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The broad-snouted caiman population recovery in Argentina. A case of genetics conservation

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Abstract. *Caiman latirostris* wild populations have suffered a drastic reduction in the past, and for that reason, a management and monitoring plan was applied since 1990 in Santa Fe, Argentina in order to achieve population recovery. Although ranching system has a noteworthy success in terms of population size recovering, there is no information about the estimation of population genetic parameters. In particular, the consequence of the bottleneck underwent by these populations has not been assessed. We evaluated variability and genetic structure of *C. latirostris* populations from Santa Fe through time, using microsatellites and mitochondrial DNA. Population genetic parameters were compared among four sites and three different periods to assess the impact of management activities, and effective population size was estimated in order to detect bottleneck events. We observed an increase in microsatellite variability and low genetic variability in mitochondrial lineages through time. Variability estimates are similar among sites in each sampling period; and there is scarce differentiation among them. The genetic background of each sampling site has changed through time; we assume this fact may be due to entry of individuals of different origin, through management and repopulation activities. Moreover, taking into account the expected heterozygosity and effective population size values, it can be assumed that bottleneck events indeed have occurred in the recent past. Our results suggest that, in addition to increasing population size, genetic variability of the species has been maintained. However, the information is still incomplete, and regular monitoring should continue in order to arrive to solid conclusions.

Keywords: *Caiman latirostris*, genetic drift, molecular markers, population genetics, sustainable use.

Introduction

The broad-snouted caiman (*Caiman latirostris*) is one of two crocodilian species in Argentina. It has a wide geographic distribution, covering southeastern Brazil, central and southern Bolivia and Paraguay, and northeast Argentina (Chaco, Corrientes, Formosa, Santa Fe, Entre Ríos, Misiones, Salta, Santiago del Estero and Jujuy provinces) (Larriera et al., 2008). Its habitat includes diverse aquatic environments of the Paraná River basin, which usually are shallow water and vegetated marshes (Larriera, 1992). It is an opportunistic carnivorous consumer at the top trophic level, and is considered a sentinel species in local ecosystems.

In Argentina, the wild populations of *C. latirostris* from Santa Fe province are subject to a management and monitoring program called Proyecto Yacaré (PY – Gob. de Santa Fe/MUPCN), devoted to the preservation and the sustainable use of this species based on its commercial interest. This program started in 1990 in response to the evident reduction of population sizes as a consequence of strong hunting pressure and habitat modification. When the activities of PY began in Santa Fe province, the broad-snouted caiman was considered endangered and it was included in the Appendix I of CITES (Convention on International Trade in Endangered Species of Wild

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Fauna and Flora). In addition, most of the literature did not include the province of Santa Fe in the range of this species, and little was known about their population status and real situation in Argentina (Larriera et al., 2008). The strategy of rational use to recover caiman populations involved the so called ranching system. It implies the collection of eggs in natural environments for captive rearing, and the releasing, after 10-months of rearing under controlled conditions, of 10% of the hatched animals to the same place where the eggs were collected. This method had a remarkable success in terms of population recovery, as periodic monitoring has shown an increase in density from 2.7 animals/km in 1990, to 8.45 animals/km in 1997, and 9.8 animals/km in 2006 (Larriera et al., 2008). Consequently, in 1997 *C. latirostris* populations of Argentina were transferred from the Appendix I to the Appendix II of the CITES. This legal framework allows the commercial use of *C. latirostris* under strict controls (Larriera et al., 2008). Due to the high quality of its leather and meat, broad-snouted caiman become an important economic resource for the region. The sustainable use of *C. latirostris* acts as a direct incentive for habitat conservation based on the community involvement, because the residents receive an economic gain from locating and protecting *C. latirostris* nests in nature.

Although the recovery of caiman populations by PY activities has been recorded, such data were accompanied only by some genetic-population studies performed by this working group using random markers and quantitative traits (Amavet et al., 2007, 2009). In particular, the consequence of the bottleneck phenomenon underwent by these populations has not been assessed.

The combination of molecular markers and robust theoretical and statistical approaches allows estimation of parameters that reflect the relative importance of recent evolutionary processes in natural populations. Bottleneck events are expected to produce dramatic effects reflected in serious loss of diversity, measured as the number of alleles and heterozygosity throughout generations (Hedrick, 2000). Therefore, the relatively fast population growth is not usually paralleled by recovery in the levels of variability, especially measured in terms of polymorphism and number of alleles. Because allelic diversity is lost at a faster rate than heterozygosity in declining populations (Cornuet and Luikart, 1996), it is useful to test heterozygosity excess relative to that expected for a population at mutation-drift equilibrium (*Heq*). If *He* value result higher than *Heq*, this fact constitutes evidence of the previous existence of bottlenecks in the studied population.

The estimation of effective population size (N_e) (Wright, 1931) is the key to evaluate the effects of selection on populations and the rate at which diversity is lost via genetic drift (Wright, 1931, 1938). Effective population size determines the strength of stochastic changes in genetic properties (Frankham et al., 2002; Hedrick, 2005). The N_e estimation is crucial to wildlife management because this parameter integrates genetic effects with the life history of the species, allowing for predictions of a population's current and future viability (Hare et al., 2011).

Genetic analysis to acquire information on population structure, patterns of dispersal and gene flow, interspecific hybridization, among other parameters, are important for conservation and management. Inferences of these patterns and the underlying evolutionary processes require the analysis of polymorphic markers. Microsatellites are widely used for many genetic studies. In contrast to single nucleotide polymorphism (SNP) and genotyping-by-sequencing methods, microsatellites are readily typed in samples of low DNA quality/concentration, and enable the quick and cheap identification of species, hybrids, clones and ploidy (Dawson et al., 2013). The application of mitochondrial DNA (mtDNA) analyses has also provided valuable tools for genetic population analysis below the species level (Avise,

2000), and has no history of previous use in broad snouted caiman.

The information about *C. latirostris* population genetic structure throughout its vast distribution range is yet relatively limited (Verdade et al., 2002; Amavet et al., 2007, 2009; Vilella et al., 2008). In relation to their migratory behavior, it is known that this is a species with low vagility, i.e. their migratory movements are not associated with their reproduction, in accordance with this fact, it has been tested that the females build their nests on the same sites every year (Larriera et al., 2008).

The aim of this study was to evaluate the levels of variability and genetic structure of broad-snouted caiman populations from Santa Fe province, Argentina, through time, using microsatellite and sequence analysis of a mitochondrial DNA fragment. Population genetic parameters were estimated and compared among sites and different years to assess the impact of management activities, in addition to estimate effective population size to detect potential bottleneck events in the past.

Materials and methods

Study sites and sample collection

We studied *C. latirostris* DNA samples from 3 different time periods (2001-2003, 2007 and 2011-2012) from 4 sampling sites of Santa Fe province, Argentina: "Estancia El Estero" (EEE) – San Javier Department (30°29′S 59°59′W), "Costa del Salado" (CSA) – San Cristóbal Department (29°58′S 60°50′W), "Estero del Paraje 114" (EDP) – San Javier Department (30°43′S 60°17′W), and "Arroyo El Espín" (AES) – Vera Department (29°58′S 60°04′W) (fig. 1).

The animals sampled were captured by hand using slipknots. All animals are treated following the Ethical Reference Framework for Biomedics Researches: ethical principles for research with laboratory, farm and wild animals (CONICET, 2005), using non-harmful techniques of blood collection and minimizing stress and suffering by suitable management methods. They were all adults, and they were measured, weighed, sexed, and marked by cuts in their tail scales to prevent the animals be resampled. The total number of individuals sampled in each period were similar: 2001-2003: n = 41; 2007: n = 56; 2011-2012: n = 57 (table 1).

Microsatellite amplification method

Genomic DNA was extracted from whole blood as described by Amavet et al. (2012).

We analyzed ten microsatellite loci employing five primer pairs designed by Zucoloto et al. (2002) for *C. latirostris* (*Cla*μ 2, *Cla*μ 6, *Cla*μ 8, *Cla*μ 9 and *Cla*μ 10), one by Glenn et al. (1998) for *Alligator mississippiensis* (*Ami*μ 20) and four primer pairs recently designed by our work group (*Cl* 58, *Cl* 279, *Cl* 309, and *Cl* 811; Amavet et al., 2015). Marker quality assurance steps were performed by the authors in each one of these cited studies.

PCR conditions for *Cla*μ 2, *Cla*μ 6, *Cla*μ 9, and *Cla*μ 10 were those described by Zucoloto et al. (2002) with modifications (Amavet et al., 2012); and for *Cl* 58, *Cl* 279, *Cl* 309, and *Cl* 811, those described in our previous paper (Amavet et al., 2015).

For amplification of locus *Ami*μ 20, amplifications were carried out in a final volume of 15 μl with 1× standard buffer; 1 μl each of the fluorescent label forward and unlabelled reverse primer (0.1 nM/μl); 1 μl of 50 mM MgCl; 1 μl of dNTP mix (200 mM); 0.1 μl of *Taq* DNA polymerase (5 U/μl) (PB-L®) and 50 ng of DNA. All programs for microsatellite amplifications were set with 30 cycles with denaturation step at 94°C for 1 minute, 1 minute annealing at optimum temperature, and extension at 72°C from 1.15 minutes at each iterative cycle. Besides an initial denaturation of 94°C for 4 min and a final extension at 72°C for 30 minutes each amplification program used.

Microsatellite amplified fragments were scored for length with an ABI 3730xl analyzer® (Applied Biosystems) using LIZ500 as the internal lane size standard (service request to the Unidad de Genómica, Instituto de Biotecnología, CICVyA-CNIA-INTA, Argentina, or to Macrogen Inc.). Fragment lengths were assigned to allelic classes with Peak Scanner® 1.0 Software (Applied Biosystems).

Genetic variability analysis

Relative probabilities based on allelic frequencies and Mendelian segregation were calculated by Micro-Checker v2.2.3 (Van Oosterhout et al. 2004). This software evaluates the excess of homozygotes for each marker due to null alleles, allele dropout or stuttering and corrects the allele frequencies. Genetic diversity was quantified by the number of alleles per locus (*A*), observed heterozygosity (*Ho*) and expected heterozygosity (*He*) for each site and sampling period of time using Arlequin 3.5 (Excoffier et al., 2005). Also we estimated allelic richness (r_g) (El Mousadik and Petit, 1996) per locus and sampling period using the package *hierfstat* (Goudet, 2014) of R (R Core Team, 2015).

We compared A, r_g, *Ho* and *He* values among sampling periods employing a generalized linear model (glm) given by

$$I_{ijk} = loc_i + samp_j + sit_k + e_{ijk}$$

where I_{ijk} is the variability measure (A, r_g, *Ho* or *He*) in the locus i, recorded in sample j at the site k, and e_{ijk} is the experimental error. The analysis was conducted using the *glm* function of the *stats* package of R. A was assumed to fit

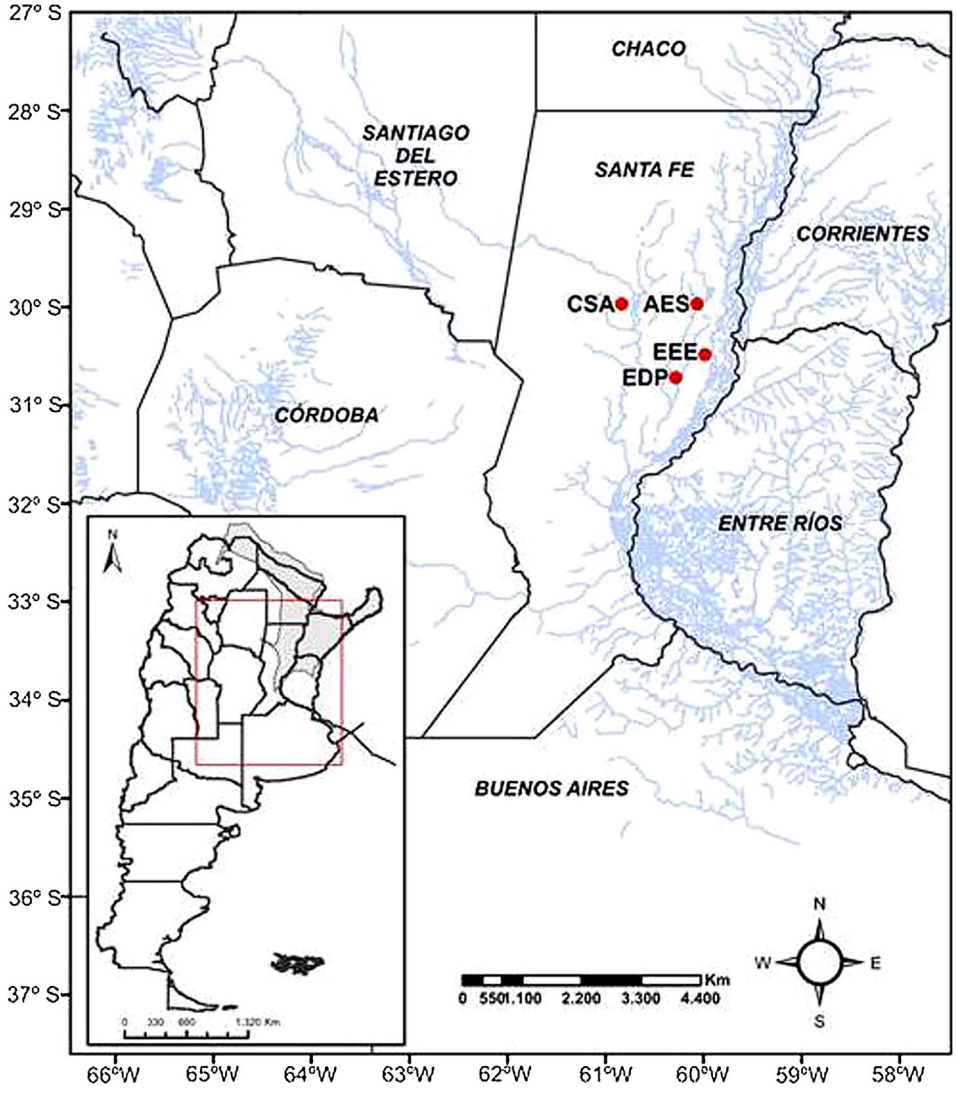

Figure 1. Geographic location of sampling sites of analyzed *Caiman latirostris* specimens in relation to the distribution area.

a Poisson distribution, what was accounted by defining the parameter *family* = "poisson".

Arlequin was used to evaluate the hypothesis of Hardy-Weinberg equilibrium (HWE), perform an analysis of molecular variance (AMOVA) (Excoffier et al., 1992), and estimate Wright's (1951) F statistics. F_{ST} estimates were calculated globally (taking all samples together) and then, analyzing differences among sites within each sampling period. Lastly, population pair wise F_{ST} were estimated to study whether the structure in each particular sampling site changes over time.

We analyzed population structure for the whole sample using the Bayesian approach implemented in STRUCTURE version 2.1 (Pritchard et al., 2000; Falush et al., 2003, 2007). The number of clusters was assigned applying the admixture model assuming independent allele frequencies, and no prior population information. The parameter settings applied were: 10 independent replicates each for a number of population (K) ranging from $K = 1$ to $K = 12$, a burn-in of 100 000 steps followed by 300 000 MCMC iterations. The most likely number of cluster was estimated with the delta K statistic (Evanno et al., 2005) using STRUCTURE HARVESTER software (Earl, 2012). Multimodality in individual and population coefficient across different runs was accounted for using the permutation procedure in CLUMPP (Jakobsson and Rosenberg, 2007). The matrix of Q values was graphically displayed through DISTRUCT (Rosenberg, 2004).

We also made a thorough study of the population structure estimating coancestry coefficients (θ) between individuals within and among sites, by an admixture F-model (*AFM*) implemented in the R package *RAFM* (Karhunen

Table 1. Variability parameters described by region and sampling period: *Ho* and *He* are respectively the observed and expected heterozygosity; *A* is the observed number of alleles. In brackets is shown the standard deviation.

Sites	Sample size	*Ho*	*He*	*A*	r_g
EEE-01/03	10	0.26 (0.32)	0.58 (0.12)	3.4 (1.0)	1.58
CSA-01/03	11	0.15 (0.19)	0.53 (0.25)	3.7 (2.0)	1.60
EDP-01/03	10	0.18 (0.20)	0.42 (0.25)	2.9 (2.16)	1.49
AES-01/03	10	0.23 (0.20)	0.63 (0.14)	4.1 (1.86)	1.63
Global 01/03	41	0.23 (0.19)	0.61 (0.10)	6.4 (3.16)	3.49
EEE-07	14	0.41 (0.32)	0.63 (0.19)	4.5 (1.5)	1.64
CSA-07	14	0.26 (0.27)	0.63 (0.13)	4.6 (1.68)	1.63
EDP-07	14	0.32 (0.31)	0.71 (0.11)	4.9 (1.92)	1.71
AES-07	14	0.29 (0.20)	0.61 (0.23)	4.9 (1.97)	1.61
Global 07	56	0.33 (0.23)	0.73 (0.08)	8.8 (2.7)	4.59
EEE-11/12	15	0.31 (0.23)	0.61 (0.23)	5.1 (2.3)	1.61
CSA-11/12	13	0.30 (0.29)	0.52 (0.27)	4.0 (2.3)	1.60
EDP-11/12	13	0.33 (0.35)	0.62 (0.14)	4.0 (1.3)	1.62
AES-11/12	16	0.40 (0.28)	0.53 (0.23)	3.8 (2.31)	1.53
Global 11/12	57	0.37 (0.24)	0.73 (0.08)	8.4 (2.87)	4.43

and Ovaskainen, 2012). *AFM* assumes that the n_y local populations derived from a common ancestral population and that the local populations may have experienced gene flow since their divergence from the common ancestral population determining an admixture of n_ε evolutionary independent lineages. We can assimilate "lineages" to different families within the hypothetical ancestral population. During the subdivision from the ancestral population, each lineage makes a differential contribution to each subpopulation. There may be admixture between lineages and also each lineage can have undergone different effects of drift, (quantified by the alpha (α) parameter), because the number of founders or survivors of each lineage or family would be variable. Furthermore, the contribution of each lineage to a certain local population is quantified by the parameter kappa (κ). Consequently, this method allows evaluating separately the effect of drift and migration. To conduct *AFM* we applied 20 000 iterations, burn-in of 10 000 and thinning of 10.

In addition, we used NE ESTIMATOR v2.01 (Do et al., 2014) to estimate effective population size ($\hat{N}_e$) by temporal method (Nei/Tajima Plan II) taking into account the data of the first and last sampling to ensure that the data belong to two different generations, due to previous data reported that *C. latirostris* specimens in the wild are able to reproduce between five to seven years old (Larriera et al., 2006).

Temporal estimators use allele frequency data from multiple samples, across generations, to calculate N_e (Nei and Tajima, 1981; Waples, 1989; Wang, 2001) being more precise and unbiased than single point estimators, especially when sample sizes are small (Waples, 1991; Tallmon et al., 2008).

We estimate real population size ($\hat{N}$) from data included in monitoring reports collected by PY staff carried out in the study area in the first and last sampling period (Siroski, 2008; Siroski and Príncipe, 2010; Príncipe 2012, 2013). Then, we use the proportion $\hat{N}_e/\tilde{N}$ to detect bottleneck events (Bishop et al., 2009).

Furthermore, we employed the software BOTTLENECK 1.2.02 (Piry et al., 1999) to detect recent effective population size reductions from allele frequency data, based on the comparison between estimated expected equilibrium gene diversity (*Heq*) and Hardy-Weinberg expected heterozygosity (*He*) under two possible models: Infinite Allele Model (IAM), and Stepwise Mutation Model (SMM). We use 70% SMM and 10 000 iterations as parameters in BOTTLENECK.

Mitochondrial DNA sequence methodology

Mitochondrial DNA (mtDNA) sequences of the cytochrome *b* gene were collected from a subsample of individuals. The mitochondrial cytochrome *b* gene was selected because of its ability to resolve questions of population structure (Avise, 2000). We amplified nearly complete cytochrome *b* mitochondrial gene in 15 samples including all sampling sites using the forward primer L14254 (from Glenn et al., 2002) and the reverse primer H15990 (from Farias et al., 2004). PCR reactions of cyt *b* gene, were carried out in a final volume of 25 μl with 1× standard buffer; 3 μl of each primer (0.1 nM/μl); 1 μl of 50 mM MgCl; 2.5 μl of dNTP mix (200 mM); 0.15 μl of *Taq* DNA polymerase (5 U/μl) (Invitrogen®) and 50 ng of DNA. A negative control containing all reagents except DNA in each set of PCR reactions performed are included. Amplifications were performed in a thermocycler with gradient Bioer Life Express® of 96 wells, with 30 cycles including denaturation step of 1 min at 94°C, annealing step of 1 min at 50°C and extension at 72°C from 2 min at each iterative cycle. Besides an initial denaturation of 94°C for 5 min and a final extension at 72°C for 10 min were used. PCR products were purified using Accuprep® PCR-purification kit. Templates were sequenced using L14254 primer and resolved on an Applied Biosystems 3730xl automated sequencer of Macrogen Inc. (Korea).

Sequences were first edited and then aligned using MUSCLE (Edgar, 2004).

Mitochondrial DNA sequence analysis

An outgroup specimen (*Alligator mississippiensis*; GenBank: AF318548.1) was added. An independent model of sequence evolution was determined using the program J-Model Test-2 (Darriba et al., 2012). Maximum likelihood phylogeny inference were carried out with 1000 bootstrap using MEGA 7 (Kumar et al., 2016) under HKY model (Hasegawa et al., 1985) for the three codon positions. In order to improve visual quality FIGTREE v.1.1 software (http://tree.bio.ed.ac.uk/software/figtree/) was used. The number of haplotypes (n), haplotype diversity (h), number of segregating sites between sequences (S) and nucleotide diversity (π), were estimated using DnaSP 5 (Librado and Rozas, 2009). A parsimony haplotype network was generated using NETWORK v. 5.0.0.1 (http://www.fluxus-engineering.com/sharenet.htm), which implements the median-joining method, in the absence of recombination.

Results

Analysis of microsatellite variability

The loci *Cla*μ 2 and *Cla*μ 6 showed signs of null alleles, as is suggested by the general excess of homozygotes detected by MicroChecker v2.2.3 (Van Oosterhout et al. 2004). No evidences of allele dropout or genotyping errors due to stuttering were found. We used the adjusted allele frequencies of amplified alleles based on Brookfield method of null allele estimation, for subsequent analyses. Allele number (A) for each sample and site are included in the Appendix.

The basic parameters of variability (table 1) show a trend towards an increase from 2001 to 2007, and similar variability estimates among sites in each year of sampling.

Estimation for allelic richness per locus for each sampling period (table 2) also showed the same trend of increasing.

Differences in A, r_g, *Ho* and *He* values among sampling periods showed that there are significant differences between 2001 and 2007 samplings whereas the differences between 2007 and 2011 are not significant, and between 2001 and 2011 are not significant except for *Ho* (table 3). We did not find deviations from Hardy Weinberg in any sampling period ($P \geqslant 0.05$). Regarding to HW test for all populations grouped, this resulted significant ($P < 0.05$).

The global F_{ST} value estimated (taking the twelve samples regardless of the sampling period) was 0.224 and highly significant ($P < 0.001$) with a confidence interval of 0.200-0.257. The differentiation among sites within each time period was about a half of the global estimate (F_{ST} 2001 $= 0.083$, $P = 0.0019$; F_{ST} 2007 $= 0.109$, $P < 0.001$; F_{ST} 2011 $= 0.156$, $P < 0.001$). Moreover, differentiation was detected for each site over time. Almost all pairwise F_{ST} values were highly significant ($P < 0.001$). The non-significant pairwise F_{ST} corresponded to estimates between sites within the period 2001/03 (table 4). The greatest pairwise F_{ST} values (all higher than 0.23), are be-

Table 2. Allelic richness (r_g) per locus estimated for each sampling period.

Locus	Sampling period		
	2001/2003	2007	2011/2012
Claμ 2	3.747	5.246	4.726
Claμ 6	1.989	4.204	4.191
Claμ 8	4.065	4.392	4.712
Claμ 9	3.064	3.503	3.791
Claμ10	3.312	4.455	3.443
Amiμ20	4.467	5.358	4.525
Cl 58	2.000	3.686	2.947
Cl 279	2.583	3.670	6.824
Cl 309	4.078	4.697	4.640
Cl 811	5.643	6.692	4.561
Mean	3.495	4.590	4.436

Table 3. Comparison of A, r_g, *Ho* and *He* values among sampling periods employing a generalized linear model.

Parameters	Statistic	2001/2003 versus 2007	2007 versus 2011/2012	2001/2003 versus 2011/2012
A	*z*	2.63	−1.06	1.59
	P	0.008	0.290	0.112
r_g	*t*	2.14	−1.49	0.45
	P	0.036	0.140	0.655
Ho	*t*	2.21	0.03	2.33
	P	0.031	0.979	0.023
He	*t*	2.63	−1.60	0.63
	P	0.11	0.115	0.530

Table 4. Population pairwise F_{ST} estimates. (*): indicate highly significant values ($P < 0.001$).

	EEE 01/03	CSA 01/03	EDP 01/03	AES 01/03	EEE 07	CSA 07	EDP 07	AES 07	EEE 11/12	CSA 11/12	EDP 11/12	AES 11/12
EEE 01/03												
CSA 01/03	0.018											
EDP 01/03	0.133	0.109										
AES 01/03	0.068	0.064	0.103*									
EEE 07	0.202*	0.246*	0.271*	0.216*								
CSA 07	0.223*	0.242*	0.238*	0.225*	0.139*							
EDP 07	0.226*	0.246*	0.260*	0.211*	0.140*	0.065*						
AES 07	0.237*	0.245*	0.285*	0.227*	0.131*	0.146*	0.124*					
EEE11/12	0.270*	0.255*	0.307*	0.247*	0.194*	0.144*	0.145*	0.178*				
CSA 11/12	0.345*	0.352*	0.370*	0.333*	0.284*	0.241*	0.264*	0.290*	0.189*			
EDP 11/12	0.294*	0.298*	0.377*	0.300*	0.215*	0.218*	0.194*	0.197*	0.117*	0.250*		
AES 11/12	0.255*	0.252*	0.317*	0.231*	0.167*	0.127*	0.137*	0.118*	0.053*	0.223*	0.096*	

tween the first and last sampling period, showing that taking these sites as a whole, populations changed their genetic composition over time.

The results of the general structure analysis using AMOVA (disregarding the sampling periods) showed that the highest percentage of variation occurs within sampling sites (78.9%) while the variation among them is much lower (21.10%). When analysis was conducted considering each sampling period separately, the results were similar.

The population structure analysis conducted applying the admixture F-model showed that the genetic background of each sampled population was changing over time. The contribution of different lineages to the sampled populations is estimated by the parameter κ (fig. 2A, B, and C).

In the first sampling the lineage that contributes most to the populations is 3. EEE, CSA and AES populations are constituted by two lineages with similar contributions (respectively 1 and 3, 2 and 3, and 4 and 3) (fig. 2A). In the sampling of 2007, (fig. 2B) the lineages that most contribute are 5 (EEE), 6 (CSA), 7 (EDP) and 8 (AES). In the third sampling, (fig. 2C) the lineages that contribute most are 9, 10, 11 and 12. In the second and third sampling populations are mainly constituted by individuals from a single lineage.

The lineage 3 is the most widely spread as the sum of its contribution ($\sum \kappa$) to each sample is 2.19 and the least expanded is the lineage 4 ($\sum \kappa = 0.55$) because it only contributes significantly to the AES 01 sampling site.

Regarding drift on each lineage as measured by α, it was observed that the lineage 1 is the one that underwent the greatest effect of the drift ($\alpha = 3.51$) and lineage 4 exhibited the lowest effect of drift ($\alpha = 1.71$).

From the symmetric coancestry matrix between different samples, a dendrogram was plotted (fig. 3), which shows that samples are not grouped by site but by the time of sampling.

The STRUCTURE analysis showed that K value with the highest likelihood was $K = 3$, with Asymmetry value (Delta K = 197.151887) (fig. 4A and B). In each period the four sites essentially share the gene pool but the genetic composition of each site has been changing among different sampling periods.

The effective population size calculated using the first and last sampling data, and Nei/Tajima method, with the lowest allele frequency of 0.05 was $\hat{N}_e = 2.1$. The harmonic mean of monitoring reported N numbers was = 61. Then, the proportion between the $\hat{N}_e$ and $\tilde{N}$ was relatively low ($\hat{N}_e/\tilde{N} = 0.034$), suggesting a low proportion of breeding individuals in the analyzed samples.

Moreover, in all periods an excess of expected heterozygosity was found, comparing

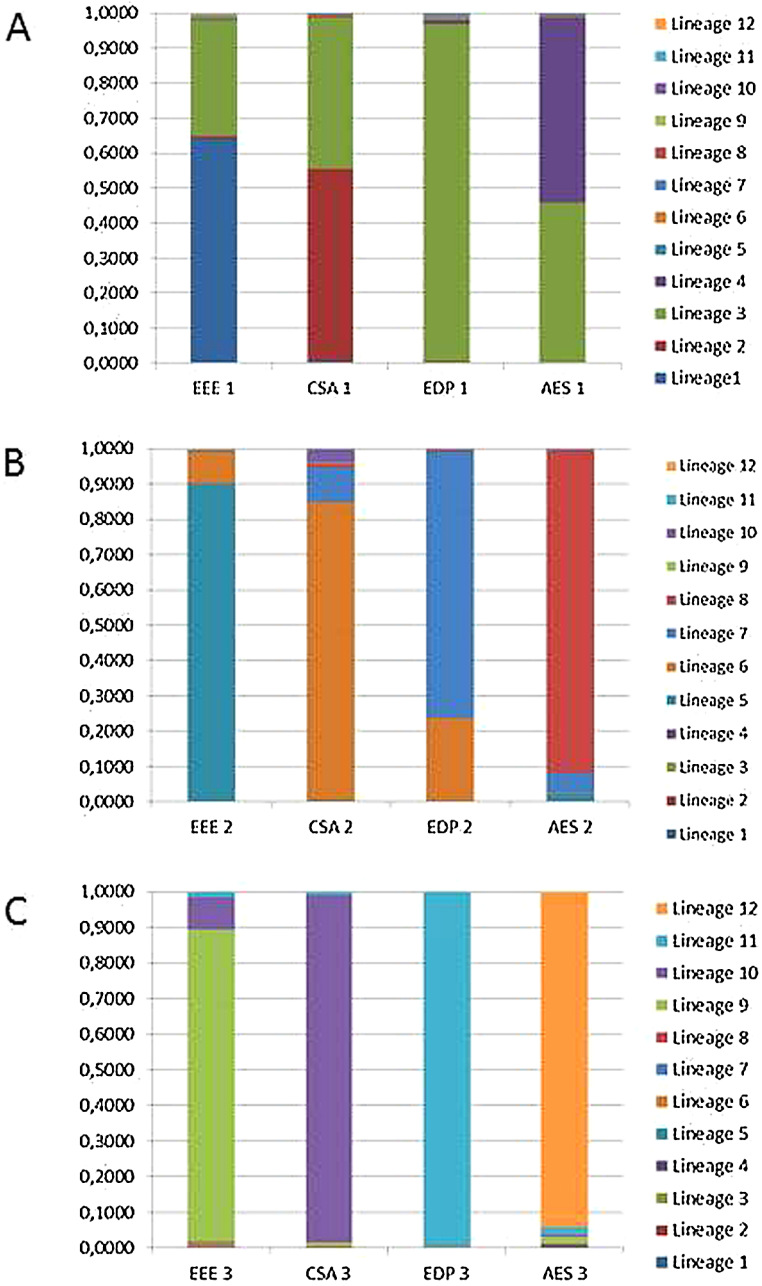

Figure 2. Population structure analysis: Twelve lineages with differential contributions were found. (A) sampling sites in the first period (2001-2003); (B) sampling sites in the second period (2007); (C) sampling sites in the third period (2011-2012).

Hardy-Weinberg heterozygosity (*He*) values versus the expected gene diversity at mutation-drift equilibrium (*Heq*), although the difference was significant only in the third sampling ($P < 0.05$) (table 5).

Analysis of mitochondrial cytochrome b sequence

After alignment, a fragment of 1077 bp was used for further analysis. All the different sequences obtained were deposited in GenBank

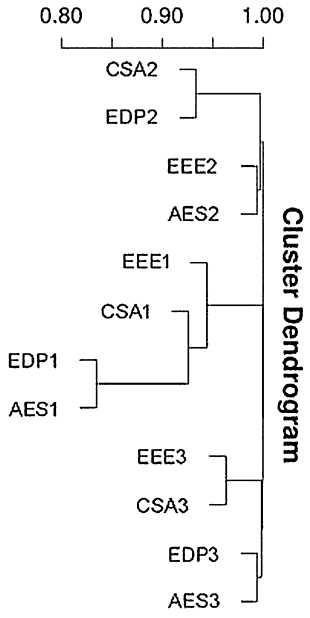

Figure 3. Phenogram constructed from matrix coancestry.

(Accession numbers: KX650391-405). Among the 15 analyzed sequences only 4 unique haplotypes, with $\pi = 0.02556$, $h = 0.450$ and $S =$

Table 5. Hardy-Weinberg heterozygosity (*He*) values, expected gene diversity at mutation-drift equilibrium (*Heq*), and probability (*P*) that all loci fit to SMM (Stepwise Mutation Model).

Sampling period	*He*	*Heq*	*Psign SMM*
2001/2003	0.61	0.590	0.085757
2007	0.73	0.618	0.186583
2011/2012	0.73	0.518	0.002048

215 were obtained. The haplotypes were identified and used to perform the haplotype network. This shows (fig. 5A) that most of specimens from 2001, 2007 and 2011 are represented by only one haplotype (H1). Two individuals from 2001 (38 AES and 14 CSA, corresponding to H4 and H5 respectively), and one for 2011 (108 AES, corresponding to H3), appear separated from the rest of *C. latirostris* individuals. H2 is the outgroup, *A. mississippiensis*. Using Mega-7 software a ML tree was constructed. The model recommended by J-model Test software based on the AICc was HKY. The resulting phylogeny is shown in fig. 5B were all samples analyzed

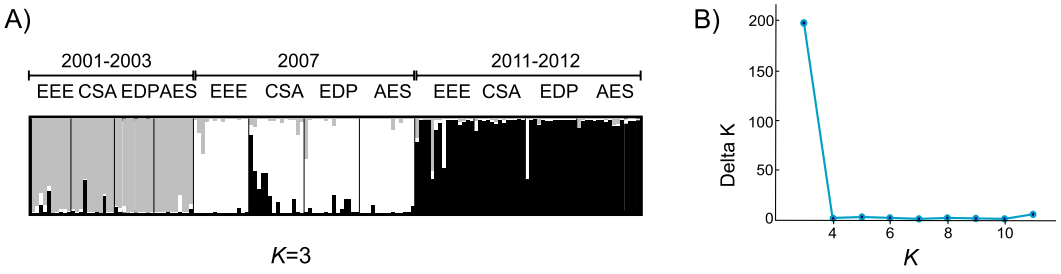

Figure 4. (A) STRUCTURE analysis, showing results with $K = 3$; (B) Delta$K$ = mean($|L''(K)|$)/sd($L(K)$).

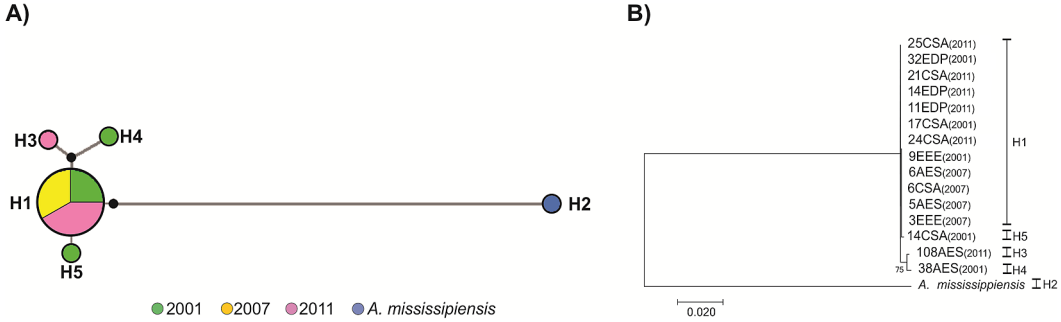

Figure 5. Results of cytochrome *b* sequences analysis using *Alligator mississippiensis* (H2) as outgroup. (A) Haplotype network, (B) Maximum Likelihood tree.

formed strongly supported (100 percent bootstrap values) reciprocally a single monophyletic group. These results show low genetic variability in mitochondrial DNA lineages, which coincides with previous data on other species included in the same family Alligatoridae (Glenn et al., 2002).

Discussion

The results obtained so far by our working group about variability in *C. latirostris* are very interesting and extend the knowledge of this scarcely studied species. Genetic variability averaged values ($Ho = 0.4415$; $He = 0.7455$) obtained here in the last sampling period are similar to previous records based on twelve families using four microsatellites (Amavet et al., 2012), even though in the present paper the sampling strategy was different and the number of microsatellite markers was higher.

Moritz (1994) and Galtier et al. (2009) claim that the use of microsatellites and mitochondrial DNA together for the analysis of population structure can make a significant contribution to the long-term planning and short-term execution of species recovery plans. In the present work, microsatellite diversity parameters are similar to those reported by other authors in *C. latirostris* (Verdade et al., 2002; Villela et al., 2008) and in other crocodilians (de Thoisy et al., 2006; Gonzalez-Trujillo et al., 2012; Bashyal et al., 2014). The cyt *b* variability results are consistent with obtained data in *A. mississippiensis* (Glenn et al., 2002) but are lower than those obtained in other caiman species (Farias et al., 2004; Vasconcelos et al., 2008), although these authors analyzed more samples that were obtained in a greater geographical extent. These data could support our hypothesis that these *C. latirostris* populations suffered bottlenecks in the past. Because mtDNA is haploid and uniparentally inherited, it is effectively a quarter of the population size of diploid nuclear DNA. There are fewer copies of mtDNA, and it is relatively sensitive to demographic events such as bottlenecks. Even if the population recovers quickly, it will have relatively low number of surviving mitochondrial haplotypes compared with nuclear genotypes (Freeland, 2005).

In the studied sites, it is observed a trend to the increase variability between 2001 and 2007 with microsatellite data. Considering the population genetic parameters and differentiation values estimated here, the studied populations appear to be part of the same evolutionarily significant unit (Crandall et al., 2000). Moreover, the genetic background of each sampling site has been changing over time. As follows from our results of population structure analysis, populations are apparently not stable. Rather, the patterns suggest that they are replaced (or repopulated) through time. Population samples taken the first sampling period are differentiated from the rest and they are more similar to each other because they share the same lineage 3 (fig. 2A). In addition, the lineage 1, present only in EEE1 is the one that underwent the greatest effect of the drift. In the second and third sampling periods, each population is mainly constituted by individuals from a single lineage. These data seem to suggest that each sampling period contains a genetic pool very different from the other two (fig. 3).

Taking into account the excess of expected heterozygosity verified in all periods it can be assumed that bottleneck events really existed in the past. Power analysis and theoretical models in BOTTLENECK showed that most of the time a heterozygosity excess can only be detected 0.5-5 N_e generations after the initiation of a population reduction, whereas a distortion of allele frequency distributions is likely to be detectable between 2 and 4 N_e generations (Nei and Li, 1976; Cornuet and Luikart, 1996). Our N_e estimated was very limited (2.1). If we consider 5 N_e generations as maximum, and taking into account that each seven years a new generation is produced, we would consider that *C. latirostris* bottleneck occurred in the last 70 years. Our data coincide with the information provided by Waller and Micucci (1995), who

stated that in the late 1970s, *C. latirostris* populations were decimated mainly due to poaching and illegal trade.

However, as BOTTLENECK test was not significant in two of the three sampling periods, further inquiries should be conducted about this indicator to corroborate this conclusion.

Because our study covered a short period of time in which it is not likely that there has been a significant variability increase through mutation; we assume that the differences in the population structure over time may be due to entry of individuals of different origin, through management and repopulation activities. We advanced this hypothesis because the natural gene flow was probably scarce due to the low vagility, characteristic of these animals. It is also likely that some adults from the first sampling have been reproduced in following sampling periods, as they may have been sexually mature at that time, but maybe these have been poorly represented in the analyzed samples.

The restocking activities carried out by the PY involve the annual releasing of 10 months old head-started animals in the same geographical sites where the eggs were collected for artificial incubation. Sometimes, pulses of floods and droughts cause environment modifications that impede making the releases into the same geographic point, as they do not represent a safety place for them. In such cases, the alternative is the nearest accessible point of the egg's collection place.

Every year in November, PY staff releases *C. latirostris* hatchlings born in February-March of the same year. Taking into account data obtained by Lovino et al. (2014), in the periods and sampling sites considered in our study, November was particularly a rainy month with an average rainfall of 182.2 mm. This value is higher than the historical monthly average calculated for these ten years (119.6 mm). The resulting increase in water levels in semi-permanent water bodies inhabited by this species often causes difficulties to reach the egg's collection sites. Therefore, with the aim of ensure animals' survival, PY activities could have contributed to produce some gene flow among sampling sites, incorporating individuals (and alleles) that were not previously represented in a particular site. We hypothesize that there may be unanalyzed populations that represent natural sources of repopulation of sites. We sampled in those four sites because they are the only ones that have been maintained over time within the PY activities, and for that reason we thought in those sites to monitor population parameters. However, the PY through time have collected eggs in different regions of the North of Santa Fe province, even to one distance of 150 km of the sampled sites, which could represent the source of repopulation of these sites. We propose for a future to expand sampling to test that hypothesis. Then, probably, as a consequence of management activities, genetic diversity increased but the local identity at each site was modified.

Our results seem to support those PY management activities, in addition to increase the population numbers, have helped to maintain genetic variability in spite of the expected consequences of bottlenecks events in the past. Considering the ratio between $\hat{N}_e$ and $\tilde{N}$, our estimates are similar to values calculated for *Crocodylus niloticus* by Bishop et al. (2009), the only previous study about N_e conducted in crocodilians. Although $\hat{N}_e$ estimation methods are different, the authors also found potential bottlenecks in the past and emphasized the need to estimate this parameter to assess the viability of crocodilian populations. Following Frankham et al. (2004) in a real population $N_e < N$ due to unequal sex ratios, high variation in family sizes, variable numbers in successive generations, and overlapping of generations. Although we do not have precise information, several of those features are likely to occur in the *C. latirostris* populations of our study.

Then, the low $\hat{N}_e$ value detected, and the excess in heterozygosity estimated here suggest that it is necessary to continue monitoring systematically these population genetic parameters

for this species. Previous studies carried out by our laboratory have demonstrated the existence of the reproductive behavior of multiple paternity in *C. latirostris* (Amavet et al., 2008, 2012), which can influence the existence of few effective breeders in this population, as a dominant males may be over represented in the reproductive group. Periodic estimates of $\hat{N}_e$ are useful for evaluating existing adaptive potential or monitoring changes in the strength of genetic drift and the population viability (Hare et al., 2011).

It is probable that the time spent since the beginning of PY activities is not enough to have fully recovered the depleted genetic variability of *C. latirostris* Argentinean populations, and the management activities implemented have been beneficial to maintain genetic viability of the species. However, as the information is still fragmentary, regular monitoring should be continued including new sites of the distribution area.

This paper provides the first evaluation of results in relation to conservation genetics of a management and sustainable use program applied to a caiman species in South America.

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Appendix. Allele number (*A*) for each sample and site.

Locus	EEE 01/03	CSA 01/03	EDP 01/03	AES 01/03	EEE 07	CSA 07	EDP 07	AES 07	EEE 11/12	CSA 11/12	EDP 11/12	AES 11/12	Mean	s.d.	Total number
Claμ 2	3	4	2	5	6	4	4	5	5	3	4	2	3.917	1.187	20
Claμ 6	2	2	2	2	4	3	2	3	6	3	3	5	3.083	1.256	10
Claμ 8	4	5	2	6	5	4	6	8	3	2	3	10	4.833	2.303	17
Claμ 9	4	3	2	3	4	4	4	3	4	4	3	3	3.417	0.640	8
Claμ10	3	3	2	4	3	6	4	5	4	2	4	4	3.667	1.106	12
Amiμ20	5	7	5	4	5	4	6	6	4	6	5	3	5.000	1.080	14
Cl 58	2	0	0	2	3	3	2	1	1	0	2	3	1.583	1.115	8
Cl 279	3	2	1	2	2	3	6	6	9	7	5	1	3.917	2.499	19
Cl 309	3	5	7	5	7	7	7	5	8	8	7	4	6.083	1.552	21
Cl 811	5	6	6	8	6	8	8	7	7	5	4	3	6.083	1.552	14
Mean	3.400	3.700	2.900	4.100	4.500	4.600	4.900	4.900	5.100	4.000	4.000	3.800	4.158	0.636	14.300
s.d.	1.020	2.002	2.166	1.868	1.500	1.685	1.921	1.972	2.300	2.366	1.342	2.315	1.871	0.400	4.584