



INTRODUCTION

The application of genetics in conservation efforts has increased dramatically over the past decades. Molecular genetic methodology has been used to address taxonomic issues, assess genetic variability and inbreeding, track gene flow and detect hybridization, all in an effort to conserve genetically healthy populations and aid in the identification of ecologically significant units (Fleischer 1998). The use of nuclear DNA (nucDNA) and mitochondrial DNA (mtDNA) sequence data in crocodylian research has increased our understanding of genetic variability (Flint et al. 2000; Ray et al. 2004; Russello et al. 2007), hybridization (FitzSimmons et al. 2002; Ray et al. 2004; Cedeño-Vásquez et al. 2008), differences between individuals (Farias et al. 2004), populations (Vasconcelos et al. 2006, 2008) and species (Li et al. 2007; Gatesy & Amato 2008; Meganathan & Dubey 2009; Meganathan et al. 2010). Microsatellites have been used to investigate population structure and gene flow in wild populations of Morelet's Crocodile *Crocodylus moreletii* Duméril & Bibron, 1851 (Dever & Densmore 2001; Dever et al. 2002), American Alligator *Alligator mississippiensis* Daudin, 1802 (Glenn et al. 1998; Davis et al. 2002) and Black Caiman *Melanosuchus niger* Spix, 1825 (de Thoisy et al. 2006). Microsatellites have also been useful in parentage analysis in Saltwater Crocodiles *C. porosus* Schneider, 1801 (Isberg et al. 2004), in determining and maintaining genetic variability

thus increasing juvenile survival rates (van de Ven et al. 2009). In 2010, 50 PWRCC captive-bred Philippine Crocodiles were released into a lake in the Divilacan municipality, geographically separated from the wild Isabela crocodile population. This release served as a pilot project to assess the adaptability of captive-bred Philippine Crocodiles under wild conditions (van Weerd & General 2003; van Weerd et al. 2010).

Recent systematics studies identified hybrids between *C. mindorensis* and *C. porosus* at PWRCC from the analyses of both mtDNA (D-loop and ND4) and nucDNA (*C-mos*) gene sequences (Louis & Brenneman 2008; Tabora et al. 2012). These studies validated previous concerns regarding reintroduction candidate purity, thus warranting forensic diagnoses prior to release. Using data generated from microsatellite loci derived from crocodilian genomes by Miles et al. (2009b,c) and this study, we address three questions regarding the Philippine Crocodile: (1) how does the genetic diversity in *C. mindorensis* compare to other crocodilian species, (2) what are the population genetic inferences of the two populations in the current range

of the Philippine Crocodile. We address these questions by comparing genetic diversity in *C. mindorensis* to other crocodilian species and by using microsatellite data to infer population structure and genetic diversity in the current range of the Philippine Crocodile. We address these questions by comparing genetic diversity in *C. mindorensis* to other crocodilian species and by using microsatellite data to infer population structure and genetic diversity in the current range of the Philippine Crocodile.

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and BayeScan 2.0 (Foll & Gaggioli 2008). Lositan implements an F_{ST} outlier method to identify loci likely under selection whereas BayeScan employs a maximum likelihood posterior probability. Relevance of the BayeScan posterior probabilities were interpreted with Jeffreys' scale of evidence (Jeffreys 1961). Considering that the extant populations are small, all within-population dyads were tested for relatedness (Queller & Goodnight 1989) using SPAGeDi (Hardy & Vekemans 2002) and compared to a simulation of 10,000 individuals of known pedigree relationships (Queller & Goodnight 1989).

Crocodylus porosus x *C. mindorensis* hybridization was identified in Tabora et al. (2012) where 57 captive crocodiles expected to be *C. mindorensis* by breeding records had inherited mtDNA haplotypes and nucDNA *C-mos* diagnostic sites found in *C. porosus*. We

C. porosus

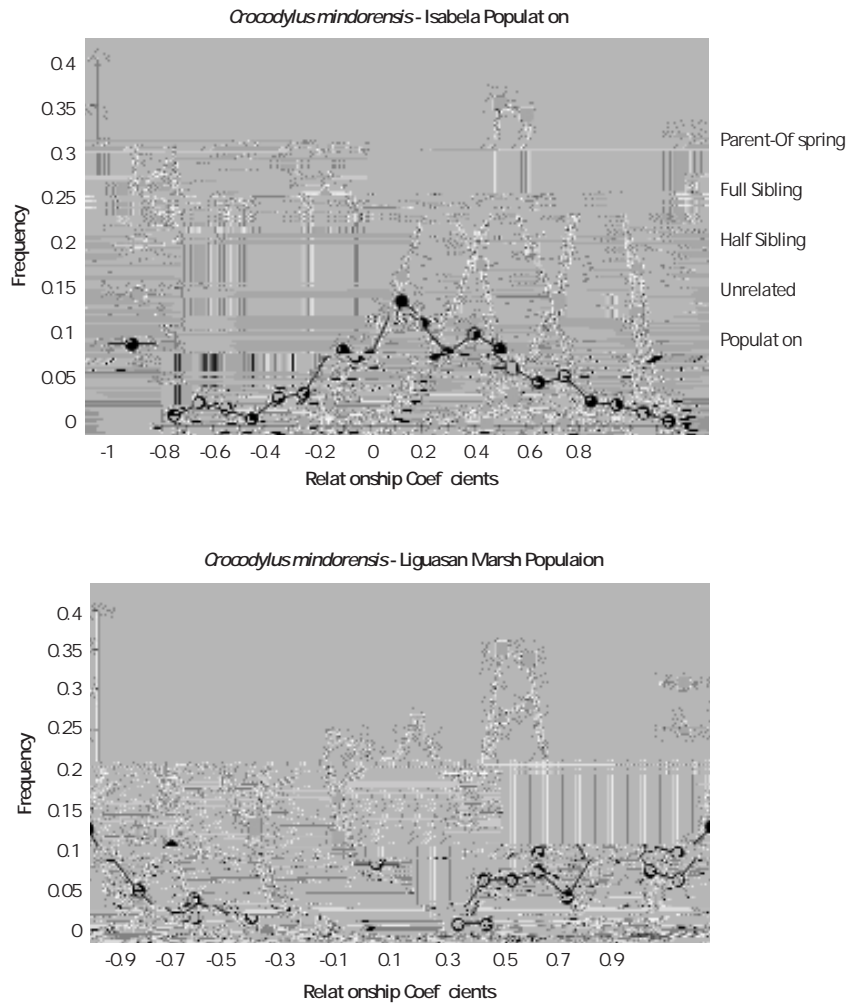


Figure 1. Relationship coefficient distributions of the two extant *Crocodylus mindorensis* populations from a - Isabela and b - Liguasan Marsh overlaid on a simulation of 10,000 individuals of known relationships by pedigree verification (Queller & Goodnight 1989).



Figure 2 Relationship coefficient distributions of the *Crocodylus porosus* population from the Republic of Palau overlaid on a simulation of 10,000 individuals of known relationships by pedigree verification (Queller & Goodnight 1989).

q -values above the noise threshold of 0.05 in the cluster represented by *C. porosus* (Fig. 4, see also Appendix 1). The PCoA suggested the same *C. mindorensis* individuals as previously identified with affinity to the *C. porosus* sample set (Fig. 5). The PCoA also identified individuals in the Isabela population that appear to group with the southern populations; a phenomenon which cannot be verified with records or observations. The PWRCC bred crocodiles reintroduced in Isabela were not included as Isabela members in this study.

DISCUSSION AND CONCLUSIONS

Previous studies have estimated genetic diversity in crocodylian species but making direct comparisons was difficult since the same marker systems were not applied across each study. Here, we used the same microsatellite loci to compare the genetic diversity of *C. mindorensis* to *C. acutus*, *C. niloticus*, *C. porosus* and *C. siamensis*. The heterozygosity estimates from our data for *C. acutus*, *C. niloticus*, *C. porosus* and *C. siamensis*

BLASTn algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_SPEC=WGS&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch) to search for potential candidate genes that might be under selection. Minimal sequence fragments ranging 25–50 bp in length were found in other species but no long sequence homologies and none of the queries returned candidates common to both flanking regions. Two short sequences were found in multiple species although corresponding to different genes. They were also found on multiple chromosomes in a single species indicating that these two sequences were both conserved and duplicated in the genome.

From the STRUCTURE analysis, $K=3$ was found to be the optimal number of clusters represented in the data by Evanno et al.'s (2005) K (Fig. 3). These clusters represent the Isabela *C. mindorensis* population, the Liguasan Marsh *C. mindorensis* population and the Republic of Palau *C. porosus* population. At K -max, a total of 59 putative *C. mindorensis* individuals had

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Tabora et al. (2012) identified a total of 57 putative hybrids in that study. From the STRUCTURE analysis of the same set of samples, we identified 59 individuals with genotypic proportions exceeding a background noise level ($q > 0.05$) in the cluster generated by the *C. porosus* samples (Appendix 1). The PCoA analysis also identified the same individuals to be closer to the *C. porosus* grouping than *C. mindorensis* below the nominal q -value threshold. Only two individuals approached the $q = 0.50$ genotypic proportions expected of an F1 individual (PWc005, $q = 0.512$; PWb097, $q = 0.409$). The former, PWc005, possesses both a *C. porosus* D-loop haplotype and the *C. porosus* C-mos diagnostic characters. We consider this individual to be an F1 from a *C. mindorensis* male and a *C. porosus* female. The latter, PWb097, possesses the *C. porosus* D-loop haplotype yet is homozygous for the *C. mindorensis* C-mos diagnostic sites. We consider this individual to be a *C. mindorensis* backcross falling in the upper tail of the backcross q -distribution. Two individuals from Abra (K7895 and K7897) exceeded the conservative 0.05 q -threshold for background noise though did not possess *C. porosus* D-loop or C-mos markers. We accept these to be *C. mindorensis* with slightly higher background noise than the conservative threshold we imposed in our criteria. The remaining 55 fell in a q -distribution around 0.25 (avg $q = 0.253 \pm 0.067$) which approximates the proportion of introgressed genes expected to be retained in the first backcross generation. Thus, we suggest one first generation hybrid cross and 56 backcross individuals only in the PWRCC-sampled group.

The morphological identification of hybrids, and particularly among the hybrids in this study, proves to be problematic. Hybrid detection through morphological characteristics is not always effective because hybrids can express mosaics of phenotypes (Campton 1987) due to incomplete penetrance or partial dominance of the diagnostic character. Hybrids in the PWRCC population were undetected since all express the post occipital scutes indicative of *C. mindorensis* (Image 1A). This suggests a single gene effect where the allele conferring the diagnostic scutes expressed in *C. mindorensis* is dominant over the allele fixed in *C. porosus* that suppresses the expression of that phenotype (Image 1B). Had F1 *inter se* mating occurred, one would expect that one fourth of the offspring should have inherited both *C. porosus* C-mos alleles and one fourth should express the absence of post occipital scutes. Neither scenario was detected in the data. Considering the multilocus allele frequency distributions, there is no indication that F1 *inter se* mating has occurred since the average of

the q -distribution of an F2 generation would be higher (A to 0.50). Then equ

indication of selection being a differentiating factor but the distance and isolation would be expected to drive genetic drift. Slightly elevated relatedness estimates suggest that future generations within both populations could face unavoidable mating of related individuals and the potential consequences of inbreeding. Genetic augmentation should be considered to offset these potential problems, whether by reintroduction from captive populations or by translocation between the populations. The most difficult constraint for successful conservation is securing the necessary funding to engage and monitor the programs. Whether genetic mixing between the two extant populations, augmentation from captive collections, or reintroduction of headstarted or captive-born candidates is decided upon, funding will be crucial to monitor the success of the effort and protect remaining habitats for the future of the species.

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Author Contribution: Ma. Rheyda Hirlo was involved with the data generation and sample collection in the Philippines and was involved in every step. John A. G. Tabora