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INTERNATIONAL WHALING COMMISSION

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ABSTRACT

Franciscanas (*Pontoporia blainvillei*) are threatened by extensive by-catch and other human-related activities. Their conservation may be even more complicated for populations that are differentiated on a microscale, between geographically close locations. Infrequent dispersal ultimately means that populations are independent from each other, and therefore they must be managed as distinct Management Units. We used genetic data to investigate the microscale population structure of franciscanas in southern Brazil, and to analyse kinship patterns, searching for evidence of philopatry in the species. Besides significant microscale population structure, we provide evidence that favours the hypothesis of philopatry of both sexes in franciscanas: a) both maternally-inherited mitochondrial DNA and biparentally transmitted nuclear microsatellites showed population differentiation between franciscanas of Babitonga Bay and nearby coastal waters; and b) in Babitonga, most dyads were of related individuals, and kinship was high, irrespective of the sex, indicating that both females and males had relatives in the local population. Those results suggest that kinship not only shapes group organisation, but is also an important feature of local populations, and that franciscanas do not disperse frequently between populations. The relevance of such findings for the conservation of franciscanas is discussed.

INTRODUCTION

The franciscana, *Pontoporia blainvillei* (Gervais & D'Orbigny, 1844) is endemic to the Southwestern Atlantic coast, ranging from Espírito Santo state, in Brazil, to the Golfo San Matías, in Argentina (Crespo 2009). Due to extensive by-catch and other human-related threats, franciscanas are recognised as endangered by IUCN (Zerbini et al. 2017). Franciscana Management Areas (FMA) were devised in order to guide research and conservation efforts for this species (Secchi et al. 2003). In recent years, genetic data have helped refining the delimitation of FMA both by unveiling microscale genetic differentiation across the species range (Mendez et al. 2008, 2010a; Costa-Urrutia et al. 2012, Cunha et al. 2014, Gariboldi et al. 2015, 2016) as well as a deep evolutionary discontinuity between franciscanas from the northern extreme of the distribution and their southern counterparts (Cunha et al. 2014). Cunha et al. (2014) made a revised FMA proposal based on genetic findings.

Recent genetic studies have also suggested that franciscanas have a matriarchal social structure (Valsecchi and Zanelatto 2003, Mendez et al 2010b, Costa-Urrutia et al. 2012), which could reflect female philopatry, as indeed was proposed by some authors (Mendez et al. 2010a). Female philopatry, as well as habitat specialisation, could explain the microscale genetic differentiation of franciscanas among geographically close localities in FMAIII and FMAIV (Mendez et al. 2008, 2010a, Costa-Urrutia et al. 2012) and would have direct consequences for conservation. In most mammals, including cetaceans, female philopatry is coupled with male biased dispersal (Greenwood 1980, Connor et al. 2000). Data concerning male dispersal in franciscanas is fragmentary: there are two reported cases of juvenile males travelling with probable mother and aunt, respectively (Valsecchi and Zanelatto 2003, Costa-Urrutia et al. 2012), but there are also some cases of adult males accompanying females that were unrelated to them (Mendez et al. 2010b, Wells et al. 2013). Those observations are not necessarily discordant, because juvenile males could have been sampled with their mother and aunt prior to leaving the natal group. In any case, the abovementioned studies evaluated kinship at group level and were not ideal for analysing philopatry or dispersal of one sex, which are population level phenomena. Besides, from the population standpoint, dispersal from the natal population, not from the natal group, is important because it will ultimately translate into immigration and gene flow.

In this study, we used population genetics and kinship analyses to address the hypothesis of female philopatry/male biased dispersal in franciscanas. Our study subjects were the franciscanas from Babitonga Bay (Santa Catarina state, southern Brazil) and adjacent coastal waters (Figure 1). Babitonga Bay houses a resident community of franciscanas, which was estimated at around 55 individuals (Cremer and Simões-Lopes 2008, Zerbini et al. 2011). Franciscanas do not frequent the areas near the bay's opening, where harbour activities are intense (Cremer et al. 2018). We investigated the existence of genetic differentiation between franciscanas collected in Babitonga and outside the bay, and compared kinship patterns in both groups.

Our results suggest that both female and male franciscanas tend to remain in their natal population after reaching sexual maturity, favouring the genetic differentiation of

populations even at small geographic scale, and increasing the risk of inbreeding, especially when the isolation and decline of populations are intensified by human interference.

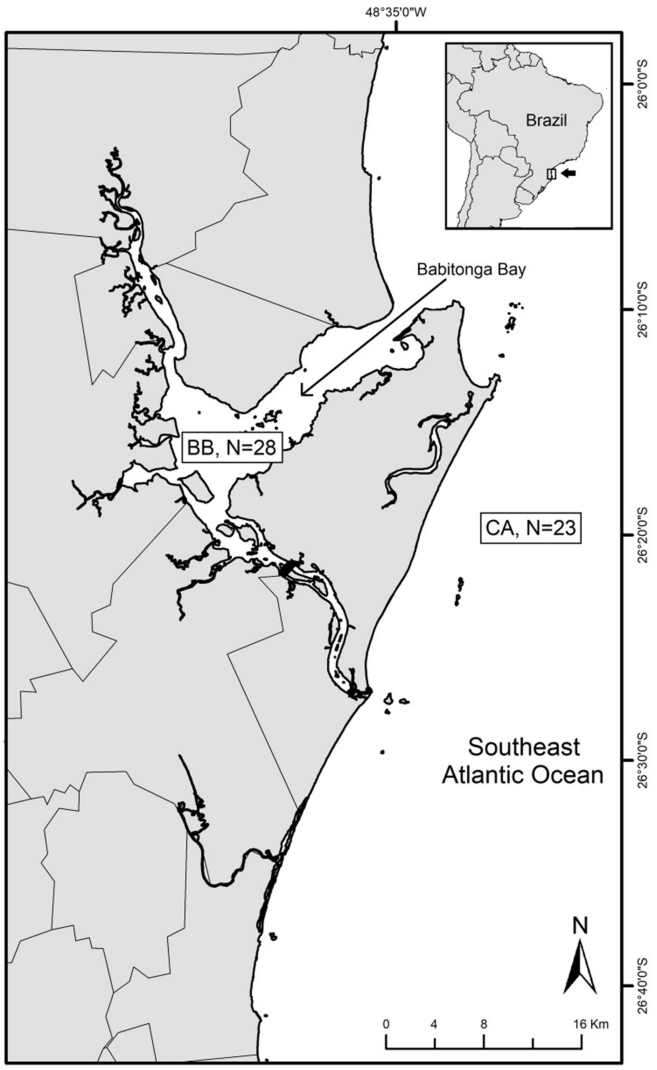

Figure 1: Sampling areas and number of franciscana samples in Babitonga Bay (BB), southern Brazil, and adjacent Atlantic coastal waters (CA).

MATERIALS AND METHODS

Sampling and DNA extraction

Franciscana samples (N = 53) were collected from carcasses (N = 6) or through skin biopsy during a capture-release procedure for tagging (N = 47) (SISBIO license #11980). Around half the samples were collected inside Babitonga Bay (BB, N = 28), and the other half were collected in adjacent Atlantic coastal waters (CA, N = 23), between 19Km north and 66Km south of the bay's mouth (Figure 1). Another two samples were collected from individuals stranded around the bay's mouth and could not

be safely assigned to be from Babitonga or from the coastal area groups, so they were considered of undetermined origin. Tissue samples were preserved in 100% ethanol and stored at –20°C. DNA was extracted using DNeasy Blood and Tissue kit (Qiagen) following the manufacturer's instructions, or a standard phenol/chloroform extraction method (Sambrook et al. 1989). When sex was unknown, molecular sexing was performed following the procedure of Rosel (2003).

Microsatellite amplification and genotyping

Individuals were genotyped at seven microsatellite *loci* (Table 1), using the tailed primer method of Schuelke (2000). PCR reactions (10 mL) contained around 30 ng of template DNA, 0.2 mM of each dNTP, 2.5 mM $MgCl_2$, 1 µg/µL BSA, 0.2 µM forward tailed primer, 0.8 µM of reverse primer, 0.4 µM of labeled M13 primer (with 6-FAM, VIC, NED or PET dyes), and 1 unit of GoTaq polymerase (Promega). All amplifications included negative controls. *Loci* were amplified following the program: 94°C for 4 min; 30X (92°C, 45 seg; T_a, 45 seg; 72°C, 45 seg), 8X (92°C, 45 seg; 53°C, 45 seg; 72°C, 45 seg); and 72°C for 30 min. The optimal annealing temperature (T_a) for each *locus* is in Table 1. PCR products were pooled and genotyped on an ABI 3500 automated sequencer using GS500-LIZ size standard. Allele sizes were determined with the software Geneious 7.1.7 (Biomatters).

Table 1: Microsatellite *loci* used in this study.

Locus	T_a (°C)	Allele size range	Reference
Ig11D2	50	289-295	Gravena et al. (2009)
Ig8H1	50	295–307	Gravena et al. (2009)
Ig2B1	54	194–220	Gravena et al. (2009)
D22	58	106-118	Shinohara et al. (1997)
Ev5Pm	58	150-166	Valsecchi and Amos (1996)
FCB5	58	121-141	Buchanan et al. (1996)
FCB17	60	171-211	Buchanan et al. (1996)

Allele frequencies, expected (H_e) and observed (H_o) heterozygosities, and the inbreeding coefficient F_{IS} were estimated in FSTAT (Goudet 1995). Deviations from Hardy-Weinberg equilibrium (HWE) and of linkage equilibrium were also tested with FSTAT, and significance levels were adjusted for multiple tests by the False Discovery Rate procedure (FDR, Pike 2011). Microchecker 2.2.0.3 (Van Oosterhout et al. 2004) was used to detect the presence of null alleles, large allele dropout and scoring errors due to stutter peaks in the microsatellite *loci*.

Genetic differentiation was investigated using the Bayesian clustering method implemented in Structure 2.3.4 (Pritchard et al. 2000). The admixture model incorporating sampling locations as prior ("locprior", Hubisz et al. 2009) was used, with the correlated allele frequencies model. MCMC were set to 900,000 steps, after a burn-in of 100,000 iterations. The number of populations (K) tested varied between 1 and 5.

Ten independent MCMC replicates were run for each value of K. Structure Harvester (Earl and von Holdt 2012) was used to build graphs of LnP(D) (Pritchard et al. 2000, Pritchard and Wen 2004) and delta (K) (Evanno et al. 2005), in order to infer the most likely number of populations. Results of independent Structure runs for the same K were summarised using CLUMPP 1.1.2 (Jakobsson and Rosenberg 2007), and graphs representing the membership coefficient (Q) of each sampled individual were drawn using Distruct 1.1 (Rosenberg 2004).

Genetic differentiation was also investigated by computing the fixation index F_{ST} and testing its significance with 10,000 permutations in Genetix (Belkhir et al. 2002). A three dimensional Factorial Correspondence Analysis (3D-FCA) was also conducted in Genetix.

The effective population size (N_e) was estimated using the Linkage Disequilibrium method (Hill 1981) in the software N_eEstimator (Do et al. 2014). Estimates were calculated using the full data set and using critical frequency values of 0.02 and 0.01 to discard rare alleles, which might influence the analysis.

The Bottleneck program (Piry et al. 1999) was used to test the hypothesis of a recent bottleneck (within the last $2N_e$–$4N_e$ generations). Coalescent simulations (1,000) were run using all three-mutation models (Infinite Alleles Model, IAM; Two-Phase Model, TPM; and Stepwise Mutation Model, SMM). For the TPM model, settings included 95% of single step mutations, and variance of 12 among multiple step mutations, as recommended by Piry et al. (1999). Significance of deviations from equilibrium heterozygosity was evaluated using the Wilcoxon signed rank test (Luikart and Cornuet 1998). The qualitative mode-shift test of Luikart et al. (1998) was also used.

Besides F_{IS}, the degree of inbreeding was investigated by calculating the Internal Relatedness (IR) index, a measure of how related the parents of an individual were (Amos et al. 2001). IR was estimated using the Excel macro IRmacroN4.xls, available at http://www.zoo.cam.ac.uk/directory/william-amos.

Kinship coefficients (r) between all pairs of individuals (dyads) were calculated by the program ML-Relate (Kalinowski 2006). The values of *r* in dyads within Babitonga, outside Babitonga and in dyads of individuals from inside and outside the bay were compared. Dyads were also stratified by sex, and their *r* values were compared.

Mitochondrial control region amplification and sequencing

The mitochondrial control region was PCR amplified using the primers designed by Cunha et al. (2014) in 15 µL reactions containing 1 unit of GoTaq polymerase (Promega); 0.20 mM dNTPs; 2.5 mM $MgCl_2$; 1 µg/µL BSA and 0.5 µM of each primer. PCR cycling was as follows: 3 min. at 93°C; 30 cycles of 1 min. at 92°C, 1 min. at 50°C and 1 min. at 72°C; plus 5 min. of final extension at 72°C. All amplifications included blank controls. PCR products were purified and sequenced in both directions in an ABI3130 or ABI3500 automated sequencer using specific chemistry and the manufacturer's instructions. Sequences were edited with program SeqMan 7 (Lasergene

Inc.), visually aligned in MEGA 4 and submitted to GenBank (accession numbers XX to YY).

Haplotype and nucleotide diversities were estimated with DNASp 5 (Librado and Rozas 2009). A haplotype network was built with PopART (Leigh and Bryant 2015). Genetic differentiation was assessed by computing and testing F_{ST} and Φ_{ST} with 10,000 permutations, using the program Arlequin (Excoffier and Lischer 2010).

RESULTS

Population structure - microsatellites

Analyses were conducted with individuals grouped as follows: from inside Babitonga Bay (BB), N = 17; from adjacent coastal waters (CA), N = 20; of unknown origin, N = 2.

All *loci* were in linkage equilibrium (P > 0.002). HWE tests indicated heterozygote deficits in *loci* Ig2B1 and D22 in BB, and the same two plus *locus* FCB17 in CA (Table 2). According to Microchecker, null alleles could be present in those same *loci* and localities.

Table 2: Diversity indices and Hardy-Weinberg equilibrium (HWE) test results for seven microsatellite *loci* in franciscanas from Babitonga Bay (BB) and coastal areas (CA). N_a = number of alleles, AR = allelic richness; H_e = expected heterozygosity; H_o = observed heterozygosity. Significance of F_{IS} values for the HWE test was assessed using a FDR procedure – significant values are marked with an asterisk.

Locus	Coastal areas (CA, N = 20)				Babitonga Bay (BB, N =17)			
	N_a	AR	H_e / H_o	F_{IS} / *P*	N_a	AR	H_e / H_o	F_{IS} / *P*
Ig11D2	3	2.796	0.350	-0.143	2	1.972	0.227	-0.100
			0.400	*1.000*			0.250	*0.400*
Ig8H1	6	5.311	0.796	0.187	5	4.518	0.764	-0.221
			0.647	*0.089*			0.933	*0.993*
Ig2B1	5	4.305	0.681	0.706*	5	5.000	0.705	0.646*
			0.200	*0.004*			0.250	*0.004*
D22	5	3.257	0.466	0.436*	4	3.061	0.324	0.455*
			0.263	*0.011*			0.176	*0.071*
Ev5Pm	6	5.135	0.693	0.278	3	2.500	0.519	-0.205
			0.500	*0.057*			0.625	*0.886*
FCB5	6	4.881	0.680	0.049	4	3.651	0.654	0.345
			0.647	*0.511*			0.429	*0.018*
FCB17	10	7.249	0.848	0.263*	4	3.234	0.360	0.258
			0.625	*0.014*			0.267	*0.021*

BB individuals had lower genetic diversity (number of alleles, allelic richness and heterozygosities) than CA (Table 2). Both groups had significant inbreeding coefficients (BB: F_{IS} = 0.175, P = 0.011; F_{IS} = 0.273, P = 0.004). Internal relatedness (IR) values indicate that around 82.35% of BB individuals were inbred (IR > 0.13, N = 14), 11.76% were born to unrelated parents (-0.13 < IR < 0.13, N = 2) and 5.88% were outbred (IR < -0.13, N = 1). CA franciscanas were also mainly inbred, but at a lower percentage: 60% (N = 12). Individuals born to unrelated parents and outbred individuals were 25% (N = 5) and 15% (N = 3), respectively.

The exploratory FCA suggested genetic differentiation of BB and CA individuals (Figure 2), and population structure between BB and CA was indicated by F_{ST} (0.089, P = 0.03). This result was better evidenced in the Bayesian clustering analyses of Structure, which showed two populations (K=2) as the most likely scenario (Figure 3a, b). The assignment coefficient Q (Figure 3c) depicts the two populations, with all individuals from CA assigned to the red population, and almost but two individuals from BB assigned to the blue population. One of the undetermined franciscanas was assigned to BB and the other to CA.

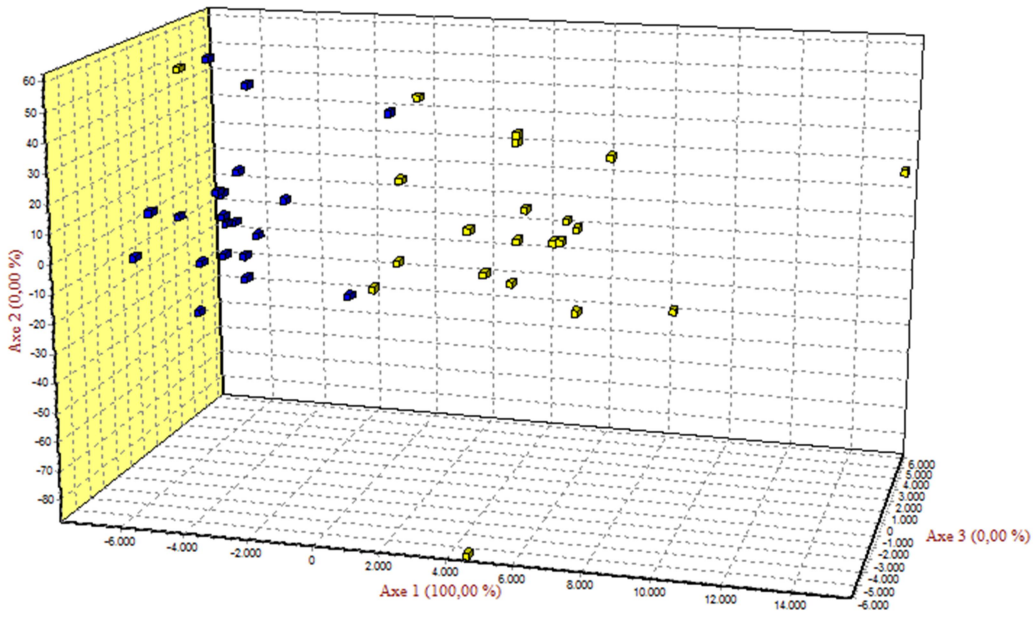

Figure 2: 3D-Factorial Correspondence Analysis of genetic differentiation between franciscanas from Babitonga Bay (blue) and the coastal area (yellow). Axe 1 explains 100% of the variation.

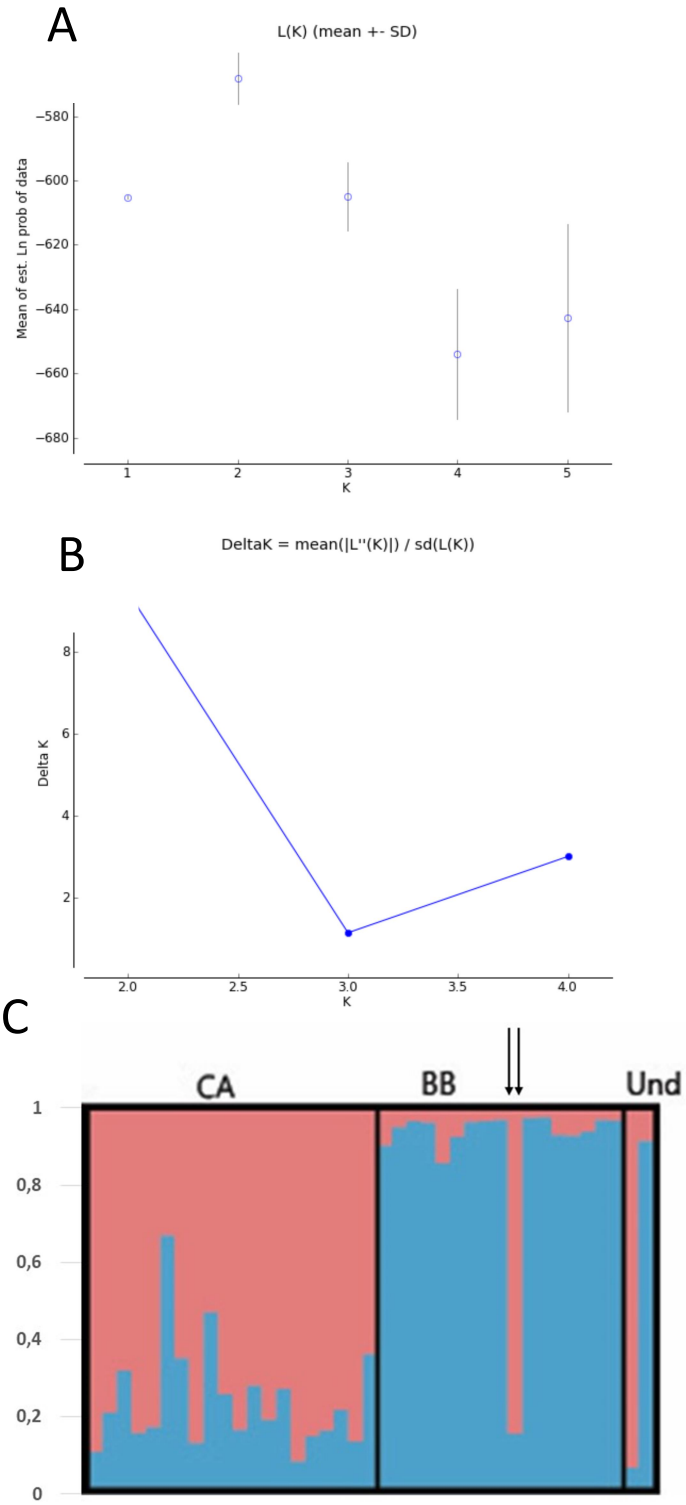

Figure 3: Results of the Bayesian assignment analysis using Structure. A) Log of posterior probability values estimated after 10 independent MCMC runs for each number of populations (K) tested (K = 1 to 5); B) Delta K values for the same analysis. Both graphs show K = 2 as the most likely scenario according to data. C) Proportion of the multilocus genotype of each franciscana (Q) that is assigned to each of the two inferred populations (red and blue). BB: Babitonga Bay; CA: coastal area; Und: individuals of undetermined origin. Arrows indicate the two franciscanas collected inside BB with a higher proportion of their genotypes assigned to CA.

Effective size (N_e) (number of breeders) of the BB population was estimated as 12.3 (CI: 2.5 – 31.2) for all three data sets (using all alleles, and discarding those with frequencies lower than 0.02 and 0.01). For CA, the three point estimates of N_e were "infinite" (CI: 66.1 – infinite).

The occurrence of a bottleneck in either population was rejected using all three mutation models (BB: IAM, P = 0.594; SMM, P = 0.945; TPM, P = 0.945 / CA: IAM, P = 0.344; SMM, P = 0.996; TPM, P = 0.992). Additionally, the "mode-shift" test showed L-shaped distributions, also suggesting that the observed allele frequency distribution fit mutation-drift equilibrium expectations.

Population structure - mtDNA

Forty-nine sequences were analysed: BB, N = 24; CA, N = 23; undetermined, N = 2. Aligned control region sequences were 614 base-pairs long, showing 18 polymorphic sites which defined six haplotypes. Of those, only two were observed in BB individuals, while all six haplotypes were found in CA (Figure 4). Although the same number of individuals was analysed inside and outside Babitonga, haplotype and nucleotide diversity values in BB (0.324 and 0.00475, respectively) were half the values found for CA (0.800 and 0.00860, respectively).

Both F_{ST} (0.194, P = 0.002) and Φ_{ST} (0.261, P = 0.002) indicated genetic differentiation of maternal lineages between BB and CA.

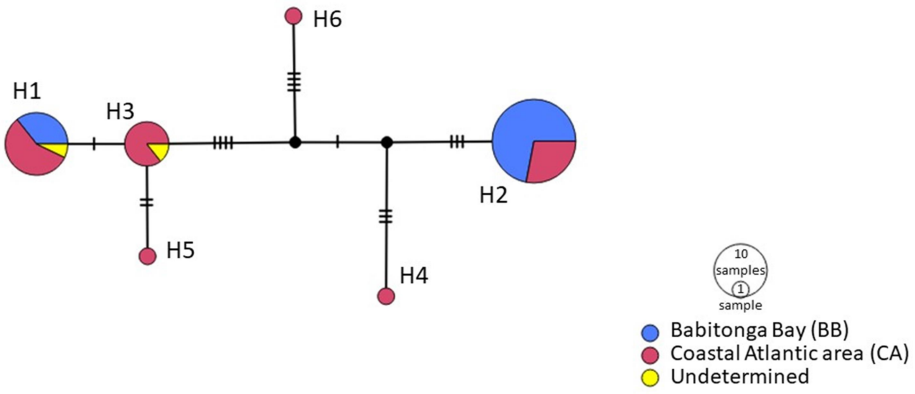

Figure 4: Haplotype network (614 bp, N = 49) built by PopART. Circle size is proportional to frequency. Branch length reflects molecular distance.

Kinship analyses

The relatedness coefficient *r* was estimated for 702 dyads, 153 between individuals from inside BB (BB x BB), 190 between individuals collected outside BB (CA x CA), and 359 between individuals from each group (BB x CA). This coefficient ranges from 0 to 0.5, with values around 0.125 corresponding to 3rd level relatives (first

cousins), around 0.250 to half-brothers or aunt-nephew relationships, and around 0.5 to parent-offspring or full-siblings.

Average *r* was higher within BB (*r* = 0.282) than outside BB (*r* = 0.108) and mixed dyads (composed of BB and CA individuals, *r* = 0.095). BB also had a higher proportion of relatives (62.08% with *r* > 0.125) than CA and BBxCA, which were mainly composed of non-relatives (Figure 5, Table 3).

Table 3: Average relatedness indices (*r*), and the number of dyads in each relationship category, in franciscanas from Babitonga Bay and the coastal Atlantic area. F: females; M: males.

r values	Babitonga Bay (BB, N = 153)	Coastal Area (CA, N =190)	BB x CA (N = 359)
Average	0.278	0.108	0.095
0 to 0.125	6	127	263
0.126 to 0.250	18	30	37
> 0.251	69	33	59
F x F			
0 to 0.125	18	26	26
0.126 to 0.250	33	3	3
> 0.251	15	7	7
M x M			
0 to 0.125	13	5	27
0.126 to 0.250	4	0	4
> 0.251	19	1	5
F x M			
0 to 0.125	35	21	85
0.126 to 0.250	11	7	10
> 0.251	35	8	22

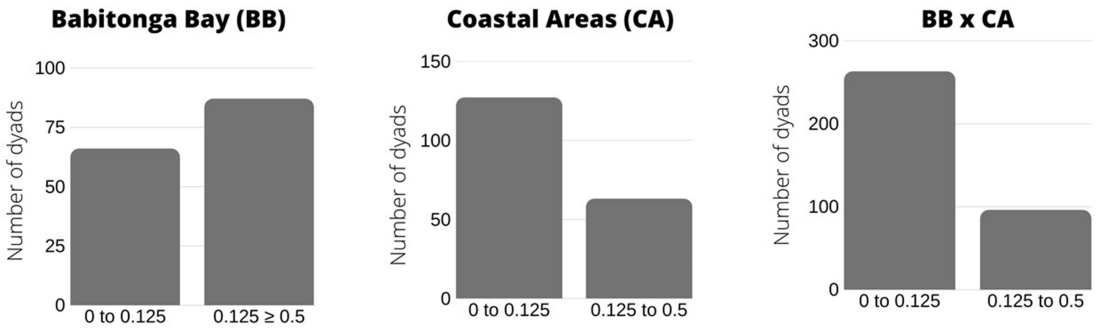

Figure 5: Relatedness indices (*r*) in franciscanas within Babitonga Bay (N = 153), outside it (coastal areas, N = 190) and mixed dyads (grouping individuals from inside and outside Babitonga Bay, N = 359).

Kinship according to sex was also investigated in 465 dyads. The same pattern described above was verified, with only BB showing more related than non-related individuals (56.83% with $r > 0.125$). Interestingly, relatedness in BB was high ($r > 0.125$) irrespective of the sex of the individuals in each dyad: F x F (50.00%, mean $r = 0.238$), F x M (56.79%, mean $r = 0.239$) and M x M (63.89%, mean $r = 0.311$) (Figure 6, Table 3).

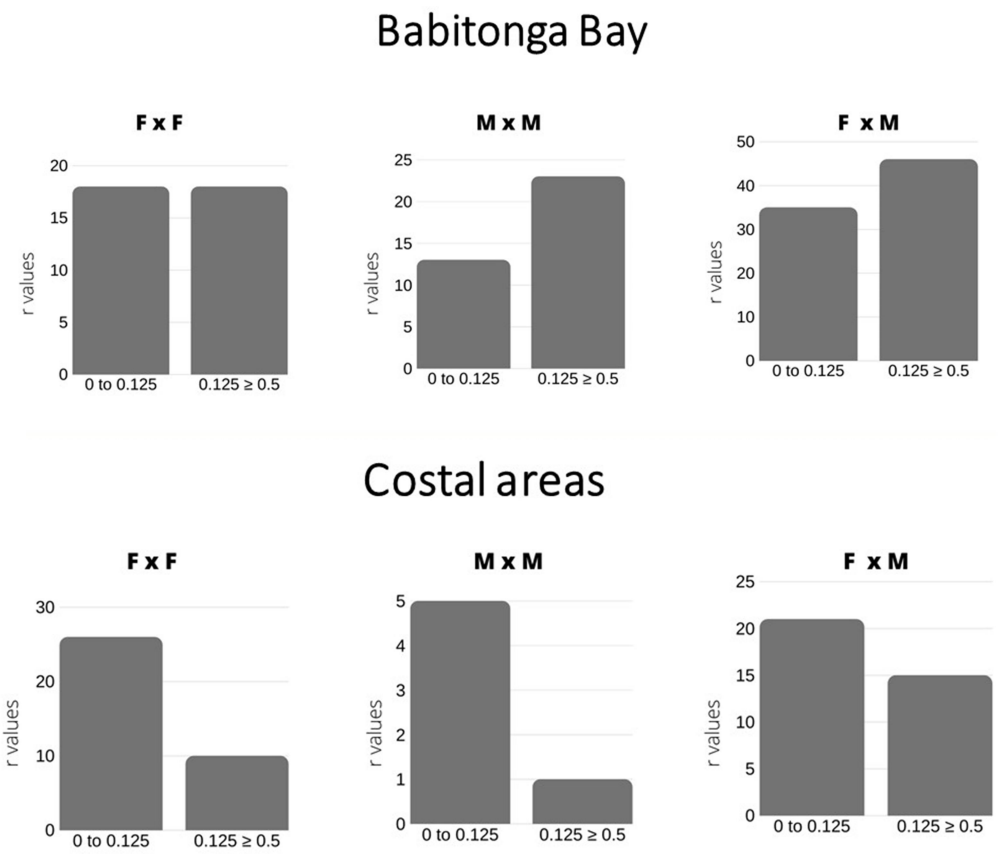

Figure 6: Relatedness indices (r) in franciscanas of known sex within Babitonga Bay (N = 153) and in coastal areas (N = 78).

In two instances franciscanas were caught in the same net during capture-release procedures for dorsal fin attachment of satellite-linked tags and at least two individuals of the group were sampled. In the first group, four franciscanas were sampled: two adult males and a mother-calf pair. One of the males could be a son of the female, and/or the father of the calf, because it could be sexually mature (it was 112 cm of total length and the smaller mature male reported for the species was 110 cm, Rosas and Monteiro-Filho 2002). The three had the same haplotype. As both dyads (pairing this male with the female and with the calf) had r around 0.5, the most plausible explanation is that the male was a son of the female, and a full-sibling of the calf. The other male was not a son of the female, not the father of the calf, and had a different haplotype. This male

was not related to any franciscana in the group, but was assigned to the Babitonga Bay population.

In another group two adult males (total length: 124 and 148cm) were sampled during capture-release procedures for attachment of satellite-linked tags. Both were tagged and they were sighted together many months following capture. Their *r* value was 0.5 and they had the same haplotype, suggesting that they could be full-siblings or father-son.

DISCUSSION

The Babitonga Bay population is small and isolated

All population structure analyses, of both nuclear and mitochondrial data, supported the existence of two populations, showing that franciscanas from Babitonga are genetically different from those in nearby Atlantic coastal waters. Thus, population structure results indicate restrictions to gene flow in a very small geographic scale, of less than 20 Km. This finding corroborates field observations, satellite-linked tracking data and photoidentification data (Cremer et al. 2012, Sartori et al. 2017, Cremer et al. 2018, Wells et al. *in prep.*) which suggested that the franciscanas in Babitonga Bay are long-term residents frequenting the interior of the bay, rarely moving to the outer areas near the mouth.

Our results suggest that both female and male franciscanas tend to remain in their natal population after reaching sexual maturity, favouring the genetic differentiation of populations even at small geographic scale, and increasing the risk of inbreeding, especially when the isolation and decline of populations are intensified by human activities.

Small-scale genetic differentiation of franciscanas has been reported in Argentina and Uruguay (Mendez et al. 2008, 2010a, Costa-Urrutia et al. 2012). Mitochondrial and microsatellite data supported the existence of three populations within FMA IV (Mendez et al. 2008, 2010a). The authors observed significant genetic structure between contiguous localities in Argentina, including two that are only 35 Km apart. Population limits correlated well with changes in environmental factors (chlorophyll concentration, water turbidity and surface temperature), which were proposed as drivers of population differentiation in franciscanas (Mendez et al. 2010a). Costa-Urrutía et al. (2011), using mtDNA and microsatellites, also found evidence of differentiation between franciscanas from the La Plata estuary and the Atlantic coast. Thus, our study is in accordance with previous findings of fine-scale structuring in the species, but at an even smaller scale. It also corroborates the possibility that differentiation results from gene flow restrictions caused by environmental discontinuities (Mendez et al. 2010a), especially between estuarine and coastal, open water habitats.

Besides the genetic distinctiveness of franciscanas from Babitonga Bay at population level, genetic data also suggest that this is a small and isolated population. The effective population size estimated with the Linkage Disequilibrium method, in our

case (iteroparous species with overlapping generations) reflects the number of individuals that contributed to the gene pool in the contemporary generation, i.e., the number of breeders (Waples and Do 2008). N_e estimates converged to 12.3 (CI: 2.6 – 32.1), which would roughly translate into a population size (N) of 53 or 87 franciscanas (considering $N_e/N = 0.14$ or 0.23; Palstra and Ruzzante 2008, Palstra and Fraser 2012, respectively). Although these N_e/N ratios, derived from empirical data, have several limitations (reviewed by Palstra and Fraser 2012), the Babitonga Bay population size estimated from N_e is close to the abundance estimates obtained by line transects in two different projects: between 2000 and 2003, 50 individuals were estimated (CV = 0.29) (Cremer and Simões-Lopes 2008), and in 2011, 55 individuals (CV = 0.24) (Zerbini et al. 2011). In any case, our N_e estimate *per se* is consistent with a small population, as made evident by the comparison with the N_e estimate for the coastal area (N_e = infinite, CI: 66.1 – infinite).

Genetic variability indices also indicate a small an isolated population in Babitonga Bay. The number of haplotypes, nucleotide and haplotype diversity, allele number, allelic richness and expected and observed heterozygosities were lower in Babitonga Bay compared to the adjacent Atlantic coastal area, despite sample sizes being equivalent. In a study of comparable scale involving franciscanas from the La Plata River estuary and adjacent costal area, expected heterozygosities were similar (0.843 and 0.832, respectively - Costa-Urrutia et al. 2012) and higher than found in Babitonga Bay and outside it (0.502 and 0.639, respectively). However, genetic diversity is higher in franciscanas in the south and decreases northwards along the species range, probably due to historical events such as the colonisation of the Atlantic by the species (Cunha et al. 2014), so comparisons between Babitonga Bay and the nearby Atlantic coastal area are more appropriate than with localities to the south.

The reduced diversity of the Babitonga Bay population may be the result of insufficient gene flow to counterbalance the eroding effect of genetic drift in a small population. Significant inbreeding ($F_{IS} = 0.175$, $P = 0.011$) and the high percentage of inbred individuals (84.21% with IR > 0.13) seem to corroborate the scenario of isolation. Alternatively, the low variability in Babitonga Bay could be due to a founder effect. This possibility is favoured by the fact that Babitonga haplotypes were a subset of those found in coastal areas, as were most of the microsatellite alleles. Only four private alleles were observed in Babitonga (versus 18 in the coastal area).

Although there was no evidence of a bottleneck, the detection window of our method ($2N_e – 4N_e$) was probably closed earlier than 50 years ago, considering the lower N_e estimate within our confidence interval and the generation time of 9.3 years estimated for franciscanas (Taylor et al. 2007). Gene flow was probably impacted by human activities in Babitonga Bay and probably this began to severely impact the franciscanas later. Originally, the bay had two channels that communicated with the Atlantic Ocean. The definitive closure of one of the channels, in 1934, left just one entrance, which became extremely busy with commercial shipping traffic in the São Francisco Harbour, which started operating in 1955. It is possible that the intense movement of vessels gradually deterred the franciscanas from using the bay access channel to the adjacent Atlantic coastal area. The monitoring of this population in the

past 20 years shows that the records of the species in this heavily trafficked region are rare and that this may be related in part to the noise pollution caused large vessels (Cremer et al., 2018).

Irrespective of whether the Babitonga Bay population was once larger and experienced a bottleneck, or has been small since its founding, our data show that gene flow with adjacent Atlantic coastal areas is negligible. In other words, migration from coastal areas into Babitonga Bay seems extremely infrequent, and the persistence of the Babitonga Bay population appears to depend entirely on adult and juvenile survival and recruitment through natality. Thus, Babitonga Bay franciscanas form a demographically independent unit (a "Management Unit" *sensu* Moritz 1994), and should be treated as such for conservation purposes. In this sense, impacts from the accidental capture in fishing nets, as reported by Pinheiro and Cremer (2003) and Cremer et al. (2018), should be considered as a very strong threat because it may be removing key individuals from the population (breeders). In addition, environmental degradation, which also includes problems related to chemical pollution (Alonso et al., 2012; De La Torre et al., 2012; Gago-Ferreiro et al., 2013), are also of concern, as they gradually reduce the conditions for the population's survival.

Kinship analyses support the philopatry of both sexes in franciscanas

Kinship analyses revealed that in Babitonga over 60% of franciscana dyads were related, while individuals collected outside the bay were not. CA individuals could belong to multiple groups that use the nearshore waters outside Babitonga, and thus relatedness among them was expected to be low. In any case the CA dyads serve as a control group, emphasising the high average relatedness among BB franciscanas. It should be noted that the high average relatedness and the fact that 62% of dyads were related do not imply that there is a single family group in Babitonga Bay (in fact, the presence of two haplotypes implies the existence of at least two matrilines in Babitonga). The same pattern could have arisen from multiple family groups, where dyads within groups would have higher *r* values than those between groups, which would tend to zero. In any case, high average relatedness, coupled with the evidence of limited gene flow with franciscanas from adjacent Atlantic coastal waters, builds a strong case for philopatry. Philopatry of one sex, usually the female, is a widespread phenomenon in mammals (Greenwood 1980), including many cetaceans (Connor et al. 2000), and has already been proposed for female franciscanas (Mendez et al. 2010a).

Another interesting finding is that kinship in Babitonga was high irrespective of the sex of the individuals (F x F, r = 0.238; M x M, r = 0.311; M x F, r = 0.239). Relatedness among females was expected, because most previous genetic and ecological studies with other small cetaceans revealed matrilineal social structure (Connor et al. 2000, Moller 2012), which has also been proposed for franciscanas (Valsecchi and Zanelatto 2003, Mendez et al. 2010b, Costa-Urrutia et al. 2012). But the presence of related adult males within their natal population, as suggested by our data, is rare. For comparison, *r* values in a resident population of *Sotalia guianensis* from Guanabara Bay are high among females, but low in female-male and male-male dyads, suggesting female philopatry and male-biased dispersal (Cunha H.A., *unpublished*

460 *data*). One possibility is that M x F dyads with high *r* values represent males that had
461 related females in Babitonga, but were not in the same family group after reaching
462 sexual maturity, i.e., that there is male exchange between family groups. But even so,
463 males would still be philopatric because they would tend to remain in their natal
464 population, raising the risk of inbreeding, further accentuated by the small population
465 size.
466 In a previous study, relatedness was reported between four members of a
467 franciscana group that were incidentally captured in the same gillnet in Southern Brazil
468 (an adult male, a lactating female, a calf and a juvenile (Valsecchi and Zanellato 2003).
469 Relatedness coefficients supported the mother-calf relationship between the lactating
470 female and the calf, an aunt-nephew relationship between the female and the juvenile,
471 and a cousins' relationship between the calf and the juvenile. The adult male was
472 unrelated to the female and juvenile, and only related to the calf. Although *r* was lower
473 than expected to support the father-calf relationship between them (r = 0.29), the
474 authors reported a 99.84% probability that the male was the father of that calf. This
475 study indicated that franciscanas travel in family groups, and that males could exhibit at
476 least short-term paternal care (of a few months). But the authors doubted the existence
477 of a longer bond between males and their offspring and respective mothers, and refused
478 the idea of monogamy (Valsecchi and Zanellato 2003).
479 Despite that, the hypothesis of a monogamic breeding system for the species has
480 been reinforced by different approaches, including the extremely small weight of
481 franciscana testes along the year (Rosas and Monteiro-Filho, 2002, Danilewicz et al.,
482 2004), the reverse sexual dimorphism and the lack of scars on males and females, that
483 could be related to conspecific aggression (Costa-Urrutia et al. 2012, Panebianco et al.
484 2012), and the prolonged or repeated close proximity of unrelated adult males and
485 females, according to data obtained from animals tagged with satellite-linked
486 transmitters (Wells et al. 2013). Two other studies investigated relatedness in
487 franciscana groups. The first study analysed eleven pairs and one trio of franciscanas
488 by-caught or captured and released together, in Argentina (Mendez et al. 2010b).
489 Results showed that three pairs were formed by mother-offspring and seven pairs by
490 unrelated adult male and female, which the authors speculated were possible
491 reproductive pairs. The trio was composed of an unrelated adult male and female and
492 her calf. Mendez et al. (2010b) argued that at least short term bonds are maintained by
493 females and their offspring and by reproductive pairs, which would tend to travel and be
494 entangled together, and discussed the genetic and demographic consequences of this
495 aspect of by-catch. According to the authors, franciscana social groups are matrilinearly
496 oriented but include unrelated reproductive adult males that could form temporary or
497 longer lasting bonds to reproductive adult females of the core of the social group.
498 In the second study, kinship was analysed in 21 groups (composed of individuals
499 either by-caught together or stranded within 1 Km in the same day) sampled in Uruguay
500 and Southern Brazil (Costa-Urrutia et al. 2012). In half of the groups, individuals were
501 relatives (r > 0.125). Related pairs involved all possible combination of sex and age.
502 The three larger groups sampled (more than 5 individuals) had *r* around 0.5 and
503 supported the presence of females with their offspring (juveniles) of both sexes, and

half and full-sibling relationships between juveniles of both sexes. Similarly to Mendez et al. (2010b), the authors proposed that the species' basic social unit is the family group, structured in matrilines. They also suggested that males may remain in their natal group, but their evidence was not strong (one juvenile male first-order related to an adult female, possibly his mother; and six juvenile males in the same group with possible half-siblings).

Thus, previous genetic data of franciscana groups supported kinship as a *criterium* for social organisation, but also revealed associations between unrelated individuals (in this case male and female pairs, Mendez et al. 2010b), although data on the temporal stability of such associations are limited. Our study did not aim to investigate group structure, but in two instances intra-group relationships could be explored. The first group seems in agreement with the hypothesis of juvenile males staying with their mothers (as suggested by Costa-Urrutia et al. 2012), and also that unrelated adult male and female may associate long enough to entangle together (as suggested by Mendez et al. 2010b). In the second group two adult males were probably full-siblings and were observed in association for at least eight months after tagging.

Kinship-based group formation implies fidelity to the natal group of at least one of the sexes, which in the case of mammals usually is the female (Greenwood 1980, Dobson 1982). Cetaceans apparently follow the rule, with only two known exceptions. Males of *Globicephala melas* and *Orcinus orca* stay in their maternal groups beyond attaining sexual maturity, but do not mate with related females. Instead, mating occurs when different groups meet (Amos et al. 1993, Pilot et al. 2010). Depending on the species' ecology, fidelity to the group corresponds to natal site fidelity, more commonly referred to as philopatry. Our results suggest that both female and male franciscanas are philopatric, because even if males do disperse from one family group to another, both sexes still remain in their natal site, and consequently in their natal population.

The possible role of bisexual philopatry in shaping population structure and consequences for the conservation of franciscanas

Evolutionary theory predicts that dispersal, and ultimately gene flow, is necessary to counterbalance the effects of genetic drift and inbreeding and maintain genetic variation within populations (Wright 1978). At the same time, gene flow is required to homogenise populations and prevent their differentiation. Philopatric species do not disperse as frequently as non-philopatric species, resulting in lack of panmixia and consequently, in population structure. Thus philopatry, as well as other phenomena that interfere with dispersal and random mating (such as habitat selection, presence of strong physical barriers, distance etc),may lead to population differentiation (Wright 1978, Avise 2004).

Philopatry of females has been evoked as an explanation for population structure in cetaceans, and in most cases is inferred by a stronger degree of structure detected with mitochondrial markers compared to nuclear *loci* (i.e. microsatellites), as a result of the strictly matrilinear mode of transmission of the former (Prugnolle & de Meeus 2002, Moller 2012). Philopatry of both sexes would have the potential to drive population differentiation faster and even at small geographic scales, and produce concordant

548 structure patterns between mitochondrial and nuclear markers. Both situations seem to
549 apply in the case of franciscanas: the first has been verified in several localities (Mendez
550 et al. 2008, 2010a, Costa-Urrutia et al. 2012, Cunha et al. 2014, this study), and the
551 second in Babitonga Bay (this study).
552 In such scenarios, all genetically differentiated populations, besides the need for
553 independent conservation actions as distinct Management Units, require measures to
554 minimise unnatural mortality rates and maintain or increase birth and survival rates.
555 Immigration should be assumed as an extremely infrequent event in this case,
556 insufficient to compensate mortality and to promote the colonisation of extirpated local
557 populations. At the same time, genetic erosion and, ultimately loss of local, genetically
558 differentiated populations could result in reduction of the adaptive potential of a
559 critically endangered species, an irreversible and unwanted outcome.
560 The Babitonga Bay population is quite unique: it is composed of resident
561 franciscanas with an overall home range of about 26 Km^2 (Cremer 2007), forming a
562 small population isolated from other franciscanas that live less than 20 Km away, in
563 Atlantic coastal waters. This isolation, coupled with the increasing anthropogenic
564 impacts in this bay, makes the persistence of this population very unlikely in the
565 medium term, unless conservation measures are adopted urgently.
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567
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569
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