Mixing rates of humpback whales of Stocks D, E and F in the Antarctic feeding grounds based on mitochondrial DNA analyses

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ABSTRACT

Genetic samples from 575 humpback whales obtained in the Antarctic during surveys of the JARPA/JARPA II and IDCR/SOWER, and from 1,057 whales from low latitude localities of the South Pacific and eastern Indian Ocean were analyzed to describe the distribution and mixing of breeding stocks in the Antarctic feeding grounds. Genetic samples from breeding grounds were obtained mainly by biopsy sampling but also from sloughed skin and beachcast whales: Western Australia (WA, n= 167, 1990-2002; n=185, 2007), Eastern Australia (Eden, Tasmania) (EA, n= 104), New Caledonia (NC, n= 243), Tonga (TG, n= 240), Cook Islands (CI, n= 56) and French Polynesia (FP, n= 62). In the Antarctic feeding grounds, samples were obtained only by biopsy sampling: Areas IIIE (n= 106), IV (n= 231), V (n= 171) and VI (n= 67). Genetic samples of both data sets were examined for approximately the first half of the mtDNA control region. Duplicated samples were excluded from the analysis. In the case of mother/calf pairs only one sequence was used. Sequences from both data sets were aligned to produce a single data set comprising 137 haplotypes. Two kinds of analyses were conducted: mixing proportion and *Fst* under two stock structure hypotheses (six stocks and four stocks). In general results were consistent with the geography. Under the six-stock hypothesis, the largest proportion in Area IIIE was of the WA stock. However this results was not consistent with the *Fst* analysis. A better interpretation of the results in Area IIIE would be possible if the analysis includes baseline samples from the eastern Indian Ocean. The largest proportion in Areas IVW and IVE was of the WA stock, and this was consistent with the *Fst* analysis. The largest proportion in Area VW was of the EA stock, and this result was consistent with the *Fst* analysis. The largest proportion in Area VE was of the NC stock. However this result was not consistent with the *Fst* analysis. The stock with the largest proportion in Area VI was the TG stock, and this result was consistent with the *Fst* analysis. None of the Antarctic Areas investigated was represented by whales of the FP and CI stocks, or just with a limited representation in Area VI (case of the CI stock). This result was consistent with the *Fst* analysis.

KEYWORDS: ANTARCTIC, FEEDING GROUNDS, BREEDING GROUNDS, GENETICS, HUMPBACK WHALE

INTRODUCTION

Humpback whales, *Megaptera novaeangliae*, are found worldwide in all major oceans. Like other Balaenopterid species, humpback whales migrate between summer feeding grounds in mid- and high latitudinal waters and winter breeding grounds in tropical or subtropical waters. Animals occur primarily in coastal and continental shelf waters. Regarding the Southern Hemisphere, Mackintosh (1965) showed that humpback whales tend to gather into five or six distinct feeding concentrations in the Antarctic during the austral summer season. These feeding concentrations were denominated as Groups I-V (with a Group IIa and IIb) corresponding roughly to IWC Management Areas I-VI. The Groups most documented are Groups IV and V.

More recently the IWC SC described the hypothetical stock structure and migratory corridors for Southern Hemisphere humpback whales based mainly on information such as Discovery tags, photo-id,

genetics and satellite track (IWC, 2005). There are seven breeding stocks named from A to G. Some of which (B, C, E and F) are further subdivided into sub-stocks. There are genetic evidences that humpback whales are genetically structured in the Southern Hemisphere and this evidence comes mainly from genetic analysis on samples in breeding grounds and migratory corridors (Baker *et al*., 1998; Olavarria *et al*., 2007; Schmitt *et al*., 2012). In the feeding grounds genetic differences have been found among Areas IIIE, IV, V and VI (Pastene et al., 2006).

In 2007 the IWC SC recommended that genetic data from breeding grounds be compared with genetic data from their associated feeding grounds in order to determine the levels of stock mixing on feeding grounds and to assist with allocation of catches. A preliminary analysis was conducted by Pastene *et al*. (2011) based on mtDNA control region sequences and samples from Western Australia (Stock D), New Caledonia (Stock E2), Tonga (Stock E3), Cook Island (Stock F1) and French Polynesia (Stock F2) (breeding grounds), and Antarctic Areas IIIE, IV, V and VI (feeding grounds). Results were discussed at the 2011 and 2012 IWC SC meetings. In 2012 the IWC SC agreed that genetic data presented in Pastene *et al*. (2011) could be used to inform relative proportions of mixing in the feeding grounds. The IWC SC noted that these data missed samples from Eastern Australia (E1) and recommended that new analyses are carried out incorporating samples from that locality (IWC, 2013).

The objective of this study therefore was to investigate the pattern of distribution and mixing of breeding stocks D, E1, E2, E3, F1 and F2 in the Antarctic feeding grounds of Areas IIIE, IV, V and VI. This objective is directly related with the recommendations from the IWC SC in 2012. Previous studies on baleen whales have used 'baseline' stock samples to estimate the mixing proportion of stocks in the feeding grounds or migratory corridors e.g. common minke whales (Pastene *et al*., 1998).

MATERIALS AND METHODS

Samples

Skin biopsy samples were obtained from free-ranging whales along the sighting surveys of the JARPA and JARPA II and IDCR/SOWER surveys in Areas IIIE, IV, V and VI, on an opportunistic basis. Biopsy samples in JARPA were collected using an air gun described in Kasamatsu *et al*. (1991) and more recently using a Paxarm system. Biopsy samples in IDCR/SOWER were collected using several methods including Paxarm system, crossbows and Larsen gun. At the laboratory all biopsy samples were checked for the possibility of re-sampling (two or more samples taken from a same individual) by comparing the genotype profiles produced by a set of six microsatellites. When mother/calf pairs were sampled, only the genetic information of the mother was used for the analysis (nine cases in Area V).

Genetic samples from breeding grounds were obtained mainly by biopsy sampling but also from sloughed skin and beachcast whales (see details in Olavarria *et al*., 2007 and Schmitt *et al*., 2012). At the laboratory the biopsy samples were checked for the possibility of re-sampling, within each of these studies (but not between the two studies).

Samples representing the Eastern Australia stock (E1) were obtained in the localities of Eden and Tasmania (Schmitt *et al*., 2012). Samples representing the Western Australia were available from two periods, 1990-2002 (Olavarria *et al*., 2007) and 2007 (Schmitt *et al*., 2012). Because the possibility of duplicate samples between the two sampling periods, these two WA samples were treated separately in the analyses.

Table 1 shows the number of samples used in the present analysis by sampling locality. A total of 1,057 samples were used from the breeding grounds and 575 from the feeding grounds. Figure 1 shows the breeding ground localities and the geographical distribution of samples in the feeding grounds of Areas IIIE, IV, V and VI.

Molecular genetic analysis

Extraction of DNA and mtDNA control region sequencing

Details of the genomic DNA extraction and sequencing of samples from breeding and feeding grounds are given in Olavarria *et al*. (2007); Schmitt *et al*. (2012), and Pastene *et al*. (2006), respectively.

MtDNA control region sequences from low latitude areas were provided to us through the Data Availability Group (DAG) under data access Protocol B (courtesy of Dr. C.S. Baker, acting Data Administrator, on behalf of the South Pacific Whale Research Consortium in case of Stock D, E2, E3, F1 and F2, and Dr. M. Double, Australian Antarctic Division, in the case of Stock D and E1).

All sequences were aligned by eye. The aligned sequences involved a common segment of 329bp of the mtDNA control region. A total of 137 unique sequences (haplotypes) were determined in the common data set of 1,632 whales.

Data analysis

Baseline stocks

During the Workshop on the Comprehensive Assessment of Southern Hemisphere humpback whales (IWC, 2006), animals from Western Australia were considered as part of a single stock (D). Regarding to Stocks E and F the Workshop listed a total of six stock structure hypotheses and assigned them different ranks of plausibility (Figure 2). In the present study we did not attempt to carry out additional analyses on stock structure on breeding grounds samples. Rather 'baseline' breeding ground samples in our analyses were defined according to some of those hypotheses.

Samples from Western Australia were considered a baseline sample for Stock D. Baseline samples for Stocks E and F were defined according to the specifications of Hypotheses 1 (medium plausibility) and 3 (high plausibility) in Figure 2, (IWC, 2006), adapted to the availability of samples:

Hypothesis 1 (medium): Eastern Australia (E1), New Caledonia (E2), Tonga (E3), Cook Islands (F1) and French Polynesia (F2) are separate stocks.

Hypothesis 3 (high): Eastern Australia+New Caledonia, Tonga+Cook Island and French Polynesia are separated stocks

In the feeding grounds, samples were grouped a) by Areas, and b) by Sectors (e.g. IIIE, IVW, IVE, VW, VE, VI).

Statistical analyses

Two kinds of analyses were conducted.

Fst analysis

The first analysis involved the estimation of *Fst* between the baseline stocks and samples of humpback whales in Areas IIIE, IV, V and VI, according to the two stock structure hypotheses and for both, Area and Sector in the Antarctic. *Fst* values were estimated using the AMOVA (Excoffier *et al*., 1992). The significance of the *Fst* values was estimated using 10,000 random permutations of the data matrix.

Mixing proportion analysis

The second analysis involved the estimation of mixing proportion of the baseline stocks in the samples of humpback whales in Areas IIIE, IV, V and VI, according to the two stock structure hypotheses and for both Area and Sector in the Antarctic. For this aim the method so-called "conditional likelihood method given the observed haplotype frequencies of the baseline populations" was used. The SE is slightly underestimated because the method did not take into account the estimation uncertainty for baseline haplotype frequencies, but the extent of underestimation should not be serious.

RESULTS

***Fst* analysis**

Results for the comparisons involving WA 'old' and WA 'new' were very similar. Results are shown only for the latter.

Analysis by Antarctic Areas

Table 2A shows the estimates of *Fst* under Hypothesis 1, by Antarctic Areas. Larger and significant *Fst* values were found in the comparison between WA and Areas IIIE, V and VI. A smaller and non-significant *Fst* value was found in the comparison WA and Area IV. EA whales were significantly

different from all Antarctic Areas although the *Fst* in the comparison with Area V was smaller. NC whales were significant different from all Antarctic Areas except Area VI. A same pattern was observed for TG whales. Whales from CI and FP were significantly different from all Antarctic Areas. The *Fst* values of the comparisons involving FP whales were particularly large.

Table 2B shows the estimates of *Fst* under Hypothesis 3, by Antarctic Area. For the case of EA+NC significant differences were found in the comparisons with Areas IIIE, IV and V (although the *Fst* value in the comparison with V was smaller). No significant difference was found in the comparison with Area VI. A similar pattern was observed in the comparisons for TG+CI. In this case the comparison with Area V showed a larger *Fst* value.

Analysis by Antarctic Sectors
Table 3A shows the estimates of *Fst* under Hypothesis 1, by Antarctic Sector. The pattern found under this hypothesis was similar to that based on Areas (Table 2A). New information is that EA whales were significantly different from Area VE, however, no significant differences were observed in the comparison with Area VW.

Table 3B shows the estimates of *Fst* under Hypothesis 3, by Antarctic Sector. The pattern found under this hypothesis was similar to that based on Areas (Table 2B).

Mixing proportion analysis
Results for the comparisons involving WA 'old' and WA 'new' were very similar. Results are shown only for the latter.

Analyses by Antarctic Areas
Table 4A shows the results of mixing proportion of stocks in the feeding grounds under Hypothesis 1, by Antarctic Area. The largest representation in Area IIIE is from WA stock (0.7112); in Area IV from WA stock (0.8351). The largest representation in Area V is from the EA (0.5011) and NC (0.4584) stocks. The largest representation in Area VI is from TG (0.4331) and NC (0.3711) stocks.

Table 4B shows the results of mixing proportion of stocks in the feeding grounds under Hypothesis 3, by Antarctic Area. In this case the largest representation in Area V is from the EA+NC stock (0.9565) and the largest representation in Area VI is from the TG+CI stock (0.7050).

Analyses by Antarctic Sectors
Tables 5A shows the results of mixing proportion of stocks in the feeding grounds under Hypothesis 1, by Antarctic Sector. The largest representation in Areas IVW is from the WA Stock (0.8450); in Area IVE from the WA stock (0.7590). The largest representation in Area VW is from the EA (0.6465) stock. The largest representation in Area VE is from the NC stock (0.8392).

Tables 5B shows the results of mixing proportion of stocks in the feeding grounds under Hypothesis 3, by Antarctic Sector. In this case the largest representation in Area VW is from the EA+NC stock (0.9412), and the same stock is the best represented in Area VE (1.0000).

DISCUSSION

The present study on southern humpback whale stock structure was one of the few cases among baleen whales in which genetic data were available from both low latitude breeding areas and high latitude feeding areas. The attainment of this comprehensive data set was possible thanks to several whale research projects being conducted on humpback whale in Australia, Oceania and the Antarctic, and access to those data was possible thanks to the IWC SC data access protocol.

Following recommendations from the IWC SC we conducted a mtDNA analysis on humpback whale samples from low and high latitudes of Breeding Stocks D, E and F to investigate distribution and mixing proportion of those stocks in the feeding grounds. The data set and analyses were similar to those used by (Pastene *et al*., 2011). This new study incorporated samples for Eastern Australia (E1), which were not available in the 2011 study.

We summarized here the main results of mixing proportion under the two hypotheses (baseline) used, noting the cases of agreement or disagreement with the *Fst* analysis.

Hypothesis 1

1) The largest proportion in Area IIIE was of the WA stock. However the *Fst* analysis indicated significant differences between WA and Area IIIE. A better interpretation of the results in Area IIIE would be possible if the analysis includes baseline samples from the eastern Indian Ocean, which were not available for this study.
2) The largest proportion in Areas IVW and IVE was of the WA stock, and this was consistent with the *Fst* analysis.
3) The largest proportion in Area VW was of the EA stock, and this result was consistent with the *Fst* analysis.
4) The largest proportion in Area VE was of the NC stock, followed by the EA stock. However this result was not consistent with the *Fst* analysis, which suggested significant differences between NC and Area VE.
5) The stock with the largest proportion in Area VI was the TG stock, and this result was consistent with the *Fst* analysis.
6) None of the Antarctic Areas was represented by whales of the FP and CI stocks, or just with a limited representation in Area VI (case of the CI stock). This result was consistent with the *Fst* analysis.

Hypothesis 3

1) The largest proportion in Areas VW and VE was of the EA+NC stock. However this result was not consistent with the *Fst* analysis. Significant differences were found in the *Fst* analysis between EA+NC and Areas VW and VE.
2) The largest proportion in Area VI was of the TG+CI stock and this result was consistent with the *Fst* analysis.

In general there was a good correlation between geographical areas and genetic signal with whales from Western Australia represented mainly in Area IV, whales from Eastern Australia represented mainly in Area VW, whales from New Caledonia represented mainly in Area VE and VI, whales from Tonga represented mainly in Area VI and whales from Cook Island and French Polynesia not represented or poorly represented in the research area investigated.

Results of the mixing proportion estimated by genetic markers in this study were consistent with previous studies on distribution and mixing based on non-genetic markers. Omura (1953) examined the distribution of humpback whale in the feeding grounds of Areas IV and V based on catch data. He suggested that two populations occur in these Areas with a boundary around 130°-142°E. He did not discard the possibility of intermingling between these two populations in the feeding ground. He also examined the pattern of distribution by month and suggested that for the month where more data were available (November-March) the boundary between these two populations changed from 120°-130°E in November to eastside of 140°E in December and to 120°-140°E in January.

Dawbin (1966) summarize the distribution and seasonal migratory movement of humpback whales from Groups IV and V, as demonstrated by mark-recapture data (Discovery-type marks). Whales from Group IV move mainly between Antarctic Area IV and Western Australia while whales from Group V move between Antarctic Area V and Eastern Australia and along the coast of New Zealand and southwest Pacific islands. Interchange of a few individuals between Groups IV and V was reported. Dawbin (1966) also reported that the boundary of Groups IV and V in the Antarctic do not correspond to the actual boundary between Areas IV and V and that some whales marked in Area VI were recovered in eastern Australia.

Previous genetic analyses in the feeding grounds have shown different pattern of distribution by sex with males being more mobile than females (Pastene *et al.*, 2006). Future analyses of distribution and mixing in the feeding grounds similar to those conducted in this study should be conducted separately for males and females.

Distribution of stocks in the feeding grounds could change yearly according to oceanographic conditions which in turn determine the distribution of krill. Analyses on a year basis (or groups of years) in the Antarctic samples would be useful. This could be possible for Areas where sample sizes are larger (e.g. Area IV). Finally the collection of additional genetic samples from Area VI and IW are recommended to further understand the pattern of distribution and mixing of Stock F (Cook Island and French Polynesia).

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Table 1. Number of samples of the humpback whale used in the present mtDNA analysis.

Breeding grounds								Feeding grounds				
WA 'old'	WA 'new'	EA	NC	TG	CI	FP	Total	IIIE	IV	V	VI	Total
167	185	104	243	240	56	62	1,057	106	231	171	67	575

Table 2A. *Fst* values between breeding stocks (under Hypothesis 1) and feeding grounds samples grouped by **Area** (significant *p* values are shown in bold).

	WA new (n=185)	EA (n=104)	NC (n=243)	TG (n=240)	CI (n=56)	FP (n=62)
IIIE (n=106)	**0.0127**	**0.0186**	**0.0213**	**0.0245**	**0.0379**	**0.0452**
IV (n= 231)	0.0001	**0.0121**	**0.0135**	**0.0128**	**0.0195**	**0.0336**
V (n=171)	**0.0175**	**0.0044**	**0.0058**	**0.0113**	**0.0279**	**0.0422**
VI (n= 67)	**0.0109**	**0.0116**	0.0003	0.0010	**0.0107**	**0.0320**

WA= Western Australia; EA= Eastern Australia; NC= New Caledonia; TG= Tonga; CI= Cook Islands; FP= French Polynesia

Table 2B. *Fst* values between breeding stocks (under Hypothesis 3) and feeding grounds samples grouped by **Area** (significant *p* values are shown in bold).

	WA new	EA+NC (n=347)	TG+CI (296)	FP
IIIE	**0.0127**	**0.0179**	**0.0245**	**0.0452**
IV	0.0001	**0.0106**	**0.0115**	**0.0336**
V	**0.0175**	**0.0028**	**0.0119**	**0.0422**
VI	**0.0109**	0.0011	0.0001	**0.0320**

WA= Western Australia; EA= Eastern Australia; NC= New Caledonia; TG= Tonga; CI= Cook Islands; FP= French Polynesia

Table 3A. *Fst* values between breeding stocks (under Hypothesis 1) and feeding grounds samples grouped by **Sector** (significant *p* values are shown in bold).

	WA new	EA	NC	TG	CI	FP
IIIE	**0.0127**	**0.0186**	**0.0213**	**0.0245**	**0.0379**	**0.0452**
IVW (n=145)	0.0015	**0.0132**	**0.0158**	**0.0156**	**0.0232**	**0.0340**
IVE (n=86)	-0.0015	**0.0112**	**0.0105**	**0.0088**	**0.0145**	**0.0346**
VW (n=110)	**0.0186**	0.0013	**0.0093**	**0.0140**	**0.0297**	**0.0413**
VE (n= 61)	**0.0222**	**0.0168**	**0.0062**	**0.0132**	**0.0324**	**0.0519**
VI	**0.0109**	**0.0116**	0.0003	0.0010	**0.0107**	**0.0320**

WA= Western Australia; EA= Eastern Australia; NC= New Caledonia; TG= Tonga; CI= Cook Islands; FP= French Polynesia

Table 3B. *Fst* values between breeding stocks (under Hypothesis 3) and feeding grounds samples grouped by **Sector** (significant *p* values are shown in bold).

	WA new	EA+NC	TG+CI	FP
IIIE	**0.0127**	**0.0179**	**0.0245**	**0.0452**
IVW	0.0015	**0.0125**	**0.0145**	**0.0340**
IVE	-0.0015	**0.0081**	**0.0073**	**0.0346**
VW	**0.0186**	**0.0044**	**0.0144**	**0.0413**
VE	**0.0222**	**0.0067**	**0.0142**	**0.0519**
VI	**0.0109**	0.0011	0.0001	**0.0320**

WA= Western Australia; EA= Eastern Australia; NC= New Caledonia; TG= Tonga; CI= Cook Islands; FP= French Polynesia

Table 4A. Mixing proportion of breeding stocks in the feeding grounds under Hypothesis 1, by **Area** (standard errors are shown in parenthesis)

Area	WA new	EA	NC	TG	CI	FP
III-E	0.7112	0.0679	0.1753	0.0000	0.0000	0.0456
	(0.0873)	(0.0840)	(0.0739)	(0.0000)	(0.0000)	(0.0424)
IV	0.8351	0.0000	0.0540	0.1108	0.0000	0.0000
	(0.0377)	(0.0000)	(0.0326)	(0.0387)	(0.0002)	(0.0000)
V	0.0294	0.5011	0.4584	0.0000	0.0112	0.0000
	(0.0202)	(0.0679)	(0.0681)	(0.0000)	(0.0156)	(0.0000)
VI	0.0000	0.0000	0.3711	0.4331	0.1958	0.0000
	(0.0000)	(0.0000)	(0.0129)	(0.0154)	(0.0968)	(0.0000)

Table 4B. Mixing proportion of breeding stocks in the feeding grounds under Hypothesis 3, by **Area** (standard errors are shown in parenthesis)

Area	WA new	EA+NC	TG+CI	FP
III-E	0.7084	0.2457	0.0000	0.0459
	(0.0784)	(0.0822)	(0.0000)	(0.0421)
IV	0.8202	0.0638	0.1159	0.0000
	(0.0404)	(0.0373)	(0.0415)	(0.0000)
V	0.0342	0.9565	0.0092	0.0000
	(0.0231)	(0.0253)	(0.0210)	(0.0000)
VI	0.0000	0.2949	0.7050	0.0000
	(0.0000)	(0.1193)	(0.1193)	(0.0000)

Table 5A. Mixing proportion of breeding stocks in the feeding grounds under Hypothesis 1, by **Sector** (standard errors are shown in parenthesis)

Area	WA new	EA	NC	TG	CI	FP
III-E	0.7112	0.0679	0.1753	0.0000	0.0000	0.0456
	(0.0873)	(0.0840)	(0.0739)	(0.0000)	(0.0000)	(0.0424)
IV-W	0.8450	0.0000	0.0659	0.0891	0.0000	0.0000
	(0.0434)	(0.0000)	(0.0410)	(0.0426)	(0.0000)	(0.0000)
IV-E	0.7590	0.0117	0.0203	0.1303	0.0786	0.0000
	(0.0920)	(0.0533)	(0.0777)	(0.0120)	(0.0881)	(0.0000)
V-W	0.0401	0.6465	0.2996	0.0000	0.0137	0.0000
	(0.0278)	(0.0744)	(0.0737)	(0.0000)	(0.0199)	(0.0000)
V-E	0.0000	0.1608	0.8392	0.0000	0.0000	0.0000
	(0.0202)	(0.0105)	(0.0105)	(0.0000)	(0.0156)	(0.0000)
VI	0.0000	0.0000	0.3711	0.4331	0.1958	0.0000
	(0.0000)	(0.0000)	(0.0129)	(0.0154)	(0.0968)	(0.0000)

Table 5B. Mixing proportion of breeding stocks in the feeding grounds under Hypothesis 3, by **Sector** (standard errors are shown in parenthesis)

Area	WA new	EA+NC	TG+CI	FP
III-E	0.7084	0.2457	0.0000	0.0459
	(0.0784)	(0.0822)	(0.0000)	(0.0421)
IV-W	0.8344	0.0729	0.0926	0.0000
	(0.0460)	(0.0449)	(0.0443)	(0.0000)
IV-E	0.7807	0.0088	0.2104	0.0000
	(0.0800)	(0.0868)	(0.1039)	(0.0000)
V-W	0.0493	0.9412	0.0095	0.0000
	(0.0338)	(0.0336)	(0.0272)	(0.0000)
V-E	0.0000	1.0000	0.0000	0.0000
	(0.0000)	(0.0000)	(0.0000)	(0.0000)
VI	0.0000	0.2949	0.7050	0.0000
	(0.0000)	(0.1193)	(0.1193)	(0.0000)

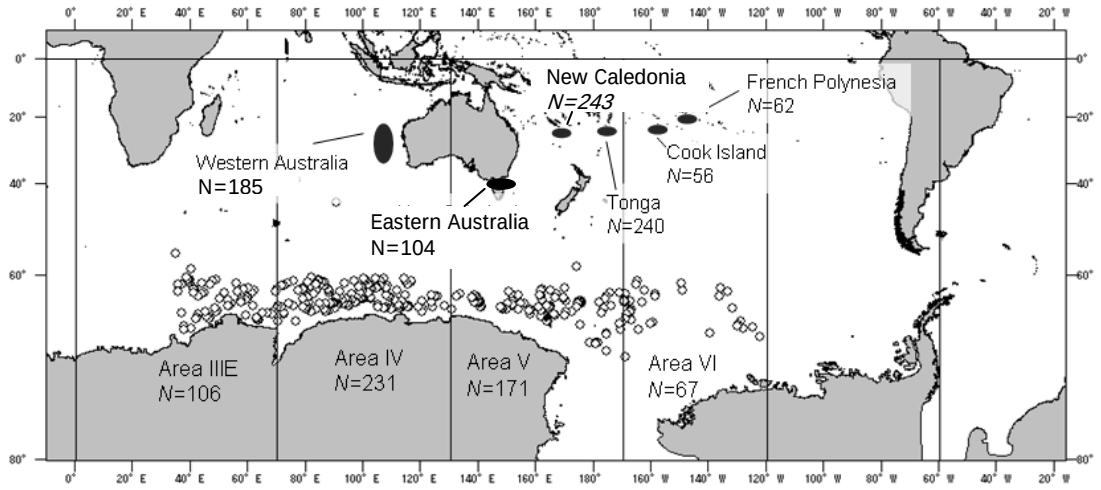

Figure 1: Geographical distribution of humpback whale samples from breeding and feeding grounds analyzed in this study.

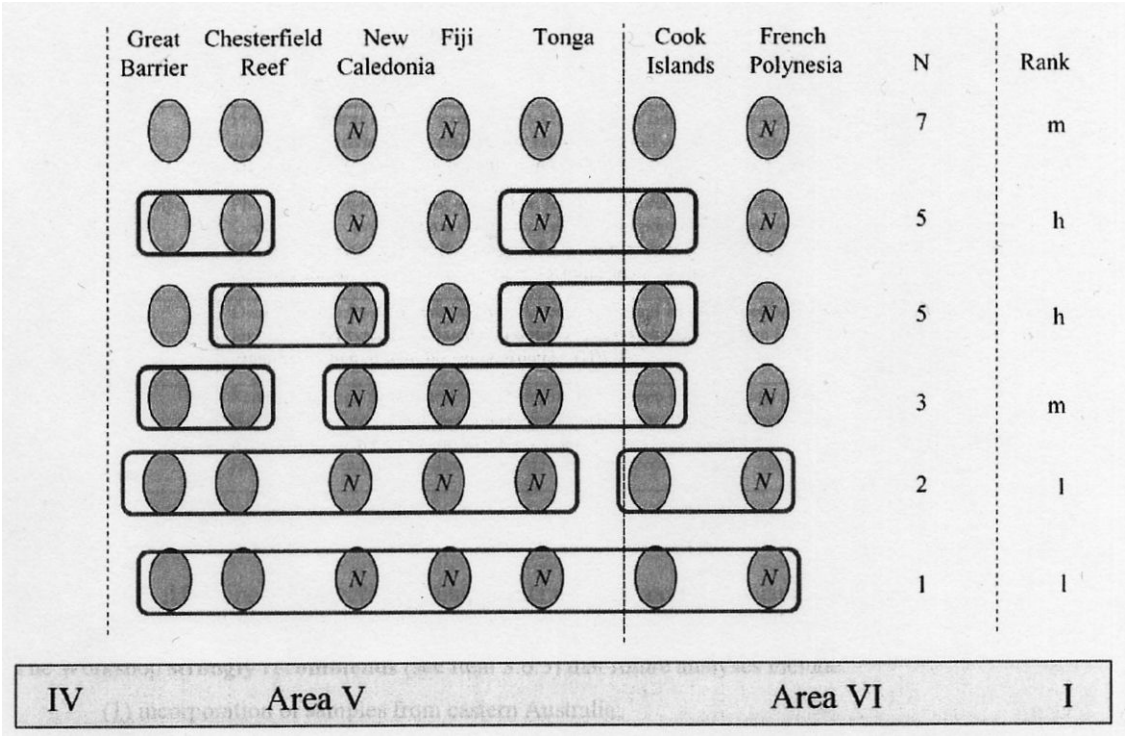

Figure 2. Schematic diagram showing possible models for stock structure in the South Pacific. N= number of breeding stocks and Rank, l= low, m= medium and h= high (taken from IWC, 2006). Samples from Eastern Australia (Eden and Tasmania), New Caledonia, Tonga, Cook Islands and French Polynesia (in addition to Western Australia), were available for the present mtDNA study.