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Population Genetics and Conservation of the Philippine Crocodile

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Abstract

The endemic Philippine crocodile (*Crocodylus mindorensis*) is considered to be one of the most highly threatened crocodylians in the world. Historically known to occur throughout the Philippine archipelago, wild populations are now confined to small and isolated populations on the islands of Luzon and Mindanao. Reintroduction is seen as an important element in the recovery of this species. Successful captive breeding programmes initiated in the 1980's increased the number to hundreds of captive Philippine crocodiles, many of which are candidates for reintroduction to suitable habitats. Preliminary genetic studies based on mtDNA found *Crocodylus porosus*-*C. mindorensis* hybrids in the biggest captive population which raises concerns on species integrity and suitability of the captive population for the reintroduction programme. In addition, unresolved issues on the extent of genetic differentiation among extant populations hampered recovery plans for many years.

To resolve these issues, a total of 618 wild and captive Philippine crocodiles were genotyped at 11 microsatellite loci to investigate genetic diversity and population structure. In addition, information from an existing mtDNA study was combined with the results from a Bayesian assignment test based on microsatellite loci to find evidence of hybridisation. A high degree of genetic differentiation across all populations was observed ($F_{ST} = 0.29$). Genetic differentiation reflected geographic structuring, with the highest F_{ST} values recorded between populations from the northern Philippines (Luzon) and southern Philippines (Mindanao). Moderate levels of genetic diversity were seen in all captive and wild populations included in the sampling, except for one captive population in Abra.

A total of 92 hybrids were identified from two captive facilities. Three of the identified hybrids in this study were part of the group released into the wild during the first reintroduction programme in 2009. These three individuals did not exhibit obvious morphological anomalies and were thought to be pure *C. mindorensis*. The results of this study have important conservation implications and will influence the management of captive and wild populations of Philippine crocodiles and the design of future reintroductions.

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A big thank you goes to Caroline Bailey, Shannon Engberg, Gary Shore, Runhua Lei, Brandon Sitzmann, Susie, Lisa and volunteers of the genetics department at HDZ. Thanks for helping me in the laboratory work and teaching me the protocols of the lab. I truly appreciate all your help. I am grateful to Paula Hubbard, Luiza Prado, Adam Smith and John Tabora for making my stay at the HDZ fun and interesting.

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Preface

This study was part of a larger research project which examined the genetics of the Philippine crocodile (*Crocodylus mindorensis*). The project was a collaboration among the following institutions: Massey University, Omaha's Henry Doorly Zoo (HDZ), the Philippine Government's Department of Environment and Natural Resources (DENR), Palawan Wildlife Rescue and Conservation Centre (PWRCC), Mabuwaya Foundation, Silliman University and the University of Southern Mindanao (USM). The need to clarify the population genetics of the Philippine crocodile was one of the key priorities outlined in the 2005 recovery plan of the species. To address this issue, the collaboration was established to facilitate sample collection, permit processing, laboratory work, data analysis and publication of results. This study looked at the population structure and genetic variation of the Philippine crocodile as revealed by microsatellite DNA loci, while another project examined phylogeography using mitochondrial DNA markers (Tabora *et al.* 2010).

Sample Collection

Tissue and blood samples used in the study were collected or provided by the following people/institutions: Glenn Rebong (PWRCC); Rainier Manalo (Conservation International), Merlijn van Weerd, Jessie Guerrero, Bernard Tarun, Willem van de Ven (Mabuwaya Foundation); Andy Ross (Silliman University), John Tabora and Cayetano Pomares (USM); Gladys Porter Zoo; and Davao Crocodile Park. I collected 465 samples from PWRCC, which were included in the most recent CITES export permit with the help of Medel Silvosa, Glenn Rebong, Renato Cornel, Ernesto Conate, Amado Mulig, Salvador Guion, Roberto Manalang, Ferdinand Palioza, William Tabinas, Alberto Guinto and Ronnie Sumiller.

DNA Extraction and Microsatellite Genotyping

The laboratory technicians at the HDZ genetics department, John Tabora (USM) and I extracted DNA from all the samples used in this research. Microsatellite marker optimization and microsatellite genotyping were carried out by me, Shannon Engbert (HDZ) and Caroline Bailey (HDZ). The DNA extractions, polymerase chain reactions

(PCR) and microsatellite genotyping were all accomplished at the genetics laboratory at the Centre for Conservation and Research, Henry Doorly Zoo, Omaha, Nebraska, USA. Data analyses were performed at HDZ and Massey University.

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List of Abbreviations

AR	Allelic richness
CFI	Crocodile Farming Institute
CITES	Convention on the International Trade of Endangered Species
CSG	Crocodile Specialist Group
DAN	Nei's improved genetic distance
DENR	Department of Environment and Natural Resources
DNA	Deoxyribonucleic acid
HDZ	Henry Doorly Zoo
IAM	Infinite allele model
IUCN	International Union for the Conservation of Nature
JICA	Japan International Cooperation Agency
K	Genetic cluster
LD	linkage disequilibrium
MC	Markov chain
MNA	Mean number of alleles
mtDNA	Mitochondrial DNA
N	Number of samples
NM	Number of migrants per generation
PCR	Polymerase chain reaction
PH	Philippines
PWRCC	Palawan Wildlife Rescue and Conservation Centre
SMM	Stepwise mutation model
TPM	Two-phase mutation model
WCSP	Wildlife Conservation Society of the Philippine

CHAPTER I: INTRODUCTION

Overview

This chapter provides a comprehensive review of the ecology and conservation of the Philippine crocodile and the use of genetics in conservation. Section 1 begins by presenting the available literature relating to the distribution, biology and ecology of the Philippine crocodile (Section 1). This is followed by Section 2 which summarises past and present efforts to conserve the species. Section 3 sets the stage for molecular genetic analysis by reviewing the current literature on the use of molecular markers in conservation, particularly in crocodylians. A brief review of the aspects of conservation genetics examined in this study is also included in this section. Finally, the research objectives of this thesis are outlined at the end of the chapter.

Section 1 Biology and Ecology of the Philippine Crocodile

1.1 Nomenclature

The Philippine crocodile, *Crocodylus mindorensis* Schmidt, was first described in 1935 from four museum specimens that originated from Mindoro Island in the Philippines (1935). The status of the species remained uncertain for many years as disagreement between its classifications persisted. Wermuth (1953) and Wermuth & Mertens (1961) classified *C. mindorensis* as a subspecies of the New Guinea crocodile, *Crocodylus novaguinae*, only to refer to it again as a distinct species in 1977 (Wermuth & Mertens, 1977). Subsequent morphological (Hall, 1989), biochemical (Densmore, 1983) and molecular studies (Densmore & White, 1991) provided support for the distinctiveness of the two species. Today *C. mindorensis* is recognised as one of the 23 species which belong to the family Crocodylidae. It is also known as the Philippine freshwater crocodile or Mindoro crocodile.

Kingdom	Animalia
Phylum	Chordata
Subphylum	Vertebrata
Class	Reptilia Laurenti, 1768
Order	Crocodylia
Family	Crocodylidae
Genus	Crocodylus Laurenti, 1768
Species	Crocodylus mindorensis Schmidt, 1935

Figure 1. Taxonomic hierarchy for *Crocodylus mindorensis* (F. King & Burke, 1989)

1.2 General Features

The Philippine crocodile is a small freshwater crocodylian which averages two metres in length when adult. Adult males can grow up to 3.5 metres long and are usually larger than adult females. The dorsal side of the animal is a dull brown colour with transverse dark stripes or bands. The ventral side is white in colour (C. Banks, 2005).

Possibly the most useful morphological character for distinguishing *C. mindorensis* from other species is the presence of enlarged post-occipital scales. Ross & Alcala (1983) used the following morphological guidelines in order to differentiate between *C. mindorensis* and the saltwater crocodile, *Crocodylus porosus*:

	<i>C. mindorensis</i>	<i>C. porosus</i>
Post-occipital scales	4 to 6 (average = 5.6)	0 to 2 (average = 0.5)
Transverse ventral scale	22 to 25 (average = 23.9)	29 to 34 (average = 31.7)
rows		
Palatine-pterygoid suture	Nearly transverse - never bisecting pterygoid	Directed posteriorly - partially bisecting pterygoid

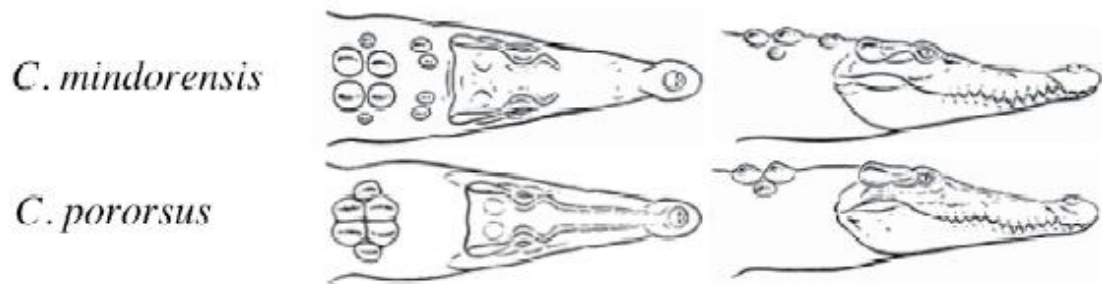


Figure 2. Dorsal and lateral views showing the presence of post-occipital scales in *C. mindorensis* and its absence in the *C. porosus* (from Schreuder, 2006).

C. mindorensis is differentiated from the New Guinea crocodile (*Crocodylus novaeguineae*) by several morphological features which include cervical scalation and the appearance of scales on the lateral sides of the body. *C. mindorensis* has prominent nuchomarginal rows (Hall, 1989) and the scales on the sides of the body are of equal size and arranged longitudinally in rows. *Crocodylus novaeguineae*, on the other hand, has reduced nuchomarginal rows and the lateral scales are of unequal size (Brazaitis, 1974; Hall, 1989).

1.3 Habitat and Distribution

The two species of crocodiles, found in the Philippines, are the estuarine or saltwater crocodile (*Crocodylus porosus*) and the smaller Philippine crocodile (*Crocodylus mindorensis*). *Crocodylus porosus* is widely distributed across the Indo-Pacific, from South Western India to Papua New Guinea and Australia. The Philippine crocodile is restricted to the Philippines (C. Ross & Alcala, 1983). Although the two crocodiles have different habitat preferences (coastlines and estuaries for *C. porosus* and inland lakes and creeks for *C. mindorensis*), the two species occur sympatrically in some areas (Van der Ploeg, Van Weerd, & Telan, 2007; Van Weerd, et al., 2006). Philippine crocodiles have been found in different habitats, including fast-flowing streams and rivers in mountains, small lowland lakes, marshes, torpid creeks and coastlines (Van Weerd, et al., 2006).

Historically known to be distributed widely across the Philippine archipelago, extant wild populations of Philippine crocodiles (based on the most recent surveys) now occur only in the islands of Luzon (specifically north eastern Luzon and Cordillera), Dalupiri (extreme northern Philippines) and Mindanao (Oliveros, Telan, & Van Weerd,

2006; Ortega, 1998; Pontillas, 2000; Van Weerd & Van der Ploeg, 2003). Based on reports, small isolated populations or individuals of *C. mindorensis* might still occur on the Ilog River in Negros, Jomalig Island near Polillo Island and Busuanga and Dipuyai Rivers in Busuanga Island (C. Banks, 2005). In 1994, Ortega *et al.* (1994) presented a report confirming the presence of *C. mindorensis* in Busuanga. A more recent survey by Pontillas (2000), however, failed to confirm any presence of *C. mindorensis* in Busuanga. Reyes (in Van Weerd & Van der Ploeg, 2003) confirmed the presence of crocodile tracks in Jomalig Island, but could not confirm whether they belonged to *C. mindorensis*. Clearly, there is a need for more extensive field surveys in order to verify reported sightings and to update the present distribution of Philippine crocodiles in the country.

Table 1 summarises the historic and present distribution of *C. mindorensis*.

Table 1. Summary of historic and present distribution of *Crocodylus mindorensis* in the Philippines

ISLAND	SPECIFIC LOCALITY	LAST SIGHTING	SOURCE
<i>Historic Distribution</i>			
Luzon	Camarines Manila Laguna de Bay	Before 1981: presumed locally extinct	Ross (1982) Ross & Alcala (1983)
Busuanga	Dimaniang River Busuanga River Dipuyai	1993	Ross & Alcala (1983) Ortega (1998) Banks (2005) Pontillas (2000) Manalo (pers. Comm.)
Masbate	Mandaon	1950's: presumed locally extinct	Ross & Alcala (1983)
Mindoro Island	Mindoro Oriental (Naujan Lake, Caituran River)	1993	Schmidt (1935), Ross (1982), Ortega (1998) Pontillas (2000)
Samar			Ross (1982) Ross & Alcala (1983)
Negros Island	Negros Oriental (Pagatban River and Sta. Catalina) Negros Occidental (Tablas area)	1990's	Ross (1982) Ross & Alcala (1983) Manalo (pers. Comm.)

Mindanao island	South and North Cotabato (Liguasan Marsh)	2008	Ross (1982), Ross & Alcala (1983), Rebong & Sumiller (2002)
	Misamis Occidental	1992	
	Lanao Del Norte		
	Lanao del Sur		
	Davao del Norte (Tagum and Nabunturan)	1981	
	Davao del Sur (Malita)	Before 1981	
	North Cotabato (Midsayap River)		
	Surigao del Norte (Placer)		
	Zamboanga City (Calarian Lake)	1981	
	Zamboanga del Sur (Pagadian City)	Before 1981	
Sulu Archipelago (Jolo)			
<i>Present Distribution</i>			
Dalupiri Island	Caucauyan Creek	2005	Oliveros <i>et al.</i> (2006)
Mainland Luzon	Abra (Binungan River)	2004	Manalo (pers. comm.) Van Weerd <i>et al.</i> (2006); Van Weerd (pers. comm.)
	Isabela (San Mariano – Dungsog Lake, Dunoy lake, Catallangan River, Disulap River, Dinang Creek, Diamallig Creek)	2008	
	Isabela (Palanan and Maconacon – Po River, Dicatian Lake, Dibukarot Creek)	2006	
Mindanao	Bukidnon (Pulangui River)	2000	Pontillas (2000); Manalo (pers. comm.) Van der Ploeg <i>et al.</i> (2007)
	North Cotabato (Liguasan Marsh)	2007	



Figure 3. Historical and current distribution of Philippine crocodiles. Historical distribution is inferred from confirmed sightings from the 1950's to the 1990's. Current distribution is based on reported sightings from 2000 to present.

1.4 Abundance

In 1982, Ross (1982) estimated the wild Philippine crocodile population at 1,000 individuals. A more recent estimate in 1998 pegged the wild population at 100 non-hatchling individuals (J. Ross, 1998) and this estimate formed the basis of the IUCN's 'Red List Status Category' for the Philippine Crocodile (Hilton-Taylor, 2000). These estimates alerted the conservation community to the critical standing of the species, but the accuracy of these numbers was doubtful, because they were not based on actual field surveys (Van Weerd & Van der Ploeg, 2003, 2004). These estimates were also made prior to the discovery of a remnant Philippine crocodile population in the remote areas of San Mariano in Isabela province in 1999 (Van Weerd & Van der Ploeg, 2004)

It is difficult to establish a dependable estimate of the wild population as of the present time. Published data on field surveys of *C. mindorensis* are few. The only wild population, which had been surveyed with regularity since 1999, is the small population of crocodiles in the foothills of the Northern Sierra Madre Mountains, in San Mariano, Isabela. The census size of this small population in 2006 was just 25 Philippine crocodiles (Van Weerd, et al., 2006). The current insurgency between the Muslim rebels and the Philippine government hampers surveys in suspected Philippine crocodile strongholds in Mindanao due to security reasons.

1.5 Ecology, Reproductive Biology, Survival and Growth Rate

The limited data on the ecology and biology of the Philippine crocodile in the wild comes primarily from the small population located on the Northern Sierra Madre in the province of Isabela, north eastern Philippines. Since its rediscovery in 1999, quarterly monitoring surveys have been conducted and eight different localities have been identified as having *C. mindorensis* (Van Weerd, et al., 2006). Studies on captive crocodiles held at Silliman University and the Palawan Wildlife Rescue and Conservation Centre (PWRCC, formerly the Crocodile Farming Institute) provide insights on the growth, survival and reproduction of the species in captivity.

Diet

Crocodylians are known as opportunistic feeders. In one study, the bulk of juvenile Philippine crocodile's diet in the wild consisted of snails (57%), small fish, dragonflies and birds. Adult crocodiles hunted larger fish (Schreuder, 2006). In captivity, a wide variety of prey items is offered and this includes marine and freshwater fish, pork, beef, chicken meat and offal. Juveniles and hatchlings are offered smaller food items such as shrimp, mince and white mice (G. Rebong & B. Tarun, pers. comm.).

Movement Patterns

The first radio telemetry study of Philippine crocodiles was completed in 2005. This study followed four female crocodiles (one adult and three juveniles) in the Catallangan River-Dunoy Lake Area in the municipality of San Mariano. Linear home ranges for the adult and one juvenile were 4.3 km and 2.9 km, respectively. The study showed seasonal movement patterns, with the crocodiles moving to Dunoy Lake at the start of the wet season and then back to the river, during the dry season (De Jonge, 2006; Van Weerd, et al., 2006). Water level and flow velocity of the river appeared to affect crocodile movement. It has been suggested that the Catallangan River might not be a satisfactory habitat for crocodiles during the rainy season due to strong currents. In a similar way, Dunoy Lake during the dry season may not be a preferred habitat for crocodiles, since the lake water level falls off to 0.5m and the lake size diminishes to half a hectare (Van Weerd, et al., 2006). Reproductive behaviour and food availability could also be reasons for the seasonal movement of crocodiles but more data are needed in order to test these hypotheses (Schreuder, 2006).

A breeding pair was monitored by Tubbs (2006) for four months along the Disulap River in San Mariano, Isabela in order to determine home range, habitat use and habitat preferences. This study revealed a core area approximately 2 km long consisting of limestone cliffs, underwater caves and beaches with vegetation. The study also showed that the water level of the river affected the crocodile's presence in this core area; heavy rainfall leading to an increase in the water level drove the crocodiles out of the core area. Radio telemetry data revealed the maximum daily movement to be 4.3 km/day for the male and 4 km/day for the female. The female moved shorter distances but more regularly, compared

to the male which moved longer distances but less frequently. Overall, the crocodiles favoured habitats characterised by average flow velocity, minimum depth and maximum width (Tubbs, 2006)

Territorial Behaviour

In a behavioural study undertaken by Schreuder (2006) on wild *C. mindorensis* in Dunoy Lake, the average distance between crocodiles regardless of age was found to be 20 metres. The least average distance could be found between hatchlings (14.1m +-12.0m) and the greatest average distance was between hatchlings and adults (27.6 +- 8.7m). There was a preference area around the lake for the different age groups. Juveniles and hatchlings preferred areas with plenty of lake edge vegetation, whereas the adults favoured open water and areas with large logs where they could bask. Schreuder (2006) observed one instance of fighting between two juveniles, during the study. This could have been an example of territoriality, although it was difficult to determine if this was the actual cause of the fight (Schreuder, 2006).

Territorial behaviour might not be important for breeding pairs of wild Philippine crocodile. Tubbs (2006) found that the core areas of a breeding pair of Philippine crocodiles, in the Disulap River, overlapped by as much as 90%. Territoriality and dominance, however, might be more pronounced in captivity, where space is extremely limited. For example, captive *C. mindorensis* breeding pairs were separated during the non-breeding season, in order to prevent fighting. Fighting and aggression have also been found to be minimised by the introduction of breeding females for pairing only in late February until early March, since pairings later than this period often led to mortality (Sumiller, 2000).

Breeding Behaviour

Information on the breeding behaviour of *C. mindorensis* comes mostly from observations of crocodiles in captivity. Female Philippine crocodiles reach breeding age when they are approximately 1.3 metres long, or around 10 years of age. Males mature later, at 15 years of age when they are about 2.1 m in length (A. Alcala, Ross, & Alcala, 1987). In Silliman University in Negros, mating and courtship occurred in the water during the dry season (December to May), with egg laying occurring between April and August.

Egg-laying at PWRCC in Palawan (formerly CFI) peaked in May to June, which is the beginning of the rainy season (A. Alcala, et al., 1987).

Females in captivity are either hole nesters or mound nesters. Breeding females may dig a hole in the ground or make a nest mound of dirt and grass into which they deposit 7-25 hard-shelled eggs (A. Alcala, et al., 1987). PWRCC reported an incubation period of 79-89 days and a mean clutch size of 25 eggs (Sibal, Sarsagat, & Satake, 1992). The lone breeding female at Silliman University had an average clutch size of 15.7 and an incubation period of 77-85 days. This female breeder, in Silliman, laid multiple clutches (up to three a year) and re-used the same mound for nesting. Parental care was observed, with the female aggressively guarding her nest, until three months after the hatching date (A. Alcala, et al., 1987). This display of parental care has yet to be observed for wild Philippine crocodiles.

Monitoring and observation of wild Philippine crocodile nests in the Northern Sierra Madre revealed that egg-laying occurs from the dry season to the onset of the rainy season (April to June), with hatchlings produced from late June to August. The average clutch size ranged from 23 to 26, which is comparable to clutches laid in captivity. All the nests found were mound nests, but an attempt at a hole (or a combination of hole and mound) nesting was also observed (Van Weerd, et al., 2006).

Hatching Success, Survival and Growth Rate

There is limited information on the rate of hatching, survival and growth of wild Philippine crocodiles. Table 2 summarises the survival of hatchlings from nests found in the Northern Sierra Madre. The mean hatching rate for five nests found in the Northern Sierra Madre (where the total number of eggs and hatchlings were reliably recorded) was 69.4% (Table 2) (Van de Ven, 2008). This is higher compared to the average hatching rate of 40.05% for artificially incubated *C. mindorensis* eggs at PWRCC, from 1988-1997 (Table 3) (Sumiller, 2000).

Hatchling mortality is high until six months of age, after which the survival rate increases (Webb, Manolis, & Whitehead, 1987). The survival rate of captive-reared hatchlings at PWRCC after six months was 60.67% (Sumiller, 2000). The survival rate in captivity is affected by a multitude of factors including stress, genetics, temperature at

incubation and management procedures (Sumiller, 2000). For one group of Philippine crocodile hatchlings (monitored in the wild), the survival rate after one year was 48.8% (Van de Ven, 2008). It has been assumed that the survival rate for wild hatchlings is lower due to predation and weather-related mortality (e.g. floods). Observed causes of mortality for *C. mindorensis* hatchlings include predation by a rufous night heron (*Nycticorax caledonicus*) and ant attacks (Van Weerd, et al., 2006).

Table 2. Survival of Philippine crocodiles in the wild (modified from Van de Ven, 2008)

Location	Hatching Date	No Eggs	No. hatchlings	Hatchling Rate (%)	Hatchling survival
Disulap	1-8-2000	25	8	32.0	One observed after one year
Dunoy	1-8-2002	Unknown	12		Nine observed after one year
Dunoy	1-8-2004	Unknown	2		Two observed after one year
Disulap	1-7-2005	23	19	82.6	10 killed by ants: nine collected for head-start
Dunoy	1-8-2005	Unknown	3		Two observed after one year
Dinang	1-7-2005	16	0		Nest accidentally destroyed by farmer
Dunoy	1-6-2006	Unknown	22		Two killed by rufous night heron: 17 collected for head-start; three left in the lake & observed after one year
Disulap	1-7-2006	26	23	88.5	18 collected for head-start: five died immediately after hatching
Dinang	1-7-2006	16	11	68.8	Three observed after one year
Dinang	1-6-2007	20	0		All eggs were stolen
Dinang	1-7-2007	16	12	75.0	12 collected for head-start
Disulap	1-7-2007	unknown	0		All eggs predated by rat & monitor lizard
		>142	112	Mean: 69.4	20 observed after one year: 56 collected for head-start; 17 died; nine unknown

Table 3. Annual captive breeding result of *C. mindorensis* at PWRCC from 1988-1997 (Source: Sumiller, 2000)

Species & Year	Female paired	Female Laid eggs	Breeding Rate	Clutch size	Hatchling per breeder	No. Of eggs	Fertility		Hatching		Survival at 6 mos.	
							No.	%	No.	%	No	%
1988	1	1	100	21	0	21	1	4.76	0	0	0	0
1989	7	5	71.43	18	1	92	25	26.20	7	10.32	5	13.33
1990	10	4	40.00	24	1	94	20	30.99	14	30.0	13	47.92
1991	19	12	63.16	29	7	352	241	63.82	136	50.70	114	73.21
1992	15	13	86.67	38	14	491	369	75.00	209	61.00	180	88.66
1993	23	12	52.17	28	4	331	246	68.46	98	37.72	88	78.99
1994	24	20	83.33	22	6	446	320	69.56	155	48.42	123	69.97
1995	22	16	72.73	25	7	397	320	74.04	160	46.74	110	64.99
1996	22	20	90.91	29	12	571	451	75.46	253	54.51	195	82.94
1997	26	21	80.77	27	10	573	416	70.58	248	61.06	218	86.72
Mean			74.12	26	6			55.89		40.05		60.67

Growth rates have been calculated for captive-bred *C. mindorensis* at PWRCC (Figure 4). Growth in crocodiles is measured in terms of weight and total length. Growth rate, especially in captivity, is affected by factors such as stocking density, nutrition, husbandry procedures, temperature, genetics and clutch to clutch variation. It is difficult to make conclusive statements on the growth of crocodiles in captivity without considering each factor (Mayer & Peucker, 1997).

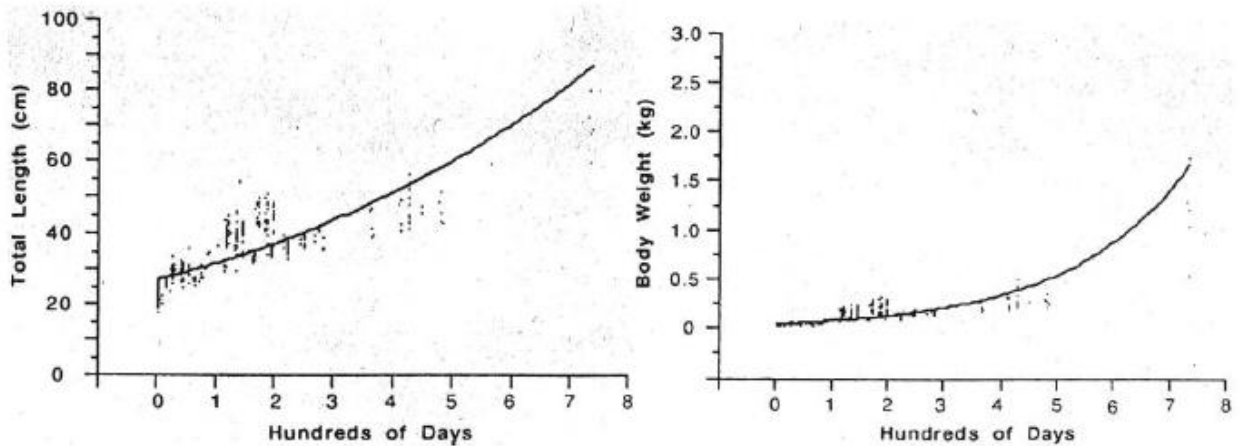


Figure.

Figure 4. Growth of farm-bred *C. mindorensis* at CFI/PWRCC (CFI, 1994)

The only data on the growth rate of wild Philippine crocodiles comes from the study of Van de Ven (2008) wherein hatchlings were gathered from wild nests as part of a head-start programme and then re-introduced back to the wild after a year in captivity. These animals were measured and weighed (prior to release) and then recaptured and measured again four to five months after release. Growth rates (measured as total length) estimated from these data differed between release sites, with the highest growth rate (0.1cm/day) exhibited by crocodiles released at a fish pond and also given supplementary feeding. The growth rates of crocodiles released at a large lake and a medium-sized artificial lake showed half the growth rate (0.4-0.5cm/day) compared to those in the fish pond. The animals released at the lakes were not supplemented with food. Van de Ven's (2008) study suggests that food availability affected the growth rate of juveniles, although external factors, such as the availability of natural prey items (at the release site) and stress, could also have played a part.

Section 2 Conservation of the Philippine Crocodile

2.1 Conservation Status

The Philippine Crocodile is listed as critically endangered on the IUCN Red List (IUCN, 2008). Trade is strictly regulated and only allowed in exceptional circumstances since the species is included in the Appendix I of the ‘Convention on International Trade in Endangered Species (CITES)’. The ‘Crocodile Specialist Group’ (CSG) considers the Philippine crocodile as the second most endangered crocodile in the world and has placed the recovery of the wild population as the highest priority for the species (J. Ross, 1998).

2.2 Threats to the Species

Direct threats

Direct threats to the Philippine crocodile include purposive killing as a result of fear and ignorance and the hunting of crocodiles for food and hides and also for the pet trade (C. Banks, 2000). Crocodiles are generally viewed by Filipinos either passively or negatively. Many consider them a danger to both people and livestock. A negative connotation is attached to the Tagalog word for crocodile - *buwaya*. Corrupt officials or crooks are termed *buwaya* by Filipinos and this image has not in any way helped to uplift the plight of the species (Banks, 2005).

Hunting for crocodile hides throughout the archipelago during the 1970’s is believed to have had a disastrous effect on Philippine crocodile wild populations (Oudejans, 2002; C. Ross & Alcala, 1983; Van der Ploeg & Van Weerd, 2005). In Northern Luzon, direct killing and hunting were the main reasons for the small population size (Van Weerd, 2002). International trade for *C. mindorensis* was banned in 1975, with the listing of *C. mindorensis* as an Appendix I species. However, the domestic trade of crocodiles and its by-products still continued (Wildlife Conservation Society of the Philippines (WCSP)).

Indirect Threats

Indirect threats to wild Philippine crocodiles include the use of illegal fishing methods (dynamite and cyanide fishing), the pollution of rivers and streams, accidental catching and the loss of freshwater habitat due to agricultural encroachment (C. Banks, 2005; Van der Ploeg & Van Weerd, 2005; Van Weerd, 2002). Probably the most important

threat to *C. mindorensis* at the present time is habitat loss, as more wetlands are converted for agricultural purposes (Banks, 2005). This has resulted in the loss of basking and breeding sites and it has also increased human-crocodile interactions, which could lead to more crocodiles being killed (Miranda, Van Weerd, & Van der Ploeg, 2004).

2.3 Environmental legislation for the protection of the species

Two fairly recent national legislations are noteworthy. These are the Republic Act (R.A.) No. 8485 and R.A. No. 9147. The Republic Act 8485 (known as the Animal Welfare Act of 1998) was put into effect in order to advance an animal welfare system in the Philippines. Section 6 of this legislation specifically states that the killing of crocodiles should be undertaken humanely. However, crocodiles are grouped with farm animals, such as swine, cattle and poultry, thus making the provisions of this Act impractical for wild crocodiles (Van der Ploeg & Van Weerd, 2004). The Act also states that: “It shall be the duty of every person to protect the natural habitat of wildlife. The destruction of said habitat shall be considered a form of cruelty to animals and its preservation is a way of protecting the animals.”

The Republic Act 9147 (or the Wildlife Resources Conservation and Protection Act of 2001) is more specific in relation to the conservation of Philippine wildlife. RA 9147 states the following objectives: i) to conserve and protect wildlife species and their habitat; ii) to regulate the collection and trade of wildlife; iii) to pursue the Philippine commitment to international conventions, and iv) to initiate or support scientific studies on the conservation of biological diversity. Crocodiles fall under the jurisdiction of the Department of Environment and Natural Resources (DENR), whilst the protection of aquatic resources and critical habitats falls under the Department of Agriculture (DA). Unlawful acts against wildlife, as outlined in the Act include killing or inflicting injury on wildlife species and the encroachment into critical habitats, which would result in adverse effects on the area. Hunting, trading, transport and the re-introduction of wildlife without permits and the destruction of nests are also considered illegal.

The following is a list of older national policies, which give a certain degree of protection to the Philippine crocodile and its habitats:

1. Presidential Decree (P.D.) 705, known as the Revised Forestry Code of the Philippines 1975
2. P.D. 1067, known as The Water Code of the Philippines of 1976
3. P.D. 1152, known as the Philippine Environment Code of 1977
4. Presidential proclamation No. 2146, on Environmental Critical Areas and Projects of 1981
5. R.A. 7586, known as the National Integrated Protected Areas System (NIPAS) Act of 1992
6. R.A. 8550, known as the Philippine Fisheries Code of 1998

2.4 Philippine Crocodile National Recovery Plan

The DENR Special Order 2000-231 created the ‘Philippine Crocodile National Recovery Team’ (PCNRT), which consisted of government officials, professionals from the academe and local and international crocodile experts. This team headed the difficult task of halting a further decline in wild populations of *C. mindorensis* and to bring the species back from the brink of extinction. The PCNRT was crucial in the review and publication of the 1st Philippine crocodile recovery plan in 2000, and also a revised edition in 2005 (Gozun, 2005 in Banks, 2005).

The team’s recovery plan outlined nine specific objectives which focused on: protecting wild populations and their habitat; basic ecological and genetic research; captive management; advocacy; obtaining funding sources; and reviewing relevant conservation policies (Banks, 2005). Given the precarious state of the wild crocodile population, the recovery team aimed to develop a Philippine crocodile release and re-stocking programme. This plan was hampered, however, by the unresolved pedigree issues of the captive stock and also uncertainties, relating to the extent of genetic isolation of the surviving *C. mindorensis* populations. The recovery team recognised the need to clarify the population genetics of *C. mindorensis* and it considered genetic research as a high priority project (Banks, 2005).

2.5 Ex-situ Conservation Approaches

Silliman University

The first captive breeding facility, for *C. mindorensis*, was established at the Silliman University Environmental Centre (SUEC) in Dumaguete City, Negros Oriental, in 1980. With funding from the World Wildlife Fund (WWF) and the Smithsonian Institution, this facility was established in order to breed *C. mindorensis* in captivity and rear the offspring for eventual release into suitable protected areas (E. Alcala, 1997; C. Banks, 2005; Groomsbridge, 1987). The centre initially started its breeding programme with three adult female crocodiles and one male, although breeding only came from one female (from the Pagatban River in Southern Negros Oriental) and the male crocodile. The lone breeding female was approximately a year old when caught in the Pagatban River and it was donated by Prof. Timoteo Oracion to the facility in 1972. The male crocodile was donated by a Zamboanga City resident in 1980 and it was believed to be 15 years old at that time (Malayang, 2007). Both crocodiles are quite old but they are still alive at the Silliman University at the time of this writing (Ross, pers. comm.).

The pair at Silliman bred continuously from 1981 to 1994, producing 354 eggs, from which 114 were successfully hatched. In 2007, twenty-seven of these crocodiles still remained in the breeding facility, whilst the remainder had been dispersed to other captive facilities, in the Philippines and overseas. Important behavioural data have been acquired by observing the crocodiles in this facility. Parental care, which has yet to be recorded for wild *C. mindorensis*, was first recorded at Silliman University (Malayang, 2007).

Palawan Wildlife Rescue and Conservation Centre (PWRCC)

The Palawan Wildlife Rescue and Conservation Centre (PWRCC), formerly the Crocodile Farming Institute (CFI), was established on 20 August 1987, with the following objectives:

1. To conserve the two species of crocodiles in the Philippines, *C. porosus* and *C. mindorensis*
2. To promote local socio-economic well-being through the development of a crocodile farming technology

The establishment of PWRCC was made possible through a joint partnership between the DENR and the Japan International Cooperation Agency (JICA). JICA provided technical and financial support to the project, from 1987-1994, after which the management was entirely transferred to the DENR (C. Banks, 2005; Sumiller, 2000). At the time of this writing, PWRCC is managed by the Natural Resources Development Corporation (NRDC), which is the commercial arm of the DENR. NRDC ensures that PWRCC is kept running by the generation of profits from *C. porosus* farming and gate receipts from the park, whilst the Protected Areas and Wildlife Bureau (PAWB) of the DENR is in charge with the captive breeding of *C. mindorensis* (C. Banks, 2005, 2006).

From 1987 to 1994, PWRCC acquired a total of 235 *C. mindorensis* individuals. These animals formed the foundation stock at the facility. Eleven of the foundation animals came from the wild, whilst the remainder came from private collections (Ortega, 1998). The IUCN's Crocodile Specialist Group (CSG) and the DENR approved the acquisition of animals from the wild because it was considered improbable at that time that crocodiles would be adequately protected and conserved in the wild. Captive breeding was seen as the best approach to the conservation of *C. mindorensis* (C. Banks, 2005; Groomsbridge, 1987; Messel, King, Webb, & Ross, 1992).

Captive breeding of Philippine crocodiles at PWRCC were successful and the first captive-bred hatchlings were recorded in 1989. From 1989 to 1997, a total of 3,368 eggs were produced, from which 1280 successfully hatched (Sumiller, 2000). A decision was made to discontinue breeding, in 2001, due to limited budget and inadequate facilities to house the animals. In addition, there were uncertainties relating to the pedigree of some Philippine crocodiles (Banks, 2005). In 2002, there were 1,276 live Philippine crocodiles at PWRCC, 87% of which were bred in captivity (Banks, 2005). By March 2009, there were only 574 *C. mindorensis* left at PWRCC, based on records. Hundreds of animals were transferred to other facilities, such as Zoobic safari, whilst many others died as a result of disease (Rebong, pers. comm.). A full inventory of the remaining *C. mindorensis* at PWRCC is warranted.

Ex-situ Captive Programmes Outside the Philippines

The DENR currently has memorandum of agreements, (MOA) for Philippine crocodile conservation, with several zoos in North America, Australia and Europe. All crocodiles outside of the Philippines (and under these MOAs) are considered the property of the Philippine government. Transfer of crocodiles from these institutions to other zoos requires prior approval from the DENR (Banks, 2005).

The Gladys Porter Zoo (GPZ) in Brownsville, Texas, USA initiated the first conservation breeding agreement with the DENR, in 1988, with the transfer of a female crocodile from Silliman to pair with a male that was already in the facility. The agreement's objective was to establish a genetically diverse population of *C. mindorensis* in North American zoos. Since 1989, GPZ has entered into breeding loan agreements with other North American zoos under the stipulations stated on the MOA with the DENR. As of 2005, GPZ has transferred Philippine crocodiles to the following institutions (Banks, 2005):

- Pittsburgh Zoo and Aquarium, Philadelphia
- The Cullen Vivarium, Wisconsin
- Alligator Adventure, South Carolina
- St. Augustine Alligator Farm, Florida
- Omaha's Henry Doorly Zoo, Nebraska

Two institutions in Australia have a pair of *C. mindorensis* each: Melbourne Zoo and Crocodylus Park in Darwin. Melbourne Zoo has been active in the *in-situ* conservation programmes for the Philippine crocodile since signing a MOA with the DENR and Silliman University in 1993 (Banks, 2005). Melbourne Zoo has also been involved in the publication of the Philippine Recovery Plan. In addition, there are currently a total of 15 *C. mindorensis*, held in six zoological institutions, across Europe as of 2006 (Banks, pers. comm.).

C. mindorensis in Europe are held at the following institutions:

- Danish Crocodile Zoo, Denmark (five crocodiles)
- London Zoo, England (one pair)
- Chester Zoo, England (one pair)

- Zurich Zoo, Switzerland (one pair)
- Cologne Zoo, Germany (one pair)
- Bergen Aquarium, Norway (one pair)

2.6. In-situ Conservation Programmes

Mabuwaya Foundation

The Mabuwaya Foundation is the only non-governmental organisation (NGO) in the Philippines which is devoted to the conservation of the Philippine crocodile. The Mabuwaya Foundation began through conservation initiatives in the Northern Sierra Madre Natural Park Conservation Programme (NSMNP-CP), which was then implemented by Plan International. In 1999, a fisherman from the Municipality of San Mariano, in Isabela province, accidentally caught a *C. mindorensis* hatchling in his fishing net. He brought this to the attention of NSMNP-CP biologists who conducted surveys around the area. These surveys led to the identification of three crocodile breeding sites: Disulap River, Dunoy Lake and Dinang Creek, which are all located in the Municipality of San Mariano (Miranda, et al., 2004).

When the NSMNP project ended in 2002, crocodile conservation was continued by the Crocodile Rehabilitation, Observance and Conservation (CROC) project. In 2003, CROC became formally recognised as an NGO - the Mabuwaya Foundation (Van Weerd & Van der Ploeg, 2004). Mabuwaya is a shortened version of the word ‘mabuhay’, which means ‘long live’ in Filipino and, ‘buwaya’, the Filipino word for crocodiles. This foundation continues the implementation of the CROC project, which is currently the only in-situ conservation programme for the species (Miranda, et al., 2004; Van der Ploeg, Cureg, & Van Weerd, 2008; Van Weerd, 2005). Apart from mobilising communities to take action to protect the crocodiles, the Mabuwaya Foundation, in partnership with the Cagayan Valley Programme on Environment and Development (CVPED) and Dutch and Filipino students, is implementing various research projects that are slowly adding to our knowledge relating to the Philippine crocodile’s ecology, behaviour and community attitudes towards the crocodiles (C. Banks, 2005; Van Weerd, 2005).

Community-based Initiatives

The Mabuwaya Foundation works closely with local and regional stakeholders including the local government unit (LGU) of San Mariano, the DENR and communities in and around the Philippine crocodile habitat (Van der Ploeg & Van Weerd, 2008). Planning and development of conservation action plans are made through a participatory approach, which is a decentralised and more community-orientated form of environmental management (Kapoor, 2001). In 2002, a Philippine crocodile workshop, held in San Mariano, brought together various stakeholders with the aim of developing a long-term strategic plan for the conservation of the Philippine crocodiles in the Northern Sierra Madre. The plan's main objective was to address the three main threats which faced the crocodiles in San Mariano: a) killing crocodiles for food, play or fear; b) illegal fishing methods; and c) loss of breeding and basking sites due to agricultural expansion into the crocodile's habitat. This conservation strategy, developed during the workshop, focused on the establishment of crocodile sanctuaries with the help and consent of the local people (Miranda, et al., 2004).

Philippine Crocodile Sanctuaries

The Municipality of San Mariano, through Ordinance No. 01-17, declared the upper 10 km of the Disulap River as the first crocodile sanctuary in the Philippines on September 7, 2001 (Miranda, et al., 2004; Van Weerd & General, 2003). The process leading to the establishment of the sanctuary had not been easy since various meetings and public consultations were necessary to ensure that all elements of the sanctuary were agreed upon by all stakeholders. One particular detail, which resulted in much bargaining and debate, was the establishment of a buffer zone. Local people strongly objected to the proposed 20-m buffer zone and therefore the proponents had to agree to a 10-m buffer zone, in order to maintain community support. This buffer zone is a point of interest, since P.D. 1067 (also known as the Water Code of the Philippines) states that "a 20 metre strip along all bodies of water should be subjected to the easement of public use." Although a 20-metre buffer zone is the legal choice under the law, the proponents had to settle for 10 metres in order to maintain community support (Miranda *et al.*, 2004).

Two other municipal orders, in favour of Philippine crocodile conservation, were approved by the Municipal Council of San Mariano. These are Municipal Order No. 1999-025 which prohibited the collection and killing of crocodiles in the municipality, and Municipal Ordinance No. 2000-02 which designated the Philippine crocodile as the flagship species of the community (Miranda *et al.*, 2004). Therefore, from being relatively unknown or regarded with fear, the Philippine crocodile was now an icon of which the community could be proud.

The CROC project began the first steps towards establishing a 2nd Crocodile Sanctuary in Dinang Creek after the establishment of the Disulap River Sanctuary. Dinang Creek was known to host the largest population of Philippine crocodiles in Luzon (Van der Ploeg & Van Weerd, 2005). Funding from the Chicago Zoological society allowed the CROC team to initiate activities, such as land surveys, in order to help farmers apply for land titles; provision of water pumps and a safe area for bathing; information and education campaign materials; and training and equipment gear for the local protection group. All these activities were initiated in order to help gain the participation and consent of the local people for the establishment of the crocodile sanctuary. Despite these efforts, which lasted for a few years, little benefit has been seen regarding crocodile conservation in Dinang Creek.

The complexity behind the establishment of a sanctuary in Dinang Creek stems from a host of issues, including ancestral domain claims (many local people belong to the Kalinga tribe), and a history of oppression and land-grabbing. The issue has become even more complicated by the involvement of the left-wing activists, the New People's Army (NPA), which spread the idea that the establishment of a crocodile sanctuary would eventually take land rights away from the indigenous Kalinga people (Van der Ploeg & Van Weerd, 2006). Although it took many years, Dinang Creek was eventually proclaimed as a Philippine crocodile sanctuary, in 2005, through an ordinance passed by the barangay council of Cadsalan (Van der Ploeg & Van Weerd, 2006). A group of trained local people, known as the Bantay Sanktuwaryo, monitor the sanctuaries and they ensure that the ordinances are observed (Van der Ploeg & Van Weerd, 2006).

Bantay Sanktuwaryo (Sanctuary Guards)

The Bantay Sanktuwaryo is a group of trained locals deputised by the barangay or municipality to ensure that ordinances in and around the sanctuary are observed. This group generally consists of farmers and fishermen who live in the three main crocodile localities in San Mariano. The group is involved in the quarterly monitoring surveys and they submit a simple report to the barangay captain and the Mabuwaya Foundation every month (Van Weerd, 2005).

The effectiveness of protected areas has been found to be strongly linked to the density of guards which patrol the area (Bruner, Gullison, Rice, & da Fonseca, 2001). Although there were only 12 members of the Bantay Sanktuwaryo patrolling the crocodile sanctuaries (as of 2008), these members have been crucial in the discovery of nests and the reporting of illegal activities in their areas (Van der Ploeg & Van Weerd, 2008). Members of the group receive a small monthly incentive for their work from the local government of San Mariano (Van Weerd, 2005).

Education and Public Awareness Campaigns

Shortly after the re-discovery of a remnant population of Philippine crocodile, in the Northern Sierra Madre, in 1999, an information and education (IEC) campaign was initiated by the NSMNP conservation project in San Mariano. This public awareness campaign was continued by the Mabuwaya Foundation/ CROC project after the phase-out of the NSMNP. They built the campaign around the concept of the Philippine crocodile as a source of pride for the community (Van der Ploeg, et al., 2008; Van der Ploeg & Van Weerd, 2005). The IEC campaign used three strategies to get the message out to the community: passive, active and interactive methods (Van der Ploeg *et al.*, 2008).

Passive Methods

Passive methods included dissemination of posters, calendars, t-shirts, newsletters and storybooks to the people of San Mariano, and the construction of informative billboards and wall paintings in different areas of the community. The first poster was a reprint of the 'Only in the Philippines' wildlife series from the DENR and Flora and Fauna International (FFI). These posters aimed at promoting the protection of endemic wildlife, including the Philippine crocodile. Development Communication (DevCom) students from

the Isabela State University (ISU) designed several posters, thereafter, which focused on the laws and ordinances prohibiting the killing of crocodiles, sustainable wetland management and the protection of crocodile nests (Figure 5). Thousands of these posters were printed in the local dialect (Ilocano) and also in English in order to reach a wider target audience (Van der Ploeg *et al.*, 2008).

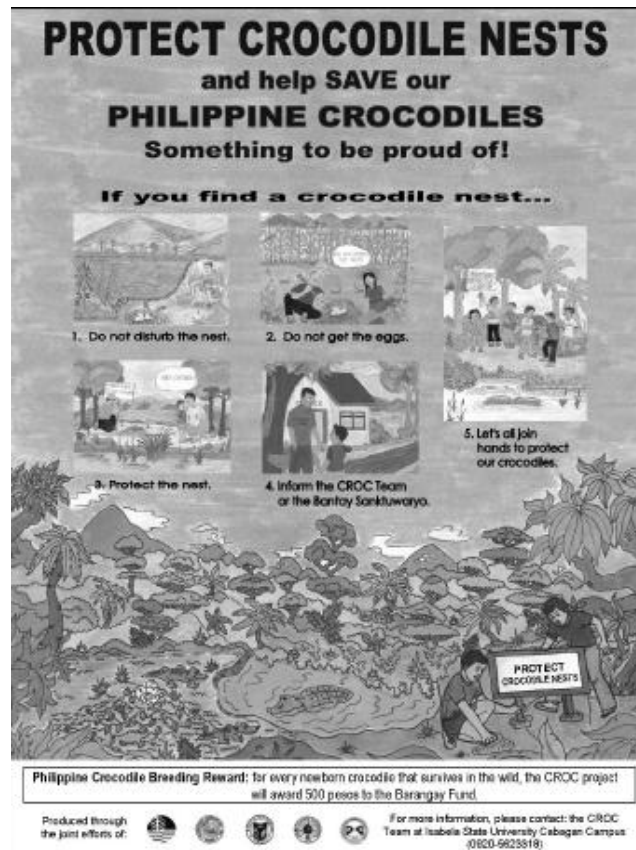


Figure 5. An example of a poster designed by Isabela State University DevCom students, for distribution across San Mariano. (From Van der Ploeg *et al.*, 2008)

The CROC project distributed a total of 9000 calendars to its conservation partners and households in San Mariano from 2004 to 2007. Calendars were made from inexpensive IEC materials which were popular within the community because of their function (Van Weerd, pers. comm.). Billboards and murals were placed or painted in strategic locations such as public markets, alongside highways, schools and town halls and in the crocodile sanctuaries to maximise their information potential. A storybook entitled, ‘Philippine

crocodile: something to be proud of!’ were distributed to schools and communities, throughout the Cagayan Valley (Van der Ploeg *et al.*, 2008).

Active Methods

Conservation Education and Public Awareness (CEPA) campaigns for Philippine crocodile conservation include puppet shows and cultural shows that are performed by DevCom students of the Isabela State University. Student dance groups perform interpretative dances showing the problems associated with illegal fishing and logging, whilst puppet shows focus on Philippine crocodile conservation. The dynamic partnership between CVPED, the Mabuwaya Foundation and the Department of Development Communication and Languages of the ISU, has allowed these shows to be presented in town fiestas and schools and during training and workshops. The Mabuwaya Foundation provides financial assistance to the students for their props, costumes and transport-related costs (Van der Ploeg *et al.*, 2008).

Aside from the activities described above, the CROC team also visit schools to give lectures and show documentaries about the Philippine crocodile. Moreover, field visits to the municipal crocodile rescue centre and Dunoy Lake are also organised by the CROC team. This is to give students the opportunity to see the foundation’s work first hand and observe crocodiles in their native habitat (Van der Ploeg *et al.*, 2008).

Interactive Methods

Community consultations are perhaps the best method of informing people about Philippine crocodile conservation (Van der Ploeg *et al.*, 2008). Public consultations allow local people to participate in the discussions and to openly raise questions and concerns, which are immediately resolved or debated on by the people present. Community dialogues are held in order to obtain the consent and participation of the community in matters such as the establishment of protected areas. DENR personnel, local barangay leaders, Mabuwaya Foundation staff, and members of the community, are usually present during these dialogues, which are held at barangay or municipal buildings (Van der Ploeg & Van Weerd, 2008).

The Mabuwaya Foundation also organises workshops for barangay and municipal officers around the Sierra Madre so that they can effectively enact environmental legislation. A lack of knowledge in environmental laws seriously hampers local leaders from effectively enforcing such laws. Thus, crimes against the environment usually go unpunished (Van der Ploeg *et al.*, 2008). Workshops and training were organised in 2004, 2006 and 2007, in order to educate local officials on environmental laws. Although interactive methods were seen as the most useful method for promoting awareness, based on an assessment of the impacts, it is also the most expensive option (Van der Ploeg *et al.*, 2008).

Section 3 Molecular Genetics in the Conservation of Endangered Species

3.1 Research using molecular markers in crocodilians

The application of molecular methods when addressing conservation issues of many animal populations has steadily increased in the past decades. Genetic analysis has been used in taxonomic identification, detection of hybridisation and assessment of genetic diversity and population structure in many endangered species (Haig, 1998). Molecular techniques have been particularly useful in clarifying ambiguities in species status and phylogenies of many organisms, including the Order Crocodylia, where discrete conservatism and unresolved relationships, amongst the different species, still abound (Densmore & White, 1991).

Population genetics is relevant to the management of captive populations; reintroductions; clarification of taxonomic relationships; detection of hybridisation; and prediction of the effects of habitat fragmentation and loss (F. W. Allendorf & Luikart, 2007). The identification of molecular or genetic markers is critical to our understanding of population structure. Molecular markers are polymorphic regions in the genome, which are chosen, in the hope that they represent the overall variation in the genomic DNA. Moreover, they are selected based on cost, the relative ease of use and development, and high polymorphism and neutrality with respect to natural selection (Beebe & Rowe, 2008). There are many types of molecular markers used in population genetic studies. Two of the most commonly used are mitochondrial DNA and microsatellites.

Mitochondrial DNA

Many research which examined genetic variation in animal populations made use of mitochondrial DNA (mtDNA). Due to its small size (16-21 kilobases average), mtDNA is easily isolated and occurs in plentiful copies in the genomic DNA (Allendorf & Luikart, 2007). The vertebrate mtDNA, in most cases, is a haploid, closed circular molecule which codes for two ribosomal RNA genes, 22 tRNAs and 13 enzymes involved in ATP synthesis. The mtDNA has coding and non-coding regions. Coding regions are areas in the gene wherein a particular protein product is produced. Thus, mutations in these regions are infrequent. Non-coding regions do not code for proteins. The major non-coding region, in the mtDNA of vertebrates, is called the D-loop or control region. It is considered to be the

most variable part of the mtDNA because of its rapid evolutionary rate (Ray & Densmore, 2002).

Mitochondrial DNA is maternally-inherited and does not undergo recombination unlike nuclear DNA. These characteristics make it a highly useful tool in phylogeography and systematics (Sunnucks, 2000). Moreover, mtDNA has a higher mutation or nucleotide substitution rate than nuclear DNA and it would, therefore, show greater interspecific variation than nuclear genes (Beebee & Rowe, 2008). Since it is maternally inherited, mtDNA can also only provide information on female dispersal or gene flow. Despite these advantages, there is evidence that strict maternal inheritance is not the norm for some species (Gyllensten, Wharton, Joseffson, & Wilson, 1991). There is also evidence that nucleotide substitution rate is higher in nuclear DNA compared to mitochondrial DNA in some taxa such as *Drosophila* (Beebee & Rowe, 2008; Shearer, van Oppen, Romano, & Worheide, 2002). Nevertheless, mtDNA markers will continue to be valuable in the reconstruction of phylogenetic trees (Sunnucks, 2000).

The gene order and the proteins coded for in the mitochondrial genome of Crocodylians are similar to that of other vertebrates, except that the tRNAPhe is inserted between tRNAPro and the 5' end of the control region, in crocodiles (Quinn & Mindell, 1996; Ray & Densmore, 2002). Figure 6 shows a diagrammatic representation of the mitochondrial genome order in Crocodylians.

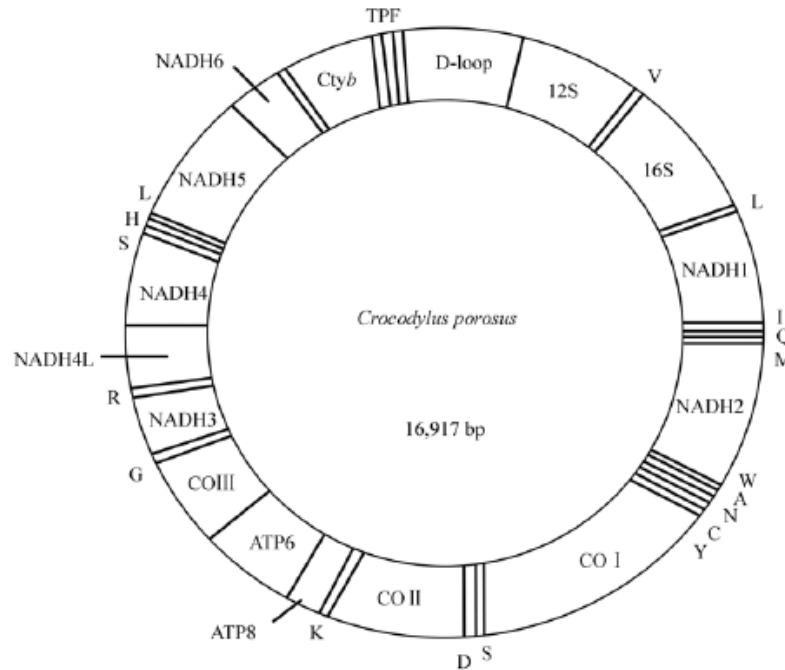


Figure 6. Mitochondrial genome order of Crocodilians (Yan Li *et al.*, 2007)

The control region, or D-loop in the Crocodylidae, is made up of three domains (Figure 7). Domain I consists of a short segment (121 bp or less) starting in the 5' end of the D-loop. This region is linked with termination associated sequences (TAS), which trigger the termination of the D-loop (Ray & Densmore, 2002). Domain II, known as the Central Conserved Domain (CCD), contains highly conserved sequences identified as the B, C, D, E and F boxes (Anderson, Bankier, Barrel, & et al., 1981). Domain II is the least variable area in the D-loop. Domain III is known as the conserved sequence block (CSB) and it is believed to be involved in mtDNA replication. In Crocodilians, the CSB consists of extensive poly-A sequences and heteroplasmic tandem repeats, which is highly variable between species (Ray & Densmore, 2002, 2003).

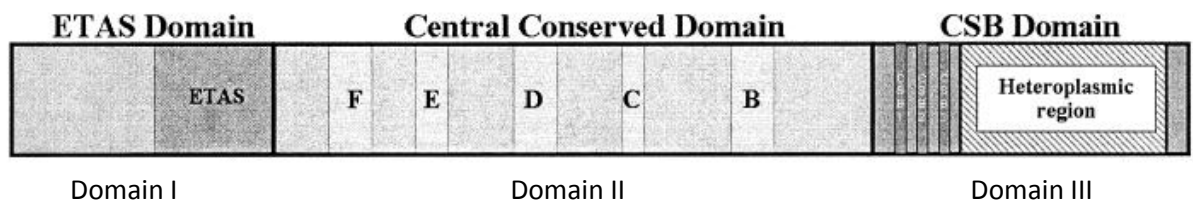


Figure 7. Diagrammatic representation of the Crocodilian D-loop (From Ray & Densmore, 2003)

Mitochondrial DNA studies have been most useful in crocodylian taxonomy. Currently, the Order Crocodylia consists of three families and eight genera based on morphological similarities. Recent evidence, based on molecular and morphological studies, has increasingly challenged this classification. The controversy has mostly been centred on the classification of four species – *Gavialis gangeticus*, *Tomistoma schlegelii*, *Crocodylus cataphractus* (Yan Li, Wu, Yan, & Amato, 2007) and *Osteolaemus* (Densmore & White, 1991; Ray, White, Duong, Cullen, & Densmore, 2000).

Using restriction fragment analyses of mitochondrial and ribosomal DNA, Densmore & White (1991) presented the first study on the molecular phylogeny of extant *Crocodylus*. Their findings suggested true crocodiles as belonging to one monophyletic group. More recent molecular and morphological research by McAilley *et al.* (2006), on the African slender-snouted crocodile, *C. cataphractus*, suggested otherwise. Comparing morphological differences and sequences from nuclear and mitochondrial genes of *C. cataphractus* with other members of *Crocodylus*, McAilley *et al.* (2006) gained support for the hypothesis that *C. cataphractus* does not belong to the genus. This finding was supported by the phylogenetic analyses of Yan Li *et al.* (2007) on the D-loop conserved regions (Domain I and II) of the Order Crocodylia.

Microsatellites

Microsatellites are short tandem repeats of sequences, about 1-6 nucleotides long. They are also known as VNTRs (variable number of tandem repeats) or SSR (simple sequence repeats). Microsatellites are highly polymorphic even in small populations of endangered species because of a high mutation rate (F. W. Allendorf & Luikart, 2007; Weber & Wong, 1993). The polymorphism is not in the sequence itself but in the number of times the short sequences are repeated (Tagu & Moussard, 2006). Microsatellites are also flanked by highly conserved and distinct sequences which serve as priming sites in Polymerase Chain Reaction (PCR) amplifications. Since these priming sites are highly conserved, microsatellite primers developed for one species can often be used for closely-related species (Allendorf & Luikart, 2007).

Microsatellite DNA loci is one of the most powerful and widely-used molecular markers in addressing population structure and genetic diversity, due to its high variability

and relative ease of scoring (Miles, Lance, Isberg, Moran, & Glenn, 2008). Microsatellites are widely distributed in the genome of eukaryotes, and thus, they require only a small amount of tissue. They are an ideal marker to use in small and inbred populations wherein allozymes and mtDNA have failed to show significant variation (Gullberg, Tegelstrom, & Olsson, 1997). One disadvantage is that the development of a microsatellite library is a relatively time-consuming and costly effort (Beebee & Rowe, 2008).

The uses of microsatellites in population genetics are varied. In the Order Crocodylia, most studies using microsatellites aim to assess genetic diversity, population structure, gene flow and parentage testing. Older studies used allozyme and isozyme data, in order to examine genetic diversity in crocodylians (Adams, Smith, & Baccus, 1980; Flint, van der Bank, & Grobler, 2000; Gartside, Dessauer, & Joanen, 1977; Menzies & Kushlan, 1991). These studies, however, failed to show significant levels of genetic diversity amongst alligator and crocodile populations, due to the low mutation rate for isozymes, thus, making it harder to assess the genetic variation actually present in populations (Dever, Strauss, Rainwater, McMurry, & Densmore, 2002). When microsatellites were used, significant variations between populations of *Alligator mississippiensis* in the United States were found, compared to the low level of genetic differentiation seen with gene products (Davis, et al., 2002; Glenn, Dessauer, & Braun, 1998).

The development of microsatellite markers, for use in population genetics studies, has increased our level of understanding of crocodile mobility, reproductive biology and the distinctiveness between populations. Microsatellites have been used to investigate population structure and gene flow in wild populations of Morelet's crocodile (*C. moreletii*) and the American alligator (*A. mississippiensis*) (Davis, et al., 2002; Dever & Densmore, 2001; Dever, et al., 2002). They have also been useful in testing for paternity and in the identification of hybrids in captive crocodiles (FitzSimmons, et al., 2002; Flint, et al., 2000). Testing for pedigree is important in the genetic management of crocodiles kept for farming purposes and in the identification of founders for reintroduction. Testing for the genetic variability present within reserve populations intended for reintroductions or supplementations would decrease the risk associated with inbreeding depression (Flint, et al., 2000).

3.2 Aspects of Conservation Genetics Examined in this Study

Population Structure

Determining the existence and scale of population subdivision or structuring is important in wildlife management. Identification of population structure leads to a better understanding of population dynamics and the identification of ecologically significant units (ESU) which are worth protecting (Fleischer, 1998). Furthermore, information on population differentiation could help conservation managers in the development of reintroduction and translocation strategies. Examination of genetic data is usually the only way to give a clear demarcation of population structure. This is where highly polymorphic molecular markers come to good use (Allendorf & Luikart, 2007).

F-statistics

Wright's F-statistics is the classical measure used in studies of genetic differentiation. F-statistics give a measure of the deficit of heterozygotes relative to expected Hardy-Weinberg proportions. Sewall Wright (1951) introduced three coefficients in order to describe differentiation between subpopulations: F_{IS} , F_{ST} and F_{IT} . The subscripts I, S and T stand for I = individual, S = subpopulation and T = total population.

F_{IS} is also known as the inbreeding coefficient because it measures the degree of inbreeding of individuals within a subpopulation. F_{IS} , therefore, measures the departure from Hardy-Weinberg proportions, within the subpopulation. A positive F_{IS} value would signify an excess of homozygotes while a negative value would indicate a deficit of homozygotes. It is given by the equation:

$$F_{IS} = 1 - (H_O / H_S)$$

H_O = average observed heterozygosity over all subpopulations

H_S = expected heterozygosity over all subpopulations

F_{ST} , also called the fixation index, measures the degree of inbreeding of subpopulations, in relation to the total population, by measuring allele frequency divergence among subpopulations. F_{ST} is a measure of population differentiation or structure. F_{ST} values range

from 0 to 1. When there is a high rate of gene flow between subpopulations, F_{ST} is low. When gene flow is low, populations diverge and F_{ST} increases.

$$F_{ST} = 1 - (H_S / H_T)$$

H_T = expected Hardy-Weinberg heterozygosity, if entire base population is panmictic

H_S = expected heterozygosity averaged over all subpopulations

The third F-statistic, F_{IT} , measures the overall level of inbreeding of an individual, relative to the total population. It is a measure of the overall departure from Hardy-Weinberg proportion in the total population, due to inbreeding of individuals, relative to their subpopulation (F_{IS}) and inbreeding of subpopulations, in relation to the total population (F_{ST}) (Allendorf *et al.*, 2007).

$$F_{IT} = 1 - (H_O / H_T)$$

Other Measures of Subdivision

Measures of subdivision similar to F_{ST} include phi-ST (Φ_{ST}), which is based on allele frequency variance and G_{ST} , which is based on gene diversities. Another measure of subdivision, R_{ST} , was developed by Slatkin (1995) to take into account allele sizes or lengths. Microsatellites are believed to follow a stepwise mutation model (SMM) whereas the F_{ST} method assumes the infinite allele model (IAM). According to the stepwise mutation model, allele sizes contain information on the relationships between alleles. The F_{ST} method disregards allele sizes, whilst R_{ST} does not. R_{ST} is considered by some as a better measure of subdivision when using microsatellite loci (Halliburton, 2004), although there are concerns that analyses following the strict SMM are prone to violations of this model and that measurements using IAM may be more reliable (Balloux & Lugon-Moulin, 2002). Computer programmes, such as RSTCALC (S. Goodman, 1997) and FSTAT (Goudet, 1995) are readily available to calculate R_{ST} and F_{ST} .

Nei's genetic distance (D_N) is another approach used to measure genetic differentiation amongst populations and species. D_N is often used to delineate populations as merely a single population or subspecies, or distinct species. Nei's genetic distance is

first calculated by getting Nei's index of genetic similarity (I_N). D_N is then calculated by the equation,

$$D_N = -\ln(I_N)$$

It is expected that genetic distance would become larger, as one move from subspecies to species, species to genera, genera to family and so forth. The degree of genetic distance, however, varies from one species to another. For example, genetic distances between subspecies of lizards range from 0.34 to 0.35, whereas the genetic distances between species of macaques only range from 0.02-0.10 (Nei, 1987). It would seem that there is no general rule that encompass all species, in relation to genetic distance.

Gene Flow

Gene flow is defined as the movement of individuals or gametes between populations. Successful reproduction is a prerequisite of gene flow, or else, movement of individuals would be aptly described as dispersal rather than gene flow (Allendorf & Luikart, 2007). Gene flow homogenises populations, whilst its absence may lead to divergence. The level of gene flow is symbolised by m , and it is defined as the proportion of alleles originating from another population in a given generation (Halliburton, 2004). There are direct and indirect ways of estimating gene flow.

Estimates of gene flow, using direct methods, apply only to the time period when the estimate was made and does not give any information on historical gene flow. Another limitation of the direct method is that pulse migrations, which occur intermittently, are not detected. Pulse migrations are driven by occurrences such as periodic weather changes or demographic reasons. The typical direct method of estimating gene flow, through the capture-mark-recapture method, only takes the assumption that migrants reproduce. The effective number of migrants per generation (NM) is very difficult to estimate in mark-recapture methods because it is extremely hard to follow all migrants in order to see if they have reproduced (Allendorf & Luikart, 2007).

Assignment tests are a useful alternative for estimating NM directly. Assignment tests determine the most likely population an individual comes from, by computing the expected frequency of its genotype (p^2) in each likely population of origin, through the use

of observed allele frequencies (p) from each population. If an individual is assigned to a population, other than the one where it was caught, then that individual is presumed to be a migrant. Assignment tests have also been used to detect interspecies crocodile hybrids in captivity (FitzSimmons, et al., 2002; Weaver, et al., 2008).

Indirect estimates of gene flow can be obtained from the allele frequency differences (F_{ST}) amongst populations. The average number of migrants per generation, moving between subpopulations (NM), can be derived from the F_{ST} using the equation,

$$NM = \frac{1}{4} (1 / F_{ST} - 1)$$

Many assumptions must be met for the above equation to remain true. Some of these assumptions state that populations must have the same size and that they should be in drift-migration equilibrium. This is rarely the case and therefore it is unlikely that natural populations will meet *all* the assumptions. In addition, a very high variance of NM is seen, when F_{ST} is low (< 0.10). This decreases the usefulness of the estimate to wildlife managers. The maximum likelihood approach, by Beerli & Felsenstein (2001), is an alternative to NM estimation. The advantage of this method is that it allows for different population sizes and different migration rates, unlike NM estimation from F_{ST} . The software MIGRATE (Beerli & Felsenstein, 2001) is freely available for this type of analysis. A disadvantage of the maximum likelihood approach is that it is computationally slow and time consuming and its methods are difficult to evaluate (Allendorf & Luikart, 2007).

Genetic Diversity

Genetic diversity is defined as the amount of genetic variation present in a population or species. Frankham (1996) described it as the raw material that allows populations to adapt to environmental change. In other words, genetic diversity is the basis for evolutionary change - for without it evolution cannot happen. The amount of genetic variation that we see today is a product of mutation, genetic drift and natural selection. Measures of genetic variation include heterozygosity, allelic diversity and a percentage of polymorphic loci (Frankham, 1996; Frankham, Ballou, & Briscoe, 2004; Lacy, 1997).

Conservation of genetic diversity is essential to the effective management of both wild and captive populations. If zoos and other captive facilities aim to be a part of species

conservation programmes, then they have to be managed in such a way that prevents or reverses the decline of genetic variability in captive populations (Lacy, 1987). In the same way, small and fragmented wild populations, which are typical of many endangered species, should be managed to prevent genetic erosion. The reason why the preservation of genetic diversity is such a crucial theme in conservation biology is due to its association with population viability or persistence in the face of a changing environment (Lande & Shannon, 1996). Genetic variation in small populations is lowered as a result of mating between close relatives (inbreeding) and genetic drift. Inbred individuals, at least in normally outbreeding species, are generally known to have reduced fitness-related traits, such as fecundity and survival rate (Soule, 1985). A decrease in fitness would affect population growth rates, thus making the population even smaller and more prone to extinction. This downward spiral of events, which eventually leads to extinction, is known as the extinction vortex (Falconer & Mackay, 1996; D. Goodman, 1987; Lacy, 1997).

We would expect that small populations of rare and endangered species have less genetic variation, compared to large populations. In the same way, widespread species will tend to have more genetic variation than restricted species. Frankham (1996) tested these assumptions and concluded that a positive correlation does exist between population size and genetic diversity. More widespread species also tend to have greater genetic diversity, than those that are restricted to smaller regions, such as island endemics. These relationships, however, are not straightforward in all cases because the extent of genetic diversity in a population, at any given time, is affected by its history and present condition (Allendorf & Luikart, 2007). For example, red pine (*Pinus resinosa*), which number in millions and is widely distributed in the northeast and north-central USA and southern Canada, has an extremely low genetic diversity as revealed by allozyme studies (Fowler & Morris, 1977; Mosseler, Egger, & Hughes, 1992; Simon, Bergeron, & Gagnon, 1986). In contrast, allozyme studies of the rare one-horned rhinoceros (*Rhinoceros unicornis*) of Nepal showed a very high heterozygosity, despite very low population numbers (60-80 individuals in 1962) (Dinnerstein & McCracken, 1990).

The relative inconsistencies, stated above, can be explained by considering the demography, distribution and life history of the species and the length and severity of the

genetic bottleneck. For the red pine, the explanation for the low genetic variation is because the species must have gone through a very small and very long bottleneck, associated with the glacial period 20,000 years ago. Since the red pine also has a lengthy generation time, it is hypothesised that the pine has not yet had sufficient time to recover the genetic variation it lost in the bottleneck (Allendorf & Luikart, 2007). The high genetic variation seen in the one-horned rhinoceros on the other hand stems from the large population size and wide distribution of the species before the bottleneck. This bottleneck is also quite recent (1950's) and the average generation time of the species is long, which accounts for the relatively high genetic variation still present within the species (Dinnerstein & McCracken, 1990). These two examples show the need to exercise caution and consider demographic and environmental factors, when interpreting genetic variation in plant and animal populations.

Inbreeding

Inbreeding is a main concern in the study of small, isolated populations because it has been shown to decrease survival and reproduction rates, both in captive and wild populations (Jimenez, Hughes, Alaks, Graham, & Lacy, 1994; Laikre & Ryman, 1991; Saccheri, et al., 1998). Inbreeding refers to mating amongst relatives, which leads to increased homozygosity in the offspring. Increased homozygosity leads to the expression of deleterious recessive alleles resulting in inbreeding depression or a decline in fitness. The level of inbreeding is traditionally expressed as Wright's inbreeding coefficient, f or F . Originally derived by Wright as a correlation, f is now defined as the probability of two alleles at a locus being identical by descent (Charlesworth & Charlesworth, 1987; Keller & Waller, 2002).

Biologists look at traits related to fitness, such as fecundity, juvenile survival and growth patterns in order to estimate inbreeding in populations (Crnokrak & Roff, 1999). The majority of the studies that investigated inbreeding depression were made on captive animals (Lacy, Petric, & Warneke, 1993; Ralls & Ballou, 1986), because studying the effects of inbreeding depression in wild populations takes considerable time and effort (Keller, 1998). Knowing the level of inbreeding and how it affects captive populations is critical, especially if the populations are likely to be sources of animals for reintroduction

or restocking programmes. Inbreeding may significantly affect the fitness and survival of the released individuals, thus, directly contributing to the success or failure of the release programme (Jimenez, et al., 1994).

Inbreeding depression may be masked in captive situations due to controlled surroundings and adequate veterinary care, but its effect may be pronounced in the wild or under more stressful conditions (P. Miller, 1994; Ralls & Ballou, 1986). Indeed, an experiment by Jimenez *et al.* (1994), on white-footed mice has shown that inbreeding adversely affected the survival of mice released into a natural habitat. Moreover, inbred mice released into natural conditions fared poorly compared to inbred mice kept in the laboratory (Jimenez et. al., 1994). This finding places emphasis on the prevention of inbreeding in captivity especially for a species wherein captive populations may be the only source for genetic restoration.

The existence of inbreeding and its effects on the persistence of natural populations has been controversial (Keller & Waller, 2002). Sceptics argued that the reasons why inbreeding might be insignificant in the wild was that animals naturally tended to avoid close mating and that they were able to deal with the genetic effects before they could be expressed at the phenotypic level (Crnokrak & Roff, 1999). Comprehensive field data from the study of song sparrows (*Melospiza melodia*) by Keller (1998), and Saccheri *et al.*'s (1998) study on the Glanville fritillary butterfly (*Militaea cinxia*), lend support to the argument that inbreeding does exist and it affects natural populations. Their studies showed that survival and reproductive traits were negatively affected by inbreeding and might even increase a population's extinction risk. The probability of extinction is greater for small and isolated populations that suffer from decreased homozygosity and an increased genetic load (Crnokrak & Roff, 1999).

The inbreeding coefficient is traditionally calculated from pedigree data. This is not a problem for captive populations wherein records on lineage are kept. On the other hand, pedigree information for wild populations is very limited and this makes the estimation of the inbreeding coefficient problematic. To solve this problem, researchers have used molecular markers to infer levels of inbreeding and inbreeding depression in wild populations (Hedrick, 2001; Keller & Waller, 2002). Ellegren (1999) compared the

inbreeding coefficient calculated from the pedigree data of captive gray wolves (*Canis lupus*) to the observed heterozygosity from 29 microsatellite loci. The author found a significant correlation between the pedigree inbreeding coefficient and heterozygosity (Ellegren, 1999; Hedrick, 2001). Nonetheless, a disadvantage when using microsatellites is that the estimates may not be precise, due to a large variance in heterozygosity values (Keller & Waller, 2002; Pemberton, 2004). For example, Slate *et al.*'s (2004) study on Coopworth sheep, using 101 microsatellite loci, showed a wide variation in heterozygosity, even for individuals with the same inbreeding coefficients (f). Pemberton (2004) suggested that linkage disequilibrium between microsatellite loci could have a local effect on fitness traits which might explain such results, but this needs further investigation. Pemberton (2004) recommended the more traditional method of pedigree analysis be used when estimating inbreeding coefficients in natural populations and to only use microsatellites, when determining parentage and rebuilding pedigrees.

Hybridisation

Hybridisation is a term often used to describe mating between individuals that come from genetically distinct populations (Rhymer & Simberloff, 1996). It is often a conservation concern because it can contribute to or be the main cause of a species' extinction. Hybridisation occurs in the natural world and it has been found to play an important role in the evolution of many plants and animals. However, habitat changes caused by people, such as the introduction of exotic species and habitat fragmentation, may increase the rate of hybridisation and put many species at risk (F. Allendorf, Leary, Spruell, & Wenberg, 2001).

In crocodiles, most instances of interspecific hybridisation are detected in captivity (FitzSimmons, et al., 2002), although recent studies on wild populations of Morelet's crocodile (*C. moreletii*) and the American crocodile (*C. acutus*) suggests that hybridisation in the wild is probably more common than originally thought (Cedeño-Vazquez, et al., 2008). Detection of hybrids in captive stocks is important because only pure-bred animals must be used to replenish wild populations (F. Allendorf, et al., 2001). It is also important to quantify the amount of genetic mixing between sympatric crocodile species in the wild in order for us to understand whether genetically different groups are products of evolutionary

processes or simply the result of increased hybridisation due to anthropogenic changes (F. Allendorf, et al., 2001; Weaver, et al., 2008). Management decisions, which consider whether hybrids are worthy of protection, often take into account whether hybridisation is natural or man-made. A paper by Allendorf *et al.* (2001) reviewed the different cases of hybridisation and suggested six hybrid categories to serve as a guideline for policy-makers and conservation managers.

In an unpublished report on Philippine crocodile systematics and population genetics using mtDNA D-loop data, Louis and Brenneman (2007) found three out of the 46 sampled *C. mindorensis* individuals had *C. porosus* maternal ancestry. These crocodiles were purported to have been bred in captivity at the Palawan Wildlife Rescue and Conservation Centre (PWRCC). The PWRCC is the most likely source of crocodiles for the Philippine crocodile reintroduction project. Although this facility has captive stocks of *both* *C. porosus* and *C. mindorensis*, it has never intentionally hybridised the two species. Louis and Brenneman's findings point to the presence of hybrids in the parental stock, thus, strengthening the necessity to genetically screen all captive animals, in order to reduce the risk of hybrid individuals becoming released into the wild.

Section 4. Research objectives

The objectives of this study are:

1. To provide a broad summary of the status and conservation of the Philippine crocodile by reviewing existing literature;
2. To assess the genetic status of wild and captive populations of *C. mindorensis*, using polymorphic microsatellite DNA loci, in order to:
 - a. Evaluate the genetic diversity present within and between populations
 - b. assess the degree of population structure/differentiation
3. To assess the species integrity of Philippine crocodile populations using microsatellites and existing mtDNA data by screening for interspecies hybrids
4. To identify captive individuals with maximum genetic diversity that could serve as founders for the reintroduction programme;
5. To present recommendations, based on the results of the study, for the development of captive management and reintroduction strategies for the Philippine crocodile;

Aside from outlining the objectives of this thesis, this chapter also presents the current knowledge on the population status and conservation of the Philippine crocodile (thesis objective no.1) and outlines the molecular genetics techniques used in this study. Chapter Two moves on to illustrate the use of molecular genetics in attaining objective two to four. Finally, Chapter Three considers the current knowledge on Philippine crocodiles and the results from Chapter Three in order to recommend specific management actions for the recovery program (thesis objective number 5).

CHAPTER TWO: THE USE OF MICROSATELLITE DNA ANALYSES IN DETERMINING POPULATION STRUCTURE AND IN DEVELOPING A CAPTIVE MANAGEMENT AND REINTRODUCTION STRATEGY FOR THE PHILIPPINE CROCODILE (*Crocodylus mindorensis*)

2.1 Introduction

The application of genetics in conservation has increased dramatically in the past decades. Genetic methods have been used to address taxonomic issues, detect hybridisation, assess genetic variability and inbreeding depression and track gene flow in an effort to conserve genetically healthy populations and to aid the identification of ecologically significant units (Fleischer, 1998). The use of nuclear and mitochondrial DNA data, in crocodylian research, has contributed to our understanding of their mobility and reproductive biology, in addition to revealing differences between individuals, populations and species.

Microsatellites have been the marker of choice in many population genetic studies because of its neutrality, relative ease of preparation and high information content (Selkoe & Toonen, 2006). Microsatellites have been used to investigate population structure and gene flow in wild populations of Morelet's crocodile (*Crocodylus moreletii*) in Belize (Dever & Densmore, 2001; Dever, et al., 2002), the American alligator (*Alligator mississippiensis*) in the southeastern United States (Davis, et al., 2002), and the Black Caiman (*Melanosuchus niger*) in South America (De Thoisy, Hrbek, Farias, Vasconcelos, & Lavergne, 2006). Microsatellites have also been useful in parentage analysis and in the determination and maintenance of genetic variability in saltwater crocodiles (*C. porosus*) bred for the leather trade (Flint, et al., 2000; Isberg, Chen, Barker, & Moran, 2004). In the case of captive crocodiles, the combination of microsatellites and mitochondrial DNA markers has proven useful in species identification and the selection of candidates for reintroduction in *C. siamensis* (FitzSimmons, et al., 2002).

Reintroduction attempts have been made with at least 16 crocodylian species (Stanley Price & Soorae, 2003), including the Chinese alligator (Hongxing, et al., 2006), the Indian Gharial (Hussain, 1999), the Orinoco crocodile (Munoz & Thorbjarnarson, 2000)

and the Siamese crocodile (FitzSimmons, et al., 2002). In many of these endeavours, captive bred animals were used to repopulate areas from where the species had previously been extirpated or were on the brink of extinction. One of the challenges associated with the use of captive animals for reintroductions include the possibility of releasing cryptic hybrids. Historically, only a few crocodile reintroduction attempts have included genetic screening of captive-bred crocodiles in order to determine the genetic integrity of the animals before release (FitzSimmons, et al., 2002; Hongxing, et al., 2006). This is unfortunate, as undetected hybrids released in the wild could have serious conservation implications.

Interspecies hybridisation of crocodiles to improve growth rates and skin quality in commercial farms is quite common (Thorbjarnarson, 1992). Unintentional hybridisation in captive breeding facilities, however, could present problems especially if captive animals will be used for wild population recovery (FitzSimmons *et al.*, 2002). Either one or a combination of morphological, mtDNA and nuclear marker data analyses are commonly used to infer hybridisation in crocodylians. For instance, Ray *et al.* (2004) and Cedeño-Vasquez *et al.* (2008) examined the mitochondrial control region to find evidence of hybridisation in wild populations of *C. acutus* and *C. moreletii*. FitzSimmons *et al.* (2002) used microsatellite and mtDNA analyses to screen *C. siamensis* for reintroduction in Vietnam.

More recent publications used a combination of morphological and molecular analyses to find evidence of hybridisation in captive and wild populations of *C. moreletii* (Weaver *et al.*, 2008; Rodriguez *et al.*, 2008). In these studies, Bayesian based statistical methods were applied on genotype data from polymorphic microsatellite markers to infer hybrid individuals and to increase the efficiency of hybrid detection. Pure individuals were expected to have high probability estimates of assignment to a species cluster whilst admixed individuals were expected to have intermediate probabilities (Rodriguez *et al.*, 2008). The method of utilizing both maternally-derived (mtDNA) and biparentally inherited molecular markers (such as microsatellites) is advantageous because it allows for a more accurate detection of hybrid types (Weaver *et al.*, 2008).

The determination of genetic structure, diversity and integrity in threatened species has many potential benefits for their conservation. For example, information on population

genetics identifies populations which should be prioritised in conservation efforts and those which could be used as a source for population augmentations. Such information would allow for more efficient planning of recovery efforts and the avoidance of costly mistakes in the future (Haig, 1998). In this study, the genetics issues in relation to the conservation of the critically endangered, Philippine crocodile, is addressed.

Crocodylus mindorensis - a crocodilian under management for reintroduction

The Philippine crocodile, *C. mindorensis*, is a species of special concern and has already been the focus of a breeding programme for many years. Hunting, persecution, habitat loss and habitat fragmentation are thought to be the reasons for decreased range of the species and reduction of the population to critically low levels (Van Weerd & Van der Ploeg, 2003). As a result, this species is currently listed as critically endangered, on the IUCN Red List (IUCN, 2008), with wild populations estimated to total fewer than 100 mature individuals (J. Ross, 1998). Recent surveys have pinpointed strongholds of free-living *C. mindorensis* at only two locations in the Philippines: Isabela in the north eastern Philippines and Liguasan Marsh in Mindanao (Van Weerd & Van der Ploeg, 2003). In 2005 a small population was discovered in the Province of Abra (Manalo, pers. comm.), and one wild female was caught on the small island of Dalupiri, north of Luzon (Oliveros, et al., 2006). These are currently the only sites in the Philippines where wild *C. mindorensis* are known to occur.

Captive breeding of the Philippine crocodile for conservation purposes was first attempted at Silliman University in Dumaguete City, Philippines in 1980. All successful breeding resulted from a single pairing between a wild-caught female from the Pagatban River in Negros Occidental (Visayas region, mid-Philippines) and a captive male from Zamboanga City in Mindanao (Malayang, 2007). The female breeder in Silliman is the only known surviving wild-caught Philippine crocodile from the Visayas region. Progeny from this pair (in Silliman) have been transferred to other locations in the Philippines and to the Gladys Porter Zoo in the USA (C. Banks, 2005).

In 1987, the Department of Environment and Natural Resources (DENR), with funding from the Japanese International Cooperation Agency, established the Crocodile Farming Institute (CFI), now known as the Palawan Wildlife Rescue and Conservation

Centre (PWRCC). One of the aims of this facility is to conserve the two species of crocodiles found in the Philippines: the saltwater crocodile, *C. porosus*, and the Philippine freshwater crocodile, *C. mindorensis* (Banks, 2005; Sumiller, 2000). Many *C. mindorensis* used as founder stock for the captive breeding programme, were acquired from private collectors, with the majority coming from a facility in Mindanao (Sumiller, 2000). PWRCC was hugely successful in captive-breeding and it currently has the largest collection of *C. mindorensis* in the world (N= 574 on March 2009), making it the most likely source of candidates for the reintroduction programme in the Philippines (Banks 2005). Captive breeding was discontinued at PWRCC in 2001 due to financial constraints, limited space and ambiguities in the pedigree of the captive stock (Banks 2005).

Major steps towards the conservation of the Philippine crocodile were taken with the drafting and publication of the species recovery plan and the formation of the Philippine Crocodile Recovery Team in 2001. Given the precarious state of the wild population, the recovery team aimed to develop a Philippine crocodile release and re-stocking programme (Banks 2005). This plan was hampered for many years by unresolved pedigree issues, the possibility of hybrids within the captive population and uncertainty about the degree of genetic isolation, amongst remaining *C. mindorensis* populations (Banks 2005). As a result of increased pressure by the conservation community, the first attempt at reintroduction took place in July 2009, with the release of fifty captive-bred *C. mindorensis* from PWRCC. The reintroduction site was Dicitian Lake, a protected area within the Northern Sierra Madre National Park, in the north eastern Philippine province of Isabela. The reintroduced crocodiles were not genetically screened (prior to release) and information on the genetics of the species was restricted to mtDNA D-loop data from forty-six Philippine crocodiles, many of which were captive (EE Louis & Brenneman, 2007).

Plans are currently in place to release more captive bred crocodiles from PWRCC, into protected habitats in the Philippines where the species once thrived. Since the genetic structure of the species still remains to be clarified, 11 polymorphic microsatellite markers were used to examine the extent of genetic differentiation within *C. mindorensis*. In addition, microsatellite genotype information was compared with existing mitochondrial D-loop sequence data, in order to identify hybrids in the captive population and present

recommendations that would aid in the development of a future reintroduction strategy for the Philippine crocodile.

2.2 Methodology

Sample collection

Tissue samples were collected from 618 Philippine crocodiles between 1999 and 2009. Following crocodile restraint, scutes were obtained by cleaning the area with 70% isopropyl alcohol and cutting with a scalpel/razor blade. The tissue samples were stored in 1.8 ml NUNC[®] tubes, which contained Seutin's solution, a preservative suitable for ambient conditions (Seutin, White, & Boag, 1991). The majority of the samples came from captive populations maintained at the PWRCC, in Puerto Princesa City, PH; Davao City Crocodile Park, PH; Calauit Game Refuge and Wildlife Sanctuary in Palawan, PH; V-square Mini Zoo in Abra province, PH; Silliman University in Dumaguete City, PH; and Gladys Porter Zoo, USA. Crocodiles from the Gladys Porter Zoo had originally come from Silliman University and thus they were pooled with the Silliman University samples. Tissue samples from wild *C. mindorensis* were taken from three locations in the Philippines: Isabela province, Dalupiri Island in the province of Cagayan and Liguasan Marsh in Mindanao (Figure 8). The single sample from Dalupiri Island was included with the samples from Isabela for statistical purposes. A list of the study areas, site descriptions and number of crocodiles sampled from each location are presented in Appendix 1.

DNA extraction

Genomic DNA from 584 *C. mindorensis* tissue samples was extracted and amplified using a whole genome amplification kit (WGA; Illustra TempliPhi[®], GE Healthcare, Piscataway, NJ) according to the manufacturer's directions. The WGA yielded an average of 500 ng of DNA per μL and all products were diluted to 50 ng/ μL . DNA from the remaining *C. mindorensis* tissue samples were extracted, using a standard phenol/chloroform/isoamyl alcohol extraction method, as described in Sambrook, Fritch & Maniatus (1989).

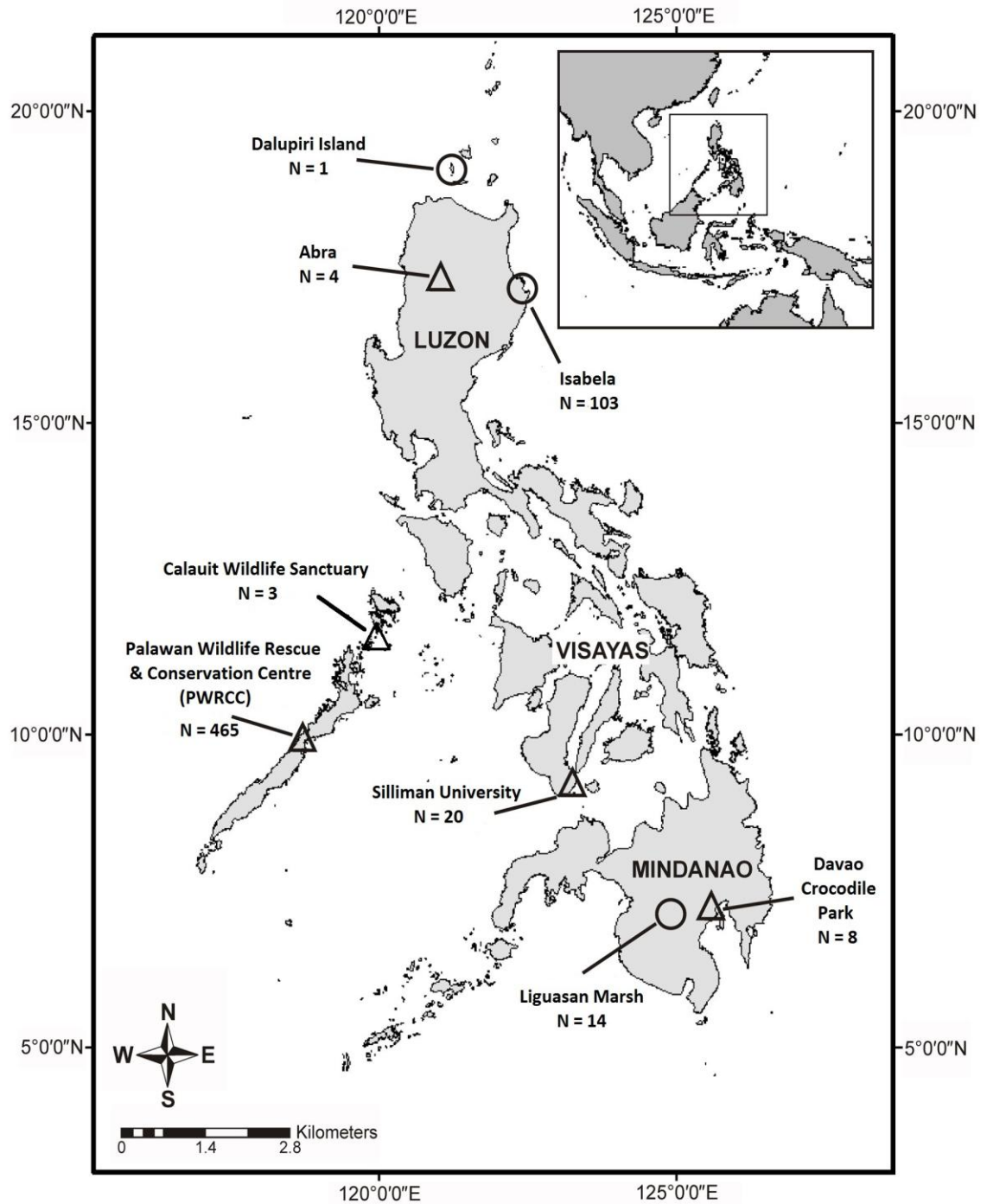


Figure 8. Location of *C. mindorensis* collection sites. Circles represent wild populations whereas triangles represent captive populations.

Microsatellite amplification

All *C. mindorensis* individuals were analysed at 13 microsatellite loci. A total of 31 microsatellite loci, developed and characterised by Miles *et al.* (2008) for *C. porosus* were tested, from which nine consistently amplified and were polymorphic in *C. mindorensis* (CpP106, CpP305, CpP801, CpP1610, CpP1708, CpP3008, CpP4004, CpP302 and CpP2516). One microsatellite locus (Ami μ 15), designed for American alligators (Glenn, *et al.*, 1998), was also polymorphic for *C. mindorensis* and this was included in the present study. In addition, three microsatellite marker loci (4HDZ27, 4HDZ35 and 4HDZ391) developed for *C. mindorensis*, following the protocol of Moraga-Amador *et al.* (2001), at Omaha's Henry Doorly Zoo (HDZ) genetics department was also used in this study. All microsatellite loci and their corresponding sequences and annealing temperature are shown in Table 4.

PCR amplifications were performed in MBA Satellite 0.2G thermal cyclers (Thermo Electron Corp., Waltham, MA), in reaction volumes of 25 μ L containing 20-50 ng of DNA template. Amplification conditions consisted of 12.5 pmol unlabelled reverse primer, 12.5 pmol fluorescently labeled forward primer, 1.5 mM MgCl₂, 200 μ M each dNTP, and 0.5 units of *Taq* DNA polymerase (Promega; Madison, WI). One of two PCR thermal cycling profiles was used, depending on the microsatellite loci amplified. Stratified touchdown programmes (TD65 and TD55) and three primer PCR conditions as described in Miles *et al.* (2008), were used for three loci (CpP302, CpP2516 and CpP4116). In the touchdown programme, the annealing temperature is reduced over a 10°C span (65°C to 55°C for TD65 and 55°C to 45°C for TD55) to correct for spurious amplifications (Don, Cox, Wainwright, Baker, & Mattick, 1991).

The TD65 programme cycling parameters used for markers CpP302 and CpP2516, were as follows: denaturation step of 95°C for three minutes followed by four cycles of (95°C for 20 s; 65°C for 20 s; and 72°C for 30 s); another four cycles of (95°C for 20 s; 62°C for 20 sand 72°C for 30 s); eight cycles of (95°C for 20 s; 60°C for 20 s and 72°C for 30 s); followed by 24 cycles of (95°C for 20 s; 55°C for 20 s and 72°C for 30 s); and concluding with a final extension step at 72°C for seven minutes. The TD55 programme used for CpP4116 had similar thermal cycling parameters except that the annealing temperature decreased from 55°C down to 52°C, 50°C and finally at 45°C.

The PCR cycling parameters, used for all other markers in this study, are described as follows: 34 cycles of 95°C for 30 s; a primer-specific annealing temperature for 45 s, and 72°C for 45 s, and a final extension step of 72°C for 10 min. Optimum annealing temperatures for microsatellite loci were determined as follows: 50°C for Ami μ 15; 56°C for CpP1610; 58°C for CpP305, CpP801, and CpP4004; 60°C for CpP1708, CpP3008, 4HDZ391; 62°C 4HDZ35; and 64°C for 4HDZ27. PCR products were visualised in order to verify amplification on 2% agarose gels stained with ethidium bromide. Allele sizes were determined, through separation of the PCR products, via POP 4 capillary buffer electrophoresed on ABI 3100/ABI 3130xl Genetic Analysers (Applied Biosystems, Inc, Foster City, CA). Fragment length genotypes were assigned by GeneScan, using GeneScan™ 500XL ROX™ size standard, in the GeneMapper software version 4.0.

Data analysis

The computer programmes MICRO-CHECKER (Van Oosterhaut, Hutchinson, & Willis, 2004) and Microsatellite Analyser (Dieringer & Schlotterer, 2003) were used to detect anomalies or possible genotyping errors in the data set. Allele frequency analyses, including null allele frequency estimation, were undertaken using CERVUS v.2.0 (Marshall, Slate, Kruuk, & Pemberton, 1998; J Slate, Marshall, & Pemberton, 2000). Loci with null allele frequencies greater than 20 % were removed from the data set before further population genetics analysis was performed.

Identification of Hybrids

The same set of individuals (N=618) have previously been screened for the displacement loop or control region (D-loop), by Tabora *et al.* (2010). Tabora *et al.*'s (2010) method consisted of amplification of the mitochondrial control region using primers CR2H and T-Phe-L (Ray and Densmore 2002) and sequencing 645bp length of the mitochondrial tRNAPro-tRNAPhe-Dloop region. For details on the methods used, see Tabora *et al.* (2010). Mitochondrial DNA data indicated the presence of *C. porosus* x *C. mindorensis* hybrids in the data set. Fifty-seven individual crocodiles had *C. porosus* (P1) haplotypes. Because mtDNA is maternally inherited, their findings show evidence of hybridisation only from a maternal lineage. In this study, I looked for evidence in

biparentally inherited nuclear markers (microsatellites), in order to determine if bidirectional hybridisation has occurred.

The programme STRUCTURE (Falush, Stephens, & Pritchard, 2002; Pritchard, Stephens, & Donnelly, 2000) was used to provide an overview of the genetic structuring for the entire data set. An initial $K=7$ (number of genetic cluster) was used, which was the same number of sampling sites in the study. The programme was run using 1.0×10^5 burn-in periods and repetitions and assuming admixture and correlated allele frequencies. The cluster where previously identified hybrids (*C. porosus* haplotype/ P1 haplotype; Tabora *et al.*, 2010) grouped under was designated as the ‘hybrid/P1 cluster’ (Figure 9). Individuals with 5% or greater ($q \geq 0.05$) assignment probability to this cluster were considered as putative hybrids.

Population Genetics Analysis

The putative hybrids identified above were removed from further analyses. Population genetic parameters were estimated for the remaining data representing pure *C. mindorensis* (N=526). The microsatellite loci were first tested for genotypic linkage disequilibrium by estimating exact P-values using the Markov chain method implemented in GENEPOP v.4.0.10 (Raymond & Rousset, 1995). Markov chain parameters were set at 10000 dememorisation steps, 100 batches and 5000 iterations per batch (default settings). Deviation from Hardy-Weinberg equilibrium was investigated using an exact Hardy-Weinberg test in Genepop v. 4.0.10. The complete enumeration method of Louis and Dempster (1987) was used to calculate the exact P-values for loci with alleles equal to or less than five. The Markov chain (MC) algorithm method (Guo & Thompson, 1992) was used to estimate exact P-values, for loci with five alleles or more. The significance of each result was assessed at $P < 0.05$ and a sequential Bonferroni correction was applied to minimise the chance of making a type 1 error (rejecting the null hypothesis when it is actually true).

Gene diversity or expected heterozygosity (H_E), observed heterozygosity (H_O), mean number of alleles (MNA), rarefacted allelic richness (AR), genic differentiation and within-population f-statistic (F_{IS}) and between population f-statistic (F_{ST}), were estimated using FSTAT v.2.9.3.2 (Goudet, 2001) and GENEPOP v.4.0.10 (Raymond & Rousset,

1995). Genetic structuring was further analysed using the Bayesian-based clustering algorithm in STRUCTURE v.2.3.1 (Falush, et al., 2002; Pritchard, et al., 2000). In order to estimate the number of genetic clusters (K) that best represent the data, the programme was run for 1-7 clusters (K=1-7) involving 1.0×10^5 burn-in periods and repetitions. The admixture model was used and correlated allele frequencies were assumed. The break in the slope of the distribution of the posterior probabilities [Pr(X/K)] was noted as described in Evanno *et al.* (2005) since this can be a good indicator of the uppermost level of structure or the true K (Evanno *et al.*, 2005).

The computer programme BOTTLENECK 1.2.02 (Cornuet & Luikart, 1996) was used to test for evidence of a severe reduction in effective population size in the wild populations included in the study (Isabela and Liguasan Marsh). The programme operates on the concept that bottlenecked populations exhibit a temporary heterozygosity excess. Average heterozygosity (H_E) was compared to the observed heterozygosity (H_O) under the infinite allele model (IAM), step-wise mutation model (SMM), and the two-Phase model (TPM), with the proportion of SMM in the TPM set at 70%. The Wilcoxon sign-rank test was used to determine statistical significance of the result.

Population Assignment for PWRCC captive crocodiles and selection of breeders for the reintroduction programme

The PWRCC population consisted of crocodiles from different sources in the Philippines. Although there was information available on which captive facility the founder stocks came from, the wild sources of these animals were uncertain. In order to determine the most likely source populations of crocodiles, the PWRCC data set was analysed using WHICHRUN version 3.2 (M. Banks & Eichert, 2000). Compared to other assignment tests, WHICHRUN uses jackknifing and critical population routines, in addition to maximum likelihood, to determine population assignment (M. Banks & Eichert, 2000). I used five putative source populations for my data: two populations from the island of Mindanao (Davao Crocodile Park and Liguasan Marsh); one source population from the Visayas region (Silliman University); and two populations from Luzon (Abra and Isabela). Genetic distance estimates, including Nei *et al.*'s (1983) improved genetic distance (DAN), were then estimated in MSA and pairwise genetic distances between male and female crocodiles

were tabulated. We considered only dyads with pairwise distances (DAN) greater than or equal to 0.60. Selected dyad members were then tested in SPAGeDi v.1.2 (Hardy & Vekemans, 2002) for relationship coefficients, which might indicate individuals with close kinship, using the relatedness estimator r from Queller and Goodnight (1989).

2.3 Results

Hybrids in the data set

Tabora *et al.* (2010) identified 57 crocodiles in the data set that have *C. porosus* (P1) D-loop haplotypes (Appendix B). The variable sites among the seven haplotypes identified are shown in Table 4. Mitochondrial tRNA^{Pro}-tRNA^{Phe}-Dloop (645 bp) sequencing data from 618 crocodiles showed 49 variable sites and 43 parsimony informative sites. The estimated difference between haplotypes M1-M6 and the P1 haplotype was 6.2– 6.4%. This is the same genetic distance estimated between the M1 to M6 haplotypes and *C. porosus*. Maximum likelihood and Bayesian analyses group the P1 haplotype with *C. porosus* whereas haplotypes M1-M6 grouped together in one distinct cluster (Tabora *et al.*, 2010).

Locus CpP302 was found to have a high frequency of null alleles ($F > 0.20$) and was excluded from analysis. STRUCTURE V.2.3.1 output from the present study, using $K = 7$, grouped all 57 individuals with a P1 haplotype in the same cluster (Figure 9). Comparison of D-loop haplotypes with the STRUCTURE output revealed that five individuals that were included and strongly assigned ($Q > 0.4$) to the ‘P1’ cluster had *C. mindorensis* (M) haplotypes (PWb054, PWb139, PWb095, PWb313, PWc006). This finding was seen as an indication of bidirectional hybridisation. The STRUCTURE output was approached conservatively by considering individuals with a proportion of membership greater than or equal to 5% in the ‘P1’ cluster as potential hybrids. A total of 92 crocodiles were thus identified, 91 of which came from PWRCC and one from the Davao City Crocodile Park (Appendix B). All ninety-two crocodiles were considered hybrids and they were removed from further population genetic analyses. One microsatellite locus, CpP2516, was identified as monomorphic once hybrids were removed from the data set and this locus was excluded from further analyses. Overall, 526 pure *C. mindorensis* were analysed at 11 microsatellite loci for population genetic estimates.

Table 4. Variable sites between D-loop haplotypes and haplotype distribution for mitochondrial tRNA^{Pro}-tRNA^{Phe}-D-loop region sequences in *Crocodylus mindorensis* (Tabora et al., 2010).

	1111111111111122222333444444444444444555555555566	IS	AB	SU	PW	CA	LM	DC	Total
	35688892223334444581255049011223334444812234456901	(104)	(4)	(20)	(465)	(3)	(14)	(8)	
	0094780125259678909575933958919679367871856760924								
M1	TGA-AACATTAT-CCCCTCGCCCTCGCTATCCTCCCAACTGTGTGGCT-	41	-	-	-	-	-	-	41
M2	...-.....-.....C.....-	63	4	-	-	-	-	-	67
M3	...-G.....-.....A.....C.....A...A...-	-	-	19	-	3	-	-	22
M4	...-G.....-.....C.A...C.....A...-	-	-	1	24	-	-	-	25
M5	...-G.....-.....A...C.....C.....A...-	-	-	-	382	-	14	8	404
M6	...-G.....-.....A.....A...A...-	-	-	-	2	-	-	-	2
P1	CA-T.GTGCA-CTTATG.TATTT.TATCGCGT.TTTGCTC.CAATATCG	-	-	-	57	-	-	-	57

P1 = *Crocodylus porosus* sequence. Sample sizes for each location or survey facility are indicated in parentheses: PW = Palawan Wildlife, Rescue and Conservation Centre (PWRCC); IS = Isabela (Isabela province, Luzon); DC = Davao City Crocodile Park (Davao City, Mindanao); SU = Silliman University (Negros, Visayas); LM = Liguasan Marsh (North Cotabato, Mindanao); AB = Binungan river (Abra, Luzon); CA = Calait Wildlife Sanctuary (Palawan, Luzon)

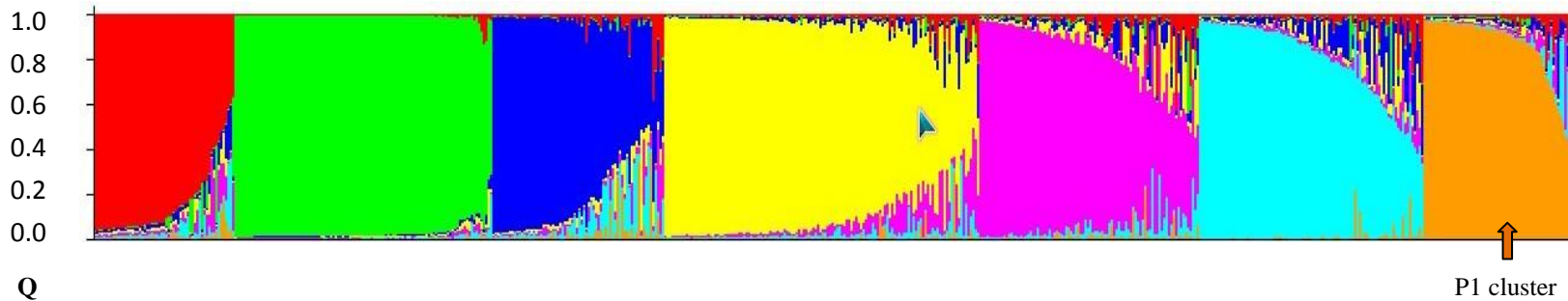


Figure 9. Graphical representation of genetic clustering from STRUCTURE v. 2.3.1 at K=7, involving 618 *Crocodylus mindorensis*. The y-axis represents the frequency (Q) of each individual's genotype in each genetic cluster. All crocodiles identified as having P1 haplotypes in Tabora *et al.*'s (2010) study grouped under the P1 cluster (arrow).

Three crocodiles identified as hybrids in this study were part of the group released in Isabela during the first reintroduction attempt in 2009. These crocodiles (samples PWb 132/SI#7275, PWb189/SI#7548 and PWb214/SI#7626) had membership proportions in the 'P1' cluster of 0.07, 0.96 and 0.97, respectively. This finding demonstrates the difficulty of recognising *C. porosus*-*C. mindorensis* hybrids in captivity.

General levels of diversity

A total of 77 alleles were scored over the whole microsatellite data set, with a mean of 7 alleles per locus. The 11 loci showed variable polymorphic information content (range = 0.45 - 0.92), with a mean PIC of 0.51 (Table 5). Allelic richness ranged from 1.58 to 2.51 and H_E ranged from 0.40 to 0.69. The Silliman University population had the highest H_E , whilst Abra had the lowest H_E and allelic richness. The remaining populations had intermediate heterozygosity values. Most populations did not deviate significantly from Hardy-Weinberg expectations. PWRCC, Silliman University & Isabela deviated significantly from HW expectations, at $\alpha = 0.001$. Mean number of alleles, allelic richness and observed and expected heterozygosities, summarised for each population, are shown in Table 6.

Table 5. Primer sequences (5' to 3') with dye label and microsatellite locus information including observed number of alleles (k), polymorphic information content (PIC) and size range in 526 *C. mindorensis*.

Locus	Primer Sequence (5'-3')	Repeat Motif	PIC	k	Size Range	Reference
CpP4116	F: CAGTCGGGCGTCATCATTTCAAATATCCGTGTCAT R: GTTTACCGCTTGAACCTTGT	(AGAT) ₁₂	0.39	3	212- 220	Miles <i>et al.</i> 2008
CpP305	F: GTTTGTAGCTGGAACCTGATAGTG R: CAGTCGGGCGTCATCAGGTTAACACGTGGTAACT ACA	(AC) ₁₆	0.73	10	189- 211	Miles <i>et al.</i> 2008
CpP801	F: CAGTCGGGCGTCATCATTTGGCATTAGATTGGTAGAC R: CAGTCGGGCGTCATCATTTGGCATTAGATTGGTAGAC	(AGAT) ₁₅	0.56	3	159- 179	Miles <i>et al.</i> 2008
CpP1610	F: CAGTCGGGCGTCATCATAGAGGGATTTTGACTGT R: GTTTGATTATTTTGTCTGGGTTCTT	(AGAT) ₅	0.59	4	296- 308	Miles <i>et al.</i> 2008
CpP1708	F: GTTTCCATTATGGCAAATCTTGTA R: CAGTCGGGCGTCATCAATTGGGATCTTGGATCTG	(ACTC) ₆	0.38	4	213- 229	Miles <i>et al.</i> 2008
CpP3008	F: CAGTCGGGCGTCATCAACAACCTGGCACATCTCA R: GTTTCCCGTAGCCTCCTACTG	(ACAG) ₁₇	0.92	31	338- 482	Miles <i>et al.</i> 2008
CpP4004	F: CAGTCGGGCGTCATCACTGAATTGGGTGGAATAG R: GTTTATCCACATTTTCCATGAC	(AGAT) ₁₀	0.61	5	398- 418	Miles <i>et al.</i> 2008
4HDZ35	F: ^{FAM} GACAGTGTGGIGGGTGC R: TGCTGGCTGCTTGGGAC	(CA) ₈ CG(CA) ₁₄	0.26	3	191- 199	HDZ lab unpublished
4HDZ391	F: ^{FAM} ATGAGTCAGGTGGCAGGTTC R: CATAAATACACTTTTGTAGCAGCAG	(GT) ₁₂	0.44	4	133- 143	HDZ lab unpublished
4HDZ27	F: ^{HEX} GCACACATTCTCTGAGTAAAAAACC R: GGCAGTGGTAGGCTTTGAAAT	(CA) ₁₇	0.35	6	147- 163	HDZ lab unpublished
Amiμ15	F: CACGTACAAATCCATGCTTTC R: GGGAGGGTTCAGTAAGAGACA	(AC) ₁₆	0.44	4	143- 157	Glenn <i>et al.</i> 1998

Table 6. Observed (H_O) and (H_E), expected Hardy-Weinberg heterozygosity, deviation from Hardy-Weinberg Equilibrium (HWE), mean number of alleles (MNA) and allelic richness (AR), in seven populations of *C. mindorensis*

Geographic region	Sampling location	N	HWE	H_O	H_E	MNA	AR
Northern Philippines (N. Luzon)	<i>Isabela</i>	104	***	0.43	0.42	3.90	2.11
	Abra	4	NS	0.16	0.19	1.64	1.58
Mid-Philippines (Palawan, Visayas)	Calauit Game Reserve	3	NS	0.64	0.47	2.00	2.00
	Silliman University	20	***	0.69	0.53	3.00	2.37
Southern Philippines (Mindanao)	Davao Crocodile Park	7	NS	0.45	0.53	3.45	2.51
	<i>Liguasan Marsh</i>	14	NS	0.40	0.43	3.18	2.17
Palawan	PWRCC	374	***	0.46	0.50	6.00	2.42

*** $P < 0.001$;

NS (not significant); wild populations are italicised

Linkage Disequilibrium (LD)

Analysis of genotypic linkage disequilibrium indicated that two populations, PWRCC and Isabela, had extensive LD ($P < 0.05$) after Bonferroni correction (21 and 8 pairs of loci in linkage disequilibrium, respectively). No significant LD was observed for the remainder of the sampling populations. The linkage disequilibrium observed in PWRCC could be explained as an artifact of mating between two or more genetically different subpopulations. When there is limited or no gene flow between subpopulations of a species for several generations, the allele frequencies at many loci become different over time (populations become genetically differentiated). When there is again interbreeding between individuals from such subpopulations, LD is created between pairs of loci that had different allele frequencies in the original subpopulations (Templeton, 2006). The founder population at PWRCC started from a few individuals that came from different sources around the Philippines, indicating that it is likely that they came from different *C. mindorensis* subpopulations.

Linkage disequilibrium may also result from sampling siblings within year classes or individuals from the same clutch (T. King, Kalinowski, Schill, Spidle, & Lubinski, 2001). This could explain the LD observed in the Isabela samples which comprise many individuals sampled opportunistically from the same clutch. When the Isabela population was trimmed down from 104 to 24 crocodiles by taking only one representative from each clutch, and LD analysis was re-run (excluding PWRCC), no significant linkage disequilibrium was observed between any pair of loci across all populations (Table 7).

Table 7. Number of loci pairs across all populations with significant linkage disequilibrium for different population scenarios. Results are shown after Bonferroni correction.

Populations included in the analysis	Sample size (N)	Number of loci across all populations with significant linkage disequilibrium ($P < 0.05$)
All seven populations (PWRCC, Davao Crocodile Park, Silliman, Calauit, Isabela, Liguasan Marsh, Abra)	526	24
Six populations (excluding PWRCC)	152	10
Six populations (excluding PWRCC but with Isabela population at $N = 24$)	72	0

Structuring of Populations

The analysis of genic differentiation (Fisher's method) revealed significant population differentiation in all population pairs except between PWRCC and Davao Crocodile Park ($P > 0.05$) (Table 8). Similarity in the ancestry of the animals from both institutions could be the reason why no significant difference was observed. This finding is supported by the maximum likelihood values estimated by WHICHRUN v.3.2, which indicated that *C. mindorensis* at PWRCC were likely assigned to either one of two source populations on Mindanao: those with ancestry similar to the animals kept at Davao Crocodile Park, or those from the Liguasan Marsh population. Animal records from

PWRCC confirmed that many of the foundation animals were acquired from Davao Crocodile Park. Foundation stock at the Davao Crocodile Park was believed to have come from Liguasan Marsh.

Table 8. P-values for genic differentiation across all loci for each population pair

Population Pair		Chi ²	df	P-value
PWRCC	Davao Croc Park	26.97	22	0.212403
PWRCC	Silliman University	infinity	22	highly significant
Davao Croc Park	Silliman University	infinity	22	highly significant
PWRCC	Calautit	infinity	22	highly significant
Davao Croc Park	Calautit	infinity	22	0.001184
Silliman University	Calautit	47.71	22	0.000131
PWRCC	Isabela	54.69	22	highly significant
Davao Croc Park	Isabela	infinity	22	highly significant
Silliman University	Isabela	infinity	22	highly significant
Calautit	Isabela	infinity	22	highly significant
PWRCC	Liguasan	105.33	22	0.000000
Davao Croc Park	Liguasan	48.74	22	0.000866
Silliman University	Liguasan	infinity	22	highly significant
Calautit	Liguasan	95.77	22	0.000000
Isabela	Liguasan	infinity	22	highly significant
PWRCC	Abra	infinity	22	highly significant
Davao Croc Park	Abra	infinity	22	highly significant
Silliman University	Abra	infinity	22	highly significant
Calautit	Abra	116.50	22	0.000000
Isabela	Abra	infinity	20	highly significant
Liguasan	Abra	infinity	22	highly significant

Weir & Cockerham's (1984) estimation of genetic differentiation (F_{ST}) across all populations and loci was 0.29. F_{ST} values between population pairs were highest when populations were paired with Abra ($F_{ST} = 0.317-0.602$) and with Isabela ($F_{ST} = 0.289 - 0.368$) (Table 9). The lowest F_{ST} value was seen between PWRCC and Davao Crocodile Park. The degree of genetic differentiation reflected the geographic distance between the sites. High F_{ST} values were recorded between populations that are geographically distant (e.g. Isabela and Abra are located in Northern Philippines, whilst the remainder are located in mid- and southern Philippines), and lower F_{ST} values were recorded for populations that are in closer proximity.

Table 9. Pairwise values of fixation indices (F_{ST} and F_{IS}) in seven populations of *C. mindorensis*. F_{ST} below diagonal, significance after Bonferroni correction above the diagonal, F_{IS} in bold on the diagonal.

	PWRCC	Davao Crocodile Park	Silliman University	Calauit Game Reserve	Isabela	Liguasan Marsh	Abra
PWRCC	0.084	NS	***	***	***	***	***
Davao Crocodile Park	-0.015	0.161	***	NS	***	NS	NS
Silliman University	0.116	0.104	-0.302	*	***	***	*
Calauit Game Reserve	0.122	0.098	0.106	-0.355	***	*	NS
Isabela	0.339	0.341	0.289	0.323	-0.012	***	***
Liguasan Marsh	0.080	0.056	0.217	0.232	0.368	0.082	**
Abra	0.492	0.531	0.444	0.602	0.317	0.587	0.176

*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$

The low degree of genetic differentiation observed among PWRCC, Davao Crocodile Park and Liguasan Marsh supports the presumption that Liguasan Marsh is the source of founder stock for PWRCC and Davao Crocodile Park. It is interesting to note the high degree of differentiation between the Abra and Isabela populations ($F_{ST} = 0.317$). Although these populations are both in the Northern Philippines, the Abra population is located further inland and is separated from the Isabela population by mountain ranges and human settlements. The high F_{ST} value observed in this study reflects the lack of gene flow between these two populations. The inbreeding coefficient (F_{IS}) indicates an excess of

homozygotes in the following populations: PWRCC, Davao Crocodile Park, Liguasan Marsh and Isabela. The high frequency of related individuals (included in the sampling) might explain the homozygote excess seen in these populations. There was no evidence of a recent bottleneck in wild *C. mindorensis* populations in Isabela and Liguasan Marsh.

Evidence of population differentiation was also found using a Bayesian population analysis assignment test implemented in STRUCTURE. The number of genetic clusters with the greatest log likelihood that best represented the underlying structure in the data set was $K = 2$. The break in the slope of the distribution of $P(X/K)$ was also seen at $K = 2$. These results group the Isabela and Abra populations into one cluster, which is a strong indication of genetic differentiation between the Luzon population (northern Philippines) and the remainder of the populations (mid and southern Philippines) (Figure 10). The Silliman University population (located in the mid-Philippines) shows admixture of Luzon genes into a primarily southern stock. The crocodiles from Silliman included in the sampling consists of a founder pair (female *C. mindorensis* from the Visayas region and male from Mindanao) and their progeny. This pairing represents the only confirmed union between crocodiles coming from two separate geographic regions. This genetic admixture is reflected in the STRUCTURE results (Fig. 10, Population #3).

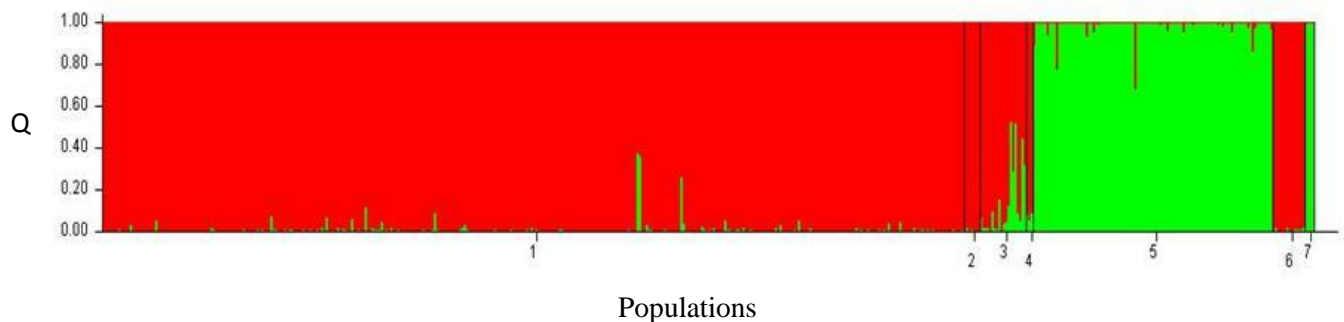


Figure 10. Genetic clustering representation from STRUCTURE v. 2.3.1 at $K=2$, involving 526 *Crocodylus mindorensis*. The y-axis represents the frequency (Q) of each individual's genotype in each genetic cluster, while the populations are on the x-axis. 1 = PWRCC; 2 = Davao Crocodile Park; 3 = Silliman University; 4 = Calauit Game Reserve; 5 = Isabela; 6 = Liguasan Marsh; 7 = Abra

DAN mating assignments

A total of 97 *C. mindorensis* pairings were identified as having pairwise DAN ≥ 0.6 (Appendix D). Three dyads, in combination with SI 2890, SI 2904 and SI 2923, produced relationship coefficients of 0.686, 0.728 and 0.805. Records show that these individuals hatched within three days of each other, suggesting that they may be clutch mates. Two male *C. mindorensis* (PwC395 and PwC426) also had identical genotypes. PWRCC records reveal that these two distinct individuals were acquired in the same year and came from the same source on Mindanao, an indication that they could be monozygotic twins.

Using an individual only once in a pairing gave a total of 16 *C. mindorensis* pairs (highlighted in Appendix C). Figure 11 shows the relationship coefficient distribution of these pairs, compared to the frequency distributions of known relationships, from a simulation of 10,000 individuals of known ancestries. The figure illustrates that most of the chosen crocodile pairs have relationship coefficients that are low, reflecting the unrelatedness of individuals in each pair. Crocodiles were paired together in this way to prevent sibling to sibling or parent to offspring mating.

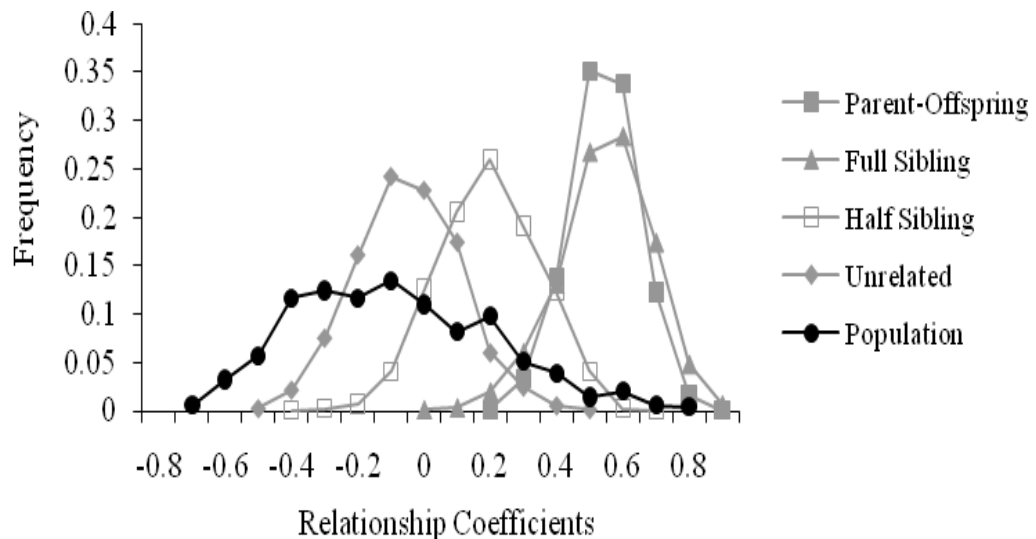


Figure 11. Relationship coefficient distribution of 16 recommended founder pairings for the reintroduction programme.

2.4 Discussion

Population Structure and genetic diversity

This study confirmed that on a broad geographic scale, genetic structuring exists within *C. mindorensis*. The degree of genetic differentiation ($F_{ST} = 0.29 - 0.60$) between the northern Philippines populations (Luzon) and the mid- and southern populations is comparable to the differentiation seen in *C. niloticus* across Africa and Madagascar ($F_{ST} = 0.17-0.59$) (Hekkala *et al.* 2009). Less intraspecific differentiation has been reported for other species of crocodylians, such as *Alligator mississippiensis* (Glenn *et al.*, 1998), *Crocodylus moreletii* (Dever, et al., 2002; Ray, et al., 2004) and *Melanosuchus niger* (De Thoisy, et al., 2006). The pattern of differentiation seen in *C. mindorensis* reflects geographic structuring, with a variation in microsatellite allele frequency that is quite distinct between populations in the two most geographically distant islands of Luzon and Mindanao.

Four distinct faunal regions based on species diversity, richness and endemism are recognized for the Philippines. These are the Luzon, Palawan, Mindanao and Negros regions, which represent the large island masses comprising the Philippines during the late Pleistocene (Heaney, 1986). Within-group faunal similarity is high in these regions, a trend that is reflected in the distribution of reptile and amphibian diversity in the country (Brown & Alcala, 1970). The pattern of genetic differentiation seen in the present study, to some degree, reflect the faunal similarity in the regions, with Philippine crocodile populations in Luzon exhibiting genetic differentiation from those found in Mindanao. Although crocodylians appear to be highly capable of dispersing large distances by sea or waterways, the climatic changes in the Pleistocene could have had an effect on the dispersal of *C. mindorensis*. Historic anthropogenic modifications of the landscape would have contributed to the fragmentation of populations leading to the current levels of geographic partitioning seen in the species.

The range of observed heterozygosity in *C. mindorensis* in this study was 0.16 to 0.69, with a mean heterozygosity of 0.46. This is within the range reported for wild populations of *C. niloticus*, $H_O = 0.25-0.61$ (Hekkala, Amato, DeSalle, & Blum, 2009) and $H_O = 0.51$ (Bishop, Leslie, Bourquin, & O'Ryan, 2009); *C. moreletti*, $H_O = 0.49$ (Dever, et

al., 2002) and *Melanosuchus niger*, $H_o = 0.47-0.70$ (de Thoisy *et al.*, 2006). The Philippine crocodile used to be widely distributed across the Philippine archipelago, but it is now limited to a few, small and fragmented wild populations in Luzon and Mindanao (Van Weerd & Van der Ploeg, 2003). Moreover, despite the severe contraction in population size and suitable habitat during the last few decades, I found that captive and wild populations of Philippine crocodiles continue to harbour intermediate levels of heterozygosity (with the exception of the Abra population). In addition, there was no evidence of a recent genetic bottleneck seen in the Isabela and Liguasan Marsh populations.

Populations which suffered from a severe decline in number may not exhibit a genetic bottleneck if past N_E has always been low due to mating systems, variability in population size or metapopulation dynamics (Pimm, 1989). Moreover, it has been shown that life history traits such as longevity and generation time buffer the loss of genetic diversity even if the species had gone through a severe reduction in population size, such as in the case of the Nile crocodile (Bishop *et al.*, 2009) and the greater one-horned rhinoceros (Dinnerstein & McCracken, 1990). Similarly, the Philippine crocodile's longevity and late sexual maturity could explain the retention of moderate levels of genetic diversity in wild populations as seen in this study. This finding, however, must not contradict the fact that severe reductions in population size has occurred, and most likely reflected a decline in the effective population size (N_E). Efforts should focus not only on maintaining a high level of genetic diversity, but also on the maintenance of N_E needed to maintain this level of diversity, or else the population will be susceptible to the negative effects of drift and selection (Bishop *et al.*, 2009).

The maintenance of genetic diversity in large captive populations such as PWRCC and Davao Crocodile Park could be explained by a large gene pool coming from founder individuals derived from subpopulations in Mindanao, but generally from Liguasan Marsh. High heterozygosity levels in the Silliman University and Calautit *C. mindorensis* could possibly reflect the high genetic variability carried by the founder stock. Liguasan Marsh, a wetland of about 220,000 ha, potentially harbours the greatest number of wild Philippine crocodiles in the country. The genetic diversity for that area may be under-estimated in this study due to a limited sampling.

The low heterozygosity ($H_O = 0.16$) seen in the Abra population is worth further investigation. Although the Abra samples analysed in this study came from a zoo in that province, records show that these crocodiles were caught from the Binungan River, one of the tributaries of the much larger Abra River. The low genetic diversity observed in the Abra population could be a consequence of the small sample size. More samples for genetic analysis from the wild crocodile population in Abra are needed in order to confirm this level of diversity. The current estimate does however suggest prioritisation of field research in that area. If indeed the Abra population is suffering from low genetic diversity, then management measures such as the supplementation of crocodiles from more diverse populations may be started as a form of genetic rescue. Nonetheless, it should be highlighted that efforts such as these would be ineffective, if the main reason for the low genetic variability observed is not dealt with first. For example, if there is inbreeding because of a low N_E due to deliberate killing of adult crocodiles, then conservation initiatives which address this issue should be undertaken first.

Caution should be exercised when interpreting the results of this study due to small sample sizes and non-random sampling in some populations. Population genetic parameters are difficult to estimate with certainty for a species such as *C. mindorensis* where captive populations seem to be more abundant than wild populations that are rare and isolated and therefore difficult to sample. Moreover, all the populations in this study, except for two, are managed populations wherein many of the basic assumptions of population genetics theory do not hold. Despite all these hindrances, a clear population structure was observed based on assignment tests and genetic differentiation estimates. Baseline genetic diversity estimates for wild and captive populations were also generated and this information will be useful for conservation planning and management.

Diversity in the PWRCC population and recommendations for captive breeding

The average observed heterozygosity, across 11 loci in the PWRCC population, is 0.46. This is in the range reported for captive purebred *C. siamensis*, $H_O = 0.42 \pm 0.17$ (FitzSimmons *et al.* 2002) and farmed *C. porosus*, $H_O = 0.59$ (Isberg *et al.* 2004). A standard approach would be to select reintroduction candidates with high heterozygosity values (Dodd & Seigel, 1991; Moritz, 1999; Snyder, *et al.*, 1996) because a higher genetic

variability is correlated with better adaptability to a changing environment and also long-term population persistence (Lacy, 1987, 1997; Lande & Shannon, 1996). Since the main goal of reintroductions is to establish self-supporting populations, the selection of *C. mindorensis* individuals with high heterozygosity is a useful basis to screen potential reintroduction candidates.

For the selection of candidate parents for the reintroduction programme, pairwise genetic distance and relationship coefficient were estimated for each pair to ensure that kinship is minimised. The method of minimising kinship has been shown to retain the highest level of allelic and gene diversity in an experimental population of *Drosophila melanogaster* (Montgomery, et al., 1997). In a broad sense wildlife managers agree that captive populations should retain at least 95% of the genetic diversity found in the wild population (W. Miller, Wright, & Zhang, 2010). In the absence of conclusive genetic diversity estimates for wild populations of *C. mindorensis*, the selection method used in this study was intended to maximise the retention of genetic diversity in the PWRCC stocks.

The population assignment test clearly reflected the origin of the founder population when compared with the animal records kept at PWRCC. Between 1987 and 1994, PWRCC acquired a total of 235 Philippine crocodiles for its captive breeding programme, 11 of which were caught in the wild in Mindanao and the rest were sourced from private collectors in Mindanao and the Visayas region (Sumiller 2000). This study identified *C. porosus* x *C. mindorensis* hybrids that were used as breeding stock; thus, explaining the high proportion of hybrids identified in the captive-bred population. It is interesting to note that none of the hybrids used as founder stock were sourced from the wild. In addition, Tabora *et al.* (2010) found no indication of hybridisation in wild *C. mindorensis* populations, even in areas where *C. mindorensis* and *C. porosus* occur sympatrically such as Liguasan Marsh. Although hybridisation of *C. mindorensis* with *C. porosus* in the wild remains inconclusive, the present study has confirmed that unintentional hybridisation has occurred in captive environments and those hybrids are difficult to distinguish morphologically. This finding makes genetic screening of all potential reintroduction candidates essential.

Ross and Alcala (1983) provided morphologic guidelines to help differentiate the Philippine crocodile from the saltwater crocodile (*C. porosus*). There are no published studies, however, on how to distinguish hybrids of the two species. We now have a good opportunity to develop such a classification using genetic data as a guide for hybrid identification. In many cases, hybrids are difficult to distinguish by morphology alone. For instance, Ray *et al.* (2004) and Cedeño-Vasquez (2008) found no morphological anomalies in some *C. moreletii* – *C. acutus* hybrids, a situation they attributed to introgression. It is cases such as these that genetic screening to aid in hybrid identification becomes essential.

A total of 460 (80.3 %) of the PWRCC crocodile population was genetically analysed in this study. Ninety-one (19.6%) of those sampled were identified as hybrids. Thus, it would be appropriate to genetically screen the remaining crocodiles at PWRCC in order to purge the entire captive stock of hybrids and identify animals which could be used for the reintroduction programme. Many Philippine crocodiles were transferred from PWRCC to various zoos around the Philippines and overseas, through conservation breeding loan agreements (Banks, 2005). In the light of these recent findings, it is important that all facilities holding *C. mindorensis* must submit tissue samples from their animals for genetic testing and that a restraint on breeding attempts is made, until the genetic integrity of stocks are confirmed. Translocations should cease, for the present time, to allow the recovery team to keep track of Philippine crocodile locations and numbers and prevent any risk of releasing hybrids into the wild.

In the present study, three hybrid crocodiles released during the first reintroduction attempt in 2009 were identified. This finding creates a dilemma for the reintroduction team because of the risk of *C. porosus* introgression into wild *C. mindorensis* populations. Although natural hybridisation has been confirmed in crocodylians, particularly between *C. moreletii* and *C. acutus* (Ray *et al.*, 2004; Cedeño-Vasquez *et al.*, 2008), anthropogenic causes of hybridisation could potentially lead to species extinction through hybridisation and introgression (Rhymer & Simberloff, 1996). In this case, the rare and endemic Philippine crocodile is at a genetic disadvantage compared to the more numerous and more widespread, *C. porosus*. Therefore, it is important that the hybrids identified in this study be removed from the reintroduced population.

Management units

A clear population structure between the northern and southern Philippine crocodile populations was seen in this study based on differences in microsatellite allele frequencies. The question of whether this genetic difference is significant to justify the management of the northern and southern populations as separate management units is still an open question. Managing *C. mindorensis* populations separately would have important management implications for future translocations within the country.

Crandall *et al.* (2000) argued that population distinctiveness should not be based solely on neutral genetic information. They proposed the concept of ecological and genetic exchangeability as a better method to assess conservation units in order to ensure retention of the adaptive evolutionary processes within the species. If individuals are genetically and ecologically exchangeable, then partitioning of the species into different management units is not necessary. Ecological exchangeability refers to similarities in adaptive traits such as life history, habitat parameters, morphological characteristics and ecological niche occupied. On the other hand, genetic exchangeability considers the gene flow between populations revealed by different genetic markers. Severely restricted gene flow that is in agreement with geographic isolation and presence of unique alleles are some of the reasons to reject genetic exchangeability. Furthermore, genetic and ecological exchangeability must be assessed in view of recent and historical time frame (Crandall *et al.*, 2000).

Although mtDNA (Tabora, et al., 2010) and microsatellite evidence (this study) for genetic partitioning within *C. mindorensis* exists, there is currently no evidence that this correlates to any evolutionary/adaptive advantage. Splitting the management of *C. mindorensis* into two entirely separate units does not present any significant advantage to the species' conservation at the present time. It is important to assess the ecological and biological differences between the northern and southern populations of the Philippine crocodile, along with the genetic findings, to clarify the issue of separate management units. In the mean time, a conservative approach to supplementations/ reintroductions should be followed. Crocodiles for supplementation of wild existing populations should come from the same geographic region but individuals with mixed geographic ancestry can be used to form new populations.

The two captive breeding facilities that are the most likely sources of candidates for the Philippine crocodile reintroduction programme are PWRCC and Silliman University. PWRCC, with almost all of the founder populations originating from Mindanao, is the best source of reintroduction candidates in the southern part of the country. Similarly, Silliman University crocodiles, which trace their lineage to the only known surviving crocodile from the Visayas region (mid-Philippines), should have the priority in reintroductions in this region. For the Philippine crocodile population in Luzon, crocodiles used for augmentation of extant wild populations should also come from within Luzon. However, crocodiles of mixed geographic ancestry can be used to create new populations in vacant suitable habitats in the absence of more suitable candidates.

The microsatellite markers used in this study have provided an overview of the species integrity and the genetic partitioning present in captive and wild Philippine crocodiles. The analysis has allowed the development of guidelines and practical recommendations, which conservation managers can use to guide recovery efforts. The greatest challenge at the present time is to shift the balance from a relatively *ex-situ* approach to crocodile conservation to an *in-situ* one – the conservation of existing wild *C. mindorensis* populations and restoration through reintroduction of genetically healthy populations that are free to fulfil their full evolutionary potential.

CHAPTER THREE: SUMMARY AND APPLICATION OF CONSERVATION GENETICS TO THE MANAGEMENT OF *C. MINDORENSIS*

3.1 Summary of Major Findings

Population Structure

Statistical tests of population differentiation (Fisher's exact test and Weir & Cockerham's F_{ST} estimation) indicated a high degree of genetic differentiation amongst *C. mindorensis*, specifically between populations from the northern Philippines and those from the southern part of the country. A Bayesian based assignment test also demonstrated the presence of geographic structuring. This test grouped the individuals from the two Northern Luzon populations together and all the remainder were placed in another genetic cluster.

Genetic Integrity of Philippine crocodile populations

Estimates of genetic diversity revealed moderate levels present in captive and wild populations of *C. mindorensis*. An exception was the captive population in Abra ($H_O = 0.16$). However, more samples are needed from this population in order to substantiate the low level of genetic diversity observed.

Ninety-two putative *C. mindorensis* – *C. porosus* hybrids were identified based on mtDNA sequencing and genotyping at 13 microsatellite loci. All hybrids came from captive breeding institutions: 91 from the Palawan Wildlife Rescue and Conservation Centre and one from the Davao Crocodile Park. I did not detect any evidence that natural hybridisation between *C. porosus* and *C. mindorensis* has occurred in the wild. Three of the hybrids identified in this study were part of the group released during the first reintroduction in Isabela in 2009. As hybrid individuals are poor candidates for re-establishment of *C. mindorensis* populations, their discovery has important implications and emphasises the difficulty of identifying *C. mindorensis*-*C. porosus* hybrids by visual assessment alone.

Founders and candidates for the reintroduction programme

By maximising the genetic distance between pairs, 16 potential founder pairings of pure *C. mindorensis* descent were identified in the PWRCC population. Heterozygosity values were also generated for all the captive Philippine crocodiles sampled in this study. These values could be used to establish guidelines for the selection of candidates for future reintroduction purposes.

3.2 Application to the Conservation and Management of the Philippine Crocodile

Captive breeding must be closely integrated with *in-situ* programmes in such a way that it supports actions aimed at conserving populations in the wild. For the Philippine crocodile, the emphasis, for the time being, should not be in the generation of more captive-bred crocodiles but in the management of the captive population in a way that genetic erosion is prevented. This can be achieved through maintenance of genetic diversity, prevention of inbreeding, and appropriate selection of founders and identification of hybrids. Once appropriate measures are in place, targets that coincide with reintroduction goals can then be set.

A review of the captive management policy for the Philippine crocodile is warranted. It is important to explicitly identify the goals that need to be achieved in a given time frame, because the maintenance of large carnivores in captivity, such as crocodiles, is an expensive undertaking (Snyder *et al.*, 1996). The primary goal of the captive programme should be to support the restoration of viable and genetically diverse populations of Philippine crocodile across its natural range, by maintaining a representative population in captivity and providing genetically and physiologically healthy animals for reintroduction.

What to do with the hybrids?

It is clear that hybrids must be removed from the conservation breeding programme in order to prevent further *C. porosus* introgression and to generate pure *C. mindorensis* for release. The large number of hybrids identified in this study, nonetheless, presents an excellent source of data for the investigation of hybrid morphological traits. The value of hides and meat from *C. porosus* - *C. mindorensis* hybrids could also be investigated. Tanned skins from Philippine crocodiles have been considered inferior compared to that of *C. porosus* (Ernesto Connate, pers. comm.). Many crocodile farms, such as those in

Thailand, are known to deliberately hybridise different species of crocodiles to improve skin characteristics for commercial trade. Better skin quality, a faster growth rate, larger clutches and a higher survival rate have been observed within interspecies hybrids, thus making them highly desirable for farming or ranching (Thorbjarnarson, 1992). There is a potential for the hybrids identified in this study to provide economic incentives as well as scientific knowledge. Studies dealing with these interspecies hybrids should be pursued.

Reintroduction Programme

The three hybrid crocodiles released in 2009 must be recaptured and removed from the population. If they are allowed to remain in the wild, they could contribute to the development of a hybrid swarm (F. Allendorf, et al., 2001). This would be of no benefit to the conservation of genetically pure *C. mindorensis* populations in the wild. At the same time, removal of the hybrids would decrease competition with *C. mindorensis* for food and territory.

Conservation managers are interested to know whether the genetic difference between the northern and southern Philippine crocodile population is substantial enough to warrant separate management. The answer to this question has implications for future re-introduction or supplementation plans for the species. Two concerns brought about by mixing genetically different populations include loss of genetic uniqueness and outbreeding depression (Bullock & Hodder, 1997). When there is differentiation caused by selection that allows adaptation in a particular environment, genetic mixing would destroy this local adaptation and could lead to a decrease in fitness (outbreeding depression) (Conner & Hartl, 2004). On the other hand, supplementation of animals from a larger and more diverse population could be highly beneficial for a small population suffering from inbreeding depression and low genetic variability. It is important to note that there is very little agreement on the outbreeding risks involved when two genetically distinct populations are mixed together (Friar, Boose, LaDoux, Roalson, & Robichaux, 2001). Decisions should be made by weighing the potential benefits of increased genetic variation against the risk presented by outbreeding depression.

As discussed in the previous chapter, evidence based on neutral genetic markers alone should not be the sole basis when deciding conservation units. Adaptive divergence

and current and historic geographic separation must be taken into account as well (Crandall *et al.*, 2000). Although a clear population structure was observed between the northern and southern *C. mindorensis* populations, it is uncertain if this genetic difference has any ecological or adaptive significance. Moreover, the difference in microsatellite allele frequencies observed in this study could very well be the result of genetic drift resulting from fairly recent fragmentation & decreased population sizes, and not because of long-term evolutionary forces. Because there is not enough evidence for ecological exchangeability, a conservative management approach would be to supplement existing populations with crocodiles from the same geographic region and use animals with mixed geographic origin to create new populations.

Although the low genetic diversity estimate for the Philippine crocodiles in Abra needs to be confirmed, this finding suggests that the urgency of management action is greater on this population, compared to other populations. Very little is known about this relatively isolated wild population; thus, determination of the current status of the Abra population is essential so that appropriate management intervention could be developed. If proven to have low genetic variability, then genetic intervention through supplementation with wild or captive-bred Philippine crocodiles could be undertaken in order to increase genetic variability.

Recovery programmes for endangered species often proceed with limited ecological and genetic information. Conservation managers frequently face the task of balancing the expected positive outcomes from recovery efforts, with the risks associated with insufficient information. The first reintroduction of the Philippine crocodile in Isabela was an example of such an effort. Despite problems such as the unintentional release of hybrid crocodiles, the reintroduction is currently generating a large amount of information about movement and survival. This valuable data can be used to improve and strategise crocodile release in the future.

3.3 Recommendation for Future Research Projects

Population Genetics

Future research projects should investigate the fine-scale genetic structure of wild *C. mindorensis*, through sampling wild crocodiles from a larger area and preferably sampling multiple individuals from the same cohort or generation. This method of sampling would be ideal, because allele frequencies change over time as well as space (Waples, 1989). The set of eleven microsatellite loci, used in this study, was sufficient to detect structuring in *C. mindorensis*, over a broad geographic scale. Testing the utility of these markers in resolving fine-scale population structure is worth investigation. Genetic structuring over a small geographic scale has been reported in American alligators (Ryberg, Fitzgerald, Honeycutt, & Cathey, 2002), broad-snouted caiman (Verdade, Zucoloto, & Coutinho, 2002) and Black caimans inhabiting different but nearby habitat types (de Thoisy *et al.*, 2006). These studies suggest that the genetic differences observed between populations of each species could be the result of selection, differences in life history parameters and mating systems, habitat types or barriers to dispersal. It is interesting to see if a similar pattern might exist in *C. mindorensis*.

Basic ecological research is important in understanding the results of molecular studies. Research focusing on movement patterns of wild Philippine crocodiles will help in the identification and subsequent protection of dispersal routes that are crucial for gene flow. A basic population estimate of the wild population, on the other hand, will help in the assessment of effective population sizes, which would be useful for establishing appropriate levels of gene diversity. Also, it is recommended that possible adaptive differences between northern (Luzon) and southern (Mindanao) wild populations of *C. mindorensis* be investigated through a comparison of life history, phenotypic traits and habitat differences.

Temporal population genetics

Preserved specimens of *C. mindorensis* in museums could be utilised in order to examine population genetic changes over time. This could be done by comparing microsatellite DNA data between samples collected within the last 100 years or so with contemporary samples. In addition, museum samples from areas where the species is now extinct may provide evidence of the historic processes that shaped the distribution of the

current gene diversity (Thomas, Paabo, Villablanca, & Wilson, 1990). Patterns of population decline and loss of genetic diversity have been deduced using historic and contemporary samples from a wide variety of threatened species. Such information could serve as guidelines for management actions (Wandeler, Hoeck, & Keller, 2007).

Hybridisation

Researchers investigating the morphology of *C. porosus* – *C. mindorensis* hybrids would be advised to generate data that will aid in the visual identification of admixed individuals. Building on the results of this study, future research should also incorporate genotype data from pure *C. porosus* using more or the same set of microsatellite loci to identify hybrid categories and determine the extent of backcrossing in captive facilities. The software NEWHYBRIDS is an example of a computer programme that can estimate the degree of backcrossing. Rodriguez *et al.* (2008) used the software NEWHYBRIDS to classify *C. moreletii* x *C. acutus* hybrids into six categories: pure *C. moreletii*; pure *C. acutus*; F1 hybrids; F2 hybrids; and backcrosses to either parental species.

Future studies should also aim to discover microsatellite loci, which are diagnostic for either *C. mindorensis* or *C. porosus*. Locus CpP2516 appears to be promising in the detection of recent *C.porosus* - *C.mindorensis* hybridisation. Nonetheless, genotype data from *C. porosus* individuals are needed, in order to verify if the alleles identified in this study are fixed for either species. Screening the remaining Philippine crocodiles in captivity and obtaining more samples from the wild will assist in characterising the full range of allelic diversity in the species.

The lack of *C. porosus* samples genotyped for the same set of microsatellite loci is a limitation of this study. For this reason, the hybrids identified herein, especially those that were not strongly assigned to the hybrid cluster, cannot be identified as hybrids with absolute certainty. Efforts are currently underway to genotype *C. porosus* individuals to support this study.

Conclusion

Genetic research of endangered species is only intended to guide management decisions and therefore, it does not in itself generate a stringent set of rules that have to be followed. Although a clear population structuring, based on regional distribution was observed in this study, *C. mindorensis* populations need not be managed strictly as separate conservation units based on differences seen with neutral genetic markers alone. The difference in allele frequencies observed in this study could very well be the result of fragmentation due to fairly recent anthropogenic changes in the landscape, and not because of long-term evolutionary forces. Consequently, the genetic differences between populations could very well reflect pre-existing structuring. Because scientific evidence at present is not sufficient to support ecological exchangeability, a conservative approach to management is recommended.

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Appendix A

Sampling sites, descriptions and number of crocodiles sampled from each site

Sampling Areas	Sitio	Barangay	Municipality/City	Province	Country	Site Description	Number of crocodiles sampled
Palawan Wildlife Rescue & Conservation Centre (PWRCC)		Irawan	Puerto Princesa	Palawan Luzon	Philippines	Government-run captive breeding facility	465
Davao Crocodile Park			Davao	Davao del Sur Mindanao	Philippines	Privately-run theme park	8
Silliman University			Dumaguete	Negros Oriental Visayas	Philippines	Research & captive breeding facility for crocodiles run within the university	14
Calauit Wildlife Sanctuary			Calauit Island	Palawan Luzon	Philippines	Government-run, 3700 hectares game preserve & wildlife sanctuary in the North-western coast of Palawan	3

Sampling Areas	Sitio	Barangay	Municipality/City	Province	Country	Site Description	Number of crocodiles sampled
Isabela Province				Luzon			
Diwagden Creek	Diwagden	San Jose	San Mariano	Isabela	Philippines	Shallow tributary of the Disulap River; lined with dense vegetation	9
Disulap River	San Isidro	San Jose	San Mariano	Isabela	Philippines	A tributary of the larger Disabungan River which joins with the Cagayan River; fast flowing river lined with limestone cliffs	32
Dunoy Lake			San Mariano	Isabela	Philippines	A 0.24 ha stagnant lake surrounded by thick vegetation and connected to the Catalangan river by a small stream	35
Dinang Creek	Lumalog	Cadsalan	San Mariano	Isabela	Philippines	A shallow creek lined with grasses and farmlands; connects with the Ilaguen River	19
		Ibujan	San Mariano	Isabela	Philippines	Fast-flowing river	1

Sampling Area	Sitio	Barangay	Municipality/ City	Province	Country	Site Description	Number of crocodiles sampled
Ibujan River		Ibujan	San Mariano	Isabela	Philippines	Fast-flowing river	1
Ilaguen River			San Mariano	Isabela	Philippines	Long, fast flowing river which connects to the Abuan river	1
Po River		Bgy. Aplaya	Maconacon	Isabela	Philippines		1
Divilacan			Divilacan	Isabela	Philippines		1
Unknown location							4
<hr/>							
Dalupiri Island							
Caucuayan River		Dalupiri Island	Babuyan Group Of Islands Luzon	Cagayan	Philippines	Caucuayan creek runs through a limestone bed, lined with dense <i>Pandanus</i> plants; elevation is 132 masl (Oliveros, Telan & van Weerd, 2006)	1

Sampling Areas	Sitio	Barangay	Municipality/ City	Province	Country	Site Description	Number of crocodiles sampled
Liguasan Marsh			Kabacan	North Cotabato	Philippines	A wetland of around 220,000 ha located in South Central Mindanao; It is a freshwater marsh with abundant growth of water hyacinth, different species of palms and a wide diversity of ferns and orchids	13
Pulangui River, Bukidnon			Don Carlos	Bukidnon Mindanao	Philippines	A 320-km long river starting in Northern Mindanao & draining SW to Liguasan Marsh	1
V-Square Mini Zoo/ Binungan River	Mabnuang	Alawa/Tapayen	Tineg	Abra Luzon	Philippines		4
Gladys Porter Zoo			Brownsville	Texas	USA		6
Total							618

Appendix B

Crocodiles identified as having 5% or greater membership in the *C. porosus* cluster and their corresponding D-loop haplotypes.

Sample no.	Serial ID No. or Cut number (Italics)	Source	D-loop Haplotype (from Tabora <i>et al.</i>, 2010)	proportion of membership in porosus cluster (STRUCTURE v.2.3.1)
PWc005,	294	PWRCC (originally from Estrella Farm, Visayas)	P1	0.891
PWc006,	371	PWRCC (originally from Estrella Farm, Visayas)	M5	0.404
PWb033,	10183	PWRCC bred	M5	0.086
PWb054,	10184	PWRCC bred	M5	0.673
PWb064,	10198	PWRCC bred	M5	0.058
PWb067,	5917	PWRCC bred	P1	0.927
PWb071,	8356	PWRCC bred	P1	0.557
PWb077,	8010	PWRCC bred	M5	0.054
PWb090,	6791	PWRCC bred	P1	0.856
PWb091,	6798	PWRCC bred	M5	0.301
PWb094,	6744	PWRCC bred	P1	0.88
PWb095,	3/9/8	PWRCC bred	M5	0.584
PWb097,	6851	PWRCC bred	P1	0.888
PWb110,	6597	PWRCC bred	M5	0.099
PWb120,	7335	PWRCC bred	P1	0.972
PWb124,	6639	PWRCC bred	M5	0.07
PWb132,	7275	PWRCC bred	M4	0.07

Sample no.	Serial ID No. or Cut number (<i>Italics</i>)	Source	D-loop Haplotype (from Tabora <i>et al.</i>, 2010)	proportion of membership in porosus cluster (STRUCTURE v.2.3.1)
PWb139,	8596	PWRCC bred	M5	0.616
PWb142,	7/0/2359	PWRCC bred	M5	0.07
PWb146,	6434	PWRCC bred	M5	0.054
PWb148,	8013	PWRCC bred	M5	0.123
PWb149,	7514	PWRCC bred	M5	0.115
PWb163,	8435	PWRCC bred	P1	0.819
PWb166,	8011	PWRCC bred	M5	0.176
PWb167,	6449	PWRCC bred	M5	0.201
PWb169,	8145	PWRCC bred	M5	0.064
PWb175,	8533	PWRCC bred	M5	0.122
PWb179,	4662	PWRCC bred	P1	0.947
PWb185,	4630	PWRCC bred	P1	0.938
PWb187,	4349	PWRCC bred	M5	0.236
PWb189,	7548	PWRCC bred	P1	0.957
PWb191,	3281	PWRCC bred	M5	0.284
PWb193,	4593	PWRCC bred	M5	0.351
PWb214,	7626	PWRCC bred	P1	0.97
PWb215,	6057	PWRCC bred	P1	0.943
PWb222,	5/7/68	PWRCC bred	M5	0.066
PWc248,	216	Captive-Maguindanao	M5	0.097
PWc250,	375	Captive-Davao; P.Dizon	M5	0.337
PWc253,	2369	PWRCC bred	M5	0.098

Sample no.	Serial ID No. or Cut number (Italics)	Source	D-loop Haplotype (from Tabora <i>et al.</i>, 2010)	proportion of membership in porosus cluster (STRUCTURE v.2.3.1)
PW255,	3/13/9	PWRCC bred	P1	0.959
PWb257,	1312	PWRCC bred	P1	0.737
PWb260,	1364	PWRCC bred	P1	0.938
PWb261,	607	PWRCC bred	P1	0.9
PWb262,	1314	PWRCC bred	P1	0.939
PWb263,	578	PWRCC bred	P1	0.941
PWb265,	775	PWRCC bred	P1	0.936
PWb266,	581	PWRCC bred	P1	0.823
PWb268,	772	PWRCC bred	P1	0.956
PWb270,	586	PWRCC bred	P1	0.968
PWb275,	2084	PWRCC bred	P1	0.927
PWb278,	1309	PWRCC bred	P1	0.961
PWb279,	642	PWRCC bred	P1	0.911
PWb281,	2155	PWRCC bred	M5	0.05
PWb282,	570	PWRCC bred	P1	0.86
PWb283,	1313	PWRCC bred	P1	0.963
PWb284,	2105	PWRCC bred	P1	0.934
PWb287,	598	PWRCC bred	P1	0.957
PWb289,	1303	PWRCC bred	P1	0.972
PWb292,	605	PWRCC bred	P1	0.87
PWc293,	429	Captive-Davao; P.Dizon	M5	0.134
PWb295,	1678	PWRCC bred	M5	0.056

Sample no.	Serial ID No. or Cut number (Italics)	Source	D-loop Haplotype (from Tabora <i>et al.</i>, 2010)	proportion of membership in porosus cluster (STRUCTURE v.2.3.1)
PWb298,	1132	PWRCC bred	P1	0.521
PWb299,	1103	PWRCC bred	P1	0.46
PWb300,	2054	PWRCC bred	P1	0.928
PWb303,	1295	PWRCC bred	P1	0.947
PWb304,	2049	PWRCC bred	P1	0.954
PWb306,	2106	PWRCC bred	P1	0.898
PWb309,	2720	PWRCC bred	P1	0.891
PWb311,	569	PWRCC bred	P1	0.669
PWb312,	599	PWRCC bred	P1	0.869
PWb313,	625	PWRCC bred	M5	0.452
PWb315,	1302	PWRCC bred	P1	0.97
PWb318,	575	PWRCC bred	P1	0.838
PWc319,	868	Captive-Davao; P.Dizon	M5	0.06
PWb320,	2023	PWRCC bred	P1	0.968
PWb322,	1307	PWRCC bred	P1	0.968
PWb354,	4786	PWRCC bred	P1	0.957
PWb355,	3566	PWRCC bred	P1	0.709
PWb364,	4350	PWRCC bred	M5	0.124
PWb367,	2853	PWRCC bred	P1	0.967
PWc439,	481	Captive- Maguindanao	M5	0.207

Sample no.	Serial ID No. or Cut number (<i>Italics</i>)	Source	D-loop Haplotype (from Tabora <i>et al.</i>, 2010)	proportion of membership in porosus cluster (STRUCTURE v.2.3.1)
PWb445,	614	PWRCC bred	P1	0.925
PWb446,	1446	PWRCC bred	P1	0.937
PWb447,	1311	PWRCC bred	P1	0.957
PWb451,	630	PWRCC bred	M5	0.151
PWb456,	1125	PWRCC bred	P1	0.968
PWb455,	1301	PWRCC bred	P1	0.769
PWb460,	638	PWRCC bred	P1	0.914
K7900,	6511	unknown	P1	0.973
K7901,	7335	PWRCC bred(F1)	P1	0.97
K7902,	7336	PWRCC bred(F1)	P1	0.962
DCc006	1/0/89	Davao Croc Park	M5	0.161

Appendix C

Ninety-eight candidate *C. mindorensis* founder pairings with $DAN \geq 0.60$. Final 16 recommended pairings as founders for the reintroduction programme are highlighted.

DAN	Male				Female			
	SI No.	Single	Left	Right	SI No.	Single	Left	Right
0.72727	10178	9	25	3	2370	0	16	26
0.70874	10178	9	25	3	8031	7	2	23
0.69822	281	2	8	1		4	8	17
0.69107	2904	1	12	28		4	4	8
0.68962	2904	1	12	28	2370	0	16	26
0.68962		0	8	0	2370	0	16	26
0.6762	10178	9	25	3		4	8	17
0.67382	281	2	8	1	8031	7	2	23
0.67382	281	2	8	1	296	2	9	6
0.66299	7388	6	6	23	2370	0	16	26
0.65656	8001	7	1	27	4470	7321	6	4
0.65656	7388	6	6	23		4	4	8
0.65656	6574	5	4	57	407	4	0	7
0.65656	2890	1	12	19	7463	6	8	24
0.65656	857	8	5	7	4490	3	7	23
0.65656		0	8	0	7437	6	7	38
0.65656		0	8	0	2392	0	16	78
0.65656	26	0	2	6	2390	0	16	68
0.65656	848	8	4	8	2390	0	16	68
0.65319	10178	9	25	3	366	3	6	6
0.6393	6596	5	5	15	407	4	0	7
0.63215	10081	9	23	6	7463	6	8	24
0.63215	10160	9	24	47		4	4	8
0.63215	10199	9	25	29		4	4	8
0.63215	9995	9	18	17	7437	6	7	38
0.63215	10176	9	25	1	2390	0	16	68
0.63215	10198	9	25	28	8031	7	2	23
0.63215	8001	7	1	27		1	2	1
0.63215	8001	7	1	27		4	4	8
0.63215	8001	7	1	27	5538	4	12	39
0.63215	4894	3	18	2		4	4	8
0.63215	6430	5	1	56	407	4	0	7
0.63215	2074	0	8	12		4	4	8

DAN	Male				Female			
	SI No.	Single	Left	Right	SI No.	Single	Left	Right
0.63215	2890	1	12	19	881	8	7	56
0.63215	2904	1	12	28	881	8	7	56
0.63215	282	2	8	2	7463	6	8	24
0.63215	26	0	2	6	407	4	0	7
0.63017	10178	9	25	3	349	3	4	9
0.62533	10199	9	25	29	2370	0	16	26
0.62533	10176	9	25	1	2370	0	16	26
0.62533	10198	9	25	28	380	3	8	0
0.62533	4894	3	18	2	2370	0	16	26
0.62533	2074	0	8	12	2370	0	16	26
0.62533	4361	3	4	39	2370	0	16	26
0.62533	282	2	8	2	2370	0	16	26
0.625	8001	7	1	27	9198	8	14	58
0.62204	10198	9	25	28	7465	6	8	26
0.62204	998	9	9	8	4470	3	6	79
0.62204	281	2	8	1	349	3	4	9
0.62204		0	8	0	4536	3	8	17
0.62204		0	8	0	4362	3	4	45
0.62064	10178	9	25	3	862	8	6	2
0.61489	10082	9	23	7	407	4	0	7
0.61489	10079	9	23	4	407	4	0	7
0.61489	9995	9	18	17	4362	3	4	45
0.61489	10297	9	267	38	407	4	0	7
0.61489	10198	9	25	28	7463	6	8	24
0.61489	8138	7	5	18	4490	3	7	23
0.61489	6447	5	2	1	407	4	0	7
0.61489	8001	7	1	27	7463	6	8	24
0.61489	8001	7	1	27	7321	6	4	28
0.61489		6	16	1	407	4	0	7
0.61489	7388	6	6	23	7463	6	8	24
0.61489	7388	6	6	23	836	8	3	6
0.61489	7388	6	6	23	406	4	59	6
0.61489	7742	6	16	29	7613	6	13	56
0.61489	7742	6	16	29	4490	3	7	23
0.61489	4894	3	18	2	7463	6	8	24
0.61489		3	3	57	407	4	0	7
0.61489	6537	5	4	2	407	4	0	7
0.61489	6282	4	35	45	407	4	0	7
0.61489	2904	1	12	28	7463	6	8	24

DAN	Male				Female			
	SI No.	Single	Left	Right	SI No.	Single	Left	Right
0.61489	2904	1	12	28	406	4	59	6
0.61489	4421	3	5	78	406	4	59	6
0.61489	4492	3	7	26	407	4	0	7
0.61489	597	5	9	7	8031	7	2	23
0.61489	282	2	8	2	7437	6	7	38
0.61489	281	2	8	1	426	4	2	6
0.61489	428	4	2	8	8031	7	2	23
0.61431	2398	1	0	1	407	4	0	7
0.61431	2398	1	0	1	2370	0	16	26
0.61431	26	0	2	6	2370	0	16	26
0.60858	6599	5	5	18	407	4	0	7
0.60774	10198	9	25	28		4	4	8
0.60774	10198	9	25	28		4	8	17
0.60774	8001	7	1	27	7542	6	12	179
0.60774		6	16	1	4490	3	7	23
0.60774	7388	6	6	23	7437	6	7	38
0.60774	7388	6	6	23	881	8	7	56
0.60774	5685	4	15	48	2923	1	12	69
0.60774	2397	1	0	0	407	4	0	7
0.60774	2890	1	12	19		4	4	8
0.60774	2904	1	12	28	2390	0	16	68
0.60774	282	2	8	2	881	8	7	56
0.60774		0	8	0	881	8	7	56
0.60774		0	8	0	383	3	18	3
0.60716	10178	9	25	3	4362	3	4	45

APPENDIX D

Genotypes of 618 Philippine crocodiles (*Crocodylus mindorensis*) at 13 microsatellite loci. Unsuccessful amplification attempts are denoted by 000000.

Samples	Loci												
	CpP1708	CpP801	CpP4004	Ami μ 15	CpP3008	CpP305	CpP1610	CpP4116	4HDZ27	4HDZ391	CpP302	CpP2516	4HDZ35
PWRCC													
PwW001,	225229	159179	406414	157157	354374	203205	308308	212212	159161	139139	186186	285285	193199
PWc002,	225225	179179	398398	157157	338338	197203	300308	212220	159159	139143	186186	285285	193193
PWc003,	225229	159179	406406	157157	350354	207207	300300	212220	159159	139139	186186	285285	193193
PWc004,	229229	159179	398402	143157	374378	197203	300300	212212	159159	139139	186186	285285	193193
PWc005,	217229	167179	398414	143143	410430	191205	300312	216220	159169	139139	186186	285285	193203
PWc006,	225225	159179	398402	157157	362362	191203	300308	212216	149159	139139	186186	285285	193193
PWc007,	225229	159179	402402	143143	374398	203207	308308	212220	159159	143143	186186	285285	193199
PWc008,	225229	159179	398398	143143	366366	203203	308308	212212	159159	139143	186186	285285	193193
PWc009,	225229	159159	398398	157157	370402	205205	296296	212212	149157	139139	186186	285285	193199
PWc010,	225229	159167	398398	157157	000000	203203	296296	212220	157157	139143	186186	285285	193193
PWc011,	225229	179179	398398	143157	358378	201203	296304	212212	157157	139143	186186	285285	193193
PWc012,	225229	159159	398398	143157	370386	197201	296304	212212	157157	143143	186186	285285	193199
PWc013,	225229	179179	398398	143157	374382	203203	296304	212212	157157	143143	186186	285285	193193
PWx014,	229229	179179	398398	143143	358378	197211	304304	212220	157157	139139	186186	285285	193193
PWc015,	225225	159159	398402	157157	000000	000000	296304	212216	149157	139139	186186	285285	193199
PWc016,	229229	159179	398398	157157	354386	203203	296296	212212	157157	139143	186186	285285	193193
PWc017,	225229	159159	398398	143157	366370	197203	304304	212220	157157	143143	186186	285285	193199
PWc018,	225229	179179	398398	145157	378386	203203	296296	212212	157159	139143	186186	285285	193193
PWc019,	225229	159179	398398	157157	354386	203205	296304	212212	157157	139143	176186	285285	193199

PWb028,	225225	159179	398402	157157	354362	203205	296296	212212	157157	139143	186186	285285	193199
PWc021,	229229	179179	398398	143157	358374	203203	296304	212220	157157	139139	186186	285285	193193
PWc022,	225225	159167	398398	143143	354358	203205	296304	212212	157157	139139	186186	285285	193193
PWc023,	225229	179179	398398	143143	378382	197203	296304	212220	157157	139139	186186	285285	193199
PWc024,	225229	159179	406406	157157	000000	000000	304304	212212	157157	139139	186186	285285	199199
PWc025,	229229	159159	398398	157157	362366	191203	296304	212212	157159	139139	186186	285285	193193
PWc026,	225225	159159	398402	143143	398398	191203	300300	212216	149157	139139	186186	285285	193199
PWb027,	225229	159159	402406	157157	366366	203203	296296	212220	157157	139139	186186	285285	193193
PWc020,	225229	159179	398398	143157	370386	201203	296304	212212	157157	143143	186186	285285	193199
PWb029,	225229	159179	398406	143157	354370	203203	300300	212212	157157	139139	176186	285285	193199
PWb030,	225229	159179	398406	143157	358370	203203	296296	212212	157157	139143	176186	285285	193199
PWb031,	225229	159179	398402	157157	338338	203205	296296	212212	157157	139139	186186	285285	193199
PWb032,	225225	159159	398406	157157	362366	203207	296304	212220	157157	139141	176186	285285	193199
PWb033,	225225	179179	398402	157157	338358	203203	296296	212212	157157	139143	186186	285285	193199
PWb034,	225225	159179	402406	157157	338350	203203	296304	212212	157157	139143	186186	285285	199199
PWb035,	213229	159159	398406	157157	354374	203203	300300	212212	157157	141143	186186	285285	193193
PWb036,	225229	159179	398398	157157	350362	191203	296304	212212	159159	139139	186186	285285	193193
PWb037,	225229	179179	402406	157157	366386	000000	296296	212220	000000	143143	186186	000000	193193
PWb038,	229229	159179	398406	157157	338366	197207	296296	212212	157157	139139	186186	285285	193193
PWb039,	225225	159179	398402	143157	354418	203203	300300	212212	157157	139143	186186	285285	193199
PWb040,	225229	159159	402406	157157	338374	203205	296296	212212	157157	139139	186186	285285	193199
PWb041,	225225	159159	398398	157157	350386	203203	296296	212220	157157	139139	176186	285285	193193
PWb042,	225225	159159	398398	157157	362366	203207	300300	212220	157157	139139	186186	285285	193193
PWb043,	225225	159179	398406	143157	354374	197203	300300	212212	157157	139139	176186	285285	193199
PWb044,	225229	159179	398402	143157	366370	207207	300308	212212	157157	139139	186186	285285	193199
PWb045,	225229	159159	402406	157157	354374	203203	296296	212212	157157	141143	176186	285285	193193
PWb046,	229229	159179	398398	157157	362366	191207	296304	212212	157157	139139	176186	285285	193193
PWb047,	225225	159159	402406	157157	350354	203207	296304	212212	157157	139139	186186	285285	199199
PWb048,	225229	159159	402406	143157	374454	203203	296304	212212	157157	139141	176186	285285	193199

PWb049,	225229	159179	398398	157157	350354	191207	296304	212212	157159	139139	186186	285285	193193
PWb050,	225225	159179	398406	157157	358374	197203	296296	212212	157157	139139	186186	285285	193199
PWb051,	225229	159167	398406	143157	374454	203203	296296	212212	157157	141143	176186	285285	193193
PWb052,	229229	159179	402406	157157	350386	203207	300300	212220	157157	139141	186186	285285	193193
PWb053,	225229	159179	398402	157157	338338	203205	296296	212212	157159	139143	186186	285285	193199
PWb054,	225229	167179	398414	157157	338362	201203	296296	216220	157157	139139	176186	285285	199199
PWb055,	225225	159179	398402	157157	338354	203203	296296	212212	157157	139139	186186	285285	193199
PWb056,	225229	159179	398402	157157	338350	203207	296304	212220	157159	139139	186186	285285	193199
PWb057,	225225	159179	398398	157157	358370	197203	296296	212212	157157	139139	186186	285285	193193
PWb058,	229229	159159	402406	143157	374454	203203	296296	212212	157157	139143	186186	285285	193193
PWb059,	225225	159159	402406	157157	350386	203207	300300	212220	157157	139139	176186	285285	193193
PWb060,	225225	159179	402406	157157	362366	203203	296304	212220	157157	139143	186186	285285	193199
PWb061,	225229	159159	000000	143157	374454	203203	296304	212212	157157	139139	186186	285285	193193
PWb062,	225229	159179	398406	143157	374454	203203	296296	212212	157157	141143	176186	285285	193193
PWb063,	229229	159159	398406	143157	374454	203203	296296	212212	157157	141143	176186	285285	193193
PWb064,	225225	179179	402406	157157	362366	203203	300308	212220	157157	141143	186186	285285	193199
PWb065,	225229	159159	398406	143157	374454	203203	296304	212212	157157	139139	176186	285285	193199
PWb066,	229229	179179	398414	157157	354354	191205	296304	212212	157157	139139	186186	285285	193193
PWb067,	225229	167179	398414	157157	362438	191205	300308	212216	157157	139139	186186	285285	193203
PWb068,	225229	159159	398398	157157	362374	203205	300300	212212	157157	139141	186186	285285	193193
PWb069,	225229	159179	398398	143157	358386	201201	296296	212212	157157	139143	186186	285285	193193
PWb070,	225225	159159	398402	143143	346370	191197	304304	212220	149157	139143	186186	285285	193199
PWb071,	225225	159179	406418	157157	354410	191203	300304	212220	157157	139143	188236	285285	199203
PWb072,	225229	159159	406406	157157	354370	203207	296304	212212	157157	139143	176186	285285	193199
PWb073,	225229	159179	398398	143143	354366	201205	296304	212212	157157	139143	186186	285285	193193
PWb074,	225225	159159	398398	143157	378386	197203	296304	212212	157157	143143	186186	285285	193193
PWb075,	225229	167179	398398	143157	354430	203203	300300	212212	157157	139143	186186	285285	193193
PWb076,	225229	159179	398398	143157	358454	203203	300300	212212	157157	139139	176186	285285	193193
PWb077,	225229	159179	398414	143157	354358	203207	304304	212212	157157	139139	186186	285285	193199

PWb078,	225229	159179	398414	157157	354374	191205	296304	212212	157157	139143	176186	285285	193193
PWb079,	225225	159179	398398	157157	374414	197203	300308	212220	157157	139143	186186	285285	193193
PWb080,	229229	159179	398406	157157	350374	203207	296296	212220	157157	139139	186186	285285	193193
PWb081,	225229	159179	398406	143143	346378	201203	304304	212212	157157	139143	186186	285285	193193
PWb082,	225229	159159	398398	143157	354370	199201	296296	212216	149157	143143	186186	285285	193193
PWb083,	225229	159167	398402	157157	366446	201201	304304	212220	157157	139139	186186	285285	193193
PWb084,	225229	159167	398402	157157	354374	203205	296296	212212	157157	139141	186186	285285	193193
PWb085,	229229	159179	398398	157157	366374	203203	304304	212212	149157	139139	186186	285285	193199
PWb086,	225225	159159	398398	143143	358366	197205	304304	212212	157157	143143	186186	285285	193193
PWb087,	225229	159179	398398	157157	354362	203205	296304	212212	157157	139141	186186	285285	193193
PWb088,	225229	159159	398414	143157	374466	197203	296304	212212	157157	139143	186186	285285	193199
PWb089,	229229	159179	398398	143157	450450	191201	296296	212212	149157	139139	186186	285285	193193
PWb090,	225225	159159	402402	157157	362430	191191	304308	216216	157157	139139	186186	285288	193193
PWb091,	225229	159179	398414	157157	354378	201205	304304	212212	157157	139143	186186	285288	193193
PWb092,	225229	159179	398398	143143	446450	191201	296304	212212	149157	139139	186186	285285	193193
PWb093,	225225	159167	398402	143157	338378	201203	296304	212212	149157	139139	186186	285285	193193
PWb094,	225229	159179	398398	157157	338378	205205	304308	220220	157157	139143	186208	285285	193199
PWb095,	217229	179179	398414	157157	362378	203203	296296	212212	157159	139139	186186	285285	193199
PWb096,	225229	159159	398406	157157	350374	197207	296296	212212	157157	139139	186186	285285	193193
PWb097,	217225	159179	398402	157157	362418	191205	300300	216216	157157	139139	186208	285288	193203
PWb098,	225229	159159	398398	143157	354358	201211	304304	212220	157157	143143	186186	285285	193193
PWb099,	225229	159159	398406	157157	350374	203207	296304	212220	157157	139143	176186	285285	193199
PWb100,	225229	159159	398398	157157	354362	203205	296296	212212	157157	139141	186186	285285	193193
PWb101,	225229	159179	398414	157157	366374	203205	300308	212212	157157	139139	186186	285285	193193
PWb102,	225229	159159	398406	157157	354374	203203	296304	212212	157157	141143	176186	285285	193199
PWb103,	225229	159159	398398	157157	354354	203205	300300	212212	157157	139139	186186	285285	193193
PWb104,	225225	159159	398398	143157	366446	191201	304304	212212	149157	139139	186186	285285	193193
PWb105,	225229	159159	398398	143157	358366	201211	296296	212220	157157	139143	186186	285285	193199
PWb106,	225229	159179	398406	143157	350374	203207	296296	212212	157157	139139	186186	285285	193193

PWb107,	225225	159159	398402	143157	366366	203203	296304	212212	149157	139143	186186	285285	193199
PWb108,	225229	159179	398398	157157	358378	205211	296296	212220	157157	139139	186186	285285	193193
PWb109,	225225	159167	398398	143157	346354	205205	296304	212212	149157	139143	186186	285285	193199
PWb110,	225229	159179	398398	157157	374430	201203	300300	212216	157159	139139	186186	285285	193193
PWb111,	225229	159179	398398	143157	354386	203205	304304	212212	157157	139143	186186	285285	193193
PWb112,	225229	159167	398398	143157	338354	201203	296304	212212	149149	139143	186186	285285	193193
PWb113,	229229	159179	398398	143157	358386	203211	296304	212220	157157	143143	186186	285285	193199
PWb114,	229229	179179	398398	143157	378386	203203	296296	212212	157157	139143	186186	285285	193193
PWb115,	225229	159159	398406	157157	362378	203203	296296	212212	157159	139143	176186	285285	193199
PWb116,	225229	159179	398406	143157	374378	207207	304304	212212	157157	139143	176186	285285	199199
PWb117,	225229	179179	000000	145145	374378	201207	304304	212212	157157	139143	186186	285285	193199
PWb118,	225225	159159	398398	157157	354386	203203	300308	212212	149157	139139	186186	285285	193199
PWb119,	225229	159159	398402	143157	414454	191211	300308	212212	149157	139143	186186	285285	193193
PWb120,	225229	175175	414418	157157	338358	205207	296308	216220	157157	139139	186186	285288	193205
PWb121,	225229	159167	398398	157157	338386	000000	296296	212212	000000	139139	186186	285285	193193
PWb122,	225229	159159	398398	143157	354366	201203	300300	212212	157157	139139	186186	285285	193193
PWb123,	225229	159179	398398	143143	354430	203205	296296	212220	149157	139143	186186	285285	193193
PWb124,	225229	159179	398398	143157	338378	201201	300308	212216	149157	143143	186186	285285	193193
PWb125,	225229	159159	398398	143157	358378	201203	296296	212216	149149	139139	186186	285285	193193
PWb126,	225229	179179	398398	143157	362378	201203	304304	212212	157157	139139	186186	285285	193193
PWb127,	225225	159159	398402	143143	378398	201203	300308	212212	157157	139139	186186	285285	193193
PWb128,	225229	159167	398402	143143	450450	201201	300308	212220	157157	139139	186186	285285	193193
PWb129,	225229	159159	398406	157157	350366	203203	296304	212212	157157	139143	176186	285285	193199
PWb130,	225225	159179	398398	145157	338374	197201	296296	212220	000000	139143	186186	285285	193193
PWb131,	225225	159159	398406	145145	374374	203207	296304	216216	157157	139139	186186	285285	199199
PWb132,	229229	159179	398398	157157	358370	197203	300308	212212	157157	143143	186186	285285	193193
PWb133,	229229	179179	398398	143157	358378	197201	296304	212220	157157	143143	186186	285285	193193
PWb134,	229229	159179	398414	157157	354354	203205	296304	212220	157157	139139	186186	285285	193193
PWb135,	225229	159159	398414	000000	366374	000000	304304	000000	000000	139143	186186	285285	193199

PWb136,	225229	159159	398398	157157	354386	203203	296296	212212	149157	143143	186186	285285	193193
PWb137,	225229	159159	398406	157157	350374	203203	300308	212212	157157	139141	186186	285285	193199
PWb138,	225229	159159	398398	143157	354370	201203	296296	212212	149157	139143	186186	285285	193193
PWb139,	225229	159179	402402	143157	438478	191203	296296	212212	149157	139143	186186	288288	193199
PWb140,	225229	167167	398402	143143	450450	191201	296296	212220	149157	139139	186186	285285	193193
PWb141,	225225	159179	402402	143157	374414	203207	300308	212212	157159	139143	186186	285285	199199
PWb142,	225225	159179	398406	157157	338354	191203	300308	212212	157157	139143	176186	285285	193199
PWb143,	225229	159159	398414	157157	354374	203205	300308	212220	157157	139141	186186	285285	193193
PWb144,	225225	159179	398398	143157	346354	203203	300308	212212	157157	143143	186186	285285	193193
PWb145,	225229	159167	398398	143157	350354	203205	304304	212212	157157	139143	186186	285285	193193
PWb146,	225229	159179	398398	143157	358430	203203	304304	212220	157157	143143	186186	285285	193193
PWb147,	229229	159179	398406	157157	354378	203207	296296	212220	157157	139139	186186	285285	193193
PWb148,	225229	159179	398406	143157	354358	203207	296304	212216	157157	139139	186186	285285	199199
PWb149,	225225	159179	398398	143157	358378	203205	300308	212212	157157	139143	186186	285285	193193
PWb150,	225229	159159	398398	145157	358366	203211	304308	212212	157157	139139	186186	285285	193193
PWb151,	225229	159179	398406	143145	374374	203203	300308	212220	157157	139143	186186	285285	193199
PWb152,	229229	159159	398406	143143	354370	197207	304304	212212	157157	139143	186186	285285	199199
PWb153,	229229	159179	402406	157157	354374	203207	296296	212220	157157	139139	186186	285285	193193
PWb154,	229229	179179	398398	143157	366366	201203	304304	212212	157157	139139	186186	285285	193193
PWb155,	229229	159159	398402	143157	370378	197203	300308	212220	157157	139143	186186	285285	193193
PWb156,	225225	159167	398398	143143	450450	201201	304304	212212	157157	139139	186186	285285	193193
PWb157,	225225	159159	398402	143157	366450	191201	296304	212212	149157	139139	186186	285285	193193
PWb158,	229229	159159	398398	157157	370386	201203	296296	212212	157157	139139	186186	285285	193193
PWb159,	225229	159179	398406	143157	374454	203207	300308	212212	157157	139139	186186	285285	199199
PWb160,	225229	159179	398414	157157	358374	203207	304304	212212	157157	139139	186186	285285	193193
PWb161,	225229	159159	402406	157157	350374	203207	300308	212212	157157	139141	186186	285285	199199
PWb162,	225225	159167	398398	143157	346354	203205	300308	212212	157157	139143	186186	285285	193193
PWb163,	225229	159179	398402	157157	362434	203205	296296	216220	149149	139139	186186	285285	193203
PWb164,	225229	159179	398406	157157	338374	203203	300308	212212	157157	139139	176176	285285	193199

PWb165,	225229	159179	398402	157157	370414	201203	296296	212212	157159	139139	186186	285285	193199
PWb166,	229229	159179	398414	143143	354454	203207	300308	212216	157157	139139	186186	285285	193199
PWb167,	229229	179179	398398	157157	338358	191203	296296	212212	157157	139139	186186	285285	193193
PWb168,	225225	179179	398398	143157	358454	203203	296296	212212	157157	139139	186186	285285	193193
PWb169,	225229	159179	398414	143157	366374	201207	304304	212216	157157	139139	186186	285285	193193
PWb170,	225229	159167	398398	157157	386386	203203	304304	212212	157157	143143	186186	285285	193193
PWb171,	225225	159159	398398	143157	338378	201201	300308	212216	149157	143143	186186	285285	193193
PWb172,	225225	159179	398398	143157	386414	203203	296296	212216	157159	139139	186186	285285	193199
PWb173,	225229	159179	398398	143145	354378	203211	304304	220220	157157	139139	186186	285285	193193
PWb174,	225229	159159	398406	143157	354374	203207	296296	212212	157157	139139	186186	285285	193193
PWb175,	225225	179179	398402	157157	378430	203203	296296	212212	157157	139143	186186	285285	193199
PWb176,	225229	159179	398398	157157	354378	203211	304304	220220	157157	143143	186186	285285	193193
PWb177,	225229	159167	398402	157157	338354	203203	296304	212212	157157	139139	186186	285285	193193
PWb178,	225229	159159	402406	143157	370374	191207	300308	212212	157157	139139	186186	285285	199199
PWb179,	225229	179179	398418	157157	358378	205205	300304	212220	157157	143143	186208	285288	193203
PWb180,	225229	159159	406406	157157	350374	203207	300308	212212	157157	139143	176186	285285	193199
PWb181,	225229	159159	398398	157157	354374	203205	300300	212220	157157	139141	186186	285285	193193
PWb182,	225225	159179	398398	157157	354370	203203	300308	212212	157157	139143	186186	285285	193193
PWb183,	225225	159159	398406	157157	354370	203205	300308	212212	157157	139139	186186	285285	199199
PWb184,	225225	159167	398398	157157	370378	203203	300308	212212	157157	139139	186186	285285	193193
PWb185,	225229	179179	414414	157157	382454	191203	304308	212220	157157	139139	186186	285288	193199
PWb186,	225229	167179	398414	143157	338370	203203	296296	212212	157157	139139	186186	285285	193199
PWb187,	225229	159167	398414	143157	358366	191191	300308	212220	149157	139143	186186	285285	193193
PWb188,	225229	159167	398406	143157	350374	191203	300308	212212	157157	139141	186186	285285	193199
PWb189,	225229	159179	398418	157157	338358	183203	296308	220220	157157	143143	186186	285288	193203
PWb190,	225229	159179	398398	143143	354366	197197	300308	212212	157157	143143	186186	285285	193193
PWb191,	225229	179179	398414	157157	354362	203205	296304	216216	157157	139141	186186	285285	193193
PWb192,	225229	159159	406406	157157	354374	203207	300308	212212	157157	139143	176186	285285	193199
PWb193,	225229	159167	398402	157157	362362	191203	296296	216220	157157	139139	176186	285285	193199

PWb194,	229229	159179	398398	157157	362374	203205	296296	212212	157157	139141	186186	285285	193193
PWb195,	225225	159159	398398	157157	354362	203205	296296	212220	157157	139139	186186	285285	193193
PWb196,	225229	159179	398398	143157	346378	205205	296296	212212	157157	139143	186186	285285	193193
PWb197,	229229	159159	398406	143157	362378	203203	296304	212212	157159	139143	186186	285285	193199
PWb198,	225229	159159	398406	143143	350366	203203	296304	212212	157157	139143	176186	285285	193199
PWb199,	225225	159179	406406	157157	354366	203203	296304	212212	157157	139143	176186	285285	193199
PWb200,	229229	159159	398406	157157	362374	203203	296296	212212	157159	139143	186186	285285	193199
PWb201,	225229	159159	402406	157157	350374	203203	296304	212220	157157	139139	186186	285285	199199
PWb202,	225225	167179	398402	143157	338370	197207	296296	212212	157157	139139	186186	285285	193199
PWb203,	225229	159179	402406	157157	350378	203207	296296	212212	157157	139139	186186	285285	193193
PWb204,	225229	159167	398398	143157	354370	203203	296304	212212	157157	139143	186186	285285	193193
PWb205,	229229	159159	398406	157157	366378	203203	296304	212212	157159	139139	186186	285285	193199
PWb206,	225229	159179	398398	143157	378386	203211	304304	212220	157157	139139	186186	285285	193193
PWb207,	225225	167179	398402	157157	378414	203205	296296	212212	157157	139139	186186	285285	193199
PWb208,	225229	159159	398406	143157	366398	197203	304304	212212	157157	139143	186186	285285	193199
PWb209,	225229	159159	398402	143143	354370	197201	296304	212220	157157	139143	186186	285285	193193
PWb210,	229229	179179	398398	143157	366386	201203	304304	212212	157157	139143	186186	285285	193193
PWb211,	225229	159167	398398	157157	370378	205205	296304	212212	157157	139143	186186	285285	193193
PWb212,	225225	159159	398398	143157	366390	203203	296296	212212	157157	143143	186186	285285	193199
PWb213,	225229	159159	398398	143157	358378	203203	296304	212220	157157	139143	186186	285285	193193
PWb214,	217229	179179	398414	157157	358378	183205	296308	220220	157157	143143	186208	285288	193193
PWb215,	213225	179179	398414	157157	378442	191203	296296	212220	157157	139143	186236	285285	199207
PWb216,	225229	179179	398398	157157	354362	191203	296296	212212	157157	139139	176186	285285	193193
PWb217,	225229	159159	406406	157157	354366	203207	296304	212212	157157	139139	176186	285285	193199
PWb218,	229229	159159	398398	157157	366386	197203	296304	212212	157157	139143	186186	285285	193193
PWb219,	225225	159179	398398	157157	366370	203203	304304	212212	157157	139143	186186	285285	193193
PWb220,	225225	159159	402414	143157	358366	201205	300300	212212	157157	139139	186186	285285	193193
PWb221,	225225	159159	398398	143143	366470	197201	296296	212220	157157	139139	186186	285285	193193
PWb222,	225229	159179	398402	157157	370430	201203	300300	212216	157157	139143	186186	285285	193199

PWb223,	225229	159167	398398	143157	354370	205205	304304	212212	157157	143143	186186	285285	193193
PWb224,	225225	159179	398398	157157	346378	205205	296304	212212	157157	143143	186186	285285	193193
PWb225,	225229	159159	398414	157157	354354	203205	296304	212220	157157	139141	186186	285285	193193
PWb226,	225229	159179	398398	157157	346354	203205	296304	212212	157157	143143	186186	285285	193193
PWb227,	225229	159159	402406	157157	338354	203205	296304	212212	157157	139139	186186	285285	199199
PWb228,	229229	179179	398398	143157	366386	201203	304304	212212	157157	139143	186186	285285	193193
PWb229,	225229	159159	402406	143143	370374	191203	296304	212212	157157	139139	186186	285285	199199
PWb230,	225229	159159	398398	157157	338386	201203	296296	212212	157157	139143	186186	285285	193193
PWb231,	225229	179179	398398	157157	338362	203203	296296	212212	157159	139139	176186	285285	193193
PWb232,	229229	179179	398398	157157	366378	197201	296304	212220	157157	139143	186186	285285	193193
PWb233,	229229	179179	398398	143157	366378	197201	304304	212212	157157	143143	186186	285285	193193
PWb234,	225225	159179	406406	157157	354370	205205	296304	212212	157157	139139	176186	285285	193199
PWb235,	225225	159167	398398	143157	354370	203205	304304	212212	157157	139143	186186	285285	193193
PWb236,	225229	159167	398398	143157	354370	203205	296296	212212	157157	143143	186186	285285	193193
PWb237,	225225	159159	406406	157157	350366	203203	296304	212212	157157	139143	176186	285285	193199
PWb238,	225225	159179	406406	157157	354374	203203	296304	212212	157157	139143	176186	285285	193199
PWb239,	225229	159179	398398	157157	354354	203205	296296	212220	157157	139139	186186	285285	193193
PWb240,	225229	159159	398398	143143	366482	201201	296304	212212	157157	139143	186186	285285	193193
PWb241,	229229	159179	398398	143157	354370	203203	296304	212212	157157	143143	186186	285285	193193
PWb242,	225225	159179	398406	157157	354366	203203	296304	212212	157157	139139	176186	285285	193199
PWb243,	229229	159179	398398	157157	358378	205211	296304	212212	157157	139139	186186	285285	193193
PWb244,	225225	159179	406406	157157	350374	203203	296304	212212	157157	139139	176186	285285	193199
PWb245,	229229	179179	398398	157157	358378	201203	296304	212220	157157	139139	186186	285285	193193
PWb246,	225229	159159	406406	157157	350374	203207	296304	212212	157157	139139	176186	285285	193199
PWc247,	225229	159159	398402	157157	366366	203203	296304	212212	149159	139139	186186	285285	193193
PWc248,	225225	159179	398406	143157	358454	191203	296304	212212	157159	139139	186186	285285	193199
PWc249,	225225	159159	398398	157157	354354	191201	300300	212212	157157	139143	186186	285285	193199
PWc250,	225225	167179	398406	157157	362362	191191	296304	212216	157159	139139	186186	285285	193199
PWc251,	225225	159159	398402	157157	362390	203203	296296	212212	157157	139143	186186	285285	193199

PWc252,	225225	159179	398402	143157	354386	203205	296296	212212	149157	139139	186186	285285	193193
PWc253,	213229	179179	398398	143157	358378	197203	304304	212212	157157	139143	186186	285285	193199
PWx254,	225229	179179	398398	143157	374378	197203	296304	212212	157157	139143	186186	285285	193193
PW255,	217229	175179	398414	157157	338358	203205	300312	212220	157157	139143	186208	285288	193193
PWc256,	225229	159179	398398	157157	374386	203203	304304	212220	157157	139143	186186	285285	193193
PWb257,	213225	179179	414414	157157	378430	191203	304308	212212	157161	139139	186236	285285	193199
PWc258,	225225	159179	398398	157157	374374	203203	304304	212220	157157	139143	186186	285285	193193
PWb259,	225229	159179	398402	157157	354362	191203	300300	212212	157159	139139	176186	285285	193199
PWb260,	225229	175179	398418	157157	338378	203205	300304	212216	157157	143143	186208	285285	199203
PWb261,	225229	179179	398402	157157	362430	203205	296296	212220	149157	139139	186186	285288	193203
PWb262,	225229	159175	398414	157157	338378	183203	296308	212220	157157	139143	186208	288288	193193
PWb263,	213225	159179	398414	157157	358358	191191	296308	212216	159159	139139	186236	285297	193193
PWx264,	225225	159179	398398	157157	362386	203203	296296	212220	157157	139141	186186	285285	193193
PWb265,	225229	159179	398418	157157	338358	203205	296308	220220	157157	139143	186186	285285	193203
PWb266,	213225	179179	398414	143143	442454	203207	300300	212220	157157	139139	186186	285297	193203
PWc267,	229229	159179	398398	143157	378378	197203	296304	212220	157157	139143	186186	285285	193193
PWb268,	225229	159179	398414	157157	358378	183205	296308	216220	157157	139143	186186	285288	193193
PWx269,	225229	159159	406406	157157	350374	203203	296304	212212	157157	139139	176186	285285	193199
PWb270,	213229	179179	414414	157157	382442	191203	304308	216220	157157	139143	188236	285285	193199
PWb271,	225225	159159	398406	157157	338354	203203	296304	212212	157159	139139	186186	285285	199199
PWc272,	225225	159159	398398	157157	338358	191203	296296	212212	149157	139139	186186	285285	193193
PWb273,	225225	179179	398398	157157	354362	203203	296296	212212	157159	139143	186186	285285	193193
PWb274,	225225	159159	402406	157157	338354	203205	296304	212212	157157	139139	186186	285285	199199
PWb275,	225229	175179	398414	157157	338378	203205	296300	212220	157157	143143	186186	285285	193203
PWb276,	225225	179179	398398	157157	354358	191203	296296	212212	157157	139143	186186	285285	193193
PWc277,	225225	159179	398398	143143	358378	197203	304304	212212	157157	139143	186186	285285	193193
PWb278,	213229	179179	398414	157157	378442	203205	296308	216220	157157	139139	186188	285285	193199
PWb279,	217225	159167	402414	157157	362410	191191	296308	212216	157157	139139	186208	285288	193193
PWb280,	225225	179179	398398	157157	354358	203203	300300	212212	157159	139143	186186	285285	193193

PWb281,	225225	159159	398414	157157	338374	203205	300308	212212	157157	139139	186186	285285	193199
PWb282,	225229	159179	398406	157157	358442	191191	300308	212220	159159	139139	186186	285297	199203
PWb283,	217229	159179	398418	157157	338358	183205	296300	216220	157157	143143	186208	285285	193203
PWb284,	225229	179179	398398	157157	362430	191191	300308	212220	157157	139139	186208	285285	193203
PWc285,	225229	159179	000000	143143	366378	201203	304304	212212	157157	143143	186186	285285	193193
PWc286,	229229	179179	398398	143143	354382	203203	296304	212220	157157	139143	186186	285285	193199
PWb287,	217225	159179	398398	157157	362430	191191	296304	216216	157157	139139	186208	285288	193203
PWb288,	225229	159159	402406	157157	338374	203205	296304	212212	157157	139139	186186	285285	199199
PWb289,	213229	179179	414414	157157	378442	191203	304308	216220	157157	143143	188236	285288	193199
PWb290,	225229	179179	398398	157157	354362	191203	300300	212212	157159	139139	176186	285285	193193
PWb291,	229229	159159	398414	143157	402402	195205	296304	212216	149157	139141	176186	285285	193199
PWb292,	217225	159179	402414	143157	362410	203205	296308	216216	149149	139139	186208	285288	193193
PWc293,	229229	159167	398398	157157	358358	203205	296304	212216	157157	143143	186186	285285	193193
PWb294,	225229	159167	398418	143157	402414	195205	296304	212216	157157	139141	176186	285285	193199
PWb295,	225229	159179	398398	143157	362454	203203	296296	212216	157157	139141	186186	285285	193193
PWb296,	225229	159159	398406	157157	354374	203207	296304	220220	157157	139143	176186	285285	193199
PWb297,	225225	159159	398406	157157	354370	203203	296304	212212	157159	139139	186186	285285	199199
PWb298,	225225	179179	406414	157157	338354	203205	296304	212220	157157	139143	186188	285288	199199
PWb299,	213225	159179	398414	143143	450454	203207	296308	212220	157157	139139	186236	285297	193193
PWb300,	217225	159179	398398	157157	362430	203205	296304	216216	149149	139139	186186	285288	193203
PWc301,	225225	167179	398414	143157	370398	197203	304304	212212	157157	139143	186186	285285	193199
PWx302,	225225	167179	398414	143157	338418	203207	296304	212220	157157	139139	186186	285285	193199
PWb303,	217229	179179	398414	157157	338358	203205	296308	212220	157157	139143	186208	285288	193193
PWb304,	225229	159175	398418	157157	338358	205205	300304	220220	157157	143143	186186	285285	199203
PWb305,	225225	159167	398398	143157	370378	205205	296296	212212	157157	139143	186186	285285	193193
PWb306,	225229	179179	398414	157157	362430	191203	296304	212216	149149	139139	186208	285285	193203
PWb308,	225229	159159	402414	157157	338354	203203	296304	212212	157159	139139	186186	285285	193199
PWb309,	225229	179179	000000	145157	382442	203205	296296	212216	157157	143143	186186	285288	199207
PWb310,	225225	159159	406406	157157	338374	203203	304304	212212	157157	139139	186186	285285	199199

PWb311,	225225	179179	398406	143143	442454	191191	296304	212216	157159	139139	186186	285285	199203
PWb312,	225229	167179	398398	157157	362430	203205	304308	212216	149157	139139	186186	285288	193193
PWb313,	225229	159167	402406	157157	362370	191203	296304	212216	159159	139139	176186	285288	199199
PWb314,	225225	159159	402406	157157	338354	203205	300308	212212	157157	139139	186186	285285	199199
PWb315,	229229	179179	398414	157157	382442	203205	300308	216216	157157	139139	186188	285285	199207
PWb316,	225225	179179	398414	143157	338374	197207	296296	212212	157157	139143	186186	285285	193199
PWc317,	225229	159179	398398	143145	358374	197203	304304	212220	157157	139143	186186	285285	193193
PWb318,	225229	179179	398398	157157	358442	201203	296296	212216	159159	139139	186186	285297	193203
PWc319,	225229	159167	000000	157161	358378	000000	304304	212220	157157	139143	000000	000000	193193
PWb320,	217229	175179	398414	157157	358378	203205	304308	216220	157157	143143	186208	285288	193199
PWc321,	225229	179179	398398	145149	358378	203203	304304	212212	157157	143143	186186	000000	193193
PWb322,	213229	179179	414414	157157	378442	191203	296304	216220	157157	143143	188236	285285	199207
PWb323,	225225	159167	398398	157157	346378	203203	296296	212212	157157	139143	186186	285285	193193
PWb324,	225229	159159	398398	143157	358366	203203	304304	212212	157157	139139	186186	285285	193193
PWb325,	229229	179179	398406	157157	354374	203207	296296	212220	157157	139139	186186	285285	193193
PWb326,	225229	159159	406406	157157	354378	197207	296296	212220	157157	139139	186186	285285	193193
PWc327,	225229	179179	398398	157157	374378	197203	304304	212212	157157	139143	186186	285285	193193
PWb328,	225229	159179	398406	143157	354354	201203	296304	212212	157157	139143	186186	285285	193199
PWb329,	225229	159167	398406	145145	350366	203203	296304	212212	000000	143143	176176	285285	193199
PWb330,	229229	159159	398398	143157	338354	201203	296304	212216	149157	139143	186186	285285	193193
PWb331,	225229	159159	398398	143157	358378	203203	296304	212212	157157	139143	186186	285285	193193
PWb332,	225229	159159	402406	143157	350374	203203	296304	212220	157157	139141	186186	285285	199199
PWb333,	229229	159179	398398	143143	354366	203203	296304	212212	157157	143143	186186	285285	193193
PWb334,	225229	159159	398406	157157	354374	197207	296296	212212	157157	139139	186186	285285	193193
PWb335,	229229	179179	402406	157157	354374	203207	300300	212220	157157	139139	186186	285285	193193
PWb336,	225225	159159	402414	157157	338354	203203	296304	212212	157159	139139	186186	285285	193199
PWb337,	225225	159159	398406	157157	350366	203203	296304	212212	157157	139139	176186	285285	193199
PWb338,	225225	167179	398414	143157	338370	197207	300300	212220	157157	139139	186186	285285	193199
PWb339,	225229	159167	398402	143143	358450	201205	296296	212220	157157	139143	186186	285285	193193

PWb340,	225229	159179	398398	143157	346354	203203	304304	212212	157157	139143	186186	285285	193193
PWb341,	225229	159167	398398	157157	370378	203205	296296	212212	157157	139143	186186	285285	193193
PWb342,	225225	159159	398398	157157	374390	203203	296304	212212	157157	139143	186186	285285	193193
PWb343,	225225	159159	398398	145157	362366	203203	296304	212212	157157	143143	186186	285285	193193
PWb344,	225225	179179	398414	143157	338370	203207	296296	212220	157157	139143	186186	285285	193199
PWb345,	225225	159179	398406	157157	354374	203207	296304	212212	157157	139139	176186	285285	193199
PWb346,	225225	159179	398398	157157	346378	203205	296296	212212	157157	143143	186186	285285	193193
PWb347,	225225	159159	398402	157157	362374	203203	296304	212212	157157	139143	186186	285285	193199
PWb348,	225229	159167	402414	143143	358450	191201	300300	212220	157157	139143	186186	285285	193193
PWb349,	225229	159159	398414	157157	354354	203205	296304	212212	157157	139141	186186	285285	193193
PWb350,	225225	159159	398414	143143	358450	201205	296304	212212	157157	139143	186186	285285	199193
PWb351,	225225	159179	398398	143157	366390	203203	296304	212212	157157	139143	186186	285285	193193
PWb352,	225225	159179	398398	157157	374390	203203	296296	212212	157157	143143	186186	285285	193199
PWb353,	225229	159167	000000	157157	370382	000000	296304	212212	157157	139139	186186	000000	193193
PWb354,	217229	179179	398414	157157	338378	205205	296308	212220	157157	143143	186208	285288	193193
PWb355,	225225	159179	414414	157157	338354	191203	300308	212220	157157	139143	186188	285285	195199
PWb356,	225229	167179	398414	143157	338418	203203	296304	212212	157157	139139	186186	285285	193199
PWb357,	225229	159179	398398	157157	370378	203205	304304	212212	157157	139143	186186	285285	193193
PWb358,	225225	167179	398414	143157	338370	203207	296296	212220	157157	139143	186186	285285	193199
PWb359,	225225	159179	402406	157157	338374	203205	296304	212212	157157	139139	186186	285285	199199
PWb360,	225229	159179	398398	143157	366378	203203	304304	212212	157157	139143	186186	285285	193193
PWb361,	225229	159167	398398	143157	346354	203205	296304	212212	157157	139143	186186	285285	193193
PWb362,	229229	179179	398398	143157	358366	203203	304304	212220	157157	139139	186186	285285	193193
PWb363,	225225	159159	398398	143157	366390	203203	296304	212212	157157	139139	186186	285285	193193
PWb364,	225229	159167	402414	143157	358366	191205	296296	212220	149157	139139	186186	285285	193193
PWb365,	225225	159179	406406	157157	350366	203203	296304	212212	157157	139143	176186	285285	193199
PWb366,	225225	159159	398398	143157	366390	191201	296304	212212	157157	139139	186186	285285	193193
PWb367,	217229	159175	398414	157157	358378	183205	296308	216220	157157	143143	186208	285288	193193
PWb368,	225225	159179	398398	157157	370378	203203	296304	212212	157157	139143	186186	285285	193193

PWb369,	229229	159159	398398	143157	354386	197201	304304	212212	157157	143143	186186	285285	193193
PWb370,	225229	179179	398402	143157	338370	203207	296296	212212	157157	139143	186186	285285	193199
PWb371,	225225	159159	398398	143157	366390	203203	300300	212212	157157	143143	186186	285285	193199
PWb372,	225225	179179	398402	143157	338370	197203	296296	212220	157157	139139	186186	285285	193199
PWb373,	225229	179179	398414	143157	338418	203203	296304	212220	157157	139139	186186	285285	193199
PWb374,	225229	167179	398414	143157	338418	203207	296296	212220	157157	139143	186186	285285	193199
PWb375,	225229	159159	398398	143143	354366	203203	304304	212212	157157	143143	186186	285285	193193
PWb376,	225229	159167	398402	143157	366390	191191	300300	212220	149157	139139	186186	285285	193193
PWb377,	225225	179179	398398	143157	338370	203203	296296	212212	157159	139139	186186	285285	193193
PWb378,	225229	167179	398402	157157	354374	203203	296304	212212	157157	139143	186186	285285	193199
PWb379,	225229	159159	398406	157157	350374	203203	296304	212212	157157	139143	176186	285285	193199
PWb380,	225229	159159	406406	143157	346354	203203	296304	212212	157157	139143	176186	285285	193199
PWb381,	225225	159179	398398	157157	354366	203207	296296	212212	157157	139139	186186	285285	199193
PWb382,	225229	159167	406406	143143	366370	197197	296304	212212	157157	139143	176186	285285	193199
PWc383,	225229	159179	398402	157157	370374	203203	304304	212220	157157	143143	186186	285285	193199
PWc384,	225229	167167	398402	143157	366374	203203	296296	212212	157157	143143	186186	285285	193193
PWc385,	229229	159179	398398	157157	358374	203203	296304	212212	157157	143143	186186	285285	193193
PWc386,	225225	159179	398398	157157	358374	203203	304304	212220	157157	143143	186186	285285	193199
PWc387,	225229	159159	398398	143157	366378	203205	304304	212220	157157	143143	186186	285285	193193
PWc388,	225229	159179	398398	143157	358370	197201	296296	212212	157157	143143	186186	285285	193193
PWc389,	225229	159159	402406	143143	350398	191203	296304	212212	149157	139139	186186	285285	193193
PWc390,	225229	159159	398398	143143	366370	203203	296304	212212	157157	143143	186186	285285	193199
PWc391,	225229	159179	398398	157157	370386	197201	296296	212212	157157	143143	186186	285285	193193
PWc392,	225229	159179	398398	143157	370374	203203	296296	212212	157157	143143	186186	285285	193193
PWc393,	225229	159179	398398	157157	374386	197203	304304	212212	157157	143143	186186	285285	193193
PWc394,	225229	159179	398398	143143	358370	197201	296296	212212	157157	143143	186186	285285	193193
PWc395,	225225	179179	398402	157157	414430	203203	296296	212212	157159	139139	186186	285285	193199
PWc396,	225229	159159	398402	143157	366370	197197	304304	212220	157157	143143	186186	285285	193199
PWc397,	225229	159179	398398	143157	358366	197201	296304	212212	157157	139143	186186	285285	193193

PWc398,	225229	159159	398402	143143	366370	197203	296296	212220	157157	143143	186186	285285	193193
PWc399,	225229	159179	398398	157157	370386	197201	296304	212212	157157	143143	186186	285285	193193
PWc400,	229229	159179	398398	143143	378386	197203	296304	212220	157157	139139	186186	285285	193193
PWc401,	225229	159159	398398	143157	354378	201203	304304	212212	157157	139143	186186	285285	193193
PWc402,	225225	159179	398398	143143	358482	197201	296304	212220	157157	139143	186186	285285	193193
PWc403,	225229	159159	398402	157157	370374	197203	296304	212212	157157	143143	186186	285285	193199
PWc404,	225229	179179	406414	157157	354386	207207	296304	212220	157159	139139	186186	285285	193193
PWc405,	225229	159159	398398	157157	354386	203203	304304	212212	157163	143143	186186	285285	193193
PWc406,	229229	159159	398398	157157	338466	205207	296304	212212	157157	139143	186186	285285	193199
PWw407,	225225	159179	398402	143157	398430	203203	300300	212212	157157	139139	186186	285285	193199
PWx408,	225229	159179	398398	143157	354386	203205	304304	212212	157157	143143	186186	285285	193193
PWc409,	225229	159179	398398	143157	000000	203203	300308	212212	149157	139143	186186	285285	193193
PWc410,	225229	159159	398398	143157	366386	201203	300300	212212	157157	139143	186186	285285	193199
PWc411,	225225	159159	402406	143157	362366	191203	300304	212212	149157	139139	186186	285285	193193
PWc412,	225225	159159	398398	157157	354374	201201	296304	212220	157157	139143	186186	285285	193193
PWc413,	225225	159159	398398	157157	358374	197203	296304	212220	157157	139143	186186	285285	193193
PWc414,	225225	159159	398402	143143	398398	203203	296296	212216	157157	139139	186186	285285	193193
PWc415,	225229	159179	398398	143143	366378	197201	304304	212212	157157	139143	186186	285285	193193
PWc416,	229229	159159	398414	143157	354366	207207	304304	212212	157157	139139	186186	285285	193199
PWc417,	225225	159179	398398	143157	374438	197203	304304	212220	157157	139143	186186	285285	193193
PWc418,	229229	179179	398398	143157	358378	197203	296304	212212	157157	139139	186186	285285	193193
PWc419,	225225	179179	398398	143143	354398	203203	296296	212216	157157	139139	186186	285285	193193
PWc420,	229229	159159	398398	143157	354386	197203	296304	212212	157157	139143	186186	285285	193193
PWc421,	225229	159179	398398	143143	366438	197203	304304	212212	157157	143143	186186	285285	193193
PWc422,	225229	159179	398398	157157	370374	197203	296296	212220	157157	143143	186186	285285	193193
PWc423,	225229	159159	398398	157157	338370	201201	296296	212216	149157	139143	186186	000000	193193
PWw424,	229229	159159	406406	143157	354374	203207	296304	212212	157157	139143	186186	285285	199199
PWc425,	225225	179179	398402	143157	398414	203203	296296	212212	157159	139139	186186	285285	193199
PWc426,	225225	179179	398402	157157	414430	203203	296296	212212	157159	139139	186186	285285	193199

PWc427,	225225	159167	398398	143157	354386	191191	304304	212212	157157	139139	186186	285285	193193
PWc428,	225225	159167	398398	145145	358378	201203	304304	212220	157157	139143	186186	285285	193193
PWc429,	229229	159179	398398	143157	354358	201211	296304	212220	157157	139139	186186	285285	193193
PWc430,	225229	159179	398402	143157	370374	197203	296304	212220	157157	143143	186186	285285	193193
PWc431,	225229	159159	398398	143143	358370	201203	296304	212212	157157	143143	186186	285285	193199
PWc432,	229229	179179	398398	157157	374378	197201	304304	212212	157157	143143	186186	285285	193199
PWc433,	225229	159179	398398	143143	378386	201211	296304	212212	157157	139143	186186	285285	193193
PWc434,	225225	159159	398398	143143	362370	191191	296296	212212	149157	139143	186186	285285	193193
PWc435,	225225	159159	398398	143157	354374	201203	296304	212212	149157	139143	186186	285285	193193
PWc436,	229229	159179	398398	143157	354358	197211	296304	220220	157157	139143	186186	285285	193193
PWc437,	225225	159179	398402	143157	398414	203203	296296	212216	157157	139139	186186	000000	193199
PWc438,	225225	159179	398402	143143	386398	203203	296296	212216	157157	139139	186186	285285	193199
PWc439,	217229	159159	406414	143157	354374	207207	304304	212216	157157	139139	186186	285285	193199
PWc440,	225225	159159	398398	143157	398414	203203	296296	212212	157157	139139	186186	285285	193193
PWc441,	225229	159167	398398	143157	354386	203205	304304	212212	157157	143143	186186	285285	193193
PWb442,	225229	167179	398402	143157	338418	197207	296304	212212	157157	139139	186186	285285	193199
PWb443,	225229	167179	398414	143157	338418	203207	296304	212220	157157	139139	186186	285285	193199
PWb444,	225229	179179	398402	143157	338370	203203	296304	212220	157157	139139	186186	285285	193199
PWb445,	217225	167179	398414	157157	362430	191205	304308	212216	157157	139139	186186	285285	193193
PWb446,	217229	175179	398418	143157	338378	205205	300304	212216	157157	143143	186208	285285	199203
PWb447,	229229	179179	414414	157157	358378	203205	304308	216220	157157	139143	186188	285285	193199
PWb448,	225225	179179	398398	157157	354358	203203	296296	212212	157159	139139	176186	285285	193193
PWb449,	225225	179179	398402	143157	338418	203207	296304	212212	157157	139143	186186	285285	193199
PWb450,	225229	159179	398398	143143	366454	203203	304304	212220	157157	139139	186186	285285	193193
PWb451,	225225	159167	398402	157157	354362	191203	300300	216220	157159	139139	186186	285285	193199
PWb452,	225225	159179	398398	143157	362366	203203	296296	212212	157157	143143	186186	285285	193199
PWb453,	225225	159179	398402	143157	366390	203203	296304	212212	157157	139143	186186	285285	193199
PWb454,	225229	159179	398402	157157	354362	191203	296296	212212	157159	139139	176186	285285	193199
PWb456,	229229	179179	414414	157157	378442	203205	304308	216220	157157	139143	186188	285288	193199

PWb455,	225225	159179	414414	157157	338354	203205	300304	212216	157157	139143	186188	285288	195199
PWb457,	225229	159159	398406	143157	370374	197203	296296	212212	157157	139143	186186	285285	193199
PWb458,	225229	159167	398406	143157	370374	203205	296296	212212	157157	143143	186186	285285	193199
PWb459,	225229	159159	398406	143157	370378	197205	296296	212212	157157	143143	186186	285285	193199
PWb460,	225229	159167	398402	157157	362410	191191	296296	216220	157157	139139	186186	285288	193203
PWb461,	225229	159159	398402	157157	362386	203203	296296	220220	157157	139139	186186	285285	193193
K7898,	225229	159179	398398	143157	366378	203205	296304	212212	157159	139143	186186	285285	193193
K7899,	225229	159179	398398	143157	370430	203205	296304	212220	149149	139139	186186	285285	193193
K7900,	217229	175179	398414	157157	338358	205207	300300	216220	157159	143143	186186	285288	199203
K7901,	225229	175175	414418	157157	338358	205207	296308	216220	157157	139143	186186	000000	193205
K7902,	217225	179179	398414	143157	358358	191205	296296	216220	157159	139139	186186	285288	199203

Davao Crocodile Park

DCc001,	225225	159159	398406	143157	354366	201201	296296	212212	157157	139143	186186	285285	193199
DCc002,	225229	159167	000000	157157	362466	201203	296296	212212	157157	139139	186186	285285	191193
DCc003,	213229	159159	398414	157157	354374	203205	296304	212212	157159	139139	186186	285285	193193
DCc004,	225229	159159	402402	157157	362446	191201	296296	212212	149157	139139	186186	285285	193193
DCc005,	225229	159179	398402	143143	338366	203203	304304	212212	157157	143143	186186	285285	193193
DCc006,	225225	179179	398414	143157	398438	203203	304304	212212	157157	139143	186186	285285	193195
DCc007,	229229	159179	398398	143157	358378	203203	304304	212220	157157	143143	186186	285285	193193
DCc008,	225229	159179	398398	157157	370374	203205	304304	212220	157157	143143	186186	285285	193199

Silliman University

SU001,	225229	159167	398414	143157	406426	203205	296304	212220	159161	139139	176188	285285	193193
SU002,	225229	159167	398414	143157	410426	203205	296296	212212	157159	139139	176176	285285	193193
SU003,	225229	159167	398414	157157	410418	203205	296304	212212	157159	139139	176176	285285	193193
SU004,	225229	159167	398414	157157	410418	203205	296296	212212	159161	139139	176188	285285	193193
SU005,	225229	159167	398398	143157	410426	203205	296296	212212	157161	139143	186188	285285	193199
SU006,	225229	159167	398414	143157	406418	203205	296296	212212	159161	139143	186188	285285	193193
SU007,	225229	179179	000000	143157	406426	203205	296304	212212	157159	139143	186186	285285	193199
SU008,	225229	159179	398414	143157	410426	000000	296304	212212	000000	139139	186186	285285	193199

SU009,	225229	159167	398414	143157	410418	000000	296304	212212	149157	139143	186186	285285	193193
SU012,	225229	159179	402406	143157	000000	000000	296296	212212	000000	139139	186186	285285	193199
SU013,	225229	159167	398402	143157	406418	000000	296296	212212	161161	139143	176188	285285	193199
SU014,	225229	159167	398414	157157	406418	203203	296304	212212	157159	139139	176188	285285	193199
SU015,	225229	159167	398414	143157	000000	203205	296296	212212	149157	139143	176176	285285	193193
SU016,	225229	167167	414414	143157	406410	205205	296304	212212	149157	139143	176188	285285	193193
K7903,	225225	159167	398414	143157	000000	195205	300300	212220	157161	133139	186188	285285	193193
K7904,	225229	167167	398414	143157	406410	195203	300300	212212	157159	139143	176186	285285	193193
K7905,	225229	159167	398414	143157	406410	199203	296296	212220	157159	133139	176188	285285	193193
K7906,	225229	159159	414414	143157	410418	199203	300300	212220	157161	139143	176188	285285	193193
K7907,	225229	167167	414414	143157	410418	195205	300300	212220	161161	139143	176176	285285	193193
K7908,	225229	167167	398414	143157	410418	195205	296296	212220	149161	139139	176188	285285	193193

Calauit Wildlife Refuge and Sanctuary

K7909,	225229	159167	398414	145157	414418	203205	300308	212212	157157	139143	176188	285285	193199
K7910,	225229	159167	398414	145157	414418	203205	300308	212212	157159	139139	176188	285285	193199
K7911,	225229	159167	414414	157157	414414	205205	304304	212212	157157	139143	000000	285285	193193

Isabela

K7912,	225225	167167	414414	143143	374426	189195	296296	216216	149149	143143	176176	285285	193193
IS1232,	225229	167167	418418	143157	350366	189189	300304	216216	149157	141143	176176	285285	193193
IS1234,	225229	167167	000000	143157	350366	195195	296304	216216	149149	141143	176176	285285	193193
IS1235,	225229	167167	414418	143145	362366	189195	300300	212216	149157	143143	176176	285285	193193
IS1236,	225229	167167	414418	143157	350362	189195	296304	212216	149149	141143	176176	285285	193193
IS1237,	225229	167167	414418	143157	350350	189189	296300	212216	149157	143143	176176	285285	193193
IS1238,	225229	167167	414414	143145	350354	189195	300304	212216	149157	141143	176176	285285	193193
IS1239,	229229	167167	414418	143157	350362	195195	296300	212216	149149	143143	176176	285285	193193
IS1240,	225229	167167	414418	143157	350350	195195	296300	212216	149149	141143	176176	285285	193193
IS1241,	225229	167167	414418	143157	362366	189195	296304	212216	149149	143143	176176	285285	193193
IS1242,	225229	167167	414418	143157	374378	189189	296296	212216	157157	143143	176176	285285	193193
IS1244,	225229	167167	414418	143157	362370	189189	300300	212212	149157	143143	176176	285285	193193

IS1245,	225229	167167	414414	143157	350362	189189	300308	212212	157157	143143	176176	285285	193193
IS1246,	225229	167167	414414	143145	362370	189189	296296	212216	149149	143143	176176	285285	193193
IS1247,	225229	167167	414414	145145	350370	189189	296308	212216	157157	143143	176176	285285	193193
IS1248,	225229	167167	414414	143157	350350	189189	296296	212216	149157	143143	176176	285285	193193
IS1249,	225229	167167	414414	143157	350370	189189	296300	212216	149157	143143	176176	285285	193193
IS1250,	225229	167167	414414	145145	350370	189189	296300	216216	149157	143143	176176	285285	193193
IS1251,	225229	167167	414414	145145	350350	189189	300308	216216	157157	143143	176176	285285	193193
IS1252,	229229	167167	414414	143157	362370	189189	296296	216216	149157	143143	176176	285285	193193
IS1253,	225229	167167	414414	143157	350370	189189	296296	216216	149149	143143	176176	285285	193193
IS1254,	225229	167167	414414	143157	350350	189189	296296	212216	149149	143143	176176	285285	193193
IS1255,	225229	167167	414414	143145	350362	189189	300308	212216	157157	143143	176176	285285	193193
IS1256,	225229	167167	398402	145145	350370	189189	296300	212216	147157	143143	176176	285285	193193
IS1257,	225229	167167	414414	143157	350370	189189	300308	216216	147147	143143	176176	285285	193193
IS1258,	225229	167167	414414	143145	350362	189189	296308	212216	147147	143143	176176	285285	193193
IS1259,	225229	167167	406414	143157	350370	189189	296296	216216	149157	143143	176176	285285	193193
IS1260,	225229	167167	414414	143157	362370	189189	296300	212216	147147	143143	176176	285285	193193
IS1272,	225229	167167	000000	143157	350366	000000	296300	212216	000000	143143	176176	285285	193193
IS1273,	225229	167167	414418	143157	350362	189195	296304	212216	149157	143143	176176	285285	193193
IS1274,	225229	167167	414418	143157	362366	189189	296300	216216	149157	141143	176176	285285	193193
IS1275,	225229	167167	414418	143143	362366	195195	296304	216216	149149	141143	176176	285285	193193
IS1276,	225229	167167	414418	143157	350366	189195	296304	212216	149149	143143	176176	285285	193193
IS1277,	225229	167167	414418	143143	362366	195195	296304	212216	149149	143143	176176	285285	193193
IS1278,	225229	167167	414418	143157	362366	195195	300300	216216	149149	143143	176176	285285	193193
IS1279,	225229	167167	414418	143157	350350	189189	296304	212216	149157	143143	176176	285285	193193
IS1280,	225229	167167	414418	143157	350366	189189	300304	216216	149149	143143	176176	285285	193193
IS1281,	225229	167167	414418	143157	350350	189189	296304	212216	149157	143143	176176	285285	193193
IS1282,	225229	167167	414418	145145	350366	195195	300300	212216	149157	141143	176176	285285	193193
IS1283,	225229	167167	418418	143145	350362	189189	296304	212216	149157	143143	176176	285285	193193
IS1284,	225229	167167	414418	143143	350366	189195	296300	216216	149149	141143	176176	285285	193193

IS1285,	225225	167167	414418	143145	362366	189195	296304	216216	149149	143143	176176	285285	193193
IS1286,	225229	167167	414418	145145	350350	195195	296300	216216	149149	143143	176176	285285	193193
IS1287,	225225	167167	414418	143157	362366	189195	296300	212216	149149	143143	176176	285285	193193
IS1288,	225229	167167	000000	143157	350374	203207	300304	216216	149157	143143	176176	285285	193193
IS1289,	225225	167167	414418	143157	362366	189195	296304	212216	149157	141143	176176	285285	193193
IS1290,	225229	167167	414414	143157	422422	189189	304304	212212	149157	143143	176176	285285	193193
IS1291,	225229	167167	414414	143143	406422	189189	304304	212216	149157	143143	176176	285285	193193
IS1292,	225229	167167	414414	143143	406406	189189	304304	212216	157157	143143	176176	285285	193193
IS1293,	225229	167167	414414	143157	406422	189189	304304	212216	149157	143143	176176	285285	193193
IS1294,	225229	167167	414414	143157	406422	189189	304304	212216	157157	143143	176176	285285	193193
IS1295,	229229	167167	414414	143143	406422	189189	304304	216216	149157	143143	176176	285285	193193
IS1296,	225229	167167	414414	143157	406422	189189	304304	212212	149157	143143	176176	285285	193193
IS1297,	225229	167167	414414	143157	406422	189189	304304	212212	157157	143143	176176	285285	193193
IS1298,	225229	167167	414414	143157	406422	189189	304304	212216	149157	143143	176176	285285	193193
IS1299,	229229	167167	414414	143157	402406	189189	304304	216216	149157	143143	176176	285285	193193
IS1300,	225229	167167	414414	143157	406422	189189	304304	212216	157157	143143	176176	285285	193193
IS1301,	225229	167167	414414	143157	406422	189189	304304	212216	149149	143143	176176	285285	193193
IS1302,	225229	159167	414414	143157	422422	189189	304304	212212	149157	143143	176176	285285	193193
IS1303,	225229	167167	414414	143157	350362	189189	300308	216216	149157	143143	176176	285285	193193
IS1304,	225229	167167	414414	143145	350362	189189	296296	212216	149157	143143	176176	285285	193193
IS1305,	225229	167167	414414	157157	350350	189189	296308	212216	157157	143143	176176	285285	193193
IS1306,	225229	167167	414414	143157	350362	189189	296300	212216	149157	143143	176176	285285	193193
IS1307,	225229	167167	414414	157157	350362	189189	296300	216216	149149	143143	176176	285285	193193
IS1308,	225229	167167	414414	143157	350370	189189	300308	212216	157157	143143	176176	285285	193193
IS1309,	225229	167167	414414	143145	362378	189189	300308	212212	149157	143143	176176	285285	193193
IS1311,	225229	167167	414414	143145	350362	189189	296300	212212	149149	143143	176176	285285	193193
IS1312,	225229	167167	414414	157157	350370	189189	300300	216216	149149	143143	176176	285285	193193
IS1314,	225229	167167	414414	143157	350370	189189	300308	216216	149157	143143	176176	285285	193193
IS1315,	225229	167167	000000	143157	000000	189189	296308	212212	149157	143143	176186	285285	193193

IS1316,	225229	167167	414414	157157	350350	189189	296308	212216	149149	143143	176176	285285	193193
IS1317,	225229	167167	414414	143157	362370	189189	300308	212216	149157	143143	176176	285285	193193
IS1318,	225229	167167	414414	143157	350370	189189	300308	216216	157157	143143	176176	285285	193193
IS1319,	225229	167167	414414	143145	350362	189189	296308	212212	149157	143143	176176	285285	193193
IS1320,	225229	167167	000000	145145	000000	189189	300308	212212	149157	143143	000000	285285	193193
IS1321,	225229	167167	414414	143145	362370	189189	296296	216216	149157	143143	176176	285285	193193
IS1322,	225229	167167	414414	143157	406422	189189	304304	212216	149157	143143	176176	285285	193193
IS1323,	225229	167167	414414	143157	362370	189189	296300	216216	149157	143143	176176	285285	193193
IS1324,	225229	167167	414414	157157	370370	189189	296300	216216	149157	143143	176176	285285	193193
IS1326,	225229	167167	414414	143157	398422	189189	304304	212212	149157	143143	176176	285285	193193
IS1327,	225229	167167	402414	143157	406422	189189	304304	212216	149157	143143	176176	285285	193193
IS1328,	225229	167167	000000	143157	350350	195195	300304	212216	149157	143143	176186	285285	193193
IS1329,	225229	167167	402402	145157	350350	189189	296300	216216	149157	141143	176176	285285	193193
IS1330,	225229	167167	418418	143145	362370	189195	300304	212216	149157	141143	176176	285285	193193
IS1331,	229229	167167	418418	143145	350362	195195	296300	216216	149149	141143	176176	285285	193193
IS1332,	225229	167167	418418	143145	362366	189195	300304	212216	149157	143143	176176	285285	193193
IS1337,	225229	167167	398414	143157	422422	189189	304304	212212	149149	141143	176176	285285	193193
K7876,	225229	167167	414414	143143	398422	189189	304304	000000	149149	143143	186186	285285	193193
K7878,	225225	167167	414414	143145	350350	189189	296296	212216	149157	143143	176176	285285	193193
K7879,	225229	167167	414418	145145	350366	189189	300308	212216	149157	141143	176186	285285	193193
K7880,	225225	167167	418418	143145	350362	189195	300300	212216	149157	141143	176186	285285	193193
K7881,	225225	167167	414414	143143	398422	189189	304304	212212	149149	141143	176176	285285	193193
K7882,	225229	167167	414418	143145	362366	189195	296300	212216	149157	143143	176186	285285	193193
K7883,	225225	159167	414414	143143	422422	189189	304304	212212	149149	143143	176176	285285	193193
K7884,	225225	167167	418418	145145	350350	195195	300304	216216	149149	141143	176176	285285	193193
K7885,	225225	167167	414414	143157	406422	189189	304304	212212	157159	139143	176186	285285	193193
K7886,	229229	159167	414414	143143	406422	189189	304304	212216	149157	143143	176176	285285	193193
K7887,	225229	167167	418418	145145	350366	189195	300300	216216	157159	141143	176176	285285	193193
K7888,	225225	167167	414418	145145	350350	189189	300304	212216	149157	141143	176176	285285	193193

K7889,	225229	167167	418418	145145	350366	189195	300300	212212	157159	143143	176176	285285	193193
K7890,	225229	167167	414418	143157	362366	195195	300304	212216	149149	143143	176186	285285	193193
K7891,	229229	167167	414418	143145	350366	189189	300304	216216	149157	143143	176186	285285	193193
K7892,	225229	167167	414414	143143	406422	189189	304304	212212	149157	143143	176186	285285	193193
K7893,	225229	167167	414414	143143	406438	189189	304304	212212	157159	143143	176186	285285	193193

Liguasan Marsh

BU001,	229229	159179	406414	157157	354374	205205	296304	212212	157157	139139	186186	285285	193199
LM001,	225225	159159	398398	143157	366390	203207	296304	212216	157157	139143	186186	285285	193193
LM002,	225225	159159	398406	143143	338366	203203	304304	212212	157157	143143	186186	285285	193199
LM003,	225225	159179	398402	143143	338366	203203	304304	212212	157157	143143	186186	285285	193193
LM004,	225229	159179	402406	143143	338366	203207	304304	212212	157157	143143	186186	285285	193199
LM005,	225225	159159	402406	143143	354354	203203	304304	212212	157157	143143	186186	285285	193199
LM006,	225225	159179	398402	143143	354366	203203	304304	212212	149157	143143	186186	285285	193193
LM007,	225225	159159	406406	143143	354366	203207	304304	212212	157157	143143	186186	285285	199199
LM008,	229229	159179	398398	157157	378386	197203	304304	212212	157157	143143	186186	285285	193199
LM009,	225225	159159	398398	143157	338366	203207	296304	212212	149157	139143	186186	285285	193193
LM010,	225225	159159	398402	143157	402430	203207	296304	212216	157157	139143	186186	285285	193199
LM011,	225225	159159	398402	157157	402430	203203	296304	212216	157157	139143	186186	285285	193199
LM012,	225225	159159	398402	143143	366402	203207	304304	212212	157157	143143	186186	285285	193193
LM013,	225225	159159	398398	143143	362370	203203	296296	212212	149157	139143	186186	285285	193193

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K7894,	221221	167167	414414	145145	422446	189189	296296	216216	149149	141141	176176	285285	193193
K7895,	221221	167167	414414	143157	442466	189189	296296	216216	149149	141141	176176	285285	193193
K7896,	221221	167167	414414	143157	446462	189189	296296	216216	149149	141141	176176	285285	193193
K7897,	221221	167167	414414	143145	442466	189189	300300	216216	149149	141141	176176	285285	193193

