CLAUDIA HOLLATZ

DIVERSIDADE MOLECULAR DO BOTO-ROSA (*INIA GEOFFRENSIS*) DA AMAZÔNIA BRASILEIRA E DO BOTO-CINZA MARINHO (*SOTALIA GUIANENSIS*) DA BAÍA DE SEPETIBA E PARATY.

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DNAmt – DNA mitocondrial
RC – Região controladora
CYTB – citocromo b
IUCN – International Union for Conservation of Nature
IWC – International Whaling Comission
CITES - Convention on International Trade in Endangered Species of Wild Fauna and Flora
MM – Mamirauá
TF – Tefé
CO – Colombian Orinoco
CA – Colombian Amazon
BA – Bolivian Amazon
SBE- Sepetiba Bay Entrance
SBI – Sepeiba Bay Interior

RESUMO

O boto-rosa (*Inia geoffrensis*) é amplamente distribuído nas bacias do Amazonas e do Orinoco ocupando uma área total em torno de 7 milhões de km^2. A estrutura filogeográfica, o tempo de divergência e a história demográfica das linhagens de *Inia* foram analisados usando sequências do DNA mitocondrial e marcadores microssatélites em diferentes populações do boto-rosa em escala micro e macro-geográfica. Os nossos resultados mostram que as populações brasileiras ocupam uma posição filogenética intermediária em relação a outras três localidades: Colômbia na bacia do Orinoco e na bacia Amazônica e Amazônia boliviana. Todas as linhagens apresentaram uma estrutura filogegráfica bem definida com linhagens divergindo no Mioceno e ao longo do Plioceno-Pleistoceno. Seis haplótipos do DNA mitocondrial foram identificados e nenhum haplótipo foi compartilhado entre as duas populações brasileiras estudadas. Um haplótipo específico do citocromo *b* (*CYTB*) foi encontrado para cada uma das localidades brasileiras. Os nossos resultados sugerem uma alta filopatria das fêmeas com uma baixa dispersão observada entre distâncias curtas e longas distâncias. A discriminação das populações brasileiras não foi evidenciada com o uso dos marcadores microssatélites, mas os mesmos dados indicaram um recente declínio populacional dentro de cada localidade no Brasil.

O golfinho de estuário (*Sotalia guianensis*) ocorre desde a América Central até o sul do Brasil, sendo que a maioria das populações desta espécie vive em estuários. Este estudo analisou a estrutura genética da maior população de *S. guianensis* já relatada para essa espécie na Baía de Sepetiba e Paraty, na costa sudeste do Brasil. Embora a análise do DNA mitocondrial não tenha detectado nenhuma variabilidade genética, uma significatica estrutura populacional ficou evidenciada entre os machos com a análise de onze *loci* de microssatélites. A análise dos microssatélites permitiu distinguir entre indivíduos de duas baías adjacentes localizados há 60 km de distância e entre indivíduos provenientes de uma mesma baía, separados por aproximadamente 15 km de distância. Os resultados sugerem que diferenças ecológicas entre hábitats e especializações comportamentais possivelmente atuam na observada estruturação populacional. Ambas as espécies relatadas neste estudo são consideradas como deficiente de dados pela União Internacional para Conservação da natureza (IUCN). Os nossos resultados devem fornecer novos dados para a definição do *status* conservacionista destas espécies além de prover informações para o delineamento de estratégias de manejo de populações expostas à crescente degradação ambiental derivadas de atividades antropogênicas.

ABSTRACT

The pink dolphin (*Inia geoffrensis*) is widely distributed along the Amazon and Orinoco basins, covering an area of about 7 million km^2. We examined the phylogeographic structure, lineage time divergence and historical demography using mitochondrial DNA sequences and autosomal microsatellites in different *I. geoffrensis* populations distributed in a large and small spatial scale. Our results show that the Brazilian populations occupy an intermediate position related to three geographic locations: Colombian Amazon, Colombian Orinoco and Bolivian Amazon. All lineages presented well defined phylogeographic structure with lineage divergence in the Pliocene and occurring throughout the Pleistocene-Holocene. Six control region (CR) haplotypes were identified with no sharing between the two populations within Brazil and a specific cytochrome *b* (*CYTB*) haplotype was detected for each location. These results also suggest a strong female phylopatry for this species due to restriction of gene flow through long and short distances. Finally, although autosomal microsatellites could not discriminate the two proximate Brazilian populations, they provided support for a recent population bottleneck in both localities. The Guiana dolphin (*Sotalia guianensis*) is widely distributed in the Atlantic coast from Central America to South of Brazil and the species is mostly confined to estuarine environments. Here we examined the genetic diversity pattern in the largest populations of *S. guinanensis* inhabiting Sepetiba and Paraty embayment at the south-eastern coast of Rio de Janeiro, Brazil. Although mitochondrial DNA (mtDNA) failed to detect any variability, we found significant evidence of male population structure on the basis of eleven nuclear microsatellite loci. Surprisingly, microsatellite alleles were able to distinguish between individuals from the two embayments located only 60 km apart, whereas a low but also significant structure was displayed for the two socially groups within Sepetiba Bay, located less than 15 km apart. The results suggest that differences in habitat type and behavioral specializations are likely to explain the patterns of genetic structure. Both species analyzed in this study are considered Data Deficient by the World Conservation Union (IUCN). These findings should add data to define the conservation status of these species and provide baselines for the management of communities exposed to increased human-driven habitat loss.

1. INTRODUÇÃO GERAL

Os cetáceos constituem uma ordem altamente diversa de animais marinhos pertencentes à classe Mammalia. Estes são divididos em duas subordens: Mysticeti, incluindo as baleias com barbatanas e Odontoceti, representando as baleias com dentes. Atualmente 90 espécies de cetáceos são reconhecidas sendo que Odontoceti é a subordem mais diversa, com aproximadamente 75 espécies distribuídas em dez famílias (Rice, 1998). Algumas espécies possuem uma distribuição muito restrita como os golfinhos de rio, atualmente apenas quatro gêneros são exclusivamente de água doce (*Inia geoffrensis, Sotalia fluviatilis, Lipotes vexilifer* e *Platanista gangetica,*) sendo que dois são endêmicos dos rios da Amazônia brasileira (*Inia geoffrensis* e *Sotalia fluviatilis*). Um quinto gênero, *Pontoporia*, tem sido descrito como espécie fluvial, entretanto a espécie além de habitar o rio da Prata, ocorre amplamente na costa do Atlântico, do Espírito Santo (ao norte) à Argentina (ao sul).

Um elemento essencial na conservação dos cetáceos é o conhecimento de sua estrutura populacional. Os esforços de conservação devem ser direcionados na manutenção de um conjunto de fatores ecológicos, comportamentais e genéticos que garantam a viabilidade da espécie (Dizon e Perrin, 1997). Um desafio para os especialistas de cetáceos é identificar e definir o que são populações, conhecer o seu *status* de conservação e desenvolver estratégias que contribuam para a permanência destas populações no ambiente.

O conhecimento da estrutura genética populacional de uma espécie, de seus padrões de dispersão e a identificação de barreiras ao fluxo gênico pode auxiliar em uma melhor compreensão da biologia das espécies (Bilgmann *et al.*, 2007). Em organismos aquáticos, diversas condições ambientais podem influenciar na estruturação social e genética de cetáceos. Um desenho esquemático exemplificando as diversas condições é dado na Figura 1 (Hoelzel, 1998).

Deste modo, é importante selecionar marcadores genéticos apropriados com diferentes modos de herança para relatar com uma maior confiabilidade a biologia de uma espécie. O uso de marcadores do DNA mitocondrial e de marcadores nucleares, tais como os microssatélites, torna possível traçar linhagens maternas e paternas, permitindo algumas vezes estimativas temporais, de dispersão e de divergência entre as populações.

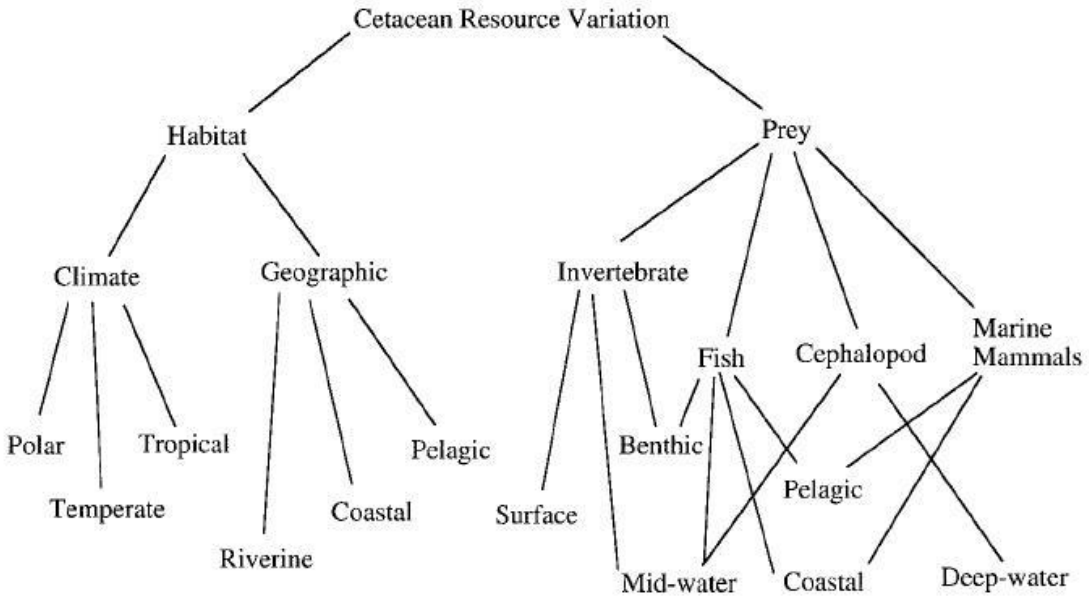

Figura 1. Representação esquemática dos tipos de variações de recursos utilizados pelos cetáceos. (Reproduzido a partir de Hoelzel *et al.*, 1998).

1.2. BREVE HISTÓRICO DA FILOGENIA DOS CETÁCEOS

Os cetáceos provavelmente surgiram há cerca de 60-55 milhões de anos atrás durante o Eoceno e por volta de 53 milhões de anos já formavam um grupo bem diversificado (Reeves *et al.*, 2008). A transição de cetáceos ancestrais, do ambiente terrestre para a água, foi acompanhada por uma série de adaptações morfológicas para uma vida aquática. Por este motivo, por um longo tempo foi difícil estabelecer a posição filogenética dos cetáceos baseando-se apenas na morfologia. Atualmente, muitos cientistas concordam que os parentes mais próximos dos cetáceos são os artiodáctilos, ungulados com número par de dedos nas patas (ex. vacas, camelos). Recentes estudos paleontológicos e genéticos sugerem que o hipopótamo é a animal vivente mais proximamente relacionado às baleias (Gatesy, 1997, 1999; Geisler and Uhen, 2003, 2005; Price *et al.*, 2005).

As relações filogenéticas entre as atuais famílias de cetáceos permanecem controversas. As possíveis reconstruções filogenéticas baseiam-se em dados morfológicos (Thewissen, 1994; Heyning, 1989), paleontológicos (Muizon 1991; Fordyce, 2001; Geisler and Sanders, 2003) e em evidências moleculares (Milinkovitch *et al..,* 1993, 1994; Arnason and Gulberg, 1996; Cassens *et al..,* 2000, Nikaido *et al.*, 2001; Arnason *et al.*, 2004; Agnarsson *et al.*, 2008) ou em uma combinação de todos (Milinkovitch 1995; Messenger and McGuire, 1998; Gatesy *et al.,* 1999, Hamilton *et al.*, 2001).

Estima-se que as duas subordens de cetáceos tenham divergido aproximadamente há 40-45 mihões de anos (McLeod *et al.*, 1993; Fordyce 2001; Xiong *et al.*, 2009). A subordem Odontoceti originou o grupo mais diverso, diferenciando-se em dez famílias. Os delfinídeos representam a família mais recente, tendo divergido dos phocenídeos no final do Mioceno, há 10-11 milhões de anos atrás (Figura 1). O escasso registro fóssil nos depósitos do Mioceno e Plioceno sugere que os delfinídeos são o provável resultado de uma radiação explosiva no final do Plioceno (Barnes, 1995).

A sistemática e as relações filogenéticas entre os golfinhos de rio permaneceram em debate ao longo de vários anos (Barnes, 1985, 1990; Heyning, 1989; Messenger and McGuire, 1998; Muizon, 1988, 1991; Yang and Zhou, 1999; Cassens *et al.*, 2000; Hamilton *et al.*, 2001; Nikaido *et al.*, 2001; Yang *et al.*, 2002, 2009; Yan, 2005). Atualmente, reconhece-se que os golfinhos de rio formam um grupo polifilético. Primeiramente, Muizon (1988, 1991) e Heyning (1989a), com base na análise de fósseis e na anatomia facial, separaram o gênero Platanista do restante das espécies de rio. Recentemente, evidências moleculares colocaram este gênero como mais proximamente relacionado à família Ziphiidae. Hoje, é amplamente aceito que o gênero *Pontoporia* e *Inia* representam grupos irmãos, ambos aparecendo em parafilia com a família Lipotidae (Figura1).

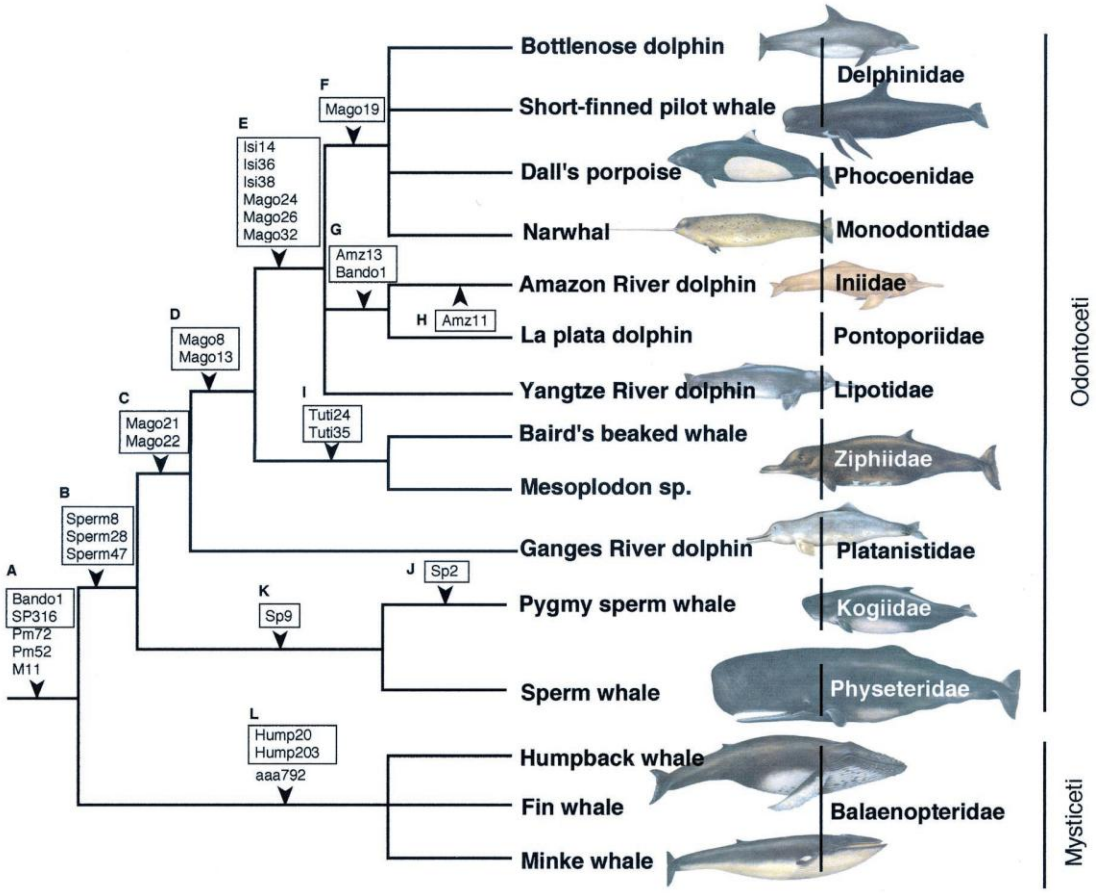

Figura 2. Relações filogenéticas entre as maiores linhagens de cetáceos. Reproduzido a partir de Nikaido *et al.* (2001) (Copyright 1998, Simon & Schuster).

1.3. BIOLOGIA DAS ESPÉCIES DESTE ESTUDO

1.3.1. Inia geoffrensis (de Blainvillei, 1817)

O boto-rosa é o maior dos golfinhos de rio, o macho pode atingir o comprimento de 255 cm e pesar até 185 kg. As menores fêmeas atingem em média 180 cm e o peso de 150 kg. A coloração varia de cinza a rosado e depende da idade do indivíduo. Uma característica interessante desta espécie é que as vértebras do pescoço não são fusionadas, permitindo o movimento da cabeça para os lados e para baixo em um ângulo de até 90˚, conferindo grande flexibilidade e vantagens de locomoção no ambiente raso onde esta espécie habita. O boto-rosa habita rios, lagos, igarapés e várzeas da bacia Amazônica e do Orinoco. São animas solitários e raramente são vistos em grupos com mais de quatro indivíduos. Grupos com dois indivíduos geralmente são constituídos por mãe e filhote. A gestação do boto-rosa dura cerca de 10-11 meses e o nascimento dos filhotes ocorre durante o período de águas baixas na Amazônia (maio, junho e julho) quando a concentração de peixes aumenta nos igarapés e várzeas tornando o acesso ao alimento mais fácil. O tempo de geração dessa espécie é relativamente longo, a maturidade sexual das fêmeas é atingida por volta dos seis anos de vida e o intervalo entre sucessivas crias é de aproximadamente três anos (Best and da Silva, 1989).

As principais ameaças para esta espécie incluem: o aumento do uso de redes de pesca com o conseqüente aumento da captura incidental dos botos; redução da fonte de alimentação do boto, devido ao aumento da demanda por peixe como conseqüência do aumento populacional na região; a poluição química como pesticidas e mercúrio: análises do leite de um boto rosa procedente da região próxima a Manaus revelaram contaminação por mercúrio (Rosas, 1996). Além disto, uma potencial ameaça é representada pela construção de hidrelétricas na Bacia do Amazonas, que pode isolar grupos e reduzir o fluxo gênico, dificultando ainda mais a reprodução entre eles. Embora as populações de botos na região amazônica ainda sejam aparentemente grandes, em comparação com outras espécies de golfinhos de água doce, desde 2000 os números vêm sofrendo quedas alarmantes. De fato, foi em 2000 que a primeira evidência de caça ao boto em larga escala apareceu. Os botos têm sido caçados à noite para o seu uso como isca para a pesca de um peixe necrófago, a piracatinga. Embora este peixe não seja apreciado no Brasil, ele é muito consumido na Colômbia para onde é exportado. Tal atividade tem se tornado uma fonte muito lucrativa para as indústrias de pesca na Amazônia Central e têm ameaçado significativamente as

populações de botos na região, sendo que uma redução de 10% nas populações ao ano foi observada a partir de 2000 (Serrano *et al.,* 2007).

O comitê científico da IWC (International Whaling Comisson) recomendou em 2000 que mais estudos sobre esta espécie devem ser conduzidos e publicados para que se possa melhor avaliar o *status* do boto-rosa na bacia Amazônica e do Orinoco. Devido a escassez de informações a cerca da biologia do *Inia,* a espécie antes classificada como vulnerável pela União Internacional para a Conservação da Natureza (IUCN) é agora declarada como deficiente de dados (2008).

1.3.2. Sotalia guianensis (Van Bénéden, 1864)

O boto-cinza, *Sotalia guianensis* é um pequeno delfinídeo cuja distribuição vai desde Florianópolis-SC, no Brasil, até Honduras, na América Central. No passado, cinco espécies foram classificadas como membros do gênero *Sotalia*, três do rio e duas da costa. Uma revisão feita Rice (1998) reconheceu apenas uma espécie, incluindo todas as formas ou ecótipos fluviais e marítimos. Entretanto, novas análises morfométricas tridimensionais de crânio de *Sotalia* no Brasil e análises genéticas reconheceram que duas espécies devem ser consideradas: *Sotalia fluviatilis*, na Bacia Amazônica e *Sotalia guianensis*, marinha (Monteiro-Filho *et al.*, 2002; Cunha *et al.*, 2005; Caballero *et al.*, 2007).

A espécie possui uma coloração cinza claro uniforme no dorso e rosa ou cinza claro no ventre. Os machos atingem 1,94 m e as fêmeas 2,06 m de comprimento. Apesar de habitar a região costeira, o boto-cinza é mais comumente encontrado em áreas protegidas como baías e estuários, onde um alto grau de residência e fidelidade ao local foi relatado para diversas baías ao longo da costa brasileira (Flores *et al.*, 2004; Azevedo *et al.*, 2005; Rossi-Santos *et al.*, 2007; Flach *et al.*, 2008; Dias *et al.*, 2009). No norte e nordeste do Brasil, a espécie se constitui de grupos pequenos de 2-6 indivíduos, mas agregações maiores são observadas quando estes desenvolvem atividades comuns tais como forrageamento (Araújo *et al.*, 2001; Rossi-Santos *et al.*, 2007). Grupos maiores são relatados para o sul e sudeste do Brasil com uma formação média de 20 a 30 indivíduos (Daura-Jorge *et al.*, 2003; Azevedo *et al.*, 2005; Flach *et al.*, 2008; Dias *et al.*, 2009). Entretanto, a maior população de botos já estimada para a costa brasileira pertence à Baía de Sepetiba (~700-1200) (Flach *et al.*, 2008), tamanho similar foi também estimado para a Baía de Ilha Grande e Parati (Lodi, 2003) na costa sudeste do Brasil.

O boto-cinza apesar de ocorrer em uma larga faixa de distribuição, é uma espécie ainda pouco conhecida, sendo que a maioria dos estudos foi realizada em áreas restritas. A espécie é listada no Apêndice I da Convenção Internacional de Comércio de espécies

ameaçadas da Fauna e Flora Silvestre (CITES) (Barros e Teixeira, 1994) e é considerada como espécie deficiente de dados pela IUCN, o que indica uma necessidade de mais estudos para definição do seu *status* de conservação.

As principais ameaças que afetam a espécie estão diretamente relacionadas com a destruição dos habitats ao longo de sua área de distribuição, incluindo a poluição por efluentes industriais e agrotóxicos. Nas regiões costeiras, o aumento do tráfego de embarcações e o desenvolvimento urbano, bem como a exploração dos mangues e estuários, vêm gradativamente afetando a estabilidade das populações. Em baías costeiras da região sudeste, como as baías de Guanabara e Sepetiba, as populações de *Sotalia* encontram-se sob forte pressão antrópica, estando expostas aos efeitos sinérgicos da poluição, perda de habitat, capturas incidentais e molestamentos intencionais por embarcações de turismo e lazer (Nery *et al.*, 2008). Análises de metais pesados mostraram altas concentrações de mercúrio em tecidos da espécie na baía de Guanabara (Seixas *et al.*, 2009). Existem registros de capturas incidentais em todo o litoral brasileiro e do uso da carne de *S. guianensis* proveniente destas capturas para uso como isca de espinhel na pesca de tubarões (Siciliano, 1994). A magnitude desses impactos é ainda desconhecida. A ocorrência de mortes nos aparelhos de pesca em toda a área de distribuição da espécie é considerada um motivo de preocupação, especialmente levando-se em conta o potencial de expansão da atividade pesqueira nas regiões estuarinas (Siciliano,1994).

1.4. MARCADORES MOLECULARES PARA ESTUDO DE POPULAÇÕES

1.4.1. DNA mitocondrial

Existem diversos tipos de marcadores moleculares, o DNA mitocondrial (DNAmt), em especial, tem sido considerado um marcador de grande valia em estudos para caracterização da variabilidade genética, estrutura de populações e na identificação espécie-específica, complementando análises ecológicas e de sistemática. O DNAmt não sofre recombinação e é transmitido somente pela mãe a seus filhos. Possui DNA circular com um tamanho de 16.569 pares de base, tendo sido completamente seqüenciado por Anderson *et al.* (1981). Este se subdivide em duas regiões maiores: a região hipervariável 1 (HVI), que compreende as posições 16.024 a 16.569, e a região hipervariável II (HVII), que compreende as posições 73 a 340 e uma região hipervariável menor (HVIII).

Na região controladora, são verificados os polimorfismos do DNAmt (Upholt & Dawid, 1977). A típica estrutura *d-loop* do DNAmt, na qual há formação momentânea de fitas simples, pode influenciar o padrão de mutação pontual (Reyes *et al.*, 1998), já que a taxa de depurinação de DNA fita-simples é quatro vezes maior que a do DNA fita-dupla (Lindahl e Nyberg, 1972). A alta taxa de substituição de bases possibilita que seja observada uma ou mais diferenças na seqüência nucleotídica (polimorfismos de DNA) das regiões hipervariáveis do DNAmt, quando indivíduos da linhagem materna de uma mesma família são comparados (Jarreta, 1999). Todos estes fatores fazem com que o DNA mitocondrial tenha uma taxa de evolução 5 a 10 vezes maior que o DNA nuclear, por isso esta região é altamente variável entre indivíduos e muito utilizada para estudos populacionais. Além disto, o DNAmt possui um quarto do tamanho populacional efetivo do DNA nuclear (diplóide), sendo mais sensível a eventos demográficos como por exemplo, *bottlenecks* (redução popualcional). Ao contrário da região controladora, o citocromo *b* (região codificadora do DNAmt) é muito utilizado para estudos filogenéticos inter-específicos.

1.4.2. Microssatélites

O genoma dos eucariotos contém seqüências repetitivas que podem ser usadas como marcadores de DNA. As seqüências simples repetidas SSR (“sequence simple repeats”) ou microssatélites são um dos marcadores mais polimórficos encontrados nos genomas de animais e plantas. Consistem de repetições de seqüências curtas de DNA, geralmente de 1-6 pares de bases. Existem quatro classes destes marcadores, os microssatélites perfeitos, em que há apenas um tipo de unidade de repetição; os microssatélites compostos, nos quais existem dois tipos de unidade de repetição; os microssatélites complexos, em que há mais de dois tipos de unidade de repetição e os microssatélites imperfeitos (interrompidos), nos quais existem uma ou mais unidades simples não repetidas dentro do arranjo do microssatélite (Weber, 1990). Os diferentes alelos de um *locus* são caracterizados pelos seus tamanhos, definidos pelo número de repetições (Estoup & Cornuet, 1999).

Atualmente são os marcadores da DNA mais utilizados em genética de populações devido às numerosas vantagens que oferecem quando comparados a outros marcadores. Os microssatélites são altamente polimórficos e informativos, a herança é codominante, o que permite a diferenciação entre homozigotos e heterozigotos; são multialélicos; ocorrem em abundância no genoma dos eucariotos; são altamente reproduzíveis e os locos são freqüentemente conservados entre espécies relacionadas. Devido ao seu alto polimorfismo,

os microssatélites são capazes, por exemplo, de capturar baixa diversidade genética em populações vulneráveis, em declínio ou isoladas (Beebee *et al.*, 2002).

Esses marcadores têm sido utilizados na análise de diferentes populações de cetáceos (vide revisão, Bourret *et al.*, 2008), sendo úteis na elucidação de estratégias de reprodução, estrutura social, análises de parentesco, taxas de migração e tamanho populacional efetivo. É importante salientar ainda que os iniciadores microssatélites desenvolvidos para uma espécie podem ser utilizados em espécies proximamente relacionadas uma vez que o sítio de ligação os iniciadores são altamente conservados. Com isso, uma ampla variedade de marcadores pode ser testada par acessar a estrutura populacional de uma espécie.

ARTIGO 1

The Amazon River system as an ecological barrier driving genetic differentiation of the pink dolphin (*Inia geoffrensis*)

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The Amazon River system as an ecological barrier driving genetic differentiation of the pink dolphin (*Inia geoffrensis*)

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Running title: High phylopatry in the *Inia geoffrensis*

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ABSTRACT

The pink dolphin (*Inia geoffrensis*) is widely distributed along the Amazon and Orinoco basins, covering an area of about 7 million km^2. We examined the phylogeographic structure, lineage time divergence and historical demography using mitochondrial (mt) DNA sequences and autosomal microsatellites in different pink dolphin populations distributed in a large and small spatial scale. Our results show that the Brazilian haplotypes occupy an intermediate position related to three geographic locations: Colombian Amazon, Colombian Orinoco and Bolivian Amazon. All lineages presented well defined phylogeographic structure with lineage divergence beginning in the Miocene and occurring throughout the Pleistocene-Holocene. Bayesian skyline plots revealed a gradual population decline through time for all lineages ending in a most recent bottleneck. The exception being a Bolivian sample, which surprisingly revealed a more recent population expansion. In addition, we have identified a prominent population structure for two neighboring populations from the Amazon basin in Brazil. Six control region (CR) haplotypes were identified with no sharing between the two populations and a specific cytochrome *b* (*CYTB*) haplotype was detected for each location. Phylogeographic study emphasizes the exceptional population structure for the pink dolphin, considering all populations in the Amazon and Orinoco river systems. These results also suggest a strong female phylopatry for this species due to restriction of gene flow through long and short distances, where isolation by distance is operating at a large spatial scale and ecological restrictions to interbreeding appears between neighboring populations. Finally, although autosomal microsatellites could not discriminate the two proximate Brazilian populations, they provided support for a recent population bottleneck in both localities.

INTRODUCTION

The pink dolphin, *Inia geoffrensis* (de Blainvillei, 1817) also called “boto” in Brazil is a freshwater river dolphin endemic to the Amazon, Orinoco, and Araguaia/Tocantins River systems of South America, an area of 7 million km^2 area (Best & da Silva, 1989). Current classification of the genus considers a single species with three geographically distinct subspecies: *I. g. geoffrensis* for most of the Amazon and Araguaia/Tocantins basin, *I. g. humboldtiana* for the Orinoco basin and *I. g. boliviensis* for the system of Bolivian rivers, with populations in the Madeira drainage area upstream of the Teotonio rapids in Brazil (Rice, 1998; Best & da Silva, 1989). The contact between *I. g. geoffrensis* and *I. g. humboldtinana* populations is constrained by Casiquiari channel between Rio Negro (a large tributary of the Amazon) and on the Orinoco River (da Silva & Martin, 2000). The Bolivian subspecies is isolated from the others by a stretch of 400 km of rapids in the Madeira drainage area, between Porto Velho and Guajará-Mirim in Brazil. However, the main streams of the Amazon River are unobstructed by physical barriers such as rapids or dams and botos occur throughout its length with an apparently decline near to the Atlantic ocean where sightings of this species are scarce (Martin & da Silva, 2004b; Emin-Lima *et al.,* 2007). The taxonomic status of Bolivian *Inia* is currently under discussion, with some authors claiming that Bolivian *Inia* should be considered a separate species (Pilleri & Gihr, 1977; da Silva, 1994, Banguera-Hinestroza-Hinestroza *et al.,* 2002). Thus, due to the recent proposal of *Inia boliviensis* as a separate species, we will use *Inia* sp within the text to refer to this species or species complex.

In the Orinoco and Amazon basins, *Inia* sp. are found in a variety of habitats including rivers, channels and lakes. In the Central Amazon, seasonal variation in water levels may influence their distribution between habitats; high densities of botos were recorded in small channels and lakes during the dry season (Martin & da Silva, 2004b) with animals moving out to the main rivers during the wet season. This pattern was also registered for Orinoco and Bolivia populations where botos were seen in oxbow lakes more often during low and falling waters (Rodriguez, 2000; Escovar, 2002; Alliaga-Rossel, 2002). Moreover, extensive boat-surveys in Brazil conducted by Martin and da Silva (2004b) have demonstrated that the most preferred habitat of the botos is the “meeting of waters”, where a channel of sediment-rich white water meets acidic black water yielding an highly productive environment.

The high density of botos found in such productive environments appears to correlate with the observation of increased entanglement of them by fishing nets employed by local fishermen in this area, although the extent of this mortality is still unknown. Direct catch of

botos in Brazilian Amazon has been forbidden by federal law since 1987; however this practice has intensified in recent years. Fishermen are using *Inia* as bait to catch catfishes, like “piracatinga” (*Calophysus macropterus*), whose meat is considered inedible by Brazilians but is exported to Colombia where it is enjoyed (Estupiñán *et al.*, 2003). This activity has become one of the main income sources of local fishermen in the Central Brazilian Amazon; therefore *Inia* sp. overexploitation is currently a focus of major concern threatening the future of this species. Indeed, there are estimates of 6-12 botos killed a day in some Amazon areas (Estupiñán *et al.*, 2003). Also, over 10% reduction a year has been recorded for *Inia* sp. populations in Mamirauá Sustainable Development Reserve, where the major concentration of this species occurs (Martin & da Silva, 2004b). In addition to fishing, *Inia* sp. is threatened by other human-related activities including habitat degradation due to expanding populations near its range area, chemical contamination by pesticides and mercury used in gold mining (Rosas & Lehti, 1996; Reeves *et al..,* 2002; da Silva, 2002). Furthermore, hydroelectric development also presents a potential threat; there are many plans to construct dams along the Amazon basin (Best & da Silva 1989a), which may interrupt gene flow between populations. Indeed, two large hydro-electrical power plants have been recently licensed and are in construction in the Madeira River, in the Brazilian Amazon drainage.

Although, some data are available on abundance, ecology and genetics of *Inia* sp, overall estimates to determine the conservation status of the Amazon River dolphin are still limited. Based on this limited information, the species which was previously listed as Vulnerable by the World Conservation Union (IUCN) is now declared as Data Deficient (2008).

The reconstruction of the evolutionary history at the intraspecific level plays a major role on the determination of suitable strategies for biological conservation (Avise, 1987). Here we examine the phylogeography and demographic history of *Inia* sp. Coalescent methods for inferring demographic histories have proved valuable for investigation of past population dynamics through time and for identification of barriers that shaped the present gene diversity of a species (Drummond *et al.,* 2005). Phylogeography studies based on mitochondrial DNA have been useful in describing the definition of management units in different cetacean species (Baker & Palumbi, 1996; Mendez *et al.*, 2007; Natoli *et al.*, 2008). To date, genetic comparisons among *Inia* subspecies have been reported by Banguera-Hinestroza *et al.* (2002), Vianna *et al.* (2010) and Ruiz-Garcia *et al.* (2010). Based on analyses of mtDNA and microsatellites, these authors indentified a strong phylogeographic pattern in a macro-geographic scale. To address the phylogenetic relationship of Brazilian Amazon populations in comparisons to previously studied populations by Banguera-Hinestroza *et al.* (2002), we have analyzed mtDNA control region (CR) sequences and the entire cytochrome *b* (*CYTB*) gene of Brazilian *Inia* sp. populations. In addition we have

analysed population diversity and demography of the two close populations in Central Brazilian Amazon using published and new standardized microsatellites.

MATERIALS AND METHODS

Sample collection and DNA extraction

Samples were collected over 14 years from a total of 43 dead animals from three different locations in the central Brazilian Amazon: Tefé Lake (n=17), Mamirauá Sustainable Development Reserve (n=16), hereafter referred to "Mamirauá", and Amanã Lake (n=10) (Figure 1 and Table S1- Supplementary Material). Samples from Mamirauá and Amanã are here treated as a single population as they form a continuous water environment at the northern margin of the Amazon River, while Tefé is located at the southern margin. Samples were obtained from animals found dead or accidentally caught in nets. Muscle and skin tissues were preserved in 95% ethanol and stored in freezer. DNA was extracted following standard phenol-chloroform extraction, as described in Sambrook *et al.* (2001).

DNA amplification, sequencing and sex determination

A 400 base pair (bp) fragment of the mtDNA Control Region (CR) was amplified via the polymerase chain reaction (PCR) using primers CR-4 (Southern *et al.,* 1988) and CR-5 (Southern *et al.,* 1988; Palumbi *et al.,* 1991). Alternatively, for samples sequenced at Oregon State University, we used primers tPro-whale M13-Dlp-1.5 (Baker *et al.*, 1996), and Dlp-8G (designed by G. Lento as reported in Dalebout *et al..,* 2005) for the amplification reactions. The PCR cycling conditions were performed following the conditions described in Caballero *et al.* (2007).

The complete mtDNA *CYTB* gene (1140 bp) was amplified using primers L14121 and H15318 (Redondo *et al.,* 2008). Each PCR mix contained 20 ng of genomic DNA, 1X Taq reaction buffer 1B (Phoneutria® - 1.5 mM $MgCl_2$, 10 mM Tris-HCl pH 8.4, 50 mM KCl, 0.1% Triton X-100), 200 µM dNTPs, 0.5 µM of each primer, and 1 unit of Taq DNA polymerase (Phoneutria®). The PCR cycling was performed in an Eppendorff Gradient thermocycler following the conditions described by Vianna *et al.* (2010).

PCR products were cleaned using 20% PEG (Polyethyleneglycol) + 2.5 % NaCl, and both strands sequenced using an ET® dye terminator kit (GE Healthcare®) on a MegaBACE automated capillary sequencer following the temperature cycling profile: 95°C for 1min, 35

cycles of 95°C at 25 sec, annealing at 50°C for 15 sec, and extension at 60°C for 3 min. Two additional primers were used to sequence the complete *CYTB* gene: XL14733 and MVZ4 (Kocher *et al.,* 1989). For samples sequenced at Oregon State University, free nucleotides and primers were removed from the PCR products using SAP (shrimp alkaline phosphatase) and ExoI (exonuclease I) and directly sequenced using the standard protocols of Big Dye terminator sequencing chemistry on an ABI 3730 automated capillary sequencer.

Individuals whose sex could not be determined by post-mortem examination, were identified by PCR amplification of a fragment of the *SRY* gene multiplexed with fragments of the *ZFY/ZFX* genes as positive control, as described by Gilson *et al.* (1998).

Microsatellite genotyping

Samples were genotyped at 14 dinucleotide microsatellite loci either developed directly for *Inia* sp. (Gravena *et al.*, 2009) or using heterologous primers isolated from the other cetacean species (Table 3) and standardized to amplify *Inia* sp. samples. Amplification reactions contained 20-40 ng DNA, 20 mM Tris-HCl, pH 8.0, 50 mM KCl, 2.5 mM $MgCl_2$, 0.4 µM of each primer, 200 µM dNTPs and 0.25 units of Platinum-Taq (Invitrogen). Each forward primer had an M13 sequence tail added to its 5′ end to allow for fluorescent labeling (HEX, NED, FAM) following Schuelke (2000). The thermocycle profile for the microsatellites isolated from another species were carried out in 10 µl volumes, with 2.5 mM $MgCl_2$ and annealing temperature varying by locus (50°C for MK5, MK6 and EV94; 55°C for PPHO142, KWM12a, TtrGT48 and GT575). Microsatellite loci isolated from *Inia* were amplified following the procedure described by Gravena *et al.* (2009). PCR products were measured using an Applied Biosystems 3730 automated sequencer. Allelic sizes were scored against the size standard GS500 LIZ (Applied Biosystems) and the peaks were analyzed using *GeneMapper* v4.0 (Applied Biosystems).

Mitochondrial DNA analysis

Sequences of the mtDNA were “base called” with the software *Phred* (Ewing *et al..,* 1998). High quality consensus sequences (400 bp for CR and 1140 bp for *CYTB*) for each individual were produced from alignments of forward and reverse strand sequences with the software *Phrap* (Green, 1994) and visualized/edited in *Consed* 12.0 (Gordon *et al.,* 1998). The consensus sequences from all individuals were aligned using the *Clustal-X* v1.83 (Thompson *et al..,* 1997) to allow the identification of nucleotide variation and the different haplotypes. In addition to our data, we included in the analysis all CR and *CYTB* sequences of *I. geoffrensis*

specimens published by Banguera-Hinestroza-Hinestroza *et al.,* 2002. To infer the relationship among the haplotypes for the populations in a geographic context, haplotype networks were constructed by the median-joining algorithm as implemented in the program *Network* v4.5 (Bandelt *et al.,* 1999).

Standard indices of genetic variation as haplotypic diversity (*h*) and nucleotide diversity (π) were estimated for each population in each geographic region using *Arlequin* v3.1 software (Excoffier *et al..,* 2005). The analysis of molecular variance (AMOVA) and population pairwise distances were calculated as $\Phi_{ST,}$ with Tamura-Nei (Tamura & Nei, 1993) substitution model of evolution (α=0.078). Exact test of population differentiation was performed with 10,000 Markov Chain steps to test significance as implemented in *Arlequin*. The number of migrants successfully entering a population per generation (N_m) was estimated following Slatkin's (1991) formula as described in *Arlequin*. Tajima's *D*-test (Tajima, 1989) and Fu's *F*s test (Fu, 1997) were also calculated for each population to evaluate the possibility of recent population expansion using 1000 bootstrap replicates.

To check the reliability of results from phylogenetics analysis of CR mtDNA sequences we assessed the substitution saturation using the software DAMBE v5.1.5 (Xia & Xie, 2001). The phylogenetic relationships of the mtDNA haplotypes were reconstructed using *Beast* v1.5.2 (Drummond & Rambaut, 2007) and the resulting trees were summarized using *TreeAnnotator* and *Figtree* programs (Drummond & Rambaut, 2007). The time of origin of each lineage was estimated using relaxed molecular clocks, an uncorrelated lognormal tree prior with a constant population size and lognormal calibration points were used to estimate the most recent common ancestor (MRCA) for each monophyletic clade. *Pontoporia* was used as calibration reference and a mean date of 11.2 (Mya) with lognormal standard deviation of 1.025 (Mya) following the results obtained by Xiong *et al.* (2009). The historical demography of each phylogeographic group was examined using Bayesian skyline plot (BSP) implemented in *Beast*. This method permits analysis of population expansions or reductions and generates a plot of effective population size (N_e) through time. The phylogenetic model HKY + G with a relaxed molecular clock was used to estimate BSP for Bolivia, Colombian Orinoco and Mamirauá+Tefé populations. All analyses in *Beast* were run for 80,000,000 generations with a burn-in of 8,000,000. Results were then visualized in Tracer 1.4 (Drummond & Rambaut, 2007).

Alternatively, Bayesian analysis (*BA*) were conducted in *MrBayes* v3.1 (Huelsenbeck & Ronquist, 2001). *BA* topology was constructed under the HKY+G substitution model as suggested by *Modeltest* approach (Posada & Crandall, 1998). Posterior probabilities with > 0.95 were considered robust. The outgroup *Pontoporia* was excluded from the analysis as the long evolutionary divergence between this species and *Inia* generated a long branch making the tree unreadable.

Microsatellite analysis

The program *Micro-checker* was used to check for likely problems due to scoring errors, large allele dropout and null alleles (Van Oosterhout *et al.*, 2004). The potential frequency of null alleles was also estimated using *Cervus* v2.0 (Marshall *et al.*, 1998). The matching genotypes and the probability of identity, the probability of two individuals sharing the same genotype across all fourteen loci was estimated in the program *GenAlEx* v6.0 (Peakall & Smouse 2006). Departures from Hardy-Weinberg equilibrium, tests of linkage disequilibrium for each locus and genetic differentiation among populations (using F_{ST} and R_{ST}) were carried out using *Arlequin* v.3.11 (Excoffier *et al.*, 2005). We calculated both F_{ST} and $R_{ST,}$ however conventional F_{ST} has been shown to be more reliable when sample size is limited (Gaggiotti *et al.*, 1999). Inbreeding coefficients (F_{IS}) and allelic richness (A), a measure of the number of alleles per locus independent of sample size (Petit *et al.*, 1998), were computed in *Fstat* v2.9 (Goudet *et al.,* 2002).

Estimates of dispersal rates between Mamirauá and Tefé populations were calculated using a maximum likelihood (*ML*) coalescent approach implemented in *Migrate* v3.0 (Beerli & Felsenstein, 1999). Initial runs were set estimating θ and M (immigration rate/mutation rate) based on F_{ST} calculations. Three replicate searches included 10 short chains with a total of 10,000 genealogies samples and 2 long chains with 1,000,000 genealogies sampled, following a burn-in of 10,000 trees. For microsatellites data, a constant mutation rate of 10^{-4} was used (Hedrick, 2005; Whitaker *et al.*, 2003) to transform estimates of θ into N_e based on formula $\theta = 4N_e\mu$ (where N_e is effective population size and μ is the mutation rate/generation). To examine whether the dispersal is sex-biased, F_{ST} estimates were calculated for males and females separately. The statistical significance was estimated using permutation simulation with default settings implemented in *Arlequin* v3.11. The potential for sex-bias was tested using the *Fstat* v2.9.2.3 (Goudet, 2002) based on sex-specific expectations with respect to F_{ST}, variance of the assignment index - vAIc and mean assignment index - *m*AIc.

The program *Bottleneck* v1.2 (Piry *et al.,* 1999) was used to evaluate deviations of mutation-drift equilibrium in the Mamirauá and Tefé populations. Three different mutation models of microsatellite evolution were employed: (1) Infinite Allele model- IAM (Ohta & Kimura, 1973); (2) Stepwise Mutation Model - SMM and (3) Two-Phased model - TPM, an intermediate to the IAM and SMM which better fit most of microsatellite datasets (DiRienzo *et al.*, 1994). The TPM parameters were set with 70% single-step mutations and 30% multiple-step mutations and a variance among multiple steps of 12. The significance was assessed with the Wilcoxon sign-rank test, a more powerful and robust test when used with few polymorphic loci (<20). We also used a qualitative graphical descriptor of the shape of the allele frequency

distribution that can differentiate between stable (L-shape indicator) and bottlenecked (mode-shift indicator) populations (Luikart *et al.,* 1998).

RESULTS

Genetic diversity

mtDNA - CR

We used 400 bp of the mtDNA CR to investigate the relationship among the three subspecies. Our CR sequences from 30 Brazilian samples were aligned to 96 CR sequences obtained by Banguera-Hinestroza *et al.* (2002). For a total sample of 126, a total of 19 haplotypes were identified. For *I. g. geoffrensis,* six from Brazil (*n*=30), two from Colombian Amazon (CA: n=38), six for *I. g. boliviensis* from Bolivian Amazon (BA: n=41) and five for *I. g. humboldtiana* from Colombian Orinoco (CO: n=17). The haplotypes show 49 variable sites (38 parsimony informative and 11 singletons). Among the four localities, Brazil displayed the highest haplotype diversity followed by CO and BA. CA was substantially lower than all other regions. Nucleotide diversity was lowest in BA haplotypes while the highest diversity was seen in CO haplotypes (Table 1). Tajima's and Fu's tests revealed no significant results for population expansions in all geographic locations except for the BA group, where the *Fs* value indicates the occurrence of expansion (*Fs*= -2.678*, p* < 0.05).

At micro-geographic scale within Brazil, we found a total of six CR mtDNA haplotypes from which five were restricted to the Central Amazon populations in Brazil. All haplotypes found in Amanã grouped with Mamirauá haplotypes, suggesting two separate Brazilian populations, northern side of the Amazon River (left margin) represented by individuals from Mamirauá and Amanã and at southern (right margin) for Tefé population. Seven polymorphic transitions were identified in the Brazilian *Inia* sp. sequences. Three haplotypes were found exclusively in the Tefé Lake and two haplotypes were found exclusively in individuals from Mamirauá and Amanã. Similar genetic diversity was displayed for Tefé samples $h = 0.5111 \pm 0.1643$ against $h = 0.4842 \pm 0.1129$ found in the samples from Mamirauá (Table 1).

Microsatellites

The potential presence of null alleles was detected in five out of fourteen microsatellites, thus the loci: KWM12a, GT575, IG2B1, IG11B1 and IG8H1 were excluded from *F*-statistics analysis. The probability of identity across all fourteen loci was highest for Mamirauá ($1 \times 10^{-}$

^{10}) and lowest for Tefé (7.4 x 10^{-11}). No matching genotypes were found among the samples. All loci were polymorphic, with a low to medium level of allelic diversity at each locus (2 to 8), no alleles were found to be unique in any population. Levels of microsatellite diversity were moderate with average gene diversity 0.5541 +/-0.3017 in Mamirauá and 0.5537 +/-0.3284 in Tefé (results over 10 loci). Negative values of F_{IS} indicate occurrence of inbreeding in either Mamirauá or Tefé populations separately. Five loci, described above, exhibit significant deviations of Hardy-Weinberg equilibrium, possibly due to null alleles (Table 3) and significant linkage disequilibrium was also observed in five out of 45 pairs of loci which may be due to the relatively limited sample sizes.

The analysis of population subdivision using F_{ST} and R_{ST} showed no significant structure across the sampled areas. F_{ST} attributed only 0.25% of the variation between populations and no significant positive values were obtained from the R_{ST} estimator.

Population differentiation

The differentiation of mtDNA among the four regions was very strong (F_{ST} = 0.53; Φ_{ST} = 0.87) of the variation occurs among populations, suggesting an extremely reduced maternal gene flow among localities. Pairwise comparisons suggested that a significant part of the diversity is due to the inclusion of the BA population whose Φ_{ST} values compared to Mamirauá, Tefé, CO and CA were close to 1, except for the latter where the value was slightly lower, 0.87 (Table 2).

Although the two Brazilian populations are only about 45 km apart (straight line between their edges), AMOVA analysis revealed a strong differentiation between both localities (F_{ST} = 0.50; Φ_{ST} = 0.70, p < 0.001) however there is still a considerable amount of variation within each locality (30%). Tajima's and Fu's tests failed to detect any significant population expansion for Mamirauá and Tefé populations. Analytical N_m estimates of gene flow were low as expected by the high population structure based on F_{ST} values. However, it is important to note that those estimates are reliable if populations fit island model assumptions, which rely on symmetrical migration rates and equal population abundance (Whitlock & McCauley, 1999).

The complete *CYTB* gene (1140 bp) was also sequenced in 20 individuals from the Brazilian Amazon. Two haplotypes were defined by a single transition. One haplotype (GenBank accession number EU554562) represents 13 individuals from Mamirauá and another one was identified in 7 individuals from Tefé (GenBank accession number EU554561). Thus, there is a specific *CYTB* haplotype for each population, Tefé and Mamirauá. This reflects the remarkable maternal isolation suggested by the control region network (Figure 2).

Phylogeographic Structure and Phylogenetics

The Median Joining Network (MJN) of CR sequences showed two population clusters in Brazil (Figure 2). Five of the six haplotypes found in Brazilian populations appeared to be unique with one haplotype shared between Mamirauá and Colombian Amazon (MM3/CA1). The star-like tree found for the Mamirauá population, showing an excess of rare haplotypes originating from the most frequent haplotype (MM3), could suggest population growth. However, this pattern was not judged significant by previous expansion tests, perhaps due to a small sample size. The haplotype MM2 connects with the remnant haplotypes from CA (CA2) and CO (CO1 to CO5). A remarkable separation between BA populations and the others is supported by 17 nucleotide substitutions. This separation was further corroborated by phylogenetic reconstructions. The MJN also indicates some heterogeneity in the Colombian Orinoco population. Phylogenetic reconstructions using *Beast* (Figure 3) and *MrBayes* (Figure S1 - Supplementary Material), based on mtDNA (CR), recovered nearly identical topologies. Colombian Orinoco, Colombian-Brazilian Amazon, and Bolivian populations reflect three clear geographic clusters. The latter appears in both trees as a monophyletic group. The Central Amazon haplotypes from Brazil fill a phylogenetic gap between CO and BA haplotypes, as it would be expected due to the intermediate geographic position between both localities, considering the connectivity of rivers and genetic divergence correlating with geographic distances.

Sex-biased gene flow and effective population size estimation based on microsatellite data

AMOVA treating males and females separately for each population reveals a significant but low structure for males (n = 9, MM and n= 6, TF) when using F_{ST} (0.014, $p < 0.05$) and no significance for females (0.003, $p > 0.05$) (n = 9, MM and n= 5, TF). Also, no significant differences on sex-biased dispersal were detected by comparisons of others estimators (R_{ST}, *v*AIc and *m*AIc scores, $p > 0.05$).

Gene flow estimates based on asymmetric migration were conducted considering the varying habitat quality between the two sampled areas. Maximum likelihood estimates (*ML*) based on microsatellite data were low, with less than two migrants per generation across both areas (Table 4). Reliable *ML* results could not be obtained for mtDNA data; according to Beerli (2006) single locus datasets with low variability (variable sites) do not allow estimating migration rates with great precision. Nevertheless, the exclusive occurrence of different mtDNA haplotypes in each area indicates a very low or insignificant recent female gene flow.

Divergence time and historical demography

The use of unreliable sequences may be one source of error to construct phylogenetic trees. Factors affecting its correct estimation can be traced by index of substitution saturation (Iss) of sequence data. The substitution saturation decreases phylogenetic information contained in the sequences and may reflect wrong phylogenetic relationships and dating estimates (Xia *et al..,* 2003). Following the procedure implemented by Xia & Xie*,* (2001) in DAMBE, no signs of substantial saturation were detected in the mtDNA CR (Iss < Iss.c; $p < 0.05$) therefore our sequence data set seems suitable for phylogenetic inference. The divergence among Bolivian *Inia* and others *Inia* populations appears to have occurred in the Miocene/Pliocene boundary around 5.8 Mya. The next earliest split is observed Colombian Orinoco and Colombian-Brazilian Amazon with an estimated mean divergence date value of 3.3 Mya. The Colombian-Brazilian group (without CA2, which is closely related to Orinoco haplotypes) shares a common ancestor at 1.9 Mya and the youngest lineage is represented by Tefé haplotypes formed in the Mid-Early Pleistocene (~0.6 Mya) (Figure 3). Although MM2 appears younger than Tefé in the tree, Drummond and Rambaut (2007) advise to avoid making statements about relationships or divergence times for a single lineage.

The Bayesian skyline plots detected gradual population declines through time for all populations analyzed and displaying a more severe bottleneck in the recent years. Indeed, the Brazilian populations (Mamirauá+Tefé) endured a bottleneck beginning ~ 8,000 years ago and a more pronounced one ~1000 years ago. In contrast, the Bolivian population underwent a surprisingly fast population growth over the last 10,000 years after an evident population decline around 50,000 years ago (Figure 4).

Bottleneck detection based on microsatellite data

Significant support ($p < 0.01$) for contemporary reductions in effective population size in Mamirauá and Tefé was found under all three microsatellite mutational models: IAM, SMM and TPM. Additionally, the graphical method indicates bottleneck-induced distortions of allele distributions (Figure 5).

DISCUSSION

Phylogeography

Our results provide further support to the remarkable macrogeographic pattern described by Banguera-Hinestroza-Hinestroza *et al.* (2002). Both network (MJN) and tree reconstructions

strongly support the occurrence of at least two Evolutionary Significant Units (ESU), which means that two independent units (Bolivia and others) should be considered for conservation purposes.

Although the phylogeographic pattern revealed a strong geographic correlation for all *Inia* sp. groups, Mantel tests using pairwise F_{ST} distances previously performed by Vianna *et al.* (2010) showed no significant correlation. If the populations had been separated for a long time, we expect that genetic drift may also erase any isolation-by-distance pattern. In the case of *Inia* sp., which migrates along the main river streams, but also through several connecting channels in the Amazon basin, it is difficult to predict the exact geographic paths facilitating gene flow. On the other hand, genetic differentiation may also be taking place between populations undergoing dissimilar environmental conditions and selection pressures as well geographic distances (Endler, 1982). Our results based on mtDNA analysis showed significant genetic differentiation between populations of Mamirauá and Tefé indicating a high phylopatry for females but low and non-significant differentiation for biparentally inherited microsatellites. While females are generally more philopatric than males among mammals (Greenwood, 1980) the degree of matrilineal phylogeography of *Inia* sp. is remarkable considering the short distance (45 km) observed between both Brazilian locations.

Sex-biased dispersal, gene flow and effective population size

In a previous long-term study of botos in the Mamirauá system, Martin and da Silva (2004b) identified a high degree of fidelity, where 90% (around 270 individuals) of the *Inia* population sighted in the system were considered to be resident of the area. These same authors (Martin & da Silva, 2004a) observed marked sexual segregation (except at low water season) in which males would preferentially remain in the main rivers' area and females would tend to inhabit the more remote and protected portions of the habitat such as lakes and várzeas (flooded forests). N_m estimates based on control region data seem to reflect these low rates of female gene flow among all populations. The shared haplotype between Mamirauá and Colombian Amazon is consistent with the lack of geographic barriers between Mamirauá (and Amanã) and Colombia Rivers that might allow occasional gene flow. Nonetheless, there is clear evidence of a strong differentiation between both areas (Φ_{ST} =0.58).

The lack of microsatellite differentiation suggests that long-term male-mediated gene flow may be maintaining the relative homogeneity between populations in Mamirauá and Tefé. However, male-biased gene flow was not detected in either AMOVA or *Fstat* analyses. An

extensive sampling and the use of a more appropriate marker as the Y chromosome would be required to better evaluate this question.

The microsatellite data using *Migrate* also showed some restricted gene flow between Mamirauá and Tefé with less than two individuals moving across both areas. In this case, the low F_{ST} values may be attributed to the increased effective population size in nuclear autosomal loci, as mtDNA presents a quarter of the effective population size of diploid markers and divergence tends to accumulate faster by drift (Palumbi *et al.*, 2001). N_e estimates of botos from Mamirauá Lake system (2,375) corresponds to ~18% of current census estimates for Mamirauá Reserve (13,000), which covers an area of 11,240 km^2 (Martin & da Silva, 2004b). Although Tefé Lake appears to harbor the highest densities of botos in the Central Amazon (M. Marmontel, personal communication), no conclusive study to estimate actual numbers of botos in this area have been published so far.

Divergence dating and historical demography with mtDNA

There is some controversy about the timing and cause of separation of Bolivian populations. Some authors claim that Bolivian population underwent a separation due to a recent barrier that consists of 400 km of rapids between Guajará-Mirim and Porto Velho in Brazil (da Silva, 1994), which would have been formed at the end of the Pleistocene (Grabert, 1967). In contrast, Hamilton *et al.* (2001) used molecular divergence to claim that the Bolivian *Inia* sp. has been isolated from the other subspecies for some million years. The allopatric separation of Bolivian *Inia* sp. would have resulted by the uplift of the Andes during Cenozoic, leading to a disruption between upper Madeira and the rest of the Amazon basin. The Bayesian trees presented robust support for Bolivian *Inia* sp. monophyly as was also demonstrated by several other authors (Hamilton *et al.,* 2001; Banguera-Hinestroza *et al.,* 2002; Agnarsson *et al.,* 2008; McGowen *et al.,* 2009) suggesting early divergence.

Ho *et al.* (2008) pointed out that the use of an external calibration point may lead to an overestimation of divergence times, thus our data may reflect this error. Although we presented here mean time estimates for comparisons between the lineages, our goal is to depict a historical trajectory of *Inia* sp. in South America without focusing on precise dates. The divergence time found between Bolivian *Inia* and the other lineages (5,78 Mya) shows that *Inia* is a very ancient species. The Madeira drainage basin is considered the most ancient and stable river of the Amazon basin, presumably having maintained its course for a long period of time (Westaway, 2006). It is conceivable that the Madeira River might have joined the proto-Amazon River before the formation of the modern Amazon River (Hoorn *et al.,* 1995 and 2006; Campbell *et al.*, 2006) thereby allowing an early path for the dispersal of *Inia* sp. along the basin.

Our data using Fu's *Fs* tests revealed significant negative values for Bolivian population indicating a possible demographic expansion (Fu *et al.*, 1997). Bayesian skyline plots analyses also demonstrated that the Bolivian population, regardless of age of origin, experienced a decline with a population growth in the recent past.

Banguera-Hinestroza *et al.* (2002) considered the hypothesis following Grabert (1984) that *Inia* sp. may have reached the Madeira drainage prior the formation of the rapids. The MJN network shows a strong genetic break with 17 substitutions separating Bolivian from Brazilian haplotypes. We could address the possibility that the unobserved intermediate haplotypes were lost due a local extinction process, but a large area has not been sampled all over the Madeira River downstream the rapids. Thus, we present here the hypothesis that such a bottleneck may be attributed to the formation of the rapids on Madeira River in the end of the Pleistocene (~40 kya, M. Cozzuol personal communication). According to Westaway (2006) the rise of barriers along this river is still an ongoing process. The subsequent demographic expansion observed in our analyses would have been led by the remnant individuals. The complete isolation is evidenced by the high level of differentiation, both genetic and morphological, between the Bolivian population and the other populations. We believe that genetic drift may not be the sole driver of the morphological distinctiveness of Bolivian group; natural selection may play a role in the adaptation of *Inia* sp. to local environments, thus driving local speciation (Endler, 1982). However, the future sampling and analysis of populations located downstream of the rapids in the Madeira River will provide a more complete picture of this scenario.

The highest nucleotide diversity found in haplotypes (Table 1) of the Orinoco basin reflects the diverse assemblage of habitat types in this region. This basin covers an area of 880,000 km² seasonally flooded (Weibezahn *et al.,* 1990) and is comprised by a network of channels, lakes and rapids, representing potential factors operating in the biological diversification of *Inia* sp. haplotypes. Banguera-Hinestroza *et al.* (2002) previously suggested that the Orinoco basin could be the original area of distribution of *Inia* sp. based on the prediction that the geographical areas with the highest genetic diversity are the central range in the distribution of a given species (Dobzhansky 1971). Conversely, our data suggest Brazilian populations with the highest genetic diversity, which points to a possible central-western origin for *Inia* sp. populations.

The TMRCA for Colombian Orinoco and Colombian-Brazilian Amazon is estimated to be 3.37 Mya in the Late Pliocene. The diversification of lineages in both groups appears to have occurred in similar time frames (1.10-1.40 Mya). Geological evidence indicates that the natural damming of Orinoco Valley persisted until the Late Pliocene (Campbell *et al.,* 2006) such landscape transformation may be associated with differentiation of Orinoco haplotypes by this time. Our analyses of time divergence indicate a recent origin to Tefé population in

the mid-Pleistocene at ~0.6 Mya. In contrast to the other *Inia* lineages, the Central Amazon lineages are relatively young (Pleistocene ~1.9 Mya). According to Campbell *et al.* (2006), the modern Amazon River drainage system was established in the early Pleistocene at 2.5 Mya. Hence, Brazilian populations living in areas bounded by the Amazon River could have been formed anytime over the past 2.5 Mya.

Interestingly, Bayesian skyline plots (BSP) based on mtDNA data analyses showed a slow population decline for Brazilian, Bolivian and Orinoco populations, which may have been caused by a reduction in available habitats probably associated with Pleistocene vicariant events that fragmented ranges of the ancestral *Inia* sp. occupying the Amazon (Haffer, 1987). In contrast, we observed a stable population size over a long period for Colombian Amazon distributed along a landscape with apparent lack of effective isolation barriers. For Mamirauá and Tefé, BSP analyses showed a past population decline for both a slow gradual decline along the past 8000 years. This period is coincident to the recorded drier climates in the early Holocene (Haffer, 1987).

Local bottleneck detected by microsatellite analysis

The bottleneck signals based on microsatellite data for Mamirauá and Tefé populations were strong enough to be detected under the three models IAM, TPM and SMM. The resident botos from Mamirauá and Tefé Lake may have undergone a past population decline in the Pleistocene around 60,000 to 145,000 kya (2-4N_e generations) to an estimated N_e of 2,375 individuals for Mamirauá and 3,050 individuals for Tefé Lake. During this period, Amazonian underwent several climate and lanscape changes (Haffer, 1987) that may have driven population declines in the past.

Conservation Implications

Human-induced impact on botos in the area of Mamirauá and Tefé is still a focus of concern. The botos have been displaced by human activities and could be suffering with reduction of food supply from competition with fisheries. Although, Mamirauá Reserve has been established since 1990 aiming the preservation of species, illegal hunting has been reported since 2000 (Serrano *et al.*, 2007). Furthermore, botos have been recently hunted and commercialized to be used as bait for “piracatinga” (Estupiñán *et al.*, 2003). This situation has been reflected on the rapid decline of botos in and around the Mamirauá Reserve (da Silva & Martin, 2007). Considering the likelihood of declining populations, small effective population size, long generation time and the unique spatial distribution scale detected for *Inia* sp., the threats pose a serious risk to this species. To minimize anthropogenic impact,

we suggest that conservation plans should consider managing local populations of dolphins as semi-independent evolutionary units in order to preserve *Inia* phylopatry in both macro and micro-geographic scales.

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FIGURE LEGENDS

Figure 1. **A**. Map showing the geographical locations studied in South America. Bars indicate barriers interrupting or constraining *Inia* sp. contact. **B**. Map showing the sampled areas in Brazil: the Tefé River and Lake, Mamirauá Reserve and Amanã Lake in the central part of the Amazon basin.

Figure 2. Median joining network based on mtDNA control region haplotypes for *Inia geoffrensis*. Circle areas are proportional to the number of individuals representing that haplotype. Branch lengths are approximately proportional to mutation events (shortest line length represents one substitution). Closed bars emphasize high number of substitutions found separating two haplotypes. Grey dots indicate median vectors.

Figure 3. The Bayesian tree based on mtDNA control region. Numbers at each branch correspond to mean divergence time dates derived from Beast. Node bars delimit the lower and upper 95% highest posterior density. BrA: Brazilian Amazon; CO: Colombian Oronoco; CA: Colombian Amazon; BA: Bolivian Amazon.

Figure 4. Bayesian skyline plots of each lineage of *Inia* sp., except for Brazilian Amazon where the two populations (Mamirauá and Tefé) were combined. The central line represents the mean divergence time value for the log of population size (N_e*τ). The area between upper and lower lines corresponds to the 95% posterior density.

Figure 5. Mode-shift distortion distribution of allele frequencies for 14 microsatellite loci in bottlenecked boto populations of Mamirauá and Tefé.

Table 1. Summary of mtDNA CR data (400 bp). n: sample size, S: polymorphic sites (parsimony informative), Ht: number of haplotypes, *h*: haplotype diversity and π: nucleotide diversity. For *h* and π it is also shown the standard deviations. BrA: Brazilian Amazon; CO: ColombianOrinoco; CA: Colombian Amazon and BA: Bolivian Amazon.

Population	n	S	Ht	*h*	π
BrA	30	7	6	0.7943+/-0.0277	0.0326+/-0.0163
- Mamirauá	20	2	3	0.4842+/-0.1129	0.0013+/-0.0012
- Tefé	10	4	3	0.5111+/-0.1643	0.0040+/-0.0029
CO	17	27	5	0.6471+/-0.1185	0.2238+/-0.1198
CA	38	6	2	0.1494+/-0.0739	0.0152+/-0.0127
BA	41	5	6*	0.5561+/-0.0755	0.0129+/-0.0115

* Bolivian haplotypes reported by Banguera-Hinestroza-Hinestroza *et al.* (2002) were originally 7 haplotypes, however further correction indicates that haplotypes BA1 and BA5 are identical sequences.

Table 2. Pairwise differentiation of mtDNA CR for populations of *Inia* sp. (Φ_{ST} -below diagonal) and estimated female migrants (*N*m-above diagonal) per generation among the four *Inia* sp. populations (including two Brazilian populations separately). All Φ_{ST} values are significant at $p < 0.0001$. BrA: Brazilian Amazon; CO: Colombian Orinoco; CA: Colombian Amazon and BA: Bolivian Amazon.

	BrA		CO	CA	BA
	Mamirauá	Tefé			
BrA					
-Mamirauá	-	0.21	0.35	0.39	0.01
- Tefé	0.70	-	0.48	0.15	0.02
CO	0.58	0.51	-	0.29	0.07
CA	0.56	0.76	0.63	-	0.01
BA	0.97	0.96	0.87	0.97	-

Table 3. Summary of microsatellite data for *Inia* sp. n: sample size for each region, *k*: number of alleles at each locus, *A*: allelic richness and H_O, H_E observed and expected heterozygosity. Significant deviations from Hardy-Weinberg equilibrium are shown in bold.

	Mamirauá						Tefé						
Locus	n	*k*	*A*	H_0	H_E	Null allele frequencies	*n*	*k*	*A*	H_0	H_E	Null allele frequencies	Source
PPHO142	21	3	2.81	0.524	0.521	+0.0015	12	3	2.83	0.583	0.507	-0.0789	Rosel *et al.*, 1999
EV94Mn	21	6	4.22	0.857	0.741	-0.0874	12	5	4.50	0.667	0.750	+0.0443	Valsecchi & Amos, 1996
KWM12a	20	5	3.90	**0.450**	**0.665**	+0.1833	12	5	4.48	**0.500**	**0.707**	+0.1188	Hoelzel *et al.*, 1998
MK5	20	3	2.90	0.600	0.617	-0.0137	12	3	2.94	0.417	0.554	+0.0981	Krutzen *et al.*, 2001
MK6	20	4	3.87	0.700	0.709	-0.0054	10	3	3.00	0.800	0.695	-0.0905	Krutzen *et al.*, 2001
GT575	21	8	5.85	**0.619**	**0.820**	+0.1282	12	6	4.99	0.500	0.761	+0.1585	Berube *et al.*, 1996
TtruGT48	18	4	3.37	0.889	0.663	-0.1820	8	5	4.87	0.875	0.800	ND**	Caldwel *et al.*, 2002
IG10A1	16	6	5.27	0.750	0.808	+0.0130	7	7	7.00	0.857	0.890	ND**	Gravena *et al.*, 2009
IG2B1	21	2	1.82	**0.095**	**0.177**	+0.2857	10	3	2.90	**0.100**	**0.426**	+0.5984	Gravena *et al.*, 2009
IG11B1	20	6	5.02	**0.500**	**0.777**	+0.2147	7	4	4.00	**0.286**	**0.703**	ND**	Gravena *et al.*, 2009
IG1F1	19	5	4.22	0.737	0.690	-0.0440	11	4	3.51	0.455	0.558	+0.0938	Gravena *et al.*, 2009
IG8H1	19	6	4.26	**0.263**	**0.523**	+0.3706	10	6	5.00	0.500	0.632	+0.1487	Gravena *et al.*, 2009
IG11D2	20	4	2.67	0.300	0.276	-0.0733	8	2	2.00	0.375	0.325	ND**	Gravena *et al.*, 2009
IG10E	19	5	4.43	0.842	0.747	-0.0888	10	6	5.58	0.600	0.821	+0.1103	Gravena *et al.*, 2009
Probability of identity			1×10^{-10}						$7{,}4 \times 10^{-11}$				
F_{IS}*			-0.177 $p < 0.05$						-0.131 $p < 0.05$				

*calculated over 10 loci that did not exhibit signs of null alleles. ** Not done, at least 10 individuals are required to perform null alleles frequencies estimates.

Table 4. Maximum likelihood estimates of effective dispersal rate among regions and effective population size (N_e) based on microsatellite data using Migrate. M_{1_2} stands for: migration from population 1 (Mamirauá) to population 2 (Tefé); M_{2_1} stands for: migration from population 2 to population 1. The confidence interval (95% CI) is also reported.

	N_e	95%CI	M_{1_2}	M_{2_1}	95%CI
Mamirauá	2375	2150-2650	-	1.31	1.17-1.46
Tefé	3050	2700-3475	1.20	-	1.07-1.34

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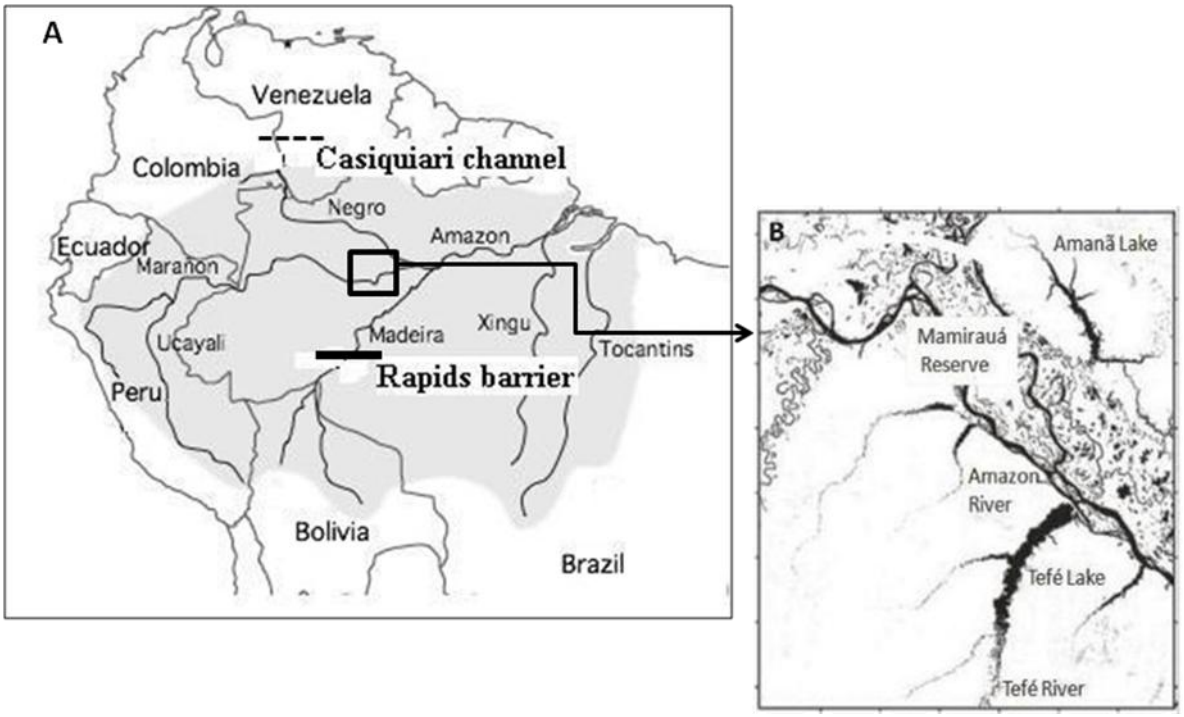
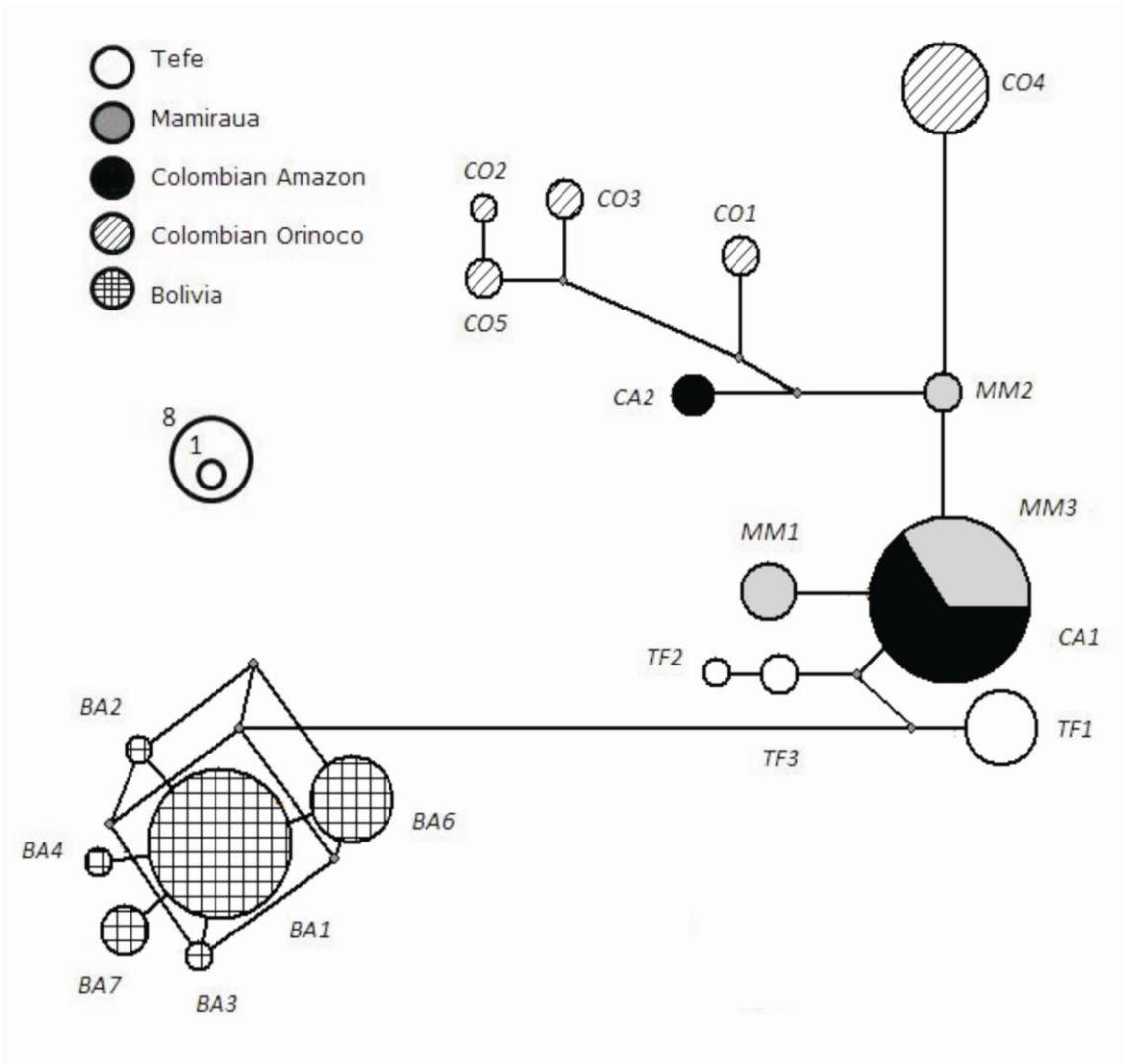
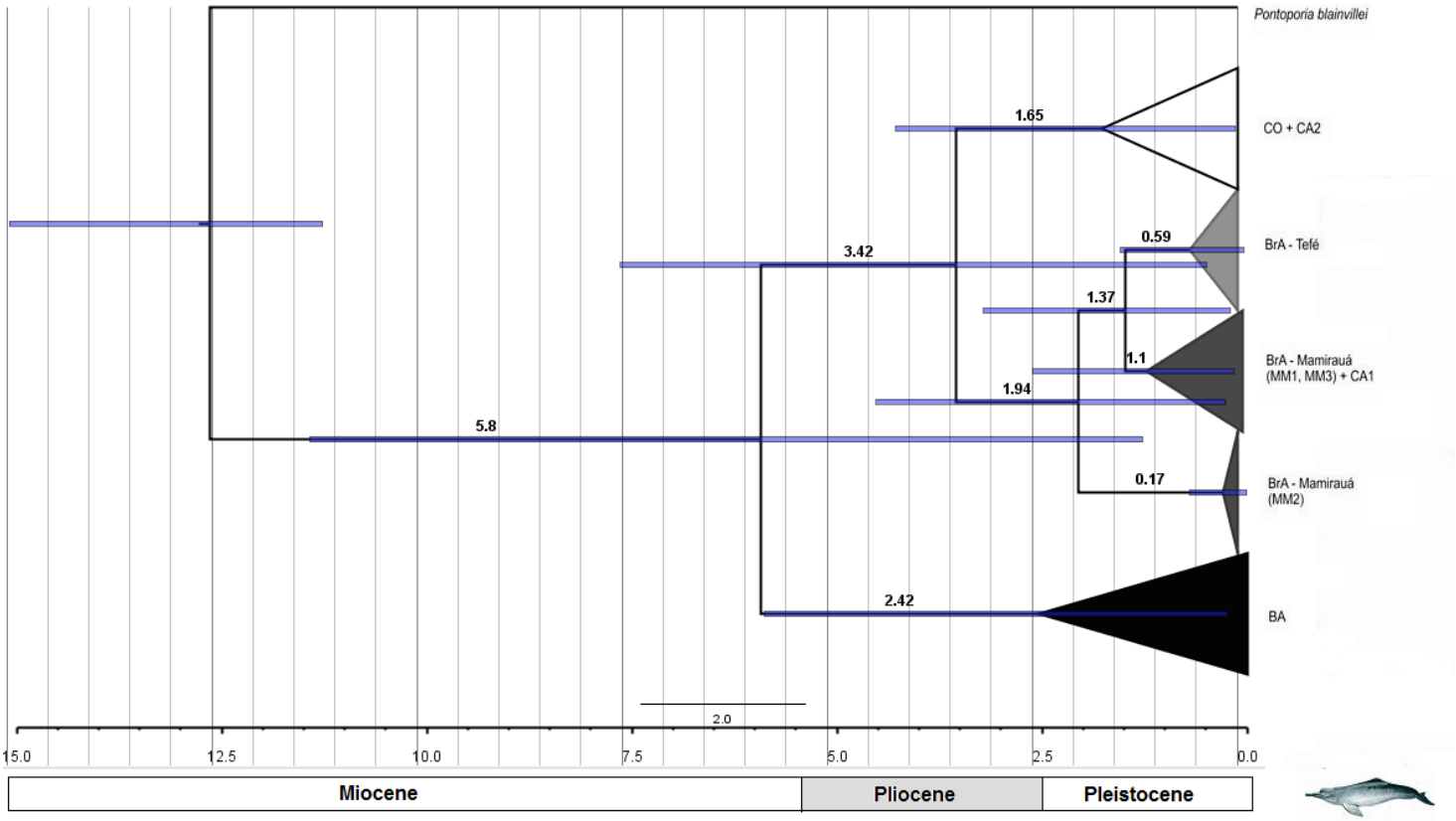


168 X 158 mm (800 X 800 dpi)



172 X 125 mm (600 X 600 dpi)



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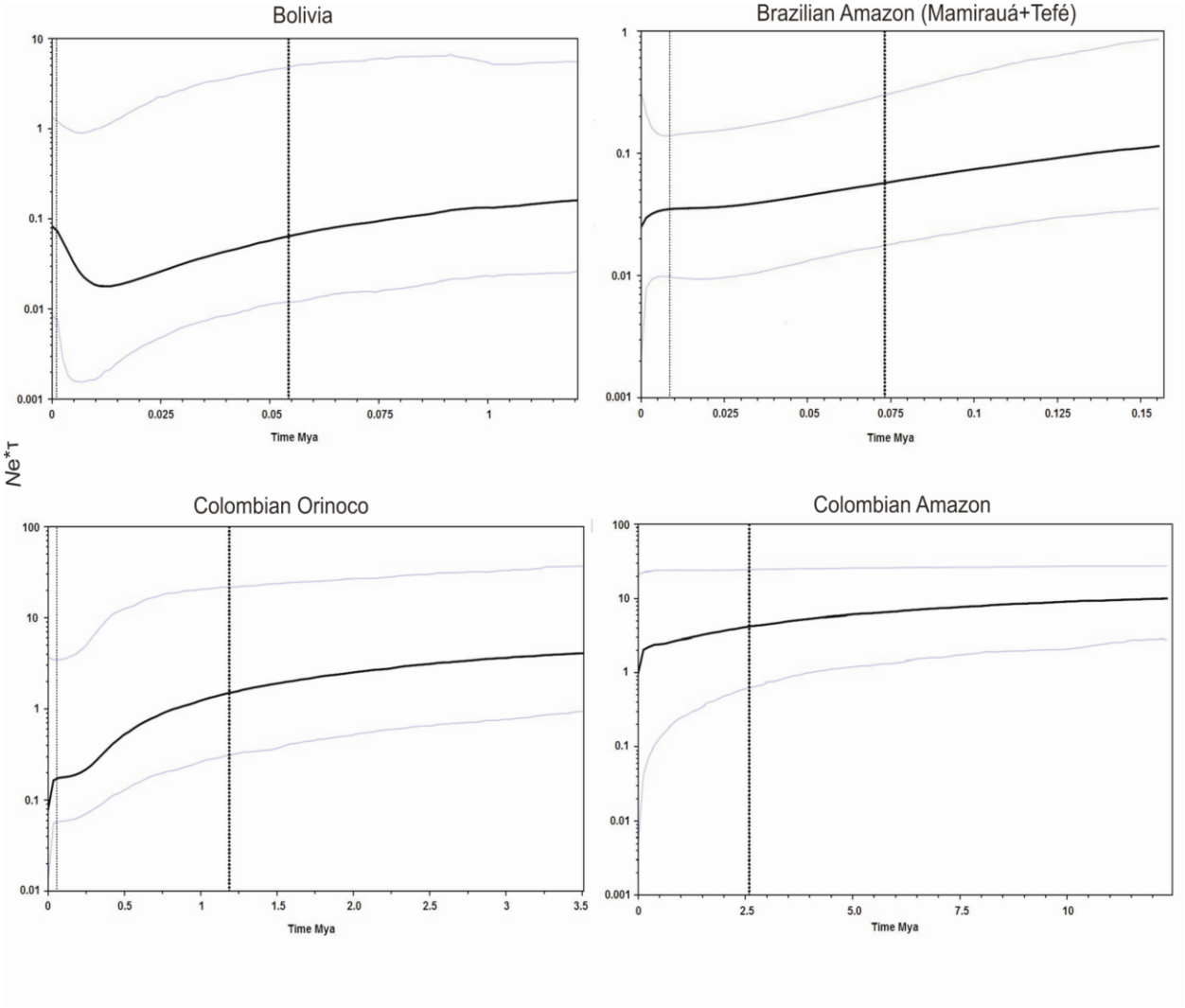

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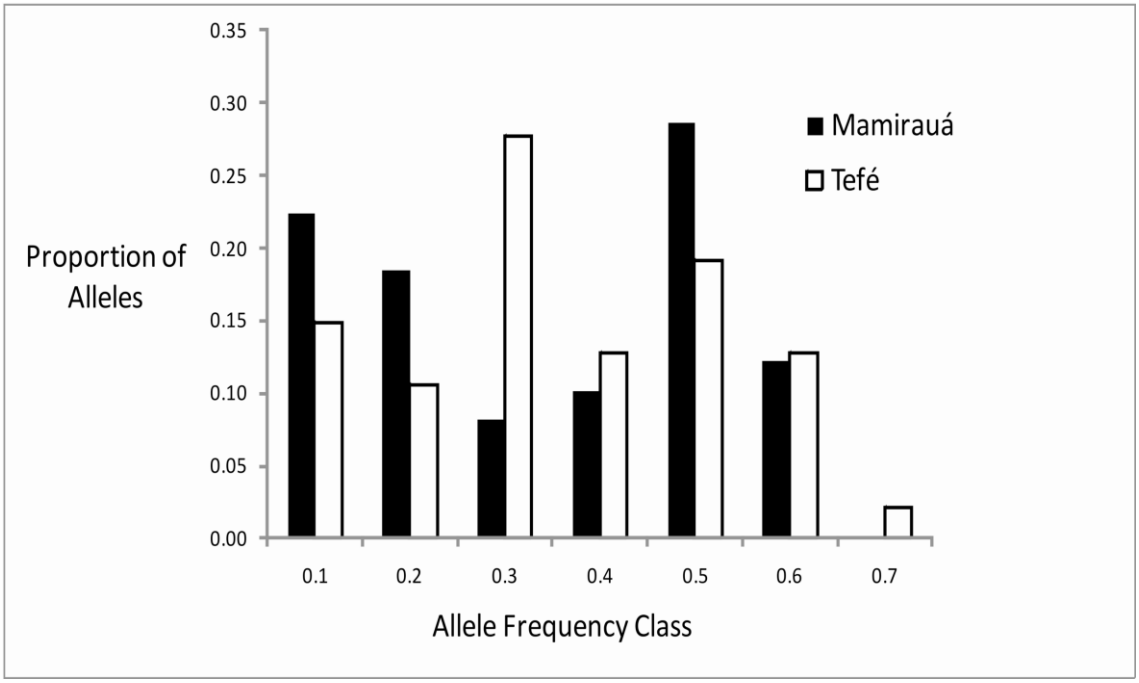

ARTIGO 2

Microsatellite data reveals fine genetic structure in male Guiana dolphins (*Sotalia guianesis)* in two geographically close embayments at south-eastern coast of Brazil

Artigo submetido para a revista *Marine Biology*

5

Short Communication to Marine Biology

Microsatellite data reveals fine genetic structure in male Guiana dolphins (*Sotalia guianesis)* in two geographically close embayments at south-eastern coast of Brazil

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Keywords: microsatellites, Guiana dolphin, population genetics, habitat, *Sotalia guianensis*.

ABSTRACT

Genetic distinctiveness among *Sotalia guianensis* populations along the Brazilian coast has been reported. However studies of genetic differentiation have not been so far investigated in closely distributed populations. Here we examined population structure for the largest populations of *S. guinanensis* inhabiting Sepetiba and Paraty embayments at the south-eastern coast of Rio de Janeiro, Brazil. Analysis of mitochondrial DNA (mtDNA) control region sequences failed to detect variability among sequences. Conversely, evidence of significant male population structure was found on the basis of eleven nuclear microsatellites loci. Surprisingly, the microsatellite markers were able to distinguish between individuals from the two embayments located 60 km apart, whereas a low but significant structure was displayed for the two socially groups within Sepetiba Bay over a distance of 15 km. The results suggest that differences in habitat type and behavioral specializations are likely to explain the patterns of genetic structure. These findings should provide baselines for the management of communities exposed to increasing human-driven habitat loss.

INTRODUCTION

Sotalia guianensis, also called Guiana dolphin, is widely distributed in the Atlantic coast from Central America to South of Brazil (Borobia et al. 1991; Carr and Bonde 2000). This species is mostly confined to estuarine environments with high site fidelity reported for different populations of these dolphins (Flores et al. 2004; Azevedo et al. 2004; Rossi-Santos et al. 2007; Flach et al. 2008; Dias et al. 2009). Along the Brazilian coast, Sepetiba Bay encloses the largest population of *S. guianensis* so far documented, which is estimated between 739 - 2196 individuals (Flach et al, 2008). Similar population size has also been reported in the neighboring Paraty embayment (Lodi 2003), making these regions an important area of occupation for this species. Moreover, both bays are of great environmental interest, comprising one of the Brazilian systems with greatest primary productivity (Nogara, 2000).

Human-driven habitat loss has been observed in the Sepetiba Bay. This region is highly degraded by chemical contamination, pesticides and mercury due its proximity to a metropolitan region with increased industrial development (Seixas et al. 2009). Potential threats to the dolphins in the Sepetiba Bay are arising from intense fishing activities and the construction of a large harbour system. Indeed, injuries and fatalities such as entanglement of these animals in fishing gear and collisions with boats have been reported in Sepetiba Bay (Nery et al. 2008). In addition, overfishing has been observed as the main threat to the dolphins in the Paraty Bay (Lodi 2003). Moreover, Paraty Bay is a popular tourism destination in Brazil and the intense recreational boating traffic is currently focus of major concern as it may influence changes in dolphin behavioral and group stability.

The species is listed in the Appendix I of the Convention in International Trade of Endangered Species (CITES) and as Data Deficient (2008) by the World Conservation Union (IUCN). Although some information regarding behavioral ecology and abundance has been reported (Flach et al. 2008; Dias et al. 2009) little is known about the genetic status of *S. guianensis* in the Brazilian coast (Cunha et al. 2005, Caballero et al. 2007).

Understanding genetic population structure plays a major role on the determination of management efforts towards conservation (Avise 1987; Frankham et al. 2003). Specific conservation plans could be developed to preserve or recover the genetic diversity of a given species. Lack or low levels of genetic variability could be perilous as those may drive species or populations to extinction. Low intra-specific variation between closely distributed populations is expected for the marine environment where the lack of geographic barriers may enhance the interchange of individuals. However, some studies have shown that marine populations are genetically more structured than might be expected even over small

geographic scales (Hoelzel 1998). Some characteristics of coastal habitats (estuaries and embayments) such as habitat type, local fidelity and behavioral specializations may drive genetic divergence among populations (Moller et al. 2007). Furthermore, oceanographic features such as surface salinity, temperature and productivity may also affect genetic differentiation (Bilgmann et al. 2007). From long-term observational studies, it appears that Sepetiba Bay harbours discrete socially structured groups of *S guianensis* along two different environments: the entrance of the bay, influenced by the open waters resulting in sandy and gravel substrates, higher salinity and water transparency and the interior part, characterized by mud and silt substrates, lower salinity and water transparency due to contact with river drainage systems (Flach et al. 2008, Dias et al. 2009). Since social structure is a potential force shaping intraspecific genetic differentiation in discrete populations of cetaceans, we have analyzed mitochondrial DNA control region sequences and microsatellite markers in order to assess the genetic structure of *S. guianensis* in Sepetiba and Paraty Bays, both located approximately 60 km apart.

MATERIALS AND METHODS

Samples were obtained from animals found dead or alternatively by biopsy darting collected between 2005 and 2009 in Sepetiba (526 km^2) and Paraty (243 km^2) Bays. Fifty individuals from both bays were sequenced at mtDNA control region (CR), including males and females. For microsatellites, only a subset of samples including all males (n=54), was genotyped for each marker due to DNA availability and quality. Samples from Sepetiba were later divided into samples coming from the entrance (n=20) and interior (n=23) of the bay; a total of 15 samples were obtained from Paraty Bay. All samples were preserved in 95% ethanol and stored in freezer. DNA was extracted following phenol-chloroform extraction protocol, as described in Sambrook et al. (2001).

A 480 base pairs (bp) fragment of mtDNA CR was amplified via the polymerase chain reaction (PCR) using primers CR-4 (Southern et al. 1988) and CR-5 (Southern et al. 1988; Palumbi et al. 1991). Each PCR mix contained 20 ng of genomic DNA, 1X Taq reaction buffer 1B (Phoneutria® - 1.5 mM $MgCl_2$, 10 mM Tris-HCl pH 8.4, 50 mM KCl, 0.1% Triton X-100), 200 µM dNTPs, 0.5 µM of each primer, and 1 unit of Taq DNA polymerase (Phoneutria®). The PCR cycling for CR was performed in an Eppendorff Gradient thermocycler following the conditions described by Vianna et al (2006). PCR products were cleaned using 20% PEG (Polyethyleneglycol) + 2.5 % NaCl, and both strands sequenced using an ET® dye terminator kit (GE Healthcare®) on a MegaBACE automated capillary

sequencer following the temperature cycling profile: 95°C for 1min, 35 cycles of 95°C at 25 sec, annealing at 50°C for 15 sec, and extension at 60°C for 3 min.

Samples were genotyped at eleven microsatellite loci either developed directly for *Sotalia guianensis*. (Cunha and Watts, 2007) or using heterologous primers isolated from the other cetacean species (Table 1) and standardized to amplify *S guianensis* samples. Amplification reactions contained 20-40 ng DNA, 20 mM Tris–HCl, pH 8.0, 50 mM KCl, 2.5 mM $MgCl_2$, 0.4 µM of each primer, 200 µM dNTPs and 0.25 units of Platinum-Taq (Invitrogen). Each forward primer had an M13 sequence tail added to its 5′ end to allow for fluorescent labeling (HEX, NED, FAM) following Schuelke (2000). The thermal cycle profile for the microsatellites isolated from another species were carried out following standard protocols, in 10 µl volumes, with 2.5 mM $MgCl_2$ and annealing temperature varying by locus (50°C for MK6, MK9, EV94 and GATA98; 55°C for PPHO142 and AAT44). Microsatellite loci isolated from *Sotalia* were amplified following the procedure described by Cunha *et al.* (2009). PCR products were measured using an Applied Biosystems 3730 automated sequencer. Allelic sizes were scored against the size standard GS500 LIZ (Applied Biosystems) and the peaks were analyzed using *GeneMapper* v4.0 (Applied Biosystem).

Individuals whose gender was previously unknown were submitted to sex identification by PCR amplification of a fragment of the *SRY* gene, multiplexed with fragments of the *ZFY/ZFX* genes as positive control, as described by Gilson et al. (1998).

Sequences of the mtDNA were “base called” with the software *Phred* 0.020425.c (Ewing et al. 1998). High quality consensus sequences (480 bp for CR) for each individual were produced from alignments of forward and reverse strand sequences with the software *Phrap* 0.990319 (Green 1994) and visualized/edited in *Consed* 15.0 (Gordon et al. 1998). The sequences were aligned using *Clustal-X* v1.83 (Thompson et al. 1997) to allow the identification of nucleotide variation and the different haplotypes. Microsatellite genotypes were screened for likely scoring errors, allele dropout and presence of null alleles using the program *Micro-Checker* (Van Oosterhout et al. 2004). The matching genotypes (to identify replicate individuals) and number of private alleles for each sampling site were estimated as implemented in the program *GenAlEx* v6.3 (Peakall and Smouse 2006). Departures from Hardy-Weinberg equilibrium, tests of linkage equilibrium for each locus, genetic differentiation among populations (using F_{ST} and R_{ST}) and inbreeding coefficients (F_{IS}) were carried out using *Arlequin* v3.1 software (Excoffier et al. 2005). The program *Bottleneck* v1.2 (Piry et al. 1999) was used to evaluate deviations of mutation-drift equilibrium in populations from the three different sites. Three different mutation models of microsatellite evolution were employed: (1) Infinite Allele model- IAM; (2) Stepwise Mutation Model – SMM and (3) Two-Phased model – TPM, an intermediate to the IAM and SMM which better fit to the most of

microsatellite datasets. The significance was assessed with the Wilcoxon sign-rank test, a more powerful and robust test when used with few polymorphic loci (< 20).

RESULTS

A fragment of 480 bp of the mtDNA CR was sequenced in 50 individuals and no variability was found across all samples. For microsatellites, all 11 loci amplified successfully in 54 individuals with no matching genotypes identified. All loci were polymorphic, except one: Sgui03 which was monomorphic for Paraty Bay. The number of alleles per locus ranged from 2 to 9. Heterozygosities were similar for all groups with low to medium level of allelic diversity at each locus. Sepetiba Bay displayed seven private alleles (3 in the entrance and 4 in the interior) compared to the five found in Paraty. Five loci exhibited significant deviations of Hardy-Weinberg equilibrium possibly due to null alleles (Table 2). However, we did not find significant differences in the *F*-statistics results when the five loci were excluded from the analyses. Also, no evidence of linkage disequilibrium was found for any locus pair. The analyses of population subdivision using F_{ST} and R_{ST} showed significant and marked genetic structure between samples from the two embayments (see Table 3). Surprisingly, F_{ST} values (1.5%) revealed a low but significant variation between the two socially groups within Sepetiba Bay (entrance and interior). In addition, negative and non-significant values of F_{IS} were obtained for the two sampling sites in Sepetiba Bay and when Paraty Bay was included in the analyses. In contrast, Paraty Bay alone displayed positive and non-significant F_{IS} values.

The program *Bottleneck* did not indicate a recent population decline in either Sepetiba or Paraty Bay. One-tailed Wilcoxon sign-rank tests for heterozygote excess indicated that each group was in mutation-drift equilibrium for all mutation models: IAM, TPM and SMM (p > 0.05).

DISCUSSION

The present study failed to find any variability in the control region over 50 individuals sampled. This result is consistent with the previous studies reported by Cunha et al (2005) and Caballero et al (2007) for the southeastern part of Rio de Janeiro. However, the number of samples analyzed by these authors was small, both analysed seven samples (n=14), considering the large population of dolphins in those areas. For marine mammals,

mitochondrial DNA is often expected to reflect population structure more rapidly than nuclear *loci*, due to its relatively low effective population size and high substitution rate (Hoelzel 2002). Our analysis based on microsatellite data found no evidence of a population bottleneck in Sepetiba or Paraty Bay, thus corroborating the hypothesis put forward by Cunha *et al.* (2005) that the reduced diversity is likely result from a founder event during the Holocene. Indeed, geological data indicates that the formation of Sepetiba embayment would have occurred during Holocene postglacial period around 6.000 BP (Pereira *et al*. 2009).Geological data indicates that the formation of Sepetiba Bay occurred during the Holocene postglacial period around 6.000 kya (Pereira et al. 2009). The closing of the bay leading to the formation of a highly productive estuarine ecosystem would have provided a suitable habitat for the establishment of *S. guianensis* in this embayment. Given the likely recent colonization of the Sepetiba Bay, we hypothesized that not enough time has elapsed to allow control region sequences to capture signals of population structure. On the other hand, the microsatellite markers used in this study seem to evolve fast enough to be reflected in patterns of genetic differentiation. Our results indicate a marked genetic structure between two localities about 60 km apart and also a low but significant differentiation between social groups in a smaller geographic scale within Sepetiba Bay. For bottlenose dolphins, several studies have shown genetically differentiated dolphin populations inhabiting different habitats over large and small distances (Krutzen et al. 2004; Natoli et al. 2005; Sellas et al. 2005; Möller et al. 2007; Bilgmann et al. 2007; Wiszniewsky et al. 2009). These studies strongly suggest that genetic distinctiveness is predominantly governed by habitat type and resource specializations. Influence of different oceanographic features (e.g. water salinity and temperature) on the genetic structure has also been reported for bottlenose dolphins (*Tursiops truncatus*) (Natoli et al. 2005; Bilgmann et al. 2007). These parameters have a direct influence on the distribution of preys and as a consequence, on the spatial distribution of the dolphins, which is observed for *S. guianensis* in Sepetiba Bay. Large dolphin aggregations have been reported at specific locations at the entrance and interior of Sepetiba Bay (Flach et al. 2008; Dias et al. 2009). Both areas have particular characteristics, such as fish species abundance and distribution (Araújo et al. 1998) and variations in topography which were observed to favor the formation of such large aggregations with up to 450 individuals (Dias *et al.* 2009). Ocean floor topography and several oceanographic features were also found to be related with population structure of common bottlenose dolphins (*T. truncatus*) in Black Sea, North Atlantic (Natoli et al. 2005). Spatial segregation and differences in habitat use and high site fidelity reported for *S. guianensis* in Sepetiba Bay indicate that habitat adaptation is likely to be influencing the distribution of genetic variation in this area. Although more information is required regarding the behavioral ecology of dolphins from Paraty Bay, it is possible that similar mechanisms are acting in population differentiation

in this location. Mating and breeding strategies are another possible explanation for the differential male genetic structure observed in both embayments. Male bottlenose population structure was also observed in Shark Bay, Australia (Krutzen et al. 2004). Connor et al (1992) suggested that alliance formation among males would facilitate the access to females more successfully. Thus, males may gain inclusive fitness benefits cooperating in this manner. Furthermore, long-range dispersal of males to other areas would minimize the chance of allying with a related partner. Most samples, acquired in social/sexual activities during this study, were from males, indicating that large groups of males around females might be commonly observed for *S. guianensis* in Sepetiba Bay, however a more detailed study is being conducted to better investigate this hypothesis.

Our results corroborate previous observations that dolphins living in protected environments with strong habitat boundaries are genetically more structured than open water populations (Möller et al. 2007). To date, localized genetic studies are mostly reported for bottlenose dolphins (*T. truncatus*) but only one study (Möller et al. 2007) showed such remarkable restricted genetic flow over scales less than 16 kilometers. Here we documented the first localized genetic study for *S. guianensis* adding evidence that this pattern of genetic differentiation is not restricted to a particular species (eg. *Tursiops* sp.) or to a particular estuarine environment.

Considering our results showing two different stocks for Sepetiba Bay dolphins and also a significant population differentiation across the embayments, in addition to the increasing human-related habitat degradation, specially in the interior of Sepetiba Bay, due to the construction of two large harbours, we suggest that specific conservation plans for each stock of Guiana dolphin should be designed in order to minimize anthropogenic disturbances in the south-eastern coast of Rio de Janeiro. This study has important implications for understanding evolutionary processes leading to genetic structuring in delphinids and for the management of Guiana dolphin populations in Brazil.

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FIGURE LEGENDS

Figure 1. Map of three sampling sites of estuarine dolphins in Sepetiba and Paraty Bay, south-eastern coast of Brazil. Numbers between parentheses indicate number of sampled individuals.

Table 1. Microsatellite *loci* used for *Sotalia guianensis* in this study.

Locus	Sequence	Repeat	Range	Specie	Author
GATA98	F-TGTACCCTGGATGGATAGATT R-TCACCTTATTTTGTCTGTCTG	(GATA)n	92-134	*Megaptera novaeangliae*	Palsboll et al. 2007
EV94Mn	F-ATCGTATTGGTCCTTTTCTGC R-AATAGATAGTGATGATGATTCACAC	(TC)n and (AC)n	198-261	*Megaptera novaeangliae*	Valsecchi and Amos, 1996
PPHO142	F-GAAGGCTCAGGGTATTG R-CAGTTACTTTCCTCGGG	(CA)n	127-161	*Phocoena phocoena*	Rosel et al. 1999
MK6	F-GTCCTCTTTCCAGGTGTAGCC R-GCCCACTAAGTATGTTGCAGC	(GT)n	145-189	*Tursiops aduncus*	Krutzen et al. 2001
MK9	F-CATAACAAAGTGGGATGACTCC R-TTATCCTGTTGGCTGCAGTG	(CA)n	168-180	*Tursiops aduncus*	Krutzen et al. 2001
Ttru AAT44	F-CCTGCTCTTCATCCCTCACTAA R-CGAAGCACCAAACAAGTCATAGA	(AAT)n	92 product size	*Tursiops truncatus*	Caldwell et al. 2002
SGUI02	F-GGATGTCACTGAACACAGAGC R: ACCTATCTACATTTCCCAGAGG	(CA)18	207–211	*Sotalia guianensis*	Cunha et al. 2007
SGUI03	F-TCCAATCTCCAACCAAATCCC R- GTCGCTAAGTTCATCATCTGC	(GT)28	148–162	*Sotalia guianensis*	Cunha et al. 2007
SGUI11	F-ACAGAGAAGCAAGTGGGAAACC R-TTCCCCGCCACTAAGATTCC	(GT)26	398–446	*Sotalia guianensis*	Cunha et al. 2007

SGUI17	F- GTGGTGGAGTAGAGGATAGG R-ACATTGGGCTTCAACGCACG	(CA)22	150–166	*Sotalia guianensis*	Cunha et al. 2007
SGUI18	F-CTGGAAAAAGAGTAGTTGGC R- GTGCAAGACCTCAAAATCC	(GT)29	234–252	*Sotalia guianensis*	Cunha et al. 2007

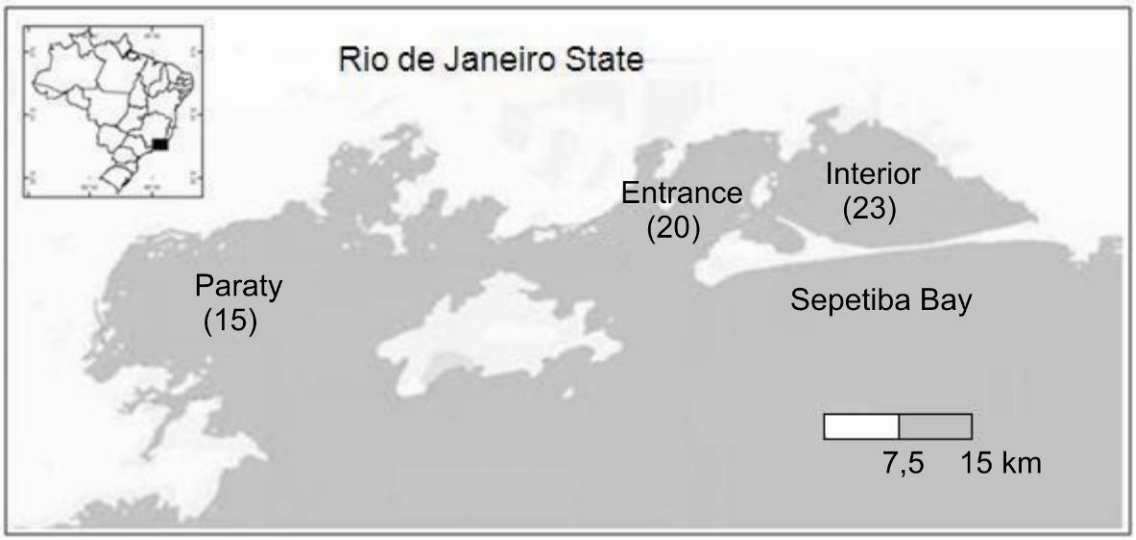
Table 2. Summary of genetic variation based on microsatellite data. n, sample size for each region; n_A, number of alleles at each locus; H_O and H_E, observed and expected heterozygosity. SBE: Sepetiba Bay Entrance, SBI: Sepetiba Bay Interior. Significant deviations from Hardy-Weinberg equilibrium are shown in bold.

Locus	**SBE**				**SBI**				**Paraty**			
	n	n_A	H_O	H_E	*n*	n_A	H_O	H_E	*n*	n_A	H_O	H_E
EV94	20	2	0.500	0.507	19	2	0.474	0.491	15	2	0.400	0.404
MK6	20	2	0.250	0.296	19	4	**0.526**	**0.627**	15	2	**0.200**	**0.508**
GATA98	20	5	0.650	0.605	19	5	0.737	0.619	15	5	0.600	0.542
PPHO142	19	2	0.474	0.462	17	2	0.588	0.513	14	3	0.500	0.632
MK9	20	2	**0.850**	**0.501**	19	2	**0.947**	**0.512**	13	2	**0.846**	**0.508**
AAT44	20	4	0.550	0.619	19	4	0.684	0.627	14	4	0.714	0.672
SGUI02	17	2	0.058	0.166	18	3	**0**	**0.209**	14	2	**0**	**0.476**
SGUI03	20	2	0.050	0.050	19	2	0.052	0.052	11	1	ND	ND
SGUI11	17	6	**0.353**	**0.531**	17	4	0.411	0.479	13	5	0.615	0.514
SGUI17	20	5	0.800	0.599	18	4	0.777	0.615	13	4	**0.308**	**0.748**
SGUI18	20	5	0.500	0.517	19	7	0.631	0.734	15	7	0.800	0.694
Average gene diversity			0.419 +/- 0.234				0.472+/- 0.260				0.502 +/- 0.279	
F_{IS} (*P* value)			-0.075 (0.849)				-0.097 (0.925)				0.072 (0.192)	

Table 3. Pairwise F_{ST} (matrix below) and R_{ST} (above matrix) values among three sampling locations based on eleven microsatellite loci. SBE: Sepetiba Bay Entrance; SBI: Sepetiba Bay Interior.

Pairwise $F_{ST,}$ R_{ST}	**SBE**	**SBI**	**Paraty**
SBE	-	0.003	0.082**
SBI	0.015*	-	0.049**
Paraty	0.060**	0.033*	-

*P value < 0.05, **P value < 0.001


Rio de Janeiro State
Entrance
(20)
Interior
(23)
Paraty
(15)
Sepetiba Bay
7,5
15 km

DISCUSSÃO FINAL

Os nossos resultados mostraram que ambos os marcadores, DNAmt e microssatélites, foram eficientes na elucidação da estruturação genética das populações. Para as populações de boto-rosa da Amazônia e Orinoco, o DNAmt foi informativo na avaliação dos níveis de diversidade, estrutura populacional e filogeografia de *I. geoffrensis* dentro dos diversos ambientes de água doce que a espécie ocupa na Amazônia brasileira. Além disto, a análise do DNAmt permitiu traçar a história biogeográfica e evolutiva da espécie ao longo de duas bacias hidrográficas no norte da América do Sul, bem como avaliar a influência de barreiras geográficas na estruturação das populações ao longo do tempo. Quatro populações foram claramente discrimadas com base no DNAmt e uma marcada separação filogeográfica ficou evidente em duas populações brasileiras distantes apenas 45 km. A análise adicional usando marcadores microssatélites corroborou o baixo fluxo gênico esperado pelos altos valores de F_{ST}.

Marcada estruturação filogeográfica foi observada em diversas espécies de cetáceos tais como em espécies de rio (ex. Família Platanistoidea), golfinhos pelágicos (ex. *Stenella clymene*, *Steno bredanensis*), em golfinhos costeiros (*Lagenorhynchus australis, Souza* sp, *Sotalia guianensis*), assim como espécies habitando ambos os ambientes (ex. *Tursiops truncatus, Stenella frontalis*, *Delphinus delphis, Pontoporia blainvillei*). Apesar de o ambiente marinho ser homogêneo o bastante para permitir a dispersão de animais em larga escala, os cetáceos apresentam-se mais estruturados do que esperado para um ambiente com suposta ausência de barreiras geográficas (Hoelzel *et al.*, 2002). Apesar de que alguns casos possam ser explicados pela separação alopátrica ou longas distâncias entre as populações, muitos outros exemplos não são explicados facilmente com base nestes mecanismos. Neste estudo, os marcadores microssatélites mostraram uma diferenciação genética significativa entre indivíduos provenientes de duas baías adjacentes. Muitos outros estudos encontraram situações semelhantes nas quais populações aparentemente panmíticas revelaram-se geneticamente estruturadas (Pichler et al., 1998; Krutzen et al. 2004; Natoli et al. 2005; Sellas et al. 2005; Adams & Rosel 2006; Möller et al. 2007; Bilgmann et al. 2007; Wiszniewsky et al. 2009b).

Em alguns casos, o uso diferencial dos recursos pode se refletir na estruturação social dentro do grupo e consequentemente na diferenciação genética em espécies ocorrendo em simpatria. As variações ecológicas que determinam o tipo de hábitat são tidas como um dos principais mecanismos na diferenciação genética dos cetáceos marinhos, uma vez que variações nas características físico-químicas da água e a topografia influenciam na

distribuição das presas e, por sua vez na distribuição e ocupação de ambientes pelos golfinhos que se especializam em determinados recursos alimentares (Hoelzel, 1998). Aspectos da história evolutiva e demografia também são determinantes na diferenciação das populações. Neste contexto, os marcadores moleculares têm um importante papel na avaliação da estrutura populacional dos cetáceos e consequentemente, no delineamento de estratégias de manejo.

Os marcadores moleculares contêm informações sobre o passado e o presente *status* da população que não podem ser obtidas por nenhum outro método (Avise, 2004). O DNAmt e os microssatélites são excelentes ferramentas para avaliar o status populacional e a combinação dos dois se mostrou particularmente interessante neste estudo uma vez que, devido à suas distintas características e modos de herança, questões diferentes puderam ser respondidas.

CONCLUSÃO FINAL

Artigo 1

1. A análise do DNAmt permitiu discriminar entre duas populações brasileiras, sendo que dois haplótipos novos foram identificados para Mamirauá e três novos para a região de Tefé. Tais resultados devem ser levados em consideração na elaboração de estratégias de manejo para ambas as populações.
2. O gene do citocromo *b* identificou um haplótipo específico para Mamirauá e outro específico para Tefé, reforçando a marcada estruturação filogeográfica revelada pela região controle.
3. As análises filogeográficas reforçam a existência de pelos menos duas unidades evolucionárias significativas, uma considerando as populações da Amazônia central brasileira e Orinoco e outra distinta para a Bolívia.
4. As análises filogenéticas usando DNAmt mostraram que as populações brasileiras preenchem uma lacuna entre as populações do Orinoco e da Bolívia, condizente com a posição geográfica intermediária entre ambas as populações.
5. As análises do tempo de divergência sugerem a presença de linhagens de *Inia* na bacia do Amazonas e do Orinoco há pelo menos 5 milhões de anos.
6. As análises demográficas ao longo do tempo mostraram que a população da Bolívia sofreu uma recente expansão populacional enquanto que para as populações brasileiras e do Orinoco uma recente redução populacional ficou evidente.

Artigo 2

1. As análises de estuturação populacional com marcadores microssatélites, usando F_{ST} e R_{ST}, detectaram uma significativa diferenciação entre duas baías adjacentes na costa sudeste do Brasil, enquanto que a análise de F_{ST} detectou ainda uma significativa diferenciação entre indivíduos dentro de uma mesma baía.

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ANEXO

Material Suplementar Artigo 1

Table 1- Supplementary Material. *Inia* sp. individuals used in this study. Sampling regions in Brazil, collection dates and genetically and morphologic indentified sex. Positive signs indicates successful amplifications for each molecular marker. The GenBank accession numbers of haplotypes compared in this study are also shown.

Sample	Locality	Date	Sex	mtDNA (CR)	mtDNA (cyt-b)	Microsatellites	Haplotypes used in this study (GenBank accession number)
Ig-02	Jurua Grande Lake - MSDR	Jan-94	F	+	+	+	**Control Region**
Ig-06	Jurua Grande Lake - MSDR	Feb-94	M	+	+	-	AF 521113
Ig-07	Anagua Lake - MSDR	Nov-94	F	+	+	-	AF 521114
Ig-08	Ressaca do Iuiri - MSDR	Nov-94	F	+	+	+	AF 521115
Ig-09	Ressaca do Iuiri - MSDR	Nov-94	M	+	+	+	AF 521116
Ig-11	Ressaca do Iuiri - MSDR	Nov-94	M	+	+	+	AF 521117
Ig-12	Ressaca do Iuiri - MSDR	Nov-94	M	+	+	+	AF 521118
Ig-13	Ressaca do Iuiri RDSM	Nov-94	M	+	+	+	AF 521119
Ig-14	Tefe Lake	Sep-95	F	+	-	-	AF 521120
Ig-15	Tefe Lake	Sep-95	M	+	+	+	AF 521121
Ig-16	Vila Nogueira - Tefe	Oct-95	M	+	+	+	AF 521122
Ig-17	Vila Nogueira - Tefe	Oct-95	?	-	-	+	AF 521123
Ig-19	Tefe Lake	Oct-95	F	+	+	-	AF 521124

Ig-20	Tefe Lake	?	F	+	+	-	AF 521125
Ig-21	Vila Nogueira - Tefe	Jan-96	F	+	+	+	AF 521126
Ig-22	MSDR	Oct-96	F	+	+	+	
Ig-27	Solimoes River - MSDR	Oct-96	F	+	-	+	**Cytochrome b**
Ig-28	MSDR	Jan-97	F	+	+	+	AF 521105
Ig-29	MSDR	Mar-97	M	+	+	-	AF 521106
Ig-30	Tefe Lake	May-97	M	+	+	+	AF 521107
Ig-31	Boca do Jarauá - MSDR	Nov-99	M	+	+	-	AF 521108
Ig-32	Boca do Jarauá - MSDR	Dec-01	F	+	+	+	AF 521109
Ig-33	Paranã do Moura - Tefe River	Jan-01	M	-	-	+	AF 521110
Ig-35	Tefe River	Feb-04	M	-	-	+	AF 521111
Ig-36	Tefe Lake	Mar-04	?	+	+	-	AF 521112
Ig-44	Amanã Lake	?	?	+	-	-	
Ig-45	MSDR	?	?	-	-	+	
Ig-46	MSDR	Nov-99	M	+	-	-	
Ig-47	Tefe River	Jan-01	M	-	-	+	

Ig-49	Amanã Lake	Aug-04	M	+	-	+
Ig-50	Amanã Lake	Jun-04	M	-	-	+
Ig-51	Amanã Lake	Jul-05	?	-	-	+
Ig-52	Amanã Lake	Aug-05	?	-	-	+
Ig-53	Amanã Lake	Aug-05	?	+	-	+
Ig-54	Tefe Lake	Oct-05	?	-	-	+
Ig-55	Tefe Lake	Oct-05	F	+	-	+
Ig-56	Tefe Lake	Mar-06	F	+	-	+
Ig-58	Tefe Lake	Jul-07	F	-	-	+
Ig-59	MSDR	Aug-07	?	+	-	+
Ig-60	Tefe River	Aug-07	F	-	-	+
Ig-61	Amanã Lake	Oct-07	F	+	-	+
Ig-62	Amanã Lake	Jan-08	M	-	-	+
Ig-63	Amanã Lake	Jan-08	M	-	-	+

220 X 170 mm (300 X 300 dpi)

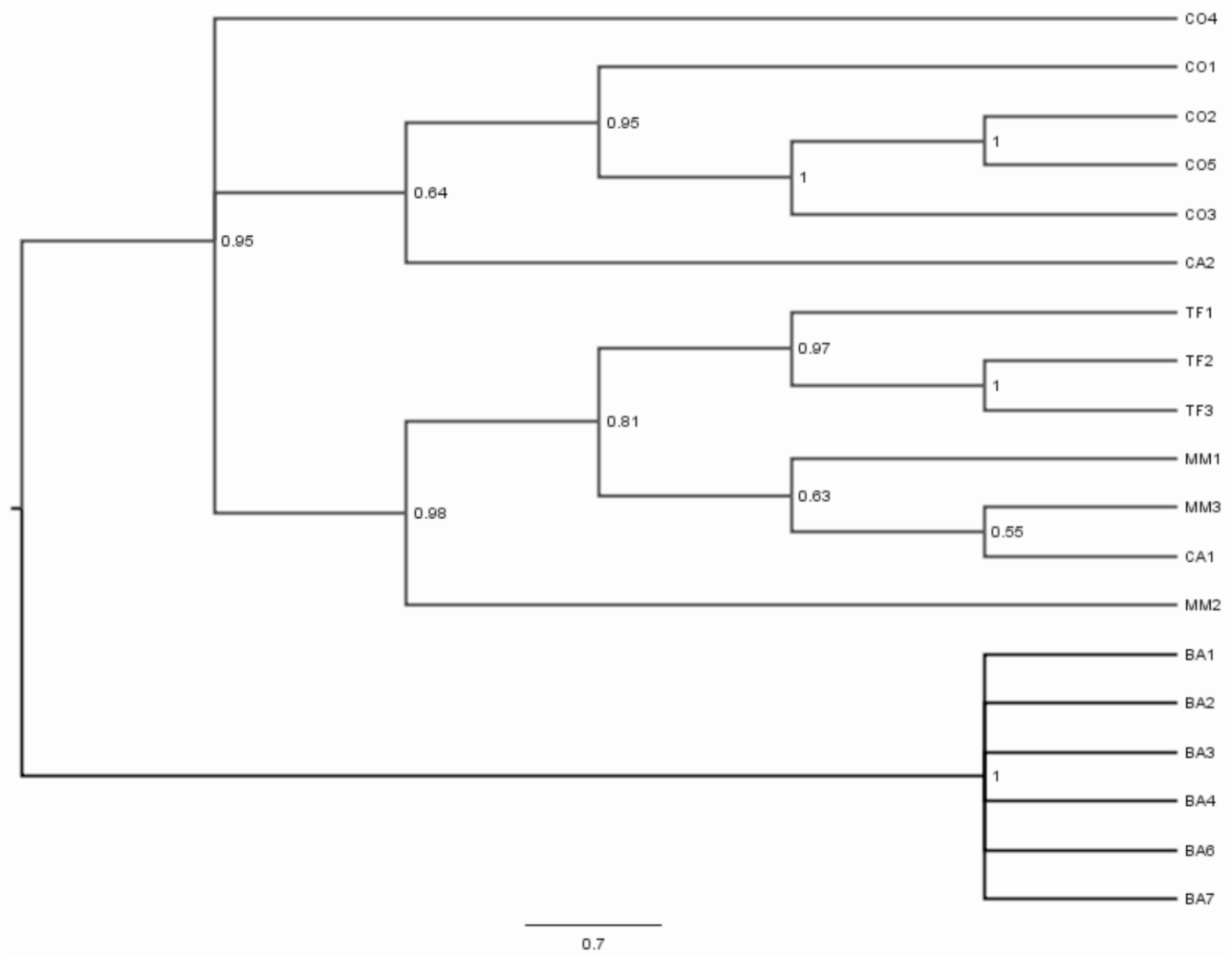

Figure S1. The Bayesian tree based on mtDNA control region. Numbers associate with nodes represent posterior probabilities from Bayesian MCMC searches conducted in MrBayes. CO: Colombian Orinoco; CA: Colombian Amazon; MM: Mamirauá-Brazil; TF: Tefé-Brazil and BA: Bolivian Amazon.

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