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Demographic and genetic isolation of New Caledonia (E2) and Tonga (E3) breeding stocks

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ABSTRACT

Historically, humpback whales migrating through Eastern Australia and New Zealand, and breeding off northeastern Australia, New Caledonia, Fiji and Tonga were assigned for management purposes to the Antarctic Area V. Under the current Comprehensive Assessment of Southern Hemisphere humpback whales these regions are considered as the Breeding Stock E. In this report we investigate the relationship between the New Caledonia and Tonga breeding grounds, based on the seasonal return and interchange of individual whales (photo-ID and genetic-ID) and population genetic differentiation (mitochondrial DNA control region sequences and nuclear DNA microsatellites). We found significantly higher recapture probabilities within each breeding ground compared to the recapture probability between them, using both photo- and genetic-ID, significant differences in the F_{ST} and Φ-$_{ST}$ for mitochondrial and nuclear markers. These analyses strongly suggest differentiation among the Breeding Stock E, supporting the proposed sub-stock division for New Caledonia (E2) and Tonga (E3).

INTRODUCTION

Historically, it was considered that the humpback whale aggregation feeding between the 130°E and 170°W form a single feeding stock; the Antarctic Area V stock (Mackintosh, 1942; Donovan, 1991). These whales corresponded to those migrating along the east coast of Australia, New Zealand and Norkfolk Island, and wintering off Eastern Australia, the Coral Sea , Fiji and Tonga (Kellogg, 1929; Townsend, 1935; Dawbin and Falla, 1949; Dawbin, 1956; Slijper, 1962; Chittleborough, 1965; Dawbin, 1966). Under the ongoing Comprehensive Assessment of Southern Hemisphere humpback whales, these breeding grounds correspond to what is called Breeding Stock E, further divisions of which are still under discussion (IWC, 2005).

Although animals from the geographically isolated wintering grounds (Eastern Australia, New Caledonia, Fiji and Tonga) were assumed to represent a single breeding unit or stock, the degree of demographic and reproductive connection/isolation between these regions was poorly understood. *Discovery* marks practically failed to reveal movements among breeding grounds even though marking was conducted in all of them (Dawbin, 1964). Some evidence of interchange was provided later by photo-identification (photo-ID) of individual whales, when some individuals were identified both in Eastern Australia and New Caledonia (Garrigue *et al.*, 2000) and in New Caledonia and Tonga (Garrigue *et al.*, 2002). Acoustic analysis of humpback whale songs in this region also suggested some migratory exchange among widely separate wintering regions (Helweg *et al.*, 1998).

The recommendation of the International Whaling Commission (IWC) were to use molecular techniques to help defining stock and to provide information for management (Donovan, 1991). Here we review the available information and present new information at the individual and population levels, to establish the degree of connection between the New Caledonia and Tonga breeding grounds (Breeding Stock E). At an individual level, we compared the rate of recapture for both photo-ID and genetic-ID, within and between breeding grounds. At a population level, we compared the genetic diversity using mitochondrial and nuclear DNA markers.

MATERIAL AND METHODS

Data collection and sampling at sea

Surveys of humpback whales have been conducted in Tonga and New Caledonia (Garrigue *et al.*, 2001) using small boats since 1991 and 1995, respectively. The analyses performed here will include only information obtained until the breeding season 2002.

Individual humpback whales were identified from photographs of the unique markings on the ventral surface of their tail flukes (photo-ID, Katona *et al.*, 1979). Small samples of skin tissue were collected using a biopsy darting and specially adapted bolt and dart, propelled by a crossbow (Lambertsen, 1987) or a biopsy rifle (Krützen *et al.*, 2002). Sloughed skin was also collected when following surface-active groups (Clapham *et al.*,

1993). When possible, skin tissue collection was combined with photo-identification (Garrigue & Greaves, 1999). All samples were preserved in 70 % ethanol and frozen until DNA extraction.

Molecular laboratory analysis

DNA extraction followed the protocol described by Sambrook *et al.* (1989) as modified by Baker *et al.* (1991). Mitochondrial DNA amplification and sequencing was as in Olavarría *et al.* (in review), which involved the amplification of 800 bp of the 5' end of the control region. A consensus region of 470 bp was defined for comparison purposes. Nuclear DNA micosatellite follows Garrigue *et al.* (2004). In summary a set of ten fluorescently labelled microsatellites loci was amplified for most samples, including three tetranucleotides (GATA28, GATA53, GATA417, Palsbøll *et al.*, 1997), one trinucleotide (TAA 31, Palsbøll *et al.*, 1997) and six dinucleotides (464/465, Schlötterer *et al.*, 1991; EV1, EV21, EV37, EV94, EV104, Valsecchi and Amos, 1996). However, GATA 53 was omitted from further analyses as it gave uncertain resolution of allele sizes in some samples.

Molecular identification of the sex was carried out using the *Sry* system, using *ZFX* as positive control (Gilson *et al.*, 1998).

Individual identification within and between breeding grounds

Whale photo-ID data were assessed within each breeding ground by direct comparison of fluke photographs. A catalogue of individual whales was compiled by removing all but one sightings in different years. Matches within region were recorded but re-captures within years were not considered for the resighting history. A comparison between the New Caledonia and Tonga catalogues was performed by three independent researchers.

Genetic-ID of individual whales was conducted within each breeding ground by comparison of microsatellite genoypes using GenAleEx (Peakall and Smouse, 2005). The probability of identity was calculated following Paetkau and Strobeck (1994). This represents the probability that two unrelated animals share the same genotype by chance alone. Additionally, the probability of identity for each genotype was calculated using GenAlEx. All matches identified using microsatellite genotypes were further corroborated utilising mtDNA sequence and the molecular sex identification, when available. All the genetic-ID whales, but one, were removed and the matches in different years within a region were recorded. Re-captures within years were not considered for the resighting history. Then, a comparison of genetic-ID whales was performed between the two regions using GenAlEx, combining both data sets.

Resight indices

To establish the degree of connection between the two regions we calculated a within-region return index and a between-region interchange index (Baker *et al.*, 1986; Calambokidis *et al.*, 2001; Garrigue *et al.*, 2002), using unique individuals based independently on genetic-ID and photo-ID.

The return index of within-region annual resights, was calculated as:

$$R = M / (A*B) *1000,$$

with A: number of whales genetic-ID/or photo-ID in all the years before 2002 (the last year considered in this analysis),

B: number of whales genetic-ID /or photo-ID in 2002,

M: number of whales genetic-ID /or photo-ID in the previous years and resighted in 2002.

An interchange index of between-region resights was calculated as:

$$R = M / (A1*B2) *1000,$$

with A1: number of whales genetic-ID /or photo-ID for New Caledonia,

B2: number of whales genetic-ID /or photo-ID in Tonga,

M: number of whales genetic-ID /or photo-ID in both regions.

The indices were considered to be zero when there were no whales sighted within or between regions (i.e., when M = 0).

Population comparison

MtDNA sequence data was compared between New Caledonia and Tonga, based on Olavarría *et al.*, (in review). Further, genetic-ID was used in the Tonga data set to identify and discard all those indivuals re-sampled within and between different years. Genetic diversity was re-estimated at the haplotype level and nucleotide level using Arlequin (version 2.0, Schneider *et al.*, 2000). The differentiation between breeding grounds was quantified using an Analysis of Molecular Variance (Excoffier *et al.*, 1992) as implemented in Arlequin. This was calculated for nucleotide differentiation (Φ_{ST}) and haplotype frequency differences (F_{ST}). The significance of the observed Φ_{ST} and F_{ST} values was tested using 5,000 random permutations of the data matrix.

Genetic differentiation based on microsatellite genotype data between breeding grounds was tested using Fisher's exact test (Raymond and Rousset 1995) as implemented in the program of Genepop, web version 3.4 (http://wbiomed.curtin.edu.au/genepop/). This procedure tests each locus for significant differences in allele frequencies between the populations, using a Markov chain to generate an exact probability distribution under the null hypothesis that there is no differentiation between populations. The estimation input parameters were 10,000 dememorization steps, 100 batches, and 5,000 iterations per batch. Population differentiation was also quantified using an Analysis of Molecular Variance AMOVA (Excoffier *et al*., 1992) as implemented in the program Arlequin (version 2.0, Schneider *et al.*, 2000).

RESULTS

Data collection

In the winter field seasons between 1995 and 2002, data were collected in New Caledonia on 375 days totaling 2577 hrs of observations at sea. During this time 191 unique individuals were photographically identified and 362 tissue samples were collected.

Individual identification: photo-ID and genetic-ID

In New Caledonia 232 unique individuals were identified using photo-ID between 1995 and 2002. In Tonga 401 unique individuals were photo-ID between 1991 and 2002.

After a preliminary review of the microsatellite genotypes, we arbitrarily decided that samples with information in four or fewer loci would be discarded of further analysis. This was supported by using the New Caledonia data set, where a high percentage of resighted individuals have been photo-ID and genetic-ID (Garrigue *et al.*, 2004). No pair of photo-ID humpback whales matched genetically when considering five or more loci. When considered only genetic-ID, false matches started to occurr only when four or less loci were compared.

Most of the New Caledonia samples included information in nine loci. For Tonga most samples included seven loci (Figure 2). The overall probability of identity for each population using nine loci was 4.3×10^{-10} for Tonga and 2.3×10^{-10} for New Caledonia (Table 1).

From 300 samples (which contained data in five or more loci) collected in Tonga a total of 255 unique genotypes was identified. Seventy-two of those were females and 179 were males. For New Caledonia, comparisons of genotypes showed that the 362 samples correspond to 249 unique individuals of which 99 were females and 149 were males. All of the matches within region shared mtDNA haplotype and sex, when available. A significant bias towards males in the number of individuals genotyped was found in both regions (60% and 70% respectively for Tonga and New Caledonia).

Comparison between regions and resight indices

The comparison of 633 (New Caledonia = 232, Tonga = 401) photo-ID whales provided only 11 matches between the two regions (Table 2). The interchange index in between the two regions was 0.12 (Table 2).

In Tonga 47 individuals (12 % of the photo-ID identified whales) were recaptured by photo-ID between 1991 and 2002. In New Caledonia 55 individuals (24 % of the photo-ID whales) were recaptured between 1995 and 2002. The number of photo-ID recaptures between years ranged between 0 and 14 for Tonga and between seven and 19 for New Caledonia. The return index of within-region annual resights using photo-ID was 0.89 and 2.72 for Tonga and New Caledonia, respectively (Table 3).

When comparing the overall 504 genetic-ID individuals (New Caledonia = 249, Tonga = 255) only two matches were found between the two regions (Table 2). Coded samples Mno96Tg07 and NC00-52, matched at all of the nine loci available (Table 4). The two other samples, coded Mno02Tg47 and NC00-20, matched at all loci, but sample Mno02Tg47 did not have information for the EV 104 locus. Additionally, these two pairs matched sex and mtDNA haplotype information. The interchange index in between the two regions was 0.03 (Table 2).

Eight genetic-ID whales were re-captured between years in Tonga (3 % of the individual genotyped whales). And 41 in New Caledonia (17 % of the individuals genotyped whales). The number of whales recaptured between year using genetic-ID ranged from 0 to 5 in Tonga and from 1 to 27 whales in New Caledonia. The return index of within-region annual resights using genetic-ID was 0.34 and 1.43 for Tonga and New Caledonia, respectively (Table 3).

The two whales observed in both regions by genetic-ID were later confirmed when a cross reference revealed that they were photo-ID in both regions.

Genetic information was available for seven of the 11 photo-ID whales observed in both regions. These seven were identified as male. Ten of 11 were classified as adults and one as sub-adult (of unknown sex). For the 21 encounters involving 11 migrants, social group information was available for 19. The adult whales have been observed in reproductive groups (eight cases one of them including a cow-calf pair), in pairs with other adult (five cases, the other adult was identified as a female when sex information available), escorting a female with calf (two cases) and as a single individual (two cases). The sub-adult was observed alone in both regions.

Population comparison

A total of 250, for New Caledonia, and 310, for Tonga, mtDNA control region sequences were compared, after the identification and discard of re-sampled individuals, based on the data set used in (Olavarría *et al.*, In review). The haplotype diversity (h) was 0.974 ± 0.003 for New Caledonia and 0.962 ± 0.004 for Tonga. Nucleotide diversity (π) was 2.12 ± 1.08 % and 2.01 ± 1.02 % for New Caledonia and Tonga, respectively.

The AMOVA for mtDNA information showed significant differences between the two breeding grounds at the haplotype and nucleotide level (F_{ST} = 0.009, p < 0.001; Φ_{ST} = 0.004, p = 0.045 ± 0.002), as in the previous analysis (Olavarría *et al.*, In review).

The exact test for population differentiation using microsatellite data indicated a highly significant overall differentiation between the two populations (χ^2 = infinity, d.f. = 18). This was the result of differentiation at five loci (GATA417, EV1, TAA31, EV94, EV104; Table 5). The AMOVA using microsatellite genotype data showed significant difference between the two breeding grounds (average for nine loci F_{ST} = 0.003, p < 0.001).

DISCUSSION

Different methods have proved to be useful in describing the movements of individuals among wintering or feeding areas. Photographic identification of individual humpback whales showed that some individuals move between different breeding grounds in the North and South Pacific in different years or even in the same year (Darling and Jurasz, 1983; Darling and McSweeney, 1985; Baker et al., 1986; Darling and Cerchio 1993; Calambokidis et al., 2001; Garrigue et al., 2002) covering great distances such as 6000 km between Japan and Hawaii (Darling and Cerchio, 1993). Infrequent cases of humpback whales switching between breeding grounds (e.g., from Western Australia to eastern Australia) were documented by *Discovery* tagging and recovery (Chittleborough, 1960 and 1965). More recently, a genetic-ID showed movement between Gabon and Madagascar (Pomilla and Rosenbaum, 2005).

A review of sighting records showed that the 11 whales observed in both New Caledonia and Tonga were sighted only once each in each of the two regions. Seven of 11 individuals were males observed in competitive groups or as escorts to mothers and calves. This suggests that interchange between regions could involve genetic mixing and play a role in the reproductive strategies of males, although the eveidence of 'gametic recapture' for New Caledonia suggests that such strategy is less sucessful than 'resident' males (Garrigue et al., 2004). Further, exchange is not limited to adult males as one mature female was also observed in both New Caledonia and Tonga previously (Garrigue *et al.*, 2002) and a number of females have been documented moving between other regions of Oceania (SPWRC, Garrigue et al., 2006).

The use of photo-ID or genetic-ID data to calculate return and interchanges indices gave similar results. Indices are greater for photo-ID data than for genetic-ID data. The return indices were greater than the interchange indices using both photo-ID recaptures or genetic-ID. The return indices reflect the size of the population and the degree of fidelity (Calambokidis et al., 2001). The return index, which is higher for New Caledonia than for Tonga, reflects the small size of the New Caledonian population estimated to be about 450 individuals compared to Tonga where the population is estimated to be more than 2000 individuals (SPWRC, Baker *et al*.,2006). It also highlights the greater rate of site fidelity rate for the New Caledonian population both by year and overall the sampling period when compare to the Tongan population.

Interchange exists between Tonga and New Caledonia but this seem to occur infrequently, as shown by the interchange index which is one or two orders of magnitude lower than the return indices within the same regions.

This strongly supports the fact that there is a higher probability of resighting an individual within a breeding ground than in another breeding ground. This is consistent with our results of significant genetic differences among breedign areas (when using mtDNA and nuclear DNA markers) at the population level.

Demographic closure is suggested by the comparison of photo-ID and genetic-ID datasets from Tonga and New Caledonia. It is clear that treating Breeding Stock E as a single unit is inappropriate given the demographic and genetic evidence at the individual and population levels. The results reported here support the proposed sub-stock division discussed in the context of the IWC Comprehensive Assessment of Southern Hemisphere humpback whales (IWC, 2005); New Caledonia as breeding sub-stock E2 and Tonga as breeding sub-stock E3.

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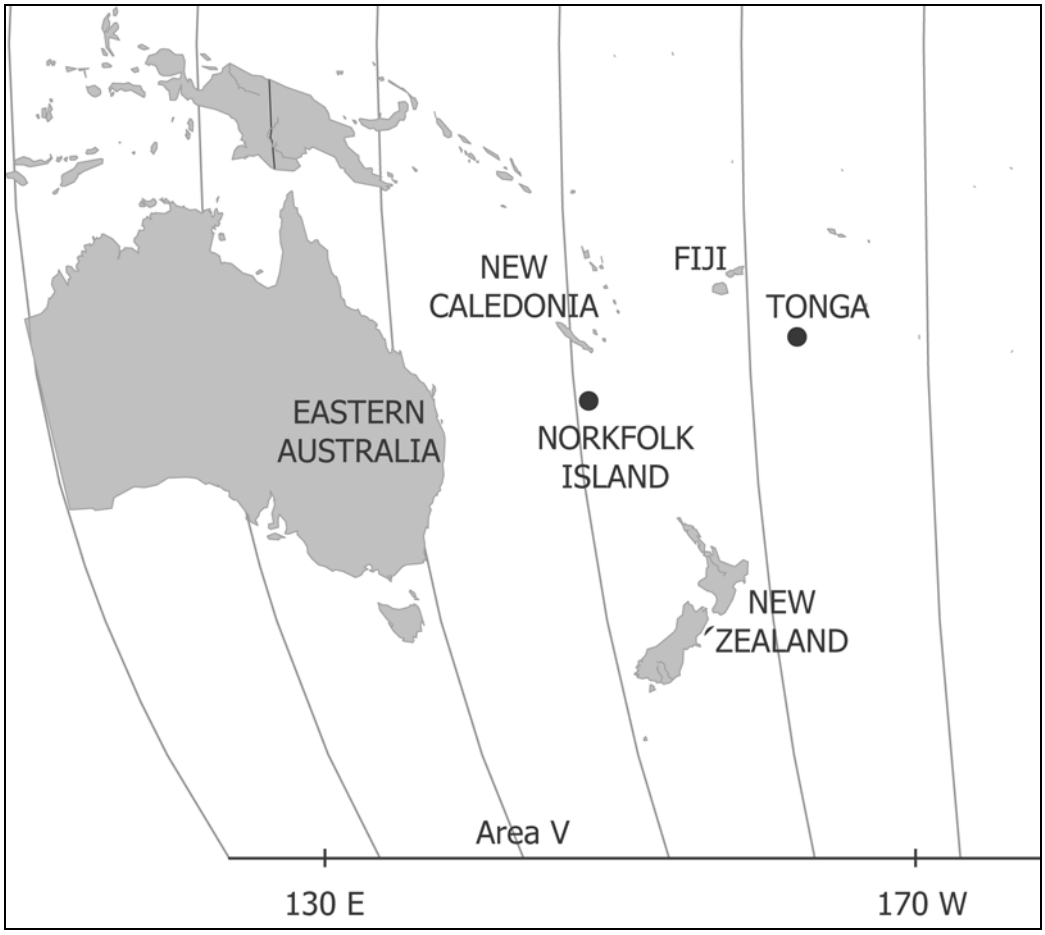

Figure 1. Map indicating the migratory corridors (Eastern Australia, Norkfolk Island and New Zealand) and breeding grounds (Eastern Australia, New Caledonia, Tonga, Vanuatu and Fiji) identified for the Breeding Stock E (IWC, 2005). It also indicates the border of the Antarctic Area V.

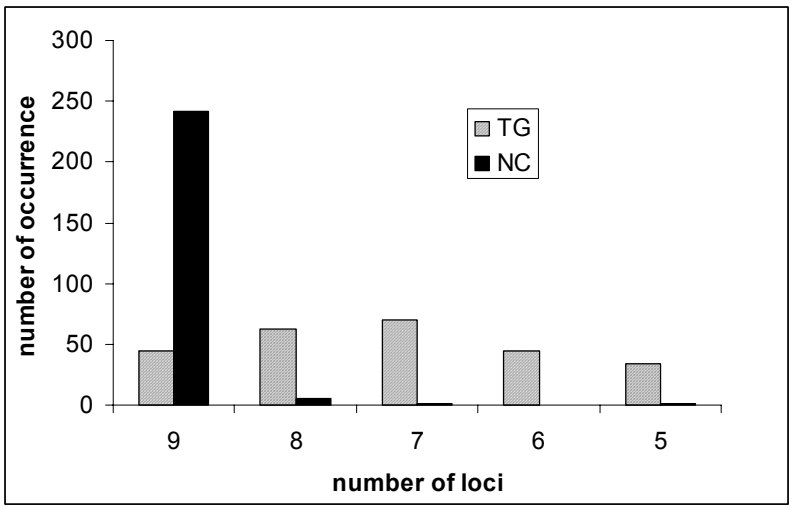

Figure 2. The number of loci available for the individual whales in the datasets of Tonga and New Caledonia.

341 Table 1. Probability of identity in the populations of Tonga and New Caledonia by locus, sample size (n =
342 individual whales) and probability of identity (PI) of microsatellite loci.

	Tonga		New Caledonia	
Loci	n	PI	n	PI
GATA28	175	0.25	248	0.216
GATA417	248	0.018	249	0.019
TAA31	95	0.029	247	0.03
464/465	202	0.204	249	0.188
EV1	251	0.293	248	0.284
EV21	254	0.131	247	0.15
EV37	249	0.012	249	0.01
EV94	248	0.065	246	0.059
EV104	102	0.558	247	0.384
Overall		4.3×10^{-10}		2.3×10^{-10}

343

344 Table 2. Interchange index (between region) for New Caledonia and Tonga using photo-ID and genetic-ID.

	Photo-ID	Genetic-ID
Number of unique photo-ID in New Caledonia in 2002	232	249
Number of unique Photo-ID in Tonga in 2002	401	255
Total number of resights by Photo-ID between the two regions	11	2
Interchange index	0.12	0.03

345

346 Table 3. Return index (within region) for New Caledonia and Tonga using photo-ID and genetic-ID resights.

	TG	NC	TG	NC
Using:	Photo-ID		Genetic-ID	
Number of unique individuals between 1995-2001	357	225	177	227
Number of unique individuals in 2002	41	18	83	34
Number of resights within region	13	11	5	11
Return index within region	0.89	2.72	0.34	1.43

347

348 Table 4. Individuals whale genotype matching between Tonga and New Caledonia.

Samples	Number of loci available	Number of loci matched	Haplotype	Sex	Probability of identity
Mno96Tg07	9	9	SP114	M	9.18×10^{-10}
NC00-52	9	9	SP114	M	1.30×10^{-10}
Mno02Tg47	8	8	SP88	M	6.09×10^{-10}
NC00-20	9	8	SP88	M	7.02×10^{-10}

349

350

351 Table 5. Genetic differentiation based on microsatellite genotype data between breeding grounds using Fisher's
352 exact test.

Locus	P-value	S.E.
464/465	0.13941	0.00596
GATA417	0.00003	0.00002
EV1	0.00769	0.00120
EV37	0.05832	0.00531
TAA31	0.03733	0.00289
EV21	0.28024	0.00993
EV94	0.00000	0.00000
GATA28	0.17189	0.00540
EV104	0.01962	0.00131

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