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Testing the Effects of Barriers on the Genetic Connectivity in *Podocnemis erythrocephala* (Red-Headed Amazon River Turtle): Implications for Management and Conservation

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Abstract. – In contrast to the other *Podocnemis* species, *Podocnemis erythrocephala* (Pleurodira: Podocnemididae) is restricted to blackwater and clearwater rivers and lakes, including the Negro River basin, and several other tributaries of the eastern Amazon River basin. In order to test the effects of geographic barriers on genetic connectivity of *P. erythrocephala*, 10 localities sampled throughout the Brazilian Amazon basin were studied. Two hundred and forty-six specimens were sequenced for the mitochondrial control region. Analysis of molecular variance confirmed the existence of population subdivision ($\Phi_{ST} = 0.28$, $p < 0.001$). In pairwise comparisons, the values of the fixation index Φ_{ST} were significant in almost all comparisons involving the São Gabriel da Cachoeira (Negro River), Jaú National Park (Jaú River), and Barreirinha (Andirá River), indicating that these populations are genetically differentiated. Genetic differentiation is most likely explained by the presence of rapids and waterfalls separating the São Gabriel da Cachoeira (Negro River) and Jaú National Park (Jaú River) populations from the remaining populations. Populations from Barreirinha (Andirá River) were also genetically differentiated, providing support for the hypothesis that the Amazon River is a geographic barrier for this species. Although distributed over a large geographic area, the remaining 7 localities were not differentiated from one another ($p > 0.005$), suggesting that these localities are part of a panmictic population distributed throughout the central Amazon basin. We recommend that the 4 structured populations be treated as separate management units.

Key Words. – Reptilia; Testudines; chelonians; Amazon basin; Irapuca; rapids; river barrier

The 7 million square kilometers of the Amazon rain forest with its numerous streams, rivers, and lakes draining a great variety of geological formations, soils, and vegetation types offer many niches for aquatic life (Smith 1979). Within the region, at least 15 species of freshwater chelonians occur (Vogt 2001). The Brazilian Amazon is also the center of diversity of the genus *Podocnemis*, with 4 of 6 recognized species occurring in the region—*Podocnemis expansa* (Schweigger 1812), *Podocnemis sextuberculata* (Cornalia 1849), and *Podocnemis unifilis* (Troschel 1848)—all 3 of which are widely distributed throughout the Amazon basin, and *P. erythrocephala* (Spix, 1824), which has a more restricted distribution in the Negro River basin and several Amazon River tributaries, including the Nhamundá, Trombetas, Tefé, and Tapajós rivers (Mittermeier and Wilson 1974; Hoogmoed and Ávila-Pires 1990; Rebêlo 1991; Andrade et al. 2007; Rueda-Almonacid et al. 2007; Mittermaier et al. 2015).

Ecologically, *P. erythrocephala* is restricted to waters lacking suspended sediment, that is, the blackwater and clearwater rivers and lakes, and it does not occur in the sediment-laden whitewater Amazon River itself (Rueda-Almonacid et al. 2007; Mittermaier et al. 2015). The species is popularly known as "irapuca," "calalumã," "tracajá-piranga," and "piranguinha." Its eggs are consumed in large quantities throughout most of the Negro River basin (Vogt 2001; Bernardes et al. 2014), in part due

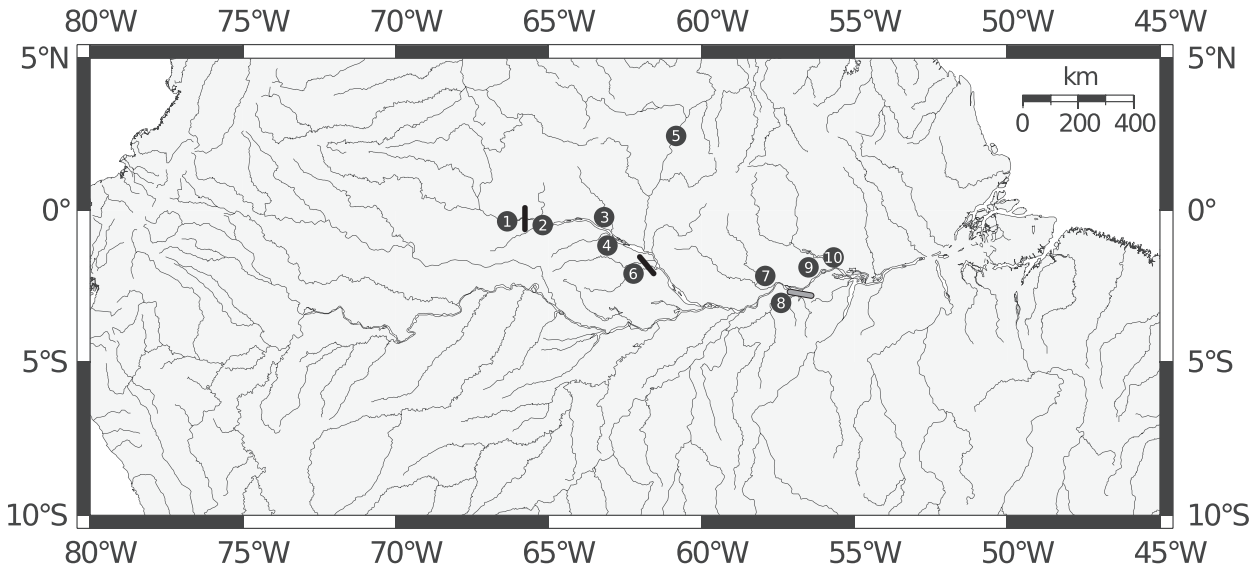

Figure 1. Localities sampled for individuals of *Podocnemis erythrocephala*. 1) São Gabriel da Cachoeira (Negro River), 2) Santa Isabel do Rio Negro (Ayuanã River), 3) Rio Cumicuri River, 4) Barcelos (Itu River), 5) Viruá National Park (Iruá Creek), 6) Jaú National Park (Jaú River), 7) Nhamundá (Paracutu River), 8) Barreirinha (Andirá River), 9) Terra Santa (Jamary Creek, community Alema), and 10) Oriximiná (Sapucuá Lake). The rapids and waterfalls are represented by a black bar, and the Amazon River as a barrier is represented by a gray bar.

to depletion of larger species of *Podocnemis* in the basin, the lack of conservation and management programs, and the lack of enforcement of existing laws in small riverine towns and communities (Mittermeier and Wilson 1974). As observed by Batistella and Vogt (2008) in the Santa Isabel do Rio Negro region, it is very common for entire riverbank families to visit nesting areas during the egg-laying season, and on these occasions it is not uncommon for 100% of the oviposited eggs to be collected. Various authors also describe similar situations for other species of chelonians and other regions of South America (e.g., Hildebrand et al. 1988; Mitchel and Quiñones 1994; Pantoja-Lima et al. 2014). During the nesting season, *P. erythrocephala* females are also captured in the nesting areas. Due to the intense pressure on eggs and on females, this species is classified by the International Union for Conservation of Nature (2015) as vulnerable. Conservation and management of this species are, however, limited by lack of ecological, population, and genetic data that are necessary for a sustainable management program (Fachín-Terán and Vogt 2004).

Several molecular studies investigating the population structure of *Podocnemis* species in the Amazon, Orinoco, and Magdalena river systems have been published (Sites et al. 1999; Bock et al. 2001; Pearse et al. 2006; Escalona et al. 2009; Silva et al. 2011; Vargas-Ramírez et al. 2012). While some noted a lack of (Bock et al. 2001; Silva et al. 2011) or weak (Vargas-Ramírez et al. 2012) population genetic structure, the studies of Escalona et al. (2009), Sites et al. (1999), and Pearse et al. (2006) suggested correlations between genetic distance and geographic distance for the species *P. unifilis* and *P. expansa*, respectively.

Genetic studies of *P. erythrocephala* to date are summarized in the chromosomal characterization of the species (Rhodin et al. 1978), the phylogenetic relationships of *P. erythrocephala* to other species of the genus *Podocnemis* (Vargas-Ramírez et al. 2008), DNA barcode characterization of the species of the genus *Podocnemis* (Reid et al. 2011), and the pattern of multiple paternity found in natural populations (Fantin et al. 2010). The present work is the first population genetic study of *P. erythrocephala* to assess genetic diversity and its distribution using an extensive data set of mitochondrial control region sequences and tests 2 a priori hypotheses to explain genetic structuring. First, because *P. erythrocephala* has a broad distribution in the tributaries of the topographically complex Negro River basin, characterized by numerous waterfalls and rapids in some of its tributaries, we predict that permanent waterfalls are geographic barriers to gene flow between upstream and downstream populations (localities 1 and 6 in Fig. 1). Second, because *P. erythrocephala* is restricted to blackwater or clearwater lakes, streams, and small rivers (Rueda-Almonacid et al. 2007; Mittermaier et al. 2015) and considering that its areas of distribution are separated by large whitewater rivers such as the Amazon (locality 8 in Fig. 1), we hypothesize that the main channel of the Amazon River may act as a geographic barrier to or restrict gene flow.

METHODS

Field Collection. — Blood samples of about 50 µl were withdrawn from each individual via femoral vein puncture with syringes containing an NE buffer (NaCl 100 mM and EDTA 10 mM, pH 8) as an anticoagulant and then preserved in tubes with absolute ethyl alcohol, labeled

with a field number, and stored in a refrigerator (4°C) until processing. Individuals were released in the same areas where they had been captured. The blood samples were deposited in the Animal Genetic Sample Collection of the Laboratório de Evolução e Genética Animal of the Universidade Federal do Amazonas (UFAM), Brazil. The collecting sites (Fig. 1) were 10 localities from throughout the known distribution of *P. erythrocephala* in Brazil: 1) São Gabriel da Cachoeira (Negro River; $n = 18$), 2) Santa Isabel do Rio Negro (Ayuanã River; $n = 34$), 3) Cumicuri River ($n = 29$), 4) Barcelos (Itu River; $n = 29$), 5) Viruá National Park (Iruá Creek; $n = 10$), 6) Jaú National Park (Jaú River; $n = 34$), 7) Nhamundá (Paracutu River; $n = 23$), 8) Barreirinha (Andirá River; $n = 33$), 9) Terra Santa (Jamary Creek, community Alema; $n = 17$), and 10) Oriximiná (Sapucuá Lake; $n = 19$).

Laboratory Methods. — Genomic DNA was isolated from blood by dissolving and digesting samples with a Proteinase K/SDS solution, followed by successive washes in phenol, phenol/chloroform/isoamyl alcohol, and hydrated chloroform; a subsequent addition of 5 M NaCl; and a final addition of 70% ethyl alcohol for DNA precipitation (Sambrook et al. 1989). The mitochondrial control region was amplified via polymerase chain reaction (PCR) using primers PRO (5′-CCCATCACCCACTCCCAAAGC-3′; Sites et al. 1999) and the conserved primer 12SR5 (5′-GGCGGATACTTGCATGT-3′; Hrbek and Farias 2008). For the amplification of each sample, we used 0.8 µl of DNA (approximately 30 ng), 2 µl of each 2-µM primer (PRO and 12SR5), 2.5 µl of buffer (200 mM Tris-KCl, pH 8.5), 1.5 µl of 25-mM $MgCl_2$, 2.5 µl of 10-mM dNTP, 0.2 µl of *Taq* polymerase enzyme (5 U/µl), and 13.5 µl of ddH_2O in a final reaction volume of 25 µl. The PCR reactions were performed under the following cycling conditions: 35 cycles consisting of 1 min at 92°C (denaturation), 35 sec at 55°C (annealing of primers), 1 min at 72°C (primer extension), and final extension at 72°C for 5 min. The PCR products were subjected to purification by precipitation with sodium acetate (3 M, pH 4.8) and 95% ethanol.

The sequence reactions were performed in a final reaction volume of 10 µl using the DYEnamic ET dye terminator kit (GE-Healthcare, São Paulo, Brazil). Each reaction had the following composition: 4 µl of amplified DNA, 2 µl of 2-µM primer, 2 µl of ET-terminator, and 2 µl of ddH_2O. The samples were then subjected to a 35-cycle thermocycling protocol with the following temperature profile: 20 sec at 95°C (denaturation), 15 sec at 55°C (annealing of primers), and 1 min at 60°C (primer extension). At the end of the PCR reaction, samples were purified according to manufacturer's instructions (GE-Healthcare). Subsequently, the samples were subjected to electro-injection and the sequences were resolved on an automatic sequencer MegaBACE 1000 DNA Analysis System (GE-Healthcare, São Paulo, Brazil) following the manufacturer's instructions.

Descriptive Statistics. — The sequences resolved on the automated sequencer were imported and manually verified in the program BioEdit (Hall 1999). Alignment was carried out in BioEdit using the program ClustalW (Thompson et al. 1996) under default conditions. Genetic diversity was estimated from gene diversity ($\hat{H}$; Nei 1987), nucleotide diversity (Π; Nei and Li 1979), the number of haplotypes of the mtDNA, and the number of segregating sites (S). These measurements of genetic variability were estimated with the programs Arlequin v3.5 (Excoffier and Lischer 2010) and DNAsp v5 (Librado and Rozas 2009). Relationships among haplotypes were estimated within a network of haplotypes based on a maximum likelihood phylogenetic tree topology using the program HAPLOVIEWER (Salzburger et al. 2011).

Population Structure and Gene Flow Estimates. — Population structure was assessed using the analysis of molecular variance (AMOVA; Excoffier et al. 1992) to test the hypotheses that rapids are barriers to gene flow and that the sediment-laden Amazon River is a barrier to gene flow for the clearwater- and blackwater-inhabiting irapuca. The AMOVA; the Φ_{ST}, which is analogous to F_{ST} (Weir and Cockerham 1984); and the Mantel test (Mantel 1967) were implemented in the software Arlequin v3.5 (Excoffier and Lischer 2010). Population structure was assessed with the Bayesian analysis of population structure in BAPS v5.2 (Corander et al. 2003), which infers the most likely number of genetic clusters within an analyzed sample. The program BAPS treats the nucleotide frequencies and the predefined number of clusters as random variables and jointly calculates the posterior distributions of the population structure and allele frequencies. Uncertainty of the unknown population structure (i.e., the probability that allele frequencies are statistically not different from each other) is estimated for all pairwise cluster comparisons, and final posterior likelihood(s) of population structure is estimated.

An approximate estimate of gene flow (Nm) between sampled localities was calculated from Φ_{ST} under the island-migration model, in which $\Phi_{ST} = 1/(Nm + 1)$. However, considering that real populations are likely to violate many of the assumptions of the above relationship (Whitlock and McCauley 1999), we also estimated female-mediated gene flow using the maximum likelihood framework in the program MIGRATE v2.4.4 (Beerli and Felsenstein 2001). We ran 10 short chains, sampling each chain 10,000 times, and then 6 long chains, sampling each chain 1,100,000 times and discarding the first 100,000 samples as burn-in.

The hypothesis of isolation-by-distance was tested by the Mantel test (Mantel 1967). Values of $\Phi_{ST}/(1 - \Phi_{ST})$ were correlated with river distances in kilometers between each pair of sampling localities (Fetzner and Crandall 2003). The significance of the Spearman correlation (Spearman 1904) was tested with 10,000 permutations in the software Arlequin v3.5 (Excoffier and Lischer 2010).

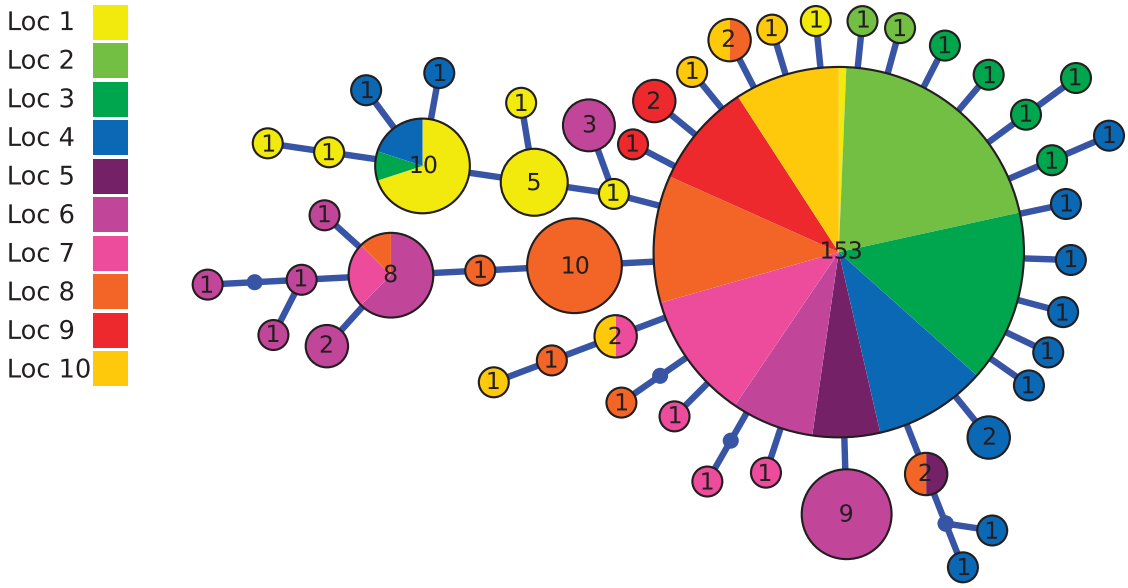

Figure 2. Haplotype network from the individuals of *Podocnemis erythrocephala*. For locality numbers, see Fig. 1. Circles with numbers represent observed haplotypes and their frequencies. Unlabeled small circles represent missing haplotypes. Haplotypes are connected by single mutational steps represented by a line. (Color version is available online.)

Demographic History. — Population demography was estimated by mismatch distribution in 2 different ways: in the program DnaSP v5 (Librado and Rozas 2009), where the distributions of pairwise differences are compared with an expected distribution under a null model of a stationary population, and in the program Arlequin v3.5 (Excoffier and Lischer 2010), which estimates the sums-of-squared deviation and Harpending's raggedness index (Harpending 1994), with significance tested through 10,000 permutations under the null model of population expansion. Tajima's *D* (Tajima 1989) and Fu's *Fs* (Fu 1997) tests were used to verify that the samples were at mutation–migration–drift equilibrium. Significant values for these tests could indicate that the mitochondrial sequences are not evolving according to the hypothesis of selective neutrality (i.e., they are not in a mutation–drift equilibrium) or that the populations were previously subdivided and/or experienced fluctuations in the past (i.e., they are not in migration–drift equilibrium; Hartl and Clark 2006). The significance of both statistics was tested by comparisons of the statistics against a distribution generated from 10,000 random samples on the assumptions of selective neutrality and population equilibrium. The tests were implemented with the program Arlequin v3.5 (Excoffier and Lischer 2010).

We estimated the female effective population sizes (*Nef* since we are dealing with an mtDNA marker) based on coalescence theory using Watterson's estimate of the population genetic parameter θ from *s* (Watterson 1975) and assuming a mutation rate of 2.48×10^{-7} mutations per site per year for the control region; this estimate was derived from *Chelonia mydas* (Chassin-Noria et al. 2004). The genetic parameter θ was derived from the analysis implemented in the program MIGRATE v2.4.4 (Beerli and Felsenstein 2001).

RESULTS

We sampled a total of 246 specimens of *P. erythrocephala*, all sequenced for 550 base pairs of the mitochondrial control region. The average base composition of this DNA fragment was 27% adenine, 32% thymine, 28% cytosine, and 13% guanine, confirming the low percentage of guanine in the nucleotide composition of the mitochondrial genome (Zhang and Hewitt 1996). Of the 550 sites analyzed, 40 sites were variable. Of these sites, 30 were transition mutations, and 10 were transversions. We found 49 haplotypes, of which 36 were unique (Fig. 2). The most frequent haplotype (H3) occurred in all localities, suggesting that this haplotype is most likely ancestral (Templeton 1998).

The genetic and demographic parameters estimated for *P. erythrocephala* localities are summarized in Table 1 and indicate different levels of genetic variability throughout the distribution of the species. The AMOVA revealed a high degree of population structuring ($\Phi_{ST} = 0.28$, $p < 0.001$); 28% of the total variance occurred among sampling localities. Some stretches of the Negro River basin cross rocky ridges and are characterized by a large number of waterfalls and rapids. These rapids separate the sampling localities of São Gabriel da Cachoeira and Jaú National Park (localities 1 and 6 in Fig. 1) from the others localities of the Negro River basin. Based on pairwise Φ_{ST} analyses (Table 2), all the comparisons involving São Gabriel da Cachoeira showed differences ($p < 0.05$). The Jaú National Park population was also found to be genetically structured in relation to the other localities analyzed, except in relation to Terra Santa (locality 9). In

Table 1. Genetic and demographic parameters estimated for *Podocnemis erythrocephala*.[a]

Localities	*n*	*S*	NH	Gene diversity ($\hat{H}$)	Nucleotide diversity per site (Π)	Mismatch distribution analysis			Neutrality tests	
						DnaSP-HRI (*p*-value)	Arlequin-HRI (*p*-value)	Arlequin-SSD (*p*-value)	Tajima's *D*	Fu's *Fs*
1	18	6	8	0.797 ± 0.074	0.00265	0.2191 (0.113)	0.0613 (0.582)	0.0015 (0.899)	−0.54150	−3.704
2	34	2	3	0.116 ± 0.074	0.00021	0.4251 (0.668)	0.6056 (0.746)	0.0002 (0.314)	−1.49966	−2.516
3	29	8	7	0.377 ± 0.115	0.00112	0.3155 (0.333)	0.1832 (0.574)	0.0125 (0.550)	−2.13647*	−4.719*
4	29	14	13	0.736 ± 0.089	0.00307	0.1817 (0.256)	0.0816 (0.975)	0.2054 (0.004)	−1.76555	−8.339*
5	10	1	2	0.200 ± 0.154	0.00036	0.3147 (0.923)	0.4000 (0.211)	0.3310 (0.102)	−1.11173	−0.339
6	34	7	9	0.813 ± 0.041	0.00289	0.1875 (0.128)	0.0557 (0.492)	0.0016 (0.746)	−0.20147	−2.824
7	23	6	6	0.458 ± 0.126	0.00109	0.3138 (0.101)	0.1039 (0.647)	0.0000 (0.999)	−1.92926	−3.700*
8	33	7	8	0.657 ± 0.066	0.00169	0.2650 (0.177)	0.1026 (0.265)	0.0067 (0.342)	−1.32833	−4.076*
9	17	2	3	0.324 ± 0.136	0.00061	0.3439 (0.363)	0.2218 (0.436)	0.0032 (0.522)	−1.06916	−1.038
10	19	6	6	0.468 ± 0.140	0.00131	0.2937 (0.186)	0.1301 (0.659)	0.0077 (0.451)	−1.86971	−3.389*
All	246	40	49	0.608 ± 0.037	0.00217	0.2155 (0.042)	0.0391 (0.908)	0.0011 (0.870)	−2.34487*	−28.964*

[a] *n* = number of individuals; HRI = Harpending's raggedness index; SSD = sum of squared deviations; * indicates level of significance after Bonferroni correction ($p < 0.005$). For locality numbers, see Fig. 1.

addition to physical barriers such as rapids and waterfalls, one of the localities (Barreirinha, locality 8) is separated physicochemically by the sediment-rich Amazon River. This effect is not as strong, however, as Barreirinha is still genetically differentiated from all but the 2 localities (Terra Santa, locality 9, and Viruá National Park, locality 5).

Analysis in BAPS (Fig. 3) partitioned the populations into 3 biological groups of populations (ln likelihood = –676.8664). The 3 groups were composed of individuals principally from São Gabriel da Cachoeira (group 1, blue), individuals principally from Jaú National Park and Barreirinha (group 2, green), and all remaining individuals (group 3, red). The isolation of these 3 localities was also evident based on Φ_{ST} estimates (Table 2), which was high between pairs of populations involving these 3 localities. Gene flow was also very restricted or nonexistent when these localities were involved, and where gene flow was detected, it tended to be from these 3 localities to the main Amazonian population (Table 3).

The Mantel test for isolation by distance indicated no correlation between genetic and geographic distances ($r = 0.17$, $p = 0.26$). Removing the Negro and Jaú River localities from the data set also resulted in no correlation ($r = 0.084$, $p = 0.33$), as did the removal of the Barreirinha locality ($r = 0.074$, $p = 0.43$).

The results of a mismatch distribution test performed in DnaSp v5 (Librado and Rozas 2009) for all localities presented a curve that fits the expected distribution of an expanding population (raggedness statistic $r = 0.039$); however, for single localities, no differences were observed (Table 1). The observed mismatch distribution tests performed in Arlequin v3.5 (Excoffier and Lischer 2010) rejected the null model of sudden population expansion, with both the observed and the expected curves indicative of constancy of population size, with the exception of Barcelos (locality 4), for which the hypothesis of population expansion could not be rejected. Tajima's *D* and Fu's *Fs* tests indicated that some localities were not in mutation–migration–drift equilibrium with respect to their mitochondrial DNA (Table 1). Whereas Tajima's *D* test was significant ($p < 0.005$) only for the Cumicuri River (locality 3), the *Fs* value of the Fu test showed significant deviation from the neutral expectation in 5 localities. However, when the localities were considered jointly, the 2 statistical tests indicated a genetic disequilibrium. The female effective population size (*Nef*) for *P. erythrocephala*, derived from its genetic diversity (Watterson's θ and Φ_{ST}), was estimated at approximately 100,000 individuals.

DISCUSSION

Genetic Diversity and Demography. — The aim of this study was to assess genetic diversity and its distribution among populations of *P. erythrocephala* and to test whether 1) rapids and waterfalls are effective

Table 2. Indirect estimates of gene flow (*Nm*; above the diagonal) and genetic differentiation (Φ_{ST}; below the diagonal) between pairs of populations of *Podocnemis erythrocephala*.[a]

Localities	1	2	3	4	5	6	7	8	9	10
1	—	**0.160**	**0.287**	**0.673**	**0.293**	**0.436**	**0.276**	**0.298**	**0.257**	**0.305**
2	0.757*	—	46.212	7.702	22.733	1.830	20.972	2.352	8.894	12.733
3	0.635*	0.010	—	27.671	∞	2.515	47.989	3.513	40.465	35.311
4	0.426*	0.060*	0.017	—	∞	3.175	13.048	4.077	14.634	16.374
5	0.630*	0.021	−0.023	−0.005	—	3.647	∞	4.965	32.832	∞
6	0.534*	0.214*	0.165*	0.136*	0.121*	—	3.433	2.243	2.663	3.296
7	0.643*	0.023	0.010	0.036	−0.016	0.127*	—	4.278	26.534	∞
8	0.626*	0.175*	0.125*	0.109*	0.091	0.182*	3.433	—	3.380	4.551
9	0.660*	0.053	0.012	0.033	0.015	0.158	0.018	0.129*	—	22.689
10	0.620*	0.037	0.014	0.029	−0.013	0.132*	−0.012	0.098	0.022	—

[a] * indicates significance level at $p < 0.001$; *Nm* values below 1 are in boldface. For locality numbers, see Fig. 1.

geographic barriers to gene flow and 2) the main channel of the Amazon River is as an effective barrier to gene flow. Average gene diversity ($\hat{H} = 0.608 \pm 0.037$) and nucleotide diversity ($\Pi = 0.00217$) observed in *P. erythrocephala* were similar to values observed by Pearse et al. (2006) in *P. expansa* ($\hat{H} = 0.65 \pm 0.305$; $\Pi = 0.00256$). However, both gene and nucleotide diversity were highly heterogeneous among the sampled localities (Table 1). The haplotype network showed a common haplotype found in most of the localities; however, groups of haplotypes separated by more than 1 mutational step from the common haplotype were observed for São Gabriel da Cachoeira (locality 1), Barcelos (locality 4), Jaú National Park (locality 6), and Barreirinha (locality 8), each of which showed a certain level of geographic structuring in relation to the other localities (Fig. 2).

The genetic diversity indices and the significantly negative values in the Tajima's *D* and Fu's *Fs* statistical tests ($p < 0.05$) observed for individuals from the Cumicuri River (locality 3) indicate that this population may be undergoing or has already undergone recent population expansion. The Barcelos (locality 4), Nhamundá (locality 7), Barreirinha (locality 8), and Oriximiná (locality 10) populations did not present significant values for the Tajima's *D* test, but Fu's *Fs* test was significant. Both tests are negative when there is an excess of recent mutations, and negative values are interpreted as evidence of population growth and/or diversifying selection (Tajima 1989; Fu 1997). According to (Ramos-Onsins and Rozas 2002), the *Fs* statistic is more sensitive to detection of population expansion and takes into account the influence of sample size in the analysis. Interestingly, the majority of samples that showed population expansion are located in the Negro River basin. Despite the fact that the neutrality tests were significant when all localities were analyzed together, the results of demographic tests (mismatch distribution) of individual localities and all localities pooled together suggest that *P. erythrocephala* is in mutation–drift equilibrium. The lack of equilibrium observed in the neutrality tests when all localities are analyzed together is an artifact of the analysis and indicates population structure (Hartl and Clark 2006), as

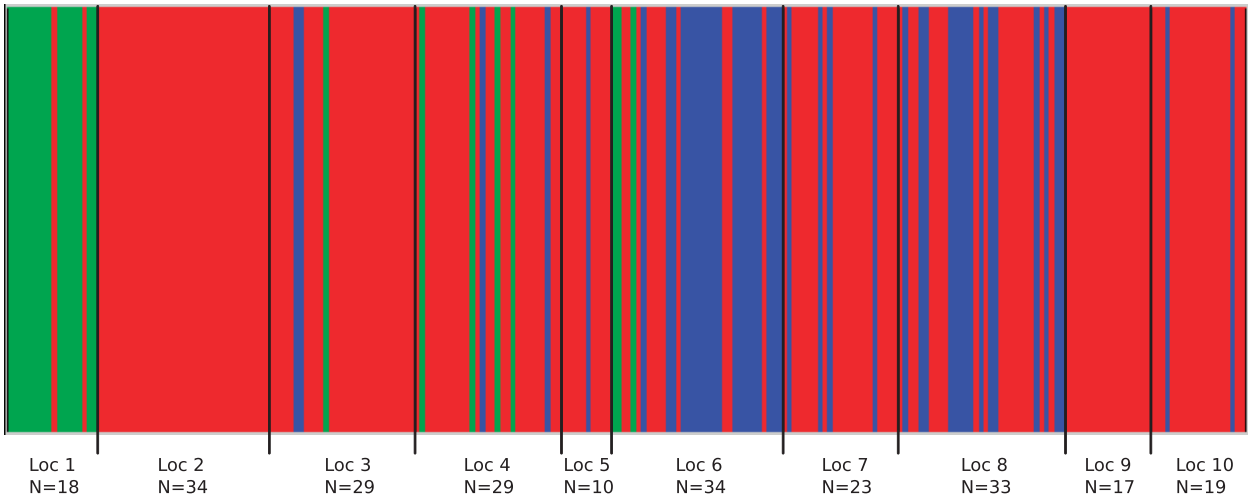

Figure 3. Biological population groups estimated by Bayesian analysis of population structure in the program BAPS. BAPS partitioned individuals into 3 biological groups (Ln likelihood = –676.8664) represented by the red, blue, and green bars; thickness of a bar is correlated to the number of individuals. Geographic localities are separated by vertical black lines. The 3 biological groups were composed of individuals principally from São Gabriel da Cachoeira (group blue), individuals principally from Jaú National Park and Barreirinha (group green), and all remaining individuals (group red). For locality numbers, see Fig. 1. (Color version is available online.)

Table 3. MIGRATE analysis showing pairwise estimates of gene flow.[a]

Localities	Theta (4*Ne* mu)	4*Nm* (*x* = receiving population) 1	6	8	Others
1	0.025	—	0.63	0.63	0.63
6	0.025	5.63	—	0.63	0.63
8	0.025	0.63	147	—	68.1
Others	0.025	0.63	53.1	0.63	—

[a] Markov chain Monte Carlo estimates Ln(L) of estimates = 7.135. For locality numbers, see Fig. 1. Others = the 7 others localities that did not show population genetic structure.

already observed in the haplotype network (Fig. 2) and BAPS analyses (Fig. 3). In summary, it is unlikely that the species as a whole or several individual populations are experiencing demographic growth.

Population Structure and Connectivity. — Based on our analyses, *P. erythrocephala* from most of the sampled localities belong to a large contiguous population occurring in the central Brazilian Amazon. No isolation by distance was observed within this main population. However, the differences showed by Φ_{ST} values for some pairwise comparisons (Table 2) and the rejection of panmixia by AMOVA suggest that some localities are genetically differentiated (Φ_{ST} = 0.27931). These genetically differentiated populations are present in the upper Negro River upstream of São Gabriel da Cachoeira (locality 1) and in the Jaú River in Jaú National Park (locality 6). Both localities are separated by rapids or small waterfalls. The Andirá River locality near the Barreirinha municipality (locality 8) is separated from the others by the main channel Amazon River and thus isolated by the physicochemical properties of this river. The genetic differentiation of these 3 localities lends considerable support to the hypothesis of geomorphological and physicochemical barriers to gene flow and the need to identify appropriate management units (MUs). Once those localities that are geographically separated by geological barriers are removed from analyses of population structure, the absence of population structuring of the remaining localities becomes apparent.

There is no correlation between pairwise Φ_{ST} and geographic distances between sampling localities; thus, the hypothesis of isolation by distance is not supported. Lack of isolation by distance has also been reported in *P. expansa* and *P. unifilis* (Bock et al. 2001) and *P. lewyana* (Vargas-Ramírez et al. 2012), although the lack of isolation by distance may be an artifact of low sampling density in both of these studies. However, 2 other studies using denser sampling and microsatellite markers suggest a possible influence of geographic distance on the pattern of genetic variability in *P. expansa* (Pearse et al. 2006) and *P. unifilis* (Escalona et al. 2009) populations.

Waterfalls and Rapids as Barriers to Gene Flow. — São Gabriel da Cachoeira (Negro River, locality 1) and Jaú National Park (Jaú River, locality 6) are separated by rapids and a small waterfall, respectively, from all other populations of *P. erythrocephala*. Both localities show differentiation considering Φ_{ST} values and are the primary contributors to the rejection of panmixia in AMOVA. This genetic differentiation, detected by 3 different methods of analysis—AMOVA, Φ_{ST}, and BAPS—reinforces the importance of the waterfalls and rapids in the Negro River basin in genetic structuring of *P. erythrocephala*.

In the upper and middle course of the Negro River, there are numerous geomorphological structures, such as waterfalls and rapids. Some waterfalls are permanent, with steeper gradients, while others present gentler slopes and become submerged during the high-water period. The river's water level, which ranges from 6 to 8 m, depends on the season. During the low-water season, the waterfalls and rapids emerge, hindering or preventing the movement of some aquatic species, while in the high-water season, currents are much stronger and may impede movement of some aquatic animals. An area of rapids separates the locality upstream of São Gabriel da Cachoeira (locality 1) and the Jaú River locality (locality 6).

The pattern of geographic structuring reported in *P. erythrocephala* was similar to that observed in *P. expansa* in the Madeira River basin (Pearse et al. 2006). In their study, Pearse et al. (2006), using control region sequences, observed that 2 populations sampled from the Madeira-Guaporé River clustered closely together and shared a private haplotype that was not found outside the Madeira-Guaporé River basin. These 2 populations are located in the upper Madeira River above the 290-km stretch of rapids (Cella-Ribeiro et al. 2013), which was then hypothesized to act as a substantial barrier to the gene flow in *P. expansa*. Nevertheless, these localities also share 2 more frequent haplotypes with other populations sampled in the Amazon region, suggesting a relatively recent structuring of these populations. In the same study, Pearse et al. (2006), using microsatellite markers, found population differentiation of *P. expansa* individuals above the falls and rapids present in the upper Madeira River and middle Tocantins-Araguaia River.

In addition to structuring populations of *P. erythrocephala* and *P. expansa*, rapids and waterfalls in the Amazon basin also structure fish species (Farias et al. 2010; Ochoa et al. 2015), crocodilians (Hrbek et al. 2008), and dolphins (Gravena et al. 2014, 2015; Hrbek et al. 2014). Rapids also structure entire fish communities (Torrente-Vilara et al. 2011). All of these examples are from the Madeira and in the case of Hrbek et al. (2014) also from the Tocantins-Araguaia River, where the effect of rapids is best studied. Studies of the effects of other rapids on aquatic fauna are effectively nonexistent, making the *P. erythrocephala* study one of the first studies focusing on the Negro River basin.

The Amazon River as a Geographic Barrier to Gene Flow. — The riverine hypothesis states that the large rivers in the Amazon basin isolate populations on opposite banks, thus limiting gene flow and allowing populations to

diverge via selection or genetic drift (Haffer 2008). Genetic evidence fully or partially supporting this hypothesis is limited to the studies of terrestrial vertebrates, such as nonvolant birds (Ribas et al. 2012), frogs (Gascon et al. 1998; Fouquet et al. 2015), and lizards (Oliveira et al. 2016). There is also evidence that rivers delimit small birds (Fernandes et al. 2012, 2014; Fernandes 2013; Weir et al. 2015). It might seem paradoxical that large rivers would delimit the distribution of aquatic organisms. However, *P. erythrocephala* is restricted to clearwater and blackwater environments (Mittermeier et al. 2015) and prefers small water bodies over main river channels (Rueda-Almonacid et al. 2007). Therefore, our results offer support for the hypothesis that major rivers can act as barriers to movement and gene flow for *P. erythrocephala*. The Andirá River locality near the Barreirinha municipality (locality 8) is the only sample from a southern tributary of the Amazon River and thus is isolated from all other localities by the main channel and the physicochemical properties of the Amazon River. Pairwise Φ_{ST} data also showed that individuals from Andirá River locality were genetically differentiated in relation to other localities, except when the comparisons involved the populations of Jamary Creek (locality 9) in the Terra Santa municipality and Iruá Creek (locality 5) in Viruá National Park. While reasons for the lack of significant differentiation between the Andirá River and Jamary Creek localities seem clear, it is unclear why no significant geographic differentiation should be observed between the Andirá River and Viruá National Park.

Despite the results presented in here, the genetic differentiation observed between *P. erythrocephala* from the Andirá River locality and most of the other localities should be viewed cautiously since it may also be the result of an ecological process, local adaptation, or some historical event that has resulted in the differentiation of the population of the Andirá River locality.

Implications for Conservation and Management. — Recent studies demonstrate that molecular data can also be used as an important component in the conservation of threatened species, as they allow the identification of population structure and subsequently of evolutionary significant units and MUs (Moritz 1994; Crandall et al. 2000; Fraser and Bernatchez 2001; Palsbøll et al. 2007). The identification of biological populations and knowing the geographic scale at which populations are demographically connected or independent are important for species monitoring and management (Moritz 1994; Palsbøll et al. 2007). From a conservation perspective, São Gabriel da Cachoeira (locality 1), Jaú National Park (locality 6), Barreirinha (locality 8), and all remaining sampling localities represent 4 distinct MUs based on the criteria of Moritz (1994), which must be administrated as separate entities, given that they comprise genetically distinct stocks.

As the São Gabriel da Cachoeira (locality 1), Jaú National Park (locality 6), and Barreirinha (locality 8) localities demonstrate high levels of genetic differentiation from the remaining centrally located population and are geographically restricted, we suggest that for any future conservation measures, the protection of only a small number of nesting beaches and the transfer of individuals between different areas would not be an appropriate management strategy for this species. Multiple protected nesting beaches and *boiadouros* (the places where females concentrate before and during the spawning period) are a priority and need to be located in the vicinity of each MU. Furthermore, any future transfer or translocation of individuals (hatchlings) needs to be done within each respective MU. With respect to the fourth MU representing the main Amazonian group, any future translocation of individuals can be done over a larger geographic area, albeit still within the geographic distribution of the MU. Future translocation plans could be valuable conservation and management actions in cases of future severe local population decline, such as in areas of the Negro River basin where indiscriminate hunting of adult females and collection of eggs is common.

While our study clearly identifies 4 MUs based on the criteria of Moritz (1994), conservation and management decisions are complex, and many other sources of information must enter into the decision-making process. Other biological sources of information, including from the nuclear genome, which may show different patterns of differentiation than the mitochondrial genome (Palsbøll et al. 2007), and the ecological role of the populations (Hunter and Hutchinson 1994) should be considered. Socioeconomic factors are also important in conservation. There are a sufficient number of highly polymorphic microsatellite loci for species of the genus *Podocnemis* (Pearse et al. 2006; Fantin et al. 2007) to follow up this study to refine our hypotheses about gene flow and population structuring, which, together with demographic and ecological data (Batistella and Vogt 2008), may be used to better delimit the number and distribution of distinct MUs of *P. erythrocephala*.

Podocnemis erythrocephala is currently considered vulnerable; however, there are no data on the effect of exploitation on the species, there are no historical data on the rate of its consumption, and there is only anecdotal evidence that it was much more abundant around 20 yrs ago (Mittermeier et al. 2015). In conclusion, there is an urgent need for more studies of all aspects of the biology of this species. We can properly conserve and manage only what we know well.

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