Molecular analysis of the social and population structure of the franciscana (*Pontoporia blainvillei*): conservation implications

E. VALSECCHI* AND R.C. ZANELATTO§

Contact e-mail: Valsecchi.Elena@libero.it

ABSTRACT

Fifteen franciscanas, including four members of a putative social group, were genetically typed in order to: (1) obtain insights into the social organisation of this poorly known dolphin species; and (2) clarify its population sub-structure across the species range. Samples were screened for 10 nuclear markers (microsatellites) and sequenced for 269bp of the mitochondrial DNA control region. The results indicate that franciscana dolphins may travel in kin groups which might include, besides mothers with their calves or juvenile offspring, the fathers of the youngest group members. All four individuals from the presumed social group shared the same mitochondrial haplotype, suggesting that the social unit might be matrilineally structured. Comparative analyses of mitochondrial data available from a previous study of two adjacent populations (19 additional haplotypes) suggest the existence of at least three distinct populations. This population fragmentation, together with the relatively low genetic variability, suggests that the franciscana dolphin is a potentially vulnerable species, which may require some management effort to ensure its preservation. Consistent with a previous study, the population occupying the northernmost extremity of the species distribution range was found to be the least variable, most isolated, and therefore potentially the most vulnerable.

KEYWORDS: FRANCISCANA; GENETICS; SOCIAL GROUPS; CONSERVATION; MANAGEMENT; SOUTH ATLANTIC; SOUTH AMERICA

INTRODUCTION

The franciscana, *Pontoporia blainvillei* (Gervais and d'Orbigny, 1844), lives in coastal waters and estuaries of the central portion of the Atlantic coast of South America. Its distribution is restricted to the stretch of coast spanning from Regência (19°S), Espirito Santo, Brazil to Golfo San Martías (42°S), Río Negro, Argentina (Rice, 1998; Fig. 1). The franciscana has been susceptible to intense incidental bycatch in gillnets throughout its distribution (e.g. Pinedo, 1994; Secchi *et al.*, 1997). However, the World Conservation Union (previously IUCN) has withheld classification on the current status of the franciscana dolphin due to the scarce information on the biology of this species.

Little is known about the social habits of the franciscana. Initially it was thought to be a solitary or non-gregarious species (Kasuya, 1984; Pinedo *et al.*, 1989), perhaps due to the difficulty of observing specimens in the wild. However, as the number of observations increased, reports of larger groups of up to 15 individuals became more frequent (e.g. Crespo *et al.*, 1998). However, no opportunity has yet arisen to investigate the sex and genetic composition of social groups. Since this species is highly evasive and maybe vulnerable, no biopsy collection from free-ranging animals has ever been attempted.

The population structure of the franciscana across its distributional range is also not fully understood. Secchi *et al.* (1998) have provided the first molecular evidence of the existence of (at least) two distinct populations, supporting Pinedo's (1991) morphological data. In the latter study, differences in osteological (mostly cranial) and morphometric parameters recorded in more than 500 specimens sampled (stranded or bycaught) over a period of 15 years along the southern coast of Brazil suggested the presence of a northern and a southern form. Most individuals surveyed in Pinedo's study were sampled south of Santa Catarina state (from Rio Grande do Sul state to Uruguay) and off Rio de Janeiro state, with little data available from animals from Parana and São Paulo states. The separation between the two forms was localised around the state of Santa Catarina, Brazil (see Fig. 1). Consistent with this observation, Secchi *et al.* (1998) found mitochondrial differentiation between franciscanas from Rio Grande do Sul and those from Rio de Janeiro. However, it still remains unclear whether the shift between the two forms is gradual (e.g. isolation by distance), with a number of local populations partially overlapping with each other (as would be expected for a resident species with low mobility of adults), or whether there are two main populations, north and south of the state of Santa Catarina, with limited genetic interchange between their members. In this case the actual geographic boundary separating the two populations remains to be identified.

This study measured genetic relatedness among four individuals sampled during the same bycatch event and putatively members of a social group. Most of the samples were collected in Parana state, which is geographically intermediate to those analysed by Secchi *et al.* (1998), and slightly north of Santa Catarina state, the boundary detected between the two morphologically distinct forms (Pinedo, 1991). This provided the opportunity to elucidate the population substructuring of the franciscana in Brazilian waters, enabling guidelines for the conservation of this species to be refined.

MATERIALS AND METHODS

Samples

In 1993, five franciscanas were found entangled in the same set gillnet (*rede de fundeio*) in the Canal da Galheta, Parana, Brazil (~25°35'S, 48°17'W). One animal was successfully disentangled and released, while the remaining four were found dead. These consisted of a lactating female, an adult

* *School of Biological Sciences, University of New South Wales, Sydney NSW2052, Australia.*
§ *Centro de Estudios do Mar, Universidade Federal do Paraná (CEM-UFPR), Pontal do Sul, Paraná, CEP 83255-000, Brazil.*

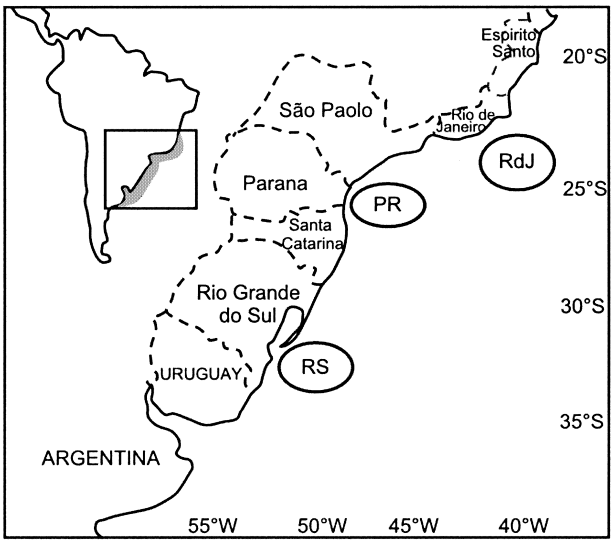

Fig. 1. Map of sampling sites in relation to the franciscana's distribution range (shaded area in the South America map on the left-hand-side of the figure). Sample sizes for Rio de Janeiro (RdJ), Parana (PR) and Rio Grande do Sul (RS) regions were 0, 13, 2 and 10, 10, 11 for nDNA and mtDNA analyses respectively. The additional mitochondrial data were available from Secchi *et al.* (1998).

male, a juvenile male and a male calf. Microsatellite and mitochondrial DNA analyses were performed on tissue samples (skin, intestine or muscle) from the four dead specimens and from 11 other conspecifics found stranded or in nets along the southern coast of Brazil between 1992 and 1998.

The 15 samples represented two main geographical areas (Fig. 1), corresponding to the central-northern coast of Parana state (PR, $n = 13$) and to the central-southern coast of Rio Grande do Sul state (RS, $n = 2$). The 13 PR samples were collected from a wider coastal range (from ~25°00'S to ~25°40'S), with a maximum distance of ~65km between the two furthermost locations. The two RS samples were both from the same location (off Rio Grande, ~32°S). Sampled individuals included seven females, seven males and one individual of unknown sex. The carcass of this specimen was too decomposed to allow direct sex determination, and its DNA was highly degraded. Samples of this type will support molecular amplification (PCR, see below) of small DNA-fragments, such as microsatellites (100-230bp), but will not support amplification of larger fragments such as the one required for sexing (~550bp). Moreover, before being molecularly analysed, the tissue sample collected from this individual had been preserved for many years in formalin, a substance which is known to inhibit molecular amplification.

In the analysis of population sub-division based on mitochondrial polymorphism (see below), data from 10 Rio de Janeiro (RdJ) and 9 Rio Grande do Sul (RS) specimens previously analysed by Secchi *et al.* (1998) were included. Consequently, this part of the analysis was based on 10 RdJ, 10 PR (potential relatives were removed) and 11 RS specimens (see Fig. 1).

Molecular screening

The 15 samples were screened for the following 10 microsatellite loci: EV5*Pm*, EV104*Mn* (Valsecchi and Amos, 1996); 199/200, 417/418, 464/465 (Schlötterer *et al.*, 1991); MK5, MK6, MK8 (Krützen *et al.*, 2001); D08 (Shinohara *et al.*, 1997) and KW12 (Hoelzel *et al.*, 1998). A single (touch-down) PCR profile allowing the simultaneous amplification of any combination of the ten loci was optimised. A starting denaturing step of 2min at 93°C was followed by seven series of 3, 5, 7, 7, 7, 7 and 10 repetitions with annealing temperatures of 61°C, 59°C, 57°C, 53°C, 49°C, 45°C and 41°C respectively. Both denaturing (30sec at 90°C) and extension (40sec at 72°C) steps were kept constant over the series. The programme terminated with a final extension step of 7min at 72°C. Amplified microsatellites were screened on an ABI-377 automated sequencer. Each locus was tested for the presence of possible null (i.e. non-amplifiable) alleles.

All individuals were sequenced for 269bp of the mitochondrial DNA control region, which was first amplified using primers Dlp-5 (Baker *et al.*, 1993) and Mt15996L (Campbell *et al.*, 1995) and subsequently sequenced using BigDye™ (Perkin-Elmer Corporation) sequencing mix, on an ABI-377 automated sequencer.

Parentage analysis

Relatedness was estimated for each pair of individuals. The average relatedness among the four individuals that may represent a social unit was compared against values from 1,000 randomisations obtained by replacing the original genotypes with alleles drawn randomly from the observed allele frequencies in the PR area. Since the adult male of the putative social group had a compatible genotype to be the father of the calf it was travelling with, the probability of paternity (W) associated with that match was measured. The formula $W = (CPI/CPI+1)\times 100$ (e.g. Brenner, 1983) was used, where CPI indicates the combined paternity index which is the product of the individual paternity index (PI) values calculated for each locus. The PI is a likelihood ratio between the chance that the alleged father may pass the paternal allele compared to the chance that a random male may pass the paternal gene to the calf. To perform such a comparison, the allele frequencies of all PR samples were used, with the exclusion of the two younger members of the putative social group (the two adult individuals were found to be unrelated).

To examine the significance of the family match, Monte Carlo simulation was also used to estimate the probability of several alternative scenarios: (1) three unrelated animals matching as a mother-father-calf trio; (2) a male matching an unrelated mother-calf pair; and (3) a male matching the sampled mother-offspring pair.

Finally, in order to test the null hypothesis that pairs of related individuals do not occur preferentially in the putative social group, the incidence of genetically related pairs ($r > 0.25$ and sharing an identical haplotype) in all the remaining individuals sampled in the PR area was examined.

Phylogenetic analysis of population structure

This part of the analysis had to be restricted to unrelated individuals, therefore only one member of the presumed social unit (which all carried the same mitochondrial haplotype) was included. Also included were mitochondrial data from 19 of the 20 individuals analysed by Secchi *et al.* (1998): haplotype H was removed as its sequence was incomplete. Since the sequences examined in the two studies were selected independently and were only partially overlapping, the analysis was restricted to the common region of 232bp. For this DNA stretch haplotypes A and B were indistinguishable, and were therefore merged in a single haplotype, here designated as haplotype AB. MtDNA polymorphism detected in the 31 individuals was measured

by estimating both gene and nucleotide diversities (Nei, 1987), computed using the software package ARLEQUIN 2.000 (Schneider *et al.*, 2000).

The relationship between haplotypes was visualised according to different phylogenetic reconstruction methods. Haplotypic phylogeny was reconstructed by building a neighbour-joining (NJ) tree (Saitou and Nei, 1987) based on distances calculated under the Jukes-Cantor (JC) model, for consistency with the previous work by Secchi *et al.* (1998), therefore allowing direct comparison. Evolutionary relationships among haplotypes were inferred by maximum-likelihood (ML) method. The significance of both branching patterns was assessed using 500 bootstrap replicates. Finally the phylogenetic relationship among haplotypes was visualised by producing a minimum spanning network (MSN) (Excoffier and Smouse, 1994) using ARLEQUIN 2.000 (Schneider *et al.*, 2000).

To estimate the extent of population differentiation between the three study areas, pairwise genetic divergence was measured using both the conventional F_{ST} (Wright, 1921), based on haplotype frequencies, and Φ_{ST} based on molecular distances between haplotypes, as in Secchi *et al.* (1998). In both cases significance was tested performing 10,000 permutations.

RESULTS

Microsatellite polymorphism

The 10 loci surveyed in this study were moderately polymorphic, with 3-9 (mean 4.9) alleles per locus found in 15 individuals (Table 1). The mean observed (H_o) and expected (H_e) heterozygosities were respectively 0.635 and 0.639. The close agreement between these values suggests that overall there is no major heterozygote deficiency and that null alleles are probably not present. Conventional Hardy-Weinberg tests are inappropriate because of the relatively small sample size and potential presence of relatives in the sample.

Table 1

Characteristics of the ten microsatellite markers used in this study.

Locus	No. of alleles	Allele size range (bp)	H_e	H_o
EV5*Pm*	3	159-163	0.585	0.643
EV104*Mn*	4	147-155	0.591	0.462
199/200	3	127-133	0.362	0.429
417/418	7	193-215	0.761	0.667
464/465	9	153-173	0.834	1.000
MK5	4	211-223	0.576	0.455
MK6	5	149-169	0.644	0.800
MK8	4	104-112	0.628	0.600
D08	6	96-110	0.797	0.583
KW12	4	154-168	0.611	0.714

Parentage analysis

The four dolphins caught in the same net were closely related (Table 2). Their genotypes are shown in Table 3. The average nuclear relatedness (*r*) measured between the four individuals was significantly higher ($r = 0.21$; $P = 0.02$) than that estimated between the remaining PR individuals ($r = -0.01$, see areas E and D in Table 2). When the six

Table 2

Pairwise nuclear relatedness among screened individuals. The r-values enclosed into boxes represent relatedness: within RS samples (box A); between RS and PR individuals (box B); between the individuals (c=calf, j=juvenile) found in the same net (box C); between these 4 individuals and the rest of the individuals sampled in Parana (box D); between all Parana samples, excluding the putative social unit (box E). Relatedness values larger than 0.25 (denoting first-to-second degree relatives) are shown in bold, and are underlined in those cases in which the two related individuals share also the same mitochondrial haplotype. The grey sector of the table highlights the average relatedness measured within each box (* = significant at the P≤0.05 level).

Sample	Hapl.	Sex	Year	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	h3	F	'92															
2	h4	M	'92	-0.41														
3	h1	M	'93	-0.28	-0.16													
4	h1	F	'93	-0.13	-0.17	-0.01												
5c	h1	M	'93	-0.38	-0.32	**0.29**	**0.49**											
6j	h1	M	'93	-0.05	-0.52	0.08	**0.27**	0.15										
7	h2	F	'94	-0.29	-0.39	**0.27**	**0.44**	**1.00**	0.06									
8	h2	-	'94	-0.06	-0.54	0.10	**0.53**	**0.41**	0.22	**0.50**								
9	h5	F	'94	-0.31	-0.49	-0.35	-0.37	-0.34	-0.11	0.01	0.13							
10	h1	F	'95	-0.26	0.08	-0.02	-0.45	-0.43	-0.08	-0.42	-0.15	0.02						
11	h2	F	'98	0.02	-0.63	-0.26	-0.10	-0.13	0.16	0.09	**0.49**	**0.36**	**0.67**					
12	h1	M	'98	-0.42	0.04	-0.36	-0.13	-0.26	0.03	-0.07	-0.09	**0.33**	**0.31**	**0.41**				
13	h2	M	'98	-0.21	-0.25	0.00	-0.17	-0.02	**0.46**	-0.26	-0.33	-0.23	0.01	-0.22	-0.03			
14	h1	M	'98	-0.11	-0.24	-0.10	-0.06	-0.11	0.01	-0.11	-0.35	-0.50	-0.06	-0.13	-0.18	-0.32		
15	h1	F	'98	0.08	-0.15	-0.38	0.04	-0.18	0.10	0.04	-0.19	**0.30**	-0.19	0.01	**0.30**	0.13	-0.11	

sample

	MM	MF	FF	All
A	-	-0.41	-	-0.41
B	-0.24	-0.25	-0.24	-0.24
C	0.17	0.25*	-	0.21*
D	-0.04	-0.05	-0.12	0.01
E	-0.22	0.03	0.09	0.01
all	-0.09	-0.05	-0.02	-0.05

pairwise relationships between the four presumed pod-members were classified according to the sex composition, and relatedness was measured and tested for significance within each category, it was found that the three male-female pairs scored a significantly higher mean relatedness ($r = 0.25$, $P = 0.01$) than the three male-male pairs ($r = 0.17$, $P = 0.18$). All four individuals shared the same mitochondrial haplotype (*h*1), although this was one of the most common (61.5%) haplotypes in the area. The incidence of individuals showing high relatedness ($r > 0.25$) and carrying identical mitochondrial haplotypes was much higher in the putative social unit (three out of six pairs, 50%), than between the remaining PR samples (four of 72 pairs, 5.6%; see Table 2).

The closest relationships were the ones between the adult female and the male calf ($r = 0.49$), between the adult male and the male calf ($r = 0.29$) and between the adult female and the juvenile male ($r = 0.27$). All these related pairs shared at least one allele per locus, with the exception of a single mismatch (at locus MK5, see Table 3) between the female and the juvenile male. It was unclear whether this was due to the presence of a null-allele (the juvenile was homozygous at locus MK5) or whether the mother was possibly the individual which had been liberated from the net, or was an uncaptured animal. The adult male and the adult female also shared one allele at each locus (not excluding a potential mother/offspring relationship), however they scored a low *r*-value ($r = -0.01$), due to the sharing of common alleles.

The genotypes of both adults were also compatible with being the calf's parents. The probability (W) of the adult male being the calf's father was estimated to be 99.84%, however this probability value was estimated under the assumption that the presumed father was not related to the calf's mother, and this can not be excluded (see above). All three simulated scenarios (three unrelated animals matching as a mother-father-calf trio; a random male matching an unrelated mother-calf pair; a random male matching the sampled mother-offspring pair) were unlikely to occur by chance ($p = 0.0002$, 0.03 and 0.025 respectively). Moreover, if the allele frequencies are recalculated without the calf and juvenile genotypes, the probabilities of the second and third scenarios are reduced by approximately a factor of two. The adult male was unlikely to be the father of the juvenile ($r = 0.08$), while the two younger individuals had genotypes compatible with being maternal half-brothers ($r = 0.15$).

Mitochondrial polymorphism and phylogenetic relationship among haplotypes

Five different haplotypes (*h*1, *h*2, *h*3, *h*4 and *h*5) were identified in the 15 study individuals (Table 4). Three of these (*h*1, *h*3 and *h*4) were also previously identified by Secchi *et al*. (1998) in the Rio Grande region (RS). When the information from the two studies was merged, a total of 11 different haplotypes could be identified over 34 individuals representing the three regions RS, PR and RdJ (Table 4). Thirteen variable sites were detected over the 232bp of comparable sequence among the two studies. All but one were transitions. The estimated haplotype (H) and nucleotide (π) diversities for the total sample were 87.2% (±3.3) and 1.5% (±0.9) respectively.

Approximately half ($n = 5$, 45.5%) of the haplotypes were unique to single individuals, while the remaining haplotypes ($n = 6$, 54.5%) were carried by 3-9 (mean 4.8) specimens. All but one (*h*1) of the 11 haplotypes were unique to a single region. Haplotype *h*1 was found mostly in the PR region ($n = 8$), but also, in one instance, in the RS area. Only one of the two diagnostic sites identified by Secchi *et al.* (1998) for distinguishing between northern (RdJ) and southern (RS) lineages (position 356 in Secchi *et al.*, 1998) was included in the analysed DNA fragment (position 168 in Table 4). Haplotypes from the PR area showed intermediate characteristics at this site: five (38.5%) individuals carried the substitution typical of the RdJ region (haplotypes *h*2 and *h*5), while the remaining eight (61.5%) carried the signature characteristic of the RS region (haplotype *h*1).

Both the NJ and the ML genealogies of the 11 haplotypes produced similar branching patterns (Fig. 2), although these results should be interpreted with caution given the restricted sample size. However, the major clades detected in both trees mostly reflected the samples' geographical origin. The most highly supported clade (AB-C-D) was found only in the RdJ region, the second well-supported clade (E-*h*2-*h*5) was mostly found in the PR region, but also (one individual) in the RdJ region. Finally, haplotypes detected in the RS area showed a less structured phylogenetic pattern. The minimum spanning network connecting the 11 haplotypes is shown in Fig. 3.

Population sub-structure

The lowest nucleotide and haplotype diversities were both found in the RdJ samples (Table 5). PR and RS samples had a similar level of nucleotide diversity, but haplotype diversity was higher in the RS region (Table 5). The three populations were all significantly distinct from each other, for both F_{ST} and Φ_{ST}, although differences were slightly more pronounced for the latter differentiation index (Table 6). The highest genetic differentiation was found between the northern (RdJ) and the central (PR) populations. The central (PR) and the southern (RS) populations were found to be the most similar (Table 6).

DISCUSSION

The present study provides the first genetic evidence suggesting that franciscanas are likely to travel in groups of (likely matrilineally) related individuals, suggesting also the possibility of father-offspring associations, which have never been previously recorded in any marine mammal species. Furthermore, the results indicate that the franciscana population is highly structured throughout its distribution range with presence of phylogeographically distinct groups, some of which seem morphologically indistinguishable.

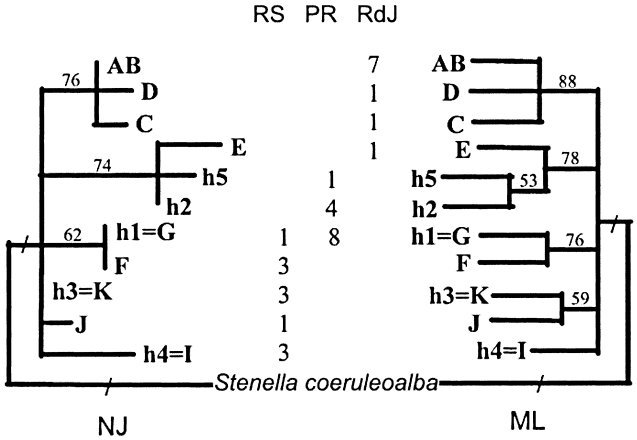

Fig. 2. Neighbour-joining (NJ) and maximum-likelihood (ML) genealogies of 11 mtDNA haplotypes detected in 34 franciscana dolphins off the southern coast of Brazil. Haplotypes are as in Table 4. Nodal numbers indicate bootstrap support (> 50%) of the observed branching patterns. The homologous sequence of a striped dolphin (*Stenella coeruleoalba*) was used as an outgroup.

Table 3

Genotypes of the four individuals caught in the same net. Allele are expressed in allele size (bp).

		loci																			
	Samples	MK6		MK5		MK8		D08		KW12		EV5		EV104		DT199		DT417		DT464	
3	Adult male	153	169	211	213	106	112	98	100	154	160	161	161	153	155	127	133	199	201	171	173
4	Adult female	149	153	211	223	104	106	98	100	154	154	161	163	153	153	133	133	199	203	161	173
5	Male calf	149	153	211	223	106	112	100	100	154	154	161	163	153	155	127	133	199	201	161	173
6	Juvenile male	153	153	213	213	106	112	100	100	154	168	161	163	153	153	133	133	199	203	161	171

Table 4

Haplotypes from franciscana dolphins identified off the southeastern coast of Brazil. Haplotypes designated with letters only (i.e. AB to K) are from Secchi *et al.* (1998). The positions of the 14 variable sites detected in the 269bp fragment are indicated by the vertical numbers at the top of the third column. Dots indicate identity at the corresponding position in haplotype h1 (complete sequences available from GenBank Accession n° AF420606-AF420610). The comparison between the two studies is based on the 232bp fragment included between positions 38 to 269 of the surveyed sequence. The occurrence of each haplotype in the Rio Grande (RS), Parana (PR) and Rio de Janeiro (RdJ) regions is shown on the right-hand side of the table.

Haplotypes *	Freq.	111111112 36669226667781 60125785890739	RS	PR	RdJ
h1 (=G)	9	GATTGCTTAAATTC	1	8	
h2	4	.G..A...G..C..		4	
h3 (=K)	3	AG........G...	3		
h4 (=I)	3	.G..AT...GG...	3		
h5	1	.G..A.C.G..C..		1	
AB	7	-G......G.G..T			7
C	1	-G.G....G.G..T			1
D	1	-G......G.G.CT			1
E	1	-G..AT.CG..C..			1
F	3	-G.......G....	3		
J	1	-GC.......G...	1		
Total	34		11	13	10

* Positions 60 and 219 correspond respectively to positions 464 and 305 in Secchi *et al.* (1998). Position 168 corresponds to one (position 356 in the original paper) of the diagnostic sites that Secchi *et al.* (1998) identified for distinguishing between northern (RdJ) and southern (RS) lineages. The other diagnostic site detected by these authors was not included in the 232bp fragment analysed here.

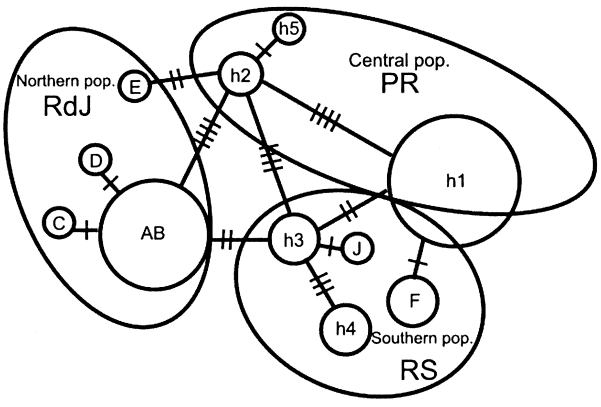

Fig. 3. Minimum spanning network (MSN) of the 11 mtDNA haplotypes from 34 franciscana dolphins. Cross hatches represent the number of point mutations between haplotypes. Circle sizes reflect haplotype frequencies. The oval-shaped lines group haplotypes found in each of the following three regions: Rio de Janeiro (RdJ), Parana (PR) and Rio Grande do Sul (RS). Only haplotype *h*1 was found both in the PR ($n = 8$) and in the RS ($n = 1$) regions.

Table 5

Nucleotide (π) and haplotype (H) diversity indices in franciscanas from the three surveyed regions off the southern coast of Brazil.

	$\pi \pm$ SD	H ± SD
Northern population (RdJ)	0.7% ± 0.5	53.3% ± 18.0
Central population (PR)	1.1% ± 0.7	64.4% ± 10.1
Southern population (RS)	1.1% ± 0.7	83.6% ± 7.0

Table 6

Indexes of mtDNA genetic differentiation between the three populations of franciscana considered in this study. Pairwise F_{ST} and Φ_{ST} values are shown above and below the diagonal, respectively. *P* values, estimated using 10,000 permutations, are shown in brackets.

	RdJ	PR	RS
Northern population (RdJ)	-	0.41 (0.0001)	0.31 (<0.0001)
Central population (PR)	0.53 (0.001)	-	0.22 (0.0012)
Southern population (RS)	0.51 (<0.0001)	0.31 (<0.0001)	-

Social structure

Although the genetic variability detected in the surveyed samples was not enough to unambiguously resolve kinship relationships, the four individuals that were found entangled in the same net were found to be closely related. Molecular evidence supports the suggestion that the group may have comprised an adult female, her male calf, a juvenile male which was, to some extent, related to the female and her calf, and an adult male which was either the female's son or the calf's father. Unfortunately, data on the age of the two adult individuals were not available, preventing distinction between the two possibilities, which will therefore both be discussed.

The combination of molecular typing and observational evidence (the female was lactating) leaves little doubt of the identity of the calf's mother. However, the implications from the molecular data that either a weaned (adult) male offspring might still swim in company of its mother or a father might have been in close proximity to its calf deserve more attention. Although the first option was not strongly supported by molecular evidence (low *r*-values and sharing of just common alleles), it is behaviourally the more convincing. Few cetacean species, such as killer and pilot whales, live in stable matrilineal groups (e.g. Amos *et al.*, 1993).

However, if the adult male was not the female's son, it was very likely the calf's father: the paternity probability associated to this match was calculated as 99.84%. Mammalian fathers do not typically maintain their bonds with their offspring, and cetaceans do not seem to be an exception (Connor *et al.*, 2000). However, Brownell (1989)

observed that the franciscana exhibits reverse sexual dimorphism (male smaller than female) and relatively small testis, both characteristics which would suggest a male reproductive strategy not based on fighting capabilities nor on sperm competition, but rather on mate guarding (see Connor *et al.*, 2000). However, Brownell (1989) concluded that, given the rarity of paternal care in mammals, these morphological traits of the franciscana are probably not to be associated with monogamy. This study provides the first molecular evidence indicating that, although not necessarily monogamous, male franciscanas might prolong their bond with their reproductive partner at least until the first months of their offspring life, providing some form of paternal care. Just why male fransiscana dolphins should remain in contact with their breeding partners remains unclear, as does the length of time they do so and the extent (if any) to which they contribute paternal care. One possibility is that the behaviour is a form of mate-guarding, perhaps evolving as an inbreeding avoidance mechanism (Clutton-Brock, 1989). This species lives at low densities in a linear, coastal habitat where population structure could be strong. The number of available, unrelated partners could be small, making it beneficial for successful pairs to stay together. This aspect of the franciscana social system is potentially extremely interesting and should be further investigated.

Although the female and the juvenile male appeared to be closely related, their genotypes did not match (not compatible for maternity) at one locus. It is unclear whether this could be due to the presence of a null allele at locus MK5, or whether the juvenile's mother remained unsampled. Unfortunately, the sex of the fifth (released) member of the pod is unknown, and it is therefore hard to speculate about its possible relationship with the rest of the (sampled) pod-members. Interestingly all pod members carried the same mitochondrial haplotype. Although the shared haplotype was one of the commonest haplotypes (together with *h*2) in the PR region, the combination of high relatedness values ($r > 0.25$) and mitochondrial identity was about 10 times more frequent among the pod members than among individuals sampled from different pods in the same region (PR). Unfortunately, the restricted sample size and amount of variation limited the application of other analysis approaches.

Population structure

MtDNA data support the suggestion that the franciscana population inhabiting the southern coast of Brazil is more structured than previously thought. A third population (PR), localised in between the two (RS and RdJ) previously found to be mitochondrially distinct (Secchi *et al.*, 1998), was detected. This 'central' population showed characteristics which do not satisfy the molecular criteria suggested by Secchi *et al.* (1998) as indicators for discriminating the two previously described populations (RS and RdJ). The diagnostic site (the only one included in the portion of mtDNA analysed) identified by Secchi *et al.* (1998) for distinguishing haplotypes from the northern and southern populations is not diagnostic in the PR region, and should therefore not be employed at these latitudes. Also, it should be considered that the sample size provided by Secchi *et al.* (1998) was probably too small to identify unambiguous diagnostic sites. In future studies larger samples should be employed before defining eventual population-specific mutations.

Osteological and morphological differences seem to mark the border between northern and southern populations at latitudes corresponding to the Santa Catarina state (Pinedo, 1991). The 'central' population considered here was sampled in Parana state, which is located north of the arbitrary borderline proposed by Pinedo (1991). Yet, the PR stock was found to be highly differentiated from the northern population (RdJ), and showed closer similarity to the southern population (RS).

All but one of the 11 haplotypes analysed in this study were found only in one of the three surveyed regions, indicating that the three geographic sets investigated were genetically differentiated from each other. However, evidence for both present and historical genetic exchange between stock was detected. The most common haplotype (*h*1) in the PR region, was found in one individual in the RS region, suggesting that a certain degree of gene flow, at least between the two less differentiated stocks, occurs. Secchi *et al.* (1998) found that haplotype E, which was sampled in the northern population (RdJ), diverged strongly from the rest of the other northern haplotypes, and even more from the southern population (RS). This study found that haplotype E clusters together with haplotypes (*h*2, *h*5) which were found only in the central population (PR). This suggests that, at least historically, genetic interchange may have occurred also between the two most differentiated stocks (RdJ and PR). However, both present and historical genetic exchanges seem to be rare, or at least not mediated by female emigrants. Nuclear DNA analysis is required to detect eventual differences in the dispersal potential of the two sexes.

The minimum spanning network (MSN) in Fig. 3 shows that in each of the three populations the most common haplotypes are simultaneously connected to each other. This effect can be used as an indicator of the degree of homoplasy among haplotypes and would suggest that the different populations have been isolated for long enough to allow haplotypes to evolve independently. However, additional samples from other areas are required to clarify whether contemporary isolation by distance or historical isolation by distance followed by contemporary fragmentation is the model that better fits the franciscana population structure.

The northern population (RdJ) was found to be the least variable. This might be associated to the marginal position of the RdJ stock in comparison to the species distribution range. Low variability in peripheral districts could be justified either by founder effects, due to colonisation in a expanding population, or by drift effects, due to 'border erosion' in a declining population. The franciscana dolphin's exploitation history is such that the latter possibility is the most likely. In a 20-year survey in the Rio Grande do Sul region, Pinedo and Polacheck (1999) noted a decreasing stranding rate in franciscanas despite a substantial increase in fishing effort, suggesting a probable decline in franciscana abundance. It would be interesting to examine the status of the franciscana population in Argentinean and Uruguayan waters, to see whether a similar effect is noticeable at the southern extremity of this species distribution or whether the franciscanas are less threatened in (or better adapted to) colder and more productive waters.

Conservation and status of the franciscana dolphin

Secchi *et al.* (1998) found franciscanas of the RdJ region to be significantly less variable than conspecifics sampled off Rio Grande do Sul (RS). These two regions are separated by about 1,600km of coastline. In this study, the RdJ samples remained the least variable population also when the comparison included a third population (PR) located approximately 600km from the first. The extent of the differentiation between RdJ and the other populations suggests that the RdJ population should be treated as a

separate management unit. Combined with the morphological differentiation, elevation to ESU (evolutionary significant unit; Moritz, 1994) status may be justified. However, further work, including the addition of data from nuclear markers, is required before such a classification could be made with confidence. Although nuclear data were not available for all three populations, it is interesting to note that in 5 of the 10 nuclear loci surveyed in this study, the two RS samples carried private alleles which were not found in any of the 13 PR specimens, suggesting nuclear (as well as mitochondrial) differentiation across regions.

On a more practical level, the data suggest that the number of genetically distinguished stocks (3) exceeds that of morphologically conserved forms (2), suggesting that the identification of appropriate units for conservation in the franciscana can not rely solely on morphological evidence. It is therefore recommended that further molecular investigation for this species, especially in the southern extremity of its distribution (Argentinean and Uruguayan waters) be undertaken.

It should also be noted, from a conservation perspective, that the implications of tight matrilineal social structure are considerable. The impact of gillnet fisheries on the genetic diversity in this species would be more serious if the franciscana social structure is confirmed to be matrilineally determined. In this case, related individuals which may share rare alleles or mitochondrial haplotypes have a higher chance of being removed from the population simultaneously than if swimming dispersed. So the near-shore fishery should be carefully regulated, at least on a local scale, where diversity is the lowest (RdJ). RdJ should have a high conservation priority.

ACKNOWLEDGEMENTS

We are grateful to Eduardo Secchi for providing the two RS samples and to Bill Amos, Simon Goodman and two anonymous referees for their valuable comments on the manuscript. This research was possible thanks to the financial and technical support of the Fundação o Boticário de Proteção à Natureza (Brazil).

REFERENCES

Amos, B., Schlötterer, C. and Tautz, D. 1993. Social structure of pilot whales revealed by analytical DNA profiling. *Science* 260(5108):670-2.

Baker, C.S., Perry, A., Bannister, J.L., Weinrich, M.T., Abernethy, R.B., Calambokidis, J., Lien, J., Lambertsen, R.H., Urbán, J., Vasquez, O., Clapham, P.J., Alling, A., O'Brien, S.J. and Palumbi, S.R. 1993. Abundant mitochondrial DNA variation and world-wide population structure in humpback whales. *Proc. Natl Acad. Sci. USA* 90:8239-43.

Brenner, C.H. 1983. Calculation of paternity index. pp. 632-8. *In:* R. Walker (ed.) *Inclusion Probabilities in Parentage Testing*. American Association of Blood Banks, Arlington. 673pp.

Brownell, R.L. 1989. Franciscana-*Pontoporia blainvillei* (Gervais and d'Orbigny,1844). pp. 45-7. *In:* S.H. Ridgway and R. Harrison (eds.) Vol. 4. *Handbook of Marine Mammals*. Academic Press, London and San Diego. 442pp.

Campbell, N.J.H., Harriss, F.C., Elphinstone, M.S. and Baverstock, P.R. 1995. Outgroup heteroduplex analysis using temperature gradient gel electrophoresis: high resolution, large scale, screening of DNA variation in the mitochondrial control region. *Mol. Ecol.* 4:407-18.

Clutton-Brock, T.H. 1989. Mammalian mating systems. *Proc. R. Soc. Lond. Ser. B.* 236:339-72.

Connor, R.C., Read, A.J. and Wrangham, R. 2000. Male reproductive strategies and social bonds. pp. 247-69. *In:* J. Mann, R.C. Connor, P.L. Tyack and H. Whitehead (eds.) *Cetacean Societies. Field Studies of Dolphins and Whales*. Chicago University Press, Chicago. 433pp.

Crespo, E.A., Harris, G. and González, R. 1998. Group size and distributional range of the franciscana, *Pontoporia blainvillei*. *Mar. Mammal Sci.* 14(4):845-9.

Excoffier, L. and Smouse, P.E. 1994. Using allele frequencies and geographic subdivision to reconstruct gene trees within a species: molecular variance parsimony. *Genetics* 136:343-59.

Hoelzel, A.R., Dahlheim, M.E. and Stern, S.J. 1998. Low genetic variability among killer whales (*Orcinus orca*) in the eastern North Pacific and genetic differentiation between foraging specialists. *J. Hered.* 89:121-8.

Kasuya, T. 1984. River dolphins. pp. 178-9. *In:* D. MacDonald (ed.) *The Encyclopedia of Mammals*. Facts on File Publications, Inc., New York. 895pp.

Krützen, M., Valsecchi, E., Condor, R.C. and Sherwin, W.B. 2001. Characterization of microsatellite loci in *Tursiops aduncus*. *Mol. Ecol. Notes* 1:10.

Moritz, C. 1994. Applications of mitochondrial DNA analysis in conservation: a critical review. *Mol. Ecol.* 3:401-11.

Nei, M. (ed.). 1987. *Molecular Evolutionary Genetics*. Columbia University Press, New York. x+512pp.

Pinedo, M.C. 1991. Development and variation of the franciscana, *Pontoporia blainvillei*. Masters Thesis, University of California, Santa Cruz. 406pp.

Pinedo, M.C. 1994. Review of small cetacean fishery interactions in southern Brazil with special reference to the franciscana, *Pontoporia blainvillei*. *Rep. int. Whal. Commn* (special issue) 15:251-9.

Pinedo, M.C. and Polacheck, T. 1999. Trends in franciscana (*Pontoporia blainvillei*) strandings rates in Rio Grande do Sul, Southern Brazil (1979-1998). *J. Cetacean Res. Manage.* 1(2):179-89.

Pinedo, M.C., Praderi, R. and Brownell, R.L. 1989. Review of the biology and status of the franciscana, *Pontoporia blainvillei*. pp. 46-51. *In:* W.F. Perrin, R.L. Brownell, K. Zhou and J. Liu (eds.) *Biology and Conservation of the River Dolphins. Occas. Pap. IUCN SSC 3*. IUCN, Gland, Switzerland.

Rice, D.W. 1998. *Marine Mammals of the World. Systematics and Distribution.* Special Publication No. 4. The Society for Marine Mammology, Lawrence, Kansas. 231pp.

Saitou, N. and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4(4):406-25.

Schlötterer, C., Amos, W. and Tautz, D. 1991. Conservation of polymorphic simple sequence loci in cetacean species. *Nature, Lond.* 354:67-8.

Schneider, S., Roessli, D. and Excoffier, L. 2000. *Arlequin ver. 2.000: A Software for Population Genetics Data Analysis*. Genetics and Biometry Laboratory, University of Switzerland, Geneva. [Available at: *http://anthro.unige.ch/arlequin*].

Secchi, E.R., Zerbini, A.N., Bassoi, M., Dalla Rosa, L., Moller, L.M. and Rocha-Campos, C.C. 1997. Mortality of franciscanas, *Pontoporia blainvillei*, in coastal gillnetting in southern Brazil: 1994-1995. *Rep. int. Whal. Commn* 47:653-8.

Secchi, E.R., Wang, J.Y., Murray, B.W., Rocha-Campos, C.C. and White, B.N. 1998. Population differentiation in the franciscana (*Pontoporia blainvillei*) from two geographic locations in Brazil as determined from mitochondrial DNA control region sequences. *Can. J. Zool.* 76:1,622-7.

Shinohara, M., Domingo-Roura, X. and Takenaka, O. 1997. Microsatellites in the bottlenose dolphin *Tursiops truncatus*. *Mol. Ecol.* 6:695-6.

Valsecchi, E. and Amos, W. 1996. Microsatellite markers for the study of cetacean populations. *Mol. Ecol.* 5:151-6.

Wright, S. 1921. Systems of mating. *Genetics* 6:111-78.

Date received: June 2001
Date accepted: July 2002