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Blue whale population structure along the eastern South Pacific Ocean: evidence of more than one population

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Abstract

Blue whales (*Balaenoptera musculus*) were among the most intensively exploited species of whales in the world. As a consequence of this intense exploitation, blue whale sightings off the coast of Chile were uncommon by the end of the 20th century. In 2004, a feeding and nursing ground was reported in southern Chile (SCh). With the aim to investigate the genetic identity and relationship of these Chilean blue whales to those in other Southern Hemisphere areas, 60 biopsy samples were collected from blue whales in SCh between 2003 and 2009. These samples were genotyped at seven microsatellite loci and the mitochondrial control region was sequenced, allowing us to identify 52 individuals. To investigate the genetic identity of this suspected remnant population, we compared these 52 individuals to blue whales from Antarctica (ANT, n = 96), Northern Chile (NCh, n = 19) and the eastern tropical Pacific (ETP, n = 31). No significant differentiation in haplotype frequencies (mtDNA) or among genotypes (nDNA) was found between SCh, NCh and ETP, while significant differences were found between those three areas and Antarctica for both the mitochondrial and microsatellite analyses. Our results suggest at least two breeding population units or subspecies exist, which is also supported by other lines of evidence such as morphometrics and acoustics. The lack of differences detected between SCh/NCh/ETP areas supports the hypothesis that eastern South Pacific blue whales are using the ETP area as a possible breeding area. Considering the small population sizes previously reported for the SCh area, additional conservation measures and monitoring of this population should be developed and prioritized.

Keywords: *Balaenoptera musculus*, biodiversity conservation, conservation genetics, eastern South Pacific, endangered species

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Introduction

The oceanic environment lacks obvious geographical barriers, presenting a challenge to understand how divergence occurs in such environments, particularly in species with high dispersal capabilities (Palumbi 1994;

Jørgensen *et al.* 2005). Thus, interpreting gene flow and population differentiation patterns is a difficult task (Waples 1998). Levels of dispersal and genetic divergence can vary among populations within a species, and it is generally thought that this variation is primarily driven by factors such as habitat differences (e.g. abundance and availability of resources), intensity of predation or habitat isolation (Bowler & Benton 2005). However, other factors such as social structure, long-term reinforced migratory behaviour and vicariant events can also have a profound effect on the degree of population structuring in marine species (Hoelzel 1998).

Large migratory baleen whales in the Southern Hemisphere represent a suitable biological model to examine population differentiation at oceanic scales and to evaluate the influence of population history, particularly regarding the recent past following cessation of whaling during the 20th century (LeDuc *et al.* 2007; Patenaude *et al.* 2007; Rosenbaum *et al.* 2009). Within the Southern Hemisphere, two different subspecies of blue whales have been recognized: true or Antarctic blue whales (*B. m. intermedia*) and pygmy blue whales (*B. m. brevicauda*) (Ichihara 1966). The two subspecies differ morphologically (Ichihara 1966) and are thought to largely remain segregated during the austral summer (Kato *et al.* 1995; Branch *et al.* 2007a,b; Attard *et al.* 2012), with Antarctic blue whales feeding south of the Antarctic convergence and pygmy blue whales feeding north of the Antarctic convergence. During the early 1900s, blue whales became one of the main target species for the whaling industry worldwide (Clapham *et al.* 1999); this exploitation continued until 1965/1966 when the International Whaling Commission (IWC) set a moratorium to cease their harvest, although illegal catches by some countries continued through the 1970s (Yablokov 1994; Ivashchenko & Clapham 2014). Before whaling ceased, more than 346 000 Antarctic blue whales were killed in the Southern Hemisphere, depleting population sizes to below 1% of the original number (Branch *et al.* 2004). The status of the pygmy blue whales is less certain, although they are currently considered to be less depleted than Antarctic blue whales (Branch *et al.* 2007a). The conservation status of the blue whale globally is 'Endangered' [IUCN Red List of Threatened Species (www.redlist.org)], although a recent analysis of whaling records has suggested that the eastern North Pacific population may have recovered to near prewhaling levels of abundance (Monnahan *et al.* 2014). The Antarctic blue whale subspecies is listed as 'Critically Endangered', while the pygmy blue whale subspecies are listed as 'Data Deficient'.

Knowledge regarding the natural history and population structure of different whale species has notably increased after the advent of highly polymorphic genetic markers (e.g. Baker *et al.* 1993; Rosenbaum *et al.* 2009); however, little is known about the biogeography of Southern Hemisphere blue whales (*Balaenoptera musculus*), although whaling data and photo-identification studies are available (e.g. Brown 1954; Mackintosh 1965; IWC 2009). The classic view of blue whale life strategy describes a seasonal migration between high-latitude summer feeding grounds and low-latitude winter breeding areas where occasional feeding can take place (Palacios 1999). In contrast to the classic view, numerous sightings and acoustic records have documented the presence of blue whales during the austral summer or even year-round in low, mid and high latitudes in the eastern South Pacific (ESP) (e.g. Donovan 1984; Reilly & Thayer 1990; Findlay *et al.* 1998; Palacios 1999; Hucke-Gaete *et al.* 2004; Širović *et al.* 2004).

Those eastern South Pacific mid-latitude areas correspond to blue whale summer feeding aggregations in southern (SCh) and northern Chile (NCh) (Findlay *et al.* 1998; Hucke-Gaete *et al.* 2004; R. Moraga, personal communication), while high-latitude feeding grounds are found in Antarctic waters. In contrast, little is known about the location of breeding ground[s] used by any of the blue whale subspecies. Based on year-round sightings in the area, Reilly & Thayer (1990) hypothesized that the Costa Rica Dome (located in the eastern tropical Pacific) could be the breeding destination of pygmy blue whales in the eastern South Pacific (ESP). Some support for this hypothesis was provided by a recent satellite tracking study in which a whale tagged off southern Chile travelled to the Nazca Ridge (*c.* 1200 km offshore) to around latitude 23°S before the tag failed (Hucke-Gaete 2004). Acoustic records obtained in waters of the eastern tropical Pacific suggest that both named subspecies (Antarctic and Pygmy) may overlap in this region for breeding (Stafford *et al.* 2004).

Some other lines of evidence suggest blue whales from Chile may be a distinct subspecies of blue whales*. Blue whale songs recorded in the waters of southern Chile are different from blue whale songs recorded in other feeding areas (McDonald *et al.* 2006; Buchan *et al.* 2010). A similar pattern using morphological traits was described, in which female blue whales captured along Chile were different from those captured in other areas of the Southern Hemisphere (including those described as pygmy blue whales) (Branch *et al.* 2007a). Finally, a recent study using molecular markers identified mitochondrial and nuclear differences between whales sampled while feeding in the Antarctic, eastern South Pacific and Indian

*Un-named subspecies accepted by the Society for Marine Mammalogy taxonomy committee (http://www.marinemammalscience.org/index.php?option=com_content&view=article&id=758&Itemid=340).

Ocean (LeDuc *et al.* 2007). Surprisingly, blue whales sampled in the eastern South Pacific and Indian Ocean were as genetically different from each other as either was to blue whales sampled in the Antarctica, despite the fact that they are considered to be part of the same subspecies.

Hence, considering that (i) the Antarctic blue whale acoustic recordings obtained in low latitudes (Stafford *et al.* 2004), (ii) the report from Aguayo (1974) that two different types of blue whales were captured in Chilean waters during the 1960s and (iii) the presence of blue whales in the ETP year-round (Reilly & Thayer 1990), we hypothesize a scenario where waters of the ESP are used by whales from more than one population. Therefore, in this study, the genetic diversity of blue whales from the ESP was characterized using both microsatellite and mtDNA markers and compared among different ESP areas (SCh, NCh and ETP) and ANT blue whale feeding grounds. The results provide new information on levels of connectivity between (i) blue whales feeding in two different areas off the coast of Chile (SCh vs. NCh), (ii) blue whales feeding off Chile and those sampled in the ETP (SCh and NCh vs. ETP) and (iii) blue whales feeding in Antarctic waters and those sampled in the ETP (ANT vs. ETP). Although genetic differences between blue whales in the Antarctic and those in the ESP have been demonstrated previously (LeDuc *et al.* 2007), we further provide a re-analysis of the level of differentiation between Antarctic and ESP blue whales using a larger and more geographically comprehensive sample set. These results provide key information for the evaluation of the population viability of this endangered species and for the identification and selection of the most appropriate conservation strategies.

Material and methods

Sample collection and DNA extraction

A total of 206 biopsy samples were obtained by various research groups and/or programs [Southwest Fisheries Science Center (SWFSC); International Whaling Commission (IWC) during the International Decade of Cetacean Research and Southern Ocean Whale and Ecosystem Research cruises (IDCR and SOWER); Universidad Austral de Chile/Centro Ballena Azul (UACh/CBA); Centro de Investigación Eutropia (CIE)] between 1993 and 2010 across four latitudinal strata: eastern tropical Pacific (ETP), Antarctica (ANT), northern Chile (NCh) and southern Chile (SCh) (Fig. 1; Table 1). All the ETP samples were collected south of the Equator.

The samples were obtained using a remote biopsy device consisting of a tip mounted on a bolt and fired from a crossbow or modified Paxarms rifle (Lambertsen 1987; Krützen *et al.* 2002). The sampling was carried out following local regulations and guidelines for each project involved. Samples were preserved in 20% dimethyl sulfoxide (DMSO), saturated with sodium chloride (NaCl; Amos & Hoelzel 1991) or in 95% ethanol and

Table 1 Number of samples obtained in different areas

Area	Sample size	Years	Months
Antarctica	96	1993/1994–2004/2005	Dec–Mar
Southern Chile	60	2002/2003–2009/2010	Jan–Apr
Northern Chile	19	1997/1998, 2007/2008	Dec–Feb
Eastern Tropical Pacific	31	1997–1998, 2000, 2003, 2006	Sept–Nov

All collections were conducted during late spring and austral summer, except for the samples at the eastern tropical Pacific (ETP) that were collected at early spring.

Fig. 1 Number of individual samples used from each study area. Blue dots represent samples. Samples of southern Chile are represented in a large circle due to the small area in which they were taken. Samples of Antarctica are not shown because the sampling area corresponded to the waters around the entire Antarctic. Red stars represent possible vagrants.

stored at −20 °C until processed. Additional information concerning samples is detailed in Table 1 and elsewhere (LeDuc *et al.* 2007; Torres 2011). Total genomic DNA was extracted from biopsy samples using the DNeasy Blood and Tissue kit (Qiagen, Chatsworth, CA, USA).

mtDNA sequencing and sexing of sampled individuals

For the samples collected off southern Chile, a fragment of the mtDNA control region was amplified using primers dlp1.5 and dlp5 (Baker *et al.* 1993), and the amplified product was cycle-sequenced in both directions with dye-labelled terminators. Sequence reactions were analysed using a 3730xl DNA Analyzer (Applied Biosystems, Inc., Foster City, CA, USA). After removal of primer sequences, the resulting 410 bp consensus fragment contained most of the polymorphic nucleotides previously identified in the mtDNA control region of blue whales (LeDuc *et al.* 2007; Sremba *et al.* 2012). Sequences were then aligned and edited manually using SEQUENCHER 3.0 (Gene Codes Corp., Ann Arbor, MI, USA) and MACCLADE v. 4.01 (Maddison & Maddison 2000). The sex of each sample was determined genetically through a male-specific multiplex amplification of a *sry* gene fragment, using fragments of the *ZFY*/*ZFX* genes as positive controls (Bérubé & Palsbøll 1996). Protocols used to sequence and sex samples from the ETP, ANT and NCh are described elsewhere (LeDuc *et al.* 2007; Sremba *et al.* 2012).

Microsatellite genotyping

Seven microsatellite loci cloned for other cetacean species were cross-amplified by PCR (Bérubé *et al.* 2000; Buchanan *et al.* 1996; Palsbøll *et al.* 1997; Valsecchi & Amos 1996; Table 2). Reactions were carried out in a 25 μL volume containing approximately 100 ng of genomic DNA, 2.5 μL of 2.0 mM $MgCl_2$ buffer, 0.6 mM of each dNTP, 0.25 units of Platinum *Taq* DNA polymerase (Invitrogen) and 0.3 μM of each primer (with the forward primer fluorescently labelled for each pair). Amplifications conditions were as follows: 90 °C for 2.5 min followed by 35 cycles of 94 °C for 45 s, 1 min at the annealing temperature depending on the locus (Table 2) and 1.5 min at 72 °C, followed by a final extension at 72 °C for 5 min. Amplicons were separated on an ABI 3100 or ABI 3130 automated sequencer. The size of allele fragments was determined by comparison to the internal lane standards, and labelling of alleles was conducted using ABI GENESCAN and GENOTYPER analysis software, followed by visual corroboration of all labelled peaks.

Table 2 Microsatellite loci used for genotyping blue whales

Locus	*k*	H_o	H_e	*T*°	Reference
ACCC392	20	0.80460	0.83343	55	Palsbøll *et al.* (1997)
DlrFCB17t	13	0.40230	0.44702	55	Buchanan *et al.* (1996)
EV37	7	0.70349	0.71340	55	Valsecchi & Amos (1996)
Gata028	12	0.79769	0.82634	52	Palsbøll *et al.* (1997)
Gata098	13	0.76437	0.75069	48	Palsbøll *et al.* (1997)
Gata417	15	0.87861	0.87694	55	Palsbøll *et al.* (1997)
Gt023	10	0.75449	0.79094	56	Bérubé *et al.* (2000)

The number of alleles per locus (*k*), observed (H_o) and expected (H_e) heterozygosities, annealing temperature for each locus (*T*°) and the source are indicated.

Nuclear genetic diversity data analyses

Scoring errors due to stutter peaks, large allele dropout and null alleles were assessed using the program MICRO-CHECKER v. 2.2.3 (Van Oosterhout *et al.* 2004). About 10% of the samples were rerun to check for possible scoring errors. GENALEX 6.4 (Peakall & Smouse 2006) was used to identify samples that shared the same multilocus genotype. To assess whether two individuals have the same genotype by chance, we estimated the probability of identity $P_{(ID)}$ for nuclear loci using two different algorithms included in the same software: the Hardy–Weinberg Equilibrium (HWE) $P_{(ID)}$ and the more conservative measure Sib $P_{(ID)}$ (Taberlet & Luikart 1999; Waits *et al.* 2001). Exact tests for HWE (Guo & Thompson 1992) and heterozygote deficiency (Raymond & Rousset 1995) were assessed for each microsatellite locus using GENEPOP v. 4.0 (Rousset 2008). Linkage disequilibrium (LD) for each pair of loci was computed using Fisher's method and the Markov chain method in GENEPOP. Deviations from HWE and LD were tested using the program settings for the Markov chain parameters (10 000 dememorization steps; 1000 batches; 10 000 iterations per batch) and corrected for simultaneous comparisons with the sequential Bonferroni test (Rice 1989). Number of alleles per locus (N_a), allelic richness (*A*) and the mean number of alleles were calculated for each locus and each locality with FSTAT v.2.9.3 (Goudet 1995) and ARLEQUIN packages. Observed (H_o) and expected (H_e) heterozygosities were all computed using the EXCEL MICRO-SATELLITE TOOLKIT 3.1 (Park 2001).

Mitochondrial genetic diversity data analyses

Matching of sequences to haplotypes was performed using COLLAPSE v1.2 (http://darwin.uvigo.es) and DNASP v5.0 (Rozas *et al.* 2003). Genetic diversity at both the haplotype (*H*) and nucleotide level (π) (Nei 1987) was estimated using ARLEQUIN v3.5.1.2 (Excoffier & Lischer 2010) and DNAsp.

Population differentiation and geneflow analyses

Population differentiation among the four strata in the southeast Pacific Ocean was assessed using both mtDNA sequences and microsatellite data following two different approaches. First, the geographic location where the samples were collected (i.e. areas) was treated as an a priori defined population. Geographic variation of haplotypes was then quantified using analysis of molecular variance (AMOVA; Excoffier *et al.* 1992) as implemented in the software ARLEQUIN. The significance of the observed F_{ST} and Φ_{ST} statistics was tested using the null distribution generated from 5000 nonparametric random permutations. No correction for simultaneous tests was applied to significance levels of pairwise comparisons (Narum 2006). Similarity among all the identified haplotypes was depicted by a median-joining network (Bandelt *et al.* 1999) implemented in the software NETWORK (http://www.fluxus-engineering.com). Population differentiation using microsatellite loci was examined by an AMOVA (5000 random permutations to test for significance) performed in ARLEQUIN. Additionally, F_{ST} statistics (Weir & Cockerham 1984) were calculated and the significance of pairwise comparisons between populations tested using 10 000 permutations.

Second, population differentiation was assessed without a priori stratification of samples. Bayesian clustering analyses were used to estimate the most probable number of populations (K) given the data as implemented in the program STRUCTURE 2.3.2 (Pritchard *et al.* 2000), using the admixture model with correlated allele frequency (Falush *et al.* 2003). This analysis assumes that within a set of samples, there are K populations and individuals are assigned to each putative population such that HWE and LD are minimized within each group. Ten independent runs were conducted for each value of K = 1–10 with a burn-in period of 10^5 steps followed by 100 000 Markov chain Monte Carlo iterations. The most likely value for K based on STRUCTURE output was determined by plotting the log probability K (Ln $P(X|K)$ of the data over multiple runs and comparing that with Δk (Evanno *et al.* 2005) as implemented in STRUCTURE HARVESTER (Earl 2009). Results of the 10 STRUCTURE runs were merged with CLUMPP (Jakobsson & Rosenberg 2007) and visualized with DISTRUCT (Rosenberg 2004).

We estimated the effective number of immigrants per generation (N_em) using the coalescence-based Bayesian inference program MIGRATE v3.2.6 (Beerli & Felsenstein 2001; Beerli 2006). We compared two scenarios: (i) a three-population model with populations ANT, a combination of NCh and SCh, and ETP; and (ii) a two-population scenario with populations ANT and the combination of the other three localities (NCh, SCh, ETP). We evaluated the immigration numbers for each data set, mtDNA and microsatellite data, separately. For each scenario and each data set, we ran the program five times using the following options: 100 replicates, 10 000 recorded samples every 500 step, and the heating scheme was set so that four parallel chains were run at temperatures of 1.0, 1.5, 3.0, 1 000 000. The runs consistently achieved effective sample size (ESS) values in the thousands or better, with consistent estimates, indicating that convergence had been achieved. We then calculated the overall number of migrants per generation for each data set following Wright *et al.* (2005), by summing the N_em in each direction.

Results

Comparison of the microsatellite genotypes, mtDNA haplotypes and sexes of samples collected within each area identified 30 of the 206 samples as duplicates. Based on seven microsatellite loci, the average probability of identity $P_{(ID)}$ was 2.5×10^{-8} and the average probability of identity assuming full-siblings Sib $P_{(ID)}$ was 1.6×10^{-3}, indicating that samples with matching genotypes belong to the same individual; thus, these duplicate samples were removed from the analysis. Due to low-quality amplification of mtDNA sequences in six samples, a total of 170 samples were actually used in the mtDNA analysis. Of the 176 samples remaining after removal of duplicates, only two failed to amplify at more than 5 loci, leaving 174 samples in the final microsatellite data set (Table 3). Of the total number of individuals sampled, 74 were identified as females and 76 as males; the sex determination of 26 samples was not possible.

Genetic diversity

A total of 53 haplotypes defined by 46 variable sites (six singleton and 40 parsimony informative sites) were detected. All haplotypes were the same as those that had been previously identified by LeDuc *et al.* (2007), Sremba *et al.* (2012) and Torres-Florez *et al.* (2014) (Genbank Accession nos HQ130726 to HQ130731; EU093921 to EU093962; JX035887 to JX035890; JN801048 to JN801070; and KC116222 to KC116224). The overall nucleotide diversity (π) was 1.51 (SD = 0.081) and the haplotypic diversity (H) was 0.954 (SD = 0.007). Similar levels of genetic diversity were observed within each sampling area, with the exception of the ETP, which had the lowest diversity (Table 3). The π values by area ranged from 0.453% to 1.819%, and H values from 0.773 to 0.968 (Table 3). To compare our data with those previously reported (LeDuc *et al.* 2007; Sremba *et al.* 2012), sequences were trimmed to 360 bp; this made haplo-

Table 3 Mitochondrial (mtDNA) control region and microsatellite diversities at each area studied: *N*, number of individual samples used; π, nucleotide diversity; H_d, haplotypic diversity; *H*, number of haplotypes; *k*, average number of alleles; H_o, observed heterozygosity; H_e, expected heterozygosity; *A*, allelic richness

	Control Region				Microsatellites				
Population	*N*	π	H_d	*H*	*N*	*k*	H_o	H_e	*A*
Antarctica	78	0.01819 (0.00102)	0.968 (0.007)	36	78	11.714	0.751	0.751	8.018
Northern Chile	19	0.01031 (0.00232)	0.906 (0.036)	9	19	6.286	0.721	0.706	6.210
ETP	25	0.00453 (0.00144)	0.773 (0.00301)	7	25	6.286	0.742	0.733	5.998
Southern Chile	48	0.01105 (0.00121)	0.895 (0.019)	12	52	7.857	0.695	0.739	6.399
Overall	170	0.01532 (0.00081)	0.955 (0.007)	51	174	8.035	0.727	0.730	7.535

ETP, eastern tropical Pacific.

types BMCH05, BMCH06, BMCH07 and BMCH08 equivalent to haplotypes previously coded in LeDuc *et al.* (2007) as P, S, O and T, respectively. The four areas (ANT, SCh, NCh and ETP) had 36, 13, 9, and 7 mitochondrial DNA lineages, respectively, with several haplotypes shared between them. Noteworthy, (i) the haplotype R was the only haplotype shared among all four areas, being present just once in ANT (and was also the most common haplotype together with haplotype Q); (ii) haplotype Q was present in all areas but ANT; (iii) almost all of the haplotypes found in the ETP and NCh were also found in SCh (except one and two, respectively); (iv) haplotype D was shared only between ANT and NCh (Table S1, Supporting information).

No evidence for null alleles, allelic dropout or stutter bands was found in the microsatellite data set. All seven loci were polymorphic within each sampling area. HWE was not significantly different from null hypothesis for all populations after corrections for multiple comparisons, and no evidence of LD was detected after the sequential Bonferroni correction was applied. The observed heterozygosity across all areas was 0.727, ranging from 0.695 in SCh to 0.751 in ANT (Table 3). No differences between expected and observed heterozygosities were found.

Genetic diversity was comparable among the four areas in terms of allelic richness (*A*) and number of alleles (*k*) (Table 3). The global allelic richness across all loci calculated for a minimum sample size of 19 individuals (the smallest sample size) was 7.486, ranging from 5.998 in the ETP to 8.018 in ANT (Table 3). No significant differences in allelic richness were found after pairwise comparisons among regions. The average number of alleles (*k*) per area ranged from 6.286 (NCh and ETP) to 11.714 (ANT). A total of 32 private alleles were observed in ANT, while four were observed in SCh, one in NCh and one in the ETP.

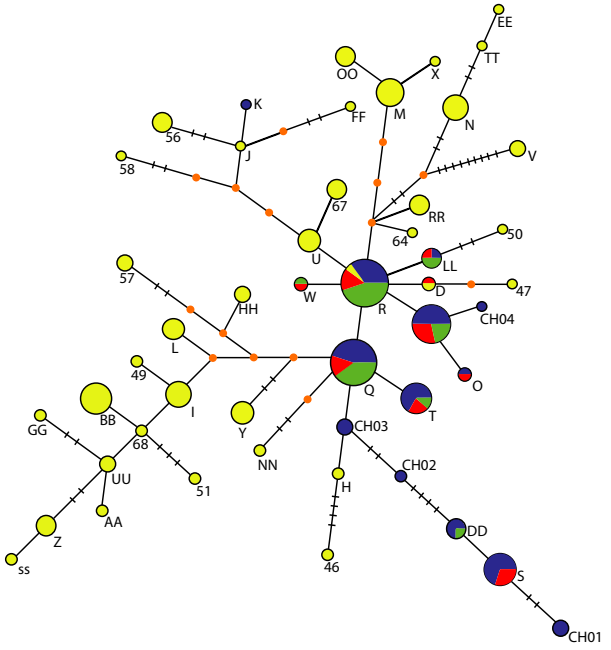

Fig. 2 Genetic relationship among blue whale populations based on 49 mtDNA haplotypes characterized using the control region. The size of each circle reflects the frequency of each haplotype, and colours represent the proportion observed in each population (Antarctica: yellow; Southern Chile: blue; Northern Chile: red; East Tropical Pacific: green). Each cross bar in connecting lines represents one additional mutational step; no bars between haplotypes represent a single mutational event.

Population differentiation and effective number of migrants

A large number of haplotypes were shared among SCh, NCh and the ETP, while only two (D and R) were shared between any of those areas and ANT. The median-joining network shows that most of the ANT haplotypes arise from haplotypes R and Q and are very dissimilar from the other SCh/NCh/ETP haplotypes (Fig. 2).

The AMOVA showed a significant differentiation for mtDNA variation among the four areas, both at the nucleotide (global Φ_{ST} = 0.138; $P < 0.01$) and haplotype levels (global F_{ST} = 0.061 $P < 0.01$). Pairwise comparisons based on Φ_{ST} showed that ANT samples differed significantly from the other three sample areas, while SCh differed slightly from ETP. The same pattern was observed for F_{ST} for the control region (Table 4).

Significant population differentiation was found among all areas (global F_{ST} = 0.018; $P < 0.05$) using the multilocus genotypes. Pairwise comparisons revealed significant differences between ANT and the other three areas ($P < 0.01$), but not among SCh, NCh and ETP (Table 4).

Bayesian clustering analyses indicated that the most likely number of population clusters present in the full data set was two (Pr $(1 \mid X)$ $K = 2$; Fig. 3A, B). These two clusters corresponded to (i) ANT and (ii) SCh, NCh and ETP. To test whether the second cluster was composed of more than one subcluster, ANT samples were removed and the analysis rerun. However, the results were inconsistent and therefore unreliable ((Pr $(1 \mid X)$ $K = 6$).

Given the two clusters previously described, most of the individuals (97%) showed a greater than 90% probability of membership to the cluster where they were sampled. A small proportion of individuals (3%) showed a probability of 90% or greater of belonging to a cluster other than that in which they were sampled and were considered possible vagrants. Two individuals from Antarctica and three from SCh/NCh/ETP fit this criterion (Table 5).

Based on the Bayesian coalescence method, the estimates of effective migrants exchanged per generation (N_em) between the areas are shown in Table 6 and Fig. 4A, B. Of the two models tested, (i) ANT vs. SCh+NCh vs. ETP and (ii) ANT vs. SCh+NCh+ETP, the former showed high confidence interval (CI) and there was no convergence for some parameters, resulting in a low migration estimates due to high gene flow between SCh+NCh and ETP at mtDNA haplotypes only, while it achieved reliable estimates at microsatellite markers. This corroborates earlier results reflecting two different clusters: ANT and SCh+NCh+ETP. For the second model tested, the CIs overlapped in all pairwise comparisons, but some general trends were evident: (i) greater migration (i.e. gene flow) rates for biparentally inherited markers than maternally inherited ones, (ii) relatively low rates of female migration between ANT and SCh+NCh+ETP and (iii) the overall number of migrants per generation was higher from SCh+NCh+ETP to ANT than vice versa.

Table 4 Pairwise comparisons of F_{ST} between areas using seven microsatellite loci (below diagonals) and the control region (above diagonals: F_{ST}/Φ_{ST})

	Eastern Tropical Pacific	Northern Chile	Southern Chile	Antarctica
Eastern Tropical Pacific		0.021 0.035	0.029 0.107*	0.117* 0.157*
Northern Chile	0.005		−0.025 −0.009	0.058* 0.139*
Southern Chile	0.004	0.0013		0.066* 0.155*
Antarctica	0.028*	0.024*	0.025*	

*$P < 0.05$.

Discussion

Genetic diversity in the Southeast Pacific and Antarctica

There is a great concern about the impact of human activities on the genetic diversity of cetacean populations (Baker *et al.* 1993; Caswell *et al.* 1999), particularly

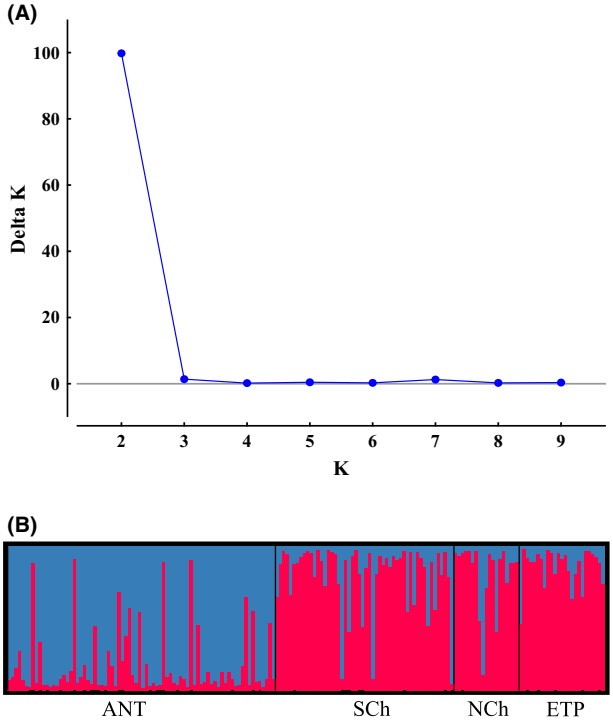

Fig. 3 Assignment probabilities of individuals to putative population clusters using an admixture model. (A) Delta K values, (B) average probability of membership (y-axis) of individuals (n = 174, x-axis) in K = 2 clusters as identified by STRUCTURE.

Table 5 Evidence of possible vagrants detected in different sampled areas

Sample id	Sampling area	Possible belonging area	Haplotype	Sex	Date	Location
13186	ANT	SEP	M	Male	01-Dec	63°05′S; 93°18′W
51458	ANT	SEP	N	Female	21-Jan	65°07′S; 135°34′E
11172	SEP	ANT	K	Male	05-Jan	39°58′S; 74°09′W
023	SEP	ANT	DD	Male	29-Mar	43°30′S; 73°30′W
034	SEP	ANT	T	Male	04-Feb	43°30′S; 73°30′W

ANT, Antarctica.

Table 6 Pairwise comparisons of N_em between the two resulted clusters (ANT and ETP-NCh-SCh) using (A) mitochondrial DNA and (B) nuclear DNA markers

(A)	N_em_{12}	N_em_{21}	97.5% CI	N_fm_{12}	N_fm_{21}
ANT	0.10		0–2.16	0.05 (0–1.08)	
ETP +NCh +SCh		6.84	2.08–14		3.42 (1.94–7)

(B)	N_em_{12}	N_em_{21}	97.5% CI
ANT	14.37		5.9–27.09
ETP+NCh +SCh		28.66	11.55–60.31

ANT, Antarctica; ETP, eastern tropical Pacific; N_em, number of migrants per generation; N_fm, number of female migrants per generation.

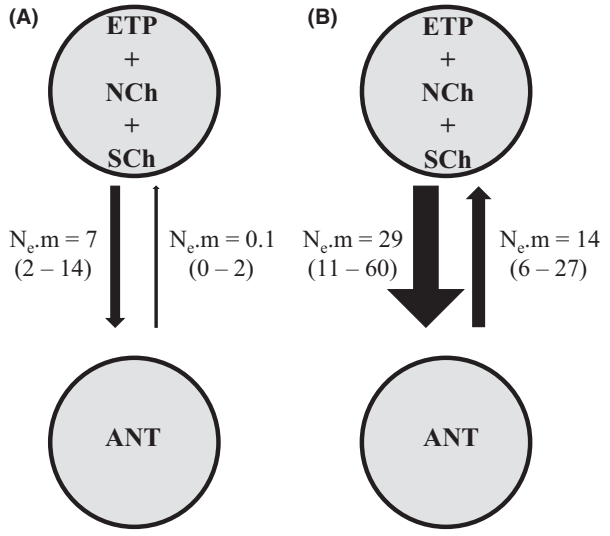

Fig. 4 Number of migrants between both clusters previously identified (ANT vs. ETP-NCh-SCh) using (A) mitochondrial DNA data and (B) Microsatellites data. N_em = total number of migrants per generation.

as small population sizes can lead to significantly reduced genetic diversity, a genetic erosion that can negatively impact the fitness of individuals when responding to diseases or heterogeneous environments (Frankham 2005; Willi *et al.* 2006; Reusch & Wood 2007).

Although blue whales were caught throughout their geographical range during the whaling era, the vast majority of captures were made in Antarctica, which reduced populations of this subspecies to less than 1% (*c.* 360 individuals) of the original numbers (Branch *et al.* 2004). Similarly, blue whales along the Chilean coast were decimated to levels thought to be between 7.2% and 9.5% of their original population size. This population was estimated to number approximately 300 animals in 1997–1998 (Williams *et al.* 2011), although this estimate must be interpreted with care given that it does not encompass the entire range of blue whales in Chilean waters. In addition, Galletti Vernazzani *et al.* (2012) reported 363 individual blue whales photo-identified off Southern Chile (Isla Grande de Chiloé west coast) from 2004 to 2010. This might suggest an increase of the Chilean blue whale population, thus highlighting that the Southern Chile feeding area comprises not just Corcovado Gulf, but also Isla Grande de Chiloé.

Although the Antarctic population was more severely depleted than the Chilean population, one would expect levels of genetic diversity in the Antarctic region ($\pi = 1.8$) to be higher than those found in southern and northern Chile ($\pi = 1.1$ and 1.0, respectively), because the prewhaling and current population sizes in Antarctica were/are possibly two or more orders of magnitude larger than the Chilean blue whale population (Branch *et al.* 2004; Williams *et al.* 2011). When this comparison is made between the two areas studied in Chile, genetic diversity indices were of about the same order of magnitude. Although whaling areas along the Chilean coast were geographically separated (Aguayo 1974; C. Allison, personal communication), the similarity in diversity indices between the northern and southern Chilean sample sets is consistent with these whales belonging to a single panmictic unit.

Although neither Antarctic nor Chilean blue whale breeding grounds are known, the ETP could be the

breeding area for blue whales coming from any of these feeding aggregation areas (e.g. Antarctica, Southern Chile, Northern Chile) (Mackintosh 1942; Reilly & Thayer 1990). If the ETP is the breeding area of whales utilizing any of those feeding grounds, we would expect to recover about the same level of genetic diversity. However, the haplotype diversity found among the ETP samples was lower than that found among any of the three feeding areas. One possible explanation for this finding could be that some Southern Chile/Northern Chile blue whales consistently utilize additional areas within the ETP that were not sampled in this study, limiting the chance of recovering the same genetic diversity at the ETP than in the feeding grounds.

The high genetic diversity observed in ANT and ESP (ETP, Northern Chile, Southern Chile) populations may be due to multiple factors, including some or all of the following: long generation times (*c.* 31 years), few generations since the end of the whaling era, substructuring of Antarctic population, hybridization between some individuals of ANT and Australia populations, and large prewhaling population size of ANT blue whales in comparison with ESP population, among other reasons (Attard *et al.* 2012; Sremba *et al.* 2012; Torres-Florez *et al.* 2014).

In terms of nDNA, the genetic diversity at the four areas exhibited similar levels of heterozygosity, although the allelic richness was higher in Antarctica, where a large number of private alleles were detected. However, the number of alleles identified among the Southern Chile samples is greater than that identified among the ETP and Northern Chile sample sets; more exhaustive sampling is needed in these two areas to establish whether alleles observed in Southern Chile are also present in the other two areas.

Population differentiation and patterns of gene flow

Both the mtDNA and nDNA analyses identified significant differences between ANT and the other three studied areas, but not between the two areas in Chile or between them and the ETP. These results suggest that Southern and Northern Chile blue whales may belong to a single population migrating from mid-latitude feeding areas to ETP waters to breed, a common behaviour observed in other whales (Stone *et al.* 1990; Rasmussen *et al.* 2007; Irvine *et al.* 2014). This association can be better visualized in the median-joining network (Fig. 2), which groups together most of the haplotypes from SCh, NCh and ETP, with all the ETP haplotypes being represented in SCh and/or NCh areas. This conclusion should be taken with some caution in the absence of samples immediately north of the equator, and taking into account other lines of evidence (satellite tagging, photo-ID, acoustics, sighting survey data) which suggest that the ETP is also being used by eastern North Pacific blue whales (ENP) (Mate *et al.* 1999; Stafford *et al.* 1999; Bailey *et al.* 2009).

When the Bayesian clustering analyses were conducted without a priori assignment to populations (i.e. areas), two distinctive clusters were revealed corresponding to (i) ANT and (ii) ESP (SCh, NCh and ETP). Interestingly, Hucke-Gaete (2004) was able to track some blue whales outfitted with satellite transmitters in southern Chile, and one individual migrated north to the Nazca Ridge before the tag failed (23°S and 1200 km offshore). Therefore, based on our results as well as on satellite tracking, one may expect that the blue whale distribution area south of the equator is more extensive than predicted and not restricted only to waters offshore of Ecuador and northern Peru. However, in order to accomplish a complete blue whale population analysis, samples from the ENP and the northern part of the ETP area (e.g. Costa Rica Dome) should be included to discriminate whether the ETP (including Costa Rica Dome, Ecuador and Northern Peru waters) is the breeding area of both northern and southern blue whale populations, as proposed by Reilly & Thayer (1990). An important consideration for testing the predictions of this hypothesis would be the timing of the sampling, which could reveal that South Pacific blue whales remain in the tropics until late austral spring, at which time they would begin their southward migration, taking advantage of the high productivity in the Humboldt Current and spreading throughout the Southeast Pacific (Hucke-Gaete 2004; Williams *et al.* 2011).

Although the differentiation analyses using F_{ST} were similar for both markers, as reported for other whale species (Hoelzel *et al.* 2002), calculations of the effective number of migrants (N_em) between the ANT and ESP clusters suggest a stronger population structure when maternally inherited markers are analysed, with only a low number of migrants estimated based on mtDNA and a larger number of migrants indicated in the nDNA analyses. As these two markers are inherited in different ways (maternal vs. biparental, respectively), the difference between the markers could be explained by the degree of philopatry to the feeding grounds by the mothers and its calves, which learn the migratory destination through maternal experience, as seen in other cetacean species such as the sperm whale and the humpback whale (e.g. Lyrholm *et al.* 1999; Baker *et al.* 2013). Thus, we would expect to observe a geographic segregation at mtDNA, but not necessarily at nDNA markers. Also, the mtDNA sequences studied may not have been sufficiently informative to estimate all the

parameters for a complete migration model (i.e. considering the four different areas independently). In addition, mtDNA is expected to produce a level of differentiation about four times greater than microsatellites as the effective population size of nuclear markers (i.e. microsatellites) is four times greater than that of mtDNA (Avise 2004). The smaller N_em values produced by the mtDNA analysis, compared to those produced by the microsatellites, suggest a higher level of ongoing or historical inter-regional male gene flow. The results suggest that very little to no female interchange occurs between Antarctica and ESP. Some males sampled throughout the ETP may feed in other grounds than NCh-SCh (e.g. Antarctica or eastern North Pacific) and can act as dispersal elements (e.g. Cassens *et al.* 2005). The latter can be supported by the sex of the vagrants (Table 5), which is biased to males and by the historical data obtained during the whaling era through the use of Discovery marks, when extensive longitudinal movements (in excess of 100°) were found among blue whales marked in Antarctica (Brown 1954; Branch *et al.* 2007b).

In addition, although ANT is different from the ESP cluster, there is still some degree of gene flow, and it is possible that some males from other areas (e.g. ANT) use the ETP to breed, which may explain the observed number of migrants between ANT and SEP. However, this last explanation needs further study. Gilpatrick & Perryman (2008) found no evidence of the Antarctic blue whale morphotype among aerial photographs of blue whales in the ETP (off northern Peru and the Galapagos), although only a small sample size was available. As well, acoustic records of Antarctic blue whales in low-latitude waters of the eastern tropical Pacific (Stafford *et al.* 2004) could correspond to vagrants or admixed individuals such as described in Australia feeding ground (Attard *et al.* 2012).

Finally, we can postulate that the two blue whale population clusters found (ANT and ESP) could be the consequence of a recent subdivision, which might ultimately lead to a subspeciation process among ESP blue whales as previously proposed by different authors (see Branch *et al.* 2007b) and seen in other whale species (e.g. Bryde's whale; Kershaw *et al.* 2013). However, the few samples from the Antarctic Peninsula and the South Pacific Gyre could be precluding us from clearly establishing that blue whales use discrete breeding areas in yet unknown locations, thus uncovering the true phylogeographic patterns in these regions.

Management implications and further research

Blue whales became one of the main whaling resources during the 20th century (Mackintosh 1942; Clapham *et al.* 1999). Despite this, population boundaries for Southern Hemisphere blue whales are not well understood. Our results suggest that the Southern Chile, Northern Chile and ETP blue whales sampled for this study may belong to a unique genetically differentiated population from the ANT (Southern Ocean) population. Furthermore, this population (ESP) may make use of resources in a different way due to alternative life history strategies; for instance, selecting and exploiting predictably productive areas located in mid-latitudes in sequential or simultaneous ways and migrating to lower latitudes during the austral winter, as previously suggested by Hucke-Gaete (2004), instead of undertaking the long conventional migrations reported for Antarctic blue whales.

Although our data support the hypothesis that blue whales sampled off SCh, NCh and the ETP belong to a unique genetically differentiated population from the Antarctic blue whale population, we strongly recommend carrying out a more systematic sampling effort, particularly throughout those areas currently represented by low sample sizes, as the South Pacific Gyre, the eastern tropical Pacific and the waters lying west of the Antarctic Peninsula. Likewise, it would be especially interesting to carry out an investigation that includes eastern North Pacific samples with the aim of better understanding the importance of the ETP for blue whale populations from both hemispheres. In perspective, conservation efforts should be focused on the maintenance of genetic diversity by minimizing anthropogenic threats and maximizing habitat protection throughout both feeding and breeding areas. Establishing steppingstones of protected habitat, probably in the form of coastal and pelagic Marine Protected Areas, would help to secure a safer and brighter future for the largest mammal ever to have existed on Earth.

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J.P.T.-F. had the original idea and designed the study under the supervision of C.C.F., R.H.-G. and H.C.R. J.P.T.-F. and R.L. performed the laboratory analyses. J.P.T.-F., L.B.-R., R.H.-G., B.T. and R.L. performed the sampling or contributed with samples. J.P.T.-F., R.L., A.L. and L.E.P. conducted the population genetic analyses. J.P.T.-F. wrote the manuscript with assistance from A.L. and H.C.R. H.C.R. and C.C.F. contributed equally as heads of their respective programs.

Data accessibility

The data used in the present study was deposited in the Dryad repository and received the DOI number doi:10.5061/dryad.bc558. The deposit file contain data of each sample with the locality (major area), latitude and longitude, multilocus genotype, sex, haplotype name and Genbank accession number. A document with the D-loop sequence of each sample was also deposited. Finally, the files (parmfile and infile) to run Migrate with the blue whale data were also deposited in the dryad repository.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Frequencies of haplotypes from each region.