PAULA LARA-RUIZ

ANÁLISE GENÉTICA DE POPULAÇÕES DE TARTARUGA DE PENTE, *ERETMOCHELYS IMBRICATA*, ENCONTRADAS EM ÁREAS DE DESOVA E DE ALIMENTAÇÃO DO LITORAL BRASILEIRO.

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Tese apresentada ao Programa de Pós-Graduação em Genética, do Departamento de Biologia Geral do Instituto de Ciências Biológicas, da Universidade Federal de Minas Gerais, como requisito parcial à obtenção do título de Doutor em Genética, área de concentração em Genética Evolutiva e de Populações.

Orientador: Prof. Dr. Fabrício Rodrigues dos Santos.

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RESUMO

Uma das sete espécies de tartarugas marinhas existentes, a tartaruga de pente (*Eretmochelys imbricata*) é considerada criticamente ameaçada (CR) de extinção pela IUCN. No Brasil, as populações de tartaruga de pente foram reduzidas quase à extinção devido à coleta de ovos e à captura de animais para a comercialização da sua carapaça. Devido ao longo ciclo de vida e o hábitat exclusivamente marinho de quase todos estágios de desenvolvimento, o que dificulta o estudo e acompanhamento destes animais na natureza, os marcadores moleculares têm sido muito úteis no estudo da história natural destes animais. Entre estes marcadores, principalmente, as seqüências do DNA mitocondrial (DNAmt) são usadas para caracterizar as colônias de desova em diversas áreas do mundo e para definir o local de origem dos indivíduos encontrados nas áreas de alimentação da espécie. Até hoje, nenhum estudo foi realizado com o intuito de caracterizar uma amostra significativa de animais provenientes das áreas de alimentação e desova no território brasileiro. No entanto, a colônia que desova no Brasil é considerada uma das maiores remanescentes no Atlântico sul. Os principais objetivos deste trabalho foram: 1) caracterizar as populações de tartarugas de pente encontradas em áreas de desova e de alimentação no Brasil, e 2) estudar em detalhe os eventos de hibridização nas populações brasileiras. Para isto, amostras provenientes das áreas de desova e de alimentação foram analisadas quanto às freqüências dos haplótipos da região controle do DNAmt encontrados, que somados a marcadores nucleares foram utilizados para melhor entender o processo de hibridização detectado. Os dados genéticos sobre populações de *E.imbricata* no Brasil sugerem que futuras estratégias de manejo com fins de conservação devem diferenciar populações de desova e de alimentação. A alta freqüência de híbridos nas áreas de desova e a introgressão unidirecional a partir de machos de tartaruga cabeçuda não deve ser ignorada na hora de estabelecer as estratégias para a conservação desta população. Nas áreas de alimentação, a origem variada dos indivíduos encontrados indica que a conservação destes agregados é de importância global, pois pode afetar populações de desova em países do Caribe, Atlântico Oriental e do Indo-Pacifico.

ABSTRACT

One of the seven extant species of marine turtles, the hawksbill turtle (*Eretmochelys imbricata*) is considered Critically Endangered (CR) by the IUCN. In Brazil, hawksbill populations were reduced almost to extinction due to egg poaching, harvest of nesting females and the slaughter of animals for the commerce of their carapace. Due to their long life cycle and the marine habitat of most life stages that difficult the study of wild animals, molecular markers have been useful in the study of their natural history. Among molecular markers, mitochondrial DNA (mtDNA) sequences have been used for the characterization of nesting colonies around the world and for the definition of the origin of individuals found in feeding aggregates. To the present no extensive study was realized to characterize the nesting and feeding populations in Brazil even though the nesting colony in this country is one of the largest remaining in the southern Atlantic. The main objectives of this work were 1) to characterize the nesting and feeding hawksbill populations found in Brazil and 2) to study in detail the hybridization events occuring in Brazilian grounds. In order to do this, both nesting and feeding aggregates were characterized by means of their mtDNA haplotype frequencies. Autosomal markers were used to understand better the ongoing hybridization process. The new genetic data about the species in Brazil suggest that management strategies for the conservation of nesting and feeding populations have to be independent. The unusual high frequency of hybrids in the nesting grounds and the unidirectional introgression process are of greater concern for managers. In feeding grounds, the multiple origin of the individuals indicate that harvest of animals in the Brazilian territory can affect nesting populations in Caribbean, East Atlantic and Indo-Pacific countries.

I) INTRODUÇÃO

Dentre as sete espécies existentes de tartarugas marinhas, a tartaruga de pente, *Eretmochelys imbricata*, é uma das espécies cujas populações se encontram mais ameaçadas devido, principalmente, à exploração comercial dos escudos da sua carapaça. Esta exploração dizimou as populações em muitos países de maneira que hoje a espécie é considerada criticamente ameaçada pela União Internacional para a conservação da Natureza (IUCN, 2006).

No Brasil, a caça de fêmeas, o roubo de ninhos, a comercialização da carapaça e a captura incidental por redes de pesca, levaram a espécie quase à extinção (Marcovaldi et al., 1999). Hoje, a tartaruga de pente está incluída na lista oficial de espécies ameaçadas e todos os estágios do seu ciclo de vida, incluindo ovos e neonatos, se encontram oficialmente protegidos (Fundação Biodiversitas, 2003). Entretanto, graças aos esforços conservacionistas realizados nas ultimas décadas, a população que desova atualmente no país representa a maior colônia de desova do Atlântico Oeste no Hemisfério Sul e é uma das poucas no mundo onde se estima que mais de 1000 fêmeas desovem cada ano (L. Soares, com pess).

Devido ao longo ciclo de vida e ao fato de passarem grande parte das suas vidas no oceano, a caracterização de populações de tartarugas marinhas através de marcadores genéticos tem se mostrado muito útil para preencher lacunas no conhecimento da história natural destes animais. Muitas das populações encontradas ao longo da distribuição da espécie, especialmente as populações do Caribe, já foram estudadas através de marcadores genéticos. Porém, as populações tanto de fêmeas encontradas em praias de desova quanto de animais de ambos os sexos encontrados nas áreas de alimentação ao longo do litoral brasileiro nunca foram objeto de um estudo abrangente visando a sua caracterização através de marcadores genéticos.

Na literatura, a única informação referente às populações brasileiras é o registro de uma alta incidência (10 de 14 amostras) de haplótipos da região controle do DNAmt de tartaruga cabeçuda (*Caretta caretta*) em amostras de *E.imbricata* (Bass, 1996), sugerindo a ocorrência de hibridização entre as duas espécies e levantando a necessidade de realizar uma caracterização mais completa desta população. A presença de haplótipos de *C.caretta* em alta freqüência numa amostra de *E.imbricata* é um fenômeno que só foi registrado nas colônias de desova no Brasil, sendo que os poucos relatos de híbridos existentes na literatura são esporádicos e referem-se a indivíduos específicos e não a grupos de indivíduos.

Em vista disso, e levando em conta que a colônia de desova brasileira é uma das maiores remanescentes no Atlântico Sul, o presente estudo foi proposto com o objetivo de realizar a caracterização das populações encontradas no Brasil, estabelecer ligações entre estas e outras populações caracterizadas previamente e estudar mais a fundo o possível processo de hibridização registrado previamente. Com esta análise pretende-se avaliar o panorama geral da situação genética da espécie no Brasil e da importância destas populações para a conservação da espécie num contexto global.

Dentre os marcadores moleculares, as seqüências da região hipervariável do DNAmt foram escolhidas por serem as mais utilizadas para realizar a caracterização de populações de tartarugas marinhas. A utilidade do DNAmt para os estudos de história natural destes animais é justificada pelo fato de que há uma alta filopatria materna nestas espécies, isto é, as fêmeas sempre voltam para desovar na praia onde nasceram (*natal homing*). Portanto, análises de haplótipos de DNAmt podem ser utilizadas para discriminar as diferentes colônias de desova e definir, por exemplo, a origem dos agregados de tartarugas em áreas de alimentação.

Tal como demonstrado em um estudo recente de populações do Caribe (Bowen et al., 2007), os agregados de indivíduos nas áreas de alimentação podem ser compostos por animais provenientes de populações de desova distantes. Portanto, se o mesmo ocorre com os agregados de alimentação no território brasileiro, sua exploração ou pesca incidental pode ameaçar ou provocar o declínio de populações cuja desova não ocorre no Brasil. Da mesma maneira, a caracterização da colônia de desova brasileira através de seqüências do DNAmt é importante para estabelecer marcadores populacionais que possam ser utilizados, no futuro, para identificar animais de origem brasileira encontrados em áreas de alimentação fora do território nacional.

Adicionalmente, para estudar os eventos de hibridização registrados na literatura, marcadores nucleares foram utilizados para entender melhor a direção dos cruzamentos entre as espécies parentais e verificar a existência de introgressão (retrocruzamento dos híbridos com uma das espécies parentais). Esta informação não pode ser obtida através do uso de seqüências de DNAmt apenas e, portanto, foi necessário utilizar marcadores autossômicos já disponíveis na literatura e desenvolver outros específicos para a diferenciação das duas espécies envolvidas nos cruzamentos.

O objetivo geral desta tese de doutorado foi produzir informação genética detalhada sobre as colônias de desova e os agregados de alimentação da tartaruga de pente no Brasil, para contribuir ao estabelecimento de estratégias adequadas de manejo visando à conservação da espécie tanto no nível nacional quanto global.

Os objetivos específicos foram:

a) Descrever e caracterizar os haplótipos da região controle do DNAmt encontrados em populações de *E.imbricata* presentes nas áreas de alimentação e de desova que ocorrem no Brasil.
b) Comparar os dados de DNAmt entre colônias de desova e agregados de alimentação para definir possíveis unidades de manejo da espécie no Brasil.
c) Comparar os haplótipos de DNAmt encontrados com os já registrados na literatura para outras populações de *E.imbricata* no mundo.
d) Identificar as possíveis populações de origem dos juvenis encontrados nas áreas de alimentação do Brasil.
e) Verificar, quantificar e qualificar a ocorrência e extensão do fenômeno de hibridização entre *E.imbricata* e *C.caretta* na costa do Brasil.
f) Desenvolver novos marcadores nucleares e aplicar os já existentes para investigar detalhes processo de hibridização e introgressão observado.

Este documento está estruturado da seguinte maneira:

1) Uma breve revisão bibliográfica enfocando os principais temas abordados na tese,
2) os artigos resultantes dos trabalhos realizados e
3) uma conclusão geral.

Os artigos apresentados são:

Artigo 1. “Extensive hybridization in hawksbill turtles (*Eretmochelys imbricata*) nesting in Brazil revealed by mtDNA analyses.”

Artigo 2. “Population structure and hybridization in hawksbill (*Eretmochelys imbricata*) feeding and nesting aggregates from Brazil.”

Artigo 3. Identification of nuclear diagnostic SNPs for the identification of hawksbill (*Eretmochelys imbricata*) and loggerhead (*Caretta caretta*) hybrids.

II) REVISÃO DA LITERATURA

II. 1) As tartarugas marinhas

As duas famílias de tartarugas marinhas (Cheloniidae e Dermochelidae) formam um grupo monofilético dentro da Sub-ordem Cryptodira (Ordem Testudines) cuja radiação data do início do Cretáceo, cerca de 110 milhões de anos atrás (MAA) (Hirayama, 1998). Atualmente, são reconhecidas seis espécies dentro da família Cheloniidae; a tartaruga cabeçuda (*Caretta caretta*), a tartaruga verde (*Chelonia mydas*), a tartaruga oliva (*Lepidochelys olivacea*), a tartaruga de Kemp (*Lepidochelys kempii*), a tartaruga de pente (*Eretmochelys imbricata*) e a tartaruga plana (*Natator depressus*). A família Dermochelidae possui como única representante, a tartaruga de couro (*Dermochelys coriacea*).

Adicionalmente, alguns autores reconhecem uma outra espécie, a tartaruga preta (*Chelonia agassizii*), mas as evidências morfológicas, bioquímicas e genéticas são conflitantes, por isso ainda é considerada pertencente à espécie *C.mydas* (Pritchard e Mortimer, 1999). Todavia, as relações filogenéticas entre os gêneros da família Cheloniidae (Figura 1) não se encontram bem definidas (Limpus et al., 1988; Bowen et al., 1993; Dutton et al., 1996; Pritchard e Mortimer, 1999).

Com exceção da tartaruga de Kemp que habita o Golfo do México e da tartaruga plana que é restrita à Austrália, as outras espécies são cosmopolitas e sua distribuição depende do seu grau de tolerância às baixas temperaturas. A tartaruga de couro apresenta a distribuição mais abrangente, tendo sido encontrada forrageando em águas polares, enquanto que a tartaruga de pente apresenta a distribuição mais restrita às águas tropicais (Meylan e Meylan, 1999).

Todas as sete espécies de tartarugas marinhas são consideradas ameaçadas de extinção. A tartaruga de Kemp, a tartaruga de pente e a tartaruga de couro são consideradas criticamente ameaçadas (CR); a tartaruga cabeçuda, a tartaruga oliva e a tartaruga verde são consideradas ameaçadas (EN), e a tartaruga plana se encontra classificada como uma espécie com dados insuficientes (DD) (IUCN, 2006). Estas categorias são baseadas em critérios tais como tamanho das populações, área de ocorrência e probabilidade de extinção na natureza, indicando a necessidade de aprofundar no conhecimento destas espécies para estabelecer planos de manejo que permitam recuperar as populações em declínio.

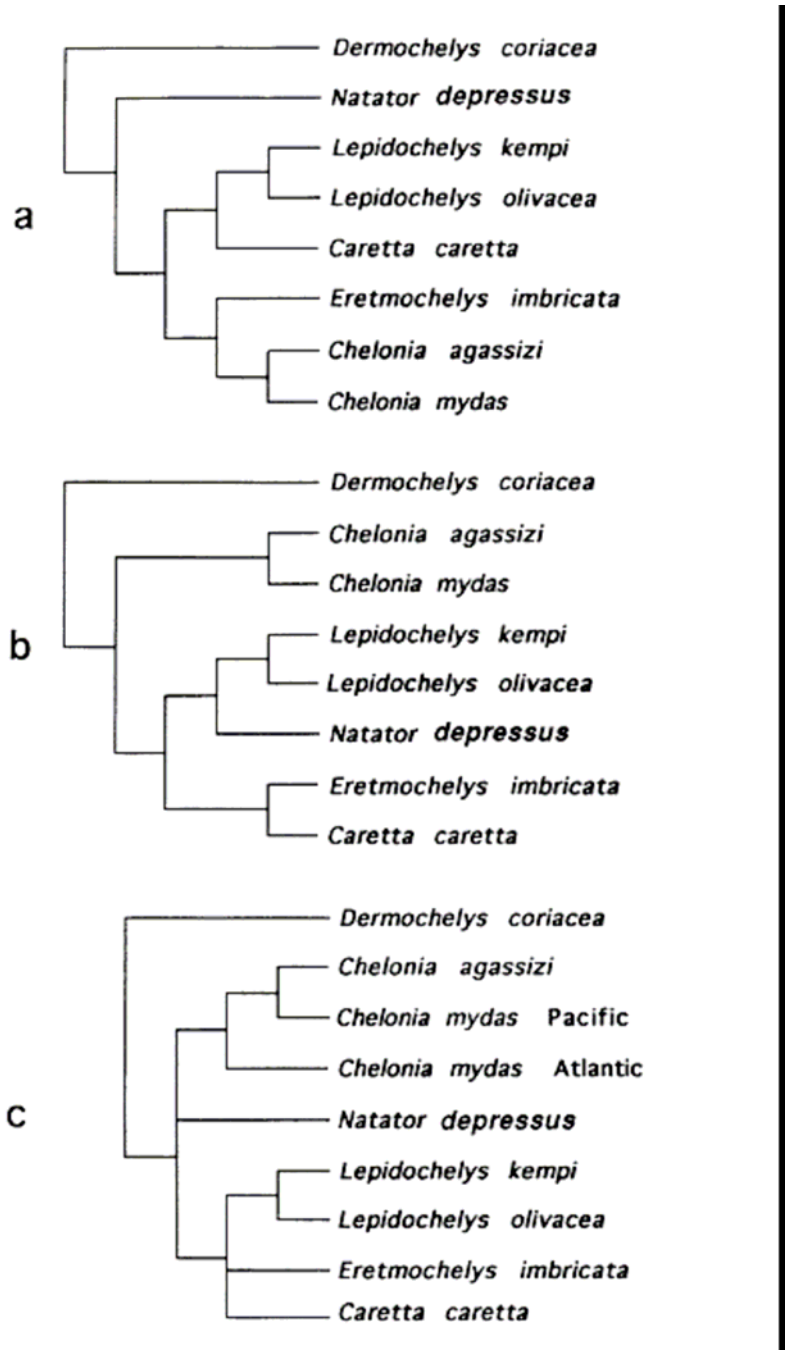

Figura 1. Algumas das hipóteses filogenéticas propostas para as relações entre as diferentes espécies de tartarugas marinhas. Baseadas em caracteres (a) morfológicos, (b) moleculares (eletroforese de proteínas) e (c) seqüências de mtDNA. Reproduzido de Dutton et al (1996).

Em geral, mudanças sazonais e etapas do desenvolvimento são consideradas os fatores principais que explicam os hábitos migratórios das tartarugas marinhas (Carr et al., 1966; Carr et al., 1978). As tartarugas verde, cabeçuda e de pente, após emergirem do ninho, começam um período pelágico que pode durar vários anos durante os quais o deslocamento dos neonatos é governado principalmente pelas correntes oceânicas. Após este período, animais juvenis (20 - 40 cm dependendo da espécie) começam aparecer nos denominados habitats de desenvolvimento onde permanecem até a maturidade. Estes

habitats são áreas de alimentação costeiras onde raramente se observam animais adultos. As estimativas de idade na maturidade, entre 15 e 50 anos, variam segundo a espécie e a região geográfica (Bjorndal e Zug, 1995). Os adultos permanecem associados às áreas de alimentação (que podem ou não compartilhar com os juvenis) e, durante o período reprodutivo migram para a região costeira associada às praias de desova (Carr et al., 1978).

Os hábitos migratórios dificultam o estudo destas espécies bem como os esforços para protegê-las, desde que medidas para sua conservação devem levar em conta todas as áreas de ocorrência das populações em seus diferentes estágios de desenvolvimento. Atualmente, a tartaruga cabeçuda e a tartaruga verde são as espécies melhor estudadas. Estudos recentes baseados em rádio-telemetria e análises moleculares permitiram aumentar o conhecimento sobre os hábitos destas, e em menor grau, de outras espécies, identificando rotas migratórias e estabelecendo relações entre colônias de desova e locais de alimentação (Lutz e Musick, 1997; Bolten e Witherington, 2003). Estas descobertas indicam que estratégias conservacionistas eficazes só podem ser feitas através de esforços conjuntos de todas as nações que abrangem a área de ocorrência de cada uma das espécies.

II.2) A espécie *Eretmochelys imbricata* (Linnaeus 1766)

A tartaruga de pente possui uma distribuição circum-global, ocorrendo em mais de 110 unidades geopolíticas em áreas tropicais dos oceanos Atlântico, Pacífico e Índico (Figura 2) (IUCN, 2006). Colônias de desova são encontradas em mais de 60 países, mas na maior parte deles se apresentam em baixas densidades como resultado da intensa exploração que foi e ainda é realizada (Meylan e Donelly, 1999), principalmente devido ao alto valor comercial de objetos fabricados com as placas da carapaça (tortoiseshell). Esta exploração diminuiu as populações drasticamente, de maneira que atualmente restam poucas áreas onde se estima que mais de 1000 fêmeas reproduzam a cada ano (Tabela 1, Figura 2).

No Caribe, acredita-se que restam apenas 5% da população original como conseqüência da sobre-exploração e da degradação dos recifes coralinos, principais habitats de alimentação desta espécie. Segundo estimativas, os corais do Caribe tiveram uma redução de 80% durante as últimas três décadas (Gardner et al., 2003). Adicionalmente, só entre 1970 e 1986, aproximadamente 250.000 indivíduos foram capturados no Caribe e exportados para o Japão onde o comércio de objetos fabricados a partir deste material ainda é comum (Meylan e Donnelly, 1999; Bjorndal e Jackson, 2003). Os recifes localizados na Austrália e no Mar Vermelho foram menos impactados nas últimas

décadas e isto, juntamente com a adoção de medidas de proteção eficazes, explica porque algumas das maiores populações remanescentes se encontram nos oceanos Índico e Pacífico (Tabela 1, Figura 2).

As fêmeas desta espécie produzem em média 140 ovos em cada postura, sendo que numa estação reprodutiva podem desovar mais de uma vez. As fêmeas adultas permanecem reprodutivamente ativas durante um longo período, porém raramente reproduzem em intervalos menores de 2-3 anos (IUCN/SSC, 2003).

Uma alta taxa de mortalidade caracteriza os primeiros estágios do desenvolvimento, sendo que menos de 1 em cada 1000 filhotes sobrevivem até a maturidade (IUCN/SSC, 2003). As taxas de crescimento variam segundo a faixa etária e a localidade onde os habitats de desenvolvimento se encontram, porém, estas taxas são lentas e indicam que a tartaruga de pente leva mais de uma década (possivelmente até duas) para atingir a maturidade (Boulon, 1994; IUCN/SSC, 2003).

O tempo estimado desde que o animal eclode até o seu retorno às praias de desova é entre 15 e 40 anos (IUCN/SSC, 2003), sendo que comportamentos como a fidelidade pelo local de nascimento e desova (*natal homing*, *spawning site fidelity*), já demonstrados para outras espécies de tartaruga marinha, também caracterizam as fêmeas da tartaruga de pente (Bass et al., 1996; Bowen et al., 2007).

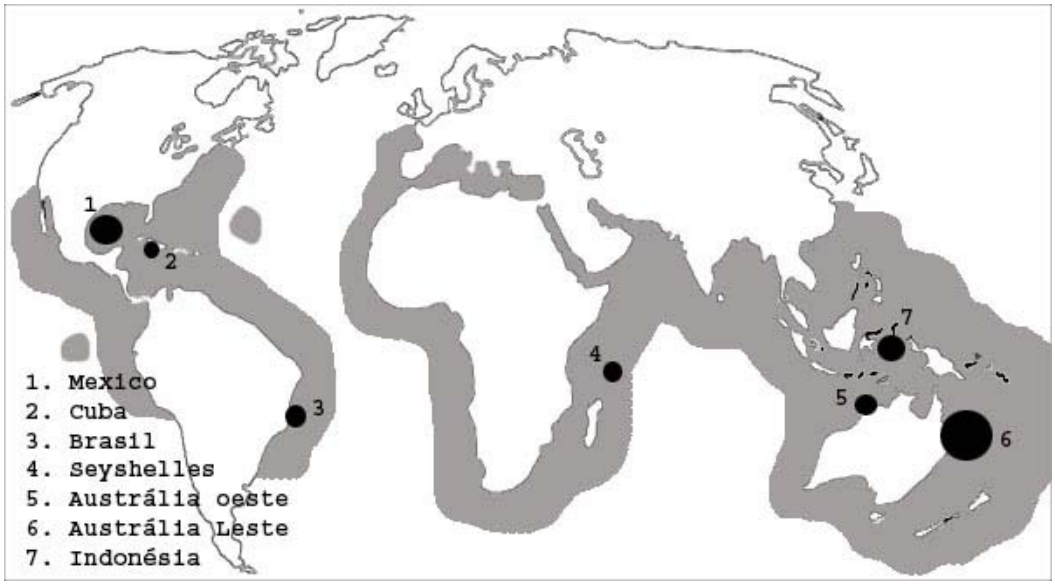

FIGURA 2. Regiões de ocorrência de *E.imbricata* (cinza) e principais localidades de desova remanescentes (preto), onde se estima que existam mais de 1000 indivíduos desovando a cada ano (ver tabela 1).

Não existe muita informação sobre os padrões de deslocamento dos neonatos que, após sua entrada na água iniciam uma fase pelágica de vários anos (Carr et al., 1966) durante a qual os movimentos dos animais são governados principalmente pelas correntes oceânicas (Carr et al., 1966; Musick e Limpus, 1997). Esta fase finaliza com o recrutamento dos juvenis (20-25 cm de comprimento da carapaça) às áreas de desenvolvimento próximas a recifes coralinos. *E. imbricata* se alimenta principalmente de diversas espécies de esponjas e cnidários, razão pela qual tanto adultos quanto juvenis se encontram frequentemente associados com recifes de coral (Meylan, 1988; Leon e Bjorndal, 2002).

Após a fase pelágica, os juvenis deslocam-se preferencialmente, para áreas de alimentação próximas às praias natais (Bowen et al., 2007) da mesma maneira que já foi demonstrado para a tartaruga cabeçuda (Laurent et al., 1998; Bowen et al 2004, 2005). Os padrões de migração e as origens dos juvenis encontrados nas áreas de alimentação não são bem conhecidos devido à dificuldade de monitorar estes animais durante um período de maturação que pode exceder 20 anos. Acredita-se que a composição das populações em áreas de alimentação pode ser influenciada pelo tamanho das colônias de desova existentes na região, pela distância até essas colônias e pelas correntes oceânicas dominantes (Bowen et al., 2007).

A tartaruga de pente já foi considerada uma das espécies com menor comportamento migratório devido ao fato dos juvenis permanecerem durante longos períodos associados aos habitats de desenvolvimento próximos da sua área de origem (Broderick et al., 1994; Bass, 1999). Porém, hoje se sabe que a espécie possui hábitos migratórios semelhantes ao das outras espécies de tartarugas marinhas. Ao longo do ciclo de vida, os indivíduos dispersam e migram ao longo de grandes distâncias, frequentemente centenas e até milhares de quilômetros (Miller et al., 1998; Meylan 1999; Horrocks et al., 2001; IUCN/SSC, 2003), existindo até registros de migrações transatlânticas (Marcovaldi e Filippini, 1991; Bellini et al., 2000).

Estudos de marcação e telemetria por satélite estão revelando padrões de movimentação dos animais entre áreas de alimentação e desova (Meylan, 1999; Horrocks et al., 2001; Projeto TAMAR-IBAMA, dados não publicados). Também se sabe que animais desta espécie podem ser sedentários e residentes numa determinada área de alimentação nos períodos precedentes e entre as migrações reprodutivas (IUCN/SSC, 2003). Adicionalmente existe informação que sugere que os juvenis, durante o desenvolvimento, alternam períodos de residência com migrações entre diversos habitats de desenvolvimento (áreas de alimentação) (IUCN/SSC, 2003).

Tabela 1. Estimativas populacionais das principais colônias de *E.imbricata* no mundo. Modificado a partir de Spotila, 2004. * Dados do Projeto TAMAR-IBAMA, 2006.

Localidade	Nº de fêmeas desovando / ano
Mar Caribe	**5000-6000**
Antígua	150
Barbados	50-60
Belize	40-50
Cuba	500-1000
Rep. Dominicana	300
Guatemala	100-200
Jamaica	200-275
Martinica	80-125
México	2800
Porto Rico	210
Turks	200-275
Ilhas Virgens	130
Venezuela	50-500
América do Sul (Norte) *	**1100**
Brasil*	800-1000
Oceano Atlântico (Leste)	**200-400**
Guiné Bissau	200
Oceano Índico	**6000-7000**
Índia	250
Austrália (Noroeste)	2000
Arquipélago de Chagos	300-700
Burma	30
Leste da África	100
Egito	500
Maldivas	300
Omã	600-800
Arábia Saudita	160
Seychelles	1000
Sudão	350
Iêmen	500
Oceano Pacífico	**10000**
Austrália	6000-8000
Indonésia	800-2000
Malásia	100-500
Palau	20-50
Papua Nova Guiné	<100
Filipinas	100-500
Ilhas Salomão	<500
Tailândia	<100

II.3) Marcadores genéticos aplicados ao estudo de tartarugas marinhas

Os estudos genéticos são de grande importância para caracterizar a estrutura populacional de tartarugas marinhas e sua dinâmica, desde que o estudo de fatores

determinantes (tais como o comportamento reprodutivo e os padrões de uso de habitat e migratórios) é muito difícil devido aos hábitos destes animais e seu longo ciclo de vida. Assim, parte da informação conhecida sobre a história natural de muitas das espécies de tartarugas marinhas foi produzida pelos estudos que utilizaram marcadores genéticos (Norman et al., 1994; Bowen e Witzell, 1996; Bowen e Karl, 1997; Bowen, 2003; Godley et al., 2004).

O comportamento de fidelidade ao local de nascimento (*natal homing*) apresentado pelas fêmeas gera uma forte diferenciação entre as colônias de desova ao longo do tempo (Meylan et al., 1990). Estas diferenças têm sido amplamente estudadas através do sequenciamento da região controle do DNA mitocondrial (DNAmt). O DNAmt é até hoje o sistema genético mais utilizado para o estudo das tartarugas marinhas, devido a sua herança uniparental materna e outras características especiais que permitem obter níveis de resolução apropriados para estudos filogeográficos baseados em herança através de matrilinhagens (Bowen et al., 1992; Bowen et al., 1994; Lahanas et al., 1994; Bass et al., 1996; Bowen et al., 1996; Encalada et al., 1996; Lahanas et al., 1998; Encalada et al., 1998; Bass, 1999; Diaz-Fernandez et al., 1999; Dutton, 1999; Bass e Witzell, 2000; Engstrom et al., 2002; Bowen, 2003).

Além disso, em muitos casos os haplótipos da região controle do DNAmt podem ser usados como marcadores populacionais, permitindo estabelecer elos entre os indivíduos encontrados em áreas de alimentação e as colônias de desova. Este fato permitiu estabelecer que indivíduos provenientes de diversas colônias compartilham áreas de alimentação bem distantes do seu local de origem (Bowen et al., 1994; Bass et al., 1996; Bass 1999; Bowen et al., 2006, Bass et al., 2006).

No passado, marcadores nucleares já foram usados para identificar eventos de hibridização entre as diversas espécies (Karl et al., 1995), e mais recentemente, estudos utilizando marcadores nucleares microssatélites estão sendo realizados com o objetivo de auxiliar no entendimento de outros aspectos da história de vida de algumas espécies (Pearse e Avise, 2001; Moore e Ball, 2002; Lee e Hays, 2004; Bowen et al., 2005; Jensen et al., 2006). Até hoje, marcadores nucleares não foram utilizados para caracterizar populações de *E.imbricata.*

II.4) Marcadores moleculares aplicados ao estudo de *E.imbricata*.

Grande parte da informação existente sobre padrões migratórios da tartaruga de pente foi obtida através de estudos genéticos realizados com marcadores da região controle do DNAmt (Tabela 2). Da mesma maneira, o DNAmt já foi utilizado para estabelecer as

origens dos animais que compõem os agregados de indivíduos presentes em áreas de alimentação (Tabela 2). Estes estudos, realizados em sua grande maioria com populações residentes no Caribe, confirmaram o comportamento de "natal homing" nesta espécie e demonstraram que a maior parte das colônias de desova de *E.imbricata* se comporta como unidades geneticamente isoladas, com diferenças significativas nas freqüências haplotípicas, e muitas vezes, com haplótipos do DNAmt característicos que podem ser usados como marcadores populacionais. Estas diferenças permitem identificar as origens de adultos e de juvenis encontrados em áreas de alimentação ou capturados pela pesca incidental, através de estudos conhecidos como análises de estoques múltiplos ou mistos (Multiple ou Mixed Stock Analysis, MSA) (Broderick et al., 1994; Norman et al., 1994; Bowen, 1995; Bowen et al., 1996; Diaz-Fernandez 1999; Troeng 2005; Bowen et al., 2007).

Os estudos genéticos realizados com populações de *E. imbricata* se limitam aos trabalhos referidos na tabela 2, realizados com populações em áreas de alimentação e desova no Caribe e no Indo-Pacífico, e ao presente estudo realizado no Brasil (Lara-Ruiz et al., 2006). Estes estudos visaram documentar a distribuição da diversidade genética nos níveis local e regional através da descrição de haplótipos e freqüências haplotípicas que caracterizam distintas colônias de desova e agregados de alimentação. Estas análises não são, de maneira alguma, tão extensas quanto as já realizadas com outras espécies, cujas populações se encontram caracterizadas no nível global (Bowen et al., 1992; Bowen et al., 1994; Dutton, 1999), mas permitiram estabelecer que, como já demonstrado para outras espécies, as colônias de desova de *E.imbricata* devem ser consideradas como unidades de manejo distintas, pois as diferenças nas freqüências haplotípicas encontradas indicam um alto grau de estruturação da diversidade genética (Bass et al 1999; Díaz-Fernández et al., 1999; Bowen et al., 2007).

A análise de composição de estoques nos agregados de alimentação de *E.imbricata* só tem sido realizada até o momento para localidades no Caribe (Bowen et al., 2007), desde que é a única região onde as colônias de desova estão relativamente bem caracterizadas. Porém, haplótipos encontrados em locais de alimentação, que não foram descritos para nenhuma das colônias de desova analisadas, devem ficar fora da análise deixando-a incompleta e limitando as conclusões que podem ser tiradas. Por isto, ainda hoje se realizam esforços para caracterizar as colônias de desova em outras regiões dentro da área de distribuição da espécie, no intuito de identificar possíveis localidades de origem de haplótipos encontrados em locais de alimentação. Esta informação é indispensável para o delineamento adequado de estratégias de manejo para a conservação da espécie.

Tabela 2. Estudos genéticos realizados em populações de *E.imbricata.*

Marcador	**Região**	**Localidades**	**N /Tipo amostra**	**Conclusões**	**Ref.**
DNAmt RFLPs	Indo - Pacífico (Austrália)	Nordeste (Pacífico) Oeste (Indico)	N=144 Desova: 4 colônias Alimentação: 2 agregados	Duas populações geneticamente distintas (oeste e nordeste – separadas por 2700 km). Colônias de desova mais próximas (< 750 km) são geneticamente semelhantes. Proporção significativa dos indivíduos em áreas de alimentação provém de áreas de desova distintas das colônias mais próximas (freqüências alélicas em áreas de alimentação são significativamente distintas das freqüências de áreas de desova próximas).	Broderick et al., 1994
DNAmt Região Controle	Indo - Pacífico	Austrália Arábia Saudita Malásia I. Salomão	N=87 Desova: 6 colônias	15 haplótipos agrupados em dois clados divergentes. Alto grau de estruturação entre colônias, caracterizada por diferenças nas freqüências haplotípicas. 13/15 haplótipos específicos de distintas populações de desova.	Broderick & Moritz, 1996
DNAmt Região Controle	Caribe	Ilha Mona, Porto Rico.	N=41 Alimentação: 1 agregado	Agregado composto por indivíduos provenientes de diversas áreas no Caribe, não só da área de desova na Ilha Mona, mas sem contribuição de populações brasileiras. A caça de tartarugas nas áreas de alimentação em Mona afeta populações de desova em todo o Caribe.	Bowen et al., 1996
DNAmt Região Controle	Caribe e Brasil	Belize México Porto Rico Barbados Cuba Ilhas Virgens Antigua Brasil	N= 103 Desova: 8 colônias	Alto grau de estruturação entre as diferentes colônias estudadas, concordante com o modelo de "natal homing" para o recrutamento de fêmeas jovens nas áreas de desova. Só dois de 21 haplótipos encontrados foram compartilhados entre distintas localidades de desova. Populações de desova efetivamente isoladas em escala de tempo ecológico (MUs), mas não em tempo evolutivo (ESUs). Existem pelo menos 6 estoques (breeding stocks) distintos que devem ser manejados como unidades independentes. Dez das 14 amostras do Brasil apresentaram haplótipos de *C.caretta*.	Bass et al., 1996
mtDNA Região Controle	Caribe	Cuba México Porto Rico	N=488 Desova 3 colônias Alimentação 3 agregados	Alto grau de estruturação. 12 haplótipos encontrados em áreas de desova (4 em México, 4 em Cuba e 4 em Porto Rico). Dezesseis haplótipos adicionais encontrados nos agregados de alimentação. 15% destes de origem desconhecida indicando a necessidade de caracterizar outras colônias ainda não estudadas. Contribuição das áreas de desova locais para as populações em áreas de alimentação é relativamente grande (41%-70%).	Díaz-Fernández et al., 1999

Continua...

Marcador	Região	Localidades	N /Tipo amostra	Conclusões	Ref.
DNAmt Região Controle	Caribe e Atlântico Norte (AN)	Anguilla Ilhas Virgens Ilhas Caiman Monserrat Turks & Caicos Bermuda (AN)	N=217 Alimentação 5 agregados N=58 Bermuda Alimentação	5 haplótipos novos (não descritos para nenhuma área de desova) encontrados nos agregados de alimentação. Isto indica que é necessário continuar a caracterização das populações de desova para permitir estabelecer o local de origem destes animais. Em Bermuda foram registrados 8 haplótipos (5 já registrados no Caribe e 3 haplótipos novos). Quatro amostras apresentaram haplótipos de *C. caretta.*	Godley et al., 2004
DNAmt Região Controle	Caribe	Costa Rica Tortuguero	N=42 Desova	Dados de telemetria e marcação indicam que adultos desovando em Tortuguero se alimentam na Nicarágua e em Honduras, enquanto que os dados das análises genéticas sugerem que podem também se alimentar em Porto Rico, Cuba e possivelmente México. Os dados genéticos proporcionam informação complementar aos dados de marcação, permitindo detectar contribuições de áreas de desova aos agregados de alimentação que a técnica de marcação não permite detectar.	Troeng et al., 2005
DNAmt Região Controle	Atlântico Oeste	Caribe e Brasil	N=347 Desova: 10 colônias N=626 Alimentação 8 agregados	Colônias de desova são geneticamente independentes. A maior parte da diversidade genética das populações do Caribe já foi identificada (*h* diminui com o aumento do N). A contribuição das colônias de desova para os agregados de alimentação não depende unicamente do tamanho das colônias ou da distância entre elas e os agregados. As correntes marinhas e outros fatores ambientais também podem ser importantes para determinar a composição dos agregados de alimentação.	Bowen et al., 2007
DNAmt Região Controle	Brasil	Bahia	Desova N=119	Alta freqüência de haplótipos de *C.caretta* (42% das amostras) em animais identificados (morfologia) como *E.imbricata*. Possível processo de introgressão. Também foi descrita pela 1ª vez a ocorrência de híbridos com *L.olivacea*. 4 haplótipos característicos de *E.imbricata*.	Lara-Ruiz et al., 2006

II.5) Hibridização em tartarugas marinhas

Além dos trabalhos de caracterização dos haplótipos da região controle do DNAmt, existem na literatura alguns outros trabalhos nos quais os autores utilizaram marcadores genéticos para confirmar a ocorrência de híbridos entre *E.imbricata* e outras espécies de tartarugas marinhas. O entrecruzamento entre várias espécies da família Cheloniidae já foi registrado em inúmeras regiões do globo (Seminoff et al., 2003). Porém, a maior parte dos registros é baseada na descrição de indivíduos com características morfológicas intermediarias (Carr e Dodd, 1983; Kamezaki, 1983; Wood et al., 1983; Frazier, 1988) e só recentemente estes eventos de hibridização foram estudados utilizando marcadores moleculares (Conceição et al., 1990; Karl et al., 1995; Seminoff et al. 2003; Lara-Ruiz et al., 2006).

A ocorrência de hibridização entre distintas espécies de tartarugas marinhas pode ser devida à inexistência de barreiras reprodutivas que permitem o intercruzamento (Seminof et al., 2003). Além disso, a hibridização natural pode estar relacionada com o pareamento meiótico normal que pode ocorrer entre espécies relacionadas (Seehausen, 2004). No caso das tartarugas marinhas, a baixa taxa de evolução cariotípica registrada permitiria a compatibilidade cromossômica (Bickham, 1981; Kamezaki, 1989, 1990; Karl *et al*., 1995) e a produção, em alguns casos, de híbridos viáveis.

Conceição et al (1990), utilizaram isoenzimas para caracterizar alguns espécimes encontrados na Bahia, Brasil, confirmando a existência de híbridos de *E.imbricata* e *C.caretta*; enquanto que Karl et al (1995) utilizaram RFLPs e DNAmt para confirmar a existência de híbridos entre *C. caretta* x *L. kempii* (n=1), *C. careta* x *E. imbricata* (n=2), *C. caretta* x *C. mydas* (n=4) e *C. mydas* x *E. imbricata* (n=1). A ocorrência de híbridos entre as diversas espécies tem sido registrada na literatura desde a primeira metade do século XX (ver Referências em Conceição et al., 1990), mas em geral, as observações são registros esporádicos de um ou poucos indivíduos. Até o presente, o único registro de ocorrência de híbridos em altas freqüências dentro de uma população de desova foi feito para a colônia do Brasil (Bass, 1996; Lara-Ruiz et al., 2006).

O estudo de processos de hibridização é importante já que auxilia no entendimento das relações evolutivas entre as espécies envolvidas (Seehausen, 2004; Allendorf et al., 2001). Considerando que a separação entre as tribos Carettini (*Caretta*, *Eretmochelys* e *Lepidochelys*) e Chelonini (*Chelonia* e *Natator*) aconteceu ao redor de 50 MAA (Bowen et al., 1993) enquanto que a separação entre as espécies destas tribos é estimada entre 10 e 20 MAA (Karl et al., 1995), estas espécies podem ser os organismos mais antigos que hibridizam naturalmente (Seminoff et al., 2003).

A hibridização entre espécies também pode ser de especial importância para a conservação desde que pode levar a extinção de espécies raras. Conseqüentemente, existem inúmeros registros de casos nos quais a hibridização levou à extinção, em espécies vegetais e animais, mesmo quando este processo não foi acompanhado de introgressão (Allendorf et al., 2001; Rhymer e Simberloff 1996; Fredrickson e Hedrick 2006; Seehausen 2006). Assim, o estudo dos padrões e processos que caracterizam um evento de hibridização pode fornecer informação indispensável para delinear estratégias de manejo adequadas para a preservação das espécies envolvidas (Allendorf et al., 2001).

III) ARTIGOS

ARTIGO 1:

Extensive hybridization in hawksbill turtles (*Eretmochelys imbricata*) nesting in Brazil revealed by mtDNA analyses

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Extensive hybridization in hawksbill turtles (*Eretmochelys imbricata*) nesting in Brazil revealed by mtDNA analyses

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Running title: Hawksbill hybrids in Brazilian nesting grounds

Key words: mitochondrial DNA, haplotype diversity, hawksbill turtles, hybridization, introgression

ABSTRACT

Bahia state hosts over 90% of hawksbill (*Eretmochelys imbricata*) nests registered in the main nesting sites monitored by Projeto Tamar-IBAMA in Brazil. The genetic diversity of this hawksbill population (N=119) was assayed through the analyses of 752 bp of the mitochondrial DNA control region in nesting females. Seven distinct haplotypes, defined by 125 polymorphic sites, were found. Most of the individuals (n = 67) display four typical hawksbill haplotypes, 50 individuals display two haplotypes characteristic of the loggerhead turtle (*Caretta caretta*) and two individuals had a haplotype affiliated with the olive ridley (*Lepidochelys olivacea*). These results demonstrate hybridization between the hawksbills and two species that nest along the Bahia coast. Of special interest is the high occurrence of loggerhead x hawksbill hybrids (42%), which display loggerhead mtDNA haplotypes but are characterized morphologically as hawksbills. The true hawksbill haplotypes present only three variable sites and low genetic diversity values (h = 0.358 +/- 0.069; π = 0.0005 +/- 0.0001). The occurrence of several nesting individuals with identical mtDNA from another species may also suggest a long history of introgression between species producing likely F2 or further generation hybrids. Marine turtle hybrids have been previously reported, but the high frequency observed in Bahia is unprecedented. Such introgression may influence evolutionary pathways for all three species, or may introduce novel morphotypes that develop apart from the parental species. The presence of a unique hybrid swarm has profound conservation implications and will significantly influence the development and implementation of appropriate management strategies for these species.

INTRODUCTION

Sea turtles nesting in Brazil have suffered under prolonged anthropogenic pressure which has caused the decline of all five species that use Brazilian beaches as nesting grounds. Under IUCN criteria, the loggerhead turtle (*Caretta caretta*), the olive ridley (*Lepidochelyes olivacea*), and the green turtle (*Chelonia mydas*), are currently considered “endangered” (EN), while the leatherback turtle (*Dermochelys coriacea*) and the hawksbill turtle (*Eretmochelys imbricata*) are classified as "Critically Endangered" (CR) (IUCN, 2004). The hawksbill turtle has a circum-global distribution in tropical areas of the Atlantic, Indian and Pacific Oceans (Groombridge & Luxmoore 1989, Pritchard & Mortimer 1999). In Brazil, slaughter of nesting females, egg poaching, traffic of shell ornaments, coastal development, and incidental capture by fisheries have reduced the species almost to extinction (Marcovaldi et al. 1999).

Hawksbill nesting in Brazil occurs mostly during the austral summer, generally from December to February, with an average of 800 nests per season. The State of Bahia, where this study was carried out, harbors ca. 90% of all hawksbill nests registered in Brazil. During the same period (1999-2002), three other species nested in northern Bahia, representing 54.8% of loggerhead nests in Brazil (ca. 2600 nests per season in bahia), 21% of the olive ridley (ca. 600 nests per season in bahia), as well as some sporadic (ca. 30 nests per season in bahia) green turtle clutches (Projeto TAMAR data bank).

Molecular markers have proven useful for resolving migration patterns, feeding ground population composition, natal homing, and the genetic composition and structure of rookeries worldwide (Bass et al. 1996, Fitzsimmons et al. 1997a, b, Bolten et al. 1998, Bowen et al. 2005). Hawksbill genetic studies, along with flipper tagging, re-capture and satellite telemetry analyses, have suggested the common use of habitats by different populations throughout the Caribbean and have provided useful information to the understanding of the species biology (Troëng et al. 2005). In Brazil, mtDNA analyses of hawksbills are restricted to the works by Bass et al. (1996) and Bass (1999) examining 14 individuals from two nesting areas in Bahia (Arembepe and Praia do Forte). These preliminary analyses revealed six haplotypes (384 bp), and a high proportion (10 of 14 samples) of loggerhead x hawksbill hybrids (morphologically diagnosed hawksbills with loggerhead mtDNA haplotypes). Evidence of hybridization was first reported by Conceição et al. (1990), who identified a likely loggerhead x hawksbill hybrid in this same population using protein electrophoresis. Despite this hybridization reports, a study on loggerheads from nesting grounds in Brazil did not registered any hawksbill mtDNA haplotypes in 81 loggerhead samples (Soares, 2004).

Here, we report the distribution and frequency of interspecific hybrids among hawksbills nesting in Bahia, Brazil, evaluated using mtDNA markers. However, the uniparental nature of mtDNA limits the inferences that can be made about this ongoing hybridization process, highlighting the need to further analyze this population using biparentally inherited nuclear markers.

METHODS

During the nesting seasons of 1999/2000, 2000/2001, 2001/2002 and 2004/2005, 117 tissue samples from nesting females and two stranded males were collected by TAMAR field staff in Bahia. The collectors are trained to identify by means of morphological diagnostic characters the different species that can be encountered at nesting beaches (based on international standards described at Eckert et al., 1999). Samples were collected using a 6 mm disposable biopsy punch, along three nesting beaches: Arembepe (n = 58), Praia do Forte (n = 53) and Costa do Sauípe (n = 8) (Figure 1). For individual identification, each turtle was tagged on the front flippers with Inconel tags (National Band and Tag Co., style 681). Sample processing, sequencing and sequence analyses were carried at the Laboratory of Biodiversity and Molecular Evolution (LBEM) at the Federal University of Minas Gerais (UFMG), Brazil. DNA extraction was performed following the standard phenol/chloroform procedure (Sambrook et al. 1989) with some modifications (detailed protocols available at http://www.icb.ufmg.br/~lbem/protocolos). PCR was performed in an Eppendorff Mastercycler gradient machine, using primers LCM15382 and H950 (F.A. Abreu-Grobois, personal communication[1]) with an amplification profile of 5 min at 94ºC, followed by 36 cycles of 30 s at 94ºC, 30 s at 50ºC and 1 min at 72ºC, and a final extension step of 10 min at 72ºC. The amplicons (~1000 bp) encompassing a portion of the $tRNA^{Thr}$, the $tRNA^{Pro}$ and a ~800 bp fragment of the control region were purified using Polyethylene Glycol 8000 20% - NaCl 2.5 M. Sequencing was conducted with the ET Dye terminator Cycle Sequencing Kit (Amersham Biosciences) following the manufacturer's recommendations for sequencing in an automated MegaBACE 1000 DNA analysis system. Sequences were read at least twice with both forward and reverse primers. For each sample, the consensus sequence for all reads was generated using the programs Phred 0.020425 (Ewing et al. 1998), Phrap 0.990319 (Green 1994) and Consed 12.0 (Gordon et al. 1998). Consed 12.0 and Sequence Analyzer 3.0 (Amersham Biosciences) were used to visualize the chromatograms and verify the quality of the sequences and the base assignment in the observed polymorphic sites.

[1] For primers sequences please contact F.A. Abreu-Grobois at: abreu@ola.icmyl.unam.mx

Defined haplotypes start at the first site after the $tRNA^{Pro}$ and encompass about 752 bp of the control region left domain. Sequences were compared with control region sequences of Cheloniidae species (Bass et al. 1996, Bowen et al. 1996, Diaz-Fernandez et al. 1999, Alberto Grobois personal communication & Archie Carr Center for Sea Turtle Research: http://accstr.ufl.edu/ccmtdna.html). The phylogenetic relationships between haplotypes were determined by Neighbor-Joining and Parsimony methods using MEGA 3.0 (Kumar et al. 2001) with 1000 bootstrap replications, and molecular diversity indexes were calculated using DNAsp 4.0 (Rozas et al. 2003).

RESULTS

The control region sequences obtained in Bahia samples revealed the presence of seven distinct haplotypes (EimBR 2, 3, 4, 8, 9, 10 and 16; Haplotypes EimBR 5-7 and 11-15 were not found in nesting grounds in Bahia and will be presented elsewhere) that are deposited in GenBank under accession numbers DQ177335 to DQ177341. The polymorphic sites that characterize the seven haplotypes are depicted in Table 1.

The phylogenetic comparison between some of the published sea turtle control region sequences and the seven haplotypes found in this study revealed that only four of them are true hawksbill sequences (Figure 2). For this analysis all hawksbill sequences available in October/2005 in the GeneBank were used, while the sequences from other species were selected based on several characteristics, including size (bp), geographic origin of the samples, and data about common and rare haplotypes. To simplify figure 2, only one characteristic haplotype was included for most of the species in the family Cheloniidae (excluding *E. imbricata* and *C. caretta*). The *D. coriacea* (Family: Dermochelidae) haplotype was used as the outgroup. Haplotypes EimBR 8, 9, 10 and 16 are analogous (comparing 339 bp) to typical hawksbill haplotypes found by Bass et al (1996) in Brazil, forming a distinctive lineage (Figure 2). Haplotypes EimBR8 and 9 exactly match the A haplotype in Bass et al (1996) and differ by one substitution at the position 660 of our alignment (Table 1). Haplotype EimBR10 is different from them (and from A haplotype in Bass et al., 1996) by one substitution at position 158 while haplotype EimBr16 has a substitution at position 363 of our alignment (Table 1). Neither EimBr10 nor EimBr16 were found by Bass et al (1996) in any of the studied populations, so this haplotypes might be exclusive of the Brazilian nesters. The group formed by these haplotypes and the ones found by Bass et al (1996) in Caribbean and Brazil populations, cluster with a group formed by the four haplotypes from the Red Sea (EimRS1 to RS4, Figure 2).

Haplotypes EimBR3 and EimBR4 differ from each other by one substitution at position 620 of our alignment (Table 1) and are closely related to typical loggerhead

haplotypes found at Brazilian nesting populations (Bahia and Espirito Santo), being identical to haplotype D (Bolten et al.,1998) (current nomenclature at Archie Carr Data Base is CC-A4). Haplotypes CC-A24 and CC-A25, found only in Brazilian loggerheads (Soares, 2004) differ from CC-A4 by one substitution. These, and haplotypes EimR, S, T and U defined by Bass et al (1996) as the Brazilian hawksbill x loggerhead hybrids all cluster together.

The haplotype named EimBR2 clustered within the olive ridley clade, being identical to haplotype F defined by Bowen et al (1997) as the only haplotype present in their Brazilian sample (n= 15) (Figure 2) and one of the two (E and F) found in Atlantic Populations.

The typical hawksbill haplotypes (EimBR 8, 9, 10 and 16) were found in 56% (67 out of 119) of the sampled individuals (Figure 1). The most common haplotype, EimBR8, was found in 44% of the samples, including the two males sampled in Arembepe. Haplotypes EimBR3 and EimBR4, related to the loggerhead sequences, comprise 42% of the studied individuals (Figure 1) (EimBR3, n = 21; EimBR4, n = 29), suggesting that we are observing hybrids of second (F2) or further generations. Only two individuals had the haplotype EimBR2 affiliated with olive ridleys, and both of them were suspected to be hybrids (based on morphological characters) suggesting that the hybridization between olives and hawksbills can be a recent and less widespread event.

These results indicate a high frequency (52 out of 119) of hybrids between hawksbill and other species of the family Cheloniidae occurring in Brazilian nesting grounds. Arembepe was the locality with the highest proportions of hybrids, and the only beach where the low frequency of *E. imbricata* x *L. olivacea* hybrids were detected.

Standard molecular diversity indexes were high, as expected, when calculated for the entire group of samples (Table 2), but decrease when only the hawksbill haplotypes are considered, revealing low levels of genetic diversity in the hawksbill populations that nest along the Bahia coast (h = 0.358 +/- 0.069; π = 0.0005 +/- 0.0001).

DISCUSSION

After 25 years of protection by Projeto Tamar (Brazilian Sea Turtle Conservation and Protection Program), there seems to be an increase in the number of nesting turtles along the Brazilian coast. However, genetic studies on loggerhead (Soares 2004), olive ridley (L. Fernandez, personal communication) and leatherback (P. Dutton, personal communication) nesting populations in this country show low genetic diversity indices. The same seems to be the case for the hawksbill population in Brazil as well, when only true hawksbill haplotypes are considered (Table 2). Diversity indices h (haplotype diversity), π (nucleotide diversity) and k (mean number of pairwise differences) are high when calculated for the entire sample (h = 0.71, π = 0.05), being similar to the highest values found by Bass et al (1996) among

seven sampled Caribbean and one Brazilian population. In Bass et al (1996) study, only the Puerto Rico population had a higher *h* value (0.78) than their Brazilian sample (0.70) that included 10 hybrid haplotypes (out of 14 samples). The π value found by these authors for the Puerto Rico population (0.006) is similar to the values found for other populations that did not presented hybrids, and is one order of magnitude smaller than the values found for the Brazilian population in that study (0.025) and in ours (0.05) showing that the hybrid contribution raise this diversity parameter. On the other hand, when only the hawksbill haplotypes are considered, our diversity estimates decrease (h = 0.36; π = 0.0005) being comparable to the low values found by Bass et al (1996) for Mexico population (h = 0.23; π = 0.0003) and the Virgin Islands population (h = 0.12; π = 0.0012).

Our results indicate high levels of hybridization in the state of Bahia, especially with loggerhead turtles; however, they also report previously-unknown hybridization with the olive ridley. Similar hybridization events have already been reported for sea turtle populations (Bowen & Karl 1996) and earlier studies have also suggested this phenomenon for the same area (Conceição 1990, Bass et al.1996), based on limited sample sizes. Hybridization events are being described for many different fauna and flora taxa, and there is a general concern about the main forces leading to these processes (Rhymer & Simberloff 1996). Anthropogenic factors such as exotic species introduction, and habitat destruction and fragmentation, are primary factors contributing to these phenomena (Rhymer & Simberloff 1996). Hybridization is of conservation concern especially when dealing with threatened species such as the hawksbill, since many studies report sterility or low fitness in hybrids (Allendorf et al. 2001). Nevertheless, hybrids can sometimes be viable, as is the case for females sampled in this study (nests are monitored until eggs hatch), so in this case, the hybridization might be accompanied by introgression, a process that implies the backcross of the hybrids with one or both parental taxa. The fact that most of the analyzed animals were undoubtedly diagnosed as hawksbills in the field argues in favor of this being a long term phenomenon, with F2 and later backcrosses. Considering the two distinct loggerhead haplotypes found, we could assume that at least two distinct events produced fertile F1 hybrids between male hawksbill and female loggerheads. However these haplotypes are very common (about 75%) in the loggerhead population nesting in the same region (Soares, 2004), thus recurrent hybridization cannot be discounted as an explanation for our results.

The breeding seasons of loggerhead, olive and hawksbill populations in Bahia overlap, although the nesting peak for hawksbill and loggerhead differ by a month. Olive ridleys are not commonly found in the study area, having a distribution biased to the extreme north of the state, where fewer hawksbills are encountered. Loggerheads in Bahia outnumber the hawksbills by a couple of thousand nests every year (Marcovaldi & Marcovaldi 1999). Besides, the studies by Marcovaldi et al. (1999) and Godfrey et al. (1999)

reported that more than 90% of hawksbill hatchlings in Bahia are females. Thus, there is a strongly female-biased sex ratio. This, in addition to the much larger number of loggerheads that choose this area to breed, and the larger body size of female loggerheads as compared to the hawksbill male, makes it hard to understand why so many interspecific cross matings are happening. However, the high frequency of loggerhead haplotypes may be the result of the occurrence of F2 or further generation female hybrids backcrossing with hawksbill males and increasing the frequency the two loggerhead mtDNA types.

Even though gene flow is a normal evolutionary process, and genes and genotypes cannot be preserved unchanged, hybridization and introgression may threaten rare species existence (Rhymer & Simberloff, 1996, Seehausen et al. 1997, Allendorf et al. 2001). Although hybridization between several Cheloniidae species has been described (Karl et al. 1995, Bowen & Karl 1996) studies with other hawksbill populations in the Caribbean (Bass et al. 1996, Díaz-Fernández et al. 1999) and Pacific (Broderick et al. 1994, Okayama et al. 1996, Broderick & Moritz, 1996) nesting grounds did not report the occurrence of hybrids. Thus the unusually high (more than 40%) proportion of hybrids in the Brazilian population is apparently unique and should represent a serious conservation concern for Brazilian hawksbills, raising the polemic about conservation efforts focusing on hybrid populations (Allendorf et al., 2001).

The introgression process indicated by the putative backcrosses described here may be related to the population decline of both species in the recent past, although it can also be evidencing an ancient process, as suggested by Karl et al. (1995), given the long generation times of sea turtles and the long evolutionary history of these species, which appear during the Miocene or earlier. Thus, these may be the oldest species that naturally hybridize.

Whether this phenomenon represents or not a threat to the hawksbill population nesting in Brazil is hard to measure. The limited information provided by maternally inherited markers makes difficult to establish how many hybridization events and how long ago these events occurred. Further studies with nuclear markers are needed to better understand the implications and causes of such events, and its impact on the genetic diversity and identity of both species. Nuclear DNA analyses may also help to determine if loggerhead males are mating with hawksbill females as well. This information added to other ecological data can provide in the future important clues about the effect of anthropogenic pressures acting on sea turtles populations.

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Figure Legends

Figure 1. Haplotype frequencies distributed along three nesting sites of *E. imbricata* in the northeastern Brazilian coast.

Figure 2. Neighbor-joining tree produced from a 339 bp alignment of D-loop sequences from all sea turtle species. Brazilian haplotypes from *E. imbricata* reported here (EimBR 2, 3, 4, 8, 9, 10 and 16, in bold) are compared to other published haplotypes (Eim A to U) obtained from Bass et al. (1996), or downloaded from the Archie Carr Center for Sea Turtle research database (Cca haplotypes) and from the GenBank (haplotype names followed by accession numbers). Bootstrap support values (>50%) are shown on the branches. Identical tree topologies were obtained using other methods like parsimony (data not shown). Dco (*D. coriacea*), Cmy (*C. mydas*), Nde (*N. depressor*), Lol (*L. olivacea*), Lke (*L. kempii*), Cca (*C. caretta*), Eim (*E. imbricata*).

Figure 1.

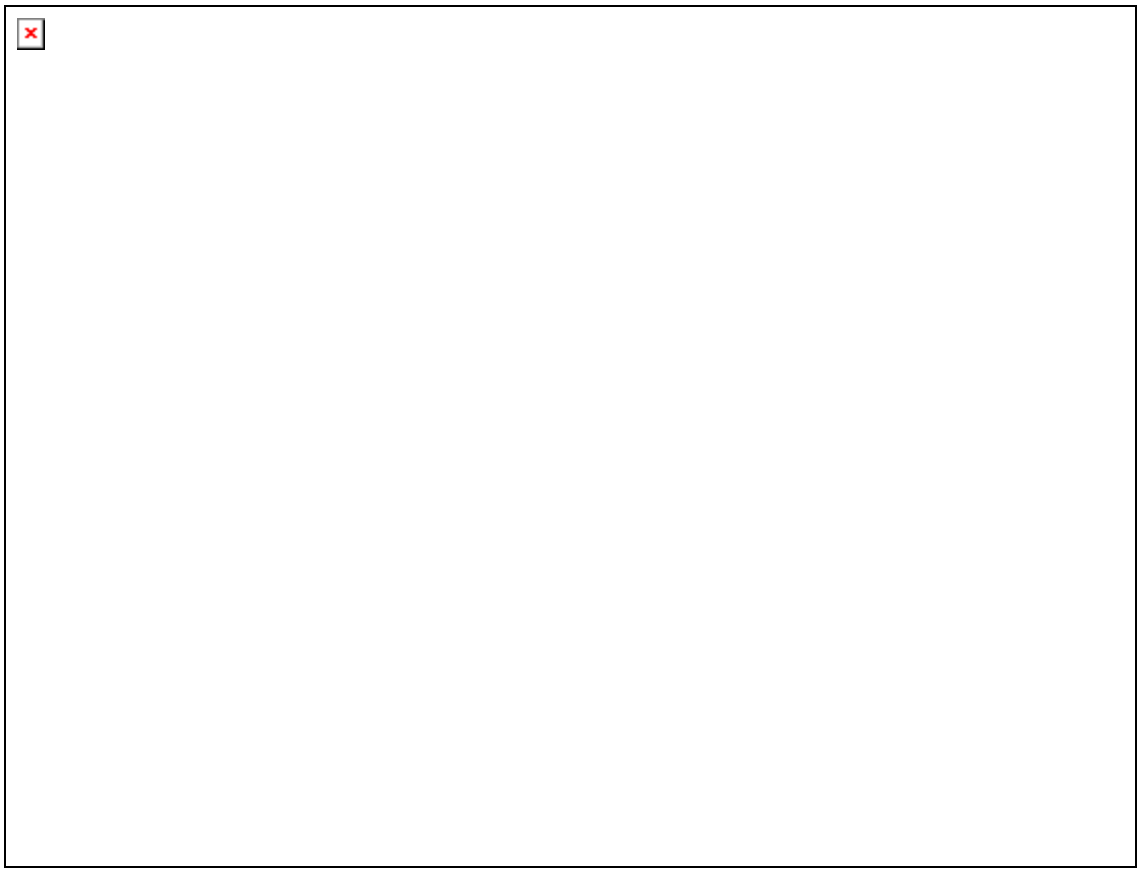

Figure 2.

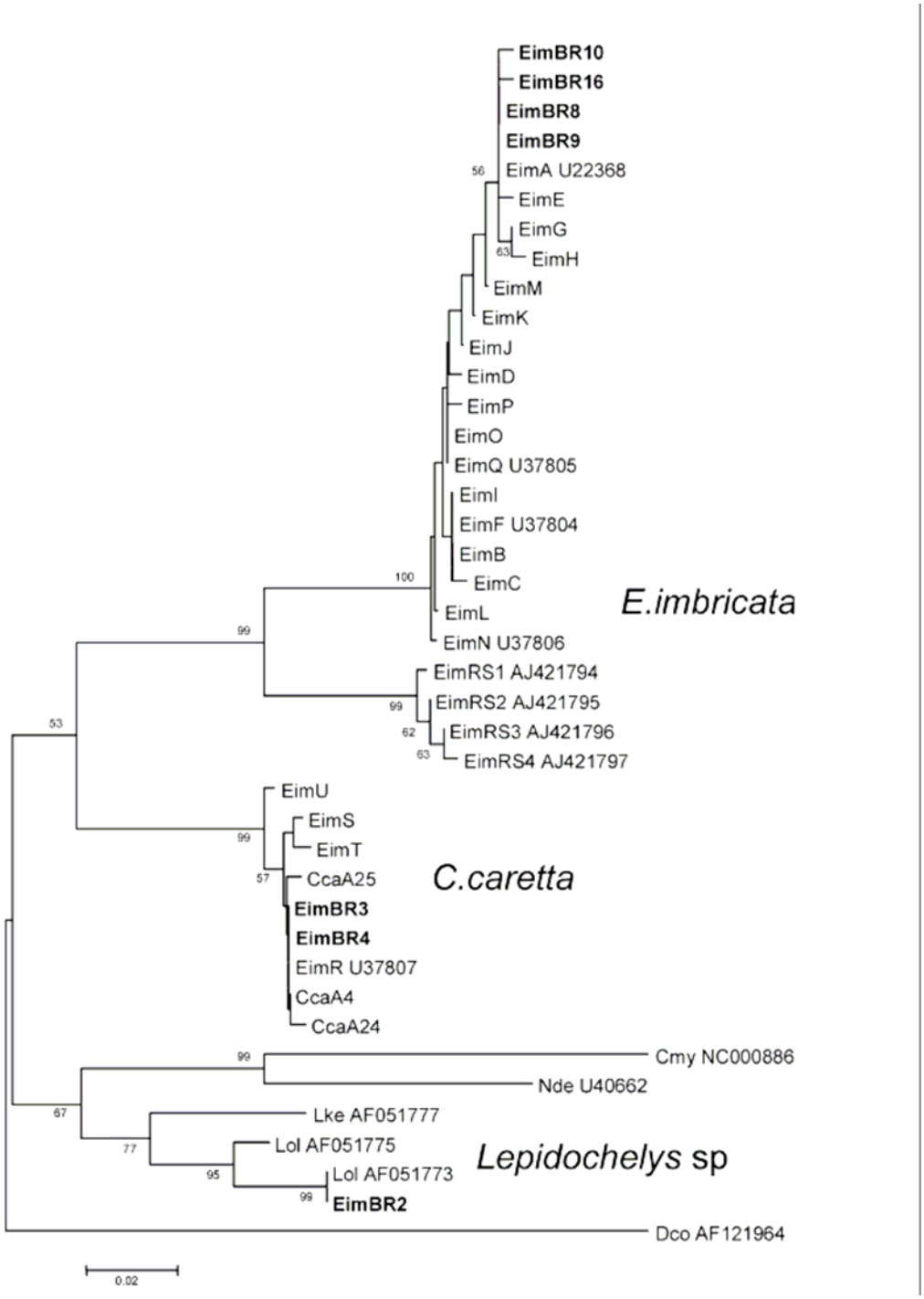
EimBR10
EimBR16
EimBR8
EimBR9
EimA U22368
EimE
EimG
EimH
EimM
EimK
EimJ
EimD
EimP
EimO
EimQ U37805
EimI
EimF U37804
EimB
EimC
EimL
EimN U37806
EimRS1 AJ421794
EimRS2 AJ421795
EimRS3 AJ421796
EimRS4 AJ421797
E.imbricata
EimU
EimS
EimT
CcaA25
EimBR3
EimBR4
EimR U37807
CcaA4
CcaA24
C.caretta
Cmy NC000886
Nde U40662
Lke AF051777
Lol AF051775
Lol AF051773
EimBR2
Lepidochelys sp
Dco AF121964
0.02

Table 1. Polymorphic sites among seven mtDNA control region haplotypes obtained from 119 *E. imbricata* individuals sampled in Bahia (Brazil) nesting grounds.

Haplotype	Polymorphic site number
	1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 2 2 2 2 2 2 2 2 2 2 2 2 2 2 3 3 3
	2 2 2 2 3 3 3 4 5 5 5 6 6 6 7 7 7 7 7 8 9 1 1 2 2 3 3 3 3 4 4 4 5 5 6 6 6 8 8 9 9 9 9 0 0 1 1 1 2 3 4 4 4 4 5 8 9 2 3 3
	1 6 8 1 2 3 8 5 6 9 4 1 4 9 5 8 9 1 2 4 5 8 6 8 0 1 2 8 0 1 4 5 3 6 8 0 8 4 8 9 7 9 0 1 3 8 6 9 1 6 8 6 9 5 6 7 8 9 1 9 1 0 1
EimBR8	A G A A C A C A T C A C G C T G C A C C T C C T A C C G A C C T C A C A A T G G A A C T C C C G A G A G T G T A A A G G T G T
EimBR9	. . . . .
EimBR10	. . . . . G . . . . .
EimBR16	. . . . .
EimBR3	. A . . T G T . . T G . A A . . T . T . C T T A . . T A G T T C T G T T . . A A T G T . . . . . A A A C C . C A . C . .
EimBR4	. A . . T G T . . T G . A A . . T . T . C T T A . . T A G T T C T G T T . . A A T G T . . . . . A A A C C . C A . C . .
EimBR2	G A G G T . . T A T G A A A G A T C T T C T T . G T T A . T T C T G T T . C A A T . . C A A A A G T G A A A C C G C T A . A C

Haplotype	Polymorphic site number *(continued)*
	3 3 3 3 3 3 3 3 4 4 4 4 4 4 4 4 4 4 4 4 4 5 5 5 5 5 5 5 5 5 5 6 6 6 6 6 6 6 6 6 6 6 6 6 7 7 7 7 7 7 7 7 7 7 7 7
	5 6 6 6 6 6 7 7 2 2 2 2 2 2 3 4 4 4 4 5 6 9 0 1 1 1 6 8 8 8 9 9 9 0 1 2 2 2 2 3 3 4 5 6 6 7 7 9 0 0 0 0 1 1 1 1 1 2 2 2 3 3
	5 3 5 6 7 9 0 1 1 3 4 6 7 9 8 1 3 4 7 3 0 1 2 0 1 7 5 3 4 8 1 3 8 8 1 0 7 8 9 2 4 9 9 0 2 3 7 8 0 6 7 9 0 1 2 3 4 2 3 6 3 4
EimBR8	G A T T T T G G G A T G G A T A C T T G C A C A G T A G A A G A A T G T A G A A C A G G A C T T C C T A C C T A T T A C G T
EimBR9	. . . . . A . . . . .
EimBR10	. . . . .
EimBR16	. G . . . . .
EimBR3	A . G . C . A T A G A A A . C G T C C A . . T C . . G A G G . G G C A C G A . T T . T T G T . C . A A . . . C . . C . . . .
EimBR4	A . G . C . A T A G A A A . C G T C C A . . T C . . G A G G . G G C A . G A . T T . T T G T . C . A A . . . C . . C . . . .
EimBR2	. . A C . C . A A G G A A G . G T C C A T G . C A C G A . . T G G C A . G A T T T G T T . T A C T A A C T T A T A C G T A C

Table 2. Standard diversity indexes calculated for each nesting beach (*Arembepe, Praia do Forte* and *Sauípe*) from Bahia (Brazil), for the entire sample (*overall*) and for the sample without the hybrid haplotypes (*Eim**). S: number of variable sites; H: number of haplotypes; *h*: haplotype (genetic) diversity; π : nucleotide diversity; *k*: mean number of pairwise differences.

Populations	**Diversity indexes**						
	n	bp	S	H	*h*	π	*k*
Arembepe	58	752	124	5	0.725 +/- 0.028	0.05763 +/-0.00338	42.355
Praia do Forte	53	752	79	5	0.649 +/-0.048	0.04812 +/-0.00451	35.509
Sauípe	8	752	79	4	0.643 +/-0.184	0.02676 +/-0.01874	19.75
Overall	119	752	125	7	0.71 +/-0.027	0.05469 +/-0.00211	40.164
*Eim**	67	752	3	4	0.358 +/-0.069	0.00051 +/-0.00011	0.37992

ARTIGO 2:

Population structure and hybridization in hawksbill (*Eretmochelys imbricata*) feeding and nesting aggregates from Brazil

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Population structure and hybridization in hawksbill (*Eretmochelys imbricata*) feeding and nesting aggregates from Brazil

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Running title: Genetic origins of hawksbill populations in Brazil

Key words: hawksbill populations, mtDNA, nuclear markers, population genetic structure, hybrids, mixed stock analysis.

ABSTRACT

The largest hawksbill nesting colony of the southern Atlantic and several foraging areas are found in Brazil. MtDNA haplotypes and genotypes of three autosomal loci were generated for nesting and feeding aggregates. As a whole, we observed 14 control region mtDNA haplotypes specific to hawksbills, two related to loggerhead sequences and one characteristic of olive ridleys. Haplotypes related to other marine turtle species are likely due to hybridization and were only found in the rookery sample (39%). The *E.imbricata* haplotypes found in the rookery are closely related to the most common Atlantic haplotype and are connected to it in a star-like pattern network suggesting a recent population expansion. The feeding ground sample presented 14 hawksbill haplotypes, matching haplotypes from Brazil, Caribbean, Australia and Kuwait nesting sites. The use of mtDNA combined with autosomal markers confirmed the presence of hybrids only in the rookery and indicated that at least 8% of hybrids are a product of introgression between F1 hybrids and the parental species *E.imbricata.* No signs of mating between *E.imbricata* or F1 hybrid females and *C.caretta* males were found, suggesting a gender biased process. Mixed stock analysis (MSA) indicated rookeries from Barbados and Cuba as the main sources of juveniles and sub-adults found in the Brazilian feeding area. However, the MSA showed to be very sensitive to the absence of hybrids at the feeding grounds, and a reanalysis assuming that hybrids do not reach these feeding grounds indicated the nesting Brazilian population as the main source.

INTRODUCTION

The hawksbill turtle *Eretmochelys imbricata* is found in tropical areas in the Atlantic and Indo-Pacific regions (IUCN 2006), associated to coral reefs and shallow environments due to its unique sponge eating habits (Meylan 1988). Rookeries can still be found in more than 60 countries worldwide but in low densities due to the intense exploitation for the carapace scutes (tortoiseshell) that are used to produce expensive luxurious items (Meylan and Donelly 1999). Egg poaching, slaughter of nesting females, habitat destruction, coastal development and incidental capture by fisheries have also contributed to dwindle populations (IUCN/SSC Marine Turtle Specialist Group 2003). Today, there are less than ten rookeries around the world where more than 1000 females are estimated to nest every year (Spotila 2004).

The species is considered critically endangered by the World Conservation Union (IUCN) and it is listed in the Appendix I of the Convention on International Trade in Endangered Species (CITES). However, non-CITES countries continue to sell and trade tortoiseshell products. This is producing an ongoing debate since the information on how the harvest in one country will affect nesting populations located outside its boundaries is only recently beginning to appear (Bowen et al. 2007, Mortimer et al. 2007).

The species shares several life history traits with other marine turtles including migratory behavior, nesting site fidelity and natal homing behavior (Carr et al. 1966, Bass et al. 1996, Broderick and Moritz 1996, Miller et al. 1998, Diaz-Fernandez et al. 1999). During its life cycle, dispersing individuals can migrate over long distances, frequently hundreds and even thousands of kilometers (Miller et al. 1998, Meylan 1999, Horrocks et al. 2001, IUCN/SSC 2003) with some reports of trans-Atlantic migrations (Marcovaldi and Filippini 1991, Bellini et al. 2000).

The long-distance migratory behavior and long life-span make it difficult to study the life history of marine turtle species and even worse to design effective conservation strategies, which need to be planed in several different geographic scales (Bowen et al. 2007). The emerging picture indicates that there is no effective conservation when individual countries protect the rookeries in-between their boundaries, but harvesting at feeding aggregates (even thousands of miles away) continues (Bowen et al. 1996, Troëng et al. 2005, Bowen et al. 2007). Thus, compiling information about single colonies, their distinctiveness and degree of isolation is mandatory for conservation purposes in a regional scale, while the knowledge about the demographic links between colonies around the world and feeding aggregates is needed to design global management strategies.

There is little knowledge about juvenile migrations and the origin and composition of feeding aggregates. Newborns enter the water to start an open ocean interval where their

movements are mainly determined by oceanic currents (Carr 1987, Musick and Limpus 1997). After a period that can last several years they migrate to shallow water feeding grounds or developmental habitats (Carr et al. 1966). The composition of these developmental habitats is believed to be determined by several factors like the size of regional nesting colonies, oceanic currents and the distance to the contributing colonies among others (Carr and Meylan 1980, Witham 1980, Bass et al. 1996, Norrgard and Graves 1996, Lahanas et al. 1998, Rankin-Baransky et al. 2001, Engstrom et al. 2002, Luke et al. 2004).

Molecular-based methodologies to analyze stock composition have been applied to determine the composition of the feeding aggregates of several marine turtle species (Bolten et al. 1998, Laurent et al. 1998, Engstrom et al. 2002, Bass et al. 2004, Luke et al. 2004, Bass et al. 2006). The original methodology (Pella and Milner 1987, Devebec et al. 2000) uses a Conditional Maximum Likelihood (CML) approach to provide estimates of the contribution of different stocks (rookeries) to a particular mixture (feeding aggregate) and has been used to determine the origins of hawksbill feeding aggregates in the Indo-pacific (Broderick et al. 1994) and the Caribbean (Bowen et al. 1996, Díaz-Fernández et al. 1999, Troëng et al. 2005). A more recent approach uses Bayesian algorithms with Markov Chain Monte Carlo (MCMC) stochastic simulation procedures (BAYES; Pella and Masuda 2001). This methodology is more accurate and less biased by small contributions, the presence of rare alleles and missing data than the CML approach (Pella and Masuda 2001, Antonovich and Templin 2003). An additional advantage of the Bayesian approach is that baseline information about the populations (such as rookery size) can be incorporated to the analysis.

Using the Bayesian approach, Bowen et al. (2007) studied juvenile migrations of Caribbean hawksbills and showed that among the factors believed to affect the composition at feeding grounds aggregates, only the distance to the contributing colonies has a significant correlation with feeding ground composition at the Caribbean (negative correlation). In a lesser extent, the size of contributing rookeries was also found to influence the composition of feeding aggregates (positive correlation), although the correlation was not so clear and could be confounded by other environmental and biological factors not evaluated by the authors. The relative influence of the three main proposed factors (colony size, distance and currents) is still unknown since results from different studies vary depending on the methods used and the species studied (Bjorndal et al. 1999, Bass and Witzel 2000, Luke et al. 2004).

The hawksbill turtle is one of the five marine turtle species that can be found in Brazilian grounds, being the fourth in terms of nesters abundance in the country (Marcovaldi and Laurent 1996). It is included in the Brazilian government (IBAMA) official list of

endangered species and protected by a law that covers all life history stages including eggs and hatchlings (Fundação Biodiversitas 2003).

Even though Brazilian rookeries had suffered a long history of exploitation that led to a great decline of nesting populations and a great reduction in the number of recorded nesting sites (Marcovaldi et al. 1999), the population nesting in Brazil is the greatest remaining in the South Western Atlantic. It is also one of the few sites where more than 1750 nests are registered every year (Marcovaldi et al. 2007). Currently, the effective conservation efforts directed by the Brazilian Project for the Conservation of Sea Turtles (Projeto TAMAR-IBAMA) have helped to stop and reverse the declining trend, leading to the growth of the Brazilian nesting population, an important stronghold for the conservation of the species.

Furthermore, considering that almost all knowledge on this species comes from the study of Caribbean populations (Bass et al. 1996, Diaz-Fernandez et al. 1999, Bowen et al. 2007), the genetic characterization of the relatively large nesting populations and the juvenile aggregates in the feeding habitats found along the Brazilian coastline can increase significantly the current knowledge on the species migratory patterns along the entire Atlantic basin.

In this study we describe the analyses of mtDNA haplotypes and autosomal loci with species-specific alleles in the feeding aggregates and the nesting colony found in Brazil. We studied the phylogenetic relationships and phylogeographic patterns that emerged from mtDNA sequences, comparing with a huge data set compiled by Bowen et al. (2007) for feeding and nesting aggregates in the Caribbean. We also performed a multiple stock analysis to ascertain the possible origin of juveniles found in the main Brazilian feeding area in order to establish links between this and other populations around the world.

The high frequency of mtDNA haplotypes from other marine turtle species observed in the Brazilian nesting site (Lara-Ruiz et al. 2006) has indicated a high incidence of hybridization, particularly with loggerheads (*Caretta caretta*). Here, we used autosomal markers to investigate this process which also detailed a biased process of hybridization and introgression where *C. caretta* females and F1 female hybrids mate preferentially with *E. imbricata* males. Based on the peculiarity of populations nesting and feeding in the Brazilian territory, some important conservation concerns are envisaged.

MATERIALS AND METHODS

Tissue samples (n=226) were collected by TAMAR-IBAMA field staff between 1999 and 2005 at several nesting localities along the Brazilian coastline and off-shore at feeding areas (Figure 1). For the nesting population we used samples from the states of Bahia (BA, n=114) (Lara-Ruiz et al., 2006), Ceará (CE, n=2), Rio Grande do Norte (RN, n=12) and

Sergipe (SE, n=4). This geographical range of our samples covers all hawksbill main nesting sites in the Brazilian territory. Samples from juveniles were collected in feeding areas near the Fernando de Noronha archipelago (FN, n=54) and Atol das Rocas island (AR, n=40), in the northeastern coast of Brazil (Figure 1). The collectors are trained to identify the different species that can be encountered in Brazilian grounds following international standards described in Eckert et al. (1999). For individual identification, all animals were tagged on the front flippers with Inconel tags (National Band and Tag Co. style 681).

Tissue samples were collected using a 6 mm disposable biopsy punch, stored in absolute ethanol or salt-saturated buffer (20% DMSO and 250 mM EDTA saturated with NaCl; pH 7.0) (Amos and Hoelzel 1991) and kept at room temperature until their processing in the laboratory. Total DNA was isolated from the samples using the standard phenol-chloroform protocol (Sambrook et al. 2001) with modifications introduced at the laboratory (available on-line at http://www.icb.ufmg.br/lbem/protocolos). A 1000 bp fragment including about 800 bp of the mtDNA control region was amplified using the primers LCM15382 and H950 (Lara-Ruiz et al., 2006) and a PCR amplification profile of 5 minutes at 94^{o}C, followed by 36 cycles of 30 seconds at 94^{o}C, 30 seconds at 50^{o}C, 1 minute at 72^{o}C and a final extension step of 10 minutes at 72^{o}C. All reactions were carried out including positive and negative controls (template-free reactions) in order to test for contamination and to assure the fidelity of the PCR amplifications.

Amplicons were purified using Polyethylene Glycol 8000 20% – NaCl 2.5M and submitted to a sequencing reaction using the ET Dye terminator Cycle Sequencing Kit (Amersham Biosciences) according to manufacturer instructions. Sequencing reactions were analyzed in an automated MegaBACE 1000 DNA sequencer. At least two independent PCR products from each sample were sequenced using both forward and reverse primers. The chromatograms were base called using Phred 0.020425 (Ewing et al. 1998) and the sequences were aligned and edited to produce a high quality consensus sequence for each individual using Phrap 0.990319 (www.genome.washington.edu/UWGC/analysis tools/phrap.htm) and Consed 12.0 (Gordon et al. 1998).

For further analyses and to characterize polymorphic sites and haplotypes the consensus sequences for all individuals were aligned using MEGA 3.1 (Kumar et al. 2004). The defined haplotypes start at the first site after the $tRNA_{Pro}$ and encompass 740 bp of the control region left domain. Haplotypes were named following the same nomenclature for *E.imbricata* samples from Brazil already published (Lara-Ruiz et al. 2006). The sequences were compared with *E.imbricata* known haplotypes (Table 1) and all the haplotypes found, even some that matched published (but shorter) haplotypes, were deposited in GenBank under the accession numbers (XXX-XXX).

The relationships among Brazilian *E.imbricata* haplotypes (740 bp) were inferred using a median joining network analysis (Bandelt et al. 1999) implemented in the program Network 4 (www.fluxus-engineering.com). In this analysis we excluded haplotypes more related to other species mtDNA, considered here as evidences of hybridization. However, sequence divergence among all haplotypes and a neighbor-joining tree were calculated using MEGA 3.1.

Estimates of haplotype (*h)* and nucleotide (π) diversity, as well as Tajima and Fu's test for detecting population expansion were generated using Arlequin 3.1 (Excoffier et al. 2005). Analyses of molecular variance (AMOVA) and exact tests of population differentiation (10,000 steps in the Markov Chain and 1,000 dememorization steps) were also carried out in the Arlequin 3.1 to evaluate several population divisions and groupings of populations according to geography. The significance of the associated *P* values were computed with 10,000 permutations for the AMOVA and population pairwise genetic distances (Φst). To evaluate differences between Brazilian populations and other known Caribbean nesting and feeding aggregations, only 380 bp haplotypes (Table 1) were used to compare with data compiled by Bowen et al. (2007). As the sequences used for these analyses were shorter, several of the described haplotypes were binned into one sequence resulting in lower (but comparable) values of haplotype diversity for the Brazilian samples. Data in Bowen et al. (2007) from Venezuela and Brazil were excluded from the analysis because of the small sample sizes. Their data sets for Puerto Rico (two data sets) and Cuba (three data sets) were pooled and the information regarding the haplotype designated "Cum" was not used since it is identical to the "DR1" haplotype (according to GenBank on February/2007).

To estimate the relative contributions of different nesting colonies to the foraging grounds (mixed stock analysis – MSA) we used the Bayesian algorithm with a Markov Chain Monte Carlo (MCMC) estimation procedure implemented in BAYES (Pella and Masuda 2001). This is expected to display a higher statistical power and more accurate estimates than the traditional maximum likelihood (ML) approach. Besides, the BAYES approach is less biased than ML estimators because it is less susceptible to uneven proportions of different stocks and it also removes automatically orphaned haplotypes (i.e. rare haplotypes found at the mixture but not present at base line stocks) (Antonovich and Templin 2003).

We ran nine MCMC chains of size 200,000, one chain per base-line stock (Antigua, Barbados, Bermuda, Belize, Brazil, Costa Rica, Cuba, Puerto Rico, US Virgin Islands) with a starting point of 0.90 for one particular stock and 0.0125 for the remaining eight stocks with equal prior parameters of Dirichlet distribution for each stock. Convergence of MCMC estimates to a desired posterior probability was assessed using the Gelman–Rubin shrink factor (Gelman and Rubin 1992), increasing the MCMC sample size until all values obtained were less than 1.2. The Brazilian feeding aggregate composition was estimated from the

mean of all nine chains after 100,000 burn-in steps. The same analysis was run not considering the hybrid haplotypes found in the Brazilian rookery.

Finally, we addressed the hybrids issue by analyzing all *E. imbricata* samples from both nesting and feeding grounds by means of three anonymous single copy nuclear DNA markers (scnDNA) previously used in the characterization of marine turtle hybrids (Karl et al. 1995, Karl and Avise, 1993). From the markers described by Karl and Avise (1993) we chose three (CM12, CM14 and CM28) known to provide species specific banding patterns for *E. imbricata* and *C. caretta* after treatment with restriction enzymes (Karl et al. 1995; S. Karl *unpublished*). The primers for these loci are described in Karl and Avise (1993). The amplification profile used for the three loci was 5 minutes at 94^{o}C, followed by 35 cycles of 20 seconds at 94^{o}C, 45 seconds at 54^{o}C, 80 seconds at 72^{o}C, and a final extension step of 9 minutes at 72^{o}C. The PCR product was treated with the enzymes *Dra*I and *Rsa*I (CM12), *Hae*III (CM14) and *Bst*OI (CM28) following manufacturer instructions. PCR-RFLP patterns were genotyped in agarose gel (0.8%, CM12 and CM28) electrophoresis stained with ethidium bromide or polyacrylamide gel (8%, CM14) electrophoresis stained with silver nitrate (Santos et al. 1996). Samples of *C. caretta* and *L.olivacea* available at our laboratory were used to compare the species specific pattern obtained with each marker.

RESULTS

The examination of the new nesting (N=18) and foraging (N=94) samples increased from 7 to 17 the number of mtDNA haplotypes described for Brazil. No new haplotypes were found in the samples from rookeries, thus all new haplotypes were contributed by samples from the foraging ground. Excluding the three hybrid mtDNA haplotypes described elsewhere (Lara-Ruiz et al., 2006), the 14 *E.imbricata* haplotypes are 740 bp long, with 47 variable sites (12 singletons) represented by 41 transitions (Ti) and 6 transversions (Tv). Haplotypes EiBR8,9, 10, 12, 13, 14, 15, 16, 17 and 19 present a deletion at site number 10, which is not present in haplotypes EiBR5, 6, 7 and 18. Compared to the available *E.imbricata* haplotypes, EiBR6 and 7 matched the EATL (384 bp) haplotype described by Bowen et al. (2007) for one sample from feeding grounds at the US Virgin Islands. According to these authors, this haplotype was previously described in a sample of animals obtained in a market at São Tomé (Eastern Equatorial Atlantic) and is more similar to the Indo-Pacific (Australian) haplotypes than to Caribbean ones. Haplotypes EiBR8 and 9 correspond to haplotype A (Bass et al. 1996), which is one of the two common haplotypes found in the Caribbean, while EiBR13 and 15 correspond to haplotype F (Bass et al. 1996), which is the other most common sequence found in the Caribbean. Haplotypes EiBR10 and 12 match haplotypes f and b, respectively, previously registered for feeding areas at Puerto Rico (Mona Island) and

Cuba (Diaz-Fernandez et al. 1999). When considering only the 384 bp available for all sequences in GenBank, haplotype EiBR14 is identical to the corresponding alignment sites of haplotypes H2 and H5 (GI:115371805 and GI:122720664) described for a sample from Kuwait (Al-Mohanna and George, *unpublished*). Haplotype EiBR17 corresponds to haplotype designated Alpha (α), which was only found in a nesting colony from Costa Rica (Troeng et al. 2005) and in several feeding grounds in the Caribbean (Bowen 1996, 2007, Diaz Fernandez et al. 1999).

Haplotype frequencies for the entire Brazilian sample, together with their homologies to previously described sequences (when considering only the 384 bp available for all the haplotypes in GenBank) are depicted in Table 1. For the comparison analysis we used the 384 bp sequences and kept the denominations used by Bass et al. (1996), Diaz-Fernandez et al. (1999) and Bowen et al. (2007) when our haplotypes matched those sequences. For this reason, haplotypes EiBR3 and 4, EIBR6 and 7, EiBR8 and 9 and EiBR10 and 19 were binned together in later analysis (see below).

Including the hybrids (EiBR2, 3 and 4) and the 14 *E.imbricata* haplotypes, the overall mean distance was 0.053. Distance between *C.caretta* and *L.olivacea* haplotypes was 0.09, between *C.caretta* and *E.imbricata* haplotypes was 0.108 and between *E.imbricata* and *L.olivacea* haplotypes was 0.143. The neighbor-joining tree constructed using sequences for all marine turtle species and some *E.imbricata* sequences chosen from the published haplotypes available (not shown) confirmed that haplotypes EiBR2, 3 and 4 belong to other marine turtle species. It also confirmed the close relationship between haplotypes found at feeding grounds and those described for rookeries in the Caribbean and Indo-Pacific. Hybrid haplotype EiBR2 is equivalent to haplotype F (Bowen et al. 1998), the most common Olive Ridley haplotype found in Brazilian rookeries, and haplotypes EiBR3 and EiBR4 are equivalent to CC-A4, the most common loggerhead haplotype found in Brazilian nesters which is also unique to the Brazilian rookery (L.S. Soares *unpublished*).

The phylogeographic analysis revealed the presence of four very distinct haplogroups (A, F, I-P1, I-P2) in the sample from feeding grounds, while only haplotypes belonging to group A are present in the sample for nesting grounds (Figure 2). Sequences in each haplogroup differed among each other for one or two substitutions (average distance within groups, d = 0.001-0.003) while 10 to 25 substitutions separated each of the groups defined (average distance between groups, d= 0.017-0.042). Of the described haplotypes, five (EiBR6, 7, 8, 18 and 14) corresponded to sequences more likely related to Eastern Atlantic and/or Indo-Pacific samples, representing almost 12% of the feeding ground samples. Only three samples from feeding grounds showed haplotypes related to the F haplotype (EiBR13 and 15) reported to be one of the two most common haplotypes in the Caribbean, while the rest of the samples (including all samples from nesting grounds) belonged to the group of

sequences related to the other most common Caribbean haplotype (A, described by Bass 1996).

No significant population differentiation was detected by AMOVA when comparing among nesting samples from different localities or between the two feeding grounds in Brazil, which also presented no significant population differentiation in the exact tests. For these reasons all samples from nesting (Brazilian coastline) or feeding (Fernando de Noronha and Atol das Rocas) grounds were considered to represent each a single demographic unit. Comparisons between these two (nesting and feeding) indicated that there are significant differences between them (Φ_{ST}= 0.29 *P*<0.0001; exact test *P* value <0.0001) even when "hybrid" haplotypes from nesting beaches were not considered (Φ_{ST}= 0.092 *P*<0.0001; exact test *P* value <0.0001). Diversity indexes (Table 2) were higher for the nesting sample when all haplotypes are considered, but when comparing these indexes without taking into account EiBR2 (*L.olivacea* haplotype) and EiBR3 and EiBR4 (*C.caretta* haplotypes), all diversity estimates obtained for the feeding aggregate were higher.

When Brazilian populations were included in the data set compiled by Bowen et al. (2007), the AMOVA indicated that there are significant differences among nesting sites (Φ_{ST}= 0.43; *P*<0.0001), with pairwise Φ_{ST} values for the comparison between the rookery in Brazil and all other Caribbean rookeries ranging from 0.25 to 0.44 (all *P* values <0.0001). These values increased when samples with *C.caretta* and *L.olivacea* haplotypes were excluded from the Brazilian sample (Φ_{ST}= 0.64; *P*<0.0001). The exact test of population differentiation (pairwise comparisons) indicated that Brazilian nesting population is significantly (*P*<0.0001) different from all other nesting populations studied in the Caribbean. The pairwise comparison of the Brazilian sample from feeding grounds with Caribbean feeding aggregations indicated that the former is significantly different from all Caribbean aggregations (Φ_{ST} values ranging from 0.20 to 0.60, all *P* values <0.0001). The AMOVA analysis indicated smaller but still significant divergence between feeding aggregations (Φ_{ST}= 0.175; *P*<0.0001) and this value increased (Φ_{ST}= 0.353; *P*<0.0001) when populations were grouped according to geography (Caribbean vs. Brazil).

The mixed stock analysis (MSA) was run using a mixture file with 80 individuals that presented the haplotypes that have been identified in hawksbill rookeries so far. Other (orphaned) haplotypes (5 haplotypes, 14 individuals) were excluded from the analysis. All chains consistently indicated that the major contribution to these feeding grounds was likely coming from Barbados rookery (ranging from 50% to 79%), followed by Cuba rookery whose contribution varied from 11% to 34% depending on the chain. The estimate for the contribution from the Brazilian rookery was low (4% – 10%) but higher than contributions from all other rookeries included in the analyses. The summary of stock assignments using results from all nine chains combined (MCMC sample = 900,000) is shown in Table 3A.

However, when hybrid haplotypes are removed from the Brazilian base-line stock, the contribution of this rookery to the feeding aggregate becomes the greatest (92% point estimate) followed by Costa Rica (3%), Cuba (2%) and Barbados (1%) and the standard deviations of the estimates are reduced substantially (Table 3B).

In order to detect likely *C. caretta* genomic ancestry in the populations, a total of 49 nesting females with morphology more similar to *E.imbricata* and bearing a *C.caretta* mtDNA haplotype were analyzed with two (8) or three (41) autosomal loci. Another 177 individuals from nesting and foraging grounds identified as *E.imbricata* by morphology and mtDNA were analyzed with two (45) or three (132) autosomal markers. None of the individuals characterized by mtDNA as *E.imbricata* presented *C.caretta* alleles at any locus. Among the samples with *C.caretta* mtDNA, four showed both alleles specific of *E. imbricata* in at least one locus, but none presented both *C. caretta* alleles in any locus indicating that mating between *C.caretta* males and *E.imbricata* females or F1 female hybrids is unlikely. Of the individuals with *C.caretta* mtDNA, 37 had alleles from both species at all three loci as expected for F1 hybrids and other eight individuals that were only genotyped with two markers also presented a pattern characteristic of F1 hybrids.

DISCUSSION

Nesting population

Our results showed that the rookery and the feeding aggregates found in Brazil are distinct demographic units. The nesting population presents few *E.imbricata* haplotypes, all derived (one mutation step) from the most common haplotype found (EiBR8), suggesting that this could be the ancestral haplotype for the Brazilian rookery population. The star shaped network obtained for the relations between these haplotypes also suggests a population that has experienced a recent bottleneck followed by population expansion likely due to an event of colonization, however Tajima and Fu´s tests of recent population expansion have shown to be non significant. If EiBR8 is the ancestral haplotype for the Brazilian rookery, and given the greater diversity of EiBR8-derived haplotypes in the Caribbean it could be suggested that the Brazilian population has a Caribbean origin or that EiBR8 haplotype is the ancestral haplotype for the entire Atlantic population. Further studies from new samplings in the Eastern Atlantic could help to sort out this issue in a macrogeographic level.

From the four rookery haplotypes (EiBR 8, 9, 10 and 16), EiBR10 and 16 differ from haplotype A when shorter (384 bp) sequences are considered and thus could be used as

population-specific markers. Consequently, the presence of haplotype EiBR10 in feeding grounds at Mona Island (haplotype f, Diaz-Fernandez et al. 1999) establishes a direct link between the Brazilian rookery and Caribbean feeding grounds The use of longer sequences to characterize a population in terms of haplotypes and their frequencies demonstrated that those display a higher discriminating power between rookeries that can improve future stock composition analysis since the accuracy of these estimations is compromised by the sharing of unresolved haplotypes between source populations.

The haplotypes named EiBR3 and 4 (*C.caretta* haplotypes) could also be used as population-specific markers since they occur in this rookery in a high frequency and have not been found in any other rookery characterized to date. To the present, no hybrid haplotypes have been found in any of the nesting colonies described for the Caribbean and there is only one report of hybrids (4 out of 58 *E.imbricata* samples with mtDNA from *C.caretta*) in a Bermuda feeding ground (Godley et al. 2004). Unfortunately, these sequences are not published so we can not ascertain the origin of the hybrid animals found in Bermuda. However, the absence of hybrid haplotypes in all other feeding aggregates already studied indicates that the contribution of hybrids from Brazilian nesting beaches, at least from the Bahia (BA) site, to the Caribbean foraging assemblages seems to be very low, unless Brazilian hybrids display a very different behavior on feeding preferences and migration pattern (see below).

Nevertheless, the presence of EiBR10 in a sample from a feeding area in Puerto Rico indicates that hawksbills nesting in Brazil can share with other populations the developmental habitats in the Caribbean. The high frequency of haplotype A, found in all feeding aggregations along the Caribbean, which matches the most frequent haplotype (EiBR8) found in Brazilian rookery, suggests that at least some of the animals in Caribbean feeding grounds can have a Brazilian origin. However, in order to find out whether the Brazilian contribution to Caribbean foraging grounds is higher, there is a need to re-analyze samples from those feeding grounds using longer sequences that can help to distinguish between common Caribbean and exclusively Brazilian haplotypes such as EiBR8 and 9.

The absence of hybrid haplotypes in other rookeries confirms once again the natal homing and spawning site fidelity of these animals and indicates that the hybridization process is local. Satellite telemetry data (N=13) have shown that adult hawksbill females (hybrids and non-hybrids) nesting in Bahia state, move either northwards or southwards after the spawning season but tend to remain in the Brazilian continental shelf (Projeto TAMAR, *unpublished data*). However, the limited transmitter life-span and the low resolution power of short DNA sequences used to characterize most feeding aggregations around the world, hinder our capability to conclude that the Brazilian rookery is an isolated population.

Hybrids

The hybridization between several sea turtle species was also previously registered (Kamezaki 1983, Frazier 1988, Conceição et al. 1990, Karl et al. 1995) and must be due to the absence of strong pre and pos-zygotic barriers that usually prevent the inter-specific mating. The lack of these barriers is likely related to the low evolutionary rate observed in this group (Karl et al. 1995). Natural hybridization in marine turtles might be related to the normal chromosome paring that can occur in close species due to low evolutionary rates allowing karyotypic compatibility (Bickham 1981). This could be the case of *E.imbricata* and *C.caretta* that display 56 chromosomes each, as for all Chelloniidae (Kamezaki 1989, 1990).

As pointed out before (Lara-Ruiz et al. 2006), the presence of two different *C.caretta* haplotypes indicates that the hybrid Brazilian population is the product of more than one hybridization event. Furthermore, the new nuclear data obtained during this study indicates introgression between parental species and hybrids due to the existence of F2 and/or further generation hybrids. Our results are based on the use of only three autosomal markers, but allowed us to have a better insight into the hybridization process.

First, we observed a likely bias due to preferential inter-species mating, which is gender-driven by the participation of only *E. imbricata* males in the process. This unidirectional introgression can be an evidence of the incapacity or failure of *E. imbricata* females or F1 female hybrids to mate with males of the parental taxa *C. caretta*. The apparently unsuccessful introgression with *C. caretta* could be due either to the low mating success of males of this species with *E. imbricata* females and hybrids or low survival/fecundity of the F1 and >F1 hybrid progeny with *C. caretta*, since all hybrids were found among nesting females. It is important to note that F1 female hybrids with *C. caretta* mtDNA are the result of the mating between a *C. caretta* female and a *E. imbricata* male and we did not observe any evidence of the opposite gender pairing. Thus, because the hybridization process seems to be biased by the gender of the parental species, it is an important factor for the directionality or trend for the introgression.

However, another hypothesis to consider is that this trend could also be due to the time of the reproductive season for both species. In Brazil the reproductive season of *E.imbricata* and *C.caretta* nesting at Bahia slightly overlaps, being the nesting peaks from October the 15th to December the 15th for loggerheads and from December the 15th to February the 15th for hawksbills (Marcovaldi et al. 1999, Marcovaldi and Chaloupka, *unpublished*). By the time the reproductive peak of *C.caretta* females is finishing, *E.imbricata* peak is starting, thus *E.imbricata* males would be expected to be at coastal waters close to the nesting beaches. This and the higher abundance of *C.caretta* in the Brazilian territory

could make the *E.imbricata* males to encounter higher numbers of *C.caretta* females facilitating the observed gender biased hybridization. Following the same reasoning the possibility of encounter between F1 female hybrids (if they behave as hawksbills) or female hawksbills and loggerhead males would be lower since loggerhead males could be leaving the Brazilian coast before the hawksbill females arrive to nesting beaches.

According to Karl et al. (1995) in a hybrid cross there is a tendency for the rare taxon to be the parental female. However, in hybrid crosses already observed in USA and Suriname the females belong to the more abundant species, such as observed in this study. As observed by Carr in 1956 (cited in Karl et al. 1995) and by surfers and SCUBA divers around the world, sea turtle males are not strict when choosing their mating pair. This, together with a greater availability of *C.caretta* females could explain the error of *E.imbricata* males in the partner choice, which is leading to more hybrids with the parental female from the most abundant species (Karl et al. 1995).

Although reproductive barriers due to pre-zygotic mechanisms seem not to be too strong among sea turtles, some post-zygotic mechanisms might be at work in this situation, since there is a gender and species biased introgression and hybrid males' contribution is also not detected in the gene pool analyzed. Female mediated gene flow introgressing *C. caretta* genes in hawksbill populations will have a likely impact on conservation management in Brazil, as at least 39% of rookery population displays *C. caretta* genes, which may theoretically lower population fitness. However, male mediated gene flow seems to be of relative small concern, since it was not detected in this study. Anyway, new studies are urgently needed to measure the real impact of hybridization in the fitness of this Brazilian nesting population. Furthermore, more detailed studies should also be done with *C.caretta* populations using nuclear markers, since male hybrids could be reproducing with loggerhead females, although not with hawksbills.

Feeding aggregation

As opposed to the rookery, the feeding aggregation presented a high number of distinct haplotypes (14 haplotypes, 4 haplogroups) and accordingly presented also high values of nucleotide and haplotype diversity, similar to values for other aggregates already studied (Bass et al. 1996, Diaz-Fernandez et al. 1999, Troeng et al. 2005, Bowen et al. 2007). This aggregate has one of the highest numbers of haplotypes registered for a single feeding assembly. Being only comparable with values found by Diaz-Fernandez et al. (1999) for some feeding aggregates in Cuba (14 haplotypes) and Mona Island (16 haplotypes), when using longer sequences for their analysis.

Interestingly, almost 15% of the samples from the feeding aggregate have a putative Eastern Atlantic or Indo-Pacific origin, reinforcing the idea that juvenile trans-Atlantic migrations in this species could be more common than was previously thought. If the animals are coming from African rookeries, they must be traveling at least 2,500 km, but if they come from the Indo-Pacific as suggested by our comparative results, the figure could rise to more than 10,000 km.

The finding of haplotypes EiBR13, 15 and 17 (Table 1), related to the F (Puerto Rico, Cuba, Virgin Islands and Belize) and α (Costa Rica) haplotypes described for Caribbean nesting colonies provides a possible link between the Caribbean rookeries and Brazilian territory, highlighting the importance of Brazilian feeding aggregates for the conservation of Caribbean populations. It also confirms that these animals are capable of migrating long distances between their natal areas and developmental habitats (3,500 km in a straight line from Barbados - 6,000 km from Costa Rica following the coast line).

This link between Brazilian feeding areas and Caribbean populations is reassured by the MSA results that indicated that more than 80% of the feeding aggregate is contributed by Insular Caribbean rookeries. The contribution of coastal Caribbean rookeries is lower, as expected because of the oceanic current patterns, but the low contribution of the Brazilian rookery to these feeding grounds (7%) is contrary to what could be expected considering the distance between Brazilian rookery and these feeding areas (ca. 540 km).

As registered elsewhere (Bass and Witzel 2000, Luke et al. 2004, Bass et al. 2006, Bowen et al. 2007), large nesting colonies are expected to contribute more than smaller ones, and closer ones more than distant ones, but the relative effect of distance is not so clear (Bowen et al. 2007). Our results agree with the expectations of larger rookeries contributing more than smaller ones since Mexico, Cuba and Barbados harbor the biggest rookeries in the Caribbean. When hybrids are excluded from the analysis (see below) the contribution of the closest colony (Brazil) is higher supporting the hypothesis that closer colonies contribute more to adjacent foraging grounds.

Even though the MSA results have to be taken with caution because of the high SD values, these values are comparable and often lower than other values already registered in the literature. The high SD values obtained when using Bayesian procedures to estimate stock composition are related to the sharing of haplotypes between base line samples (rookeries). In this case, haplotype A is the most common for rookeries in Brazil, Barbados, Cuba and Antigua. Nevertheless, there are other facts that can explain the low Brazilian contribution found by the MSA. More prevalent is the absence of hybrid haplotypes (EiBR3 and 4) that together account for almost 40% of the rookery samples (they were binned for the analyses), while EiBR8 represents 50% of these samples. Thus, if the contribution to the feeding grounds was from the Brazilian rookery, it would be reasonable to expect at least a

similar proportion of hybrid and EiBR8 haplotypes in the aggregate. The Bayesian estimate of the Brazilian contribution is highly influenced by this absence and this estimate could have been lower if it was not for the presence of two haplotypes exclusively found in the nesting population, which were also found in the feeding grounds (EiBR 10 and 16).

However, when the MSA analysis were ran disregarding the hybrid haplotypes from the Brazilian rookery, the point estimate for its contribution to the studied feeding grounds rises up to 92% and the accuracy of all estimates is greatly improved (see SD values in table 3B). This is expected since 100% (3) of the haplotypes present in the Brazilian rookery appear at the feeding aggregates while no other base-line stock used had 100% of its haplotypes represented at the mixture. These contrasting results demonstrate the sensitivity of the MSA analysis to the presence or absence of haplotypes in the base line data. In the case of the first analysis, the absence in the mixture of one of the high-frequency haplotypes in Brazilian rookery leads the analysis to an extremely low contribution estimate. In the light of our previous findings this results could suggest that: *i*) most of the individuals born in Brazil do not migrate to the feeding areas sampled during this study or *ii*) only the non-hybrid animals from the Brazilian rookery are to be found feeding at these areas. If the latter situation is the case, then there are two possible explanations for this. First, it is possible that only the closest nesting population (RN), which presents no hybrids, is contributing young individuals to FN and AR feeding sites. Second, the largest nesting population in Brazil (BA) where the hybrids occur also contribute individuals to FN and AR, but hybrid individuals display different feeding or migratory behavior, keeping them away from the hawksbill juvenile feeding areas. Although this later hypothesis is tentative, it seems plausible because hybrids are formed from species with very different characteristics, e.g. *E. imbricata* feeds almost exclusively on sponges and *C. caretta* feeds on crustaceans and mollusks. Thus, if hybrids are escaping the outbreeding depression that is expected after the interbreeding of species with very different adaptive genes, and are not found using the parental species' niche, they could be fitting *C.caretta*'s niche.

Implications for conservation

Our results revealed striking differences between the two Brazilian populations studied. They showed to be independent and deserve to be treated as different management units with different conservation strategies directed to each one. These data highlight the importance of the main Brazilian feeding aggregates found in Atol das Rocas (AR) and Fernando de Noronha (FN) for populations all around the world since these areas are developmental areas where juveniles are recruited to spend several years until they migrate to other developmental areas or to nesting sites. Harvest in these areas could affect

considerably the populations in the Caribbean but also other populations from Western Atlantic and Indo-Pacific regions. Fortunately, both feeding areas are protected biological reserves in Brazil. For the nesting population, there is still little evidence of connectivity between the Brazilian rookery and feeding aggregates elsewhere, although a small contribution to the Caribbean feeding grounds could be inferred by the presence of a Brazilian haplotype in Caribbean feeding aggregations.

Because MSA analysis is incapable to deal with orphaned haplotypes they were excluded from the analysis. However, the proportion of these haplotypes is considerable (14.9%) and if they have an Indo-Pacific or Western Atlantic origin, long trans-oceanic migrations might not be as infrequent as it was believed before. The multiple origins of the individuals at these feeding grounds imply that harvest and/or incidental capture of animals in Brazilian grounds could be affecting several nesting populations around the world, besides the Caribbean.

The absence of observed hybrids between *E.imbricata* females and *C.caretta* males or between male hybrids with *E.imbricata* females could suggest that malformed embryos, stillborns or individuals that do not reach reproductive age are being produced, representing a waste of reproductive effort. The considerable portion of likely F1 hybrids in the Brazilian population might not threaten seriously the conservation of the parental species, but further studies and special management measures should be taken to decrease this reproductive waste, whether it is confirmed. It would be of special interest to identify the causes of this hybridization event and to characterize this hybrid swarm in terms of reproductive and survivorship parameters to establish if the process could result in an eventual decline of the sea turtle population. In addition, genetic monitoring of this rookery in the long term would be advisable to asses if hybrid proportions are rising in the population.

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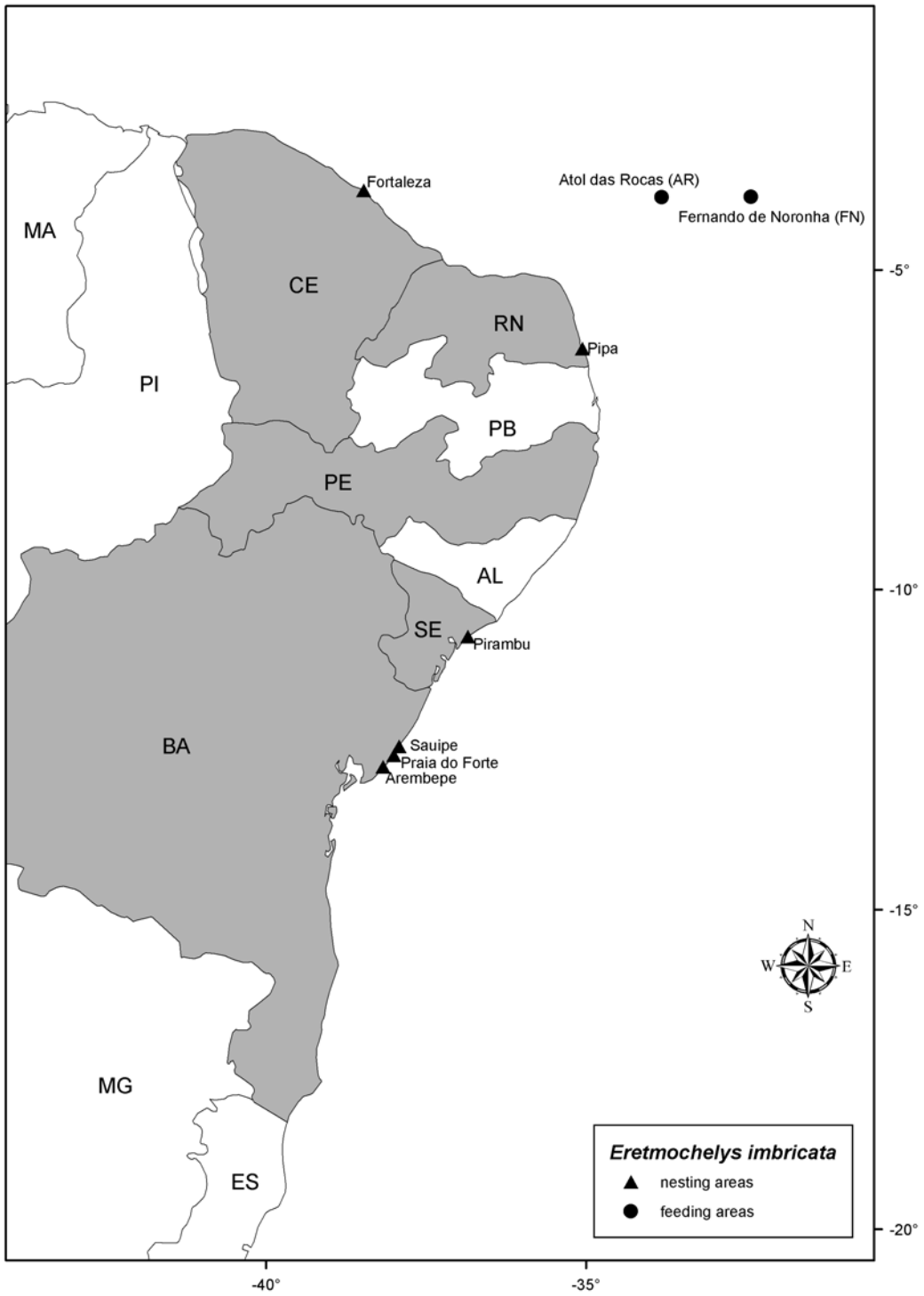
Figure legends

Figure 1. Location of nesting beaches and feeding areas where hawksbill's samples were collected.

Figure 2. Median joining network showing the relationships between *E. imbricata* mtDNA control region haplotypes described for Brazilian samples. Haplogroups were named following the most common haplotypes registered in the literature (A and F) or I-P1 for the group related with Kuwait sequences and I-P2 for the sequence that matches the EATL haplotype that has been related with Australian samples. The area of the circle is proportional to haplotype frequencies in all data set. The detail depicts the relationships among haplotypes from haplogroup A. Black: samples from nesting grounds. Light Grey: samples from feeding grounds. For clarity, only the numbers that identify each haplotype were included.

Figure 1.


Fortaleza
Atol das Rocas (AR)
Fernando de Noronha (FN)
MA
CE
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Pipa
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-5°
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Eretmochelys imbricata
nesting areas
feeding areas

Figure 2.

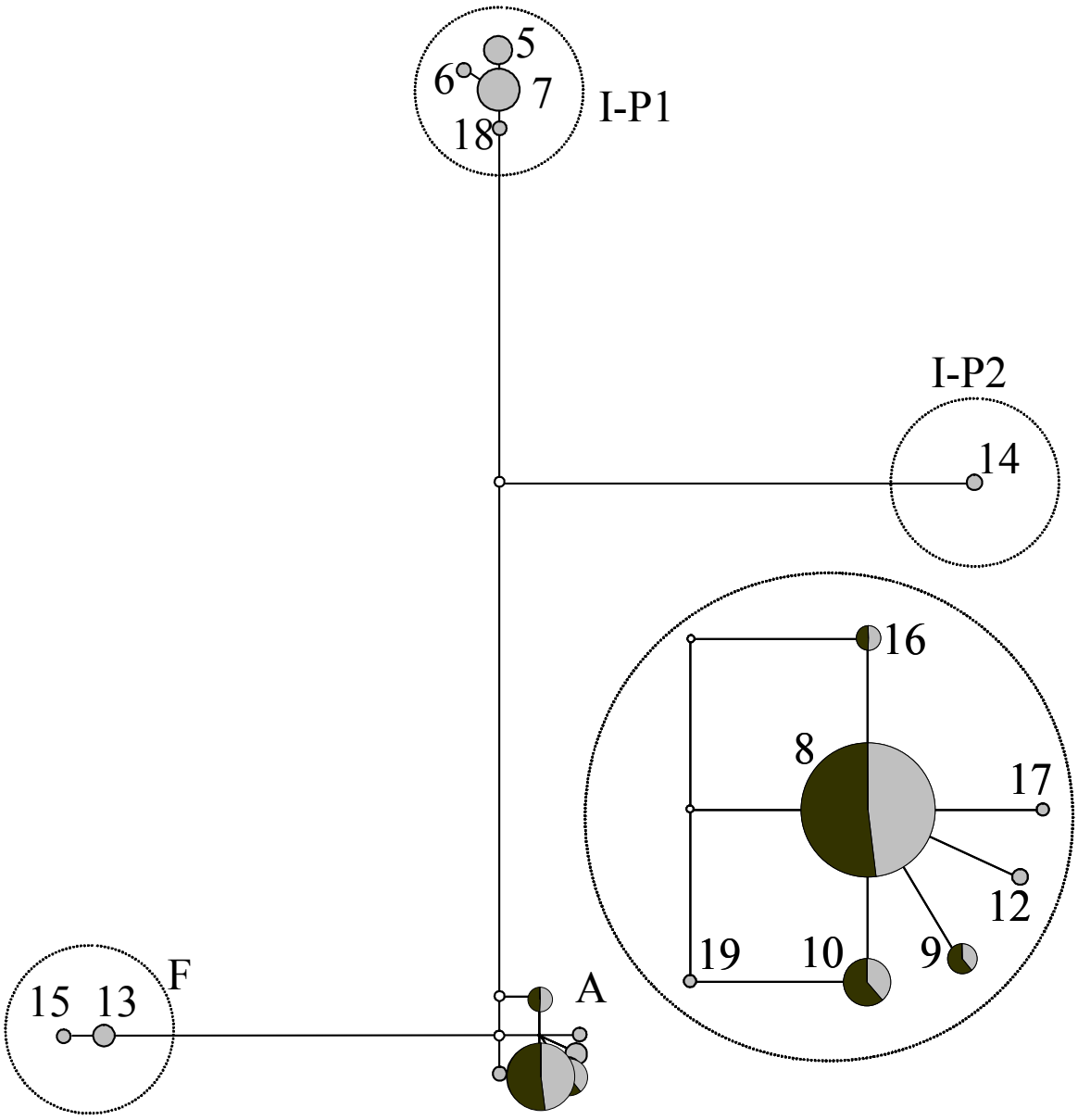

Table 1. Absolute frequencies of control region mtDNA haplotypes described for Brazilian samples from nesting (Lara-Ruiz et al., 2006) and feeding grounds (this study) together with the corresponding 384 bp sequence matches from sequences deposited in GenBank (February 2007) and the designation of the haplotype in the literature.

Haplotype (740 bp)	Observed Frequencies			Matching shorter sequences (384 bp)
	Feeding	Nesting	Total	ID
EiBR2		2	2	*L.olivacea*[1]
EiBR3		24	24	*C.caretta* EiBR3[1]
EiBR4		26	26	*C.caretta* EiBR4[1]
EiBR5	3		3	none[2]
EiBR6	1		1	EATL[3]
EiBR7	6		6	EATL[3]
EiBR8	65	65	132	A[4]
EiBR9	2	4	6	A[4]
EiBR10	6	9	15	EiBR19[2] and f[6]
EiBR12	2		2	b[6]
EiBR13	2		2	F[4]
EiBR14	1		1	H2-H5[5]
EiBR15	1		1	F[4]
EiBR16	2	2	4	none[2]
EiBR17	1		1	α[6]
EiBR18	1		1	none[2]
EiBR19	1		1	EiBR10[2]
	94	132	226	

Data sources are from Lara-Ruiz et al. 2006[1], this study[2], Bowen et al. 2007[3], Bass et al. 1996[4], Al-Mohanna and George (GenBank)[5] and Diaz-Fernandez et al. 1999[6]. None means that the large haplotype does not match any short sequence.

Table 2. Standard and molecular diversity indexes generated by Arlequin for nesting (with and without "hybrid" haplotypes considered) and feeding aggregates sampled in Brazil.

	Nesting		Feeding
	With hybrids	Without hybrids	
N	132	80	94
Nº of loci	750	739	740
Nº of polymorphic sites (S)	139	3	48
Nº of haplotypes	7	4	14
Ts	98	3	41
Tv	33	0	6
indels	15	0	1
h	0.6795 +/- 0.0314	0.3213 +/- 0.0630	0.5157+/- 0.0625
π	0.0544 +/- 0.0263	0.0005 +/- 0.0005	0.0094 +/- 0.0049
k	40.814 +/-17.830	0.3413 +/- 0.3414	6.9286 +/- 3.2873

Table 3. Stock assignments using results from all nine chains combined (MCMC sample = 900,000) when considering hybrid haplotypes described for the Brazilian rookery (A) and when the hybrid haplotypes are removed from the analysis (B).

	STOCK	MEAN	SD	2.5%	MEDIAN	97.5%
A						
	Brazil	0.0733	0.1134	0.0000	0.0056	0.3745
	Cuba	0.2155	0.3354	0.0000	0.0042	0.9568
	Puerto Rico	0.0026	0.0076	0.0000	0.0000	0.0251
	US Virgin Islands	0.0076	0.0160	0.0000	0.0002	0.0572
	Antigua	0.0148	0.0921	0.0000	0.0001	0.0704
	Barbados	0.6542	0.3984	0.0000	0.8880	0.9895
	Costa Rica	0.0251	0.0246	0.0000	0.0191	0.0859
	Belize	0.0055	0.0130	0.0000	0.0001	0.0463
	Mexico	0.0014	0.0042	0.0000	0.0000	0.0132
B						
	Brazil	0.9155	0.0676	0.7220	0.9329	0.9861
	Cuba	0.0181	0.0506	0.0000	0.0002	0.1737
	Puerto Rico	0.0028	0.0080	0.0000	0.0000	0.0266
	US Virgin Islands	0.0089	0.0173	0.0000	0.0003	0.0612
	Antigua	0.0043	0.0139	0.0000	0.0000	0.0408
	Barbados	0.0120	0.0367	0.0000	0.0001	0.1152
	Costa Rica	0.0306	0.0258	0.0000	0.0261	0.0917
	Belize	0.0063	0.0139	0.0000	0.0001	0.0499
	Mexico	0.0014	0.0041	0.0000	0.0000	0.0131

ARTIGO 3:

Identification of autosomal SNPs to use in the characterization of *Eretmochelys imbricata* x *Caretta caretta* hybrids.

Artigo aser submetido para publicacao em Setembro de 2007.

Identification of autosomal SNPs to use in the characterization of *Eretmochelys imbricata* x *Caretta caretta* hybrids.

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Running Title: Diagnostic SNPs for identifiction of marine turtle hybrids.

Key words: Marine turtles, hybrids, nuclear markers, SNPs, hawksbills, loggerheads.

ABSTRACT

The hawksbill turtle, *Eretmochelys imbricata* (Reptilia: Chelonidae), is considered a critically endangered species by the IUCN. The population nesting in the Brazilian territory has been subjected to mtDNA analysis that evidenced an ongoing hybridization process between this species and the loggerhead turtles, *Caretta caretta*, which can increase the extinction risk of the former population. However, in order to better understand this hybridization process there is a need to study autosomal markers that can help to evaluate the number of F1 hybrids, the degree of introgression and to estimate the time since the first hybrids appeared. Using available sequences for five autosomal loci from the two target species we identified variable nucleotide sites and characterized intra and inter-specific polymorphisms. The sites identified as variable between species but not within species can be used as diagnostic characters to analyze putative hybrid populations. These markers can be used in the future to improve the understanding of the hybrid populations, particularly in Brazil where ca. 40% of the nesting hawksbill turtles are likely products of hybridization.

INTRODUCTION

The study of hybridization is important since it can help us to better understand interspecies relationships and horizontal evolutionary processes (Seehausen 2004; Allendorf et al., 2001). The interbreeding among five of the six chelonid species has been registered historically in several areas around the world (Seminoff et al., 2003). However, most reports are based solely on the description of individuals with intermediate morphological characters (Carr and Dodd, 1983; Kamezaki, 1983; Wood et al., 1983; Frazier, 1988) and only recently these hybridization events have been studied by the use of molecular markers (Conceição et al., 1990; Karl et al., 1995; Seminoff et al. 2003; Lara-Ruiz et al., 2006). On the other hand, hybridization can also have extreme conservation implications when the patterns and extent of it lead to the extinction or replacement of a rare species. The harmful effects of hybridization have led to the extinction of many populations and species in many plant and animal taxa even when the process does not imply gene flow between the populations that are hybridizing (i.e. introgression) (Allendorf et al., 2001; Rhymer and Simberloff 1996; Fredrickson and Hedrick 2006; Seehausen 2006).

The phylogenetic relationships between all marine turtles are still not conclusive (Dutton et al., 1996), however the separation between the tribes Carettini (*Caretta*, *Eretmochelys* and *Lepidochelys*) and Chelonini (*Chelonia* and *Natator*) may have occurred as long as 50 MYA (Bowen et al., 1993) while the first split among species from each tribe was estimated to be at least 10 to 20 MYA (Karl et al., 1995). Thus, the crossbreeding between marine turtle species provides a good case study on the evolutionary relationships between interbreeding ancient lineages.

Molecular genetic studies allow the study of hybridization events with greater precision than the morphologically based approach (Seminoff et al., 2003). The first molecular genetics approach involved the use of mtDNA markers to characterize different parental species and to screen populations with putative hybrids in search for mtDNA haplotypes from another species (Rhymer and Simberloff, 1996). This provides also information on the gender of the parental species in the crossing generating the F1 hybrids, which can also be complemented with morphological analysis. However, the analysis of maternally inherited DNA (such as mtDNA) alone does not provide a complete picture on the extent of the hybridization or introgression processes. For example, if the F1 or >F1 hybrids are morphologically more similar to the female genitor species, no further information can be recovered from mtDNA analysis. Besides, it will also very difficult to identify hybrid individuals in a hybrid swarm (i.e. where a high proportion of individuals are introgressed due to mating between hybrids and backcrossing with the parental species). Thus, there is a need to

analyze biparentally inherited autosomal markers such as allozymes, microsatellites and single nucleotide polymorphisms (SNPs).

Hybrid populations will carry genes from both parental taxa but may still be morphologically indistinguishable from one of the parental species. Considering that a moderate influx of a foreign species' genes in a population will lead to a percentage of hybrids that still appear to be genetically pure based on the analysis of few individual diagnostic loci (Rhymer and Simberloff, 1996; Allendorf, 2001), the use of several independent sections of the genome will allow a better understanding of the genetic pattern and implications of the process.

Additionally, when several independent markers are available to analyze the distribution of gametic disequilibria, it is possible to describe the distribution of hybrid genotypes in order to estimate the age of the hybridized population (Forbes and Allendorf, 1991), an important parameter when assessing conservation concerns (Allendorf et al., 2001).

To the present, the hybrids between *E.imbricata* and *C.caretta* found in Brazil have been studied by means of mtDNA haplotypes (Lara-Ruiz et al., 2006) and three RFLP markers (Lara-Ruiz et al., to be submitted). These data suggest a unidirectional process since no *C.caretta* individuals with *E.imbricata* mtDNA haplotypes have been identified, but 40% of morphologically identified *E.imbricata* display *C.caretta* mtDNA. These findings also indicated an ongoing introgression process between *E.imbricata* males and *C. caretta* females, but to better evaluate the extent of this introgression and the possibility of inter-hybrid crossings there is a need to analyze the population with more nuclear markers. Moreover, more nuclear markers are also needed to analyze the *C.caretta* population nesting in Brazil to rule out the possibility of introgression in this parental species.

This work aimed to identify interspecific SNPs in five different single copy nuclear genes, standardize their detection and to asses their suitability as diagnostic characters for the target species. In the future this new set of nuclear markers can be used together with the mtDNA analysis and three RFLPs already available, as well as microsatellite markers, to provide a better picture of the ongoing hybridization process registered in Brazil.

MATERIALS AND METHODS

Protocol standardization and sequencing of PCR products

E.imbricata and *C.caretta* single copy nuclear sequences of four exons (Brain-derived neurotrophic factor – *BNDF*, Oocyte maturation factor Mos – *Cmos* and two Recombination activating genes - *Rag1* and *Rag2*) and one intron (RNA fingerprint protein 35 gene - *R35*)

were kindly provided by E. Naro-Maciel and collaborators. These genes have been previously used to address phylogenetic relationships between fresh water turtles and tortoises (Krenz et al. 2005, Le et al. 2006) and more recently for a phylogenetic analysis of marine turtles (Naro-Maciel et al., *unpublished*).

For each of the five nuclear genes, sequences available in the GenBank, EMBL, DDBJ and PDB data bases, representative of several (20) distinct lineages of fresh water turtles and tortoises were used to construct alignments together with the Chelonian sequences. The sequences were chosen by their degree of similarity with the Chelonian sequences as indicated by a BLAST search (Altschul et al., 1997).

The alignments constructed using the Clustal W algorithm implemented in MEGA 3.1 (Kumar *et al*., 2004) were used to search for conserved regions, potential variable sites and diagnostic characters for the target marine turtle species *E.imbricata* and *C.caretta*.

Once the most variable regions were identified for each gene, the conserved regions flanking the variable sites, mainly the ones found to be different between the target species (i.e. putative diagnostic characters), were used to design primers for the amplification of fragments including these variable sites. Primers were designed using the software Primo Pro 3.4 (http://www.changbioscience.com), modified manually to increase their specificity and checked for dimmer, hairpins, cross dimmer and palindromes using NetPrimer (http://www.premierbiosoft.com).

The optimal amplification conditions for each gene segment amplified by the designed primers were searched by varying several conditions in the amplification mix and testing in gradient thermo cycling machines for the optimal annealing temperature. Several magnesium ($MgCl_2$ 0.75, 1.5 and 2.0 mM), template DNA (10, 50, 100ng) enzyme (0.5 – 1 U/reaction) and primer concentrations (0.1 to 1 μM), as well as the use of PCR enhancers (DMSO, Gelatin and BSA) were tested to obtain the maximum efficiency and specificity of PCR products.

For the purification of amplicons before sequencing reaction, alcohol and PEG precipitation protocols available at our laboratory (www.icb.ufmg.br/lbem/protocols) were tested. Independent sequencing reactions were performed using both forward and reverse primers following the instructions of the ET Dye Terminator Cycle Sequencing Kit (Amersham Biosciences) manufacturer and read in a MegaBACE 1000 automated sequencing machine.

Identification of diagnostic sites and hybrid diagnosis

The individuals selected as representatives of *E.imbricata* (n = 25) were chosen from a sample from feeding areas in Brazil where no hybrids were registered after mtDNA

analysis and a preliminary RFLP screening with three anonymous nuclear markers (Lara-Ruiz et al., *to be submitted*). Care was taken to include samples representative of the most divergent haplotypes found which we believe to be individuals that come to feed in Brazilian grounds from locals in the Caribbean and Indo-Pacific where no hybrids between these species have been identified. The selected *C.caretta* samples (n= 25) were chosen from a sample of 93 animals captured off-shore that were characterized by mtDNA control region at our laboratory (*unpublished*). We used representatives of the five mtDNA haplotypes registered for the Brazilian sample that include a sequence only described for Brazilian rookeries and other sequences related to haplotypes previously described for samples from the East Atlantic and Indo-Pacific oceans (*unpublished data*).

For the five nuclear loci analyzed, sequences were generated (with both forward and reverse primers) from phenotypically and mitochondrially pure parental-type individuals of the two species from areas where no hybrids have been recorded (see above). High quality consensus sequences were obtained using the programs Phred 0.020425 (Ewing et al. 1998), Phrap 0.990319 (www.genome.washington.edu/UWGC/analysis tools/phrap.htm) and Consed 12.0 (Gordon et al. 1998).

The consensus sequences were aligned in MEGA together with the two *E.imbricata* and *C.caretta* reference sequences provided by Naro-Maciel et al. (*unpublished*). Polymorphic sites were identified by visual inspection and using Polyphred 6.11 (Nickerson et al., 1997; Stephens et al., 2006). All variable characters were recorded and considered as diagnostic only the characters varying between species that appeared to be fixed in each species.

After defining the diagnostic characters for each species we analyzed sequences from hybrids and “pure” *E.imbricata* and *C.caretta* individuals with the program Polyphred to verify its power to detect both parental alleles using only one forward and one reverse sequence from each sample.

RESULTS

Protocol standardization

The primers were designed to amplify of 400-600 bp PCR fragments that contained most of the possible variable sites. As our objective was to develop a quick and efficient method for identifying hybrids, we chose short PCR fragments which are easier to amplify and sequence even in slightly degraded samples. In addition, as sequencing analysis requires both alleles to be of the same size, we excluded regions in the reference sequences where indels were detected.

The sequences of the designed primers are shown in Table 1. After standardization, the thermocycling protocol for the amplification of the five segments in samples from the two species was defined as 94ºC for 3 minutes followed by 35 cycles of 94ºC for 40 seconds, annealing temperature (see Table 1) for 45 seconds and 72ºC for 50 seconds, with a final extension step of 72ºC for 10 minutes.

The final composition of the PCR mix for the amplification of each of the five targeted sequences was standardized for a volume of 15 µl and 50ng template DNA. All standardized reactions were carried out using the Phoneutria© PCR Buffer IB 10X (500 mM KCl, 100 mM Tris-HCl pH 8.4, 1% Triton X-100, 15 mM $MgCl_2$) diluted 10 times, 1 unit Taq Polymerase Phoneutria© per reaction and 200 µM dNTP's. Final primer concentrations and PCR enhancers are shown in table 1. The PCR products were visualized in a 0.8% agarose gel stained in ethidium bromide (Figure 1).

For sequencing with the ET Dye Terminator Kit (Amersham Biosciences) we used PCR products purified by precipitation with PEG 8000 20%, NaCl 2.5 M and ethanol in 96-well plates. In both cases, the purified amplicon was resuspended in 1.5 the original volume of PCR product used. Both purification protocols rendered DNA of suitable quality for the sequencing reaction that was prepared using 2µl of purified PCR product in a final reaction volume of 10 µl. The thermocycling program used for all sequencing reactions was 95ºC for 2 seconds followed by 35 cycles of 95ºC for 25 seconds, 55º for 15 seconds and 60ºC for 100 seconds. After the sequencing reaction the product was purified by ethanol precipitation, injected in a MegaBACE 1000 (100 seconds – 2 Volts) and ran for 240 minutes at 6 Volts. One sequence obtained for each gene fragment per species was deposited in GenBank (XXXXXXX).

Identification of diagnostic sites

The number of polymorphic characters identified in each gene segment after sequence alignment analysis is shown in table 2. This table also shows the size of the PCR products obtained after the amplification with the primers designed in this study, the diagnostic characters in each gene fragment and the number of samples sequenced of each parental species.

The observed polymorphic characters identified after the analysis and their position in the produced sequences are shown in Table 3. Some of the characters were found to be polymorphic among *E.imbricata* samples while appeared to be fixed in *C.caretta.* As expected for an intron, the R35 gene fragment was the one that displayed the higher variation, while in the BNDF exon fragment no variation was found between the two species. Thogether, these results allowed the identification of 19 SNPs of which 13 were inter-specific

polymorphic characters (i.e. species diagnostic characters) observed in Rag1 (3), Rag2 (4), R35 (4) and Cmos (2) sequences, while six were shown to be polymorphic for *E.imbricata* (2 in Rag1, 2 in R35 and 2 in Cmos).

Hybrid diagnosis using PolyPhred

After the identification of the diagnostic and polymorphic sites we use the Polyphred software and the Phred Prap Consed package to analyze sequences of previously described hybrids (samples from specimens with *E.imbricata* morphology, *C.caretta* mtDNA and scnDNA alles of both species in at least one of three locus) in order to verify the capability of PolyPhred to identify both alleles in a sequence. In all cases the software was capable to detect the heterozygous sample (hybrid) in which the heterozygous position was represented by two peaks of approximately half the size of the peak displayed in the homozygous sample (Figure 2). In some cases, the software did not recognize the position as heterozygous but indicated the discrepancy between reads by highlighting the base in each read (Figure 3A). We found that this happened when few samples were being analyzed, and the capacity of the software to detect the heterozygous individuals increased when the number of homozygous individuals used to create the assemblage was increased (Figure 3B).

When only three samples were used in the alignment (one of each parental species and the putative hybrid, each one with forward and reverse reads), PolyPhred was capable to detect half of the diagnostic characters identified in the previous analyses, usually the ones located in the central regions of the consensus sequence where sequence quality (i.e. Phred scores) is better. As the number of samples was increased, the quality of the bases in the consensus sequence also did and consequently, the ability of PolyPhred to identify the polymorphic sites. No false positives were identified in the sequences analyzed indicating that the algorithm correctly differentiates between sequencing errors and / or poor base qualities and real heterozygous positions as demonstrated by Stephens et al. (2006). It also was found that if the aligned reads are of good quality, only two reads (Forward and Reverse) per sample are needed in order for the software to identify the polymorphic positions.

DISCUSSION

Of the five nuclear regions chosen for this work, only the BNDF sequence was not found to have any polymorphic or diagnostic characters. According to the BNDF sequences used for reference, an A rather than a G (supposedly a private allele for loggerheads) should represent the hawksbill samples; however the 22 samples sequenced (that represent all the

haplogroups previously registered for the species' feeding grounds in Brazil) presented a G in that position suggesting that this character is also fixed for hawksbills. Thus the BNDF sequence is not suitable for hybrid identification. As expected for an intron sequence, the R35 fragment was the one where more polymorphic characters were found, including some variable characters not present in the two reference sequences used for primer design. This and the fact that our analysis detected several "new" (i.e. not found to be variable between species in the sequences used for primer design) sites in Rag1, Rag2 and Cmos sequences, indicates that our *E.imbricata* sample may contain a substantial portion of the genetic variability that can be found in this species.

The allelic variation found in the four sequences studied (excluding BNDF) was low enough to allow a straightforward inference of the sequences of both alleles in heterozygotes as well as the numerous homozygous individuals. However, the variation found can be enough to allow the use of the methods developed here in future phylogeographic studies at least in hawksbills (since no variation was apparent in loggerhead samples). The use of nuclear markers for phylogeographic studies has been suggested as a powerful tool to better understand historical demographic and selective processes (Hare, 2001; Morin et al., 2004). As for the hybrid analysis there is no need to score all of the 13 SNPs identified since the most recommended approach is to cover the greater amount of genomic regions rather than concentrate in one of them (Allendorf et al., 2001; Seehausen, 2004). Thus the identification of at least one SNP in each sequence must be enough to indicate the hybrid character of the sample.

As a method for quickly identify heterozygotes / hybrid samples, PolyPhred showed to be reliable but dependant on the quality of the sequences produced and the amount of samples used in the analysis, effects that have already been described (Stephens et al., 2006). Thus, in order to facilitate the identification of heterozygous or hybrid sequences, the analysis can concentrate on the diagnostic positions identified at the central parts of the sequences where the two reads (forward and reverse) overlap producing higher Phred scores.

During our analysis, PolyPhred accurately found all the diagnostic positions identified, provided that the sequence quality for each sample was good enough. Stephens et al. (2006) suggested that a quality of 30 is ideal for heterozygote identification by PolyPhred. The methods here presented allowed us to obtained high >20 Phred scores in the consensus sequence for each sample using only two reads (one Forward and one Reverse). In such case, the analysis by sequencing is not as expensive and is easier than the restriction enzyme analysis. Moreover, sequencing methods allow the analysis of higher number of samples in less time also providing information that is easier to analyze. However, in laboratories where sequencing is not ready available or where high Phred scores for

consensus sequences only are reached after the production of several reads with each F and R primers, the digestion with restriction enzymes could be an alternative for hybrid diagnostics. Another advantage of the diagnostic analysis presented here is that is cheaper than the traditional analysis of SNPs using fluorescent primers.

From the analysis of samples from supposedly "pure" *C.caretta* and *E.imbricata* samples, our results suggest that before performing a phylogenetic analysis it is advisable to sequence several samples from a species, preferably from specimens from distinct geographical locations (or at least from specimens that display very divergent mtDNA haplotypes) in order to first identify hidden intraespecific variation that can affect posterior phylogenetic inference. This was the case with four of the expected diagnostic characters that turned out to be polymorphic in our sample of *E.imbricata* specimens and thus cannot be considered diagnostic for the two species analyzed or used for further phylogenetic analysis.

The fact that all the polymorphisms found were observed in our *E.imbricata* sample can be explained by our sample selection. As mentioned above, the samples from *E.imbricata* were selected to represent several very distinct mtDNA haplotypes found only in feeding areas of the species in Brazil characterized by high haplotype and nucleotide diversity values (Lara-Ruiz et al., *to be submitted*). In contrast, our sample of *C.caretta* is more limited, and restricted to a subset of the samples available at our laboratory that do not belong to feeding aggregations (which in all Chelonid populations are believed to harbor greater diversity than the rookeries). Even though our sample contains individuals with haplotypes believed to be characteristic of populations from the indo pacific and both eastern and western Atlantic, it present much lower values of haplotype and nucleotide diversity as a whole (5 mtDNA haplotypes in 93 individuals characterized so far as opposed to 14 haplotypes characterized in 94 *E.imbricata* samples from feeding grounds in Brazil). Alternatively, the higher diversity registered in the hawksbill sample can be related to the nesting behavior of the species which is characterized as not being as colonial as the other Chelonids, being commonly referred as a solitary nester (Hirth, 1980; Ehrhart, 1995; Spotila, 2004). Thus, considering that i) one of the main threatens for all marine turtles is the human activities in nesting beaches, ii) these activities are responsible for the extermination of several colonies around the world during the past two centuries (IUCN/SSC Marine Turtle Specialist Group, 2003; IUCN, 2006) and iii) that the homing behavior of marine turtles causes strong genetic structure between different colonies, it could be possible that the more scattered nesting behavior of the hawksbills allowed the species to retain more of the pre-exploitation genetic diversity levels.

CONCLUSION

We provided here an efficient method to analyze putative hybrids simples using sequence analysis to detect nuclear SNPs from four different autosomal regions in loggerhead and hawksbill turtles. Our results indicate that there are 13 fixed SNPs that can be used in the identification of hybrid loggerhead x hawksbill individuals. We had not tested the usefulness of these markers in polymorphism analyses of other marine turtle species but as the primers were designed to anneal in conserved regions shared between Chelonians and Testudines, it is possible that they can be used in the other marine species.

We hope that in the future, the use of these markers, together with microsatellite, RFLP and mtDNA analyses can provide a better understanding of the hybridization and introgression processes taking place in the Brazilian territory.

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Figure Legends

Figure 1. PCR products after standardization of PCR conditions. From left to right and top to bottom: BNDF, RAG2, Cmos, RAG1, R35. All showing the molecular weight marker (1 Kb Promega) followed by the amplified product of four *E.imbricata* samples, four C*.caretta* samples, four hybrid samples and a negative control. 0.8% agarose gel with ethidium bromide staining.

Figure 2. Example of the "aligned reads" window (top) and the correspondent "trace window" (bottom) displayed by PolyPhred after the alignment of forward and reverse reads from RAG1 sequences of *C. caretta* (A34R0369F and A34R0399R), *E.imbricata* (Ei7R0036F and Ei7R0036F) and a putative hybrid (HR0061R and HR0061F). The highlighted position corresponds to position 102 in the Rag1 PCR product (Table 3). In blue, the homozygous positions identified by PolyPhred (T for *C.caretta* and C for *E.imbricata*) and in pink indicating the "heterozygous" hybrid with T/C.

Figure 3. Example of the aligned reads window (top) and the correspondent trace window (bottom) displayed after the alignment of forward and reverse reads (only traces of forward reads shown) from Rag2 sequences of *C. caretta* (A33R0330), *E.imbricata* (Ei17R0227) and a putative hybrid (HR0059). In this case, PolyPhred identified the heterozygote (A/G) and the homozygotes at the position 282 (as defined in table 3) but failed to detect the polymorphic (G/C) character at position 274 (left of the A/G site) which appears highlighted in red but without color tags indicating a polymorphic site. The heterozygous character of the hybrid in the latter position is clearly visible in the trace window and was detected by PholyPhred when more samples were added to the alignment (B).

Figure 1.

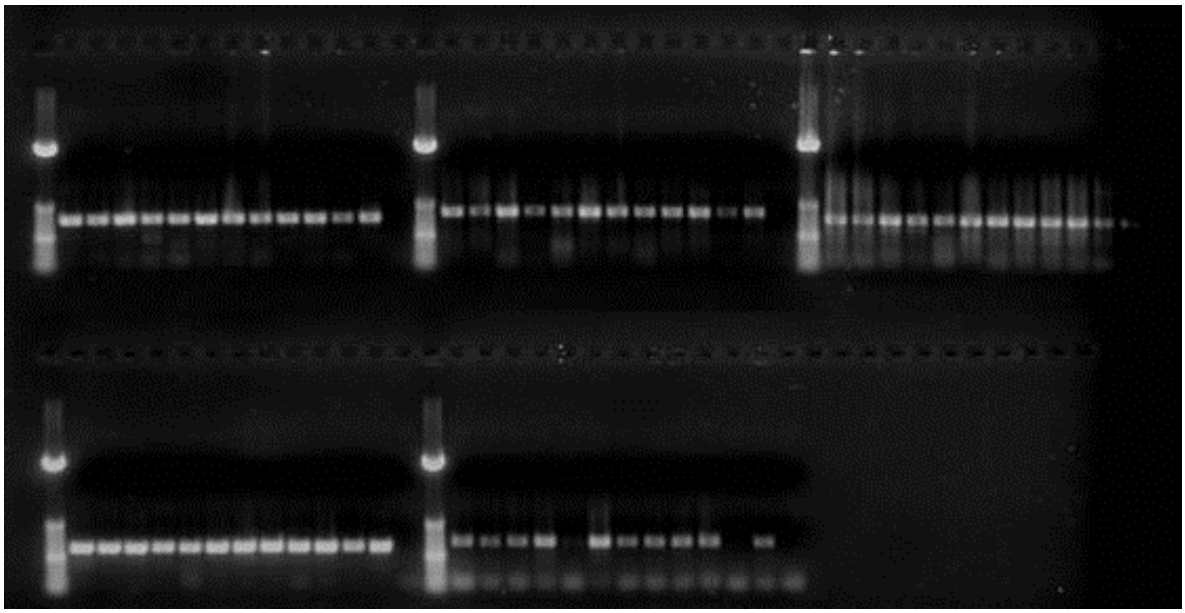


Figure 2.

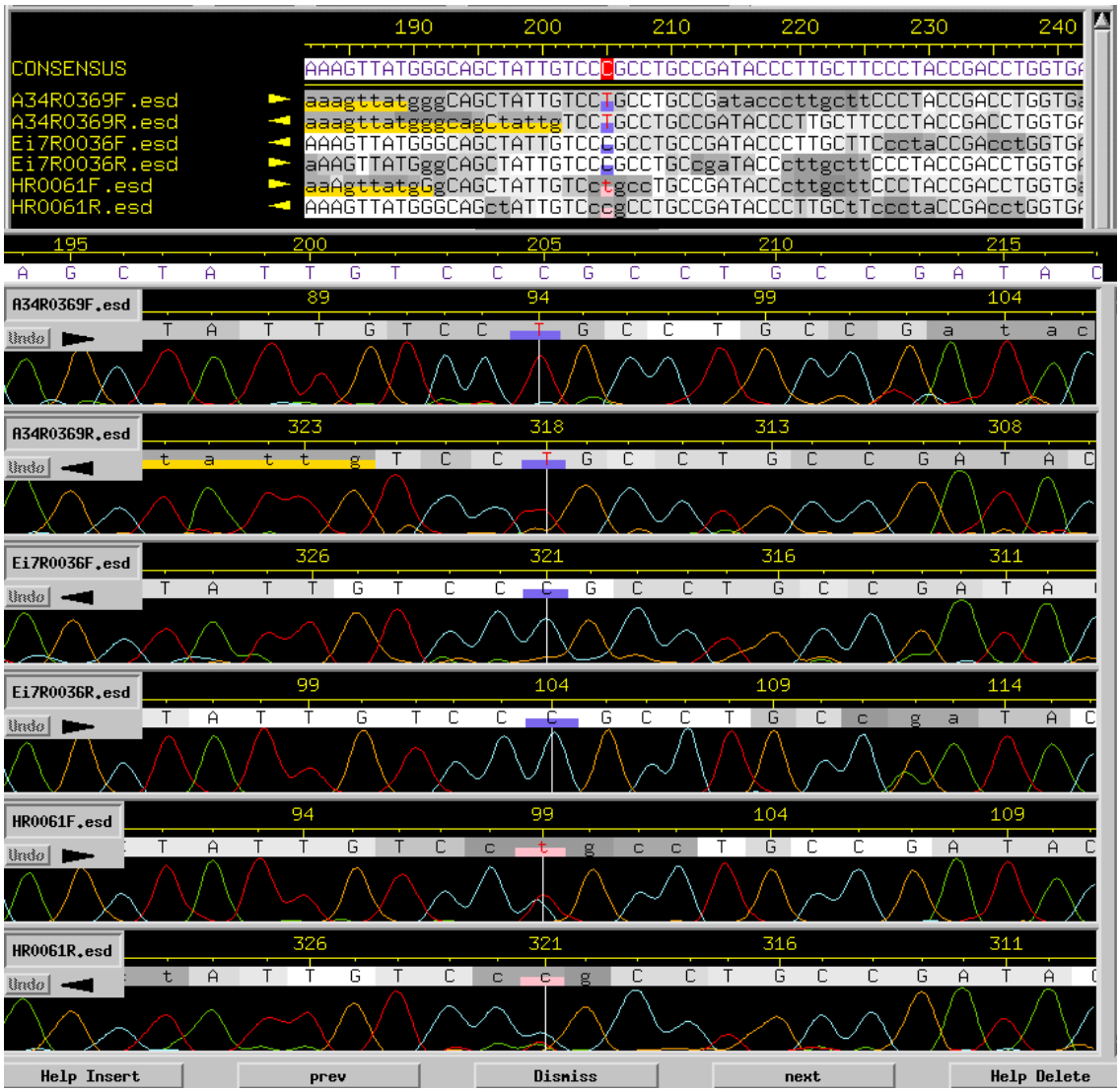
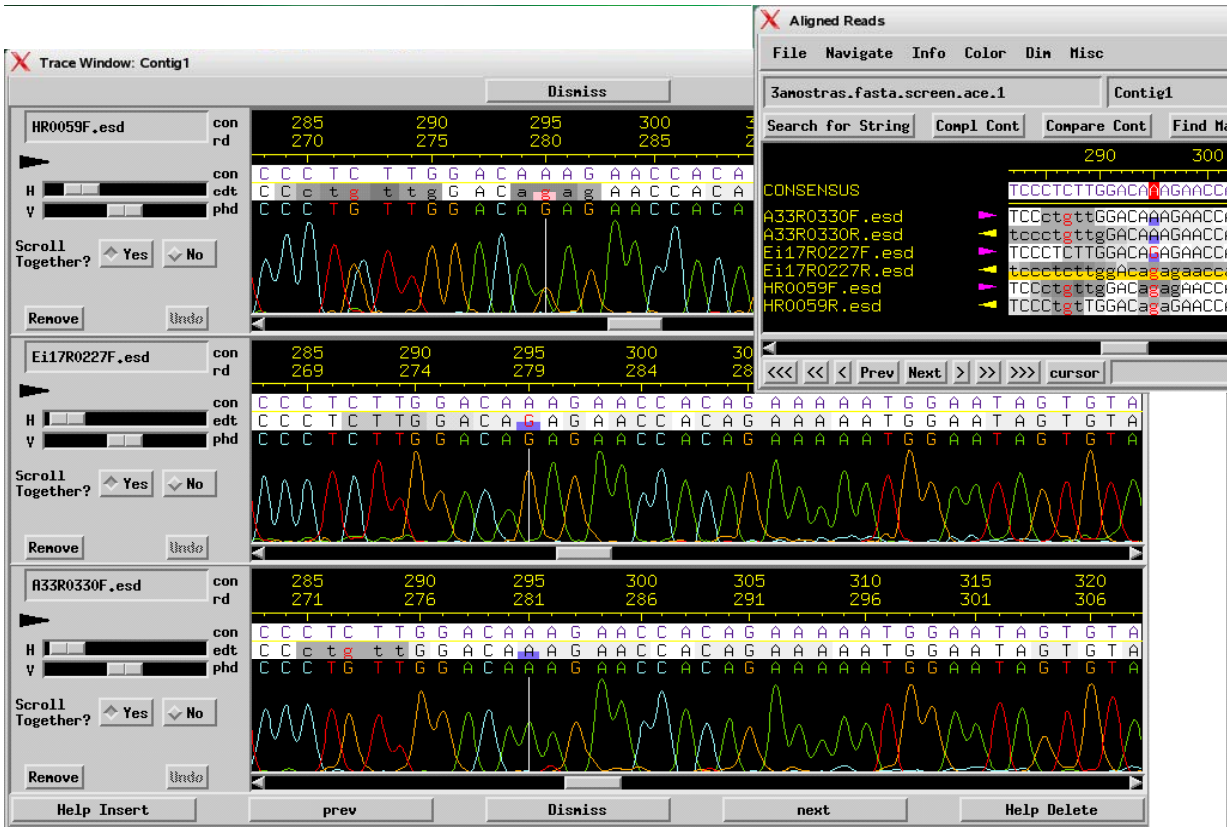

CONSENSUS
A34R0369F.esd
A34R0369R.esd
Ei7R0036F.esd
Ei7R0036R.esd
HR0061F.esd
HR0061R.esd
Undo
Help Insert
prev
Dismiss
next
Help Delete

Figure 3.

A.


Trace Window: Contig1
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Ei17R0227F.esd
A33R0330F.esd
Scroll Together?
Yes
No
Remove
Undo
Help Insert
prev
Dismiss
next
Help Delete
Aligned Reads
File Navigate Info Color Dim Misc
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Contig1
Search for String
Compl Cont
Compare Cont
CONSENSUS
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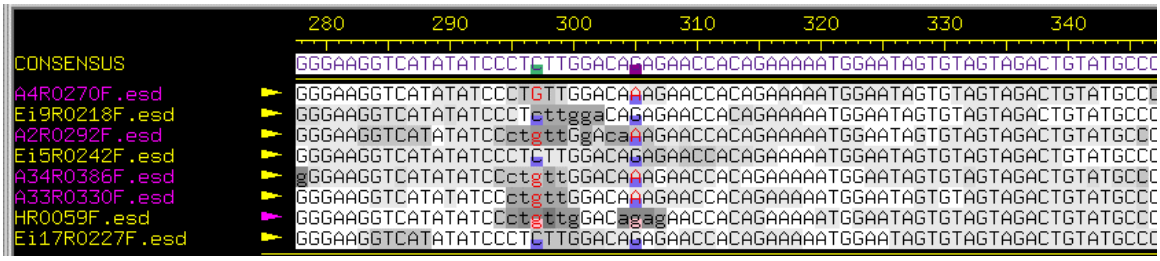
cursor

B.


CONSENSUS
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Ei9R0218F.esd
A2R0292F.esd
Ei5R0242F.esd
A34R0386F.esd
A33R0330F.esd
HR0059F.esd
Ei17R0227F.esd

Table 1. Primers designed for the amplification of targeted nuclear sequences, melting temperature (Tm) as calculated by NetPrimer software, annealing temperature (At) after cycling protocol standardization, final primer concentrations and final concentration of the enhancers used in PCR mix.

Primer	Sequence (5'- 3')	Tm (ºC)	At (ºC)	Primer concentration (µM)	Enhancers
RAG1F	AGTCCATCTCTTGCCAGGTC	56,16	61.5	0.25	BSA[1] 1X
RAG1R	CAGCAGGAACAAAGTTAGGC	55,26			
RAG2F	CTGCTATCTTCCCCCTCTCC	57,8	68	0.35	
RAG2R	GTTGTCACACTGGTAGCCCC	57,3			
R35F	CAAGTGAGTCCTTTGCTGG	53,41	53.5	0.20	
R35R	CAGCCATCTGTATCTGAAAGG	54,96			Gelatin[2]
CmosF	ATTGTGCCTACTACAGCCCC	56,78	68	0.25	0.001%
CmosR	ATATGTGCCCCCCTGCTG	58,59			
BNDFF	TCTGGAGAGCCTAAGTGGG	54,81	65.5	0.25	
BNDFR	TAAACCGCCAGCCAACTC	56,7			

[1] 100X stock solution, PROMEGA; [2] 1% stock solution; SIGMA.

Table 2. Size of the reference *E.imbricata* and *C.caretta* sequences together with the size of the PCR products obtained with the designed primers, the number of substitutions found in them and the number of samples of each parental species that were analyzed.

Marker	**RAG1**	**RAG2**	**R35**	**Cmos**	**BNDF**
Size of reference sequences (bp)	2165	1000	1073	629	724
Size of PCR product (bp)	440	607	484	567	526
Observed diagnostic sites	3	4	4	2	0
E.imbricata samples sequenced (N)	25	16	18	17	22
C.caretta samples sequenced (N)	19	13	16	16	19

Table 3. Observed substitutions in the samples sequenced during this work for the five autosomal regions studied. In all cases position 1 corresponds to the first nucleotide of the sequence amplified by the primers designed in this work.

Marker	Position	*C. caretta*	*E. imbricata*	Character
Rag1	33[4]	G	G (11)A (14)	Polymorphic in *Ei*
	85	A	A (11)T (14)	Polymorphic in *Ei*
	102	T	C	diagnostic
	175	T	C	diagnostic
	379[4]	A	G	diagnostic
Rag2	115[4]	A	G	diagnostic
	274[4]	G	C	diagnostic
	282	A	G	diagnostic
	520[4]	T	G	diagnostic
R35	46	C	G	diagnostic
	47	A	G	diagnostic
	114	G	A	diagnostic
	136[2]	C	C (16) T (2)	polymorphic in *Ei*
	389	T	C	diagnostic
	450[2]	A	A (16)G (2)	polymorphic in *Ei*
CMOS	176[2,4]	A	A (13)G (4)	polymorphic in *Ei*
	366	T	A (2)T (15)	Polymorphic in *Ei*
	522	T	C	diagnostic
	531[2]	T	C	diagnostic
BNDF	-	-	-	None

[1] When indicated, the numbers in parenthesis represent the number of samples observed having that specific nucleotide at each referred position. [2] Indicate sites not expected to be variable according to the reference sequences provided by Naro-Maciel et al. [3] Indicates a site expected to be variable according to Naro-Maciel et al. sequences but not found to be variable in the set of samples analyzed. [4] Indicate substitutions that produce an amino acid change.

IV) CONCLUSÕES FINAIS

Segundo as análises realizadas, tanto a colônia de desova quanto os agregados de alimentação encontrados no Brasil são significativamente distintos de outras colônias e agregados já estudados em outras regiões. As análises também demonstraram que estas duas populações são significativamente diferentes entre elas e, portanto, devem ser manejadas separadamente.

Foram descritos 17 haplótipos, sendo sete encontrados nas áreas de desova e 14 nas áreas de alimentação. Conseqüentemente, os valores de diversidade haplotípica e nucleotídica encontrados foram baixos na amostra que representa as áreas de desova, enquanto que os valores encontrados para as áreas de alimentação se encontram entre os mais altos já registrados para agregados de alimentação da espécie.

Dos sete haplótipos encontrados nas áreas de desova, três correspondem a seqüências descritas para outras espécies de tartarugas marinhas, verificando os relatos registrados na literatura sobre a ocorrência de híbridos no Brasil. A ocorrência do fenômeno de hibridização foi verificada tanto através da utilização de seqüências do DNA mitocondrial quanto através de marcadores nucleares (RFLPs e SNPs). Os resultados indicaram uma alta freqüência de híbridos principalmente de primeira geração, mas também indicaram a presença de animais de >F2 que são produto de cruzamentos com a espécie parental *E.imbricata*. Isto sugere um processo de introgressão unidirecional que deverá ser estudado com marcadores autossômicos adicionais.

Os polimorfismos de seqüência (SNPs) interespecíficos descritos poderão ser utilizados em conjunto com outros marcadores nucleares de tipo microssatélites para poporcionar uma visão mais detalhada do processo de introgressão e uma estimativa da idade da população híbrida encontrada no Brasil.

Todos os haplótipos descritos são “inéditos” devido ao tamanho das seqüências produzidas. A comparação destes haplótipos com seqüências já registradas na literatura indicou que dois destes podem ser utilizados como marcadores da colônia de desova brasileira já que seqüências semelhantes não foram descritas para nenhuma outra área de desova da espécie. Alternativamente, os haplótipos relacionados com a espécie *C.caretta* também podem ser usados como marcadores da população que desova no Brasil.

Nas áreas de alimentação, foram encontradas seqüências que podem ser agrupadas em quatro grupos distintos: 1) seqüências relacionadas com o haplótipo mais comum encontrado no Atlântico Oeste; 2) seqüências características de populações do Caribe; 3) haplótipos relacionados com seqüências descritas para o Mar Vermelho e Kuwait; e 4) uma seqüência possivelmente característica de áreas de desova na Austrália. Estes resultados

indicam que nas áreas de alimentação de Fernando de Noronha e do Atol das Rocas no Brasil podem ser encontrados indivíduos provenientes de localidades distantes e sugerem que a tartaruga de pente pode realizar migrações mais longas do que o esperado entre o seu local de origem e as áreas de desenvolvimento.

Devido à presença de haplótipos de outras espécies, principalmente de tartaruga cabeçuda, e às diferenças na diversidade e freqüências haplotípicas encontradas na colônia de desova e nas áreas de alimentação, estas devem ser consideradas unidades independentes e os planos de manejo para a conservação delas devem ser diferenciados.

Para estabelecer medidas eficazes para a conservação da população que desova no Brasil é de fundamental importância continuar a caracterização dos haplótipos de DNAmt em áreas de alimentação em toda a área de distribuição da espécie. Isto permitirá a identificação dos locais de alimentação destes animais em outros paises. O plano de manejo desta colônia de desova também deve levar em conta o processo de hibridização registrado e propor medidas para diminuir o impacto que este processo pode ter na manutenção da identidade da espécie no Brasil. Já o plano de manejo visando à preservação dos agregados de alimentação deve incluir parcerias internacionais que levem em conta o fato de que a exploração em águas brasileiras pode afetar populações de desova em diversos continentes.

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