Genetic structure of common bottlenose dolphins (*Tursiops truncatus*) inhabiting adjacent South Florida estuaries – Biscayne Bay and Florida Bay

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ABSTRACT

Coastal common bottlenose dolphins show a variety of migration and residency patterns adding to the difficulty of defining stocks for management purposes. Genetic structure plays an important role in identifying population stocks of dolphins. This study examines genetic differentiation in common bottlenose dolphins both between two social groups occurring in Biscayne Bay, Florida and between Biscayne Bay and an adjacent group of dolphins in Florida Bay. Skin biopsy samples were sequenced at the mitochondrial DNA (mtDNA) control region and genotyped at microsatellite loci. Significant genetic differentiation was found between bottlenose dolphins in Biscayne Bay and Florida Bay (mtDNA $F_{ST} = 0.139$, $p \leq 0.001$; microsatellite $F_{ST} = 0.042$, $p \leq 0.001$) supporting independent management stock status for these two populations. Within Biscayne Bay, evidence of weak but significant population differentiation was found between the two social groups using microsatellite markers ($F_{ST} = 0.0149$, $p \leq 0.009$); however, differentiation was not evident from the mtDNA-based estimates of F_{ST} and ϕ_{ST}. The lack of differentiation at mtDNA coupled with field observations indicating overlapping home ranges for these two groups suggests ongoing, though perhaps low, levels of interbreeding. These data are insufficient to warrant splitting the Biscayne Bay management stock at this time.

KEYWORDS: GENETICS; BIOPSY SAMPLING; SITE FIDELITY; NORTH AMERICA; ATLANTIC OCEAN; COMMON BOTTLENOSE DOLPHIN

INTRODUCTION

The common bottlenose dolphin (*Tursiops truncatus*) is found throughout temperate and tropical waters worldwide (Reynolds *et al.*, 2000). Two morphologically and genetically distinct bottlenose dolphin ecotypes exist in the western North Atlantic, a deep water ecotype (offshore) and a shallow water ecotype (coastal) (Hersh and Duffield, 1990; Hoelzel *et al.*, 1998; Mead and Potter, 1995; Rosel *et al.*, 2009). Coastal bottlenose dolphin populations vary extensively in residency patterns, migration and site fidelity (Hohn, 1997; Wells and Scott, 1999). For example, a seasonally migrating population of bottlenose dolphins spends winter months in the coastal waters of central North Carolina and migrates as far north as Long Island, New York during the summer (Rosel *et al.*, 2009; Waring *et al.*, 2008), while other bottlenose dolphins are year-round residents of embayments and estuaries along the southeast US Atlantic and Gulf of Mexico coasts (Rosel *et al.*, 2009).

Identifying population structure and distinguishing resident estuarine stocks is important for effective management and conservation of bottlenose dolphins. In the USA, the Marine Mammal Protection Act (MMPA) mandates that human-caused mortality and serious injury of a specific management stock should not exceed a level that would cause the stock to decline and/or prevent recovery of a depleted stock. The accurate identification and delineation of stocks for management purposes is critical to both determining population abundance status and in assigning human-caused mortalities to the correct stock. Within estuarine systems, resident populations may be particularly susceptible to chronic impacts on survival and productivity associated with factors such as environmental toxins, disease and harmful algal blooms (Reeves and Ragen, 2003; Schwacke *et al.*, 2004). Hence, understanding the population boundaries and residence patterns is critical for understanding the exposure of stocks to these environmental stressors.

Photo-identification studies have been useful in determining residence patterns of dolphins; however, there is no consistent definition used to distinguish resident from non-resident groups. Residency has been described as a group of dolphins having stable home ranges or repeated occurrences in a given area over a period of years (Wells and Scott, 1999). Some estuarine populations have been studied long term (> 10 years) using photo-identification techniques and have animals that meet the above definition of residency; these include Charleston, South Carolina (Speakman *et al.*, 2006; Zolman, 2002), the Indian River Lagoon system on the Florida east coast (Mazzoil *et al.*, 2005) and Sarasota Bay on the Florida west coast (Wells, 1991; 2003). The variability of residency and migratory patterns observed for bottlenose dolphins, combined with a continuous distribution throughout the species' range, make it difficult to clearly define and distinguish resident populations.

In addition to other methods, genetic markers are commonly used to investigate population structure in dolphins (e.g. Curry and Smith, 1997; Rosel *et al.*, 1999; Wade and Angliss, 1997). Sellas *et al*. (2005) found

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significant genetic differentiation between resident bottlenose dolphins in Sarasota Bay, Florida and those found in nearshore coastal Gulf of Mexico waters just outside of Sarasota Bay. Their results indicate that little interbreeding is occurring, despite sightings of mixed groups of resident dolphins from Sarasota Bay with those primarily sighted in the nearshore Gulf of Mexico (Sellas *et al.*, 2005). Several other studies also have found genetic structure on a remarkably small geographic scale in bottlenose dolphins inhabiting unobstructed inshore habitats such as Little Bahama Bank, Bahamas (Parsons *et al.*, 2006). Rosel *et al* (2009) found significant genetic differentiation among five populations of dolphins in the western North Atlantic spanning from Jacksonville, Florida north to New Jersey. Two of these populations were separated by as little as 80km (Georgia and Jacksonville) while others were thought to seasonally migrate and potentially overlap in space and time.

This study examines genetic differentiation both within bottlenose dolphins occurring in Biscayne Bay and between these and an adjacent group of dolphins in Florida Bay, Florida. Biscayne Bay is a shallow subtropical estuary located along the east coast of Miami-Dade County, Florida (Fig. 1). Northern Biscayne Bay is extensively developed and separates the cities of Miami and Miami Beach. The Bay opens to the Atlantic Ocean in the centre through a series of tidal channels and then extends south where it is less developed and connects to Florida Bay through Barnes and Blackwater Sounds. The National Marine Fisheries Service, Southeast Fisheries Science Center (NMFS/SEFSC) has been conducting a photo-identification (photo-ID) project of bottlenose dolphins in Biscayne Bay since 1990 (Litz, 2007). To date, over 200 individual dolphins have been catalogued and many of these appear to be long-term residents with sightings across multiple years and seasons (NOAA Fisheries, unpublished data). Analyses of the sighting histories and association patterns of known individuals from the Biscayne Bay photo-ID data demonstrated that there are at least two overlapping social groups of animals in the Bay; those that are sighted primarily in northern Biscayne Bay and those that are sighted primarily in southern Biscayne Bay (Litz, 2007).

Florida Bay is bounded by the mainland of Florida to the north, the Florida Keys to the east and south, and is open to the Gulf of Mexico to the west (Fig. 2). It is divided into a series of semi-isolated shallow basins by mudbanks and mangrove islands that restrict circulation (Torres and Urban, 2005). Studies suggest that bottlenose dolphins are present throughout Florida Bay year-round (Engleby *et al.*, 2002; McClellan *et al.*, 2000). In May of 2003, a targeted mark-recapture study was conducted and estimated the abundance of bottlenose dolphins using Florida Bay during that month as 514 (Read *et al*., pers. comm.).

Biscayne and Florida Bays have no geographic barriers preventing bottlenose dolphins from travelling throughout or beyond the Bays; therefore, resident dolphins from either Bay could mix and possibly interbreed with neighbouring dolphin communities. However, if mating between social groups or embayments is rare, genetic divergence could develop over time. This study used both maternally inherited mitochondrial DNA and biparentally inherited microsatellite markers to investigate genetic differentiation of dolphins within Biscayne Bay, particularly between the identified northern and southern social groups. In addition, samples from dolphins inhabiting Biscayne Bay were compared to those from Florida Bay to investigate the genetic differentiation between dolphins inhabiting these adjacent embayments.

METHODS

Biopsy sample collection and sighting histories

Skin samples were obtained from common bottlenose dolphins in Biscayne Bay using remote biopsy techniques with a dart fired from a modified .22 caliber rifle (Hansen *et al.*, 2004). Samples were primarily collected between May 2002 and April 2003 (n = 63) with 19 additional samples collected during November 2003 and March 2004. Field days were rotated throughout the Bay and survey effort was varied by time of day and location to minimise the chance of encountering the same dolphins. This sampling regime was designed to ensure the samples collected reflected the true diversity of the Biscayne Bay community. Biopsy darts were quickly retrieved and the samples were removed and processed immediately. Skin was separated from the blubber and stored at room temperature in 20% dimethyl sulfoxide (DMSO) saturated with sodium chloride. The blubber was placed in cryogenic Teflon vials in and stored in a –80°C freezer for storage for organohalogen pollutant analyses (Litz *et al.*, 2007). Darts, forceps and scalpel handles were cleaned using a method similar to that described by Hansen *et al.* (2004).

During biopsy collection, the dorsal fin of each sampled animal was photographed using digital video and/or still photography. These dorsal fin photos were compared to the NOAA Fisheries, SEFSC Biscayne Bay bottlenose dolphin photo-ID catalogue (Litz, 2007). For each sampled animal that was matched to the catalogue, the mean latitude and mean longitude of the animal's sighting history was calculated and used as the geographic reference for the sample. If an animal was sighted more than once during a survey day, only the first sighting of that day was used for that individual. The mean was chosen because it is weighted towards the majority of the animal's sightings and can be used as a continuous variable. For any tests that required an *a priori* geographic division of the data, animals with mean latitudes north of 25.61°N were considered northern and animals with mean latitudes south of 25.61°N were considered southern. If a sample could not be matched to the catalogue, the sample collection site was used for its geographic reference. Sample sizes are listed in Table 1.

Skin biopsy samples were collected from bottlenose dolphins in Florida Bay using similar methods in 1998 and 2002 during a collaborative study among the National Ocean Service, the Dolphin Ecology Project and NOAA Fisheries (Fair *et al.*, 2003). All skin samples were stored at room temperature in 20% DMSO saturated with sodium chloride.

DNA extraction and sexing

Skin (15–25mg) was minced and digested in 250µl of extraction buffer [10mM Tris HCl (pH 8), 2mM EDTA (pH 8), 10mM NaCl, 1% SDS, 8mg/ml DTT, and 0.2mg/ml proteinase K] overnight at 50°C (Rosel and Block, 1996). The DNA was extracted from the homogenised tissue using two

phenol-chloroform (v/v 1:1) extractions and one chloroform extraction in Phase Lock gel® tubes (Eppendorf). The DNA was ethanol-precipitated and re-suspended in 10mM Tris HCl (pH 7.6), 1mM EDTA (pH 8), and stored at –20°C.

Molecular sexing of the Biscayne Bay samples was completed using a multiplex PCR reaction that targets both the ZFXY genes from the X chromosome and the SRY gene from the Y chromosome (Rosel, 2003). The primers, PCR reaction and cycling profile used were the same as those described by Rosel (2003) with the exception that the concentration of DNA in the samples was unknown. Therefore, 2.0µl of DNA template was added to each 25µl reaction. Florida Bay biopsies were sexed in one of three ways: as in Rosel (2003) directly from skin or from DNA, or under identical conditions of Rosel (2003) but using only three primers: ZFX0923R, ZFY00767R, ZFYX0582F (Bérubé and Palsbøll, 1996).

Mitochondrial DNA sequencing

Biscayne Bay samples were sequenced at a laboratory within the University of Miami. A 356 base pair segment of the control region of the mitochondrial DNA was amplified using the primers L15824 and H16265 (Rosel *et al.*, 1999). Samples collected in Biscayne Bay were amplified in 25µl PCR reactions containing 20mM Tris HCl pH 8.0, 50mM KCl, 0.1% Tween 20, 1.5mM $MgCl_2$, 0.25µM of each primer, 200µM dNTPs, 1 unit of Taq DNA polymerase, and 2µl of DNA template. The thermal cycler profile consisted of initial denaturation at 94°C for 2 minutes, 30 cycles of 94°C for 10 seconds, 50°C for 10 seconds, and 72°C for 20 seconds, followed by a final extension of 5 minutes at 72°C. PCR products were purified by ExoSAP-IT® (USB Corporation) by adding 2µl of ExoSap-IT® to 5µl of PCR product and incubating at 37°C for 15 minutes followed by 80°C for 15 minutes. PCR products were cycle-sequenced using the same forward primer and 2µl of purified product following protocols supplied by the manufacturer of the Big Dye® terminator v1.1 cycle sequencing kit (Applied Biosystems, Inc.). Approximately one-third of the DNA samples were also cycle-sequenced using the reverse primer to verify sequence accuracy. Products were cleaned with Sephadex columns (Princeton Separations) according to manufacturer's directions and resolved using an ABI Prism® 310 Genetic Analyzer (Applied Biosystems, Inc.). Sequences were edited and aligned using Bioedit v5.0.9 (Hall, 2001).

Florida Bay samples were amplified and sequenced at the NOAA Fisheries SEFSC Marine Mammal Molecular Genetics Laboratory using the same primers as the Biscayne Bay samples. Concentrations of the DNA extractions from Florida Bay were measured using a fluorometer (Amersham Biosciences). Samples were amplified in 25µl PCR reactions containing 20mM Tris HCl pH 8.4, 50mM KCl, 1.5mM $MgCl_2$, 0.3µM of each primer, 150µM dNTPs, 1.25 unit of Taq DNA polymerase, and 25ng of DNA template. The thermal cycler profile consisted of initial denaturation at 94°C for 30 seconds, 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, followed by a final extension of 7 minutes at 72°C. PCR products were purified by gel purification (1% SeaPlaque® GTG® Agarose in 1×TAE) followed by agarase treatment. PCR products were cycle-sequenced in both the forward and reverse directions using 1µl of purified product following protocols supplied by the manufacturer of the Big Dye® terminator v1.1 cycle sequencing kit (Applied Biosystems, Inc.). Cycle sequencing products were cleaned by ethanol precipitation and resolved using an ABI Prism® 3130 Genetic Analyzer (Applied Biosystems, Inc.). Sequences were edited in Sequence Navigator (Applied Biosystems, Inc.), and aligned in SeqPup v0.6 (Gilbert, 1995).

Microsatellites

Biscayne Bay samples were genotyped at 14 loci and Florida Bay samples were genotyped at 10 of the same loci. For logistical reasons, the genotyping occurred in two different laboratories. Three loci were analysed from different samples in both laboratories. Raw data from these loci were analysed in allelogram (available at: *http://code.google.com/p/allelogram/*) with binning normalised by a control sample. The Allelogram analysis confirmed that there were no scoring differences between the two laboratories. At the University of Miami, Biscayne Bay samples were PCR amplified at seven microsatellite loci (Appendix 1) developed by Caldwell *et al*. (2002). Each PCR reaction contained 20mM Tris-HCl, pH 8.0, 50mM KCl, 0.1% Tween 20, 1.5mM $MgCl_2$, 0.25µM of each primer, 200µM dNTPs and 1 unit of Taq DNA polymerase. 2µl of DNA template was added to each 25µl reaction. The thermal cycler profile consisted of initial denaturation at 94°C for 2 minutes, followed by 30 cycles of 94°C for 10 seconds, annealing temperature (Appendix 1) for 10 seconds, and 72°C for 20 seconds, followed by a final extension of 5 minutes at 72°C. Each locus was amplified alone and then TtruGT6, TtruGT48, TtruGT39, TtruAAT40, TtruAAT44, and TtruGT162 were diluted at a v/v 1:20 ratio with water and co-loaded for genotyping. TtrGT51 was loaded independently. All samples were genotyped on an ABI Prism® 310 Genetic analyzer at the University of Miami using the Genescan-500 Tamara size standard (Applied Biosystems, Inc.). Genotyping used the Genotyper 2.1 and Genescan Analysis 3.1 software (Applied Biosystems, Inc.).

The Biscayne Bay samples were genotyped at seven additional loci (Ttr04, Ttr11, Ttr19, Ttr34, Ttr48, Ttr58, Ttr63) (Rosel *et al.*, 2005) at the NOAA Fisheries Laboratory. Twenty-five microliter amplification reactions consisted of 20mM Tris-HCl, pH 8.4, 50mM KCl, 1.5mM $MgCl_2$, 200µM dNTPs, 1 unit of Taq DNA polymerase, 25ng of DNA template, and primer concentrations varied from 0.16µM to 0.4µM as listed in Appendix 1. Thermal cycler profiles are listed in Appendix 2. Three pairs of loci were multiplexed (Ttr04 and Ttr11; Ttr34 and Ttr48; Ttr58 and Ttr63) and each pair was loaded separately for genotyping. Ttr19 was PCR amplified and loaded independently.

These seven loci were also used to genotype the Florida Bay samples along with TtruGT39, TtruGT48 and TtruGT51 (Caldwell et al., 2002) (Appendix 1). DNA from one sample was used as a positive control and a negative control with no DNA was run with each set of amplifications. All Florida Bay samples and these seven loci for Biscayne Bay samples were genotyped on an ABI Prism® 310 Genetic analyzer using the Genescan 500 Tamara size standard (Applied Biosystems, Inc.). Genotyping used the Genotyper 2.1 and Genescan Analysis 3.1 software (Applied Biosystems, Inc.).

Statistical analyses

Genetic structure within Biscayne Bay was investigated by comparing northern Biscayne Bay dolphins (NBB, mean latitudes north of 25.61°N) to southern Biscayne Bay dolphins (SBB, mean latitudes south of 25.61°N). Florida Bay data were compared to Biscayne Bay as a whole and to each of the Biscayne Bay subgroups, NBB and SBB. There were seven pairs of animals sampled in Biscayne Bay that were known from the photo-ID study to be mother/calf pairs. Data from the known mother/calf pairs were compared to ensure they had shared at least one allele at each locus. Calves were excluded from all other analyses.

For the mtDNA data, haplotype (h) and nucleotide (π) diversity (Nei, 1987) were calculated using the program Arlequin (Nei, 1987; Schneider *et al.*, 2000). Pairwise F_{ST} and ϕ_{ST} values between Florida Bay and Biscayne Bay and within Biscayne Bay were estimated using an analysis of molecular variance (AMOVA) in Arlequin (Excoffier *et al.*, 1992; Schneider *et al.*, 2000; Weir and Cockerham, 1984). Evolutionary distances between the sequences were estimated using the Tamura-Nei model (Tamura and Nei, 1993) with no gamma correction. The significance values for both F_{ST} and ϕ_{ST} were obtained by 10,000 permutations; sequential Bonferroni corrections were applied to the p values (Rice, 1989). To represent the differences among haplotypes, a phylogenetic network was constructed using the software Network and the median-joining algorithm. The recommended default settings were used (weights 10, epsilon 0). The network was re-calculated with increasing epsilon values (by increments of 10 up to 60) to confirm the full median network had been calculated with the default parameters (Bandelt *et al.*, 1999).

For the microsatellite data, Hardy-Weinberg Equilibrium and linkage disequilibrium tests were conducted on Biscayne Bay data (14 loci) and Florida Bay data (10 loci) using GENEPOP (Raymond and Rousset, 1996). A Markov chain method was used to estimate p values using the following parameters: dememorisation of 1,000, 1,000 batches and 1,000 iterations per batch with the exception of the linkage disequilibrium test where 2,000 batches were run (Guo and Thompson, 1992). Sequential Bonferroni corrections were applied to all p values (Rice, 1989). Tests for duplicate samples were carried out using the program Identity (Amos, 2000). Probabilities of identity (P_{ID}) were estimated using the software Gimlet (Valiére, 2003). Gimlet provides both an unbiased estimate of P_{ID} and P_{IDsibs}, which is a more conservative measure of the power of the microsatellite data to resolve siblings. Expected and observed heterozygosities were calculated in GENALEx 6 (Peakall and Smouse, 2006). GENALEx 6 was also used to estimate F_{ST} (Wright, 1965) by AMOVA (Excoffier *et al.*, 1992; Weir and Cockerham, 1984). F_{ST} was calculated between Florida Bay and Biscayne Bay using 10 loci. F_{ST} was also calculated within Biscayne Bay using all 14 loci genotyped and results were very similar. Therefore, the results from the tests using the 10 loci in common between Biscayne Bay and Florida Bay are presented. The significance values were obtained by 10,000 permutations and sequential Bonferroni corrections were applied to the p values (Rice, 1989).

Pairwise relatedness values were estimated among all individuals within each sampling location (Biscayne Bay and Florida Bay) using the web based software RERAT (Lynch and Ritland, 1999; Schwacke and Rosel, 2005). The average r value for the known mother/calf pairs was 0.507. As a result one member of each pair with an $r > 0.5$ was removed in addition to the seven known calves. Pairwise F_{ST} and ϕ_{ST} were re-estimated from the mtDNA data and pairwise estimates of F_{ST} were recalculated from the microsatellite data using the same methods described above.

The software 'STRUCTURE' (Pritchard *et al.*, 2000) was used to investigate population structure using the microsatellite data without requiring *a priori* divisions of the data. STRUCTURE uses a Bayesian clustering technique to probabilistically assign individuals with multilocus genotypes to one or more populations based on Hardy-Weinberg expectations and linkage equilibrium (Pritchard, 2004; 2000). Models were run under the admixture ancestry model and the no admixture model. Results from the two ancestry models were similar and results from the admixture model are presented. The correlated allele frequency model was applied, which assumes that the frequencies in the different populations are likely to be similar, probably due to migration or shared ancestry (Falush *et al.*, 2003; Pritchard, 2004). The results presented were obtained with a burn-in length of 100,000 followed by a run length of 100,000. The models were run for several values of K (1, 2, 3, 4 and 5 populations) using the microsatellite data from 10 loci with both Biscayne Bay and Florida Bay samples combined. The model for each K was run independently five times to verify stability in results. The model gives the log likelihood of the data conditional on the specified K and the posterior probability of each K was calculated assuming a uniform prior of K (Pritchard, 2004). A larger posterior probability indicates the best fit model.

RESULTS

Sample collection and sex determination

Sixty-five survey days were completed in Biscayne Bay during which 135 biopsy attempts were made. A total of 82 skin samples were collected; 17 of which were duplicates as determined by photo analysis. An additional nine skin samples were obtained during preliminary sampling in 2000 and four samples were obtained from animals that stranded in Biscayne Bay, for a total of 78 samples (Fig. 1). Seventy-four percent of samples collected were matched to the NOAA, SEFSC Biscayne Bay photo-ID catalogue. The remaining 26% of sampled animals could not be matched to the catalogue because they either had a distinct fin not recognised in the catalogue, a non-distinct fin, or poor photos and/or video of the biopsy attempt prevented identification. A total of 53 samples were available from Florida Bay (Fig. 2).

Mitochondrial DNA sequencing identified a total of 10 samples (2 from Biscayne Bay and 8 from Florida Bay) with offshore haplotypes (details discussed below). These animals are not likely to be residents of the embayments and were therefore removed from all statistical analyses. In addition, the Identity (Amos, 2000) program indicated eight pairs of identical samples from the microsatellite data. The agreement of sequence and sex information for these pairs was verified. In each case, at least one member of the pair had not been identified or matched to the photo-ID catalogue,

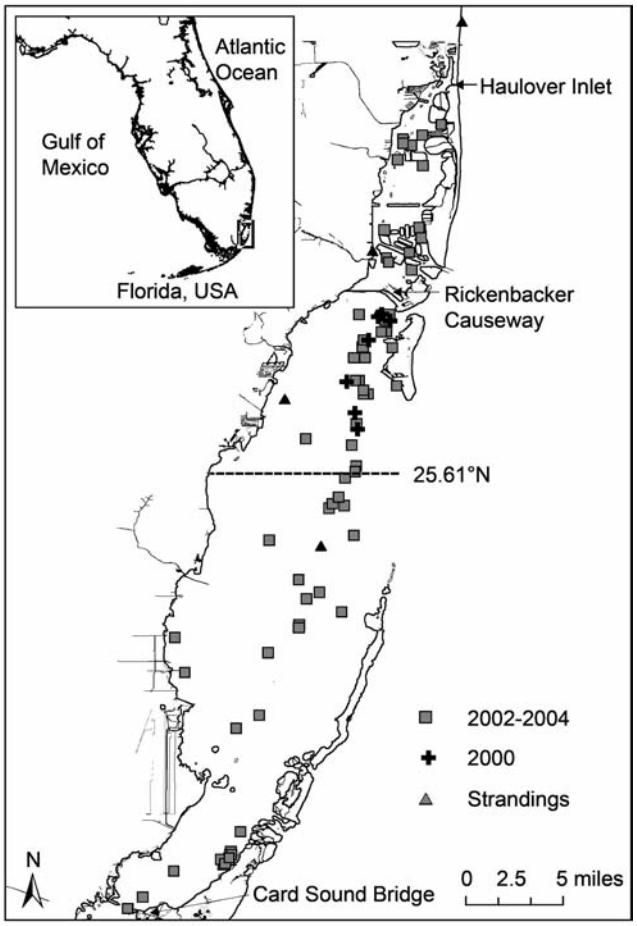

Fig. 1. Location of skin biopsy samples and four samples from stranded dolphins collected from Biscayne Bay, FL.

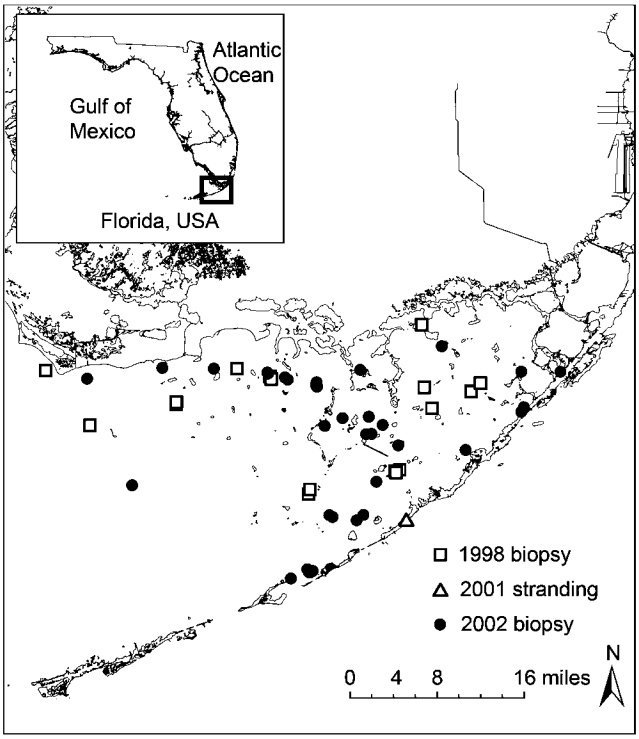

Fig. 2. Location of skin biopsy samples and one sample from a stranded dolphin collected from Florida Bay, FL.

such that it was possible that the same animal was sampled twice. One member from each of these pairs (6 from Biscayne Bay and 2 from Florida Bay) was removed from all data analyses. Of the remaining 70 samples from Biscayne Bay, 26 were females, 42 were males and two samples could not be sexed due to poor DNA quality. Thirty-six of the samples were from dolphins from northern Biscayne Bay and 34 were from southern Biscayne Bay. Of the remaining 43 samples from Florida Bay, 31 were males and 12 were females. The probability of two individuals having identical genotypes (P_{ID}) in Biscayne Bay (14 loci) is 7.86×10^{-12} and P_{IDsib} is 4.34×10^{-5}. In Florida Bay (10 loci) the P_{ID} is 1.57×10^{-8} and P_{ID}sib is 8.86×10^{-4}.

Mitochondrial DNA sequences

The mitochondrial control region was sequenced and aligned from all Biscayne Bay and Florida Bay samples. Offshore haplotypes were identified based on fixed site differences in the sequences and phylogenetic analysis. Four offshore haplotypes were found with eight variable sites, two insertion/deletions and six transitions (Appendix 3, Genbank accession numbers GQ504085, GQ504087, HQ383684 and HQ383685). Three of the offshore haplotypes were found in eight Florida Bay samples and one was found in two samples from dolphins stranded in Biscayne Bay. Seven coastal haplotypes were found with 11 variable sites consisting of one insertion/deletion and 10 transitions (Appendix 3, Genbank accession numbers AY997307 – AY997309, GQ504101, GQ504103, GQ504049 and HQ383686). Three of the coastal haplotypes were found in both Bays, two were unique to Biscayne Bay, and two were unique to Florida Bay (Table 1). The two most common haplotypes in Florida Bay were not found in Biscayne Bay and the two most common haplotypes in Biscayne Bay were found in Florida Bay at the lowest frequencies. The median-joining network of the seven coastal haplotypes is shown in Fig. 3.

Both haplotype and nucleotide diversity based on coastal haplotypes were higher in Florida Bay than Biscayne Bay (Table 1). While samples from each Bay consisted of five coastal haplotypes, more than 70% of the Biscayne Bay samples consisted of two haplotypes (Ttr32 or Ttr15), whereas the haplotypes were more evenly distributed in Florida Bay. The mtDNA sequence data indicate significant differentiation between Florida Bay and Biscayne Bay as a

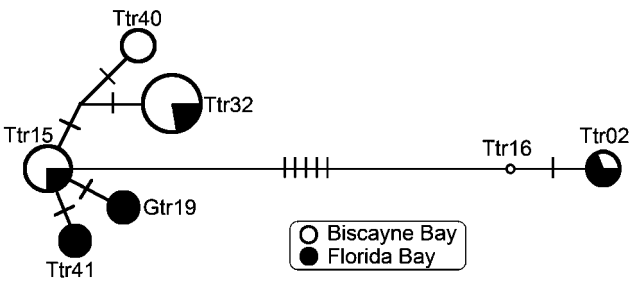

Fig. 3. Median-joining network of coastal haplotypes generated by the median-joining algorithm (Bandelt *et al.*, 1999). The size of the circle representing each haploype is proportional to the frequency of that haplotype in the total sample. The colours represent the proportion of the haplotypes found in each population (Florida Bay in black and Biscayne Bay in white). The branch lengths are proportional to the number of changes between the haplotypes and each hash mark represents one change. One intermediate ancestral node is indicated between Ttr15, Ttr40, and Ttr32.

Table 1

mtDNA coastal haplotypes; number of samples per haplotype (*n*) and frequency (Freq.) per population. Numbers in parentheses indicate the number of calves from known mother/calf pairs removed from the analyses. The frequencies were calculated from the data excluding these seven calves.

mtDNA coastal haplotypes	All Biscayne Bay (*n* = 70)		North Biscayne Bay (*n* = 36)		South Biscayne Bay (*n* = 34)		Florida Bay (*n* = 43)	
	n	Freq.	*n*	Freq.	*n*	Freq.	*n*	Freq.
Ttr02	4	0.064	4	0.133	0	0	9	0.209
Ttr15	17(1)	0.270	6(1)	0.200	11	0.333	6	0.140
Ttr16	1	0.016	0	0	1	0.031	0	0
GTtr19	0	0	0	0	0	0	11	0.256
Ttr32	29(4)	0.460	17(3)	0.567	12(1)	0.364	8	0.186
Ttr40	12(2)	0.190	3(2)	0.100	9	0.273	0	0
Ttr41	0	0	0	0	0	0	9	0.209
Haplotype diversity	0.6856 ± 0.0357		0.6322 ± 0.0772		0.7027 ± 0.0295		0.8117 ± 0.0174	
Nucleotide diversity	0.0061 ± 0.0038		0.0073 ± 0.0045		0.0047 ± 0.0032		0.0096 ± 0.0056	

whole (F_{ST} = 0.1388, $p \leq 0.0001$; ϕ_{ST} = 0.1677, $p \leq 0.0001$) and also between Florida Bay and each of the Biscayne Bay subgroups (Table 2). No significant difference was found between the two geographic subgroups of Biscayne Bay (F_{ST} = 0.0463, p = 0.0684; ϕ_{ST} = 0.0344, p = 0.1034). Results did not change after estimating relatedness and removing 10 individuals from Biscayne Bay and 5 individuals from Florida Bay (Biscayne Bay vs. Florida Bay: F_{ST} = 0.1305, $p \leq 0.0001$; ϕ_{ST} = 0.1810, $p \leq 0.0001$; Within Biscayne Bay: F_{ST} = 0.0159, p = 0.2226; ϕ_{ST} = 0.0350, p = 0.1200).

Microsatellite loci

The Biscayne Bay samples were genotyped at 14 loci and the Florida Bay samples were genotyped at 10 loci. Sixteen private alleles were found across the 10 loci in common, 13 of which were found only in Biscayne Bay and three only in Florida Bay. All loci were in Hardy-Weinberg Equlibrium (HWE) after sequential Bonferroni correction, and pair-wise tests for linkage showed no significant linkage disequilibrium. The number of alleles per locus, observed vs. expected heterozygosity and HWE *p*-values are listed in Table 3. Analyses reveal significant differentiation between Florida Bay and Biscayne Bay as a whole (F_{ST} = 0.0416, $p \leq 0.001$), and also between Florida Bay and each of the Biscayne Bay subgroups (Table 2). A significant F_{ST} was also found between the northern and southern Biscayne Bay subgroups (F_{ST} = 0.015, p = 0.009). Results did not change after estimating relatedness and removing one animal from each pair where $r > 0.5$ (Biscayne Bay vs. Florida Bay: F_{ST} = 0.0380, $p \leq 0.001$; within Biscayne Bay: F_{ST} = 0.0138, p = 0.024).

Table 2

mtDNA F_{ST} and Φ_{ST} statistics and microsatelite F_{ST} statistics for pairwise comparisons between Florida Bay (FB), Biscayne Bay as a whole (BB), northern Biscayne Bay dolphins (NBB), and southern Biscayne Bay dolphins (SBB).

	mtDNA		Microsatellite
	F_{ST}	Φ_{ST}	F_{ST}
BB vs. FB	0.1353, p ≤ 0.0001	0.1658, p ≤ 0.0001	0.0407, p ≤ 0.0001
NBB vs. FB	0.1357, p ≤ 0.0001	0.1396, p = 0.0011	0.0509, p ≤ 0.0001
SBB vs. FB	0.1437, p ≤ 0.0001	0.1788, p ≤ 0.0001	0.0380, p ≤ 0.0001
NBB vs. SBB	0.0463, p = 0.0638	0.0344, p = 0.1034	0.0149, p = 0.0074

The results from the STRUCTURE model runs indicate the best fit model for the Biscayne Bay and Florida Bay samples combined is the two population model (K = 2; Table 4). The two population model (K = 2; Fig. 4), shows a split that corresponds exactly to the division of Florida Bay and Biscayne Bay samples in the data. The three population model (K = 3; Fig. 4) was unable to differentiate a third population division. The results from testing four and five populations (K = 4 and K = 5, respectively) were similar to that of three populations and are not shown.

DISCUSSION

Haplotype diversity found in the Biscayne Bay mtDNA sequences was similar to that found in other inshore resident dolphin populations in Sarasota Bay, FL, Charlotte Harbor, FL, Matagorda Bay, TX and Abaco Island, Bahamas (Parsons *et al.*, 2006; Sellas *et al.*, 2005) and was higher than that found in three communities of dolphins in Jacksonville, FL (Caldwell, 2001). In a study of five bottlenose dolphin populations in the northwest Atlantic, Rosel *et al.* (2009) found inshore resident populations had lower diversity than nearshore coastal dolphin populations. The haplotype diversity of Biscayne Bay was higher than those found in the inshore populations in Rosel *et al.* (2009) but still lower than the nearshore coastal animals. Florida Bay's haplotype diversity was slightly higher than Biscayne Bay and very similar to that found in a nearshore coastal Gulf of Mexico dolphin population off Sarasota, Florida (Sellas *et al.*, 2005). The haplotype diversity was also higher than the nearshore coastal bottlenose dolphins along the US Atlantic Coast (Rosel *et al.*, 2009). The higher diversity in Florida Bay compared to Biscayne Bay may be explained by the distribution of haplotypes. Florida Bay haplotypes were more evenly distributed across samples, whereas the majority of Biscayne Bay samples (73%) had one of two haplotypes. The greater haplotype diversity found in Florida Bay and the higher presence of offshore haplotypes implies that there may be a greater degree of mixing, and possibly a larger population size, in Florida Bay than Biscayne Bay. Future studies of residency patterns in Florida Bay dolphins may help verify this.

Significant genetic differentiation was found between Biscayne Bay and Florida Bay in both the mtDNA control region (F_{ST} and $\phi_{ST)}$ and the microsatellite loci (F_{ST}).

Table 3

Number of microsatellite alleles (Na), observed heterozygosity (H_O), expected heterozygosity (H_E), and Hardy-Weinberg Equilibrium p-value (p) per locus and population.

	All	Biscayne Bay				Florida Bay			
Locus	Na	Na	H_O	H_E	p	Na	H_O	H_E	p
Ttr04	7	7	0.705	0.743	0.095	6	0.744	0.720	0.350
Ttr11	6	6	0.787	0.794	0.900	6	0.744	0.768	0.284
Ttr19	4	4	0.246	0.237	0.208	3	0.535	0.501	1.000
Ttr34	5	5	0.667	0.607	0.825	4	0.465	0.513	0.413
Ttr48	5	5	0.300	0.323	0.409	3	0.163	0.226	0.115
Ttr58	4	3	0.459	0.493	0.250	4	0.535	0.574	0.012
Ttr63	14	13	0.869	0.850	0.280	10	0.907	0.852	0.810
TtruGT39	4	4	0.656	0.591	0.450	4	0.535	0.526	0.800
TtruGT48	6	6	0.610	0.594	0.543	3	0.571	0.544	0.641
TtruGT51	9	8	0.787	0.725	0.576	8	0.791	0.771	0.635
TtruAAT40	–	5	0.656	0.614	0.629	–	–	–	–
TtruAAT44	–	4	0.567	0.518	0.565	–	–	–	–
TtruGT142	–	6	0.869	0.788	0.235	–	–	–	–
TtruGT6	–	7	0.733	0.677	0.260	–	–	–	–

STRUCTURE also differentiated the two populations without requiring *a priori* assignments. The estimates of F_{ST} from the microsatellite data and the mtDNA data were similar to F_{ST} values found between bottlenose dolphins in other regions (including between Sarasota Bay, FL and the nearshore coastal Gulf of Mexico and between populations around Abaco Island Bahamas; Table 5) (Parsons *et al.*, 2006; Sellas *et al.*, 2005). The microsatellite F_{ST} was also similar to that found between bottlenose dolphins in other parts of the world including between those in the Western and Eastern Mediterranean Sea (Natoli *et al.*, 2005) and between the United Kingdom and Northeast Scotland (Nichols *et al.*, 2007). The genetic differentiation found between Florida Bay and Biscayne Bay in both maternally inherited mtDNA and biparentally inherited nuclear markers suggests both male and female philopatry to their respective Bays.

It has been suggested that complex social structure, differential habitat utilisation and foraging specialisation may all contribute to natal site fidelity and thus reduced dispersal in both sexes (Natoli *et al.*, 2005; 2004; Parsons *et al.*, 2006; Rosel *et al.*, 2009; Sellas *et al.*, 2005). For example, significant genetic differentiation among five populations of bottlenose dolphins along the US east coast was attributed to habitat differences and social facilitation of foraging strategies (Rosel *et al.*, 2009). It is possible that both social structure and differential habitat utilisation play a role in the site fidelity observed in both Biscayne Bay and Florida Bay. Social structure analysis of Biscayne Bay dolphins showed strong evidence of long term social bonds (Litz, 2007). Female bottlenose dolphins have been shown to strongly associate with other females in groups called bands (Connor *et al.*, 2000). Analysis confirmed the presence of female bands in Biscayne Bay and identified at least one female calf who rejoined her natal group (Litz, 2007). Several long-term male pair bonds were also identified in Biscayne Bay, supporting the idea that lack of dispersal of both sexes could be linked to complex social bonds. While Biscayne Bay and Florida Bay do not have vastly different habitat types, there are subtle differences. Northern Biscayne Bay has poor water circulation within largely manmade shorelines (mostly seawalls). Southern Biscayne Bay is much more open with natural mangrove shorelines and Florida Bay is divided into semi-isolated basins divided by mangrove islands and mud banks. While bottlenose dolphins in general show a wide range of foraging behaviours, some specialised behaviours have been observed in these areas. For example, dolphins in northern Biscayne Bay have been observed using the seawall to help catch fish (NOAA, unpublished data). Individual dolphins in Florida Bay have been shown to specialise in one of several foraging tactics, including a very specific mud-ring feeding behaviour rarely seen elsewhere (Torres and Read, 2009). These authors found strong evidence that dolphins in Florida Bay limited their spatial distribution to habitats that are most suitable for that foraging type leading to strong site fidelity. The strong genetic differentiation found between Biscayne Bay and Florida Bay indicates restricted genetic exchange between them. This result, coupled with distinct foraging strategies in both locations further supports the growing body of evidence that bay and estuarine populations of bottlenose dolphins exhibit strong site fidelity and limited genetic exchange with nearby populations despite a lack of barriers to movement and genetic exchange.

Table 4

Estimated posterior probabilities of K [Pr (K/X)] calculated from the estimated prior distributions of K [ln Pr(X/K)] from the outputs of the STRUCTURE model runs. The K with the greatest probability represents the best fit model and is indicated in bold font.

Number of populations	Florida Bay and Biscayne Bay (10 microsatellite loci)	
	ln Pr (X/K)	Pr (K/X)
K = 1	–2,707	~0
K = 2	–2,604	**1**
K = 3	–2,658	~0
K = 4	–2,673	~0
K = 5	–2,811	~0

At least two social groups of bottlenose dolphins are present in Biscayne Bay, a northern (NBB) and southern (SBB) group (Litz, 2007). Analysis of organic pollutants in the dolphins' blubber provides evidence that these social groups are foraging in different areas of Biscayne Bay (Litz *et al.*, 2007). Despite these differences, many of the animals have overlapping sighting histories in the centre of the Bay

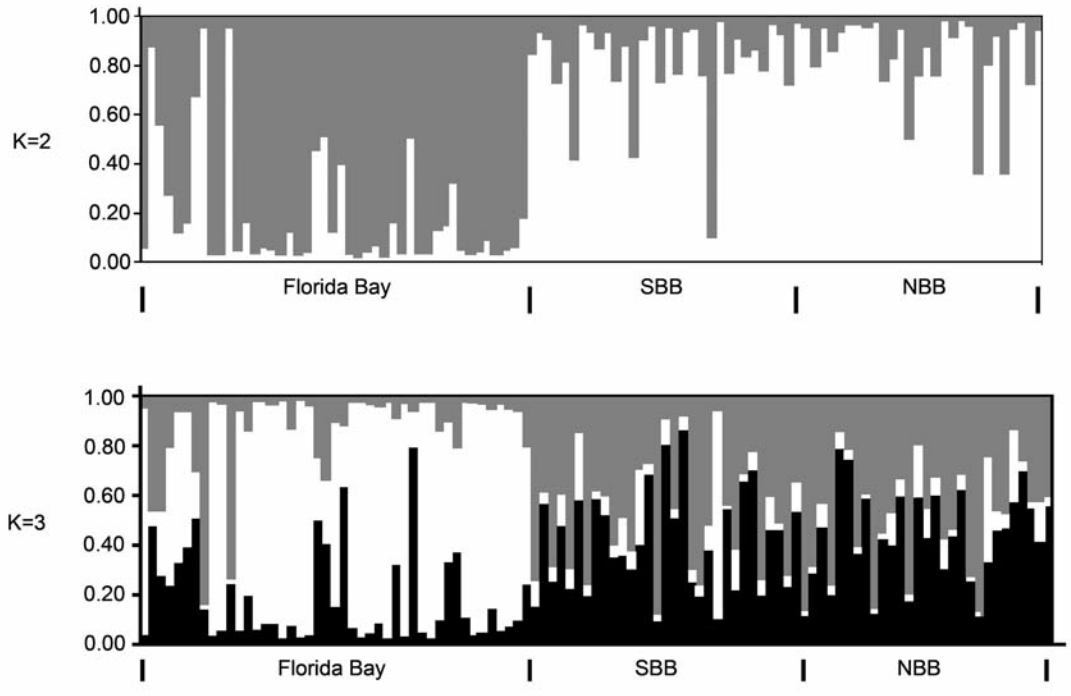

Fig. 4. Output from STRUCTURE runs for two and three populations (K = 2 and K = 3, respectively) using microsatellite data from 10 loci with Biscayne Bay and Florida Bay samples combined. Each bar represents an individual and the shading represents the proportion (y-axis) of the individual's genome drawn from each putative population. The regional affiliations of the samples [Florida Bay, southern Biscayne Bay (SBB), and northern Biscayne Bay (NBB)] are labelled below the x-axis.

and about a third of the photo-ID sightings contain animals from both social groups providing opportunity for interbreeding (Litz, 2007). The social groups are weakly, but significantly differentiated at the microsatellite markers (F_{ST} = 0.0149, p ≤ 0.009), however the mtDNA based estimates of F_{ST} and ϕ_{ST} within Biscayne Bay were not significant. The lack of significant population structure at the maternally inherited mitochondrial locus within Biscayne Bay is possibly a result of low statistical power. The mtDNA is a single locus, and in this case, seven haplotypes were found but only two were common in Biscayne Bay samples. On the other hand, microsatellite data are highly polymorphic and each locus acts as an independent marker. Therefore, they have the power to describe small genetic differences between populations (Kalinowski, 2002). While no strong evidence of significant population structure within Biscayne Bay was found, the possibility that structure exists but there was insufficient power to detect it cannot be excluded. Additional studies should be conducted to increase the sample size.

Population differentiation runs on a continuum from complete isolation to complete panmixia (Waples and Gaggiotti, 2006). Determining at what point on the continuum two groups should be managed as separate stocks is difficult. The differences in haplotype and genotype frequencies found between Biscayne Bay and Florida Bay and the stable residency patterns observed in Biscayne Bay dolphins (Litz, 2007) provide strong evidence that Biscayne Bay and Florida Bay should be managed as separate biologically-relevant stocks. Within Biscayne Bay, the significant but low level of genetic differentiation at microsatellite markers indicates limited levels of genetic exchange between the two social groups. However, given that the two groups share a single embayment and have overlapping sighting histories, the low value of the F_{ST} (0.01) and the lack of a significant F_{ST} value from the mtDNA marker does not provide enough evidence to warrant managing the two social groups as separate biologically-relevant stocks at this time.

Table 5

Comparisons of mtDNA and microsatellite F_{ST} values for Biscayne and Florida Bays compared to published studies on other bottlenose dolphin populations.

Study areas	mtDNA F_{ST}	Microsat. F_{ST}	Reference
Biscayne Bay vs. Florida Bay	0.139	0.042	This study
Sarasota Bay vs. Gulf of Mexico	0.113	0.042	Sellas *et al.* (2005)
3 locations in Abaco, Bahamas	0.192	0.040	Parsons *et al.* (2006)
Sarasota Bay vs. Tampa Bay	0.137	0.027	Sellas *et al.* (2005)
Sarasota Bay vs. Matagorda Bay	0.284	0.043	Sellas *et al.* (2005)
Northern vs. southern Jacksonville	0.698	0.044	Caldwell *et al.* (2001)
Northern vs. coastal Jacksonville	0.456	0.042	Caldwell *et al.* (2001)
Eastern vs. western Mediterranean	0.032	0.045	Natoli *et al.* (2005)
Western United Kingdom vs. NE Scotland	0.049		Nichols *et al.* (2007)

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Appendix 1

GENBANK ACCESSION NUMBERS, FLUORESCENT DYE LABELS, ANNEALING TEMPERATURES, PRIMER CONCENTRATIONS AND ALLELE SIZE RANGES FOR MICROSATELLITE PRIMER PAIRS

			Biscayne Bay			Florida Bay		
Locus	GenBank Accession no.	Dye label	Anneal Temp.	Primer Conc. (μM)	Allele size range	Anneal Temp.	Primer Conc. (μM)	Allele size range
Ttr04*	DQ018982	6-FAM	62	0.20	109–123	62	0.16	109–119
Ttr11*	DQ018981	TET	62	0.20	203–215	62	0.20	203–215
Ttr19*	DQ018980	6-FAM	60	0.15	183–197	60	0.24	183–197
Ttr34*	DQ018984	TET	58	0.15	183–193	58	0.30	183–193
Ttr48*	DQ018983	TET	58	0.20	130–140	58	0.20	130–138
Ttr58*	DQ018985	HEX	63	0.16	179–187	60	0.16	179–197
Ttr63*	DQ018986	6-FAM	63	0.40	102–136	60	0.40	102–134
TtruGT39#	AF416504	6-FAM	55	0.50	154–160	55	0.20	154–160
TtruGT48#	AF416505	HEX	55	0.50	185–223	55	0.24	193–199
TtruGT51#	AF416506	6-FAM	60	0.50	201–217	61	0.28	203–221
TtruAAT40#	AF416500	TET	60	0.50	155–164	–	–	–
TtruAAT44#	AF416501	HEX	60	0.50	82–94	–	–	–
TtruGT142#	AF416507	6-FAM	60	0.50	195–205	–	–	–
TtruGT6#	AF416503	TET	55	0.50	193–214	–	–	–

*Rosel *et al.* (2005); #Caldwell *et al.* (2002).

Appendix 2

PCR THERMAL CYCLER PROFILES RUN FOR FLORIDA BAY SAMPLES (10 LOCI) AND BISCAYNE BAY SAMPLES (7 Ttr LOCI ONLY)

	94°C initial denaturation	No. of cycles	94°C	Annealing temp, time	72°C	72°C final extension
Ttr04 and Ttr11	30 sec	30	20 sec	62°C, 20 sec	40 sec	10 min
Ttr19	30 sec	30	20 sec	60°C, 20 sec	40 sec	10 min
Ttr34 and Ttr48	30 sec	28	20 sec	58°C, 20 sec	20 sec	10 min
Ttr58 and Ttr63	30 sec	28	30 sec	60°C, 40 sec	40 sec	15 min
TtruGT39 and TtruGT48	30 sec	30	20 sec	55°C, 20 sec	1 min	15 min
TtruGT51	30 sec	30	20 sec	61°C, 20 sec	40 sec	15 min

Appendix 3

POLYMORPHIC SITES IN mtDNA SEQUENCE FOR COASTAL AND OFFSHORE HAPLOTYPES WITH THE SITE NUMBER LISTED AT THE TOP OF EACH COLUMN

Site number 1 is equivalent to site #62 in the published sequence for GTtr19, Genbank accession number AY997307 (Sellas *et al.*, 2005). A dash indicates a gap and a dot represents identity with the first sequence.

	Genbank accession no.	27	74	98	121	152	196	285	286	296	327	328
Coastal haplotypes:												
Ttr32	GQ504101	T	–	T	A	C	G	T	C	T	G	A
Ttr02	AY997308	C	C	.	G	T	A	C	T	.	A	.
Ttr15	GQ504049	.	–	.	.	.	A	.	.	.	A	.
Ttr16	AY997309	C	C	.	G	T	A	C	.	.	A	.
GTtr19	AY997307	.	–	C	.	.	A	.	.	.	A	.
Ttr40	GQ504103	.	–	.	.	.	A	.	.	C	.	.
Ttr41	HQ383686	.	–	.	.	.	A	.	.	.	A	G

		47	105	111	276	277	286	306	332
Offshore haplotypes									
OTtr21	GQ504085	A	A	G	T	C	C	G	C
OTtr23	GQ504087	.	G	.	.	–	T	.	T
OTtr69	HQ383684	.	G	A	–	.	.	A	.
OTtr49	HQ383685	G	.	.	.	.	.	.	.