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Estimating population structure under nonequilibrium conditions in a conservation context: continent-wide population genetics of the giant Amazon river turtle, *Podocnemis expansa* (Chelonia; Podocnemididae)

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Abstract

Giant Amazon river turtles, *Podocnemis expansa*, are indigenous to the Amazon, Orinoco, and Essequibo River basins, and are distributed across nearly the entire width of the South American continent. Although once common, their large size, high fecundity, and gregarious nesting, made *P. expansa* especially vulnerable to over-harvesting for eggs and meat. Populations have been severely reduced or extirpated in many areas throughout its range, and the species is now regulated under Appendix II of the Convention on International Trade in Endangered Species. Here, we analyse data from mitochondrial DNA sequence and multiple nuclear microsatellite markers with an array of complementary analytical methods. Results show that concordance from multiple data sets and analyses can provide a strong signal of population genetic structure that can be used to guide management. The general lack of phylogeographic structure but large differences in allele and haplotype frequencies among river basins is consistent with fragmented populations and female natal-river homing. Overall, the DNA data show that *P. expansa* populations lack a long history of genetic differentiation, but that each major tributary currently forms a semi-isolated reproductive population and should be managed accordingly.

Keywords: conservation, phylogeography, *Podocnemis*, population genetics, South America

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Introduction

The identification of genetic 'breaks', evolutionarily significant unit (ESU) or management unit (MU) boundaries, is a central problem in population genetics and an important component in the conservation of endangered species (Waples 1995; Hughes *et al*. 1997; Crandall *et al*. 2000; Fraser & Bernatchez 2001; Pearman 2001; Moritz 2002; reviews in Frankham *et al*. 2002; DeSalle & Amato 2004). In widely distributed species with high vagility, population genetic differentiation may be low, but nonetheless may be an important biological consideration for species management. In many marine fishes, for example, high juvenile dispersal and few biogeographic barriers greatly limit the formation of population genetic structure (e.g. Bernardi *et al*. 2001), yet design of Marine Protected Areas must still to take into account patterns of dispersal and gene flow in a metapopulation context (Kritzer & Sale 2004). Similarly, genetic isolation by distance in highly mobile and dispersive riverine species depends on their life history and dispersal patterns as well as on the strength of physical geographic structuring in the river systems (e.g. barriers, such as waterfalls vs. floodplain dispersal corridors). When such species are declining, it is important to carefully evaluate population structure and conduct conservation measures at appropriate spatial scales.

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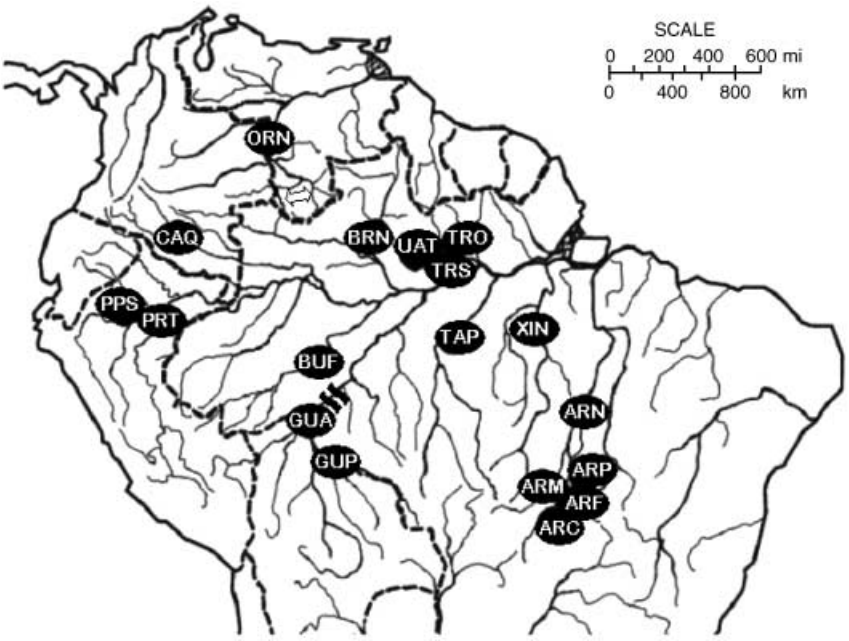

Fig. 1 Map of northern South America with sample sites labelled as in Table 1. Each site is considered a single population sample, identified by the major river basin of origin; ⇔ identifies location of Brazo Casiquiare, and ⤡ identifies a major cataract on Madeira River. The four Caquetá samples (all < 100 km apart) are indicated by the single 'CAQ' and were grouped for some analyses. See text for details.

Evaluating population structure and gene flow in species that have experienced recent demographic changes presents special challenges because the populations may not be in mutation–drift equilibrium, and thus violate the assumptions of most common analytical methods (Whitlock & McCauley 1999). Recently, a variety of approaches have been developed that are appropriate to evaluate population genetic structure under nonequilibrium conditions (reviewed by Pearse & Crandall 2004; Manel *et al*. 2005). Although their apparent ability to define genetic boundaries makes these methods promising for conservation genetic management, their performance has been largely evaluated through simulations (e.g. Cornuet *et al*. 1999; Abdo *et al*. 2004). Nonetheless, empirical studies have shown the advantages of using multiple complementary analytical methods to detect different signals in genetic data sets (e.g. Austin *et al*. 2004; Cassens *et al*. 2004; Lemaire *et al*. 2004; Hickerson & Cunningham 2005), and we take this approach here. In the same way that concordant results across genes or species strengthen the support for phylogenetic or phylogeographic hypotheses (Avise 2000), concordant results obtained from analysis of the same data with complementary population genetic methods reinforces support for a given hypothesis by providing increased confidence that the results reflect the true signal present in the data and are not influenced by violations of method-specific assumptions (Jones *et al*. 2004; Hickerson & Cunningham 2005).

Giant Amazon river turtles (*Podocnemis expansa*) are indigenous to the Amazon, Orinoco, and Essequibo River basins (Iverson 1986), with a range spanning South America east of the Andes (Fig. 1). *P. expansa* is the largest *Podocnemis* species, and constitute an important protein source for peoples living along lowland tropical rivers since precolonial times (Smith 1974, 1979; Pritchard & Trebbau 1984; Licata & Elguezabal 1997; Páez & Bock 1997; Thorbjarnarson *et al*. 1997; von Hildebrand *et al*. 1997). Although once common, their large size, relatively high fecundity, and gregarious nesting behaviour (Vanzolini 1967, 2003; Ojasti 1967, 1971; Alho & Padua 1982a, b; von Hildebrand *et al*. 1997), made *P. expansa* especially vulnerable to over-harvesting of eggs and meat, and populations have been severely reduced or extirpated in many areas throughout their range (Bates 1863; IUCN/SSC Tortoise and Freshwater Turtle Specialist Group 1989; Moll & Moll 2004). All but one of the recognized *Podocnemis* species are now listed at some level of conservation concern by the IUCN (Moll & Moll 2004; Table 2.1), and *P. expansa* is regulated under Appendix II of the Convention on International Trade in Endangered Species (CITES), and listed as endangered under the US Endangered Species Act.

Since 1975, the Brazilian agency Instituto Brazileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA) has identified and protected important nesting beaches of *P. expansa*, and collected biological information needed for their conservation and management (Alfinito 1975; Cantarelli 1993, 1997). This surveillance has produced over 30 million hatchlings on protected beaches in Brazil, and increased the number of nesting females at some localities (Cantarelli 1997). In Venezuela, *P. expansa* nesting is protected by the Ministry of the Environment on a single beach on the Orinoco River (Licata & Elguezabal 1997) currently used by ~1000 females. Nests are relocated to higher grounds and the hatchlings are raised in captivity by the Fundacion para el Desarrollo de las Ciencias Fisicas

Matematicas y Naturales (FUDECI), for release after one year. This programme has head-started ~210 000 juvenile *P. expansa* over the past 13 year (O. Hernandez, personal communication). Although this population appears stable, nests on other Venezuelan beaches are almost completely sacked by poachers, even in remote areas (D.E.P., personal observation). In Colombia, protection has varied in space and time since the 1960s, but takes place mainly in beaches along the Caquetá River, some within the Cahuinarí National Park (von Hildebrand *et al*. 1997), and has included nesting and incubation surveillance plus intermittent head-start programs similar to those mentioned above.

Mark–recapture data indicate that female *P. expansa* can travel long distances (> 400 km) between nesting beaches and feeding areas, and between nesting beaches (> 200 km) in consecutive years (von Hildebrand *et al*. 1997). Additionally, individuals in some populations return to the same nesting beach in successive years with high fidelity (Roze 1964; Ojasti 1971; Alho *et al*. 1979), while others do not (von Hildebrand *et al*. 1997). Philopatry to natal sites may generate significant genetic differentiation among nesting beaches, especially in matrilineally inherited DNA markers. Alternatively, if philopatry is socially facilitated (as when primiparous females follow experienced females to a nesting location and then return to that site in future nesting episodes; Hendrickson 1958), it is not expected to result in marked genetic differentiation among nesting beaches because females would not necessarily nest on their natal beach under this scenario (Bass *et al*. 1996). The 'natal homing' hypothesis has been verified in several species of sea turtles (Bowen & Karl 1997; FitzSimmons *et al*. 1997a, b; Roberts *et al*. 2004; Bowen *et al*. 2005), whose hatchlings are hypothesized to imprint on some cue of their natal beach with sufficient entrainment to permit their return as nesting adults (Carr 1967).

Compared to the spectacular long-distance migrations of marine turtles, migrations of freshwater turtles are more modest (Kuchling 1999), but one other parallel between *P. expansa* and marine turtles is biologically significant. *P. expansa* is one of only two known riverine turtles in which both sexes migrate between feeding and nesting areas (the other is the Asian 'tutong', *Batagur baska*). Such regular migrations in *P. expansa* may render populations vulnerable to poaching far from their nesting beaches (as true for marine turtles; Bowen 1995; Laurent *et al*. 1998), even if they are strictly protected during nesting. Given their adult herbivory and formerly high densities (von Humboldt 1814; Bates 1863), *P. expansa* likely played important roles in energy flow, nutrient cycling, and seed dispersal for many terrestrial plants in Neotropical river ecosystems (Moll & Moll 2004), so declines of *P. expansa* populations have probably had ecosystem-level effects.

Previous genetic studies on *P. expansa* have found consistent patterns of genetic structure, but have all been limited geographically as well as by the sampling capacity and power of the genetic markers used. For example, Sites *et al*. (1999) developed *P. expansa*-specific microsatellite markers to infer gene flow among nesting areas and river basins, and a pilot study of the utility of six of these markers for inferring metapopulation structure suggested substantial gene flow within one river system (among three nesting beaches separated by up to 280 km in the upper Araguaia River basin), but little gene flow between beaches in two widely separated river basins (Araguaia River and Tapajós River, > 2400 km; Sites *et al*. 1999). Sites *et al*. (1999) also presented the first mitochondrial DNA (mtDNA) sequence data for *P. expansa* (354 bp of the control region), and results were congruent with the microsatellite data; all samples were fixed for a single haplotype within the Araguaia River, while samples from the Tapajós River contained that haplotype as well as three others. A second study, of four nesting sites within the middle Caquetá River basin of Colombia, found significant among-beach genetic differentiation using eight microsatellite loci (Valenzuela 2001). However, due to the small sample sizes (12–15 individuals/site), the results were inconsistent and left open the question of whether biologically significant (as opposed to statistically significant; Waples 1998) structure existed among nesting beaches within the single basin. Additional studies using allozymes and mtDNA have found concordant results, but were similarly limited to small geographic areas (Bock *et al*. 2001; Viana *et al*. 2004).

The present study is a range-wide evaluation of population genetic structure and gene flow based on a total of 453 samples. These include samples analysed by Sites *et al*. (1999), Valenzuela (2001), and Viana *et al*. (2004), and samples from nine new localities throughout the range of *P. expansa*, providing a comprehensive representation of the species and allowing analysis at several geographic distances within and among sub-basins (which we define as single river systems tributary to the main Amazon River). We use nine microsatellite markers for samples from all but one river basin, and complete mitochondrial control region sequences from a subset of these. We analyse these data using complementary methods that carry different assumptions and rely on different properties of the data, both to guard against the biases inherent to any single method and because commonly used gene flow estimators may be biased under nonequilibrium conditions expected of declining or expanding populations (Whitlock & McCauley 1999; Pearse & Crandall 2004). Our extensive sampling and use of nuclear microsatellite loci in combination with mitochondrial sequence data provide a means for estimating the cumulative effects of gene flow over large geographic distances and longer time frames than is practical for field studies based on mark–recapture or radio-tracking data (long-distance dispersal is especially difficult and expensive to measure directly; Koenig *et al*. 1996). Here, we compare the

Table 1 Sample information and summary of DNA data used in this study. Population codes refer to the map in Fig. 1; Ar is allelic richness (rarefied by sample sizes as described in Methods), and H_E is heterozygosity for microsatellites

Country	River	Population code	*N* for microsatellites	Ar	H_E	No. of mtDNA sequences
Brazil	Faz, Araguaia River	ARF	23	5.0	0.68	—
Brazil	Praias, Araguaia River	ARP	22	5.7	0.72	—
Brazil	Crixas-Açu, Araguaia River	ARC	23	5.4	0.75	—
Brazil	Rio das Mortes, Araguaia River	ARM	24	5.5	0.70	22
Brazil	Araguaia National Park	ARN	24	4.9	0.62	20
Brazil	Xingu River	XIN	24	4.4	0.66	23
Brazil	Tapajós River	TAP	24	8.4	0.83	21
Brazil	Trombetas River	TRO	—	—	—	8
Brazil	Terra Santa, Amazon River	TRS	37	6.0	0.78	18
Brazil	Uatuma River	UAT	24	7.7	0.79	18
Brazil	Pimentieras, Guapore River	GUP	24	6.8	0.82	22
Brazil	Guapore River	GUA	24	7.1	0.83	22
Brazil	Abufari, Purus River	BUF	30	5.4	0.75	12
Peru	Peru	PRT	16	8.7	0.84	10
Peru	Pacaya-Samiria Reserve	PPS	23	7.6	0.81	—
Colombia	Caquetá River	CAQ	47	6.8	0.81	55
Brazil	Branco River	BRN	24	7.8	0.79	24
Venezuela	Playa Medio, Orinoco River	ORN	40	4.7	0.62	18
	Total		453			293
	Mean		26.6	6.4	0.75	20.9

results of several equilibrium and nonequilibrium population genetic methods to (i) evaluate broad-scale patterns of population genetic variation; (ii) estimate the extent of population declines and their effect on the loss of genetic diversity; and (iii) contrast biparental and female-inherited genetic markers to discriminate between male- and female-mediated genetic effects and to test for natal homing, as has been documented in sea turtles (FitzSimmons *et al*. 1997a, b; Roberts *et al*. 2004; Bowen *et al*. 2005). We then consider the conservation implications of these findings for current management activities, at both metapopulation and local demographic scales. Our overarching goal is to provide information that will enhance the probability of protecting the genetic variance within *P. expansa*, in the 'adaptive evolutionary conservation' (AEC) framework as defined by Fraser & Bernatchez (2001).

Materials and methods

Sample collection, DNA extraction, and molecular data

Specimens from most Brazilian sample sites were obtained by IBAMA staff during monitoring of protected nesting beaches. A single hatchling was sacrificed from up to 24 nests per beach, and liver tissue was immediately preserved in 95% ethanol, or blood was drawn and stored in Queen's lysis buffer following Valenzuela (2000). Sampled beaches include the four locations in two river basins sampled by Sites *et al*. (1999), the four Caquetá River (CAQ) sites sampled by Valenzuela (2001), the three sites sampled by Viana *et al*. (2004), as well as 10 new sites throughout the Amazon (Brazilian and Peruvian localities) and Orinoco basins (Table 1; Fig. 1). One of the Peruvian samples (PRT) was a mixture of locations collected by Tag Engstrom, and is included for general comparison with the other Peruvian site (PPS). For most analyses, the four sites sampled by Valenzuela (2001) on the Caquetá River were treated as a single location (CAQ) due to the small sample sizes from the individual beaches. In analyses where they were treated separately, they are indicated as CAQ-Cent, -Tam, -Guad, and -Yaru. Of the new Brazilian samples, two represent additional sites in the Araguaia River system [Parque Nacional do Araguaia (ARN) and Mortes River (ARM)], while the rest are from other major tributaries of the Amazon River (Fig. 1). All samples were collected between 1994 and 2002; interannual variation among samples is expected to be extremely low due to the long lifespan, high annual survival, and repeated breeding cycles of *Podocnemis expansa*. Voucher specimens for these samples were deposited in the Herpetology collection at the Universidade Catolica de Goias (UCG, Goiania, Brazil), and tissue samples were exported from host countries and imported into the United States under the appropriate CITES import and export permits. Samples from one population, Trombetas Reserve ('TRO') were used only for the mtDNA analysis due to the small sample size ($n = 8$) and their late acquisition to the study.

Total genomic DNA was extracted from blood and tissue samples using the protocol of Fetzner (1999) and

resuspended in ~100 μL of either dH_2O or TE buffer. Nine microsatellite loci were used in the present study; six (Pod 1, 62, 79, 91, 128, and 147) isolated by Sites *et al*. (1999), and three (PE 344, 519 and 1075) isolated independently by Valenzuela (2000). Microsatellite loci were amplified individually using fluorescently labelled primers (HEX TET or FAM). Typically, 1 μL of resuspended DNA solution was placed in a 25-μL reaction containing 0.5 U of *Taq* DNA polymerase (Promega), 2 mM $MgCl_2$, and 10 pmol of each primer. Amplification profiles began with a 95 °C denaturing step for 2 min, followed by 30 cycles as follows: 95 °C for 30 s, annealing temperature for 30 s, and amplification at 72 °C for 45 s. Further details of microsatellite amplification are described elsewhere (Sites *et al*. 1999; Valenzuela 2000). One microlitre of a 1/10 dilution of the amplification reactions was dried under vacuum and submitted for automated analysis to the DNA Sequencing Centre at Brigham Young University. Alleles were scored using the software programs GENESCAN and GENOTYPER (Applied Biosystems).

Mitochondrial control region fragments were amplified in two overlapping polymerase chain reactions (PCRs), and sequences were generated using the four terminal primers (*Pro*: 5′-CCCATCACCCACTCCCAAAGC-3′; *DLR*: 5′- GGGATGCTGGTTTCTTGAG-3′; *CSB*: 5′-TTATAGTGCTCTTCCCCATATTATG-3′; *PodF*: 5′-TAATCTATCGCATCTTCAG-3′). Sequences were electrophoresed on an ABI 3100 capillary sequencer, and were checked and aligned using the software SEQUENCHER (Applied Biosystems). Sequences for each individual were then assembled into the full consensus, and final alignment of insertions/deletions (indels) and mini- and microsatellite repeats for all individuals was done by eye using the PAUP (Swofford 2001) editor. At each stage, putative point mutations were checked back in the original sequence data file to confirm the difference.

Data analysis

Microsatellite DNA variation. Genetic variation within and among populations was summarized using GDA (Lewis & Zaykin 2001) and HP-RARE (Kalinowski 2004, 2005). Tests of Hardy–Weinberg equilibrium, linkage disequilibrium, and isolation by distance were performed by Markov chain permutation using GENEPOP on the Web (Raymond & Rousset 1995; www.cefe.curs-mop.fr).

Population structure. Population structure was initially assessed among all sampled nesting populations using pairwise F_{ST} values (Weir & Cockerham 1994), calculated with the program GENETIX (Belkhir *et al*. 2000), and Cavalli-Sforza-Edwards distances (Cavalli-Sforza & Edwards 1967) calculated using the PHYLIP computer package (Felsenstein 2004). Genetic relationships among populations were graphically represented with neighbour-joining networks based on the calculated distances, and significance of the relationships was evaluated using 1000 bootstrap replicates of the data (PHYLIP; Felsenstein 2004). To test for isolation by distance among major river sub-basins, values of $[F_{ST}/(1-F_{ST})]$ were fit to distance in kilometres using Mantel's test in the program ISOLDE in GENEPOP 3.2a (Mantel 1967; Rousset 1997). Distances were measured as the shortest river path between each pair of populations (Fetzner & Crandall 2003), and Spearman's rank correlation with 10 000 permutations was used to assess significance.

Two recently developed methods were used to complement the traditional population genetic analyses described above and evaluate their concordance in assessing the genetic distinctness of the sampled nesting populations. The model-based clustering method STRUCTURE (Pritchard *et al*. 2000) was used to estimate the maximum-likelihood value for k, the number of genetically distinct populations from which our sampled individuals were drawn. This program assigns individuals to the k populations based on Bayesian estimation of conformity to equilibrium expectations, allowing the data to define the populations with no a priori information about the source population of each individual. Another model-based clustering method, BAPS (Corander *et al*. 2003), was used as an alternative to the distance-based analysis at the population level. This program uses a Bayesian algorithm to estimate the allele frequency distributions of all populations, then pools any population with nonsignificant allele frequency differences and recalculates the distributions using the pooled populations. F_{ST} values or Nei's genetic distances are then calculated from the pooled allele frequency distributions, allowing the construction of neighbour-joining networks like that produced using PHYLIP.

Genotypic methods. Two individual-based assignment tests were implemented in the program GENECLASS-2 (Piry *et al*. 2004) as alternative indicators of population differentiation and current gene flow among populations. The proportion of individuals correctly assigning to their source populations was estimated using both the frequency-based test of Paetkau *et al*. (1995) and the Bayesian method of Rannala & Mountain (1997). Correct assignment rates generated using these programs have been shown to provide good estimates of relative dispersal among populations (Berry *et al*. 2004), and are robust to violations of some assumptions about mutation mechanisms for microsatellite loci. In addition, the Bayesian estimator of migration rates implemented in the program BAYESASS+ (Wilson & Rannala 2003), was used to estimate migration among all populations based on the proportion of individuals sampled in each population that assign to another population. All programs were run using the default parameters unless otherwise stated.

Demographic analyses. To test for genetic evidence of historical changes in population size and deviations from

Fig. 2 Diagrammatic representation of mtDNA control region variation in *Podocnemis expansa*; vertical lines toward 5′ end mark sites of single-base indels, and solid rectangle denotes 67-bp indel. Thick lines at 3′ end indicate minisatellite region composed of two to five 75-bp repeats, each with an inserted dinucleotide microsatellite of variable size ($TA_{6–7}$).

equilibrium conditions, we applied two methods designed to detect recent reductions in population size. The first method, implemented in the program BOTTLENECK (Piry *et al*. 1999), tests for excess heterozygosity as compared with that expected under mutation–drift equilibrium. When populations decline in size, rare alleles are expected to be lost quickly, while heterozygosity will decline much more slowly because rare alleles have little effect on it (Spencer *et al*. 2000). For each sample, bottleneck calculates the per-locus deviation from expected heterozygosity under a mutational model, and then averages across all loci. Significance of observed deviations was determined by a two-tailed Wilcoxon signed rank test (Luikart & Cornuet 1998), under the two-phase mutation model for microsatellites. A second method, the '*M* ratio' test of Garza & Williamson (2001), exploits a different feature of the behaviour of microsatellite loci in restricted demographic situations ('bottlenecks'), and is thus complementary to the BOTTLENECK method. Microsatellite loci do not always conform to a strictly stepwise pattern of mutation (Ortí *et al*. 1997; Culver *et al*. 2001; Webster *et al*. 2002), and as a result, rare alleles may be intermediate in size rather than only among the largest or smallest size classes under a normal distribution. Consequently, when rare alleles are preferentially lost during a population size reduction, the *number* of allele size classes is reduced to a greater extent than the *range* in allele sizes. Garza & Williamson (2001) have shown that the test statistic $M = k/r$, where k is the number of alleles and r is the range of allele sizes, is reduced in populations known to have declined in size.

mtDNA sequence analysis. The mtDNA control region of *P. expansa* contained a repeated minisatellite with an inserted microsatellite of variable size (Fig. 2), which could violate independent site assumptions in some analyses. Although homoplasy is likely in such repeated DNA motifs (especially in microsatellites; Li *et al*. 2002), recent studies of chloroplast microsatellites suggest that their inclusion in haplotype studies of population diversity is informative, while this is not true for estimates of genetic distance and phylogenetic inference (Navascués & Emerson 2005). We thus conducted two sets of analyses on the mtDNA sequences. First, to provide a graphical representation of the haplotype relationships, a median-joining haplotype network (Bandelt *et al*. 1999) was constructed using the software NETWORK (Fig. 3; www.fluxus-engineering.com). Second, to incorporate the repeat variation into our analysis, a post hoc treatment of the sequence data was devised as follows. Oligonucleotides at variable sites were coded as 1 = A, 2 = C, 3 = G, 4 = T, 5 = N, or 0 = gap. The minisatellite region was coded by the number of TA microsatellite repeats it contained (6 = TA_6, 7 = TA_7) or 0 if the minisatellite was absent. This procedure generated a 95-linked-character haplotype for each sequence (haplotypes available from the authors upon request). We then summarized this variation and assessed female metapopulation structure using frequency statistics based only on these coded haplotypes.

Nucleotide diversity (π; Nei 1987) and Tajima's *D* were calculated using the program DNASP (Rozas *et al*. 2003), while haplotype diversity (Nei 1973), Fu (1997) *Fs* test, pairwise F_{ST} values, significance of among-locality haplotype frequency data, and hierarchical analyses of molecular variance were calculated using ARLEQUIN (Schneider *et al*. 2000). The standardized number of haplotypes per population (allelic richness) was calculated using CONTRIB (Petit *et al*. 1998). Tests for isolation by distance among river sub-basins were conducted as for the microsatellite data.

Significant values for either Tajima's *D* or Fu's *Fs* test statistics may indicate that sequences are evolving non-neutrally (are not in mutation–drift equilibrium), or that populations were previously subdivided and/or have experienced past fluctuations (are not in migration–drift equilibrium). Neutrality is an implied assumption in molecular studies of population history and structure, so statistical tests of this assumption are necessary (Rand 1996; Ballard & Whitlock 2004). The *Fs* test appears to be especially sensitive to detection of population expansion (Fu 1997), and we tested its significance by comparing the *Fs*-statistic against a distribution generated from 1000 random samples under the hypotheses of selective neutrality and population equilibrium.

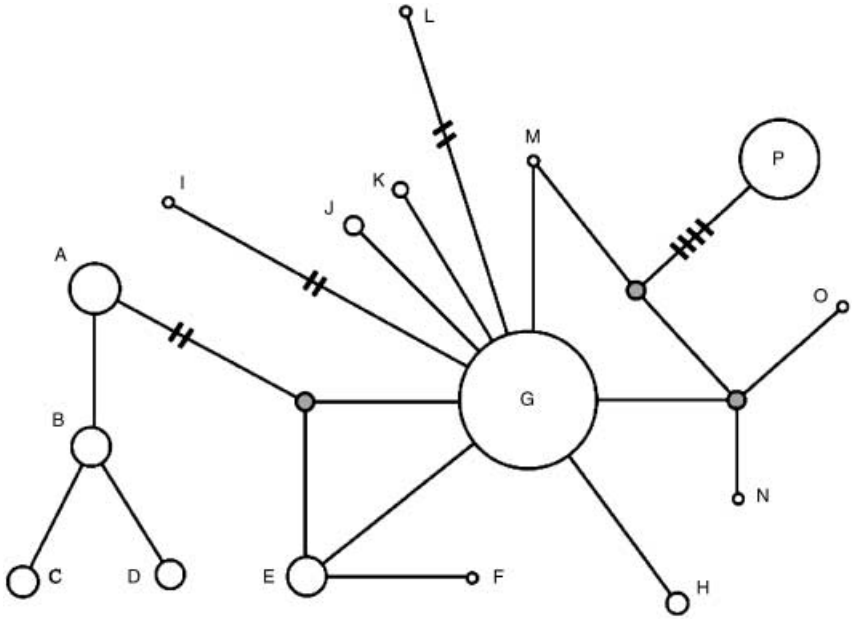

Fig. 3 Median-joining network of mtDNA haplotypes based on only the polymorphic nucleotides and indels, excluding the repeat variation coded in the mtDNA analyses. Haplotype letters correspond to designations in Table 6. All branch lengths represent a single change unless additional hatch marks indicate otherwise, and circle size corresponds to overall haplotype frequency. Grey haplotypes were not observed.

Results

Microsatellites

Within-population statistics. Hardy–Weinberg equilibrium probability tests performed in GENEPOP found 23 locus-population comparisons to be significantly out of Hardy–Weinberg equilibrium after Bonferroni's correction for multiple comparisons (alpha = 0.05, $P < 0.0003$). Of these, the most consistent patterns were in the population Abufari (BUF; significant for seven of the nine loci), and loci Pod91 and Pod147 (significant in six and seven out of 17 populations, respectively). The five other significant locus–population combinations were distributed across four other loci and three separate populations. Exact tests for heterozygote deficiency and excess found 10 and 1 significant population/locus combinations, respectively. Sample sizes, average heterozygosity (H_E), and allelic richness (Ar), are shown for all populations in Table 1. Heterozygosity values ranged from 0.62 (ARN and ORN sites) to 0.84 (PPT site), and averaged 0.75. Notably, significant differences in allelic richness were found among populations (Table 1; ANOVA, $P < 0.001$), with an almost twofold difference between the minimum and maximum mean Ar per population. Mean values of Ar were lowest for the Xingu (XIN; 4.4) and Orinoco (ORN; 4.7) sites, the five localities within the Araguaia basin (4.9–5.7), and the Abufari site (BUF; 5.4) on the Purus River. Allelic richness was highest at the 'Peru' (PPT; 8.7) and Tapajós (TAP; 8.4) sites (Table 1). As expected, mean Ar values were highly correlated with average heterozygosity for each population ($R^2 = 0.76$, $P < 0.001$). Mean Ar values were also strongly correlated with the mean value of pairwise F_{ST} calculated for each population ($R^2 = 0.74$, $P < 0.0001$). This last result supports the general observation that populations of reduced size experience increased genetic drift, which both lowers Ar and leads to higher mean pairwise F_{ST} values for that population.

Table 2 Tests for recent population declines using *M* ratio and BOTTLENECK for microsatellite data

Population	*M* ratio†	BOTTLENECK‡
All Araguaia§	0.464*	0.018*
Xingu	0.437*	0.001*
Tapajós	0.474*	0.213
Terra Santa	0.459*	0.019*
Uatuma	0.477*	0.500
Guapore/Pimentieras§	0.442*	0.187
Abufari	0.440*	0.014*
Peru§	0.465*	0.630
Caquetá	0.560*	0.064
Branco	0.492*	0.064
Orinoco	0.555*	0.014*

†Significance of *M* ratio results based on simulations with 48 alleles, $4N_e\mu = 4$, 10% non-stepwise mutations, and size of non-stepwise mutations = 3 bp. See text for details.
‡BOTTLENECK results reported under the two-phase model of microsatellite mutation.
§The five populations from the Araguaia River, the Guapore and Pimentieras populations, and the two Peru populations, respectively, were pooled by sub-basin in *M* ratio, but run separately in BOTTLENECK; mean values are reported for the BOTTLENECK results.

Demographic tests using both the *M* ratio statistic and the heterozygote excess test found strong evidence for recent reductions in population sizes (Table 2). Since the

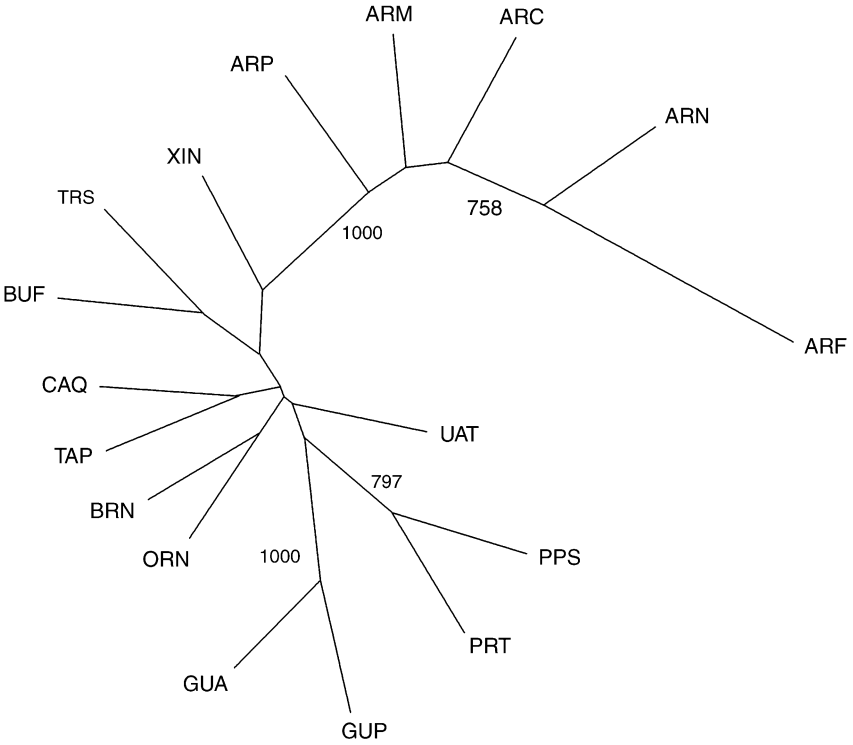

Fig. 4 Unrooted neighbour-joining network based on Cavalli-Sforza–Edwards distances calculated from the microsatellite data, with support (out of 1000 bootstrap resamplings) indicated for each node with a value greater than 500. Localities abbreviated as in Table 1.

M ratio statistic is a relative value, the program CRITICAL_M was used to simulate expected values for the *M* ratio under realistic demographic and mutational parameters. Using values of $4N_e\mu = 4$, proportion of non-stepwise mutations of 0.1, and an average size of non-stepwise mutations of three steps, as suggested by Garza & Williamson (2001), produced a critical value of 0.74. Based on the simulation parameters, populations with *M* ratios below this value show evidence of population decline with 95% probability, and *M* ratio values were < 0.56 in all *Podocnemis expansa* populations tested (Table 2). Similarly, the heterozygote excess test using the two-phase model of microsatellite evolution in BOTTLENECK found significant evidence for a bottleneck in all Araguaia River populations, and separately in the Abufari (BUF), Orinoco (ORN), Terra Santa (TRS), and Xingu (XIN) samples (Table 2).

Among-population differentiation. Overall F_{ST} across all populations was 0.112 (95% CI 0.970–0.129). All pairwise F_{ST} values among populations from separate sub-basins were significant, but only 2 out of 11 values among populations within river basins [i.e. all Araguaia (AR-) and both Madeira/Guapore (GU-) River populations] were not significantly different from zero (Table 3). Neighbour-joining trees based on Cavalli-Sforza–Edwards (CSE) distances grouped all populations within river sub-basins with strong bootstrap support (five within the Araguaia, two within Guapore, and two in Peru), but there was little support for relationships among river basins (Fig. 4).

Distances among populations ranged from 410 to 4970 km between sub-basins, and from 84 to 566 km within sub-basins. Tests for isolation by distance were significant when all populations were included in the analysis ($F_{ST}/(1 - F_{ST})$ vs. distance in kilometres; $R^2 = 0.25$, $P < 0.001$). However, when the comparison was limited to only populations from different sub-basins, the relationship was not significant ($R^2 = 0.078$, $P > 0.2$; Table 3; Fig. 5a). Similarly, although the largest distance between within-sub-basin nesting populations is greater than the smallest distance between different sub-basins, all among sub-basin pairwise F_{ST} values were significantly different from zero, while only 2 of the 11 total pairwise F_{ST} values among populations within sub-basins were significantly nonzero (Table 3). Overall, these patterns reflect a structure characterized by limited current dispersal among sub-basins, high gene flow and low differentiation among populations within a single sub-basin.

We made multiple runs of the Bayesian partitioning method of Pritchard *et al.* (2000) using various burn-in lengths and repetitions to estimate the maximum-likelihood value for *k*, the number of genetically distinct populations from which our sampled individuals were drawn. However, the results from STRUCTURE were not statistically informative due the flatness of the likelihood curve around the maximum-likelihood value of $k = 12$, making it impossible to conclude with confidence that this is the correct value (data not shown). Nonetheless, a value of $k = 12$ populations corresponds to the number of river sub-basins sampled,

Table 3 Matrix of pairwise F_{ST} values calculated from the microsatellite data (below diagonal) and mtDNA coded haplotype frequencies (above diagonal). F_{ST} values significantly different from zero (calculated using GENETIX) are shown in bold. Pairwise F_{ST} values between populations within a single sub-basin are underlined

	ARF	ARP	ARC	ARM	ARN	XIN	TAP	TRO	TRS	UAT	GUP	GUA	BUF	PPS	PRT	CAQ	Cent	Tam	Guad	Yaru	BRN	ORN
Araguaia-F	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Araguaia-P	0.005	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Araguaia-C	0.019	0.009	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Araguaia-M	0.013	–0.003	0.021	—	–0.004	**0.578**	**0.486**	**0.607**	**0.688**	**0.688**	**0.587**	**0.621**	**0.882**	**0.681**	—	—	**0.593**	**0.58**	**0.588**	**0.557**	**0.503**	**0.61**
Araguaia-N	0.015	0.012	**0.044**	**0.027**	—	**0.622**	**0.522**	**0.667**	**0.728**	**0.728**	**0.62**	**0.655**	**0.938**	**0.735**	—	—	**0.636**	**0.625**	**0.63**	**0.599**	**0.534**	**0.648**
Xingu(XIN)	**0.185**	**0.171**	**0.158**	**0.169**	**0.241**	—	**0.207**	**0.303**	**0.467**	**0.447**	**0.157**	**0.134**	**0.628**	**0.312**	—	—	**0.35**	**0.33**	**0.344**	**0.314**	**0.305**	**0.39**
Tapajós(TAP)	**0.141**	**0.113**	**0.097**	**0.113**	**0.171**	**0.088**	—	0.019	**0.239**	**0.233**	**0.109**	**0.132**	**0.392**	**0.127**	—	—	**0.109**	**0.085**	**0.103**	**0.072**	**0.083**	**0.16**
Trombetas(TRO)	—	—	—	—	—	—	—	—	**0.25**	**0.244**	**0.114**	**0.158**	**0.47**	0.042	—	—	**0.089**	0.033	**0.074**	0.029	0.006	**0.091**
Terra Santa(TRS)	**0.158**	**0.142**	**0.123**	**0.143**	**0.195**	**0.115**	**0.053**	—	—	**0.431**	**0.344**	**0.38**	**0.586**	**0.361**	—	—	**0.305**	**0.284**	**0.3**	**0.268**	**0.266**	**0.35**
Uatuma(UAT)	**0.145**	**0.121**	**0.107**	**0.12**	**0.179**	**0.088**	**0.019**	—	**0.07**	—	**0.327**	**0.362**	**0.604**	**0.35**	—	—	**0.302**	**0.284**	**0.297**	**0.259**	**0.245**	**0.35**
Pimentieras(GUP)	**0.155**	**0.128**	**0.118**	**0.132**	**0.189**	**0.127**	**0.032**	—	**0.083**	**0.041**	—	–0.004	**0.499**	0.071	—	—	**0.218**	**0.196**	**0.213**	**0.182**	**0.14**	**0.266**
Guapore(GUA)	**0.158**	**0.132**	**0.118**	**0.135**	**0.189**	**0.141**	**0.035**	—	**0.082**	**0.051**	0.003	—	**0.537**	0.059	—	—	**0.256**	**0.234**	**0.251**	**0.22**	**0.156**	**0.302**
Abufari(BUF)	**0.161**	**0.144**	**0.132**	**0.143**	**0.182**	**0.159**	**0.061**	—	**0.087**	**0.079**	**0.09**	**0.087**	—	**0.568**	—	—	**0.483**	**0.466**	**0.478**	**0.444**	**0.413**	**0.515**
Peru(PPS)	**0.151**	**0.122**	**0.102**	**0.128**	**0.183**	**0.118**	**0.031**	—	**0.063**	**0.043**	**0.045**	**0.047**	**0.073**	—	—	—	**0.213**	**0.188**	**0.207**	**0.172**	**0.068**	**0.267**
Peru(PRT)	**0.157**	**0.132**	**0.102**	**0.134**	**0.187**	**0.141**	**0.036**	—	**0.09**	**0.036**	**0.04**	**0.036**	**0.086**	**0.028**	—	—	—	—	—	—	—	—
Caquetá(CAQ)	**0.146**	**0.121**	**0.106**	**0.124**	**0.173**	**0.127**	**0.034**	—	**0.077**	**0.043**	**0.056**	**0.058**	**0.078**	**0.037**	**0.054**	—	—	—	—	—	—	—
Cent	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	–0.010	–0.056	–0.025	**0.118**	**0.219**
Tam	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	–0.029	–0.041	**0.074**	**0.089**
Guad	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	–0.04	**0.105**	**0.184**
Yaru	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	**0.072**	**0.118**
Branco(BRN)	**0.155**	**0.125**	**0.114**	**0.132**	**0.19**	**0.117**	**0.034**	—	**0.08**	**0.043**	**0.056**	**0.066**	**0.091**	**0.024**	**0.047**	**0.048**	—	—	—	—	—	**0.135**
Orinoco(ORN)	**0.229**	**0.194**	**0.205**	**0.188**	**0.257**	**0.194**	**0.123**	—	**0.154**	**0.135**	**0.145**	**0.162**	**0.198**	**0.133**	**0.158**	**0.134**	—	—	—	—	**0.118**	—

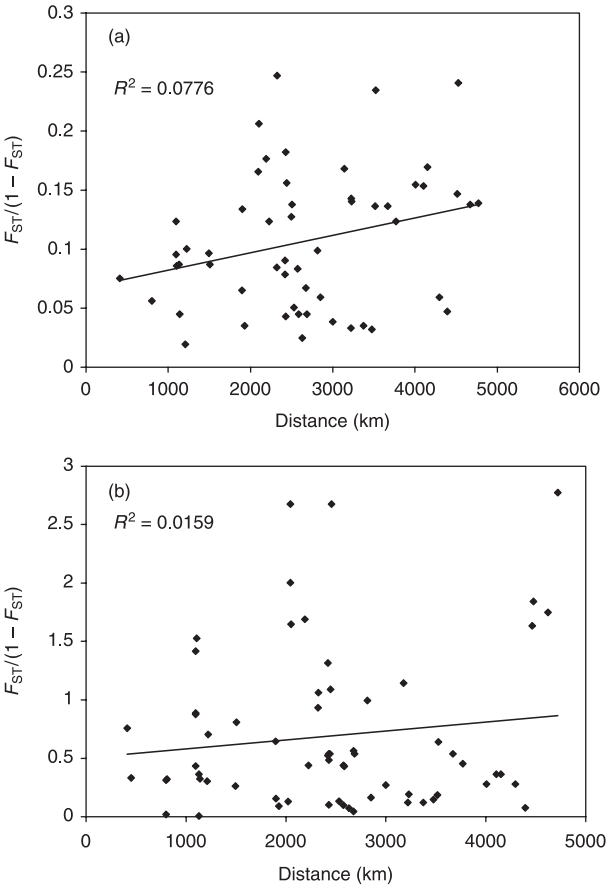

Fig. 5 Relationship between genetic differentiation [$F_{ST}/(1 - F_{ST})$] and river distance for all pairwise combinations of populations for: (a) microsatellites, and (b) mtDNA haplotypes. The point BUF-ARN [3090 km, $F_{ST}/(1 - F_{ST}) = 15.1$] was removed from the mtDNA plot to preserve the scale, but was included in the calculations. Only a single population from each separate sub-basin was included.

and is thus consistent with both the pairwise F_{ST} values and distance-based findings. The other clustering method, BAPS, clearly supported eight clusters (Table 4); two clusters grouped populations together by sub-basin (five in the Araguaia River and two in the Madeira/Guapore River), while a third cluster contained populations spread throughout the Amazon basin, from the upper reaches in Peru (PPS, PRT), and including BRN, UAT, and TAP in central Amazonia (Table 4). The remaining five sub-basins, each represented by a single population sample, were supported as unique populations (BUF, CAQ, ORN, TRS, and XIN).

Genotypic methods. The frequency-based and Bayesian assignment tests provided very similar average self-assignment rates (Table 5a). Both methods were highly concordant in relative assignment values for all populations, and correct assignment rates using the Bayesian method ranged from

Table 4 Clusters of populations supported by the program BAPS; this arrangement of population clusters was supported with a probability of 1.00 (abbreviations as in Table 1)

Cluster	Populations
Araguaia	ARF, ARP, ARC, ARM, ARN
Xingu	XIN
Amazon	TAP, UAT, PPS, PRT, BRN
Guapore	GUP, GUA
Terra Santa	TRS
Abufari	BUF
Caquetá	CAQ
Orinoco	ORN

a low of 0.63 in the Branco population up to more than 95% for the Araguaia (all five sites combined), Xingu (XIN), Terra Santa (TRS), the two Madeira/Guapore River sites combined (GUA and GUP), Abufari (BUF), the Caquetá sites combined, and the Orinoco (ORN) (Table 5a).

Current migration rates among populations were also estimated using the program BAYESASS+ (Wilson & Rannala 2003). However, although likelihood-ratio tests for all populations indicated that significant information about current migration rates could be calculated from the data (data not shown), estimates of 'self-migration' rates, or the proportion of individuals in a sample which derived from the sampled location, varied widely among repeated runs of the program, and a consistent signal of migration among populations could not be determined (Table 5b).

mtDNA sequences

Molecular structure. A total of 293 complete mtDNA control region haplotypes (1343 base pairs) were sequenced for individuals from 17 populations (Tables 1 and 6). Full sequences have been deposited in the EMBL/GenBank Data Libraries (Accession nos AF361951-361997, AY572978-572985, DQ352567–352804). The general structure of the control region consists of 916 bp of sequence starting at the 5′-end, including five unique indels; four single-base indels, and one 67-bp deletion (Fig. 2) present only in 13 individuals from the Uatuma population (UAT) and a single individual from the Branco River (BRN). Following this initial sequence segment are from two to five copies of a 75-bp region, each of which contains a dinucleotide microsatellite of six or seven TA repeats within it (Fig. 2). Excluding the repeat regions, the mtDNA control region exhibited 20 polymorphic sites (15 nucleotide polymorphisms and 5 indels), which resulted in a total of 16 haplotypes (Fig. 3). The greatest number of differences among any of these haplotypes was 8 (~0.8%; Fig. 3), and the most common haplotype was found in 14/17 populations (haplotype G; Table 6). Given the low observed variation and presence of homoplasy among nucleotide sites in the

Table 5 Results from genotypic approaches. (a) Results from GENECLASS using the assignment method of Rannala & Mountain (1997). Only individuals which amplified at five or more loci were included in this analysis. Mean overall assignment rate to sub-basin was 0.87. For the four sub-basins with multiple populations, Araguaia, Madeira/Guapore, Peru, and Caquetá, average correct assignment to beach within sub-basin was 0.41, and to sub-basin were 1.00, 0.96, 0.71, and 0.94, respectively

	Assigned Population																			
	Araguaia									Madeira			Peru		Caquetá					
Sampled population	ARF	ARP	ARC	ARM	ARN	XIN	TAP	TRS	UAT	GUP	GUA	BUF	PRT	PPS	Cent	Tam	Guad	Yaru	BRN	ORN
Araguaia-F	10	1	4	4	4															
Araguaia-P	5	6	3	5	3															
Araguaia-C	5	4	9	4	1															
Araguaia-M	3	8	5	5	3															
Araguaia-N	4	3	1	3	10															
Xingu						24														
Tapajós							16		1	2				1					3	
Terra Santa							1	30												
Uatuma							3		16	1		1		1					2	
Pimentieras							1			14	9									
Guapore										9	14								1	
Abufari												27							1	
Peru(PRT)							1		1	1		1	6	3	1				1	
Peru(PPS)							1		1		1		2	17				1		
Caquetá-C										1					—	1	2	1		
Caquetá-T																7	1	3	1	
Caquetá-G															2	1	2	2		
Caquetá-Y															1	2		5		
Branco								1	3	1			1	3					15	
Orinoco																				38
% Correct	0.43	0.27	0.39	0.21	0.48	1.00	0.70	0.97	0.67	0.58	0.58	0.96	0.40	0.74	0.00	0.58	0.29	0.63	0.63	1.00

(b) Current migration rates estimated using BAYESASS+ for all 17 populations. Shown are the proportions of individuals in each population derived from that source location (the 'self-migration' rate). Mean and standard deviation of five independent runs of the program are given.

Run	ARF	ARP	ARC	ARM	ARN	XIN	TAP	TRS	UAT	GUP	GUA	BUF	PRT	PPS	CAQ	BRN	ORN
1	0.99	0.68	0.68	0.68	0.68	0.99	0.68	0.68	0.69	0.96	0.68	0.68	0.70	0.68	0.89	0.68	0.99
2	0.68	0.99	0.68	0.68	0.68	0.68	0.84	0.99	0.68	0.68	0.97	0.68	0.70	0.68	0.97	0.69	0.99
3	0.68	0.68	0.68	0.68	0.68	0.99	0.69	0.99	0.68	0.68	0.68	0.81	0.70	0.68	0.98	0.97	0.68
4	0.99	0.68	0.68	0.68	0.68	0.68	0.68	0.99	0.68	0.68	0.95	0.99	0.71	0.97	0.95	0.76	0.99
5	0.68	0.68	0.99	0.68	0.68	0.68	0.68	0.86	0.68	0.68	0.98	0.99	0.71	0.68	0.85	0.68	0.99
Mean	0.80	0.74	0.74	0.68	0.68	0.80	0.71	0.90	0.68	0.74	0.85	0.83	0.70	0.74	0.93	0.76	0.93
SD	0.17	0.14	0.14	0.00	0.00	0.17	0.07	0.14	0.01	0.13	0.16	0.16	0.01	0.13	0.05	0.12	0.14

Table 6 Observed mtDNA haplotype counts in populations of *Podocnemis expansa* sampled for this study. Haplotype letters refer to nucleotide variation as in Fig. 3; numbered haplotypes were coded as described in the text. Bold haplotypes are those private to a single population or sub-basin, and underlined denotes haplotypes shared among nesting beaches within a sub-basin. The '*' indicates known transplantations between TAP and TRO (see text for details). Bottom rows summarize the following: N, number of individuals sequenced; # of haplotypes, total number of haplotypes observed; Ar(hap), number of haplotypes per population standardized for sample size; haplotype diversity, ð (Nei 1987); and private alleles, unique haplotypes (bold types in first column)

		Araguaia							Madeira				Caquetá					
Haplotypes		ARM	ARN	XIN	TAP*	TRO*	TRS	UAT	GUA	GUP	BUF	PERU	Cent	Tam	Guad	Yaru	BRN	ORN
P	1	21	20	7	—	—	—	—	—	—	—	—	—	—	—	—	—	—
G	2	—	—	15	3	—	—	1	11	9	—	2	—	—	—	—	—	—
G	3	—	—	—	—	1	—	—	7	5	—	5	—	—	—	—	6	—
G	4	—	—	—	—	1	—	—	—	—	—	—	—	3	1	2	3	9
G	5	—	—	—	—	—	—	—	—	—	—	—	5	4	5	4	1	—
E	6	—	—	—	—	—	—	12	—	—	—	—	—	—	—	—	1	—
A	7	—	—	—	—	—	10	—	—	—	1	—	—	—	—	—	—	—
B	**8**	—	—	—	—	—	—	—	—	—	**11**	—	—	—	—	—	—	—
G	9	—	—	—	—	—	—	—	—	—	—	—	3	1	3	1	1	—
C	**10**	—	—	—	—	—	—	—	**2**	**6**	—	—	—	—	—	—	—	—
D	**11**	—	—	—	—	—	**7**	—	—	—	—	—	—	—	—	—	—	—
A	**12**	—	—	—	—	—	—	—	—	—	—	—	**2**	**1**	**2**	**1**	—	—
G	**13**	—	—	—	—	—	—	—	—	—	—	—	**2**	**1**	**1**	**1**	—	—
G	**14**	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	**5**	—
H	15	—	—	—	—	—	—	1	—	—	—	—	1	—	—	2	—	—
G	16	—	—	—	—	1	—	1	—	1	—	1	—	—	—	—	—	—
A	17	—	—	—	1	1	—	—	—	—	—	—	—	—	—	—	2	—
G	**18**	—	—	—	**4**	—	—	—	—	—	—	—	—	—	—	—	—	—
G	19	—	—	—	—	1	—	—	—	1	—	2	—	—	—	—	—	—
G	20	—	—	—	—	—	—	1	—	—	—	—	—	—	1	1	—	—
G	**21**	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	**3**	—
P	22	1	—	—	2	—	—	—	—	—	—	—	—	—	—	—	—	—
G	**23**	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	**3**
B	24	—	—	—	1	1	—	—	—	—	—	—	—	—	—	—	—	—
J	**25**	—	—	—	**2**	—	—	—	—	—	—	—	—	—	—	—	—	—
G	**26**	—	—	—	—	—	—	—	—	—	—	—	—	—	**1**	**1**	—	—
G	27	—	—	—	1	—	—	—	1	—	—	—	—	—	—	—	—	—
K	**28**	—	—	—	**2**	—	—	—	—	—	—	—	—	—	—	—	—	—
G	29	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	**2**
G	**30**	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	**2**
G	**31**	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	**1**	—
I	**32**	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	**1**
O	**33**	—	—	—	**1**	—	—	—	—	—	—	—	—	—	—	—	—	—
F	**34**	—	—	—	—	—	—	**1**	—	—	—	—	—	—	—	—	—	—
N	**35**	—	—	—	—	—	—	**1**	—	—	—	—	—	—	—	—	—	—

Table 6 *Continued*

		Araguaia							Madeira				Caquetá					
Haplotypes		ARM	ARN	XIN	TAP*	TRO*	TRS	UAT	GUA	GUP	BUF	PERU	Cent	Tam	Guad	Yaru	BRN	ORN
J	**36**	—	—	—	—	**1**	—	—	—	—	—	—	—	—	—	—	—	—
L	**37**	—	—	**1**	—	—	—	—	—	—	—	—	—	—	—	—	—	—
P	**38**	—	—	—	**1**	—	—	—	—	—	—	—	—	—	—	—	—	—
P	**39**	—	—	—	**1**	—	—	—	—	—	—	—	—	—	—	—	—	—
M	**40**	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	**1**	—
A	**41**	—	—	—	—	**1**	—	—	—	—	—	—	—	—	—	—	—	—
G	**42**	—	—	—	—	—	—	—	—	—	—	—	**1**	—	—	—	—	—
G	**43**	—	—	—	—	—	—	—	—	—	—	—	—	**1**	—	—	—	—
G	**44**	—	—	—	**1**	—	—	—	—	—	—	—	—	—	—	—	—	—
G	**45**	—	—	—	—	—	—	—	—	—	—	—	—	**1**	—	—	—	—
G	**46**	—	—	—	—	—	—	—	**1**	—	—	—	—	—	—	—	—	—
G	**47**	—	—	—	**1**	—	—	—	—	—	—	—	—	—	—	—	—	—
G	**48**	—	—	—	—	—	—	—	—	—	—	—	—	**1**	—	—	—	—
G	**49**	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	**1**
G	**50**	—	—	—	—	—	—	—	—	—	—	—	—	—	—	**1**	—	—
G	**51**	—	—	—	—	—	**1**	—	—	—	—	—	—	—	—	—	—	—
	N =	22	20	23	21	8	18	18	22	22	12	10	14	13	14	14	24	**18**
	# of haps	2	1	3	13	8	3	7	5	5	2	4	6	8	7	9	10	6
	Ar (hap)	0.364	0.000	1.322	5.599	7.000	1.441	2.667	2.313	2.607	0.667	2.756	3.755	4.650	4.063	5.084	4.516	3.153
	haplotype	0.0909	0.0000	0.5020	0.9524	1.0000	0.5686	0.5686	0.6667	0.7359	0.1667	0.7333	0.8352	0.8636	0.8462	0.9143	0.8841	0.7451
	diversity	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±	±
		0.0809	0.0000	0.0829	0.0298	0.0625	0.0707	0.1378	0.0741	0.0549	0.1343	0.1199	0.0704	0.0786	0.0742	0.0519	0.0381	0.1006
	Private	0	0	1	8	2	2	2	1	0	1	0	1	3	0	1	4	5
	Alleles	0							2				8					
	Pi†	0.00000	0.0000	0.00214	0.00248	0.00200	0.00500	0.00036	0.00074	0.00187	0.00015	0.00000	0.00085	0.00057	0.00072	0.00059	0.00041	0.00011
	Fu's F_S	—	—	4.02	−0.75	−2.83**	−102	−4.37**	2.12	2.92	∞	0.42	0.71	1.05	2.03	0.05	−2.46**	−0.79

†Overall nucleotide diversity, Pi = 0.00256.

nonrepeat region, we elected to code the haplotypes as described in Methods and use only frequency statistics. Our coding method identified 51 distinct haplotypes (available by request), summarized by population in Table 6. Tajima's *D* was not significant (–0.0679, $P > 0.10$), consistent with the hypothesis that sequence evolution approximates neutrality in this region of the mtDNA genome, but Fu's *Fs* test was significant at a $P < 0.01$ for TRO, UAT, and BRN (Table 6).

Within-population statistics. Populations contained between one (ARN) and 13 (TAP) haplotypes, and the Trombetas population (TRO) was unique in that every individual had a different haplotype ($N = 8$). Nei's haplotype diversity (the probability that two sampled alleles are different) ranged from 0.00 to 1.00 (mean = 0.65), and was highest in the TRO, TAP, BRN, Caquetá, and ORN samples (Table 6). Similarly, allelic richness (Ar, the number of haplotypes detected, standardized for sample size), calculated by rarefaction to a sample size of 8 (TRO), ranged from 0.00 to 7.00 (mean = 3.06) and was highest in the TRO, TAP, and Caquetá samples (Table 6). These two genetic diversity measures were highly correlated ($R^2 = 0.85$, $P < 0.001$).

Twelve of the 17 populations had unique (private) haplotypes. Five and three of these 12 had only one or two private alleles, respectively, but CAQ-Tam, BRN, and ORN had three, four, and five, respectively. Tapajós (TAP) and the combined Caquetá River populations each had eight private haplotypes. Most private haplotypes were present at low frequencies (haplotypes 19, 20, 22, and 24–51; Table 6), but several were present at moderate to high frequencies, including: haplotype 8 in BUF ($11/12 \approx 0.92$); haplotype 11 in TRS ($7/18 \approx 0.39$); haplotype 14 in BRN ($5/24 \approx 0.21$); and haplotype 18 in TAP ($4/21 \approx 0.19$).

Three other general patterns emerge from this summary. First, several haplotypes were not private, but were nevertheless present in high frequencies in single populations while being much less common elsewhere, e.g. haplotype 4 in ORN ($9/18 = 0.50$), haplotype 6 in UAT ($12/18 \approx 0.67$), and haplotype 7 in TRS ($10/18 \approx 0.56$). Second, in river basins represented by multiple population samples, haplotypes were either unique (haplotype 10 in GUA and GUP; haplotypes 12 and 13 in the four CAQ sites), or shared at higher frequencies than elsewhere (haplotype 1 in ARM and ARN; haplotypes 2 and 3 in GUA and GUP (but see also XIN and BRN, respectively); and haplotypes 5 and 9 in the four CAQ sites). Finally, two haplotypes were uniquely shared (haplotype 24) or nearly so (haplotype 17) between TAP and TRO samples, which is consistent with known transplantations of females from Trombetas to Tapajós ~30 years ago (Alfinito *et al*. 1976).

Among-population differentiation. The global mtDNA F_{ST} value estimated from the haplotype frequencies using the program ARLEQUIN was 0.34, with significant differentiation in almost all (121/128) pairwise comparisons among populations from different sub-basins (Table 3). On the other hand, no significant differentiation was detected within sub-basins (Araguaia, Madeira/Guapore, and Caquetá Rivers, Table 3). Thus populations are structured among river basins, but interconnected within basins. Among sub-basins across the entire region, pairwise F_{ST} values calculated from mitochondrial haplotype frequencies were not significantly correlated with geographic distance between populations, and distance explained less than two percent of the variance in F_{ST} values among populations ($R^2 = 0.016$, $P > 0.4$; Fig. 5b).

Correlation between marker types

If historical biogeographic and demographic effects had a strong influence on present levels of population genetic diversity within and among populations in both sexes, patterns in one type of DNA marker should correlate with similar patterns in other marker types. This was true for both pairwise F_{ST} values among populations (Table 3; $R^2 = 0.45$, Mantel test, Spearman's rank correlation, $P < 0.0001$) and for genetic diversity measures within populations. Correlations between microsatellite and mtDNA diversity indexes within populations were positive and statistically significant, consistent with the hypothesis that the observed patterns of genetic diversity in the two marker classes result from common historical demographic effects (Tables 1 and 6: microsatellite Ar vs. mtDNA Ar, $R^2 = 0.49$, $P < 0.01$; microsatellite Ar vs. mtDNA haplotype diversity, $R^2 = 0.41$, $P < 0.05$; microsatellite heterozygosity vs. mtDNA haplotype diversity, $R^2 = 0.34$, $P < 0.05$).

Discussion

The use of both maternally and biparentally inherited molecular markers to investigate the current and historical population genetics of *Podocnemis expansa* allows us to apply and compare the results from diverse analytical methods. Many recently developed methods do not rely on the equilibrium assumptions required by traditional methods, or make different use of the data than traditional methods (Pearse & Crandall 2004; Manel *et al*. 2005). We emphasize, however, that equilibrium and nonequilibrium methods should be considered complementary, such that concordance of results across methods provides a measure of the strength of a given signal (Hickerson & Cunningham 2005). Here, we discuss our results for *P. expansa*, and relate the populations genetic interpretations to the biology and historical exploitation of the species. Below, we compare the patterns of genetic differentiation of *P. expansa* throughout its range with results from similar studies of other widely distributed aquatic taxa endemic to the same

geographic region, and summarize the implications of our findings for current conservation and recovery efforts.

Population structure

Taken together, our results show a pattern of isolation at the level of each individual river basin, but no divergence among individual nesting beaches within any given river basin. The lack of differentiation seen at both nuclear and mitochondrial loci among populations within the Araguaia, Madeira/Guapore, and Caquetá suggests that populations within sub-basins are essentially panmictic, and lack significant natal homing to individual nesting beaches by either sex. Bayesian clustering analysis (Table 4) and the relatively high rate of cross-assignments between sample sites within sub-basins (Table 5a) also support this result. Although Valenzuela (2001) detected genetic differences among the four Caquetá River using microsatellites, the differentiation was low and not consistently significant, and is not supported by our analysis of new mtDNA data. Thus, in general, it appears that *P. expansa* mix freely among nesting areas within any given sub-basin, but that some degree of natal-river fidelity or other behavioural mechanism maintains differentiation between rivers, even when they are geographically close.

At a larger scale, all pairwise microsatellite F_{ST} values for populations from separate sub-basins were significant (Table 3), indicating limited gene flow between the tributary rivers. However, this pattern was not always concordant across methods. Although the most highly diverged populations, from the Araguaia and Orinoco, were uniquely identifiable using all analytical methods (Tables 4 and 5a; Fig. 2), several populations with significant pairwise F_{ST} values were grouped together by BAPS in a single 'Amazon' cluster (Table 4) had and relatively low correct self-assignment rates (Tables 3 and 5). Nonetheless, the overall patterns of genetic differentiation were highly concordant across both methods and markers, and below we discuss some of the most important finding with respect to conservation management.

One notable pattern in our data was that some populations separated by large geographic distances (> 3000 km, e.g. Tapajós, Peru, Guapore) were only slightly more differentiation than populations in neighbouring river basins. This finding parallels the patterns of mtDNA haplotype structure found in both manatees (*Trichechus inunguis*; Cantanhede *et al*. 2005) and pirarucu (*Arapaima gigas*; Hrbek *et al*. 2005) sampled from the main-stem Amazon over similar geographic distances. Table 7 compares population genetic data for *P. expansa* and three other aquatic Amazonian vertebrates with comparable geographic sampling of mtDNA diversity. In black caiman (*Caiman niger*), mtDNA haplotype data suggest a pattern of restricted gene flow with isolation by distance, whereas in river dolphins (*Inia geoffrensia*), populations have apparently been isolated above the cataract in the Madeira/Guapore basin (Fig. 1) long enough for mtDNA haplotypes to segregate to reciprocal monophyly with dolphins in the remaining Amazon + Orinoco basins. However, patterns of geographic variation within each haploclade suggest a similar metapopulation structure (restricted gene flow with isolation by distance; Banguera-Hinestroza *et al*. 2002). The general pattern is that for all of these large, highly vagile species, migration in the Amazon is sufficiently free to prevent development of a strong relationship between gene flow and geography, even over long distances.

Consistent with the above observations, isolation by distance among populations of *P. expansa* nesting in different sub-basins was not significant for either microsatellites or mtDNA, and explained only 7.8% and 1.2% of the variance in F_{ST} values, respectively (Fig. 5a, b). For both marker types, this is due primarily to the presence of pairs of populations that are geographically distant, but genetically very similar (Tables 2 and 3; Figs 1, 5a, b). This lack of association between genetic structure and geography is true even for the Orinoco River population, which is only tenuously connected to the Amazon system through the Brazo Casiquiare (Fig. 1). Although this waterway may constitute a significant gene flow barrier for river dolphins (*Inia geoffrensis*; Banguera-Hinestroza *et al*. 2002), unlike dolphins, *P. expansa* may traverse potential shallow-water barriers which dolphins cannot cross. While it is impossible to draw strong conclusions from the nonassociation of geography and genetic differentiation, it is consistent with a hypothesis of recent divergence driven by independent drift following fragmentation and population size reductions. Under such a scenario, isolation and fragmentation of formerly connected breeding areas, possibly strengthened by female fidelity to particular sub-basins and directed exploitation of nesting females, could cause the idiosyncratic pattern of genetic divergence seen in *P. expansa* populations. Further, this scenario would not produce the deeper genetic divergences among groups of populations expected of historical phylogeographic patterns or long-term demographic effects. This hypothesis is supported by the strong, highly significant correlation between allelic richness and mean among-basin pairwise F_{ST} values for all populations, which suggests that drift, driven by population size reductions, is the primary force influencing genetic differentiation among these populations of *P. expansa*.

One alternative to the above hypothesis is that selection on either the microsatellite or mtDNA loci may have affected the distribution of genetic variation in these populations. Selection acting on particular loci has been hypothesized to affect phylogenetic interpretations in many taxa (Pogson & Fevolden 2003), including Amazonian fishes sympatric with *P. expansa* (Turner *et al*. 2004; Moyer *et al*. 2005). Although we cannot rule out selection completely, two lines of

Table 7 Summary of population genetic variation and metapopulation structures estimated for several approximately codistributed, large-bodied, aquatic, tetrapod Amazonian vertebrates, on the basis of mtDNA gene regions

Taxon	# of sites	Mean sample size (range)	DNA region	Nucleotide diversity (π)	Haplotype diversity (h)	River basin	Inferred population structure
Caiman niger[1] (black caiman)	4	11.25 (8–17)	cyt *b*	0.715 (± 0.049)	—	Amazon + French Guiana	Restricted gene flow with isolation-by-distance
Inia geoffrensis[2] (pink river dolphin)	15	6.7 (1–28)	D-loop	0.567	0.931 (± 0.072)	Amazon + Orinoco river	Reciprocal monophyly of Bolivian
			Cyt *b*	0.338	0.778 (± 0.109)	basins	Amazon vs. Amazon + Orinoco haplotypes
Trichechus inunguis[3] (Amazon manatee)	6	11.3 (8–18)	D-loop	0.624 (± 0.384)	0.887 (± 0.026)	Main Amazon basin only	Restricted gene flow, some long-distance dispersal
Arapaima gigas[4] (Pirarucu)	8	17.4 (13–33)	NADH1 ATPase	—	0.554 (± 0.341)	Main Amazon basin only	Restricted gene flow, some long-distance dispersal
Podocnemis espansa (this study)	14	20.9 (8–24)	*D*-loop	0.00256	0.65 (± 0.305)	Amazon + Orinoco river basins	Restricted gene flow, fragmented populations

[1]Farias *et al.* (2004).
[2]Banguera-Hinestroza *et al.* (2000).
[3]Cantanhede *et al.* (2005).
[4]Hrbek *et al.* (2005).

evidence suggest that it is not the major force driving the distribution of genetic variation in *P. expansa*. First, Tajima's *D* and Fu's *Fs* test are consistent with neutrality for the mtDNA data, suggesting that recent selective sweeps are unlikely. Second, the overall differentiation among populations estimated by F_{ST} values followed the expected pattern of an approximately fourfold greater value for the maternally inherited marker vs. the nuclear markers. Although the power of neutrality tests is low, and stochastic variation in coalescence can be high (Hudson & Turelli 2003), these observations suggest that the patterns of genetic variation reflect past demographic influences on both classes of loci rather than selective effects on any single locus.

Given the above, the strongest inferences can be drawn from the populations exhibiting concordant results from both nuclear and mtDNA data analysed with a variety of methods. By these criteria, the most clearly differentiated group of populations are the five Araguaia River populations, which are differentiated from all other populations at the nuclear loci, and nearly fixed for mtDNA haplotype 1/P), which is rare elsewhere (Table 6). The position of the Araguaia River near the mouth of the Amazon system suggests that the divergence of these populations may stem from a Miocene separation between the small Atlantic proto-Amazon drainage, and the western-central Amazon–Orinoco that drained north into the Caribbean (Hamilton *et al*. submitted). The divide separating the Atlantic palaeo-Amazon from the western-central Caribbean palaeo-Amazon drainages may have been present from 20 to 10 million years ago, and have breached about 10 to 9 million years ago, when the transcontinental drainage of today's Amazon basin was established (Hamilton *et al*. submitted). Alternatively, *P. expansa* may have colonized this basin after breaching, and then differentiated in isolation due to subsequent Pliocene–Pleistocene marine incursions (Nores 2004), during which high sea levels may have separated the Araguaia River from the Amazon River. Similarly, the two population samples from the Madeira/Guapore River (GUA and GUP) are grouped by the microsatellite loci and share an mtDNA haplotype (haplotype 10/C, Table 6) not found outside that sub-basin. Both of these populations are upstream of the large Madeira River cataract, below the Guapore River nesting beaches (GUA and GUP; Fig. 1), which could be a substantial barrier to gene flow in *P. expansa*. Nonetheless, they also share the common haplotypes 2 and 3 (G; Table 6) at high frequency, suggesting a relatively recent divergence from the other Amazonian populations.

Population size reductions

The microsatellite data are consistent with historical accounts of *P. expansa*'s extensive population declines due to hunting over the past two centuries (von Humboldt 1814; Bates 1863; Ramirez 1956; Dixon & Soini 1977; Pritchard &

Trebbau 1984). Tests for recent population reductions were significant for most populations examined (Table 2). *M* ratio values for all sampled *P. expansa* populations are among the lowest observed, and similar to values from the drastically reduced salmonid populations on the west coast of North America (J.C. Garza, personal communication). This consistent signal across all *P. expansa* populations suggests a widespread hunting pressure not limited to areas surrounding major population centres. In fact, as turtle populations near major human settlements were decimated, remote populations (e.g. Caquetá) became an alternative supply for large markets (von Hildebrand *et al*. 1997), thus increasing their exploitation despite low human densities nearby and the long distances to major markets. Values of standardized allelic richness follow a similar pattern, but may suggest somewhat reduced hunting in remote or protected regions, with an almost twofold difference in diversity levels between sites. Values of Ar should be the more sensitive of the variability estimators we employed due to loss of rare alleles (Spencer *et al*. 2000; England *et al*. 2003). Populations with the lowest Ar values (XIN, ORN, PNA, ABU; with Ar = 4.4, 4.7, 4.9, and 5.4, respectively) are known or strongly suspected to have been intensively exploited. Conversely, the two samples in which allelic diversity is highest (PPS = 8.7, TAP = 8.4) are either known or strongly suspected to represent admixed populations (see Alfinito *et al*. 1976; for TAP; T. Engstrom, personal communication for PPS). Importantly, historical hunting pressure primarily targeted nesting females, which theoretically could have reduced female effective population size further than overall N_e. However, reductions in female numbers generally reduce overall N_e along with female N_e, such that the effects should be seen in nuclear markers unless counteracted by increased polyandry (which reduces the variance in male reproductive success and increases N_e; Sugg & Chesser 1994). Multiple paternity is not common in *P. expansa* in Venezuela (Pearse *et al*. in press), but may be more frequent in other populations (Valenzuela 2000), and the extent of variation among populations and its impact on effective population size remains unknown.

Conservation implications

The overall implications of our study are encouraging for genetic management of this species. At the within sub-basin level, the relatively extensive gene flow within the Araguaia, Madeira/Guapore, and Caquetá basins argues against strict natal homing among females. However, lack of genetic differentiation does not imply complete demographic connectedness. Bock *et al*. (2001) recommended that, until more information is available on nest-site fidelity of *P. expansa* throughout its range, restoration programmes should not release hatchlings long distances from their natal beaches. Our study endorses this, but also suggests that, at least within sub-basins, *P. expansa* does not suffer a high 'evolutionary handicap' (Schroth *et al*. 1996) resulting from strict natal homing by either sex. This provides some flexibility in release of hatchlings away from their natal beach but within the same sub-basin. Nonetheless, populations within sub-basins should be treated as demographically independent unless dispersion data indicate otherwise. Furthermore, the effects of social facilitation among nesting females (where, how, and when these occur) requires study before optimal release strategies can be determined. Although only limited dispersal data exist from natural populations of *P. expansa* (Pritchard & Trebbau 1994; Cantarelli 1997; von Hildebrand *et al*. 1997), the inference of dispersal and gene flow patterns by indirect molecular methods can provide critical data on dispersal among populations at a variety of scales (Berry *et al*. 2004), and our data thus provide guidelines for such future studies.

Perhaps of more concern is that holding hatchlings in captivity may disrupt imprinting, learning, or social facilitation experiences with adults that may be needed to develop appropriate migratory behaviour (Bock *et al*. 2001). Understanding these behaviours may facilitate the establishment or augmentation of nesting sites from nearby source populations in the absence of the evolutionary handicap (Schroth *et al*. 1996). It is encouraging, for example, that *P. expansa* nests on beaches constructed around artificial ponds (R. Vogt, personal communication), suggesting that migration per se is essential for successful nesting. Furthermore, releasing hatchlings and yearling juveniles, as done in Brazil and Venezuela, respectively, are better long-term genetic strategies for population recovery and persistence than rearing and releasing adults because they maximize within-population fitness heterogeneity and enhance the purging of deleterious alleles (Robert *et al*. 2004). Nonetheless, protection of adults, particularly nesting females, remains paramount to avoid the demographic declines associated with adult harvesting in species with Type III survivorship curves (Spencer & Thompson 2005).

Patterns of mtDNA haplotype diversity (Table 6) likely reflect differences in long-term population sizes, and populations with low mtDNA diversity coupled with reduced heterozygosity and allelic richness at nuclear microsatellite loci may be predicted to have reduced fitness and lowered evolutionary potential (Frankham *et al*. 2002; Reed & Frankham 2003). However, this threat is lessened in migratory species lacking strict natal homing; individuals may move between demes or be transplanted as with the Trombetas to Tapajós example (Alfinito *et al*. 1976). Following transplantations from Trombetas, nesting females observed at Tapajós (TAP, Fig. 1) increased from only ~300 females in 1979 to ~3000 females today (V.H.C., unpublished data). Tapajós displays much higher genetic diversity than most other Brazilian populations at nuclear and mtDNA loci (Tables 1 and 6), including two haplotypes shared only

with Trombetas. The dramatic recovery by the Tapajós nesting aggregation may be due a combination of genetic 'rescue' of a bottlenecked population by the transplantation (Westemeier *et al*. 1998; Madsen *et al*. 1999) as well as post-bottleneck recruitment from nearby sources not sampled in this study. In either case, this rebound reflects the efficacy of approximately two decades of IBAMA protection.

Lastly, our genetic data suggest that several river basins have distinct genetic 'signatures', but additional markers and sampling within some sub-basins is needed to resolve these boundaries sharply and to develop statistically solid forensic resources to assign unknown individuals to their basin of origin (DeSalle & Amato 2004). The latter would be useful for detection of illegal harvests and transport routes.

What the data don't tell us

The maintenance of diversity among populations is recognized as a central conservation problem (Hughes *et al*. 1997), and the structure diagnosed by neutral genetic markers is generally assumed to correlate with demographic or ecological variation among populations and/or local adaptation (Crandall *et al*. 2000). If true, then the use of neutral loci to delimit ESUs or MUs (Moritz 1995) can contribute to recovery efforts, but the presumed correlation between population variation at neutral molecular markers and variation in 'persistence value' is not firmly established (Pearman 2001). While some recent studies do suggest that molecular markers are useful indicators of population persistence-extinction probabilities (reviewed in Pearman 2001; Frankham *et al*. 2002), others report substantial differentiation of fitness correlates between populations connected by gene flow over very short geographic distances (e.g. sprint speed, endurance, and wariness in the lava lizards *Microlophus albemarlensis* across an environmental gradient of < 1.0 km; Jordan *et al*. 2005). Unfortunately, the heritability of most ecological and life history traits is unknown. However, assuming that population differences are not entirely due to phenotypic plasticity, patterns of divergence in adaptive differences among populations could differ substantially from patterns of genetic variation at neutral loci, like those reported here, and the former are more likely to determine vital rates and long-term population viability (Holsinger *et al*. 1999).

This final point underscores that absence of population structure, as in the central-Amazon *P. expansa* populations (Fig. 4), does not imply absence of conservation value. Population structure in quantitative traits that reflect local adaptations and/or persistence value will be undetected in this or equivalent (Lynch 1995), and future efforts to characterize variation in such traits are not only warranted but essential. The range of *P. expansa* spans a huge region characterized by pronounced differences in wet/dry seasons across the Amazon and Orinoco basins, and river basins that further vary in geological substrates, sediment loads, and biological productivity (Goulding *et al*. 2003). Nesting populations differ substantially in body size, clutch size, daily nesting activity (e.g. nocturnal vs. diurnal nesting, V.H.C., personal observation), and timing and success of hatching and emergence. Indeed, significant between-basin differences in clutch size exist, but no significant variation *within* river basins has been found (Vanzolini 2003). Thus, data from many sources, including ecology, should be thoroughly integrated across the geographic range of species of conservation concern before time and resources are irrevocably committed to a particular strategy (Johnson *et al*. 2004). In this context, the population differences delimited in this study present a working hypotheses about the conservation value of the interconnected groups (Pearman 2001), rather than the last word about identification of the management units (ESUs or MUs; Moritz 1995) of conservation value.

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