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Low levels of genetic differentiation characterize Australian humpback whale (*Megaptera novaeangliae*) populations

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Abstract

Humpback whales undertake long-distance seasonal migrations between low latitude winter breeding grounds and high latitude summer feeding grounds. We report the first in-depth population genetic study of the humpback whales that migrate to separate winter breeding grounds along the northwestern and northeastern coasts of Australia, but overlap on summer feeding grounds around Antarctica. Weak but significant differentiation between eastern and western Australia was detected across ten microsatellite loci (F_{ST} = 0.005, P = 0.001; D_{EST} = 0.031, P = 0.001, n = 364) and mitochondrial control region sequences (F_{ST} = 0.017 and Φ_{ST} = 0.069, P = 0.001, n = 364). Bayesian clustering analyses using microsatellite data could not resolve any population structure unless sampling location was provided as a prior. This study supports the emerging evidence that weak genetic differentiation is characteristic among neighboring Southern Hemisphere humpback whale breeding populations. This may be a consequence of relatively high gene flow facilitated by overlapping summer feeding areas in Antarctic waters.

Key words: mtDNA, microsatellites, population genetic structure, conservation, management, *Megaptera novaeangliae*.

For many marine species, ecological and environmental discontinuities such as ocean currents, changes in bathymetry and ocean temperature are increasingly being identified as cryptic barriers to gene flow and dispersal (Kaschner *et al.* 2006, Knutsen *et al.* 2009, Unal and Bucklin 2010, Mikkelsen 2011, Shen *et al.* 2011). The

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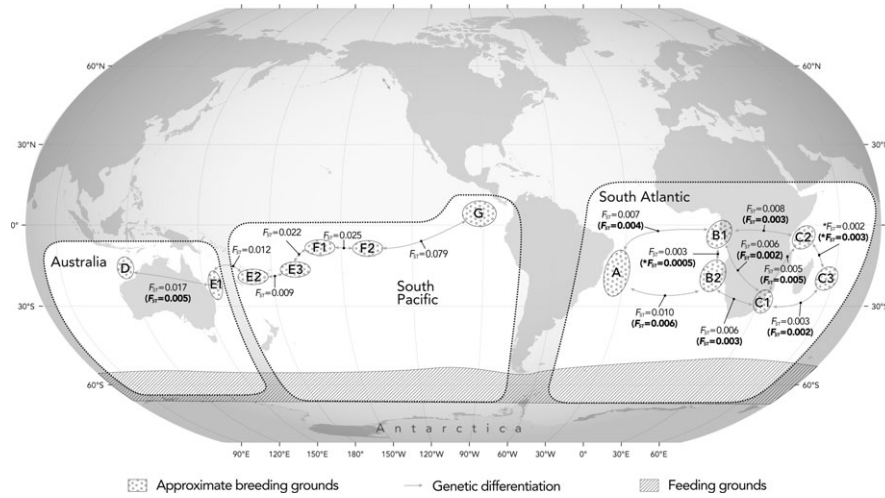

Figure 1. Estimates of genetic differentiation among selected humpback whale populations in the Southern Hemisphere. Pairwise estimates of F_{ST} based on microsatellite data (boldface; Australia comparisons = ten loci, this study, South Atlantic = nine loci, Pomilla 2005) and mtDNA control region sequence data (Australia and South Pacific = 470bp, South Atlantic = 486bp, Baker *et al.* 1998a, Olavarría *et al.* 2007, Rosenbaum *et al.* 2009). All *P*-values were less than 0.05 except those indicated by an asterisk.

influence of social and learned behaviors that may also establish or reinforce population boundaries are less understood. Such factors may be highly relevant to cetacean species that exhibit complex communication and social behaviors and where migratory behavior is thought to be learned through social inheritance from the mother to the calf (Clapham 1996, Hauser *et al.* 2007). Therefore, despite their high vagility, cetaceans may exhibit highly structured populations primarily driven by nonphysical barriers (Hoelzel 1998).

Like other balaenopterid species, humpback whales undertake long-distance seasonal migrations between low latitude winter breeding and calving grounds and high latitude summer feeding grounds (Fig. 1; Mackintosh 1965). These whales also exhibit a large range of social and sexual behaviors, have strong maternal fidelity, and are renowned for their repertoire of complex culturally acquired "songs" and calls (Clapham 1996, Noad *et al.* 2000, Valsecchi *et al.* 2002, Smith *et al.* 2008). Historically, humpback whale populations have been defined based on the distribution of calving areas and migratory routes and these populations have been treated as management units in the apportionment of catch quotas for commercial whaling (Kellogg 1929, Chittleborough 1965, Mackintosh 1965, Dawbin 1966). More recently, because demographic studies are difficult to undertake, genetic analysis of mitochondrial (mtDNA) and nuclear markers has been applied to gain insights on population structure, dispersal and mating systems.

In the Northern Hemisphere, humpback whale populations are geographically separated by the American and Asia–European continents (Baker *et al.* 1986; Palsbøll *et al.* 1995; Calambokidis *et al.* 1996; Clapham 1996; Palsbøll *et al.* 1997*a*; Clapham *et al.* 1999; Calambokidis *et al.* 2001, 2008) and within each ocean basin,

individuals from common breeding grounds can show strong fidelity to different discrete foraging areas (Calambokidis *et al.* 2001, Stevick *et al.* 2006). In contrast, the high latitude feeding grounds of humpback whales in the Southern Hemisphere are circumpolar with individuals from different breeding populations intermingling while in Antarctic waters (*e.g.*, Chittleborough 1961, Dawbin 1966, Robbins *et al.* 2011). On their annual migration, they segregate into at least seven low latitude breeding areas, which are widely distributed around oceanic islands and specific coastal regions proximate to continental shelf areas (Mackintosh 1965). With no continental barriers to movement while on feeding grounds, there is the potential for permanent migration among populations as described for other marine megafauna (Bonfil *et al.* 2005, Boyle *et al.* 2009). Recent studies have shown relatively low levels of differentiation between neighboring humpback whale populations in the Southern Hemisphere (Baker *et al.* 1998*a*, Olavarría *et al.* 2007, Rosenbaum *et al.* 2009, Cypriano-Souza *et al.* 2010).

Two recognized populations of humpback whales occur along the coasts of Australia. One migrates along the eastern seaboard and is thought to mate and calve within the Great Barrier Reef (Smith *et al.* 2012), the other migrates along the western seaboard and mates and calves off the Kimberley coast of western Australia (Jenner *et al.* 2001). During the 20th century, Australian humpback whales were hunted along both the eastern and western migratory corridors and intensively in their Antarctic feeding grounds (Mackintosh 1965). By the time commercial whaling ceased in 1963, the western Australian population was estimated to be fewer than 500 animals, down from approximately 17,000 prior to 1934 (Chittleborough 1965, Bannister 1994), and the eastern Australian population was reduced to as few as 100 individuals, down from a preexploitation abundance estimate of between 16,022 and 22,957 (Chittleborough 1965, Paterson *et al.* 1994, Jackson *et al.* 2008). Recent data have shown that both populations are recovering strongly with the current rate of increase at about 10%–11% per annum (Noad *et al.* 2011, Paxton *et al.* 2011, Salgado Kent *et al.* 2012). Absolute abundance for western Australian humpback whales is currently estimated at 21,750 (95% CI 17,550–43,000) (Hedley *et al.* 2011) and 14,522 (95% CI 12, 777–16,504) for eastern Australia (Noad *et al.* 2011).

Although the approximate migration routes and distribution of breeding activity is reasonably well described for the two Australian populations, the degree of connectivity is less known. During the 1950s and 1960s stainless steel "Discovery" marks were shot into whales, some of which were later recovered when the whales were killed and flensed (Mackintosh 1965, Dawbin 1966). These studies showed that whales from the separate breeding grounds mix in Antarctica, and there were even two cases where individuals moved between breeding grounds (see below), but it is difficult from such data to estimate the magnitude of gene flow or whether the populations are likely to be demographically independent. Here, based on extensive sampling, we specifically evaluate (1) the population genetic structure among the eastern and western populations of Australian humpback whales by examining variation in both maternally inherited mtDNA and biparentally inherited microsatellite markers for both sexes, (2) extend previous analyses of mtDNA variation among humpback whales in Oceania and western Australia by combining our data with Olavarria *et al.* (2007) to include eastern Australia, and (3) compare and contrast our findings with other studies of humpback whales and consider the ecological implications of the emerging genetic patterns.

Table 1. Samples collected from individual (*n*) humpback whales from three locations off the east and west coast of Australia. The number of duplicate samples is also shown, and the number of known female (F) and male (M) individuals.

Region Sampling site	Sampling period	Samples	No. of duplicates	*n*	F	M
Eastern Australia		141	10	131	50	81
Eden	2008	63	2	61	14	47
	June	45	2	43	8	35
	October, November	18	0	18	6	12
Tasmania	2006–2008	78	8	70	36	34
	July	1	0	1	0	1
	November, December	77	8	69	36	33
Western Australia		223	18	205	89	116
Exmouth	2007 September, October					
Total		364	28	336	139	197

Methods

Population Definition in the Southern Hemisphere

The International Whaling Commission (IWC) currently recognizes seven breeding populations in the Southern Hemisphere (named A to G), although it is unclear whether further subdivision is appropriate for African, Australian, and South Pacific populations (Chittleborough 1965, Mackintosh 1965) (Fig. 1). This uncertainty has led to the description of "subpopulations" (with the addition of a numerical suffix), although this term has never been strictly defined. The humpback whales that migrate along the west and east coasts of Australia are recognized as population D and E1, respectively. Subpopulations E2 and E3 (New Caledonia and Tonga), and F1 and F2 (Cook Islands and French Polynesia) are often referred to in IWC literature as "Oceania," which is listed separately by the IUCN as endangered (IWC 1998, Childerhouse *et al.* 2008).

Sample Collection, DNA Extraction and Sex Identification

A total of 364 biopsy samples were collected from humpback whales. These samples were collected from eastern (Eden, New South Wales; eastern Tasmania) and western Australia (Exmouth). The timing and location of the sampling is presented in Table 1. Samples were collected using a biopsy dart propelled by a modified 0.22 caliber rifle and then stored in 70% ethanol at −80ºC (Krützen *et al.* 2002). Total cellular DNA was extracted from skin tissue using a standard salt extraction technique (Aljanabi and Martinez 1997), or an automated Promega Maxwell 16 System. Sex was determined using a fluorescent 5′ exonuclease assay producing PCR product from the ZFX and ZFY orthologous gene sequences (Morin *et al.* 2005).

Microsatellite Loci

Samples were genotyped at ten polymorphic microsatellite loci including nine dinucleotide repeats [EV1, EV14, EV37, EV94, EV96 (Valsecchi and Amos 1996);

GT211, GT23, GT575 (Bérubé *et al.* 2000); rw4-10 (Waldick *et al.* 1999)] and one tetranucleotide repeat [GATA417 (Palsbøll *et al.* 1997*b*)]. To allow simultaneous amplification of several loci in one PCR reaction, we used a Qiagen Multiplex Kit for the following sets of loci: set 1 (EV37 and GT23); set 2 (EV14, EV96, and GATA417); set 3 (EV1, EV94, and GT575) and GT211 and rw4-10 individually. For each locus, one of the primers within each pair was labeled fluorescently at the 5′ end to allow for visualization of alleles on an automated sequencer. Each PCR had a final volume of 12.5 μL and included: 1× Qiagen Multiplex PCR Master Mix (containing HotStarTaq DNA Polymerase, Multiplex PCR Buffer, $MgCl_2$ and dNTP mix), 2 μM of each primer (labeled and nonlabeled) and 1–8 ng of template DNA (estimated using a NanoDrop spectrometer 3300). The thermocycling profile consisted of an initial denaturing step of 95°C for 15 min, 30 cycles (30 s at 94°C, 90 s at 58°C annealing, 60 s at 72°C) followed by a final extension step of 30 min at 60°C, with the exception that the optimal annealing temperature for the single locus reactions (GT211 and rw4-10) was 53°C. The annealing temperature for the single locus reactions was lowered to 53°C because null alleles were detected when run at 58°C.

Fluorescently labeled PCR products were resolved on an ABI 3130 automated sequencer. Allele sizes in base pairs (bp) were determined using the LIZ-500 size standard run in each lane. Microsatellite alleles were visualized and scored using GeneMapper v3.7 (Applied Biosystems).

Microsatellite Validation

Four steps were taken to ensure a robust microsatellite analysis. (1) To estimate genotyping error rate (Bonin *et al.* 2004) a subset of 16 samples was randomly selected, DNA extracted and genotyped at all ten loci individually by an independent geneticist. (2) Samples with identical matching genotypes across all ten loci were assumed to be due to repeated sampling and were removed from the data set (see Results). The average probability that two unrelated animals share the same genotype by chance alone, PI (probability of identity), and the more conservative probability, PI_{SIBS} (probability of identity siblings), were calculated following Peakall *et al.* (2006). (3) MICROCHECKER version 2.2.3 (van Oosterhout *et al.* 2004, 2006) was used to screen the microsatellite data set for genotyping errors such as null alleles, stuttering and large allele dropout. (4) Using Arlequin 3.1 (Excoffier *et al.* 2005), we tested for deviation from Hardy-Weinberg equilibrium at each locus and for linkage disequilibrium between loci within each population and among populations. Sequential Bonferroni correction was applied to all multiple pairwise comparisons (Rice 1989).

mtDNA

We amplified an approximately 700bp fragment of the control region proximal to the Pro tRNA gene via PCR reaction using primers light-strand M13Dlp1.5 and heavy strand Dlp8 (Garrigue *et al.* 2004). Amplifications were conducted in a final volume of 10 μL at the following concentrations: 2.5 mM $MgCl_2$, 200 μM dNTP, 0.4 mM each primer, 0.25U Taq (New England BioLabs Inc.), 1 × PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$) and 1 μL DNA (approximately 10–50 ng). Temperature profiles consisted of an initial denaturing period of 2 min at 94°C, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 54°C for

40 s, and extension at 72ºC for 40 s. A final extension period for 10 min at 72ºC was also included. Unincorporated primers were removed from PCR products using ExoSAP-IT or Agencourt AMPure XP. Sequencing reactions with the PCR primers were run using a Big Dye terminator cycle sequencing kit v3.1 (Applied Biosystems) followed by the use of Agencourt CleanSEQ to remove unincorporated primers. PCR products were sequenced on an ABI 3130 automated sequencer.

Forward and reverse sequences were manually edited, trimmed, and aligned within Sequencher 4.8 (Gene Codes Corp.) against sequences of 470bp in length, representing the panel of haplotypes previously defined from the South Pacific (Olavarría *et al.* 2007). This region started at position six of the reference humpback whale control region sequence (GenBank X72202; see Baker and Medrano-Gonzalez 2002, Olavarría *et al*. 2007), and is considered to include more than 85% of the variation in the entire control region. Comparisons of sequences to identify polymorphic sites and haplotypes were conducted using GenAlEx 6.5 (Peakall and Smouse 2006, 2012).

Statistical Analysis

For the purpose of presenting summary statistics, the samples from Eden and Tasmania were pooled and are collectively referred to as eastern Australian samples. For each microsatellite locus, the number of alleles, the number of private alleles, the observed heterozygosity, and the expected heterozygosity for each geographic region were calculated using GenAlEx 6.5. Arlequin 3.5 (Excoffier and Lischer 2010) was used to determine standard measures of mtDNA genetic diversity including haplotype frequencies, the number of unique haplotypes, the number of shared haplotypes, haplotype (Nei 1987) and nucleotide (Tajima 1983) diversity, and the number of sequence polymorphic sites. Haplotype and nucleotide diversity estimates were also recalculated following bootstrap resampling of the western Australian data set to generate ten data sets of the same size as eastern Australia.

The extent of genetic differentiation among the Eden and Tasmania sampling locations was initially evaluated using an Analysis of Molecular Variance (AMOVA) (Excoffier *et al.* 1992) with statistical testing by random permutation (999 permutations). Based on the outcome of this analysis, genetic differentiation was also calculated at a population level (*i.e.,* western Australia *vs*. eastern Australia). For microsatellite data, an estimate of F_{ST} (infinite allele model) was calculated using GenAlex 6.5 as per Weir and Cockerham (1984), Peakall *et al.* (1995) and Michalakis and Excoffier (1996). Recent analyses suggest that these standard measures of differentiation may be poorly suited as estimators of population divergence for data sets in which allelic diversity is high (Hedrick 2005, Jost 2008, Meirmans and Hedrick 2011). Given the high variability of the markers used here, Jost's D_{EST}, an unbiased estimator of divergence, was calculated using a modified version of the R package DEMEtics V0.8.0 (Jueterbock *et al.* 2010), with overall estimates of D_{EST} calculated from individual loci using a harmonic mean approximation and statistical testing by bootstrapping with 1,000 permutations. Compared with F_{ST}, D_{EST} partitions diversity based on the effective number of alleles rather than on the expected diversity to give an unbiased estimation of divergence (Jost 2008). For mtDNA data, an AMOVA was performed at both the nucleotide and haplotype level. GenAlEx 6.5 was used to estimate F_{ST} based on haplotype frequencies (Griffiths *et al.* 2011). For the nucleotide level analysis, MODELTEST 2.1.1 (Guindon and Gascuel 2003, Posada 2008, Darriba *et al.* 2012) identified Tamura and Nei (1993), assuming equal base frequencies with gamma correction ($\alpha = 0.12$), as the most appropriate model

of DNA evolution given the sequence data. Arlequin 3.5 was used to calculate individual pairwise nucleotide distances under this model of sequence evolution.

In keeping with the common practice in similar studies of humpback whales (Olavarría *et al.* 2007, Rosenbaum *et al.* 2009) we use the notation F_{ST} for haplotype frequency differentiation and Φ_{ST} for nucleotide differentiation (*e.g.* Weir and Cockerham 1984, Takahata and Palumbi 1985, Hudson *et al.* 1992).

To evaluate the genetic data without the need to impose *a priori* population structure, we applied the Bayesian clustering approach implemented in the software STRUCTURE version 2.3.1 (Pritchard *et al.* 2000) to the microsatellite data set. We also repeated the analysis using the three sampling locations as priors to assess the influence of geography (LocPrior model; Hubisz *et al.* 2009). This method attempts to partition samples into K group(s) such that the loci in those groups are in Hardy-Weinberg equilibrium, and linkage equilibrium. An ancestry model of admixture and correlated allele frequencies were assumed among populations with 10,000 burn-in steps and 300,000 Markov Chain Monte Carlo repetitions. Five replicates for each number of populations ($K = 1$ to 6) were performed to verify that the number of populations identified was consistent between runs. STRUCTURE output was summarized and evaluated using the software *CorrSieve* (Campana *et al.* 2011).

Potential differences in female and male dispersal rates between eastern and western Australia were investigated using both genetic markers by calculating pairwise estimates of F_{ST} among populations for each sex. For comparative purposes, Jost's D_{EST} was also calculated for microsatellite data. D_{EST} was not calculated for mtDNA data as the method is based on differences in interpopulation gene diversity (Jost 2008), and as such, does not take into account the evolutionary relationships between haplotypes (Meirmans and Hedrick 2011).

To investigate genetic structure between the Australian populations and those of the South Pacific (including New Caledonia, Tonga, Cook Islands, French Polynesia, and Colombia we combined our mtDNA data with those presented by Olavarría *et al*. (2007) and calculated F_{ST} and Φ_{ST} for pairwise comparisons. The correlation between geographic and genetic distances was analyzed using a Mantel test with statistical testing based on 999 random permutations conducted in GenAlEx 6.5 (Smouse *et al.* 1986, Smouse and Long 1992). Correlation coefficients were calculated between F_{ST} and Φ_{ST}, and the geographic distances between all sampling locations.

Results

Each of the 10 microsatellite loci were found to be in Hardy-Weinberg equilibrium (Table 2) and pairwise comparisons between loci revealed no linkage disequilibrium (all values of $P > 0.01$) after sequential Bonferroni correction. MICROCHECKER found no evidence of null alleles or stutter/short allele dominance effects across microsatellite loci, with null allele frequency estimates listed for each region in Table S1, Supplementary information. Repeat genotyping of 16 samples by an independent geneticist revealed two inconsistencies across 320 alleles–an error rate of 0.6%. This rate is lower than suggested by the guidelines of the IWC (2008) for systematic quality control in the use of microsatellite markers ($\leq$10% error rate) for management decisions. This low error rate does not guarantee that these genotypes, are in fact correct, but provides a significant increase in probability that they are correct compared to a single genotyping event (Pompanon *et al.* 2005).

Table 2. Genetic diversity in humpback whales from eastern and western Australia genotyped at ten loci. *n* = number of genotyped individuals per locus and HW = deviation from Hardy-Weinberg equilibrium (*P*-value); no HWE *P*-values were significant at a level of 0.05 after adjustment for multiple comparison with the sequential Bonferroni test (Rice 1989) (standard errors in parentheses).

	n		Number of alleles		Number of private alleles		Observed heterozygosity		Expected heterozygosity		HW (*P*-value)	
Locus	East	West	East	West	East	West	East	West	East	West	East	West
EV14	131	203	9	8	1	0	0.725	0.754	0.748	0.778	0.747	0.459
EV37	131	202	19	19	2	2	0.916	0.931	0.913	0.904	0.364	0.316
EV96	131	202	13	12	1	0	0.863	0.876	0.848	0.869	0.872	0.682
GATA417	131	203	15	15	2	2	0.870	0.911	0.890	0.903	0.622	0.751
GT211	130	203	10	10	0	0	0.785	0.803	0.820	0.836	0.685	0.030
GT23	131	204	9	9	0	0	0.763	0.838	0.797	0.821	0.192	0.621
rw4-10	131	203	12	12	1	1	0.786	0.877	0.831	0.854	0.567	0.809
EV1	130	203	4	4	0	0	0.523	0.567	0.552	0.526	0.429	0.427
EV94	130	202	9	9	0	0	0.792	0.827	0.807	0.809	0.769	0.352
GT575	130	203	14	14	1	1	0.815	0.788	0.811	0.803	0.801	0.260
All loci	130.6	202.8	11.4	11.2	8	6	0.784	0.817	0.802	0.810	0.605	0.471
	(0.2)	(0.2)	(1.3)	(1.3)			(0.034)	(0.033)	(0.031)	(0.034)	(0.069)	(0.077)

Table 3. Variability in the mtDNA control region of humpback whales sampled along the east and west coasts of Australia (h = haplotype diversity and π = nucleotide diversity), n = number of samples used in analyses.

Region	n	No. of haplotypes	No. of unique haplotypes	No. of shared haplotypes	$h \pm$ SD	$\pi \pm$ SD
East	104	33	17	16	0.961 ± 0.006	0.018 ± 0.009
West	185	56	40	16	0.972 ± 0.004	0.019 ± 0.010
Total	289	73			0.975 ± 0.003	0.019 ± 0.010

Sample Size and Sex Ratio

The 364 samples generated 336 unique microsatellite genotypes suggesting the sample set included 28 duplicate samples (resampling the same individual within a pod) (Table 1), with no matches between sampling locations. After removal of the duplicate genotypes the average probability of identity calculated using all remaining genotyping was 6.8×10^{-14} ($PI_{SIBS} = 3.3 \times 10^{-5}$) as calculated from the formulas shown in Peakall *et al*. (2005). These values indicate identical genotypes are most likely to be due to resampling the same individual and therefore duplicates should be removed from the sample. Also for each of the 28 duplicate sets the pair of samples was always of the same sex and haplotype.

The sex ratio of the overall sample was significantly biased toward males (197 males to 139 females, $\chi^2 = 10.39$, $P < 0.01$) as were the eastern Australian samples separately (81 males to 50 females, $\chi^2 = 7.34$, $P < 0.01$). The sex ratio of the western Australian samples did not differ significantly from parity (116 males to 89 females, $\chi^2 = 3.56$, $P = 0.06$) (Table 1).

Genetic Diversity

Summary data for each microsatellite locus are presented in Table 2. Across all ten loci, the mean number of alleles per locus was 11.4 and 11.2 for eastern and western Australia, respectively, ranging from four (EV1) to 19 alleles (EV37). There were 120 alleles in total, eight of which were private to eastern Australia with six private to western Australia. Mean expected heterozygosity across loci was similar for both western and eastern Australia (0.81 ± 0.03 and 0.80 ± 0.03, respectively).

Of the 336 samples representing unique genotypes, 289 sequences, of 470bp in length were used in all subsequent analyses (104 from eastern Australia and 185 from western Australia); 33 could not be sequenced and 14 samples produced ambiguous base calls within the target sequence. Within these sequences 65 polymorphic sites were identified (two indels, two transversions and 61 transitions), which defined 73 haplotypes (Fig. S1). Of these 73 haplotypes, 40 were found only in western Australia and 17 only in eastern Australia (Table 3). Overall haplotype and nucleotide diversities were 0.98 ± 0.003 and 0.02 ± 0.01, respectively. The haplotype and nucleotide diversity for western and eastern Australia are presented in Table 3. Bootstrap resampling of the western Australian data set to generate ten data sets of equivalent size to the eastern Australian data set showed similar diversity estimates (haplotype diversity = 0.97 ± 0.01, nucleotide diversity = 0.02 ± 0.01).

Genetic Differentiation and Population Structure Analysis

There was no significant differentiation between Eden and Tasmania in an AMOVA analysis for either the microsatellite (infinite allele model F_{ST} = –0.0003, P = 0.5; D_{EST} = –0.002, P = 0.6) or the mtDNA (haplotype level F_{ST} = –0.0001, P = 0.5; nucleotide level Φ_{ST} = –0.01, P = 0.9) data sets. This result, together with the known timing of migration and satellite tracking data (Gales *et al.* 2009), suggests the whales sampled off Eden and Tasmania are likely to be from the same population and were therefore combined in all subsequent analyses to represent the eastern Australian population.

The AMOVA analysis found significant structure between the eastern and western Australian populations for mtDNA at the haplotype and nucleotide level (F_{ST} = 0.017, P = 0.001; Φ_{ST} = 0.069, P = 0.001). For microsatellite data, there was also significant but low differentiation between populations using the infinite allele model of mutation (F_{ST} = 0.005, P = 0.001) and Jost's D_{EST} (D_{EST} = 0.031, P = 0.001).

When the STRUCTURE simulation was run without any priors on the geographic origin of samples, only one population was detected for microsatellite data [$Pr(k) > 0.99$]. When the three sampling locations were provided as priors, however, the results indicated evidence (highest posterior probability) for two populations consisting of western Australia *vs*. the two eastern sampling locations combined (average estimated *ln* probability: K = 1: –13,270; K = 2: –13,250; K = 3: –13,677; K = 4: –13,503; K = 5: –13,674; K = 6: –13,990) (Fig. 2a). This result was confirmed by the *CorrSieve* calculation of ΔK and ΔF_{ST}, with maximum values for both equations at K = 2 (Fig. S2).

Pairwise analyses for microsatellite data showed significant structure between the two populations for males (F_{ST} = 0.007, P = 0.001; D_{EST} = 0.04, P = 0.001) but not for females for F_{ST} (F_{ST} = 0.002, P = 0.07) after sequential Bonferroni correction. Significant differentiation, however, was detected for females between populations using Jost's D_{EST} (D_{EST} = 0.02, P = 0.01). In pairwise analyses of mtDNA, both males and females showed significant structure between populations at the haplotype and nucleotide level (females: F_{ST} = 0.02, P = 0.002; Φ_{ST} = 0.10, P < 0.0001 and males: F_{ST} = 0.01, P = 0.002; Φ_{ST} = 0.05, P < 0.0001 after sequential Bonferroni correction). Due to the low levels of differentiation detected in these analyses, we did not examine whether the difference between F_{ST} male and F_{ST} female could be attributed to chance sampling, as the differences are unlikely to be significant.

After merging the data sets described here with mtDNA data described by Olavarría *et al*. (2007), which had no data from eastern Australia, we found low but significant differentiation between the eastern Australia population and all six breeding populations represented from Oceania at both the haplotype and nucleotide level after sequential Bonferroni correction (Table 4). The Mantel test revealed significant correlation between genetic and geographic distances suggesting a pattern of increasing genetic differentiation with increasing geographic separation (F_{ST}: R_{XY} = 0.70, P = 0.03; Φ_{ST}: R_{XY} = 0.67, P = 0.04).

Discussion

Both nuclear and mtDNA markers revealed low but significant differentiation between the eastern and western Australian humpback populations. This finding was supported by the detection of two populations using a Bayesian clustering analysis of

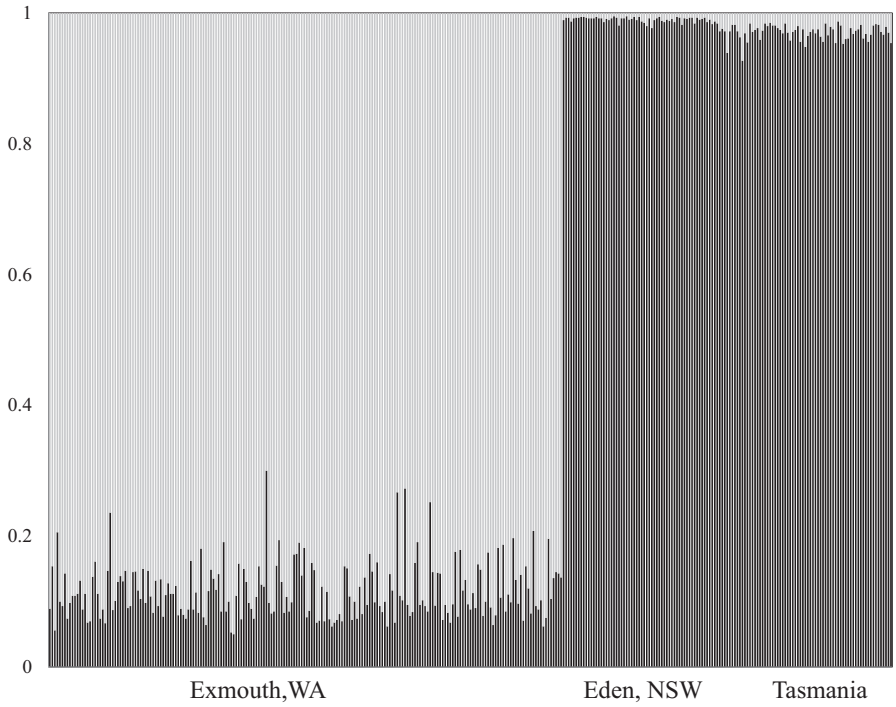

Figure 2. Proportional assignment of individual genotypes to each of the $K = 2$ inferred clusters in the STRUCTURE admixture analysis. Black and gray bars represent proportions of membership to the eastern Australian and western Australian clusters, respectively, using the three sampling locations as priors.

Table 4. Pairwise comparisons (F_{ST} and Φ_{ST}) among the Australian populations and those of the South Pacific with respective stock definitions in parentheses. Data combines mtDNA control region sequences trimmed to a 470bp consensus region from the present study with those of Olavarría *et al.* (2007). *P*-values based on statistical testing of 999 random permutations were all significant at $P < 0.005$.

F_{ST}			Φ_{ST}		
Region (stock)[a]	WA (D)	EA (E1)	Region (stock)[a]	WA (D)	EA (E1)
WA (D)			WA (D)		
EA (E1)	0.014		EA (E1)	0.053	
NC (E2)	0.015	0.012	NC (E2)	0.011	0.036
TG (E3)	0.017	0.011	TG (E3)	0.018	0.018
CI (F1)	0.028	0.031	CI (F1)	0.018	0.032
FP (F2)	0.040	0.044	FP (F2)	0.045	0.063
COL (G)	0.057	0.063	COL (G)	0.051	0.060

[a]WA = western Australia, EA = eastern Australia, NC = New Caledonia, TG = Tonga, CI = Cook Islands, FP = French Polynesia, COL = Colombia.

the microsatellite data with sampling location provided *a priori*. However, without priors the Bayesian clustering analysis failed to detect population subdivision which, as noted by other studies (Berry *et al.* 2004, Latch *et al.* 2006), is likely to be a consequence of the relative insensitivity of this approach when population differentiation is weak.

This low level of differentiation is perhaps surprising given the clear separation of breeding areas by the Australian continent and a distance between breeding areas of approximately 2,500 km. The geographic distribution of these breeding populations contrasts with many other recognized breeding populations in the Southern Hemisphere, particularly those in Oceania, which have been reported to have similarly low levels of differentiation (Fig. 1, Olavarría *et al.* 2007). There the land masses are relatively small and distances between breeding areas are smaller (although still sometimes over 1,500 km). Therefore in this region, and perhaps unlike the Australian scenario, it would be reasonable to expect frequent movements of individuals between breeding areas and thus low levels of differentiation or even panmixia.

Despite their geographical separation, movements of individual humpback whales between the Australian breeding populations have been documented. During the 1950s and 1960s stainless steel "Discovery" marks were shot into whales and later recovered when the whales were killed and flensed (Mackintosh 1965, Dawbin 1966). This era of marking revealed two cases where humpback whales were tagged near the breeding area off northeastern Australia and then killed in later breeding seasons off northwestern Australia (Chittleborough 1961, 1965; Dawbin 1966). Similarly, in a preliminary comparison of fluke images from eastern and western Australia, Kaufman *et al.* (2011) reported a match between catalogs, again providing evidence of at least temporary movement between breeding populations. These non-genetic data of course do not confirm gene flow but do show that movements occur; also given the low statistical resolution of both studies such movements may be frequent enough to mediate at least low levels of gene flow between the populations.

Another study that inferred movement between the Australian humpback whale populations was based on song. Noad *et al.* (2000) reported that over three breeding seasons the humpback whale song characteristic of western Australia replaced the song of eastern Australian whales. The authors suggested that this song evolution is mediated by the movement of a small number of males between populations although they recognized it was possible that singing on feeding grounds may also transfer song types between populations without the movement of individual whales (Mattila *et al*. 1987).

Genetic differentiation between the eastern and western Australian humpback populations was stronger for mtDNA than nuclear DNA. Several factors can contribute to this common pattern including the larger effective population size of nuclear genes, differences in the rate and mode of mutation (Palumbi and Baker 1994, Baker *et al.* 1998*a*), and sex-biased dispersal (Avise 1995, Balloux *et al.* 2000). In this study, when the sexes were analyzed separately, we found similar levels of genetic differentiation between the Australian humpback whale populations indicting little evidence for strong sex-biased dispersal despite the expectation of female philopatry and male-driven gene flow displayed by many migratory marine vertebrates (Greenwood 1983, Pardini *et al.* 2001, Bowen and Karl 2007, Engelhaupt *et al.* 2009).

Collectively the genetic and nongenetic evidence suggest the low genetic differentiation between the Australian populations is likely to be a consequence of low levels of ongoing gene flow, mediated by the occasional movement of individuals between breeding populations. However, it is possible that the low differentiation is due to

recent isolation of the two Australian populations. This isolation could have been driven by the severe depletion of these populations during the era of industrial whaling. This depletion together with strong genetic drift while numbers were low may have resulted in the genetic differentiation apparent today. If the former is the most likely scenario then quantifying the contemporary magnitude of gene flow is notoriously difficult at such low levels of differentiation. Allendorf *et al*. (2013) suggest that for reliable estimates of Nm based on F_{ST}, the levels of differentiation need to be moderate to large ($F_{ST} > 0.05$–0.10). Furthermore, they warn against interpreting Nm values literally at the low F_{ST} values as found in this study. Similarly, more complex methods for estimating migration, such as the coalescent- and assignment-based approaches are equally unreliable at low levels of genetic divergence (Faubet *et al.* 2007, Palsbøll *et al.* 2010). Consequently we can only suggest that the rate of gene flow is likely to be relatively low (no more than a few tens of individuals per generation), otherwise any genetic differentiation between populations would be eroded rapidly.

Elsewhere in the Southern Hemisphere there are low levels of differentiation between neighboring humpback whale populations. Pomilla (2005), and Rosenbaum *et al.* (2009) reported low genetic differentiation between populations separated by the African landmass (see Fig. 1). Among the breeding populations of the South Pacific mtDNA analyses (microsatellite studies have yet to be published) also showed weak structure (Olavarría *et al.* 2007) (Fig. 1). Similarly, here we report that even between distant breeding populations, such as eastern Australia *vs*. Colombia, (see Table 4), F_{ST} values are low ($F_{ST} \sim 0.06$). Thus, the available evidence suggests that most if not all humpback whale populations of the Southern Hemisphere are characterized by weak genetic differentiation. This indicates that at least historically, if not presently, gene flow occurs between neighboring humpback whale populations in the Southern Hemisphere, but again, is not sufficiently high to erode all genetic differentiation.

Similar to the Australian populations there is also nongenetic evidence for ongoing and wide-ranging movement between breeding grounds throughout the Southern Hemisphere. Garrigue *et al.* (2011) assessed the movement of humpback whales throughout the Oceania region over a 6 yr period using regional catalogs of fluke photographs representing 776 annual sightings of 659 individual whales. Resightings mostly occurred within breeding areas but 20 of the 98 resightings occurred outside the original region and almost all were resighted in neighboring breeding areas between seasons. Only one whale was resighted in more than one region during the same winter breeding season and there was no evidence of sex-biased dispersal. This is a remarkably high level of movement between breeding areas reported to be genetically differentiated based on mtDNA (Olavarría *et al.* 2007). Long distance contemporary movements have also been reported. For example, Stevick *et al*. (2011) described the movement of an individual female humpback whale from the breeding grounds off Brazil to Madagascar, which are separated by a distance of nearly 10, 000 km. Such long distance movements have also been reported between breeding and feeding areas; (Robbins *et al.* 2011) reported a round trip migration of some 18, 000 km between American Samoa and the Antarctic Peninsula. Such movements show the capacity for extensive intermingling of humpback whale populations in Antarctic waters.

Much stronger population differentiation has been detected among breeding populations within the North Pacific. Between the wintering grounds of the Hawaiian archipelago and the coast of Mexico the genetic differentiation for mtDNA

(F_{ST} = 0.11) and nuclear intron alleles (F_{ST} = 0.07) was comparably high, suggesting longer-term isolation or very infrequent gene flow (Baker *et al.* 1998*b*, Baker and Steel 2010). Interestingly, analyses of mtDNA sequences revealed strong differentiation between feeding areas in the Northern Hemisphere within both the North Pacific (F_{ST} = 0.18), and the North Atlantic (K_{ST} = 0.04) (Palsbøll *et al.* 1995, Larsen *et al.* 1996, Baker *et al.* 1998*b*). The feeding areas in the Northern Hemisphere are often localized and discrete (Calambokidis *et al.* 1996) and long-term fidelity by both males and females to these disparate feeding grounds, combined with strong natal philopatry, may explain the comparatively high levels of genetic differentiation between both breeding and feeding populations.

In contrast, in the high latitudes of the Southern Ocean, prey density is high and widely distributed throughout a broad, uninterrupted circumpolar region (Williams *et al.* 2010) where glacial barriers have not fluctuated to the same extent (Barker *et al.* 2009). Therefore the extent to which humpback populations mix on these feeding grounds is more likely to depend merely upon the distance between them (Hoelzel 1998). Intermingling of populations, however, may not necessarily increase gene flow. Copulations in humpbacks whales are rarely observed but it is thought they occur exclusively within the low latitude calving regions and associated migratory routes (Clapham 1996). Therefore, for gene flow to occur, individuals must change their migration behavior which is thought to be socially inherited from the mother to her calf (Clapham 1996). The ease at which breeding populations in the Southern Ocean mix may reduce the strength of natal fidelity and explain the relatively low differentiation compared to populations in the Northern Hemisphere. Although it is expected juveniles rather than adults are more likely to move between populations (Clapham 1996), there is growing evidence of adult movements. In addition to the Discovery marking and recovery described earlier (Chittleborough 1961, Dawbin 1966), photo-identification of humpback (Garrigue *et al.* 2000, 2002; Kaufman *et al.* 2011) and other baleen whales (Pirzl *et al.* 2009, Carroll *et al.* 2011) have all revealed movement of mature whales between breeding populations.

This study has revealed low differentiation between the Australian humpback whale populations, which appears to be characteristic of most, if not all, neighboring populations in the Southern Hemisphere. We suggest this low differentiation is a consequence of the erosion of natal philopatry due to the intermingling of populations in the circumpolar Antarctic feeding areas. Although this intermingling may facilitate gene flow, it is not sufficiently frequent to remove all genetic population differentiation and so would not be sufficiently frequent to suggest demographic interdependence. We therefore suggest each Australian humpback whale population should remain a separate management unit.

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Supporting Information

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Figure S1. Geographic distribution and relative position of variable nucleotides in humpback whale mtDNA control region defining 73 haplotypes. Dots (.) indicate matches with published reference sequence X72202 (Genbank), dashes indicate insertion/deletion events (Position 1 of alignment corresponds with position 6 of the reference sequence). The total number of each haplotype is indicated for both regions. *For consistency with the South Pacific haplotype data set, this polymorphic site was not included in the genetic analyses, however, including this locus does not change the number of haplotypes.

Figure S2. Delta K and delta F_{ST} values (ΔK and ΔF_{ST}) calculated using *CorrSieve* for each of the K inferred clusters in STRUCTURE, with a maximum value achieved at $K = 2$.

Table S1. MICROCHECKER (van Oosterhout *et al.* 2004) null frequencies for all microsatellite loci by population.