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(NEED CAPTION)

GENETIC POPULATION STRUCTURE OF THE YELLOW ANACONDA (*EUNECTES NOTAEUS*) IN NORTHERN ARGENTINA: MANAGEMENT IMPLICATIONS

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ABSTRACT: We present the first study of genetic population structure of Yellow Anacondas (*Eunectes notaeus*). Partial cytochrome B (cyt-*b*) and ND4 mitochondrial DNA sequences were analyzed to evaluate population structure in *E. notaeus* in northern Argentina. Our mtDNA analysis of the geographical distribution of genetic variation suggests that genetically isolated populations of *Eunectes* exist in the study area. Populations in northern Formosa, in eastern Formosa along the Paraguay River, and in southwestern Formosa exhibit moderate to substantial levels of genetic divergence. Our analysis does not provide evidence of significant genetic isolation between populations in southeastern Formosa and Corrientes, but this relationship should be closely monitored as more samples from these regions become available. Such genetic differentiation results from the interaction between prominent geographic features in the region and issues inherent to the species' natural history. Because this species is being commercially hunted, management recommendations are timely. The genetic structure among the analyzed samples suggests that the genetically differentiated groups should be considered distinct management units.

INTRODUCTION

The Yellow Anaconda (*Eunectes notaeus*) is the largest snake inhabiting Argentina. The species is distributed along the Paraguay River in the Pantanal Region in Bolivia, Brazil, and Argentina, where it reaches its southernmost distribution (Henderson et al., 1995). Anacondas are largely aquatic trophic generalists restricted mainly to river floodplains and wetlands (Strüssmann and Sazima, 1993; Henderson et al., 1995). *Eunectes notaeus* shows marked sexual size dimorphism, with females attaining larger sizes than males (to 4 m in total length and ca. 30 kg of mass; Micucci et al., 2006; Henderson et al., 1995; Dirksen, 2002).

The trade of *E. notaeus* skins has been among the most extensive of any Neotropical species (Waller and Micucci, 1993), and Argentina has been the second highest supplier (second only to Paraguay). After a complete ban was effectively implemented in 1999, the government of Argentina established a sustainable-harvest program for *E. notaeus* in 2001 (Micucci et al., 2006). In light of this reality, conservation genetics offers an appropriate perspective, through the characterization of management units (MU), to identify and prioritize entities for management and conservation (Frankham et al., 2002; Vogler and DeSalle, 1994; Goldstein et al., 2000; King and Burke, 1999).

Herein we describe the genetic diversity of *E. notaeus* in northeastern Argentina, where the species has been hunted for decades. Because this is the first population-level genetic assessment of this species, it is also our objective to contribute to the general scientific knowledge of *Eunectes* in the study area.

MATERIALS AND METHODS

Study Area

Our study area is situated in the provinces of Formosa and northwestern Corrientes, Argentina (22°28'–30°46' S; 55°40'–62°30' W; Fig. 1), where *Eunectes notaeus* has been taken historically for commercial purposes and a harvest program was recently established. The site lies in the Chaco Savanna and Humid Chaco ecoregions (Dinerstein et al., 1995) and can be described as a vast sedimentary plain with high morphological instability and modeled by Andean rivers. Its most conspicuous and relevant geographic elements are the Pilcomayo and the Bermejo rivers, which currently flow in a NW–SE direction. The bed of the Bermejo River moved 35–50 km north about 150 years ago, and the bed of the Pilcomayo River began regressing to the west about 30 years ago, creating a 300,000-ha marshland locally known as the "Bañado la Estrella" (Adámoli, 2001). This wetland comprises one of the most densely populated *Eunectes* habitats in Argentina (P. Micucci and T. Waller, unpublished). Smaller rivers in the eastern portion of the region flow in the same general direction. The

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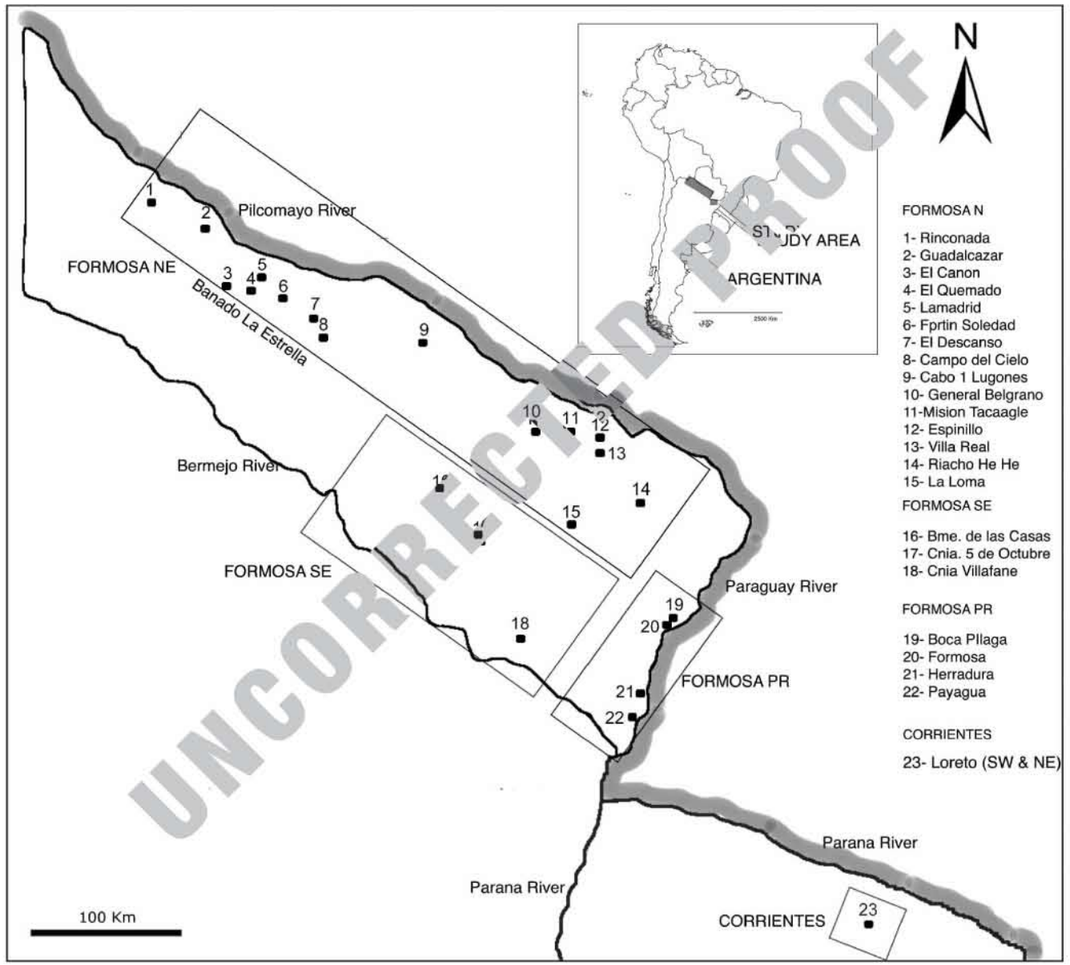

Fig. 1. Study area map showing location of sampling sites. Rectangles delimit groups of sampling sites pooled for the population genetic analysis.

Paraguay River is the only potential natural corridor for aquatic snakes that connects suitable habitats in the northeastern and southeastern regions of Formosa Province.

Sampling Sites

Sampling sites were established in northern and southeastern Formosa Province and in northern Corrientes Province. These sites cover the region in which the species has been commercially harvested and where the sustainable harvest program occurs. Our sampling effort was dependent on available logistic resources to collect animal samples, but should provide a fair representation of snake abundance in the region.

From 2000 to 2002, blood samples were extracted from animals found at 21 sampling sites in Formosa Province and 2 sampling sites in the western tip of Corrientes Province (Table 1). We grouped the sampling sites *a priori* for the population genetic analysis. The grouping into Formosa N, Formosa SE, Formosa PR, and Corrientes took into account landscape features that we suspected might influence movement of snakes between sites (i.e., rivers and streams serve as passive movement agents or corridors, whereas extensive dry uplands impede or prevent dispersal; Waller, 1986; Waller et al., 1993).

Table 1. Sampling sites with their coordinate position and the number of samples analyzed for each gene region in each site.

Site name	Province	Latitude	Longitude	N (cyt-*b*)	N (ND4)
La Rinconada	Formosa	-23°29'39"	-61°34'35"	4	3
Guadalcazar	Formosa	-23°40'24"	-61°09'49"	4	1
El Quemado	Formosa	-24°00'00"	-60°59'12"	1	1
Lamadrid	Formosa	-24°04'36"	-60°41'30"	3	1
Fortín Soledad	Formosa	-24°08'20"	-60°35'17"	4	3
El Descanso	Formosa	-24°12'43"	-60°29'02"	2	3
Campo del Cielo	Formosa	-24°22'28"	-60°25'33"	2	2
Cabo 1° Lugones	Formosa	-24°24'40"	-59°45'26"	4	4
General Belgrano	Formosa	-24°55'23"	-58°59'49"	1	1
Misión Tacaaglé	Formosa	-24°57'22"	-58°48'11"	4	4
Espinillo	Formosa	-24°58'47"	-58°33'29"	2	0
Villa Real	Formosa	-25°06'20"	-58°34'45"	4	3
Riacho He He	Formosa	-25°26'22"	-58°15'21"	5	3
La Loma	Formosa	-25°27'42"	-58°46'16"	1	1
Bartolomé de las Casas	Formosa	-25°21'31"	-59°37'27"	3	3
Colonia 5 de Octubre	Formosa	-25°50'41"	-59°19'45"	0	1
Colonia Villafañe	Formosa	-26°12'16"	-59°04'52"	3	4
Boca Pilagá	Formosa	-26°04'08"	-57°58'37"	4	3
Formosa	Formosa	-26°09'19"	-58°08'38"	2	1
Herradura	Formosa	-26°28'16"	-58°14'11"	1	3
Payagua	Formosa	-26°43'10"	-58°17'20"	4	3
Loreto SO	Corrientes	-27°42'35"	-57°11'20"	2	3
Loreto NE	Corrientes	-27°34'58"	-57°09'51"	2	3

Laboratory Procedures

Due to its relatively rapid mutation rate, mitochondrial DNA (mtDNA) is frequently used as a genetic marker for population-level genetic analyses in vertebrates (Brown et al., 1979; Kocher et al., 1989; Vigilant et al., 1997; Avise, 2000). In particular, cytochrome B (cyt-*b*) and ND4 sequences have often been used to study population genetics in reptiles (e.g., Rodríguez-Robles et al., 1999, 2001; Keogh et al., 2001; Janzen et al., 2002). In our study, we sequenced partial regions of the cyt-*b* and ND4 genes in the mtDNA to analyze population structure among our samples of *E. notaeus* in northern Argentina.

We extracted blood samples from animals in the field and preserved them in 96% ethanol or EDTA until the purification protocol was carried out in the genetic laboratories at the Center for Environmental Research and Conservation, Columbia University, New York. We followed standard protocol for DNA-extraction from blood (Sambrook et al., 1989), using the DNeasy® Tissue extraction kit (QiaGen, Valencia, California). We verified the presence and size of DNA fragments using 1% Agarose-TBE gels (Promega®, Madison, Wisconsin), and then amplified the cyt-*b* and ND4 genes by PCR reactions using the following primers: LGLU (5' TGA TCT GAA AAA CCA CCG TTG TA 3'), and H15544 (5' AAT GGG ATT TTG TCA ATG TCT GA 3') for the cyt-*b* gene (Janzen et al., 2002); DW1641 (5' TGA CTA CCA AAA GCT CAT GTA GAA GC 3') and DW1642 (5' TAT TAG TAG GTG TTC TCG 3') for the ND4 gene (Janzen et al., 2002). PCR amplifications for both mtDNA regions were carried out in a 50-µl reaction volumes using the following conditions: 1 pM of each primer, 11.25 µl of H_2O, 25 µl of FailSafe® buffer (Epicentre®), and 11.5 µl of template DNA (1–2 ng DNA). Thermal profiles for the cyt-*b* region were as follows: initial denaturation for 3 min at 94 °C followed by 40 amplification cycles (60 sec of denaturation at 94 °C, 90 sec of annealing at 50 °C, and 120 sec of extension at 72 °C), and a final 5-min extension at 72 °C. Thermal profiles for the ND4 region were as follows: initial

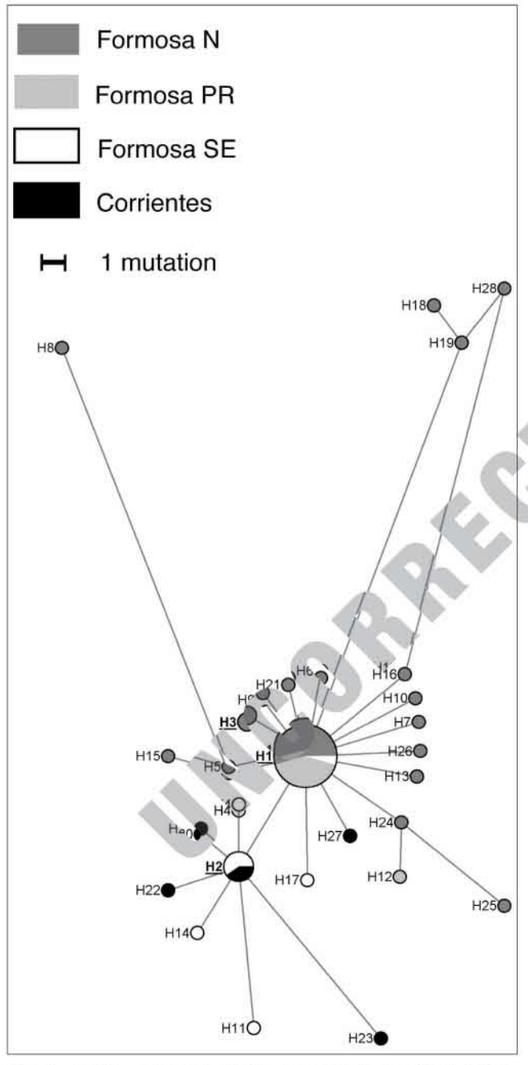

Fig. 2. Median Joining haplotype network for the ND4 region. Haplotypes are colored according to population, and haplotype sizes are proportional to their frequency. Underlined haplotypes are represented by more than one sample.

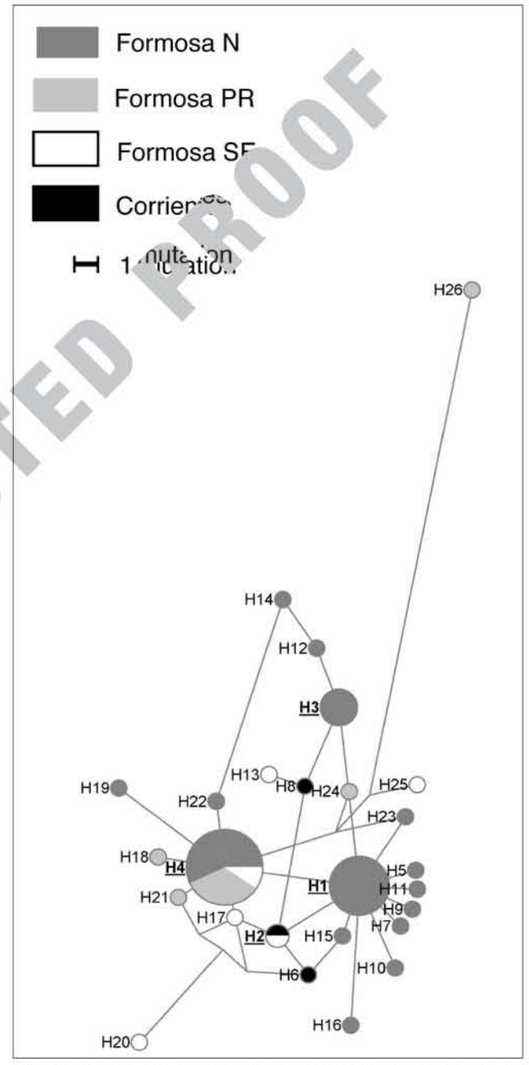

Fig. 3. Median Joining haplotype network for the cyt-*b* region. Haplotypes are colored according to population, and haplotype sizes are proportional to their frequency. Underlined haplotypes are represented by more than one sample.

denaturation for 3 min at 94 °C followed by 35 amplification cycles (30 sec of denaturation at 94 °C, 60 sec of annealing at 50 °C, and 60 sec of extension at 72 °C), and a final 5-min extension at 72 °C. We then followed standard purification protocol using the QIAquick Spin Purification kit (QiaGen). Cycle sequencing was carried out with the BigDye-DNA sequencing kit (Applied Biosystems, Foster City, California). The thermal profiles used were as follows for both mtDNA regions: 30 cycles (10 sec at 96 °C, 5 sec at 50 °C, and 4 min at 60 °C). The samples were then cleaned by filtration in a matrix of Sephadex™/water and run in an automatic sequencer (ABI Prism 377 DNA Sequencer, Perkin Elmer, Wellesley, Massachusetts). Parallel and anti-parallel strands were obtained and assembled using the Autoassambler software (ABI Prism software, Applied Biosystems).

Data Analysis

We extracted the targeted portions of DNA and partially sequenced them in 54 and 62 samples for the ND4 and cyt-*b* genes, respectively. We analyzed and compared 486 bp of the ND4 gene and 390 bp of the cyt-*b* gene. Patterns of genetic variation between the identified haplotypes for both mtDNA gene regions were depicted using Median Joining networks (Bandelt et al., 1999) as implemented in Network (www.fluxus-engineering.com). We also constructed statistical parsimony haplotype networks for comparison (Templeton et al., 1992; Posada et al., 2001) using the software TCS (Clement et al., 2000).

We sought to visualize relationships between the different haplotypes of *E. notaeus* in the spatial context of our study area through the use of haplotype networks. Haplotype networks are more appropriate than gene trees when studying patterns of intraspecific genetic variation and gene flow. For instance, the use of trees assumes that ancestral haplotypes are no longer present in the population, although those are the haplotypes most frequently sampled in population-level studies (Clement et al., 2000).

Population genetic analyses were carried out using the Arlequin (Scheider et al., 2000) and DNAsp (Rozas et al., 1995; 1997; 1999; 2003) packages. Once sequences were aligned with the software Mac Clade v. 4 (Madison et al., 2005), we used DNAsp to identify haplotypes for subsequent population analyses. We performed ANOVA (Excoffier et al., 1992) and computed global F_{st} indices in order to analyze genetic structuring among our samples. Subsequently, we made comparisons between groups of sampling sites or "populations" using the F_{st} index (Weir et al., 1984; Slatkin, 1987, 1991). The statistical significance of the extent of differentiation between populations was assessed using non-parametric permutations of haplotypes between those populations (Excoffier et al., 1992). As an approach to test for a random distribution of individuals between pairs of populations, an exact test of population differentiation (Raymond et al., 1995) was used. The significance of this test (analogous to a Fisher's test) is an indication of non-random association of individuals among populations. We further characterized our proposed populations by computing the number of segregating sites (S) and number of haplotypes (h), and by estimating haplotype diversity (Hd; Nei, 1987), the mean number of pairwise differences among sequences (K; Tajima, 1983, 1993; Kimura, 1980), and the nucleotide diversity (π; Tajima, 1983; Nei et al., 1975; Kimura, 1980). Finally, we used Mantel tests (Mantel, 1967) as implemented in Arlequin to assess the significance of correlations between geographical and genetic distance matrices, which is a method to explore patterns of isolation by distance among our study sites.

RESULTS

Both of the partial gene regions we examined exhibited moderate amounts of variation. We identified 28 haplotypes generated by 61 segregating sites for the ND4 region, and 26 haplotypes and 65 segregating sites for the cyt-*b* region. We observed a clear geographical distribution of the genetic variation in the study area, as depicted in both of the median-joining haplotype networks (Figs. 2, 3). Although the ND4 and cyt-*b* gene regions do not show exactly the same patterns, some generalizations can be made. In both networks, the highest frequency haplotype (H1 for the ND4 gene region, and H4 for the cyt-*b* gene region) is widely distributed across the study area. Unique haplotypes are common for both gene regions, mainly occurring in Formosa N, followed by Formosa SE, Corrientes, and Formosa PR. The ND4 gene region network shows a clustering of the haplotypes occurring in Formosa SE and Corrientes. Such segregation is less obvious in the cyt-*b* gene network, which shows a clear separation of a group of haplotypes occurring in Formosa N. Overall, the ND4 gene region network displays more obvious geographical patterns of genetic variation than the cyt-*b* gene region. Such patterns appear to be consistent with the proposed division of sampling sites for the population genetic analysis. Statistical parsimony networks displayed analogous patterns of genetic variation, and are not shown here because they do not provide additional information.

Both global ANOVAs resulted in significant structuring of the samples according to the population scheme we proposed ($F_{st} = 0.154$, $P < 0.005$ for the ND4 gene region; $F_{st} = 0.089$, $P < 0.005$ for the cyt-*b* gene region). Almost all pairwise F_{st} values were significant ($P < 0.005$) for the ND4 region; Formosa SE versus Corrientes being the only exception. Pairwise exact differentiation comparisons were also mostly significant for this gene region ($P < 0.005$; Table 2). For the cyt-*b* region, only the pairwise F_{st} value for the Formosa N–Formosa PR comparison was significant ($P < 0.005$). However, other values approached sig-

Table 2. Below diagonal are pairwise F_{st} values for the ND4 gene region between defined populations. Significant values ($P < 0.05$) are in bold. Above diagonal, marked as positive are significant P values for the exact test of population differentiation. Non-significant values are marked as negatives.

	Formosa N	Formosa RP	Formosa SE	Corrientes
Formosa N	0	-	+	+
Formosa RP	**0.12046**	0	+	+
Formosa SE	**0.08827**	**0.41045**	0	-
Corrientes	**0.1193**	**0.49871**	0	0

Table 3. Below diagonal are pairwise F_{st} values for the cyt-*b* gene region between defined populations. Significant values ($P < 0.05$) are in bold. Above diagonal, marked as positive are significant P values for the exact test of population differentiation. Non-significant values are marked as negatives.

	Formosa N	Formosa RP	Formosa SE	Corrientes
Formosa N	0	+	+	+
Formosa RP	**0.10491**	0	-	+
Formosa SE	0.04511	0.05362	0	-
Corrientes	0.12432	0.26119	0	0

nificance (e.g., Formosa N–Corrientes, $P = 0.08$), and almost all pair-wise exact differentiation contrasts were significant ($P < 0.005$; Table 3).

Formosa N had the greatest number of haplotypes for both gene regions, more than double that of the other populations (Tables 4, 5.) This population also exhibited larger values of genetic and nucleotide diversity for the ND4 gene region, but lower values of genetic and nucleotide diversity for the cyt-*b* region (Figs. 4, 5). This situation was opposite that for the Formosa PR population, which exhibited larger values of genetic and nucleotide diversity in the cyt-*b* gene region and significantly lower values in the ND4 gene region (Figs. 6, 7). Corrientes and Formosa SE had similar values of genetic and nucleotide diversity for both gene regions.

The Mantel tests, performed to assess significance in correlations between geographical and genetic distances, were not significant, indicating no correlation between the genetic and geographical distances of our populations ($P = 0.25$ for the cyt-*b* gene region and $P = 0.46$ for the ND4 gene region). Only 0.04% and 0.001% of the genetic divergence (respectively for the cyt-*b* and ND4 gene regions) between populations were determined by geographical distances.

DISCUSSION

We found significant genetic structure among populations of *Eunectes notaeus* in our study area. Although not absolutely concordant in every aspect of our analysis, both gene regions exhibited similar patterns of gene flow and genetic structuring. Both haplotype networks displayed clustering of haplotypes of Formosa N and of Formosa SE together with

Table 4. Population parameters for the ND4 gene region. N = number of samples, S = number of segregating sites, h = number of haplotypes, Hd = haplotype diversity, K = mean number of pairwise differences among sequences, and π = nucleotide diversity. Standard deviations are in parentheses.

Population	N	S	h	Hd (SD)	K (SD)	π (SD)
Formosa N	30	41	18	0.8460 (0.0665)	5.967816 (2.927681)	0.013471 (0.007354)
Formosa RP	10	2	2	0.2000 (0.1541)	0.400000 (0.402885)	0.000823 (0.000938)
Formosa SE	8	12	6	0.8929 (0.1113)	4.964286 (2.700353)	0.010257 (0.006355)
Corrientes	6	13	5	0.9333 (0.1217)	4.333333 (2.492210)	0.009782 (0.006496)
Total	54	61	28			

Table 5. Population parameters for the cyt-*b* gene region. N = number of samples, S = number of segregating sites, h = number of haplotypes, Hd = haplotype diversity, K = mean number of pairwise differences among sequences, and π = nucleotide diversity. Standard deviations are in parentheses.

Population	N	S	h	Hd (SD)	K (SD)	π (SD)
Formosa N	42	28	15	0.8211 (0.0403)	3.265970 (1.715948)	0.008374 (0.004887)
Formosa RP	11	39	5	0.6182 (0.1643)	8.072727 (4.062254)	0.020699 (0.011751)
Formosa SE	7	22	6	0.9524 (0.0955)	6.666667 (3.582114)	0.017094 (0.010521)
Corrientes	3	7	3	1.0000 (0.2722)	4.666667 (3.126944)	0.011966 (0.010000)
Total	63	65	26			

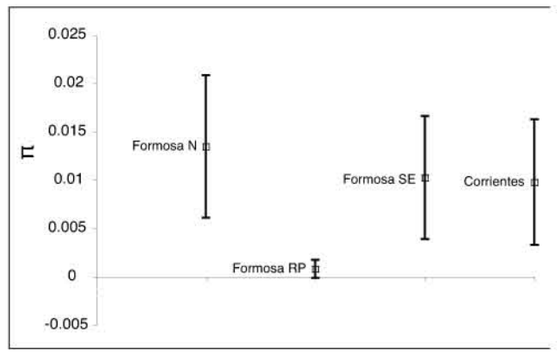

Fig. 4. Nucleotide diversity values (π) and standard deviations for each population for the ND4 gene region.

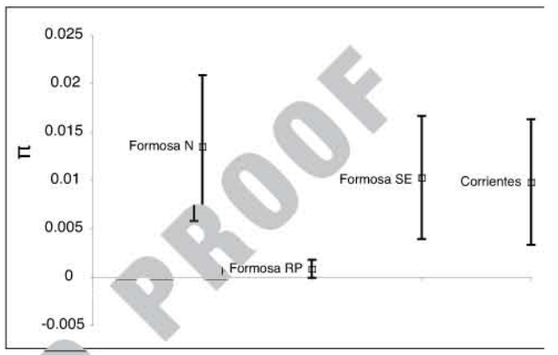

Fig. 5. Nucleotide diversity values (π) and standard deviations for each population for the cyt-*b* gene region.

Corrientes. These are expected patterns, giving that no suitable corridors connect the habitats in northern and southern Formosa, or in northern Formosa and Corrientes. Haplotypes in Formosa PR associated with those in Formosa N and with those in Formosa SE and Corrientes, which also was expected, given the intermediate location of this population. Although dispersion is facilitated downstream, this species is known to disperse upstream as well (Waller, 1986; Waller et al., 1993). The most frequent haplotypes (H1 and H3 in the ND4 and cyt-*b* gene regions, respectively) are also the most widespread in the study area. Since ancestral lineages are usually preferentially sampled in population-level studies (Clement et al., 2000), we suggest that haplotypes H1 and H3 are the oldest in our sample. Both gene regions also have unique haplotypes, which supports assumptions of recent divergence within populations (Avise, 2000; Rodriguez-Robles et al., 2001; Janzen et al., 2002).

The visualuzation of haplotypic relationships in a geographical context with the use of networks is important in recognizing general patterns of gene flow, as well as identifying geographical factors influencing these patterns. Statistical analyses of population structure in the form of panmictic demographic groups and analyses of non-random associations of individuals target a different issue. Here, we were particularly concerned with identifying groups of individuals that exhibited reproductive isolation and a non-arbitrary association. Our population genetic analyses and that for the ND4 gene region in particular suggest that all proposed populations behave effectively as independent demographic groups, except populations Formosa SE and Corrientes. The cyt-*b* gene region exhibited the same general patterns of associations, but with less statistical power. An explanation for the discrepancy between the probability values of the fixation indices reported for both gene regions could be that, despite more samples for the cyt-*b* region, a better representation of the data existed in the ND4 region (compare sample sizes of popu-

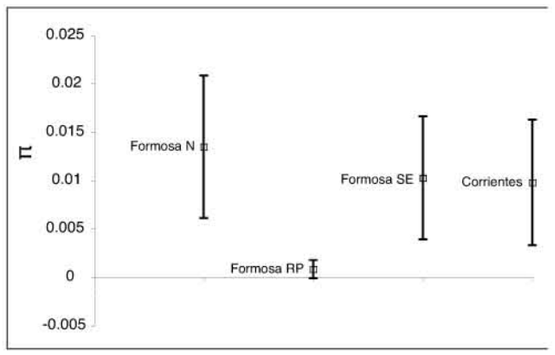

Fig. 6. Mean number of pair-wise differences among sequences with standard deviations for each population for the ND4 gene region.

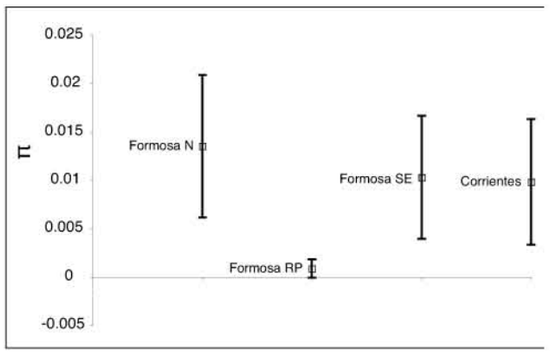

Fig. 7. Mean number of pair-wise differences among sequences with standard deviations for each population for the cyt-*b* gene region

lations Formosa SE and Corrientes in Tables 2 and 3). The exact tests of population differentiation for both gene regions suggest a non-random association of individuals in almost all proposed populations, in accordance with the population structure analysis. The greatest genetic differentiation emerges between populations Formosa PR and Corrientes, and between Formosa PR and Formosa SE for the ND4 gene region (we omit a detailed comparison with the cyt-*b* region here, since most fixation values in that gene are not statistically significant). These two values for the fixation indices are considered to represent "very great" genetic differentiation (Hartl et al., 1997). F_{st} values from 0.05–0.15 are considered "moderate" differentiation, which is the case in all other comparisons we presented. Although comparisons with related taxa addressed by other studies is difficult, given the lack of comparable information, the reported F_{st} values are typically considered adequate evidence for population isolation (Hartl et al., 1997; Frankham et al., 2002). Therefore, we suggest that Formosa N, Formosa PR, and Formosa SE together with Corrientes comprise three genetically isolated *E. notaeus* populations. The lack of significance in the Mantel tests suggested that the reported patterns of genetic isolation do not fully respond to a process of isolation by distance. Although an analysis of this kind that actually compares genetic and geographical distances along river streams at a finer scale would provide more resolution, the present results suggest that other mechanisms (i.e., habitat use and habitat preference) are involved.

Comparisons to other studies regarding levels of genetic diversity in snakes are difficult, primarily because few genetic studies of snakes address population genetic questions (e.g., Rodriguez-Robles et al., 2001; Janzen et al., 2002). Although the values we report for genetic and nucleotide diversity have large standard deviations, some internal comparisons can be made. In particular, the significantly lower levels of diversity for Formosa PR in the ND4 region are attributable to its low haplotype diversity. Formosa SE and Corrientes have similar levels of diversity, with Corrientes having the smaller values, probably a consequence of smaller sample size and lower number of haplotypes. In general, the reported number of haplotypes is in agreement with other studies presenting these kinds of data. Rodriguez-Robles et al. (2001) found 32 unique haplotypes in a sample of 38 individuals of North American Rubber Boas (*Charina bottae*) distributed across a much greater geographical area.

Management Implications

The management unit concept (see Avise, 2000, 1987; Oveden, 1990; Ryman and Utter, 1987) states that "any population exchanging so few migrants with others as to be genetically distinguishable will be usually demographically independent, and should therefore be considered as a distinct management unit." Palimbi and Cipriano (1998) suggested that matrilineal subdivisions are relevant to conservation objectives because studies of the mtDNA region are so sensitive to population subdivision.

Our study supports the matrilineal subdivision of populations Formosa N, Formosa PR, and Formosa SE-Corrientes. We propose that these three population groups be considered as independent management units. However, because Formosa SE and Corrientes were the most poorly sampled populations, a more comprehensive sampling scheme could shed further light on their relationship, perhaps disagreeing with our tentative suggestion that they be grouped.

The harvesting of a species following a management unit approach is of key importance when such units exist. Discrete populations, being demographically independent, would not have much chance of recovery by immigration from other populations if an adverse environmental event should deplete them (Avise, 2000). Sudden harvesting events can be as deleterious as outbreaks of diseases or stochastic climatic changes (Meffe et al., 1997). Therefore, if the harvest is not in accordance with a management approach that takes population discreteness into account, unique populations could be lost.

Because our study analyzed gene regions in the mtDNA, our results do not necessarily represent a full description of the population structure of the species. Since mtDNA is maternally inherited, its study addresses questions related to matrilineal phylopatry and maternal lineage distribution and structure. This issue becomes especially important in species like anacondas, in which sexual dimorphism can result in differential habitat preferences and differential dispersal abilities. Analyses of nuclear gene regions and patrilineal lineages would certainly contribute to a more detailed description of the processes favoring the isolation of populations in this species.

Eunectes notaeus is not currently an endangered species. However, because its populations are subject to hunting pressures and exhibit marked genetic differentiation, the species should be closely monitored, and harvesting should be mitigated by rigorously enforced regulations. Conservation genetics can play an important role in anticipating population decreases and fragmentation, genetic impoverishment, and other population parameters of vital importance for species persistence.

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