UNIVERSIDAD SAN FRANCISCO DE QUITO

Colegio de Ciencias Biológicas y Ambientales

Genetic Diversity And Connectivity Of Eastern Pacific Humpback Whales (*Megaptera Novaeangliae*, Borowski, 1781) Off The Coast Of Esmeraldas, Ecuador.

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Tesis de grado presentada como requisito para la obtención del título de Licenciatura en Biotecnología.

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HOJA DE APROBACIÓN DE TESIS

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ABSTRACT

The genetic diversity and connectivity of humpback whales (*Megaptera novaeangliae*) off the coast of Esmeraldas, Ecuador is analyzed. A total of 35 samples of skin tissue were collected using a biopsy darting method during the 2010 and 2011 breeding seasons. DNA was extracted following a modified CTAB protocol. A variable section of the mitochondrial DNA control region was amplified by means of the Polimerase Chain Reaction (PCR). The PCR products were sequenced and a total of sixteen different haplotypes were determined. One of the identified haplotypes corresponded to a new unreported sequence and another one has not been reported in the Southeast Pacific but in Western Australia, New Caledonia and French Polynesia. The remaining 14 sequences were matched to previously reported haplotypes in the Southern Hemisphere and some showed a high identity level with sequences from Alaska, the South Atlantic and Indian Oceans. Haplotype diversity (h±SD) was estimated to be 1.0000 ± 0.0068 and the nucleotide diversity (π±SD) 0.020931 ± 0.010994, which reflected high genetic diversity in the Ecuadorian population of humpback whales. The Analysis of Molecular Variance (AMOVA) showed no significant differences with the populations of humpback whales of Santa Elena-Ecuador, Colombia, and the Antarctic Peninsula but significant differentiation was found between whales of Esmeraldas in the two years of the surveys and between whales of Esmeraldas and the Magellan Strait in terms of haplotype frequencies and nucleotide composition. The phylogenetic reconstruction grouped the 35 haplotypes in three of the four clades present in the Southern Hemisphere. The results of this study will make possible the understanding of connectivity of humpback whales that visit the coastal area of Ecuador with those from Galápagos and other ocean basins.

RESUMEN

Se presenta información sobre la diversidad genética y la conectividad de las ballenas jorobadas (*Megaptera novaeangliae*) que visitan la costa de Esmeraldas, Ecuador durante la época reproductiva. Un total de 35 muestras de piel se obtuvieron mediante dardos de biopsia durante las temporadas de reproducción de los años 2010 y 2011. El ADN de las muestras fue extraído siguiendo un protocolo CTAB modificado. Una sección variable de la región control del ADN mitocondrial se amplificó por medio de la Reacción en Cadena de la Polimerasa (PCR). Los productos de la PCR fueron secuenciados y un total de dieciséis diferentes haplotipos fueron determinados. Uno de los haplotipos identificados correspondió a una secuencia nueva no reportada anteriormente mientras que otro no ha sido reportado antes en el Pacífico Sudeste pero sí en el oeste de Australia, en Nueva Caledonia y en Polinesia Francesa. Las restantes 14 secuencias coincidieron con haplotipos previamente reportados paras el Hemisferio Sur y algunas de ellas mostraron un alto nivel de identidad con secuencias de Alaska, el Atlántico Sur y el Océano Índico. Se estimó un valor de 1,0000 ± 0,0068 para la diversidad de haplotipos (h ± SD) y de 0,020931 ± 0,010994 para diversidad de nucleótidos (π ± SD) lo que refleja una alta diversidad genética en la población ecuatoriana de ballenas jorobadas. El análisis de varianza molecular (AMOVA) no mostró diferencias significativas con la población de ballenas de Santa Elena-Ecuador, Colombia, y la Península Antártica, pero sí se encontró diferencias significativas entre las ballenas de Esmeraldas en los dos años de muestreo y entre las ballenas de Esmeraldas y el área de alimentación del Estrecho de Magallanes, tanto en términos de frecuencias de haplotipos como de composición de nucleótidos. La reconstrucción filogenética agrupó a los 35 haplotipos en tres de los cuatro clados presentes en el hemisferio sur. Los resultados de este estudio facilitarán la comprensión de la conectividad de las ballenas jorobadas que visitan las aguas costeras del Ecuador con aquellas de Galápagos y de otras áreas oceánicas.

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1. INTRODUCTION

1.1. *Natural History of the humpback whale*

The humpback whale (*Megaptera novaeangliae*, Borowski, 1781) is a migratory marine mammal of the family *Balaenopteridae* within the suborder Mysticeti (baleen whales) and is the only species of its genus. This species is characterized by the presence of fleshy protuberances on head and jaws called tubercles and long and knotty pectoral flippers, which are approximately one third the length of the body (Clapham & Mead, 1999; Perrin *et al.*, 2008). Its common name refers to the characteristic hump at the base of the dorsal fin (Olavarría, 2008). The body is black dorsally while the pigmentation on the underside can vary among black, white or mooted. The underside of the tail exhibits a pattern of white and black pigmentation that is unique to each individual (Perrin *et al.,* 2008).

The size of adult whales varies depending on the geographical area but in average is 14-15m and calves are approximately 4 m at birth and 8-10 m when they leave their mothers. Adult female humpback whales are typically 1-1.5 m longer than males (Perrin *et al*., 2008), however it is difficult to visually differentiate between males and females as the only external morphological difference is seen in the genital region, a hemispheric lobe in females and a greater distance between the genital opening and the anus in males (Perrin *et al*. 2008; Olavarría, 2008).

Humpback whales are seasonal breeders of a cosmopolitan distribution. They are known to travel great distances; from their wintering areas in warmer waters at low latitudes, where

they give birth and breed, to their feeding areas of cold water at high latitudes near the polar and sub-polar regions during summer periods (Baker *et al*., 1986; Craig & Herman, 1997; Olavarría, 2008).

Reproductive cycles of the humpback whales are closely related with their seasonal migration (Baker, Perry & Herman, 1987). Mature females conceive on the breeding grounds during winter and give birth to a single calf after a gestation period of about 11.5 months (Clapham & Palsbol, 1997; Perrin *et al*., 2008). When the calf is weaned the mother begins a round trip migration to the breeding grounds again. There is no paternal investment in the rearing of offspring (Clapham & Palsbol, 1997). The calf remains in maternal groups in the feeding areas until they reach the reproductive maturity. Humpback whales do not live in stable groups and they are widely scattered throughout an extensive breeding range in winter (Clapham, 1996).

A mature female can give birth to a calf once every 2 or 3 years and more rarely can do so in consecutive years. It depends on whether she conceives immediately after parturition, after the weaning of their calf, or one year after the weaning of a calf respectively (Baker, Perry & Herman, 1987).

1.2. *Population Structure and Distribution*

The world population of humpback whales is distributed in all the major ocean basins except the Arctic (Clapham & Mead, 1999). The humpback whales are found in three oceanic regions: North Pacific, North Atlantic and the Southern Oceans (Baker *et al.* 1993;

1998a). Most populations migrate for feeding during summer to high latitude areas and then during winter to low latitude breeding grounds (Kellogg, 1929 cited in Olavarría, 2008). Subpopulations of humpback whales (*Megaptera novaeangliae*) are named as stocks (Baker *et al.* 1986). The International Whaling Commission (IWC) Scientific Committee currently recognizes seven humpback whale breeding stocks (labeled A to G) and six distinct Antarctic or feeding areas (labeled I-VI) in the Southern Hemisphere (IWC, 2011).

Whales of the breeding stock of the southwest Atlantic (stock A, coast of Brazil) are connected by migration with Area II. Whales of the southeast Atlantic (stock B, coast of West Africa from the Gulf of Guinea down to South Africa) are probably connected with Areas II and III. The breeding stock of the southwestern Indian Ocean (stock C, coasts of eastern South Africa, Mozambique, and Madagascar) has a migratory relationship with Area III. Whales from the southeastern Indian Ocean (stock D, northwestern Australia) are connected with Area IV. Whales breeding in the southwest Pacific (stock E, northeastern Australia, New Caledonia, Tonga and Fiji.) have a migratory relationship with Area V. The breeding stock of the central south Pacific (stock F, Cook Island and French Polynesia) is connected with Area VI (Olavarría, 2008; Reilly *et al.* 2012). Finally, the South Eastern Pacific whales move along the west coast of Central and South America (Costa Rica, Panama, Colombia and Ecuador) (Olavarría, 2008). Humpback whales encompassed within this area are designated as the Breeding Stock G (IWC 1998) and have a migratory connection with the Antarctic Area I including the Antarctic Peninsula, Bellingshausen Sea, South Shetland Islands (120 ° W - 60 ° W) and the area of Chile Patagonian channels (49° S – 53 ° S) (Acevedo, Aguayo-Lobo & Pastene, 2006; Acevedo *et al.*, 2007; Flores-González *et al.,* 2007; Olavarría, 2008).

It is widely believed that breeding areas in the Southeast Pacific are connected to the feeding areas on the west side of the Antarctic Peninsula and the Magellan Strait in Southern Chile. Furthermore there exists a connection with areas further east of the Antarctic Peninsula into the Southwestern Atlantic Ocean (Acevedo *et al.*, 2007; Félix, Caballero & Olavarría, 2012). Finally, Caballero *et al.* (2001); Acevedo *et al.*, 2007; Félix, Caballero y Olavarría (2007) and Olavarría *et al*. (2007) suggest there is no genetic differentiation between whales from the Antarctic Peninsula feeding area and the Colombian and Ecuadorian breeding grounds and that whales from the Magellan Strait conform a genetically different aggregation from the Antarctic feeding area.

It has been established that the mitochondrial DNA (mtDNA) diversity of humpback whales is relatively high in the Southern Hemisphere (Baker *et al.* 1993). Haplotypes based on mtDNA sequences could be classified into one of four primary clades: *AE*, *CD, IJ and SH* (Baker *et al*., 1993). Each clade is present predominantly in a specific ocean basin population; the clade AE predominates in the North Pacific, IJ in the North Atlantic, CD in the Southern Hemisphere (Baker *et al.*, 1993; Olavarría, 2008) and SH has been registered among Southern Hemisphere populations (Engel *et al.*, 2008; Olavarría *et al.*, 2007).

1.3. *Humpback whales off Ecuador*

Humpback whales that visit Ecuador belong to the G reproductive stock of the Southern Hemisphere (Scheidat *et al.*, 2000, Olavarría, 2008). Genetic analysis and photo identification have shown migratory relationship heterogeneity of this population compared with other stocks in the South west Pacific like those from the breeding grounds from New

Caledonia, Tonga, Cook Islands, eastern Polynesia (Félix, Caballero & Olavarría, 2007; Olavarría *et al.,* 2007).

Variation in pigmentation patterns in the ventral area of the tail have been used to characterize different stocks in the Southern Hemisphere (Baker, 1986), including humpback whales of Ecuador. These patterns have revealed that there is an exchange of individuals between Colombia and Ecuador (Félix & Haase, 1998) and Colombia and the Antarctic Peninsula (Acevedo *et al.*, 2007; Félix, Caballero & Olavarría*,* 2012). This was confirmed by genetic analysis (Caballero *et al.*, 2001; Olavarría *et al.*, 2007) which also established the existence of a link between the breeding areas of Colombia and Ecuador with the Magellan Strait at the southern tip of Chile (Olavarría, 2008).

Genetic studies have been conducted in different parts of the Southeast Pacific including breeding grounds from the coast of Ecuador and the Galapagos Islands (Felix *et al*., 2006; 2007). Ecuadorian whales display a slightly higher diversity compared with other feeding and breeding areas of the Southeast Pacific and the Antarctic Peninsula (Olavarría *et al.*, 2006; 2007; Félix, Caballero & Olavarría*,* 2012). However the haplotype composition and migratory behavior of humpback whales visiting Ecuadorian waters is not completely determined.

1.4. *Molecular Genetic Analysis*

The mitochondrial DNA has been chosen as a genetic marker for the study of population genetics of humpback whales because it is a haploid molecule of maternal inheritance that

apparently does not recombine (Sasaki *et al.*, 2005). The distribution of mtDNA haplotypes is influenced by maternally fidelity to migratory destinations due to experience during calf first year of life (Baker, *et al.*, 1994; 1998b). Therefore, mtDNA describes the genetic structure of maternal lineages within populations and shows high sensitivity to demographic changes in populations (Baker, 1993). To evaluate the possible effects caused by commercial whaling in genetic variation of whale populations, standardized techniques using mitochondrial DNA have been implemented in order to recognize possible variations in the mitochondrial control region or "D-loop" sequence, a non coding region that is highly variable in most vertebrates (Baker *et al.*, 1993). Based on these analyzes, the humpback whale is currently considered a species of least concern by the standards of IUCN (Reilly, *et al.* 2012).

By means of the Polymerase Chain Reaction (PCR), specific DNA fragments can be amplified from very small amounts of tissue, resulting in large amounts of DNA fragments (Necochea & Canul, 2004). DNA sequencing and genotyping of the PCR products are techniques that can be used to characterize the sequence variation between individuals (Palumbi 1966 cited in Olavarría, 2008). These techniques are used in this study to analyze the mtDNA control region of humpback whales of the area of "Bajos de Atacames", Esmeraldas, in order to provide an overview of the genetic diversity and connectivity of humpback whales (*Megaptera novaeangliae*) off the coast of Esmeraldas, Ecuador. Molecular techniques based upon sex-specific DNA sequences were also used to identify the sex of the sampled individuals since there is no visual sexual dimorphism in humpback whales.

2. OBJECTIVES

2.1. General Objective

The aim of this project is to analyze the genetic diversity and connectivity of humpback whales from the breeding area of Esmeraldas, Ecuador, by mtDNA control region analyses.

2.2. Specific Objectives

- To determine the mitochondrial DNA (mtDNA) control region genetic diversity of humpback whales from the coast of Esmeraldas, Ecuador, from samples collected during the field season of 2010 and 2011.
- To analyze and expand the mtDNA haplotype bank of Ecuadorian humpback whales and to compare their frequencies with other coastal and insular areas.
- To compare the genetic diversity of humpback whales from Ecuador with that from other populations.

3. JUSTIFICATION

The humpback whale is a cosmopolitan species that was hunted intensively in the Southern Hemisphere (Baker & Clapham 2004), exposing the populations to a bottleneck effect that could reduce their genetic variability, resulting in reduced fertility rates and juvenile mortality (Baker, 1987). Although since 1966 the humpback whales are internationally protected against hunting (Baker *et al.,* 1986; 1998a) and are no longer considered a threatened species, other problems such as pollution of the oceans, shipping, fishing nets, etc. can affect the growth of their populations and their migratory routes (Frisch, 2002). Researches in the fields of molecular biology and ecology have yielded information on the population status and conservation of humpback whales in different regions of its distribution. Thus, several aspects of migration, gene flow and geographic distribution of populations and subpopulations of humpback whales have been clarified. This information is essential to the achievement of an international agreement in terms of investigation and conservation of this species. Molecular methods such as the sequencing of mitochondrial DNA and the use of molecular markers are needed to expand the available knowledge concerning the population of humpback whales from the coasts of Ecuador and other South Pacific areas (Constanza, 2003). These methods are a tool to determine their migratory routes, habitat displacement and genetic variability (Constanza, 2003). Genetic analyzes of this study will determine the genetic heterogeneity of humpback whales that visit the coastal area of Esmeraldas and will provide useful information in order to monitor them over time. To enrich the information about the haplotype composition and to determine the genetic variability of this population is also a tool for understanding mating systems and dispersal of this species (Baker *et al.*, 1998a).

4. STUDY AREA

Humpback whales visit the coastal waters of the province of Esmeraldas during the months of June through September and part of October (Scheidat *et al.*, 2000; Félix, 2003). The whale biopsies collection for this study was conducted during August 2010 and July 2011 in the coastal area of Cantón Atacames in the province of Esmeraldas (0º20`N), area known as "Bajos de Atacames" (Figure 1). The average sea temperature in this zone is 26 °C. Prevailing currents come from the northeast, registering an average speed of 0.6 knots. There is a slight weakening of currents intensity in the months of June, July and August, but then stays at an average of 0.8 knots (Inocar 2012). The genetic analyzes of the samples were performed in the Laboratory of Plants Biotechnology at San Francisco de Quito University.

5. MATERIALS

5.1. *Sampling*

- Modified 0.22 calibre rifle (Paxarm)
- Biopsy darts
- Forceps
- Collection tubes
- Ethanol, 70%
- Freezer

5.2. *Laboratory and molecular analyses*

5.2.1. *DNA extraction*

- Humpback whale epithelial tissue sample
- Liquid nitrogen
- CTAB Buffer (Tris-HCl (Invitrogen), NaCl, EDTA (Invitrogen), CTAB (Sigma))
- B-mercaptoethanol (Sigma)
- Chloroform/isoamyl alcohol, 24:1 (HVO)
- Isopropanol (HVO)
- Ethanol, 76%
- TE Buffer (Tris 10mM (Invitrogen), 1mM EDTA (Invitrogen), pH 7,5)
- Thermo scientific 2052 Sand Bath
- Eppendorf 5415 D Centrifuge

5.2.2. *Sex determination of the sampled whales*

- Humpback whales DNA (40 ng)
- 20 mM Tris pH 8.4 (Invitrogen)
- 50 mM KCl
- 2 mM $MgCl_2$ (Invitrogen)
- 200 µM dNTPs (Invitrogen)
- 0.5 mg/ml BSA (Promega)
- 0.5 µM from each primer (SFY1204 / SFY0097)
- 0.4 U of Taq DNA Polymerase (Invitrogen)
- 3 U of Taq 1 restriction endonuclease (Invitrogen)
- T-Personal Biometra Thermo cycler

5.2.3. *Mitochondrial DNA analyses*

- Humpback whales DNA (50 ng)
- 1X PCR Buffer (Invitrogen)
- 2 mM $MgCl_2$ (Invitrogen)
- 200 µM dNTPs (Invitrogen)
- 20 pmol of each primer (t-Pro whale Dlp 1.5 / Dlp 8)
- 1 U of Taq DNA Polymerase (Invitrogen)
- T-Personal Biometra Thermo cycler

5.2.4. *DNA cuantification*

- Thermo Scientific NanoDrop 1000
- TE Buffer (Tris 10mM, 1mM EDTA, pH 7,5)
- Ultra Pure Distilled Water (GIBCO)

5.2.5. *Electrophoresis*

- Agarose (SeaKem)
- TBE 1X
- SYBR Safe 10000X (Invitrogen)
- Blue Juice 10X (Invitrogen)
- 100 bp Ladder (Axygen)
- Electrophoresis chamber Scientific Co. MGU-502T
- Power supply Scientific Co. EPS-300 II C.B.S
- Gel documentation system (Molecular Imager: BIO-RAD; Gel Doc XR)

5.3. *Sequence and statistical analyses*

- Software Mega version 5 (Tamura *et al.*, 2011)
- Software Arlequín version 3.5. (Excoffier, Laval & Schneider, 2005)

6. METHODS

6.1. *Sampling*

Twenty humpback whale skin samples were obtained during August 2010 and 15 skin samples were obtained during July 2011 off the coast of Esmeraldas, Ecuador by means of the Paxarm Biopsy Sampling System (Krutzen *et al*., 2002). When the target whale was sighted, the biopsy dart was shot at the whales' flank region. On impact the dart tip penetrated the skin and retained a skin sample of approximately 3 cm, which was then collected from water (Russell, 2002).

The tip of the dart was removed with forceps and the adipose and epithelial tissues were obtained. The tissue sample was stored in sample collection tubes with 70% ethanol. Each sample was labeled with the corresponding code, date and location of the point of collection along with the photograph code if applicable. Sampled whales were photographed (Annexes 1 to 3) in order to avoid possible re-sampling and duplicates of the same individual. Samples were refrigerated in the field and later transported to the laboratory and stored at -20°C for future processing.

6.2. *Laboratory and molecular analyses*

6.2.1. DNA extraction

Extraction of genomic DNA from samples followed the CTAB (hexadecyltrimethylammonium bromide) DNA extraction protocol (Shangai-Maroof *et al.*, 1984) which is as follows: 100 mg from each tissue sample was grinded in liquid nitrogen using a clean pestle for every sample ensuring tissue was frozen the entire time. 800 µl of CTAB buffer and 10 µl of β-mercaptoethanol were added and the mixture was macerated to homogenize. The macerated tissue was then transferred to labeled eppendorf tubes. Each tube was incubated at 62°C in a sand bath for one hour, mixing every 15 minutes. After incubating, 500 µl of chloroform-isoamyl alcohol (24:1) was added; after shaking the mixture was centrifuged for 20 minutes at 13200 rpm. The aqueous phase (top layer) was then transferred into a new individually labeled tube. 500 µl of cold isopropanol (4°C) were added. The tubes were mixed by inverting them several times and then centrifuged for 5 minutes at 5000 rpm. The supernatant was discarded without dislodging the pellet. Subsequently, 800 µl of 76% ethanol were added in order to wash the pellet. Ethanol was removed with a micropipette and the tubes with pellet were inverted on a clean wipe and allowed to dry. Finally, pellets were hydrated and re-suspended with 70-100 µl TE.

6.2.2. *Sex determination of the sampled whales*

The SFY/SFX regions of the genome were amplified using the Polimerase Chain Reaction (PCR) (Saiki *et al.*, 1988) on a T-Personal Biometra Thermo cycler. Approximately 40 ng of DNA template was amplified in 20 µl reaction volume containing 20 mM Tris pH 8.4, 50mM KCl, 2mM $MgCl_2$, 200 µM dNTPs, 0.5 mg/ml BSA, 0.5 µM from each primer (SFY1204/SFY0097) and 0.4 U of Taq DNA Polymerase (Invitrogen) (Pallsboll *et al.*, 1992). Temperature profiles consisted of an initial denaturation at 94°C for 2 minutes, 35 cycles of 94°C for 1 minute, primer annealing at 60°C for 1 minute, polymerase extension at 72°C for 4 minutes and a final extension at 72°C for 5 minutes. DNA from a random female person was included as a positive control. Gel electrophoresis was run with 5 µl of each PCR product on a 1% agarose gel at 100 V for 45 minutes in order to verify the amplification of a fragment of approximately 1100bp (Pallsboll *et al.*, 1992 with modifications).

10 µl of the PCR products was digested with 3 U of Taq1 restriction endonuclease (Invitrogen) for 1 hour at 60°C. The restriction fragments of each whale sample were separated and visualized by electrophoresis on a 3% agarose gel (50 ml of TBE 1X, 1.5 g of agarose and 2 µl of SYBR safe) at 100 V for 1 hour. Sex was determined according to the restriction-fragment patterns; the enzymatic digestion of DNA of female whales produces fragments of 439 pb while digestion of DNA of males produces fragments of two different sizes, one of 439 pb and another one of 621 pb. The restriction-fragment pattern of the positive control was similar to male humpback whales (Pallsboll *et al*. 1992, with modifications).

6.2.3. *Mitochondrial DNA analyses*

From the extracted DNA, a fragment of approximately 500bp length of the mitochondrial DNA control region (CR) was amplified via the PCR (Saiki *et al*. 1988). For the PCR the following primers were used: t-Pro-whale Dlp1.5 (5'-TCACCCAAAGCTGRARTTCTA-3') and Dlp8 (5'CCATCGWGATGTCTTATTTAAGRGGAA-3') (Baker *et al.*, 1998a; Olavarría *et al.*, 2007, Félix, Caballero & Olavarría*,* 2012).

Each PCR reaction of 50 µl total volume contained 1X PCR Buffer, 2 mM $MgCl_2$, 200 µM of dNTPs, 20 pmol of each primer, 1 U Taq polymerase (Invitrogen), and 50 ng DNA. The reactions were conducted on a T-Personal Biometra Thermo cycler. Temperature profiles consisted of an initial period of denaturation at 95°C for 2 minutes, 36 cycles of 94°C for 30 seconds, primer annealing at 55.5°C for 1 minute and polymerase extension at 72°C for 1 minute and 30 seconds, and a final extension at 72°C for 5 minutes (Félix, Caballero & Olavarría, 2007 with modifications).

In order to verify the amplification of the specific fragment of 500bp of the mitochondrial DNA, a gel electrophoresis was performed with 10 µl of the PCR product and 3 µl of Blue Juice 10X (Invitrogen) on a 1% agarose gel (50 ml of TBE 1X, 0.5 g of agarose and 2 µl of SYBR safe) at 80 V for 1 hour (Figure 2).

The PCR products were quantified in a Thermo Scientific NanoDrop 1000 and then submitted to Functional Biosciences, Inc. (Madison USA), where they were cleaned using

the Exo/Sap protocol (Affymetrix, Inc,. 2010) and then DNA was sequenced in both directions with an ABI 3730xl DNA Sequencer with 50cm arrays.

The sequences were aligned using the ClustalW system of the computer program MEGA 5 (Tamura *et al.*, 2011), which uses progressive alignment methods. In these methods, the most similar sequences, those with the best alignment score, are aligned first. Then progressively more distant groups of sequences are aligned until a global alignment is obtained (Thompson *et al*., 1994). Sequences were trimmed to 470 bp (beginning at position 6 of the reference humpback whale control region sequence X72202) in order to match a consensus region analyzed previously (Olavarria *et al.*, 2006; 2007; Félix, Caballero & Olavarría, 2012). This region includes more than 85% of the variation of the mtDNA control region of humpback whales (Baker & Medrano-González, 2002).

A search of the Genbank was made with the Basic Local Alignment Search Tool (BLAST) provided by the National Center for Biotechnology Information (NCBI) web page. The algorithm for highly similar sequences (megablast) was selected in order to identify the haplotypes present among the humpback whales of this study and to detect unique or unreported haplotypes. A phylogeny tree was constructed using the Neighbor Joining method also with the software MEGA 5 using the Kimura 2-parameter model and 1000 bootstrap replications. The fin whale sequence (Genbank accession number X61145; Árnason *et al*., 1991) was included in the tree for comparison because of its taxonomic relationship. The haplotypes previously identified in South Pacific populations as well as sequences from Alaska (Jackson *et al*., 2009), Brazil (Engel *et al.,* 2008), and the South

Atlantic and Indian Oceans (Rosenbaum *et al*., 2009) were included for the phylogeny reconstruction in order to obtain a clear distribution of the existent clades.

6.3. *Statistical analyses*

The following diversity indices were computed using the software Arlequin 3.5: gene diversity ($\hat{H}$), defined as the probability that two randomly chosen haplotypes are different in the sample; nucleotide diversity $(\hat{\pi}_n)$, which expresses the probability that two randomly chosen homologous nucleotide sites are different; number of polymorphic sites (*S*), namely the number of usable loci that show more than one allele per locus (Shane, 2005).

An Analysis of Molecular Variance (AMOVA) was ran in Arlequin 3.5 to calculate the differences in haplotype frequency (FST) and nucleotide differentiation (ΦST) between the breeding ground of Esmeraldas with other areas of the Southern Hemisphere. The significance of the observed ΦST and FST values was tested using 5,040 random permutations of the data matrix.

7. RESULTS

7.1. *Sex determination*

From the 35 humpback whale samples that were analyzed, only four were identified as females. 2 from the 20 whales from 2010 were females and 2 from the 15 whales of 2011 were females which resulted in a sex ratio of 8:1, males: females ($\chi^2 = 20.82$, $p < 0.001$) (Annex 4).

7.2. *Genetic diversity*

Sixteen haplotypes were identified among the 35 samples of Esmeraldas, Ecuador (Table 1, Annex 4). One of them was a new sequence not previously recorded in the world. This new haplotype corresponded to the sample 33 collected during 2011. The other fifteen matched with haplotypes from other Southern Hemisphere breeding grounds and feeding areas; one of them was identified for the first time in Ecuador (SP89).

The variable nucleotides include 32 polymorphic sites, 1 insertion/deletion, 2 transversions and 30 transitions. The overall gene diversity (H ± sd) was 1.0000 ± 0.0068. Nucleotide diversity (π ± sd) was 0.020931 ± 0.010994. The mean number of pair-wise differences was 8.811765 ± 4.163525.

7.3. *Inter annual genetic diversity between whales of 2010 and 2011 off the coast of Esmeraldas*

Through the AMOVA significant differences were found between whales sampled during 2010 and 2011 in both haplotype frequency and nucleotide composition (F_{ST}=0.08778, $p<0.05$; ΦST =0.08778, $p<0.00001$) (Table 2). From the 16 identified haplotypes only 3 (SP25, SP32 and SP90) were found in both years, 2010 and 2011, 9 haplotypes were found only during 2010 and 4 haplotypes including the new one corresponded to whales of 2011.

7.4. *Connectivity or similarities with other Southeast Pacific Areas and stocks of Brazil, Alaska and South Atlantic and Indian Oceans*

The predominant haplotype found in humpback whales of the coast of Esmeraldas was SP90 with 17.1%, which has been reported as the most common haplotype in the Southeastern Pacific (Félix *et al*., 2006; Olavarría, 2008). The second most common haplotype was SP62 with 14.3%, which has been reported in Colombia, the Antarctic Peninsula, the Magellan Strait and also in Salinas, Ecuador but in a lower percentage. SP32, the second most common haplotype in Salinas, Colombia and the Antarctic Peninsula, was the third most frequent in Esmeraldas with 11.4% (Table 1). The haplotype SP89, recorded twice in this study, has not been reported before in other breeding grounds or feeding areas of the Southeastern Pacific; however it has been reported in Western Australia, New Caledonia and French Polynesia (Olavarría *et al*., 2007).

Humpback whales of the coastal area of Esmeraldas shared 14 haplotypes of 41 reported for Salinas, Ecuador (34%) (Félix, Caballero & Olavarría*,* 2012), 13 of 27 haplotypes

reported for Colombia (48%), 12 of 34 in Antarctic Peninsula (35%) and the 4 haplotypes reported for Magellan Strait (100%) (Olavarría, 2008).

When comparing haplotypes from Esmeraldas with those of other areas of the Stock G, the AMOVA revealed a between variance of 5.49% and a within variance of 94.51%. No significant differences were found between the population of Esmeraldas, and the populations of Santa Elena, Colombia and the Antarctic Peninsula, however, as has been reported previously, a significant difference was found between Ecuadorian and Magellan Strait whales in both haplotype frequency and nucleotide composition (F_{ST}=0.19654, ΦST =0.19654, $p<0.00001$) (Félix, Caballero & Olavarría, 2007; 2012) (Table 3).

A high identity level was found when comparing sequences from Esmeraldas with haplotypes reported for populations of Alaska (Jackson *et al*., 2009), Brazil (Engel *et al.,* 2008), and the South Atlantic and Indian Oceans (Rosenbaum *et al*., 2009). This confirms a potential genetic exchange between the populations of the Southern Hemisphere but also between the populations of the Northern and Southern Hemisphere that converge in their breeding grounds. However, by means of the AMOVA significant differences were determined between the haplotype frequencies and nucleotide composition of whales of Ecuador and whales of Alaska (F_{ST}=0.72580, ΦST =0.72590, $p<0.00001$), Brazil (F_{ST}=0.10863, $p=0.00159$; ΦST=0.10863, $p<0.00001$), and South Atlantic and Indian Oceans (F_{ST}=0.02668, $p=0.01429$; ΦST =0.03206, $p<0.00001$) (Table 4).

7.5. *Phylogenetic reconstruction*

The Neighbor-Joining reconstruction grouped the 16 haplotypes into 3 clades: CD, IJ and AE though with a low bootstrap support (<50%) (Figure 3). The use of alternative models had little impact over the resulting topology of the phylogenetic tree. As expected, the CD clade predominated (63%) followed by the IJ clade (31%) and AE (6%). Two of the whales from 2011 corresponded to the clade AE, which is characteristic of humpback whales of North Pacific but has been registered in Colombia and Ecuador (Table 5). The fourth clade reported for Southern Hemisphere referred to as SH, was not found between whales from this study (Figure 3).

8. DISCUSSION

The overall gene diversity (H ± sd) of humpback whales at the Esmeraldas breeding site was 1.0000 ± 0.0068. This value is higher in comparison with gene diversity values of other whales' populations from the Southeast Pacific like the Gorgona Island in Colombia, Santa Elena in Ecuador and the Antarctic Peninsula (Olavarría, 2008; Félix, Caballero & Olavarría, 2012). The fact that 16 different haplotypes were identified in only 35 samples also confirms the high haplotype diversity of the population of whales of Ecuador.

When comparing the haplotypes of the whales off the coast of Esmeraldas between the two years of surveys, little overlap of haplotypes was found because only 3 of the 16 haplotypes were identified in both years. Likewise, molecular analysis of variance showed significant differences between whales of 2010 and 2011 both in haplotype frequency as in nucleotide composition. The whales' samples were taken at different months each year which could explain these differences. In 2010 the surveys were made during august, when mothers and calves are more abundant and probably resident groups are more common (Félix & Haase, 2001; Flórez-González *et al.*, 2007). By the other side, in 2011 the whales' samples were taken during July, when whales in general are more abundant and are still in transit. Moreover, Félix and Haase (2001) have found that during July groups are significantly larger and formed mostly by adult males (escorts) in pursuit of receptive females.

The results of the photo-identification revealed no re-sighting of individuals between 2010 and 2011, which might suggest that different individuals and consequently, different haplotypes reach the Ecuadorian coast at different times of the breeding season. However it should be taken into account the limited number of samples obtained each year, which can override these inferences.

14 out of the 16 haplotypes identified in Esmeraldas have been also found in other sites of the Southeast Pacific and the east of Antarctic Peninsula, the main feeding area of this stock. SP90 was the most common haplotype between whales of Esmeraldas, the same as in Santa Elena, Colombia and the Antarctic Peninsula. Nevertheless, SP62 was the second most common haplotype in Esmeraldas and it has never been dominant in the other areas.

It was remarkable to find the haplotype SP89 between whales of Esmeraldas since this haplotype has been found before in West Pacific populations such as New Zealand, Western Australia, New Caledonia and French Polynesia (Olavarría, 2008) but never before in the East Pacific populations. This suggests that there is some dispersal of females across the South Pacific and that most likely; whales from breeding areas of Eastern Pacific are not greatly isolated from whales of Oceania.

When comparing whales from Esmeraldas with the populations of whales of Santa Elena, Colombia, the Antarctic Peninsula and the Magellan Strait, only 5.49% of the total genetic variation was distributed among these subpopulations of the Stock G and 94.51% of the variation was found within subpopulations. The high proportion of the

within variance and the low variation among the compared sites indicate that these whales populations present a high genetic similarity and confirms the statement from Flórez-González *et al.* (1998), Félix, Caballero & Olavarría*,* (2007; 2012) and Olavarría (2008) that humpback whales from the South East Pacific establish a panmictic population. The values of F_{ST} and Φst between Esmeraldas and the populations of Santa Elena, Colombia and the Antarctic Peninsula were lower than 0.01 that suggests little genetic differentiation (Wright, 1978 cited in Shane, 2005) most probably as a result of the existence of gene flow between whales visiting these areas.

The highest value of F_{ST} calculated between the Stock G was the one between whales from Esmeraldas and the Magellan Strait that involves a substantial differentiation between these two whales' subpopulations. Although the four haplotypes reported in the Magellan Strait were identified in Esmeraldas (and have also been reported in Santa Elena), this study confirmed the differentiation between whales of Ecuador and the Magellan Strait. These differences could mean heterogeneity among humpback whales populations of the Stock G and stratification in the feeding areas. Despite the presence of gene flow during the breeding periods in breeding areas, the humpback whales have preferences for one or another feeding areas of the South Pacific, either the Antarctic Peninsula or the Magellan Strait, and these preferences determine some level of genetic differentiation (Félix, Caballero & Olavarría*,* 2007; 2012).

The high level of identity in the whales samples of this study with some sequences of populations of other oceans and Stocks such as Alaska (Jackson *et al.*, 2009), Brazil (Engel *et al.*2008), and both the Indian and South Atlantic Oceans (Rosenbaum *et. al.*,

2009) could be the result of transoceanic migrations of individuals moving through greater ranges of the oceans basins (Baker *et al.*, 1998a; Pomilla & Rosenbaum, (2005); Stevick *et al.*, 2010; 2011). These stocks could be closely related as can be observed in the Phylogenetic reconstruction.

Furthermore, as Baker *et al.* (1994) has established, whales may share stock G ranges with North Pacific groups and hence there is some genetic exchange between populations of the Northern and Southern Hemispheres. The results of this study confirmed that the AE clade is not only characteristic of the North Pacific as it is also present in populations of the South Pacific. This clade has been reported before in the feeding areas of Antarctic Peninsula and the Magellan Strait and in the breeding grounds of Colombia (Olavarría, 2008), Santa Elena-Ecuador (Félix, Caballero & Olavarría, 2007; 2012) and now in the area of Esmeraldas.

It has been reported that the clade SH is present in the population of Brazil as well in all breeding grounds of South Pacific except the breeding areas of Colombia and Santa Elena, Ecuador (Engel *et al.*, 2008; Olavarría, 2008; Félix, Caballero & Olavarría, 2007; 2012). None of the haplotypes found in Esmeraldas corresponded to this clade, what supports the absence of this clade in the breeding grounds of South East Pacific; however, it must be considered the reduced number of whales from Esmeraldas that were analyzed.

As has been reported previously for other breeding areas, the sex ratio in this study was biased towards males. The reduced number of humpback whales that were sampled in

this study increases the probability of a sex bias (Félix *et al.* 2009; Olavarría *et al.* 2003; Palsboll *et al*., 1997). A major proportion of males could be the result of different temporal and spatial patterns of migration between females and males (Olavarría, 2008). It has been suggested that females spend more time in Antarctic feeding areas than males (Brown, *et al.,* 1995; Olavarría, 2008) provoking by the contrary, a bias towards females in the Antarctic areas (Craig & Herman, 1997; Félix & Haase, 2005). If females are delayed in feeding areas, this can explain why more males have been sampled in breeding areas in the early months of the breeding period resulting in a sex bias towards males. Moreover, it should be considered that competitive groups are those most likely to be spotted and sampled as they exhibit more surface activity. These groups are usually formed by a core female surrounded by males (escorts) competing to approach her (Tyack, P. & Whitehead, H. (1982); Félix & Haase, 2001); due to this structure and conformation it is much more accessible obtaining skin samples from males than from females.

9. CONCLUSIONS AND RECOMMENDATIONS

Humpback whales that visit the coastal area of Ecuador present a high genetic diversity given the low number of analyzed samples and the high haplotype diversity that was found.

The haplotype SP89 was registered for the first time in Ecuador and one new haplotype for Ecuador and the world wide population of humpback whales was identified. These two new haplotypes found in Ecuador give a total of 43 haplotypes for the Ecuadorian breeding areas.

The CD and IJ were the predominant clades in the population of humpback whales of Esmeraldas. The presence of the AE clade in Esmeraldas, as in Santa Elena and Colombia and the high level of identity of haplotypes from Esmeraldas with some haplotypes of Brazil, Alaska and the Indian and South Atlantic Oceans support the thesis of transoceanic migration of some individuals and the gene flow across the equator.

Through this study, the molecular protocols were modified and standardized in order to apply them in future projects, especially those with the aim of understanding the connectivity of humpback whales between the main land and Galápagos. For future analyses much larger sample sizes and a wider geographic coverage is recommended.

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11. TABLES

Table 1. Haplotypes diversity of humpback whales off the coast of Esmeraldas. The frequency and percentage of each mtDNA haplotype is shown by sex and year (2010 and 2011). N refers to the total of individuals (males and females) of each mtDNA haplotype.

Haplotype	**MALES**				**FEMALES**						
	August 2010	July 2011	2010 & 2011	% of males	August 2010	July 2011	2010 & 2011	% of females	N (males & females)	% of whales	Clade
SP1		1	1	3.2		1	1	25.0	2	5.7	CD
SP8	1		1	3.2			0		1	2.9	AE
SP10	2		2	6.5			0		2	5.7	IJ
SP14		2	2	6.5			0		2	5.7	IJ
SP25	1		1	3.2		1	1	25.0	2	5.7	IJ
SP32	2	2	4	12.9			0		4	11.4	IJ
SP50			0	0.0	1		1	25.0	1	2.9	IJ
SP52	1		1	3.2			0		1	2.9	CD
SP62	5		5	16.1			0		5	14.3	CD
SP68			0	0.0	1		1	25.0	1	2.9	CD
SP73	3		3	9.7			0		3	8.6	CD
SP89	1		1	3.2			0		1	2.9	CD
SP90	1	5	6	19.4			0		6	17.1	CD
SP98		2	2	6.5			0		2	5.7	CD
Mno03MA02	1		1	3.2			0		1	2.9	AE
Nuevo033		1	1	3.2			0		1	2.9	CD
TOTAL	18	13	31	100.0	2	2	4	100.0	35	100.0	

Table 2. Pair-wise test of differentiation for mtDNA control region sequence between humpback whales sampled in 2010 and 2011 in Esmeraldas, Ecuador. The significance was analyzed by 5040 non-parametric permutations using the project distance matrix. The significant *p-values* are shown in **bold**.

	Esmeraldas 2010	
Esmeraldas 2011	0.08778	$\mathbf{F_{ST}}$
	0.02778±0.00257	***p-value***
	0.08778	$\mathbf{\Phi_{ST}}$
	<0.00001	***p-value***

Table 3. Pair-wise test of differentiation for mtDNA control region sequence between humpback whales of Esmeraldas, Ecuador and humpback whales of other Stock G locations. The significance was analyzed by 5040 non-parametric permutations using the project distance matrix. The significant *p-values* are shown in **bold**.

	Santa Elena, Ecuador	**Colombia**	**Magellan Strait**	**Antarctic Peninsula**
$\mathbf{F_{ST}}$	-0.00643	-0.00074	0.19654	0.00115
p-value	0.73829±0.00564	0.42520±0.00751	**<0.00001**	0.34464±0.00643
$\mathbf{\Phi_{ST}}$	-0.00643	-0.00074	0.19654	0.00109
p-value	0.92976	0.52242	**<0.00001**	0.37282

Table 4. Pair-wise test of differentiation for mtDNA control region sequence between humpback whales of Esmeraldas, Ecuador and humpback whales of other stocks. The significance was analyzed by 5040 non-parametric permutations using the project distance matrix. The significant *p-values* are shown in **bold.**

	South Atlantic & Indian Oceans	**Brazil**	**Alaska**
$\mathbf{F_{ST}}$	0.02668	0.10863	0.72580
p-value	**0.01429±0.00200**	**0.00159±0.00053**	**<0.00001**
$\mathbf{\Phi_{ST}}$	0.03206	0.10863	0.72590
p-value	**<0.00001**	**<0.00001**	**<0.00001**

Table 5. Summary of sampling periods (years) and number of samples including gene diversity (H), nucleotide diversity (π), number and percentage of individuals in each clade (CD, IJ, AE or SH).

Stock	**Year**	**Samples**	**# of haplotypes**	**H ± SD**	**π ± SD**	**Number of individuals in each clade**			
						CD (%)	**IJ (%)**	**AE (%)**	**SH (%)**
G	2010	20	12	1.0000 ± 0.0158	0.023119 ± 0.012333	12 (60)	6 (30)	2 (10)	0 (0)
G	2011	15	7	1.0000 ± 0.0243	0.014635 ± 0.008238	10 (67)	5 (33)	0 (0)	0 (0)
G	2010 & 2011	35	16	1.0000 ± 0.0068	0.020931 ± 0.010994	22 (63)	11 (31)	2 (6)	0 (0)

12. FIGURES

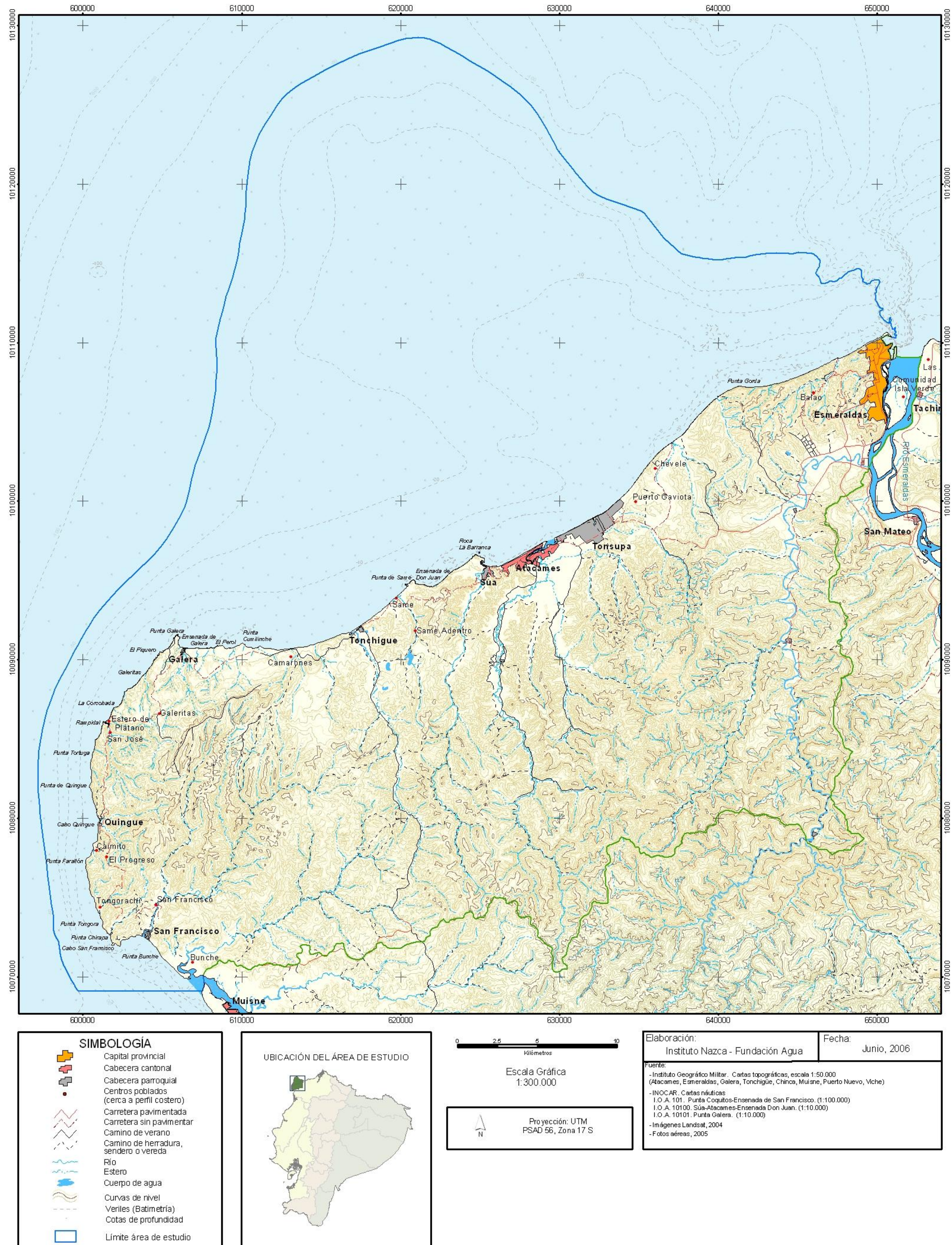

Figure 1.- Study area on the coast of Esmeraldas, Ecuador. The blue line represents the limits of the study area. (Denkinger, *et al.* 2006)

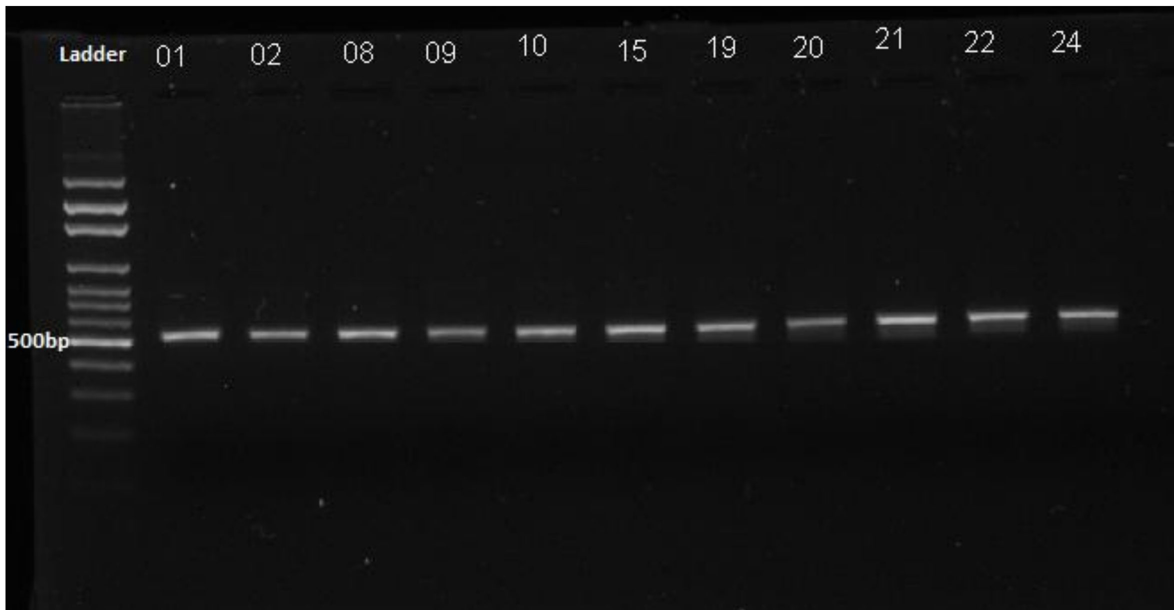

Figure 2. Electrophoresis gel of amplified mtDNA from 11 of the 35 humpback whales samples. Approximate size: 500 pairs of bases. The wells 01, 02, 08, 09, 10, 15, 19 and 20 correspond to amplified mtDNA from humpback whales surveyed during 2010. The wells 21, 22 and 24 correspond to samples of 2011.

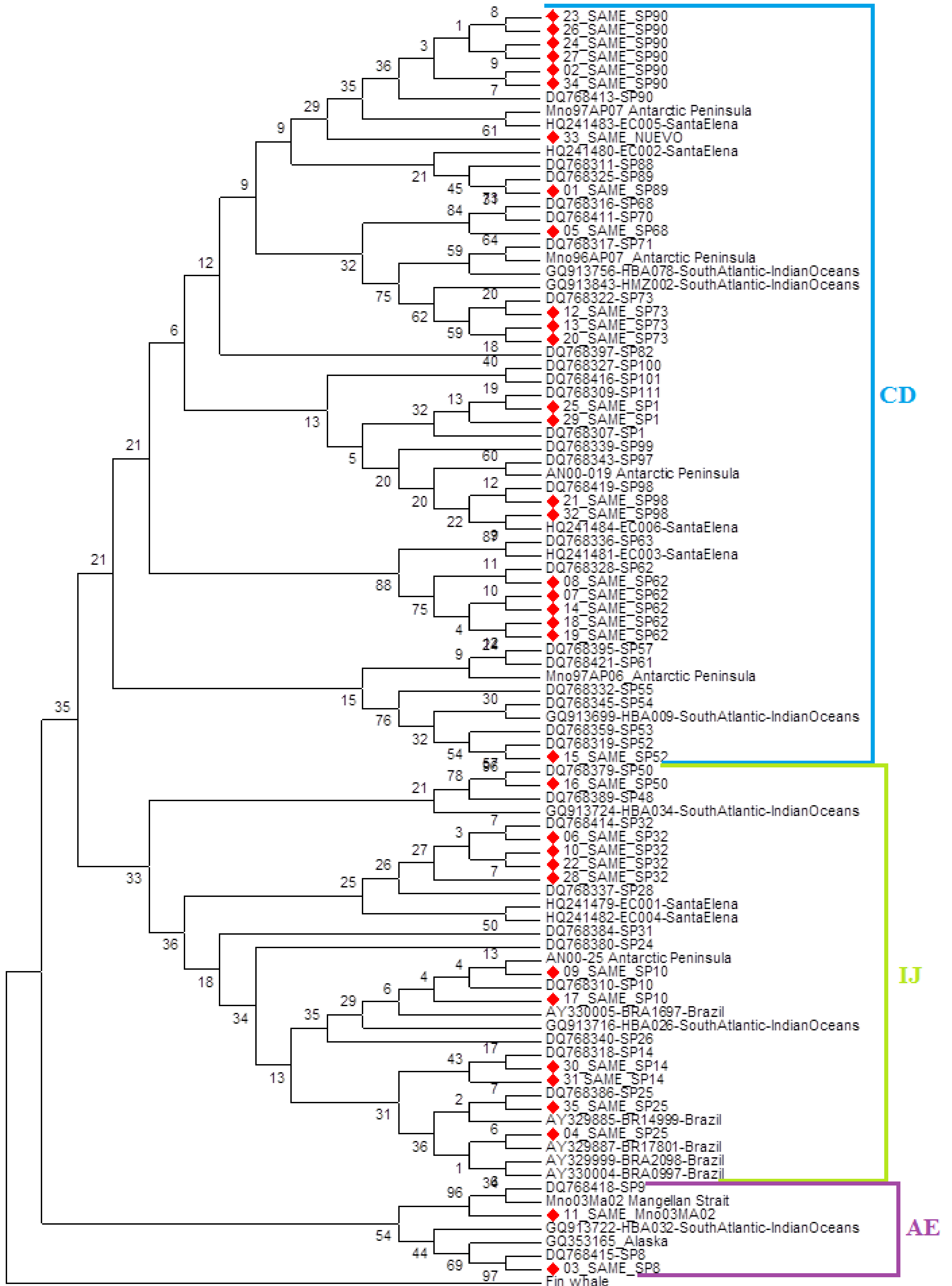
23_SAME_SP90
26_SAME_SP90
24_SAME_SP90
27_SAME_SP90
02_SAME_SP90
34_SAME_SP90
DQ768413-SP90
Mno97AP07 Antarctic Peninsula
HQ241483-EC005-SantaElena
33_SAME_NUEVO
HQ241480-EC002-SantaElena
DQ768311-SP88
DQ768325-SP89
01_SAME_SP89
DQ768316-SP68
DQ768411-SP70
05_SAME_SP68
DQ768317-SP71
Mno96AP07_Antarctic Peninsula
GQ913756-HBA078-SouthAtlantic-IndianOceans
GQ913843-HMZ002-SouthAtlantic-IndianOceans
DQ768322-SP73
12_SAME_SP73
13_SAME_SP73
20_SAME_SP73
DQ768397-SP82
DQ768327-SP100
DQ768416-SP101
DQ768309-SP111
25_SAME_SP1
29_SAME_SP1
DQ768307-SP1
DQ768339-SP99
DQ768343-SP97
AN00-019 Antarctic Peninsula
DQ768419-SP98
21_SAME_SP98
32_SAME_SP98
HQ241484-EC006-SantaElena
DQ768336-SP63
HQ241481-EC003-SantaElena
DQ768328-SP62
08_SAME_SP62
07_SAME_SP62
14_SAME_SP62
18_SAME_SP62
19_SAME_SP62
DQ768395-SP57
DQ768421-SP61
Mno97AP06_Antarctic Peninsula
DQ768332-SP55
DQ768345-SP54
GQ913699-HBA009-SouthAtlantic-IndianOceans
DQ768359-SP53
DQ768319-SP52
15_SAME_SP52
DQ768379-SP50
16_SAME_SP50
DQ768389-SP48
GQ913724-HBA034-SouthAtlantic-IndianOceans
DQ768414-SP32
06_SAME_SP32
10_SAME_SP32
22_SAME_SP32
28_SAME_SP32
DQ768337-SP28
HQ241479-EC001-SantaElena
HQ241482-EC004-SantaElena
DQ768384-SP31
DQ768380-SP24
AN00-25 Antarctic Peninsula
09_SAME_SP10
DQ768310-SP10
17_SAME_SP10
AY330005-BRA1697-Brazil
GQ913716-HBA026-SouthAtlantic-IndianOceans
DQ768340-SP26
DQ768318-SP14
30_SAME_SP14
31_SAME_SP14
DQ768386-SP25
35_SAME_SP25
AY329885-BR14999-Brazil
04_SAME_SP25
AY329887-BR17801-Brazil
AY329999-BRA2098-Brazil
AY330004-BRA0997-Brazil
DQ768418-SP9
Mno03Ma02 Mangellan Strait
11_SAME_Mno03MA02
GQ913722-HBA032-SouthAtlantic-IndianOceans
GQ353165_Alaska
DQ768415-SP8
03_SAME_SP8
Fin whale
CD
IJ
AE

Figure 3. Bootstrap consensus tree of the Phylogenetic reconstruction of humpback whales using the Neighbor-Joining and Kimura 2-parameter model. Bootstrap support after 1000 replications is indicated above or below branches. The sequences with red markers correspond to the 35 humpback whales of this study. Brackets on the right indicate the grouping of haplotypes in CD, IJ and AE clades. The SH clade is not shown as it was absent between the humpback whale samples of Esmeraldas.

13. ANNEXES

Annex 1. Photograph of the humpback whale to which the skin sample #26 corresponds.

Annex 2. Photograph of the humpback whale to which the skin sample # 29 corresponds.

Annex 3. Photograph of the humpback whale to which the skin sample #32 corresponds.

Annex 4. Data from humpback whales sampled in the coast of Esmeraldas during 2010 and 2012.

Area	Sample #	Location		Collection date	Haplotype	Sex
		UTMN	UTME			
Same-Ecu	1	631313	120753	12/08/2010	SP89	M
Same-Ecu	2	611727	105596	14/08/2010	SP90	M
Same-Ecu	3	610589	109582	12/08/2010	SP8	M
Same-Ecu	4	622697	113794	13/08/2010	SP25	M
Same-Ecu	5	622697	113794	13/08/2010	SP68	F
Same-Ecu	6	631313	120753	12/08/2010	SP32	M
Same-Ecu	7	610589	109582	12/08/2010	SP62	M
Same-Ecu	8	627678	109262	13/08/2010	SP62	M
Same-Ecu	9	610589	109582	12/08/2010	SP10	M
Same-Ecu	10	617162	119409	12/08/2010	SP32	M
Same-Ecu	11	611727	105596	14/08/2010	Mno03Ma02	M
Same-Ecu	12	619801	113242	13/08/2010	SP73	M
Same-Ecu	13	626116	112144	14/08/2010	SP73	M
Same-Ecu	14	617162	119409	12/08/2010	SP62	M
Same-Ecu	15	631313	120753	12/08/2010	SP52	M
Same-Ecu	16	627140	113206	13/08/2010	SP50	F
Same-Ecu	17	630780	113893	13/08/2010	SP10	M
Same-Ecu	18	630780	113893	13/08/2010	SP62	M
Same-Ecu	19	630427	111875	13/08/2010	SP62	M
Same-Ecu	20	627140	113206	13/08/2010	SP73	M
Same-Ecu	21	0058546	08000599	06/07/2011	SP98	M
Same-Ecu	22	0059332	08000275	07/07/2011	SP32	M
Same-Ecu	23	0059874	08000004	07/07/2011	SP90	M
Same-Ecu	24	0059874	08000004	07/07/2011	SP90	M
Same-Ecu	25	0058394	08001008	07/07/2011	SP1	F
Same-Ecu	26	0100854	07953622	09/07/2011	SP90	M
Same-Ecu	27	0100854	07953622	09/07/2011	SP90	M
Same-Ecu	28	0101472	07954715	09/07/2011	SP32	M
Same-Ecu	29	0101981	07952914	09/07/2011	SP1	M
Same-Ecu	30	0101297	07951561	09/07/2011	SP14	M
Same-Ecu	31	0101297	07951561	09/07/2011	SP14	M
Same-Ecu	32	0056358	08001358	10/07/2011	SP98	M
Same-Ecu	33	0057125	08001918	10/07/2011	NUEVO	M
Same-Ecu	34	0057877	08000283	10/07/2011	SP90	M
Same-Ecu	35	0057877	08000283	10/07/2011	SP25	F