Phylogeography of the West Indian manatee (*Trichechus manatus*): how many populations and how many taxa?

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Abstract

To resolve the population genetic structure and phylogeography of the West Indian manatee (*Trichechus manatus*), mitochondrial (mt) DNA control region sequences were compared among eight locations across the western Atlantic region. Fifteen haplotypes were identified among 86 individuals from Florida, Puerto Rico, the Dominican Republic, Mexico, Colombia, Venezuela, Guyana and Brazil. Despite the manatee's ability to move thousands of kilometres along continental margins, strong population separations between most locations were demonstrated with significant haplotype frequency shifts. These findings are consistent with tagging studies which indicate that stretches of open water and unsuitable coastal habitats constitute substantial barriers to gene flow and colonization. Low levels of genetic diversity within Florida and Brazilian samples might be explained by recent colonization into high latitudes or bottleneck effects. Three distinctive mtDNA lineages were observed in an intraspecific phylogeny of *T. manatus*, corresponding approximately to: (i) Florida and the West Indies; (ii) the Gulf of Mexico to the Caribbean rivers of South America; and (iii) the northeast Atlantic coast of South America. These lineages, which are not concordant with previous subspecies designations, are separated by sequence divergence estimates of $d = 0.04–0.07$, approximately the same level of divergence observed between *T. manatus* and the Amazonian manatee (*T. inunguis*, $n = 16$). Three individuals from Guyana, identified as *T. manatus*, had mtDNA haplotypes which are affiliated with the endemic Amazon form *T. inunguis*. The three primary *T. manatus* lineages and the *T. inunguis* lineage may represent relatively deep phylogeographic partitions which have been bridged recently due to changes in habitat availability (after the Wisconsin glacial period, 10 000 BP), natural colonization, and human-mediated transplantation.

Keywords: conservation genetics, marine mammal, mitochondrial DNA, molecular systematics, zoogeography

Introduction

Two species of manatee (Family Trichechidae) occur in the western Atlantic: the West Indian manatee (*Trichechus manatus*) and the Amazonian manatee (*T. inunguis*) (Husar 1978). The former species occupies coastal habitats while the natural range of the latter is restricted to the Amazon basin. However, both species require abundant aquatic vegetation and fresh water. Two subspecies have been proposed for the West Indian form: the Florida

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manatee (*T. m. latirostris*), restricted to the Florida peninsula, and the Antillean manatee (*T. m. manatus*), distributed throughout the Caribbean, Central and South America (Hatt 1934; Domning & Hayek 1986). The Florida and the Antillean subspecies are similar in terms of external morphology, and are distinguished primarily by morphometric analyses of cranial characters (Domning & Hayek 1986).

The West Indian manatee occurs from the southeastern USA to the northeastern coast of Brazil, but this distribution is patchy. *T. manatus* typically occupy grazing pastures in shallow coastal waters and adjacent freshwater ecosystems (Best 1981), and are rare or absent from areas which lack these two habitats. Seasonal migrations are documented in Florida (Reid & O'Shea 1989; Rathbun *et al.* 1990), presumably in response to intra-annual changes in climate (Raymond 1981; Reid *et al.* 1991; Reid *et al.* 1995).

In recent decades, manatee populations have been affected by habitat degradation (O'Shea *et al.* 1995), occasional cold weather (O'Shea *et al.* 1985), red tide outbreaks (Buergelt *et al.* 1984; O'Shea *et al.* 1991), hunting and incidental catch (Thornback & Jenkins 1982; Mignucci-Giannoni 1990), and collisions with boats (O'Shea *et al.* 1985). Unfortunately, a low reproductive rate limits the ability of this species to recover from population reductions (Thornback & Jenkins 1982; Marmontel 1995), and some populations have been reduced relative to historical levels (Lefebvre *et al.* 1989). The manatee is a specialized feeder and reduction of seagrass beds due to coastal development poses an especially serious threat (Reynolds 1995). For these and other related reasons, the International Union for Conservation of Nature and Natural Resources (IUCN) recognizes *T. manatus* as a vulnerable taxon (Thornback & Jenkins 1982).

The physiology, social behaviour and seasonal migratory patterns of the Florida manatee have been studied intensively over the past 30 years (O'Shea *et al.* 1995) and this information provides a substantial scientific foundation for management programs. However, the fundamental units of wildlife management, the populations, have not been adequately defined. Previous studies with protein electrophoresis indicated genetic homogeneity in manatees around the Florida peninsula; McClenaghan & O'Shea (1988) concluded that there is a strong intermixing of individuals among Florida populations, consistent with the migratory habits of this species along coastal corridors. In a more-recent study using sequences of the cytochrome *b* region of the mitochondrial genome, three Florida manatee samples had the same haplotype (Bradley *et al.* 1993). To enhance population resolution, the present study focuses on the mitochondrial DNA (mtDNA) control region. This segment of the mitochondrial genome is known to accumulate mutations at a more rapid pace than coding sequences such as cytochrome *b* (Quinn 1992; Encalada *et al.* 1996), and is expected to provide a more sensitive assay of mtDNA diversity in the West Indian manatee. The resulting gene genealogies are used to illuminate aspects of evolution and biogeography that are pertinent to sirenian systematics, ecology, and wildlife management programs.

Materials and methods

Sample distribution

A total of 86 *Trichechus manatus* individuals was sampled from eight locations (Table 1) including Florida east and west coasts (*n* = 23), Chiapas and Quintana Roo, Mexico (*n* = 6), the Dominican Republic (*n* = 6), the southeast and northeast coasts of Puerto Rico (*n* = 12), the Magdalena Basin, San Jorge River and Sinu River, Colombia (*n* = 22), the Orinoco Basin and Lake Maracaibo, Venezuela (*n* = 4 semicaptive individuals), Guyana (*n* = 7) and the northeast coast of Brazil (*n* = 6). In addition, 16 samples from *T. inunguis*, collected near Tefé (Amazonas, Brazil), were included to provide a yardstick for interpreting intraspecific partitions within *T. manatus*. Samples were collected as blood or skin biopsies from live individuals, and bone samples from carcasses (Table 1). In cases where captive individuals were sampled, care was taken to assure that multiple samples were not drawn from the same matriline.

DNA isolation

Approximately 0.5 mL of blood or 1–4 g of skin was collected for genetic analysis. Skin subsamples were minced and added to 1 mL of STE buffer (100 mM NaCl; 10 mM EDTA, pH 8.0; 50 mM Tris-HCl, pH 8.0), 12.5 μL of proteinase K (200 mg/mL) (Sigma Chemical Co.), and 0.11% [v/v] sodium dodecyl sulphate (SDS). Aliquots were incubated overnight at 50 °C, then macerated with a glass homogenizer, and genomic DNA was purified with a standard phenol–chloroform procedure (Hillis *et al.* 1996). Samples from rib, vertebra and ear (periotic) bone were extracted by drilling at slow speed. Thirty to 50 mg of bone powder was incubated in extraction buffer (0.5 M EDTA, 10% SDS and 25 μL of proteinase K (100 mg/mL)) at 50 °C overnight (Rudin *et al.* 1995). A standard phenol–chloroform extraction was performed and the final aqueous layer was further processed as follows: a small amount of siliconized glass wool was inserted into the bottom of a 1 mL syringe barrel, which was then placed in a 15 mL conical centrifuge tube. The syringe was filled with a slurry of P6DG resin (3% w/v in water, Bio-Rad) and then centrifuged for 3 min at 8000 *g*, and the liquid was removed from the bottom of the tube. Fifty μL of

Table 1 List of samples used in the study

Species	No. of individuals	Site	Tissue sample	Observations
T. manatus	14	Florida, West Coast	Blood	Wild born
T. manatus	7	Florida, East Coast	Blood	Wild born
T. manatus	2	Florida, East Coast	Blood	Captive born East Coast
T. manatus	4	Dominican Republic	Bone	Wild born
T. manatus	2	Dominican Republic, Baraona	Blood	Wild born
T. manatus	10	Puerto Rico, Ceiba	Blood	Wild born
T. manatus	1	Puerto Rico, Toa Baja	Blood	Wild born
T. manatus	1	Puerto Rico, San Juan	Blood	Wild born
T. manatus	1	Mexico, Quintana Roo	Blood	Wild born
T. manatus	5	Mexico, Chiapas	Blood	Wild born
T. manatus	1	Colombia, Rio Sinu	Blood	Wild born
T. manatus	12	Colombia, Magdalena River	Blood	Wild born
T. manatus	1	Colombia, Villa Luz Lake	Blood	Captive born
T. manatus	2	Colombia, Villa Luz Lake	Blood	Wild born
T. manatus	5	Colombia, San Jorge River	Blood	Wild born
T. manatus	1	Colombia (unknown)	Blood	Wild born
T. manatus	1	Venezuela, Maracaibo Lake	Blood	Wild born
T. manatus	1	Venezuela, Portugueza River	Skin	Wild born
T. manatus	2	Venezuela, Apure	Blood	Wild born
T. manatus	7	Guyana (unknown)	Serum	Wild born
T. manatus	6	Brazil (unknown)	Blood	Wild born
T. inunguis	3	Amazon, Amana Lake	Blood	Captive since 18 April 1984
T. inunguis	1	Amazon, (unknown)	Blood	Captive since 9 July 1974
T. inunguis	1	Amazon, Mamori	Blood	Captive since 24 July 1985
T. inunguis	1	Amazon, Ariau River	Blood	Captive since 16 May 1978
T. inunguis	1	Amazon, (unknown)	Blood	Captive since 22 September 1977
T. inunguis	1	Amazon, Anagua Lake	Blood	Captured and released
T. inunguis	2	Amazon, Saracura Lake	Blood	Captured and released
T. inunguis	2	Amazon, Acacio Lake	Blood	Captured and released
T. inunguis	1	Amazon, Arati Lake	Skin	Captured and released
T. inunguis	2	Amazon, Acacio Lake	Skin	Captured and released
T. inunguis	1	Amazon, Apara Channel	Skin	Hunting mortality

extract was added to the column and centrifuged for 3 min as above. Water (50 μL) was then added to the column which was centrifuged again. The sample was recovered from the centrifuge tube and used as a template for PCR amplifications.

PCR procedures

Approximately 0.1 μg of double-stranded DNA was used as a template for amplification in a 50 μL volume reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.01% gelatin, 4 mM $MgCl_2$, 150 μM of each dNTP, 0.3 μM of each primer, and 2.5 units of Taq DNA polymerase (Gibco BRL). The PCR conditions used were: 94 °C for 3 min, followed by 35 cycles at 94 °C for 1 min, 47 °C for 1 min, and 72 °C for 1 min; a final extension period at 72 °C for 1 h completed the reaction. The heavy-strand primer (CR-5: 5'-TCACCATCAACACCCAAAGC-3'), was designed from the tRNA-Pro consensus sequences for dolphin, cow

and mouse (Southern et al. 1988; Palumbi et al. 1991). The light-strand primer (CR-4: 5'-AGATGTCTTATTTAAGAG-GAA-3') was designed from a conserved segment of the control region (sequence C, Fig. 4, in Southern et al. 1988) based on 100% homology between cow and dolphin sequences. PCR-amplified products were removed from the reaction mixture with 30 000 M_r filters (Millipore Ultrafree-MC) following the manufacturer's protocol. DNA sequencing was accomplished in the DNA Sequencing Core at the University of Florida with the Taq DyeDeoxy terminator protocol developed by Applied Biosystems Inc. using fluorescently labelled dideoxynucleotides. Sequences were aligned and edited with SEQUENCHER version 3.0 (Gene Codes Corporation). Control region fragments were initially sequenced in the 5' to 3' heavy-strand orientation. Ambiguous sequences, and at least one representative of each haplotype, were sequenced in the opposite direction to ensure the accuracy of nucleotide sequence designations.

Data analysis

Nucleotide diversity (π: eqn 10.5 in Nei 1987) and haplotype diversity (h: eqn 8.5 in Nei 1987) within each region and overall were calculated with the computer program REAP version 4.0 (McElroy *et al.* 1992). The sequence divergence (d) within and between populations was calculated with a Kimura 2-parameter algorithm (Kimura 1980) using REAP. For populations with a sample size of $n \geq 6$, a chi-square test of haplotype frequencies among regional populations was performed using the program CHIRXC (Zaykin & Pudovkin 1993), using 250 randomizations of the original data matrix to estimate an approximate probability distribution for each test (see Roff & Bentzen 1989). The proportion of genetic diversity within and among regional samples was estimated with an analysis of molecular variance (AMOVA version 1.5, Excoffier *et al.* 1992). Estimates of gene flow (migration: Nm) based on the distribution of haplotypes and the divergence among haplotypes were generated with the F_{ST} analogue in AMOVA (Φ_{ST}). The computer program PAUP (version 3.1.1; Swofford 1993) was used to determine the number of site differences between haplotypes. Evolutionary relationships among mtDNA haplotypes were assessed with a neighbour-joining tree (Saitou & Nei 1987) using PHYLIP (version 3.5; Felsenstein 1993).

Results

A 410 bp fragment was resolved for *Trichechus manatus* and *T. inunguis* samples, containing 51 polymorphic sites, 47 transitions and six transversions (Table 2). Two positions, 328 and 332, contained both a transition and a transversion. No insertions or deletions were observed among the sequences of *T. manatus* and *T. inunguis* in this control region fragment, a finding that contrasts with control region sequence comparisons in other taxonomic groups (Lee *et al.* 1995). Sixteen haplotypes were observed within *T. manatus*, and an additional eight haplotypes were observed in the single population sample of *T. inunguis* (Table 3).

The level of mtDNA diversity varied widely between regional samples (Table 4). *T. manatus* samples from Florida ($n = 23$), Mexico ($n = 6$) and Brazil ($n = 6$) contained no polymorphism, while samples from Colombia and adjacent Guyana contained more than 20 polymorphic sites. Overall haplotype diversity within *T. manatus* was relatively high ($\pi = 0.839$). Overall nucleotide diversity was relatively high for a large vertebrate ($\pi = 0.040$), a finding which may be attributed to the deep evolutionary partition observed among clusters of haplotypes (see below). Maximum sequence divergence among pairwise

Table 2 Polymorphic sites observed in *Trichechus manatus* and *T. inunguis* haplotypes. These polymorphisms include 47 transitions and six transversions. Both transitions and transversions are found in sites 328 and 332. Haplotypes A–O are *T. manatus*, haplotypes Q–X are *T. inunguis*, and P represents possible hybrids (see Discussion). Question marks indicate unresolved nucleotides. Representative sequences (from haplotypes A, J, M and T) have been submitted to GenBank, Accession nos: AF046157, AF046158, AF046159 and A046160, respectively

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          0 0 0 0 0 0 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 2 2 2 2 3 3 3 3 3 3 3
          0 4 5 5 8 8 2 2 2 3 4 5 5 5 5 5 5 6 6 7 9 9 9 9 0 1 2 2 2 3 3 4 4 5 5 5 5 5 5 6 7 9 9 9 2 3 3 3 3 3 6
          4 8 2 5 1 7 1 4 5 9 5 0 4 5 6 7 8 5 7 3 0 2 4 7 8 6 6 6 8 9 0 4 5 6 0 1 3 4 5 6 7 7 8 1 4 6 8 0 1 2 3 8 8
Haplotype
B         T T T T A A C C A T A A T G A G T C T G C G T C G T C T T C G G C T T T G C T C A T C C T T G A T C A C
A         ? . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . C . . . . . . . . . . . . . . .
C         . . . . . . . . . . . . . . . . . . . C . . A . . . . . . . . . . . . . C . . . . . . . . . . . . . . .
D         . . . . . . . . . . . . . . . . . . . C . . A . . . . . . . . . . . . . C . . . . . . . . . A . C T . .
E         . . C C T . T T . C . . . A . A . T . . . A . . . . . C C T A A T . . C A . . . . C T . C A A . C T . T
F         C . C C T . T T . C . . . A . A . T . . . A . . . . . C C T A A T . . C A . . . . C T . C A A . C T . T
G         . . C C T . T T . C . . . A . A . T . . . A . . . . . C C T A A T . . C A . . . . C T . C A A . C T G T
H         C . C C T . T T . C . . . A . A . T . . . A . . . . . C C T A A T . . C A . . . . C T . C A A . C T G T
I         ? . C C T . T T . C . . . A . A . T . . . A . T . . . C C T A A T . . C A . . . . C T . C C A . C T . T
J         . . C C T . T T . C . . . A . A . T . . . A . . . . . C C T A A T . . C A . . . . C T . C C A . C T . T
K         . . C C T . T T . C . . . A . A . T . . . A . . . . . C C T A A T . . C . . . . . C T . C C A T C T . T
L         ? . . . T . . T . . . . . A G A C . . . . A C . A C T C . . A . . . . . . . T . . . C T T C . . . ? T . T
M         . . . . T . . T . . . . . A G A C . . . . A C . . C T C . . A . . . . . . . T . . . C T T C . . . C T . T
N         . . . . T . . T . . . . . A G A C . . . . A C . . C T C . . A . . C . . . . T . . . C T T C . . . G T . T
O         . . . . T . . T T . . . . A G A C . . . . A C . . C T C . . A . . C . . . . T . . . C T T C . . . G T . T
P         . C C C . G . T . . G . C A . . . . C A T A . . A . T C . . . . . . . . C C A . C T C C T . . . . . . . T
Q         . C C C . G . T . . G . C A . . . . C A T A . . . . T C . . . . . . C . A . C T C C T . . . . . . . T
R         . . C C . G . T . . G . C A . . . . C A T A . . . . T C . . . . . . C . A . C T C C T . . . . . . . T
S         . . C C . G . T . . G . C A . . . . C A T A . . . . T C . . . . . . C C A . C T C C T . . . . . . . T
T         . C C C . G . T . . G . C A . . . . C A T A . . . . T C . . . . . . C C A . C T C C T . . . . . . . T
U         . C C C . G . T . . G G C A . . . . C A T A . . . . T C . . . . . . C . A . C T C C T . . . . . . . T
V         . C C C . G . T . . G . C A . . . . C A T A . . . . T C . . . . . . C . A . C . C C T T . . . . . . . T
W         . C C C . G . T . . G . C A . . . . C A T A . . . . T C . . . . . . C . A . C . C C T . . . . . . . . T
X         . C C C . G . T . . G . C A . . . . C . . T A . . . . T C . . . . . . C . A . C . C C T T . . . . . . . T
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Table 3 Distribution of manatee mtDNA haplotypes. Cluster 1 represents haplotypes from Florida, the Caribbean and Colombia. Cluster 2 represents haplotypes from Mexico, Colombia and Venezuela. Cluster 3 represents haplotypes from Guyana and Brazil. Cluster 4 represents haplotypes from Brazil and Guyana. The letters A to O represent haplotypes from *T. manatus*. The letters Q to X represent haplotypes from *Trichechus inunguis*. The letter P represents possible hybrids between *T. manatus* and *T. inunguis*. Brazilian samples ($n = 22$) include the coastal *T. manatus* ($n = 6$) and the Amazonian form ($n = 16$)

Species	Cluster	Haplotype	Florida ($n = 23$)	Puerto Rico ($n = 12$)	Dominican Republic ($n = 6$)	Mexico ($n = 6$)	Colombia ($n = 22$)	Venezuela ($n = 4$)	Guyana ($n = 7$)	Brazil ($n = 22$)
T. manatus	1	A	23	5	2					
	1	B		7	4					
	1	C					4			
	1	D					1			
	2	E					7			
	2	F					2			
	2	G					5			
	2	H					2			
	2	I						1		
	2	J				6	1	2		
	2	K						1		
	3	L							1	
	3	M							1	6
	3	N							1	
	3	O							1	
	4	P							3	
T. inunguis	4	Q								1
	4	R								1
	4	S								3
	4	T								5
	4	U								1
	4	V								2
	4	W								1
	4	X								2

comparison of haplotypes ($d = 0.074$) was high relative to the level observed in congeneric control region comparisons in other marine mammals (Baker *et al.* 1994; Rosel *et al.* 1994).

Among the 16 haplotypes in *T. manatus* samples, 12 were observed in only one sample location (private or endemic haplotypes; Slatkin 1985a) and four were shared among sample sites (Table 3). Despite the sharing of haplotypes among locations, there was strong geographical structuring of mtDNA diversity (Fig. 1). Haplotype frequencies were significantly different in 20 of 21 pairwise comparisons (Table 5), and analysis of molecular variance (AMOVA) indicated that 79.8% of the total mtDNA variation in *T. manatus* was partitioned among sample locations.

Migrations (in the sense of gene flow) between locations can be expressed as Nm, the number of migrants per generation (Slatkin 1985b). In general, values of $Nm > 1$–4 indicate that gene flow is sufficient to homogenize haplotype frequencies among locations. Values below this threshold may indicate that populations will diverge

Table 4 Haplotype (h) and nucleotide diversity (π) for *Trichechus manatus* and *T. inunguis*. The Venezuelan population ($n = 4$) is not included in location-specific estimates of genetic diversity due to small sample size, but is included in overall estimates of genetic diversity. The Guyana sample includes three putative hybrids (see Discussion)

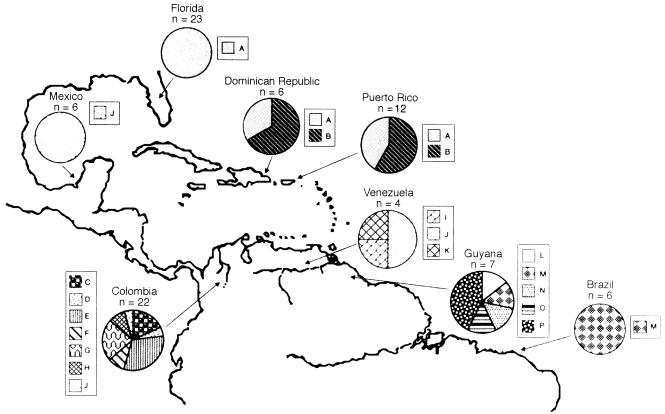| Population	Sample size (n)	Haplotype diversity (h)	Nucleotide diversity (π)
T. manatus			
Overall	86	0.839 ± 0.028	0.040
Florida	23	0.000	0.000
Puerto Rico	12	0.530 ± 0.076	0.001
Dominican Republic	6	0.533 ± 0.172	0.001
Mexico	6	0.000	0.000
Colombia	22	0.831 ± 0.047	0.027
Guyana	7	0.857 ± 0.137	0.044
Brazil	6	0.000	0.000
T. inunguis			
Brazil	16	0.875 ± 0.059	0.005

Fig. 1 Distribution of manatee samples. Pie charts indicate the frequency of haplotypes in each location.

through the process of genetic drift (but see Allendorf 1983). Estimates of manatee migration among localities based on the F_{ST} analogue in AMOVA (Φ_{ST}), using the equation $Nm = 1/2(1/F_{ST} - 1)$ from Takahata & Palumbi (1985), ranged from $Nm = 0.0$ to 3.5 (Table 5).

Phylogenetic and phenetic analyses revealed three primary mtDNA lineages within *T. manatus*, separated from each other by sequence divergence estimates of $d = 0.04–0.07$ (Table 6). Haplotypes A–D, corresponding to all samples from Florida, Puerto Rico, the Dominican

Table 5 Population parameters for *Trichechus manatus*. Above the diagonal: migration rates (Nm) between sample locations based on Φ_{ST} estimator. Below the diagonal: pairwise haplotype frequency comparisons based on χ^2 statistics. Chi-square and P-values appear in bold for one population pair which did not differentiate (NS, nonsignificant). The Venezuelan population is excluded from the analysis because of small sample size ($n = 4$)

Population	Florida	Puerto Rico	Dominican Republic	Mexico	Colombia	Guyana	Brazil
Florida	–	0.3	0.1	0.0	0.1	0.1	0.0
Puerto Rico	17.38 $P < 0.001$	–	3.5	0.0	0.2	0.2	0.0
Dominican Republic	18.46 $P = 0.002$	**0.117** **$P = 0.792$** **NS**	–	0.0	0.3	0.4	0.0
Mexico	30.00 $P < 0.001$	18.00 $P < 0.001$	12.00 $P = 0.002$	–	3.5	0.3	0.0
Colombia	46.00 $P < 0.001$	34.00 $P < 0.001$	28.00 $P < 0.001$	23.00 $P < 0.001$	–	0.6	0.3
Guyana	31.00 $P < 0.001$	19.00 $P < 0.001$	13.00 $P = 0.011$	13.00 $P = 0.001$	29.00 $P < 0.001$	–	1.1
Brazil	30.00 $P < 0.001$	18.00 $P < 0.001$	12.00 $P = 0.005$	12.00 $P = 0.004$	28.00 $P < 0.001$	9.55 $P = 0.015$	–

Republic, and five of 22 specimens from Colombia, constitute the 'Florida and West Indies Cluster' or Cluster 1 in Fig. 2. A second grouping, defined as 'the Gulf of Mexico to Caribbean rivers of South America Cluster' or Cluster 2 (haplotypes E–K), includes all samples from Mexico, 17 of 22 specimens from Colombia, and all four samples from Venezuela. The third grouping, defined as the 'northeast Atlantic coast of South America Cluster' or Cluster 3 (haplotypes L–O), includes all samples from Brazil and all but three individuals from Guyana (see below). Bootstrap support for these distinct lineages ranged from 87 to 100% in the neighbour-joining analysis. Despite the overlap of these lineages in Colombia, the results indicate strong geographical structuring among mtDNA lineages in *T. manatus*, defining three phylogeographic units which are not concordant with the accepted subspecies designation.

T. manatus and *T. inunguis* samples were distinguished by $d = 0.04$–0.08 (Table 6), values which parallel the range of divergences among the three geographical clusters of *T. manatus* ($d = 0.04$–0.08). Hence the anticipated genetic distinction of *T. inunguis* and *T. manatus* was not supported by mtDNA data. Furthermore, three individuals from Guyana, identified as *T. manatus* by field researchers, contained haplotype P which is affiliated with *T. inunguis* in phylogenetic analyses. This finding was unexpected because *T. inunguis* is not known to occur outside the Amazon Basin.

Discussion

Manatees are characterized by significant haplotype frequency shifts between most locations, low mtDNA diversity in some locations, and strong (but incomplete) geographical partitioning of three relatively deep mtDNA lineages corresponding approximately to the West Indies (and Florida), the Caribbean mainland coast, and the Atlantic coast of South America. What aspects of climate, geography, and natural history could produce these phylogeographic patterns? Whitehead (1977) suggested that the range of *Trichechus manatus* falls within the northern and southern limits of the 24 °C mean annual isotherm. The manatee's low metabolic rate (15–22% of predicted weight-specific values) and high thermal conductance have prompted suggestions that the manatees could not survive winter water temperatures in latitudes north of central Florida (Irvine 1983). Shallow coastal areas, shelter from oceanic wave action, and availability of vegetation and freshwater are also important elements of manatee habitat (Lefebvre *et al.* 1989).

Population genetic structure in T. manatus

Patches of manatee habitat are characterized by population-level separations, yet dispersal events over great

geographical scales have occurred, as indicated by sharing of haplotypes A and J among widely separated locations (Table 3). How can these apparently conflicting observations be explained? One consideration is the preference of *T. manatus* for near-shore habitat. These animals are rarely observed in deep water, where appropriate food and freshwater are unavailable and where the risk of shark predation may be high (Mou Sue *et al.* 1990). Therefore the strict coastal habitat of *T. manatus* may be a primary regulator of population partitions. A second consideration is the evidence that long-distance movements are rare, based on direct observations. Suspected strays from Florida have been recorded as far north as Rhode Island (Reid 1996), as far east as the Bahamas (Odell *et al.* 1978; Domning & Hayek 1986) and as far west as Texas (Wright 1997). Reynolds & Ferguson (1984) sighted two manatees 61 kms northeast of the Dry Tortugas, and
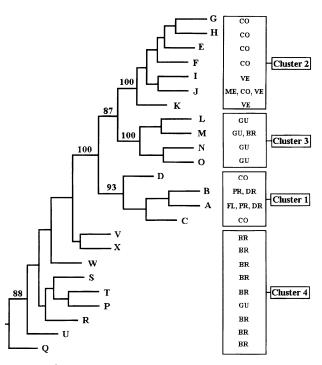

Fig. 2 Neighbour-joining tree describing the relationships among clusters of mtDNA haplotypes in *Trichechus manatus* and *T. inunguis*. Cluster 1, the Florida and West Indies (haplotypes A–D); Cluster 2, the Caribbean coast and rivers of Central America to the northern Atlantic coast of South America (haplotypes E–K); Cluster 3, the northeast Atlantic coast of South America (haplotypes L–O); and Cluster 4, Atlantic coast of South America and rivers of the Amazon Basin. Parsimony analysis (not shown) yields an identical topology with minor rearrangements of twigs within the four primary branches. Bootstrap values for critical nodes are based on 100 replicates. Sample locations are indicated in boxes to right of haplotypes (CO = Colombia; VE = Venezuela; ME = Mexico; GU = Guyana; BR = Brazil; PR = Puerto Rico; DR = Dominican Republic; FL = Florida).

Table 6 Genetic distances between haplotypes calculated with the Kimura 2-parameter algorithm

Haplotype	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
A	0.0000																							
B	0.0026	0.0000																						
C	0.0052	0.0073	0.0000																					
D	0.0130	0.0148	0.0073	0.0000																				
E	0.0726	0.0659	0.0685	0.0607	0.0000																			
F	0.0726	0.0685	0.0712	0.0633	0.0024	0.0000																		
G	0.0754	0.0685	0.0712	0.0633	0.0024	0.0049	0.0000																	
H	0.0754	0.0712	0.0738	0.0659	0.0049	0.0024	0.0024	0.0000																
I	0.0752	0.0717	0.0744	0.0662	0.0051	0.0051	0.0077	0.0077	0.0000															
J	0.0724	0.0658	0.0684	0.0605	0.0024	0.0049	0.0049	0.0074	0.0026	0.0000														
K	0.0726	0.0659	0.0685	0.0607	0.0074	0.0098	0.0098	0.0123	0.0077	0.0049	0.0000													
L	0.0586	0.0539	0.0565	0.0539	0.0539	0.0539	0.0565	0.0565	0.0579	0.0538	0.0539	0.0000												
M	0.0559	0.0502	0.0528	0.0502	0.0502	0.0528	0.0528	0.0554	0.0552	0.0501	0.0502	0.0025	0.0000											
N	0.0587	0.0529	0.0555	0.0555	0.0555	0.0580	0.0580	0.0607	0.0607	0.0554	0.0555	0.0075	0.0049	0.0000										
O	0.0616	0.0555	0.0581	0.0581	0.0581	0.0608	0.0608	0.0634	0.0635	0.0580	0.0581	0.0100	0.0074	0.0024	0.0000									
P	0.0614	0.0605	0.0528	0.0605	0.0713	0.0739	0.0739	0.0766	0.0773	0.0712	0.0712	0.0699	0.0712	0.0739	0.0767	0.0000								
Q	0.0559	0.0554	0.0476	0.0554	0.0713	0.0739	0.0739	0.0766	0.0773	0.0712	0.0766	0.0699	0.0659	0.0686	0.0714	0.0049	0.0000							
R	0.0531	0.0528	0.0451	0.0528	0.0686	0.0713	0.0713	0.0739	0.0746	0.0685	0.0739	0.0673	0.0633	0.0660	0.0687	0.0073	0.0024	0.0000						
S	0.0559	0.0554	0.0476	0.0554	0.0660	0.0686	0.0686	0.0713	0.0718	0.0659	0.0713	0.0699	0.0659	0.0686	0.0714	0.0049	0.0049	0.0024	0.0000					
T	0.0586	0.0580	0.0502	0.0580	0.0686	0.0713	0.0713	0.0739	0.0746	0.0685	0.0739	0.0726	0.0685	0.0713	0.0740	0.0024	0.0024	0.0049	0.0024	0.0000				
U	0.0586	0.0580	0.0502	0.0580	0.0739	0.0766	0.0766	0.0792	0.0801	0.0738	0.0792	0.0726	0.0685	0.0713	0.0740	0.0073	0.0024	0.0049	0.0049	0.0024	0.0000			
V	0.0559	0.0554	0.0476	0.0554	0.0713	0.0739	0.0739	0.0766	0.0773	0.0712	0.0766	0.0646	0.0607	0.0634	0.0661	0.0098	0.0073	0.0073	0.0073	0.0098	0.0073	0.0000		
W	0.0531	0.0528	0.0451	0.0528	0.0686	0.0713	0.0713	0.0739	0.0746	0.0685	0.0739	0.0673	0.0633	0.0660	0.0687	0.0073	0.0049	0.0049	0.0073	0.0073	0.0049	0.0024	0.0000	
X	0.0531	0.0528	0.0451	0.0528	0.0686	0.0713	0.0713	0.0739	0.0746	0.0685	0.0739	0.0619	0.0580	0.0608	0.0635	0.0123	0.0098	0.0098	0.0123	0.0098	0.0098	0.0123	0.0049	0.0000

suggested that they could be wanderers from Florida, Cuba, or the Yucatan Peninsula. A third consideration is the temporal scale and frequency of long-distance colonization. Population genetic theory and empirical studies both demonstrate that even rare migration events can be sufficient to homogenize populations (Slatkin 1985b). Hence the population structure of *T. manatus* probably reflects the balance between habitat requirements which limit dispersal, and the swimming ability of manatees which permits rare colonization events.

Does this confluence of genetic and distribution data allow predictions about population structure in unsurveyed manatee aggregates? While the exact relationships among populations may be difficult to predict, the mtDNA data support a model in which manatee aggregates separated by large tracts (hundreds to thousands of km) of inappropriate habitat are probably distinct demographic units. This conclusion must be considered to be provisional, however, because mtDNA surveys include only the maternal lineages. Evidence of sex-specific dispersal has been reported (Bengtson 1982) and nuclear DNA assays will be necessary to evaluate this aspect of manatee population structure.

Low mtDNA diversity as an indicator of population history

Samples from east and west Florida ($n = 23$) contained only a single haplotype, so we were unable to determine whether population structure exists across the Florida peninsula. However, the relatively continuous distribution of manatee habitat in southern Florida, and the strong swimming capacity of these animals (Reid 1996; Wright 1997), diminish the possibility of strong reproductive isolation on this geographical scale (McClenaghan & O'Shea 1988). What could explain the low level of mtDNA diversity in Florida manatees? Two historical explanations to account for the low mtDNA diversity in Florida manatees merit consideration. First, perhaps the Florida population passed through a bottleneck (severe reduction in population size) in recent evolutionary time. Any number of biological or physical stresses could induce this phenomenon, such as extreme winter seasons (O'Shea et al. 1985; Ackerman 1995) and epizootics (Buergelt et al. 1984; O'Shea et al. 1991; Bossart et al. 1998). For example, in 1996 about 5–10% of the Florida manatees perished due to brevetoxin exposure caused by a severe red tide (Bossart et al. 1998). Alternatively, the lack of mtDNA diversity may be the result of a recent colonization event from the West Indies. Geochemical evidence indicates that the tropical Atlantic was 5 °C cooler during the most recent (Wisconsin) glacial period, ≈ 18 000–12 000 years BP (Guilderson et al. 1994), and palaeontological records show that the temperate fauna of the mid-Atlantic bight

(Massachusetts to North Carolina, USA) extended south of the Florida peninsula during this interval (Hedgpeth 1954). Hence, it is unlikely that a manatee population persisted in Florida through the Wisconsin glacial period. Florida was probably colonized (or recolonized) during the last 12 000 years, an interval too short to allow accumulation of equilibrium levels of diversity in the mitochondrial genome. The phylogenetic affiliation of Florida samples with the West Indies cluster, and the fixation of one haplotype which also occurs in the Dominican Republic and Puerto Rican populations, indicate this geographically proximate source of colonizers.

No mtDNA diversity was observed for the Brazilian samples of *T. manatus* ($n = 6$), contrasting with the other populations surveyed in South America (Guyana with five haplotypes, Venezuela with three, and Colombia with seven). Similar to the Florida example discussed above, the Brazilian population occupies a high-latitude extreme of the range, and is known to have a relatively small population size (Domning 1982a; Lefebvre et al. 1989). These factors could certainly influence the level of observed genetic diversity. However, the alternative explanation, recovery from a recent bottleneck effect, bears consideration. Manatee populations in Brazil (both *T. manatus* and *T. inunguis*) have been commercially exploited for 300 years (Domning 1982a). While historical records are scarce, current populations are almost certainly a fraction of pre-exploitation levels. Hence three centuries of exploitation might also contribute to the low haplotype diversity observed in Brazilian samples of *T. manatus*.

Phylogeography of T. manatus

The mtDNA phylogeny for *T. manatus* includes three primary lineages corresponding to Florida and the West Indies (Cluster 1), the Gulf of Mexico and Caribbean rivers of South America (Cluster 2), and the NE Atlantic coast of South America (Cluster 3; see Fig. 2). These groupings are not completely segregated, as haplotypes from Clusters 1 and 2 co-occur in Colombia (Table 3).

The sequence divergence between the three primary *T. manatus* lineages is comparable to the level of control region divergence between genera of whales (Baker et al. 1993), raising the possibility that these partitions reflect deep evolutionary separations (see Moritz 1994). How old are these three lineages? While a control region clock for manatees is unavailable, estimated rates of change reported for terrestrial mammals range from ≈ 8 to 15%/Myr between lineages (Vigilant et al. 1991; Stewart & Baker 1994). Estimated rates for baleen whales, based on fossil calibrations, are ≈ 1%/Myr (Hoelzel et al. 1991; Baker et al. 1993) and an estimate rate for dugong (*Dugong dugon*, a Pacific sireniar) is 2%/Myr based on fossil evidence (Tikel 1997). Using the provisional range of values for terrestrial

mammals, the three lineages in *T. manatus* diverged in the order of 0.5–1 Myr BP. The time frame based on the marine mammals (1–2%/Myr) is ≈ 3.5–7 Myr BP. This slower molecular clock (and correspondingly greater time estimate) may be more appropriate for manatees, as: (i) long generation time and low metabolic rate are correlated with slower molecular clocks in other vertebrates (Avise *et al.* 1992; Martin & Palumbi 1993); and (ii) this range of estimates is calibrated for another member of the Sirenia. Notably, the range of estimates based on this clock bracket the period inferred from the fossil record for the origin of *T. manatus* (within the last 4 Myr; Domning 1982b).

What could account for the distribution of the three primary branches in the mtDNA phylogeny? The rapid lineage sorting of maternally inherited mtDNA diminishes the possibility that all three lineages coexisted in the same populations through the last few million years. A more probable scenario involves isolation for an extended period followed by recent range extensions and admixture. Because manatees do not readily cross wide stretches of open water, the isolation of a Florida–West Indies cohort (Cluster 1) can be explained in terms of habitat distribution and the isolation of the Greater Antilles from continental coastlines. The separation of Caribbean–Continental (Cluster 2) and South American (Cluster 3) cohorts (with a putative break in the vicinity of Venezuela and Guyana) is consistent with the boundaries of marine biogeographic provinces. In particular, Briggs (1974) considers the coastal habitats of Venezuela to be the southernmost outpost of the Caribbean province. Hence the phylogeographic break between Caribbean–Continental and South American mtDNA lineages is concordant with a discontinuity in the distribution of other marine faunas.

The spread of the West Indies mtDNA lineage into Florida is probably a range expansion after the Wisconsin glaciation. The extension of the West Indies lineage into South America probably occurred in recent evolutionary time, based on the similarity of mtDNA haplotypes A and B in the West Indies vs. C and D in Colombia. While a specific route of colonization cannot be inferred from the molecular data, it is notable that archaeological and early historic evidence indicates that manatees occurred in the Lesser Antilles during the age of European exploration (Lefebvre *et al.* 1989). Thus, the link between haplotypes observed in the West Indies (Puerto Rico and the Dominican Republic) and the northern rim of South America (Colombia) may have been established by now-extinct populations that inhabited the Lesser Antilles.

Manatee evolution and systematics

Fossil evidence indicates that the ancestor of extant manatees arose during the Miocene, possibly in coastal rivers and lagoons of South America, and never expanded beyond the Atlantic Ocean and adjacent tributaries (Domning 1982b). The Amazonian *T. inunguis* may have arisen in isolation after the Miocene origin of the Andes (Domning 1982b). This species is distinguished from *T. manatus* by a range of morphological characters including lack of nails on the pectoral flippers, reduced number of dorsal vertebrae, smaller and more complex molars, and thickened supraoccipital (Domning & Hayek 1986). In addition, *T. inunguis* has a higher diploid chromosome number (56 compared to 48 in *T. manatus*; Loughman *et al.* 1970; White *et al.* 1976). *T. inunguis* is distributed along the Amazon River and adjacent coastal estuaries, and it is not known what factors maintain the apparent parapatry of *T. inunguis* and *T. manatus* at the mouth of the Amazon River (Domning 1981).

The mtDNA phylogeny of Western Atlantic manatee species does not readily align with the current taxonomy of *Trichechus* spp. The control region phylogeny contains four primary branches, including three in *T. manatus* and one in *T. inunguis*. While this does not necessarily contradict the species status of the Amazonian manatee, it does indicate that the two putative western Atlantic species shared a maternal ancestor more recently than was previously suspected. Molecular clocks must be interpreted with caution, but the range of dates estimated from mtDNA data (2–4 Myr based on the dugong calibration of 2%/Myr) are consistent with a Pliocene (rather than a Miocene) origin for the Amazonian form.

Subspecies designations based on cranial characters in *T. manatus* also do not coincide with the intraspecific partitions based on mtDNA sequences. Domning & Hayek (1986) used the name *T. m. latirostris* to distinguish the Florida morphotype from *T. m. manatus* populations through the rest of the range. In contrast, the mtDNA data link the Florida manatee with those from the West Indies. The molecular data do not strictly contradict the accepted taxonomic assignments, but bolster the hypothesis that the Florida form is a very recent derivative of West Indies populations. As in the comparison of *T. manatus* and *T. inunguis*, the mtDNA data are useful for placing an approximate evolutionary time frame on the documented morphological differentiation.

One surprising result is the phylogenetic affiliation of a haplotype of *T. manatus* from the Guyana population with the endemic Amazonian form, *T. inunguis*. These findings invoke a range of interesting possibilities, including an enormous range expansion of *T. inunguis*, retention of an ancestral haplotype since the *manatus–inunguis* split, a re-alignment of manatee taxonomy, or a hybrid zone in northern South America. Nuclear DNA sequence and chromosome analyses will doubtless prove useful in sorting out these possibilities, but a simpler explanation must be considered here. All three individuals with the putative *T. inunguis* haplotype came from a system of

freshwater canals located within the Guyana Zoological Gardens. While zoo records are incomplete, manatees were stocked into this area 30 years ago from adjacent regions to enhance the zoo collection and to control aquatic vegetation (Allsopp 1969). It is possible that a few *T. inunguis* individuals were acquired and released with *T. manatus*. However, the contemporary individuals used for genetic analysis were identified during the preparation of this report as *T. manatus* based on morphometric characters (P. MacWilliams, personal communication). This finding raises the possibility of human-induced hybridization between *T. manatus* and *T. inunguis* in Guyana. Notably, *T. inunguis* has also been introduced into manatee habitats in Panama (Mou Sue *et al.* 1990), invoking the possibility of a second hybrid zone.

Overall, the mtDNA data prompt a recognition that taxonomic designations for *Trichechus* may not reflect evolutionary partitions. Although the information from a single line of evidence (such as mtDNA) is not sufficient to overturn robust taxonomic classifications based on a suite of morphological characters, it is certainly sufficient to identify points for further inquiry. Research efforts in progress (L. Parr, S. Fain, and D. Duffield, personal communication) should clarify the higher level systematics of manatees. Nuclear DNA assessment and morphometric analyses, as well as additional sampling, are also desirable to resolve remaining evolutionary and systematic issues.

Conservation implications

Intensive research in Brazil, Mexico, the USA, Puerto Rico and elsewhere has provided a scientific foundation for efforts to preserve remaining manatee populations (O'Shea *et al.* 1995). The data presented here enhance this conservation foundation in at least three ways. First, mtDNA haplotype frequency comparisons provide the first robust assessment of the interaction between habitat restrictions (which presumably limit movement) and dispersal events in defining population genetic units. Long-distance migration does occur, but is evidently not sufficient to homogenize populations separated by large tracts of inappropriate habitat. The results from surveyed manatee populations directly confirm the demographic independence of isolated populations, and, perhaps more importantly, provide an approximate yardstick to define management units throughout the range of *T. manatus*.

A second conservation consideration is that previously unrecognized evolutionary partitions may exist within *T. manatus*. The mtDNA data alone are not sufficient to resolve this question, but provide strong motivation for additional investigations. If the separations defined in a mtDNA phylogeny are supported by other lines of evidence, then conservation priorities may be realigned to include three or more taxonomic and evolutionary units within *T. manatus*.

Finally, a relatively thorough catalogue of genetic diversity in manatees can provide a foundation for forensic investigation. Based on the highly structured nature of manatee populations, it is feasible to identify suspected manatee products to species and regions of origin. This would enhance the arsenal for enforcement of wildlife protection regulations, illustrating the value of range-wide genetic inventories of endangered species.

Acknowledgements

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