

Population genetics of the endangered South American freshwater turtle, *Podocnemis unifilis*, inferred from microsatellite DNA data

Tibisay Escalona · Tag N. Engstrom ·
Omar E. Hernandez · Brian C. Bock ·
Richard C. Vogt · Nicole Valenzuela

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Abstract We studied the population genetics of *Podocnemis unifilis* turtles within and among basins in the Orinoco and Amazon drainages using microsatellites. We detected high levels of genetic diversity in all sampled localities. However, ‘*M*-ratio’ tests revealed a substantial recent population decline in ten localities, in accord with current widespread exploitation. Our results reveal a consistent pattern across multiple analyses, showing a clear subdivision between the populations inhabiting the Amazon and Orinoco drainages despite a direct connection via the Casiquiare corridor, and suggesting the existence of two biogeographically independent and widely divergent lineages. Genetic differentiation followed an isolation-by-distance model concordant with hypotheses about

migration. It appears that migration occurs via the flooded forest in some drainages, and via river channels in those where geographic barriers preclude dispersal between basins or even among nearby tributaries of the same basin. These observations caution against making generalizations based on geographically restricted data, and indicate that geographically proximate populations may be demographically separate units requiring independent management.

Keywords Population structure · Biogeography · Gene flow · Landscape genetics · Conservation · Evolution

Introduction

Anthropogenic harvesting, and particularly commercial harvesting, has contributed to the population decline and even extinction of many species (Mills 2006). By changing the distribution and abundance of populations, extreme harvesting can affect dynamic processes such as natural selection, gene flow and genetic drift. Therefore, harvesting could modify the structure of diversity within and between populations of these exploited species (Vrijenhoek 1998) and consequently, their genetic diversity and ultimate fitness (Stockwell et al. 2003). Indeed, because genetic diversity may ensure the success of species in variable environments, the loss of alleles is of particular concern in declining taxa as genetically depauperate species may have a reduced ability to adapt to new evolutionary challenges (Write 1978).

Many turtle species are threatened by harvesting and habitat degradation by humans (IUCN 1994; Seminoff and Shanker 2008). For such species, molecular studies are an important tool, not only to improve our understanding of the role of biogeographic processes and population level

T. Escalona (✉) · N. Valenzuela
Department of Ecology, Evolution and Organismal Biology,
Iowa State University, 253 Bessey Hall, Ames, IA 50011-1020,
USA
e-mail: escalona@iastate.edu

T. N. Engstrom
Department of Biological Sciences, California State University,
Chico, CA 95929-0515, USA

O. E. Hernandez
FUDECI, Fundación para el Desarrollo de las Ciencias
Físicas, Matemáticas y Naturales. Av. Universidad, Bolsa a
San Francisco, Palacio de Las Academias, Edf. Anexo,
Piso 2, Caracas, Venezuela

B. C. Bock
Instituto de Biología, Universidad de Antioquia,
Calle 67 No 53-108, Medellín, Colombia

R. C. Vogt
Instituto Nacional de Pesquisas da Amazônia (INPA),
Coordenação de Pesquisas em Biologia Aquática (CPBA),
CP 478, Manaus, AM 69083-000, Brazil

phenomena in shaping genetic structure and demography in these taxa, but also for assessing the effects of anthropogenic harvesting on genetic diversity in order to help the implementation of effective management programs. Here we investigate a widely distributed freshwater turtle, *Podocnemis unifilis*, inhabiting the Orinoco, Amazon, and Essequibo river basins and eastern Guianas (Pritchard and Trebbau 1984; Fig. 1). This turtle represents an important economic and cultural resource for local people in Colombia, Bolivia, Brazil, Ecuador, Peru, Venezuela, and the Guianas (Mittermeier 1978). Unfortunately, anthropogenic overexploitation (meat and eggs) and habitat destruction has caused drastic population declines (e.g. Soini 1996; Thorbjarnarson et al. 1997; Escalona and Fa 1998). Today, *P. unifilis* is classified as Vulnerable by the IUCN Red Data Book (IUCN 1994), as endangered by the US Endangered Species Act (US Fish and Wildlife Service http://ecos.fws.gov/tess_public/SpeciesReport.do), and it is included in Appendix II of CITES (<http://www.cites.org/eng/app/appendices.shtml>). Thus, concern exists about whether this human-induced population decline may cause loss of genetic variation and fixation of deleterious alleles, with consequent inbreeding depression and reduced adaptive potential (Cornuet and Luikart 1996). Unfortunately, despite *P. unifilis*' economic and cultural value, little is known about its population genetic structure (but see Bock et al. 2001; Engstrom 2003), unlike its congener, *P. expansa* (e.g. Sites et al. 1999; Valenzuela 2001; Pearse et al. 2006).

Additionally, because *P. unifilis* and *P. expansa* are widely distributed and largely sympatric, a comparative study of their population structure will also contribute to

answer long standing questions about evolutionary biogeography of the South American biota, such as the role of geography in species formation and distribution (e.g. Patton et al. 1994; Rosales et al. 1999; Hall and Harvey 2002). For instance, river drainages represent major conduits for migration and gene flow in aquatic taxa thus contributing to determine patterns of population distribution, genetic structure and speciation (Lovejoy and De Araujo 2000; Sivasundar et al. 2001; Turner et al. 2004; Willis et al. 2007). In South America, the Orinoco and Amazon river basins are geologically complex and have experienced drastic hydrological changes over evolutionary time (Weitzman and Weitzman 1982; Lundberg et al. 1998). This resulted in well documented biological diversification and population geographic structure of Neotropical fish (Winemiller et al. 2008) but to what extent the same has occurred for other aquatic organisms such as turtles, remains understudied. Importantly, migration and gene flow between the Amazon and Orinoco basins may occur via the geologically recent Casiquiare corridor, considered hydrologically to be a stream capture in progress which links the upper Orinoco and the Rio Negro in the Amazon basin (Sternberg 1975; Turner et al. 2004; Willis et al. 2007; Winemiller et al. 2008). Significant migration may also occur within and between the Amazon and Orinoco basins through the contiguous flooded habitat that is available permanently or seasonally in the region, as exemplified by the Amazonian *várzea* and *igapó* ecosystems (Junk 1993; Vasconcelos et al. 2006; Hrbek et al. 2007).

In this preliminary study we use microsatellites to examine *P. unifilis* population genetic structure as a first step to obtain genetic information that can be used to develop successful conservation strategies, including knowledge about the extent of population bottlenecks, the conduits for gene flow among populations, and the effect that both may have on the genetic diversity of this South American turtle. We also examined the biogeography of the Orinoco and Amazon river systems in order to understand the relationship between geography and genetic patterns. By combining this information we provide a more robust explanation of the genetic patterns detected in this species.

Materials and methods

Sample and data collection

Blood, skin or muscular tissue samples were taken from natural populations of *P. unifilis* from 11 localities, including six from Venezuela (CAU, NICH, MAT, RFTA, MET, CQ), one from Colombia (CUT), and three from Peru (MC, PI, SC) corresponding roughly to a North–South transect, plus one farther eastern locality in Brazil (TRO)



Fig. 1 Distributional range of *Podocnemis unifilis* in South America (shaded area), and sampling localities used in this study (dots) and labeled as in Table 1. Relevant rivers are noted

(Fig. 1; Table 1). CUT and MC samples came from a previous allozyme study by Bock et al. (2001), while MC, PI, and SC samples came from a mtDNA study by Engstrom (2003), and were collected under permits described therein (i.e., one hatchling per nest). Non-terminal sampling of skin or blood from adults was used in Venezuela and Brazil. Adults were marked before release to avoid duplicate sampling. Samples were preserved in 95% ethanol, stored at 4°C or room temperature prior to arrival at the laboratory and at -80°C thereafter. Transportation of samples was conducted under appropriate CITES import and export permits.

Most sample sizes were 28–30 individuals per locality (range: 14–55, total: 312; Table 1). Samples were collected between 1994 and 2007, and inter-annual variation among samples is expected to be low because of *P. unifilis* longevity, high annual survival and iteroparity, similarly to *P. expansa* (Pearse et al. 2006). Genomic DNA was extracted using Puregene DNA isolation kit (Gentra Systems) and stored at

-20°C. DNA concentration was measured with a NanoDrop Spectrophotometer (ND-1000 v3.2.1) and standardized to 10 ng/μl per sample.

The polymerase chain reaction (PCR) was used for individual amplification of nine dinucleotide-repeat microsatellites originally designed for *P. expansa*: Sat1, Sat62, Sat79, Sat91, Sat128 and Sat147 (Sites et al. 1999), PE344, PE519 and PE1075 (Valenzuela 2000) using fluorescently labeled primers (HEX or FAM). In general, 1 μl of re-suspended DNA was amplified in a 12.5 μl reaction containing 0.2 μM of each primer, 1× Premix and 1× Enzyme Poly Mix of the Failsafe™ PCR system from Epicentre®. Amplification profiles consisted of 94°C for 1 min, followed by 30 cycles of 94°C for 30 s, 55–57°C for 30 s, 72°C for 45 s, and a final elongation at 72°C for 2 min. Alleles were genotyped in an ABI 3100 automated sequencer at the DNA Sequencing Facility of Iowa State University, and scored manually using GENOTYPER (Applied Biosystems).

Table 1 Sample localities of *Podocnemis unifilis* and summary genetic parameters inferred from microsatellite data using FSTAT as described in the text

Country	Basin	Locality	N	Allelic diversity		Genetic diversity		PA	F _{is}	Cluster ID(%)
				A _{ob}	A _R	H _o	H _E			
Venezuela	Orinoco-Caura	CAU: Lower Caura River, middle Orinoco River tributary	20	6.60	5.78	0.63	0.59	0	-0.066	1 (70)
Venezuela	Orinoco-Caura	NICH: Nichare River, upper Caura tributary	30	9.40	6.79	0.73	0.69	3 (2)	-0.060	1 (80)
Venezuela	Orinoco-Caura	MAT: Mato River, lower Caura tributary	55	11.40	7.49	0.63	0.68	3 (2)	0.067	2 (43) 1 (39)
Venezuela	Orinoco	RFTA: Arrau turtle refuge, middle Orinoco River	30	10.60	8.07	0.76	0.75	0	-0.012	2 (57) 1 (22)
Venezuela	Orinoco	MET: Meta River, middle Orinoco tributary	14	5.80	5.65	0.63	0.67	0	0.066	2 (72)
Venezuela	Orinoco	CQ: Casiquiare River, upper Orinoco tributary	29	5.40	4.43	0.53	0.54	1 (1)	0.004	3 (81)
Colombia	Amazon	CUT: Caquetá River, Tres Islas, upper Amazon tributary	29	9.60	7.55	0.74	0.74	13 (3)	-0.010	4 (93)
Peru	Amazon-Pacaya Samiria	MC: Manco Capac, upper Amazon	28	10.60	8.41	0.74	0.79	3 (2)	0.066	5 (91)
Peru	Amazon-Pacaya Samiria	PI: Pithecia-Samiria River, upper Amazon tributary	29	11.20	8.71	0.75	0.78	4 (2)	0.043	5 (85)
Peru	Amazon-Pacaya Samiria	SC: Santa Cruz-Pacaya River, upper Amazon tributary	30	12.00	9.01	0.78	0.77	4 (4)	-0.018	5 (88)
Brasil	Amazon	TRO: Trombetas River, lower Amazon tributary	18	9.40	7.90	0.64	0.68	18 (2)	0.051	6 (80)
Total			312							
Mean				9.272	7.255	0.69	0.70			

Locality abbreviations as in Fig. 1

N sample size; A_{ob} mean observed number of alleles per locus; A_R allelic richness; H_o observed heterozygosity; H_E unbiased expected heterozygosity; PA number of private alleles per locality (number of loci); F_{is} Weir and Cockerham's (1984) inbreeding coefficient. Significance levels were calculated after Bonferroni correction (α = 0.0045) as described in the text. Cluster ID membership of a locality to a population cluster (% = mean proportion of membership of individuals from a locality to a given cluster) as described in the text

Data analyses

Genetic diversity within localities

The number of alleles per population and locus, observed heterozygosity (H_O), Nei's (1987) unbiased expected heterozygosity (H_E) under Hardy–Weinberg equilibrium, and private alleles (PA) were calculated using GenAlEx 6.1 (Peakall and Smouse 2005). Allelic richness (A_R) was calculated as described by El Mousadik and Petit (1996) using FSTAT version 2.9.3.2 (Goudet 2001).

Pairwise linkage disequilibrium (LD) among loci was tested using GENEPOP version 4 (Rousset 2008), and significance levels were evaluated using a Markov-chain randomization procedure (Guo and Thompson 1992) with 10,000 dememorization steps, 500 batches, and 5,000 iterations per batch. Deviations from Hardy–Weinberg equilibrium (HWE) were tested using F_{is} (Weir and Cockerham 1984) within and across loci for each locality and globally, with significance levels calculated with a randomization procedure as implemented in FSTAT. Additionally, we used MICRO-CHECKER 2.2.3 (van Oosterhout et al. 2004) to assess the presence of null alleles (NA) that could also cause departures from Hardy–Weinberg expectations (HWE). In cases when null alleles were likely, we estimated an unbiased F_{st} (Weir 1996) after correcting for null alleles following the ENA method and using a NA-corrected genotype dataset obtained with the INA method as implemented in FreeNa (Chapuis and Estoup 2006). All estimates of population differentiation were carried out using both the original and the NA-corrected datasets. Significance levels for all multiple comparisons in this and all other tests described below were Bonferroni corrected (Rice 1989).

Genetic differentiation among localities

Genetic divergence among populations within and across loci was assessed using θ_{st} (Weir and Cockerham 1984) unbiased estimator of Wright's F_{st} , ρ_{st} (Rousset 1996) estimator of R_{st} (Slatkin 1995), and G_{st} multiallele estimator of F_{st} (Nei 1973) corrected for heterozygosity as implemented in FSTAT (Goudet 2001). While R_{st} incorporates microsatellite-specific modes of mutation (Slatkin 1995) it suffers from lower statistical power partly due to the relative higher variance of its estimator (Valenzuela 2001). On the other hand, although F_{st} 's assumptions (e.g. infinite-allele or k-allele mutation model) may be violated for microsatellite loci, F_{st} outperforms R_{st} when sample sizes and the number of loci are moderate (Gaggiotti et al. 1999). Finally, G_{st} is a multiallele F_{st} estimator equivalent to a weighted average of F_{st} across loci (Takahata and Nei 1984). Using ARLEQUIN version 3.11 (Excoffier et al. 2005) we statistically compared pairwise F_{st} among

localities both from the original data and from the NA-corrected dataset. We calculated the number of migrants per generation using GENETIX (Belkiri et al. 2000) by the indirect estimator of gene flow given by $N_m = (1 - F_{st})/4F_{st}$ (Wright 1969). The relationship between genetic distance [$F_{st}/(1 - F_{st})$] and geographic distance (km), measured as the shortest river channel path between populations using ArcView version 9.2 (ESRI), was calculated to test for isolation-by-distance using PASSaGE 2 (Rosenberg 2008), and the significance was tested through normalization by the Mantel statistic Z with 10,000 permutations of the data (Mantel and Valand 1970).

Genetic relationship among localities

We applied a principal coordinate analysis (PCoA) to the matrix of Nei's genetic distance among the 11 localities to visualize their genetic relationships using GenAlex. Cavalli-Sforza and Edwards (1967) genetic distances (CSE) were calculated using PHYLIP (Felsenstein 2004) for both the original and NA-corrected datasets. A consensus unrooted neighbor-joining (NJ) tree (Saitou and Nei 1987) was constructed from these genetic distances using CONSENSE (Margush and McMorris 1981), with 1,000 bootstrap iterations computed using Seqboot (Felsenstein 1985), as implemented in PHYLIP, and then visualized using Tree-View version 1.6.0 (Page 1996). Because the assumption of F_{st} estimators that populations are in mutation-drift equilibrium may be violated in some populations that have suffered drastic declines, we further tested the population structure via a Bayesian clustering method implemented in STRUCTURE version 2.2 (Pritchard et al. 2000; Falush et al. 2003, 2007) run for $k = 1-8$ clusters. The posterior probability of the data [$\ln \Pr(x/k)$] for each value of k was inferred from multilocus genotypes 20 times with 500,000 repetitions each and a burn-in of 100,000, using the admixture ancestry model and the assumption of correlated allele frequencies among samples (Falush et al. 2003). Statistical significance of adding each new cluster to the model was assessed using a likelihood ratio test (Goldman 1993). An assignment test of populations to clusters based upon proportional membership (q) was conducted using STRUCTURE. We also calculated the number of clusters following Evanno et al. (2005). An individual-based assignment test was used to calculate the probability that an individual's multi-locus genotype derives from alternative populations (Paetkau et al. 1995), using the Bayesian method implemented in GENECLASS 2 (Piry et al. 2004).

Population reduction tests

Evidence of recent population bottlenecks was assessed with BOTTLENECK (Piry et al. 1999) using the two-phase

model (TPM) (Di Rienzo et al. 1994) assuming 90–95% of the microsatellite mutations followed the strict SMM and 5–10% produced multiple steps changes, and setting the variance among multiple steps to 12, following Piry et al. (1999). To determine whether deviations of observed heterozygosity relative to that expected at drift-mutation equilibrium were significant we used a two-tailed Wilcoxon signed-rank test ($\alpha = 0.05$) (Luikart and Cornuet 1998), and also tested for a mode-shift away from an L-shaped distribution of allelic frequencies (Luikart et al. 1998). Additionally, we used the ‘M-ratio’ test to detect reductions in effective population size, and calculated M values across loci using the software *M_P_Val.exe* and *Critical_M.exe* available at <http://swfsc.noaa.gov/textblock.aspx?Division=Fed&id=3298> to simulate expected values for the ‘M-ratio’ under demographic and mutational parameters ($4N_e\mu = 4$, non-stepwise mutations = 0.1, average size of non-stepwise mutations = 3.5 steps), following Garza and Williamson (2001). We then compared the observed M values to the critical value (M_c) in order to detect recent population bottlenecks (Garza and Williamson 2001).

Results

Genetic diversity within localities

Nine microsatellite loci originally designed for *P. expansa* were subjected to PCR, of which six cross-amplified successfully in *P. unifilis*, and five exhibited appropriate levels of polymorphism and were used to estimate population structure. Sequencing confirmed the presence of the microsatellite repeats in *P. unifilis* at these five loci. Number of alleles per locus averaged 24.5 (range = 11–37; Table 2). Out of 120 total alleles, 31 were private alleles found at very low frequency (mostly < 0.03 ; average = 0.046, data not shown). Private alleles were highest in TRO and CUT (Table 1) with high frequencies at Sat62 (0.361 and 0.194)

and at Sat128 (0.103), respectively, (data not shown). No significant linkage disequilibrium between any pair of loci was detected at any locality nor across localities (data not shown).

Observed allelic diversity (A_{ob}) per population ranged from 5.4 to 12 (average = 9.30), and allelic richness (A_R) was slightly lower (average = 7.25; range = 4.43–9.01; Table 1). Expected heterozygosity was high across all localities (average = 0.697; range = 0.537–0.789; Table 1). Overall, these indices indicated that high levels of genetic diversity exist across all localities. The lowest genetic diversity was detected in CQ (Venezuela), and the highest in Peru (MC, PI, SC). No significant differences in A_R and H_E were found among localities (ANOVA, $P > 0.7$), and the two measures were highly correlated ($R^2 = 0.773$, $P < 0.001$). As expected, mean A_R values were highly correlated with average heterozygosity ($R^2 = 0.82$, $P < 0.0001$). Given the range of sample sizes, we also tested the correlation between sample size and number of alleles. Two loci (Sat128 and PE1075) showed a significant association ($P = 0.04$ and $P = 0.05$, respectively). However, when using the average number of alleles, no relationship was detected.

The multilocus estimates of F_{is} per locality obtained in FSTAT ranged from -0.066 to 0.076 , and none differed significantly from HWE (Table 1). Furthermore, no deviation from HWE was detected for the entire dataset ($F_{is} = 0.012$, 95% CI: -0.129 to 0.126 ; $P > 0.1$) (Table 2). When considering F_{is} for each locus and locality, however, locus Sat62 exhibited significant heterozygote deficiency at MAT ($P < 0.00001$). Heterozygote excess was found at locus PE1075 in NICH, RFTA and CQ ($P < 0.00001$). We detected potential null alleles at NICH, MAT, RFTA, MET, CQ, MC for locus Sat62, CQ for Sat91, and PI for Sat128, as estimated with MICRO-CHECKER. To account for the potential impact that the presence of null alleles could have on the population structure estimates, an NA-corrected dataset for loci Sat62, Sat91 and Sat128 was generated and used in follow up analyses as described below.

Table 2 Summary fixation indexes across localities of *Podocnemis unifilis* for five microsatellite loci calculated in FSTAT

Locus	Allele size	Size range (bp)	F_{is}	F_{it}	F_{sta}	F_{stb}	G_{st}	R_{st}
Sat1	37	(143–243)	0.017	0.067	0.051	0.050	0.053	0.367
Sat62	26	(173–257)	0.230	0.401	0.222	0.183	0.206	0.340
Sat91	11	(100–122)	-0.055	0.161	0.204	0.192	0.224	0.087
Sat128	33	(137–219)	0.060	0.144	0.090	0.087	0.092	0.089
PE1075	13	(165–253)	-0.246	-0.134	0.089	0.088	0.091	0.105
All loci	120		0.012	0.137	0.127	0.114	0.128	0.221

bp base pairs. F -statistics within samples based on 5,000 randomizations as described in the text. Bold numbers indicate values significantly different from zero after Bonferroni correction ($\alpha < 0.01$). $F_{sta} = F_{st}$ values estimated for the original dataset, $F_{stb} = F_{st}$ values estimated for the dataset corrected for null alleles

Genetic differentiation among localities

Estimates of population differentiation were very similar for the original and the NA-corrected datasets (t -test = 0.2079, $P > 0.05$) and highly correlated (Mantel correlation $r_M = 0.99$ $P < 0.0001$). Additionally, results did not change considerably when removing PE1075 from the analysis of population differentiation. Thus, because null alleles and heterozygote excess appear to have no significant effect, the results presented here correspond to the original dataset unless specified otherwise. The three measures of genetic differentiation across localities (F_{st} , G_{st} and R_{st} ; Table 2) gave statistically similar results (G-test of overall values per locus, $P = 0.999$) even though some values for R_{st} appear much higher than the corresponding F_{st} or G_{st} . Globally, all measures detected a moderate genetic structure (Table 2). Here, we focus on F_{st} as this estimator performs better for small numbers of loci (Gaggiotti et al. 1999). Overall F_{st} across localities was 0.127 for the original data (95% CI: 0.071 to 0.192) and 0.114 for the NA-corrected dataset (95% CI: 0.069 to 0.165), indicating that genetic differentiation among localities was significant ($P < 0.001$; Table 2). Consistently, many pairwise F_{st} values were statistically significant (Table 3). Non-significant values were observed among some (but not all) geographically proximate localities within river basins or between nearby basins (e.g. CAU-MAT and CAU-NICH within the Caura basin; MC-PI-SC within the Pacaya-Samiria reserve; MAT-RFTA between the Caura and Orinoco rivers, and RFTA-MET between the Orinoco and Meta rivers). As expected, the corresponding pairwise migration estimates (N_m) revealed low gene flow between the most distant localities (e.g. $N_m = 0.50$ for

CQ-TRO), compared to neighboring tributaries or basins (e.g. $N_m = 21.36$ for CAU-MAT; Table 3) in accord with the pattern detected by the F-statistics. Geographic distances among localities along river channels ranged from 214 to 7682 km between basins, and from 86 to 690 km within basins. A significant positive correlation was found between genetic distance and geographic distance across all localities ($r_M = 0.5019$, $P < 0.003$; Fig. 2), as well as within the Orinoco drainage alone (CAU, NICH, MAT, RFTA, MET, CQ) ($r_M = 0.8342$, $P < 0.002$; Fig.2).

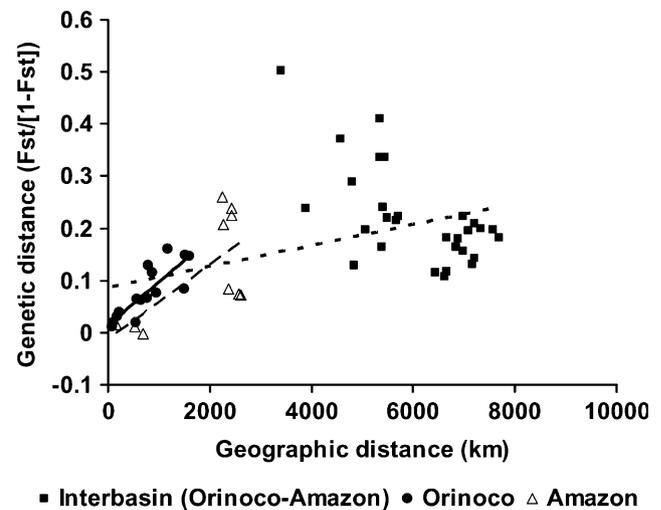


Fig. 2 Relationship between pairwise genetic [$F_{st}/(1 - F_{st})$] and river distance in *Podocnemis unifilis*. Isolation-by-distance was tested using pairwise distances between populations within **a** the Orinoco (circles) and **b** Amazon (triangles) basins alone, and **c** overall, combining these two with pairwise distances for populations between basins (squares). Isolation-by-distance is evident within the Orinoco (solid line), Amazon (dashed line) and overall (dotted line)

Table 3 Pairwise F_{st} values (below diagonal) and N_m values (above diagonal) among *Podocnemis unifilis* localities calculated in Arlequin and Genetix, respectively, from the microsatellite original dataset

	Orinoco basin						Amazon basin				
	Caura basin			RFTA	MET	CQ	Pacaya-Saimiria basin				
	CAU	NICH	MAT				CUT	MC	PI	SC	TRO
CAU	–	12.94	21.36	3.87	1.92	1.70	1.04	1.12	1.26	1.20	0.61
NICH	0.018	–	8.03	3.98	2.17	1.73	1.13	1.28	1.38	1.25	0.74
MAT	0.016	0.031	–	12.22	3.68	3.09	1.48	1.56	1.86	1.70	0.74
RFTA	0.067	0.063	0.017	–	6.68	3.38	1.93	2.13	2.32	2.10	0.87
MET	0.116	0.103	0.058	0.039	–	1.58	1.26	1.37	1.51	1.38	0.67
CQ	0.136	0.129	0.081	0.071	0.137	–	1.03	1.12	1.15	1.10	0.50
CUT	0.193	0.181	0.126	0.107	0.162	0.174	–	3.01	3.33	3.50	0.97
MC	0.188	0.164	0.120	0.098	0.156	0.165	0.078	–	16.59	20.94	1.21
PI	0.168	0.155	0.102	0.091	0.143	0.165	0.070	0.017	–	∞	1.12
SC	0.172	0.166	0.109	0.098	0.149	0.165	0.066	0.012	–0.002	–	1.05
TRO	0.291	0.252	0.241	0.222	0.270	0.325	0.206	0.175	0.187	0.193	–

Bold F_{st} values are significantly different from zero. $N_m = ∞$ correspond to $F_{st} ≤ 0$

The same pattern was found within the Amazon drainage (CUT, MC, PI, SC, TRO, $r_M = 0.6682$; Fig. 2), but the significance test was precluded by the small number of localities.

Genetic relationship among localities

Consistent with the F_{st} analyses, populations sampled from adjacent localities showed low genetic divergence as measured by CSE genetic distance (e.g. CAU-NICH, SC-PI) compared to localities farther apart. The CSE-based unrooted NJ tree separated the localities into two major groups, Orinoco and Amazonian populations, with strong bootstrap support (Fig. 3). Within the Amazonian population, all Peruvian localities (SC, PI, MC) clustered together and differentiated largely from Colombia (CUT) and Brazil (TRO), but these latter were not separated by a large bootstrap support. In contrast, within the Orinoco cluster, the Caura basin (NICH, CAU, MAT) differentiated from the Arrau turtle refuge, Meta and Casiquiare (RFTA, MET, CQ, respectively). Interesting, within the Caura basin, the separation of MAT from CAU and NICH (Fig. 3) was strongly supported. Similar results were obtained with the NA-corrected dataset (not shown).

Likewise, the scattergram of the first two PCoA axes of Nei's genetic distance among localities (which together accounted for 78.24% of the total variance) identified a clear divergence among the Amazonian (Brazil, Peru and

Colombia) and Orinoco populations along the PCoA1. Two clusters were detected within the Orinoco population: the Caura basin and the MET-RFTA-CQ, and two clusters in the Amazonia: the Pacaya-Samiria (MC-PI-SC)-CUT and the TRO (Fig. 4a). When the Brazilian population was removed from the PCoA, the test identified the Peruvian (MC-PI-SC) and Colombian (CUT) groups as distinct clusters (Fig. 4b).

Bayesian clustering algorithms distinguished at least six ($k = 6$) genetically distinct populations with confidence, a result statistically supported by the likelihood ratio test (Table 4). The six clusters were defined by the proportion of membership (Table 1)—three of which included samples from more than one locality (NICH-CAU, MET, CQ, CUT, MC-PI-SC and TRO) and two localities each grouped with the same two clusters (MAT and RFTA). By contrast, the Δk statistic (Evanno et al. 2005) recognized only the main subdivision of $k = 2$ corresponding to the Amazon and Orinoco populations ($\Delta k_2 = 20.01$, $\Delta k_3 = 0.16$ and $\Delta k < 0.16$ for $k > 3$, Table 4). Individual-based assignment analyses produced similar results. Individuals were accurately assigned to the population from which they were sampled (59.1% overall or 165 out of 279 individuals), or to a different one. The latter was more frequent among neighboring rivers and tributaries, rather than among widely separated populations (Table 5).

Population reduction tests

We obtained contrasting results for the bottleneck tests. On the one hand, we found that the mean A_R values for each population were not correlated with the mean pairwise F_{st} values ($R^2 = 0.29$, $P = 0.111$) indicating that there was no evidence that *P. unifilis* has experienced population bottlenecks that would increase genetic drift. Similarly, the TPM model detected no significant evidence of bottleneck relative to drift–mutation equilibrium in any locality, except for TRO, which was due to heterozygote deficiency (Wilcoxon one tailed test, $P = 0.03$), rather than heterozygote excess ($P = 0.98$). In contrast, the 'M-ratio' test revealed M values were below the critical value (M_c) of 0.56 with 95% probability in all localities except for SC from Peru, indicating strong evidence for recent reductions in population size (Table 6), although the exact timeframe of such reductions cannot be ascertained.

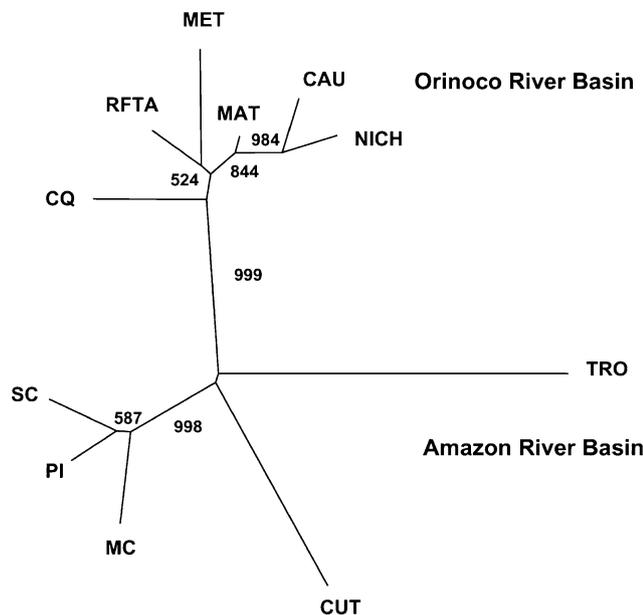


Fig. 3 Unrooted NJ tree based on Cavalli-Sforza distances among all *Podocnemis unifilis* localities. Nodes with support of >500 are shown. Localities are labeled as in Table 1. Procedures are described in the text

Discussion

Population decline increases the chances of genetic erosion by promoting inbreeding and accelerating the loss of alleles which can reduce the evolutionary potential of populations in the face of environmental change (Stockwell et al. 2003).

Fig. 4 Scatterplot of PCoA scores from microsatellite allele frequencies of *Podocnemis unifilis* for, **a** all sampled localities, and **b** excluding Brazil

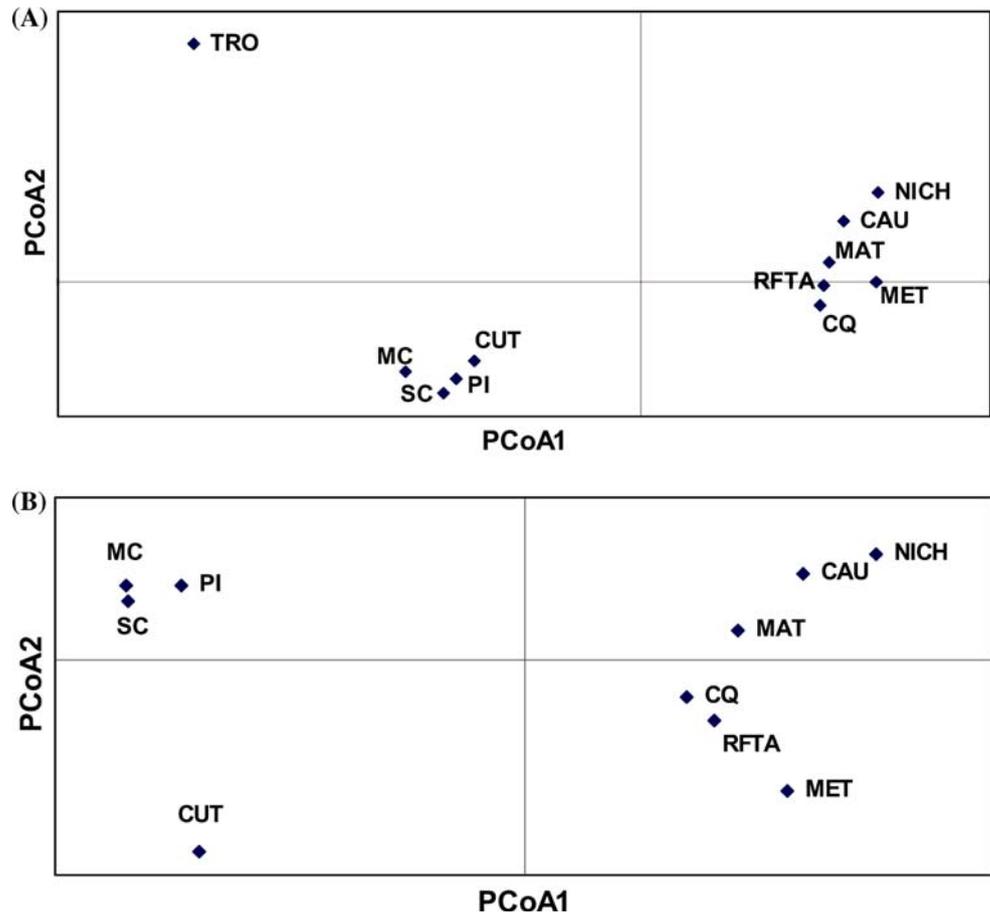


Table 4 Results of the likelihood ratio (Goldman 1993) and ad hoc (Evanno et al. 2005) tests of significance of adding each new cluster to the population structure model obtained from STRUCTURE as described in the text

Cluster (<i>k</i>)	Goldman likelihood ratio test						Evanno (ΔK)
	Mean LnP(x/k)	#Parameters	2LnP(x/k)	Difference	DF	<i>P</i>	
1	-6997.270	1	13994.54				
2	-6313.750	2	12627.50	1367.04	1	<0.05	20.01
3	-6185.125	3	12370.25	257.25	1	<0.05	0.16
4	-6081.370	4	12162.74	207.51	1	<0.05	1
5	-5991.560	5	11983.12	179.62	1	<0.05	0.17
6	-5911.225	6	11822.45	160.67	1	<0.05	0.21
7	-5915.760	7	11831.52	-9.07	1	NS	
8	-5916.580	8	11833.16	-1.64	1	NS	

NS non significant

Such population declines are recurrent for South American biota and many are induced by human activity as is the case of the riverine turtle, *Podocnemis unifilis* (e.g. Soini 1996; Thorbjarnarson et al. 1997; Escalona and Fa 1998). Here we studied patterns of genetic diversity and subdivision of *P. unifilis* turtles mostly along a North–South transect of its distributional range in the Orinoco and Amazon drainages using microsatellites, as a first step to gather information

about the role of natural processes and anthropogenic activities in structuring its populations that can be used to implement effective conservation programs. Our results are consistent across multiple analyses, revealing a clear subdivision of the populations inhabiting the Orinoco and Amazon drainages, and support some but not other patterns from previous smaller scale studies that used allozymes and mtDNA as described below.

Table 5 Assignment test of *Podocnemis unifilis* individuals to localities

Sampled Population	Assigned population											Correctly assigned (%)
	Orinoco population						Amazon population					
	NICH	CAU	MAT	RFTA	MET	CQ	SC	MC	PI	CUT	TRO	
NICH	16	7	7									53
CAU	4	10	4	1	1							50
MAT	6	10	16	6	3	1						38
RFTA			5	12	4	3						50
MET			2	3	7	1						54
CQ			1	2	1	17						81
SC							17	3	8			61
MC							6	14	4			61
PI							9	5	11	1		42
CUT										29		100
TRO								1	1	1	15	83

Only individuals that amplified at all five loci were included in the analysis. Shaded values derived from localities within the same river basin (i.e. Caura basin: CAU-MAT-NICH; Pacaya-Samiria reserve: MC-PI- SC). Bold values represent individuals correctly assigned to the population from which they were sampled.

Table 6 Results of the tests for population decline in *Podocnemis unifilis* as described in the text

Population	Bottleneck	M-ratio
Caura (CAU)	0.0625	0.3508*
Nichare (NICH)	0.0937	0.4512*
Mato (MAT)	0.0937	0.5044*
Arrau Turtle Refuge (RFTA)	0.1562	0.5051*
Meta (MET)	0.6250	0.3797*
Casiquiare (CQ)	0.1562	0.4566*
Caquetá (CUT)	0.2187	0.5438*
Manco Capac (MC)	0.8125	0.4537*
Pithecia (PI)	0.6250	0.5594*
Santa Cruz (SC)	0.6250	0.7202
Trombetas (TRO)	0.0312*	0.5252*

Asterisks denote significant evidence of a population decline from BOTTLENECK or values of $M < 0.56$, the M_c (critical value)

Genetic diversity within localities

We detected high levels of genetic diversity in all localities of *P. unifilis*, as measured by gene diversity and allelic richness, suggesting that the human-induced population decline (Soini 1996; Thorbjarnarson et al. 1997; Escalona and Loiselle 2003) has not yet affected the genetic variability of the populations examined in a significant way (e.g. Ellstrand and Elam 1993). Similar high genetic diversity has been found in other heavily exploited turtles (e.g. Kuo and Jansen 2004) including its congener *P. expansa* (Pearse et al. 2006), and may reflect historic variability that has been buffered from recent bottlenecks by the species longevity, overlapping generations, and multiple paternity (Valenzuela 2000; Garza and Williamson 2001). Genetic diversity levels obtained from microsatellites contrast with those reported for *P. unifilis* from mtDNA for Peru (40.5%, Engstrom 2003) and from allozymes (mean $H_E = 7.1\%$, 5.9 and 6.9% for Peru, Brazil, and Colombia, respectively, Bock et al. 2001).

Given that we used the same Peruvian and Colombian samples for our analyses, differences are likely due to the properties of the molecular markers used for the three studies. For instance, allozymes are under strong selection and less variable than microsatellites due to lower mutation rates and hidden heterogeneity (Karl and Avise 1992). Additionally, the fragment of mtDNA used by Engstrom (2003) was the 5'-end of the control region which contains little variation as seen in *P. expansa* (Pearse et al. 2006). By contrast, *P. expansa* exhibited similar diversity indexes when a larger fragment of the mtDNA control region and microsatellite markers were examined (Pearse et al. 2006). Further, our results also resemble genetic diversity found in many other studies of turtle populations using microsatellite data, in that they also detected high values of heterozygosity and number of alleles (e.g. Hauswaldt and Glenn 2003; Mockford et al. 2005; Tessier et al. 2005).

Genetic differentiation among localities

We detected a complex geographical pattern of genetic variation at different scales that appears highly influenced by landscape features. At the continental level, genotypic estimates revealed the existence of high inter-locality differentiation throughout the geographic range studied, with a strong genetic structure among watersheds that is geographically consistent with current hydrological connections, and follows an isolation-by-distance model (Wright 1943). The clear genetic separation between the Orinoco and the Amazonian river basins was highly supported by the dichotomy observed in the NJ tree, PCoA, Bayesian analysis and assignment test, and indicates that there is very limited gene flow among populations from the Amazon and Orinoco, and that populations in these two watersheds are demographically independent. This result may be expected considering that the Amazonian and Orinoco drainages have been separated for 8–11 MY (although repeated fragmentation, reconnection, and separation may have

occurred), and each drainage reflects differential landscape evolution, water quality as well as hydrological and geo-morphological processes (Hoorn et al. 1995; Lundberg et al. 1998; Rosales et al. 1999; Albert et al. 2006). However, *P. unifilis* inhabits white-water rivers, as well as clear-water and black-water rivers, flooding areas, and lakes (Pritchard and Trebbau 1984; Rueda-Almonacid et al. 2007). Such tolerance to varied water conditions may enable dispersion between the Orinoco and Amazon basins via the Casiquiare corridor or the flooded forest. On the other hand, because the connection through the Casiquiare riparian corridor appears to be very recent as estimated from fish data, although no exact estimates exist (Turner et al. 2004; Willis et al. 2007; Winemiller et al. 2008), gene flow may be too recent to have resulted in widespread homogenizing effect. Alternatively, ecological differences between populations of *P. unifilis* occupying black-waters (Río Negro) and white-waters (Río Orinoco) with varying hydrochemical gradients may result in dissimilarities in migration and life history that constrain genetic exchange between these populations, and thus may act as an ecological barrier promoting population structuring. For instance, low-pH nutrient-poor black-water rivers sustain different biotic communities than neutral-pH nutrient-rich white-water rivers (Sioli 1984). Such ecological difference among populations has been reported for Amazonian crocodylians (e.g. Farias et al. 2004; De Thoisy et al. 2006; Vasconcelos et al. 2006), but whether a similar phenomenon occurs among populations of *P. unifilis* from these two drainages remains uncertain and warrants further research. Interestingly, molecular data from *P. expansa* over a large geographical scale revealed lack of phylogeographic structure (Pearse et al. 2006), suggesting significant historical gene flow and free dispersal through the Casiquiare river. On the contrary, the Casiquiare river acts as a partial barrier to dispersion for other vagile aquatic organisms, such as some fish (Winemiller et al. 2008).

Geographic separation was also observed in the pattern of isolation-by-distance within the Orinoco and Amazon basins, in contrast to previous allozyme (Bock et al. 2001) and mtDNA (Engstrom 2003) studies. Within the Amazon drainage most methods (PCoA, Bayesian, and assignment test) identified three distinct populations, i.e. TRO (Brazil), CUT (Colombia), and MC-PI-SC (Peru), while CUT was indistinguishable from MC by allozymes from the same samples (Bock et al. 2001). Isolation-by-distance has been reported for other aquatic Amazonian vertebrates including black caiman, river dolphins, and fish (Farias et al. 2004; Banguera-Hinestroza et al. 2002; Willis et al. 2007). Conversely, giant Amazon river turtles, *P. expansa* (Pearse et al. 2006), manatíes, *Trichechus inunguis* (Cantanhede et al. 2005), spectacle caiman, *Caiman crocodilus* (Farias et al. 2004; Vasconcelos et al. 2006) and some vagile fish (e.g. Hrbek et al. 2007; Santos et al. 2007) display higher genetic connectivity that is enabled by the contiguous

flooded habitat provided permanently or seasonally by the Amazonian *várzea* (white-water) and *igapó* (black-water) ecosystems (Junk 1993; Vasconcelos et al. 2006; Hrbek et al. 2007). Within the Orinoco drainage we also identified three distinct populations, one represented by the CQ (upper Orinoco), one by two localities restricted to the middle Orinoco (MET-RFTA), and the other by the Caura river basin localities (NICH-CAU-MAT). However, mixing was observed between the lower section of the Caura (MAT) and the middle Orinoco (RFTA), perhaps reflecting the isolation by distance effect. Genetic subdivision in this region is expected given the environmental heterogeneity of the Orinoco basin and the presence of physical barriers to dispersal such as water falls and the Guyana shield belt in the southern and southeastern margins of the Orinoco River. This belt together with the Salto Pará waterfall, separates the Caura from the Casiquiare River, thus forcing the exchange among populations to occur via a large distance of ~1600 km along the river channels rather than through flooded areas. Contrastingly, floodplains within the rest of the Orinoco basin (Junk 1997; Berry et al. 1995), may allow greater gene flow between neighboring watersheds, as occurs for the lower tributaries of the Caura, i.e. MAT with RFTA and MET. It is interesting to note that this gene flow does not appear to extend southward to connect CQ with MET-RFTA in a significant way.

Estimates of migration among the 11 sampled sites corroborated the general pattern of isolation-by-distance, with little migration detected between separate and distant river basins, and extensive migration among tributaries within basins, or between neighboring river basins (Table 3). Additionally, within the two major populations (Orinoco and Amazon basins), migration estimates revealed cases where additional factors are needed to explain the genetic structure. For instance, MAT (Caura basin) shares greater gene flow with RFTA (middle Orinoco) located ~547 km away, than with NICH located only ~189 km away in the same basin, likely because the mountain range separating MAT and NICH (Cerro Mato) precludes gene flow through floodplains unlike between MAT and RFTA. Gene flow through the Caura River may also be impacted by the presence of rapids which could represent a substantial barrier that allows only occasional gene flow. In contrast, extensive migration occurs among MC, PI and SC which are located in three Peruvian rivers within the Reserva Nacional Pacaya-Samiria and are separated by 460–700 km along the river channel. This is likely due to the complete connectivity through flooded forest during the rainy season (Engstrom 2003). This pattern in the Pacaya-Samiria is consistent for microsatellites (our study), and mtDNA (Engstrom 2003).

Contrary to *P. unifilis*, *P. expansa* shows no significant effect of isolation-by-distance (Bock et al. 2001; Pearse

et al. 2006), revealing a greater dispersal capability of *P. expansa* perhaps because its larger body size allows individuals to travel larger distances (Ojasti 1967; von Hildebrand et al. 1997) as proposed for marine turtles (FitzSimmons et al. 1997). Considerable differences in the degree of population structure among other turtle species (Walker and Avise 1998; Pearse et al. 2006) and regions (Starkey et al. 2003) are well documented.

Not surprisingly, TRO showed the highest degree of differentiation from other localities in accord with the large geographic distance that separates this locality from all others, and with the high frequency of private alleles it contained (Table 1). However, further sampling at intermediate locations is required to better understand gene flow between TRO and other populations. Notably, CUT was identified as the second most genetically divergent population by all analytical methods, a site from a Colombian region where localities along a 60 km stretch of the Caquetá River showed as much genetic differentiation among themselves as they did to localities in Peru and Brazil based on allozymes (Bock et al. 2001). Interestingly, *P. expansa* inhabiting this region also exhibited genetic differentiation among nesting beaches <100 km apart along the same river. The extreme genetic diversification within this region warrants further sampling to test whether features such as the Jutai tectonic ridge, which defined boundaries and separated the basins in the past but is not evident today (Räsänen et al. 1987; Da Silva and Patton 1998), could have contributed to this biogeographic region behaving as an evolutionary independent unit. The alternative that low dispersal capability in *P. unifilis* could cause the pattern in the Caquetá can be ruled out given the gene flow detected among other, more distant, localities for both *P. unifilis* (this study) and *P. expansa* (Pearse et al. 2006), and the lack of female philopatry in *P. expansa* (Pearse et al. 2006), which remains untested for *P. unifilis*. Behavioral or ecological differences in the level of fidelity to either nesting or natal areas, or in the partitioning of niche resources among populations, may also explain variation in migration levels among localities at any geographic scale.

Population size reductions

Contrasting results were observed among the analytical approaches used in this study to test for population declines and genetic bottlenecks. On the one hand, the ‘*M*-ratio’ test revealed a substantial reduction in population size in all but one locality (SC), consistent with current widespread hunting of this species throughout its range (Mittermeier 1978; Soini 1996; Escalona and Fa 1998; Escalona and Loiselle 2003). Observed *M* values are indicative of a population contraction and are similar to those reported for other exploited species, such as *P. expansa* (Pearse et al.

2006). However, evidence of population decline was detected in one of eleven localities with BOTTLENECK, a result that could occur by chance in populations under mutation-drift equilibrium with no population decline. Similarly, no signs of erosion of genetic diversity as measured by allelic richness (A_R) were found, nor did we detect any signs of reduction of heterozygosity. Although the high number of rare alleles seen in our data may mask the heterozygosity excess caused by a bottleneck (Cornuet and Luikart 1996), this would not explain the results of the ‘*M*-ratio’ test which measures the loss of rare alleles relative to their size range (Garza and Williamson 2001). Alternatively, the ‘*M*-ratio’ test may be revealing a very recent bottleneck [more likely since we compared the observed *M* values to the critical value (M_c)] or a more ancient bottleneck that is undetectable by the other methods. Finally, the number of loci used here may not provide sufficient power for these methods to detect bottlenecks conclusively.

Conclusions and conservation implications

Podocnemis unifilis populations have recently declined dramatically in many places, particularly following the overexploitation of the larger-bodied *P. expansa* (Mittermeier 1978; Thorbjarnarson et al. 1997). The results of our preliminary population genetic study of this species have important implications for conservation. For instance, although we found no indication of dramatic losses of genetic diversity at the level of gene diversity and allelic richness, other analysis (i.e., ‘*M*-ratio’) showed a signature of genetic vulnerability due to population bottlenecks, which may impair future genetic integrity of *P. unifilis* if anthropogenic exploitation persists, leading to decreased fitness (Gautschi et al. 2002; Beheregaray et al. 2003) and increased extinction probability (Newman and Pilson 1997; Frankham 1998; Saccheri et al. 1998). Importantly, we found that in some drainages migration via the flooded forest contributes to the demographic and genetic structure (e.g. MAT-RFTA, MC-PI-SC), while in others gene flow occurs only via river channels because geographic barriers exist that prevent free dispersal between basins and even among nearby tributaries of the same basin (e.g. MAT-NICH). Such differences in the level of population differentiation among regions caution against making broad scale generalizations based on data from a restricted area because some geographically proximate localities may be demographically and evolutionarily separate units requiring independent management, while in other areas widely separated localities may be demographically linked by migration. Although the patterns detected in our study are clear and expected, our results also indicate that further geographic sampling is necessary. Our sampling

concentrated mostly on localities forming a North-to-South transect along the western edge of the distributional range (Fig. 1), with the exception of TRO. Future sampling should include the Río Negro (black water) and other tributaries of the Amazon and Essequibo river basins, as well as other drainages in the Guianas, to fully describe the complete pattern of population structure for *P. unifilis* that may be used for management plans. Such sampling will enable testing the intriguing genetic distinctiveness detected in the Caquetá river in Colombia, as well as the gene flow between peripheral and central localities within the distributional range. Until more genetic, ecological and behavioral information is available, including aspects of nest site fidelity and fine scale genetic structure, populations (as defined by their significant genetic differentiation from other such units determined by this or future studies) within basins should be treated as demographically independent management units. In the absence of such additional information transplant of eggs among rivers or the release of hatchlings away from their natal beach should be avoided. This should help maintain the ecological function and the suite of genetic local variability present in distinct populations.

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