PONTIFICIA UNIVERSIDAD CATÓLICA DEL ECUADOR

FACULTAD DE CIENCIAS EXACTAS Y NATURALES

ESCUELA DE CIENCIAS BIOLÓGICAS

Population Structure and Conservation Status of the Bottlenose Dolphin Population in the Gulf of Guayaquil, Ecuador.

Tesis previa a la obtención del título de

Magíster en Biología de la Conservación

ROSA DE LOS ÁNGELES BAYAS REA

Quito, 2015

Certifico que la Tesis de Magíster en Biología de la Conservación de la candidata Rosa de los Ángeles Bayas Rea ha sido concluida de conformidad con las normas establecidas; por lo tanto, puede ser presentada para la calificación correspondiente.

Rommel Montúfar, Ph.D.

Director de la Tesis

Quito, 13 de octubre de 2015

AGRADECIMIENTOS

En primer lugar, agradezco al Dr. Rommel Montúfar por aceptar dirigir la presente investigación relacionada con delfines. Muchas gracias por las facilidades para realizar la parte práctica del estudio y la ayuda para conseguir financiamiento, el mismo que fue otorgado por la Maestría de la Biología de la Conservación. Sin ese apoyo, este trabajo no hubiese sido posible. Le agradezco por todas las revisiones y sugerencias para mejorar el manuscrito.

Gracias al Dr. Fernando Félix por la idea de la investigación y todo su apoyo en varias fases del estudio. Desde su gran ayuda en el campo durante la recolección de muestras; así como, en su importante aporte durante las correcciones del manuscrito. Agradezco al Ministerio de Ambiente de la provincia del Guayas por otorgar el permiso de investigación (No 004-IC-FAU-DPG/MAE).

Un agradecimiento especial a todos los amigos que me acompañaron al campo a colectar las muestras. A Andrea Torres, Karina Ponce, Andrea Marquínez, Mercedes Serrano y César Yumiseva, muchas gracias. También quisiera agradecer a las personas que manejaron los botes tanto en Posorja, Isla Puná como en Bajo Alto, por todo el apoyo y paciencia para buscar a los delfines.

A Ben Haase por permitir el acceso a la colección de las muestras óseas de delfines depositadas en el Museo de Ballenas de Salinas. A César Yumiseva por la elaboración del mapa de los puntos de colección. A Oscar Pérez por el préstamo de la incubadora para el procesamiento de las muestras óseas.

A los lectores tanto externos como internos, Dra. Johanna Alfaro, Dr. Fernando Félix y M.Sc. Santiago Burneo, muchas gracias por sus valiosos

comentarios en la revisión final del presente manuscrito. A Doyle Beaty por las correcciones del idioma, las mismas que son importantes para mejorar la presentación del manuscrito, muchas gracias.

Agradezco a todas las personas que forman o formaron parte del laboratorio de Genética y Ecología de la PUCE y a todas las personas que de alguna u otra forma apoyaron esta bonita experiencia de trabajar con delfines. Gracias a Gabriel Rivadeneira Gallegos, Ana María Troya, Andrés Recalde, Sebastián Escobar, Nelson Dueñas e Isabel Ojeda. Siempre se aprende algo nuevo de cada persona, gracias.

Gracias a mi familia; en particular, le agradezco mucho a mi hermano Marco por apoyarme en cada una de mis propósitos y lo más importante porque siempre puedo contar con él, en las buenas y en las malas.

Muchas gracias Fer por la ayuda y apoyo en la última etapa de la tesis.

Por último pero no menos importantes, un gracias enorme a todos los delfines que nos permitieron tomar su muestra de piel, GRACIAS…

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El presente trabajo y la literatura citada se encuentran redactados bajo el formato de la revista PLOS ONE.

1. RESUMEN

El delfín nariz de botella, *Tursiops truncatus*, tiene una amplia distribución a lo largo de la costa oeste de Sudamérica. En Ecuador, una población residente de delfines habitan el estuario interno del Golfo de Guayaquil, localizado en la parte sudoeste del país. Para evaluar el estado de conservación de esta población, se analizó la estructura poblacional, los patrones filogeográficos y las relaciones filogenéticas en base a microsatélites y genes codificantes y no codificantes del ADN mitocondrial. Para el estudio se colectaron 31 muestras de piel de delfines de tres localidades del estuario interior del Golfo de Guayaquil. Adicionalmente, para los análisis filogenéticos y filogeográficos se incluyeron nueve muestras de piel y 17 de hueso de animales varados. Como resultado, los análisis filogenéticos y filogeográficos revelaron que la población de delfines del estuario constituye un grupo genéticamente diferente, separándose de otras poblaciones de *T. truncatus*. Además, los datos a nivel de microsatélites, región control y del gen citocromo oxidasa I mostraron una estructura poblacional significativa. La diversidad genética fue alta en base a los datos de los microsatélites y el gen citocromo oxidasa I; mientras que, con la región control fue baja. Por otro lado, los resultados con los microsatélites indican altos niveles de endogamia y ausencia de dispersión mediada por el sexo. En base a los resultados se sugiere que la población de delfines del estuario interior del Golfo de Guayaquil representa una unidad evolutiva significativa. Debido a que los delfines del estuario enfrentan una variedad de amenazas antropogénicas, se enfatiza la fragilidad de la población y se sugiere a las autoridades gestionar un manejo y medidas de conservación a corto plazo.

2. ABSTRACT

The Bottlenose Dolphin, *Tursiops truncatus*, has a wide distribution along the west coast of South America. In Ecuador, a resident population of Bottlenose Dolphins inhabits the inner estuarine area of the Gulf of Guayaquil, located in the southwestern part of the country. In order to evaluate the conservation status of this population, population structure, phylogeographical patterns, and phylogenetic relationships were assessed using loci microsatellites and non-coding and cording-protein gene of the mitochondrial DNA. Therefore, 31 epidermal samples were collected from free-ranging dolphins in three localities in the inner estuary of the Gulf of Guayaquil. In addition, 9 skin and 17 bone samples from stranded dolphins were included into phylogenetic and phylogeographic analysis. Our phylogenetic analysis indicated that this estuarine Bottlenose Dolphin population constitutes an isolated group with high levels of genetic differentiation from other populations of *T. truncatus*. The results also showed a significant population structure with microsatellite loci, control region, and the cytochrome oxidase I gene. In addition, the results show a high genetic diversity with microsatellites and the cytochrome oxidase I gene, and low genetic diversity with mitochondrial control region. Moreover, high levels of inbreeding, and no evidence of sex-bias dispersal were identified in microsatellite data. Based on the results, we suggested that the estuarine Bottlenose Dolphin population of the inner estuary in the Gulf of Guayaquil represents a distinct Evolutionary Significant Unit. Since these estuarine Bottlenose Dolphins face a variety of anthropogenic threats in this area, we highlight the fragility of the dolphin population and urge authorities to issue management and conservation measures promptly.

3. INTRODUCTION

Monitoring the conservation status of coastal, small, and resident populations of marine mammals is very important for conservational and management purposes [1]. Three of the main genetic issues in conservation biology are phylogenetic status, genetic diversity, and population structure [2]. Phylogenetic status allows the understanding of the taxonomic relationships of the species. Genetic diversity is one of the three levels of diversity that requires conservation since it represents the ability to adapt and evolve in response to environmental changes [2, 3]. Population genetic structure provides insight into the impacts of mutation, genetic drift, selection, and migration on population dynamics [3, 4]. Altogether, the evidence provides Evolutionary Significant Units (ESUs) and Management Units (MUs) [5]. ESU is a population or group of populations with adaptive divergence and reproductive isolation whereas MUs is associated with demographically isolated populations [3] in which the most important is the rate of births ands deaths [5]. Thus, information on phylogeny, genetic diversity, and population structure of threatened coastal wildlife populations are essential to develop appropriate conservation and management strategies.

The genus *Tursiops* includes at least two accepted species based on molecular and morphological differences [6]. The species are *T. aduncus* (Ehrenberg, 1833) and *T. truncatus* (Montagu, 1821) with two subspecies, *T. truncatus ponticus* and *T. truncatus truncatus* [6]. The aduncus-type dolphin from South Africa has been proposed as different species [7]; currently, its status as species is not accepted [6]. Recently, an endemic small population of the Australian continent, the Burrunan dolphin (*T. australis*), also has been suggested as a new species based on morphological, genetic differentiation [8-10], and phylogenetic analysis [11]. Nevertheless, its status as distinct species is not confirmed due

to the insufficient supporting evidence [6]. Although several subspecies were reported in the Mediterranean [12, 13], only two subspecies of *T. truncatus* are recognizes nowadays [6].

The Bottlenose Dolphin, *Tursiops truncatus,* is widely distributed in pelagic and coastal waters; including sounds, bays, and estuaries [6]. Its adaptation to different environmental conditions has generated two different ecotypes, coastal and offshore [7, 14]. The two ecotypes can be in sympatry or parapatry [15]. The offshore ecotype is present in pelagic, coastal and insular waters [14, 16, 17] whereas the coastal ecotype was reported only in coastal, estuarine, bays, and continental areas [18, 19]. The offshore ecotype is highly dispersed, has high levels of genetic diversity and a lack of population structure [7, 14, 20]. On the contrary, most of the coastal populations show population structure at small geographical scales with low genetic diversity at the regional level worldwide [21, 22, 23].

Although the Bottlenose Dolphin is present along the west coast of South America [6], only one genetic study was carried-out for this species in this wide region. Sanino et al. [16] evaluated the genetic diversity and the phylogenetic relationships of two groups of offshore and two coastal dolphin populations from Peru and Chile. Among these populations, three different evolutionary units, one offshore Peruvian-Chilean population and two different coastal Peruvian and Chilean populations, were found. In Ecuador, information on the Bottlenose Dolphin is scarce. The presence of the offshore ecotype has been reported in the Galápagos Islands and in pelagic waters [24, 25]. The coastal ecotype was documented in the inner estuary of the Gulf of Guayaquil, including Jambelí Channel, Puná Island [26, 27], Morro Channel, Posorja Harbor, and Bajo Alto mangroves [28]. Two studies were carried out in the 90's based on behavioral ecology, organization, and social

structure of the Bottlenose Dolphin [26, 27]. These studies reported on the presence of a single resident coastal population of around 600 dolphins divided into at least five communities in Jambelí Channel and Puná Island. A more recent study described a single resident population of around 45 dolphins inhabiting Morro Channel within the Morro Mangrove Wildlife Refuge [28]. The social organization of the Bottlenose Dolphin in the Gulf of Guayaquil was characterized as a hierarchically structured society in which females are organized in bands while males form alliances in order to obtain a dominant status and access mature females [27]. In addition, Bottlenose Dolphins in the Gulf of Guayaquil form small or big groups so they can forage at a specific site [26, 28].

The Bottlenose Dolphins are listed as a species of Least Concern (LC) in the Red List of the International Union of Conservation of Nature (IUCN) [29] and in Appendix II of the Convention on International Trade in Endangered Species of wild Fauna and Flora (CITES) [30]. In Ecuador, this species is considered Vulnerable (VU) according the Ecuadorian Mammal Red List, and protected by Ecuadorian law so that hunting and trade is prohibited in the country indefinitely [31]. Although the species is classified as protected, the Bottlenose Dolphin population in the inner estuary of the Gulf of Guayaquil (hereafter estuarine Bottlenose Dolphin) is one of the most vulnerable to threats of human activities, including intense fishing, vessel collision [32, 33], water pollution, popular tourism [28, 31], habitat degradation, and dredging activities [28].

In order to assess the population status of the estuarine Bottlenose Dolphin population in the inner estuary of the Gulf of Guayaquil in terms of phylogeny, population structure, and genetic diversity, a genetic study was conducted using different molecular markers. This type of information is important to improve local management strategies for such discrete populations and to ensure their long-term conservation.

4. MATERIALS AND METHODS

4.1. ETHICS STATEMENT

No ethical approval was considered necessary due to the fact that the animals were not handled directly and a non-invasive sampling technique was used; for that reason, the sampling technique was not submitted for an ethical analysis. The samples of free ranging dolphins were collected following the guidelines specified by the research permits given by the Department of Guayas, Environmental Ministry of Ecuador (permit number No 004-IC-FAU-DPG/MAE).

4.2. STUDY AREA

The Gulf of Guayaquil, the largest estuary of the southeast Pacific coast, is located in the southwestern part of Ecuador (3˚S and 81˚W) (Fig. 1). The entrance of the gulf is 200 km wide, stretching from Santa Elena (2˚12'S), Ecuador to near Máncora (4˚07'S), north of Peru, and extending 130 km inland. The Gulf of Guayaquil includes an outer estuary and an inner estuary. The outer estuary begins at the west side of Puná Island (80˚15'W) and ends 81˚W; whereas, the inner estuary extends inland in several directions, embracing a complex of islands covered in mangroves and channels. Two large branches, Estero Salado and the Guayas River, extend northeastward. Puná Island lies between two channels: Morro Channel, stretching 3 km northeast, and Jambelí Channel, a channel 11-28 km wide that connects the outer estuary with the Guayas River [34]. Morro Channel is part of a protected area of the Morro Mangroves Wildlife Refuge, (2˚39'S and 80˚ 11'W), a reserve of approximately 10,130 hectares of mangrove forest [35]. In the north part of Puná Island, the Cascajal Channel connects the Estero Salado to the Guayas River [34].

The climate of the study area is regulated by two seasons: one dry and cold, and one warm and rainy. In the outer estuary, the surface water temperature varies from 21.5ºC to 25ºC during the dry and warm seasons respectively. Whereas, in the inner estuary, the water surface temperature fluctuates between 25ºC during the dry season to 28ºC during the warm season. Rainfall is seasonal with more than 95 % of the precipitation occurring from December to May, causing seasonal river discharge [36]. Guayas River is the main contributor of freshwater to the estuary [34].

The Guayas estuary ecosystem is characterized by high biological productivity in which there are around 148,000 hectares of mangrove forest. The mangrove is characterized by water saturated with poor drainage, has and by the presence of a high biodiversity of fish, aquatic birds, and marine invertebrates [37].

4.3. SAMPLE COLLECTION

4.3.1. ECUADOR DATABASE

A total of 31 epidermal cell samples were collected from free-living dolphins in three different sites (Posorja Harbor n = 22, Morro Channel n = 7, and Puná Island n = 2) in the inner estuary of the Gulf of Guayaquil (Fig. 1). The collection of the epidermal samples took place between March and August 2013. Swab samples were taken from the dorsal-lateral region of the dolphins based on the non-invasive technique reported by Harlin et al. [38] with a few modifications. A sterilized square piece of sand paper 5 cm x 5 cm was used instead of a nylon scrub pad wrapped around the tip of a pole. Epidermal cells samples were removed with sterilized forceps and stored in 100 % ethanol for subsequent genetic analysis.

In addition, 38 museum samples were included into the study; of which, 37 were obtained from stranded dolphins (8 skin and 29 bone samples), and one skin sample from a Galápagos free-living dolphin (permit number No PC-13-05). The stranded samples were collected from different localities of the Gulf of Guayaquil; principally from Mar Bravo and Punta Carnero seashores located in Salinas. These samples are available at the collection of the "Museo de Ballenas" (Whale museum) in the city of Salinas. Skull and mandible bone powder was gathered based on the technique reported by Morin et al. [39]. All genetic analysis was performed in the Laboratory of Ecology and Genetic at the Pontifical Catholic University of Ecuador. The data of all samples was included into the study and its distribution is summarized in S1 Table.

4.3.2. WORLDWIDE DATABASE

In order to perform a phylogenetic analysis, a total of 22 mitogenomic sequences belonging to *Tursiops truncatus* (n = 13), *T. aduncus* (n = 3), Burrunan dolphin (*T. australis*, n = 2), *Delphinus capensis* (n = 1), *Sousa chinensis* (n = 1), *Stenella attenuata* (n = 1), and *S. coeruleoalba* (n = 1) were taken from the GenBank database (S2 Table) and included in the present study. The sequences of the harbor porpoise, *Phocoena phocoena* (accession number NC_005280.1), and narwhal, *Monodon monoceros* (accession number NC_005279.1), were included as outgroups. Additionally, 91 haplotype sequences of 400 bp from different geographical regions made available at the GenBank database were included to investigate the phylogeographic relationships among haplotypes located in the Gulf of Guayaquil and other populations of *T. truncatus* (accession numbers, S3 Table) elsewhere.

4.4. DNA EXTRACTION

Total genomic DNA from 31 epidermal cells and 9 skin samples was isolated using a modified proteinase K digestion protocol and two clorophorm:isoamyl (24:1) extractions followed by ethanol precipitation [40]. DNA from the bone powder of 20 samples was extracted using a Wizard Genomic DNA Purification Kit (Promega), following the manufactures' protocol. The concentration and purity of genomic DNA was analyzed using a Nanodrop spectrophotometer (Thermo Scientific).

4.5. MOLECULAR SEX DETERMINATION

Dolphin sex was identified from 31 epidermal samples from free-ranging dolphins with a duplex Polimerase Chain Reaction (PCR) amplification using two sets of primers, ZFX0582F and ZFX0923R [41], to target a partial fragment of the ZFX gene, and PMSRYF [42] and TtSRYR [43] to amplify a partial fragment of the SRY gene. PCR amplification reactions were carried out based on those reported by Rosel [43], with a few modifications detailed in Materials and Methods S1.

4.6. MITOCHONDRIAL DNA SEQUENCING

Partial mitochondrial DNA from 31 epidermal samples from free ranging dolphins and nine skins museum samples were amplified by PCR using seven sets of primers. (i) A fragment of ~1,062 bp comprising partial regions of 12S rRNA, tRNA-Val, and a 16S rRNA was amplified using the primers mt12F-12S RNA and mt12R-12S RNA [44]. (ii) A partial fragment of 16S rRNA (~590 bp) was amplified using the primers 16SarL and 16SbrH [45]. (iii) The region composing partial fragments of NADH dehydrogenase subunit I y II (ND1 y ND2) genes, tRNA-Ile, tRNA-Gln, and tRNA-Met was amplified using the ND1F and ND1R [46]. (iv) A partial region of the cytochrome oxidase I (COI,

~830 bp) gene was acquired with the primers COXI F and COXI R [47]. (v) A fragment of ~ 800 bp including the complete cytochrome oxidase II (COII) gene, and a partial tRNA-Asp and tRNA-Lys was achieved using primers CO2LCet and CO2RCet [48]. (vi) A fragment of ~ 450 bp of partial tRNA-Glu and cytochrome b (Cytb) gene was amplified with the primers L14724 [45] and H15149 [49]. (vii) The control region (CR, ~ 800 bp) was amplified using the primers dLp1.5t-pro [50] and dLp8G [51]. For the 29 bone DNA samples, the hypervariable region of CR (~ 500 bp) was amplified with the primers dLp1.5t-pro and dLp5 [50].

The details of each set of primers and PCR conditions are in the S4 Table. All PCR products were purified for sequencing using exonuclease I and shrimp alkaline phosphatase (ExoSap-IT®) and then by incubating at 37˚C for 30 minutes and 80˚C for 15 minutes. Both strands were sequenced by MACROGEN (Seul, Korea). All sequences were manually edited using BioEdit 7.2.3 software [52]. In order to validate the data, all sequences were analyzed by using the BLAST algorithm in GenBank [53]. Multiple alignments were performed for each gene using CLUSTAL W implemented in MEGA 6 software [54].

4.7. MICROSATELLITE GENOTYPING

A total of 31 epidermal samples from free-ranging dolphins were genotyped at 10 microsatellite loci. Primer sets for loci D8, D22, TexVet5, TexVet7, Ttr11, and TtrRC12 were derived from *Tursiops truncatus* [55-57]; MK6, MK9, and Tur4_91 from *T. aduncus* [58, 59]; and EV37 from humpback whales (*Megaptera novaeangliae*) [60]. The details of each set of primers and conditions of PCR are in the S5 Table. The PCR products were separated by electrophoresis in 6 % denaturing polyacrylamide gels and visualized by silver nitrate staining, following the protocol described by Benbouza et al. [61].

4.8. MITOCHONDRIAL DNA DATA ANALYSIS

4.8.1. PHYLOGENETIC ANALYSIS

For the phylogenetic analysis, 40 Ecuadorian samples (31 free-ranging dolphin samples and 9 skin museum samples) and 24 sequences from GenBank (22 from different species of Delphinidae and two as outgroups) were used. A Bayesian inference was achieved in concatenated sequences of seven fragments of mitochondrial DNA. Additionally, the analysis was done in four groups: (i) a partial fragment of 12S rRNA, tRNA-Val, and a partial fragment of 16S rRNA, (ii) protein-coding genes with its tRNAs, (iii) CR, and (iv) the COI partial gene. To perform the phylogenetic analysis, mitochondrial data was analyzed based on two partitioning schemes to apply the substitution model unto non-coding and protein-coding regions. Non-coding regions were concatenated in three groups: (i) the two partial ribosomal RNA genes (12S rRNA and 16S rRNA), (ii) the seven partial tRNA genes (tRNA-Val, tRNA-Ile, tRNA-Gln, tRNA-Met, tRNA-Asp, tRNA-Lys, and tRNA-Glu), and (iii) CR. The protein-coding genes (ND1, ND2, COI, COII, and Cytb) were divided into three partitions considering the first, second, and third codon positions [62]. The best-fitted model of nucleotide evolution under the Akaike Information Criterion (AIC) was inferred using jModelTest v.2.1.4 software [63].

All phylogenetic analysis was performed using MrBayes v.3.2.2 [64]. Posterior probabilities of the trees and parameters in the evolutionary model were approximated with Markov Chain Monte Carlo (MCMC). Two independent runs of four chains were carried out to 20,000,000 and 10,000,000 generations for complete and individual analyses respectively with a 200,000 and 100,000 burn-in, sampling every 5,000 generations. In order to ensure mixing and convergence of the posterior distribution and parameters,

effective sample size (ESS) values were evaluated using Tracer v.1.6 software [65]. The tree was visualized and edited using the FigTree v.1.4.2 [66].

4.8.2. PHYLOGEOGRAPHIC ANALYSIS

In the phylogeographic analysis, we included 57 samples (31 epidermal cells samples from free-ranging dolphins, 9 skin and 17 of 29 bone samples from the museum) that amplified ~ 500 bp belonging to the hypervariable region within CR. Additionally, 91 haplotypes from different geographic localities were in alignment with Ecuadorian samples. The genealogical relationships at the haplotype level were inferred using median-joining network implemented in Network v.4.6.0 software [67].

4.8.3. POPULATION STRUCTURE

To investigate population structure and genetic variability within and among sampling sites, 31 epidermal samples from free-ranging dolphins were used to analyze the CR (~ 800 bp) and the COI gene (~ 830 bp) as they were the most variable sequences. Population genetic differentiation was calculated using an analysis of molecular variance (AMOVA) [68] based on haplotype data (F_{ST}), haplotype frequency and genetic distance (φ_{ST}) via Arlequin v.3.5 [69]. The Tamura-Nei [70] model with a gamma correction of 0.5 was used to estimate the distance between sequences. F_{ST} and φ_{ST} significance levels were tested with 10,000 permutations.

The variability of both mitochondrial regions (CR and COI) was compared to haplotypes of *Tursiops truncatus* from the Gulf of California (accession number KF570389.1). Genetic diversity was estimated by calculating the number of haplotypes and haplotype diversity (h) using DNAsp v.5.10.01 [71], and nucleotide diversity (π) with the Arlequin v.3.5 software [69]. Tajima's D test [72] and Fu's Fs test [73] of selective

neutrality were estimated with Arlequin v.3.5. Significance of both neutrality tests was inferred by randomization (10,000 steps).

4.9. MICROSATELLITE DATA ANALYSIS

4.9.1. POPULATION STRUCTURE

As for the analysis of population structure, we used 31 free-ranging dolphin samples from the inner estuary of the Gulf of Guayaquil. A Bayesian model-based clustering method was used to determine the most probable number of distinct nuclear genetic clusters and assign individuals to each one using STRUCTURE v.2.3.3 [74]. The admixture model with correlated allelic frequencies was selected, without specifying the sampling location. The model was run with the most probable number of clusters (K) set to values of 1 to 5 with a burn in period of 10,000 iterations followed by 100,000 MCMC iterations. Five independent runs for each number of clusters were carried out for each K value. The real K value was detected by calculating the modal value of ΔK, a quantity based on the second order rate of change with respect to K of the likelihood function [75] using Structure Harvester web v.0.6.93 [76]. The five independent runs from STRUCTURE were analyzed with the CLUMPP 1.1.2 [77]. The results were visualized using DISTRUCT 1.1 [78].

The degree of genetic differentiation among clusters was tested by calculating pairwise F_{ST} using Tamura Nei distances [70] with Arlequin v.3.5 software [69]. An analysis of molecular variance (AMOVA) was implemented to compute significance with 10,000 permutations. Cluster interrelationships were estimated with the Neighbor Joining (NJ) algorithm as implemented in PAUP v.4.0b10 [79]. Bootstrap confidence estimates were based on 1,000 replications [80].

Measurements of genetic diversity were based on three clusters and evaluated by calculating the number of private alleles (PA), the inbreeding coefficient (F_{IS}), observed and expected heterozygosity (Ho and He) and the number of alleles (NA) using FSTAT v.2.9.3.2 [81]. Significant deviations from the Hardy-Weinberg Equilibrium (HWE) were tested using Markov Chain Parameters with 1,000 iterations with Genepop web v.4.2 software [82].

The evidence of sex-biased dispersal of the three clusters was estimated according to the corrected assignment index (AIc) using GENALEX 6 [83]. In addition, sex-biased dispersal was evaluated by analyzing the differences in F_{IS}, F_{ST}, relatedness, mean assignment index, and variance of assignment indices [84] using FSTAT v.2.9.3.2 [81] and 10,000 permutations.

5. RESULTS

5.1. PHYLOGENETIC ANALYSIS

Of a total of 40 sequences, 29 non-shared haplotypes were identified in Ecuador (21 from the Gulf of Guayaquil, one from Galápagos Islands, and seven from stranded dolphins). Fifty-three concatenated haplotype sequences of ~5,268 bp from seven mtDNA fragments, 29 no shared haplotypes from Ecuador and 24 haplotypes sequences from GenBank, were included into the phylogenetic analysis. The mtDNA sequences of 53 haplotypes presented 1,008 variable sites of which 527 were informative sites and 43 included gaps. Details from sequence variation, the substitution model, and tree characteristics are shown in Table 1.

Bayesian inference with concatenated sequences produced a well-defined and strongly supported tree with posterior probability values > 90 for most nodes. All *Tursiops truncatu*s sequences formed a single clade with a probability value of 100. The 21 haplotypes from the free-ranging dolphins pertaining to the inner estuary and from the two stranded animals (from Peru and a locality in Mar Bravo) presented a posterior probability value of 100, grouping them into a cluster separate from other coastal and pelagic *T. truncatus* populations. Additionally, four haplotypes from the stranded dolphins (one from Punta Carnero and three from the Mar Bravo locality) were clustered with the single haplotype from the Gulf of California (accession number KF570389.1), whereas two haplotypes (from the Galápagos Islands and the Mar Bravo locality) were clustered with the haplotypes from the Atlantic Ocean, the Black Sea, and the Mediterranean Sea (Fig. 2).

The four individual phylogenetic analyses reveled three different results. The Bayesian inference with non-coding genes (12S rRNA, tRNA-Val, and a partial fragment

of 16S rRNA), and protein coding genes (ND1-ND2, COII, and Cytb) demonstrated that Ecuadorian dolphin sequences cluster together within the *Tursiops truncatus* cluster (S1 and S2 Figs.). Whereas the phylogenetic analysis of the CR showed that the estuarine Bottlenose Dolphins of the inner estuary of the Gulf of Guayaquil and the two samples of stranded dolphins (Peru and the Mar Bravo locality) are grouped into the clade conformed by *T. aduncus* (accession number KF570362.1)*, Delphinus capensis* (accession number NC_012061.1)*,* and *Stenella coeruleoalba* (accession number NC_012053.1) (S3 Fig.). Finally, the analysis of the COI gene indicated that most of the free ranging dolphins' sequences from the inner estuary of the Gulf of Guayaquil clustered into a single clade, separate from other species of the family Delphinidae (S4 Fig.).

5.2. PHYLOGEOGRAPHIC ANALYSIS

A total of 57 sequences of 400 bp of the CR from Ecuador (31 samples from free-ranging dolphins, plus 9 skin and 17 bone museum samples) and 91 sequences from different geographic localities were aligned. The median-neighbor joining method showed the relationships among haplotypes from different populations of Bottlenose Dolphins, revealing evidence of a phylogeographic pattern. The network analysis indicated that the group of haplotypes from the free-ranging dolphins plus the haplotypes from the stranded dolphins collected at different areas of the Gulf of Guayaquil (H_94, H_100, H_101, H_103, and H_104) and one from Peru (H_105) clustered together and diverged from the other haplotypes of different geographic localities. The main characteristic of this group was the presence of a central haplotype (H99) from the inner estuary of Gulf of Guayaquil. The central haplotype was connected to other haplotypes from the inner estuary, one from Peru, and one from the Gulf of California (H_47, accession number HE617284.1). The other haplotypes (H92, H_93, H_94-H98, and H_102) were more related to other

populations of dolphins, mainly from the Northeast Pacific. Additionally, the network revealed several mutational steps (~16) away from the main group indicating a divergence of haplotypes (Fig. 3). Overall, the Median-joining network method was consistent, showing a close relationship among CR haplotypes of the free-dolphins of the inner estuary of the Gulf of Guayaquil, indicating phylogeographic separation.

5.3. POPULATION STRUCTURE

Two approaches with different genetic information were used in order to evaluate population structure. Thirty-one epidermal cell samples from free-ranging dolphins were genotyped at 10 cetacean microsatellite loci. No matching genotypes were identified in microsatellite data; furthermore, microsatellite D22 was monomorphic. The Bayesian analysis performed using STRUCTURE proposed three clusters (K) of estuarine dolphin samples: cluster 1 (n = 11), cluster 2 (n = 8), and cluster 3 (n = 12) (Fig. 4). The pattern of genetic structure in the population was inferred from Bayesian clustering and confirmed with Neighbor Joining (S5 Fig.). The AMOVA based on the three clusters identified by the Bayesian analysis identified an 18.61% percentage of variation among groups. Fixation indices among clusters indicated a structured population ($F_{ST} = 0.186$, $P < 0.05$).

Genetic diversity was calculated based on the three clusters identified by STRUCTURE with microsatellite data. The mean number of alleles ranged between 3.3 and 4.1, and expected heterocigosity varied from 0.52 to 0.63. Overall, genetic diversity ranged from 0.35 to 0.49 within groups whereas the global high was 0.50. Global inbreeding values were high and significant over all loci and samples ($F_{IS} = 0.51$, $P < 0.001$). All clusters exhibited significant deviations from the Hardy-Weinberg Equilibrium (Table 2).

The AMOVA performed with 694 bp of the CR and 839 bp of COI indicated genetic structuration between sites (CR: percentage of variation among groups = 16.96 %, F_{ST} = 0.169, $P < 0.05$, and COI: percentage of variation among groups = 11.78 %, F_{ST} = 0.117, $P < 0.05$). However, the analysis of genetic distances (ϕ_{ST}) was unable to detect differences between localities (CR: percentage of variation among groups = 18.59 %, ϕ_{ST} = 0.186, $P = 0.074$, and COI: percentage of variation among groups = -4.03 %, ϕ_{ST} = -0.04, $P = 0.483$).

The analysis of genetic diversity showed seven polymorphic sites, revealing seven haplotypes not previously described (Table 3). Three haplotypes were shared among the sites Posorja Harbor and Morro Channel; three haplotypes were unique to Posorja Harbor, and one to Puná Island (S6 Fig.). The analysis of the COI gene revealed 54 polymorphic sites defining eleven haplotypes (Table 3). Three haplotypes were common between Posorja Harbor and Morro Channel, one haplotype shared between Puná Island and Morro Channel; five haplotypes were unique to Posorja Harbor, and two to Morro Channel (S7 Fig.). Overall, haplotype diversity (h) was low ($h = 0.7$) and high ($h = 0.83$) for CR and COI respectively while nucleotide diversity (CR: $\pi = 0.002$, COI: $\pi = 0.016$) was low for both mitochondrial markers (Table 3).

5.4. SEX-BIAS DISPERSAL

A total of 31 epidermal cells samples from free-ranging Bottlenose Dolphins in the inner estuary of Gulf of Guayaquil were identified by molecular sex analysis. Molecular sex determination showed a sampling bias in favor of males (22) over females (9) (sex ratio 2.4:1). Sex-biased dispersal was estimated across all dolphin populations in terms of the corrected assignment index (AIc). The mean assignment bias (AIc) was 0.325 for female and – 0.108 for male, the AIc values were not significant ($P > 0.05$), suggesting no

sex bias dispersal. Sex-biased dispersal tests showed lower estimated F_{IS}, F_{ST} values, or relatedness for females (F_{IS} = 0.357, F_{ST} = 0.082, and Relatedness = 0.116) than for males (F_{IS} = 0.431, F_{ST} = 0.178, and relatedness = 0.232); nevertheless, values were not significant ($P > 0.05$). The mean assignment index (female = -0.123 and male = 0.05), and variance of assignment (female = 5.83 and male = 6.79) indices were non-significant ($P > 0.05$). Overall, the assignment tests and the sex-biased dispersal tests were unable to detect any sex-biased differences in the dispersal behavior of males and females.

6. DISCUSSION

6.1. GENETIC DIVERGENCE OF THE ESTUARINE BOTTLENOSE DOLPHIN POPULATION OF THE GULF OF GUAYAQUIL

The phylogenetic relationships of the species of the genus are controversial. Our phylogenetic analysis revealed a clear separation of *Tursiops truncatus* from the Burrunan dolphin (*T. australis*) population and the accepted species *T. aduncus*. In addition, the phylogenetic reconstruction did not support the monophyly of the genus similar to other studios based on mtDNA [9, 11 46]. The relationships inside the genus can be related to the rapid radiation of the species [6]. This pattern was evidenced in the discordance the phylogeny based on individual genes. This could be attribute to the incomplete lineage sorting or events of the hybridization between species in the entire family [85]. However, the most relevant pattern was the genetic divergence between the estuarine Bottlenose Dolphin from the Gulf of Guayaquil and the coastal and offshore *T. truncatus* populations. This adaptive divergence may be as a consequence of environmental drivers as it has been suggested to explain the diversification of the genus [11]. The coastal species *T. aduncus* presents a similar pattern, two genetically diverging populations originating from the Indo-Pacific Ocean and South Africa [7, 11]. In order to understand the taxonomic status of the estuarine Bottlenose Dolphins in the Gulf of Guayaquil a combination of environmental, genetic, ecological, and morphometric studies are required.

At an intraspecific level, the systematics of *Tursiops truncatus* is not well defined. Several studies have suggested that coastal and offshore ecotypes could constitute different linages of *T. truncatus* [11]. In particular, the coastal populations are genetically well differentiated between each other worldwide [17]. The high levels of genetic

differentiation reported by mitochondrial data suggest that the estuarine Bottlenose Dolphins inhabiting the inner estuary of the Gulf of Guayaquil have a different evolutionary pattern. This could be a consequence of geographic isolation, territorial behavior [86], and the form in which the population is structured in semi-closed resident communities, precluding any genetic influx from nearby areas of the Gulf of Guayaquil. Thus, this population constitutes an isolated population with high levels of genetic differentiation.

It has been suggested that limited interaction or gene exchange supports genetic variation and isolation that may lead to speciation [7, 12, 87, 88]. The lack of gene exchange between estuarine Bottlenose Dolphins and other populations of *T. truncatus* could have occurred after a possible founder effect of coastal dolphins in embayment areas [17, 18, 87]. A similar pattern was proposed for the Mediterranean Sea subpopulations of *T. truncatus* [11]. Founder events play an important role in adaptation to new environments [88] with local specializations for resources [14]. It was suggested that habitat specialization in *T. truncatus* occurred independently in different areas over a wide range of distribution [88] and was driven by environmental factors such as salinity, temperature and productivity [12]. The inner estuary of the Gulf of Guayaquil has a high level of ecological diversity with specific abiotic and biotic characteristics. For example, the estuary contains a network of islands and channels, sand and mud banks, sandy beaches, as well as a high tidal range of 2-3 m, among other features. The mangrove forests and the oceanographic characteristics of the estuary such as temperature and salinity contribute to the high diversity of prey species [36]. The especial characteristics of the estuary could have driven genetic differentiation and adaptive divergences as occur with other marine species (see [89]). As a consequence, this estuarine Bottlenose Dolphin population could have been confined to small areas within the inner estuary and have adapted to the habitat

with specialized foraging behavior thus generating possible isolation from other populations of *T. truncatus*.

6.2. POPULATION GENETICS OF THE ESTUARINE BOTTLENOSE DOLPHIN

Coastal dolphin populations present genetic differentiation at fine geographical scale in different parts of the world [21, 22, 23]. This pattern is also observed in the estuarine Bottlenose Dolphin population from the Gulf of Guayaquil, which presents population genetic structure. In our analysis, F_{ST} values (0.186; $P < 0.05$) of microsatellites based on three genetic groups identified by STRUCTURE and the CR (0.169) suggested high population structure over small geographical scales (see [90]). Those values were higher than other populations of *Tursiops truncatus* in which the F_{ST} values ranged between 0.002-0.071 [23, 91]. Similar high levels of structure have also been reported from two communities of coastal *T. truncatus* in Ireland [22]. This high value of genetic differentiation is expected in large geographical scales [20, 91].

The sex-biased dispersal pattern plays an important role in determining the genetic structure of the population. In mammalians, male dispersion is expected whereas females tend to be philopatric [92]. Male dispersion and female philopatricy have been reported in some coastal populations of Bottlenose Dolphins [93]. In contrast, our assignment test and sex-biased dispersal test were unable to detect any sex-biased difference in the dispersal behavior of males and females. The lack of evidence for sex-biased dispersal for the estuarine Bottlenose Dolphins reported in this study could be related to the small sample size. However, these results are similar to other coastal populations in which both males and females present some degree of philopatry [12, 18, 19, 23]. The advantage of philopatry might help social facilitation of foraging and transferable communication over

generations [87]. This fidelity site may also promote inbreeding as suggested by the high inbreeding coefficient levels (F_{IS} = 0.51, $P < 0.001$) and would explain the deviation from the HWE found in this estuarine Bottlenose Dolphin population.

Bottlenose Dolphins in the Gulf of Guayaquil also showed low and high levels of genetic diversity. The low levels of genetic diversity were revealed by the mtDNA control region (CR: h = 0.7, π = 0.002). Similar values were documented in coastal dolphins [7, 15, 17, 21] and estuarine resident populations [23] in contrast to offshore dolphins. The results of the present research can be compared with those from the study described by Parson and colleagues [93] who worked with 29 samples in Bahamas and reported low genetic diversity (h = 0.69 and π = 0.0009 to 0.0164). On the contrary, high levels of genetic diversity were found in the study analyzing mtDNA COI and nuclear DNA (COI: h = 0.83 π = 0.016; mean He: 0.57). The high values of COI genetic diversity are related to offshore populations [7, 17, 20] and a few coastal small populations of New Zealand [17]. High mitochondrial diversity levels could be the result of population expansion [88], generating an excess of haplotypes distinguishable from each other by one or a few mutations [94]. However, the population expansion hypothesis is not supported by the Tajima's and Fu's Fs selective neutrality tests (CR: Tajima's D = 0.632 ns; Fu's F_s = -0.655 ns; COI: Tajima's D = -0.324 ns; Fu's F_s = 4.074 ns). The low nucleotide diversity values (CR: π = 0.002, COI: π = 0.016) found in the present study are similar to populations that have experienced a population bottleneck [95]; for instance, strong reduction of genetic diversity has been previously reported in other cetaceans, such as *Orcinus orca* [96].

6.3. CONSERVATION IMPLICATIONS

Based on the results in the present study, the pattern of genetic divergence and the lack of gene flow suggest that the Bottlenose Dolphin population inhabiting the inner estuary of the Gulf of Guayaquil is an isolated population with a unique genealogical and adaptive legacy. Therefore, management and conservation strategies need to be developed for the conservation of this population soon, before current activities cause irreversible damage. Such strategies have to be different to those applied to other populations of *Tursiops truncatus* due to the particular genetic diversity. Besides the low genetic diversity, another aspect of major concern is the high level of inbreeding, which makes this population more vulnerable to environmental stochasticity. This population must be considered as an Evolutionary Significant Unit (ESU) and its conservation status should be reevaluated. Although the sample size is small, our findings provide information on the molecular baseline for management; therefore, actions should not be delayed.

Ecological and evolutionary processes acting on this population may be affected by human activities, such as intense fishing, vessel traffic, water pollution, tourism, dredging, and habitat destruction, among others. Under this uncertain scenario, a priority for dolphin conservation requires that strategies should be orientated to minimize the impact of the above-mentioned human activities [97]. Improving conservation of the mangrove ecosystem is a key part of a future strategy because it constitutes a refuge for dolphins, but it will not sufficient if direct threats such as bycatch, collision with ships and irresponsible tourism are not addressed simultaneously.

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8. FIGURES

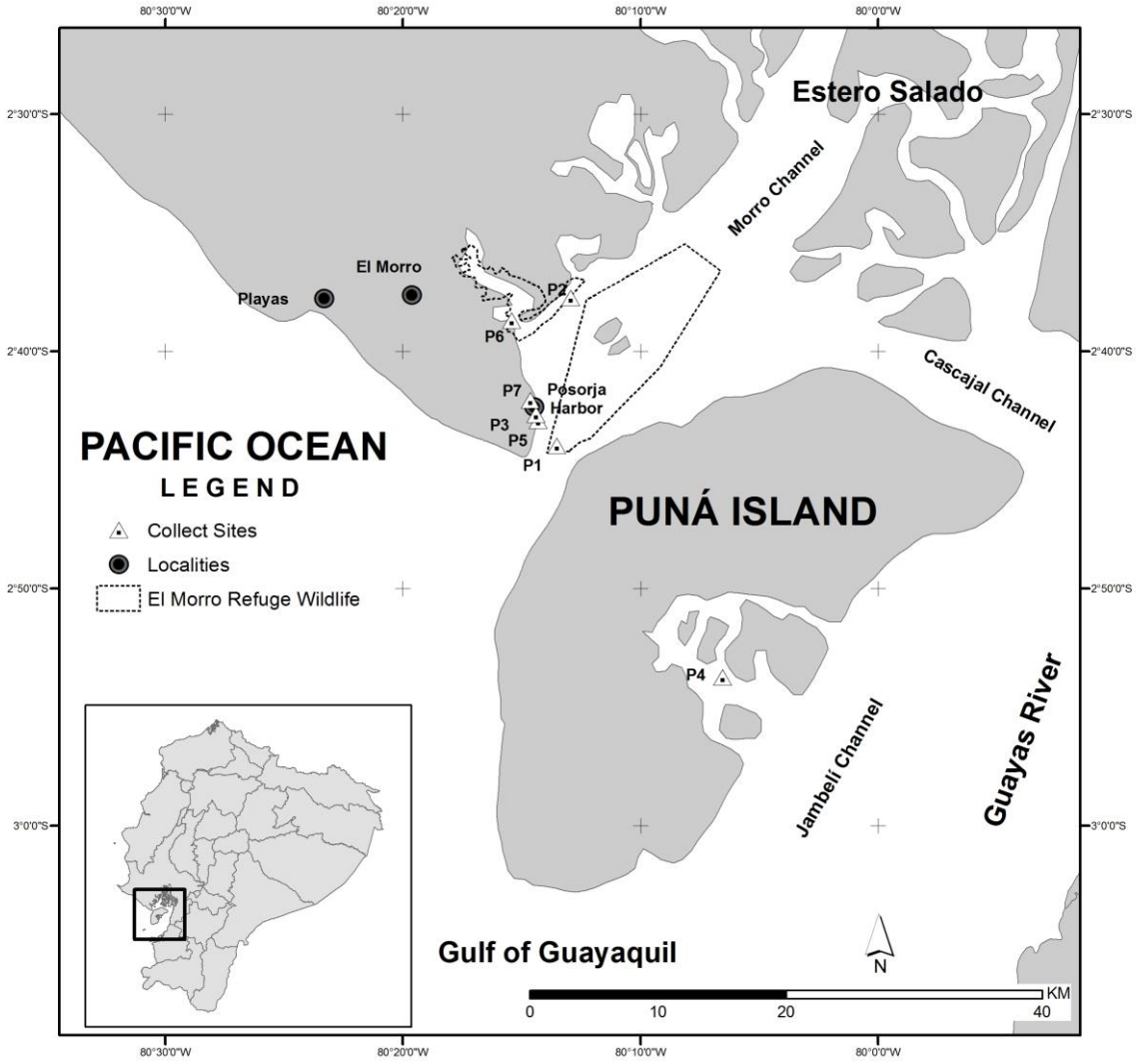


Fig. 1. Map of the Gulf of Guayaquil showing the three sampling sites. The sites are represented by the letter P, which indicates the GPS points where collection of the epidermal cells of free-ranging dolphins took place. Posorja Harbor (P1: Ttr38-Ttr46, P3: Ttr49-Ttr52, P5: Ttr55-Ttr57, P7: Ttr63-Ttr68), Morro Channel (P2: Ttr47-Ttr48, P6: Ttr58-Ttr62), and Puná Island (P4: Ttr53-Ttr54).

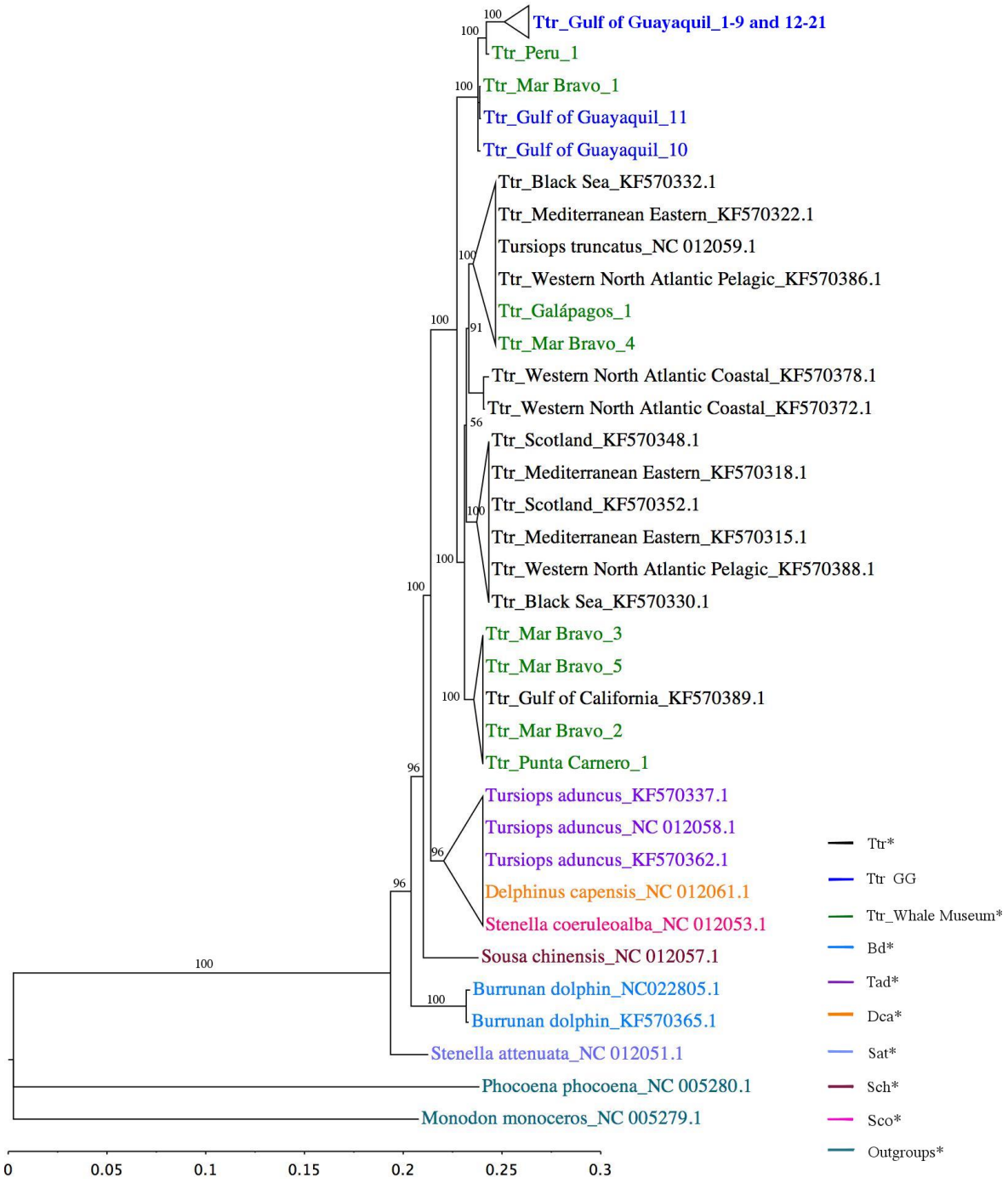


Fig. 2. Bayesian phylogenetic tree inferred from the analysis of ~ 5,268 bp of mtDNA. The phylogram shows the relationship between the Bottlenose Dolphins from the inner estuary of the Gulf of Guayaquil and the haplotypes from different localities. Numbers next to or above the main branches represent the posterior probability values

as a result of the search of MCMC. The haplotypes identified in this study are termed Ttr (*Tursiops truncatus*) plus Galápagos, Mar Bravo, Gulf of Guayaquil or Peru. The sequences obtained from GenBank are labeled with their accession number. The name of haplotype are colored according to species shown in the legend. (*) represents the haplotypes obtained from the Whale Museum or GenBank database. Outgroups include the harbor porpoise, *Phocoena phocoena*, and the narwhal, *Monodon monoceros*. Acronyms: Ttr_GG = *Tursiops truncatus* of Gulf of Guayaquil, Bd = Burrunan dolphin (*Tursiops australis*), Tad = *Tursiops aduncus*, Dca = *Delphinus capensis*, Sat = *Stenella attenuata*, Sch = *Sousa chinensis*, Sco = *Stenella coeruleoalba*.

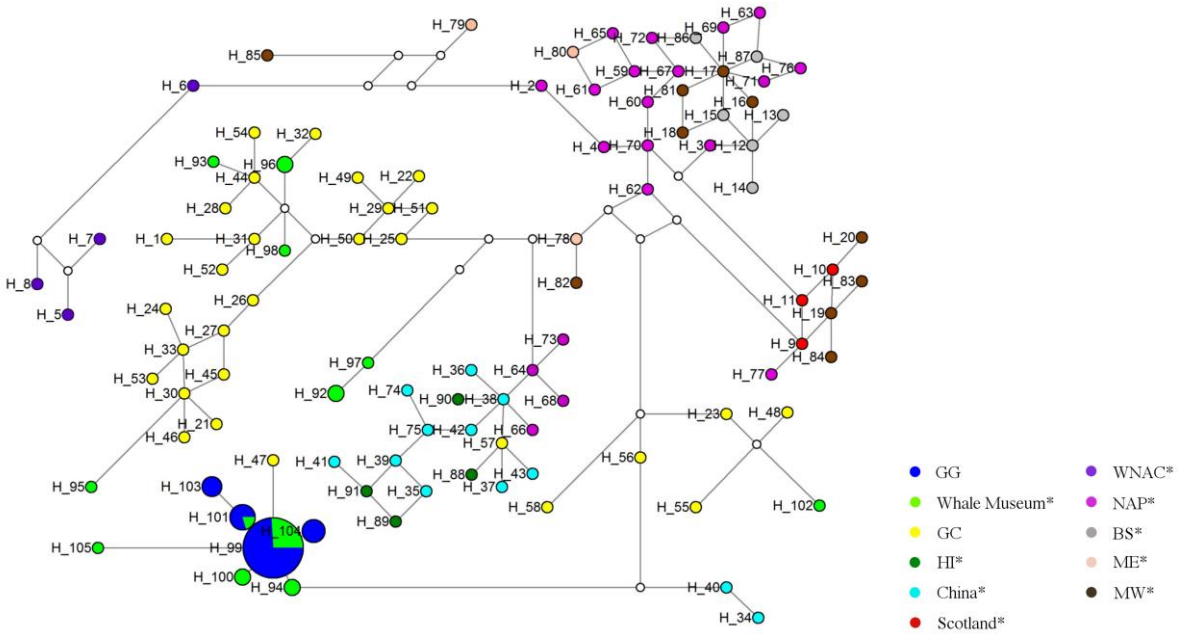


Fig. 3. Genealogical relationships among the ~ 400 bp sequences of the hypervariable control region. Circle size is related to the number of individuals that share the same haplotype. The circles are colored according to the geographical region shown in the legend. The white circle corresponds to the missing or intermediated haplotype. The longitude of the branch is proportional to the number of mutational steps among haplotypes. (*) represents the haplotypes obtained from the Whale Museum or GenBank database. Acronyms: GG = Gulf of Guayaquil, GC = Gulf of California, HI = Hawaiian Islands, WNAC = Western North Atlantic Coastal, NAP = North Atlantic Pelagic, BS = Black Sea, ME = Mediterranean Easter, MW = Mediterranean Western.

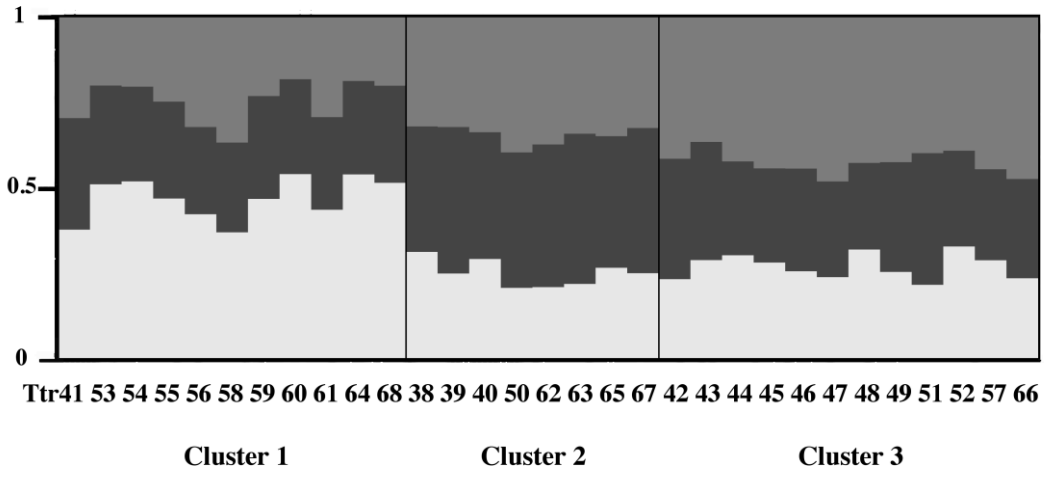


Fig. 4. Bayesian clustering assignment of individual Bottlenose Dolphins to three clusters based on different genotypes at ten microsatellite DNA loci. Each color represents an inferred genetic cluster (K). Each column symbolizes individual dolphins grouped into a genetic cluster according to its genetic identity.

9. TABLES

Table 1. Sequence variation and tree characteristics of combined phylogeny.

Mitochondrial region	N	Length (bp)	Substitution model	S	Parsimony informative sites	Indels
12S rRNA-16S rRNA	53	~1,558	TrN+I	163	65	13
tRNA genes	53	~ 392	TIM3+I	56	25	1
Control region	53	~ 701	GTR+I+G	156	81	29
First position protein-coding genes	53	~ 873	TrN+I	116	67	-
Second position protein-coding genes	53	~ 873	TPM3uf	27	9	-
Third position protein-coding genes	53	~ 871	TIM+I+F	490	280	-
Total	53	~ 5,268		1,008	527	43

Total number of haplotype sequences (N), polymorphic sites (S).

Table 2. Genetic diversity estimates based on clusters inferred by STRUCTURE with ten microsatellites loci.

Cluster	N	Ne	P	A	PA	Ho	He	F_{IS}	Gene diversity
1	11	2.49	7	4.7	0.3	0.30	0.57	0.47 ***	0.497
2	8	2.13	6	3.3	0	0.29	0.52	0.42***	0.350
3	12	2.39	7	4.1	0.4	0.44	0.63	0.30***	0.442
Total	31	2.3	8	4.7	-	0.30	0.57	0.51 ***	0.503

Cluster 1 (Ttr41, Ttr53-Ttr56, Ttr58- Ttr61, Ttr64, and Ttr68), Cluster 2 (Ttr38- Ttr40, Ttr50, Ttr62, Ttr63, Ttr65, and Ttr67); Cluster 3 (Ttr42. Ttr49, Ttr51, Ttr52, Ttr57, and Ttr66); Number of individuals genotyped (N); mean number of effective alleles (Ne); number of polymorphic loci (P); mean numbers of alleles (A); mean number of private alleles (PA); expected heterozygosity (He); observed heterozygosity (Ho); the inbreeding coefficient (F_{IS}); *** $P < 0.001$.

Table 3. Genetic diversity indices based on localities of the Gulf of Guayaquil with ~ 694 bp mitochondrial DNA control region (CR) and ~ 839 bp of a partial sequence of the cytochrome oxidase I (COI) gene.

Collection Sites	CR								COI							
	N	**S**	**H**	**Hd**	**K**	**Π**	**Tajima's D**	**Fu's Fs**	**N**	**S**	**H**	**Hd**	**K**	**Π**	**Tajima's D**	**Fu's Fs**
Morro Channel	7	2	3	0.52	0.57	0.001	- 1.006 ns	-0.921 ns	7	14	6	0.95	4.0	0.005	- 0.59 ns	-1.59 ns
Puná Island	2	0	1	0	0	0	0	0	2	0	1	0	0	0.000	0	0
Posorja Harbor	22	7	6	0.72	1.96	0.003	0.893 ns	-0.153 ns	22	54	8	0.86	15.6	0.019	0.212 ns	6.84 ns
Total	31	7	7	0.71	1.84	0.002	0.632 ns	-0.655 ns	31	54	11	0.83	12.3	0.016	- 0.324 ns	4.074 ns

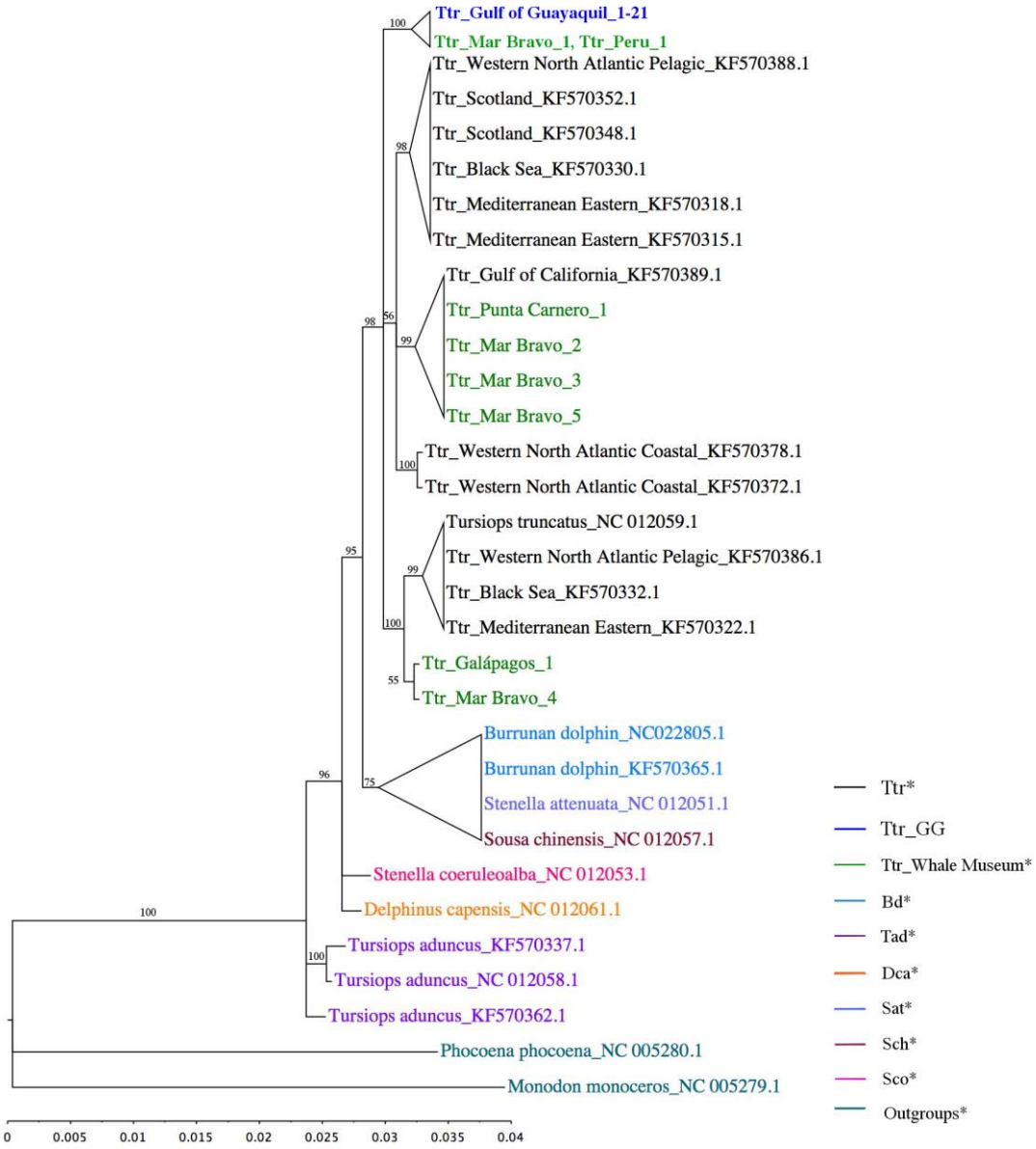
Number of samples by locality (N), number of haplotypes (H), polymorphic sites (S), haplotype diversity (Hd), nucleotide diversity (π), and average number of nucleotide diversity (K).

10. SUPPORTING INFORMATION

MATERIALS AND METHODS S1

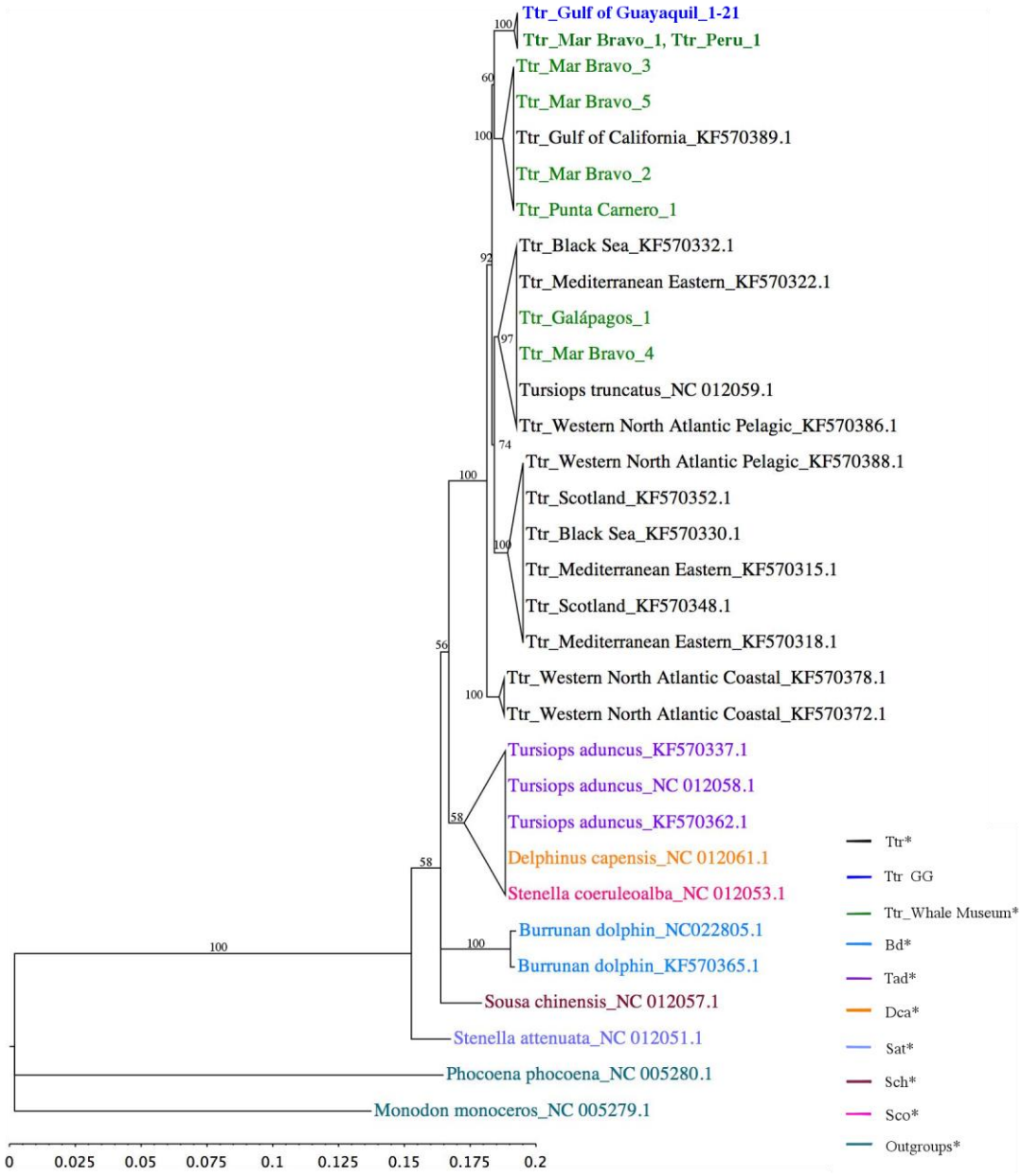
MOLECULAR SEX DETERMINATION

PCR amplification reactions were carried out in 20 µl reaction mixture containing 20-30 ng of DNA, 1mM of PCR buffer, 1.5 mM $MgCl_2$, 0.3 µM of each primer, 150 µM dNTPs, and 1.0 U of Taq DNA polymerase (Promega). PCR thermo-cycling conditions consisted of an initial denaturation step at 95˚C for 5 minutes, followed by 35 cycles of denaturation at 94˚C for 30 seconds, annealing at 51˚C 45 seconds, and extension at 72˚C for 45 seconds with a final extension step at 72˚C for 10 minutes. The sex was determined by the banding pattern on a 2.5 % agarose gel, stained with ethidium bromide, and visualized under ultraviolet light.



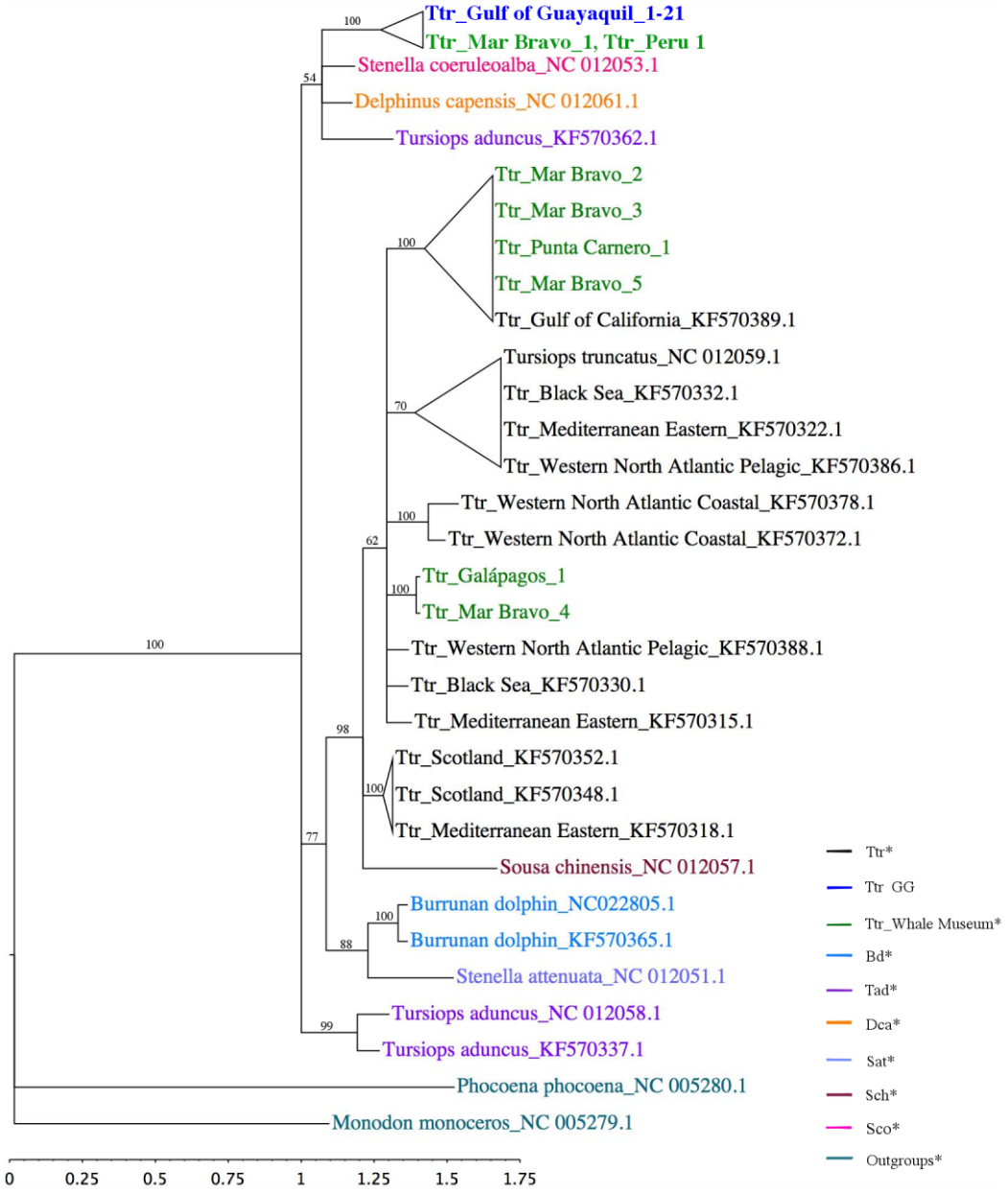
S1 Fig. Bayesian phylogenetic tree based on the analysis of ~ 1,625 bp of the partial region 12S rRNA, tRNA-val, and a partial region of 16S rRNA. The phylogram shows the relationship of the Bottlenose Dolphins of the inner estuary of the Gulf of Guayaquil with other haplotypes from different localities. Numbers above the main branches represent the posterior probability values as a result of the search for MCMC. The haplotypes identified in this study are termed Ttr (*Tursiops truncatus*) plus Galápagos, Mar Bravo, Gulf of Guayaquil or Peru. The sequences obtained from GenBank are labeled with their accession number. The name of haplotype are colored

according to species shown in the legend. (*) represents the haplotypes obtained from the Whale Museum or GenBank database. Outgroups include the harbor porpoise, *Phocoena phocoena*, and the narwhal, *Monodon monoceros*. Acronyms: Ttr_GG = *Tursiops truncatus* of Gulf of Guayaquil, Bd = Burrunan dolphin, Tad = *Tursiops aduncus*, Dca = *Delphinus capensis*, Sat = *Stenella attenuata*, Sch = *Sousa chinensis*, Sco = *Stenella coeruleoalba*.



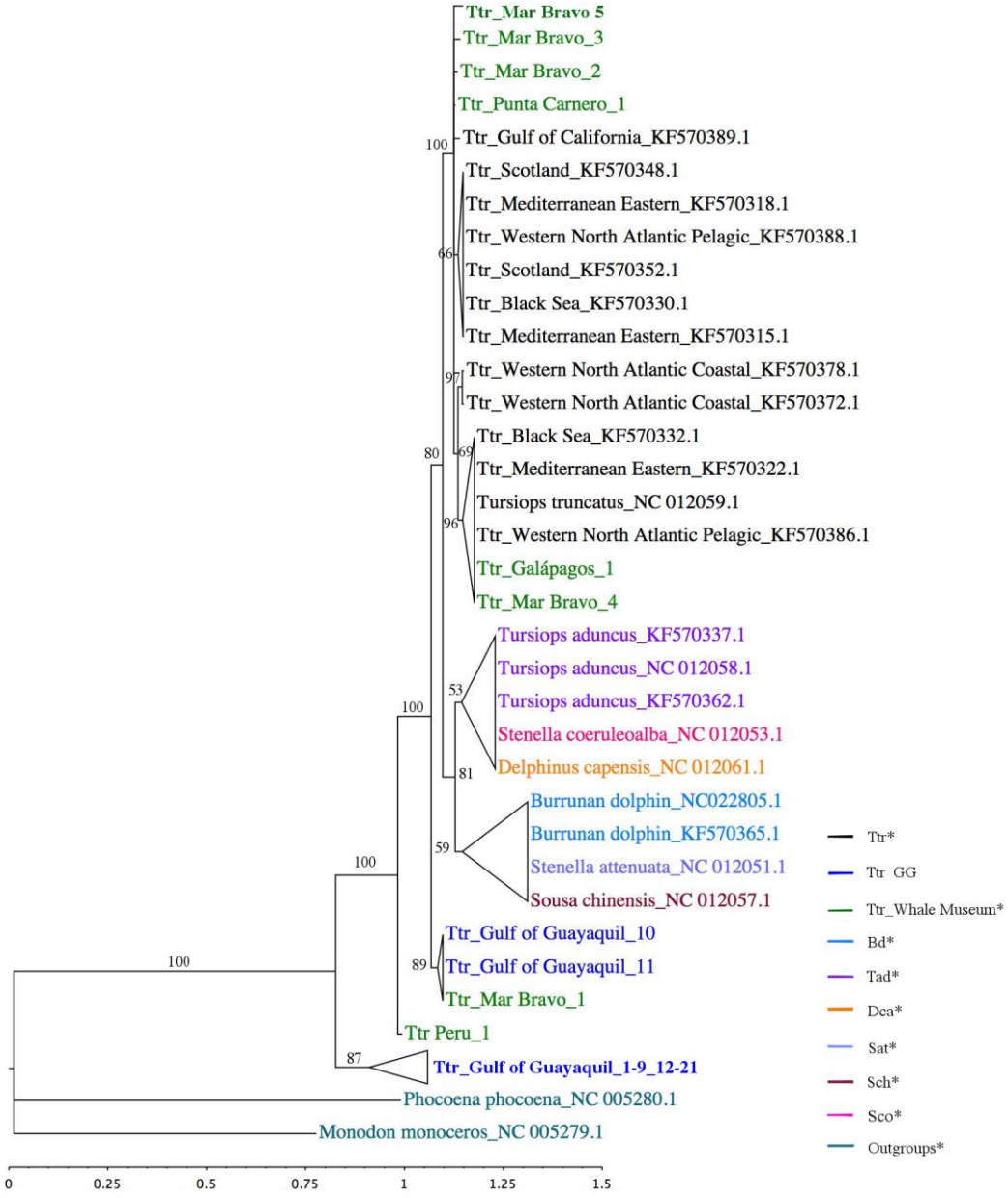
S2 Fig. Bayesian phylogenetic tree based on the analysis of ~ 2,103 bp of the partial protein-coding genes ND1, ND2, COII, and Cyt b, and six tRNAs. The phylogram shows the relationship of the Bottlenose Dolphins of the inner estuary of the Gulf of Guayaquil with other haplotypes from different localities. Numbers next to or above the main branches represent the posterior probability values as a result of the search for MCMC. The haplotypes identified in this study are termed Ttr (*Tursiops truncatus*) plus Galápagos, Mar Bravo, Gulf of Guayaquil or Peru. The sequences

obtained from GenBank are labeled with their accession number. The name of haplotype are colored according to species shown in the legend. (*) represents the haplotypes obtained from the Whale Museum or GenBank database. Outgroups include the harbor porpoise, *Phocoena phocoena*, and the narwhal, *Monodon monoceros*. Acronyms: Ttr_GG = *Tursiops truncatus* of Gulf of Guayaquil, Bd = Burrunan dolphin, Tad = *Tursiops aduncus*, Dca = *Delphinus capensis*, Sat = *Stenella attenuata*, Sch = *Sousa chinensis*, Sco = *Stenella coeruleoalba*.



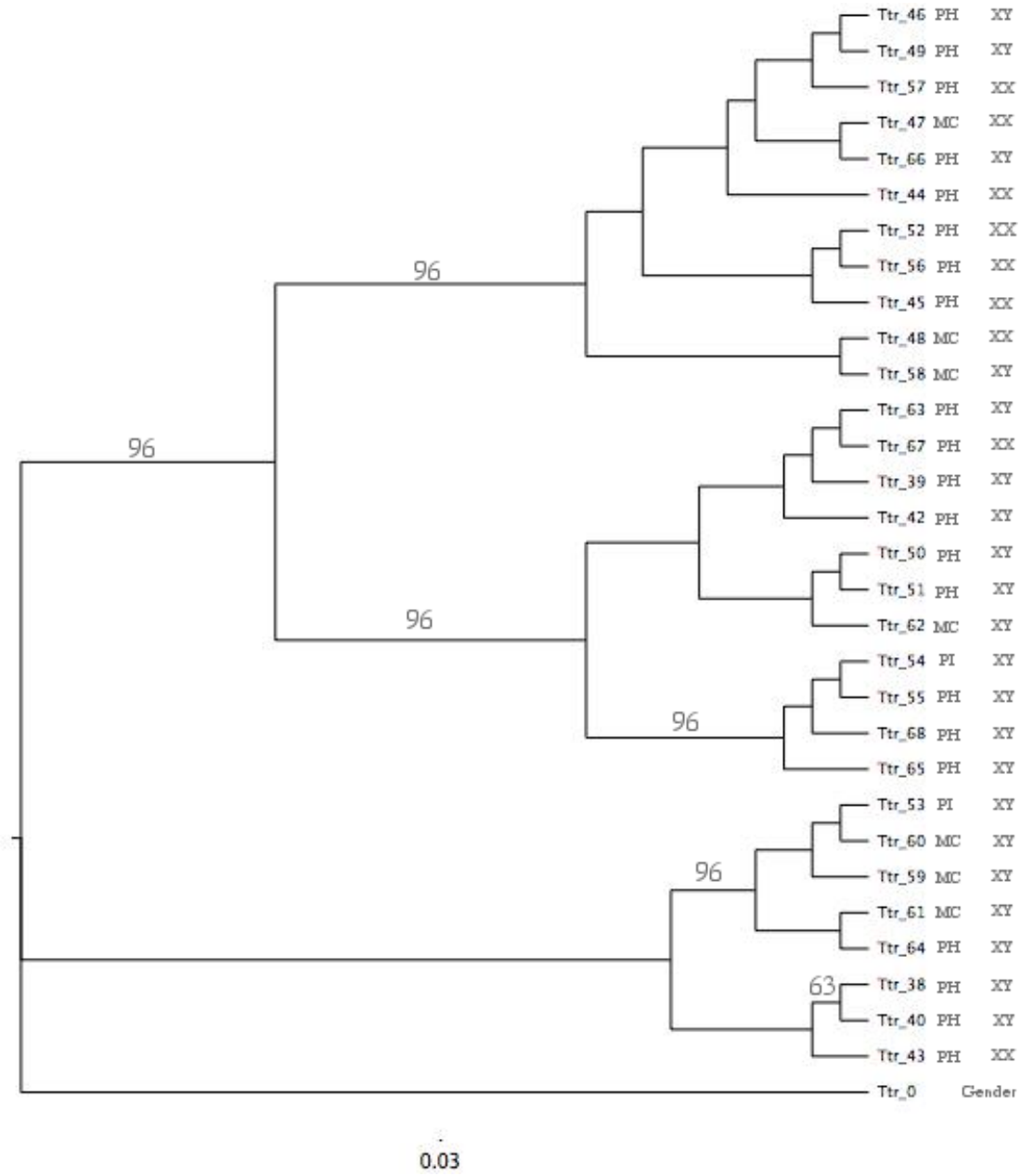
S3 Fig. Bayesian phylogenetic tree inferred on the analysis of ~ 694 bp of the mtDNA control region. The phylogram shows the relationship of the Bottlenose Dolphins of the inner estuary of the Gulf of Guayaquil with other haplotypes from different localities. Numbers next to or above the main branches represent the posterior probability values as a result of the search for MCMC. The haplotypes identified in this study are termed Ttr (*Tursiops truncatus*) plus Galápagos, Mar Bravo, Gulf of Guayaquil or Peru. The sequences obtained from GenBank are labeled with their

accession number. The name of haplotype are colored according to species shown in the legend. (*) represents the haplotypes obtained from the Whale Museum or GenBank database. Outgroups include the harbor porpoise, *Phocoena phocoena*, and the narwhal, *Monodon monoceros*. Acronyms: Ttr_GG = *Tursiops truncatus* of Gulf of Guayaquil, Bd = Burrunan dolphin, Tad = *Tursiops aduncus*, Dca = *Delphinus capensis*, Sat = *Stenella attenuata*, Sch = *Sousa chinensis*, Sco = *Stenella coeruleoalba*.



S4 Fig. Bayesian phylogenetic tree inferred on the analysis of ~ 839 bp of mtDNA cytochrome oxidase I (COI). The phylogram shows the relationship of the Bottlenose Dolphins of the inner estuary of the Gulf of Guayaquil with other haplotypes from different localities. Numbers next to or above the main branches represent the posterior probability values as a result of the search for MCMC. The haplotypes identified in this study are termed Ttr (*Tursiops truncatus*) plus Galápagos, Mar Bravo, Gulf of

Guayaquil or Peru. The sequences obtained from GenBank are labeled with their accession number. The name of haplotype are colored according to species shown in the legend. (*) represents the haplotypes obtained from the Whale Museum or GenBank database. Outgroups include the harbor porpoise, *Phocoena phocoena*, and the narwhal, *Monodon monoceros*. Acronyms: Ttr_GG = *Tursiops truncatus* of Gulf of Guayaquil, Bd = Burrunan dolphin, Tad = *Tursiops aduncus*, Dca = *Delphinus capensis*, Sat = *Stenella attenuata*, Sch = *Sousa chinensis*, Sco = *Stenella coeruleoalba*.



S5 Fig. Neighbor Joining tree displaying the relationships among microsatellite loci of the Bottlenose Dolphin in the inner estuary of the Gulf of Guayaquil. Numbers above the branches are the bootstrap values acquired from 1000 replications. Acronyms: XX = female, XY = male, MC = Morro Channel, PI = Puná Island, PH = Posorja Harbor.

	Polymorphic sites																																																
	5	6	1	2	3	4	8	9	9	1	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3	3	3	3	3	3	4	4	4	4	4	5	5	6	6	6	Collection localities			
			9	9	2	6	9	0	6	0	0	1	4	0	3	4	4	4	4	6	7	7	7	8	8	8	8	9	9	0	5	6	7	8	8	0	5	5	7	9	2	5	0	3	5				
										5	9	3	7	3	2	3	5	6	9	3	5	6	7	0	1	2	8	1	2	1	5	8	1	8	9	7	4	5	6	9	3	4	2	2	3	MC	IP	PH	Total
Hap_GC	A	-	G	C	C	A	T	T	T	A	G	G	C	C	T	C	T	G	C	T	T	T	T	C	T	T	A	C	T	G	C	T	T	A	T	T	C	T	T	A	T	C	G	G	-				
Hap_1	.	-	A	T	T	.	.	C	.	.	A	.	.	T	.	.	C	A	T	A	.	.	.	T	C	.	.	.	C	A	T	.	.	.	.	.	.	.	C	.	C	T	.	.	-			4	4
Hap_2	.	-	A	T	T	.	.	C	.	.	A	.	.	T	.	.	.	A	T	A	.	.	.	T	C	.	.	.	C	A	T	.	.	.	.	.	.	.	.	.	C	T	A	.	-	5		11	16
Hap_3	.	-	A	T	T	.	.	C	.	.	A	.	.	T	C	.	C	A	T	A	.	.	.	T	C	.	.	.	C	A	T	.	.	.	.	.	.	.	C	.	C	T	.	.	-			3	3
Hap_4	.	-	A	T	T	.	.	C	.	.	A	.	.	T	.	.	.	A	T	A	.	.	.	T	C	.	.	.	C	A	T	.	.	.	.	.	.	.	.	.	.	T	A	.	-	1		2	3
Hap_5	-	-	A	T	T	.	.	C	.	.	A	.	.	T	.	.	.	A	T	A	.	.	.	T	C	.	.	.	C	A	T	.	.	.	.	.	.	.	.	.	C	T	A	.	-	1		1	2
Hap_6	.	-	A	T	T	.	.	C	.	.	A	.	.	T	.	.	.	A	T	A	.	.	.	T	C	.	.	.	C	A	T	.	.	.	.	.	.	.	.	.	C	T	A	.	T			1	1
Hap_7	-	-	A	T	T	.	.	C	.	.	A	.	.	T	.	.	.	A	T	A	.	.	.	T	C	.	.	.	C	A	T	.	.	.	.	.	.	.	.	.	.	T	A	.	-		2	0	2
																																														7	2	22	31

S6 Fig. Mitochondrial DNA of control region haplotypes found in the inner estuary of the Gulf of Guayaquil. Polymorphic sites and haplotype frequency identified within ~ 694 bp sequence. The identity of the sequences was compared to the haplotypes from the Gulf of California (Hap_GC, GenBank accession number KF570389.1). Dots (.) show the identity with the Hap_GC and the dash (-) represents a gap or deletion. The numbers under each collection locality represent the number of individuals with this haplotype. Acronyms: MC = Morro Channel, PI = Puná Island, PH = Posorja Harbor, T = Total.

	Polymorphic sites	Collection localities			
	1 5 7 9 9 9 1 1 1 1 1 2 2 2 2 2 2 2 2 2 2 3 3 3 3 3 4 4 4 4 4 4 5 5 5 5 5 6 6 6 6 6 6 6 6 6 6 6 7 7 7 7 7 7 7 7 7 7 8				
	5 7 8 1 3 9 0 3 6 8 8 1 3 3 4 5 5 6 6 7 9 2 4 6 9 9 1 2 4 5 7 8 0 3 5 7 9 0 0 0 1 2 3 3 7 8 9 9 0 1 2 4 5 5 5 7 8 9 1				
	8 5 2 0 9 3 4 7 3 0 5 1 7 3 1 1 2 3 0 3 4 0 4 6 1 6 1 7 2 3 1 1 6 9 0 7 4 9 2 4 4 6 6 1 9 2 0 3 9 4 3 2 0	MC	IP	PH	T
Hap_GC	T C C G A C T A G G T A A T G T C A C T C C A G C C G T C G A A C T C A T T T T C T C C G T T A T C T T A T T C T A T				
Hap_1	A T . . G T C G A A C G G A A C T G T C T T G A A T A C T . A . T C T G C C C C T C T T A . C . C . C C G C C T C G C	1		1	2
Hap_2	A T . . G T C G A A C G G A A C T G T C T T G A A T A C T . A G T C T G C C C C T C T T A G C G C . C C G C C T C G C			6	6
Hap_3	A T . . G T C G A A C G G A A C T G T C T T G A A T A C T . A G T C T G C C C C T C T T A . C . C . C C G C C T C G C	1		4	5
Hap_4	A T . . G T C G A A . G G A A C T G T C T T G A A T A C T . A G T C T G C C C C T . T T A . C . C . C C G C C T C G C			2	2
Hap_5	A T . . G T C . A A . G G A A C T G T C T T G A A T A C T A A . T C T G C C C C T . T T A . C . C . C C . C C T . G .			1	1
Hap_6	A T . . G T C G A A . G A A A C T G T C T T G A A T A C T A A . T C T G C C C C T . T T . . C . C . C C . C C T . G .	1			1
Hap_7	A T . . G T C . A A . G A A A C T G T . T T G A A T A C T A A . T . T G C C T C . . T T . . C . . . . C . . C T . . .			1	1
Hap_8	A . T A . . . . A . . . . A T T . . T A . . . . T			3	3
Hap_9	A T . . G T C G A A C G G A A C T G T C T T G A A T A C T . A G T C T G C C C C T C T T A . C G C . C C G C C T C G C	1	2		3
Hap_10	A T . . G T C G A A C G G A A C T G T C T T G A A T A C T . A G T C T G C C C C T C T T A G C . C . C C G C C T C G C	1		4	5
Hap_11	A T . . G T C G A A C G G A A C T G T C T T G A A T A C T . A G T C T G C C C C T C T T A C C G C . C C G C C T C G C	2			2
		7	2	22	31

S7 Fig. Mitochondrial DNA of partial region of cytochrome oxidase I haplotypes found in the inner estuary of the Gulf of Guayaquil. Polymorphic sites and haplotype frequency identified within ~ 839 bp sequence. The identity of the sequences was compared to the haplotypes from the Gulf of California (Hap_GC, GenBank accession number KF570389.1). Dots (.) show the identity with the Hap_GC. The numbers under each collection locality represent the number of individuals with this haplotype. Acronyms: MC = Morro Channel, PI = Puná Island, PH = Posorja Harbor, T = Total.

S1 Table. Sample information.

Sampling location	Code	GPS collection position	Year
Galápagos Islands	Ttr1		2005
Punta Carnero	Ttr2		2006
Mar Bravo	Ttr3		2006
Mar Bravo	Ttr4		2007
Mar Bravo	Ttr5		2007
Mar Bravo	Ttr6		2008
Mar Bravo	Ttr7		2009
Mar Bravo	Ttr8		2010
Isla Puná	Ttr9		1992
Isla Jambelí	Ttr10		1990
Isla Puná	Ttr11		1991
Isla Puná	Ttr12		1994
Unknown	Ttr13		1995
Unknown	Ttr14		1994
Isla Puná	Ttr15		1991
Isla Puná	Ttr16		1993
Playas	Ttr17		1996
Punta Carnero	Ttr18		2005
Isla Puná	Ttr19		1994
Isla Puná	Ttr20		1991
Isla Puná	Ttr21		1991
Unknown	Ttr22		1993
Isla Puná	Ttr23		1991
Unknown	Ttr24		1992
Isla Puná	Ttr25		1993
Unknown	Ttr26		1995
Galápagos Islands	Ttr27		2001
Unknown	Ttr28		1992
Unknown	Ttr29		1994
Unknown	Ttr30		1992
Isla Puná	Ttr31		1992
Mar Bravo	Ttr32		1995
Isla Jambelí	Ttr33		1990
Mar Bravo	Ttr34		1997
Mar Bravo	Ttr35		1995
Mar Bravo	Ttr36		1993
Mar Bravo	Ttr37		2009
Posorja Harbor	Ttr38	P1: S2.73330 W80.22536	2013
Posorja Harbor	Ttr39	P1: S2.73330 W80.22536	2013
Posorja Harbor	Ttr40	P1: S2.73330 W80.22536	2013
Posorja Harbor	Ttr41	P1: S2.73330 W80.22536	2013
Posorja Harbor	Ttr42	P1: S2.73330 W80.22536	2013
Posorja Harbor	Ttr43	P1: S2.73330 W80.22536	2013
Posorja Harbor	Ttr44	P2: S2.73330 W80.22536	2013
Posorja Harbor	Ttr45	P2: S2.73330 W80.22536	2013

S1 Table. Continued…

Sampling location	Code	GPS collection position	Year
Posorja Harbor	Ttr46	P2: S2.73330 W80.22536	2013
Morro Channel	Ttr47	P3: S2.62933 W80.21539	2013
Morro Channel	Ttr48	P3: S2.62933 W80.21539	2013
Posorja Harbor	Ttr49	P4: S2.71116 W80.23975	2013
Posorja Harbor	Ttr50	P4: S2.71116 W80.23975	2013
Posorja Harbor	Ttr51	P4: S2.71116 W80.23975	2013
Posorja Harbor	Ttr52	P4: S2.71116 W80.23975	2013
Isla Puná	Ttr53	P5: S2.895870 W80.109010	2013
Isla Puná	Ttr54	P5: S2.895870 W80.109010	2013
Posorja Harbor	Ttr55	P6: S2.71543 W80.8023821	2013
Posorja Harbor	Ttr56	P6: S2.71543 W80.8023821	2013
Posorja Harbor	Ttr57	P6: S2.71543 W80.8023821	2013
Morro Channel	Ttr58	P7: S2.64513 W80.25678	2013
Morro Channel	Ttr59	P7: S2.64513 W80.25678	2013
Morro Channel	Ttr60	P7: S2.64513 W80.25678	2013
Morro Channel	Ttr61	P7: S2.64513 W80.25678	2013
Morro Channel	Ttr62	P7: S2.64513 W80.25678	2013
Posorja Harbor	Ttr63	P8: S2.70135 W80.24386	2013
Posorja Harbor	Ttr64	P8: S2.70135 W80.24386	2013
Posorja Harbor	Ttr65	P8: S2.70135 W80.24386	2013
Posorja Harbor	Ttr66	P8: S2.70135 W80.24386	2013
Posorja Harbor	Ttr67	P8: S2.70135 W80.24386	2013
Posorja Harbor	Ttr68	P8: S2.70135 W80.24386	2013
Santa Rosa-Perú	TtrP		2013

S2 Table. Accession numbers belonging to mitogenome sequences obtained from the GenBank database for the phylogenetic analysis.

Species	**Locality**	**Accession number**	**Reference**
Tursiops truncatus	Gulf of California	KF570389.1	Moura et al., 2013
Tursiops truncatus	Western North Atlantic Pelagic	KF570388.1	Moura et al., 2013
Tursiops truncatus	Western North Atlantic Pelagic	KF570386.1	Moura et al., 2013
Tursiops truncatus	Western North Atlantic Coastal	KF570378.1	Moura et al., 2013
Tursiops truncatus	Western North Atlantic Coastal	KF570372.1	Moura et al., 2013
Tursiops truncatus	Scotland	KF570352.1	Moura et al., 2013
Tursiops truncatus	Scotland	KF570348.1	Moura et al., 2013
Tursiops truncatus	Black Sea	KF570332.1	Moura et al., 2013
Tursiops truncatus	Black Sea	KF570330.1	Moura et al., 2013
Tursiops truncatus	Mediterranean Eastern	KF570322.1	Moura et al., 2013
Tursiops truncatus	Mediterranean Eastern	KF570318.1	Moura et al., 2013
Tursiops truncatus	Mediterranean Eastern	KF570315.1	Moura et al., 2013
Tursiops truncatus	Sequence reference	NC_012059.1	Xiong et al. 2009
Tursiops aduncus	South Africa	KF570362.1	Moura et al., 2013
Tursiops aduncus	Australia Southeast	KF570337.1	Moura et al., 2013
Tursiops aduncus	Sequence reference	NC_012058.1	Xiong et al. 2009
Tursiops australis	Australia South	NC_022805.1	Moura et al., 2013
Tursiops australis	Australia South	KF570365.1	Moura et al., 2013
Delphinus capensis	Sequence reference	NC_012061.1	Xiong et al. 2009
Stenella attenuata	Sequence reference	NC_012051.1	Xiong et al. 2009
Sousa chinensis	Sequence reference	NC_012057.1	Xiong et al. 2009
Stenella coeruleoalba	Sequence reference	NC_012053.1	Xiong et al. 2009
Phocoena phocoena	Sequence reference	NC_005280.1	Arnason et al., 2004
Monodon monoderos	Sequence reference	NC_005279.1	Arnason et al., 2004

S3 Table. Accession numbers belonging to the mitochondrial CR of *Tursiops truncatus* obtained from the GenBank database for the phylogeographic analysis.

Haplotype	Locality	Accession number	Reference
Hap_1	Gulf of California	KF570389.1	Moura et al., 2013
Hap_2	Western North Atlantic Pelagic	KF570388.1	Moura et al., 2013
Hap_3	Western North Atlantic Pelagic	KF570384.1	Moura et al., 2013
Hap_4	Western North Atlantic Pelagic	KF570381.1	Moura et al., 2013
Hap_5	Western North Atlantic Coastal	KF570374.1	Moura et al., 2013
Hap_6	Western North Atlantic Coastal	KF570372.1	Moura et al., 2013
Hap_7	Western North Atlantic Coastal	KF570371.1	Moura et al., 2013
Hap_8	Western North Atlantic Coastal	KF570370.1	Moura et al., 2013
Hap_9	Scotland	KF570352.1	Moura et al., 2013
Hap_10	Scotland	KF570351.1	Moura et al., 2013
Hap_11	Scotland	KF570350.1	Moura et al., 2013
Hap_12	Black Sea	KF570334.1	Moura et al., 2013
Hap_13	Black Sea	KF570333.1	Moura et al., 2013
Hap_14	Black Sea	KF570329.1	Moura et al., 2013
Hap_15	Black Sea	KF570328.1	Moura et al., 2013
Hap_16	Mediterranean Eastern	KF570323.1	Moura et al., 2013
Hap_17	Mediterranean Eastern	KF570321.1	Moura et al., 2013
Hap_18	Mediterranean Eastern	KF570320.1	Moura et al., 2013
Hap_19	Mediterranean Eastern	KF570319.1	Moura et al., 2013
Hap_20	Mediterranean Eastern	KF570318.1	Moura et al., 2013
Hap_21	CICIMAR_Ttr36	DQ105733.1	Segura, 2006
Hap_22	CICESE_UPO3	DQ105731.1	Segura, 2006
Hap_23	CICIMAR_Tt005	DQ105730.1	Segura, 2006
Hap_24	CICIMAR_Tt14	DQ105725.1	Segura, 2006
Hap_25	CICIMAR_Tt04	DQ105723.1	Segura, 2006
Hap_26	CICESE_S02	DQ105722.1	Segura, 2006
Hap_27	CICIMAR_Tt003	DQ105719.1	Segura, 2006
Hap_28	CICESE_BTS02	DQ105717.1	Segura, 2006
Hap_29	SWFSC_z13426	DQ105714.1	Segura, 2006
Hap_30	SWFSC_z4675	DQ105710.1	Segura, 2006
Hap_31	SWFSC_z4668	DQ105705.1	Segura, 2006
Hap_32	SWFSC_z13430	DQ105704.1	Segura, 2006
Hap_33	SWFSC_z13455	DQ105703.1	Segura, 2006
Hap_34	China	AB303174.1	Kita et al., 2008
Hap_35	China	AB303173.1	Kita et al., 2008
Hap_36	China	AB303170.1	Kita et al., 2008
Hap_37	China	AB303167.1	Kita et al., 2008
Hap_38	China	AB303166.1	Kita et al., 2008
Hap_39	China	AB303164.1	Kita et al., 2008
Hap_40	China	AB303163.1	Kita et al., 2008
Hap_41	China	AB303162.1	Kita et al., 2008
Hap_42	China	AB303157.1	Kita et al., 2008
Hap_43	China	AB303156.1	Kita et al., 2008

S3 Table. Continued…

Haplotype	Locality	Accession number	Reference
Hap_44	Gulf of California	HE617288.1	Segura et al., 2012
Hap_45	Gulf of California	HE617287.1	Segura et al., 2012
Hap_46	Gulf of California	HE617286.1	Segura et al., 2012
Hap_47	Gulf of California	HE617284.1	Segura et al., 2012
Hap_48	Gulf of California	HE617276.1	Segura et al., 2012
Hap_49	Gulf of California	HE617275.1	Segura et al., 2012
Hap_50	Gulf of California	HE617269.1	Segura et al., 2012
Hap_51	Gulf of California	HE617268.1	Segura et al., 2012
Hap_52	Gulf of California	HE617259.1	Segura et al., 2012
Hap_53	SWFSC:z62354	HQ206697.1	Perrin et el., 2011
Hap_54	SWFSC:z67823	HQ206691.1	Perrin et el., 2011
Hap_55	SWFSC:z26316	HQ206685.1	Perrin et el., 2011
Hap_56	SWFSC:z44082	HQ206684.1	Perrin et el., 2011
Hap_57	SWFSC:z74964	HQ206675.1	Perrin et el., 2011
Hap_58	LACM:84059	HQ206673.1	Perrin et el., 2011
Hap_59	Madeira	DQ525384.1	Querouil et al., 2009
Hap_60	Madeira	DQ525380.1	Querouil et al., 2009
Hap_61	Madeira	DQ525370.1	Querouil et al., 2009
Hap_62	Madeira	DQ525364.1	Querouil et al., 2009
Hap_63	Azores	DQ525361.1	Querouil et al., 2009
Hap_64	Azores	DQ525358.1	Querouil et al., 2009
Hap_65	Portugal	DQ073728.1	Querouil et al., 2009
Hap_66	Azores	DQ073714.1	Querouil et al., 2009
Hap_67	Azores	DQ073711.1	Querouil et al., 2009
Hap_68	Azores	DQ073710.1	Querouil et al., 2009
Hap_69	Azores	DQ073709.1	Querouil et al., 2009
Hap_70	Azores	DQ073707.1	Querouil et al., 2009
Hap_71	Azores	DQ073705.1	Querouil et al., 2009
Hap_72	Azores	DQ073702.1	Querouil et al., 2009
Hap_73	Azores	DQ073701.1	Querouil et al., 2009
Hap_74	China	AF459523.1	Ji et al., 2002
Hap_75	China	AF459522.1	Ji et al., 202
Hap_76	East North Atlantic	AY963626.1	Natoli et al., 2004
Hap_77	East North Atlantic	AY963619.1	Natoli et al., 2004
Hap_78	West Mediterranean	AY963615.1	Natoli et al., 2004
Hap_79	West Mediterranean	AY963608.1	Natoli et al., 2004
Hap_80	West Mediterranean	AY963605.1	Natoli et al., 2004
Hap_81	East Mediterranean	AY963601.1	Natoli et al., 2004
Hap_82	East Mediterranean	AY963598.1	Natoli et al., 2004
Hap_83	East Mediterranean	AY963596.1	Natoli et al., 2004
Hap_84	East Mediterranean	AY963595.1	Natoli et al., 2004
Hap_85	East Mediterranean	AY963594.1	Natoli et al., 2004
Hap_86	Black sea	AY963593.1	Natoli et al., 2004
Hap_87	Black sea	AY963589.1	Natoli et al., 2004
Hap_88	Hawaiin Island	EF672724.1	Martien et al., 2011

S3 Table. Continued…

Haplotype	Locality	Accession number	Reference
Hap_89	Hawaiin Island	EF672713.1	Martien et al., 2011
Hap_90	Hawaiin Island	EF672704.1	Martien et al., 2011
Hap_91	Hawaiin Island	EF672703.1	Martien et al., 2011

S4 Table. Primers for mitochondrial DNA used in the present study.

Primer	Sequence	Size pb	Mitochondrial region	Ta ˚C	$MgCl_2$ mM	Reference
mt12F-12S RNA	5′-TTA CAC ATG CAA GCA TCC GC-3′	~ 1062	12S rRNA-16S rRNA	55	1.5	Cunha et al., 2011
mt12R-12S RNA	5′-GGT ACT CTC TCT ATA GCG CC-3′					
16SarL	5'-CGC CTG TTT ATC AAA AAC AT-3′	~ 590	16S rRNA	55	1.5	Palumbi et al., 1991
16SbrH	5′-CCG GTC TGA ACT CAG ATC ACG T -3′					
ND1F	5′-TCA GAA CTC GTA TCT GGC-3′	~ 956	NADH1-NDH2	51	1.5	Xiong et al., 2009
ND1R	5′-ATT AGT CCT GTG CTT AGG G-3′					
COX1F	5′-TGC CTA CTC GGC CAT TTT AC-3′	~ 756	COI	52	1.5	Amaral et al., 2007
COX1R	5′-TGA AAC CCA GGA AGC CAA TA-3′					
CO2LCet	5′-TAA ART CTT ACA TAA CTT TGT C-3′	~ 684	COII	50	2.0	McGowen et al., 2008
CO2RCet	5′-TCT CAA TCT TTA ACT TAA AAG G-3′					
L14724	5’ –TGA CTT GAA RAA CCA YCG TTG- 3’	~ 465	Cytochrome b	48	1.5	Palumbi et al., 1991
H15149	5’ –CAG AAT GAT ATT TGT CCT CA -3’					Kocher et al. 1989
dLp1.5t-pro	5'-TCA CCC AAA GCT GRA RTT CTA-3'		D-loop	55	1.5	Dalebout et al., 1998
dLp8G	5’-GGA GTA CTA TGT CCT GTA ACC A-3’	~ 800				Lento et al., 1997
dlp5	5’- CCA TCG WGA TGT CTT ATT TAA GRG GAA - 3’	~ 550				Dalebout et al., 1998
dlp4	5'-CGG GTT GCT GGT TTC ACG-3'	~ 400				Pichler et al., 2000

PCR amplification reactions were carried out in 25 µl reaction mixture containing 20-30 ng of DNA, 1mM of PCR buffer, 1.5–2.0 mM$MgCl_2$ (specific to each primer), 0.4 µM of each primer, 200 µM dNTPs, and 1.0 U of Taq DNA polymerase (Promega). PCR thermo-cycling conditions consisted of 94˚C for 2 minutes, followed by 35 cycles of 94˚C for 30 seconds, annealing temperature (specific to each primer) for 60 seconds, and 72˚C for 60 seconds with a final extension step at 72˚C for 10 minutes. The PCR products were electrophoresed on a 1-1.5 % agarose gel contained ethidium bromide, and visualized under ultraviolet light.

S5 Table. Microsatellite DNA loci and additional information used in the present study.

Locus	Sequence	Size	Repeat motif	Ta (˚C)	$MgCl_2$	Reference
EV37F	5'-AGCTTGATTTGGAAGTCATGA-3'	178-224	$(AC)_{26}$	55˚C	1.0 mM	Valsecchi y Amos, 1996
EV37R	5'-TAGTAGAGCCGTGATAAAGTGC-3'					
D8F	5'-GATCCATCATATTGTCAAGTT-3'	103	$(TG)_{18}$	57˚C	2.0 mM	Shinohara et al., 1997
D8R	5'-TCCTGGGTGATGAGTCTTC-3'					
D22F	5'-GGAAATGCTCTGAGAAGGTC-3'	135	$(CA)_3TA-(CA)_{21}$	56˚C	2.0 mM	Shinohara et al., 1997
D22R	5'-CCAGAGCACCTATGTGGAC-3'					
TexVet5F	5'-GATTGTGCAAATGGAGACA-3'	236-260	$(CA)_{24}$	51˚C	1.5 mM	Rooney et al., 1999
TexVet5R	5'-TTGAGATGACTCCTGTGGG-3'					
TexVet7F	5'-TGCACTGTAGGGTGTTCAGCAG-3'	155-163	$(CA)_{12}$	57˚C	2.0 mM	Rooney et al., 1999
TexVet7R	5'-CTTAATTGGGGGCGATTTCAC-3'					
Ttr11F	5'-CTTTCAACCTGGCCTTTCTG-3'	193-223	$(CA)_{21}$	60˚C	1.0 mM	Rosel et al., 2005
Ttr11R	5'-GTTTGGCCACTACAAGGGAGTGAA-3'					
TtrRC12F	5'-GAAAAATGCTTCATGCAAC-3'	125-141	$(TA)_{19}$	52˚C	1.0 mM	Rosel et al., 2005
TtrRC12R	5'-GTTTCATGATGGCAAAATGATAC-3'					
MK6F	5'-GTCCTCTTTCCAGGTGTAGCC-3'	145-189	$(GT)_{17}$	52˚C	2.0 mM	Krützen et al., 2001
MK6R	5'-GCCCACTAAGTATGTTGCAGC-3'					
MK9F	5'-CATAACAAAGTGGGATGACTCC-3'	168-180	$(CA)_{17}$	55˚C	1.5 mM	Krützen et al., 2001
MK9R	5'-TTATCCTGTTGGCTGCAGTG-3'					
Tur4_91F	5'-GTTGGCTCTCCAGCTCTCAGGT-3'	207-235	$(GATA)_{14}$	60˚C	2.0 mM	Nater et al., 2009
Tur4_91R	5'-CAGTGGCTCCCATCTGTATTAGTCA-3'					

PCR amplification reactions were carried out in 20 µl reaction mixture containing 10-15 ng of DNA, 1mM of PCR buffer, 1.0 -2.0 mM $MgCl_2$ (specific to each primer), 0.3 µM of each primer, 200 µM dNTPs, and 1.0 U of Taq DNA polymerase (Promega). PCR thermo-cycling conditions consisted of 94˚C for 2 minutes, followed by 35 cycles at 94˚C for 40 seconds, 52˚C - 60˚C for 60 seconds, and 72˚C for 60 seconds with a final extension step at 72˚C for 10 minutes. The PCR products were electrophoresed on a 2.0 % agarose gel contained ethidium bromide, and visualized under ultraviolet light.

DECLARACIÓN Y AUTORIZACIÓN

Yo, Rosa de los Ángeles Bayas Rea, C.I. 0201588548, autora del trabajo de graduación intitulado: "Population Structure and Conservation Status of the Bottlenose Dolphin Population in the Gulf of Guayaquil, Ecuador", previa a la obtención del grado académico de MAGÍSTER DE BIOLOGÍA DE LA CONSERVACIÓN en la Facultad de Ciencias Exactas y Naturales:

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Quito, 13 de octubre de 2015

Srta. Rosa de los Ángeles Bayas Rea
C.I. 0201588548