TEMPORAL PATTERNS OF POPULATION STRUCTURE OF HUMPBACK WHALES IN WEST COAST OF AFRICA (B1-B2 SUB-STOCKS) BASED ON MITOCHONDRIAL DNA AND MICROSATELLITE VARIATION

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ABSTRACT

In the eastern South Atlantic Ocean (Region B) humpback whales are distributed along the west coast of South Africa and wintering in the Gulf of Guinea. The most recent data available suggest that Breeding stock B is possible sub-structured, considering B1as a breeding ground and B2 as a summer feeding ground and a winter migration corridor. However questions regarding to the population structure of B stock remain unclear. Here, we present an approach of temporal population structure in humpback whales in west coast of Africa using maternally (mitochondrial DNA control region) and bi-parentally (10 microsatellites) inherited markers. We amplified, sexed, genotyped and sequenced 1947 samples from Gabon, Angola, São Tomé and West South Africa. The results showed significantly differentiation based on haplotype frequencies (F_{ST}) and molecular distances (Φ_{ST}) between B1 and B2; similar results were obtained with the microsatellite data. For the temporal analysis, significant results were obtained only for haplotype frequency statistics (F_{ST}), where west South Africa seasons were significantly different from seasons in Gabon. When the samples were stratified by sex, significant differentiation at the haplotype level were found for both sexes and nucleotide level only for females. The direct detection of movements by genetically identified individuals, females and males, suggests that interchange occurs between regions. However, all movements to dates are from a northbound to southbound direction. The results presented here indicate that there is some spatial and temporal population substructure in humpback whales in B stock.

INTRODUCTION

Southern Hemisphere humpback whales (*Megaptera novaeangliae*) undertake annual migrations from summer high latitude feeding grounds to winter breeding grounds in tropical and sub-tropical waters (Mackintosh, 1942). In the Southern Hemisphere, humpback whale distribution on wintering grounds occurs within seven Wintering Regions (A-G) based on low latitude distributions (IWC, 1997).

In the eastern South Atlantic Ocean (Region B) whales are distributed from the west coast of South Africa (Olsen, 1914; Matthews, 1938) to wintering areas in Gulf of Guinea (Budker and Collignon, 1952; Van Waerebeek *et al*. 2001; Walsh *et al*, 2000). Recent studies have been conducted in Gabon and Angola confirming that these areas are used as breeding grounds by these animals (Rosenbaum *et al.*, 2006; Best *et al*, 1995; Best *et al,* 1999). Humpback whales have been reported in

areas off the West coast of Africa, and it seems that some proportion of the animals that pass through coastal waters of Angola and Gabon may be visiting other known humpback areas in the region, including the Bight of Benin, São Tomé, Bioko, Equatorial Guinea, Cameroon and Congo (Best *et al.*, 1995; Best *et al.*, 1999; Rosenbaum *et al.*, 2001; Van Waerebeek *et al.*, 2001, Carvalho, 2004, Picanço *et al. in press*).

Recently it has been postulated that there may be two breeding stocks off West Africa: Breeding Stock B1 which is thought to winter (June-October) along central African coasts and around islands of the Gulf of Guinea and Breeding Stock B2 which is thought to winter off the west coast of South Africa, Namibia, and possibly some part of the Angolan coast, though its northerly extent remains undefined (SC/58/Rep.5). The most recent data available suggest that Breeding stock B is possibly sub-structured (Barendse *et al.* 2006; Pomilla *et al.* 2006, Rosenbaum *et al.* 2006; SC/58/Rep.5; Rosenbaum et al. submitted) with B1 as a breeding ground and B2 as a summer feeding ground and a winter migration corridor. Some degree of movements of animals has been detected between Gabon (B1) and west South Africa (B2): two females (one with a calf) were sighted in Gabon and a few months later they were re-sighted off the west coast of South Africa. Significant differences have been found in mitochondrial DNA haplotype frequencies between these two regions (Rosenbaum *et al.* 2006; Rosenbaum et al. submitted). On the west South African coast, recent observations indicate a regular and extended presence of whales as late as February and 19th century catches during the summer season off Namibia suggest that this area besides functioning as a migration corridor, may also serve as a summer feeding ground at least for some animals (Barendse *et al*., 2006; Best *et al*. 1995: Findlay, 2000).

A number of questions regarding the population structure of B stock remain unclear. One possible explanation for some of these differences could be a temporal structuring of this stock. Evidence of temporal and geographic sub-structure has been found in other wintering grounds (Smultea, 1994; Medrano-Gonzalez et al. 1995, Brown *et al.*, 1995, Dawbin, 1997). There are also significant differences in the time period that the whales arrive at and depart from a breeding area based on their reproductive status (Dawbin, 1997). Differences in group size and composition during a season (Smultea, 1994) and temporal distribution of matrilines at breeding grounds have also been demonstrated (Medrano-Gonzalez et al., 1995).

The present report is an update of SC60/SH44 (Carvalho *et al*., 2008) report, using a larger number of samples and using mitochondrial DNA and microsatellites markers, and provides greater resolution concerning population structure of humpback whales on the west coast of Africa.

MATERIALS AND METHODS

- ***Data collection and molecular analysis***

Sample collection, DNA extraction and sex determination

Samples were collected from humpback whales at five sampling sites on the west coast of Africa (Fig. 1, table I). Skin tissues were mostly obtained using the biopsy dart procedure (Lambertsen 1987), but included sloughed skin and stranded specimens. Samples were preserved in 95% Ethanol or salt saturated 20% Dimethyl Sulfoxide solution (DMSO) and later stored at –20ºC until processed. Total genomic DNA was extracted from the skin tissues using proteinase K digestion followed by a standard Phenol/Chloroform extraction method (Sambrook et al. 1989) or using DNAeasy tissue kit (Qiagen) following manufacturer's protocol. Sex determination was carried out by PCR amplifications, followed by TaqI digestion, of the ZFX/ZFY region of the sex chromosomes (Palsbøll *et al*., 1992) or using multiplex PCR amplification of the ZFX/ZFY sex linked gene (Bérubé and Palsbøll, 1996).

Mitochondrial DNA sequencing

A segment of 550 bp fragment within the mtDNA control region (Kocher *et al*., 1989; Baker *et al*., 1993) was amplified by the Polymerase Chain Reaction (PCR) with primers Dlp 1.5 and Dlp 5 described by Baker *et al*. (1993). Reactions of 25µL total volume containing 50mM KCL, 10mM Tris-HCL pH 8.8, 2.5 mM MgCl2, 200µM of each dNTP, 1.0 µM of each primer, and 0.05 U/µl Taq polymerase were conducted either on a Perkin-Elmer thermocycler or an Eppendorf Gradient Mastercycler following the

standard PCR procedures (94˚C for 4 min for initial denaturing, followed by 30 cycles of 94˚C for 45 s, 54˚C for 45 s, and 72˚C for 45 s). Amplified PCR products were cycle sequenced with dye-labeled terminators. Reactions were run on an ABI3700 or ABI3730 DNA analyzer (Applied Biosystems, Inc). PCR amplifications included negative control reactions to check for exogenous contamination.

Microsatellite genotyping

Ten microsatellite loci, which have proven to be polymorphic in humpback whales has used in this study: GATA028, GATA053, GATA417 (Palsbøll *et al.* 1997), 199/200, 417/418, 464/465 (Schlötterer *et al.*, 1991), EV1Pm, EV37Mn, EV94Mn, EV96Mn (Valsecchi & Amos, 1996). The 5'-end of the forward primer from each locus was labeled with a fluorescent tag (HEX, 6-FAM, and TET, Qiagen-Operon; NED, Applied Biosystems, Inc). PCRs were carried out in a 20µl volume with the following conditions: 50 mM KCl, 10 mM Tris-HCl pH 8.8, 2.5-3.5 mM $MgCl_2$, 200 µM of each dNTP, 0.4 µM of each primer, and 0.025 U/µl *Taq* Gold polymerase (Perkin-Elmer). Amplifications were completed in either a Perkin-Elmer 9600 thermocycler or an Eppendorf Gradient Mastercycler, after optimization of published annealing temperatures and profiles. PCR products were loaded with the addition of an internal standard ladder (ROX, Applied Biosystems, Inc) on an ABI 3700 or ABI 3730 DNA analyzer (Applied Biosystems, Inc). Microsatellite alleles were identified by their sizes in base pairs using the software GENEMAPPER 4.0 (Applied Biosystems, Inc).

- ***Statistical analysis***

Sample size

To limit genotyping errors, some specific guidelines were used during laboratory work and scoring procedures. First, given the elevated number of samples, automation was introduced whenever possible during PCR setup and manipulation of genomic DNA or PCR products. Negative controls were run at the PCR step to control for exogenous contamination. Two reference samples of known allele size were added to each amplification and subsequent analyses to standardize scoring. Scoring was automated in GENOTYPER 2.1 and GENEMAPPER 4.1, and allele sizing was successively checked by hand. Samples that yielded ambiguous allele peaks were repeated a second time. The presence of null alleles was be monitored comparing the genotypes of known mother-offspring pairs. For detecting errors in our database, we will used DROPOUT 1.3 program (McKelvey & Schwartz, 2005), that identifies both loci and samples that likely contain errors affecting capture-mark-recapture; this program uses a "*bimodal test*" that enumerates the number of loci different between each pair of samples, and provides information to determine the source of the errors, and uses a "*difference in capture history test*" to determine those loci producing the most errors, this test allows one to determine that a data set is error-free.

Duplicate samples within each population were detected from microsatellite genotype identity using MS_TOOLKIT (Park, 2001) and DROPOUT 1.3, and were excluded from the analysis. The probability of different individuals in each population sharing the same genotype by chance (Probability of Identity, PI) was estimated using the Excel add-in GENEALEX 5.1 (Peakall and Smouse, 2001).

Data analysis- Genetic variation

DNA sequence variation patterns were characterized into mtDNA haplotype definitions for this species as previously recorded in Rosenbaum *et al*. (2002). From the 520 bp mtDNA Control Region fragment, a 477 bp consensus region that contains the majority of variable nucleotide positions in the mtDNA control region of humpback whales was examined for all samples (Baker *et al*., 1993). Sequences were aligned and assembled using *Sequencher 4.5* (Gene Codes, Inc). Sequences for this portion of the mtDNA Control Region were maintained for each individual in *MacClade 4.08*. Matching of sequences to a haplotype was done using *Collapse 1.2.*

The diversity of humpback whale mtDNA sequences was estimated at both the haplotype and nucleotide level (Nei, 1987) using *Arlequin 3.01.* (Schneider *et al*., 2000). At the haplotype level, diversity and its standard error were calculated without reference to the genetic distance (i.e., number of

nucleotide substitutions) between two mtDNA sequences. At the nucleotide level, diversity (Nei, 1987) and its standard error for both sampling and stochastic processes were calculated from the pairwise differences between the mtDNA sequences.

Microsatellite variation was measured using the mean number of alleles per locus (*K*), the observed heterozygosity (H_o), and the heterozygosity expected (H_e) under Hardy–Weinberg assumptions (Nei, 1987) using the Excel add-in MS_TOOLKIT package (Park, 2001). Deviations from HWE and LD were tested by the Markov chain method implemented in GENEPOP (Guo and Thompson, 1992; Raymond and Rousset, 1995).

Data analysis-Population Structure

For mtDNA sequences, genetic variation was estimated as haplotype diversity (*h*) and nucleotide diversity (π) (Nei, 1987) over all samples, for each sampling site using ARLEQUIN 3.0.1 (Excoffier *et al.,* 1992, Schneider *et al.,* 2000). Nucleotide diversity and its standard error were calculated from pairwise differences between mtDNA sequences and haplotype diversity and its standard error were calculated without regard to the genetic distance. The diversity and geographic variation of haplotypes were quantified using the Analysis of Molecular Variance procedure (AMOVA; Excoffier *et al.,* 1992) as implemented in the software ARLEQUIN 3.01. AMOVA was performed incorporating both Φ_{ST} and F_{ST}. The Φ_{ST} takes into account the relationships between haplotypes based on molecular distance (Excoffier *et al.,* 1992), whereas the F_{ST} considers only the difference in overall haplotype frequencies (Weir and Cockerham, 1984). The statistical significance of these values was tested by 10000 permutations.

For microsatellites, AMOVA was performed using ARLEQUIN 3.01.The distances between microsatellites genotypes were estimated with F_{ST} (Weir and Cockerham, 1984) and R_{ST} (Slatkin 1995; Michalakis and Excoffier, 1996).

For tests of spatial structure, we tested the B1 (samples from Gabon, São Tomé) and B2 (samples from west coast of South Africa) division, adding Angola samples (Cabinda, n=12) to one or another sub-stock. For the temporal analysis the data were partitioned according to the seasons for each area, taking into account the data available for these regions and the sampling months that we have for these analyses we only used Gabon and South Africa data. Based on distributional data, whales sampled off the coast of Gabon were divided into "Early" season (July and August) and "Late" season (September and October). For west South Africa the season was divided as "Regular" season (July- October) and "Late" season (November-February). In this way the regular season in South Africa corresponded to the entire season in Gabon. Data were further divided into strata to evaluate the effect of sex (males, females).

Direct measure of dispersal

Movements of specific individuals between different areas are suggestive of current interchange. To document such movements we used a genetic capture –recapture approach based on the attainment of unique individual genetic profiles, and consisting in searching for genotype matches between different areas (Waits *et al*, 2001). We used the Excel add-in MS_TOOLKIT package (Park, 2001) and DROPOUT 1.3 program (McKelvey & Schwartz, 2005).

RESULTS

Sample size and sex ratio

Table I illustrates the sample sizes for each sampling site and for the divisions described above, and the number of known males and females. The 1947 tissue samples were inferred to represent 1718 whales. Average probability of identity (PI) for each population was small enough to exclude within-site re-samples with confidence. In Gabon the sex ratio is biased towards males (2.1M:1F), while in South Africa the sex ratio is close to parity (1M:1.1F).

Genetic variation

A consensus region of 477 bp of the mitochondrial DNA control region was assembled in which 145 maternal haplotypes were detected from 19-82 polymorphic sites. Haplotype diversity ranged between 0.9737-1.0000 and nucleotide diversity estimates ranged between 0.02095-0.02245 (Table II).

For microsatellite data, the mean number of alleles (K) across areas averaged 8.3, the largest number of alleles (22) was found in GATA417 for Gabon, the smallest (3) was recorded at locus EV1Pm for Sao Tome and Angola. The mean expected heterozygosity ranged from 0.67 in west South Africa to 0.7819 in Angola. No significant differences were found between the observed heterozygosity (H_0) and the heterozygosity expected (H_e) under Hardy-Weinberg assumptions across loci (Table III).

Population Structure

For B1-B2 analysis, significant differentiation occurred between areas at haplotype level (F_{ST}= 0.068, p=0.0000 for B1 with Angola; F_{ST}= 0.004, p=0.0234 for B2 with Angola) and nucleotide level (Φ_{ST}= 0.0034, p=0.03030 for B1 with Angola; Φ_{ST}= 0.00389, p=0.0284 for B2 with Angola). Similar results were obtained with microsatellites, when we tested in the AMOVA, both F_{ST} (0.00201, p=0.0000 for B1 with Angola and 0.0017, p=0.0000 for B2 with Angola) and R_{ST} (0.00200, p=0.0001 for B1 with Angola and 0.00176, p=0.0000 for B2 with Angola).

For the temporal analysis, significant differentiation was founded between seasons at haplotype level (F_{ST}= 0.00261, p=0.0000), but not at nucleotide level. In the pairwise comparisons based on haplotype frequencies differentiation was founded in all seasons between the two regions. (Table IV), and between "Late" season in west South Africa (WSA) and "Early" season in Gabon related with molecular distances (Table IV).

When the samples were stratified by sex, we founded significant differentiation at the haplotype level (F_{ST} =0,0048) and nucleotide level (Φ_{ST}= 0,00728) for females; for males only haplotype frequencies (F_{ST} = 0,00183) showed differentiation. In pairwise comparisons Females from "Late" season in west South Africa were significantly different from Gabon females (independent of season) and females from "Regular" season in South Africa were different from females in "Late" season in Gabon (Table V). For males (Table VI), the differences were only between males from the two regions.

Direct measure of dispersal

The genetic capture-recapture approach recovered a total of 10 matches (Table VII) between Gabon and west South Africa coast. Two of these had already been described by Pomilla *et al*. (2006). All the movements were from a northbound to southbound direction when considering month of year (and not year itself). Four of them were re-sampled in the same year and the other six were re-sampled in different years (maximum interval of four years between the capture and re-capture). Six were females (one of them with a small calf) and four males. Two of the individuals sampled in Gabon were re-sampled in South Africa in a situation related with feeding behavior (feeding or defecation). Two individuals were sampled in west South Africa and then were re-sampled later (five year's interval) in Gabon. One animal was sampled in west South Africa feeding in a group of 20 whales.

DISCUSSION

The tests for population differentiation of B1 and B2 were significant, based on haplotype frequencies and molecular distances. Similar results have been reported for comparison of regions B1 with B2 using only mtDNA (Rosenbaum *et al.* submitted), with very low estimates of gene flow between the two areas. Based on catch histories, Findlay (2000) suggested a degree of spatial segregation of this stock with some whales using alternate migration routes and thus avoiding capture, (e.g. some animals migrate further offshore and others inshore. Recent telemetry data indicate the same pattern (Rosenbaum & Mate, submitted). Besides the low values, we have got similar population differentiation with the microsatellite data; this result is different from what Pomilla *et al* (2006) described. We found significant differences irrespective of where Angola (Cabinda) samples were grouped, either with Gabon or west

South Africa, as well as for mitochondrial DNA and microsatellites. However the values of F_{ST} were higher, for both markers, when Angola is grouped with Gabon, these are in agreement with Pomilla *et al.* (2006). These differences in results with previous data could be explained since we are using a larger number of samples especially from Gabon, and using the only available samples from Angola (N=12).

For the temporal structure, we founded significant differentiation only at haplotype level, the differences were founded among seasons between areas. This result could be directly affected by how the seasons were divided and the different size of the sample sizes between seasons in Gabon and west South Africa. However in pairwise comparisons among the seasons we founded that animals that are in Gabon in June-July (Early season) were significantly different from the ones that are in summer (December to February) in west South Africa, for both statistics. This means that west South Africa is not a feeding ground destination for the majority of whales migration from Gabon.

When we analyzed the data stratified by sex, significant results were obtained among seasons between areas, for females and males. Based on haplotype frequencies and molecular distances, females that occurred in summer months in west South Africa were different from females from Gabon, independent of season. In Gabon the sex ratio is highly biased toward males, especially in the Late season (2.4M:1F). This could be explained by sampling bias (because big competitive groups are easier to detect), group structure segregation (because not all females migrate to breeding areas each year) (Chittleborough, 1965: Dawbin, 1997; Brown et al 1995) or by some spatial structure among groups within the Gulf of Guinea breeding area, where some groups of animals (like mother-calves or pregnant females) prefer some areas more than others (Smultea, 1994; Morete *et al.*, 2007). In fact differences occur between percentages of mother-calves pairs observed in Gabon (4.8%) (Rosenbaum & Collins, 2006) and off shore in São Tomé and Principe archipelago (65.15%) (Carvalho, 2004). Conversely, the sex ratio on the west coast of South Africa (1M:1,1F – Winter season and 1M:1,2F – Late season) is much more like that of a feeding area, where normally it is biased toward females (Mackintosh,1942; Brown *et al.* ,1995) A lower or even a lack of differentiation for male was expected since the sex with higher dispersal (males in the case of humpback whales) will have a lower between-subpopulations F_{ST} value than the sex that is dispersing less (Prugnolle & Meeus, 2002). For species in which females are philopatric and males disperse, genetic differentiation between populations is expected to be higher when estimated using mtDNA (or another maternal marker) (Prugnolle & Meeus, 2002); comparisons with microsatellite data may be more useful, but contain bi-parental contributions to genetic variance.

Some degree of connectivity between Gabon and west South Africa was demonstrated with direct detection movements by genetically identified individuals between these two areas, both for females and males. All the movements detected are from a northbound to southbound direction (when considering month of recapture). Therefore when recaptures did not occur in the same year, the presumption is that the monthly nature of recaptures (July/Aug in Gabon and October-November-December in South Africa) reflects an overall pattern of movement of some animals from B1 to B2. However this fact could be an artifact of the sample effort in west South Africa, since less effort is done when the animals are migrating in northerly direction (prior to the Regular season in West South Africa). These findings coupled with the feeding observations suggest that while significant differences between B1 and B2 exist, some animals do migrate and feed in the waters off South Africa.

The results presented here indicate that there is some temporal and spatial population substructure in whales in B stock. Additional resolution has resulted from the inclusion of additional samples and molecular data. Forthcoming analyses will help illuminate population-level differences and connectivity between animals from B1 and B2 regions.

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LITERATURE CITED

Baker, C.S., Perry, A., Bannister, J.L., Weinrich, M.T., Abernethy, R.B., Calambokidis, J., Lien, J.,Lambertsen, R.H., Urbán, J., Vásquez, O., Clapham, P.J., Alling, A., O´Brien, S.J and Palumbi, S.R. 1993. Abundant mitochondrial DNA variation and world-wide population structure in humpback whales. *Proceedings Natural Academic Science* 90: 8239-8243.

Barendse J, Best PB, Thornton M. (2006). Preliminary results of photo-identification of humpback whales on the west coast of South Africa. Paper SC/AO6/HW4 presented to the IWC Scientific Committee Workshop on the Comprehensive Assessment of Southern Hemisphere humpback whales. April 2006, Hobart, Australia (unpublished).

Berubé, M. & Palsbøll, P. 1996. Identification of sex in Cetaceans by multiplexing with three ZFX and ZFY specific primers. *Molecular Ecology*, 5, 283-287.

Best, P. B., D. Reeb, M. Morais, and A. Baird. 1999. A preliminary investigation of humpback whales off northern Angola. Paper SC/51/CAWS33 presented to the IWC Scientific Committee, 1999 (unpublished).

Best, P. B., K. Sekiguchi, and K. P. Findlay. 1995. A suspended migration of humpback whales Megaptera novaeangliae on the west coast of South Africa. *Marine Ecology Progress Series* 118, 1-12.

Brown, M.; Corkeron, P.; Hale, P.; Schultz. K. & Bryden, M. 1995. Evidence for a sex-segregated migration in the humpback whale (*Megaptera novaeangliae*). *Proceedings of Royal Society of London, Serie B*, 259, 229-234.

Budker P, Collignon J (1952) Trois campagnes balenieres au Gabon: 1949-1950-1951. *Bulletin de l'Institut d'etudes centrafricaines*, **3**, 75-100.

Carvalho, I. (2004). Ocorrências e comportamentos das baleias corcundas (*Megaptera novaeangliae*), Borowski, 1781), no sul de S. Tomé e Príncipe**.** Dissertação do Mestrado em Etologia. Instituto Superior de Psicologia Aplicada. [In Portuguese]

Carvalho, I; Loo, J.; Pomilla, C.; Leslie, M.; Collins, T.; Barendse, J.; Best, P. & Rosenbaum, H. (2008). Preliminary analysis on temporal variation on mitochondrial DNA diversity of humpback whales on B1 and B2 sub-stocks. Paper SC_60_SH44 presented to the IWC the Scientific Committee, (unpublished).

Chapman, D.G. 1974. Status of Antarctic rorqual stocks. Pages 218-238. In: W.E. Schevill (ed.) *The Whale Problem*. Harvard University Press, Cambridge. 419pp .

Dawbin, W. 1997. Temporal segregation of humpback whales during migration in southern hemisphere waters. *Memoirs of the Queensland Museum*, 42, 105-138.

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

Excoffier, L., P. E. Smouse, and J. M. Quattro. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* 131, 479-491.

Findlay K.P, (2000) A Review of humpback whale caches by modern whaling operations in the Southern Hemisphere. *Memoirs of the Queensland Museum* **47,** 411-420.

Guo S, Thompson E (1992) Performing the exact test of Hardy Weinberg proportion for multiple alleles. *Biometrics*, **48**, 361-372.

IWC. 1997. Report of the scientific committee. Report of the International Whaling Commission 47.
IWC. 2000. Report of the scientific committee. Report of the International Whaling Commission 52.
IWC. 2007. Report of the Workshop on the Comprehensive Assessment of Southern Hemisphere Humpback Whales. Paper SC/58/Rep5.
Lambertsen, R. H. 1987. A biopsy system for large whales and its use for cytogenetics. *Journal of Mammalogy* 68, 443-445.
Mackintosh, N. A. 1942. The southern stocks of whalebone whales. *Discovery Reports* 22, 197-300.
Matthews LH (1938) The humpback whale, *Megaptera nodosa*. *Discovery Reports*, **17**, 7-92.
McKelvey, K.S. & Schwartz, M.K. (2005). DROPOUT: a program to identify problem loci and samples for noninvasive genetic samples in a capture-mark-recapture framework. *Molecular Ecology Notes*, 5: 716-718.
Medrano-González, L.; Aguayo-Lobo, A.; Urbán-Ramirez, J. & Baker, S. 1995. Diversity and distribution of mitochondrial DNA lineages among humpback whales, *Megaptera novaeangliae*, in the Mexican Pacific Ocean. *Canadian Journal of Zoology*, 73, 1735-1743.
Michalakis Y, Excoffier L (1996) A generic estimation of population subdivision using distance between alleles with special reference for microsatellite loci. *Genetics*, **142**, 1061-1064
Morete, M.; Bisi, T. & Rosso, S. 2007. Temporal pattern of humpback whale (*Megaptera novaeangliae*) group structure around Abrolhos Archipelago breeding region, Bahia, Brazil. *Journal Marine Biological Association of United Kingdom*, 87, 87-92.
Nei, M. 1987. *Molecular Evolutionary Genetics*. Columbia University Press, New York.
Nielsen, R., Wakeley, J. 2001. Distinguishing Migration From Isolation: A Markov Chain Monte Carlo Approach. *Genetics* 158:885-896.
Palsbøll PJ, Bérubé M, A.H. L, Jørgensen H (1997b) Primers for the amplification of tri- and tetramer microsatellite loci in baleen whales. *Molecular Ecology*, **6**, 893-895.
Palsbøll PJ, Vader A, Bakke I, Raafat El-Gewely M (1992) Determination of gender in cetaceans by thepolymerase chain reaction. *Canadian Journal of Zoology*, **70**, 2166-2170.
Palsbøll, P. J., P. J. Clapham, D. K. Mattila, F. Larsen, R. Sears, H. R. Siegismund, J. Sigurjonsson, O. Vasquez, and P. Arctander. 1995. Distribution of mtDNA haplotypes in North Atlantic humpback whales: the influence of behavior on population structure. *Marine Ecology Progress Series* 116:1-10.
Park S (2001) MS_Toolkit: Excel add-in tool package for microsatellite data. Genetics Department, Trinity College, Dublin.
Peakall, R and P Smouse. 2001. GenAlEx V5: genetic analysis in Excel. Population genetic software for teaching and research,. Canberra, Australian National University
Picanço, C.; Carvalho, I. & Brito, C. (*In press*). Occurrence and distribution of cetaceans in Sao Tome and Principe tropical archipelago and their relation to environmental variables. *Journal of the Marine Biological Association of the United Kingdom*.
Pomilla C., Best P.B., Findlay K., Kotze P.J.H., Engel M.H., Barendse J., and Rosenbaum H.C. 2004. Population structure of Southern Hemisphere humpback whales from wintering Regions A, B, and C based on nuclear microsatellite variation, pp. 12, Paper SC/56/SH4 presented to the IWC the Scientific Committee, (unpublished).
Pomilla C., Best P.B., Findlay K.P., Collins, T., Engel. M., Minton, G., Ersts, P., Barendse, J., Kotze, P.G.H., Razafindrakoto, Y., Ngouessono, S., Meyer, M., Thornton, M. and Rosenbaum, H. 2006. Population structure and sex-biased gene flow in humpback whales from Wintering Regions A, B, C, and X based on nuclear microsatellite variation. SC/A06/HW38 presented to the IWC Scientific Committee Workshop on the Comprehensive Assessment of Southern Hemisphere humpback whales. April 2006, Hobart, Australia (unpublished).
Prugnolle, F. & Meeus, T. 2002. Inderring sex-biased dispersal from population genetic tools: a review. *Heredity*, 88, 161-165.
Raymond M and F Rousset. 1995. GENEPOP: population genetic software for exact tests and ecumenicism. *Journal of Heredity* **86**: 248-249.
Rosenbaum H.C., Pomilla C.C, Leslie M.C., Mendez, M.C., Best P.B., Collins, T., Engel. M., Ersts, P., Findlay K.P., Bonato, S., Kotze, P.G.H., Meyer, M., Minton, G., Barendse, J., Thornton, M., Razafindrakoto, Y., and Ngouessono, S. 2006. MtDNA diversity and population structure of humpback whales from their wintering areas in the Indian and south Atlantic Ocean (Breeding regions A, B, C and X). IWC Scientific Committee Workshop on the Comprehensive Assessment

of Southern Hemisphere humpback whales. presented to the IWC Scientific Committee Workshop on the Comprehensive Assessment of Southern Hemisphere humpback whales. April 2006, Hobart, Australia (unpublished).

Rosenbaum, H. C., P. B. Best, and C. Pomilla. 2001. A preliminary analysis of mtDNA variation among humpback whales of the southeastern Atlantic Ocean from the wintering grounds along the coast of West Africa. Pp. 8. Paper SC/53/IA32 presented to the IWC Scientific Committee, (unpublished).

Rosenbaum, H. C., P. B. Best, K. P. Findlay, M. H. Engel, C. Pomilla, Y. Razafindrakoto, M. E. Morete, A. C. Freitas, C. S. Baker, C. Jenner, M.N. Jenner, and J. Bannister. 2000. Mitochondrial DNA variation among humpback whales from the wintering grounds in the South Atlantic and Southwestern Indian Oceans. Pp. 13. Paper SC/52/IA11 presented to the IWC The Scientific Committee, 2000 (unpublished).

Rosenbaum, H. C., P. Ersts, Y. Razafindrakoto, G. Sounguet, C. Pomilla, S. Ngouessono, V. Rasoamampianina, and L. White. 2002. Population characteristics, distribution, and relative abundance of humpback whales off the coasts of Madagascar and Gabon: an update on recent and planned research. Pp.13. Paper SC/54/H20 presented to the IWC Scientific Committee, (unpublished).

Rosenbaum, H.C. and Collins, T. 2006. The Ecology, Population Characteristics and Conservation Efforts for Humpback Whales (Megaptera novaeangliae) on Their Wintering Grounds in the Coastal Waters of Gabon. pp. 425-433. In: *Natural History of the Gamba Complex*. Alonso, A. and Campbell, P. (eds). Smithsonian Press, Washington D.C.

Rosenbaum, H.C. and Mate, B. From north of the equator to the Antarctic: unique and unexpected movements for humpback whales off the coast of West Africa and throughout the eastern South Atlantic Ocean. (*Submitted*).

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: a Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press, New York.

Schlötterer C, Amos B, Tautz D (1991) Conservation of polymorphic simple sequence loci in cetacean species., *Nature*, **354**, 63-65

Schlötterer, C., B. Amos, and D. Tautz. 1991. Conservation of polymorphic simple sequence loci in cetacean species. *Nature* 354:63-65.

Schneider S, Roessli D, Excoffier L (2000) ARLEQUIN (Version 2.0): A software for population genetic data analysis. University of Geneva, Geneva, Switzerland.

Schneider, S., D. Roessli, and L. Excoffier. 2000. ARLEQUIN (Version 2.000): A software for population genetics data analysis. University of Geneva, Geneva, Switzerland.

Slatkin M (1995) A measure of population subdivision based on microsatellite allele frequencies. *Genetics*, **139**: 457-462.

Smultea, M. 1994. Segregation by humpback whale (*Megaptera novaeangliae*) cows with a calf in coastal habitat near the island of Hawaii. *Canadian journal of Zoology*, 72, 805-811.

Takahata, N., and S R. Palumbi. 1985. Extranuclear differentiation and gene flow in the finite island model. *Genetic* 109:441-457.

Valsecchi E, Amos W (1996) Microsatellite markers for the study of cetacean populations. *Molecular Ecology*, **5**,151-156.

Van Waerebeek K, Tchibozo S, Montcho J, Nobime G, Sohouhoue P, Dossou C (2001) The Bight of Benin, a North Atlantic breeding ground of the Southern Hemisphere humpback whale population, likely related to Gabon and Angola substocks. Paper SC/53/IA21 presented to the Scientific Committee of the IWC, (unpublished).

Waits L, Luikart G, Taberlet P (2001) Estimating the probability of identity among genotypes in natural populations: cautions and guidelines. *Molecular Ecology*, **10**, 249-256.

Weir BS, Cockerham CC (1984). Estimating F-statistics for the analysis of population structure. *Evolution*, **38**:1358-1370.

Figure 1- B1 and B2 areas in the South Atlantic Ocean.

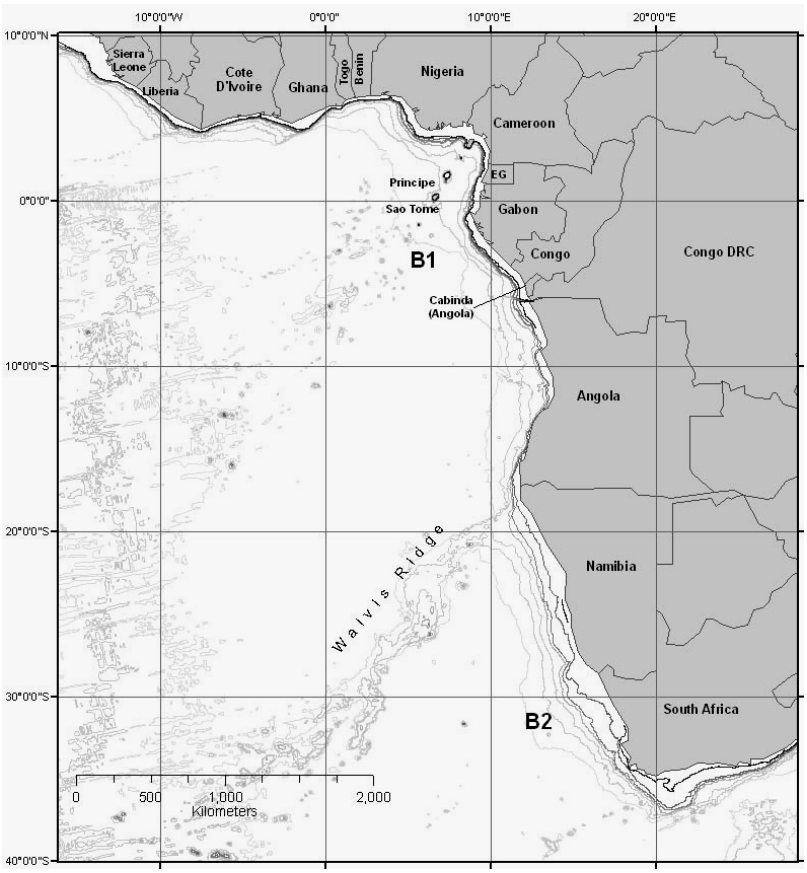

Table I – Years of sampling and number of samples collected from individual (N) humpback whales in west coast of Africa. Number of known males (M) and female (F) individuals is also shown.

Sampling Regions	Years	Samples	N	M	F
Angola (ANG)	1998	13	12	8	4
São Tomé (ST)	2004-2005	5	5	1	4
Gabon (GA)	2000-2006	1696	1496	992	460
West South Africa (WSA)	1990-2008	233	205	93	109

Table II – Mitochondrial DNA control region variability. Sampling site, number of individuals (N), number of haplotypes, polymorphic sites, haplotype (*h*) and nucleotide (π) diversities, as well as their standard deviations (SD).

Sampling site	**N**	**Haplotypes**	**Polymorphic sites**	***h* ± SD**	**π ± SD**
GA	1332	139	82	0,980±0,000	0,0214±0,012
ST	4	4	19	1,000±0,177	0,022±0,016
ANG	12	11	32	0,985±0,040	0,021±0,012
WZA	191	63	61	0,974±0,004	0,021±0,011

Table III – Genetic variability in humpback whale genotyped at ten loci. *N* = mean number of genotyped individuals per locus; *K* = mean number of alleles per locus; H_o and H_e =observed and expected heterozygosities (SD- standard deviation). *PI* = average probability of identity.

Sampling site	*N*	*K*	H_o ± SD	H_e ± SD	PI
GA	1490	13.00	0,74±0,003	0,74±0,049	1.9×10^{-12}
WSA	202	11.30	0,74±0,009	0,73±0,052	3.9×10^{-12}
ST	4.9	5.1	0,77±0,060	0,74±0,069	5.9×10^{-10}
ANG	12	7.4	0,80±0,036	0,781±0,044	6.3×10^{-12}

Table IV – Pairwise comparisons between Seasons in Gabon and West South Africa, using molecular distances (ϕ_{ST}) (above the diagonal in italic) and haplotype frequencies (F_{ST}) (below the diagonal). Significant values are highlighted in bold (p <0.05).

	Early Gabon	Late Gabon	Late WSA	Regular WSA
Early Gabon		*-0.00076*	***0.00524***	*-0.00012*
Late Gabon	0.0000		*0.00450*	*-0.00105*
Late WSA	**0.00857**	**0.00979**		*-0.00123*
Regular WSA	**0.00313**	**0.00430**	-0.00037	

Table V – Pairwise comparisons, for females, between different seasons in Gabon and West South Africa, using molecular distances (ϕ_{ST}) (above the diagonal in italic) and haplotype frequencies (F_{ST}) (below the diagonal). Significant values are highlighted in bold (p <0.05).

	Early Gabon	Late Gabon	Late WSA	Regular WSA
Early Gabon		*0.0015*	***0.0104***	*0.0099*
Late Gabon	0.0013		***0.0111***	***0.0235***
Late WSA	**0.0079**	**0.0127**		*0.0059*
Regular WSA	0.0041	**0.0105**	-0.0019	

Table VI – Pairwise comparisons, for males, between different seasons in Gabon and West South Africa, haplotype frequencies (F_{ST}) (below the diagonal). Significant values are highlighted in bold (p <0.05).

	Early Gabon	Late Gabon	Late WSA	Regular WSA
Early Gabon				
Late Gabon	-0.0003			
Late WSA	**0.0055**	**0.0066**		
Regular WSA	**0.0078**	**0.0078**	0.0048	

Table VII – Matches founded between Gabon (GA) and west coast of South Africa (WSA). Sex, date of collection in each site and the situation when the sample was collected. (*) matches already described by Pomilla *et al.*(2006).

Matches	Sex	GA date	Situation	WSA date	Situation
1 *	**F**	08/09/2001	Pair	16/12/2001	2 Adults/ Defecation
2 *	**F**	09/09/2002	M-C pair	10/01/2003	M-C pair
2	**F**	09/09/2002	M-C pair	25/10/2004	2 adults
3	**M**	07/08/2001	?	21/11/2005	Male with a M-C pair
4	**F**	16/08/2002	Pair	07/11/2006	2 adults
5	**M**	04/09/2003	Competitive	18/01/2003	2 adults
6	**F**	26/09/2004	Competitive	08/11/2004	2 Adults/ Defecation
7	**M**	14/08/2005	Competitive	17/10/2005	3 adults
8	**F**	20/08/2006	Competitive	12/10/2001	2 ind in mixed group of whales
9	**M**	04/09/2006	Competitive	17/12/2001	20 ind./milling and feeding