

# Population Genetic Structure and Social Kin Associations of Franciscana Dolphin, *Pontoporia blainvillei*

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## Abstract

We investigated population and social structure of the franciscana dolphin, *Pontoporia blainvillei*, an endemic and the most endangered cetacean of the southwestern Atlantic Ocean. We analyzed samples from the Rio de la Plata estuary obtained in Uruguayan waters and from the Atlantic Ocean obtained in both Uruguayan and Brazilian waters. Mitochondrial and microsatellite DNA markers were used to study differentiation between the estuary and the ocean and the association between kinship and social group structure. Although multilocus analyses suggested that franciscanas are structured into 2 subpopulations ( $K = 2$ , divergence among clusters:  $F_{ST} = 0.06$ ,  $P = 0.002$ ;  $R_{ST} = 0.3$ ,  $P = 0.001$ ), mitochondrial markers did not support such divergence ( $F_{ST} = 0.02$ ,  $P = 0.12$ ;  $\Phi_{ST} = 0.06$ ,  $P = 0.06$ ). However, these units are not entirely segregated geographically. Regarding social structure, some groups are composed by first-order related individuals ( $R \geq 0.5$ ,  $P < 0.5$ ). Overall, the data suggest that matrilineal units could be the social unit in this species. We argue that the divergence found could be associated to local adaptation and social structure, resulting from either feature leading to a recent divergence or reflecting equilibrium between local differentiation and gene flow. This evidence supports considering franciscanas from the Rio de la Plata estuary a discrete management unit.

**Key words:** fine-scale population structure, local adaptation, matrilineal units, social structure

A deep understanding of population structure, social kin associations, and the factors that influence them is needed for effective management and conservation of cetacean populations. In general, we have limited understanding on what barriers constrain the movement of highly mobile animals, such as cetaceans, and thus, it is difficult to know where one population ends and the next one begins (Rosel et al. 1999). Thus, assignment of individuals to populations based on sampling origin may, or may not, have biological significance (Pritchard et al. 2000), specially in this kind of populations. Overall, cetaceans are considered to present population structure (Hoelzel et al. 1998), possibly due to a combination of factors, such as behavioral specialization to local resources and social structure (Hoelzel 1998). These factors are not mutually exclusive; rather, they are expected to result in a trade-off between local adaptation and minimization of inbreeding (Hewitt and Butlin 1997). In this context, molecular analyses have great importance in the study of cetacean populations and their social structure. Establishing kinship patterns within a social group helps in

understanding the origin and stability of groups and making inferences about the population mating system (Amos et al. 1993; Whitehead et al. 2000; DeWoody 2005).

The franciscana dolphin, *Pontoporia blainvillei*, is an endemic species of the southwestern Atlantic Ocean, whose distribution extends from Itaúnas, Brazil (lat 18°25'S, long 30°42'W) (Siciliano 1994), to northern Patagonia, Argentina (42°10'S) (Crespo et al. 1998). This species is primarily restricted to coastal waters, within the 30 nautical miles and up to 35 m deep. (Danilewicz et al. 2009). The franciscana has high incidental mortality as a result of entanglement in fishing gillnets. It is considered the most endangered small cetacean in the southwestern Atlantic Ocean (Secchi 2010), with high estimated mortality of several hundred up to a few thousands individuals per year along its distribution (Secchi, Ott, et al. 2003). The potential demographic and genetic impact of group bycatch (mostly composed of 2 individuals) on franciscana populations have been highlighted in previous studies (Mendez, Rosebaum, Subramaniam, et al. 2010).

The franciscana dolphin has recently been listed as a “vulnerable” species by the International Union for Conservation of Nature (IUCN 2009).

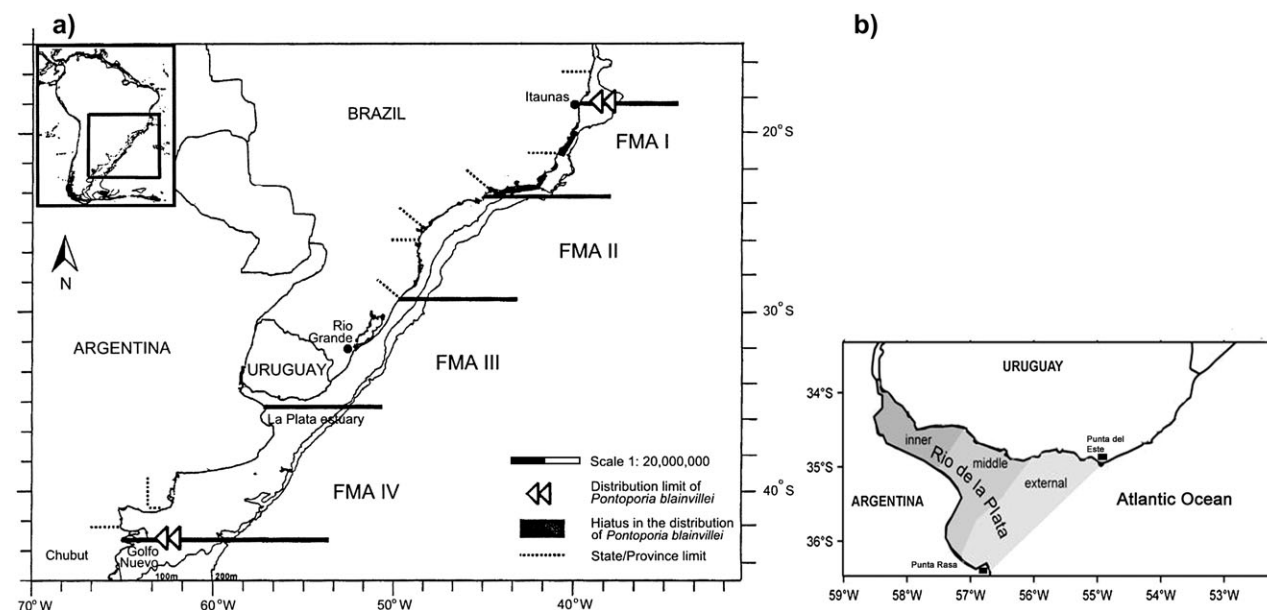
Currently, 4 management areas are recognized for franciscana (Franciscana Management Area, FMAs), as proposed by Secchi, Danilewitz, et al. (2003): FMA I ranging from Espírito Santo to Rio de Janeiro (18°25' 22°25'S), FMA II from São Paulo to Santa Catarina (23°20' 29°20'S), FMA III from Rio Grande do Sul to Uruguay (29°20' 35°00'), and FMA IV Argentina (35°00' 42°35'S) (Figure 1a). These areas were proposed based on distribution, vital rates, and feeding-related ecology (diet parasites and pollutants) as well as phenotype and genotype data (see details and references in Secchi, Danilewitz, et al. (2003)).

Based on mitochondrial DNA (mtDNA) data, 3 genetically distinct population units can be recognized from north to south of the species distribution: 1) Rio de Janeiro, Brazil; 2) São Paulo and Paraná, Brazil; and 3) southern Brazil (southern Santa Catarina and Rio Grande do Sul states), Uruguay, and Argentina, with this later unit presenting subdivision within Argentina, though less evident (Secchi et al. 1998; Lázaro et al. 2004; Mendez et al. 2008; Mendez, Rosenbaum, Wells, et al. 2010). Those studies agreed that isolation by distance explains the population structure found with mtDNA along its range. A multilocus fine-scale population structure analysis found divergence between adjacent areas of the Atlantic Ocean and the Rio de la Plata estuary in Argentine waters, which is supported by environmental patterns (Mendez, Rosenbaum, Wells, et al. 2010). Thus, an estuarine population unit (stock) was proposed for Argentine waters taking into account behavioral and molecular evidence (Bordino et al.

1999; Mendez et al. 2008; Mendez, Rosenbaum, Wells, et al. 2010).

Although the studies of social structure are an important tool for conservation, little is known about the social habits of this species due to the difficulty associated to observing this dolphin in the wild. Initially, the franciscana was suggested to be a solitary species (Pinedo et al. 1989). However, groups of 2–6 dolphins are commonly observed in Bahía Samborombon, Argentina (Bordino et al. 1999), and groups of up to 15 animals were observed during aerial surveys (Crespo et al. 1998). A group of 4 individuals sampled in the same bycatch event was analyzed using 10 microsatellite loci and was inferred to be composed by a mother, 2 offspring, and a putative father of the youngest offspring, suggesting that the franciscana could travel in groups of related individuals on the basis of a matrilineal structure (Valsecchi and Zanelatto 2003). Four different cases of mother–calf pairs entangled were recently reported and taken as evidence of franciscana traveling in family groups (Mendez, Rosenbaum, Subramaniam, et al. 2010).

In this paper, the population and social structure of franciscana dolphins from FMA III were analyzed by means of variation in microsatellites and mtDNA. We provide insights about fine-scale population divergence and social organization of this endemic and endangered southwestern Atlantic species by testing the hypotheses that 1) franciscanas from FMA III particularly associated to the Rio de la Plata, and neighboring Atlantic Ocean waters are genetically distinct; and 2) the social groups consist in part of related individuals and structured on the basis of matrilineal. Finally, we made inferences on the franciscana mating system.



**Figure 1.** Franciscana range distribution. (a) FMAs along all are range, the study area of this work is located in FMA III (modified from Secchi, Danilewitz, et al. 2003). (b) Map showing the Rio de la Plata zones.

## Materials and Methods

### Study Area, Samples, and DNA Extraction

Samples of franciscana dolphins were collected from the Río de la Plata estuary (RP) ( $n = 21$ ) and the Atlantic Ocean coast (AO) from both Uruguay ( $n = 28$ ) and Rio Grande southern Brazil ( $n = 36$ ) between the years 1998 and 2007 (Figure 1a,b). The Río de la Plata is funnel shaped 320-km long and oriented southward, with an open mouth of 230 km along the line that joins Punta Rasa (Argentina) and Punta del Este (Uruguay) (Guerrero et al. 1997). Considering its main features and dynamics, the system can be classified into inner, middle, and outer zones (Figure 1b), which can fluctuate and overlap depending on the dominant wind and on the river discharge. Samples used in this study were taken from the middle and outer zone. In the adjacent Atlantic coast, wind regimes, river discharge, and the displacement of the Brazil–Malvinas Confluence generate seasonal variability, with cold and nutrient-rich subantarctic waters dominating in the winter and warm and nutrient-poor subtropical waters during the summer (Piola et al. 2000).

Muscular tissue samples were collected from stranded individuals, and animals incidentally caught by artisanal and industrial fisheries. Sex was recorded and the standard length was measured in the field as a part of necropsy procedure, following the American Society of Mammalogy (1961) guidelines. All samples were preserved in ethanol at 95%. Age classes were assigned according to Kasuya and Brownell (1979) for Uruguayan samples and according to Danilewicz (2003) and Danilewicz et al. (2004) for Brazilian samples. Three age classes determined according to individuals mean lengths were used: 1) adults, sexually mature females and males from Uruguay (140 and 131.4 cm) and southern Brazil (138.9 and 127.4 cm); 2) calves, supposed newborns, up to 85 cm in both countries;

3) juveniles, sexually immature individuals of intermediate lengths between calves and adults. Total DNA was extracted using a proteinase K digestion, extraction of proteins with sodium chloride, and alcohol precipitation following a protocol modified from Miller et al. (1988).

### Microsatellite Typing

A total of 85 samples were analyzed. The 12 most polymorphic dinucleotide-repeat microsatellites loci previously used for this species were chosen for this study (Table 1). All forward primers were labeled with a fluorescent dye. DNA was amplified by PCR using the following final concentrations in a volume of 9  $\mu$ l: 3:100 (~30 ng) DNA dilution, 0.5 U Taq polymerase (Applied Biosystems), 2.5 mM MgCl<sub>2</sub>, 1  $\mu$ M of each primer, and 0.2 mM of dNTP. PCR cycling profile consisted on an initial denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 94 °C, annealing at 50–63 °C for 30 s (see Table 1) and polymerase extension at 94 °C for 30 s, and a final extension at 72 °C for 5 min. Amplified DNA was analyzed for length variation on an automated ABI PRISM 377 DNA sequencer using Genescan 3.7 and Genotyper 3.7 software (Applied Biosystems). Loci were tested for genotyping errors using Micro-Checker software (Van Oosterhout et al. 2004). All analyses were performed with and without the loci presenting possible null alleles.

### Mitochondrial Control Region Sequencing

Based on microsatellite results, for a total of 16 samples (see Results for further details), amplification of a 500-bp fragment of the hypervariable region I of the mtDNA was done using primers THR: L15926 5' TCA AAG CTT ACA CCA GTC TTG TAA ACC (Kocher et al. 1989) and TDKD 5' CCT GAA GTA GGA ACC AGA TG (Kocher et al. 1993). Details of final concentration conditions and PCR amplification are in Lázaro et al. (2004). Purification

**Table 1** Microsatellite loci and their genetic diversity shown as number of alleles ( $N^{\circ}A$ ), observed ( $H_O$ ), and expected heterozygosity ( $H_E$ ) per locus in Río de la Plata estuary (RP) and Atlantic Ocean coast.

Locus	$T_a$ (°C)	$N^{\circ}A$	Size (bp)	RP		AO		References
				$H_O$	$H_E$	$H_O$	$H_E$	
417/418	54	11	165–204	0.76	0.83	0.85	0.82	Amos et al. (1993)
464/465	58	12	140–186	1.00	0.91	0.87	0.82	
D22	58	12	100–130	0.85	0.89	0.85	0.89	Shinohara et al. (1997)
EV14	54	10	120–168	0.66	0.85	0.60	0.83	Valsecchi and Amos (1996)
EV5	54	13	150–200	0.66	0.84	0.80	0.88	
EV94	54	11	202–240	0.85	0.79	0.82	0.83	
FCB17	63	14	175–230	0.80	0.85	0.85	0.88	Buchanan et al. (1996)
FCB2	54	11	150–198	0.87	0.88	0.85	0.75	
FCB5	58	11	120–156	0.72	0.88	0.85	0.87	
MK5	56–64	8	205–250	0.80	0.81	0.61	0.72	Krutzen et al. (2001)
MK6	56–64	12	160–210	0.80	0.86	0.77	0.88	
MK8	58	11	87–112	0.76	0.73	0.88	0.82	

$T_a$  (°C) is the annealing temperature, and Size (bp) is the observed range in fragment size.

and sequencing reactions were conducted by a specialized service (Macrogen Inc.).

## Data Analysis

### Population Structure

**Microsatellite loci.** Variation at these markers was studied using 21 samples from RP and 23 from AO. Aiming at minimizing the probability of including close relatives in the analysis, only one individual from multiple incidental captures and strandings was included.

The level of polymorphism was estimated as the number of alleles per locus, observed ( $H_O$ ), and expected ( $H_E$ ) heterozygosities using Arlequin 3.1 (Excoffier et al. 2005). Evaluation of possible deviations from Hardy–Weinberg (HW) equilibrium and linkage disequilibrium (LD) was performed using Fisher's Exact test and Markov chain methods (iterations per batch set at 10 000; Guo and Thompson 1992) implemented in Genepop 4.0 (Raymond and Rousset 1995). Significance levels ( $P = 0.05$ ) for departure from HW and LD were corrected for multiple comparisons with Bonferroni correction (Rice 1989). Population structure analyses were performed in 2 different ways. In the first analysis, a priori stratification of the data was based on the geographical limit between the estuary and the ocean. The level of differentiation between RP and AO populations was estimated through  $F_{ST}$  (Wright 1978) and  $R_{ST}$  (Slatkin 1995) using Arlequin 3.11 (Excoffier et al. 2005). The number of migrants per generation " $N_m$ " could be changed to " $N_m$ " throughout the article. Please check and correct if necessary. ( $N_m$ ) was calculated under private allele methods (Slatkin 1985) using Genepop 4.0 (Raymond and Rousset 1995). In the second analysis, an alternative Bayesian approach was used to estimate population differentiation using the Structure 2.2 software (Pritchard et al. 2000), which applies a Markov Chain Monte Carlo (MCMC) algorithm. The number of subpopulations ( $K$ ) represented by the samples is a user-specified prior distribution. We performed 10 preliminary runs in order to know the maximum value  $K$  to be used and the length of the chain not to affect the results. The number of iterations used was between  $10^6$  and  $5 \times 10^6$ , and  $K$  ranged from 5–15. Finally,  $K = 5$  was tested through 10 independent MCMC runs of  $10^6$  iterations, following a burn-in period of 60 000 iterations. We recalculated  $F_{IS}$ ,  $F_{ST}$ , and  $R_{ST}$  based on the clusters detected through the Bayesian approach.

**Mitochondrial DNA.** The sequences obtained were compared with those available from Uruguayan and Brazilian Atlantic coasts. We also reported 17 additional unpublished sequences from Argentina (FMA IV) (Abud 2005). Electropherograms were scored and analyzed using Proseq (Filatov 2002) and aligned using CLUSTAL X as implemented in MEGA 4.0.2 (Tamura et al. 2007). Haplotypes were defined using DNAsp v5 (Librado and Rozas 2009) and confirmed by direct observation.

These sequences were combined with all previous franciscana mtDNA haplotypes reported in GenBank. We

used 11 haplotypes reported by Secchi et al. (1998), 22 haplotypes reported by Lázaro et al. (2004) (5 of them match those of Secchi et al. 1998), and 19 reported by Mendez et al. (2008). A median-joining network was implemented in Network 4.5.1.0 software. Maximum parsimony approach was used to minimize the tree connections. Transversions were weighted twice as transitions (2:1), epsilon was set at 15 and the remaining parameters were set at default (www.fluxus-engineering.com/network\_terms.htm).

Divergence between the Rio de la Plata estuary and the neighboring Atlantic Ocean from FMA III was studied using only the samples from this zone ( $n = 16$ ) in diversity and population structure analyses. Nucleotide ( $\pi$ ) and haplotype (H) diversity were estimated, and population structure was analyzed through  $F_{ST}$  and  $\Phi_{ST}$  statistics using Arlequin 3.11 (Excoffier et al. 2005).  $\Phi_{ST}$  incorporates information of both the degree of genetic distance between haplotypes and their frequencies in each population. Migration rates ( $Nm$ ) between populations were estimated using a maximum-likelihood (ML) approach based on coalescent theory as implemented in MDIV software (Nielsen and Wakeley 2001). To calculate  $Nm$ , we first obtained  $M$  as migration rate per gene per generation ( $M = m/\mu$ ) and  $\theta = 4N\mu$  where  $N$  is the effective population size and  $\mu$  de mutation rate of the studied gene ( $\mu = 1 \times 10^{-8}$  were used for mtDNA). Preliminary multiple runs with increasing Markov chain lengths were conducted ( $10^6$ – $10^8$ ) and different burn-in ( $10^4$ – $10^6$ ) iterations to determine if parameters estimated reached convergence. When all runs resulted in the same mode for each parameter, we considered that convergence was reached. Results are presented from 10 independent runs of  $1 \times 10^8$  iterations, each with  $1 \times 10^5$  of burn-in, and the remaining values required by the software were set as default. The maximum value of the integrated likelihood function of each parameter was used as an estimator of the parameter ( $M$  and  $\theta$ ) in each run. The mode of these parameters values was used to obtain the final value  $Nm$ .

### Kinship Patterns

Kinship analysis was conducted on the 12 loci based on 21 groups ( $n = 64$  samples), all of them from AO. A group was defined putatively either as 2 or more animals caught in the same net and fishing operation or as 5 or more stranded animals found in less than 1 km in the same day. Each group included one sample used in the population structure analysis.

There are basically 2 types of approaches to estimate relatedness indexes for inferring pairwise relationships: moment-based methods (Moment based Estimators [ME], e.g., Queller and Goodnight 1989) and Maximum Likelihood Estimator [ML] estimators (e.g., Kalinowski et al. 2006). The performance of both estimators indicates that the major discrepancy between them is due to uncertainty about the actual allele frequencies in the population of interest (Milligan 2003; Bink et al. 2008). Although ML estimators are robust, ME methods adjust better when there is no reliable

estimate of allele frequencies, for example, because of small sample size. Because no reliable allele frequency estimate was available, ME was used as the main estimator, and the pairwise comparisons were made with ML approaches.

Levels of kinship (estimated as the coefficient of relatedness,  $r$ ) were assessed by comparisons of allele frequencies at each microsatellite locus, and comparative estimates based on allele sharing between individuals of the same group and of different groups (Queller and Goodnight 1989). Overall  $r$  for the whole sample ( $n = 66$ ) and average  $r$  between groups were estimated using Relatedness 4.2 software (Queller and Goodnight 1989), and pairwise  $r$  within groups were estimated using Kinship 1.2 software (Goodnight and Queller 1999). The expected mean value for this statistic is 0.5 for first-order relatives (parents–offspring and full siblings), 0.25 for second-order relatives (half siblings, grandsons and nephews), 0.125 for third-order relatives (cousins), and 0 for unrelated individuals. Close relative designation was substantiated using ML methods via ML-relate (Kalinowski et al. 2006) by 1) determining the most likely relationship (first order, second order, or unrelated) for within-group pairwise comparisons and 2) testing the specific hypothesis test of relatedness (software provided option) in decreasing order, for example, if the most likely relationship obtained was full sibling, the alternative hypothesis to test would be that of half sibling. An important advantage of this program is that it can discriminate parent–offspring from full sibling. Simulations ( $n = 10^5$ ) of random genotype pairs were used to specify the probability of the ML estimate for the relationship obtained.

## Results

### Population Structure

#### Microsatellite Loci

The 12 loci studied were polymorphic within the 2 populations analyzed, and the number of alleles per locus ranged from 8 to 14 (Table 1). Evidence for possible null alleles was detected for *EV5* for RP population and for *EV14* for both populations. Because there were changes when analyses were performed with 12 and 10 loci, results of population comparisons were based on 10 loci.

The 2 groups showed high and similar levels of heterozygosity, the observed mean heterozygosity for RP was 0.82 and for AO was 0.80 (Table 1).  $F_{IS}$  values were near zero for both RP and AO ( $F_{IS} = 0.027$ ,  $P = 0.3$  for RP;  $F_{IS} = 0.009$ ,  $P = 0.5$  for AO) suggesting random mating and no significant deviations from HW equilibrium. Two pairs of loci for RP (*MK6-FCB17*; *MK8-EV94*,  $P = 0.03$  in both cases) and a pair for AO (*464/465-FCB17*,  $P = 0.03$ ) showed linkage disequilibrium but was not significant after Bonferroni correction ( $P = 0.005$ ).

Differentiation between RP and AO was not strong ( $F_{ST} = 0.02$ ,  $P < 0.01$ ;  $R_{ST} = 0.042$ ,  $P < 0.01$ ). However, gene flow from private alleles approaches ( $Nm = 1.86$ ) indicated moderate population subdivision.

In contrast, Bayesian clustering showed clear population subdivision. Considering a range of 5 populations, the posterior probability of the data strongly suggested the existence of 2 populations ( $K = 2$ ,  $\ln\text{Pr}(X/K) = 0.99$ ). The other probabilities ranged from  $10^{-16}$  to  $10^{-67}$  (Table 2). Assuming 2 populations ( $K = 2$ ) for each individual, the ancestry assignment analysis indicated that all samples from AO belong to the same group ancestry (AO) with over 0.90 probability for all individuals (except 2 individuals with 0.81 and 0.83 of probability). However, for RP samples, 7 showed ancestry in RP, 12 in AO, and 2 of them showed mixed ancestry (Table 3).

The amount of divergence from inferred clusters by both  $F_{ST}$  and  $R_{ST}$  increased notably in comparison with the first analysis ( $F_{ST} = 0.06$ ,  $P = 0.002$ ;  $R_{ST} = 0.3$ ,  $P = 0.001$ ) showing a strong population structure between the 2 clusters.

#### Mitochondrial Sequences

Based on the Structure results described above, we sequenced 6 individuals who showed ancestry in Rio de la Plata estuary (hereafter RP for practical purposes) and 10 individuals which showed ancestry in the Atlantic Ocean (hereafter AO). On comparing our sequences with the haplotypes reported for the distribution range of the species (Secchi et al. 1998; Lázaro et al. 2004; Mendez et al. 2008; Mendez, Rosenbaum, Wells, et al. 2010), we found 6 new haplotypes (GenBank accession numbers: JN129291–JN129296), which defined one new variable site, totalizing 46 variable sites that define the haplotypes of the data set. One of the new haplotypes was obtained from a sample collected at RP, 2 from AO, and the other 3 from Argentina (FMA IV). The remaining 13 samples collected at FMA III matched previously defined haplotypes as follows: 3 to H1(K), 3 to H10(G), 3 to M6, and 1 to each of the following: H17, H3(J), H8(I), H9, and M1. The haplotypes defined by the remaining 14 samples collected at FMA IV matched previously reported haplotypes as follows: 5 to H5, 2 to H10(G), and 1 to each of the following: H1(K), H6, H15, H19, M3, M10, and M11. We also found that 3 haplotypes previously reported (M4, M5, and M12; Mendez et al. 2008; Mendez, Rosenbaum, Wells, et al. 2010) were shorter sequences that matched one previously reported haplotype (J in Secchi et al. 1998; 3J in Lázaro et al. 2004),

**Table 2** Inferences of population structure following a Bayesian approach.

K	Ln P(D)	P(k)
1	−2042.1	$7.7 \times 10^{-16}$
2	<b>−2007.3</b>	<b>0.99</b>
3	−2058.7	$4.8 \times 10^{-23}$
4	−2126.2	$2.3 \times 10^{-52}$
5	−2161.4	$1.2 \times 10^{-67}$

K is the number of population used as prior information. Ln P(D) is the logarithm of the data probability, and P is the posterior for each K. Bold represents the highest posterior probability observed for K=2.

**Table 3** Inferred probability for each inferred group and ancestry for each of the individuals sampled in the Rio de la Plata Estuary (RP) using the Bayesian approach. AO refers Atlantic Ocean.

Sample	Probability		Ancestry
	AO	RP	
1	0.41	0.58	Mixed
2	0.95	0.05	RP
3	0.98	0.01	RP
4	0.97	0.02	RP
5	0.97	0.02	RP
6	0.97	0.02	RP
7	0.98	0.01	RP
8	0.96	0.03	RP
9	0.33	0.66	Mixed
10	0.01	0.98	AO
11	0.01	0.99	AO
12	0.02	0.98	AO
13	0.07	0.92	AO
14	0.01	0.99	AO
15	0.08	0.91	AO
16	0.06	0.94	AO
17	0.01	0.98	AO
18	0.01	0.98	AO
19	0.01	0.99	AO
20	0.02	0.97	AO
21	0.03	0.96	AO

totalizing 50 haplotypes for the entire distribution range of the species up to date.

Our most common haplotypes matched the commonest haplotypes (G and K) in the southern range, previously reported by Secchi et al. (1998). The median-joining network showed a complex phylogeographic signal (Figure 2). No haplotypes were shared between Rio de Janeiro and southern locations, and as expected, new haplotypes found in this study were closely related to haplotypes of southern clusters and not to northern haplotypes, as shown in Figure 2. Shared haplotypes within southern regions were common; the 3 southern clusters of haplotypes as previously defined by Lázaro et al. (2004) were still recognizable.

Haplotype and nucleotide diversity were high (RP:  $H = 1$ ,  $\pi = 6.6$ ; AO:  $H = 0.86$ ,  $\pi = 5.1$ ). Regarding population structure, in contrast to microsatellite loci, divergence between RP and AO was weak and not statistically significant ( $F_{ST} = 0.02$ ,  $P = 0.12$ ;  $\Phi_{ST} = 0.06$ ,  $P = 0.06$ ). The migration rate based on ML approach was high ( $Nm = 10.6$ ), as expected by the low structure reflected in the fixation indices.

### Kinship Group Patterns

All analyses were conducted with 12 and 10 loci. Because there were no changes in the overall results, data from all 12 loci were presented. The coefficient of relatedness ( $r$ ) for the whole sample was near zero ( $r = 0.084$ ,  $P = 0.02$ ). Thirteen groups showed average  $r$  higher than the value for the whole population, and 6 groups showed  $r \geq 0.125$ , although none of them were significant (see pairwise  $r$  values from all groups analyzed in Supplementary Material 1).

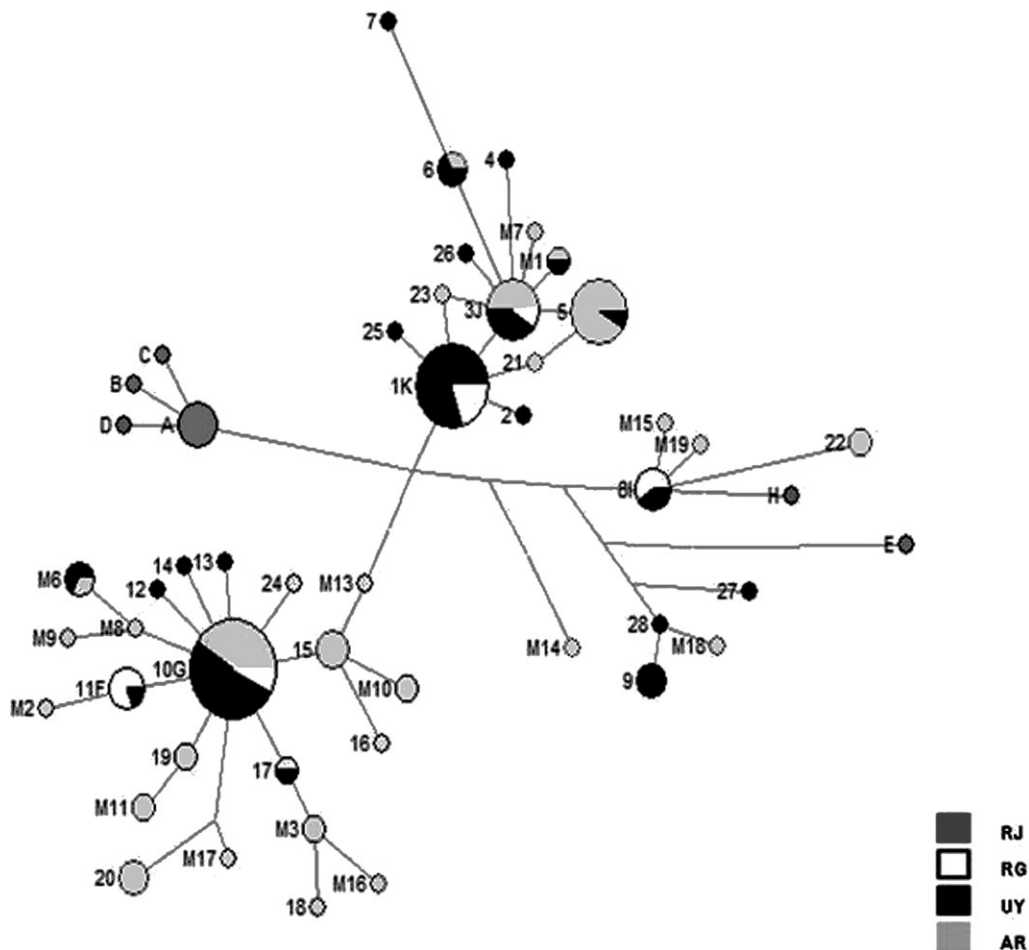
Putative close relatives were found in 11 of 21 groups ( $r \geq 0.125$ ,  $P \leq 0.05$ ). Within the groups of 2 and 3 individuals, relationships detected ( $r \geq 0.125$ ,  $P \leq 0.05$ ) included all possible combinations (between males or females, individuals of different sexes, and between adults and juveniles). The ML-relate model provided consistent results with those of the Kinship model with a few exceptions. In 7 of 11 groups, putative close relatives were the most likely relationships obtained through ML estimates. It is worth noting that ML-relate program finds up to second-order relatives, thus third-order relatives found by Kinship program were assigned as unrelated by this program (see pairwise  $r$  values from all groups analyzed through ML-relate program in Supplementary Material 2).

The larger groups (1,  $n = 8$ ; 4,  $n = 5$ ; and 5,  $n = 10$ ) showed the highest pairwise  $r$  ( $r \geq 0.5$ ,  $P \leq 0.05$ ) and appeared to be a complex network of relatedness. It is worthwhile noting that the kinship pattern may be underestimated due to the sample source (strandings and bycatch). Details of some significant ( $P < 0.05$ ) relationships from larger groups are described below.

Group 1 consists of 8 bycaught individuals, 5 females, and 3 males. Three independent significant kinship relationships were found among them. The first one involved an adult (151 cm) and a young (132 cm) female ( $r = 0.29$ ), the second one a juvenile female (114 cm) and male (105 cm) ( $r = 0.36$ ), and the third one a male (127 cm) and a weaned (99 cm) female ( $r = 0.22$ ). The last 2 were presumably half siblings. All these pairwise comparisons were supported by ML approaches. However, they differed in that the  $r = 0.36$  relationship was assigned as full-sibling relationship by ML-relate.

Group 4 is composed by 5 individuals found stranded, a juvenile male, 2 adults, and 2 juveniles of unknown sex. A probably adult individual (138 cm) held a first-order relationship with 2 juveniles (121 and 124 cm) ( $r = 0.51$  and  $r = 0.43$ , respectively). Second-order relationships were found between both juveniles ( $r = 0.20$ ) and between one juvenile (124 cm) and the other adult (144 cm) ( $r = 0.22$ ). All these pairwise comparisons were supported by ML approaches, except for the  $r = 0.22$  relationship that was assigned as unrelated. First-order relatives were assigned as full siblings by ML approaches. A complementary analysis of exclusion by eye was conducted to confirm whether the putative parent shared at least one allele per locus screened with the offsprings. We found that the adult shared one allele in the 11 locus but no shared alleles in one locus, with the 2 juveniles, although that exception can be either a point mutation or a genotyping error, and so it does not rule out the possibility to be a parent-offspring relationship.

Group 5 consists of 10 individuals, 1 female, 4 males, and 5 of unknown sex, all of them engaged in a total of 15 significant kinship relationships. Ten of 15 pairwise comparisons were support by ML approaches. An individual (132 cm) was related to 5 individuals showing varied levels of kinship. An adult female (146 cm) held a first-order relationship with a juvenile male (124 cm,  $r = 0.5$ ) and with a weaned individual (103 cm,  $r = 0.70$ ), and among them,



**Figure 2.** Median-joining network of haplotypes. Circles are proportional to haplotype frequency. Branch lengths are proportional to haplotype distance. Transverse marks in branches indicate median vectors. Colors indicate geographic location (from North to South RJ: Rio de Janeiro; RG: Rio Grande; UY: Uruguay; and AR: Argentina). Haplotypes A–K were reported by Secchi et al (1998), 1–22 reported by Lázaro et al. (2004), M1–M19 (except M4, M5, and M12) reported by Mendez et al. (2008), and 23–28 reported in this study.

kinship was also high ( $r = 0.68$ ). All these 3 first-order relationships were assigned as full-sibling relationships by ML approaches. Although  $r = 0.70$  relationship was assigned with ML as parent–offspring, a specific hypothesis test failed to reject the full-sibling alternative hypothesis. This suggests that these 3 individuals could be 3 full siblings; under this scenario, the same couple should be maintained for at least 3 breeding season, which is rare for mammals. However, it cannot be discarded because the exclusion analysis showed that the adult shared one allele in 10 of the 12 locus with the juvenile female (124 cm) and the weaned individual. Probability of mutations and genotyping errors in 2 alleles in the same individual is almost insignificant, suggesting that neither scenario considered can be ruled out.

## Discussion

We investigated the genetic subdivision and kinship association of franciscana dolphin between southwestern

Atlantic Ocean and Rio de la Plata estuarine coast. From multilocus analyses, we provide evidence that 1) franciscana dolphin are structured into 2 subpopulations, which have correspondence with different environmental areas (estuarine and oceanic coast) and 2) some groups are composed, in part, by first-order related individuals, suggesting that matriline could be the social unit in this species.

## Genetic Diversity

As expected from previous studies (Lázaro et al. 2004; Mendez et al. 2008; Mendez, Rosenbaum, Wells, et al. 2010), nuclear genetic diversity estimates for franciscana are high for the Río de la Plata estuary and the Atlantic Ocean. Similar values were reported for a marine species, *Phocoena phocoena* from Northwest Atlantic (mean  $H_O = 0.8$ , Rosel et al. 1999). Lower values were reported for other species of river dolphins, namely *Inia geoffrensis* from the Amazon River system (mean  $H_O = 0.56$ , Hollatz et al. 2011) and *Sotalia fluviatilis* (mean  $H_O = 0.53$ , Cunha and Watts 2007).

However, it is worth noting that values for *I. geoffrensis* do not come from a set of the most polymorphic loci for the species. Estuarine mitochondrial nucleotide diversity estimates were higher than values reported others localities for La Plata-estuarine Argentine waters (e.g.,  $\pi = 3.0$  San Clemente,  $\pi = 4.3$ ; values reported as  $K$  in Mendez et al. 2008). Values of Oceanic mitochondrial nucleotide diversity were similar to those reported (Lázaro et al. 2004; Mendez et al. 2008).

### Population Structure

Studies on both broad and fine scales have shown strong evidence of genetic structuring in franciscana dolphin populations. A clear separation was detected using morphological and mitochondrial genetic data distinguishing franciscana between areas to the north and to the south of Santa Catarina (ca. 28oS), Brazil (Pinedo 1991; Secchi et al. 1998; Lázaro et al. 2004). Mitochondrial genetic studies suggest that the southern part of the distribution exhibits greater homogenization than the northern part of the distribution. In particular, the highest gene flow for the distribution range is recorded between Rio Grande do Sul and Uruguay, considered to represent a subpopulation (Secchi et al. 1998; Lázaro et al. 2004). However, fine-scale structure was reported for Argentine waters since divergence was found between contiguous distributed populations differing in their environmental landscape (Mendez, Rosenbaum, Wells, et al. 2010). The analysis of data from mitochondrial and microsatellite markers suggested the existence of an estuary subpopulation (at San Clemente, the southern part of the Rio de la Plata estuary) differing from ocean populations (Mendez et al. 2008; Mendez, Rosenbaum, Wells, et al. 2010). Our results from microsatellite data support this pattern for Uruguayan and Brazilian waters. Strong population structure was detected when Bayesian clustering and standard approaches were used to distinguish estuarine from oceanic populations. The natural boundary between the Rio de la Plata estuary and the Atlantic Ocean is a highly dynamic frontal system, whose location depends on the river discharge and winds, varying among seasons and years (Guerrero et al. 1997). Thus, it can be expected to collect samples from the ocean in the estuary and vice versa. In this study, we found that 12 individuals sampled in the estuary had their inferred ancestry in the ocean, suggesting overlapping populations at least in the border zone.

At first glance, in this work, there appears to be discordance between microsatellite and mitochondrial results. However, mtDNA showed isolation by distance pattern along the franciscana range (Lázaro et al. 2004); thus, it is expected that a mitochondrial marker does not indicate strong divergence between adjacent areas. In our study, mtDNA data showed lack of population structuring between Rio de la Plata estuary and the Atlantic Ocean; it might suggest that current or past female-mediated gene flow is or was greater than that mediated by males. Furthermore,  $F_{ST}$  value from mtDNA falls within the range

of individual  $F_{ST}$  (range  $F_{ST} = 0-0.2$ ) value from microsatellite loci; thus, the lack of significance may be because the sample size used in this study was small, considering the high haplotype diversity, preventing us to account for significant divergence. Finally, departure from neutrality at the mtDNA control region should not be discarded.

There are also ecological evidences supporting the genetic population separation between franciscanas from the estuary and the ocean (Mendez et al. 2008; Mendez, Rosenbaum, Wells, et al. 2010; this study). Quantitative and qualitative prey differences among individuals sampled in the Rio de la Plata estuary (Samborombón bay) and portions of the ocean coast of Argentina were found (Rodríguez et al. 2002). Samborombón bay is an important spawning and nursing ground for several fish species preyed by franciscana (Rodríguez et al. 2002). Its residency at the area might indicate niche occupation related to local adaptation to feeding resources (Mendez et al. 2008). There are several cases of cetacean population divergence that suggest adaptation to a local environment such as those of orcas, *Orcinus orca* (Hoelzel et al. 1998); long-finned pilot whales, *Globicephala melas* (Fullard et al. 2000); and bottlenose dolphins, *Tursiops* sp. (Natoli et al. 2004). A well-known case is the divergence between pelagic and coastal bottlenose dolphin populations in western North Atlantic, with values of  $F_{ST} = 0.205$  and  $R_{ST} = 0.236$ , for 9 microsatellite loci (Hoelzel et al. 1998; Natoli et al. 2004).

### Social Structure

Franciscanas are seen in aggregations from 2 to 30 individuals, though small groups of 2–3 animals are more frequent (Crespo et al. 1998; Bordino et al. 1999; Danilewicz et al. 2009). Large groups have been proposed to be aggregations of smaller social units and have been associated with foraging activities mainly in winter and with reproduction in spring and summer (Bordino et al. 1999). High kinship asymmetry, from first-order relationships to unrelated individuals, was found within and among the 21 groups analyzed. Since they were found in summer, such asymmetry could represent aggregations of some social units for breeding purposes.

Due to family-origin uncertainty of samples (e.g., mother–calf pair confirmed in field), we did not perform parentage analysis, and it is expected that some relationships remain vague. However, we found mature females and more than one immature individual engaged in a first-order relationship, supported by the 2 approaches used, which suggests natal group philopatry. In addition, other 6 second-order relationships involving sexually immature dolphins were found suggesting half-sibling relationships within groups (1 in each of groups 4, 5, 7, 11, and 15). It might indicate that the basic social unit for franciscanas could be the family group, particularly of matrilineal structure. This sort of social structure was previously suggested for the species based on the finding of a single group of 4 individuals, which included a sexually mature female, 2 male offspring ( $r = 0.49$  and  $r = 0.27$ ), possibly half brothers



( $r = 0.15$ ) and an adult male, the putative father of one of them ( $r = 0.29$ ) (Valsecchi and Zanelatto 2003). Such finding and our results indicate that males and females could stay with their mothers. Lack of dispersal from the natal group, neither by female nor by male offspring, is extremely rare in mammals. For cetacean species, the exceptions are the resident and transient morph types of killer whales from British Columbia (Baird 2000) and pilot whales (Amos et al. 1993). Franciscana dolphins may be a third case.

Matrilines as a social unit in cetaceans was suggested for several species, but the stability of the social unit varies among species and populations (Bigg et al. 1990; Amos et al. 1993, Gero et al. 2008). The removal of breeding females changes the basic initial genetic makeup and the structure of the group, increasing the asymmetry in the relationships within groups (Emlen 1995). Theory predicts that replacement families are less stable than intact families, in terms of the increase of dispersal tendency of mature individuals and the evolutionary conflict of interest between parents and offspring, such as sexual conflict (Emlen 1997). The franciscana dolphin suffers from long-term removal of both mature and immature individuals due to bycatch along all its range (Secchi, Ott, et al. 2003; Secchi 2010), including the estuarine and oceanic waters (Franco-Trecu et al. 2009). Moreover, there are several records of entanglement of pregnant females or female–calf pairs (Danilewicz 2003; Mendez, Rosenbaum, Wells, et al. 2010). The high rate of bycatch in this species could be a selection pressure favoring weaker bond or associations of unrelated animals.

The permanence of the mature male with reproductive females is a rare behavior in mammals (Greenwood 1980), and cetaceans appear to follow the rule (Connor 2000). However, it suggests that some adult males could stay in their social unit beyond the reproductive period (Valsecchi and Zanelatto 2003; this study). Franciscana dolphins show a very low testis relative weight in relation to body weight ratio compared with other dolphins (Danilewicz et al. 2004), suggesting that sperm competition is unlikely to occur (Brownell and Ralls 1986; Danilewicz et al. 2004). Under this scenario, guarding appears to be a suitable mating tactic for franciscana males. Valsecchi and Zanelatto (2003) suggested that there could be a low probability to find reproductive partners in the population and thus staying in the group could be evolving as a mate-guarding behavior. We suggest an alternative but not exclusionary explanation in which the adult male could provide offspring extra parental care. It is possible that franciscana calves represent a great female energy expenditure as their length at birth is 70–80 cm (Danilewicz et al. 2004), approximately 50% the length of their mothers. It was observed that monogamy concerns small species where the weight of the litter is high compared with the mother body weight in primates (Leutenegger 1980). For some primates, such as the genus *Callitrichis*, in which the offspring represents excessive energy expenditure for their mothers, mate guarding has been suggested as a strategy to increase offspring survival (Garber 1997). Weaning occurs gradually in franciscanas, and it has been found that nursing calves have milk as well

as solid food in their stomachs (Rodríguez et al. 2002). Gradual weaning represents a major stress for a female, as it would have to simultaneously teach hunting, wean the offspring, and feed itself (Clutton-Brock 1989). Thus, for franciscana, the presence of the father in the family group could be a facultative property of males that could influence the chance of offspring survival.

Despite constraints imposed by the sample size and precedence from dead animals, it is likely that all these ecological, behavioral, and physiological variables converge to a more complex social structure of franciscana than previously thought.

At this time, we can suggest that environmental features and social behavior influence the shaping of population structure. The fine-scale population divergence, the low dispersal of males and females found in franciscana dolphin (Bordino et al. 1999; Mendez et al. 2008; this study), and the intense incidental mortality of the species (Secchi et al. 2003a), including entanglements of mother–calf pairs and possible reproductive partners (Mendez, Rosebaum, Subramaniam, et al. 2010; this study) could lead us to think, at least in theory, loss of genetic variability through inbreeding depression. However, the high diversity showed by franciscana populations might indicate that inbreeding is minimized somehow by their mating system, for example, the avoidance of close relatives during mating. In addition, considering a broad scale, mitochondrial haplotypes are shared among females at the southern range of the distribution, and there is a high gene flow among localities (Secchi et al. 1998; Lázaro et al. 2004; Mendez et al. 2008; this study). Thus, we might suggest that some females could play a role as founders of social groups in other neighboring areas of the distribution rather than their local natal site, minimizing the chances of mating kin partners.

In summary, we suggest that both local adaptation to a particular environment and social structure influence population structure. Either feature could lead to think of a recent divergence or could be reflecting equilibrium between local differentiation and gene flow. In a conservation context of relevance to this species, the implications of our results and their plausible explanations agree with the idea that franciscanas associated to the Rio de la Plata estuary could be treated as a different management stock.

## Supplementary Material

Supplementary material can be found at <http://www.jhered.oxfordjournals.org/>.

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