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

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Running title: Genetic differentiation among Australian humpback whales

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 north-western and north-eastern 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} = 0.058, P = 0.001, n = 364). For both marker types, Bayesian clustering analyses 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 Southern Hemisphere humpback whale populations. This may in part reflect the circumpolar distribution of summer feeding grounds that lack continental barriers, allowing for extensive whale movement.

Keywords: mtDNA, microsatellites, population genetic structure, conservation, management, *Megaptera novaeangliae*

INTRODUCTION

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 influence of social and learnt 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 non-physical 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 (Figure 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 so 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.

Much of what we know about population differentiation among humpback whales has resulted from studies in the Northern Hemisphere, where whales 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.* 1997a, Clapham *et al.* 1999, Calambokidis *et al.* 2001, Calambokidis *et al.* 2008). Within each ocean basin, individuals show strong fidelity to specific foraging areas and mix on common breeding grounds (Calambokidis *et al.* 2001, Stevick *et al.* 2006).

In contrast to the Northern Hemisphere where foraging areas are numerous and discrete, humpback whales in the Southern Hemisphere have a circumpolar distribution on high latitude feeding grounds in the Southern Ocean. On their annual migration, they segregate onto 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 dispersal on feeding grounds, there is the potential for frequent movement between populations as described for other marine megafauna (Bonfil *et al.* 2005, Boyle *et al.* 2009).

Two putative 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, the other migrates along the western seaboard and mates and calves off the Kimberley coast off 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 from approximately 17,000 prior to 1934 (Chittleborough 1965, Bannister 1994), and the eastern Australian population was reduced to as few as 100 individuals from a pre-

exploitation abundance estimate of between 16,022 and 22,957 (Chittleborough 1965, Paterson *et al.* 1994, Jackson *et al.* 2008). Recent data has shown that both populations are recovering strongly with the current rate of increase at about 10-11% per annum (Bannister and Hedley 2001, Noad *et al.* 2008, Salgado Kent *et al.* In press). Absolute abundance for western Australian humpback whales is currently estimated at 21,750 (95% CI 17,550-43,000) (Hedley *et al.* 2009) and 14,522 (95% CI 12,777-16,504) for eastern Australia (Noad *et al.* 2011).

The degree of connectivity between the Australian populations is poorly understood but migration between the populations has been documented. During the 1950s and 1960s stainless steel 'Discovery' marks were shot into whales and some were then recovered when the whales were killed and flensed. This approach provided the first means of tracking the movement of whales over large distances and periods of time (Mackintosh 1965, Dawbin 1966). For example, in the summer of 1958-59, from a total of ten whales originally marked in the Southern Ocean south of eastern Australia, two were recovered later that winter off the western Australian coast, indicating some movement between breeding populations (Chittleborough 1961, 1965; Dawbin 1966).

Here, based on extensive sampling, we specifically evaluate (i) 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, (ii) 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, (iii) compare and contrast our findings with other studies of humpback whales and consider the ecological implications of the emerging genetic patterns.

METHODS

Population definition in the Southern Hemisphere

The International Whaling Commission (IWC) currently recognizes seven breeding aggregations in the Southern Hemisphere as 'populations' A to G, with questions remaining about further subdivision around Africa, Australia, and the South Pacific (Chittleborough 1965, Mackintosh 1965). The humpback whales that migrate along the west and east coasts of Australia are recognized as putative population D and subpopulation E1 respectively. Subpopulations E2 and E3 (Tonga and New Caledonia), 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.* 2009).

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 -80˚C (Krützen *et al.* 2002). Total cellular DNA was extracted from skin tissue using a standard salt extraction technique (Aljanabi 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

(Berube *et al.* 2000); rw4-10 (Waldick *et al.* 1999)] and one tetranucleotide repeat [GATA417 (Palsbøll *et al.* 1997b)]. 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 labelled fluorescently at the 5' end to allow for visualization of alleles on an automated sequencer. Each PCR had a final volume of 12.5µl and included: 1x Qiagen Multiplex PCR Master Mix (containing HotStarTaq®DNA Polymerase, Multiplex PCR Buffer, $MgCl_2$ and dNTP mix), 2µM of each primer (labelled and non-labelled) and 1 to 8ng of template DNA (estimated using a NanoDrop spectrometer 3300). The thermocycling profile consisted of an initial denaturing step of 95˚C for 15 minutes, 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 minutes at 60˚C, with the exception that the optimal annealing temperature for the single locus reactions (GT211 and rw4-10) was 53˚C.

Fluorescently labelled 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 were 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 dataset (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, van Oosterhout *et al.* 2006) was used to screen the microsatellite dataset for genotyping errors such as null alleles, stuttering and large allele dropout. 4) Using Arlequin 3.1 (Schneider *et al.* 2000), 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 tPro RNA 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 10µl at the following concentrations: 2.5mM $MgCl_2$, 200µM dNTP, 0.4mM each primer, 0.25U Taq (New England BioLabs ®Inc.), 1 X PCR reaction buffer and 1µl DNA (approximately 10-50ng). Temperature profiles consisted of an initial denaturing period of 2 minutes at 94˚C, followed by 35 cycles of denaturation at 94˚C for 30 seconds, annealing at 54˚C for 40 seconds, and extension at 72˚C for 40 seconds. A final extension period for 10 minutes at 72˚C 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 sequencing kit (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 470bps 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, Olavarria 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 6.3 (Peakall and Smouse 2006).

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 was calculated using GenAlEx 6.3. Arlequin 3.1 (Schneider *et al.* 2000) was used to determine standard measures of mtDNA genetic diversity including haplotype frequencies, the number of unique haplotypes, the number of shared haplotypes, haplotype and nucleotide diversity, and the number of sequence polymorphic sites. Haplotype and nucleotide diversity was calculated according to Nei (1987) and Tajima (1983).

The extent of genetic differentiation among sampling locations and among the two putative populations was evaluated using Analysis of Molecular Variance (AMOVA) (Excoffier *et al.* 1992) as implemented in GenAlEx 6.3, with statistical testing by random permutation (999 permutations). For microsatellite data, an estimate of F_{ST} (infinite allele model) was calculated 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 datasets 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 1000 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. For these analyses, genetic distance matrices were constructed using individual pairwise differences at all polymorphic nucleotide sites, or haplotype differences among all individuals (Griffiths *et al.* 2011). In keeping with the common practice in similar studies of humpback whales (Rosenbaum *et al.* 2004, Olavarria *et al.* 2006, Olavarría *et al.* 2007) we use the notation F_{ST} for haplotype 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 dataset. 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 assesses the probability that K populations are represented within the data and assumes the loci are at 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 by calculating pairwise estimates of F_{ST} among populations within and between each sex using an AMOVA in GenAlEx 6.3 for both genetic markers. 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 Columbia), we combined our mtDNA data with those presented by Olavarria *et al*. (2007) and calculated F_{ST} and Φ_{ST} for pairwise comparisons. A Mantel test was used to determine whether genetic differentiation increases with geographic distance between populations, with statistical testing based on 999 random permutations conducted in GenAlEx 6.3 (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 ten 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 (IWC 2008) for systematic quality control in the use of microsatellite markers (≤ 10% error rate) for management decisions.

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 very low probabilities and each of the 28 pairs were also of the same sex and mtDNA haplotype justify the removal of the putative duplicate samples.

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. Average expected heterozygosity across loci was similar for both western and eastern Australia (0.81 ± 0.03 and 0.80 ± 0.03, respectively). Random resampling of the western Australian dataset was conducted to generate ten datasets of the same size as eastern

Australia. The datasets showed average heterozygosities (0.81 ± 0.03) similar to eastern Australia.

Of the 336 samples representing unique genotypes, 289 sequences were used in all subsequent analyses (104 from eastern Australia and 185 from western Australia). Within these sequences 65 polymorphic sites were identified (two indels, two transversions and 61 transitions) which defined 73 haplotypes (Fig. S1, Supporting information). 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. Resampling of the western Australian dataset to generate ten datasets of equivalent size to the eastern Australian dataset showed similar diversity estimates (haplotype diversity = 0.97 ± 0.01, nucleotide diversity = 0.02 ± 0.01).

Genetic differentiation and population structure analysis

Pairwise comparisons between sampling locations in an AMOVA analysis found no significant differentiation between Eden and Tasmania for either the microsatellite (infinite allele model $F_{ST} < 0.001$, $P = 0.5$; $D_{EST} < 0.001$, $P = 0.6$) or the mtDNA (haplotype level $F_{ST} < 0.001$, $P = 0.4$; nucleotide level $\Phi_{ST} < 0.001$, $P = 0.4$) datasets. In contrast, significant differentiation was found between Eden and western Australia, and Tasmania and western Australia (see below). 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} = 0.058, 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 versus the two eastern sampling locations combined (average estimated *ln* probability: K = 1: -13270; K = 2: -13250; K = 3: -13677; K = 4: -13503; K = 5: -13674; K = 6: -13990) (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. 2b).

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} = 0.08, P < 0.001 and males: F_{ST} = 0.01, P = 0.002; Φ_{ST} = 0.04, P < 0.001 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 datasets described here with mtDNA data described by Olavarria *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} = 0.74, P = 0.04).

DISCUSSION

Patterns of genetic differentiation among Australian humpback whales

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 by the Bayesian clustering analysis for both markers when using *a priori* information on sampling location. However, without priors the Bayesian clustering analysis failed to detect population subdivision which, as noted by other studies (e.g. 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.

High genetic diversity was found within both the eastern and western Australian populations for each marker. Such high diversity may be surprising given the known population bottlenecks, however, industrial whaling in the Southern Hemisphere was intense but relatively brief (approximately four decades) and the rates of recovery for both populations have been rapid (Bannister and Hedley 2001, Noad *et al.* 2008, Sremba *et al.* 2012, Salgado Kent *et al.* In press). These factors together with the long generation time and age structure of

humpback whales have all contributed to minimising the loss of genetic diversity (see Baker *et al*. 1993 for a similar conclusion).

As expected, genetic differentiation between the eastern and western Australian humpback populations was stronger for mtDNA than nuclear DNA. Several factors likely 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), and sex-biased dispersal (Avise 1995, Balloux *et al.* 2000). However, among Australian humpback whale populations there is limited 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), with similar levels of genetic differentiation at both marker types evident between the sexes.

Is low population genetic differentiation a characteristic of Southern Hemisphere humpback whales?

A summary of the known patterns of inter-population genetic differentiation among humpback whales of both the Northern and Southern Hemispheres is shown in Figure 1. In the Northern Hemisphere there is strong differentiation among the feeding areas of the North Pacific based on informative RFLP mtDNA 'haplogroups' (F_{ST} ~ 0.5-0.6), and the North Atlantic (K_{ST} ~ 0.04) (Palsbøll *et al.* 1995, Larsen *et al.* 1996, Baker *et al.* 1998) (Figure 1). Strong population differentiation has also been detected among breeding aggregations within the North Pacific for both RFLP 'haplogroups' (F_{ST} ~ 0.3) and nuclear intron alleles (F_{ST} ~ 0.1), reflecting long-term isolation between the Hawaiian archipelago and the coast of Mexico (Baker *et al.* 1998) (Fig. 1).

By contrast to the Northern Hemisphere, we have shown that differentiation among the Australian populations is weak at both nuclear microsatellites and mtDNA. Similarly, in the South Atlantic the degree of genetic differentiation between putative breeding populations is also weak at nuclear and mtDNA markers (Rosenbaum *et al.* 2004, Pomilla *et al.* 2005). Studies of divergence among the breeding subpopulations of the South Pacific have not yet included nuclear markers. Nonetheless, mtDNA analysis indicates patterns of weak structure (Olavarría *et al.* 2007) (Fig. 1). Even among distant breeding grounds, such as between eastern Australia versus Columbia F_{ST} values are low at mtDNA (F_{ST} ~ 0.06 compared to a mean F_{ST} ~ 0.3 in the North Pacific). Thus, the emerging evidence suggests that humpback whale populations of the Southern Hemisphere are characterized by weak differentiation. This indicates that at least historically, if not presently, there c extensive movement of humpback whales among populations in the Southern Hemisphere.

Is non-genetic evidence consistent with the genetic evidence for extensive movement among Southern Hemisphere humpback whales?

For both males and females there is non-genetic evidence for ongoing and wide-ranging movement across the Southern Hemisphere. For example, based on fluke matching, Stevick *et al.*(2011) reported the movement of an individual female humpback whale between the breeding grounds off Brazil and Madagascar, representing a distance of nearly 10,000 km. A photo-identification study over a six year period in the South Pacific reported four instances of movement of four out of six male humpback whales between eastern Australia and the breeding grounds of New Caledonia (Garrigue *et al.* 2011). Similarly, using photo-identification, Kaufman *et al.* (2011) reported movement of a single whale between the eastern and western Australian populations.

The analysis of humpback whale song also provides another line of non-genetic evidence in support of gene flow among Southern Hemisphere populations. Male humpback whales sing throughout their migration from the feeding grounds to breeding grounds where the song is transmitted culturally among individuals and is thought to be a form of sexual display (Noad *et al.* 2000). All males in a population produce the same song, which changes over time, and all singers maintain the changes (Winn and Winn 1978, Payne *et al.* 1983). The differences in the theme composition of male song between humpback breeding populations within the same ocean basin are found to increase with distance, possibly reflecting at least historical migratory exchange between geographically close populations (Helweg *et al.* 1998).

Humpback whale song from western Australia was found to replace the song of eastern Australia over only three breeding seasons in an analysis of song evolution (Noad *et al.* 2000). Noad *et al.* (2000) suggested that this song evolution is mediated by the movement of a small number of males between populations although it is possible that singing on feeding grounds may also transfer song types between populations without the movement of individual whales (Mattila 1987). Nonetheless, this rapid transmission of song and change in theme composition support contact among males of these two populations somewhere in their annual migratory cycle or on the feeding grounds.

It is evident that the non-genetic evidence is consistent with the genetic evidence for long-range movement among Southern Hemisphere humpback populations and limited movement among populations of the Northern Hemisphere. However, neither photo-ID or song analysis are accurate indices in estimating the magnitude of interchange among populations.

Why are the patterns of genetic variation among humpback whale populations different between the Northern and Southern Hemispheres?

In the Northern Hemisphere, coastal wind-driven and curl-driven upwelling from continental land barriers have resulted in localized areas of nutrification and biological production (Chechley Jr. and Barth 2009), creating opportunities for the formation of segregated humpback whale feeding areas. These localized feeding areas are likely to have emerged through the formation of the Panama Land Bridge and as a result of long glacial and interglacial periods in the Arctic (Kojima *et al.* 2009). Long-term preference of males and females to localized feeding grounds combined with natal philopatry may explain the comparatively high levels of genetic differentiation between breeding and feeding populations. By contrast, in the high latitudes of the Southern Ocean, prey density is high and widely distributed throughout a broad, circumpolar area (Williams *et al.* 2010) where glacial barriers have not fluctuated to the same extent (Barker *et al.* 2009), increasing the potential for mixing and therefore gene flow among breeding populations. The extent to which humpback populations mix on these feeding grounds is therefore more likely to depend merely upon the distance between them (Hoelzel 1998).

Although migratory behavior is thought to be socially-inherited from the mother to her calf (Clapham 1996), the mixing of breeding populations in the Southern Ocean may weaken natal fidelity, relative to that found in the Northern Hemisphere. Also, although it is expected juveniles rather than adults are more likely to move between populations while on the feeding grounds in the Southern Ocean (Clapham 1996), there is growing evidence of adult movement too. In addition to the Discovery marking and recovery described earlier (Chittleborough 1961, Dawbin 1966), photo-identification of humpback (Garrigue *et al.* 2000, Garrigue *et al.* 2002, Garrigue *et al.* 2007, Kaufman *et al.* 2011) and other baleen

whales (Pirzl *et al.* 2009) have all revealed movement of mature whales between breeding populations.

Estimating gene flow in the Southern Hemisphere?

The very low genetic differentiation that appears to characterize humpback populations of the Southern Hemisphere, presents challenges for reliable estimates of the magnitude of gene flow. Allendorf *et al*. (2007) 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 to 0.10). Furthermore, they warn against interpreting *Nm* values literally at the low F_{ST} values 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) such as those that characterize Southern Hemisphere humpback populations. For this reason, along with the fact that the accuracy of coalescent-based methods can also be seriously compromised by errors in sample size and mutation rate estimates (Karl *et al*. 2012), gene flow estimates were not included in the present study.

We suggest that while the emerging evidence for gene flow among populations is compelling, additional development is required before we can quantify the magnitude of gene flow with any degree of certainty. With further development, novel approaches such as the kinship-based analyses of the spatio-temporal distribution of related individuals may be able to yield more reliable estimates of current migration rates even at low levels of differentiation (Palsbøll *et al.* 2010). Alternatively, although requiring considerable time and effort, multi-state capture-recapture models of current movement using photo-identification and/or genotype data may prove to be the most reliable method for quantifying the magnitude of gene flow.

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TABLES

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
	Oct, Nov	18	0	18	6	12
Tasmania	2006-2008	*78*	*8*	*70*	*36*	*34*
	July	1	0	1	0	1
	Nov, Dec	77	8	69	36	33
Western Australia		**223**	**18**	**205**	**89**	**116**
Exmouth	2007					
	Sept, Oct					
total		**364**	**28**	**336**	**139**	**197**

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); significant at $P < 0.05$ 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 (*0.2*)	202.8 (*0.2*)	11.4 (*1.3*)	11.2 (*1.3*)	8	6	0.784 (*0.034*)	0.817 (*0.033*)	0.802 (*0.031*)	0.810 (*0.034*)	0.605 (*0.069*)	0.471 (*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 **± SD**	π **± 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

1
2

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 Olavarria *et al.* (2007). *P* -values based on statistical testing of 999 random permutations were all significant at $P < 0.005$.

WA = western Australia; EA = eastern Australia; NC = New Caledonia; TG = Tonga; CI = Cook Islands; FP = French Polynesia; COL = Columbia.

a) F_{ST}

Region/Stock	WA (D)	EA (E1)
WA (D)		
EA (E1)	0.014	
NC (E2)	0.015	0.012
TG (E3)	0.017	0.011
CI (F1)	0.028	0.031
FP (F2)	0.040	0.044
COL (G)	0.057	0.063

b) Φ_{ST}

Region/Stock	WA (D)	EA (E1)
WA (D)		
EA (E1)	0.032	
NC (E2)	0.015	0.024
TG (E3)	0.018	0.013
CI (F1)	0.023	0.027
FP (F2)	0.043	0.046
COL (G)	0.049	0.061

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

Locus	Null Present	Null Frequency	
		East	West
EV14	no	0.0208	0.008
EV37	no	-0.0008	-0.0143
EV96	no	-0.0072	-0.0054
GATA417	no	0.0117	-0.0041
GT211	no	0.0216	0.02
GT23	no	0.0232	-0.0124
rw4-10	no	0.0283	-0.0137
EV1	no	0.032	-0.0467
EV94	no	0.0094	-0.0132
GT575	no	-0.0067	0.009

FIGURES

Figure 1. Worldwide humpback whale population structure and geographic distribution for both nuclear and mitochondrial DNA (mtDNA) markers (Baker *et al.* 1993, Palsbøll *et al.* 1995, Larsen *et al.* 1996, Baker *et al.* 1998, Calambokidis *et al.* 2001, Pomilla *et al.* 2004, Rosenbaum *et al.* 2004, Pomilla *et al.* 2005, Stevick *et al.* 2006, Olavarría *et al.* 2007).

(Three shared haplotypes between the North Pacific, North Atlantic and Southern Ocean from a total of 22. All *P*-values less than 0.05).

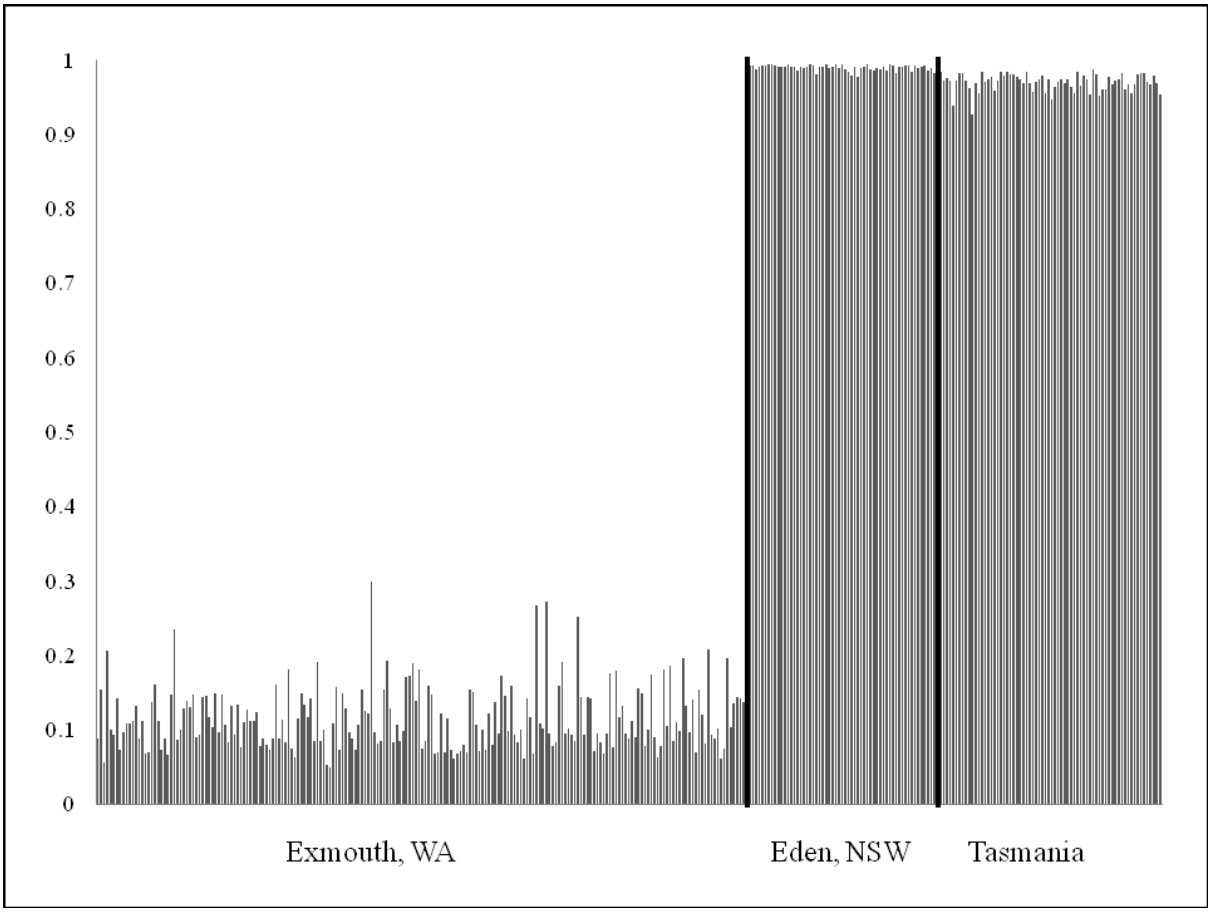
* F_{ST} values in the North Pacific, reported in Baker et al. (1998), are likely to be inflated due to a reduced diversity within regions resulting from the choice of informative RFLPs or sequences.

Figure 2. A. Proportional assignment of individual genotypes to each of the $K = 2$ inferred clusters in the STRUCTURE admixture analysis. Grey and white bars represent proportions of membership to the eastern Australian and western Australian clusters, respectively, using the three sampling locations as priors. B. Delta *K* and delta F_{ST} values (ΔK and ΔF_{ST}) for each of the *K* inferred clusters, with a maximum value achieved at $K = 2$.

Figure S1. Geographic disribution 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.

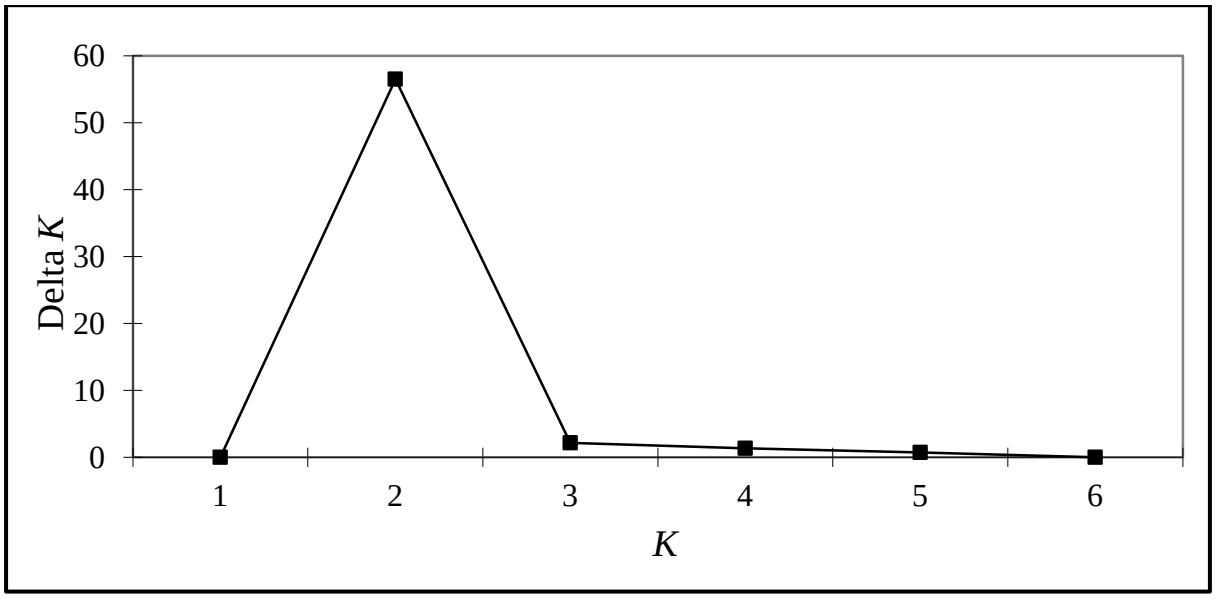
* For consistency with the South Pacific haploytpe dataset, this polymorphic site was not included in the genetic analyses however, including this locus does not change the number of haplotypes

1 A

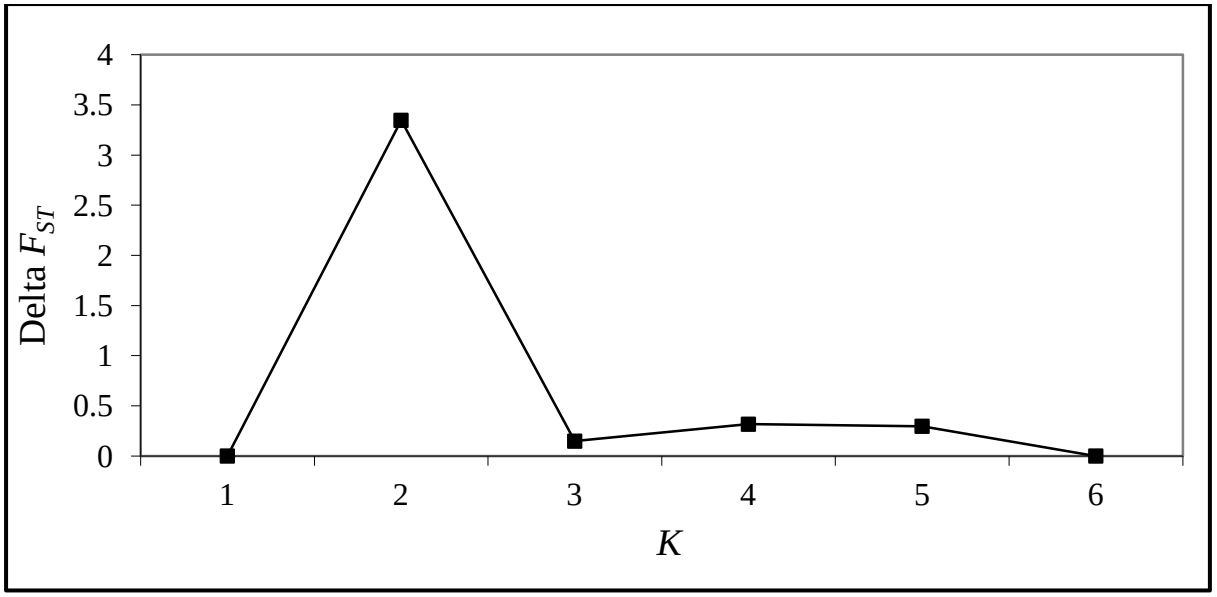

1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Exmouth, WA
Eden, NSW
Tasmania

2

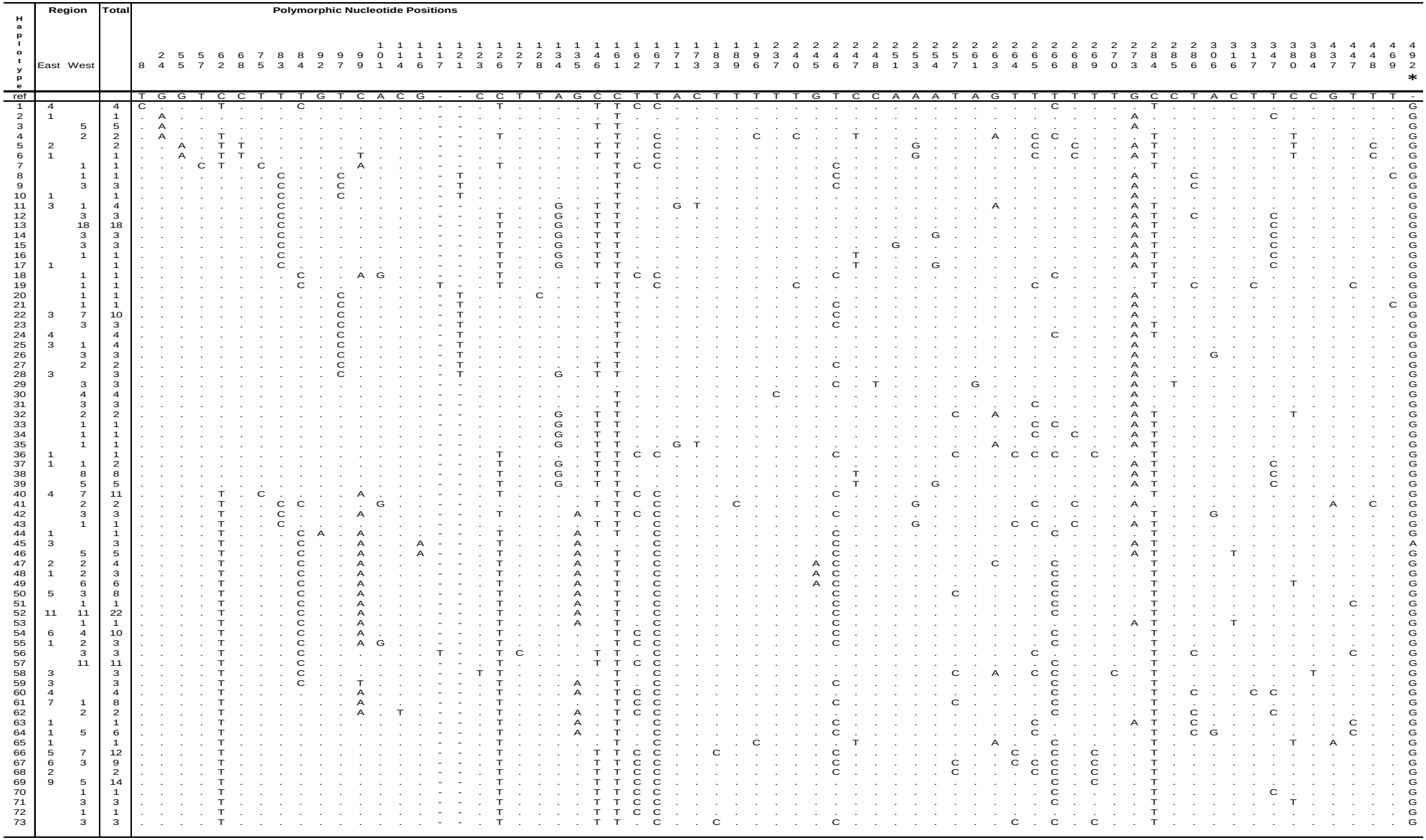
3 B

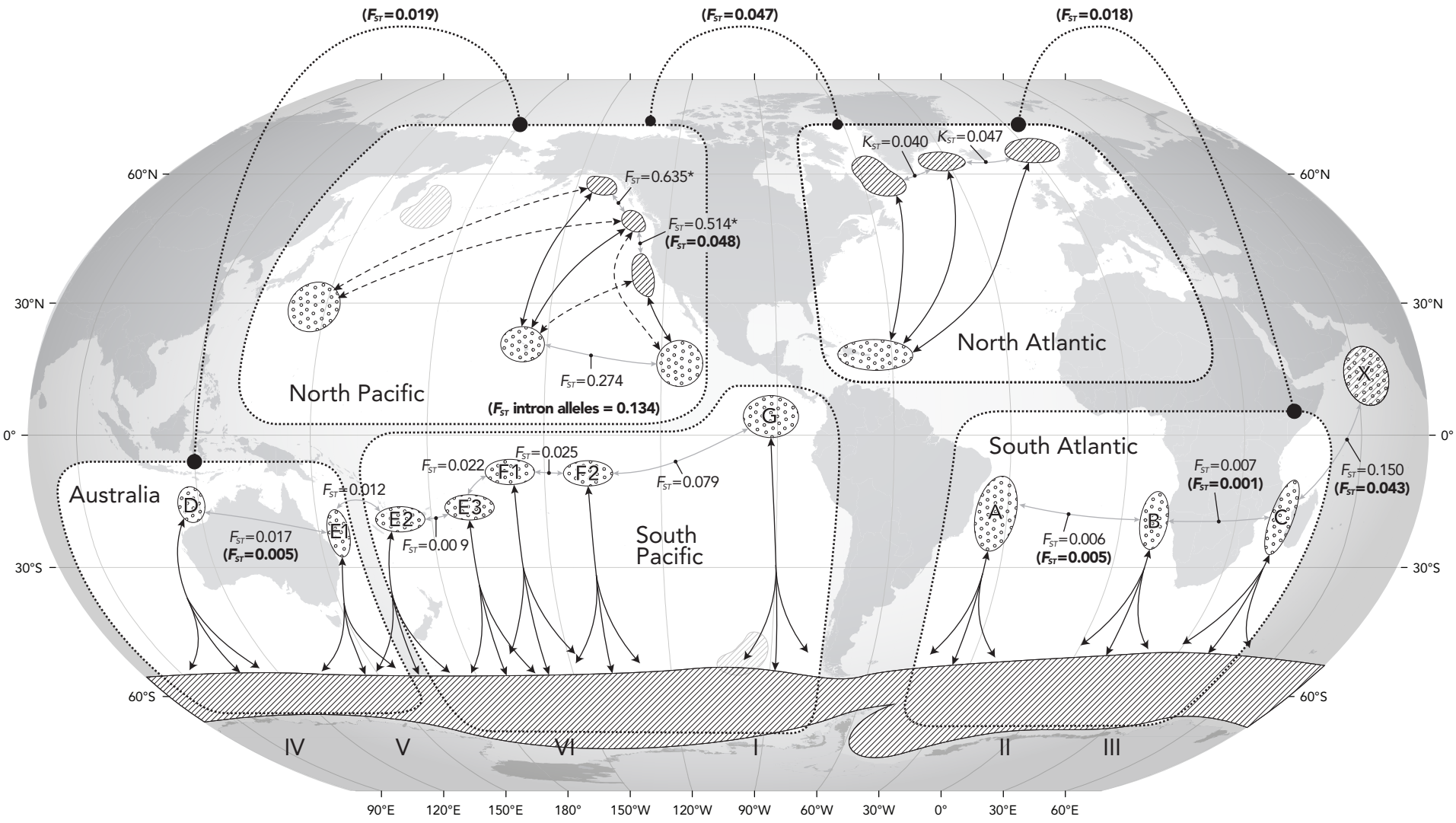

60
50
40
30
20
10
0
Delta K
1
2
3
4
5
6
K

4



1


Haplotype
Region
East West
Total
Polymorphic Nucleotide Positions
ref


(F_{ST}=0.019)
(F_{ST}=0.047)
(F_{ST}=0.018)
60°N
30°N
0°
30°S
60°S
90°E
120°E
150°E
180°
150°W
120°W
90°W
60°W
30°W
0°
30°E
60°E
F_{ST}=0.635*
F_{ST}=0.514*
(F_{ST}=0.048)
F_{ST}=0.274
(F_{ST} intron alleles = 0.134)
North Pacific
K_{ST}=0.040
K_{ST}=0.047
North Atlantic
X
F_{ST}=0.150
(F_{ST}=0.043)
South Atlantic
F_{ST}=0.007
(F_{ST}=0.001)
A
B
C
F_{ST}=0.006
(F_{ST}=0.005)
G
F_{ST}=0.025
F_{ST}=0.022
F1
F2
F_{ST}=0.079
Australia
D
F_{ST}=0.012
E1
E2
E3
F_{ST}=0.017
(F_{ST}=0.005)
F_{ST}=0.00 9
South Pacific
IV
V
VI
I
II
III
Breeding grounds
Feeding grounds
F_{ST}= Pairwise genetic distances between microsatellite alleles
Genetic differentiation
Putative feeding grounds
F_{ST}= Pairwise genetic distances between mtDNA haplotypes
Migration route
Limited movement
K_{ST}= Average number of differences between mtDNA sequences