Mixed-stock analysis of humpback whales (*Megaptera novaeangliae*) on Antarctic feeding grounds

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ABSTRACT

In understanding the impact of commercial whaling, it is important to estimate the mixing of low latitude breeding populations on Antarctic feeding grounds, particularly the endangered humpback whale populations of Oceania. This paper estimates the degree of genetic differentiation among the putative populations of Oceania (New Caledonia, Tonga, the Cook Islands and French Polynesia) and Australia (western Australia and eastern Australia) using ten microsatellite loci and mtDNA, assesses the power of the data for a mixed-stock analysis, determines ways to improve statistical power for future studies and estimates the population composition of Antarctic samples collected in 2010 south of New Zealand and eastern Australia. A large proportion of individuals could not be assigned to a population of origin (> 52%) using a posterior probability threshold of > 0.90. The mixed-stock analysis simulations however, produced accurate results with humpback whales reapportioned to their population of origin above the 90% threshold for western Australia, New Caledonia and Oceania grouped using a combined mtDNA and microsatellite dataset. Removing the Cook Islands, considered a transient region for humpback whales, from the simulation analysis increased the ability to reapportion Tonga from 86% to 89% and French Polynesia from 89% to 92%. Breeding ground sample size was found to be a factor influencing the accuracy of population reapportionment whereas increasing the mixture or feeding ground sample size improved the precision of results. The mixed-stock analysis of our Antarctic samples revealed substantial contributions from both eastern Australia (53.2%, 6.8% SE) and New Caledonia (43.7%, 5.5% SE) [with Oceania contributing 46.8% (5.9% SE)] but not western Australia. Despite the need for more samples to improve estimates of population allocation, our study strengthens the emerging genetic and non-genetic evidence that Antarctic waters south of New Zealand and eastern Australia are used by humpback whales from both eastern Australia and the more vulnerable breeding population of New Caledonia, representing Oceania.

KEYWORDS: HUMPBACK WHALE; CONSERVATION; FEEDING GROUNDS; GENETICS; MIGRATION; SOUTHERN OCEAN; ANTARCTIC

INTRODUCTION

In conservation and resource management there is often a requirement to assess how breeding populations are impacted either through deliberate or accidental removals. Management tends to focus on breeding units but for migratory species, exploitation or mortality often occurs in other parts of the range. In cases where only a single population is impacted this can be relatively straight forward, but the situation can become complicated when removals occur where populations mix. The assessment then requires an understanding of the degree of mixing and the relative impact on each population contributing to a mix, referred to as mixed-stock analysis. The classic example of this is the impact of pelagic fishing on salmon populations that exhibit natal philopatry but following smoltification return to the ocean where the mixing of genetically distinct stocks occurs simultaneously with commercial exploitation (e.g. Beacham *et al.,* 2011; Beacham *et al.,* 2008; Cadrin *et al.,* 2005; Olsen *et al.,* 2000; Utter and Ryman, 1993; Waples *et al.,* 1993). Under such conditions, the development of a mixed stock analysis model has been useful in minimising the risk of overexploiting less productive stocks in the mixed stock fishery.

During the era of industrial whaling in the Southern Hemisphere, > 2,000,000 whales were killed, driving some populations to near extinction (Clapham and Baker, 2002). The waters of Antarctica were heavily targeted because many baleen whale populations migrate to and mix in these krill-rich high-latitude waters during the summer. They subsequently return to their low-latitude breeding and calving grounds in the winter. Based on catch records corrected for illegal Soviet whaling, some 200,000 humpback whales were killed by pelagic whaling operations around Antarctica after 1900 (Allison, 2010; Clapham and Baker, 2002), driving a massive population decline (from an estimated pre-whaling population of 125,000) of this species.

The impact of whaling and the recovery of whale populations is a key focus of the International Whaling

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Commission (IWC) Scientific Committee. This Committee takes estimates of historical population abundance and trends and combines them with estimates of catches and population dynamic models to predict recovery rates. The models require an ability to accurately allocate catch to a source breeding population, estimates of biological parameters such as population structure, and abundance estimates, all of which are subject to considerable uncertainty (Baker and Clapham, 2004; Jackson *et al.,* 2008).

For whales hunted in the Southern Ocean, where the mixing of two or more breeding populations is suspected, the application of population dynamic models is particularly problematic. First pass attempts to allocate catches to a source population have been made using individual catch data collated and coded by the IWC from commercial whaling operations in Antarctic waters (Allison, 2010). For example, the first model (denoted 'Naïve') assumed that the breeding populations corresponded to a single feeding area along arbitrary lines of longitude (Areas I–VI) (IWC, 1998; Mackintosh, 1942;1965). As new information has emerged on the mixing of populations on the feeding grounds (Anderson *et al.,* 2010; Franklin *et al.,* 2008; Gales *et al.,* 2009; Steel *et al.,* 2008), alternative catch allocation models have been developed to include areas of mixing where whales are expected to be equally drawn from adjacent populations (e.g. IWC, 2010).

To date, assessments have been completed for several humpback whale breeding populations thought to have both simple and complex relationships between feeding areas and breeding grounds, e.g. those whales that winter in the south-western Atlantic (Zerbini *et al.,* 2006) and the south-eastern Pacific around Columbia, Panama and Ecuador (Johnston *et al.,* 2011) versus those that winter along the west and east coast of South Africa (IWC, 2009; 2011; 2012). The humpback whales that breed off eastern Australia and around the low latitude island groups of the South Pacific defined as 'Oceania', however present major challenges. This is largely due to uncertainties about both the population structure on the breeding grounds and the mixing of these populations in Antarctic waters.

As a further complication, recovery for the eastern Australian and Oceania populations has been variable. While the humpback whales migrating along eastern Australia have shown a high annual rate of population increase (10–11% Noad *et al.,* 2011), Oceania humpback whales are yet to show signs of recovery (Childerhouse and Gibbs, 2006; Gibbs *et al.,* 2006; Paton *et al.,* 2006). This lack of recovery has prompted the relisting of the population as Endangered on the IUCN Redlist (Childerhouse *et al.,* 2008; IWC, 1998). The IWC Scientific Committee have (IWC, 2011) therefore recommended an assessment of the mixing between eastern Australia and Oceania in Antarctic feeding Area V (130°E–170°W – south of New Zealand and eastern Australia), as well as eastern Australia and western Australia in Antarctic feeding Area IV (80–130°E – south of western Australia).

In light of the uncertainty about the population structure of the humpback whales of Australia and the South Pacific, many different structure hypotheses have been proposed for consideration (IWC, 2006; 2011). The present study examines different hypotheses that deal with this uncertainty by keeping the low latitude island groups of Oceania either separated or combined. Hypothesis 1 proposes a population structure where Oceania is sub-divided into four populations including New Caledonia, Tonga, Cook Islands and French Polynesia, with eastern Australia also considered a demographically independent population. This hypothesis was ranked 'medium' in plausibility as a realistic biological model during an IWC Scientific Committee workshop on the Comprehensive Assessment of Southern Hemisphere Humpback Whales, based on available biological evidence (Garrigue *et al.,* 2011; IWC, 2006; Olavarría *et al.,* 2007). Hypothesis 2 combines New Caledonia, Tonga and French Polynesia to represent the 'combined' Oceania population, while retaining eastern Australia as a separate population and eliminating the Cook Islands. This hypothesis was proposed for 'priority' consideration in the comprehensive assessment as a simple but plausible population structure scenario to deal with the limitations of catch allocation, which excludes the Cook Islands as it lacks a viable abundance estimate and is considered a transitory region as opposed to a true population (Garrigue *et al.,* 2002; Hauser *et al.,* 2010; IWC, 2011; Jackson *et al.,* 2006; Jackson *et al.,* 2009). Although these hypotheses include groupings that do not necessarily represent the true population structure, they can be used in catch allocation scenarios for population modelling (IWC, 2011).

Population genetic analysis has the potential to test these two hypotheses. Furthermore, genetic analysis can also assist in determining the degree of mixing of Australian and Oceania humpback whale populations on their feeding grounds. Population-level genetic methods hold promise for these tasks, i.e. using genetic information to ascertain population membership of groups of individuals (Manel *et al.,* 2005). Mixed-stock analysis uses allele frequencies in all potential contributing (baseline) populations and maximum likelihood or Bayesian methods to estimate proportional contributions of each population to a mixture (Pella and Milner, 1987). Here, the question of interest is not the population origin of individual whales in a feeding ground mixture, but rather the population composition of a feeding ground mixture and how it changes in space and time.

Several studies of humpback whales have employed maternally inherited mitochondrial DNA (mtDNA) in mixed-stock analysis to estimate population mixing within Antarctic feeding areas (Albertson-Gibb *et al.,* 2008; Pastene *et al.,* 2011; Pastene *et al.,* 2013), the last being most comprehensive as it includes over 1,000 high latitude samples. However, no study to date has combined mtDNA and nuclear markers to investigate humpback whale population allocation on the feeding grounds nor assessed statistical power to conduct a mixed-stock analysis. The present study draws on the most comprehensive dataset of mtDNA and nuclear microsatellite markers presently available for humpback whales of Australia and Oceania. The dataset stems from a large scale collaborative effort between two laboratories, with a total of more than 1,300 samples obtained over eleven years (Fig. 1). This extensive dataset provides us with an unprecedented opportunity to investigate the mixing of humpback whales on the feeding grounds.

Specific objectives of the present study were to: (1) assess the patterns and extent of genetic differentiation among the

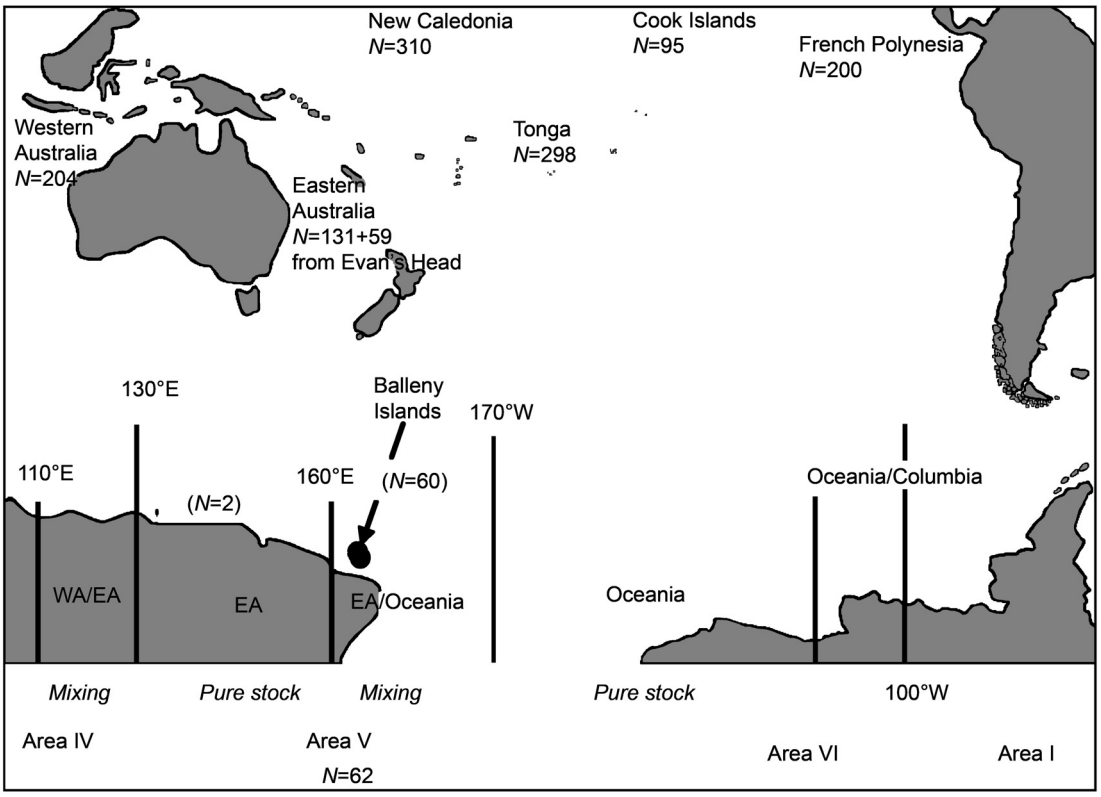

Fig. 1. Australian and Oceania humpback whale breeding/migratory populations and their associated feeding areas (Area IV, V, VI and I) divided into 'Pure Stock' and 'Mixing' areas (IWC, 2009). We also include the number of individuals sampled from each region (N) with Area V samples divided into Area VW ($N = 2$) and Area VE ($N = 60$). 'Oceania' combines New Caledonia, Tonga and French Polynesia in a population structure hypothesis proposed for the Comprehensive Assessment of Southern Hemisphere humpback whales (IWC 2006). WA = western Australia; EA = eastern Australia.

populations; (2) apply a series of simulations to evaluate the power of the microsatellite and mtDNA datasets for mixed-stock analysis given available sample size and the patterns of genetic divergence among populations under the two hypotheses on population structure; (3) extend the simulations to determine ways in which we can improve the accuracy and precision of mixed-stock analyses for these priority populations for future studies; and (4) estimate the population composition of Antarctic Area V samples collected during the Australia/New Zealand Antarctic Whale Expedition (AWE) in 2010, and interpret the findings in light of the simulation outcomes.

METHODS

The sample collection

Antarctic Area V sampling

Skin biopsy samples from 64 animals were collected from humpback whales in Antarctic feeding Area V (130°E–170°W), south of New Zealand and eastern Australia (where the mixing of breeding populations is expected) during a six week Australian–New Zealand Expedition (AWE) conducted in February and March 2010. The majority of samples were collected from adult whales between 162˚E and 179˚E around the Balleny Islands (specific sampling locations are available from the authors). Samples were obtained using a biopsy dart propelled by a modified .22 calibre rifle and stored in 70% ethanol at –80˚C. All pods were sampled opportunistically with every effort made to sample all individuals within a pod, weather and time permitting.

Seven additional samples collected during the IWC's IDCR/SOWER surveys[13] of Antarctic Area V from 1999–2004 were also included in the sample dataset, with five samples from Area VE and two samples from Area VW (Table 1; Fig. 1) (described in Albertson-Gibb *et al.,* 2008; Steel *et al.,* 2008).

Source data

Mixed-stock analysis assumes that all potential populations contributing to a mixture have been sampled. For the source dataset, we included six breeding/migratory populations which are likely to mix in Antarctic feeding Area IV or V both frequently and sporadically (Albertson-Gibb *et al.,* 2008; Chittleborough, 1965; Gales *et al.,* 2009; Steel *et al.,* 2008; Steel *et al.,* 2011): western Australia, eastern Australia and Oceania (New Caledonia, Tonga, Cook Islands and French Polynesia). Oceania samples were obtained by members of the South Pacific Whale Research Consortium during synoptic surveys dating back to 1999 (described in Constantine *et al.,* 2014). Australian samples were collected off Exmouth (Western Australia), Tasmania and Eden (NSW) between 2006 and 2008 (described in Schmitt *et al.,* 2014). An additional 59 samples from eastern Australia were collected off Evan's Head (NSW), 560km north of Eden and were included in the second mixed stock analysis of the Area V samples (see METHODS section III).

See Table 1 for a summary of the sample details. Fig. 1 provides a map of the sampling locations and the feeding areas.

[13] *http://iwc.int/SOWER*

Table 1

Samples from individual whales (assumed from unique genotypes) genotyped at ten microsatellite loci and sequenced at the mtDNA control region for Antarctic feeding Area V and the six source populations. M = males; F = females; ? = unknown.

		N							
			Sex				Sex		
Region	Sampling period	Microsatellites	M	F	?	mtDNA	M	F	?
Antarctica Area V	1999–2010	**62**	31	31	–	**62**	31	31	–
Western Australia									
Exmouth	2007	204	116	88	–	189	107	82	–
Eastern Australia		190	127	62	1	180	141	59	–
Eden	2008	61	47	14	–	57	44	13	–
Tasmania	2006–08	70	34	36	–	66	31	35	–
*Evans Head**	2009	59	46	12	1	57	66	11	–
Total Australia		**394**	243	150	1	**369**	248	141	–
New Caledonia	1999–2005	310	172	123	15	299	170	121	8
Tonga	1999–2005	298	196	97	5	292	193	95	4
Cook Islands	1999–2005	95	47	42	6	91	46	42	3
French Polynesia	1999–2007	200	100	83	17	190	93	81	16
Total Oceania		**903**	515	345	43	**872**	502	339	31
Total		**1,359**	789	526	44	**1,303**	781	511	31

*Evans Head samples only used in the mixed-stock analysis of Antarctica Area V samples.

Molecular genetic analysis

The DNA extraction, sex-typing, microsatellite genotyping and mtDNA sequencing have been described fully elsewhere. See Schmitt *et al*. (2014) for the Australian samples, and Constantine *et al.* (2014) for the samples from Oceania. Note that due to minor differences in the laboratory procedures, it was necessary to standardise the allele sizes for the microsatellite loci before the datasets could be combined. The standardisation was achieved by re-analysis of 22 reference samples drawn from the Constantine *et al*. (2014) study for which DNA was re-extracted and genotyped by the methods of Schmitt *et al.* (2014). The lack of unresolvable discrepancies between the datasets allowed us to combine them for all subsequent analyses. The final combined microsatellite dataset consisted of ten loci genotyped for 335 samples from Australia (plus 59 samples from Evans Head), 903 from Oceania and 62 from Antarctic Area V (Table 1).

For the mtDNA analysis, DNA sequences from the control region were truncated and aligned with a 470bp consensus region starting at position six of the reference humpback whale control region sequence (GenBank X72202: see Baker and Medrano-Gonzalez, 2002; Olavarría *et al.,* 2007). In total, 312 sequences from the Australian samples, 872 from Oceania and 62 from Antarctic Area V were used in all subsequent analyses (Table 1), with 57 sequences from the Evan's Head samples used in the mixed-stock analysis estimation.

Statistical analysis

Genetic structure

For an initial evaluation of the two hypotheses for population structure; hypothesis 1 (H1) where the Oceania populations are considered separately, and hypothesis 2 (H2) where New Caledonia, Tonga and French Polynesia are combined (IWC, 2006; 2011; Jackson *et al.,* 2006), genetic differentiation was calculated among each population pair for both scenarios using an Analysis of Molecular Variance (AMOVA; Excoffier *et al.,* 1992) as implemented in GenAlEx 6.5 (Peakall and Smouse, 2006; 2012) with statistical testing by random permutation (999 permutations). For microsatellite data, an estimate of F_{ST} (infinite allele model) was calculated as in Weir and Cockerham (1984), Peakall *et al*. (1995) and Michalakis and Excoffier (1996). Given the high variability of microsatellite markers, Jost's D_{EST} (Jost, 2008; Meirmans and Hedrick, 2011), an unbiased estimator of divergence, was also calculated using a modified version of the R package DEMEtics V0.8.0 (Jueterbock *et al.,* 2010), with 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. For these analyses, genetic distance matrices were constructed using individual pairwise differences at all polymorphic nucleotide sites (following Excoffier *et al.*, 1992), or haplotype differences among all individuals (Nei, 1987). In keeping with the common practice in similar studies we use the notation F_{ST} for haplotype differentiation and Φ_{ST} for nucleotide differentiation (Olavarria *et al.,* 2006; Olavarría *et al.,* 2007; Rosenbaum *et al.,* 2009). For all marker sets we also estimated Shannon's Mutual Information index, ${}^{S}H_{UA}$ among population pairs as offered in GenAlEx 6.5 (Sherwin *et al.,* 2006). This analysis provides a G-test of allele frequency differences, but with tests of significance performed by random permutation rather than by the more conservative chi-square.

Simulations

This is the first study to attempt a mixed stock analysis of Antarctic Area V humpback whale samples using both mtDNA and microsatellite markers. The assembled genetic data are the most comprehensive presently available for putative source populations, although the sampling of the potential source population is not uniform across the study system. Computer simulations were used to assess our ability to estimate mixing on the feeding grounds.

In these simulations, the source data are analogous to a predictive model. A high degree of correct apportionment (see below for thresholds) would suggest that the level of genetic differentiation is sufficient to distinguish the populations in a mixture. The term 'confidence' comprises two components: accuracy and precision. 'Accuracy' in these simulations is defined as the agreement between the simulated value and the expected value, and 'precision' refers to the repeatability of the simulated value as measured by a confidence interval (i.e. the smaller the confidence interval, the more precise the value). The square root of the mean square error (RMSE) is also reported in evaluating the resolution of the source datasets for a mixed-stock analysis which incorporates both the standard deviation and bias (Estimate–Expected) of an estimate.

Three factors are known to be important for effectively estimating mixture proportions using mixed-stock analysis: the degree of differentiation among source populations and stocks, adequate sampling of all contributing source populations and a sufficient number of genetic markers (Epifanio *et al.,* 1995; Kalinowski, 2004; Pella and Milner, 1987). These factors are considered below.

EVALUATING THE RESOLUTION OF SOURCE DATASETS FOR A MIXED-STOCK ANALYSIS

For the mixed-stock analysis, the program SPAM, v. 3.7 (Alaska Department of Fish and Game, 2003; Debevec *et al.,* 2000) was used as it is currently the only mixed-stock simulation software that can accommodate both microsatellite and mtDNA data combined. SPAM employs a maximum likelihood approach, with allele frequency distributions modelled using the Rannala-Mountain posterior (Rannala and Mountain, 1997). This approach allows for the estimation of allele frequencies for loci with many low-frequency alleles that can cause bias and/or imprecision in stock-composition estimates.

The 100% simulation feature in SPAM was used to assess the ability of mixed-stock analysis to accurately reapportion populations, assuming there is no mixing. This approach simulates a sample composed of 100% of each population from the source data and then attempts to reapportion simulated individuals to their population of origin. The simulation uses bootstrap resampling of allele frequencies from the source data based on a user nominated sample size of the 'pure stock'. This provides an initial benchmark with which to test the statistical power of our datasets for mixed-stock analysis and is a widely reported method in demonstrating the apportion accuracy for genetic stock identification applications (e.g. Beacham *et al.,* 2006; Smith *et al.,* 2005).

The simulations were performed on three separate datasets: microsatellites, mtDNA, and microsatellites + mtDNA combined. All 100% sample reapportioning simulations were conducted with 40 simulated individuals per iteration and 1000 bootstrap resamplings were used to calculate all mean proportional contribution estimates with 95% symmetric bootstrap confidence intervals. The sample size of 40 was chosen as a conservative sample number that might be collected on a six week voyage in the Southern Ocean. A population was considered identifiable if 90% or more of the simulated 'pure stock' was correctly identified to have originated from within the population (e.g. as demonstrated by Albertson-Gibb *et al.,* 2008; Anderson *et al.,* 2008; Hess *et al.,* 2011; Smith *et al.,* 2005; VanDeHey *et al.,* 2010).

It is well established that sampling from populations, particularly when sample sizes are small, can lead to bias in allele frequency estimation where the sample allele frequencies differ by more than the real (unknown) allele frequencies of the populations being sampled (Anderson *et al.,* 2008). This in turn leads to an over inflation of F_{ST} which may yield overly optimistic conclusions about the accuracy of a mixed-stock analysis. For example, Anderson *et al.* (2008) showed that for co-dominant genetic markers, raw F_{ST} is inflated by a magnitude of approximately $1/(2S)$, where S is the population sample size.

Therefore, in a further test of the ability of mixed-stock analysis to accurately reapportion populations, mixed-stock outcomes were evaluated using the 100% simulation feature in SPAM for 'mtDNA only test sample sets' under H1 in which the degree of genetic differentiation (F_{ST}) among the populations was on average less than observed for the real data. To obtain these test sample sets, customised routines in GenAlEx 6.5 (Peakall and Smouse, 2012) were used that drew on the simulation routines described in Banks and Peakall (2012). In brief, test sample sets of $N = 200$ for each of the six source populations were obtained by randomly drawing mtDNA haplotypes from respective population haplotype frequency distributions. To reduce pairwise F_{ST} values relative to the original data, haplotypes that were drawn from the baseline haplotype frequency distribution of the combined populations at defined rates (effectively simulating 'migration') were incorporated. Subsequent AMOVA analyses confirmed that this approach had the desired outcome of reducing average pairwise F_{ST} values, while at the same time broadly retaining the pairwise patterns of population genetic structure (See Appendix Table 4 for details). The performance of five-test sample sets for each of the three new levels of F_{ST} were then evaluated for each source population as described above.

IMPROVING THE RESOLUTION OF OUR SOURCE DATASETS FOR MIXED-STOCK ANALYSIS

A mixed-stock analysis combining microsatellites + mtDNA for the grouped Oceania populations (H2) correctly assigned the greater proportion of samples and therefore was used in subsequent mixed-stock analyses. The influence of source data sample size on the ability of mixed-stock analysis to accurately reapportion a 100% sample of each putative population was examined by changing the number of samples in the source dataset to $N \geq 200$, 250, 300 and 400 for all populations (e.g. any population with less than 200 individuals in the source dataset would be increased to $N = 200$ and any population with $N \geq 200$ would remain at the current sample size) and the 100% simulation was repeated in SPAM using 40 simulated individuals and 1,000 bootstrap resamplings. When simulating an increase in source population sample sizes, the original baseline allele frequency was used to generate new individuals and therefore was not taken into account the increased likelihood of rare alleles appearing if larger sample sizes were available. Despite the bias associated with sampling error (Anderson *et al.,* 2008), the result will help determine whether the

differences in sample size between populations have a strong influence on the ability to identify each population in a mixture. Larger baseline samples are expected to increase accuracy in population identification (Beacham *et al.,* 2011; Kalinowski, 2004).

Given extensive sampling in the Southern Ocean can be both expensive and time consuming, it is also useful to determine whether feeding ground sample size is important for an accurate mixed-stock analysis. Different sample sizes of a 'pure stock' were simulated, representing samples on the feeding grounds, to determine the minimum number of samples required to confidently reapportion one population from its neighbouring population, as likely to occur in a feeding ground mixture (i.e. western Australia and eastern Australia; Oceania and eastern Australia). Using the 100% simulation feature in SPAM, various feeding ground sample sizes (i.e. N = 40, 65, 80, 120, 160, 200, 280, 400) were simulated and mean correct assignment of all individuals compared to their population of origin. By plotting these estimates for one population from each potential mixture and identifying the inflection point where the mean correct assignment begins to stabilise, the minimum sample size required for that given level of accuracy can be determined.

The Antarctic feeding grounds are likely to be a mixture of neighbouring humpback whale breeding populations (e.g. 50% from western Australia and 50% from eastern Australia; 60% from eastern Australia and 40% from Oceania; 10% from western Australia, 50% from eastern Australia and 40% from Oceania). By simulating realistic population mixtures with user defined proportions in SPAM, the degree of confidence in the source dataset to determine the proportion of humpback whales assigned to each population in a mixture can be assessed. Predicted precision and accuracy was assessed from the difference between predicted and observed population proportions. An error rate of ≤10% outside the predicted proportion was used to imply confidence in the source data in predicting stock contribution in a mixture (VanDeHey *et al.,* 2010). Two possible mixture combinations of N = 40 with expected proportional contributions varying from 0.0 to 1.0 were simulated. The influence of the sample size of the source data and the number of simulated individuals (mixture or feeding ground sample size) on our ability to estimate different population contributions in the two mixtures was investigated by repeating the analysis using a population sample size of $N \geq$ 200 and a mixture sample size of N = 160 based on results from previous analyses.

Mixed-stock estimation of the 62 individual samples collected from Antarctic feeding Area V

The program SPAM and the source data was used to calculate maximum likelihood estimates of humpback whale population contributions (with Oceania populations grouped and not grouped) to the Antarctic feeding Area V samples using all three genetic marker sets and 10,000 bootstrap re-samplings. Based on the outcome of the mixed-stock analysis simulations (see below), the estimation both with and without the 59 samples from Evan's Head in eastern Australia was carried out. It was decided not to conduct an individual assignment as most individual whales from the source data could not be assigned to a population of origin.

RESULTS

The data set consisted of 1,309 individuals, including microsatellite data for 1,300 samples, and mtDNA sequences for 1,246 samples. Summary genetic data for each microsatellite locus as well as variability in the mtDNA control region for all samples are presented in Appendix Tables 1 and 2. There was only one case out of the 80 tests (10 loci × 8 datasets) for which significant departure from Hardy-Weinberg equilibrium expectations was detected at $P < 0.01$ (Appendix Table 1). There was some evidence for a non-random association of alleles between EV14 and rw4-10, and EV37and GT23 for New Caledonia but not elsewhere. The sex ratio was significantly biased towards males (743 males to 514 females, $\chi^2 = 54.6$, $P < 0.0001$) for all sampling locations with the exception of the Cook Islands (47 males to 42 females, $\chi^2 = 0.3$, $P = 0.60$) (Table 1).

Statistical analysis

Genetic structure

The patterns of genetic diversity for the Oceania population sub-divided (H1) versus grouped (H2) were examined.

Under H1, genetic differentiation was weak but significant (microsatellites: F_{ST} = 0.004, P = 0.001; D_{EST} = 0.004, P = 0.035, mtDNA: haplotype level F_{ST} = 0.018, P = 0.001; nucleotide level Φ_{ST} = 0.025, P = 0.001). Pairwise comparisons for the ten microsatellite loci detected no significant difference between Antarctic Area V and eastern Australia (infinite allele model of mutation F_{ST} and Jost's D_{EST} = 0, $P > 0.05$), and Antarctic Area V and New Caledonia (F_{ST} and $D_{EST} < 0.005$, $P > 0.05$). Using the D_{EST} index there was also no significant structure detected between the Cook Islands and New Caledonia, Tonga, and French Polynesia respectively ($P > 0.05$) (Table 2a). For mtDNA there was low but significant structure detected at the haplotype level between all comparisons except Antarctic Area V and eastern Australia, and French Polynesia and the Cook Islands ($P > 0.05$). At the nucleotide level twelve pairwise comparisons were significantly different ($P < 0.05$) and nine were not including Antarctic Area V and eastern Australia, New Caledonia, Tonga and the Cook Islands respectively ($P > 0.05$) (Table 3a).

With the Oceania populations grouped (H2), genetic differentiation was similarly weak but significant across the entire dataset, but with a substantial increase in D_{EST} (microsatellites: D_{EST} = 0.017, P = 0.001, mtDNA: haplotype level F_{ST} = 0.012, P = 0.001; nucleotide level: Φ_{ST} = 0.020, P = 0.001). Pairwise genetic differentiation was weak but significant between all comparsions (P = 0.001) except Antarctic Area V and eastern Australia for all statistics, and Antarctic Area V and Oceania for Φ_{ST} (Tables 2b and 3b).

The Shannon's *Mutual Information Index* supported the AMOVA results but also found no significant difference in allele frequencies between Tonga and the Cook Islands (${}^{S}H_{UA}$ = 0.009, $P > 0.05$) and the Cook Islands and French Polynesia for the ten microsatellite markers (${}^{S}H_{UA}$ = 0.012, $P > 0.05$) (Appendix Table 3).

Simulations

EVALUATING THE RESOLUTION OF SOURCE DATASETS FOR A MIXED-STOCK ANALYSIS

Overall, the mtDNA alone reapportions a 'pure stock' with a slightly greater accuracy than did the combined and

Table 2

Pairwise F_{ST} and D_{EST} values among source populations and Antarctic feeding Area V under: (a) H1; and (b) H2 based on ten microsatellite loci. F_{ST} values are given below the diagonal and D_{EST} values are given above the diagonal. Significant *P*-values ($P < 0.05$ after sequential Bonferroni correction) for F_{ST} , based on statistical testing of 999 random permutations and for D_{EST}, based on 1,000 bootstrap resamplings, are shown in **bold**. Area V = Antarctic feeding Area V; WA = western Australia; EA = eastern Australia; NC = New Caledonia; TG = Tonga; CI = Cook Islands; FP = French Polynesia.

Population	Area V	WA	EA	NC	TG	CI	FP
(a) H1							
Area V		**0.028**	0.000	0.004	**0.019**	0.020	**0.035**
WA	**0.006**		**0.031**	**0.024**	**0.023**	**0.026**	**0.026**
EA	0.000	**0.005**		**0.014**	**0.023**	**0.020**	**0.023**
NC	0.002	**0.006**	**0.003**		**0.009**	0.008	**0.022**
TG	**0.016**	**0.014**	**0.015**	**0.013**		0.000	**0.007**
CI	**0.007**	**0.010**	**0.009**	**0.004**	**0.013**		0.000
FP	**0.008**	**0.005**	**0.005**	**0.005**	**0.009**	**0.004**	

Population	Area V	WA	EA	Oceania
(b) H2				
Area V		**0.028**	0.000	**0.013**
WA	**0.006**		**0.031**	**0.021**
EA	0.000	**0.005**		**0.016**
Oceania	**0.005**	**0.01**	**0.01**	

Table 3

Pairwise F_{ST} and Φ_{ST} values among source populations and Antarctic feeding Area V under: (a) H1; and (b) H2 based on mitochondrial DNA control region sequences. F_{ST} values are given below the diagonal and Φ_{ST} values are given above the diagonal. Significant *P*-values ($P < 0.05$ after sequential Bonferroni correction) based on statistical testing of 999 random permutations are shown in **bold**. Area V = Antarctic feeding Area V; WA = western Australia; EA = eastern Australia; NC = New Caledonia; TG = Tonga; CI = Cook Islands; FP = French Polynesia.

Population	Area V	WA	EA	NC	TG	CI	FP
(a) H1							
Area V		**0.02**	0.001	0.003	0.005	0.018	**0.05**
WA	**0.016**		**0.042**	**0.017**	**0.022**	**0.045**	**0.082**
EA	0.002	**0.015**		**0.021**	0.012	0.012	**0.036**
NC	**0.005**	**0.016**	**0.010**		0.005	**0.019**	**0.042**
TG	**0.015**	**0.016**	**0.010**	**0.009**		0.007	**0.035**
CI	**0.033**	**0.031**	**0.025**	**0.030**	**0.010**		0.005
FP	**0.037**	**0.034**	**0.031**	**0.031**	**0.017**	0.003	

Population	Area V	WA	EA	Oceania
(b) H2				
Area V		**0.024**	0.001	0.006
WA	**0.016**		**0.042**	**0.027**
EA	0.002	**0.02**		**0.013**
Oceania	**0.010**	**0.02**	**0.010**	

microsatellite datasets (Fig. 2). Furthermore, the patterns of high population reapportionment for the mtDNA dataset under H1 were largely maintained across the 15 'test sample sets' in which the average pairwise F_{ST} values were reduced (see Appendix Fig. 1), relative to the original data set used in the simulations shown in Fig. 2, particularly those populations that achieved a correct reapportionment above the 90% threshold. The combined mtDNA + microsatellite dataset however, showed a consistent trend of more precise estimates, characterised by smaller Coefficient of Variation, CV (e.g. for populations grouped under H2, mtDNA mean $CV = 0.06$; mtDNA + microsatellites mean CV = 0.05) with a lower RMSE for all populations except eastern Australia and the Cook Islands (e.g. for populations grouped under H1, EA: mtDNA RMSE = 0.113, mtDNA + microsatellites RMSE = 0.135, CI: mtDNA RMSE = 0.315, mtDNA + microsatellites RMSE = 0.352). Overall, confidence in reapportioning a 'pure stock' was highest when populations were grouped as for H2. Under this grouping, mixed stock apportionment was correct 90% or more of the time for mtDNA and the combined marker set with the exception of eastern Australia (average ~ 94%). By contrast when populations were grouped as for H1, correct reapportionment was lower than the 90% threshold (between 77.2% to 86.7%, with the exception of western Australia, eastern Australia and French Polynesia for mtDNA, and western Australia and New Caledonia for the combined marker set). Given the improved confidence under the H2 groupings and consistent trend of improved precision (lower confidence intervals and RMSE), all subsequent simulations were carried out using the combined marker set and grouped as for H2 (with Oceania populations combined).

IMPROVING THE RESOLUTION OF OUR SOURCE DATASETS FOR MIXED-STOCK ANALYSIS

The cost involved in obtaining whale samples is high. Simulations allowed us to evaluate the potential benefits of increasing sample size from putative breeding populations and

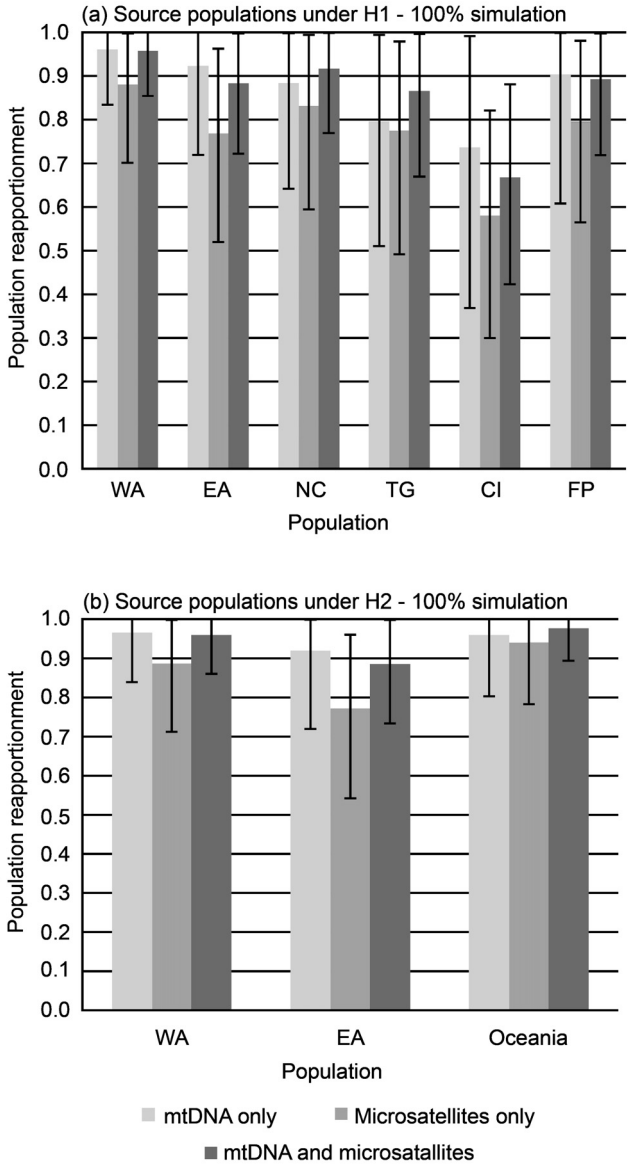

Fig. 2. Results of 100% simulations for humpback whale populations under (a) H1 and (b) H2. The program SPAM was used to simulate a 'pure stock' (N = 40) consisting of 100% of each source population. We then assessed the ability of mixed stock analysis to correctly estimate the reapportionment of each population using mitochondrial DNA (mtDNA) control region sequences, ten microsatellite loci, and mtDNA + microsatellites combined. We expect to see 100% of the correct population. Error bars represent 95% confidence intervals. WA = western Australia; EA = eastern Australia; NC = New Caledonia; TG = Tonga; CI = Cook Islands; FP = French Polynesia.

on the feeding grounds under the assumptions that present allele frequencies are representative of the source populations.

Increasing the simulated sample size for eastern Australia had the greatest effect on our ability to reapportion a 'pure stock' using mixed-stock analysis, increasing the mean correct assignment in the 100% sample reapportioning simulations to over 93.9% (an increase of 5.4%, Fig. 3). Accuracy and precision of assignment improved only marginally for western Australia and eastern Australia when sample sizes were increased from ≥ 250 to ≥ 400 (WA by 1.7% and EA by 3.1% overall).

The number of simulated individuals, representing feeding ground individuals, had minimal impact on the accuracy of reapportioning a 'pure stock' using mixed-stock analysis, although precision increased (Figs 4a and b). Confidence

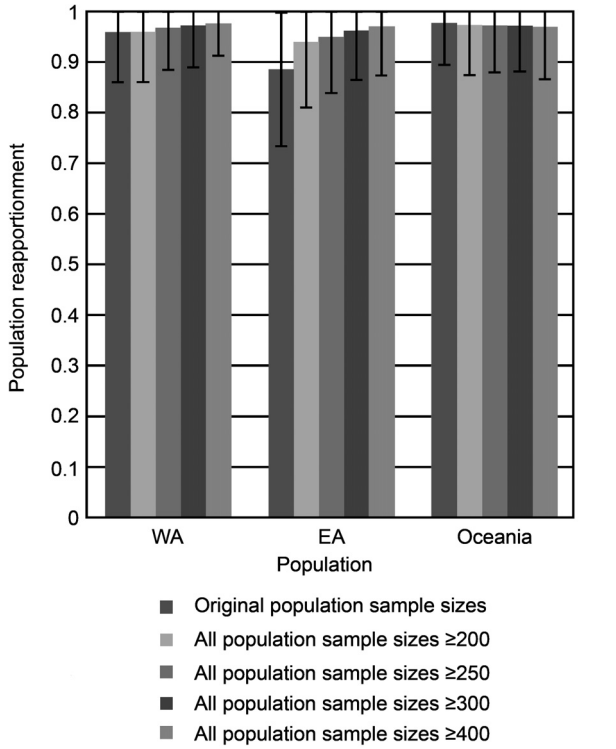

Fig. 3. The effect of increasing population sample size on the genetic distinctiveness of populations under H2 for the combined mtDNA + microsatellite dataset using the 100% simulation analysis in SPAM and a simulation sample size of N = 40. Error bars represent 95% confidence intervals.

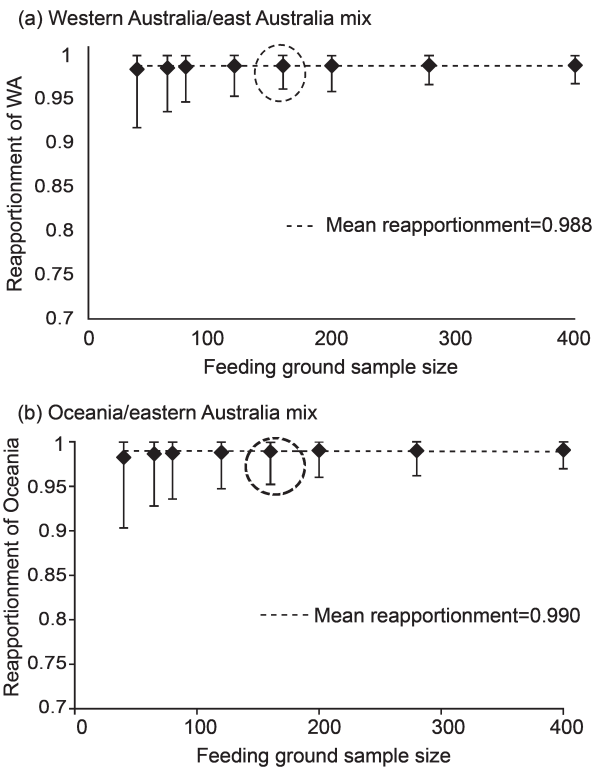

Fig. 4. Mean reapportionment for (a) western Australian humpback whales in a western Australia/eastern Australia mix, and (b) Oceania humpback whales in a Oceania/eastern Australia mix, simulated as a function of feeding ground (mixture) sample size using the 100% simulation feature in the program SPAM and the combined microsatellite and mtDNA control region dataset. Error bars represent 95% confidence intervals. The horizontal line represents where the mean correct assignment begins to stabilize. In both figures the dashed circle encompasses the feeding ground sample size where the mean reapportionment matches the horizontal line value and confidence intervals begin to stabilize (N = 160).

intervals around the mean correct assignment begin to stabilise at a sample size of 160 for both feeding area mixes.

In simulating mixtures of different proportions using our original sample sizes and a mixture sample size of $N = 40$, the differences between expected and estimated proportions for both feeding ground mixes were all within the 10% threshold error rate (3.4 to 3.6%) for identity except when eastern Australia was at an expected proportion of 90% (11.3%, Figs 5a and b). Confidence intervals however, were all outside the 10% threshold error rate and at their largest for a 50:50 mixture.

When sample size was increased from 131 to 200 for eastern Australia, the accuracy of proportion estimates increased for both mixtures. Differences between the expected and estimated proportions of western Australia (and likewise for eastern Australia) were reduced by an average of 0.9% (all differences within 1.8%) while for eastern Australia (and likewise Oceania) the differences were reduced by an average of 3.0% (all differences within 6.3%) with estimates improving by as much as 5% for expected proportions of 50–90% (Figs 5c and d). Increasing the source data sample size had little effect on confidence intervals.

When mixture sample size was also increased to $N = 160$, confidence intervals were reduced by half and were within the 10% threshold error rate for all expected proportions for both feeding area mixtures (Figs 5e and f).

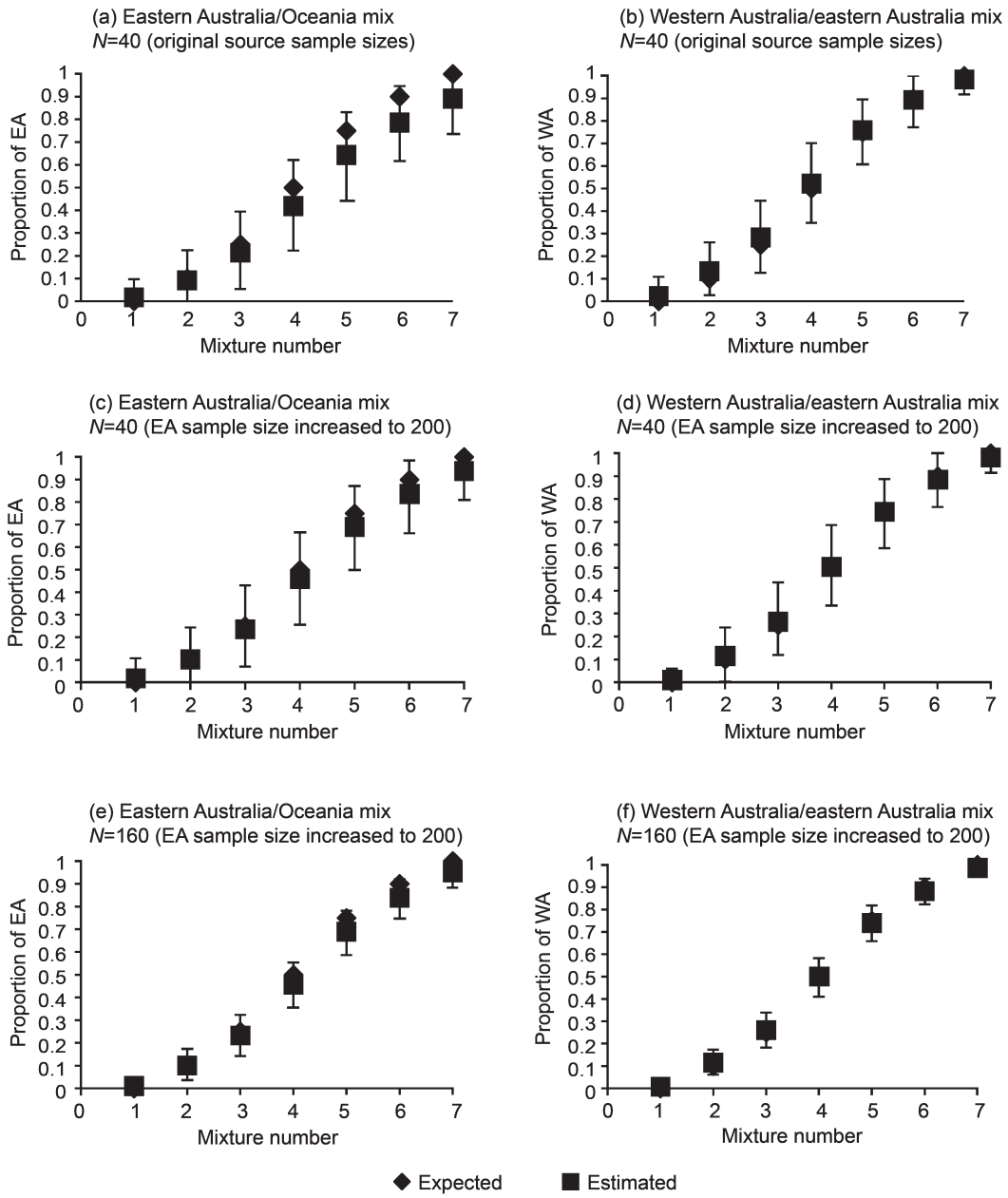

Fig. 5. Results of simulated mixture analyses for two humpback whale feeding ground mixtures; eastern Australia/Oceania and western Australia/eastern Australia. We used the program SPAM to simulate mixtures with expected proportional contributions varying from 0.0 to 1.0 (e.g. mixture 1 = 0% eastern Australia/100% Oceania; 0% western Australia/100% eastern Australia). We then performed mixed stock analysis, calculated maximum likelihood estimates of the proportional contributions in each simulated mixture, and compared estimated values to the true expected proportions using the combined microsatellite + mtDNA control region datasets. (a) and (b) used original population sample sizes and a mixture sample size of $N = 40$; (c) and (d) used population sample sizes ≥ 200 and a mixture sample size of $N = 40$; (e) and (f) used population sample sizes ≥ 200 and a mixture sample of $N = 160$.

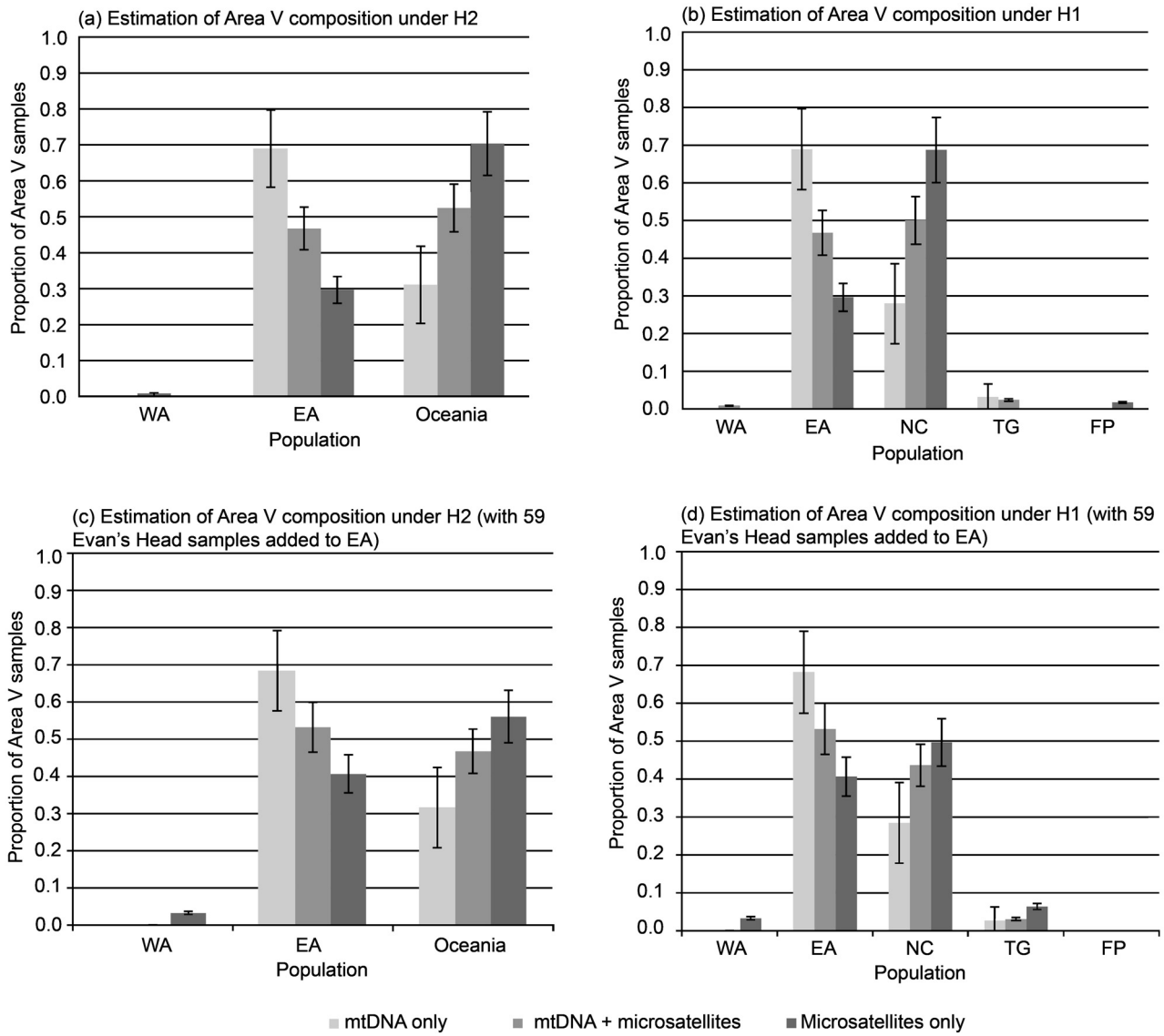

Fig. 6. SPAM maximum likelihood estimates of humpback whale feeding Area V composition (N = 62) under H1 (b) and (d) and H2 (a) and (c) using all three genetic marker sets. Error bars represent jackknife standard errors. (a) and (b) show estimates using source data and (c) and (d) show estimates using source data with eastern Australia supplemented by 59 Evan's Head samples.

Mixed-stock estimation of the 62 individual samples collected from Antarctic feeding Area V

With populations grouped as for H2, eastern Australia accounted for 46.8% (SE = 5.9%), Oceania accounted for 52.4% (SE = 6.7%) and western Australia for 0.8% (SE = 0.1%) of the 62 Antarctic feeding Area V samples using the combined mtDNA + microsatellite dataset. Eastern Australia accounted for as much as 68.9% (SE = 10.8%) using the mtDNA only dataset (Oceania: 31.1%, SE = 10.8%) but only 29.6% (SE = 3.7%) using the microsatellite dataset (Oceania: 70.4%, SE = 8.9%) with no contribution from western Australia (Fig. 6a).

Although signals in the above simulations appear to favour a grouping of populations in Oceania, under H1, three populations for mtDNA and two for the combined dataset performed above the 90% threshold in the 100% sample reapportioning simulations. Based on this result, a mixed-stock analysis was also carried out with Oceania as separate populations. In this case, contributions from Oceania to the Antarctic samples were found to be predominantly drawn from New Caledonia across all three marker sets (combined: 50.0%, SE = 6.4%; mtDNA: 27.9%, SE = 10.6%; microsatellites: 68.7%, SE = 8.6%) with close to negligible contributions from Tonga (Fig. 6b).

At the beginning of the simulation study, the 59 samples from Evan's Head (eastern Australia) were not available, and were therefore not included in the simulations. As increasing the number of samples from eastern Australia was found to improve the accuracy of population allocation estimates, the mixed-stock analysis for the Area V samples with these additional 59 samples was repeated. An AMOVA analysis between the three eastern Australian sampling locations found no significant differentiation for either the microsatellite (F_{ST} = 0.000, P = 0.5; D_{EST} = 0.000, P = 0.5) or the mtDNA (F_{ST} = 0.003, P = 0.2; Φ_{ST} = 0.000, P = 0.5) datasets, thereby justifying the pooling of the data. The addition of these 59 samples had little effect on apportionment for the Area V samples using the mtDNA data only however, the estimated contribution of eastern Australia for both microsatellite datasets increased, while the contribution of New Caledonia and Oceania as a whole decreased (combined: EA, 53.2%, SE = 6.8%; NC, 43.7%,

SE = 5.5%; Oceania, 46.8%, SE = 5.9%; microsatellites: EA, 40.6%, SE = 5.1%; NC = 49.6%, SE = 6.3%; Oceania = 56.1%, SE = 7.1%) (Figs 6c and d).

DISCUSSION

This study combined genetic analysis of mtDNA and microsatellite DNA data and simulations to explore the role of differentiation among source populations, as well as source and feeding ground sample size in effectively identifying pure samples and estimating mixture proportions. Consistent with other humpback whale studies in the Southern Hemisphere, weak but significant differentiation was detected between populations (e.g. Olavarría *et al.,* 2007; Rosenbaum *et al.,* 2009; Schmitt *et al.,* 2014). In cases such as this where differentiation is low, simulations are particularly important to evaluate the statistical power given the available genetic markers and the sample size. The discussion below examines the strengths and limitations of our current datasets for drawing conclusions about population allocations on the feeding grounds. Recommendations on sampling gaps and adjustments that can be implemented to ensure robust estimates are provided.

Genetic structure

This is the first study to assess the patterns of genetic differentiation at nuclear loci across the populations of Australia and Oceania. The degree of differentiation among pairwise comparisons was consistently low for both marker sets (microsatellites: H1 D_{EST} from 0.000 to 0.035, H2 D_{EST} from 0.000 to 0.031; mtDNA: H1 F_{ST} from 0.002 to 0.037, H2 F_{ST} from 0.002 to 0.016; Table 2 and 3). Differentiation was particularly weak among the ungrouped populations of Oceania (H1), with a D_{EST} of only 0.009 between New Caledonia and Tonga, 0.000 between Tonga, the Cook Islands and French Polynesia and 0.009 between Tonga and French Polynesia (mtDNA F_{ST} = 0.009, 0.010, 0.003 and 0.017 respectively). In spite of this result, differentiation between New Caledonia, Tonga and French Polynesia was found to be significant for both markers across all statistics ($P < 0.05$), suggesting demographic independence among these breeding grounds. These results are consistent with recent findings based on multi-state measurements of genotype exchange within the ungrouped populations of Oceania and between these populations and eastern Australia i.e. eastern Australia may not be more isolated from Oceania than animals within Oceania are from one another (Jackson *et al.,* 2012).

Feasibility of a mixed stock analysis

The ability to confidently reapportion the populations of Australia and Oceania using a mixed-stock analysis was influenced by the choice of genetic markers and the degree of differentiation. The 100% sample reapportioning simulation results suggest that the ten microsatellite loci alone did not allow accurate reapportion of each population using a 90% identity threshold, and may therefore give a poor estimate of population apportionment on the feeding grounds. This finding is likely to have been influenced by the low differentiation at the microsatellite loci. Although the mtDNA control region dataset alone could accurately discriminate among most populations at the 90% identity threshold and a mixture sample size of 40, the combined mtDNA + microsatellite dataset could do so with a greater precision (smaller confidence intervals and RMSE). Given that the increase in precision was not substantial however, mtDNA control region sequences alone may be sufficient for mixed-stock analysis in humpback whales. Furthermore, the levels of population reapportionment under H1 were largely maintained for the mtDNA data even when pairwise F_{ST} values were reduced in the 'test sample sets'. Collectively, these results are consistent with the highly discriminatory nature of mtDNA due to their haploid nature and uniparental inheritance which are expected to result in a larger genetic drift compared to nuclear loci and a smaller effective population size (e.g. Avise, 1995; Sunnucks, 2000). Although nuclear loci can provide greater resolution in discriminating between populations due to the variable nature of some markers (Angers and Bernatchez, 1998; Selkoe and Toonen, 2006), the AMOVA results in this study suggest that the ten microsatellite loci may add little to the discriminatory power of mtDNA. This result may also be a direct consequence of male-driven gene flow which, given the male-bias sex ratios in our datasets, would act to reduce structure between the breeding populations and, in turn, impact the accuracy of the feeding ground mixed-stock analysis. However, a previous study focused on Australian humpback whales found little evidence to support male biased dispersal (Schmitt *et al.,* 2014) and there is still debate over whether the male biased sex ratio is an artefact of behaviour or sampling (Brown *et al.,* 1995). Therefore, given this and the low differentiation that characterises Oceania and Australian humpback whales at the nuclear level, combining both marker types may not necessarily improve the ability to estimate the mixture contributions of populations on the feeding grounds.

The 100% sample reapportioning simulations showed that western Australia, eastern Australia, New Caledonia and French Polynesia could be reapportioned above the 90% threshold for either the mtDNA or combined dataset using original sample sizes and a simulation sample size of 40. The lack of power to reapportion Tonga and the Cook Islands is likely to be a consequence of the interplay between the smaller sample size of the Cook Islands and the weak differentiation between them. Yet when the Cook Islands was removed from the simulation analysis, the ability to reapportion Tonga increased from 86% to 89% and French Polynesia increased from 89% to 92% using the combined mtDNA + microsatellite dataset (data not shown). This result and the lack of significant differentiation between Tonga, the Cook Islands and French Polynesia, is consistent with the suggestion that the Cook Islands aggregation is transient (Garrigue *et al.,* 2002; Hauser *et al.,* 2010).

Improving the accuracy and precision of mixed-stock analyses

The sample size of the source populations was found to influence the accuracy of the predicted population identification with feeding ground sample size impacting the precision of each estimate. For example, a moderate increase in the sample size of eastern Australia from 131 to 200 had the greatest effect on our ability to reapportion the population, increasing the accuracy from 88.5% to 93.9%; above the 90% threshold for identity. Increasing the sample size of eastern

Australia and the feeding ground mixture also produced estimates and confidence intervals within the stringency threshold for all expected proportions for both feeding area mixtures. Indeed a moderate increase in source sample size has been found to have greater gains in the statistical power of the data than moderate increases in the number of nuclear loci (up to 20 loci; 8–33 alleles each), particularly when F_{ST} is low (< 0.01) and the average correct assignment to a population is greater than 80% (Hess *et al.,* 2011; Kalinowski, 2005; Morin *et al.,* 2009). However, given that the increase in source sample sizes did not take into account the increased likelihood of rare alleles appearing, these results should be interpreted with caution. Therefore for this study we can say that increasing the sample size of eastern Australia will improve the accuracy of apportioning these populations on the Antarctic feeding grounds but cannot determine with sufficient certainty the specific number of samples required.

Simulation limitations

There are several ways in which our analyses may bias estimates of simulation performance for all three marker sets.

SPAM has been found to overestimate the predicted accuracy and precision of mixed-stock analysis by resampling from the baseline with replacement, particularly for closely related populations (Anderson *et al.,* 2008). Different types of simulation software have attempted to address this problem (Anderson *et al.,* 2008; Banks and Eichert, 2000; Piry *et al.,* 2004) but there is still no software available yielding unbiased estimates of genetic stock identification that can accommodate combined mtDNA and nuclear data. Preliminary analyses using the 100% sample reapportioning simulation feature in ONCOR which attempts to reduce this bias (Anderson *et al.,* 2008) and the microsatellite data only, produced similar estimates when mean correct assignment was greater than 90% (e.g. SPAM estimate for Oceania under H2 = 94.1%; ONCOR estimate = 92.4%). However, when the SPAM estimates dipped below 80–85%, the ONCOR estimates dropped below 70% (e.g. SPAM estimate for New Caledonia under H1 = 83.2%; ONCOR estimate = 67.5%). Nonetheless, it is uncertain what effect sampling the baseline with replacement might have on mtDNA or combined mtDNA and nuclear data. Despite the bias associated with SPAM, our results provide information on the relative accuracy and precision of marker types, and the effect increasing marker number and sample size has on genetic stock identification in humpback whales.

It should also be noted that all mixed-stock analysis simulations assume that the source samples and allele frequency estimates are representative of the populations present in the mixture and, therefore, do not take account of unrepresentative baseline sampling or omitted source populations. Presently there is no way to systematically account for the possibility of individuals from unsampled populations in the mixture, although computer intensive models have attempted to address this issue (e.g. Smouse *et al.,* 1990).

Estimates of both nuclear allele frequencies and mtDNA haplotype frequencies are inevitably a biased estimate of the true global but unknowable frequencies. This bias has the potential to lead to underestimation of genetic differentiation despite implicit corrections for sample size in F-statistics estimation via the methods of Weir and Cockerham (1984), or the Analysis of Molecular Variance framework (Excoffier *et al.,* 1992). Simulation and estimation results should therefore be interpreted with a degree of caution.

Population composition of Area V samples

Our simulations offer important clues about the degree of confidence that can be expected in estimating the population apportionment of Australia and Oceania humpback whales on the Antarctic feeding grounds using a mixed-stock analysis. It is evident that these estimates are influenced by the genetic distinctiveness among source populations, choice of genetic markers, and both source and mixture sample sizes.

Notwithstanding the value of increasing sample sizes, the simulations indicate that our mtDNA and combined mtDNA + microsatellite datasets have the potential to provide estimates that are close to satisfying the 90% stringency threshold for both hypotheses of population structure.

In light of these simulations, what can be safely concluded about the population mixture of the Antarctic Area V sample? Two important insights emerge from the simulations. The first is that the contribution of western Australia is small to negligible. We have a high degree of confidence in this conclusion given both the small mixed-stock analysis estimated error (Figs 6a to d) and the outcomes of the simulations that predict close agreement between estimated and expected proportion mixtures when the contribution from western Australia is low (Fig. 5b). The second insight is that both eastern Australia and Oceania (largely represented by New Caledonia) make substantial contributions to the Area V sample. What is less certain is the exact apportionment of feeding Area V samples to these populations. This uncertainty arises from the discrepancy between the mixed-stock analysis estimates between the three different marker sets, and the error surrounding these estimates (Fig. 6a and b). The simulations also indicated the largest errors are predicted when there are more or less even contributions of eastern Australia and Oceania (Fig. 5a). With the addition of the 59 Evan's Head samples to eastern Australia however, the discrepancy between estimates for each dataset was reduced (Fig. 6c and d). Thus, while we can be confident that there is a substantial contribution of both populations to Antarctic feeding Area V, whether or not the contributions are equal will require more discriminatory genetic markers.

Collectively, these results add support to individual connection studies using Discovery tags, photo-identification and genotype matches, as well as satellite tags linking eastern Australia to Antarctic Area V (Constantine *et al.,* 2014; Dawbin, 1966; Franklin *et al.,* 2008; Gales *et al.,* 2009; Olavarria *et al.,* 2006; Rock, 2006). From the Antarctic Area V whales used in this study, Constantine *et al.* (2014) found the majority of fluke and genotype matches were to eastern Australia, with one match to New Caledonia and the New Zealand migratory corridor. Despite the low number of matches with Oceania, their result nonetheless implies that Area V is a region where mixing between eastern Australia and Oceania occurs. Their study also corroborates our findings of a negligible contribution from western Australia in Area V, with no matches found. This is consistent with the results of Discovery tag recoveries, implying humpback whales breeding in western Australia are more likely to mix

with those of eastern Australia east of 115°E in Antarctic Area IV (Chittleborough, 1965; Dawbin, 1966).

Our findings are broadly consistent with the mixed-stock analysis study of Pastene *et al.,* (2013). That study, which also focused on the Australian and Pacific region, used only mitochondrial data from a greater number of feeding ground samples but fewer samples from the breeding grounds (1,057 vs 1,297). The mixed-stock analysis from the study indicated a very high proportion of eastern Australian and New Caledonian whales allocated to Area V whereas breeding grounds further east or west were not represented or were at very low frequencies. However, by sector the analyses indicated a high proportion of New Caledonian whales in Area VE ($N = 61$; ~0.84) whereas our study, (using combined marker data) suggested approximately equal proportions allocated to eastern Australia and New Caledonian whales ($N = 62$). Interestingly, our analyses of mitochondrial data alone showed a greater discrepancy with the Pastene *et al*., (2013) study despite using an identical marker (~0.84 NC vs ~0.3 NC). This discrepancy may simply be a consequence of substructure within feeding grounds and the localised nature of the sampling in this study. However, as per our simulations it remains difficult to estimate mixing proportions in this sector. Such difficulties were recognised by the IWC Scientific Committee during the recent in-depth assessment of breeding stocks D, E and F (IWC, 2014; Annex H) and 'significant differences between the high latitude catch allocations that best fitted a three stock population model and the results of a mixed stock analysis' were noted. Consequently IWC (2014) recommended *inter alia* further studies to examine the influence of sample sizes from the breeding grounds in mixed-stock analyses. Our study will contribute to addressing this recommendation.

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APPENDIX

Appendix Table 1

Genetic diversity in humpback whales sampled from Antarctic feeding Area V, the six source populations, and the Evan's Head data from eastern Australia genotyped at ten loci. *N* = number of genotyped individuals per locus, *Na* = number of alleles, *Ho* = observed heterozygosity, *He* = expected heterozygosity, and HW = deviation from Hardy-Weinberg equilibrium (p-value); significant at $P < 0.01$ (standard errors in parentheses).

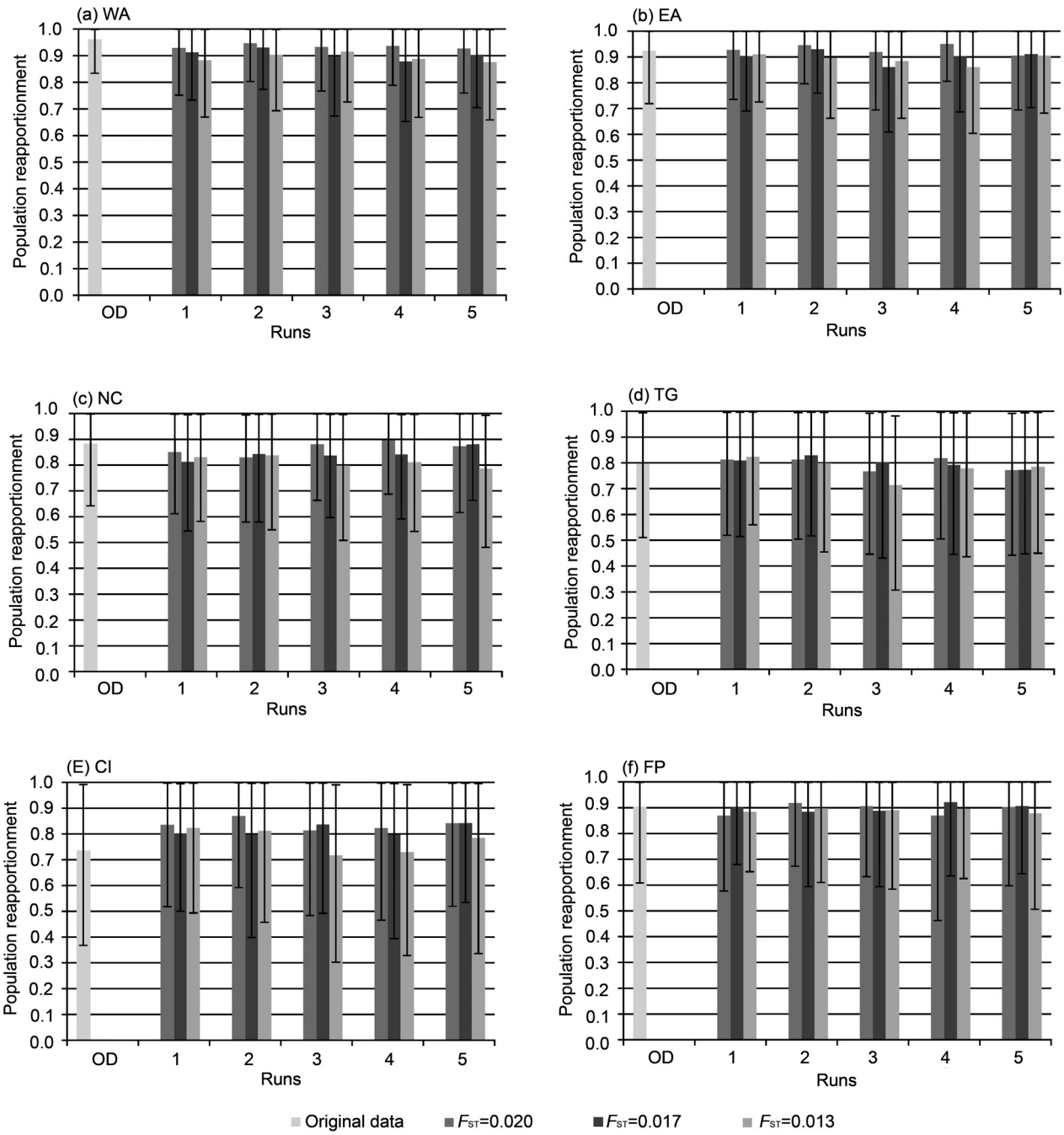
	Antarctic Area V					Western Australia				Eastern Australia				Evan's Head (eastern Australia)				New Caledonia				Tonga				Cook Islands				French Polynesia			
Locus	*N*	*Na*	*Ho*	*He*	HW	*N*	*Na*	*Ho*	*He*	HW	*N*	*Na*	*Ho*	*He*	HW	*N*	*Na*	*Ho*	*He*	HW	*N*	*Na*	*Ho*	*He*	HW	*N*	*Na*	*Ho*	*He*	HW	*N*	*Na*	*Ho*	*He*	HW
Ev14	62	9	0.758	0.723	0.426	203	8	0.754	0.778	0.458	131	9	0.725	0.748	0.742	59	11	0.729	0.757	0.367	309	9	0.725	0.729	0.558	188	10	0.766	0.773	0.344	94	9	0.830	0.798	0.120	189	9	0.735	0.761	0.014
Ev37	62	16	0.935	0.909	0.372	202	19	0.931	0.904	0.322	131	19	0.916	0.913	0.360	59	15	0.898	0.910	0.043	292	21	0.932	0.925	0.910	288	19	0.913	0.921	0.025	90	17	0.867	0.921	0.011	199	19	0.920	0.909	0.761
Ev96	62	13	0.823	0.852	0.049	202	12	0.876	0.869	0.688	131	13	0.863	0.848	0.867	59	12	0.915	0.864	0.641	310	13	0.910	0.880	0.375	292	12	0.839	0.878	0.303	87	12	0.770	0.864	0.039	183	12	0.869	0.867	0.309
GATA417	58	15	0.862	0.883	0.420	203	15	0.911	0.903	0.741	131	15	0.870	0.890	0.631	59	13	0.898	0.877	0.043	275	18	0.931	0.904	0.378	294	21	0.925	0.909	0.935	75	17	0.920	0.898	0.790	197	21	0.873	0.905	0.590
GT211	62	9	0.758	0.787	0.868	203	10	0.803	0.836	0.033	130	10	0.785	0.820	0.675	59	9	0.644	0.849	0.024	310	10	0.852	0.831	0.425	290	10	0.824	0.825	0.637	94	10	0.840	0.816	0.869	199	9	0.804	0.823	0.437
GT23	62	8	0.823	0.759	0.492	204	9	0.838	0.821	0.618	131	9	0.763	0.797	0.185	59	8	0.797	0.792	0.410	309	9	0.822	0.790	0.883	292	9	0.818	0.795	0.226	95	9	0.811	0.778	0.771	196	9	0.806	0.811	0.265
rw4-10	59	10	0.932	0.835	0.819	203	12	0.877	0.854	0.798	131	12	0.786	0.831	0.582	59	10	0.847	0.825	0.123	307	11	0.834	0.844	0.144	292	13	0.795	0.824	0.289	77	12	0.805	0.813	0.938	196	11	0.801	0.822	0.941
Ev1	62	4	0.452	0.565	0.006	203	4	0.567	0.526	0.428	130	4	0.523	0.552	0.429	59	4	0.694	0.581	0.434	300	4	0.500	0.506	0.057	288	4	0.479	0.480	0.244	94	4	0.511	0.501	0.624	193	4	0.430	0.455	0.510
Ev94	62	10	0.823	0.801	0.182	202	9	0.827	0.809	0.357	130	9	0.792	0.807	0.760	59	8	0.780	0.831	0.081	309	10	0.848	0.807	0.461	293	9	0.775	0.805	0.194	90	9	0.711	0.813	0.020	192	9	0.802	0.803	0.034
GT575	61	14	0.803	0.783	0.703	203	16	0.788	0.804	0.292	130	14	0.815	0.811	0.824	59	11	0.847	0.819	0.578	308	15	0.828	0.814	0.417	292	15	0.815	0.833	0.391	95	12	0.758	0.817	0.300	197	14	0.838	0.806	0.688
All loci	61.2	10.8	0.80	0.79	0.43	202.8	11.4	0.82	0.81	0.47	130.6	11.4	0.78	0.80	0.61	59	10.1	0.81	0.81	0.27	302.9	12.0	0.82	0.80	0.46	280.9	12.2	0.80	0.80	0.36	89.1	11.1	0.78	0.80	0.45	194.1	11.7	0.79	0.80	0.46
		(0.5)	*(1.2)*	*(0.04)*	*(0.03)*	*(0.10)*	*(0.20)*	*(1.4)*	*(0.03)*	*(0.03)*	*(0.08)*	*(0.2)*	*(1.3)*	*(0.03)*	*(0.03)*	*(0.07)*	*(0.0)*	*(2.9)*	*(0.09)*	*(0.09)*	*(0.08)*	*(3.6)*	*(1.6)*	*(0.04)*	*(0.04)*	*(0.09)*	*(10.3)*	*(1.6)*	*(0.04)*	*(0.04)*	*(0.08)*	*(2.3)*	*(1.2)*	*(0.04)*	*(0.04)*	*(0.12)*	*(1.6)*	*(1.6)*	*(0.04)*	*(0.04)*	*(0.10)*

*Of the 13,590 genotypes in total we failed to genotype 384, with only two of 1,359 individuals missing genotypes from three or more loci and no individuals missing data from more than four of ten loci.

Appendix Table 2

Variability in the mtDNA control region of humpback whales sampled from Antarctic feeding Area V, the source populations, and Evan's Head (eastern Australia) (h = haplotype diversity and π = nucleotide diversity). Area V = Antarctic feeding Area V; WA = western Australia; EA = eastern Australia; NC = New Caledonia; TG = Tonga; CI = Cook Islands; FP = French Polynesia.

	Region/population	No. of haplotypes	No. of unique haplotypes	$h \pm$ SD	$\pi \pm$ SD
Antarctica	Area V	30	3	0.969 ± 0.008	0.141 ± 0.072
	WA	56	23	0.971 ± 0.004	0.132 ± 0.067
	EA	40	4	0.966 ± 0.005	0.126 ± 0.065
	NC	64	9	0.973 ± 0.002	0.138 ± 0.070
	TG	53	4	0.964 ± 0.003	0.136 ± 0.069
	CI	29	1	0.925 ± 0.016	0.125 ± 0.065
	FP	30	3	0.919 ± 0.010	0.114 ± 0.059
	Oceania	80	30	0.986 ± 0.009	0.133 ± 0.067
Evan's Head	EA	27	1	0.946 ± 0.018	0.199 ± 0.103
Total		114	48	0.973 ± 0.002	0.133 ± 0.067



Appendix Fig. 1 Results of 100% simulations conducted in the program SPAM comparing 'mtDNA only test sample sets' with our original mtDNA only dataset under H1 for (a) western Australia, (b) eastern Australia, (c) New Caledonia, (d) Tonga, (e) Cook Islands and (f) French Polynesia. With the 'mtDNA only test sample sets' the degree of genetic differentiation (F_{ST}) among the populations was on average similar or less than observed for the real data (F_{ST} = 0.020, F_{ST} = 0.017, F_{ST} = 0.013; original mtDNA only dataset, F_{ST} = 0.018) over five simulated runs. In SPAM we simulated a 'pure stock' (N = 40) consisting of 100% of each source population. We then assessed the ability of mixed stock analysis to correctly estimate the reapportionment of each population. We expect to see 100% of the correct population. Error bars represent 95% confidence intervals.

Appendix Table 3a

Pairwise comparisons of allele frequency differences (Shannons mutual information index, *sHua*) among source populations and Antarctic feeding Area V under: (a) H1; and (b) H2 based on ten microsatellite loci. Significant p-values ($P < 0.05$ after sequential Bonferroni correction) based on statistical testing of 999 random permutations are shown in **bold**. Area V = Antarctic feeding Area V; WA = western Australia; EA = eastern Australia; NC = New Caledonia; TG = Tonga; CI = Cook Islands; FP = French Polynesia.

Population	Area V	WA	EA	NC	TG	CI
(a) H1						
WA	**0.020**					
EA	0.012	**0.023**				
NC	0.010	**0.017**	**0.013**			
TG	**0.012**	**0.019**	**0.017**	**0.010**		
CI	**0.027**	**0.023**	**0.024**	**0.013**	0.009	
FP	**0.021**	**0.026**	**0.024**	**0.019**	**0.012**	0.012

Population	Area V	WA	EA
(b) H2			
WA	**0.020**		
EA	0.012	**0.023**	
Oceania	**0.005**	**0.011**	**0.008**

Appendix Table 3b

Pairwise comparisons of haplotype frequency differences (Shannons mutual information index, *sHua*) among source populations and Antarctic feeding Area V under: (a) H1; and (b) H2 based on mitochondrial DNA control region sequences. Significant p-values ($P < 0.05$ after sequential Bonferroni correction) based on statistical testing of 999 random permutations are shown in **bold**. Area V = Antarctic feeding Area V; WA = western Australia; EA = eastern Australia; NC = New Caledonia; TG = Tonga; CI= Cook Islands; FP = French Polynesia.

Population	Area V	WA	EA	NC	TG	CI
(a) H1						
WA	**0.367**					
EA	0.165	**0.378**				
NC	**0.149**	**0.374**	**0.193**			
TG	**0.321**	**0.376**	**0.255**	**0.215**		
CI	**0.430**	**0.375**	**0.369**	**0.246**	0.089	
FP	**0.370**	**0.434**	**0.397**	**0.319**	**0.168**	**0.112**

Population	Area V	WA	EA
(b) H2			
WA	**0.367**		
EA	0.165	**0.378**	
Oceania	**0.091**	**0.254**	**0.127**

Appendix Table 4

Average pairwise F_{ST} values among source populations and Antarctic feeding Area V under H1 for the 'mtDNA only test sample sets', where genetic differentiation among the seven regions was on average similar or less than observed for the real data. Pairwise F_{ST} comparisons are shown for a total, average F_{ST} of: (a) 0.020; (b) 0.017; and (c) 0.013 across five simulation runs. Area V = Antarctic feeding Area V; WA = western Australia; EA = eastern Australia; NC = New Caledonia; TG = Tonga; CI = Cook Islands; FP = French Polynesia.

Population	Area V	WA	EA	NC	TG	CI
(a) F_{ST} = 0.020						
WA	0.019					
EA	0.011	0.016				
NC	0.011	0.014	0.012			
TG	0.018	0.015	0.013	0.010		
CI	0.035	0.028	0.027	0.026	0.012	
FP	0.038	0.032	0.032	0.029	0.018	0.007
(b) F_{ST} = 0.017						
WA	0.017					
EA	0.008	0.014				
NC	0.009	0.013	0.010			
TG	0.018	0.013	0.011	0.011		
CI	0.029	0.024	0.020	0.024	0.009	
FP	0.030	0.024	0.024	0.022	0.014	0.007
(c) F_{ST} = 0.013						
WA	0.014					
EA	0.006	0.012				
NC	0.008	0.011	0.008			
TG	0.012	0.012	0.008	0.005		
CI	0.020	0.018	0.015	0.014	0.006	
FP	0.025	0.022	0.020	0.018	0.013	0.004