GENETIC ANALYSIS OF THE CAPTIVE BREEDING PROGRAM FOR THE CRITICALLY ENDANGERED PAINTED TERRAPIN, *BATAGUR BORNEOENSIS*

by

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Introduction

Captive breeding is frequently suggested as a way to preserve endangered species (Soule et al. 1986; Ebenhard 1995; Mallinson 1995a,b; Bowkett 2009; Griffiths and Pavajeau 2008). Captive breeding aims to increase the population size of a species by breeding animals outside of their natural environment and then reintroducing them to the wild once conditions have improved (Bowkett 2009; Robert 2009; Williams and Hoffman 2009). In the past, captive breeding and reintroduction efforts have been successful only 11- 38% of the time and have been criticized as being too expensive given their low success rate (Griffith et al. 1989; Beck et al. 1994; Snyder et al. 1996). Reasons for failure included difficulties replicating species-specific breeding requirements, adaption to captive conditions that reduce reintroduction success, and genetic problems such as inbreeding depression (Snyder 1996; Lynch and O'Hely 2001; Frankham 2008). Nevertheless, some highly endangered species such as Peregrine falcons, *Falco peregrinus,* California condors, *Gymnogyps californianus,* large blue butterflies, *Phengaris arion*, and golden lion tamarins, *Leontopithecus rosalia*, have benefitted from captive breeding (World Wildlife Fund 2007; California Condor Conservation 2010). Increasing pressures on wild populations have renewed interest in captive breeding and reintroduction, especially for some groups such as amphibians and turtles that may be easier to breed and house in captivity than some other taxonomic groups (Russello and Amato 2007; Bowkett 2009). Using captive individuals to increase population numbers may also be the only option left for species that have gone extinct in the wild or for those that are critically endangered (Russello and Amato 2007).

When a species is bred in captivity for eventual reintroduction, care must be taken to maintain the genetic diversity of the founders and create breeding groups that maintain the evolutionary distinctiveness of populations in their native range (Lacy 1995; Fernandez and Caballero 2001; Williams and Osentoski 2007; Tallmon et al. 2004). The best method for maintaining genetic diversity in captivity utilizes pedigrees to pair animals for mating so that mean group kinship is minimized and inbreeding is avoided (Ballou and Lacy 1995). Determining the evolutionary distinctiveness of captive individuals ultimately requires knowledge of a species' population genetic structure in the wild and determination of the geographic origin of the founders. Unfortunately, relatedness and geographic origins of captive individuals are often unknown for some or all individuals in captivity. Molecular markers have recently been used successfully in these situations to determine relatedness and geographic origins of the founders and to help guide the management of captive breeding programs (e.g. Milinkovitch et al. 2004; Russello and Amato 2004; Russello et al. 2007; Tzika et al. 2009; Ivy et al. 2009; Alcaide et al. 2010).

All turtle species in Southeast Asia are facing extinction if current human activities in the region are not curtailed (Altherr and Freyer 2000). Turtles in Southeast Asia are disappearing due to a number of factors including habitat destruction and harvest for food, medicine, religious ceremonies, and the pet trade (Williams 1999; Gong et al. 2009). Groups such as the Turtle Survival Alliance (TSA, Hudson and Buhlmann 2002), Asian Turtle Consortium (ATC, Schaffer 2004), and the Fort Worth Zoo in Fort Worth, Texas, USA have started captive breeding programs for Asian turtles. Captive populations of these turtles consist of individuals donated from private collections or confiscated from illegal trafficking,

and thus levels of relatedness and the geographic origins of individuals are often unknown (Williams and Osentoski 2007; Fong et al. 2007). A lack of knowledge about the population structure of wild populations for most Asian turtle species presents an additional challenge to managing captive breeding programs (Fong et al. 2007). These problems will need to be addressed if the descendants of these captive individuals are to be successfully repatriated to their native habitat.

The painted terrapin *(Batagur borneoensis)* is a large (50 cm in length) freshwater turtle that lives in Southern Thailand, Malaysia, Borneo, and Sumatra (Ernst and Barbour 1989). Populations of this turtle have been rapidly decreasing due to the harvesting of adults and eggs for food and the construction of beach front property causing the loss of nesting areas (CITES 2006). As a result of this population decline, *B. borneoensis* is listed by the IUCN as critically endangered, indicating a high risk of extinction in the wild in the near future (www.iucnredlist.org).

B. borneoensis is unusual in that females move from freshwater estuaries to ocean-side beaches to lay their eggs in the sand (Dunson and Moll 1980). The eggs hatch after an incubation time of 70-90 days, and then the hatchlings swim back to freshwater estuaries (Bonin et al. 2006). Hatchlings can tolerate 100% salinity for up to two weeks, allowing them to survive during their swim to freshwater (Dunson and Moll 1980).

Captive breeding has been suggested as one of the few methods left for preserving *B. borneoensis*. The third edition of *B. borneoensis* studbook lists 170 specimens at 28 institutions. Some specimens have died or were acquired by a private holder and lost to follow up. Lost to follow up is the status given to individuals that cannot be tracked because their

current location and status is unknown. As of April 2007, there were 114 individuals at 14 institutions and 2 private collections in the United States and Canada that are being managed as part of a Species Survival Plan. Species Survival Plans are species specific plans developed by the Association of Zoos and Aquariums for *ex situ* population conservation. Species Survival Plans require AZA Zoos and Aquariums and other participating zoos to follow the species specific conservation plan (www.aza.org). Fifty-seven of these individuals were wild-caught adults (founders), whereas the remaining 57 were born in captivity. Most (75%) of the founders have unknown origins and little is known about this species population structure in its native range.

Breeding of *B. borneoensis* in captivity has been successful, with the Fort Worth Zoo, having clutches produced in seven different years (Barber, 2007). *B. bornoensis* breeds best when kept in mixed groups of multiple males and females in large ponds, making accurate determination of genetic parentage and hence genetic management of captive individuals difficult (Barber, 2007). I developed molecular markers for *B. borneoensis* to 1) determine parentage in breeding groups, 2) compare levels of genetic diversity in the remaining founders and their offspring, and 3) determine phylogenetic relationships among founders that might indicate if the captive population is composed of individuals from highly differentiated populations. These data will be used to help maximize the success of the captive breeding program.

Materials and Methods

DNA Extraction

Blood samples from 54 turtles from 10 institutions were used in this study (Table 1).

Blood (~ 100 µl) was placed in 400 µL lysis buffer (75 mM NaCl, 25 mM EDTA, 1%SDS) with 7 µl Proteinase K (20 mg/ml), then incubated at 55°C overnight. 1.5 volumes of 7.5 M ammonium acetate were then added to precipitate proteins which were pelleted by centrifugation for 10 minutes. DNA was precipitated by adding 0.7 volumes of isopropanol to the supernatant and centrifuging for 15 minutes to pellet the DNA. The DNA pellet was washed with 70% ethanol then allowed to dry before resuspending in 100 µL 10 mM Tris-HCl pH 8.5.

Table 1. Number of *Batagur borneoensis* genetically sampled, not sampled, or dead/lost to follow up in institutions and private collections included in the SSP (Species Survival Plan).

		Founders			**Captive**			
Location	Abb	Sampled	Not Sampled	Dead/LTF	Sampled	Not Sampled	Dead/LTF	Total
Private	SC	0	2	0	0	0	0	2
Toronto	TZ	5	0	4	1	2	3	15
Indian River	IR	0	0	0	0	1	0	1
Birmingham	BZ	1	0	0	1	3	0	5
San Diego	SDZ	3	0	2	1	1	0	7
Denver	OJ	0	0	0	1	3	2	6
Miami	MZ	0	0	0	2	2	0	4
St Augustine	SAF	3	0	2	0	0	0	5
Newport	NA	0	0	0	0	5	0	5
Detroit	DZI	0	0	2	0	3	0	5
Columbus	CZ	1	0	0	0	1	0	2
Oklahoma City	OKZ	5	0	0	0	0	1	6
Fort Worth	FWZ	11	0	0	9	4	4	28
San Antonio	SAZ	2	1	2	8	6	1	18
Dallas	DWA	0	0	0	0	3	2	5
Bronx	NYB	0	0	0	0	0	3	3
Amherst	UMA	0	0	1	0	0	0	1
Santa Barbara	SBZ	0	0	2	0	0	0	2
Washington DC	DCZ	0	0	2	0	0	0	2
Orlando	GAT	0	0	0	0	0	4	4
Atlanta	ZA	0	0	1	0	0	0	1
Wichita	SCZ	0	0	4	0	0	0	4
Providence	RWZ	0	0	0	0	0	3	3
Riga, Latvia	RZ	0	0	0	0	0	5	5
Private	SURY	0	23	0	0	1	0	24
Unknown		0	0	1	0	0	1	2
Private	HJ	0	0	1	0	0	3	4
Total		31	26	24	23	34	32	170

Microsatellite development

Microsatellite loci were isolated using the protocol of Glenn and Schable (2005). DNA was co-digested with *RsaI* and *XmnI* and the resulting fragments were ligated to SuperSNX linkers. Fragments were then hybridized to groups of biotinylated oligos ($AAAC_8$, $AAAG_8$, $GATA_8$, AC_{12}, GA_{12}, $ACTC_8$, $AGAT_8$, and $AATG_8$) and captured with streptavidin coated magnetic beads (Promega USA). The enriched fragments were cloned with the pGEM-T Easy Vector System (Promega USA). We sequenced 600 clones using primers SP6 and T7. Sequences were electrophoresed on an ABI 3130 Genetic Analyzer (Applied Biosystems USA) using ABI Big Dye Terminator Cycle Sequencing v 3.1 chemistry (Applied Biosystems USA). We screened all sequences with MSATCOMMANDER (Faircloth 2008) for dinucleotide loci that had >8 repeats and tri and tetranucleotide loci that had >6 repeats and found 44 AG_n loci, nine GAA_n or ATC_n loci, and one CTTT locus. We designed primers for 34 clones that contained sufficient flanking regions using Primer 3 (Rozen and Skaletsky 2000). From these 34 loci, 21 failed to amplify, eight did not produce a specific product, and five were polymorphic, amplified consistently, and had scorable profiles (Table 2).

Table 2. Polymorphic microsatellite loci developed for *Batagur borneoensis*.

Locus	Sequence (5'-3')	Repeat in Original Clone	Size in Original Clone (bp)	Size Range (bp)	Number of Alleles
AC01	F: TACACACCCACACACACGTT	AC_{13}	223	213-230	7
	R: ACAGTGAGTTGGCTGCCTTT				
CT06	F: TGCACTGTTTCTCATACAGTCCTC	CT_{13}	129	112-126	3
	R: CATGTGTTGGAAGTTTAGTGTGAA				
CTTT	F: CTGGGGACAGAACTTTGGAG	$CTTT_{14}$	202	175-239	16
	R: TCATCTGCAGTCCAATGAGC				
GT1	F: TTGACTCCTCCCCCTCAAC	$GT_5AT(GT)_{10}$	191	193-213	8
	R: GGGGCTAGGTTCCAGTGAA				
GT16	F: CACTGCCCCATTACCTTGTT	AC_{13}	101	88-97	4
	R: TGGCCATTCTTTTAGATTTCG				

I amplified all five loci in one multiplex set using Qiagen's Multiplex Reaction Kit. Polymerase chain reactions (PCR) (10 µL) contained 10-50 ng DNA, 0.2 µM of each primer, 1X Qiagen Multiplex PCR Master Mix with HotStarTaq, Multiplex PCR buffer with 3 mM $MgCl_2$ pH8.7, and dNTPs. Reactions were cycled in an ABI 2720 thermal cycler. The cycling parameters were one cycle at 95^{o}C for 15 min, followed by 30 cycles of 30 s at 94^{o} C, 90 s at 60^{o}C, 90 s at 72^{o}C, then a final extension at 60^{o}C for 30 min. The resulting multiplexes were diluted 10X with dH_2O. For each sample 0.5 µl of diluted product was loaded in 10 µl HIDI formamide with 0.1 µl LIZ-500 size standard (Applied Biosystems USA) and electrophoresed on an ABI3130 Genetic Analyzer (Applied Biosystems USA). Genotypes were scored using Genemapper v4.0 (Applied Biosystems USA).

Mitochondrial DNA

The 5' part of the mitochondrial control region was amplified using primers Des1 and Des2 (Starkey et al. 2003). To increase specificity, I redesigned the primers for *B. borneoensis*

and used these to amplify all individuals (Bb_dlF – 5'- TCTCTACATGACTTCACAGAGGAT - 3', Bb_dlR – 5' – TGTTGCTTTAACGGGGGTAG – 3'). Polymerase chain reactions (PCR) (10 µL) contained 50 ng DNA, 50mM Tris-HCl (pH 9.2), 16 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.1% Tween, 200 µM each dNTP, 0.5 µM primers, and 0.4 U *Taq* DNA polymerase. Reactions were cycled in an ABI 2720 thermal cycler. The cycling parameters were one cycle at 95^{o}C for 15 min, followed by 30 cycles of 30 s at 94^{o} C, 15 s at 55^{o}C, 1 min. at 72^{o}C, then a final extension at 72^{o}C for 5 min. PCR products were sequenced using ABI Big Dye Terminator Cycle Sequencing v 3.1 chemistry (Applied Biosystems USA) using the PCR primers and an internal primer (Bb_dliR – 5' – AAAAACAACCAGAGGCCAGA – 3'). Sequences were electrophoresed on an ABI3130 Genetic Analyzer (Applied Biosystems USA). Sequences were trimmed, edited, and contiged using Sequencher v. 4.8 then aligned in Bioedit using Clustal X v2.0 (Larkin et al. 2007).

Genetic diversity

I tested microsatellite loci for deviation from Hardy-Weinberg equilibrium (HWE) and genotypic linkage equilibrium using Genepop v 4.0 (Rousset 2008). For the microsatellite loci the number of alleles, observed heterozygosity (H_O), and expected heterozygosity (H_E), were calculated for each locus and population using GenAlEx6.2 (Peakall and Smouse 2006). Allelic richness was calculated for each population using rarefaction to control for differences in sample sizes (as described in El Mousadik and Petit 1996) and implemented in FSTAT v 2.9.3.1. I used Wilcoxon sign-rank tests to determine if founder and captive born individuals differed in allelic richness, observed heterozygosity, and F_{IS}.

For the mitochondrial haplotypes, I used GenAlEx6.2 to calculate the frequency of haplotypes and haplotype diversity (h). Haplotype diversity can be interpreted as the probability that two individuals from a given sampling unit will have different haplotypes. The Kimura 2-parameter genetic distance measure (K2P) (Kimura 1980) was calculated between all unique haplotypes in MEGA v 4 (Tamura et al. 2007).

I calculated the genetic distance between all pairwise comparisons of wild-caught individuals using 1 – the proportion of microsatellite alleles shared between individuals (Bowcock et al. 1994). These inter-individual distances were then used to build a neighbor joining tree using PHYLIP (Felsentein 1995) to look for evidence of distinct clusters (Felsenstein 1987). Relationships among mitochondrial haplotypes were visualized by constructing a neighbor-joining tree using MEGA v 4. Branch points were tested with 5000 bootstraps. Relationships among haplotypes were also visualized as a statistical parsimony network using the program TCS (Templeton et al. 1992, Clement et al. 2000).

Parentage

I used CERVUS 3.0 (Kalinowski et al. 2007), a likelihood-based program, to determine genetic parentage for 20 of the 23 individuals born in captivity. We did not have candidate parents for the other three individuals because they either died or were lost to follow up 10 years ago. The likelihood ratio is the ratio between the likelihood that a candidate parent is the true parent over the likelihood that the candidate parent is not the true parent, given the observed frequency of alleles in the population. The LOD score is the natural logarithm of the likelihood ratio. Positive LOD scores indicate a candidate parent is more likely to be the true parent than a randomly chosen individual, while a zero or negative number indicates the

candidate is as likely or less likely to be the true parent as a randomly chosen individual. CERVUS simulates the critical difference in LOD scores (delta score) between the most-likely candidate parent and the second-most-likely candidate that is required to assign parentage at a specified confidence level.

To simulate the critical delta scores, I calculated allele frequencies using all 31 adult individuals. I assumed a genotyping error rate of 0.01 and that 82% of candidate parents were sampled (the average number sampled per zoo). Candidate mothers were the sampled adult females who were in the same location as the eggs at the time of hatching. I assigned a female as the candidate mother to a hatchling if she was assigned at the ≥ 80% confidence level by CERVUS. After I assigned the mother, I then used each assigned mother as a known candidate and assigned or excluded males in the same zoo as the sire of the hatchling.

Using the estimated variance in reproductive success from the parentage analysis I calculated the effective population sizes of females and males using the following equations (Lande and Barrowclough 1987):

$$N_{ef} = N_f k_f - 1/[(k_f - 1) + (V_{kf}/k_f)]$$

$$N_{em} = N_m k_m - 1/[(k_m-1) + (V_{km}/k_m)]$$

N_f and N_m represent the number of females and males, k_f and k_m are the mean offspring number per male and female and V_{km} and V_{kf} represent the variances in offspring number per male and female. The total effective population size was then calculated using the following equation that takes into account sex ratio bias (Wright 1931):

$$N_e = 4N_m N_f / N_m + N_f$$

Results

Microsatellite Genetic Diversity

All loci in all individuals amplified successfully. Observed heterozygosity ranged from 0.52 – 0.94 and there were 3 to 16 alleles (n = 31 founders) at the five microsatellite loci (Table 3). There was no significant heterozygote deficit at any locus (P > 0.05 in all cases) and MICROCHECKER did not indicate the presence of null alleles. All loci were also in genotypic linkage equilibrium. Observed heterozygosity was similar between the founders and captive born individuals (Wilcoxon, P = 0.50). Allelic richness for first generation captive born individuals was 20% lower than the founders, although the difference was not significant (Wilcoxon, P = 0.13). The average F_{IS} of the founders was 0.062 and -0.108 for captive born individuals (Wilcoxon, P = 0.13; Table 3).

Table 3. Genetic diversity estimates for five microsatellite loci for founders (n = 31) and captive-bred (n = 23) *Batagur borneoensis*. Na is number of alleles, H_O is observed heterozygosity, H_E is expected heterozygosity, F_{IS} is the inbreeding coeffiecient, allelic richness is the number of alleles adjusted for the smallest sample size (n = 23).

Pop	Locus	Na	H_O	H_E	F_{IS}	Allelic Richness
Founders	*AC01*	7	0.71	0.72	0.01	5.4
	AC06	3	0.52	0.64	0.19	3.0
	CTTT	16	0.94	0.92	-0.01	15.3
	GT1	8	0.74	0.75	0.02	6.2
	GT16	4	0.58	0.67	0.13	4.0
Average			0.69±0.07		0.068±0.04	6.79±2.20
Captive	*AC01*	5	0.65	0.66	0.01	5.0
	AC06	2	0.61	0.49	-0.24	2.0
	CTTT	12	0.91	0.85	-0.08	12.0
	GT1	4	0.61	0.51	-0.19	4.0
	GT16	4	0.57	0.54	-0.05	4.0
Average			0.67±0.06		-0.11±-0.08	5.4±1.72

There were 11 private alleles in the founders that were not represented in captive born individuals. There were no private alleles in captive born individuals (Table 4). The Fort Worth Zoo, San Diego Zoo, and the Toronto Zoo had the highest number of alleles not found in captive born individuals (n = 5, 4, and 4 alleles respectively).

Table 4. Private microsatellite alleles found in the founders across sampled zoos. The first number indicates the number of private alleles for each locus present at that zoo. The second number is the number of private alleles present at only at that zoo and no other zoo. A dash indicates no private alleles for that locus at that zoo.

Zoo	*AC01*	*AC06*	*CTTT*	*GT1*	*GT16*	Total per zoo
AF	-	-	2/1	-	-	2/1
BZ	-	1/0	-	1/1	-	2/1
CZ	-	1/0	-	-	-	1/0
FWZ	1/0	1/0	1/0	1/1	-	5/1
OKZ	1/0	1/0	-	1/1	-	3/1
SA	-	1/0	-	-	-	1/0
SDZ	1/1	1/0	2/0	-	-	4/0
TZ	-	1/0	2/1	1/1	-	4/2
Total unique alleles	2	1	4	4	-	

Mitochondrial Genetic Diversity

There were a total of 13 haplotypes, with the founders having seven private haplotypes that were not found in the captive-born population. Haplotype sequences were very similar to each other and were on average only 0.5% divergent (range 0.2% - 1.3%, Kimura-2 parameter distance). Haplotype diversity was 0.92 for the founders and 0.76 for captive-bred individuals. The Fort Worth Zoo and the San Diego Zoo had the most haplotypes (n=10 and 4 haplotypes respectively). Of the seven private mitochondrial haplotypes found

only in the founders and only at one zoo, four were found at the Fort Worth Zoo, one was found at the Oklahoma City Zoo, and one was found at the San Diego Zoo (Table 5).

Table 5. Haplotype distribution among sampled zoos. All haplotypes present are listed followed by the number of private haplotypes that are present in only one zoo.

Zoo	Haplotypes	Total	Private
AF	H6	1	0
BZ	H4, H9	2	0
CZ	H7	1	0
FWZ	H1, H3, H4, H6, H8, H9, H10, H11, H12, H13	10	4
MZ	H8, H9	2	0
OKZ	H5, H7	2	1
OJ	H9	1	0
SA	H6, H9, H11	3	0
SDZ	H2, H6, H7, H11	4	1
TZ	H6, H9, H10	3	0

Evidence for structure in captive population

There was little evidence for strong genetic breaks or clear groupings of founder individuals using either the microsatellite loci or mitochondrial haplotypes. The positive F_{IS} values for four of the five microsatellite loci in the founders (Table 3) may indicate a Wahlund effect from the mixing of slightly differentiated populations. A neighbor-joining tree of the proportion of alleles shared between individuals at microsatellite loci revealed what might be considered two main clusters of individuals with two individuals (11 and 53) in an intermediate position between the two clusters (Fig. 1).

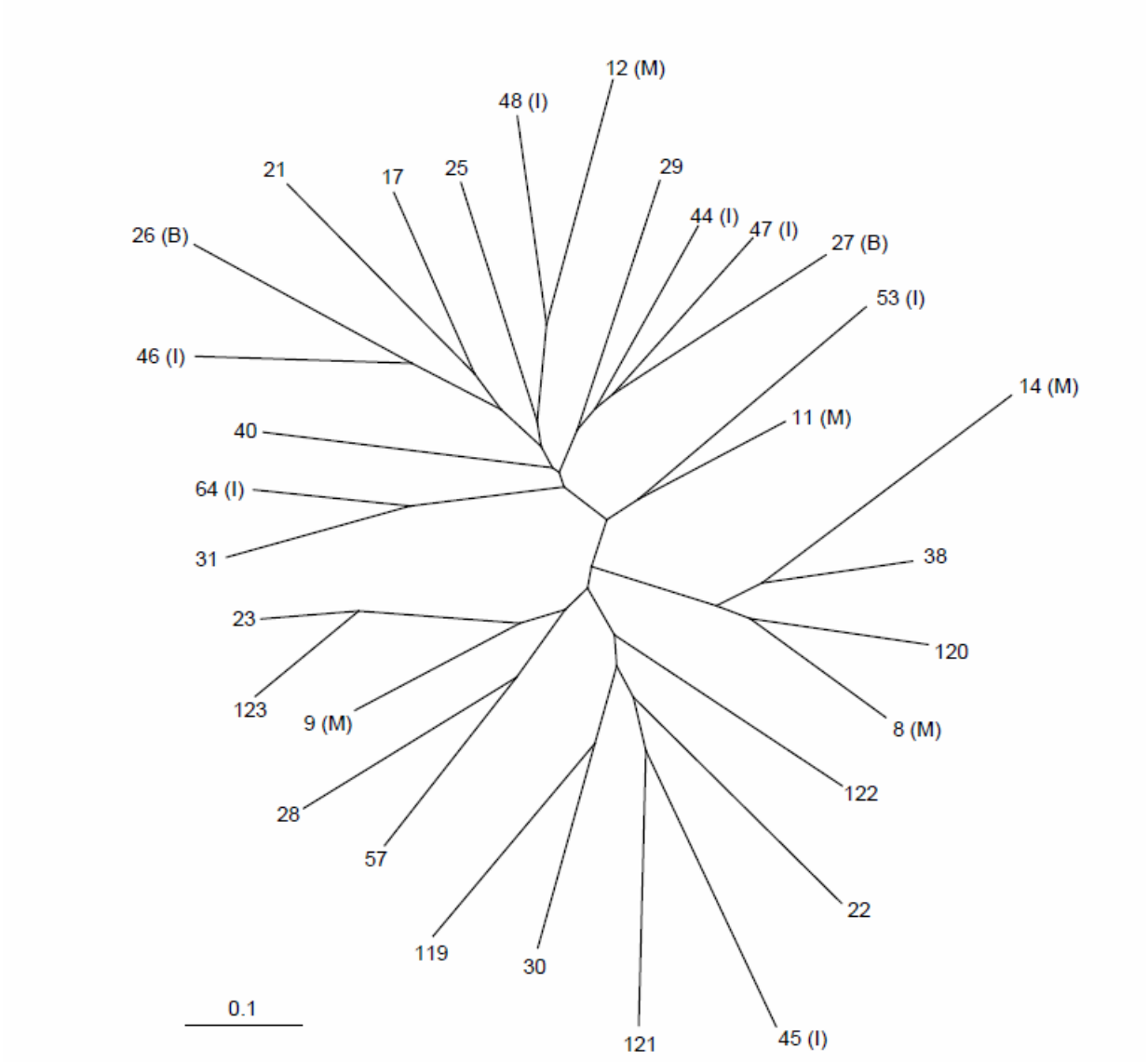

Figure 1. Neighbor-joining tree between individuals (stud book numbers) constructed using [1 – (the proportion of microsatellite alleles shared between individuals)]. Geographic origin is in parentheses, I – Indonesia, M – Malaysia, B – Borneo.

The haplotype network had a star shape with four groups radiating out from the center H6 haplotype (Fig. 2).

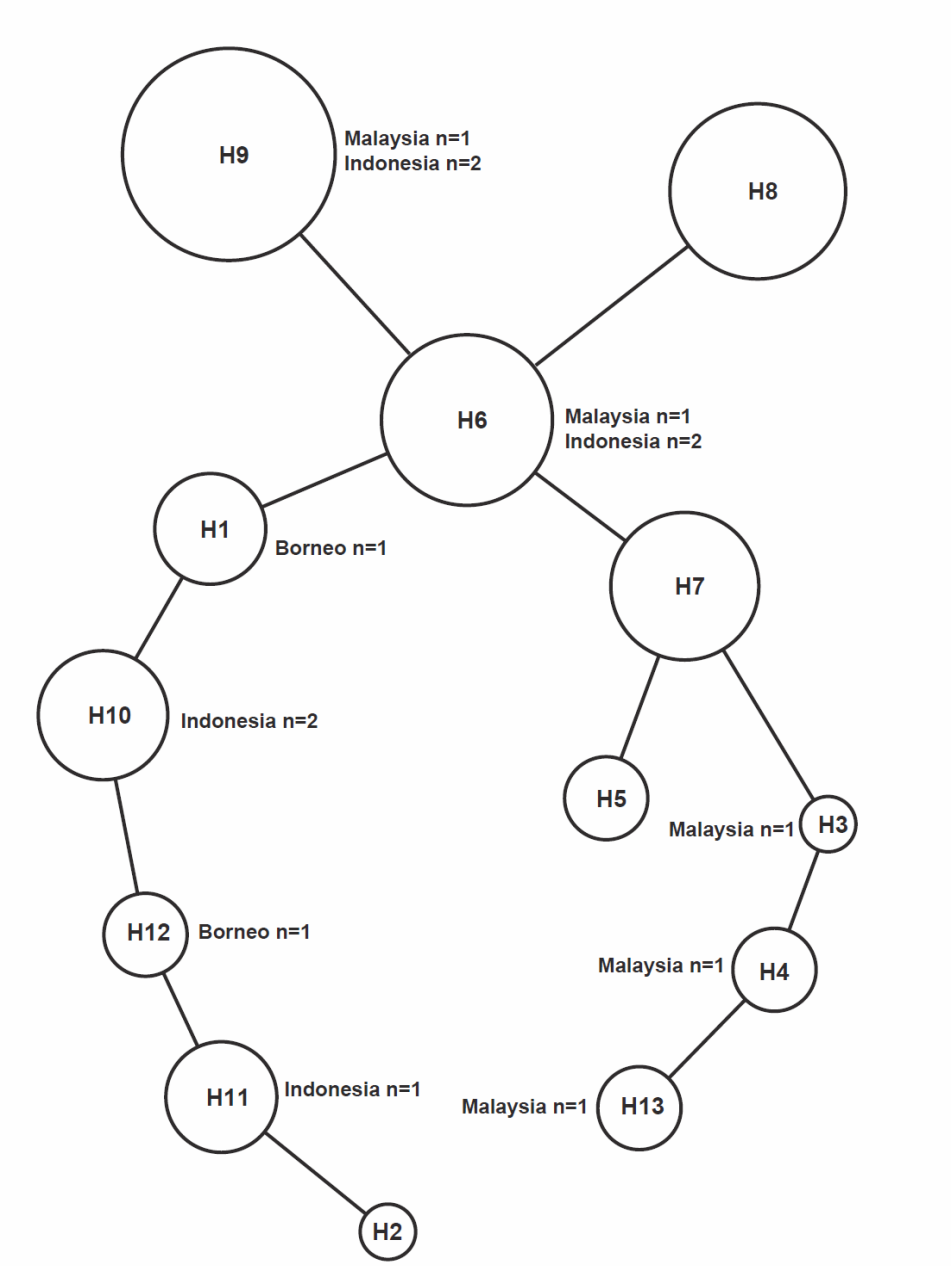

Figure 2. Statistical parsimony haplotype network for the mitochondrial control region of *Batagur borneoensis*. The size of circles is proportional to the representation of that haplotype. Each line represents one mutational difference between haplotypes.

When the capture locations of the wild-caught turtles were added to the neighbor-joining tree, there was weak evidence for a division of the haplotypes between Malaysia and the islands of Borneo and Indonesia. The neighbor-joining tree also revealed a similar weak division between Malaysia (H3, 4, 13) and Indonesia/Borneo (H1, 10, 11, 12), but the bootstrapping values were very low (<70) (Fig. 3). Haplotypes 6 and 9 were found in both Indonesia and Malaysia. Adding the haplotypes or geographic origin to individuals in the microsatellite tree did not reveal any clear pattern between haplotypes (not shown) or between Malaysia, Borneo, and Indonesia.

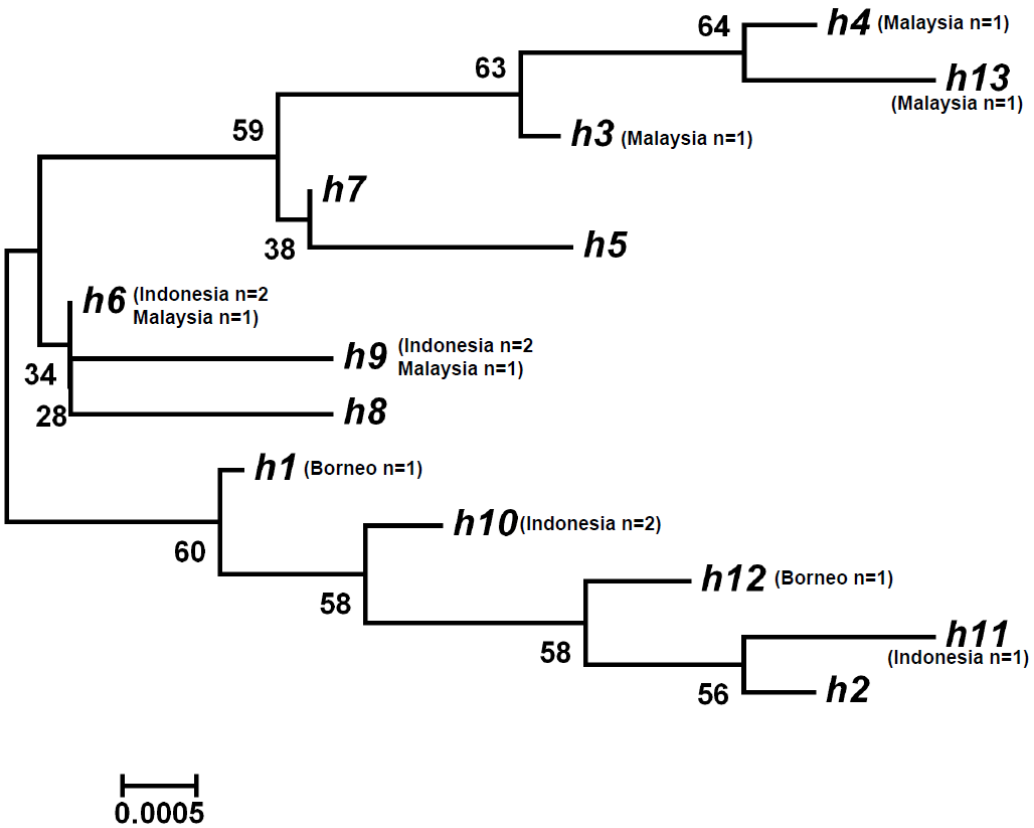

Figure 3. Neighbor-joining tree of mitochondrial haplotypes for the control region of *Batagur borneoensis*. Numbers at nodes are bootstrap values. Numbers next to geographic locations are the number of founders from that particular region.

Parentage

I assigned 20 offspring to five mothers. Fourteen offspring were assigned mothers at the 95% confidence level, five at 80% confidence level, and one at < than 80% confidence. All assigned females had zero mismatches with the offspring except for the one female assigned at less than 80% confidence (Fig. 4). This female had one mismatch with the hatchling but was the assigned mother of two other hatchlings from the same clutch with zero mismatches. She also had the same mitochondrial haplotype as the hatchling in question.

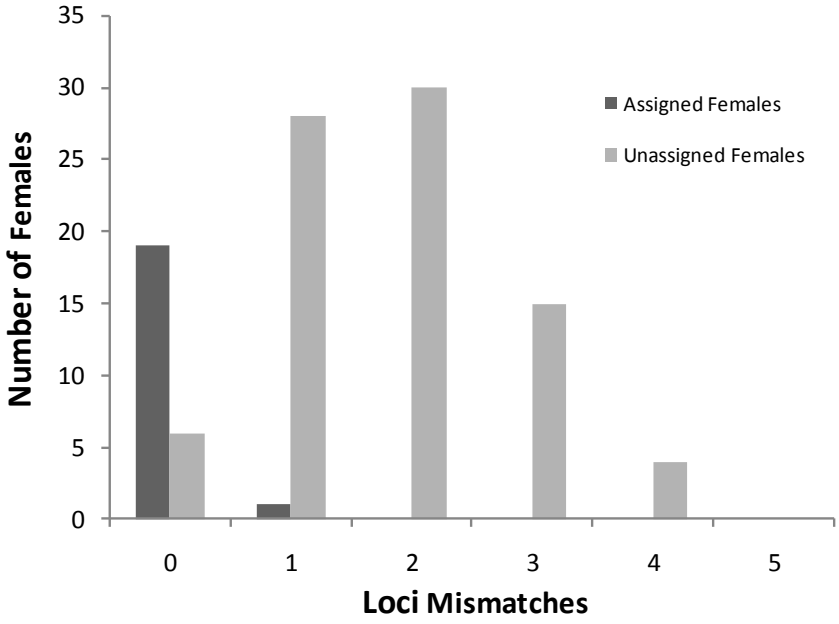

Figure 4. The number of mismatches across loci between potential female breeders and captive-born individuals. Assigned females are the most likely mother as determined by CERVUS.

The next most likely female had two mismatches and a different haplotype than the hatchling thus she was not assigned maternity. For the five females assigned maternity at the 80% confidence level and one female assigned at the 95% confidence level there was another female in the zoo that also had zero mismatches with the hatchlings. Five of these females

were from the Fort Worth Zoo and one was from the Birmingham Zoo. The mitochondrial haplotypes of all of the most likely females matched that of the offspring (while the other females did not), thus the most likely female was assigned maternity. These females also had the highest LOD scores for each offspring (Fig. 5).

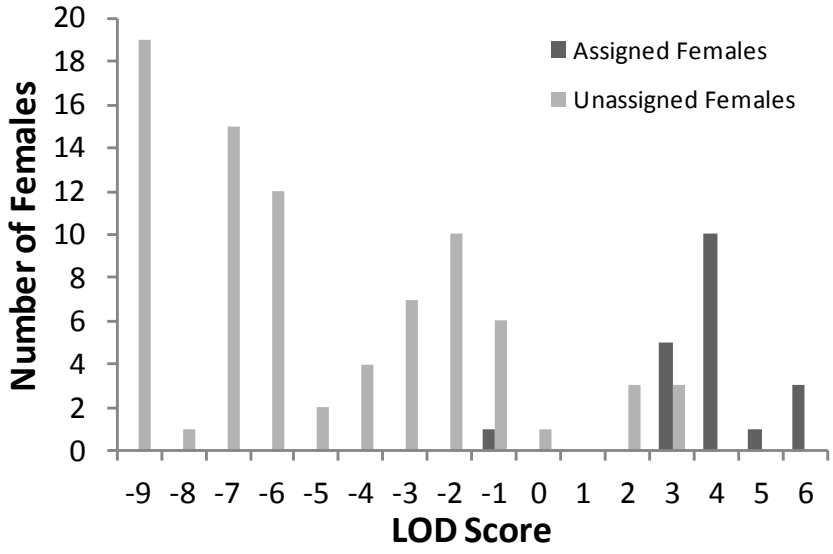

Figure 5. Distribution of LOD scores (natural log of the likelihood ratio), calculated in CERVUS, for female *Batagur borneoensis*. Scores are of females that were assigned genetic maternity and those that were excluded from genetic maternity.

Table 6. Maternity for 20 captive-born individuals. Possible mothers are individuals present at the zoo who could have laid eggs. Red stud book numbers indicate that the female is listed as the dam for that offspring. Mothers were assigned using CERVUS and mitochondrial haplotype matching (see methods). Mismatches are the number of loci at which the genotype of the mother and the offspring did not match. The LOD score is the likelihood of parentage. Females sampled/unsampled represents the numbers of possible mothers for which samples were obtained/number of samples that were not obtained. *=died in March 2006

Offspring	Hatch	Clutch	Hatch Zoo	Possible Mothers	Assigned Mother	Female Mismatches	Female LOD Score	Female Sampled/ Unsampled
61	27-May-95	A	BZ	8,9,11,**12**	11	0	3.08	4/0
102323	30-May-95	A	BZ	8,9,11,**12**	11	0	2.42	4/0
103	29-Aug-02	G	TZ	44,**45**,47,49	45	0	5.83	3/1
81	2-Aug-00	C	SA	20,21,24,25	21	0	2.92	2/2
159	12-May-06	J	SA	20,21,**24***,25	21	0	3.79	2/2
160	13-May-06	J	SA	20,21,**24***,25	21	0	3.79	2/2
161	13-May-06	J	SA	20,21,**24***,25	21	1	-1.07	2/2
163	28-Jun-06	K	SA	20,21,25	21	0	3.79	2/1
157	12-May-06	J	SA	20,21,**24***,25	21	0	3.76	2/1
66	9-Jun-98	B	FWZ	8,9,**12**,17,26,27,28,29	12	0	3.65	8/0
89	4-Apr-01	E	FWZ	8,9,12,**17**,26,27,28,29	17	0	4.45	8/0
148	12-Aug-05	I	FWZ	8,9,11,12,17,26,27,28,29	17	0	3.68	9/0
150	12-Aug-05	I	FWZ	8,9,11,12,17,26,27,28,29	17	0	2.66	9/0
154	13-Aug-05	I	FWZ	8,9,11,12,17,26,27,28,29	17	0	2.32	9/0
167	2-Aug-06	L	FWZ	8,9,11,12,17,26,27,28,29	17	0	1.00	9/0
168	13-Aug-06	L	FWZ	8,9,11,12,17,26,27,28,29	17	0	2.66	9/0
205048	3-Sep-07	M	FWZ	8,9,11,12,17,26,27,28,29	17	0	2.92	9/0
205049	3-Sep-07	M	FWZ	8,9,11,12,17,26,27,28,29	17	0	3.41	9/0
205075	3-Sep-07	M	FWZ	8,9,11,12,17,26,27,28,29	27	0	5.78	9/0
205076	3-Sep-07	M	FWZ	8,9,11,12,17,26,27,28,29	27	0	5.28	9/0

Of the nine captive born individuals for whom the stud book listed a possible mother, only three (33%) assigned mothers matched the mother listed in the studbook (Table 6). Using the assigned mothers, I was able to assign two males to 16 of the offspring. All of these males but one had zero mismatches with the offspring, while unassigned males had from 1-4 mismatches (Fig. 6).

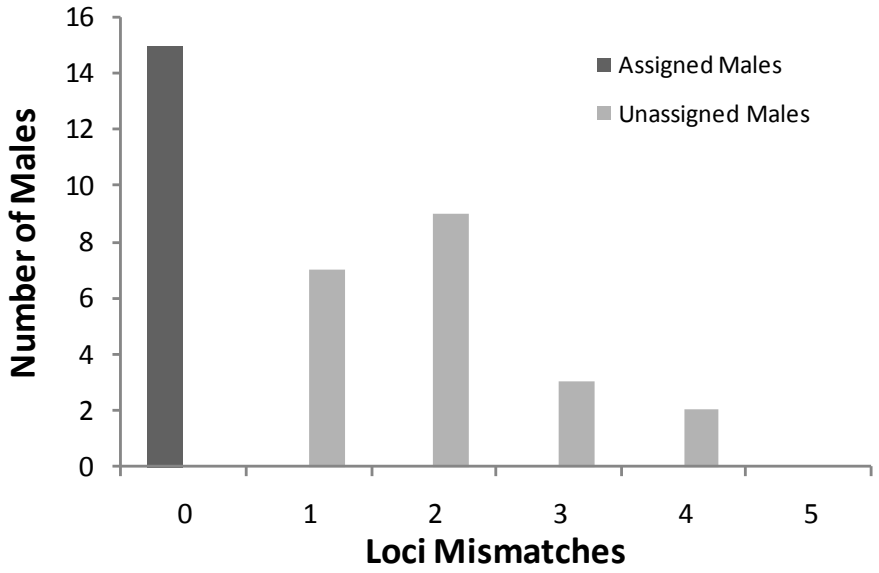

Figure 6. The number of mismatches across loci between potential male breeders and captive-born individuals. Assigned males are the most likely father as determined by CERVUS.

For two of the hatchlings (61, 102323), there was only one possible father yet he was unsampled, thus he was assigned as the father. For two other hatchlings (103, 66), only one of two males was sampled yet they mismatched the hatchlings at two and three loci. I tentatively assigned the unsampled males as the true fathers in these cases. The male LOD scores were highest for the males that were assigned paternity (Fig. 7).

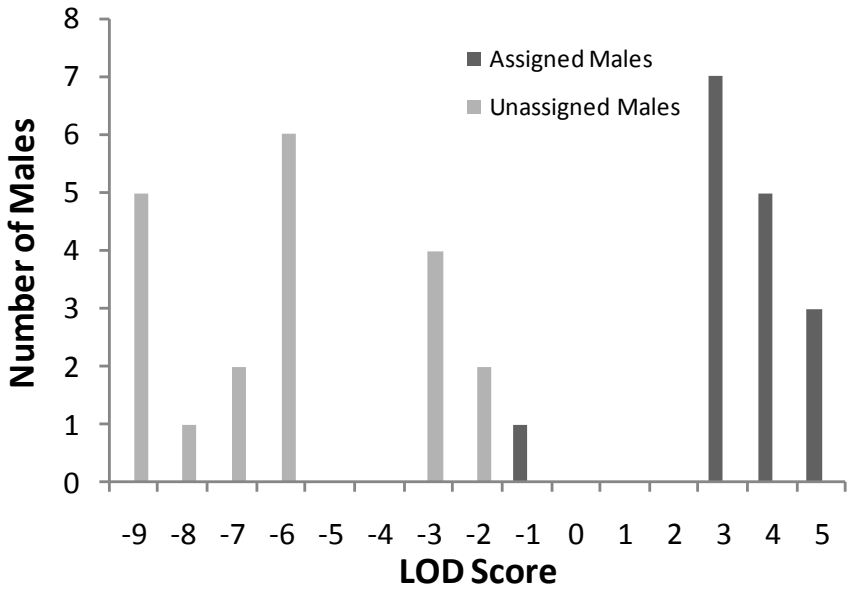

Figure 7. Distribution of LOD scores (natural log of the likelihood ratio), calculated in CERVUS for male *Batagur borneoensis*. Scores are of males that were assigned genetic paternity and those that were excluded from genetic paternity.

Most captive breeding of *B. borneoensis* in North America occurs at the Fort Worth Zoo and San Antonio Zoo (N = 60 of 79 individuals born in captivity, 43 of which are still in zoos), and it appears that only one male at each zoo is currently reproducing. Male 23 fathered all sampled hatchlings at the Fort Worth Zoo (n = 10). Male 41 fathered all sampled hatchlings at the San Antonio Zoo (n = 6) (Table 7).

Effective Population Size

The effective population size was calculated for the captive population of breeding individuals. The total breeding population was considered to be 29 individuals, 10 males and 19 females. When using the equations considering variance in reproductive success, $N_{ef} = 4$ and $N_{em} = 3$. The total effective population size was seven individuals.

Table 7. Paternity for 20 captive-born individuals. Possible fathers are adult males at the zoo who could have mated. Fathers were assigned using CERVUS (see methods). Mismatches are the number of loci at which the genotype of the father and the offspring did not match. The LOD score is the likelihood of parentage. Males sampled/unsampled represents the numbers of possible fathers for which samples were obtained/number of samples that were not obtained. Blank cells under mismatches and LOD occur due to the inability to calculate these values for the assigned father due to lack of a DNA samples (see results).

Offspring	Hatch	Clutch	Hatch Zoo	Possible Fathers	Assigned Father	Male Mismatches	Male Sampled/ Unsampled	Male LOD Score
61	27-May-95	A	BZ	10	10		0/1	
102323	30-May-95	A	BZ	10	10		0/1	
103	29-Aug-02	G	TZ	46,50	50		1/1	
81	2-Aug-00	C	SA	23,41,42,43	41	0	4/0	2.87
159	12-May-06	J	SA	41,43	41	0	2/0	2.31
160	13-May-06	J	SA	41,43	41	0	2/0	2.31
161	13-May-06	J	SA	41,43	41	0	2/0	2.38
163	28-Jun-06	K	SA	41,43	41	0	2/0	3.19
157	12-May-06	J	SA	41,43	41	1	2/0	-1.45
66	9-Jun-98	B	FWZ	16,22	16		1/1	
89	4-Apr-01	E	FWZ	22,23	23	0	2/0	3.46
148	12-Aug-05	I	FWZ	22,23	23	0	2/0	4.06
150	12-Aug-05	I	FWZ	22,23	23	0	2/0	4.11
154	13-Aug-05	I	FWZ	22,23	23	0	2/0	2.23
167	2-Aug-06	L	FWZ	22,23	23	0	2/0	3.38
168	13-Aug-06	L	FWZ	22,23	23	0	2/0	4.11
205048	3-Sep-07	M	FWZ	22,23	23	0	2/0	2.99
205049	3-Sep-07	M	FWZ	22,23	23	0	2/0	2.74
205075	3-Sep-07	M	FWZ	22,23	23	0	2/0	3.08
205076	3-Sep-07	M	FWZ	22,23	23	0	2/0	3.59

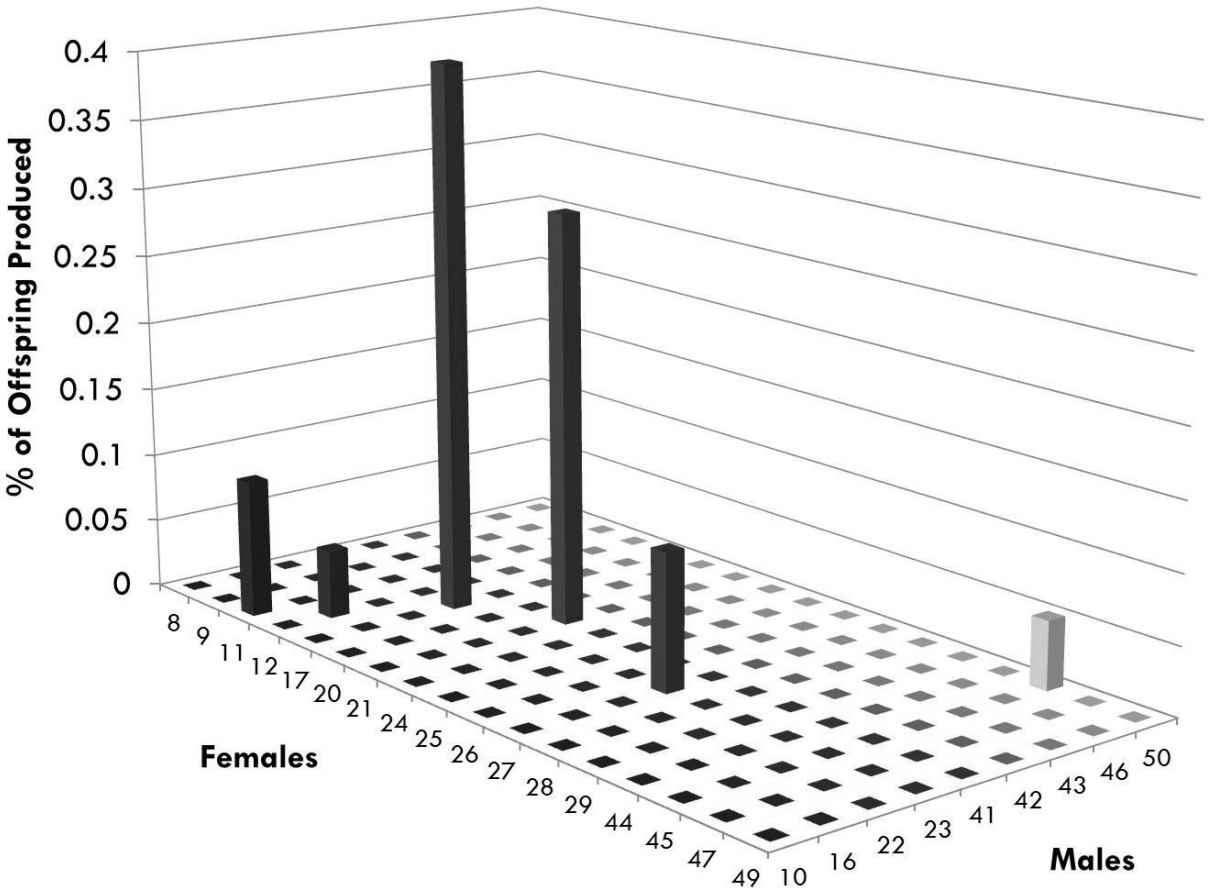

Figure 8. Percentage of total offspring produced by breeding males and females. Listed males and females were present at the location and hatch date of the offspring hatchings.

Discussion

The captive breeding program for *B. borneoensis* suffers from two uncertainties that are common to many captive breeding programs: 1) founders are often from unknown origins and 2) genetic parentage is often uncertain, especially paternity. The second of these is especially problematic for species like *B. borneoensis* when animals must be kept in multi-male-female groups. These uncertainties can make genetic management of captive populations difficult and can potentially jeopardize the long term success of reintroductions if the captive population becomes inbred or if the hybridization of unique populations results in outbreeding depression. Molecular data can provide information on these uncertainties for captive breeding programs (e.g. Milinkovitch et al. 2004, Russello and Amato 2004, Fong et al. 2007; Williams and Osentoski 2007, Tzika et al. 2009, Ivy et al. 2009, Alcaide et al. 2010).

The majority (73.4%) of the captive-born offspring of *B. borneoensis* come from the Fort Worth Zoo and the San Antonio Zoo. While the zoos' successful captive breeding programs are a great step towards conserving this species, it is clear that the parentage of all offspring must be determined genetically. I found that the potential mothers listed in the studbook were frequently (67% of offspring) incorrect. Overall, 29% of females and 56% of males have produced at least one offspring (Table 1, Fig. 8), and yet breeding appears to be highly skewed to a few male-female pairs. Two pairs produced 70% of the offspring and two males sired 80% of the offspring (Fig. 8). The reasons for high skew in offspring production are unknown and have been observed in other captive breeding programs of reptiles (e.g. Milinkovitch et al. 2004; Moore et al. 2008; Tzika et al. 2009). Dominance interactions between males, female choice for particular males, or fertility problems have all been suggested as possibilities for

observed patterns of reproductive skew (Milinkovitch et al. 2004). Dominance interactions between males are observed in some turtles (e.g. Gailbraith 1991; Kaufmann 1992), although there is not good evidence for overt female choice (Pearse and Avise 2001) or for fertility problems in captivity. *B. borneoensis* males and females are dichromatic, a trait that is unusual among turtles. Females have olive colored heads, whereas males have charcoal colored heads that turn white with a red stripe between the eyes during the breeding season (Moll et al. 1981). It is unknown if male color change is a sexually selected trait that may be important for female mate choice or male dominance interactions. Further study of breeding behavior in captive populations would be useful for determining the relative importance of female mate choice and dominance in *B. borneoensis*.

Multiple paternity is common in turtles (Pearse and Avise 2001; Uller and Olsson 2008) and would be expected to increase the representation of the founders in captive breeding programs (Williams and Osentoski 2007). In our study, however, usually only one offspring per clutch was sampled and so we could not adequately test this possibility. Nevertheless, two males (23 and 41) were consistently identified as sires across clutches suggesting that if other males were breeding, their relative numbers were low.

Allelic richness in the first captive generation was slightly lower than in the founders for four of the five loci. Observed heterozygosity was similar between the two groups. A number of the founders have not contributed offspring to the first captive-bred generation. The high observed skew in reproductive success and resulting low effective population size (7 individuals) will result in the loss of genetic diversity and contribute to inbreeding in the captive

population over successive generations. High skew in reproductive output may also select for individuals that breed well in captivity (Frankham 2008). Adaptation to captive conditions can significantly reduce reproductive fitness upon reintroduction (Ford 2002; McPhee 2004; Araki et al. 2007; Fritts et al. 2007; Araki et al. 2009). Increasing the representation of the founders will be an important issue to resolve because avoiding inbreeding, maintaining evolutionary potential, and avoiding adaptation to captive conditions are some of the top concerns when breeding for repatriation (Fraser 2008). Based on these results, I recommend that 1) males and females be moved between zoos using group management techniques (e.g. Princée 1995, Wang 2004) to avoid inbreeding; and 2) mating between new pairs of individuals is encouraged to promote the highest level of genetic diversity in future generations. Artificial insemination may be necessary to increase the number of breeding males and females.

Captive breeding programs should manage individuals from unique populations separately and then reintroduce them and their offspring to their location of origin. This strategy assumes that populations are locally adapted and so returning them to their region of origin would increase the success of reintroduction and avoid possible outbreeding depression (Williams and Osentoski 2007). Unfortunately, we still do not have the ability to predict the risk of outbreeding depression for most taxa and there is disagreement on its potential importance for reintroduction efforts (Edmands 2007, Tallmon et al. 2004, Frankham et al. 2009). Williams and Osentoski (2007) suggests managing captive individuals separately if there is evidence for strong phylogenetic structure (e.g. reciprocal monophyly) between captive individuals. If the genetic structure of wild populations can be reconstructed using wild-caught individuals and

museum specimens, then the offspring from these reproductive units could then be returned to their region of origin.

I examined the data to determine if there were any phylogenetic patterns within the captive population that might reveal the origins of the founders and indicate if any came from highly differentiated populations in the wild. The mitochondrial haplotype network and neighbor-joining tree revealed a weak association with geography; however, there was no evidence of reciprocal monophyly or high genetic differentiation between haplotypes. Similarly, the neighbor-joining tree based on the proportion of shared alleles at microsatellite loci did not reveal any patterns consistent with geographic origin or mitochondrial haplotypes. A recent study compared genetic variation at the mitochondrial cytochrome b and control regions for female *B. borneoensis* and its congener *B. baska* on the east and west coasts of Peninsular Malaysia (Duli 2009). There was evidence for significant genetic structure between the east and west coasts for *B. baska* but not for *B. borneoensis* (Duli 2009). Nothing is known about dispersal patterns in *B. borneoensis.* Adult *B. borneoensis* may be capable of long distance dispersal along the coast or possibly between islands resulting in low population differentiation. When hatchlings leave the beach they can withstand sea water for up to two weeks (Dunson and Moll 1980) which means that they could be dispersed by ocean currents. Whether studies of wild populations across the entire range of *B. borneoensis* may reveal more genetic structure is a subject that needs further study. The lack of strong genetic differentiation between captive individuals suggests they can probably be managed as a single unit at this time.

Appendices

Appendix 1. Clutches of captive-born individuals with members of each clutch, hatch date and location of clutch when hatched. Bold numbers indicate deceased terrapins *=terrapins with samples included in this study

Clutch ID	Offspring Members	Hatch Date	Hatch Location
A	60	27-May-95	NY Bronx
	61*	27-May-95	NY Bronx
	62	30-May-95	NY Bronx
	63*	30-May-95	NY Bronx
B	66*	9-Jun-98	Fort Worth
	67	19-Jun-98	Fort Worth
	68	20-Jun-98	Fort Worth
	69	20-Jun-98	Fort Worth
	72	22-Jun-98	Fort Worth
	73	23-Jun-98	Fort Worth
C	80	1-Aug-00	San Antonio
	81*	2-Aug-00	San Antonio
	82	3-Aug-00	San Antonio
D	***84***	5-Aug-00	Fort Worth
	85	5-Aug-00	Fort Worth
	86	6-Aug-00	Fort Worth
E	87	1-Apr-01	Fort Worth
	88	1-Apr-01	Fort Worth
	89*	4-Apr-01	Fort Worth
	90	4-Apr-01	Fort Worth
	91	4-Apr-01	Fort Worth
F	92	23-Apr-01	Fort Worth
	93	24-Apr-01	Fort Worth
	94	25-Apr-01	Fort Worth
	95	26-Apr-01	Fort Worth
	96	28-Apr-01	Fort Worth
G	101	28-Aug-02	Toronto
	102	28-Aug-02	Toronto
	103*	29-Aug-02	Toronto
H	***105***	13-Sep-02	Fort Worth
	110	19-Sep-02	Fort Worth
	111	20-Sep-02	Fort Worth
I	148*	12-Aug-05	Fort Worth
	149	12-Aug-05	Fort Worth
	150*	12-Aug-05	Fort Worth
	151	12-Aug-05	Fort Worth

	152	12-Aug-05	Fort Worth
	154*	13-Aug-05	Fort Worth
	155	15-Aug-05	Fort Worth
J	157*	12-May-06	San Antonio
	158	12-May-06	San Antonio
	159*	12-May-06	San Antonio
	160*	12-May-06	San Antonio
	161*	12-May-06	San Antonio
K	162	28-Jun-06	San Antonio
	163*	28-Jun-06	San Antonio
	164	30-Jun-06	San Antonio
	165	2-Jul-06	San Antonio
	166	18-Jul-06	San Antonio
L	167*	2-Aug-06	Fort Worth
	168*	13-Aug-06	Fort Worth
M	205048	3-Sep-07	Fort Worth
	205049	3-Sep-07	Fort Worth
	205075	3-Sep-07	Fort Worth
	205076	3-Sep-07	Fort Worth

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ABSTRACT

GENETIC ANALYSIS OF THE CAPTIVE BREEDING PROGRAM FOR THE CRITICALLY ENDANGERED PAINTED TERRAPIN, *BATAGUR BORNEOENSIS*

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Captive breeding is an often suggested method for preventing the extinction of species. The painted terrapin (*Batagur borneoensis)* is a critically endangered turtle from Southeast Asia that is currently the focus of a captive breeding program at zoos across the USA and Canada. In this study, I used genetic markers to determine: 1) phylogenetic relatedness between wild-caught captive individuals (founders); 2) parentage within zoos; and 3) current levels of genetic diversity in the captive population. Our data did not identify genetic structure among the founders, indicating that they are not mixture of individuals from genetically distinct populations in the wild. The parentage analysis indicated that only a few founders are currently breeding and so breeding strategies need to be revised in order to promote a more equitable distribution of parentage across individuals in order to maintain the genetic diversity of the founders.