FOR CONSIDERATION BY THE SCIENTIFIC COMMITTEE OF
THE INTERNATIONAL WHALING COMMISSION
BERLIN, GERMANY
26 May – 6 June 2003

Population differentiation of humpback whales from far Polynesia (Group F breeding grounds) based on mitochondrial DNA sequences

CARLOS OLAVARRÍA[*], MICHAEL POOLE[+], NAN HAUSER[#], CLAIRE GARRIGUE[¥], SUSANA CABALLERO[*†], MURIEL BRASSEUR[±], KAREN MARTIEN[£], KIRSTY RUSSELL[*], MARC OREMUS[*+], REMI DODEMONT[¥], LILIAN FLÓREZ-GONZÁLEZ[†], JUAN CAPELLA[†], HOWARD ROSENBAUM[‡], DORIAN MORO[±], CURT JENNER[§], MICHELINE-NICOLE JENNER[§], JOHN BANNISTER[¶] & C. SCOTT BAKER[*]

[*]*School of Biological Sciences, University of Auckland, Private Bag 92019, Auckland, New Zealand*
[+]*Centre de Recherches Insulaires et Observatoire de l'Environnement, BP 1013, Moorea, Polynésie Française*
[#]*Cook Islands Whale Research, P.O. Box 3069, Avarua, Rarotonga, Cook Islands*
[¥]*Opération Cétacés, BP 12827, 98802 Nouméa, Nouvelle-Calédonie*
[†]*Fundación Yubarta, Carrera 24F Oeste No. 3-110, Tejares de San Fernando, Cali, Colombia*
[±]*Centre for Ecosystem Management, Edith Cowan University, Joondalup, Perth, WA 6027, Australia*
[£]*Southwest Fisheries Science Centre, 8604 La Jolla Shores Drive, La Jolla, CA 92037, USA*
[‡]*American Museum of Natural History, Molecular Systematics laboratory, New York, NY 11024, USA*
[§]*Centre for Whale Research (Western Australia), P.O. Box 1622 Freemantle WA 6959, Australia*
[¶]*Western Australian Museum, Francis Street, Perth, WA 6000, Australia*

Contact e-mail: c.olavarria@auckland.ac.nz

ABSTRACT

It has been proposed that South Pacific humpback whales (*Megaptera novaeangliae*) are divided into three main stocks (E, F and G) based on the distribution of breeding grounds. Recently photo-identification data have shown a limited degree of demographic interchange between adjacent breeding grounds of the western stocks and important segregation with the G stock (Colombia, Eastern Pacific). Here we investigate maternal gene flow among six regional breeding grounds: Western Australia (D stock), New Caledonia (Eii1 sub-stock), Tonga (Eii2 sub-stock), Cook Islands (F stock), French Polynesia (F stock) and the Pacific coast of Colombia (G stock). Mitochondrial DNA control region sequences (n = 1069, 447 bp) identified 138 haplotypes defined by 85 polymorphic sites. An analysis of molecular variance (AMOVA, F_{ST} and Φ_{ST}) showed significant differentiation at both the haplotype and nucleotide level between all the breeding grounds considered in this study, (with the exception of Tonga and the Cook Islands at the nucleotide level, Φ_{ST}). This suggests that the Cook Islands and French Polynesia should be considered two stocks, not the single F stock proposed previously. These analyses also indicate that New Caledonia and Tonga should be considered two stocks (Eii1 and Eii2), as suggests by recent photo-identification analysis of these two regions. The data were also analysed using Boundary Rank (BR), a geographically constrained clustering method. The results of the BR analysis agreed with the AMOVA results in suggesting that both the stocks E and F may not reflect fully the population structure in the western South Pacific. The extensive data set presented here could be used for an improved allocation of feeding grounds to breeding grounds. A proposal to use genetic assignment test to allocate humpback whales samples from the IDCR/SOWER cruises to South Pacific breeding grounds was submitted and approved two years ago at SC53. To date, these samples have not been received for analysis.

KEY WORDS: HUMPBACK WHALE, BREEDING GROUNDS, SOUTHERN HEMISPHERE, GENETICS

INTRODUCTION

The humpback whale (*Megaptera novaeangliae*) is a widely distributed species that undertakes migrations between high-latitude summer feeding grounds and tropical winter breeding grounds. Three main ocean basin populations have been described (North Pacific, North Atlantic and Southern Ocean), with several sub-populations recognised within them (Mackintosh 1965).

In the South Pacific, three main populations, or stocks, have been proposed (stocks E, F and G), including several breeding grounds or sub-stocks which are considered to correspond to specific Antarctic feeding grounds (IWC 2001). The western stock (E) comprises the breeding grounds of East Coast, Australia, around New Caledonia and the Kingdom of Tonga. The central stock (F) comprises the recently described Cook Islands (Hauser et al. 2000) and French Polynesian breeding grounds (Poole 2002). An eastern stock (G) includes whales breeding along the coasts of Colombia and Ecuador, in western South America. The relationships between these stocks and among the breeding grounds remain unclear, although there has been some investigation of several breeding grounds (SPWRC 2003).

In the past, *Discovery* tagging provided evidence of interchange between the breeding grounds of western South Pacific (Baker 2000). More recently, photo-identification methods have suggested significant demographic segregation between the G stock and other South Pacific stocks, and low-level interchange between adjacent breeding grounds within the E and F stocks (Rosenbaum et al. 1995; Garrigue et al. 2002; SPWRC 2003). Genetic analyses support the segregation of G stock in the South Pacific, and interchange between breeding grounds within the E stock (Baker et al. 1998; Rosenbaum et al. 1998; Caballero et al. 2000).

Here we investigate the genetic relationship among components of the F stock, the Cook Islands and French Polynesia breeding grounds, using mitochondrial (mt) DNA sequences, and compare them to other South Pacific breeding grounds, as well as stock D, the Western Australia breeding ground, from the Indian Ocean.

MATERIALS AND METHODS

Skin samples were collected from free-ranging whales, using either a crossbow (Lambertsen 1987), a PAXARM biopsy system (Krützen et al. 2002), or as sloughed skin (Amos et al. 1992). Additionally, tissue samples from a small number of dead stranded animals were collected. The samples were stored in 70% ethanol or in a solution of 20% DMSO saturated with NaCl at -15° C for subsequent analysis.

Genomic DNA was extracted using a standard phenol/chloroform extraction protocol (Davies et al. (1986) as modified by Baker et al. (1994)). Symmetrical amplification of the mtDNA control region, proximal to the tPro RNA gene, was performed via the Polymerase Chain Reaction (PCR, Saiki et al. (1988)) following standard protocols (Palumbi 1995). 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 (Lento et al. 1997). Amplification and subsequent cycle sequencing were improved by the addition of an M13 tag to the 5' end of the Dlp-1.5 primer. Temperature profiles consisted of a preliminary denaturing period of 2 minutes at 94°C followed by 35 cycles of denaturation for 30 seconds at 94°C, primer annealing for 40 seconds at 54°C and polymerase extension for 40 seconds at 72°C. A final extension period for 10 minutes at 72°C was included at the end. PCR products were cleaned with ExoSAP-IT (USB) and sequenced in one direction with BigDye™ terminator chemistry on an ABI3100 DNA sequencer (Applied Biosystem), using M13Dlp-1.5 as the sequencing primer.

Sequencher™ (version 4.1.2, Genes Codes Co.) was utilized for the alignment, and possible sequencing errors were corrected by eye. Comparisons to identify polymorphic sites and haplotypes were performed using MacClade version 4.0 (Maddison and Maddison 2000).

Geographic differentiation among haplotypes frequencies and nucleotide variation was quantified using an Analysis of Molecular Variance (AMOVA; Excoffier et al. (1992)) as implemented in Arlequin version 2.0 (Schneider et al. 2000), using nucleotide differentiation (Φ_{ST}) and haplotype frequency differences (F_{ST}). The significance of the observed Φ_{st} and F_{ST} values were tested using 5000 random permutations of the data matrix. Genetic diversity was estimated at haplotype and nucleotide level using Arlequin version 2.0 (Schneider et al. 2000). At the haplotype level, diversity was calculated without regards to the genetic distance (i.e., number of nucleotide substitutions); at the nucleotide level, diversity was calculated using pair-wise differences between sequences.

The relationships among the six breeding grounds were examined using Boundary Rank (BR, Martien and Taylor (In review)), a geographically constrained clustering algorithm designed to generate population structure hypotheses that are consistent with the available genetic data. The analysis was initialised with six units corresponding to the six breeding grounds (Table 1). BR requires specifying a connectivity matrix, which constrains the geographic shape of the units generated by BR to be consistent with what is known about the behaviour and movement patterns of the species in question. We used a connectivity matrix that reflected a simple stepping-stone model in which each breeding ground was 'connected' to the two breeding grounds on either side of it.

RESULTS

Over a thousand skin samples were obtained from six Southern Hemisphere breeding grounds. In this analysis, a total of 1069 mtDNA control region sequences were used (Table 1, Figure 1). Over a 447 bp consensus region of the mtDNA control region, 85 polymorphic sites identified a total of 138 haplotypes. Haplotype diversity ranged between 0.974 and 0.913. Nucleotide diversity ranged between 0.023 and 0.021 (Table 1).

In comparisons among the six breeding grounds, significant differences at the haplotype (F_{ST}) and nucleotide level (Φ_{ST}) were found for all the pair-wise comparisons, except for Tonga and the Cook Islands at nucleotide level (Table 2).

The BR analysis showed that all of the breeding grounds are more similar to the breeding ground to the west of them than they are to the breeding ground to their east (Figure 2). New Caledonia is more similar to Western Australia than it is to Tonga and the Cook Islands are more similar to the breeding grounds in stocks D and E than it is to French Polynesia. The χ^2/dof values (the measure of genetic differentiation used by BR) between all of the breeding grounds were quite high, which is consistent with the highly significant differentiation detected in the AMOVA analyses.

Table 1. Locations and years of sample collection, number of sequences analyzed, haplotypes and polymorphic sites (pol. sites), and diversity at the haplotypic (h) and nucleotide (π) level, including standard deviations, for each breeding ground.

Region	Stock (sub-stock)	Years collection	N sequences	N haplotypes	N pol sites	h±SD	π±SD
Western Australia (WA) *off Tantabiddi, Exmouth*	D	2002	123	47	53	0.970 ± 0.006	0.023 ± 0.012
New Caledonia (NC) *Grande Terre*	E (ii 1)	1995-2002	251	60	59	0.974 ± 0.003	0.023 ± 0.011
Tonga (Tg) *Vava'u, Ha'apai, Tongatapu*	E (ii 2)	1991-2002	348	48	50	0.961 ± 0.003	0.021 ± 0.011
Cook Islands (CI) *Rarotonga, Aitutaki, Palmerston*	F	1998-2002	122	24	41	0.925 ± 0.01	0.021 ± 0.011
French Polynesia (FP) *Moorea, Huahine, Rurutui*	F	1997-2002	84	20	40	0.913 ± 0.013	0.021 ± 0.011
Colombia (Col) *Gorgona Island, Malaga Bay*	G	1991-1999	141	50	41	0.937 ± 0.013	0.023 ± 0.012

Table 2. Pair-wise comparisons of six breeding grounds or stocks/sub-stocks of Southern Hemisphere humpback whales showing Φ_{ST} (under the diagonal) and F_{ST} (above the diagonal) values. Values shown in bold are significantly greater than those found in 5% of 5,000 random permutations of the data matrix ($P < 0.05$). The probability (p) of obtaining greater values by chance alone is shown in italics. No adjustment was undertaken for multiple comparisons.

Region - Stock	WA - D	NC - E (ii 1)	Tg - E (ii 2)	CI - F	FP - F	Col - G
WA - D		**0.01247** *0.0 ± 0.0*	**0.01674** *0.0 ± 0.0*	**0.02316** *0.0 ± 0.0*	**0.03484** *0.0 ± 0.0*	**0.04023** *0.0 ± 0.0*
NC - E (ii 1)	**0.00574** *0.04602 ± 0.0029*		**0.00836** *0.0 ± 0.0*	**0.03042** *0.0 ± 0.0*	**0.04490** *0.0 ± 0.0*	**0.03755** *0.0 ± 0.0*
Tg - E (ii 2)	**0.01101** *0.00635 ± 0.0011*	**0.00830** *0.00417 ± 0.0010*		**0.02320** *0.0 ± 0.0*	**0.03228** *0.0 ± 0.0*	**0.04342** *0.0 ± 0.0*
CI - F	**0.01118** *0.02995 ± 0.0023*	**0.01458** *0.00417 ± 0.0009*	0.00492 *0.07756 ± 0.0037*		**0.02533** *0.0 ± 0.0*	**0.05831** *0.0 ± 0.0*
FP - F	**0.02173** *0.00516 ± 0.0010*	**0.02305** *0.00119 ± 0.0005*	**0.02641** *0.00079 ± 0.0004*	**0.01419** *0.03888 ± 0.0027*		**0.06584** *0.0 ± 0.0*
Col - G	**0.09100** *0.0 ± 0.0*	**0.10233** *0.0 ± 0.0*	**0.10514** *0.0 ± 0.0*	**0.11439** *0.0 ± 0.0*	**0.10953** *0.0 ± 0.0*	

DISCUSION

High levels of genetic variation were observed in the Southern Hemisphere breeding grounds. Genetic variation was greatest in the western South Pacific (New Caledonia) and in Western Australia, as has been described previously (Baker et al. 1998). The lower genetic diversity found in the Cook Islands and French Polynesia may reflect smaller effective population sizes in these areas, compared with those of Western Australia or more intense hunting. Alternatively, it may be due in part to sampling methods, as some of the samples collected in these areas consisted of sloughed skin, during the collection of which unrecognised replicate sampling of the same individuals can occur.

The result of the AMOVA analysis over such a large number of mtDNA control region sequences suggests that humpback whales breeding grounds are strongly structured in the South Pacific, requiring further modification to the stock definition model currently considered in the Southern Hemisphere assessment.

These analyses confirm that there are marked differences between the eastern stock (G) and the western stocks (E and F) in the South Pacific, as suggested by photo-identification studies (Rosenbaum et al. 1995; Garrigue et al. 2002; SPWRC 2003) and previous genetic analysis (Baker et al. 1998; Rosenbaum et al. 1998; Caballero et al. 2000).

Both the AMOVA and BR analyses suggested that the E and F stock must be sub-divided. Within the E stock, significant differences were found between the New Caledonia and Tonga breeding grounds, supporting the proposed existence of two genetically distinct populations within this stock (Baker 2000). The separation of New Caledonia and Tonga is also supported by the BR results, which show that New Caledonia is actually less similar to Tonga than it is to Western Australia. Similarly, significant differentiation was observed between the Cook Islands and French Polynesia, the putative central Pacific F stock. Both AMOVA and BR showed that Cook Islands is actually less similar to French Polynesia than it is to the breeding grounds to the west of it, lending further support to the notion that Cook Islands and French Polynesia should not be combined in a single stock.

Nuclear DNA genotyping (microsatellites) is in progress and will detect possible duplicate sampling of individuals and address the question of whether the observed mtDNA differentiation between breeding grounds may be sex-biased.

Finally, a comprehensive understanding of population structure in the South Pacific requires allocation of feeding grounds to breeding grounds. A proposal to use genetic assignment test to allocate humpback whales samples from the IDCR/SOWER cruises to South Pacific breeding grounds was submitted two years ago (Olavarría et al. 2001) and approved at SC53, where the Committee recommended that tissue samples should be provided to the principal investigator if possible (IWC 2002). To date, these samples have not been received for analysis. This delay could result in the loss of information critical to completion of the South Hemisphere assessment.

ACKNOWLEDGEMENTS

Many people and institutions have contributed to this research. For support in French Polynesia we thank West Marine Products, M. Poliza and the Starship Millennium Voyage, Office des Postes et Télécommunications, Sin Tung Hing Marine, Mercury Outboards, West Coast Whales Research Foundation, the Foundation Naturalia et Biologia, Hardy Jones-Julia Whitty Productions, the Raie Manta Club, Cine Marine, Eco-media Productions, Canal+ and the BBC. In the Cook Islands, we thank to the Cook Island Government, Tap Pryor, Phil Clapham, The People of Rarotonga, Aitutaki and Palmerston, Joan Daeschler, Helen Hauser, and Doug & Julie Macrae. In New Caledonia, we thank to Inco, Provinces Sud, Nord and Iles, and Les Editions Catherine Ledru. Research in the Kingdom of Tonga was conducted under special scientific permit from the Tongan Ministry of Fisheries and His Majesty, the King of Tonga, with funding from the International Whaling Commission, Auckland University Research Council, Whale and Dolphin Conservation Society, South Pacific Regional Environment Program, Whale and Dolphin Adoption Project, Pacific Development Trust of New Zealand, Cetacean Society International and International Fund for Animal Welfare. We thank B. Abernethy, N. Patenaude, R. Constantine, S. Childerhouse, N. Gibbs, T. O'Callaghan, M. Donoghue and the sailing vessel *Nai'a* for field support. In Colombia, financial support for the research of Fundación Yubarta was given by Colciencias and Ecofondo. For support in Western Australia we thank Shell Oil, Chevron, Texaco, Apache Energy, EDR-Hydrosearch, and Strike Oil.

Funding for genetic analysis was provided by the New Zealand Marsden Fund, International Fund for Animal Welfare (IFAW) and a University of Auckland International Doctoral Scholarship (to C. Olavarría). We thank J. Murrell, D. Steel, A. Chupón for laboratory assistance, B. Taylor for assistance in BR analysis and S. Lavery and M. Dalebout for comments to this manuscript. This analysis is the result of a large-scale survey made possible through the generous collaboration of members and affiliates of the South Pacific Whale Research Consortium (SPWRC), with support of the International Fund for Animal Welfare (IFAW).

LITERATURE CITED

Amos W, Whitehead H, Ferrari MJ, Glockner-Ferrari DA, Payne R, Gordon J (1992) Restrictable DNA from sloughed cetacean skin: its potential for use in population analysis. Marine Mammal Science 8: 275-283

Baker CB (2000) New evidence for stock boundaries of humpback whales in the proposed South Pacific Whale Sanctuary. SC/52/IA20

Baker CS, Flórez-González L, Abernethy B, Rosembaum HC, Slade RW, Capella J, Bannister JL (1998) Mitochondrial DNA variation and maternal gene flow among humpback whales of the southern hemisphere. Marine Mammal Science 14: 721-737

Baker CS, Slade S, Bannister JL, Abernethy RB, Weinrich MT, Lien J, Urbán J, Corkeron P, Calambokidis J, Vásquez O, Palumbi SR (1994) Hierarchical structure of mitochondrial DNA gene flow among humpback whales *Megaptera novaeangliae*, world-wide. Molecular Ecology 3: 313-327

Caballero S, Hamilton H, Flórez-González L, Capella J, Olavarría C, Rosenbaum HC, Baker CS (2000) Stock identity and diversity of humpback whale mitochondrial DNA lineages on the Colombian winter breeding grounds. SC/52/IA14

Davis LG, Dibner MD, Battey JF (1986) Basic methods in Molecular Biology. Elsevier Science Publishing Co.

Excoffier L, Smouse PE, Quatro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131: 479-491

Garrigue C, Aguayo A, Amante-Helweg VLU, Baker CS, Caballero S, Clapham P, Constantine R, Denkinger J, Donoghue M, Flórez-González L, Greaves J, Hauser N, Olavarría C, Pairoa C, Peckham H, Poole M (2002) Movements of humpback whales in Oceania, South Pacific. Journal of Cetacean Reserach and Management 4: 255–260

Hauser N, Peckham H, Clapham PJ (2000) Humpback whales in the southern Cook Islands, South Pacific. Journal of Cetacean Research and Management 2: 159-164

IWC (2001) Report of the Scientific Committee. Journal of Cetacean Research and Management 3 (Suppl.): 1-76

IWC (2002) Report of the Scientific Committee. Journal of Cetacean Research and Management 4 (Suppl.): 1-453

Krützen M, Barré LM, Möller LM, Heithaus MR, Simmer C, Sherwin WB (2002) A biopsy system for small cetaceans: darting success and wound healing in *Tursiops* spp. Marine Mammal Science 18: 863-878

Lambertsen RH (1987) A biopsy system for large whales and its use for cytogenetics. Journal of Mammalogy 68: 443-445

Lento GM, Patenaude NJ, Baker CS (1997) Molecular genetic identification of whale and dolphin products for sale in Japan and Korea, 1995-97. Submission to the Scientific Committee of the International Whaling Commission. SC/49/O21

Mackintosh NA (1965) The stocks of Whales. Fishing News (Books) Ltd., London

Maddison DR, Maddison WP (2000) MacClade: Analysis of phylogeny and character evolution. Sinauer, Sunderland, Massachusetts

Martien KK, Taylor BL (In review) A new method using genetic data to generate hypothesized population structures for continuously distributed species

Olavarría C, Baker CS, Garrigue C (2001) Genetic "assignment" of humpback whales from the Southern Hemisphere feeding grounds to breeding grounds: a no-cost request for access to IDCR-SOWER biopsy samples (1996-2000). Research Proposal to the International Whaling Commission, pp 12 pp.

Palumbi SR (1995) Nucleic Acids II: The polymerase chain reaction. In Hillis D, C Moritz, BK Mable (eds) Molecular Systematics. Sinauer Associates, Sunderland, Masachusetts: 205-248

Poole M (2002) Occurrence of humpback whales (*Megaptera novaeangliae*) in French Polynesia in 1988-2001. SC/54/H14

Rosenbaum H, Clapham P, Allen J, Jenner MN, Jenner C, Flórez-González L, Urbán J, Ladrón de Guevara P, Mori K, Yamaguchi M, Baker CS (1995) Geographic variation in ventral fluke pigmentation of humpback whale *Megaptera novaeangliae* populations worldwide. Marine Ecology Progress Series 124: 1-7

Rosenbaum H, Razafindrakoto Y, Flórez-González L, Capella J, Garrigue C, Greaves J, Jenner C, Jenner MN, Robles-Saavedra MR, DeSalle R, Baker CS (1998) Variation and geographic structure of humpback whale mitochondrial DNA from the wintering grounds of Areas III, IV, V and VI in the Southern Hemisphere. SC/50/CAWS35

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239: 487-491

Schneider S, Roeslli D, Excoffier L (2000) Arlequin. A software for population genetics data analysis

SPWRC (2003) Report of the Annual Meeting of the South Pacific Whale Research Consortium. 13-17 February 2003, Auckland, New Zealand. SC/55/SH2

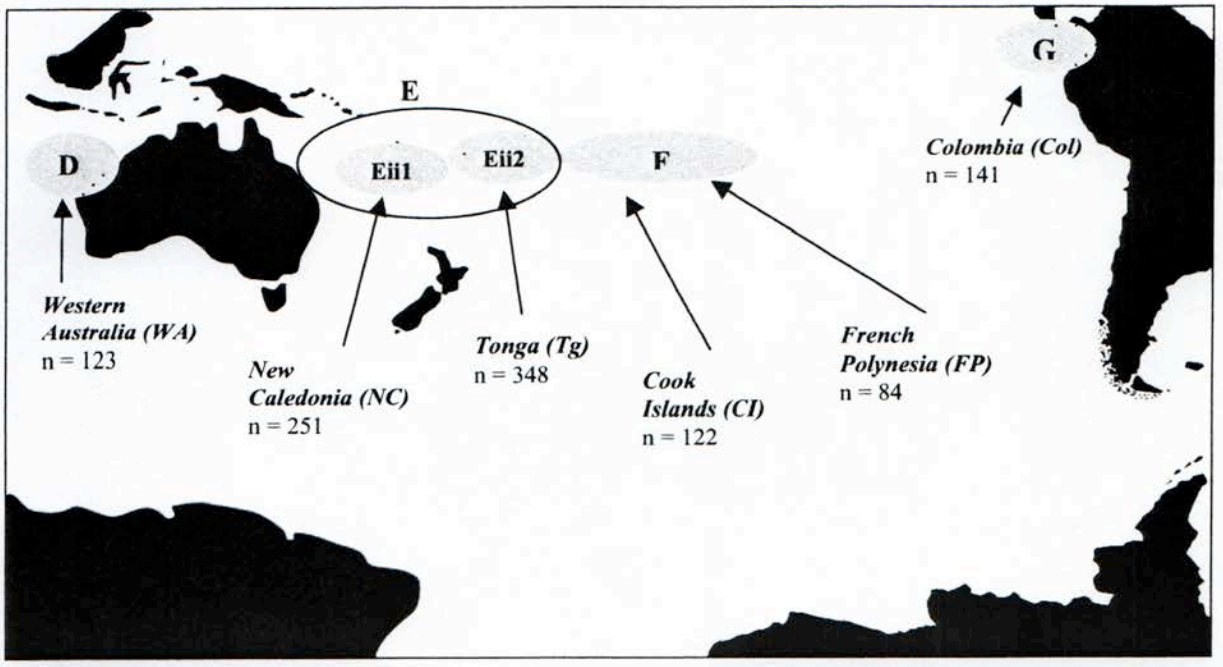

Figure 1. Map showing sampling areas and number of sequences used in this analysis for each breeding ground.

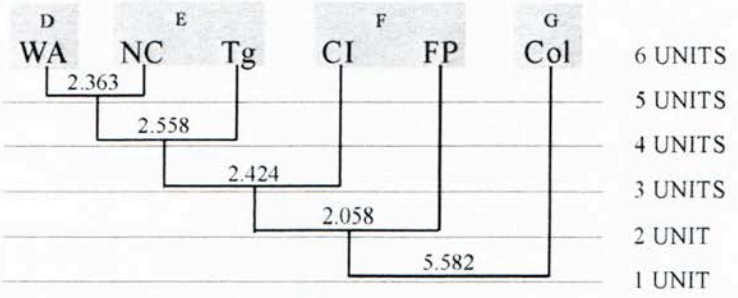

Figure 2. Dendrogram representing the results of the Boundary Rank analysis, with the initial units shown at the tips. Nodes indicate that two units are combined into a single unit, with the depth of the node indicating at what point in the analysis the units were combined. The number associated with each node is the degree of genetic differentiation (as measured by χ^2/dof) between the units being combined at that node. The number of hypothesized units remaining at a given point in the analysis is indicated along the right side of the dendrogram. WA = Western Australia, NC = New Caledonia, Tg = Tonga, CI = Cook Islands, FP = French Polynesia and Col = Colombia. The shaded boxes at the top of the figure indicate which stock (D, E, F or G) each breeding ground is currently part of.