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**Isolated in the Caribbean: Low genetic diversity of bottlenose dolphin population in Bocas del  
Toro, Caribbean Panama**

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**Abstract**

The global conservation status of the bottlenose dolphins (*Tursiops truncatus*) by the IUCN is as “Least Concern”. However, bottlenose dolphins seem to form a global metapopulation consisting of a widely distributed genetic form (“worldwide”) and many localized “inshore forms” that live relatively isolated in small populations and that are more at risk of decline and extinction due to their overlap with human activities. In this study we used previously published data from bottlenose dolphins in the Caribbean to assess the degree of genetic isolation of bottlenose dolphins from Bocas del Toro (BDT), Panama. A previous study found a described a unique mtDNA haplotype for this population, not found anywhere else in the Caribbean, suggesting high degree of isolation. Using microsatellites data we compared the BDT population to the neighboring population in Costa Rica and other populations in the Caribbean. Our finding support that BDT dolphins are isolated from other Caribbean populations, and that despite no photoID evidence there is small genetic flow from Panama to Costa Rica. This population is threaten by intense dolphin watching that affecting their foraging time, communication, and even causing death due to collisions with boats. The whale-watching **Resolution ADM/ARAP NO. 01** was established 8 years ago, but there is neither enforcement nor a transparent certification process. The dolphin Bocas needs urgently need the Panamanian government to follow their commitment to protect their marine mammals.

## Introduction

Currently, the global conservation status of the bottlenose dolphins (*Tursiops truncatus*) by the IUCN is as 'Least Concern' (IUCN, 2015). However, this categorization does not represent the conservation status of many inshore bottlenose dolphin populations that are generally small, show high site fidelity (Natoli et al., 2004; Tezanos-Pinto et al., 2009; Caballero et al., 2011), and inevitably interact with a number of human activities (Parsons et al., 2006, Segura et al., 2006; May-Collado and Quiñones, 2014). Together, these conditions make inshore bottlenose dolphin populations highly vulnerable to local extinction (Gerrodette and Gilmartin, 1990; Caley et al., 2001; Lusseau, 2003, 2003b, 2004, 2005; Culik, 2004; May-Collado et al., SC/65b/WW06, SC/64/WW2; Rowe et al., 2010; see reports presented to the WW subcommittee). Given that a 'Least Concern' categorization is viewed as of low conservation priority, the protection and research of bottlenose dolphin populations worldwide is put at risk. Many bottlenose dolphin populations are facing similar situations to that of the Fiordland in New Zealand. In this area, the occurrence of bottlenose dolphins was common into the fiord, until dolphin-watching activities increased without control. As result of this rapid increase in dolphin-boat interactions, the soundscape of these animals' habitat changed affecting calving (Lusseau 2003) and increasing emigration rate exposing them to higher predation risk (Lusseau 2003, 2003b, 2004, 2005), resulting in a substantial population decline (Lusseau 2004, 2005). Furthermore, Tezanos-Pinto et al. (2009) found that the bottlenose dolphins in New Zealand have two distinct genetic forms: "worldwide" and "inshore". The inshore form showed high levels of genetic isolation. Together, these factors lead to important change in the conservation status of this population by the IUCN from least concern to critically endangered.

For a decade the Panacetacea research team has been studying the bottlenose dolphins of Bocas del Toro. This population is small with both males and females showing high levels of philopatry (Barragán-Barrera et al., SC/65a/SM15). This small population is divided into two communities: a large community consisting of dolphins with larger home ranges and low residency rate and a small community of 37 dolphins with small home ranges and high residency rate (see May-Collado et al. report to WW). Because of its high predictability the small community has been experiencing an exponential increase in exposure to dolphin watching activities which has led to 10 deaths (Trejos and May-Collado report to WW), a significant reduction in foraging time (May-Collado et al., SC/65b/WW06), particularly in groups with nursing mothers (Kassamali-Fox et al. report to WW),

and modifications to their communicative signals (May-Collado and Quiñones-Lebrón, 2014; May-Collado and Wartzok to WW; May-Collado to WW). In addition, like the New Zealand dolphins, the bottlenose dolphins of the Caribbean also showed the two genetically distinct worldwide and inshore forms (Caballero et al., 2011). Bocas del Toro dolphins not only belong to the inshore form but also has a unique mtDNA haplotype not found anywhere else in the Caribbean (Barragán-Barrera et al., SC/65a/SM15) suggesting genetic isolation from neighboring populations including the Gandoca-Manzanillo population in Costa Rica only 35 km north of Bocas del Toro.

Following the recommendation of the IWC Scientific Committee meeting (St Kitts, 2006) to increase understanding about how bottlenose dolphin populations are genetically structured in the Caribbean we collected 25 skin biopsies from dolphins in Bocas del Toro to conduct genetic analyses. We use nine microsatellite loci to (1) determine if this bottlenose dolphin population genetically structured or not and (2) confirm its genetic isolation or genetic flow with other Caribbean populations in Colombia, Costa Rica, Honduras, Bahamas, Mexico, Cuba and Puerto Rico.

## **Methods**

Skin samples were obtained from dolphins by firing remote biopsy darts with a modified rifle (PAXARMS) from a distance of approximately 10 m from the research boat (Krützen et al., 2002). The biopsy system uses a modified 0.22 veterinary rifle with adjustable pressure. The biopsy darts have a hollow polycarbonate body and a small stainless steel biopsy tip (5 mm diameter, 9 mm length) (Krützen et al., 2002; Tezanos-Pinto and Baker, 2011). This system allows penetration of the dolphin epidermis leaving behind a small wound (Tezanos-Pinto and Baker, 2011). The effect on the dolphins is expected to be low, because the polycarbonate body of dart to spread the impact over a wider area and therefore, reducing the risk of injury when penetrating the skin (Krützen et al., 2002; Parsons et al., 2003; Tezanos-Pinto and Baker, 2011). The biopsies were taken only if the individual was photo-identified previously to avoid sampling the same dolphin repeatedly (Krützen et al., 2002). Samples were preserved in alcohol 70% and stored at -20 °C (Amos and Hoelzel, 1991) for subsequent laboratory analysis. A total of 25 samples were collected from six locations within the Archipelago including Dolphin bay, Almirante bay, Pastores islands, Tierra Oscura, Loma Partida, Popa, Shark Hole and Zapatilla cay.

DNA was extracted from skin samples using the DNeasy kit (QIAGEN, Valencia, CA, USA). A 650pb *D-loop* hypervariable portion of the *mtDNA CR* was amplified by the polymerase chain reaction (PCR), using two pairs of primers, t-Pro-whale M13Dlp1.5 (5'-TGTAACGACAGCCAGTTCACCCAAAGCTGRARTTCTA-3') and Dlp8G (5'-GGAGTACTATG TCCTGTAACCA-3'), following amplification conditions proposed by Baker et al. (1998). All samples were sexed following the protocol by Gilson et al. (1998).

Fragment analysis of 11 polymorphic microsatellite loci was achieved following Caballero et al. (2011). Microsatellite loci include: *D08*, *D22* (Shinohara et al., 1997), *TexVet7*, *TexVet5* (Rooney et al., 1999), *MK6*, *MK8*, *MK9* (Krützen et al., 2001), *EVI* (Valsecchi and Amos, 1996), *Tur48*, *Tur91* and *Tur117* (Nater et al., 2009). Primers were fluorescently labeled for detection on an ABI 3100 at Universidad de los Andes, Bogotá. All individuals were genotyped for at least nine loci. Alleles were visualized and subsequently binned using GeneMapper Software.

To determine the genetic structure of bottlenose dolphins from Bocas del Toro, we compared our microsatellite results ( $n = 25$ ) with nuclear data of samples from Costa Rica ( $n = 5$ ) and previously published nuclear data from other seven Caribbean geographic locations, which include Honduras ( $n = 6$ ), Colombia ( $n = 3$ ), Puerto Rico ( $n = 20$ ), Bahamas ( $n = 11$ ), Cuba ( $n = 53$ ), and México ( $n = 30$ ) (Caballero et al. 2011) for a total of nine microsatellite loci from 153 individuals.

The program TANDEM was used to conduct the binning of the microsatellite data. This software is based on a heuristic search with the Nelder-Mead Downhill Simplex algorithm, and to calculate allele number applies a least-square minimization of rounding errors (Matschiner and Salzburger, 2009). In order to evaluate the presence of null alleles, large allele dropout and scoring errors due to stutter peaks, we used the software MICRO-CHECKER version 2.2.3 with Bonferroni correction (Van Oosterhout et al., 2004).

To determine the number of alleles ( $N_A$ ) per locus, the observed heterozygosity ( $H_o$ ), the expected heterozygosity ( $H_E$ ), levels of polymorphism, deviation from Hardy-Weinberg equilibrium (HWE) (Markov chain 100000, dememorization steps 100), and linkage disequilibrium (LD) (permutations 10000), the fixation index ( $F_{IS}$ ) we used the software ARLEQUIN version 3.5.1.2 (Excoffier et al., 2000). To identify gene flow we calculated pairwise  $F_{ST}$  for each pair of population. Genetic subdivision among Caribbean populations was identified conducting an analysis of molecular

variance (AMOVA), to compare variations between and within groups. ARLEQUIN v. 3.5.1.2 was also used to conduct these analyses.

To evaluate the patterns of genetic structure we used the software STRUCTURE v. 2.3.4 (Pritchard et al., 2000). We set the burn in period to 10000 iterations and we determine the probability estimates using 100000 Markov chain Monte Carlo (MCMC) iterations. To infer the true K from the log probability of the data  $\text{LnP(D)}$  (Evanno et al., 2005), first we conducted the runs with K set from 1 to 10 for each value of K with the admixture model and correlated frequencies on the software Structure. After, using the program STRUCTURE HARVESTER, we compare the log probability  $\text{LnP(D)}$  of different values for K using an ad hoc statistic  $\Delta K$ , which calculates the second order rate of change of  $\text{Ln P(D)}$ . Finally, the corresponding values for each K were plotted to determine the uppermost level of population structure for our dataset.

We assessed differentiation and genetic diversity among population units determined by STRUCTURE using the software ARLEQUIN v. 3.5.1.2 (Excoffier et al., 2000). With this program, we calculated pairwise  $F_{ST}$  and  $R_{ST}$  values, deviation from both Hardy–Weinberg equilibrium (HW) and linkage disequilibrium, and genetic diversity as expected and observed heterozygosity (HE and HO).

## **Results**

### *Population structure*

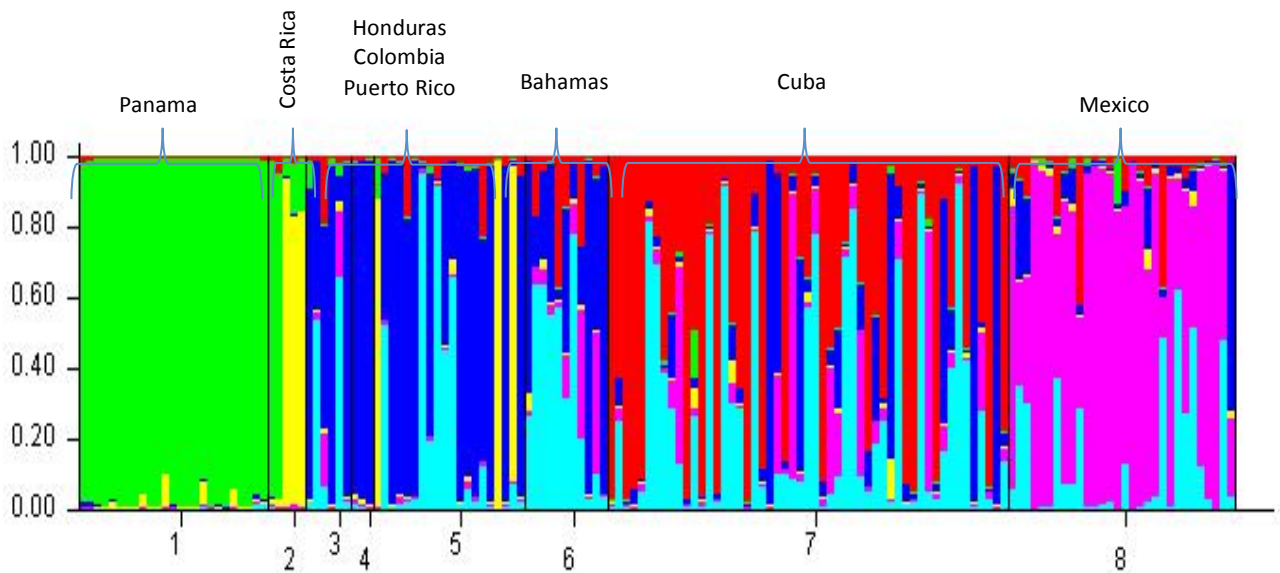
Comparisons of  $F_{ST}$  and  $R_{ST}$  between geographically sampled populations produced high values of  $F_{ST}$  for both Panama and Costa Rica populations compared with the others ones in the Caribbean, suggesting certain degree of isolation of both populations in the Caribbean. Additionally, the comparison between these both populations produced a non-significant negative value of  $F_{ST}$  (-0.055) suggesting that Panamanian and Costa Rican bottlenose dolphins could conform one population unit (Table 2). On the other hand, the other populations in the Caribbean show relatively low but not significant  $F_{ST}$  values, which suggest that these populations could share genetic flow among them.

Evaluation of the K values produced two interesting scenarios: using the  $\Delta K$  method, the software identified  $K = 2$  as the most likely number of groups present in the data ( $\Delta K = 267.317$ ), but using

the LnPK the true K identified is between 6 and 7, since they had the highest likelihood ( $-3141.500 \pm 15.918$  and  $-3112.460 \pm 7.974$ , respectively). Excluding the Bocas del Toro and Costa Rica populations, Caballero et al. (2011) found four population units, thus we believe that  $K = 6$  is the most likely number of populations in the Caribbean (see Figure 1). In this barplot, where axis X corresponds the each individual, it is notable that Panama cluster (Bocas del Toro) conforms a solid population unit, where there are only a few migrants toward Costa Rica, but not in the other direction. This plot shows that Bocas del Toro population is highly structured and suggest that the individuals do not appear to maintain genetic flow with other populations except with Costa Rican dolphins. However, to confirm this assumption, we are running migration analyses using MIGRATE software in order to determine the number of migrant among populations.

**Table 2.** Population differentiation between pairwise populations with nine microsatellites. High and significant scores are in bold and the P-value is shown below them.  $F_{ST}$  values are below diagonal.  $R_{ST}$  values are above diagonal.

$F_{ST}$	$R_{ST}$	Panama	Costa Rica	Colombia-Honduras-Puerto Rico	Bahamas	Cuba	Mexico
Panama	-	-	-0.052 (0.952)	<b>0.368</b> (0.000)	<b>0.388</b> (0.000)	<b>0.392</b> (0.000)	<b>0.335</b> (0.000)
Costa Rica	-0.055 (0.712)	-	-	<b>0.201</b> (0.002)	<b>0.231</b> (0.001)	<b>0.238</b> (0.000)	<b>0.142</b> (0.000)
Colombia-Honduras-Puerto Rico	<b>0.479</b> (0.000)	<b>0.609</b> (0.003)	-	-	0.042 (0.031)	0.075 (0.000)	0.081 (0.000)
Bahamas	<b>0.440</b> (0.000)	<b>0.488</b> (0.003)	0.091 (0.052)	-	-	0.061 (0.000)	0.085 (0.000)
Cuba	<b>0.327</b> (0.000)	<b>0.183</b> (0.003)	0.044 (0.054)	0.013 (0.277)	-	-	0.082 (0.000)
Mexico	<b>0.514</b> (0.000)	<b>0.480</b> (0.000)	0.039 (0.049)	0.038 (0.087)	0.073 (0.005)	-	-



**Figure 1.** Barplot of the likelihood (Y-axis) of each individual's (X-axis) assignment to a particular population units for  $K = 6$ .

#### *Genetic diversity*

When analyzing the Bocas del Toro population in conjunction with samples from other Caribbean locations we found that these dolphins form a separate unit maybe with Costa Rican individuals. Table 1 shows that heterozygosity values are high and similar for most of the Bocas del Toro population loci. In general, heterozygosity values of Bocas del Toro population are higher than the others bottlenose dolphin populations in the Caribbean. Particularly for Bocas del Toro population,  $H_E$  was higher than  $H_O$  for most of the loci except for loci D08, MK6, Tur117 and Tur91. However, none of these values are significant ( $P > 0.05$ ), which means that there is not a significant reduction of diversity. However, although this population shows high diversity, this diversity may be lost through the time because this population is isolated in the Caribbean.



**Table 1.** Genetic diversity for nine nuclear microsatellites in Bocas del Toro population. For each locus: total number of alleles (n), expected (HE) and observed (HO) heterozygosity.

<b>Locus</b>	<b>n</b>	<b>HE</b>	<b>HO</b>	<b>P</b>
D08	4	0.160	0.258	0.400
D22	5	0.792	0.701	0.833
TexVet5	5	0.880	0.721	1.000
MK6	4	0.250	0.409	0.222
MK8	4	0.800	0.731	0.500
MK9	4	0.625	0.579	0.667
Tur117	3	0.560	0.654	0.429
Tur91	4	0.521	0.554	0.444
Tur48	2	0.120	0.115	0.667

## **Discussion**

Here we present additional evidence that bottlenose dolphins from Bocas del Toro are highly philopatric and they are highly isolated from neighboring populations. Caballero et al. (2011) described two forms of bottlenose dolphins found in the Caribbean: the “inshore” and “worldwide distributed form”. Particularly the “inshore” ecotype is commonly found in the Bahamas, Mexico, and the Western North Atlantic, and based on mitochondrial data the “inshore” is also present in Bocas del Toro (Barragán-Barrera et al., SC/65a/SM15). In fact, bottlenose dolphins from Bocas del Toro share a unique haplotype not reported before in the Caribbean, which nested only with “inshore” haplotypes from Cuba, Bahamas, Mexico and Puerto Rico (Barragán-Barrera et al., SC/65a/SM15). Microsatellite data also support the assumption that bottlenose dolphins from Bocas del Toro correspond to the “inshore” ecotype, since the microsatellite data show high HE values. According to Caballero et al. (2011), high values of nuclear genetic diversity of bottlenose dolphins in the Caribbean indicates the population is entirely constituted by “inshore” ecotype, despite other studies in other geographic areas found that these high values correspond to populations entirely constituted by the “worldwide distribution form” (Natoli et al. 2004; Quérrouil et al. 2007; Tezanos-Pinto et al. 2009).

Additionally, the barplot supports the high level of isolation from other bottlenose dolphins in the Caribbean. This population conform a strong population unit, and they do not share genetic flow

with any other Caribbean population. However, it is notable that there are only a few individuals that may be moving from Bocas del Toro to Costa Rica (Gandoca-Manzanillo), but not from Costa Rica to Bocas del Toro. It is possible that both Bocas del Toro and Costa Rica conform an unique population unit, but it is necessary to get more samples from Costa Rica to test this hypothesis.

These genetic results agree with ten years of Photo-ID data that indicate this population is a small and relatively isolated population (May-Collado et al., SC/65b/WW06). This situation has great implications for these dolphins, since boat traffic seems to be negatively affecting this population, and boat traffic could be an important factor in bottlenose dolphin diseases associated to environmental pollution, which may produce stress, skin lesion, and even the death (Lusseau, 2003, 2003*b*, 2004). To date, there has been reported twelve deaths of animal associated to collision with propeller engines boats and asphyxiation in fishing nets (Trejos and May-Collado report to WW). Therefore, our results demonstrate the high vulnerability of these dolphins to disease-related morbidity or mortality, anthropogenic activities and climate change. Because these dolphins are genetically isolated, it is necessary to conduct genetic analyses in the Major Histocompatibility Complex in order to establish if the animals have an immune system which may respond to diseases. The results to be obtained from these analyses could be decisive in protecting this population of bottlenose dolphins. On the other hand, it is necessary to get more samples from adjacent areas between Bocas del Toro and Gandoca-Manzanillo (Costa Rica) in order to establish the real genetic status of these both populations in the Caribbean.

The situation of these dolphins in Bocas del Toro is critical. It is urgent to establish regulations to reduce boat traffic impacts on dolphins, principally because our results show bottlenose dolphin from Bocas del Toro would have to be managed like a population in risk and as an independent stock for conservation.

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