operate throughout the species' range.

Some *E. minor* colonies have undergone declines and even extinction during recorded history (Dann 1992, 1994; Dann et al. 2004; Stevenson and Woehler 2007) while others have increased (Dann 1994; Johannesen et al. 2003). Known contributors to declines include predation by introduced carnivores (Dann 1992; Overeem and Wallis 2003, 2007), mass mortality of important prey species (Dann et al. 2000) and an oil spill (Goldsworthy et al. 2000). Human settlement has also greatly modified breeding habitat in a few areas via agriculture, housing, recreational activities and coastal erosion (Harris and Bode 1981). Given the geographic isolation of some *E. minor* colonies, and the low fecundity and fledgling success of this species (~1 individual per season per breeding pair; Marchant and Higgins 1990), a paucity of migration among colonies would elevate their conservation concern as it may be insufficient to offset a localised decline.

Eudyptula minor appears to be strongly philopatric (Dann 1992) but a few individuals have been recorded breeding in locations other than their natal colony (Reilly 1977; P. Dann, unpublished data). In southeast Australia, movement of adults between colonies seems to be relatively infrequent, for example, approximating one out of every 200–500 breeders between Phillip and Gabo Islands (Dann 1992). However, the genetic exchange associated with fledglings breeding at non-natal colonies on maturity, and the movements of adults between colonies, is unclear because breeding status of observed 'immigrants' was not known and search effort has been concentrated at Phillip Island, possibly resulting in underestimation of inter-colony movements and overestimation of natal philopatry. However, differences exist among southeast Australian colonies with respect to individual morphology, diet and breeding (Reilly and Cullen 1981; Cullen et al. 1992; Overeem and Wallis 2003; Arnould et al. 2004; Overeem 2005; Overeem et al. 2006), which may reflect genetic isolation or environmental variation. For example, within southeast Australia, individuals east of Cape Otway have significantly smaller beaks than those to the west (Overeem et al. 2006). In New Zealand, analysis of blood proteins revealed population-specific loci distinguishing colonies separated by >250 km, but not for two colonies separated by ~75 km (Meredith and Sin 1988). However, these genetic differences may represent comparisons among different species or subspecies, and not a lack of gene flow among conspecific populations. A mitochondrial DNA (mtDNA) phylogeographic analysis also suggested a deep genetic difference between two individuals sampled towards the southern limit of the species distribution in New Zealand (Otago), and those sampled elsewhere in New Zealand, but this difference may reflect a recent dispersal event from Australia, rather than long-term isolation within New Zealand (Banks et al. 2002).

Herein we employed three molecular approaches to test for genetic isolation among *E. minor* colonies: phylogeography of mtDNA control region sequences, variation in mtDNA haplotype frequencies and variation in microsatellite allele frequencies. These three approaches have different sensitivities to detect genetic isolation among colonies, and their combined application should provide more information than if any single approach was employed (Avise 2004). MtDNA phylogeography will test for prolonged genetic isolation among colonies and the accumulation of region-specific mutations. Across shorter time scales, the haploid and maternal inheritance of mtDNA results in rapid rates of genetic drift relative to diploid, nuclear markers and hence analysis of mtDNA haplotype frequency heterogeneity among colonies will have greater sensitivity to detect genetic isolation than any single nuclear locus. However, mtDNA variation will only reflect movement and breeding patterns of females. In contrast to mtDNA, any variation detected in the frequencies of nuclear DNA variants, such as microsatellite alleles, will be reflective of genetic isolation in both sexes, and several independent markers (loci) can also be surveyed. Higher polymorphism at microsatellite loci may also increase the power of statistical tests for population isolation (Goudet et al. 1996).

The aim of this study was to determine the spatial structuring of population genetic variation among seven southeast Australian colonies of *E. minor*, and hence test the null hypothesis that there is exchange of reproductive individuals among colonies. Southeast Australia, and Bass Strait in particular, represent the region of highest abundance for *E. minor* (Dann et al. 1996), and spatial differences with respect to individual morphology and ecology have been observed therein. Assessment of inter-colony movement can be more effective via molecular approaches than traditional banding studies for several reasons; data can be obtained from a much larger number of colonies for a given effort, and movement patterns are averaged over longer time-scales and therefore less likely to be biased by rare observations (Mundy et al. 1997; Roeder et al. 2001). Genetic surveys are also less intrusive than flipper banding studies (Jackson and Wilson 2002). Despite these potential advantages, and the observation of genetic structuring over large geographical scales in several other penguin species (Triggs and Darby 1989; Ritchie et al. 2004; Jouventin et al. 2006), this is the first published study of genetic variation among Australian colonies of *E. minor*.

Materials and methods

Sampling and DNA extraction

Genetic data were collected from seven colonies from southeast Australia (Fig. 1). Blood samples were taken from ~50 breeding individuals at each colony during 2003 or 2004, except for the majority of samples collected from Middle Island which were tissues from fox-predated individuals killed during the 2002/2003 *E. minor* breeding season. A standard method for drawing blood was utilised (Ellegren 1996; Radford and Blakey 2000), of which ~75 μL was added to 1 mL of Longmire's buffer (Longmire et al. 1991) and stored at room temperature. Total genomic DNA extraction was undertaken following the protein precipitation method of Crandall et al. (1999), employing either 100 μL of resuspended blood or ~50 mg of tissue.

Mitochondrial DNA

For the testing of phylogeographic structuring among colonies (i.e. geographic clustering of closely related genetic variants) a 655 basepair (bp) fragment of the mitochondrial control region was sequenced from 2 to 3 individuals per colony. The control region fragment was amplified using the primers 'C L-tRNAglu' and 'D H-Dbox' (Roeder et al. 2002) following Banks et al. (2002). Only 'D H-Dbox' was employed for sequencing, owing to the presence of length heteroplasmy at the opposite end of the fragment (Roeder et al. 2002; Banks et al. 2002), yielding sequences of 417 bp.

While DNA sequence analysis of a small number of individuals per colony is sufficient to test for phylogeographic structuring among colonies, as it will be readily refuted if absent, tests of more subtle population structuring require larger sample sizes to reveal heterogeneity in the frequencies of genetic variants. Consequently, the sequences obtained above were aligned visually and a search was performed for commercially available restriction enzymes that would distinguish nucleotide states at polymorphic sites. Three restriction enzymes were identified that would survey four polymorphic sites: *Tsp*R I, *Bsm* I, *Bsi*HKA I (New England Biolabs, Beverly, MA, USA). To provide robust estimates of nucleotide frequencies at these polymorphic sites, ~50 individuals per colony were amplified as above and digested independently with the three enzymes. Digests employed 9 μL of the amplified PCR product and 1 U of enzyme with appropriate buffer at 65°C overnight. Restriction products were run on a 1.5% agarose gel and visualised by ethidium bromide staining. Products were scored against a HAE III ladder and samples of known haplotypes (based on direct sequencing).

Microsatellites

Frequency variation of nuclear microsatellite alleles among colonies was tested to assess genetic isolation. Seventeen microsatellite PCR primer pairs previously employed on other penguin species were initially assessed for amplification and scorability in 12–24 individuals of southeast Australian *E. minor*, and 11 of these loci were already documented to exhibit polymorphism in this species by Roeder et al. (2002) and Schlosser et al. (2003) (Table 1). PCR reactions were prepared in 10 μL volumes containing 1× PCR buffer (Invitrogen, Carlsbad, CA, USA), dNTPs (0.2 mM), 1.0–2.5 mM $MgCl_2$, 0.05 U of *Taq* polymerase (Invitrogen) and 1 μL DNA template.

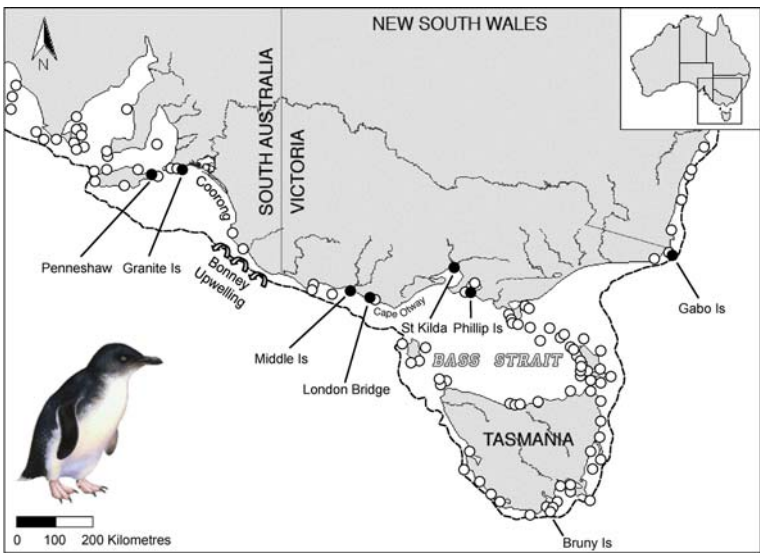

Fig. 1 Locations of *Eudyptula minor* colonies in southeast Australia based on Marchant and Higgins (1990), Robinson et al. (1996) and Brothers et al. (2001). *Closed circles* represent sampled colonies, *open circles* represent un-sampled colonies. The *dashed line* represents the approximate location of the coastline during the most recent Pleistocene Glaciation (~25,000 bp, Lambeck and Chappell 2001)

Table 1 Microsatellite amplification conditions and polymorphism in *Eudyptula minor*

Locus	Source	Reported motif	Reported size (bp)	Annealing (°C)	$MgCl_2$ (mM)	Multiplexing (in 72 μL)	Dye label	Polymorphism in *E. minor* (number of individuals)	
								Previously	This study
AM12	Roeder et al. (2002)	$(CA)_{13}$	140–154	55	2.5			Y (3)	N (24)
AM13	Roeder et al. (2002)	$A_{11}N_{33}(GT)_9(GC)_4(GT)$	110–135	55	2.5	6	HEX	Y (3)	Y (24)
RM6	Roeder et al. (2002)	$(CA)_{10}$	168–180	55	1.5			Y (3)	N (12)
TP500	Roeder et al. (2002)	$(CA)_{14}$	106–126	[b]	[b]			Y (3)	[b]
B3-2	Akst et al. (2002)	$(GT)_{12}$	292–314	55	1.5	2	FAM[c]	[a]	Y (24)
G3-6	Akst et al. (2002)	$(TG)_{10}$	258–308	55	1.5			[a]	N (12)
G3-11	Akst et al. (2002)	$(TG)_{12}$	300–306	55	1.5	3	HEX	[a]	Y (24)
G2-2	Akst et al. (2002)	$(GT)_{13}$	367–387	55	1.5	1	FAM	[a]	Y (24)
H2-6	Akst et al. (2002)	$(TG)_{15}$	282–296	55	1.5			[a]	N (24)
M1-11	Akst et al. (2002)	$(TG)_7CGTGC(GT)_3$	128	60	1.5			[a]	N (12)
Sh1Ca9	Schlosser et al. (2003)	$T_5(GT)_{17}$	128–139	55	1.5	2	FAM[c]	Y (5)	Y (24)
Sh1Ca12	Schlosser et al. (2003)	$(T)_7(CTA)(T)_4$	123–147	50	2.5			Y (5)	N (12)
Sh1Ca16	Schlosser et al. (2003)	$(CCCT)_2(CT)_1(CA)_{20}$	98–116	55	1.5			Y (5)	N (12)
Sh1Ca17	Schlosser et al. (2003)	$(CA)_{15}TATGCAA(CA)_4$	105–121	55	1.5			Y (5)	N (24)
Sh2Ca12	Schlosser et al. (2003)	$T_3(CA)_{14}A_3$	99–111	55	1.5			Y (5)	N (24)
Sh2Ca21	Schlosser et al. (2003)	$(CA)_{11}$	106–128	55	1.5			Y (5)	N (24)
Sh2Ca22	Schlosser et al. (2003)	$(CA)_{17}A_2(CA)_5$	95–129	55	1.5			Y (5)	N (24)

[a] Successfully amplified, but did not test polymorphism

[b] Unable to amplify during this study

[c] 5′ end of forward primer appended with an 18 bp 'M13' sequence (TGTAAAACGACGGCCAGT), to facilitate the incorporation of a dye label during PCR (Schuelke 2000)

Two strategies were employed for the incorporation of dye labels into PCR products for subsequent scoring on an automated fragment analyser. For all loci the forward primer was initially 5′ appended with an 18 bp 'M13' sequence (TGTAAAACGACGGCCAGT), and employed at 0.03 μM, while the reverse primer and a FAM- or HEX-labelled 'M13 only' primer were employed at 0.5 μM. This method is more economical than individually labelling a primer for each locus (Schuelke 2000). However, label incorporation via this method was not always successful, and in such instances the original forward primer was labelled directly. The 5′ end of the reverse primer was modified by adding 'GTTT'; this modification aimed to alleviate A-tailing effects and improve scorability of alleles (Brownstein et al. 1996).

Under the 'M13' approach, thermal cycling conditions were employed as follows; initial denaturation for 3 min at 95°C; 8 cycles of 30 s denaturation at 95°C, 30 s annealing (locus specific, Table 1) and 1 min extension at 72°C; and then 33 cycles as above but with annealing at 53°C (specific for dye-labelled 'M13 only' primer). A final extension of 10 min at 72°C was employed. Where the M13 approach was not employed, a single annealing temperature was retained throughout all cycles with forward and reverse primers at 0.5 μM. Locus-specific annealing temperatures were optimised in the range of 45–65°C, and $MgCl_2$ concentrations in the range of 1.0–2.5 mM. Scoring difficulties owing to partial A-tailing of PCR products were absent for all loci except AM13, and an increased final extension time (2 h) alleviated this problem.

PCR products were separated on a 6% denaturing polyacrylamide gel using an ABI 373 (Applied Biosystems Inc., Warrington, UK) automated fragment analyser, following manufacturer's instructions. Lengths of PCR products were determined relative to the GS400 size standard (ABI). Polymorphic loci were subsequently multiplexed and scored for ~50 individuals per colony, to assess allele frequency homogeneity among colonies.

Data analysis

Evolutionary relationships among mtDNA sequences were reconstructed via tree-building (maximum parsimony, maximum likelihood, Bayesian inference) and network analyses. Sequences obtained from ten New Zealand individuals during the study of Banks et al. (2002) were employed as outgroups. The nucleotide substitution model

for maximum likelihood and Bayesian analyses was selected from a set of 56 hierarchically nested candidates using ModelTest 3.7 and likelihood ratio tests (Posada and Crandall 1998). Parsimony and likelihood trees were constructed using PAUP*4.0b10 (Swofford 2003) under the heuristic search algorithm with ten random sequence additions. Gaps were treated as missing data. Bootstrap analysis (Felsenstein 1985) was conducted under the parsimony criterion with searches as above. Bayesian consensus trees were constructed using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003), with the same nucleotide substitution model as employed for maximum likelihood, but model parameters were derived from the default prior distributions. Duplicate Monte Carlo Markov chain searches were performed, each with four chains of 1,000,000 generations, and trees were sampled every 100 generations. Three of the chains were heated according to 'Temp = 0.1' to improve mixing among chains. The first 1,000 trees sampled were discarded as 'burn in', based on attainment of asymptotes for LnL and other parameters, and the remainder of trees were employed for the calculation of a consensus tree and bipartition posterior probabilities. Network analysis was performed to explore relationships among mtDNA sequences at shallow levels, without the assumption of strictly bifurcating relationships and extinction of ancestral forms (Posada and Crandall 2001). Networks were constructed using TCS 1.21 (Clement et al. 2000).

Polymorphic microsatellite loci were tested for Hardy–Weinberg equilibrium using Fisher's exact test, implemented in Genepop 3.3 (Raymond and Rousset 1995), such that any allele frequency differences among colonies can be considered the product of genetic isolation, rather than selection or other factors. For the same reason, non-neutrality of mtDNA haplotype frequencies was assessed with the Ewens–Watterson–Slatkin test (Slatkin 1996) using Arlequin 2.001 (Schneider et al. 2000). Genotypic disequilibrium among microsatellite loci was tested using Fisher's exact test in Genepop.

Homogeneity of mitochondrial haplotype, microsatellite allele and microsatellite genotype frequencies between colonies was assessed by exact tests using Genepop, with microsatellite results combined across loci using Fisher's method, following Ryman and Jorde (2001). To provide a measure of genetic structuring, variance of allele and haplotype frequencies among samples (F_{ST}, Wright 1978) was estimated by calculating θ (Weir and Cockerham 1984) with FSTAT 1.2 (Goudet 1995). Significance of observed θ-values were estimated from 1,000 permutations of mitochondrial haplotypes and microsatellite alleles or genotypes (in the case of deviation from Hardy–Weinberg expectations) among colonies. The presence of a significant relationship between θ and geographic distance between colonies (shortest ocean distance) was assessed using a Mantel test (1,000 randomisations) implemented in IBD 1.52 (Bohonak 2002). We also conducted Spatial Analysis of Molecular Variance (SAMOVA, Dupanloup et al. 2002) of the microsatellite data using Arlequin, to determine the maximally differentiated groups of populations without any assumptions of Hardy–Weinberg or genotypic equilibrium. Critical significance levels were adjusted for simultaneous tests, using the sequential Bonferroni procedure where necessary (Rice 1989).

The Bass Strait region has undergone recent and dramatic change, representing a dry land connection or marshy/estuarine habitat as recently as 25,000 BP (Lambeck and Chappell 2001). To assess whether the genetic structuring among colonies in the Bass Strait region (Middle Island–Gabo Island) could reflect recent colonisation and population expansion, we tested the mitochondrial control region variation for signatures of such an event by calculating Fu's (1997) F_s and Tajima's (1989) D using Arlequin. Mismatch distribution analysis (Rogers and Harpending 1992; Harpending 1994) was also performed, and estimates of τ were converted into years since the onset of population expansion by inferring a 2–3 years generation time for *E. minor* (Dann and Cullen 1990; Dann et al. 1995), and mutation rate estimates for the same part of the mitochondrial control region derived from temporally separated Adélie penguin individuals ($0.53–1.43 \times 10^{-6}$ changes per site per year; Lambert et al. 2002). These mutation rates are more rapid than those traditionally employed (e.g. 0.208×10^{-6} changes per site per year, Quinn 1992), but seem more appropriate for population-level analyses (Burridge et al. 2006; Ho and Larson 2006; Waters et al. 2007).

Results

mtDNA phylogeography

Among the 22 individuals analysed from southeast Australia, 13 polymorphic nucleotides were observed among the 417 bp sequenced, and five of these were parsimony-informative (sequences available from GenBank, accession numbers AF468954, AF468956, EU043384-403). The tree topologies derived from Bayesian, likelihood (-LnL = 987.34145) and parsimony (86 steps) analyses were largely concordant, with one of the parsimony topologies depicted in Fig. 2. Two deep and well-supported lineages were observed, one represented only by New Zealand individuals, the other containing both Australian and New Zealand individuals, consistent with Banks et al. (2002). Three haplotypes were shared among southeast Australian colonies and there was no evidence for phylogeographic

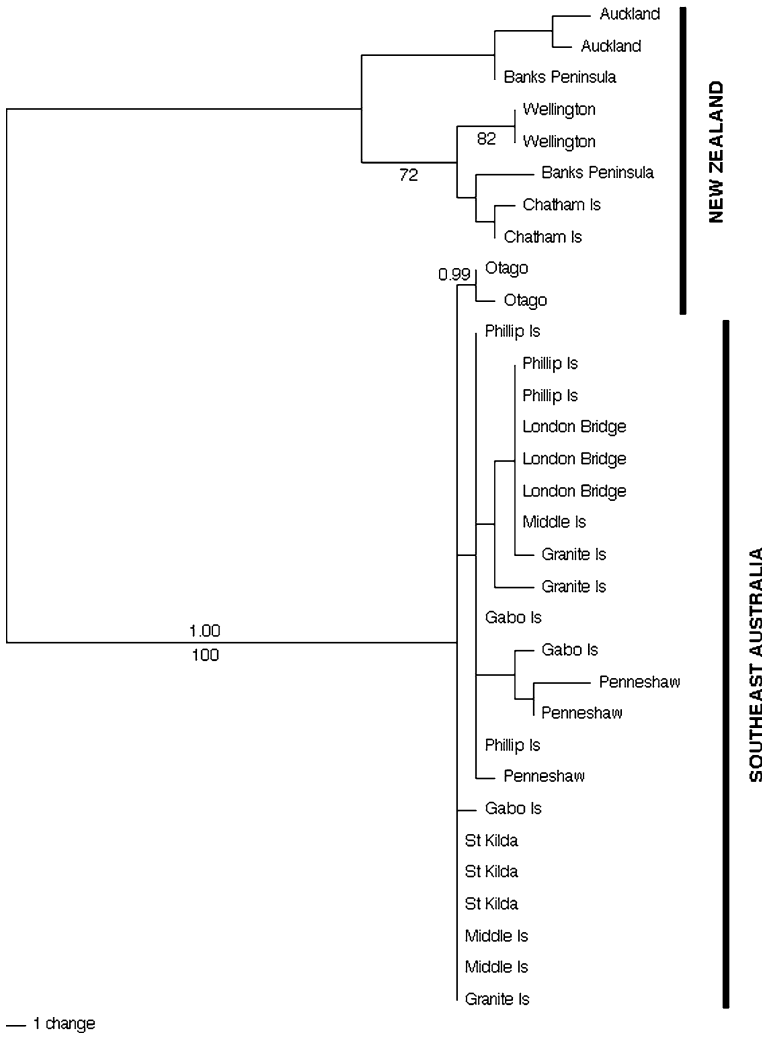

Fig. 2 One of 86 equally most parsimonious topologies recovered from the analysis of mitochondrial DNA control region sequences. Numbers below branches represent bootstrap percentages derived from 500 pseudoreplicate datasets (when exceeding 75%), while the numbers above branches represent Bayesian posterior probabilities (when exceeding 0.95) derived from duplicate analyses of 1×10^6 generations. New Zealand haplotypes were derived from Banks et al. (2002) and were included for comparative purposes

structuring in this region, based on a lack of well-supported, geographically concordant clades. Network analysis produced the same conclusions (data not shown).

Microsatellite and mtDNA RFLP variation

PCR amplification was achieved in *E. minor* for 16 of the 17 microsatellite loci, but only five of these loci gave repeatable and scorable polymorphic patterns: AM13, B3-2, G2-2, G3-11, Sh1Ca9. Approximately 350 adults were genotyped for these five microsatellite loci across the seven study colonies. All seven colonies exhibited polymorphism at all five loci (Table 2), and the mean number of alleles per colony across loci ranged from 3.6 to 4.4, with mean expected heterozygosity ranging from 0.412 to 0.435 (Table 3). There was no evidence of genotypic disequilibrium within samples ($P > 0.05$). The only locus deviating from Hardy–Weinberg expectations was G2-2 at Penneshaw, following Bonferroni correction for simultaneous tests ($P < 0.001$). Restriction analysis of the mitochondrial control region fragment with three enzymes yielded 13 haplotypes, and all colonies were polymorphic (Table 2). The Ewens–Watterson–Slatkin test failed to reject selective neutrality of haplotype frequencies following Bonferroni correction ($P > 0.04$).

Based on exact tests of microsatellite allele frequencies, Penneshaw was found to be genetically distinct from all other colonies ($P < 0.004$), which in turn were genetically homogeneous ($P > 0.006$) (Table 4). Analysis of microsatellite genotype frequencies avoids any potential problems surrounding the inclusion of loci deviating from Hardy–Weinberg equilibrium (such as locus G2-2 at Penneshaw), and produced the same results as analysis of allele frequencies apart from the Penneshaw–Granite Island comparison, which was no longer significant ($P = 0.007$) (Table 4). Likewise, SAMOVA, which does not make any assumptions of Hardy–Weinberg or genotypic equilibrium, revealed maximum Φ_{CT} for clustering of populations into only two groups (Table 5); these

Table 2 Microsatellite allele and mitochondrial haplotype frequencies for seven southeast Australian colonies of *Eudyptula minor*

Locus	South Australia		Victoria				
Allele	Penneshaw	Granite Island	Middle Island	London Bridge	St. Kilda	Phillip Island	Gabo Island
AM13							
n	51	50	50	50	52	50	51
117	0.588	0.770	0.663	0.735	0.735	0.690	0.725
118	0.275	0.130	0.214	0.184	0.206	0.230	0.167
119	0.137	0.100	0.122	0.082	0.059	0.080	0.108
B3-2							
n	50	50	50	50	51	50	51
307	0.010	0.040	–	0.031	0.020	0.080	0.010
313	–	0.010	–	–	–	–	–
315	0.286	0.340	0.500	0.429	0.350	0.390	0.333
317	0.061	0.140	0.083	0.020	0.040	0.040	0.020
319	0.429	0.250	0.208	0.224	0.290	0.160	0.186
321	0.214	0.180	0.146	0.276	0.280	0.230	0.324
325	–	0.040	0.063	0.020	0.020	0.100	0.118
329	–	–	–	–	–	–	0.010
G2-2							
n	50	50	48	50	52	50	51
377	0.612	0.410	0.280	0.235	0.350	0.260	0.265
379	0.010	–	–	–	–	–	–
381	0.133	0.170	0.110	0.163	0.130	0.170	0.176
383	0.143	0.280	0.427	0.398	0.400	0.330	0.382
385	–	0.020	–	–	–	–	–
387	–	–	0.012	–	–	–	–
389	0.092	0.110	0.146	0.153	0.100	0.230	0.137
391	–	–	–	0.031	–	–	–
393	0.010	0.010	0.012	0.010	0.020	0.010	0.039
395	–	–	0.012	0.010	–	–	–
G3-11							
n	51	50	50	50	52	50	51
320	–	–	0.020	0.020	–	0.030	–
324	0.069	0.030	0.031	0.031	0.010	0.010	0.020
326	0.931	0.970	0.949	0.949	0.990	0.960	0.980
Sh1Ca9							
n	51	50	50	50	52	50	51
132	0.108	0.100	0.061	0.031	0.088	0.040	0.088
134	0.892	0.880	0.939	0.949	0.912	0.960	0.902
136	–	–	–	0.020	–	–	–
138	–	0.020	–	–	–	–	0.010
mtDNA							
n	51	50	43	48	51	50	51
AAA	–	0.120	–	–	0.020	0.020	–
AAB	0.078	0.040	0.023	0.021	0.039	0.020	0.078
AAC	0.176	0.160	0.116	0.021	0.039	0.140	0.176
AAD	0.059	0.200	0.698	0.521	0.725	0.420	0.569
ABA	0.118	0.020	–	–	–	–	–
ABB	0.412	0.200	0.047	0.042	–	0.020	0.020
ABC	0.059	0.100	0.023	0.063	0.020	0.120	0.039

Table 2 continued

Locus	South Australia		Victoria				
Allele	Penneshaw	Granite Island	Middle Island	London Bridge	St. Kilda	Phillip Island	Gabo Island
ABD	0.059	0.080	0.023	0.021	0.020	–	0.020
BAA	–	–	–	0.021	–	0.060	–
BAB	0.020	0.040	0.047	0.292	0.118	0.180	0.078
BAC	0.020	–	0.023	–	–	–	0.020
BAD	–	–	–	–	0.020	0.020	–
BBD	–	0.040	–	–	–	–	–

Table 3 Microsatellite variability for each *Eudyptula minor* colony (averaged across loci) and each locus (averaged across colonies)

	Population						
	Penneshaw	Granite Island	Middle Island	London Bridge	St. Kilda	Phillip Island	Gabo Island
n (total)	51	50	50	50	52	50	51
H_o	0.352	0.420	0.380	0.400	0.415	0.400	0.427
H_e	0.435	0.431	0.423	0.412	0.404	0.427	0.428
A	3.600	4.200	4.000	4.400	3.600	3.800	4.000
	Locus						
	AM13	B3-2	G2-2	G3-11	Sh1Ca9		
H_o	0.441	0.685	0.646	0.077	0.148		
H_e	0.458	0.724	0.708	0.075	0.150		
A	3.000	6.000	5.857	2.429	2.429		
A (total)	3	8	10	3	4		

n number of individuals scored, H_o observed heterozygosity, H_e expected heterozygosity, *A* number of alleles observed

Table 4 Pair-wise exact test probabilities of frequency homogeneity among colonies for mitochondrial DNA haplotypes and microsatellite alleles and genotypes

	Penneshaw	Granite Island	Middle Island	London Bridge	St. Kilda	Phillip Island	Gabo Island
Penneshaw		0.007	0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]
Granite Island	0.003[a]		0.000[a]	0.000[a]	0.000[a]	0.000[a]	0.000[a]
	0.007						
Middle Island	0.000[a]	0.063		0.058	0.287	0.057	0.872
	0.000[a]	0.082					
London Bridge	0.000[a]	0.007	0.218		0.087	0.369	0.019
	0.000[a]	0.011	0.232				
St. Kilda	0.000[a]	0.198	0.072	0.281		0.009	0.183
	0.002[a]	0.246	0.100	0.237			
Phillip Island	0.000[a]	0.007	0.110	0.420	0.038		0.085
	0.000[a]	0.012	0.143	0.410	0.045		
Gabo Island	0.000[a]	0.032	0.077	0.153	0.526	0.178	
	0.000[a]	0.049	0.124	0.144	0.569	0.202	

Mitochondrial DNA-values are given above the diagonal, and microsatellite values (probabilities combined across loci using Fisher's method) are below the diagonal, with the upper element representing genic homogeneity based on all loci, and the lower value genotypic homogeneity based on all loci. Populations are ordered from west to east

[a] $P < 0.05$ following sequential Bonferroni correction for 21 simultaneous tests (and more than one standard error less than the critical *P* in the case of mitochondrial DNA)

Table 5 Fixation indices corresponding to the groups of populations inferred by the SAMOVA algorithm

Number of groups	2	3	4	5
Φ_{ST}	0.083[a]	0.060[a]	0.046[a]	0.043[a]
Φ_{SC}	0.016[a]	0.009[a]	0.007	0.010[a]
Φ_{CT}	0.068	0.051[a]	0.053[a]	0.053[a]

[a] $P < 0.05$ (proportion of randomised permutations of populations to groups yielding Φ greater than or equal to observed Φ)

corresponded to Penneshaw in one group, and the remaining six colonies in the other group. Although the Φ_{CT} for these two groups of populations was not significantly >0 (Table 5), this reflects the fact that one-in-seven random permutations of seven populations into two groups, when they contain one and six populations each, will return a Φ_{CT} equal to the observed Φ_{CT} (i.e. Penneshaw versus the remaining populations). Hence, the proportion of randomised groups achieving Φ_{CT} greater than or equal to the observed Φ_{CT} cannot be any <0.14 given the limitations imposed by the number of populations analysed and an inferred 'six plus one' grouping. mtDNA RFLP haplotype frequencies distinguished both Penneshaw and Granite Island from all other colonies ($P < 0.001$), but not from each other ($P = 0.007$) (Table 4).

Estimates of F_{ST} (θ) for each microsatellite locus over all samples ranged from 0.003 to 0.032, with those for B3-2 and G2-2 significantly >0 when permuting either alleles or genotypes among samples ($P < 0.001$), although that for G2-2 was not significant after the removal of Penneshaw ($P = 0.132$), which deviated from Hardy–Weinberg expectations at this locus (Table 6). θ derived from all microsatellite loci and all samples was 0.018, and was significantly >0 ($P < 0.001$); θ across all microsatellite loci dropped to 0.005 when Penneshaw was removed from the analysis, but remained significantly >0 ($P = 0.018$) (Table 6). θ derived from mitochondrial RFLP haplotype frequencies was higher than any of the microsatellite loci (0.102) and significantly >0 ($P < 0.001$) (Table 6). Mantel tests did not reveal a significant relationship between θ and geographic distance between colonies based on microsatellites, regardless of whether employing all loci ($r = 0.4079$, $P = 0.138$), excluding locus G2-2 from all sites ($r = 0.3755$, $P = 0.131$), or excluding Penneshaw ($r = 0.3475$, $P = 0.194$). Similarly, mtDNA RFLP also lacked such a relationship based on all colonies ($r = 0.4708$, $P = 0.064$).

Table 6 Structuring of genetic variation (θ, Weir and Cockerham 1984) among colonies of *Eudyptula minor* in southeast Australia for microsatellite and mitochondrial loci

Locus	θ	P (θ not > 0)
AM13	0.004	0.175
B3-2	0.018	<0.001
G2-2	0.032 (0.005)	<0.001 (0.132)
G3-11	0.004	0.171
Sh1Ca9	0.003	0.264
All microsatellites	0.018 (0.005)	<0.001 (0.018)
G2-2 excluded	0.011	0.001
mtDNA	0.102	<0.001

The significance of θ-values was estimated from 1,000 permutations of alleles, genotypes, or haplotypes among colonies. Values in parentheses represent the exclusion of the Penneshaw colony, which deviated from Hardy–Weinberg equilibrium for locus G2-2

Both Fu's (1997) F_s and Tajima's (1989) D were not significant ($F_s = 0.31$, $D = -0.18$, $P > 0.46$) for individuals from the Bass Strait region (Middle Island–Gabo Island), and hence not indicative of recent population expansion. In contrast, a smooth unimodal mismatch distribution was observed, suggesting population expansion. Estimates of time since the onset of population expansion derived from τ were 6,500 to 17,500 BP, accommodating both uncertainty in mutation rate and generation time, with 95% confidence intervals spanning 800–63,400 BP.

Discussion

Genetic homogeneity among colonies

The dominant finding of this study was the expansive genetic homogeneity among colonies of *E. minor* within southeast Australia. Five colonies between Gabo Island in the east and Middle Island in the west (~860 km apart) were not distinguished from one another by either microsatellite or mtDNA analyses. This is in contrast with the sometimes-large distances (up to 555 km between adjacent sampled colonies), and the ecological and morphological differences among individuals from different colonies (Reilly and Cullen 1981; Cullen et al. 1992; Overeem and Wallis 2003; Arnould et al. 2004; Overeem 2005; Overeem et al. 2006). While this result hinges on variation in mtDNA and only five microsatellite loci, we screened a total of 17 microsatellite loci observed to exhibit polymorphism in penguin species, including 11 loci that were reported to be polymorphic in *E. minor* (Roeder et al. 2002; Schlosser et al. 2003). Ideally, additional polymorphic markers should be surveyed to confirm our results, but other genetic studies of seabirds have also documented a lack of genetic differentiation among colonies in contrast to that anticipated based on variation in morphology, colouration, ecology, or behaviour (e.g. Randi et al. 1989; Avise et al. 2000; Genovart et al. 2003).

The lack of genetic structuring among *E. minor* colonies throughout the majority of the study range may reflect either historical or contemporary gene flow. With respect to

contemporary gene flow, there is evidence of some, albeit occasional, movement of fledglings from their natal colony to other colonies to breed. Reilly (1977) reported the discovery of an individual that was banded as a chick on Bruny Island in southern Tasmania and found breeding at Port Campbell National Park in Victoria (proximate to the London Bridge site) (Fig. 1). In addition, there have been two chicks banded on Phillip Island that have been reported breeding at St. Kilda Breakwater, several years later (P. Dann, unpublished data). There is also some evidence of movements by adults between colonies. For example, in an analysis of re-traps of over 5,505 adults flipper-banded on Phillip Island, Dann (1992) found that 0.2% were re-trapped on Gabo Island. However, it is not known if these adults bred in both locations or were breeding in a location other than their natal colony. In summary, there is observational evidence of some genetically effective movement between colonies via fledglings, but not as yet for adults. Contemporary observations of rapid increases in population size following founding or a period of decline in some *E. minor* colonies (Dann 1992; Johannesen et al. 2003) suggest a large contribution by immigrants, rather than growth entirely via self-recruitment, and this has been suggested for other seabirds (Brit-Friesen et al. 1992; Austin et al. 1994).

Population expansions over longer time scales may have contributed to the lack of genetic structuring among contemporary colonies. Sea level rise subsequent to the most recent Pleistocene glaciation (~25,000 BP) dramatically increased the area available for *E. minor* foraging and nesting in southeast Australia (Fig. 1; Galloway and Kemp 1981; Carter 1994; Lambeck and Chappell 2001). The results from mitochondrial mismatch distributions were entirely consistent with this possibility, suggesting population expansion at 6,500–17,500 BP (95% confidence intervals: 800–63,400 BP). Contemporary Bass Strait colonies may have been derived from a genetically homogenous source population, and any concomitant increases in population numbers and general abundance would also tend to hinder the development of population genetic structuring (McCauley 1991). While our results cannot reject population expansion associated with other, unknown events falling within the broad confidence intervals reported above, Pleistocene fluctuations in availability of nesting habitat have also been implicated for a lack of genetic differentiation among colonies of short-tailed shearwater (*Puffinus tenuirostris*) in this region (Austin et al. 1994), and among other seabird populations elsewhere (Roeder et al. 2001; Peck and Congdon 2004), as has post-Pleistocene re-colonisation of a wide area from a single, homogeneous source population (Friesen et al. 1996; Liebers and Helbig 2002). Consequently, such climatic fluctuations may represent a major contributor to contemporary genetic structuring of near-shore nesting seabirds.

Genetic distinction of western colonies

While genetic homogeneity existed across the majority of the study range, the two western-most colonies, Penneshaw and Granite Island, are distinct from the eastern colonies based on mitochondrial haplotype frequencies. Penneshaw was also distinguished from all other colonies, including Granite Island, based on microsatellite allele frequencies, but distinction from Granite Island disappeared when analysing genotype frequencies—to compensate for deviation from Hardy–Weinberg equilibrium at locus G2-2 in Penneshaw.

The genetic distinctiveness of the two western-most samples is coincident with a putative biogeographic break immediately east of Granite Island—an expansive region of beach without offshore islands, termed the Coorong (Fig. 1). While this region has been hypothesised as a barrier to dispersal in several near-shore taxa (Edgar 1986), genetic tests to date do not provide support, even in obligate inter-tidal or reef dwellers (Waters et al. 2004, 2005). It is also difficult to imagine how a long stretch of beach could be a barrier to *E. minor*, which forages widely in open water (Collins et al. 1999), and breeds on a range of coastal habitats, including sand dunes (Stahel and Gales 1991). This result supports the observation that geographic isolation alone appears to be a poor predictor of genetic differentiation among populations of seabirds (Friesen et al. 2007).

A more likely explanation for the genetic break towards the western limit of the sampling distribution is a difference in breeding phenology (Lack 1966; Thumser and Karron 1994; Roeder et al. 2001). South Australian *E. minor* breed earlier than Victorian individuals (Dann 1992; Overeem and Wallis 2003; Overeem 2005; A. Wiebkin, personal communication). For example, during 2002 the mean egg laying date for successful breeders at Granite Island (June-24) was significantly earlier than those for Middle Island, London Bridge, St. Kilda and Phillip Island, which spanned August-27 to September-22 (Overeem 2005). During 2003, mean egg-laying dates for successful breeders were later relative to 2002, but Granite Island was still significantly earlier than London Bridge, St. Kilda and Phillip Island (May-30 versus October-3 to November-30; no data for Middle Island) (Overeem 2005). A marked decline has been reported in breeding success with delayed onset at some colonies (Rogers et al. 1995; Johannesen et al. 2003; Overeem 2005), such that any immigrants arriving late in a colonies' breeding season will have a lower chance of successfully raising offspring even if they succeed in finding an available partner and nesting site.

Recent evidence suggests that cooler sea surface temperatures in the autumn preceding breeding lead to a

delayed breeding season in *E. minor* (Chambers 2004), and hence support previous hypotheses that inter-regional differences in *E. minor* breeding phenology may also be explained by sea surface temperatures—and probably—food availability and quality (Reilly and Cullen 1981; Gales 1984; Mickelson et al. 1992). Sea surface temperatures are typically 2°C warmer in the vicinity of Granite Island and Penneshaw relative to those colonies we sampled further east, and a localised region of cold water upwelling to the east of the Coorong—the Bonney Upwelling (Fig. 1; Butler et al. 2002)—may also help reinforce significant breeding phenology differences and genetic isolation of *E. minor*. Further data on breeding phenology in these two areas, plus genetic data from additional colonies and markers, are required to test this hypothesis. Differences in breeding phenology and oceanography have also been invoked for genetic isolation among colonies of rockhopper penguins, *Eudyptes* (Jouventin et al. 2006; Banks et al. 2006), and oceanographic differences also appear related to genetic structuring among *Calonectris* shearwaters (Gómez-Díaz et al. 2006).

Low microsatellite polymorphism

It was surprising that only five of the 17 microsatellite loci tested were found to be polymorphic in southeast Australian *E. minor*, as 11 of these loci were previously reported to be polymorphic in this species (Table 1; Roeder et al. 2002; Schlosser et al. 2003). It is unlikely that these discrepancies could be explained by sampling intensity, as the surveys of Roeder et al. (2002) and Schlosser et al. (2003) employed three and five individuals, respectively, whereas we surveyed 12–24 individuals from across the southeast Australian study range. Scoring errors may have artificially increased the amount of polymorphism previously reported (Harker 2001; Hoffman and Amos 2005). Alternatively, the source of samples may be responsible. Schlosser et al. (2003) surveyed captive *E. minor* from North American zoos, which could be of wider geographically origin than those analysed herein. Roeder et al. (2002) surveyed three *E. minor* individuals from New Zealand, where five subspecies have been recognised, as opposed to Australia where only one has been considered (Kinsky and Falla 1976). One might also expect greater microsatellite polymorphism for New Zealand rather than Australian *E. minor* based on the comparative depth of mtDNA variation in these regions (Fig. 2). The paraphyletic relationship of New Zealand haplotypes with respect to Australian haplotypes may reflect a recent colonisation of Australia from New Zealand (Banks et al. 2002), through which some microsatellite polymorphism could have been lost.

Conservation implications

The genetic data at hand suggests that, minimally, the South Australian colonies of Penneshaw and Granite Island are distinct from those we analysed in Victoria, and that this distinction should be accommodated in management practices. While microsatellites suggest that Penneshaw is distinct from Granite Island, this result hinges on a locus that deviates from Hardy–Weinberg expectations, and requires verification. Given that differences in breeding phenology exist among colonies in South Australia (A. Wiebkin, personal communication), it is possible that the deviation from Hardy–Weinberg at Penneshaw reflects the presence of individuals sourced from a genetically distinct population further west (i.e. Wahlund effect).

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