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Conservation Genetics of the Giant Amazon River Turtle (*Podocnemis expansa*; Pelomedusidae) – Inferences From Two Classes of Molecular Markers

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ABSTRACT. – **The giant Amazon river turtle (*Podocnemis expansa*) is poorly known ecologically, and like marine turtles, is a potentially long-lived colonial nester that may migrate over large geographic distances. In the absence of long-term tagging studies and other ecological information, we infer between-rookery gene flow and population structure from investigating the geographic distribution of alleles at unlinked nuclear and mitochondrial loci. We established a microsatellite library and targeted six loci for the analysis of genotypes across 94 individuals collected from four different rookeries in two river systems (Araguaia and Tapajós) in the Brazilian Amazon Basin. Mitochondrial DNA sequences (354 bp) were obtained using control region primers for a subset of 73 turtles, allowing an assessment of within- versus between-river system structure. Patterns of genetic variability at both nuclear and mitochondrial loci suggested extensive within-system gene flow (up to 275 km) but very little gene flow between river systems (2400 km). These results suggest that intensive sampling within river systems (< 200 km) is probably not necessary to elucidate gene flow and metapopulation structure, but that effort should be focused on sampling across a broad geographic range and between major river systems.**

KEY WORDS. – **Reptilia; Testudines; Pelomedusidae; *Podocnemis expansa*; turtle; conservation genetics; population structure; microsatellite; mitochondrial DNA; Amazon Basin; Brazil**

In the last decade, the use of various classes of molecular markers to address questions of gene flow and population structure has become widespread, due primarily to techniques that permit assessment of multiple gene loci evolving at varying rates (Avise, 1994; Ferraris and Palumbi, 1996; Hillis et al., 1996). The combined use of nuclear and cytoplasmic markers is especially instructive in assessing sex-biased gene flow, particularly in species that exhibit a high degree of structure in female lineages (Karl et al., 1992; Melnick and Hoelzer, 1992; Palumbi and Baker, 1994). Mitochondrial DNA (mtDNA) markers are valuable for inferring maternal genealogies and demographic units (Moritz, 1994; Avise, 1995), while microsatellite loci, because of their high mutation rates and high allelic diversity are ideal for many population questions (Ashley and Dow, 1994; Schlotterer and Pemberton, 1994), particularly in species of conservation concern where limited ecological information may be available. For example, in marine turtles mtDNA markers have been used to test female (and male, FitzSimmons et al., 1997a) fidelity to breed at natal beaches (reviewed by Bowen and Karl, 1997), and comparisons to nuclear markers have allowed tests of male-mediated gene flow (Karl et al., 1992; FitzSimmons et al., 1997b). Almost all aspects of the population biology of marine turtles previously inferred from tagging studies, and some not previously suspected, have been clarified or extended by molecular population studies (Bowen and Karl, 1997).

Here we present results of a pilot study of the population genetic structure of the giant Amazon river turtle (*Podocnemis expansa*), a species indigenous to the Amazon, Essequibo, and Orinoco River basins of northern South America (Iverson, 1986). This species resembles marine turtles in its large size, in nesting in large numbers on selected exposed sand beaches, and having a poorly known life cycle spread across potentially very large spatial and temporal scales. Also like marine turtles, *P. expansa* nests colonially and because in some places it is heavily harvested for meat and eggs, indiscriminate collecting has severely reduced the size of some nesting populations in all three river basins (IUCN/SSC Tortoise and Freshwater Turtle Specialist Group, 1989). Unlike marine turtles, however, few long-term tagging studies have been undertaken to reveal migratory habits or nest-site fidelity (but see Ojasti, 1967, for results of tagging studies of nesting females in the Orinoco River). Nevertheless, extensive efforts by the Brazilian government have been devoted to identification and monitoring of important nesting areas, and safe-guarding adults and nests from poachers (Cantarelli, 1993, 1997).

In this paper we describe the isolation and characterization of microsatellite loci from a gene library developed for *P. expansa*, and contrast patterns of variability in several of these markers with sequence data from the mtDNA control region, within and between two river systems in Brazil's portion of the Amazon Basin. This study is intended to

address questions of immediate conservation concern regarding the geographic scale at which gene flow defines populations. Further work will aim to elucidate metapopulation structure and migratory habits, and to identify independent demographic units for conservation management.

Biology and Conservation of *Podocnemis expansa*

Podocnemis expansa (*arrau* in Venezuela; *tartaruga* in Brazil) can grow to a carapace length of 89 cm (Ojasti, 1967) and weigh up to 90 kg (Vogt, in press). Because it nests in colonies, it has been heavily harvested for both meat and eggs over much of its range (Mittermeier, 1975, 1978; Smith, 1979; Pritchard and Trebbau, 1984; Johns, 1987). Consequently, nesting populations at some locations have been extirpated or severely reduced (IUCN/SSC Tortoise and Freshwater Turtle Specialist Group, 1989; Licata and Elguezabal, 1997; Thorbjarnarson et al., 1997; Vogt, in press). In the mid-1970s, the Brazilian government realized the need for protective measures for conservation and sustainable management of *P. expansa* populations in Brazil's part of the Amazon Basin (Alfinito, 1975). The agency in charge of wildlife resources, IBAMA (Instituto Brasileiro do Meio Ambiente; Brazilian Institute for the Natural Environment), worked for over a decade to identify important nesting beaches, and to guard these during nesting emergence of adult females (Projeto Quelônios da Amazônia; Cantarelli, 1997). In 1990, IBAMA committed additional resources to this effort by dedicating a unit called CENAQUA (Centro Nacional dos Quelônios da Amazônia; National Center for Amazonian Turtles), strictly to this mission. CENAQUA efforts from 1979–92 have resulted in the release of over 18,000,000 young turtles back into rivers after they hatched from nests on protected beaches (Cantarelli, 1993, 1997). CENAQUA fields a crew of over 70 people during the nesting seasons, and teams monitor nesting activity on about 115 beaches in 15 regions on 12 rivers flowing through 9 Brazilian states (Fig. 1); the ultimate goal is restoration and long-term monitoring of *P. expansa* rookeries on about 500 beaches if support becomes available (Cantarelli, 1993, 1997).

Superficially, some aspects of the nesting biology of *P. expansa* resemble that of marine turtles (Carr and Giovannoli, 1957; Vanzolini, 1967; Alho et al., 1979; Ehrenfeld, 1979). Alho and Pádua (1982) described nesting at a rookery on the Río Trombetas (site 1 in Fig. 1) and showed that adult females aggregated offshore of a sand bar, then gathered close to and basked on the beach (the *boia-douro*) at midday, while occasionally touching their noses to the sand. At this particular site, fidelity to a single nesting beach was high, even though three similar beaches were nearby. Evidence for this was the appearance of adult females only on this beach for the nesting seasons of 1978, 1979, and 1980, and their complete absence on the other nearby beaches (distances not given); this interpretation is not based on long-term tagging studies (Alho and Pádua, 1982). Unlike marine turtles, however, *P. expansa* shows no evidence of multiple clutches deposited by the same female in the same

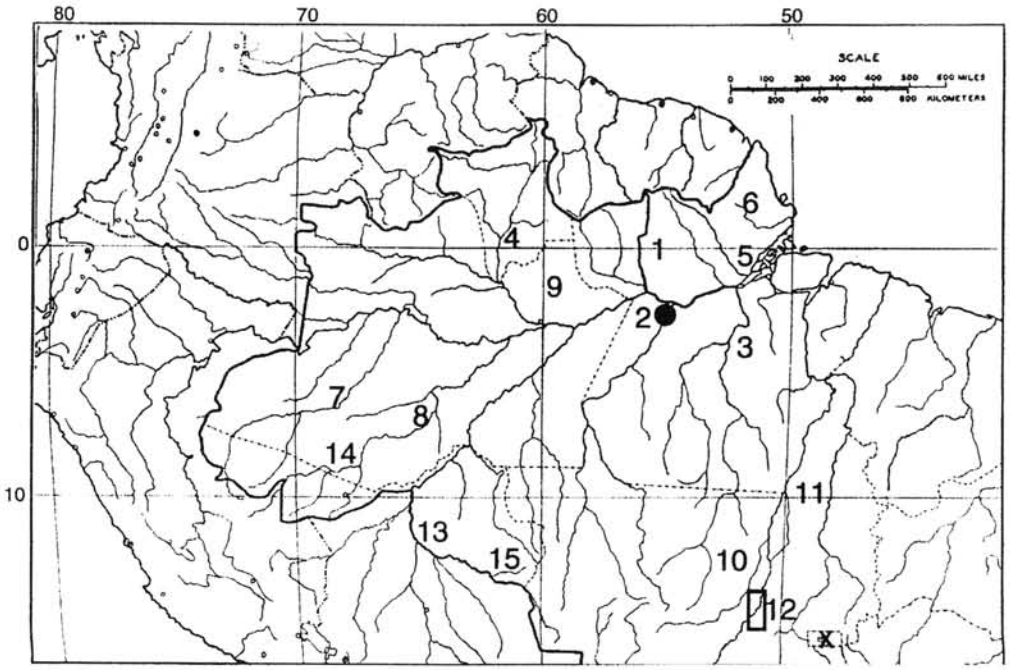

Figure 1. Approximate locations of *P. expansa* rookeries monitored by CENAQUA: 1. Río Trombetas (state of Pará); 2. Río Tapajós (Pará); 3. Río Xingu (Pará); 4. Río Branco (Roraima); 5. Río Amazonas (Amapa); 6. Região dos Lagos (Amapa); 7. Río Juruá (Amazonas); 8. Río Purus (Amazonas); 9. Río Uatuma (Amazonas); 10. Río das Mortes (Mato Grosso); 11. Río Araguaia (Tocantins); 12. Río Araguaia (Goiás); 13. Río Guaporé (Rondonia); 14. Río Purus (Acre); 15. Río Pimenteiras (Rondonia). The solid circle marks the Río Tapajós rookery (site no. 2) sampled for this study, the rectangle marks the 3 nesting beaches sampled from the upper reaches of the Río Araguaia (site no. 12; see also Fig. 2); "X" marks Brasilia.

nesting season; Alho and Pádua (1982) recorded only single nestings in a sample of 168 females throughout one nesting season at the Río Trombetas site. Comparison of clutch sizes and mean incubation times across the range of *P. expansa* suggests considerable between-population variation in at least some life history traits (Alho and Pádua,1982; Pritchard and Trebbau, 1984; Hildebrand et al., 1988; Vogt, in press), although growth rates and time required to attain sexual maturity are unknown for any natural population (Vogt, in press). *Podocnemis expansa* exhibits TSD (temperature-dependent sex determination), with a higher proportion of females emerging at higher temperatures (Pádua and Alho, 1982; Alho, 1984; Alho et al., 1985; Valenzuela et al., 1997).

Few data are available on long-term movements in *P. expansa* (but see Pritchard and Trebbau, 1984), but Vogt (in press) summarized studies made on females outfitted with radio-transmitters at the Río Trombetas rookery. Females nested, and then remained for at least two months in the deep pools adjacent to the nesting beach, and did not feed. With the onset of the rainy season and rising water levels, adult females moved on and were tracked for up to 45 km in two days, but were then lost from the study area after entering the main channel of the Amazon River (Vogt, in press). Hatchlings emerge also with the onset of rising water levels, and turtles of all sizes enter oxbow lakes and flooded forests (*varzéa*) to feed, although the distances and directions moved from the nesting beaches are unknown. Little information exists on diet of *P. expansa* in the wild, but available observations indicate that they are herbivorous (Pritchard and Trebbau, 1984; Guerreiro de Carvalho, 1992), and adults may be important seed dispersers (Vogt, in press). Given the high reproductive potential of *P. expansa* and its herbivorous diet (food is not likely limiting to population sizes), it may be possible to restore this species in parts of its range if human exploitation can be kept in check (Cantarelli, 1993, 1997; Vogt, in press).

MATERIALS AND METHODS

Sampling Design. — Turtles were collected by CENAQUA personnel during routine field work. Liver samples were taken from between 22 and 25 hatchlings at each of four different rookeries from two river systems within the Amazon Basin. Each hatchling represented a single clutch deposited by a known female, and the specimens were catalogued into the research collection of the Centro de Estudos e Pesquisas Biológicas [CEPB], Universidade Catolica de Goiás (NJdS, curator).

In 1995, three different rookeries were sampled from the upper reaches of the Río Araguaia in the state of Goiás (region 12 in Fig. 1), under the following design. Two rookeries were sampled along the main river channel of the Araguaia, downstream and upstream, respectively, from the mouth of a major tributary, the Río Crixás Açu (Fig. 2). The two main river sites were designated (1) Ara-Praia (n = 22; vouchers CEPB/CH0024-0046) and (2) Ara-Faz (Fazenda

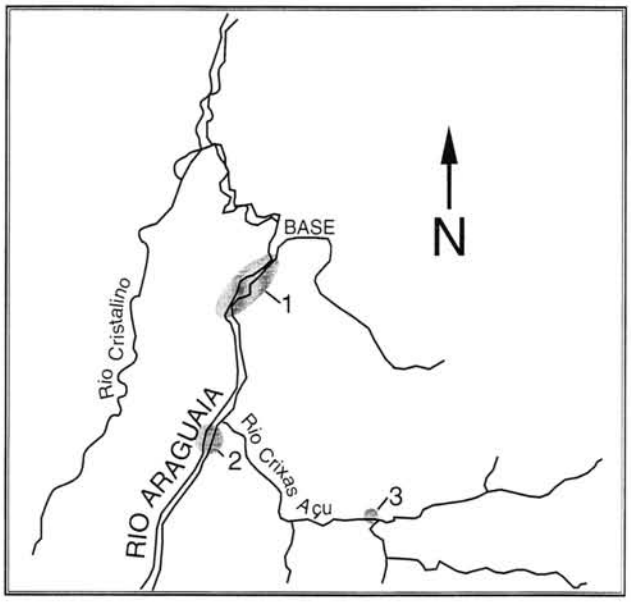

Figure 2. Detailed map of three nesting beaches sampled from the upper Río Araguaia watershed in the state of Goiás (site 12 in Fig. 1). Locality 1 is the Ara-Pra site in which 22 nests were sampled from five separate sandbars (see text); locality 2 is the Ara-Faz site (n = 24); and locality 3 is the Ara-Crix site (n = 23).

Montaria, n = 24; CEPB/CH0047-0071), and are separated by approximately 100 river km. The Ara-Praia collections were taken from five separate sandbars (Praia Gaviota [1], Praia Cascalho [1], Praia dos Três Furos [1], Praia Volta Grande [2], and Praia Rebojinho [17]), each with small nesting populations, scattered over a region of about 60 river km. The mid-region of this stretch of river was used to measure distances from this site to the other rookeries, all of which came from single beaches. The third rookery, designated (3) Ara-Crix (Praia Barreira Branca, n = 23; CEPB/CH0001-0023), was sampled from a beach along the Río Crixás Açú about 200 km upstream from its confluence with the Río Araguaia (Fig. 2). In 1996, samples were collected from a second river basin, near Santarém along the Río Tapajós (region 2 in Fig. 1) approximately 2400 km from the midpoint of the Río Araguaia samples; this sample is designated (4) Rio-Tap (n = 25; CEPB/CH0072-0096). The sampling design thus permits assessment of the potential for within- vs. between-river system gene flow, across geographic scales differing by an order of magnitude. Samples were exported from Brazil and imported into the US, exported to Australia and finally back to the US under approprite CITES permits from all three nations, and with a second permit from the U.S. Department of Interior for handling species listed by the U.S. Endangered Species Act.

DNA Extraction. — Liver samples were stored in vials of 70% ethanol and total genomic DNA was extracted by digesting approximately 0.50 g of tissue for 3 hrs at 55°C with proteinase K (final concentration of 0.5 mg/ml) in 300 µl of lysis solution containing 40 mM Tris (pH 8.0), 20 mM EDTA-Na2, 100 mM NaCl, and 1.5% SDS. This was followed by standard phenol/chloroform extractions (Sambrook

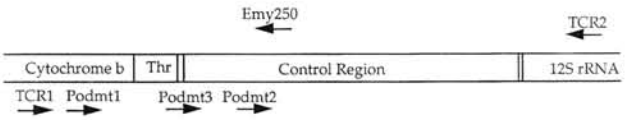

Figure 3. Location of PCR primers used to generate mtDNA sequences in *P. expansa*.

et al., 1989). The DNA precipitate was resuspended in 200 µl of 1x TE buffer, and samples (5 µl) were run on 1.2% agarose gels to check concentrations and purity.

MtDNA Control Region. — Mitochondrial control region sequences were initially amplified using a conserved external primer pair (TCR1/TCR2) developed for the marine turtle *Chelonia mydas* by Norman et al. (1994). Further amplifications used a *P. expansa* primer we designed in the cytochrome-b gene, Podmt1 (5'-CAATGCTGCGATCCATCC-3'), and one in the control region designed for the pleurodire genus *Emydura* (Emy250; B. Shaffer and T. Engstrom, *pers. comm.*). These primers amplified the 5' end of the control region (Fig. 3), and PCR conditions included an initial denaturation at 94°C for 90 sec followed by 33 cycles of 94°C (30 sec), 50°C (50 sec), 72°C (50 sec) and a final extension at 72°C for 2 min. Amplified products were cycle sequenced (35 cycles; 55°C annealing) using both the Emy250 primer and internal *P. expansa* primers Podmt3 (5'-TCACAGACATAACCATAAGCAC-3') and Podmt2 (5'-TTGCTGTAGAATCTGACATCC-3'). Sequences were run on standard denaturing 6% acrylamide gels, and aligned in the CLUSTAL W program (Thompson et al., 1994).

Microsatellites. — To prepare the gene library, approximately 5 µg of total genomic DNA was digested in two aliquots; one with 12 units of Sau3A1, and the second with a three-enzyme combination (AluI, HaeIII, and RsaI) for 3 hrs. Both were run on 1.2% low melt agarose gels for size separation, and the 350–600 bp fragments were cut from the gels and purified with a Prep-A-Gene kit (Bio-Rad), following the manufacturer's suggestions. Turtle template DNA that had been digested with Sau3A1 was ligated into pUC18 BamHI/BAP plasmids (Pharmacia), and the 3-enzyme digests were blunt-end ligated into pUC18 SmaI/BAP plasmids. Ligation reactions included 1.5 units of ligase, 1x ligase buffer, 25 ng plasmid, and purified target DNA amounts of 3, 6, and 9 µl. Fresh competent *E. coli* bacteria were obtained following the procedures of Inoue et al. (1990), and transformation of ligated DNA into competent cells was achieved by heat shocking. To assess transformation efficiency, 50 µl of each transformed solution was plated onto LBamp plates and grown overnight at 37°C. Remaining solutions were stored overnight at 4°C.

Colonies were grown on Hybond N+ membranes (Amersham, Sydney) and duplicate transferred to Zeta-Probe blotting membranes for hybridization. A synthetic oligonucleotide microsatellite probe (dA-dC)n-(dG-dT)n (Pharmacia) was radioactively labeled by random-priming (Feinberg and Vogelstein, 1983) using a Mega-Prime kit (Amersham) and 32P-dCTP. Filters were hybridized overnight at 65°C with shaking, then washed for 20 min each in

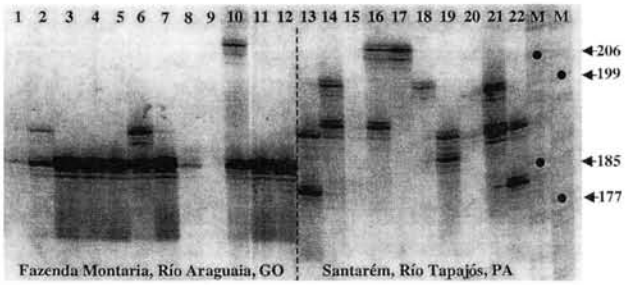

Figure 4. Between-river system allelic variation at locus Pod62 [repeat motif = $(GT)_{11}$; see Table 1] in *P. expansa*, showing representative individuals from the Ara-Faz (site no. 2 in Fig. 2) and Rio-Tap (site no. 2 in Fig. 1) rookeries. In this and all other loci, we consistently scored the darkest band present when more than one was evident ("stutter" bands), and any questionable genotypes or weak amplifications were rerun and independently scored. Genotypes are: *186/186* homozygotes for lanes 1, 3, 4, 5, 7, 8, 11, and 12; *186/192* heterozygotes in lanes 2, 6, and 19; *186/210* in lane 10; *182/190* in lanes 13 and 22; *192/200* in lane 14; *192/210* in lane 16; *210/210* in lane 17; *200/200* in lane 18; and *190/198* in lanes 20 and 21. Dots in the right lanes identify size markers.

a solution of 2x SSC and 0.1% SDS, first at room temperature, and then with fresh solution at 65°C. A final wash was done in 0.2x SSC and 0.1% SDS at 65°C. Filters were exposed to X-ray film at -80°C with intensifying screens for approximately 4 hrs. Positive colonies were selected from the original Hybond colonies after alignment to autoradiography images and colonies were grown overnight. This library of potentially positive clones was subjected to a second round of hybridization to confirm positive status, and the ligated target-plasmid DNA was isolated by a miniprep method that lysed cells in boiling water (Sambrook et al., 1989).

Miniprep template DNA was screened for microsatellites by PCR cycle sequencing with 33P-ATP (Murray, 1989). A total of 68 clones was sequenced, and 10 of these, representing a diversity of sizes, were originally selected for primer design and amplification. Primers were designed to flank loci containing at least 10 uninterrupted dinucleotide repeats, using OLIGO 4.0-s software (Rychlik, 1992), and amplified loci were then tested on at least 10 turtles representing both river systems to assess variability at each locus. This initial screening was carried out on small 8% polyacrylamide gels, and staining with EtBr. PCR conditions were optimized for six loci that varied in repeat length and allelic diversity (Table 1), and all samples were then screened for these markers by using a primer end-labeled with 33P-ATP or by incorporating 33P-dATP into PCR products. Products were run on 6% denaturing sequence gels, and allele sizes determined by comparison to a sequenced size standard run at several places on the same gel (see Fig. 4). Questionable genotypes were rerun along side the size standard marker and unambiguously scored individuals, and virtually every individual was scored for its genotype at all six loci.

Statistical Analyses. — Nucleotide diversity (Pi; Nei, 1987) and the average number of nucleotide differences (k; Tajima, 1983) were estimated for mtDNA haplotypes using the program DnaSP (ver. 2.52; Rozas and Rozas, 1997). As suggested by Rand (1996), we tested for mtDNA sequence

deviation from neutral expectations using algorithms of Tajima (1989) and Fu and Li (1993). Divergence in haplotype frequencies at both scales was tested by Monte Carlo randomizations in REAP (McElroy et al., 1992), and population subdivision between river systems was estimated by an estimator of Wright's (1931) F_{st} (θ; Weir and Cockerham, 1984) using FSTAT (Goudet, 1994). F_{st} and Nm were also estimated using the expressions of Lynch and Crease (1990) and Hudson et al. (1992), as implemented in DnaSP. An analysis of molecular variance (AMOVA; Excoffier et al., 1992) was used to partition variance (Φst) within and between river systems, using the observed number of substitution differences among sequences as the distance measure. The significance of observed values was compared to a null distribution of the test statistic generated by 3000 permutations.

Variation at microsatellite loci was summarized from observed and expected heterozygosities and tested for deviation from Hardy-Weinberg expectations, linkage disequilibrium, and allelic and genotypic frequency divergence among rookeries by Markov chain permutations in the program GENPOP (Raymond and Roussett, 1995). Pairwise estimates of population subdivision were obtained for rookeries within the Araguaia system, and between the Araguaia and Tapajós systems using unbiased estimates of F_{st} in FSTAT (Goudet, 1994) and Slatkin's (1995) R_{st} using R_{st}CALC (Goodman, 1997). The significance of both estimates was assessed by permutation tests (3000 replications). Both θ and R_{st} were used to derive gene flow estimates (Nm; Slatkin and Barton, 1989), as described by Slatkin (1995) and Goodman (1997). All significance levels for tests involving multiple comparisons were adjusted following the sequential Bonferroni correction described by Rice (1989).

RESULTS

Variation in mtDNA Control Region Sequences. — A total of 354 bp of control region sequence (both strands) was obtained from 73 turtles, sampled from the four rookeries in the following numbers: Ara-Praia (n = 17), Ara-Faz (n = 16), and Ara-Crix (n = 15), and (4) Rio-Tap (n = 25). Four

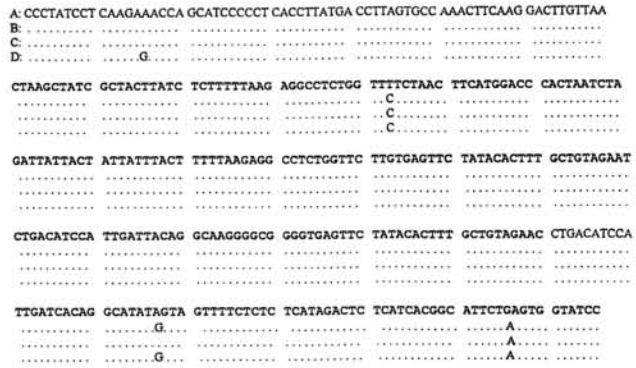

Figure 5. Sequence variation in a 346 bp region of the 5' end of the mtDNA control region, among four haplotypes (A–D) identified from 73 *P. expansa* sampled from all four rookeries.

haplotypes were present and are designated A – D (Fig. 5). Samples from the three upper Araguaia rookeries were fixed for mtDNA haplotype A, but the sample from the Tapajós rookery contained all four haplotypes in the following frequencies: A (16%), B (68%), C (12%), and D (4%). Nucleotide diversity (Pi) in the Tapajós sample was 0.0032, and the average number of nucleotide differences (k) was 1.113. Patterns of sequence variation conformed to expectations of neutral evolution by both Tajima's ($D = 0.14$, $p > 0.10$) and Fu and Li's tests ($D = 1.08$, $p > 0.10$). The nested analysis of molecular variance (AMOVA) showed that most of the variation was found between the two river systems (87.1%) rather than within systems (13.8%; $p < 0.0003$, with 3000 randomizations). This finding is supported by estimates of Nm that revealed very limited gene flow between the Araguaia and Tapajós river systems (Nm = 0.14, using both the N_{st} and F_{st} estimators described by Lynch and Crease [1990] and Hudson et al. [1992], respectively).

Variation in Microsatellite Loci. — Microsatellite loci were found in 37 of the 68 clones selected for sequencing. Of those, 22 were "perfect," or uninterrupted dinucleotide repeat motifs $(AC)_{6-32}$, and the rest were "compound" – two motifs of different dinucleotide repeats were juxtaposed. Each of the six loci scored in this study had at least 11 simple (dinucleotide) repeats, and all but Pod1 were compound (Table 1). All were moderately to highly polymorphic across all samples, with alleles ranging from 9–40 per locus (Pod62

Table 1. Summary of microsatellite loci and primer sequences used to screen all samples of *P. expansa*; all primer sequences are given in the 5' to 3' direction.

Locus	Primer Sequence	Repeat Motif	Size (bp) Range	No. of Alleles
Pod1	F-GATCTTTCTTTACAGGTGCAGTTC R-CACAACTAAATTACAGCACTCCG	$(CA)_{32}$	154-204	21
Pod62	F-ATGAGTGTGGAATGAGAGGAAC R-CCCATCCACAGAAGCAAATTCC	$(GT)_{11}(TA)_5$	182-214	9
Pod79	F-GGGAGAGCATTGCTGGTTGGTG R-CAATGTCATCACCGCAGAACCC	$(CT)_{13}(CA)_{16}$	220-260	16
Pod91	F-TCATTTTGGTTAGAAGTGAAGGC R-GGTTGTTCATCTTTTAGATTCACC	$G_9(GT)_{17}(GA)_8$	111-255	40
Pod128	F-GTGTCAGGGCTACCATCAAGATTG R-CCAGTAAAATTCACTACCAGCATG	$(GT)_{27}(GC)_7$	140-209	23
Pod147	F-GTGACAGCAGCATCTCATTTTCTC R-ATGACACATTACCATCCCATAGG	$(GT)_{16}(A)_{20}$	181-249	19

Table 2. Number of alleles per locus (A) and expected heterozygosity (H) at six microsatellite loci across four rookeries of *P. expansa* from the Brazilian Amazon Basin; numbers in parentheses are sample sizes.

Locus	Rookery			
	Ara - Praia (22)	Ara - Faz (24)	Ara - Crix (23)	Rio – Tap (25)
Pod1				
A	7	8	7	19
H	0.73	0.58	0.74	1.00
Pod62				
A	5	5	6	8
H	0.59	0.50	0.69	0.80
Pod79				
A	9	6	6	13
H	0.86	0.79	0.69	0.80
Pod91				
A	15	19	14	29
H	0.82	0.87	0.69	0.92
Pod128				
A	5	4	3	20
H	0.41	0.96	0.30	0.92
Pod147				
A	7	6	8	15
H	0.73	0.75	0.78	0.68
All loci				
A	7.3	8.0	7.3	17
H	0.69	0.74	0.65	0.85

and Pod91, respectively; Table 1). Individual rookeries had a range of 3–29 alleles per locus, and expected heterozygosities (H, averaged across all loci) ranged from 0.65 to 0.85 (Table 2). All three samples collected from the upper reaches of the Río Araguaia had consistently lower A and H values compared with the single sample collected from the downstream region of the Río Tapajós (Table 2). The distribution of alleles by size classes in the two river systems revealed two patterns of variation (Fig. 6). The allele distribution was characterized by a large number of unique alleles (82 of 127 total alleles), predominantly occurring within the Tapajós rookery (46.5% of all alleles). This pattern is particularly striking given that the total sample size for the Araguaia system was almost three times the size of the Tapajós sample, which affords a much greater probability of sampling unique alleles within the Río Araguaia.

Tests for deviation from Hardy-Weinberg equilibrium were not significant when corrected for multiple comparisons (i.e., corrected for six tests, based on six loci scored per sample), except for the Pod128 locus at the Rio-Crix rookery that showed a significant deficit of heterozygotes ($p < 0.01$; corrected $\alpha[0.01] = 0.0016$). This may indicate the presence of a non-amplifying (null) allele at this particular rookery (Pemberton et al., 1995). Linkage disequilibrium tests were carried out for all 15 pairwise comparisons at each of the four sites, and across the Araguaia samples com-

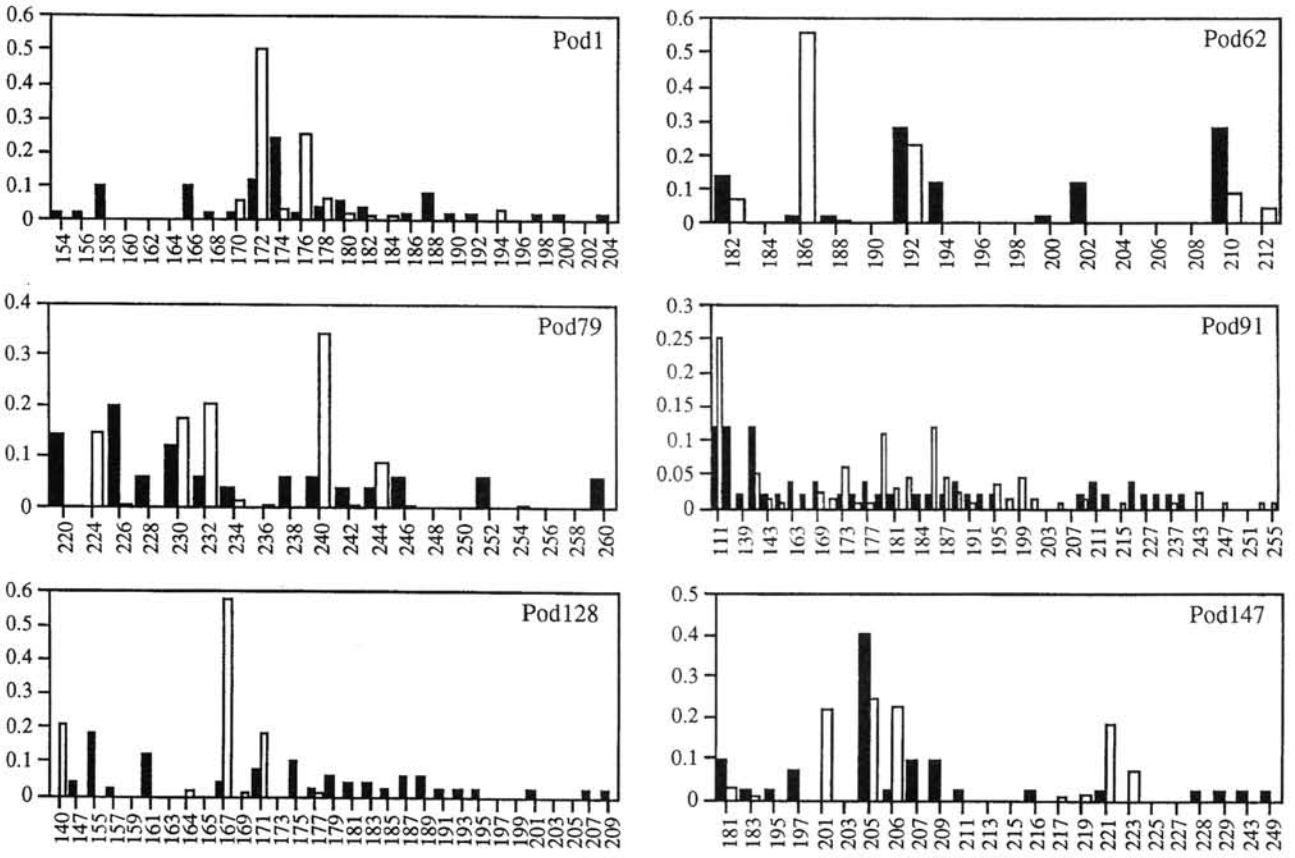

Figure 6. Distribution of allele frequencies between the Araguaia (three rookeries combined, open bars) and Tapajós (closed bars) river systems, for the six microsatellite loci resolved in *P. expansa*.

Table 3. Estimates of population subdivision and gene flow within and between river systems, across four rookeries of *P. expansa*, as estimated by F_{st} (Weir and Cockerham, 1984) and R_{st} (Slatkin, 1995), and Nm is estimated from the equation $Nm = 1/4\ (1/F_{st} - 1)$.

	Within Araguaia		Araguaia vs. Tapajós	
Locus	F_{st}	R_{st}	F_{st}	R_{st}
Pod1	0.002	0.021	0.15***	-0.018
Pod62	0.040*	0.023	0.20***	0.203***
Pod79	0.005	-0.005	0.098***	-0.018
Pod91	0.002	0.001	0.024**	-0.014
Pod128	-0.013	-0.018	0.23***	0.224***
Pod147	0.003	-0.007	0.084***	-0.002
All loci	0.006	0.002	0.130***	0.063***
	$Nm(R_{st}) = 125$		$Nm(R_{st}) = 3.72$	
	$Nm(F_{st}) = 41.4$		$Nm(F_{st}) = 1.67$	

* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

bined. None of these revealed significant disequilibrium after corrections for multiple tests. Thus the six microsatellite loci were considered to be statistically independent in further tests.

Within the Río Araguaia, tests for allele frequency differences at five of six loci, and all loci combined, revealed no significant divergence across the three rookeries sampled, as estimated by both F_{st} and R_{st} (Table 3). In contrast, all allele frequency tests showed significant differences between the Araguaia and Tapajós rookeries ($R_{st} = 0.063$, and $F_{st} = 0.13$, $p < 0.001$). Estimates of Nm revealed a high level of nuclear gene flow within the Río Araguaia ($Nm_{[Rst]} = 125$; $Nm_{[Fst]} = 41.4$), but between river systems gene flow was substantially reduced ($Nm_{[Rst]} = 3.72$; $Nm_{[Fst]} = 1.67$). In each of these comparisons, estimates of Nm based on R_{st} were higher than those based on F_{st}.

DISCUSSION

Allele frequency distributions at five of the six microsatellite loci reflect a pattern expected of extensive nuclear gene flow among rookeries in the upper Río Araguaia system. Nm estimates of between-population migration per generation were high and the same common alleles were shared across all three rookeries. All individuals assayed for mtDNA all shared the same haplotype (A), so we could not distinguish between male and female-biased contributions to population gene flow at this scale.

The approximate distances between the Ara-Pra and Ara-Faz rookeries is about 100 km by river, and the distance between each of these two and the Ara-Crix site is about 275 and 205 km, respectively (Fig. 2). It would certainly not be surprising for turtles of the size and probable longevity of *P. expansa* to (at least) migrate this far. In the Orinoco River, for example, Ojasti (1967) reported results of a study in which 2576 adult female *P. expansa* were tagged on nesting beaches, and returns reported on 316 turtles captured in subsequent months showed extensive movements both upstream (to a distance of ca. 100 km) and downstream (up to a few hundred km in a few cases) from the main rookery (Playa del Medio). Many recaptures also came from smaller tributaries of the Orinoco, suggesting widespread dispersal during the rainy season following nesting. Similarly, limited radio-tracking data for *P. expansa* in the Río Trombetas in Brazil, show movements of up to 45 km in two days by adult females after nesting (Vogt, in press). These data suggest that either the use of non-natal nesting beaches by some females is enough to produce a single, random-mating population among these three rookeries within the upper Río Araguaia system, or that the time frame of colonization has been too recent to allow the generation of unique mtDNA haplotypes. The lack of observed genetic structure at microsatellite loci may result from either male or female migratory behavior.

In strong contrast to this regional pattern, however, both classes of markers show strong structure between the Araguaia and Tapajós rookeries. The F_{st} and R_{st} average values show significant structure at the $p < 0.001$ level (Table 3), as does the distribution of mtDNA haplotyes. Estimates of Nm (derived from F_{st} and R_{st}) are of similar magnitude and are much smaller than the "within Araguaia" gene flow rates. For reasons given below, we suspect that the F_{st} estimator is more accurate, and note that the low $Nm_{[Fst]}$ of 1.67 is likely to allow fixation of alleles within each river system (reviewed by Mills and Allendorf, 1996). This view is supported by the high proportion of unique alleles observed within each river system (35.2% of 128 total alleles). If the populations sampled here are in approximate mutation-drift equilibrium, then these two river systems are only occasionally interconnected by gene flow, and behave as demographically independent units over ecological time (Moritz, 1994, 1995). Further, a reduced number of alleles was observed within the Araguaia in comparison to the Tapajós (67 and 104, respectively), suggesting variation in historic population size or colonization history.

From a conservation perspective, demographically independent units should be managed as separate entities, but the sampling design we used is inadequate to define the geographic limits of these units. Turtles may move considerable distances within river systems but rarely move between them, in which case most of the genetic structure will be partitioned between river systems. In this case, the individual watersheds become the units of management. Alternatively, the Araguaia and Tapajós rookeries may simply reflect isolation-by-distance, which would be expected in a river-dwelling organism characterized by linear "stepping-stone" population structure in which gene flow is largely confined to exchanges between adjacent breeding units in a one-dimensional space (i.e., up- or downstream; Kimura and Weiss, 1964; Slatkin, 1993). The river distance (ca. 2400 km) between the Tapajós rookery and confluence of the Río Crixas Açu with the Río Araguaia (Fig. 2) is an order of magnitude greater than distances among the Araguaia rookeries, but given our sampling design, we cannot yet test this possibility.

One issue requiring comment is the large discrepancy in F_{st} and R_{st} estimators, with the F_{st} estimate approximately twice that of R_{st} across loci between the two river systems (Table 3). These estimators are based on different models of mutation, and their usefulness is affected by the relative influences of gene flow, mutation rate, and coalescence time of the populations being compared. The F_{st} (θ) estimate of Weir and Cockerham (1984) is based on the "infinite alleles model" (IAM; Kimura and Crow, 1964) wherein each new mutation produces a novel allele, and allelic similarity between samples is inferred to result either from gene flow or a shallow coalescence time. In contrast, Slatkin's (1995) R_{st} estimator takes into account allele size differences under a "stepwise mutation model" (SMM; Ohta and Kimura, 1973), wherein mutational events retain a "memory" of allelic relationships (Slatkin, 1995). If coalescence time is recent, an IAM-based F_{st} estimator is expected to be more accurate because genetic drift would be more important than mutation in creating local differentiation (Slatkin, 1995).

We can gain some insight into the relative importance of these processes (drift, gene flow, and mutation) at the within- versus between-river system scales by considering both classes of markers collectively. Two different patterns are evident. First, within the upper Araguaia system, the absence of any mtDNA divergence among the three rookeries suggests a relatively recent colonization of this area, and both the high Nm estimates (Table 3) and widespread sharing of the same common alleles suggests recent gene flow or recent coalescence as the overriding force structuring these rookeries. Between river systems, however, mtDNA haplotype divergence implies a much longer coalescence time, and inspection of microsatellite allele distributions (Fig. 6) reveals the presence of many low frequency unique alleles in the Tapajós sample that are absent from the Araguaia samples. Further, alleles that are shared between river systems often differ by large frequencies (Fig. 6). These patterns imply that both drift and mutation strongly override gene flow as the dominant forces structuring the Araguaia and Tapajós populations.

The pattern of variation in allele frequency between these two river systems is also consistent with expectations of a "cryptic" bottleneck (i.e., a genetic bottleneck not observable from decreased census sizes) within the Araguaia rookeries. Such bottlenecks may result from either high variance in family sizes, and/or skewed sex ratios (Luikart et al., 1998). We suggest that both of these factors may be operating to create an "attenuation effect" associated with smaller turtle populations nesting in the upper reaches of the large Amazonian rivers, relative to the lower reaches. In absolute terms, rookeries near the mouths of the large rivers may draw on a much larger total population of turtles, and thereby be expected to segregate for more alleles at a given locus. We suggest that the absence of rare alleles among the Araguaia rookeries is due to either a cryptic genetic bottleneck resulting from upstream attenuation of population size, and/or a stepping-stone structure and isolation-by-distance with the smaller populations being further upstream. This proposition could be easily tested by more extensive sampling within and between other river systems in the Amazon Basin. Further, because of the difficulty of estimating dispersal in long-lived species that roam over large areas (Koenig et al., 1996), and in determining natal fidelity through tagging studies, these genetic markers should be ideal for determining the metapopulation structure of *P. expansa* throughout its range.

Resumo

A tartaruga da Amazônia (*Podocnemis expansa*) é pouco conhecida ecologicamente e, como as tartarugas marinhas, é uma espécie colonial de alta longevidade que pode migrar grandes distâncias geográficas. Na ausência de estudos de marcação a longo prazo e de outras informacões ecológicas, nós inferimos o fluxo gênico e a estrutura de populacão entre áreas de postura a partir de estudos de distribuição geográfica de alelos nucleares e mitocondriais em loci independentes. Foi estabelecida uma biblioteca genética para essa espécie sendo otimizadas as condições de PCR para 6 loci de microsatélites, os quais foram então testados em 94 indivíduos coletados em quatro áreas de postura diferentes em duas bacias fluviais (Araguaia e Tapajós) da Amazônia brasileira. Foram obtidas sequências de DNA mitocondrial da parte 5' da região de controle (354 pb) de uma amostra de 73 tartarugas, e a estratégia amostral permitiu uma comparação entre a estrutura populacional intra e inter-bacias fluviais. Os padrões de variabilidade genética nos loci de genes nucleares sugerem um extenso fluxo gênico intra-bacia fluvial mas muito pouco entre bacias. Esses resultados sugerem que uma extensa amostragem intra-bacias fluviais provavelmente não é necessária para elucidar o fluxo gênico e a estrutura de metapopulação. Contudo, é necessário es forço de coleta abrangendo uma ampla área geográfica bem como entre as principais bacias fluviais.

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