Journal of Helminthology, page 1 of 9
doi:10.1017/S0022149X13000540

Genetic diversity and population structure of *Synthesium pontoporiae* (Digenea, Brachycladiidae) linked to its definitive host stocks, the endangered Franciscana dolphin, *Pontoporia blainvillei* (Pontoporiidae) off the coast of Brazil and Argentina

J. Marigo[1,2,4*†], H.A. Cunha[3], C.P. Bertozzi[4], S.P. Souza[5], F.C.W. Rosas[6], M.J. Cremer[7], A.S. Barreto[8], L.R. de Oliveira[9], H.L. Cappozzo[10], A.L.S. Valente[11], C.P. Santos[2] and A.C.P. Vicente[1]

[1]Laboratório de Genética Molecular de Microorganismos, Instituto Oswaldo Cruz, FIOCRUZ, Rio de Janeiro, RJ, Brazil: [2]Laboratório de Avaliação e Promoção da Saúde Ambiental, Instituto Oswaldo Cruz, FIOCRUZ, Rio de Janeiro, RJ, Brazil: [3]Laboratório de Biodiversidade Molecular (UFRJ)/Laboratório de Mamíferos Aquáticos e Bioindicadores (MAQUA, UERJ), Rio de Janeiro, RJ, Brazil: [4]Projeto BioPesca, Praia Grande, SP, Brazil: [5]Instituto Terra & Mar, Ilhabela, SP, Brazil: [6]Instituto Nacional de Pesquisas da Amazônia, Manaus, AM, Brazil: [7]Universidade da Região de Joinville, Joinville, SC, Brazil: [8]Universidade do Vale do Itajaí, Balneário Camboriú, SC, Brazil: [9]Laboratório de Ecologia de Mamíferos Universidade do Vale do Rio dos Sinos (UNISINOS), Grupo de Estudos de Mamíferos Aquáticos do Rio Grande do Sul (GEMARS), Porto Alegre, RS, Brazil: [10]Museo Argentino de Ciencias Naturales 'Bernardino Rivadavia' (MACN-CONICET)/Universidad Maimónides y Fundación Azara, CABA, Buenos Aires, Argentina: [11]Universidade Federal de Pelotas, Pelotas, RS, Brazil

(Received 17 January 2013; Accepted 9 July 2013)

Abstract

Pontoporia blainvillei (Gervais and d'Orbigny, 1844) is an endangered small cetacean endemic to South America with four Franciscana Management Areas (FMA) recognized as different population stocks. The role of the intestinal parasite *Synthesium pontoporiae* (Digenea: Brachycladiidae) as a possible biological marker to differentiate *P. blainvillei* stocks was evaluated using

[†]Present address: Laboratório de Patologia Comparada de Animais Selvagens (LAPCOM), Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Av. Prof. Dr. Orlando Marques de Paiva 87, 05508-270, Cidade Universitária, São Paulo, Brazil.
*E-mail: jumarigo@hotmail.com

nuclear and mitochondrial DNA markers. Internal transcribed sequence 1 and 2 (ITS1 and ITS2) regions of *S. pontoporiae* did not show intraspecific variability. The mitochondrial NADH dehydrogenase subunit 3 (ND3) and cytochrome oxidase subunit I (COI) gene sequences suggested lack of population structure in *S. pontoporiae* and population expansion. The apparent panmixia of *S. pontoporiae* may be due to the high mobility of one or more of its intermediary hosts. Alternatively, it may be due to the small sample size. This result is incongruent with the previously proposed FMA.

Introduction

The Franciscana, *Pontoporia blainvillei* Gervais and d'Orbigny 1844, is a small dolphin endemic to the western South Atlantic, whose occurrence ranges from Espírito Santo, Brazil (18°25′S), to Golfo San Matias, Argentina (42°35′S) (Siciliano *et al.*, 2002). The greatest threat to *P. blainvillei* is its incidental capture in coastal gillnets throughout most of its distribution and it is one of the most threatened small cetacean species in the western South Atlantic (IBAMA, 2001; Secchi *et al.*, 2003). However, the comprehensive impact of the by-catch on populations is still unknown due to uncertainties about stock structure and lack of abundance estimates for most of the areas. These topics have been considered research priorities for this species in several meetings, workshops and action plans during the past decades (Secchi *et al.*, 1998, 2002; Mendez *et al.*, 2007, 2010; Barbato *et al.*, 2011). Secchi *et al.* (2003) proposed four management stocks (known as Franciscana Management Areas or FMAs), based on data available on life history, parasite load and genetics. Recently, the subdivision of Franciscanas off the coast of Santa Catarina into two stocks (Ott *et al.*, 2008) and from Argentina and Uruguay into at least two genetically recognizable populations each (Mendez *et al.*, 2007; Costa-Urrutia *et al.*, 2011) was verified. This demonstrates a significant genetic subdivision at regional levels and fine-scale structure within *P. blainvillei* populations.

Multidisciplinary approaches have been used to investigate host population structure, using a variety of parameters, including biological tags such as parasites. Parasites can often be used to identify subpopulations even where genetic studies fail to do so (e.g. MacKenzie, 2002). The term 'ecological stock' is used to describe subpopulations which are distinguished by behavioural differences, but there is still a considerable amount of gene flow among them (MacKenzie, 2002). It differs from the 'genetic or biological stock' that would represent reproductively isolated units that are genetically different from each other (Altukhov, 1981; Wang, 2002). Up to the present moment, parasite load has been recommended (Reeves & Leatherwood, 1994; IBAMA, 2001) and used to identify ecological stocks of *P. blainvillei* (Aznar *et al.*, 1995; Andrade *et al.*, 1997; Marigo *et al.*, 2002; Secchi *et al.*, 2002), corroborating the hypothesis of segregated populations.

Recently, studies based on parasite genes have been used to elucidate the history or demography of host populations, revealing useful markers to indicate the source of host populations. Given that parasites are closely linked to their hosts, it might be expected that hosts would have similar phylogeographical patterns. Indeed, there is a relationship between host and parasite genetic differences related to their population historical biogeography (for a review see Criscione *et al.*, 2005) and this is influenced by transmission and which host's life cycle is analysed. Also, parasite populations can be more structured than their hosts, and through one parasite's molecular data it could be possible to identify its host population better than using the host's genotypes (Criscione *et al.*, 2006).

Molecular studies on helminths have increased over the past decade, especially among parasites of medical importance and also those of fish and other marine organisms (e.g. Semyenova *et al.*, 2003; Théron *et al.*, 2004; Morgan *et al.*, 2005; Shrivastava *et al.*, 2005; Mattiucci & Nascetti, 2007; Zhu *et al.*, 2007; Attwood *et al.*, 2008; Mattiucci *et al.*, 2008; Klimpel *et al.*, 2010). From the Brachycladiidae family (Trematoda: Digenea), the marine mammal parasites, only the 18S rDNA gene and mtDNA NADH dehydrogenase subunit 3 (ND3) gene sequences are available and have been used in phylogenetic studies (Fernández *et al.*, 1998a, b). In a recent phylogenetic study using nuclear 18S and mitochondrial ND3 genes, the genus *Synthesium*, represented by *Synthesium pontoporiae* (Raga, Aznar, Balbuena and Dailey, 1994) and *S. tursionis* (Marchi, 1873), was positioned among brachycladids (Marigo *et al.*, 2011), but no genetic intraspecific studies have been performed up to the present moment.

Synthesium pontoporiae is a small intestinal trematode parasite with adult worms exclusively in *P. blainvillei* which has been suggested as a biological marker for the species and shows differences in prevalence and mean intensity along its distribution (Aznar *et al.*, 1995; Marigo *et al.*, 2002). Marigo *et al.* (2002) reported significant differences in the prevalence and mean intensity of *S. pontoporiae* among three areas with a small sample size (12–17 intestines) as preliminary data; however, with a larger number of intestines analysed, these numbers greatly changed (Marigo, pers. obs.), possibly due to seasonality. The next step was to investigate the parasite morphometrics, and no significant difference was found among areas that could indicate different ecological parasite stocks (Marigo *et al.*, 2008) Thus, based on the main aim of this study, which was to identify Franciscana stocks through parasites, we decided to use the molecular approach. For the investigation of large-scale evolutionary patterns and the internal structure of this species, molecular markers have been used with increased frequency and robustness. Since studies on the intraspecific level usually deal with recent evolutionary processes, the most useful markers, in these cases, are rapidly

evolving DNA regions, such as mitochondrial DNA (mtDNA) (Avise, 2008).

Here, the first internal transcribed sequence (ITS) and cytochrome oxidase I (COI) sequences of the genus *Synthesium* were recovered from *S. pontoporiae* from different geographical areas. Also, the presence of polymorphisms in these markers and ND3 was investigated in order to relate them to intraspecific variation that could indicate stock differentiation of their host populations. This is the first study on population genetics of a marine mammal trematode.

Materials and methods

Sample collection, DNA extraction, amplification and sequencing

Synthesium pontoporiae specimens were collected from the intestine of *P. blainvillei* individuals stranded or incidentally captured off the south-eastern and southern Brazilian and Argentinian coasts (fig. 1). Their collection and transport for research were authorized by local environmental agencies. Intestines were frozen after necropsy and thawed for parasite inspection. Trematodes were fixed and conserved in 70% ethanol for up to 10 years. Each parasite used was collected from a different individual host and named after host number and location ($N = 131$). Seven areas were sampled: São Paulo North (SPN); São Paulo Central (SPC); São Paulo South (SPS); Paraná (PR); Santa Catarina (SC); Rio Grande do Sul (RS) and Argentina (ARG) (table 1).

DNA was extracted according to the QIAamp DNA Mini Kit (Qiagen, Los Angeles, California, USA) protocol. ITS regions were amplified using two sets of primers, for ITS1: 5′-GACGACCAAACTTGATCATT-3′ and 5′-TGCGCTCTTCATCGACACACGA-3′ (this study); and for ITS2: NC13F 5′-ATCGTGAAGAACGCAGC-3′ and NC2R 5′-TTAGTTTCTTTTCCTCCGCT -3′ (Zhu *et al.*, 2000).

The ND3 gene was amplified using two sets of primers:

(a) ND3A 5′-GCGTTAGCAGGATCCTGTGATATAG-3′ and ND3B 5′-CCAAAGCTTAAATCATCGTTAGCAG-3′; and
(b) ND3C 5′-CTACTAGTGAGATTGATCTYCGTCGGT-3′ and ND3D 5′-CTACTAGTCCCACTCAACRTAACCYT-3′ (Fernández *et al.*, 1998a).

The COI gene was amplified by polymerase chain reaction (PCR) using primers COIPRA 5′-TGGTTTTTTGTGCATCCTGAGGTTTA-3′ and 5′-AGAAGAACGTAATGAAAATGAGCAAC-3′ (Bessho *et al.*, 1992).

Amplification reactions contained 400 ng of DNA, 0.2 mM of deoxynucleoside triphosphates (dNTPs), 200 ng of each primer, 3 mM of $MgCl_2$ and 2 U of *Taq* DNA polymerase, in a final volume of 50 µl. PCR conditions were: initial denaturation at 94°C for 5 min, followed by 40 cycles at 94°C for 30 s, 47°C for 30 s; 72°C for 30 s. PCR amplicons were purified using Perfect Cleanup (Eppendorf, Hamburg, Germany) and directly sequenced on both strands in an ABI PRISM 3730 Genetic

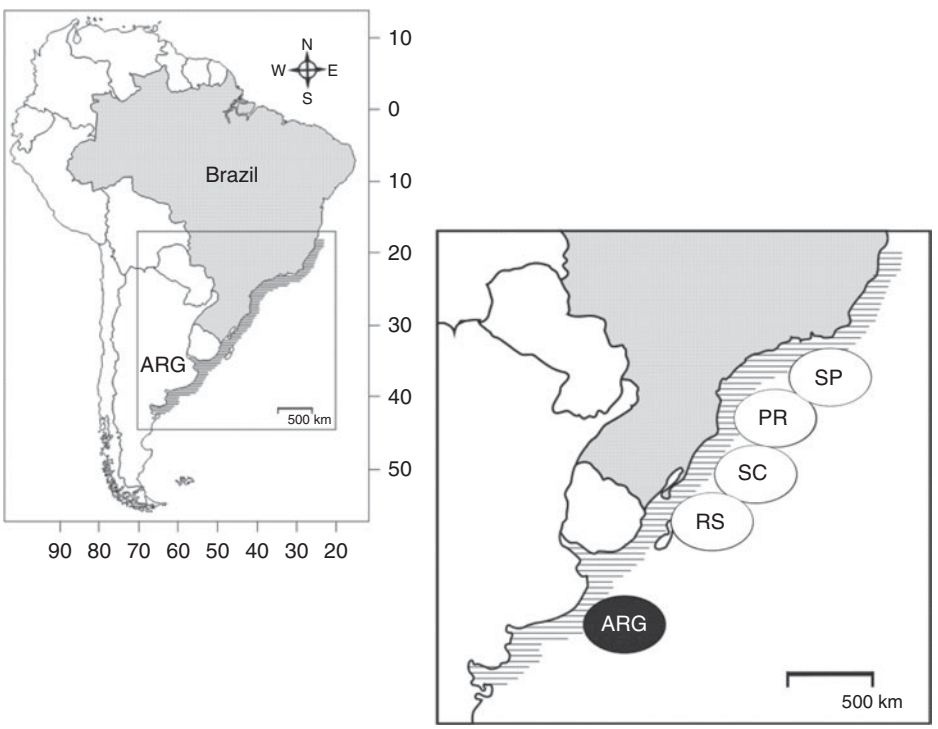

Fig. 1. Sampling sites of *Synthesium pontoporiae* from the endangered Franciscana dolphin off the coast of Brazil and Argentina: SP, São Paulo North, Central and South; PR, Paraná; SC, Santa Catarina; RS, Rio Grande do Sul; ARG, Argentina.

Table 1. The occurrence of *Synthesium pontoporiae* sequences in each amplified DNA region, relative to geographical location; see fig. 1 for full names of locations.

	Amplified DNA region				
Location	ITS1	ITS2	ND3	COI	COI + ND3
SPN	6	6	6	6	6
SPC	7	7	8	6	6
SPS	3	4	6	6	5
PR	2	1	2	2	1
SC	7	6	6	6	4
RS	3	3	3	3	3
ARG	3	2	6	5	5
No. of sequences	31	29	37	34	30

Analyzer (Applied Biosystems, Foster City, California, USA) from both strands. Raw sequences were transferred to DNASTAR software (Lasergene software, Madison, Wisconsin, USA) and the consensus sequences were determined. Sequences were aligned on CLUSTAL W (Thompson *et al.*, 1994). The ND3 amplicon included a tRNA sequence prior to the ND3 gene sequence; the analysis was performed using only the ND3 gene sequence.

Data analysis

Haplotypes were identified and the genetic diversity within populations was estimated using the DnaSP software (Rozas *et al.*, 2003). COI and ND3 sequences were concatenated for population analyses. The Arlequin 4.5 software (Excoffier *et al.*, 2005) was used for population structure analyses. Pairwise fixation indexes based on the haplotype diversity (F_{ST}) were estimated and their significance was ascertained by 10,000 random permutations. The variation distribution of the genetic diversity within and between populations was inferred and tested for different scenarios (two to five populations, see table 3) using an analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992). Spatial AMOVA (SAMOVA, Dupanloup *et al.*, 2002) was also used to search groupings of geographically adjacent locations that had the highest amount of variation among them.

The *S. pontoporiae* population size oscillations over time were also investigated through a Bayesian skyline plot method implemented in the BEAST v.1.3 software (Drummond & Rambaut, 2007) using COI sequences. Demographic reconstruction used the above-mentioned evolutionary rate, the HKY +G mutation model as selected using ModelTest (Posada, 2008) and 10 group intervals. Ten million Markov chain Monte Carlo (MCMC) steps and a burning of 1,000,000 were used to achieve effective sample size (ESS) values > 200 for all parameters.

Statistical parsimony networks for each mitochondrial gene and the concatenated sequence were built using TCS 1.21 (Clement *et al.*, 2000). Ambiguities in the network of concatenated COI and ND3 haplotypes were solved according to criteria of Crandall & Templeton (1993). The new nucleotide sequences reported in this paper for *S. pontoporiae* are available in GenBank under accession numbers: JX644084 (ITS1); JX6440845 (ITS2); JX644086–JX644122 (ND3) and JX644086–JX644156 (COI).

Results

All 31 ITS1 (534 bp) and 29 ITS2 sequences (447 bp) of *S. pontoporiae* were identical (monomorphic) in all locations studied, so they were not used for population analyses. A total of 37 sequences of the mtDNA ND3 of *S. pontoporiae*, spanning 331 bp, were analysed, revealing ten haplotypes defined by 13 variable sites. The G + C content was 0.351. Haplotype (Hd) and nucleotide diversity (per site, π) were 0.577 and 0.003, respectively. Thirty-four 416 bp long COI sequences were also analysed, revealing 24 haplotypes and 30 variable sites. The G + C content was 0.381. Haplotype diversity (Hd) was 0.932 and nucleotide diversity (π) was 0.007.

For population analyses, the ND3 and COI sequences of 30 single parasites were concatenated (747 bp). The unique sequence from PR was joined with sequences from SPS ($N = 5$), the closest locality (77 km). The highly divergent BP06SPC sequence was excluded during population differentiation analysis.

The F_{ST} pairwise values were non-significant for all site comparisons (table 2). Population differentiation scenarios based on stocks previously proposed for Franciscana dolphins in the literature were tested using AMOVA. No significant separation of populations was supported by AMOVA in any scenario proposed, suggesting the absence of geographical structuring and no restriction of gene flow among the parasites, despite their different locations. Hypothetical population divisions were also evaluated, but none of them was statistically significant (table 3). SAMOVA also failed to detect any significant grouping of geographically adjacent sampling localities. However, due to the small sample size, there is insufficient evidence to infer population structure.

Table 2. Pairwise F_{ST} values of *Synthesium pontoporiae* for COI and ND3 concatenated sequences relative to geographical location (SPN, SPC, SPS + PR, SC, RS, ARG; see fig.1 for the full names of locations).

Location	SPN	SPC	SPS + PR	SC	RS	ARG
SPN	–					
SPC	−0.062	–				
SPS + PR	−0.057	−0.028	–			
SC	−0.040	0.019	−0.084	–		
RS	−0.067	−0.029	−0.051	−0.125	–	
ARG	0.041	−0.018	−0.027	−0.020	−0.004	–

Table 3. Analysis of genetic population subdivisions (Φ_{sT}) through AMOVA, using COI and ND3 concatenated sequence data, for each population differentiation scenario (*P* values indicate the proportion of random values larger than observed values).

Number of population scenarios	Φ_{sT}	*P*
Two populations		
(SPN + SPC + SPSPR + SC) × (RS + ARG)	0.004	0.39
(SPN + SPC + SPSPR) × (SC + RS + ARG)	0.02	0.10
(SPN + SPC + SPSPR + SC + RS) × (ARG)	0.0006	0.48
Three populations		
(SPN + SPC + SPSPR) × (SC) × (RS + ARG)	0.02	0.21
(SPN + SPC + SPSPR) × (SC + RS) × (ARG)	0.02	0.15
(SPN + SPC + SPSPR + SC) × (RS) × (ARG)	0.001	0.48
Four populations		
(SPN + SPC + SPSPR) × (SC) × (RS) × (ARG)	0.02	0.27
(SPN + SPC) × (SPSPR + SC) × (RS) × (ARG)	0.03	0.14
(SPN) × (SPC) × (SPSPR + SC) × (RS + ARG)	0.02	0.27
Five populations		
(SPN + SPC) × (SPSPR) × (SC) × (RS) × (ARG)	0.03	0.25
(SPN) × (SPC) × (SPSPR) × (SC) × (RS + ARG)	0.006	0.30
(SPN) × (SPC) × (SPSPR + SC) × (RS) × (ARG)	0.026	0.30
(SPN) × (SPC) × (SPSPR) × (SC + RS) × (ARG)	0.02	0.34

In the TCS haplotype network, each connection is a single mutational step, with white circles representing inferred haplotypes (extinct/unsampled), and coloured circles representing the actual observed haplotypes. The sizes of circles are proportional to the number of individuals that have that haplotype. The networks of both markers and the concatenated sequence also suggest a lack of population structure, since samples from all localities are scattered throughout the networks. However, another pattern was observed in the haplotype networks: a star-like conformation usually attributed to recent population expansion in both markers studied, with the exception of COI (fig. 2). The Bayesian skyline plot (fig. 3) also indicates population expansion, and estimates that all *S. pontoporiae* sequences coalesced around 167,840 (87,590–269,360) years ago.

Discussion

Molecular studies on digenean trematodes with an indirect life cycle are more often motivated by the need to understand the epidemiology of parasites of medical and economical importance, such as *Schistosoma* (Shrivastava *et al.*, 2005; Attwood *et al.*, 2008). Additionally, other studies have focused on the genetic structure of digeneans from marine habitats (Vilas *et al.*, 2003; Prugnolle *et al.*, 2005a; Criscione *et al.*, 2006, 2011; Hansen & Poulin, 2006; Criscione & Blouin, 2007). The present study analysed *S. pontoporiae*, aiming to reveal polymorphism labelling the previously recognized stocks of its definitive host, the endangered *P. blainvillei* dolphin.

Preliminary data on infection levels of *S. pontoporiae* along its host distribution (Marigo *et al.*, 2002; Secchi *et al.*, 2002) corroborated the hypothesis of different *P. blainvillei* stocks (Pinedo, 1991; Secchi *et al.*, 1998; Mendez *et al.*, 2007) and the putative stock subdivision proposed by Secchi *et al.* (2003). However, this conclusion changed later when more intestinal samples were analysed (Marigo, pers. obs.) and the morphometry of the parasite was assessed. We searched for possible genetic differences among these morphologically homogeneous parasites that exhibited latitudinal variation in infection level. The ITS, COI and ND3 sequences of *S. pontoporiae* from three Franciscana management stocks did not show any polymorphism that could be associated with the genetic structure of the species.

Host vagility should be a major determinant of parasite gene flow because many parasites have low dispersal capacity in their free-living stages, comparing the limited mobility of molluscs to that of rats as intermediate hosts for *Schistosoma mansoni*, for instance (Prugnolle *et al.*, 2005b). As a consequence, the most mobile host can control the gene flow in a parasite with a complex life cycle (Jarne & Théron, 2001; Criscione *et al.*, 2005; Prugnolle *et al.*, 2005b). Although dolphins are considered highly mobile, *P. blainvillei* individuals satellite-tracked in Argentina exhibited very restricted and localized movements, with small home ranges in bay areas (Bordino *et al.*, 2008). Furthermore, there are at least two genetically recognizable local populations of Franciscana dolphins in Argentina, and also in Uruguay (Mendez *et al.*, 2010; Costa-Urrutia *et al.*, 2011). If the life cycle of *S. pontoporiae* was restricted to *P. blainvillei*, or if intermediary hosts had lower vagility compared to genetically structured Franciscana, the parasite population would also be subdivided. However, it is important to mention that ecological stocks do not always reflect their differences of behaviour, seasonality, maturity and also morphology in their genes, consequently they will not be recognized as genetic stocks (Meyer *et al.*, 1990).

In this context, taking into account only the genetics of the parasites of Franciscana dolphins, our results showed no structure due to: (1) the existence of apparently a single parasite population; or (2) the small sample size that would have caused lack of power in pairwise comparisons and hierarchical AMOVA. When discussing stock identification results and inadequate statistical power, Wang (2002) mentioned that in this case, 'the appropriate conclusion would be that differences in the characters examined were not detected rather than differences do not exist between the units being studied'. Therefore, we would have to improve the sample size of our parasites to meet the first explanation. However, if the first explanation was right (a single parasite population) we suggest that this lack of structure could be due to some prey of *P. blainvillei* involved in the *S. pontoporiae* life cycle that is more mobile than the definitive host and therefore could carry the parasites across FMAs.

The life cycle of *Synthesium* is unknown and, for digeneans in marine mammals, larval stages including metacercariae are inadequately described. The digenean life cycle involves two asexual generations in a molluscan and a sexual one in a vertebrate host and, in the majority of cases, one or more intermediary hosts are needed to complete the life cycle (Gibson, 2002). The Franciscana diet includes cephalopods, crustaceans and fish, and many of those are species that spend most of their adult lives offshore and migrate to estuarine and protected areas to spawn (Sardiña & Lopez Cazorla, 2005; Rodrigues & Gasalla, 2008). Any of these animals may be part of the cycle, although no brachycladiid cercaria or metacercariae

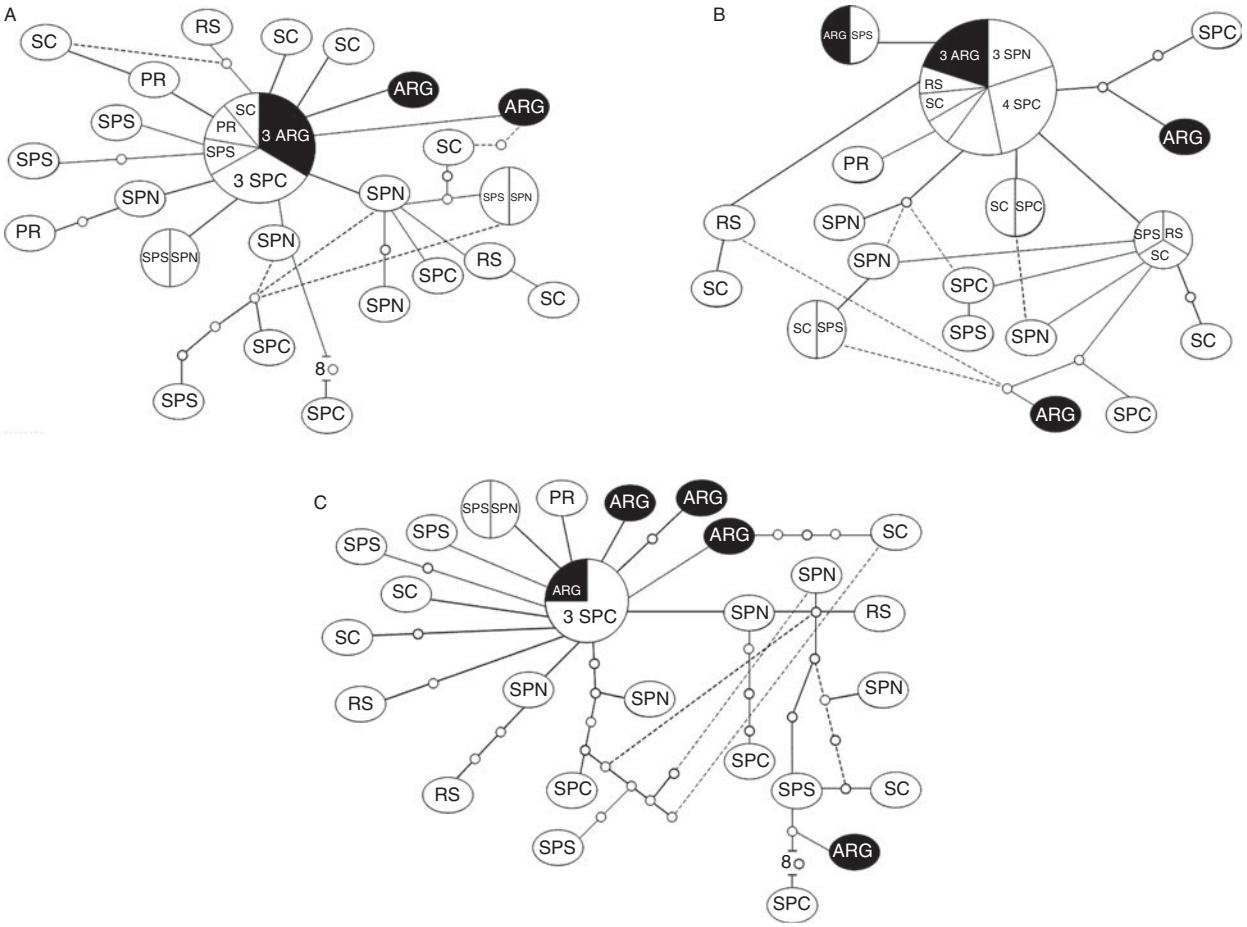

Fig. 2. Haplotype network drawn from (A) 331 bp of ND3; (B) 416 bp of CO1 and (C) 747 bp of ND3 and CO1 gene sequences from seven geographical locations: SP, São Paulo North, Central and South; PR, Paraná; SC, Santa Catarina; RS, Rio Grande do Sul; ARG, Argentina. Each cross bar along network connectors represents one mutational step, and small white circles represent extinct/unsampled haplotypes.

have been found inside them so far. In coastal areas, *S. pontoporiae* adults present in Franciscanas, as regular digenean trematodes, shed their eggs and the cycle is completed in the presence of these molluscs and fish.

Considering that the possible intermediary hosts are more mobile than the Franciscana dolphin, our data would support the hypothesis that the most mobile host can influence the gene flow in a parasite with complex life cycle (Jarne & Théron, 2001; Criscione *et al.*, 2005; Prugnolle *et al.*, 2005b). In a similar study, Baldwin *et al.* (2011) found that anisakid nematodes were not applicable as markers to identify population subdivision of Pacific sardines, possibly due to the migration of both fish and cetacean hosts mixing the haplotypes. Therefore, in view of the assumed vagility of intermediary hosts and/or the small sample size, precise conclusions on the structure of single populations of *S. pontoporiae* cannot be made.

In relation to the population expansion scenario, since *S. pontoporiae* occurs exclusively in Franciscanas, one potential explanation would be that the expansion reflected the colonization of the species by the parasite. Within the Brachycladiidae, *Synthesium* is a genus that exhibits a worldwide distribution and occurs in a number of odontocete families (Pontoporiidae, Delphinidae, Monodontidae and Phocoenidae), suggesting a long period of host–parasite interactions, with many potential host captures among different odontocete groups (Fernández *et al.*, 1998a). However, when and which *Synthesium* species adapted to its first odontocete host cannot be defined. It is possible to suggest that the ancestor of the *Synthesium* species could adapt first to other odontocete families and later to *Pontoporia*. The evolutionary and biological peculiarities of Franciscanas may be responsible for *S. pontoporiae* exclusivity as a parasite found only in *P. blainvillei*. The anatomical characteristics of *P. blainvillei* would have resulted in adaptations of the ancestral *S. pontoporiae*, and the reproductive isolation that followed caused the speciation.

Acknowledgements

This work is part of a PhD dissertation of J.M. developed at Instituto Oswaldo Cruz, FIOCRUZ, RJ, Brazil. We thank Katia Groch and Heloisa M. Diniz (Laboratório de

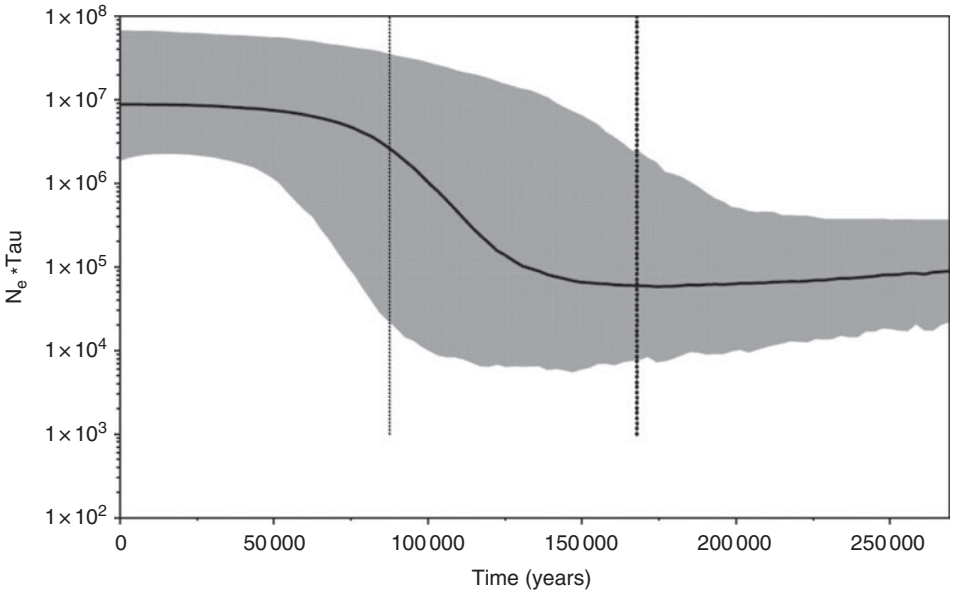

Fig. 3. Bayesian skyline plot with COI *Synthesium pontoporiae* sequences for the entire species (Ne, effective population size; tau, generation length in years).

Produção e Tratamento de Imagem-IOC) for technical assistance and the PDTIS/FIOCRUZ genomic platform for DNA sequencing. We are particularly grateful to those who provided intestines for parasite sampling: Projeto BioPesca, SP (V. Ruoppolo, J.A.Ribeiro, J.V.S. Lima, M.B. Alonso, F. Marcatto and B. Henning); Instituto Terra & Mar, São Paulo; Instituto de Pesquisas de Cananéia (IPeC); Centro de Estudos do Mar (CEM-UFPR); Universidade da Região de Joinville (UNIVILLE); Universidade do Vale do Itajaí (UNIVALI); Grupo de Estudos de Mamíferos Aquáticos do Rio Grande do Sul (GEMARS) (D. Danilewicz, I.B. Moreno, P.H. Ott, M. Tavares, R. Machado, S.B. Nakashima, C.C. Trigo); and Museo Argentino de Ciencias Naturales (MACN-CONICET) (M.F. Negri, M.N. Paso Viola, M.V. Panebianco, F. Pèrez).

Financial support

The project 'Research and Conservation of Aquatic Mammals and Sea Turtles in the Rio Grande do Sul' ('Pesquisa e Conservação de Mamíferos Marinhos e Tartarugas Marinhas no Litoral Norte do Rio Grande do Sul') was supported by Fundação O Boticário de Proteção à Natureza and Fundo Nacional do Meio Ambiente – FNMA/MMA (Convenio 094/2001), which provided financial support especially for sampling Franciscanas along the coast of Rio Grande do Sul. We also thank the Cetacean Society International, the Society for Marine Mammalogy, Yaqu Pacha, Project AWARE-PADI, MacArthur Foundation, Fundação O Boticário de Proteção à Natureza and PROBIO/FNMA for the financial support of the collaborating institutions.

Conflict of interest

The authors declare no conflict of interest.

Ethical standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals.

References

Altukhov, Y.P. (1981) The stock concept from the viewpoint of population genetics. *Canadian Journal of Fisheries and Aquatic Sciences* **38**, 1523–1538.

Andrade, A.L.V., Pinedo, M.C. & Pereira, J. Jr (1997) Franciscanas from southern Brazil, Uruguay and North Argentina coasts: are one or more ecological stocks? *Reports of the International Whaling Commission* **47**, 669–673.

Attwood, S.W., Fatih, F.A. & Upatham, E.S. (2008) DNA-sequence variation among *Schistosoma mekongi* populations and related taxa; phylogeography and the current distribution of Asian schistosomiasis. *PLoS Neglected Tropical Diseases* **2**, e200.

Avise, J.C. (2008) Phylogeography: retrospect and prospect. *Journal of Biogeography* **36**, 3–15.

Aznar, F.J., Raga, J.A., Corcuera, J. & Monzón, F. (1995) Helminths as biological tags for franciscana (*Pontoporia blainvillei*) (Cetacea, Pontoporiidae) in Argentinian and Uruguayan waters. *Mammalia* **59**, 427–435.

Baldwin, R.E., Rew, M.B., Johansson, M.L., Banks, M.A. & Jacobson, K.C. (2011) Population structure of three species of *Anisakis* nematodes recovered from Pacific sardines (*Sardinops sagax*) distributed throughout the California current system. *Journal of Parasitology* **97**, 545–554.

Barbato, B.H.A., Secchi, E.R., Di Beneditto, A.P.M., Ramos, R.M.A., Bertozzi, C., Marigo, J., Bordino, P. & Kinas, P.G. (2011) Geographical variation in franciscana (*Pontoporia blainvillei*) external morphology. *Journal of the Marine Biological Association of the United Kingdom* **92**, 1645–1656.

Bessho, Y., Ohama, T. & Osawa, S. (1992) Planarian mitochondria II. The unique genetic code as deduced from cytochrome *c* oxidase subunit I gene sequences. *Journal of Molecular Evolution* **34**, 331–335.

Bordino, P., Wells, R.S. & Stamper, M.A. (2008) Satellite tracking of Franciscana Dolphins *Pontoporia blainvillei* in Argentina: preliminary information on ranging, diving and social patterns. *International Whaling Commission Scientific Committee Paper SC/53/IA32 presented to the IWC Scientific Committee (unpublished manuscript), June 2008, Santiago, Chile*.

Clement, M., Posada, D. & Crandall, K.A. (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology* **9**, 1657–1659.

Costa-Urrutia, P., Abud, C., Secchi, E.R. & Lessa, E.P. (2011) Population genetic structure and social kin associations of franciscana dolphin, *Pontoporia blainvillei*. *Journal of Heredity* **103**, 92–102.

Crandall, K. & Templeton, A.R. (1993) Empirical tests of some predictions from coalescent theory with applications to intraspecific phylogeny reconstruction. *Genetics* **134**, 959–969.

Criscione, C.D. & Blouin, M.S. (2007) Parasite phylogeographical congruence with salmon host evolutionarily significant units: implications for salmon conservation. *Molecular Ecology* **16**, 993–1005.

Criscione, C.D., Poulin, R. & Blouin, M.S. (2005) Molecular ecology of parasites: elucidating ecological and microevolutionary processes. *Molecular Ecology* **14**, 2247–2257.

Criscione, C.D., Cooper, B. & Blouin, M.S. (2006) Parasite genotypes identify source populations of migratory fish more accurately than fish genotypes. *Ecology* **87**, 823–828.

Criscione, C.D., Vilas, R., Paniagua, E. & Blouin, M.S. (2011) More than meets the eye: detecting cryptic microgeographic population structure in a parasite with a complex life cycle. *Molecular Ecology* **20**, 2510–2524.

Drummond, A.J. & Rambaut, A. (2007) BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology* **7**, 214.

Dupanloup, I., Schneider, S. & Excoffier, L. (2002) A simulated annealing approach to define the genetic structure of populations. *Molecular Ecology* **11**, 2571–2581.

Excoffier, L., Smouse, P.E. & Quattro, J.M. (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* **491**, 479–491.

Excoffier, L., Laval, G. & Schneider, S. (2005) Arlequin (version 3.0): an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online* **1**, 47–50.

Fernández, M., Aznar, F.J., Latorre, A. & Raga, J.A. (1998a) Molecular phylogeny of the families Campulidae and Nasitrematidae (Trematoda) based on mtDNA sequence comparison. *International Journal for Parasitology* **28**, 767–775.

Fernández, M., Littlewood, D.T., Latorre, A., Raga, J.A. & Rollinson, D. (1998b) Phylogenetic relationships of the family Campulidae (Trematoda) based on 18S rRNA sequences. *Parasitology* **117**, 383–391.

Gibson, D.I. (2002) Trematodes in marine mammals: morphology, systematics and origins. pp. 59–63 *in Proceedings of the Tenth International Congress of Parasitology*. Vancouver, Canada. Bologna, Italy, Monduzzi Medimond Editore.

Hansen, E.K. & Poulin, R. (2006) Spatial covariation between infection levels and intermediate host densities in two trematode species. *Journal of Helminthology* **80**, 255–259.

IBAMA, (2001) *Mamíferos Aquáticos do Brasil: Plano de Ação – Versão II*. 706 pp. Brasília, IBAMA (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis, Ministério do Meio Ambiente).

Jarne, P. & Théron, A. (2001) Genetic structure in natural populations of flukes and snails: a practical approach and review. *Parasitology* **123**, S27–S40.

Klimpel, S., Busch, M., Kuhn, T., Rohde, A. & Palm, H. (2010) The *Anisakis simplex* complex off the South Shetland Islands (Antarctica): endemic populations versus introduction through migratory hosts. *Marine Ecology Progress Series* **403**, 1–11.

MacKenzie, K. (2002) Parasites as biological tags in population studies of marine organisms: an update. *Parasitology* **124**, S153–S163.

Marigo, J., Rosas, F.C.W., Andrade, A.L.V., Oliveira, M.R., Dias, R.A. & Catão-Dias, J.L. (2002) Parasites of Franciscana (*Pontoporia blainvillei*) from São Paulo and Paraná States, Brazil. *Latin American Journal of Aquatic Mammals* **1**, 115–122.

Marigo, J., Vicente, A.C.P., Valente, A.L.S., Measures, L. & Santos, C.P. (2008) Redescription of *Synthesium pontoporiae* n. comb. with notes on *S. tursionis* and *S. seymouri* n. comb. (Digenea: Brachycladiidae Odhner, 1905). *Journal of Parasitology* **94**, 505–514.

Marigo, J., Thompson, C.C., Santos, C.P. & Iñiguez, A.M. (2011) The *Synthesium* Brachycladiidae Odhner, 1905 (Digenea) association with hosts based on nuclear and mitochondrial genes. *Parasitology International* **60**, 530–533.

Mattiucci, S. & Nascetti, G. (2007) Genetic diversity and infection levels of anisakid nematodes parasitic in fish and marine mammals from Boreal and Austral hemispheres. *Veterinary Parasitology* **148**, 43–57.

Mattiucci, S., Farina, V., Campbell, N., Mackenzie, K., Ramos, P., Pinto, A., Abaunza, P. & Nascetti, G. (2008) *Anisakis* spp. larvae (Nematoda: Anisakidae) from Atlantic horse mackerel: their genetic identification and use as biological tags for host stock characterization. *Fisheries Research* **89**, 146–151.

Mendez, M., Rosenbaum, H.C. & Bordino, P. (2007) Conservation genetics of the franciscana dolphin in northern Argentina: population structure, by-catch impacts, and management implications. *Conservation Genetics* **9**, 419–435.

Mendez, M., Rosenbaum, H.C., Subramaniam, A., Yackulic, C. & Bordino, P. (2010) Isolation by

environmental distance in mobile marine species: molecular ecology of franciscana dolphins at their southern range. *Molecular Ecology* **19**, 2212–2228.

Meyer, A., Kocher, T.D., Basasibwaki, P. & Wilson, A.C. (1990) Monophyletic origin of Lake Victoria cichlid fishes suggested by mitochondrial DNA sequence. *Nature* **347**, 550–553.

Morgan, J.A., Dejong, R.J., Adeoye, G.O., Ansa, E.D.O., Barbosa, C.S., Brémond, P., Cesari, I.M., Charbonnel, N., Corrêa, L.R., Coulibaly, G., *et al.*, (2005) Origin and diversification of the human parasite *Schistosoma mansoni*. *Molecular Ecology* **14**, 3889–3902.

Ott, P.H., Oliveira, L.R., Barreto, A.S., Secchi, E.R., Almeida, R., Moreno, I.B., Danilewicz, D. & Bonatto, S. (2008) Unidades de manejo da toninha, *Pontoporia blainvillei*: uma avaliação molecular do limite entre as FMAS II e III p. 81 in *XIII Reunión de Trabajo de Especialistas em Mamíferos Acuáticos de América del Sur y 7º. Congresso SOLAMAC (Sociedade Latino Americana de Especialistas em Mamíferos Aquáticos),* Montevideo, Uruguai, 13–17 October.

Pinedo, M.C. (1991) Development and variation of the franciscana (*Pontoporia blainvillei*). PhD thesis, University of California, Santa Cruz.

Posada, D. (2008) jModelTest: phylogenetic model averaging. *Molecular Biology and Evolution* **25**, 1253–1256.

Prugnolle, F., Liu, H., de Meeus, T. & Balloux, F. (2005a) Population genetics of complex life-cycle parasites: an illustration with trematodes. *International Journal of Parasitology* **35**, 255–263.

Prugnolle, F., Theron, A., Pointier, J.P., Jabbour-Zahab, R., Jarne, P., Durand, P. & de Meeûs, T. (2005b) Dispersal in a parasitic worm and its two hosts and its consequence for local adaptation. *Evolution* **59**, 296–303.

Reeves, R. & Leatherwood, S. (1994) *Dolphins, porpoises and whales: 1994–1998 Action Plan for the Conservation of Cetaceans*. Gland, Switzerland, IUCN (International Union for Conservation of Nature).

Rodrigues, A.R. & Gasalla, M.A. (2008) Spatial and temporal patterns in size and maturation of *Loligo plei* and *Loligo sanpaulensis* (Cephalopoda:Loliginidae) in southeastern Brazilian waters, between 23°S and 27°S. *Scientia Marina* **72**, 631–643.

Rozas, J., Sánchez-Delbarrio, J.C., Messeguer, X. & Rozas, R. (2003) DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics* **19**, 2496–2497.

Sardiña, P. & Lopez Cazorla, A. (2005) Feeding interrelationships and comparative morphology of two young sciaenids co-occurring in South-western Atlantic waters. *Hydrobiologia* **548**, 41–49.

Secchi, E.R., Wang, J.Y., Murray, B.W., Rocha-Campos, C.C. & 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. *Canadian Journal of Zoology* **76**, 1622–1627.

Secchi, E.R., Danilewicz, D., Ott, P.H., Ramos, R., Lazaro, M., Marigo, J. & Wang, J.Y. (2002) Report of the working group on stock identity. *Latin American Journal of Aquatic Mammals* **1**, 47–54.

Secchi, E.R., Danilewicz, D. & Ott, P.H. (2003) Applying the phylogeographic concept to identify franciscana dolphin stocks: implications to meet management objectives. *Journal of Cetacean Research and Management* **5**, 61–68.

Semyenova, S.K., Morozova, E.V., Chrisanfova, G.G., Asatrian, A.M., Movsessian, S.O. & Ryskov, A.P. (2003) RAPD variability and genetic diversity in two populations of liver fluke, *Fasciola hepatica*. *Acta Parasitologica* **48**, 125–130.

Shrivastava, J., Gower, C.M., Balolong, E., Wang, T.P., Qian, B.Z. & Webster, J.P. (2005) Population genetics of multi-host parasites – the case for molecular epidemiological studies of *Schistosoma japonicum* using larval stages from naturally infected hosts. *Parasitology* **131**, 617–626.

Siciliano, S., Di Beneditto, A.P.M. & Ramos, R.M.A. (2002) A toninha, *Pontoporia blainvillei* (Gervais and d'Orbigny, 1844) (Mammalia, Cetacea, Pontoporiidae), nos estados do Rio de Janeiro e Espírito Santo, costa sudeste do Brasil: Caracterizaçãoo dos habitats e fatores de isolamento das populações. *Boletim do Museu Nacional, Serie Zoologia* **476**, 1–15.

Théron, A., Sire, C., Rognon, A., Prugnolle, F. & Durand, P. (2004) Molecular ecology of *Schistosoma mansoni* transmission inferred from the genetic composition of larval and adult infrapopulations within intermediate and definitive hosts. *Parasitology* **129**, 571–585.

Thompson, J.D., Higgins, D.G. & Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**, 4673–4680.

Vilas, R., Paniagua, E. & Sanmartin, M.L. (2003) Genetic variation within and among infrapopulations of the marine digenetic trematode *Lecithochirium fusiforme*. *Parasitology* **126**, 465–472.

Wang, J.Y. (2002) Stock identity. pp. 1115–1118 *in* Perrin, W.F., Würsig, B. & Thewissen, J.G.M. (*Eds*) *Enciclopedia of marine mammals*. San Diego, Academic Press, Elsevier.

Zhu, X., Gasser, R.B., Jacobs, D.E., Hung, G.C. & Chilton, N.B. (2000) Relationships among some ascaridoid nematodes based on ribosomal DNA sequence data. *Parasitology Research* **86**, 738–744.

Zhu, X.Q., Podolska, M., Liu, J.S., Yu, H.Q., Chen, H.H., Lin, Z.X., Luo, C.B., Song, H.Q. & Lin, R.Q. (2007) Identification of anisakid nematodes with zoonotic potential from Europe and China by single-strand conformation polymorphism analysis of nuclear ribosomal DNA. *Parasitology Research* **101**, 1703–1707.