# Eastern Australia humpback whale genetic diversity and their relationship with Breeding Stocks D, E, F and G

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ABSTRACT

Humpback whales off the east coast of Australia were heavily exploited by commercial whaling operations. It has been documented in recent years that this population is growing; however, it is considered that still is below pre-exploitations levels. Here we investigate the genetic diversity of Eastern Australian humpback whales, comparing mitochondrial DNA control region sequence data with that of breeding grounds across the South Pacific (New Caledonia, Tonga, Cook Islands, French Polynesia and Colombia) and eastern Indian (Western Australia) oceans. We compared 156 sequences, representing individual whales sampled off Byron Bay (northbound migration 2002-2003) and Ballina (southbound migration 2003), with 1,112 samples from breeding grounds, comparing a 470 bp fragment of the mtDNA control region consensus sequence. The analysis revealed 42 haplotypes in Eastern Australia, with five unique haplotypes. The Eastern Australian humpback whale haplotype diversity (h) was 0.962 ± 0.005, and the nucleotide diversity ($\pi$) was 2.32 ± 1.18%. These levels were similar to those from the compared breeding grounds, but were significantly different only at haplotype level with New Caledonia, Cook Island, French Polynesia and Colombia breeding grounds. We found significant differences at haplotype and nucleotide levels with all the breeding grounds when a pair-wise AMOVA was performed, except with Tonga at nucleotide level. The genetic differentiation observed here and our previous analyses presented to the SC/IWC support the proposed stock sub-division of the breeding stock E into three sub-stocks, E1 (Eastern Australia), E2 (New Caledonia) and E3 (Tonga).

## INTRODUCTION

The humpback whale (*Megaptera novaeangliae* Borowski, 1781) is distributed worldwide, with populations in all the major oceans (Kellogg, 1929; Clapham and Mead, 1999). During the last two centuries humpback whales were hunted intensively, especially in the Southern Hemisphere, where it was estimated that the population was reduced perhaps only to a few percent of its pre-exploitation abundance (Chapman, 1974). Based on catch records corrected for illegal Soviet whaling, a total of more than 200,000 humpback whales were killed from 1904 to 2000 (Baker and Clapham, 2002; Clapham and Baker, 2002).

The Eastern Australian humpback whale stock, as a result of commercial whaling, declined to levels estimated to be a few hundred individuals by 1962 (Paterson *et al.*, 1994). However, this stock has shown signs of recovery after more than 40 years since the end of commercial exploitation (Paterson and Paterson, 1984;1989; Paterson *et al.*, 1994), with a current estimated abundance of about 7,000 individuals (Noad *et al.*, 2006; Paton *et al.*, 2006).

Historically, humpback whales migrating through and/or wintering at Eastern Australia, New Zealand and the western South Pacific islands were considered to form the Group V stock (Mackintosh, 1965). It seemed that these whales spent the summer feeding in Antarctic waters east and west of the Balleny Islands (Mackintosh, 1965), but for management purposes they were considered to form the Antarctic Area V stock, which was limited by 130°E and 170°W, in the Southern Ocean (Donovan, 1991).

Recently, under the Comprehensive Assessment of Southern Hemisphere humpback whales, the stock structure of this whale species has been reviewed and this breeding stock (BS) has been re-named E (IWC, 1998). Sub-divisions within this stock have been proposed, but they have not been completely agreed. These correspond to the sub-stock E1 (Eastern Australia), E2 (New Caledonia) and E3 (Tonga) (IWC, 2005). The putative subdivisions between E2 and E3 have been based on restricted movements of individuals and population level

analysis of molecular variance (Garrigue *et al.*, 2002; Olavarría *et al.*, 2003; Garrigue *et al.*, 2006). The relationship of the Eastern Australia component, however, has been only partially addressed using photo-ID and genetic analysis (Baker *et al.*, 1998; Garrigue *et al.*, 2000).

Here we extend our previous analyses by comparing Eastern Australian mtDNA control region sequence data with information from the South Pacific humpback whale breeding grounds (New Caledonia, Tonga, Cook Islands, French Polynesia and Colombia), including additionally the breeding ground off Western Australia representing the eastern Indian Ocean.

## MATERIALS AND METHODS

### Study area and sampling methods

Slough skin samples (Amos *et al.*, 1992) were collected from humpback whales off Byron Bay and Ballina, Eastern Australia (Figure 1). All samples were stored in the field in 70% ethanol at room temperature and transferred in the laboratory to -70°C for long-term storage.

### Laboratory analyses

Genomic DNA was extracted following Elphinstone *et al.* (2003). Before mtDNA sequencing was undertaken, samples were genotyped for 13 microsatellite loci for individual identification (M Anderson, unpublished data).

Symmetrical amplification of the mtDNA control region (proximal to the tPro RNA gene) of humpback whale individuals, was performed via the Polymerase Chain Reaction (PCR, Saiki *et al.*, 1988), following standard protocols (Palumbi, 1996). An 800 base-pair (bp) portion of the mtDNA control region was amplified using the primers, light-strand tPro-whale Dlp-1.5 (Baker *et al.*, 1998) and heavy strand Dlp-8G (Pichler *et al.*, 2001). This region extended across the two shorter and partially overlapping fragments used in past analyses, referred to as the North Atlantic and the Worldwide consensus regions (Baker and Medrano-González, 2002). Amplification and subsequent cycle sequencing were improved by the addition of an M13 forward sequence to the 5' end of the Dlp-1.5 primer. Temperature profiles consisted of a preliminary denaturation period of 1 minute at 92ºC, followed by 35 cycles of denaturation for 10 seconds at 92ºC, primer annealing for 30 seconds at 55ºC, and polymerase extension for 1 minute at 75ºC. A final extension period for 5 minutes at 75ºC was included.

Primers and nucleotides were cleaned from PCR products, using a Qiaquick PCR purification kit as per the manufacturers instructions (QIAGEN), and sequenced on an ABI3730 DNA sequencer (Applied Biosystem), using the primer M13Dlp1.5. Sequences were aligned and edited using FINCHTV (version 1.3.1, Geospiza) and SEQUENCHER™ (version 4.1.2, Genes Codes Co.). Chromatographs were checked visually for possible sequencing errors. All variable positions were checked by comparison to other chromatographs using SEQUENCHER™. Comparisons of sequences to haplotypes were performed using ARLEQUIN (version 2.0, Schneider *et al.*, 2000).

### Data analyses

Genetic diversity was estimated at haplotype level (without regard to the genetic distance or number of nucleotide substitutions) and nucleotide level (using unadjusted pair-wise differences between sequences) using the program ARLEQUIN (version 2.0, Schneider *et al.*, 2000).

Eastern Australian haplotypes were compared with a extensive data set of mitochondrial DNA control region sequences (n = 1,112) from five South Pacific humpback whale breeding grounds, and one breeding ground of the eastern Indian Ocean (Olavarría *et al.*, In review). The differentiation with the breeding grounds was quantified using an Analysis of Molecular Variance (Excoffier *et al.*, 1992) as implemented in ARLEQUIN. This was calculated for nucleotide differentiation ($\Phi_{ST}$) and haplotype frequency differences ($F_{ST}$). The significance of the observed $\Phi_{ST}$ and $F_{ST}$ values was tested using 20,000 random permutations of the data matrix.

## RESULTS

### Sampling

Overall, over 1,500 skin samples have been collected in Eastern Australia by Southern Cross University Whale Research Centre over six years (2000-2005). To date at least 800 individual whales have been identified by microsatellite genotyping (M. Anderson, unpublished data). From that, 156 individual whale samples have been sequenced, 131 from Byron Bay (collected during the northbound migration of 2002 and 2003) and 25 from Ballina (collected during the southbound migration of 2003).

**Genetic diversity**

A 470 bp consensus sequence of the mtDNA control region was defined for analysis. This fragment corresponds with the segment analyzed in previous analyses of Southern Hemisphere humpback whales (Olavarría *et al.*, In review). A comparison of the 156 mt DNA control region sequences from Eastern Australian humpback whales revealed 42 haplotypes (Table 1). The haplotype diversity (h) was 0.962 ± 0.005 and the nucleotide diversity ($\pi$) was 2.32 ± 1.18%. These genetic diversities were similar to those previously reported from breeding grounds of the South Pacific and Indian oceans (Olavarría *et al.*, In review), but high in comparison to some populations in other oceans (Baker and Medrano-González, 2002). A modified t-test (Nei, 1987) revealed a significant difference only at haplotype diversity with New Caledonia, Cook Island, French Polynesia and Colombia breeding grounds.

**Differentiation with breeding grounds**

The pair-wise AMOVA between Eastern Australia and each breeding ground from D, E, F and G stocks showed significant differences at haplotype and nucleotide levels with all stocks, except with Tonga at nucleotide level ($p<0.05$; Table 2).

## DISCUSSION

Several analyses have suggested segregation among regions of the Southern Hemisphere humpback whale stock E. *Discovery* marking provided the first evidence (Dawbin, 1959); however, recovery of a small number of tags in different region to where they were originally fired into the whales, suggested a limited degree of connectivity among these close areas (Chittleborough, 1959; Dawbin, 1964). Photo-ID comparisons confirmed this scenario, with a large number of whales being recaptured within areas they were observed, but a few interchanges were recorded (Garrigue *et al.*, 2000; Garrigue *et al.*, 2002). Our analysis here shows a differentiation to a population level of stock E, which together with available demographic information, support the stock sub-division into three sub-stocks: E1 (Eastern Australia), E2 (New Caledonia) and E3 (Tonga) (IWC, 2005).

Our analysis supports the reproductive isolation of the two Australian humpback whale stocks (D and E1), originally suggested to be a result of the separation by the two stocks by the Australian continent during the breeding season and revealed by *Discovery* marking data (Chittleborough, 1965). However, given that both Australian stocks are considered to comprise more than 7,000 individuals (Noad *et al.*, 2006; Paxton *et al.*, 2006) it would be desirable to increase the sample size for each stock before attempting to quantify the rate of exchange between them.

In the near future, a comparison of humpback whale photo-ID across the region covered in this study is planned by members of the South Pacific Whale Research Consortium. The degree of demographic interchange able to be quantified by that study, complemented with our analysis, will provide the necessary information to delineate the management units of humpback whales in the Western South Pacific.

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224 Table 1. Frequencies of mtDNA control region sequences from Eastern Australian humpback whales, including
225 for comparison those from breeding grounds of the South Pacific and Indian Oceans (Olavarría et al., In review).
226 The division of breeding stocks (D - G) follows the model of stock structure currently in discussion by the
227 Scientific Committee of the International Whaling Commission (IWC, 2005).

| Region | Western Australia | Eastern Australia | New Caledonia | Tonga | Cook Islands | French Polynesia | Colombia |
|---|---|---|---|---|---|---|---|
| *Stock* | *D* | *E1* | *E2* | *E3* | *F1* | *F2* | *G* |
| EA004 | | 1 | | | | | |
| EA025 | | 1 | | | | | |
| EA066 | | 1 | | | | | |
| EA093 | | 1 | | | | | |
| EA105 | | 1 | | | | | |
| SP1 | 16 | 5 | 3 | 19 | 2 | 17 | 5 |
| SP2 | | 4 | 6 | 1 | | | |
| SP3 | | 6 | 6 | 12 | 2 | 2 | |
| SP10 | 1 | 4 | 6 | 2 | | | 4 |
| SP11 | | 6 | 4 | | | | |
| SP13 | | 5 | 8 | 1 | 2 | | |
| SP14 | 6 | 5 | 17 | 17 | 8 | 1 | 4 |
| SP19 | 3 | 1 | | 9 | 4 | 14 | |
| SP26 | | 14 | 2 | 4 | | | |
| SP27 | 6 | 1 | 4 | 8 | | | |
| SP29 | | 1 | 3 | 4 | | 6 | 1 |
| SP38 | | 1 | 4 | 4 | 2 | | |
| SP39 | | 1 | | 3 | | | |
| SP41 | 2 | 2 | 2 | | | | |
| SP46 | | 4 | 1 | | | | |
| SP49 | 1 | 1 | 1 | | | | |
| SP52 | 8 | 6 | 11 | 5 | 8 | | 9 |
| SP63 | | 10 | 3 | | | | 4 |
| SP65 | | 1 | 3 | | | | |
| SP68 | 1 | 5 | 14 | 7 | | | 1 |
| SP71 | 9 | 14 | 9 | 2 | | | |
| SP72 | | 1 | 1 | 9 | 2 | 11 | 2 |
| SP73 | 2 | 7 | 14 | 34 | 8 | | 2 |
| SP76 | 8 | 3 | 4 | 2 | | | |
| SP78 | 1 | 2 | 5 | 2 | | | |
| SP85 | | 3 | 1 | | | | |
| SP87 | | 5 | 1 | | | | |
| SP88 | 6 | 2 | 13 | 13 | 1 | 1 | |
| SP89 | 3 | 1 | 4 | | | 1 | |
| SP91 | 7 | 1 | 7 | 4 | | 1 | |
| SP93 | 3 | 1 | 2 | | | | |
| SP94 | 7 | 1 | 1 | 4 | | | |
| SP96 | | 4 | 5 | | 3 | 1 | |
| SP102 | | 11 | 5 | 8 | | 2 | |
| SP107 | 2 | 3 | 2 | | | | |
| SP114 | | 1 | 2 | 5 | | | |
| SP115 | 2 | 8 | | | | | |
| *Total Nº sequences* | *174* | *156* | *250* | *310* | *131* | *99* | *148* |
| *Total Nº haplotypes* | *53* | *42* | *61* | *48* | *23* | *21* | *27* |
| *Total Nº unshared haplotypes* | *27* | *5* | *17* | *4* | *1* | *2* | *9* |
| *Total Nº whales with unshared haplotypes* | *58* | *5* | *33* | *6* | *2* | *6* | *85* |

228

Table 2. Pair-wise test of differentiation for mtDNA control region sequence between the Eastern Australia stock and six breeding grounds of humpback whales in the Southern Hemisphere based on $\Phi_{ST}$ and $F_{ST}$ indexes. Unadjusted *p*-values based on 20,000 random permutations of the data matrix are shown in italics. The division of breeding stocks (D - G) follows the model of stock structure currently in discussion by the Scientific Committee of the International Whaling Commission (IWC, 2005).

| Region | Western Australia | New Caledonia | Tonga | Cook Islands | French Polynesia | Colombia |
|---|---|---|---|---|---|---|
| Stock | D | E2 | E3 | F | F | G |
| $F_{ST}$ | 0.01946 | 0.01084 | 0.01938 | 0.04211 | 0.05243 | 0.06168 |
| | *0.00000* | *0.00000* | *0.00000* | *0.00000* | *0.00000* | *0.00000* |
| $\Phi_{ST}$ | 0.01843 | 0.00596 | 0.00217 | 0.01049 | 0.03028 | 0.04689 |
| | *0.00085* | *0.04000* | *0.16181* | *0.02387* | *0.00050* | *0.00000* |


Figure 1. Sampling locations in Eastern Australia.