Habitat and population structure of the Morelet’s crocodile (*Crocodylus moreletii*) in Calakmul Biosphere Reserve, Campeche, Mexico

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This thesis is submitted to the School of Science, Engineering and Environment, University of Salford, in fulfilment of the requirements for the degree of Doctor of Philosophy 2021.

I dedicate this work to my mother, Maria José Barão, and my late grandfather, José Carlos Barão, as without encouragement and tireless support I would not have had the opportunity to first be a Portuguese in the Amazon and then a Portuguese in Mayan jungles of Mexico.

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Abstract

The Yucatan Peninsula (YP) is one of the most recognizable parts of Mexico and is well known as one of the significant areas in Mesoamerica which supported the Mayan civilization. In the southern portion of the Peninsula, natural aquatic habitats occur at relatively low densities across the region in the form of semi-temporary ponds (aguadas) sustained by rainfall, and constitute the only local source of standing water during most of the year to both the fauna and human communities. The Morelet's crocodile (*Crocodylus moreletii*) is an apex predator and a flagship species inhabiting these aquatic environments, but only rudimentary information about this species exists in the region.

This PhD project contributes to current knowledge on aquatic habitat in Calakmul Biosphere Reserve (CBR) and characterizes aguada habitats across the region through information on their general structure and water quality. Such inferences provide a baseline to investigate the population structure of *C. moreletii* within and around CBR through *N*-mixture modelling to estimate local abundances and population sizes, and population genetic analyses using SNPs generated through a double-digest RADseq approach (dd-RADseq).

A total of 85 waterbodies were located and described throughout CBR, out of which 64 were annually monitored for water level and vegetation cover from 2016 to 2019, providing detailed data on shape and size variation in response to fluctuations in annual precipitation. A citizen science project was also designed for long-term monitoring of aguadas using standardised data entry forms and information uploaded through a mobile app to a centralized online database. *N*-mixture models applied to count data (n = 1,105 crocodiles) from 256 spotlight surveys conducted across 50 representative aguada locations have resulted in estimates of crocodylian detection and abundance, with covariates enabling insights into population responses to local environmental conditions. Extrapolating abundance estimates across Calakmul yielded in ~12,000 *C. moreletii* present, demonstrating that the region is an important global stronghold for this species. Population structure assessment of *C. moreletii* showed that CBR still harbours genetically pure individuals, whereas genetic pollution through increased hybridization with the American crocodile (*Crocodylus acutus*) is common across other parts of its range. This assessment also revealed that the six main geographical regions within Calakmul where samples originated from to a large extent represent own genetic clusters, and spatial population structure is highly linked to extended family networks within each region.

Taken together, the findings of this PhD will play an essential role in management actions towards the conservation of *C. moreletii* populations in aguadas, establishing *C. moreletii* as an umbrella species for the biodiversity that occurs in them.

Chapter 1 – General Introduction

1.1 Environmental change and herpetofauna

Variations in climate are due to occur naturally across seasons, years and millennia. However, the unprecedented rate of warming observed in recent decades poses a significant threat to ecosystems, especially when combined with the vast range of other anthropogenic stress sources to which water sources are subjected to (Malmqvist et al., 2008). On a global scale, temperature is increasing due to the effects of climatic change (Kerr, 2001), which is currently recognised as one of the largest threats to biodiversity as environmental homeostasis is required for many species to persist (Margalef, 1963; Omann et al., 2009). Many species are unable to adapt quickly enough with the current pace of environmental change. Humans, with their ability to manipulate the environment, are themselves feeling the impacts of these changes (Jarvis et al., 2012), the effects of which are magnified on species which lack the capability to respond.

Increasing temperatures due to global warming pose a threat to regions that are heavily reliant on rainfall, such as tropical forests. These habitats typically experience two well established seasons over the year, categorized as dry and rainy season related to the abundance of rain (Murphy & Lugo, 1986). A seasonal cycle is essential to the existence of these habitats, as rainfall patterns directly influence the life-cycle of flora and fauna that occur in these regions (Leng, 1990). If rainfall is insufficient, drought effects are more pronounced as the rate of evaporation is amplified due to the high temperatures natural to tropical and sub-tropical regions (Trenberth, 2011). Therefore, a lack of rainfall in drier regions may result in the loss of habitat to aquatic species and a lack of drinking water for many terrestrial organisms (Taylor, 1970). Moreover, loss of available habitat due to desiccation can result in reduced feeding grounds, breeding areas and the ability to persist in the area (Lake, 2003; Matthews & Marsh-Matthews, 2003). Climate change can also contribute to excessive rainfall (Trenberth, 2011), which results in flooding which may block terrestrial migration routes, temporarily or permanently deplete available food resources (Mwalyosi, 1991), or result in death due to drowning of less mobile species (Ahlers et al., 2010). Increased rates of weather fluctuations have been observed at various tropical locations around the globe (Condit et al., 1996; Still et al., 1999; Williams et al., 2003), with weather patterns becoming more variable in the future (Woodward et al., 2010). Continued weather fluctuations could be devastating to global biodiversity, as a larger number of species is reliant on tropical climates compared to temperate ecosystems (Brooks et al., 2002). This is because tropical environments are rich in biodiversity, with many of the world's biodiversity hotspots being located in them (Myers et al., 2000).

Herpetofauna represents a paraphyletic clade containing reptiles and amphibians. Although phylogenetically rather distant, these two taxa share many traits that allow them to be grouped together, such as their ectothermic nature and reliance upon specific biological niches to thrive (Vitt & Caldwell, 2013). Amphibians and reptiles play integral roles in food webs as herbivores, predators, and prey, as well as connecting aquatic and terrestrial ecosystems (Schenider et al., 2001; Vitt & Caldwell, 2013). Some of the most important factors that have affected herpetofauna during the last four decades are change in land use (habitat deforestation, fragmentation and deterioration), emergence of infectious diseases (e.g. chytridiomycosis), toxin release into the environment (and toxin-accumulation in trophic chains), overexploitation (by illegal trafficking or unmeasured scientific collecting), exotic species introduction (competitors or predators), and synergetic interactions with environmental change (e.g. see Urbina-Cardona, 2008; Johnson et al., 2017; García-Padilla et al., 2020; and references therein).

Environmental change itself represents an intricate association of stressors, which include alterations in temperature and rainfall patterns. Interaction between stress factors may occur and enhance climate change effects. For example, prolonged summer droughts may lead to an increase in temperature and may also amplify the effects of eutrophication and pollution by increasing the concentrations of nutrients and toxins, which can result in the alteration or disappearance of local herpetofauna communities (Woodward et al., 2010). Any change to the equilibrium of their habitats could be devastating, as many species lack the means to disperse easily to new habitats (Row & Blouin-Demers, 2006), and as a result an extended drought or excessive rainfall could be potentially fatal. Additionally, weather changes are particularly important to them, as their ectothermic ("cold blooded") lifestyle makes them reliant on ambient temperature (Gardner, Barlow, & Peres, 2007; Laurance et al., 2011). In particular amphibians have suffered dramatic losses to their numbers in recent years (Beebee & Griffiths, 2005), with habitat loss being a leading cause of this downturn (Stuart et al., 2004), due to their reliance on freshwater for metabolic processes and reproduction (Vitt & Caldwell, 2013). It is necessary to increase our understanding of how habitats are affected by global warming and how that affects their usage by herpetofauna.

1.2 Modern Crocodylians – Crocodiles, Alligators and Gharials

Crocodylians are group of aquatic reptiles adapted to the water-land interface for more than 200 million years (Brochu, 2003). There are currently 27 recognized species (Figure 1), distributed across three families (Crocodylidae, Alligatoridae, Gavialidae - Figure 2; Grigg & Kirshner, 2015), although data from recent molecular studies indicate that this number might increase (e.g. see Muniz et al., 2018; Pacheco-Sierra et al., 2018; Roberto et al., 2020). All extant species inhabit tropical and subtropical lakes, rivers and coasts, spending most of their time in or close to water; some alligatorids may extend their distribution range into temperate habitats. Although crocodylians exhibit morphological features typically associated with modern reptiles (e.g. scales; Figure 3), their phylogenetically closest relatives are birds and extinct groups belonging to the clades Crurotarsi and Ornithodira (such as dinosaurs, pterosaurs and other “crocodile-like reptiles”), which together form the Superorder Archosauria (Grigg & Kirshner, 2015).

All crocodylians share a range of distinctive traits, such as having a heart with four chambers which allows efficient respiration underwater, and the possession of a sublingual valve that prevents water entering the oesophagus when submerged. All species have an almost exclusive carnivorous diet, generally preying upon unaware animals at the interface between water and land (Grigg & Kirshner, 2015). Reproduction is oviparous with rather large clutches per nest (usually between 20 and 90 eggs, depending on species, female size and body condition), and females nesting every one or two years. Sexual dimorphism is well defined, with males being larger than females. Mating occurs in water, before females usually leave the aquatic environment to lay eggs in mound-shaped nests constructed by accumulation of leaf-litter, vegetation and branches (all Alligatoridae, the false gharial and about half of the Crocodylidae species, including *C. moreletii*) or hole-shaped nests excavated in a suitable substrate (the gharial and the remaining Crocodylidae species). Contrary to lepidosaurs which represent the majority of extant reptiles, female crocodylians exhibit intensive parental care, which includes construction, maintenance, vigilance and defence of the nest and hatchlings for an extended period of up to two years (Grigg & Kirshner, 2015). Nesting females of more terrestrial species (e.g. spectacled caiman - *Caiman crocodilus)* remain in proximity to their nest during egg incubation, during which females alter their feeding frequency and dietary composition (Barão-Nóbrega et al., 2016a), at an associated metabolic cost (Barão-Nóbrega et al., 2017).

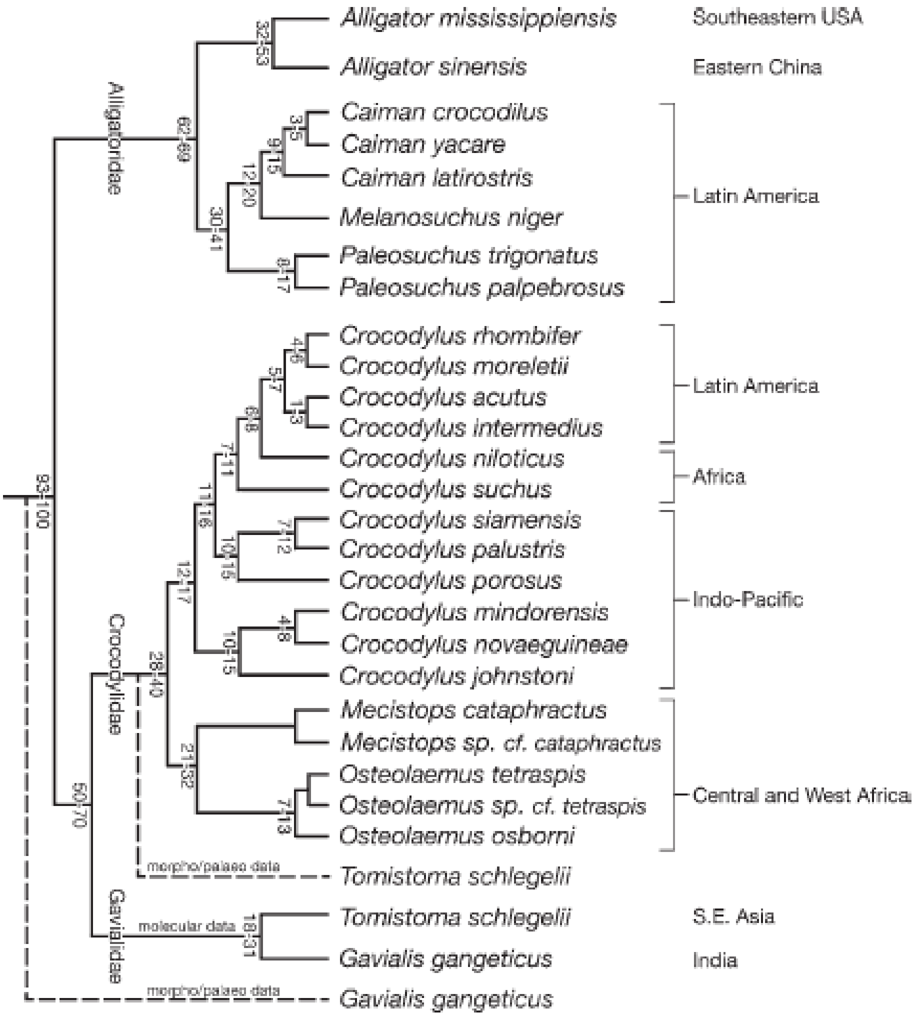
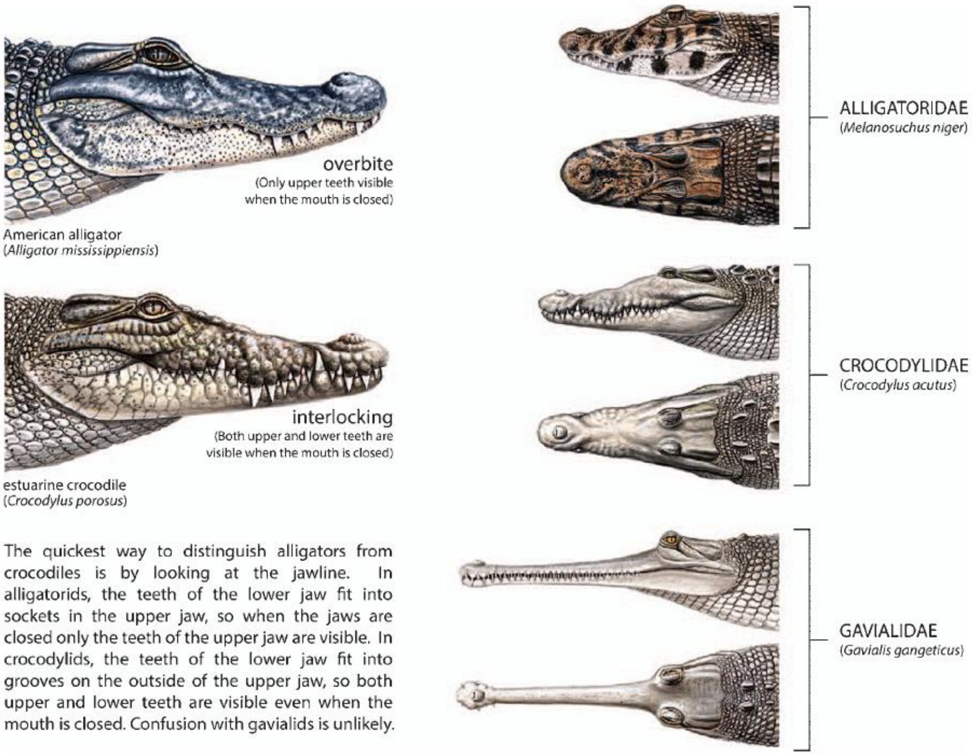


Figure 1. Cladogram exhibiting the phylogenetic relation between modern crocodylians and the geographical regions where these species occur. Adapted from Grigg and Kirshner (2015).



The quickest way to distinguish alligators from crocodiles is by looking at the jawline. In alligatorids, the teeth of the lower jaw fit into sockets in the upper jaw, so when the jaws are closed only the teeth of the upper jaw are visible. In crocodylids, the teeth of the lower jaw fit into grooves on the outside of the upper jaw, so both upper and lower teeth are visible even when the mouth is closed. Confusion with gavialids is unlikely.

Figure 2. General morphology of the head and dentition examples of the three living crocodylian families (dorsal and side views). Taken from Grigg and Kirshner (2015).

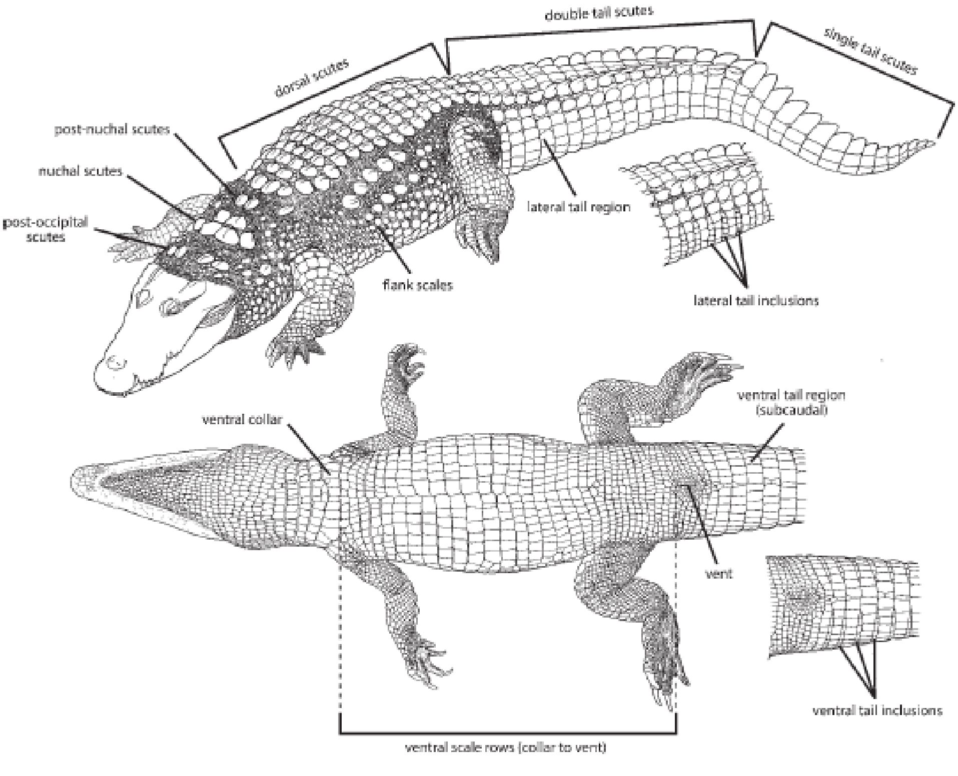


Figure 3. General morphology of a crocodylian. Adapted from Grigg and Kirshner (2015).

Crocodylians are amongst the largest and most abundant vertebrates in many ecosystems around the world (Ross, 1989; Grigg & Kirshner, 2015). In the Amazon basin, for example, caimans are considered to be amongst the predators with the highest biomass (Magnusson & Lima, 1991; Campos et al., 2016). Despite their important ecological, cultural and economic role (Grigg & Kirshner, 2015; Sigler & Gallegos, 2017), crocodylians are an understudied group, and little is still known about key aspects of their life history (Somaweera et al., 2020). Crocodylians have been reported to influence the ecosystems they inhabit by modifying the habitats during drought periods (e.g. digging holes and dens), and by regulating the population size of their prey (Goldschmidt et al., 1993; Grigg & Kirshner, 2015). In central Amazonia, it is believed that caimans (Alligatoridae) have a high contribution to recycling processes of vital nutrients through consumption and excretion in areas where primary production is low, enhancing the growth and development of fish (Fittkau, 1973; Rosenblatt et al., 2013). In the coastal Everglades in Florida, alligators dig and maintain water-filled holes in freshwater marsh areas, which provide refuge to the alligators themselves during dry periods but can also be used by other aquatic organisms (Palmer & Mazzotti, 2004).

Information regarding population structure and density is essential for conservation planning in crocodylian species (Zamudio et al., 2004; Balaguera-Reina & Densmore III, 2014). As apex predators their disappearance can induce alterations in the balance of the food chain in aquatic ecosystems (Rosenblatt et al., 2013), as they occupy different trophic levels as they grow in size (Grigg & Kirshner, 2015). Thus, understanding their response to habitat alteration is necessary to improve our efforts towards species conservation and ecosystem integrity.

1.3 Morelet's crocodile (*Crocodylus moreletii*, Duméril and Duméril 1851)

The Morelet's crocodile (Figure 4) is a medium-to-large species that can grow up to maximum size of 4 metres, with adult individuals averaging a total length of between two and 2.5 metres (Sigler & Gallegos, 2017). This species inhabits mainly freshwater areas such as marshes, swamps, ponds, rivers, lagoons and manmade waterbodies (Alvarez del Toro, 1974). *Crocodylus moreletii* can be found in Belize, Guatemala and Mexico, with the latter accounting for about 88% of the species' distribution area (Sánchez-Herrera et al., 2011). Populations of *C. moreletii* were greatly reduced in many areas due to unregulated skin hunting, which occurred principally in the 1940s and 1950s (Sigler & Gallegos, 2017). Due to severe sanctions, illegal hunting is now thought to be minimal, but still considered to be the principal threat to population recovery in some areas (Zamudio et al., 2004; Platt et al., 2010).

Figure 4. Four individuals of Morelet's crocodile (*Crocodylus moreletii*, Duméril and Duméril 1851). Photos by Edward Gilbert and Maurício González-Jáuregui.

In Mexico, *Crocodylus moreletii* is protected by law and is included in the Official Mexican Directive (NOM-059-ECOL-2001), under the list of species that are subject to special protection. It is also listed in CITES (www.cites.org/eng/app/appendices.php) and as Least Concern in the IUCN Red List (https://www.iucnredlist.org/species/5663/3045579), which reports that its conservation depends on the preservation of its habitat (Cedeño-Vázquez, Platt, & Thorbjarnarson, 2012). A monitoring programme (Morelet's Crocodile Monitoring Program Mexico-Belize-Guatemala) was established a decade ago aiming to ascertain the conservation status of this species based on an estimation of population numbers and abundance (Sánchez-Herrera et al., 2011). The results obtained suggested that *C. moreletii* was not in danger of extinction, and was recently removed from CITES Appendix I to Appendix II, and also removed from the Endangered Species Act (ESA) for the trade of skins in North America (http://www.fws.gov/endangered/laws-policies/).

Although hybridization is considered a potential threat to endangered crocodylians (e.g. Milian-Garcia et al., 2016), the frequency, geographical extent and drivers of hybridization among wild crocodylians remain poorly understood (Hekkala et al., 2015). In the case of *C. moreletii*, hybridization with *C. acutus* has been reported in coastal regions of sympatry between both species within the Yucatan Peninsula (Cedeño-Vázquez et al., 2008; González-Trujillo et al., 2012). It is also known from inland sites currently outside the distribution range of *C. acutus* in Belize (Ray et al., 2004) and Mexico, including remote locations up to 450 km from the coastline (Pacheco-Sierra et al., 2016). Consequently, individuals and populations routinely identified as *C. moreletii* are very likely to be admixed (i.e. of hybrid origin), although conservation considerations for *C. moreletii* largely do not take this into account (Figure 5; Pacheco-Sierra et al., 2016). Pacheco-Sierra et al. (2016) also identified only three pure (non-admixed) populations of *C. moreletii* in Mexico, occurring in locations that represent mainland "islands", supporting the species original status as endangered. These locations are characterised by geographical barriers to gene flow with other populations, and non-hybrid individuals have a decreased chance of contact with hybrid individuals (Pacheco-Sierra et al., 2016).

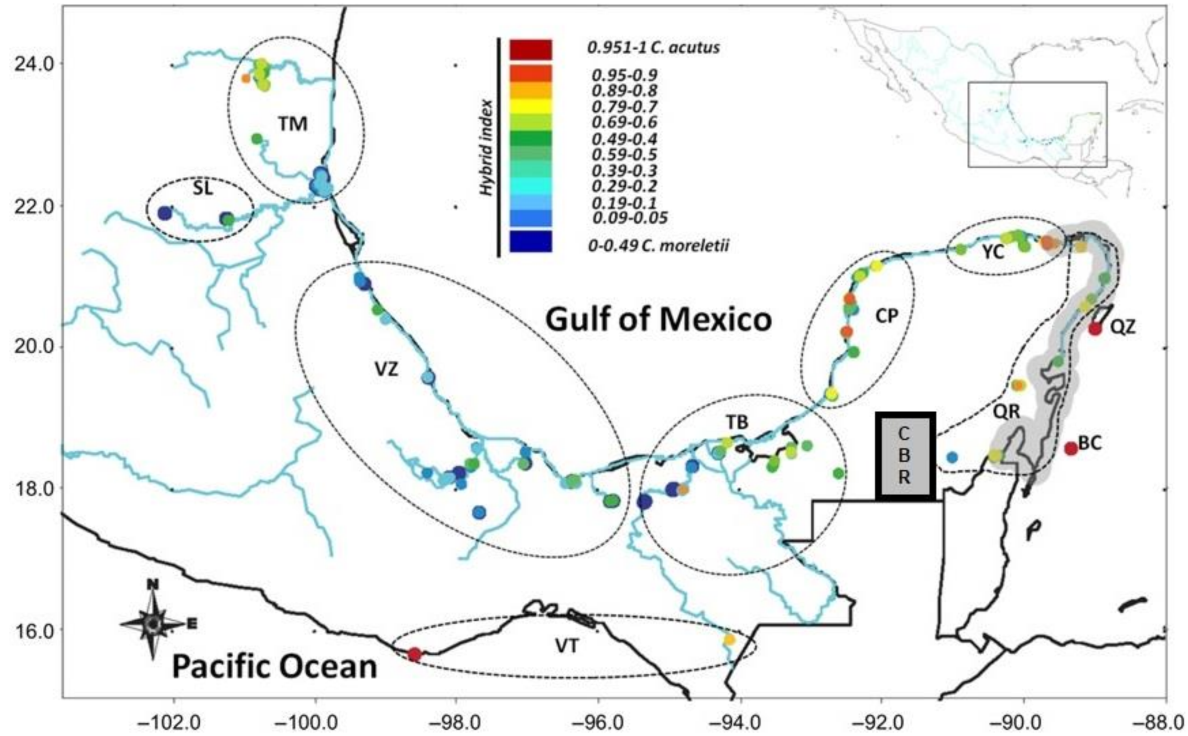


Figure 5. Map of sampling localities for *Crocodylus acutus* and *Crocodylus moreletii* throughout Mexico and the Caribbean. Circles indicate sampling localities; the blue lines represent the river system and possible routes of migration, while the grey shadow is the historical sympatric zone between the two species. Dark red and dark blue dots indicate the localities where non-admixed individuals of *C. acutus* and *C. moreletii* are present, respectively. Admixed individuals are represented by circles, which are coloured to reflect the gradient in hybrid indexes with a colour gradient (shown in the insert scale from blue to orange, with the corresponding hybrid index value). Letters under the dashed circles indicate the name of the Mexican State where samples were collected: TM - Tamaulipas, SL - San Luis Potosi, VZ - Veracruz, TB - Tabasco, CP - Chetumal, YC - Yucatan, QR - Quintana Roo, QZ - Cozumel, BC - Banco Chinchorro, VT – Ventanilla. The location of Calakmul Biosphere Reserve (CBR) is highlighted in grey. Adapted from Pacheco-Sierra et al. (2016).

1.4 Calakmul Biosphere Reserve (CBR)

Calakmul Biosphere Reserve (18.60583 N, 89.94444 W; WGS84; Figure 6) is an expanse of tropical forest that covers an area of 723,000 hectares, and is part of the Selva Maya that encompasses Mexico, Guatemala and Belize, spanning over 10.6 million hectares and representing the largest continuous section of tropical forest in Mesoamerica (Vester et al., 2007). The climate in CBR is warm and sub-humid with a mean annual temperature between 24 and 25 ºC. The topography is relatively flat with shallow rocky soils, although altitudes range from 100 to 380 m (García-Gil et al., 2002). There is a rainy season between June and October with an annual rainfall average of 1,076 mm, varying between 700 mm in the north and more than 2,000 mm in the south (García-Gil et al., 2002; Reyna-Hurtado et al., 2010), with significant effects on forest structure and tree species composition (Vester et al., 2007). The majority of the reserve is composed of tropical semi-deciduous forest with a canopy ranging from 15 to 40 meters, with tropical deciduous forest and a canopy height of 8 – 20 meters in the north (Chowdhury, 2006). Being one of the last remaining stands of primary forest in Mexico, CBR is classified by UNESCO as World Heritage Site of Culture and Nature (UNESCO, 2016). More than 35,000 people inhabit almost 100 communities dispersed around the protected area and depend on the extraction of natural resources such as water from the aguadas that persist in the dry season (Gunn et al., 2002; Faust & Folan, 2015).

Figure 6. Location and geopolitical structure of Calakmul Biosphere Reserve (CBR) in southern portion of the Yucatan Peninsula (Campeche, Mexico).

1.5 Freshwater habitats in CBR

In the CBR, most of the substrate consists of carbonated rocky materials ($CaCO_3$), which facilitates water filtration and underground movement due to its permeability (García-Gil et al., 2002), which difficulties the existence of permanent waterbodies (Gunn et al., 2002). Most of the rainwater percolates into the underground, leading to the absence of a permanent surface hydrological network in the form of rivers and streams. Surface water occurs only in areas where the terrain allows the accumulation of rainfall to create semi-temporary natural ponds locally known as aguadas (García-Gil et al., 2002; Reyna-Hurtado et al., 2010; Delgado-Martínez & Mendoza, 2020). Aguadas are an essential resource for a wide variety of species (Hernández-Huerta et al., 2000; Martínez-Kú et al., 2008). They offer drinking and bathing spots for large mammals such as tapirs and peccaries (Reyna-Hurtado et al., 2016), allowing the occurrence of fish (Valencia Díaz, 2001; Vega-Cendejas et al., 2013) and herpetofauna species that require aquatic habitat such as frogs, freshwater turtles, and *C. moreletii* (Cedeño-Vázquez, Mandujano, & Pozo, 2006a; Colston et al., 2015; Barão-Nóbrega et al., 2020).

The shape and size of aguadas varies seasonally in response to fluctuations in annual precipitation (García-Gil et al., 2002; Gunn et al., 2002; Barão-Nóbrega, 2019). Aguadas represent the only local source of standing surface water during most of the year (Reyna-Hurtado et al., 2010; Faust & Folan, 2015), but occur at relatively low densities across the region (on average less than one non-dry waterbody per 535 hectares, García-Gil, 2000; Delgado-Martínez & Mendoza, 2020). Coupled with the dispersed distribution of aquatic habitat across CBR, annual water availability depends on precipitation patterns which significantly fluctuated over the last decades (Figure 7; Cuervo-Robayo et al., 2020). Data from the Mexican Government Weather Department (*Comisión Nacional del Agua, Servicio Meteorológico Nacional* - CONABIO) show that CBR experienced a 16% reduction in annual median precipitation values during the last 50 years (Reyna-Hurtado et al., 2010; Márdero et al., 2012; Morales & Pantí, 2015; Márdero et al., 2019). For example, during the dry season of 2006, Aguada Calakmul (one of the biggest aguadas in the reserve's southern Core Zone) entirely dried up for the first time in at least 16 years, forcing several peccary groups (*Tayassu pecari*) to abandon the area and heading 15 km south looking for water (Reyna-Hurtado et al., 2009). Fluctuations in occurrence and abundance of the endangered Baird's tapir (*Tapirus bairdiii*) are also associated with variation in water availability in the CBR (Figure 8; Reyna-Hurtado et al., 2019). A decrease in general herpetofauna abundance and species diversity has also been linked with periods prolonged drought (Figure 9; Slater et al., 2020). No information on how drought effects local *C. moreletii* populations in CBR is however yet available.

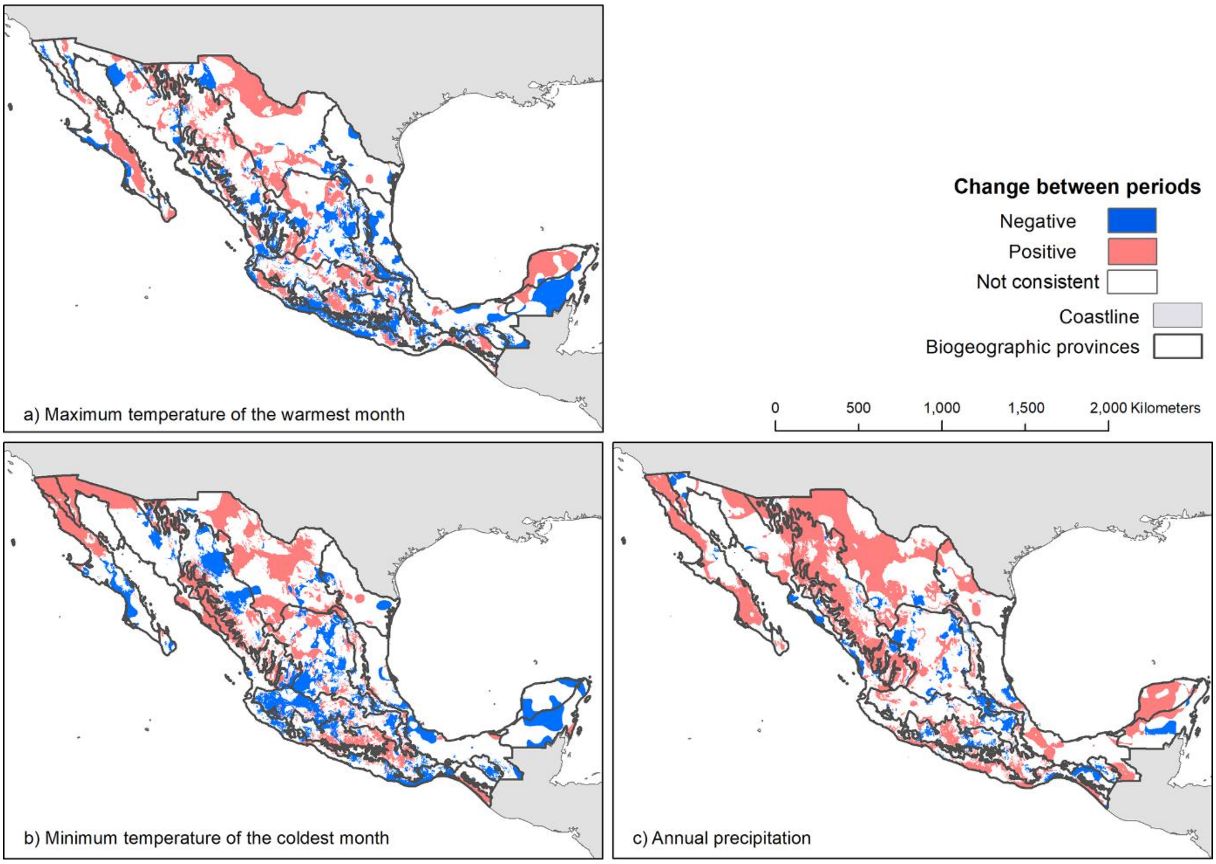


Figure 7. Climate change data in Mexico between 1910 and 2000. Negative and positive areas represent decreases and increases changes, respectively, in maximum temperature of the warmest month (a), minimum temperature of the coldest month (b) and annual precipitation (c). Blue areas indicate negative signs (decrease) for the difference between 1940 –1970 and 1970 – 2000. Red areas indicate positive signs (increase) for the difference between the same periods. White areas indicate an inconsistent sign. Adapted from Cuervo-Robayo et al. (2020).

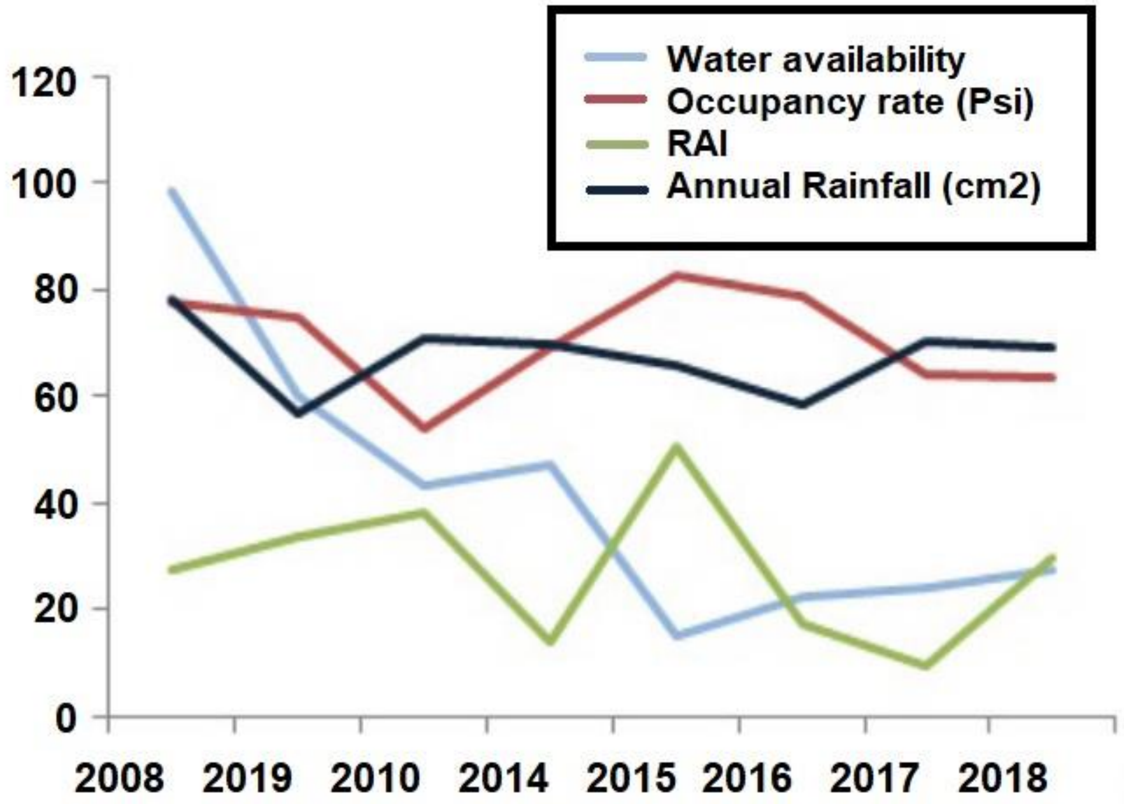


Figure 8. Annual rainfall and water availability (as percentage of aguadas with water) and average and Baird's tapir (*Tapirus bairdiii*) relative abundance index (RAI) and occupancy rate (Psi) in ten aguada habitats in the Southern Core Zone of Calakmul Biosphere Reserve. Image taken from Reyna-Hurtado et al. (2019).

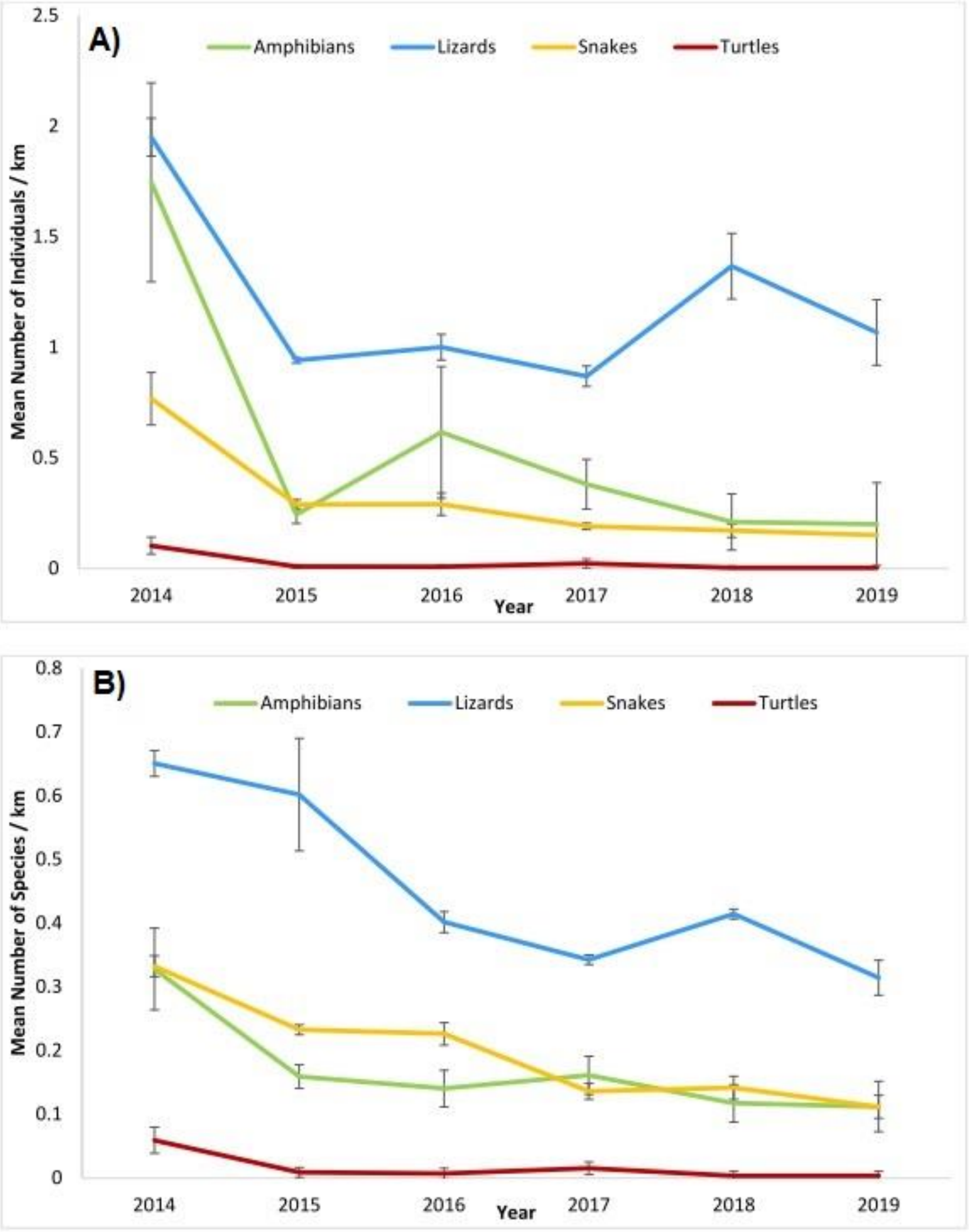


Figure 9. Mean relative abundance (A) and species richness (B) of herpetofauna in Calakmul Biosphere Reserve between 2014 and 2019, based on visual encounter surveys. Image taken from Slater et al. (2020).

1.6 Herpetofauna in Calakmul

Calakmul contains 109 species of amphibians and reptiles, including the Peten centipede snake (*Tantilla cuniculator*), the Yucatan's mushroomtongue salamander (*Bolitoglossa yucatana*), the spiny-tailed iguana (*Cachryx defensor*) and a box turtle (*Terrapene yucatana*) which are endemic to the Yucatan peninsula (Colston et al., 2015; Barão-Nóbrega et al., 2020). The number species of amphibians and reptiles found in CBR and surrounding areas represent, respectively, approximately 84% and 75% of total species richness registered for the state of Campeche and Yucatan Peninsula (González-Sánchez et al., 2017). Furthermore, it also encompasses approximately 60% of the observed herpetofauna richness in the entire Mayan jungle, which has one of the richest assemblages in the Americas (188 species; Lee, 2000).

Herpetofauna are considered excellent biological indicators because they tend to be abundant and diverse, occupy a wide range of habitat niches and are particularly sensitive to changes in their environment (Vitt & Caldwell, 2013). Consequently, as habitats become more disturbed, herpetofauna diversity is expected to decrease and species that are no longer present reflect the loss of a specific habitat niche. Environmental change and the associated droughts observed in the CBR in the past decades is a major concern, as the presence and availability of water in aguadas is under threat due to insufficient rainfall (Barão-Nóbrega, 2019; Reyna-Hurtado et al., 2019; Slater et al., 2020). Monitoring data from 2014-2019 indicate a decline in overall herpetofauna abundance and richness of both reptiles and amphibians paired with a significant reduction in water surface (Slater et al., 2020). Indeed, it has been shown that amphibian diversity to be highly influenced by local habitat factors such as hydroperiod (Pechmann et al., 1989; Snodgrass et al., 2000; Couto et al., 2017)

1.7 *Crocodylus moreletii* in Calakmul

Although our knowledge of *C. moreletii*'s life history across its distribution range has increased considerably over the last decades (Sigler & Gallegos, 2017), aspects such as population size, structure and genetic diversity are still unknown in remote areas of the Selva Maya. Calakmul is no exception, and little to no information is available on crocodiles inhabiting local aguadas across the region. *Crocodylus moreletii* is relatively easy to survey, and, similar to other crocodylians, is vulnerable to anthropogenic activities (Kushlan, 1988; González-Trujillo et al., 2014) and changing environmental conditions (Mazzotti et al., 2009). Therefore, *C. moreletii* represents a valuable model species to assess the impact of habitat alteration on semi-aquatic species in inland waterbodies (Mazzotti et al., 2009; González-Trujillo et al., 2014). Due to the geographical remoteness and topographic profile of aguada habitats in CBR, the region may also still hold a non-admixed *C. moreletii* crocodile population (i.e. genetically pure, without the intrusion of *C. acutus* DNA), and might therefore represent a location of great importance to the conservation of this species. Observed disruptions in rainfall patterns due to change in climate (Márdero et al., 2019; Cuervo-Robayo et al., 2020) are impacting aguada availability by shifting water accumulation patterns during the dry and even early-mid rainy season in CBR (Barão-Nóbrega, 2019; Slater et al., 2020). As such, it is of the utmost importance to better understand how these changes to the environment affect long-lived species such as *C. moreletii*, which can serve as umbrella species for the biodiversity that occurs in them.

1.8 Project rationale and major aims

The Yucatan Peninsula is one of the most recognizable parts of Mexico and is well known as one of the significant areas in Mesoamerica that supported the Maya civilization (Sharer, 1998; González-Sánchez et al., 2017). Current knowledge on the location and characteristics of small to medium sized waterbodies within and around CBR is still very incipient, and in great part based on an analysis of aerial photographs from 1995-1996 (García-Gil, 2000). More than 20 years later, no or limited further information exists on aspects such as general structure (e.g. dominant vegetation and vegetation cover), water quality and water availability across seasons and years.

It is imperative to update the available information on the dynamic changes to structural characteristics of waterbodies in CBR, due to shifts in water availability patterns which influence occurrence and abundance of local aquatic fauna. Research on the location and characteristics of waterbodies across the reserve, and how long-lived vertebrate species like *C*. *moreletii* respond to such alterations, are important when assessing conservation management challenges to counteract alterations in water distribution in waterbodies across the reserve. Crocodiles are the only long-lived large bodied aquatic species in the region and a very charismatic species in general, which makes them a good umbrella species for other biodiversity occurring in these aquatic habitats.

For top predators such as *C*. *moreletii* in CBR, more information is required on the factors that shape the structure and persistence of population structure under the influence of prolonged periods of drought. Adding information to this knowledge gap and providing information on the population status of *C*. *moreletii* in CBR is not only important for local aguada habitat conservation, but also at a national level (Álvarez, 2005; Sánchez-Herrera et al., 2011; Cedeño-Vázquez et al., 2012) as most studied populations across the species' distribution range are largely composed of admixed individuals (Pacheco-Sierra et al., 2016; Pacheco-Sierra et al., 2018).

Based on what was described above, this PhD project has three major aims:

1) Update current knowledge on aquatic habitat in CBR, by providing descriptive information characterizing waterbodies in the region (aguadas) through details on their general structure and water quality;

2) Provide baseline data on *C. moreletii* populations in CBR by estimating local abundances in aguadas and total population sizes across the region;

3) Describe the population structure of *C. moreletii* in Calakmul (CBR and surrounding areas) by using a genotyping by sequencing (ddRAD-Seq) approach to characterize the species genetic profile across the region.

1.9 Thesis structure

This PhD thesis is structured into a general introductory chapter, followed by three data chapters, and a final chapter summarizing the presented information.

Chapter 2 expands our existing knowledge on aquatic habitats in CBR by providing a characterization of aguadas through details on their general structure and water quality parameters. It also presents monitoring data on water levels and vegetation cover between 2017-2019, revealing the effect of a prolonged drought and presenting a citizen science project designed for aguada surveillance

Chapter 3 focuses on estimating local abundances and total population sizes across Calakmul by employing a set of binomial *N*-mixture modelling approaches in comparison with more traditionally used methods.

Chapter 4 employs a SNP dataset generated through a double-digest RADseq approach (dd-RADseq) to detect the presence of *Crocodylus moreletii* – *Crocodylus acutus* hybrids and investigate the population structure of *C. moreletii* across Calakmul.

Chapter 5 consists of an overall discussion providing a final overview on this thesis project and highlighting its key findings.

Chapter 2 – Distribution and characterization of aguadas in the Calakmul Biosphere Reserve

Unpublished data and information

2.1 Introduction

Wetlands have been important to human societies on all continents for millennia (Millennium Ecosystem Assessment, 2005). Although substantial wetland resources still exist worldwide, extensive areas have largely disappeared or have been transformed by human influence, especially in the tropics (Moreno-Casasola et al., 2012). Ecological monitoring is a key part of adaptive management efforts (Lovett et al., 2007). Representative indicators which are able to show clear trends in response to change, and which are easily and efficiently communicated, need to be selected for monitoring purposes (Schiller et al., 2001; Doren et al., 2009; Mazzotti et al., 2009).

Understanding natural conditions of an aquatic system allows predictions on whether an ecosystem is able to support significant change in its general composition without affecting its ecological functions, or if it is sensitive to small physicochemical fluctuations that may result in ecosystem degradation (Bolpagni, 2020; Boon & Baxter, 2020). It is therefore important to identify whether water degradation is part of a natural cycle, or whether it is a consequence of human activities in the region (Chapman et al., 1996; Ritchie et al., 2003). Assessing the "health" of an aquatic system requires information on external factors, such as human activities, changes in seasonal hydric flow patterns, physicochemical characteristics of the water and information on presence of contaminants (Hirsch et al., 1982; Smith et al., 1997; Hawkins et al., 2010). The latter factor acquires higher relevance when considering the effects of short-term (seasonal) and medium to long-term (e.g. prolonged droughts) climatic changes and alterations in land use (Chapman et al., 1996; Burton Jr, 2002).

When measuring water quality, it is important to consider that pure water (i.e. water that contains nothing but hydrogen and oxygen) does not naturally exist in the environment (Prior, 2013). Natural water is affected by contact with bedrock or erosion, evapotranspiration, deposition of dust particles, percolation of organic matter and soil nutrients, and hydrological factors such as currents (Nollet & De Gelder, 2000). Furthermore, biological processes in aquatic (e.g. role of caimans in nutrient recylcing in the Amazon Basin; Fittkau, 1973) and terrestrial environments (Mitchell & Soga, 2005) may alter the physical and chemical composition through the presence of dissolved or suspended matter (Chapman et al., 1996; Moreno-Casasola et al., 2012). While minerals and salts are necessary to maintain certain ecological processes, others components such as metals, pesticides and organic toxins can have negative impacts (Chapman et al., 1996; Burton Jr, 2002).

Drought can be defined as a prolonged period of moisture deficiency and abnormally low water levels, often triggered by disruption in precipitation and temperature patterns (Dai, 2011) and impacting species and human communities alike by reducing food production and increasing environmental temperatures (Rymer et al., 2016). Although periods of drought are common occurrences, many ecosystems (both arid and non-arid; IPCC, 2007) are experiencing recurrent negative anomalies in water availability, with the frequency of extreme drought events predicted to increase in the next decades (Easterling et al., 2000; Dai, 2011).

Calakmul Biosphere Reserve (CBR) in Mexico is part of the Selva Maya that spans over 10.6 million hectares across Mexico, Guatemala and Belize, making it the largest section of neotropical forest north of the Amazon. Most of the CBR is situated on a plateau, originated by uneven limestone erosion shaping the region in a heterogenic mixture of small mountains and micro-valleys (Gunn et al., 2002). Most of the substrate consists of carbonated rocky materials ($CaCO_3$), which facilitates water filtration and underground movement due to its permeability (García-Gil et al., 2002). Soil composition allowed the development of a karst system, where rainwater is filtered, dissolving the rock and creating underground tunnels where water can flow (Porter Bolland et al., 2006; Pérez-Cortez et al., 2012). Such tunnel systems drain water to the west and northwest of the Yucatan peninsula, leading to the formation of cenotes, aguadas, wetlands, basins, caverns and springs (García-Gil et al., 2002; Gunn et al., 2002). In Calakmul itself, above-ground waterbodies (locally known as aguadas) occur in low densities, and represent areas of reduced water permeability due to a layer of soil rich in organic material and clay (García-Gil et al., 2002; Reyna-Hurtado et al., 2010). Aguadas can range from around 100 m^2 up to several hectares in area, although the vast majority is relatively small (less than one hectare), not very deep (under 1 m) and dries up throughout the dry season (García-Gil, 2000; Valencia Díaz, 2001; García-Gil et al., 2002).

Aguadas play an important role in the CBR as they represent sources of water to village communities and also serve as vital habitats to aquatic species, as well as charismatic terrestrial mammals such as peccaries *Tayassu pecari* (Martínez-Kú et al., 2008; Reyna-Hurtado et al., 2010) and the endangered tapir *Tapirus bairdii* (Reyna-Hurtado et al., 2016). Aguadas are influenced by soil permeability, climatic variation, and intensity of usage by both fauna (domestic and wild) and human communities. Temporal and spatial variation of structural characteristics of aguadas influence the surrounding forest, and seasonal patterns of water availability affect activity patterns and movements of local fauna (Reyna-Hurtado et al., 2010; Briceño-Méndez et al., 2014; Carrillo-Reyna et al., 2015; Reyna-Hurtado et al., 2016). Despite their importance, insufficient knowledge still exists regarding these aquatic habitats (Reyna-Hurtado et al., 2010; Slater et al., 2020).

Water in aguadas is largely sustained by annual rainfall, and the maintenance of water levels heavily depends on precipitation patterns (Valencia Díaz, 2001; O'Farrill et al., 2014). Insufficient rainfall may lead to desiccation, which often results in the breakdown of impermeable membranes due to the rapid proliferation of fast-growing herbaceous vegetation, hampering rainfall accumulation in the next rainy season due to water loss by infiltration (Reyna-Hurtado et al., 2009; Barão-Nóbrega, 2019; Slater et al., 2020). Over the last 50 years, the region has seen a 16% reduction in annual mean precipitation (Márdero et al., 2012; Márdero et al., 2019), resulting in an overall decrease in aguada abundance (Reyna-Hurtado et al., 2019). More recently, the 2015 El Niño event resulted in virtually no rain during the rainy season, disrupting aguada maintenance cycles in Calakmul and, affecting habitat factors and species' distributions (Reyna-Hurtado et al., 2019; Slater et al., 2020). Shortage of precipitation persisted over the following years. 2017 saw another period of drought (Márdero et al., 2019) prompting aguadas to dry in many locations across Calakmul (Barão-Nóbrega, 2019) and impacting on spatial and temporal patterns of species which depend on these water sources (Sánchez-Pinzón et al., 2020; Slater et al., 2020).

The scale and complexity of current environmental problems pose serious challenges to conservation biology, natural resource management, and environmental protection (Bonney et al., 2009; Bonney et al., 2014). In the last decades, scientific and technological advances increasingly offer powerful tools for tackling these conservation and management challenges (McKinley et al., 2017). Successful conservation efforts should also consider social, cultural, and political factors that affect natural ecosystems (e.g. Mascia et al., 2003; Balmford & Cowling, 2006). Effective conservation efforts should further incorporate public input and engagement in creating solutions (Eden, 1996; Germain et al., 2001; Steelman, 2005). Citizen science, although not a perfect system (Callaghan et al., 2019), is becoming increasingly used as a powerful approach to significantly boost the availability of data for research (Phillips et al., 2019; Fraisl et al., 2020). Through the implementation of user-friendly platforms, multiple citizen science projects have thrived in maintaining public interest and continuous engagement (e.g. iNaturalist community; iNaturalist, 2019).

This chapter aims to contribute to the current knowledge on aquatic habitats in the region of Calakmul by: 1) characterizing aguadas across the region through providing information about their general structure and water quality; 2) evaluating the effects of prolonged droughts on local aguada structures by monitoring water levels and vegetation cover between 2017 and 2019; and 3) designing a standard monitoring protocol for aguada surveillance by combining digital data and citizen science implemented and managed by local environmental authorities in collaboration with local communities.

2.2 Material and Methods

2.2.1 Surveying for aguada locations

Aguadas within and around CBR were located based on previous information compiled by Mexico's National Commission for the Knowledge and Use of Biodiversity – CONABIO (García-Gil, 2000), satellite imagery in *Google Earth Pro* (Gorelick et al., 2017) and knowledge from local communities and authorities (National Commission of Protected Natural Areas – CONANP and the NGO Pronatura Peninsula Yucatan). Exploratory surveying to locate waterbodies were conducted over 35 days (June – July 2016, and August – September 2017). To ensure a representative distribution of survey sites, location effort was spread across all four sectors of the CBR (Core Zone North, Core Zone South, Buffer Zone North, Buffer Zone South) and adjacent areas outside the reserve (Figure 10). Survey areas were selected based on their geographical location and accessibility. Daylight reconnaissance surveys along survey sites were performed to determine feasibility, locate possible hazards and characterize habitats. The selection of waterbodies was further based on their relative importance to ongoing monitoring activities (e.g. aguadas used for crocodile surveys – Chapter 3; Operation Wallacea's annual monitoring and camera-trap grid), accessibility (access by vehicle and by foot), importance to local communities, and landowner permission within and outside CBR's buffer zones.

Each located aguada had its location recorded and the margins at maximum flooding capacity tracked with a GPS unit (Garmin 64s). Data were uploaded into *Google Earth Pro* for initial spatial visualisation and preparing the base shapefiles for the creation of aguada polygons. At each visit, photos were taken to provide visual information on the general structure of waterbodies for monitoring purposes. The dataset and photos collected during this study was made available online (Appendix II - Figure 47; Barão-Nóbrega, 2019) using Google's Spreadsheet Mapper v3.2 tool (Gorelick et al., 2017). More comprehensive datasets, containing additional information such as GIS aguada polygons, GPS tracks of access routes, water quality parameters and sediment contamination data will be made available to the public for easy visualisation in Google Earth and GeoNet (https://community.esri.com/), an ESRI community platform that allows GIS data to be shared for easy public visualization in ArcGIS online (https://www.arcgis.com/).

2.2.2 Aguada general structure characterization

An initial baseline characterization of an aguada was produced during the first visit to the site (during the exploratory field surveying; see above). Based on general structure and vegetation cover, aguadas were identified as pond (maximum area < 1 hectare), laguna (maximum area > 1 hectare) or jaguey (artificial pond excavated by local communities). Surface area (m^2) and maximum flooding capacity perimeter polygons (m) were calculated using satellite imagery in QGIS Desktop software version 3.0.2 (QGIS Development Team, 2019). Vegetation structure was classified based on general appearance and species composition within the waterbody (e.g. reeds, bushes, floating) and further classified as swamp (if dominant plant community is arboreal or shrubs), marsh (if dominated by emergent/submergent herbaceous vegetation coupled with low water depth) or floating vegetation (Moreno-Casasola et al., 2012). Vegetation cover was estimated visually including desiccated aguadas and categorized as (1) Low (0 - 30%), (2) Moderate (30 - 60%) or (3) High (>60%). Aguada water level (relation between current water surface and maximum flooding capacity) was again estimated visually and categorized as (1) Dry, (2) Mud, (3) Drying (0-25%), (4) Stable (25-75%) or (5) Full (>75%). An aguada was considered Dry when upon inspection the soil was desiccated (Figures 11A, 11D).

Monitoring of water level and vegetation cover was then carried out by revisiting sites in March (mid-dry season) and July (early-mid rainy season), 2018 and 2019. Depending on hydroperiod, aguadas were further classified as (1) temporary (i.e., dries up during dry season), (2) semi-temporary (i.e., often retains water all year round) or (3) permanent.

2.2.4 Aguada water level and vegetation cover analysis

Temporal and spatial patterns of water availability and vegetation cover across the region of Calakmul were separately investigated through a multi-model inference (MMI) on generalised linear models (GzLMs) with a Poisson error structure, including the interaction effect of year and zone on water level and vegetation cover (Zar, 2013; Slater et al., 2020) in the MuMIn package for *R* statistical software (*R* Development Core Team, 2019) in *R*Studio version 1.1.456 (*R*Studio Team, 2016).

The MMI ranked all potential models using Akaike Information Criterion (AIC) values and measured information loss ($^{\Delta}$AIC) between models to determine best fit to the data

(Akaike, 1974). The top model set was determined by using a threshold of $^{\Delta}$AIC < 2 (Burnham & Anderson, 2002; Burnham et al., 2011). Model-averaged effect sizes and 95% confidence intervals (CI) were calculated for the top model set variables. Variables with 95% CI not overlapping zero were assumed to significantly affect the response variables water level and vegetation cover.

2.2.5 Aguada water quality parameters

Physical-chemical parameters, including pH, water temperature, dissolved O_2, conductivity and salinity were measured during each visit (unless the waterbody had dried) with the use of the multi-parametric instruments *HACH-HQ40d* and *HANNA HI 98130*. Water samples (2L) were collected to evaluate water quality parameters (Table 1), following the standard guidelines of environmental health in freshwater ecosystems in Mexico (Chapman et al., 1996; Jiménez-Cisneros et al., 1997; Burton Jr, 2002; CONAGUA, 2019). After collection, water samples were frozen and transported back to EPOMEX Institute (Campeche, Mexico), where they were deep-frozen (-20ºC) until processed. Ammonium (NH_4^+), Cyanides ($CN^–$), Colour, Nitrites ($NO_2^–$), Nitrates ($NO_3^–$), Nitrogen (N), Total Phosphorous (P) and Sulphides ($S^{2–}$) were analysed using colorimetric measurement using UV-visible spectrophotometry, following the methods of the Environmental Protection Agency – EPA (1997), and the corresponding Official Mexican Standards (NOM127-SSA1-1994).

Superficial sediment samples (0 – 20 cm) were also collected between August and September 2017 in 66 aguadas for a screening study of Persistent Organic Pollutants (POPs) (Barão-Nóbrega et al. manuscript in preparation; Appendix IV).

2.2.3 Setting up an aguada long-term monitoring citizen science project

Taking advantage of the fact that smartphones are easy to acquire also for people living in rural areas such as Calakmul (Heimerl et al., 2015), a citizen science project for long-term aguada monitoring using digital data collection was designed and trailed using Epicollect5 (https://five.epicollect.net/, Aanensen et al., 2009; Aanensen et al., 2014), a mobile application developed by the Centre for Genomic Pathogen Surveillance (https://www.pathogensurveillance.net/) in collaboration with the Big Data Institute (https://www.bdi.ox.ac.uk/). This software is widely used worldwide in several fields of study, and in 2018 alone over 14,000 projects collected over 2.9 million data points (https://five.epicollect.net/projects/search).

Table 1. Variables used to characterize general structure and water quality of waterbodies in the region of Calakmul. Minimum threshold values for water quality follow the Mexican governmental guidelines for aquatic fauna protection in freshwater environments (CONAGUA, 2019).

Local characteristics	Units
General structure	
Classification	Category
Flooding capacity (perimeter)	metres
Hydric coverage	Score (1-4)
Dominant vegetation	Category
Vegetation cover	Score (1-3)
Water Physical parameters	
Acidity	pH
Dissolved O_2	mg/L
Conductivity	mS/cm
Salinity	ppt (mg/L)
Water quality parameters (permitted limits)	
Ammonium	(0.06) mg/L
Cyanides	(0.005) mg/L
Colour	(15) units
Nitrites	(0.01) mg/L
Nitrates	(0.04) mg/L
Nitrogen (total)	(0.05) mg/L
Phosphorous (total)	(0.05) mg/L
Sulphides	(0.002) mg/L

2.3 Results

2.3.1 Aguada general structure characterization

Twenty-four different areas across the three main zones in the region of Calakmul (Core, Buffer and Outside) were visited between June and July 2016 and August and September 2017, and a total of 85 waterbodies were identified (Figure 10). In total, 68% of aguadas were classified as pond (area < 1 ha), 22% as laguna (area > 1 ha) and 10% as Jaguey (artificial ponds, Figure 11). Nine (13%) waterbodies were dominated by emergent vegetation, six (9%) by floating vegetation, 39 (56%) by marshland and 35 (50%) by swamp vegetation (Figure 11).

Figure 10. Spatial distribution of all visited aguadas in the region of Calakmul. Each dark blue dot represents an aguada.

Figure 11. An example of a A) high vegetation cover dry pond (marsh); B) the same pond at full flooding capacity; C) pond covered in floating vegetation (*Salvinia* sp.); D) low vegetation cover dry pond (swamp); E) laguna close to full flooding capacity; F) dry laguna.

2.3.2 Aguada water level and vegetation cover analyses

The categorical value Dry was the overall modal water level across all aguada monitoring visits (2016-2019, Figure 12). Between survey years, the categorical value Stable was the modal water level in 2016, whilst Dry was the modal value in 2017-2019. The modal water level value was Dry in the Core Zone South, Buffer Zone North and Buffer Zone South, and Stable in the Core Zone North, Outside North and Outside South. The categorical value High was the overall modal vegetation cover across all aguadas, with no variation between survey years (Figure 13). The modal water level value was High for both Core and Buffer zones, but Low and Moderate for Outside North and Outside South, respectively.

To investigate the influence of survey year and zone on water level and vegetation cover, two GzLM model sets were investigated; the first considered the cumulative effects of surveyed year and zone, and the second accounted for possible interactions between these two variables. No significant interactions between survey year and surveyed zone, and no differences between model sets were observed for both water level ($p = 0.268$) and vegetation cover ($p = 0.94$). Water level variation was negatively affected by survey year ($p < 0.05$; Figure 14) and zone, with the lowest water level categorical values more often found in the southern Core and Buffer Zones (Figure 14). Vegetation cover was largely unaffected by both survey year and zone (Figure 15).

Table 2. Models used to investigate the effect of survey year and zone on water levels and vegetation cover of aguada habitats in Calakmul.

Model	R^2	AIC	LogLike	ΔAIC	AIC weight
Water level ~ Survey Year + Zone	0.23	774	-379.92	0	0.99
Water level ~ Zone	0.16	796	-392.13	22	<0.01
Water level ~ Survey Year	0.09	807	-401.90	33	<0.01
Water level ~ (.)	0.00	830	-414.11	55	<0.01
Vegetation Cover ~ Survey Year + Zone	0.04	765	-376.43	0	0.38
Vegetation Cover ~ Zone	0.00	765	-381.77	0	0.32
Vegetation Cover ~ Survey Year	0.04	766	-376.22	1	0.16
Vegetation Cover ~ (.)	0.00	767	-381.56	2	0.14

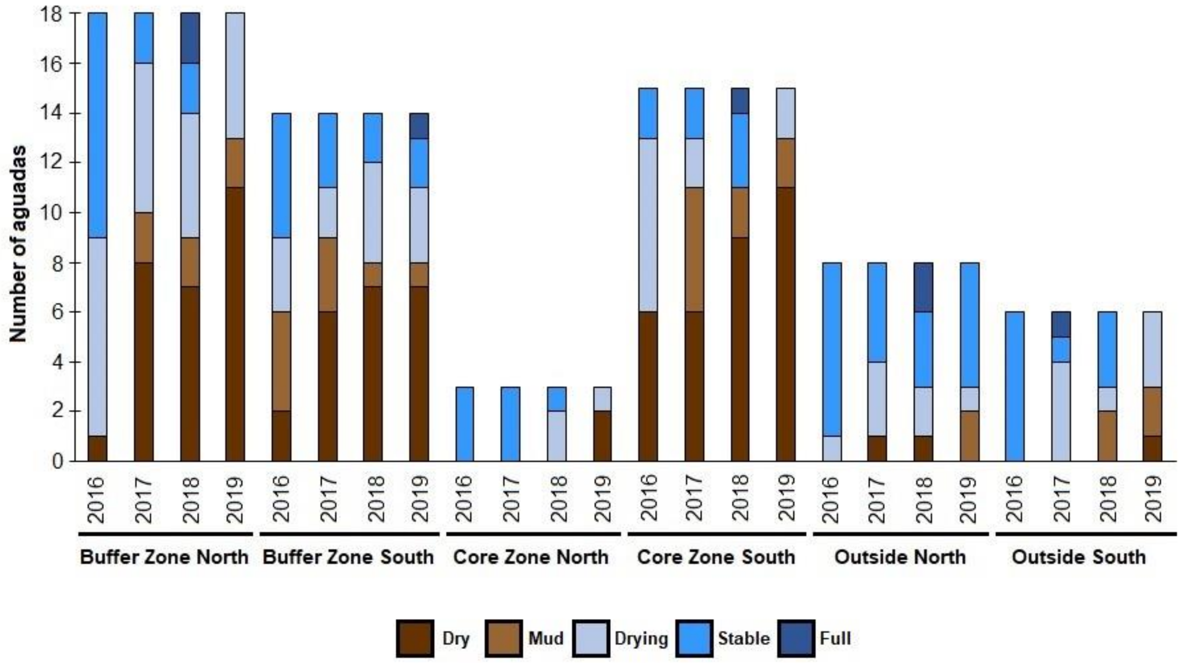


Figure 12. Categorical hydric state of aguadas across surveyed zones and years within and around Calakmul Biosphere Reserve. Bars indicate number of waterbodies exhibiting different categories of water level Dry = Dark brown; Mud = Light brown; Drying (less than 25%) = Light blue; Stable (25 – 75%) = Mid blue; Full (more than 75%) = Dark blue.

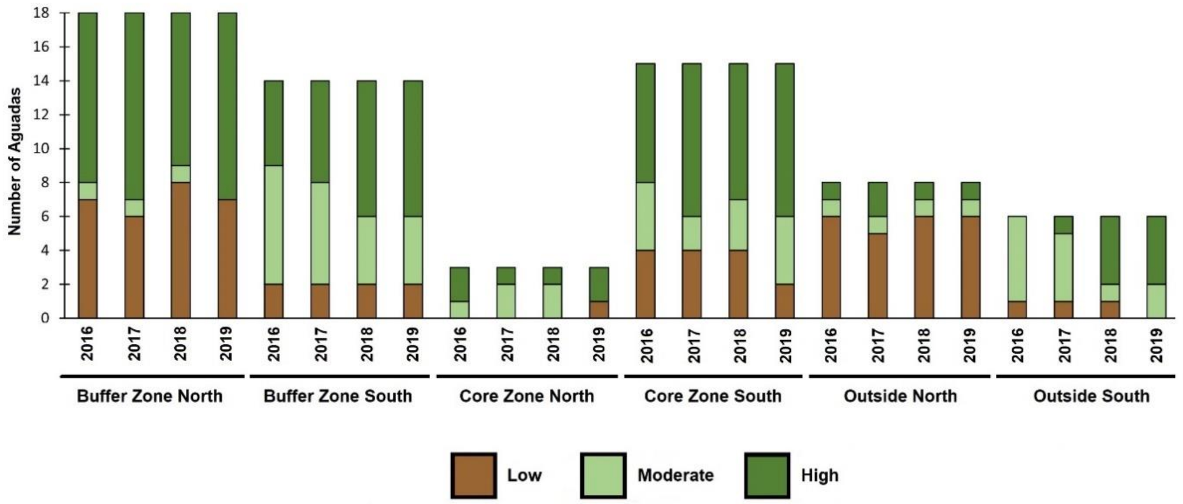


Figure 13. Categorical vegetation cover of aguadas across surveyed zones and years within and around Calakmul Biosphere Reserve. Bars indicate number of waterbodies exhibiting different categories of vegetation cover. Low (less than 30%) = Light brown; Moderate (30 – 60%) = Light Green; High (more than 60%) = Dark Green.

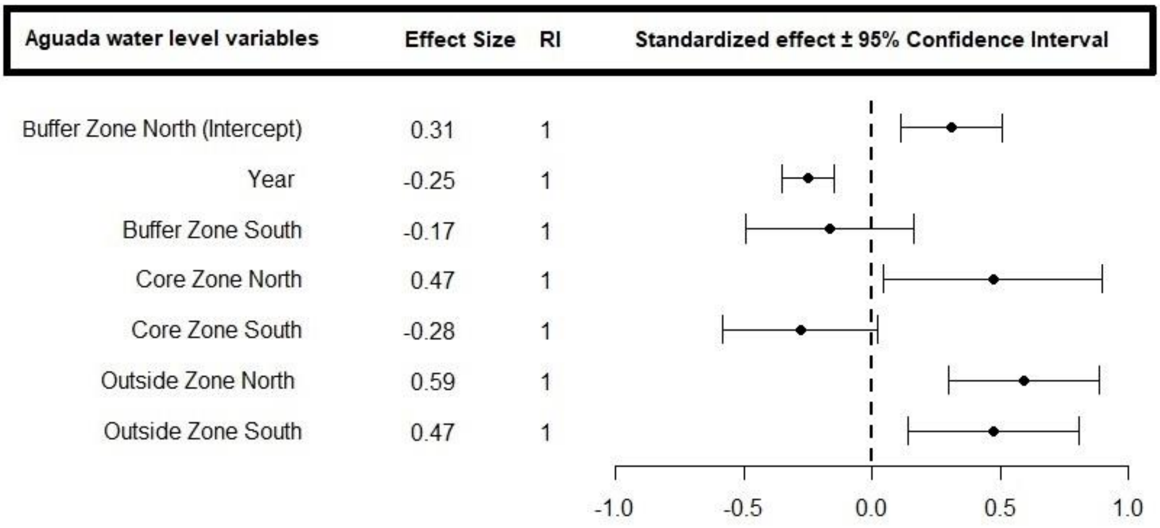


Figure 14. Multi-model inference used to investigate the effect of survey year and zone on water levels of aguada habitats in Calakmul. Points represent averaged standardised effect sizes from the top model set ($^{\Delta}$AIC < 2), and error bars indicate ± 95% confidence intervals. Relative importance (RI) is the proportion of models in the top set that include each variable.

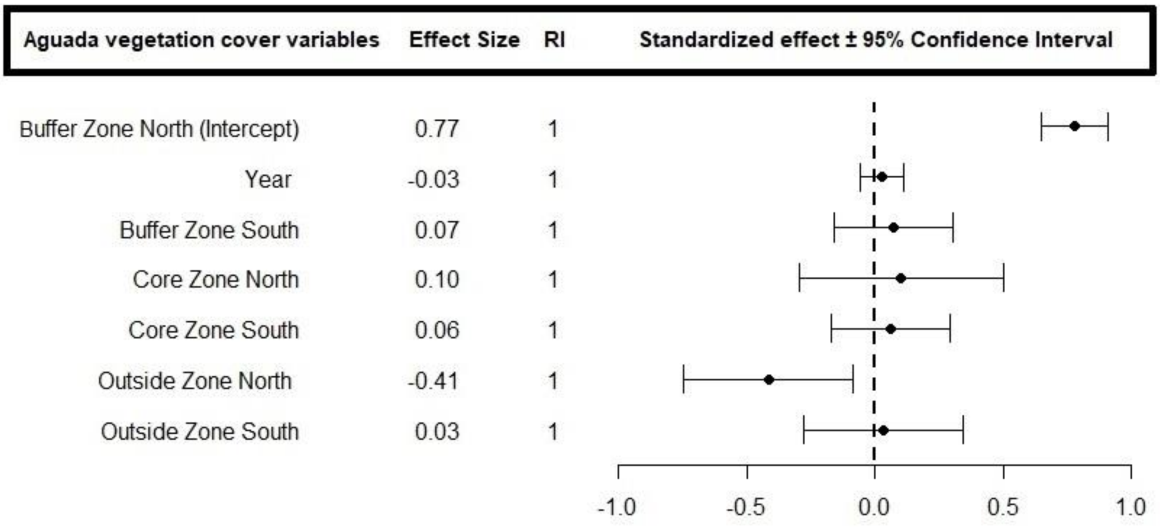


Figure 15. Multi-model inference used to investigate the effect of survey year and zone on vegetation cover of aguada habitats in Calakmul. Points represent averaged standardised effect sizes from the top model set ($^{\Delta}$AIC < 2), and error bars indicate ± 95% confidence intervals. Relative importance (RI) is the proportion of models in the top set that include each variable.

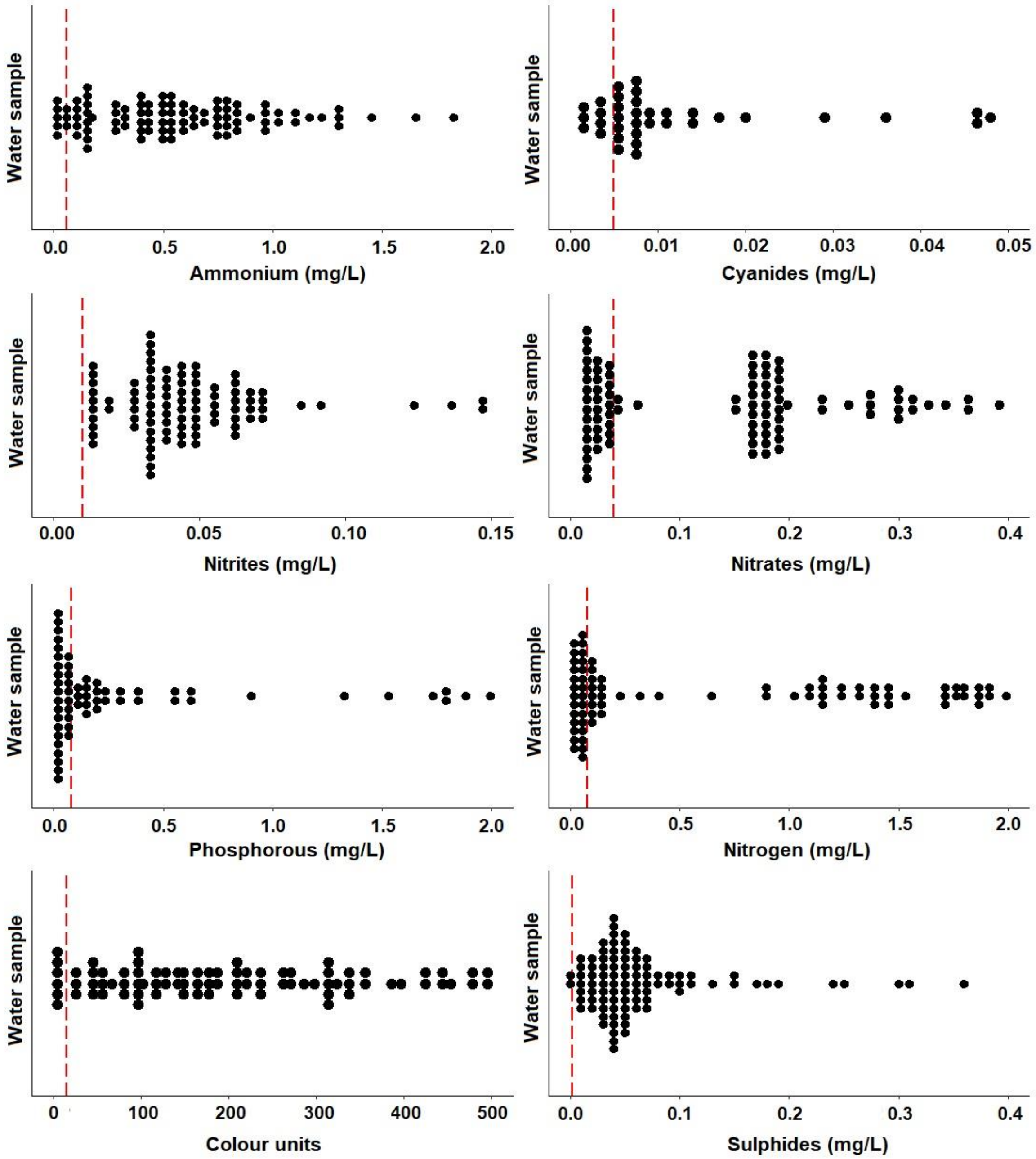


Figure 16. Water chemistry parameters measured in aguadas in Calakmul Biosphere Reserve. Each black circle represents a water sample from an aguada. Red dashed line indicates the minimum threshold value listed in the Mexican governmental guidelines for aquatic fauna protection in freshwater environments (CONAGUA, 2019).

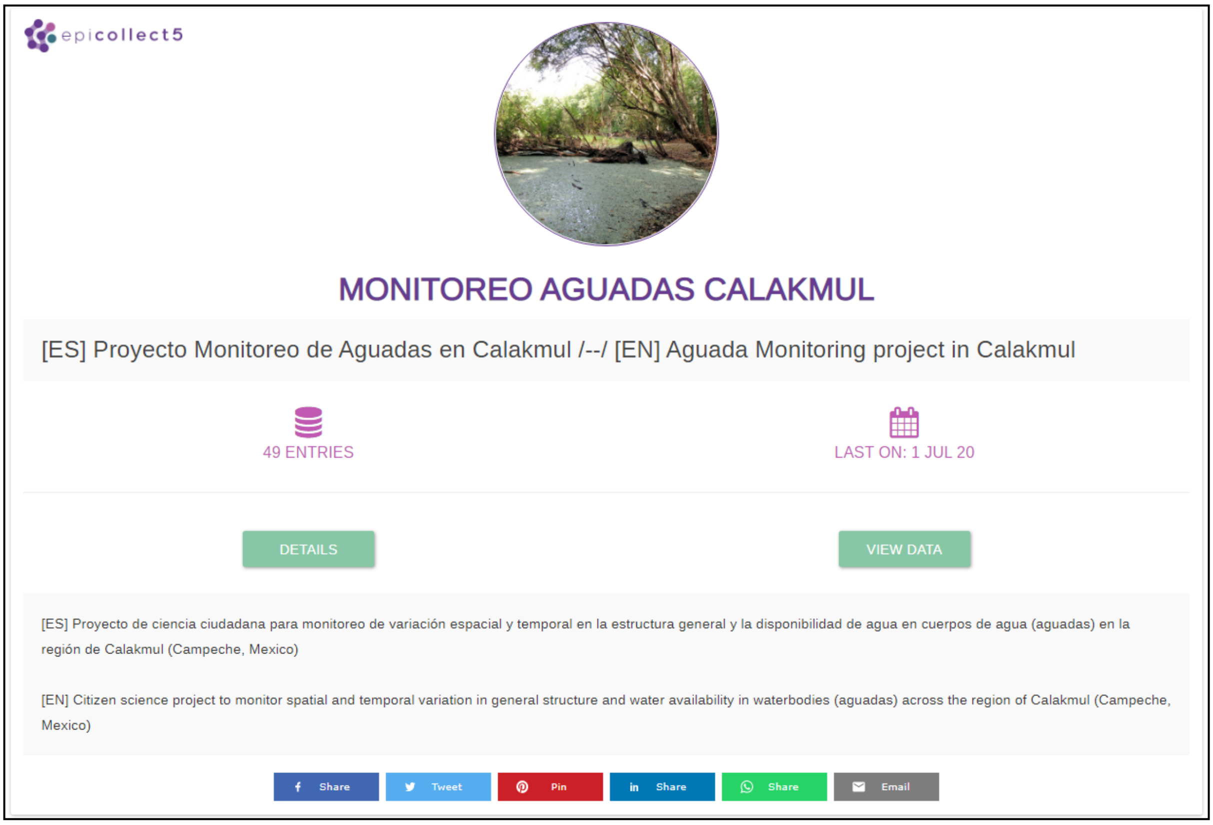
2.3.3 Water quality parameter analysis

Physical water parameters were available for 75 waterbodies (33 in September 2017 and 33 in March 2017 and 9 in July 2018). Equipment malfunctioned during the fieldwork in July 2018, and only nine aguadas could be measured during this month. Conductivity ranged from 0.11 to 2.44 mS (mean ± SD = 0.35 ± 0.41), salinity from 0.06 to 0.32 ppt (mean ± SD = 0.13 ± 0.06), pH from 3.5 to 8.9 (mean ± SD = 6.6 ± 1.0), and dissolved O_2 from 0.1 to 15.8 mg/L (mean ± SD = 3.4 ± 3.6). The range of values of chemical parameters are showed in Figure 16.

2.5.2 Long-term aguada monitoring

The baseline version of a digital data collection system for aguada monitoring using the Epicollect5 platform was set-up in February 2018, and different versions were field-tested in 25 aguadas in March and July 2018. The final version was presented to the CBR management authorities in July 2019 as the basis for a long-term citizen science project for aguada monitoring (Figures 17, 18), allowing information to be collected by park-wardens, local tour guides, field biologists and other people living or operating in the region using standardised data entry forms and data uploaded to a centralized online database. The data entry form takes under 5 minutes to complete and contains the following sections: 1) General Information – Q1: Geographical zone; Q2: Aguada location; Q3: Surveyor name; Q4: Date of visit; 2) Site location – Q1: Waterbody type; Q2: Surrounding forest structure; Q3: GPS coordinates (automatically obtained through the mobile device); 3) Hydric State – Q1: Water level; Q2: Estimated depth; 4) Vegetation Cover – Q1: Type of dominant vegetation; Q2: Vegetation cover density; 5) Presence of Aquatic Fauna (Crocodiles, Turtles or Fish); 6) Presence of Mammal Tracks; 6) Photographic Material (up to three photos of the aguada can be attached); 7) General Comments.

Unfortunately, I had plan to initiate the aguada monitoring using citizen science campaign in the summer of 2020 to capacitate local authorities and personnel in this digital data collection and lead the aguada monitoring efforts using citizen science, but unfortunately, I was not able to travel to Mexico due to the COVID-19 pandemic.



MONITOREO AGUADAS CALAKMUL

[ES] Proyecto Monitoreo de Aguadas en Calakmul /--/ [EN] Aguada Monitoring project in Calakmul

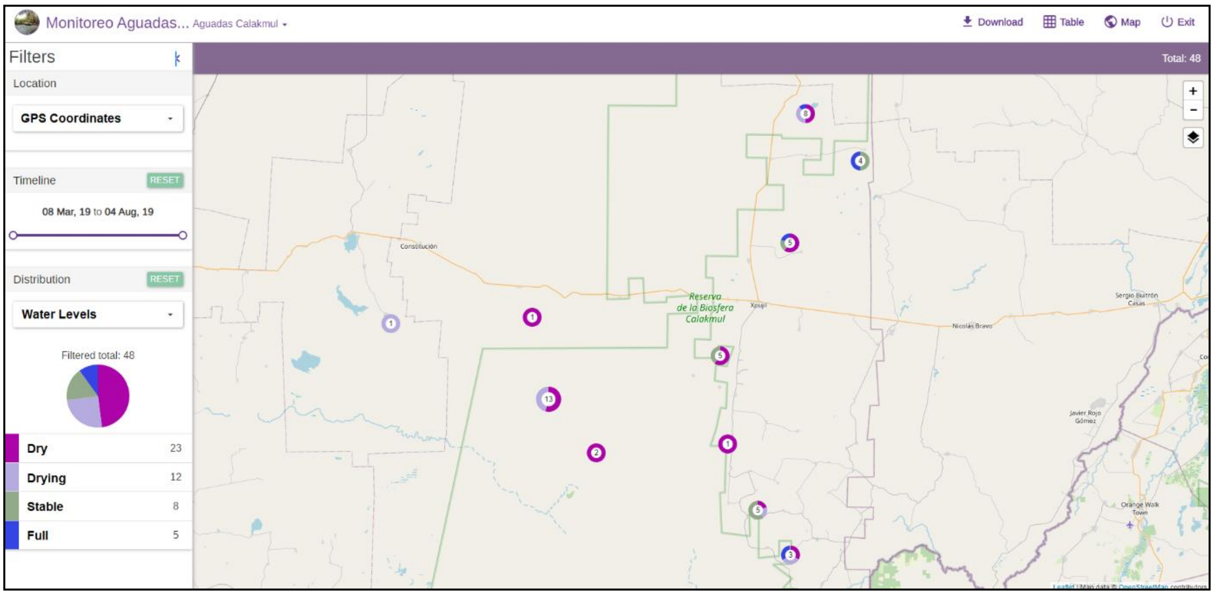
49 ENTRIES

LAST ON: 1 JUL 20

[ES] Proyecto de ciencia ciudadana para monitoreo de variación espacial y temporal en la estructura general y la disponibilidad de agua en cuerpos de agua (aguadas) en la región de Calakmul (Campeche, Mexico)

[EN] Citizen science project to monitor spatial and temporal variation in general structure and water availability in waterbodies (aguadas) across the region of Calakmul (Campeche, Mexico)

Figure 17. Digital data collection project for long-term aguada monitoring in the region of Calakmul using Epicollect5 (https://five.epicollect.net/project/monitoreo-aguadas-calakmul).



Location

GPS Coordinates

Timeline

08 Mar, 19 to 04 Aug, 19

Distribution

Water Levels

Filtered total: 48

Dry	23
Drying	12
Stable	8
Full	5

Figure 18. Spatial visualisation of aguada water level data collected in 2019 across Calakmul using Epicollect5. Adapted from https://five.epicollect.net/project/monitoreo-aguadas-calakmul.

2.4 Discussion

Aguadas constitute an important resource across Calakmul, serving as a source of water for local communities and providing habitats and food resources to many aquatic and terrestrial species, some of which are endangered (e.g. Baird's tapir – *Tapirus bairdii*; Narrow-bridged Musk Turtle – *Claudius angustatus*). This chapter adds to the current knowledge of the general structure of aguada habitats in Calakmul, and demonstrates how the recent prolonged droughts severely impacted water sources across the region, impacting both wildlife and human communities.

2.4.1 General structure monitoring

The abiotic and biotic conditions that dominate the region facilitate habitat structures that do not occur elsewhere within the Yucatan Peninsula (García-Gil et al., 2002; Moreno-Casasola et al., 2012). Aguadas represent important landscape elements as they maintain locally humid conditions during the annual dry season and play an important role for wildlife occurring in the region (Reyna-Hurtado et al., 2019; Sánchez-Pinzón et al., 2020; Slater et al., 2020).

Drought considerably influenced water levels in aguadas across Calakmul between 2016 and 2019, but its impact varied by zone, supporting that worsening drought conditions would further reduce water availability depending on the region (Slater et al., 2020). Although waterbodies in the northern portion of the reserve are more likely to desiccate when compared to the south due to their sizes and the precipitation gradient across the reserve (García-Gil et al., 2002), the southern Core and Buffer zones were worse affected. Aguada monitoring data combined with information gathered from informal interviews of local guides suggest that resistance to water level decline in a following dry season is only observed in locations that retain water or elevated soil moisture (i.e. mud) until the beginning of the rainy season, but not in sites entering the rainy season completely desiccated. These waterbodies continue to exhibit a temporary hydroperiod on a yearly basis even when previously classified as semi-temporary (García-Gil, 2000; Barão-Nóbrega, 2019) until unusually intense precipitation hits the region (e.g. a hurricane) and hydrologically saturates multiple layers of soil. This is due to the proliferation of soil cracks and the colonization of fast growing herbaceous and arbustive vegetation which rupture the impermeable layers that retain water. Cracks extend the soil – air interface deeper into the soil profile, and induce water loss via evaporation (Somasundaram et al., 2018). They also facilitate rainwater infiltration down the soil profile, hampering the ability of the superficial layer of soil to act as a natural barrier retaining water on the surface (Coleman et al., 2017), causing rainfall intensity to no longer correlate with aguada water levels (Reyna-Hurtado et al., 2019).

A possible mitigation measure would involve the scything of the secondary vegetation and adding of water to the dry soil to create mud to which leaf-litter is added to achieve a re-impermeabilization of the sediment. This method is efficient (Slater et al., 2020) but limited to aguadas that are relatively close to a main road to allow access by a water truck. Potential alternative intervention methods could include the use of impermeable artificial lining, which have a long history of use in African game reserves (Smit et al., 2007) and have also have been successfully used to create and maintain vernal pools in the USA (Biebighauser, 2002).

2.4.3 Water quality assessment

The pH of the majority of natural aguadas in Calakmul was previously measured to fall between 6.0 and 8.5, with the lowest pH values occurring in water with high values of dissolved organic matter and the highest values in eutrophic waters (Chapman et al., 1996). This range of values agrees with what was previously reported in a variety of aquatic systems in the northeast of the Yucatan Peninsula (cenotes, caverns, rivers, aguadas and wetlands; Pearse et al., 1936; Alcocer et al., 1998; Herrera-Silveira et al., 1998). In the present study, mean aguada pH was 6.6, supporting a tendency for slightly acidic conditions due to high levels of dissolved organic matter (see also CONAGUA, 2019). Indeed, the majority of aguadas in Calakmul are small to medium sized ponds (García-Gil, 2000) and form lentic ecosystems for extended periods (Reyna-Hurtado et al., 2010; Moreno-Casasola et al., 2012; Barão-Nóbrega, 2019).

Observed values of dissolved oxygen averaged 3.4 mg/L, at however significant variation between waterbodies (minimum and maximum values of 0.1 to 15.8 mg/L, respectively). This variation is not surprising, as it reflects the observed variation in general structure between aguadas in Calakmul. Overall, lower values are associated with biological activity (respiration and microbial oxidation of organic matter) which generates chemical oxidation (Pearse et al., 1936; Alcocer et al., 1998). Higher values may be explained the presence of aquatic macrophytes which increase oxygen concentrations (Alcocer et al., 1998).

Coinciding with previous studies for the Yucatan Peninsula, aguadas in Calakmul exhibited relatively low salinity and conductivity values, which may be explained by the presence of dissolved $CaCO_3$ (Alcocer et al., 1998). Indeed, most of the subtract in the region is mainly composed of carbonated rocky materials (García-Gil et al., 2002).

The dissolved nutrient values obtained in the present study were compared with the official water quality criteria set by Mexican legislation for aquatic fauna protection in freshwater environments (CE-CCA-001/89; CONAGUA, 2019), and demonstrate that concentrations of Ammonium (NH_4^+), Cyanides (CN^-),Nitrites (NO_2^-), Nitrates (NO_3^-), Nitrogen (N), Total Phosphorous (P) and Sulphides (S_2^-) overall exceeded the minimum thresholds. This regulation is generally applied to all types of waters in Mexico without consideration of hydrodynamic or biogeochemical differences (Camacho-Cruz et al., 2020), and is therefore not necessarily fully adequate to assess ecological quality. Nonetheless, this study shows that aguadas in Calakmul are generally nutrient rich, likely due to their lentic nature (Valencia Díaz, 2001; Reyna-Hurtado et al., 2010; Moreno-Casasola et al., 2012) combined with high levels of recycling of vital nutrients through consumption and excretion by residing fauna (see also Fittkau, 1973; Rosenblatt et al., 2013). The measurements of water quality parameters will provide reference values for future studies that should also include landscape analysis to shed further light into any possible spatial patterns associated with, for example, dominant vegetation, surrounding forest structure, habitat heterogeneity and proximity anthropogenic structures and agriculture fields.

2.4.4 Long-term aguada monitoring

Scientific research conducted in whole or in part by people for whom science is not their profession is now considered mainstream, with thousands of participants worldwide contributing observations of the natural world to various citizen science projects every day (Callaghan et al., 2019). These projects are valuable for society and grow in scientific importance with time (Dickinson et al., 2012; McKinley et al., 2017), as they provide educational opportunities, increase scientific knowledge, and collect immense amounts of biodiversity information (Flemons et al., 2007; Chandler et al., 2017; Soroye et al., 2018; iNaturalist, 2019). Projects employing citizen science and aiming at broadscale data collection fall into a continuum, from unstructured to structured, based on the objectives, survey design, flexibility, rigorousness, and detail collected about the observation process (Welvaert & Caley, 2016; Kelling et al., 2019). Projects with clear objectives, planned data analysis and rigorous protocols like the UK Butterfly Monitoring Scheme (Fox et al., 2011) are classified as structured projects. On the other hand, projects like iNaturalist (iNaturalist, 2019) with open and flexible recruitment and an overall general lack of protocols are classified as unstructured (Callaghan et al., 2019; Kelling et al., 2019). Regardless of the level of structure in data collection, citizen science projects generally aim to collect observations of a unique topic along with spatial and temporal data.

Given the vast potential of citizen science monitoring schemes (Tulloch et al., 2013; Chandler et al., 2017), methods to decrease patchiness and increase information in the data are crucial (Callaghan et al., 2019). A common bias associated with citizen science is inter-observer skill differences, which influences data quality and validation and particularly affects biodiversity sampling projects (Callaghan et al., 2019; Kelling et al., 2019) . While the aguada surveillance scheme in Calakmul should be less affected by such biases, sampling strategies and protocols should be monitored throughout the life of such projects (Kelling et al., 2019). At this initial stage, the aguada monitoring project is aimed to facilitate structured data collection by personnel from the CBR’s reserve management team (e.g. park wardens) and other relevant authorities which will have received appropriate training.

The long-term aguada surveillance using Epicollect5 was proposed by Operation Wallacea in June 2020 to integrate Calakmul's action plan for aguada monitoring and conservation, and became approved by the CBR's management and environmental authorities. The action plan was further presented to UNESCO and IUCN during the annual status review meetings, where logistical details for data collection campaigns were further discussed. Plans for future efforts to also deploy the Epicollect5 digital collection tool in adjacent projected areas was also discussed, aiming to set the foundation for standardized data collection across the entire Selva Maya (Mexico, Guatemala and Belize) by 2030.

By involving local communities across the region through field guides and elected community representatives (ejidal commissary), additional surveillance projects can be further developed on a wider scale across Calakmul. This unlocks the potential to provide valuable information to better understand the hydrological characteristics and its respective variation over time, which will help to better direct conservation efforts towards communities worst affected by prolonged drought periods. The long-term goal is to empower reserve management teams across many areas, by providing their personnel with the baseline working tools for future monitoring projects using digital collection linked to centralized databases (e.g. road-kill surveillance to identify important areas for wildlife crossing; illegal logging activity surveillance).

Chapter 3 – N-mixture models provide informative crocodile (*Crocodylus moreletii*) abundance estimates in dynamic environments

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Abstract

Estimates of animal abundance provide essential information for population ecological studies. However, the recording of individuals in the field can be challenging, and accurate estimates require analytical techniques which account for imperfect detection. Here, we quantify local abundances and overall population size of Morelet's crocodile (*Crocodylus moreletii*) in the region of Calakmul (Campeche, Mexico), comparing traditional approaches for crocodylians (Minimum Population Size – MPS; King's Visible Fraction Method – VFM) with binomial *N*-mixture models based on Poisson, zero-inflated Poisson (ZIP) and negative binomial (NB) distributions. A total of 256 nocturnal spotlight surveys were conducted across 50 representative locations (hydrologically highly dynamic aquatic sites locally known as aguadas) over a period of five years (2015-2019). Population size estimates through MPS and VFM revealed a median of 0.4 (min – max: 0 – 67; Q1 – Q3: 0 – 3) and 0.95 (0 – 159; 0 – 8) non-hatchling *C. moreletii* for each aguada, respectively. The ZIP based *N*-mixture model shown overall superior confidence over Poisson and NB, and revealed a median of 5 ± 2 individuals (min = 0; max = 116 ± 15; Q1 = 0.9 ± 0.6; Q3 = 23 ± 4) jointly with high detectabilities in drying aguadas with low and intermediate vegetation cover. Extrapolating these inferences across all waterbodies in the study area yielded in an estimated ~12,000 *C. moreletii* present, demonstrating that Calakmul is an important global stronghold for this species. Because covariates enable insights into population responses to local environmental conditions, we confirm that *N*-mixture models applied to spotlight count data result in particularly insightful estimates of crocodylian detection and abundance.

3.1 Introduction

Measures of abundance and population size are key to understanding the ecology and natural history of wild animals, and form a basis for the implementation of conservation management plans. However, due to elusive behaviours and logistic constraints, researchers are usually unable to record all individual animals in a given location. Because detectability also interacts with for example local environmental conditions, precise abundance estimates based on census data alone are generally difficult to obtain (e.g. Sutherland, 2006; Mazerolle et al., 2007).

An emerging approach to estimate population sizes from repeated standard count surveys is represented by *N*-mixture models, which jointly quantify a measure of abundance (λ) with the probability of detecting an individual (p) (Royle, 2004; Zipkin et al., 2014; Denes et al., 2015; Kéry & Royle, 2016). Binomial *N*-mixture models, for example, treat λ as a random independent variable generated from a statistical distribution to estimate p (Royle, 2004; Kéry et al., 2005). *N*-mixture models are particularly promising for wildlife studies because they have the potential to reveal estimates which are comparable to those obtained by more labour-intense (and often more invasive) capture-mark-recapture approaches, and because explanatory variables that may influence λ and p can be investigated in a straightforward way using generalized linear models (GLMs; Priol et al., 2014; Courtois et al., 2016; Ficetola et al., 2018). *N*-mixture models have already been applied to a wide range of wildlife species (e.g. Belant et al., 2016; Hunter et al., 2017; Romano et al., 2017; Ward et al., 2017; Kéry, 2018; Kidwai et al., 2019; Manica et al., 2019), but are still considered as an emerging framework with ongoing extensions to original parameterizations (Denes et al., 2015; Kéry & Royle, 2016; Barker et al., 2018; Bötsch et al., 2019).

Despite their large size, crocodylians are an example taxonomic group for which imperfect detection during surveys is common (e.g. Hutton & Woolhouse, 1989; Da Silveira et al., 2008; Balaguera-Reina et al., 2018). Historically, crocodylian population size estimations outside the capture-recapture framework have largely been based on spotlight surveys to reveal minimal counts, or accounting for visible fractions (Bayliss, 1987; King et al., 1990; Balaguera-Reina et al., 2018). Although not yet widely used, *N*-mixture models have already been explored to investigate the influence of covariates on both local abundance and crocodile detection (Fujisaki et al., 2011;

Waddle et al., 2015; Gardner et al., 2016; Lyet et al., 2016; Mazzotti et al., 2019; Naveda-Rodriguez et al., 2020), but detailed comparisons with more traditional methods particularly in hydrologically dynamic habitats are as yet lacking.

The Morelet's crocodile (*Crocodylus moreletii*) is a medium-to-large crocodile species occurring in Atlantic lowlands surrounding the Gulf of Mexico (Belize, Guatemala and Mexico; Sigler & Gallegos, 2017). Our knowledge of the population ecology and status of *C. moreletii* has markedly increased over the last decades, and a standard international survey program to monitor its wild populations was developed in 2010 (Sánchez-Herrera et al., 2011). However, only rudimentary information about this species is available for the southern region of the Yucatan Peninsula, an area which is characterized by very dynamic hydrological regimes and which harbours parts of the largest remaining expanse of tropical forest in Mesoamerica (Carr III, 1999; Vester et al., 2007). In the present study, we estimate both local abundances as well as the total population size of *C. moreletii* in this region, using a set of binomial *N*-mixture modelling approaches for comparison with more traditionally used methods. Because *C. moreletii* locally inhabits particularly unstable and heterogeneous waterbodies, the study area provides an excellent opportunity to probe the versatility of *N*-mixture models under highly variable levels of detectabilities.

3.2 Material and Methods

3.2.1 Study area and data collection

Calakmul Biosphere Reserve (CBR) is located within the southern portion of the Yucatan Peninsula in Campeche, México (18°21.921' N, 089°53.220' W; Figure 19), and together with the adjacent state reserves Balam-Ku and Balam-Kin encompasses more than 1.2 million hectares of protected forest for which *C. moreletii* represents one of the main flagship species. CBR is part of the Selva Maya, which was home to the ancient Mayan civilization and covers 10.6 million hectares of forest across Mexico, Guatemala and Belize (Vester et al., 2007). Precipitation gradually increases from 900 mm annually in the north to 1,400 mm in the south, with significant effects on local forest structure and tree species composition (Martínez & Galindo-Leal, 2002; Vester et al., 2007). The majority of the reserve is composed of tropical semi-deciduous forest with a canopy ranging from 15 to 40 m in height, with the northern parts containing deciduous forest with canopy heights of 8 to 20 m (Chowdhury, 2006). The geological characteristics of the CBR result in rapid rainwater belowground runoff, and non-permanent as well as semi-permanent small to medium-sized waterbodies, locally known as aguadas, represent the only source of water during the dry season (García-Gil et al., 2002; Reyna-Hurtado et al., 2010; Barão-Nóbrega, 2019). The distribution, prevalence and morphology of aguadas across Calakmul is strongly influenced by annual precipitation cycles, resulting in high levels of seasonal and yearly variation in their general structure (hydric coverage, vegetation communities and cover; García-Gil et al., 2002; Barão-Nóbrega, 2019; Márdero et al., 2019).

Candidate aguadas for *C. moreletii* surveys were identified using existing information (García-Gil, 2000), *Google Earth Pro* (Gorelick et al., 2017), and local knowledge by environmental authorities (*Comisión Nacional de Áreas Naturales Protegidas* - CONANP and *Pronatura Península Yucatán*), local guides and community representatives to define a total of 50 survey locations spread across the CBR and its surroundings. While accessibility by vehicles as well as landowner permissions were a prerequisite for field surveys, information on the presence or absence of *C. moreletii* was largely unavailable for survey site selection. Exhaustive nocturnal spotlight counts were performed in July (wet season) 2017 and in March (dry season) as well as July 2018-2019 for all 50 waterbodies whenever possible, supplemented with additional surveys for a subset of 20 waterbodies in July 2015 and 2016. Water levels for each aguada were classified into Dry, Drying, Stable, or Full, and vegetation cover was defined as Low, Medium or High (for details see Barão-Nóbrega, 2019; Chapter 2 – Figure 11). Avoiding days of full moon, high winds and heavy rain, surveys were conducted by systematically traveling along the perimeter of the waterbody on foot or by paddling along the shoreline aboard a 3.5 meters aluminium boat. Individual *C. moreletii* were located by their eyeshine reflection (Sánchez-Herrera et al., 2011). If the waterbody was empty, the number of detected crocodiles was assumed zero. Crocodile hatchlings (TL < 30 cm) have high mortality rates (Grigg & Kirshner, 2015) and were excluded from the count data (Balaguera-Reina et al., 2018). Research permits for fieldwork activities in CBR were issued annually by Mexico's Secretariat of Environment and Natural Resources (SEMARNAT; SGPA/DGVS/03030/17;

SGPA/DGVS/005403/18) and National Commission of Natural Protected Areas (CONANP; D-RBC-118/2017; D-RBC-030/2018; D-RBC-087/2019).

Figure 19. Location of the Calakmul Biosphere Reserve (CBR) in southern-central region of the Yucatan Peninsula (Mexico). The area delimited inside the inner lines within the grey and dark grey areas (CBR) represent, respectively, the politically established buffer and core zones of the biosphere reserve. White squares represent *C*. *moreletii* survey locations.

3.2.2 Abundance estimation

As abundance estimation methods which are in common use for many crocodylians including *C. moreletii* (Cedeño-Vázquez et al., 2006b; Tellez et al., 2017), I used a Minimum Population Size (MPS) approach as well as the King's Visible Fraction Method (VFM). MPS quantifies the number of recorded individuals per spatial unit to obtain baseline data (Sánchez-Herrera et al., 2011). VFM makes use of repeated counts per site, estimating the percentage of the total population observed during a single count (the visible fraction) as $vf = \frac{\overline{x}}{1.05(2\sigma+\overline{x})}$, where σ is the standard deviation and $\overline{x}$ is the mean number of crocodiles counted across replicates at each site (King et al., 1990; Balaguera-Reina et al., 2018). Local absolute abundances (n) at given waterbodies can therefore be expressed as $n = \frac{RA}{vf}$, with RA being the observed local relative abundance.

Aside of these traditionally applied methods, we also used binomial *N*-mixture models and explored three alternative statistical distributions: Poisson, Negative Binomial (NB), and Zero-Inflated Poisson (ZIP). Poisson distributions are generally applied to describe relative density, but have a variance which is equal to its mean and therefore do not conform well to under- or over-dispersed data (Denes et al., 2015). Both Poisson and NB distributions tend to perform poorly in the presence of a significant number of true zeros in the dataset (Wenger & Freeman, 2008; Joseph et al., 2009), whereas the ZIP distribution is generally able to better accommodate both true and false zeros (Denes et al., 2015). Both ZIP and NB distributions allow for over-dispersion (Kéry & Royle, 2016; Mazzotti et al., 2019). All *N*-mixture models were fitted to the dataset using the *pcount()* function in the unmarked package in *R* (Fiske & Chandler, 2011; *R* Development Core Team, 2019), using *R*Studio version 1.1.456 (*R*Studio Team, 2016). At first, we allowed λ to vary as a linear function of year to investigate the general assumption of population closure and presence of any temporal trend in the dataset. As no significant difference in abundance values was observed between years (F = 1.7; p = 0.151; Figure 20), we then assumed that λ remained overall constant with respect to survey year within survey locations in the final models. Waterbody perimeter (in metres) was used as a covariate for λ, in addition to a geographic specifier to account for possible spatial differences in abundance within Calakmul. Waterbody hydric state (Dry, Drying, Stable, Full) and vegetation cover (Low, Medium, High) were used as covariates of *p*. Waterbody perimeter was scaled and centred by subtracting the mean from each value, divided by the standard deviation (Kéry & Royle, 2016). We discarded models that failed to converge, and used Akaike's Information Criterion (AIC, Akaike, 1974) to identify the best models from each *N*-mixture approach. The *predict*() function in unmarked was used to generate plots of estimated relationships with predictors for each covariate. Parametric bootstrapping (1000 simulations) was conducted using the *parboot*() function (Fiske & Chandler, 2011), to calculate p-values from sums of squares (SSE), Pearson's Chi-square and Freeman-Tukey fit statistics that quantified the fit of models to the dataset. A dispersion parameter (ĉ) was calculated as the ratio of the observed fit statistic to the mean of the simulated distribution (Kéry & Royle, 2016). As caution is often advised when using NB, even when it is greatly favoured by AIC, particularly when this distribution produces substantially higher estimates than Poisson and ZIP distributions (Kéry & Royle, 2016; Kéry, 2018), we've further investigated which model structure would provide most overall confidence in crocodile abundance estimations by running a residual diagnostic analysis using the *plot.Nmix.resi*() function available in the *AHMbook* package for R (Kéry & Royle, 2016).

To assess the total number of *C. moreletii* present in the study area ($\widehat{N}$), we extracted the total number of semi-temporary and permanent aguadas in the study area (CBR and surroundings) from an existing GIS-based dataset (García-Gil, 2000), and calculated their perimeters using QGIS Desktop software version 3.0.2 (QGIS Development Team, 2019). $\widehat{N}$ was assessed through four local abundance estimators (n^{MPS}, n^{VFM}, *N*-mixture NB and *N*-mixture ZIP). For n^{MPS} and n^{VFM}, the relationship between local *C. moreletii* abundance and the perimeter of each surveyed waterbody was determined through a GLM and extrapolated to all waterbodies. Total population sizes based on MPS and VFM were also calculated by solely multiplying average local abundance estimations with the total number of waterbodies.

3.3 Results

A total of 256 surveys were conducted between 2015 and 2019 (20 surveys in July of both 2015 and 2016, 42 surveys in July 2017, 47 and 48 surveys in March and July 2018, as well as 50 and 47 surveys in March and July 2019), yielding a total of 1,105 *C. moreletii* records (Total waterbody perimeter = 24,980 metres; Total survey distance covered = 127,897 m). *Crocodylus moreletii* was detected at least once in 32 of the 50 surveyed sites (64%). Counts per spotlight survey ranged between zero and 89 (median = 0; Q1 = 0; Q3 = 2) and relative abundance per waterbody (mean number of individuals counted) averaged between 0 and 67 (median = 0.4; Q1 = 0; Q3 = 3). Across sites, vf varied between 0.16 and 0.95 (mean ± SD = 0.48 ± 0.24), with the resulting local population size estimates (n^{VFM}) ranging from 0 to 159 individuals (median = 0.95; Q1 = 0; Q3 = 8).

Considering all possible combinations of covariates, twelve *N*-mixture models were generated for each distribution (Poisson, ZIP and NB). Overall, NB distributions yielded the lowest AIC values across all parameter combinations, although Poisson and ZIP models achieved a better agreement between both observed and expected data as well as between residuals and fitted values (Table 3, Figure 21). The models which accounted for waterbody location and perimeter on λ as well as the cumulative effects between co-variates on *p* exhibited the lowest AIC values amongst all possible *N*-mixture combinations (Table 3). Median abundance estimates for NB, ZIP and Poisson were 10 ± 9 (min–max = 0 – 308 ± 264; Q1 = 1 ± 1; Q3 = 31 ± 16), 5 ± 2 (min–max = 0 – 116 ± 15; Q1 = 0.9 ± 0.6; Q3 = 23 ± 4) and 6 ± 3 (min–max = 0 – 123 ± 13; Q1 = 0.9 ± 0.6; Q3 = 26 ± 4) individuals per waterbody, respectively. Taken together, we considered that *N*-mixture ZIP provided superior model confidence over the Poisson and NB models, as particularly the latter showed poor residual diagnostic performance and yielded unrealistically high abundance estimates with wide-ranging confidence intervals, in comparison to the other two (Figure 22). Based on the *N*-mixture ZIP model, the highest probabilities of detection ($p = 0.70$) were observed in Drying waterbodies with Low to Moderate vegetation cover, whereas low detectability was associated with High vegetation cover ($p < 0.25$, Figure 23). Bootstrap p-values for the best-fit *N*-mixture ZIP model based on SSE, Freeman-Tukey, and Chi-square statistics were 0.14, 0.01 and 0.20 (Figure 24), respectively, at evidence for over-dispersion (ĉ = 2.70).

The relationships between waterbody perimeter and local abundance estimates (n^{MPS}, n^{VFM}, *N*-mixture NB and *N*-mixture ZIP) were used to provide an estimate for the total number of non-hatchling *C. moreletii* in the study area (Table 4). Analyses of the GIS-based dataset revealed a total of 1,663 aguadas, which had a median perimeter of 139 (min = 40 m; max = 3639 m; Q1 = 98 m; Q3 = 207 m; Figure 25). Based on these numbers, $\mathrm{MPS}^{\mathrm{GLM}}$ defined a minimum *C. moreletii* population size of 4,273 non-hatchling individuals, which is considerably lower than the values obtained by approaches which take detectability into account (Table 4). The *N*-mixture ZIP model revealed a total population size of 11,681 crocodiles, which was slightly below the value obtained by $\mathrm{VFM}^{\mathrm{GLM}}$ (12,177 crocodiles). The highest population size estimate of 15,184 individuals was produced by VFM without taking waterbody perimeter into account.

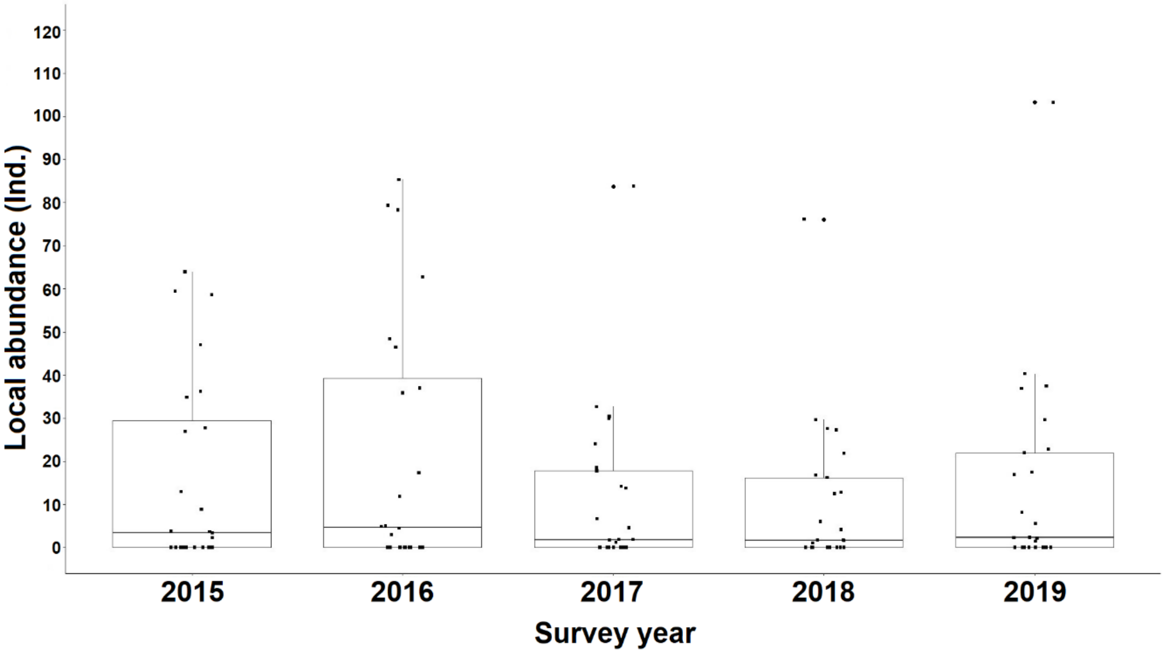


Figure 20. Variation in local *Crocodylus moreletii* abundances in Calakmul in relation to survey year. Local abundances were estimated through binomial *N*-mixture modelling approach, and five summary statistics are visualised (the median, two hinges and two whiskers). Lower and upper hinges correspond to the first and third quartiles, whilst the whiskers extend from the hinge to the largest value no further than 1.5 * IQR (inter-quartile range, or distance between the first and third quartiles) from the hinge.

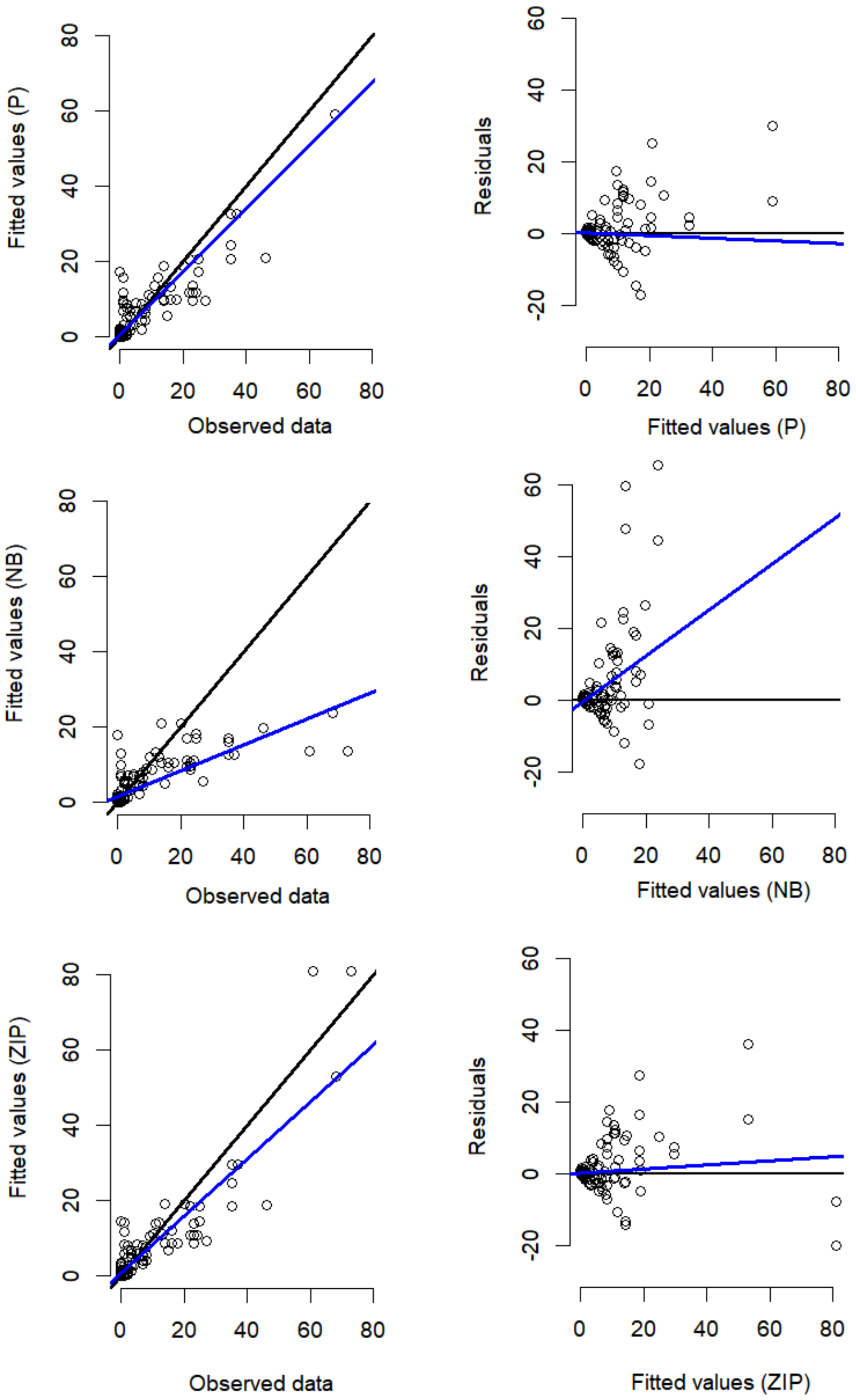


Figure 21. Residual diagnostics for the three *N*-mixture models fitted to the *Crocodylus moreletii* spotlight count dataset. Left hand side figures represent Poisson, NB and ZIP *N*-mixture fitted values vs observed crocodile counts, where the black line shows a 1:1 relationship and the blue line is the linear regression line of best fit. Right hand side figures represent residuals vs fitted values (blackline denotes a zero residual and the blue line is the linear regression line).

Table 3. Abundance estimation models of *Crocodylus moreletii* abundance in Calakmul using three different *N*-mixture model approaches (Poisson, Negative Binomial - NB and Zero Inflated Poisson - ZIP), with different combinations of waterbody perimeter as covariate of abundance (λ) and vegetation cover (Low, Moderate, High) and water level (Dry, Drying, Stable, Full) as categorical (ordinal) co-variables of detection (*p*).

Model Structure	**Model covariates**	**AIC**	**LogLike**	**ΔAIC**	**AIC weight**
Poisson	*p* (water + veg), λ (per + region)	748	493	0	0.99
	p (water + veg), λ (region)	776	515	27	< 0.01
	p (water * veg), λ (perimeter)	1056	528	307	< 0.01
	p (water + veg), λ (perimeter)	1082	544	333	< 0.01
	p (water), λ (perimeter)	1185	592	436	< 0.01
	p (veg), λ (perimeter)	1388	710	639	< 0.01
	p (water * veg), λ (.)	1457	717	709	< 0.01
	p (water + veg), λ (.)	1481	729	732	< 0.01
	p (water), λ (.)	1668	813	919	< 0.01
	p (.), λ (perimeter)	1709	885	960	< 0.01
	p (veg), λ (.)	1892	940	1143	< 0.01
	p (.), λ (.)	2401	1197	1652	< 0.01
NB	*p* (water * veg), λ (perimeter)	629	248	0	0.45
	p (water + veg), λ (perimeter + region)	630	299	1	0.27
	p (water * veg), λ (.)	631	297	2	0.18
	p (water + veg), λ (region)	632	310	3	0.11
	p (water + veg), λ (perimeter)	645	315	16	< 0.01
	p (water + veg), λ (.)	650	315	21	< 0.01
	p (water), λ (perimeter)	673	318	44	< 0.01
	p (water), λ (.)	681	319	52	< 0.01
	p (veg), λ (perimeter)	862	391	233	< 0.01
	p (veg), λ (.)	868	394	239	< 0.01
	p (.), λ (perimeter)	107	452	448	< 0.01
	p (.), λ (.)	1087	457	457	< 0.01
ZIP	*p* (water + veg), λ (perimeter + region)	740	403	0	0.99
	p (water + veg), λ (region)	770	412	29	< 0.01
	p (water * veg), λ (perimeter)	882	417	142	< 0.01
	p (water + veg), λ (perimeter)	902	424	162	< 0.01
	p (water), λ (perimeter)	969	438	229	< 0.01
	p (water * veg), λ (.)	1137	438	397	< 0.01
	p (water + veg), λ (.)	1156	568	415	< 0.01
	p (veg), λ (perimeter)	1175	580	435	< 0.01
	p (water), λ (.)	1274	630	534	< 0.01
	p (.), λ (perimeter)	1469	716	728	< 0.01
	p (veg), λ (.)	1526	756	785	< 0.01
	p (.), λ (.)	1967	1197	1226	< 0.01

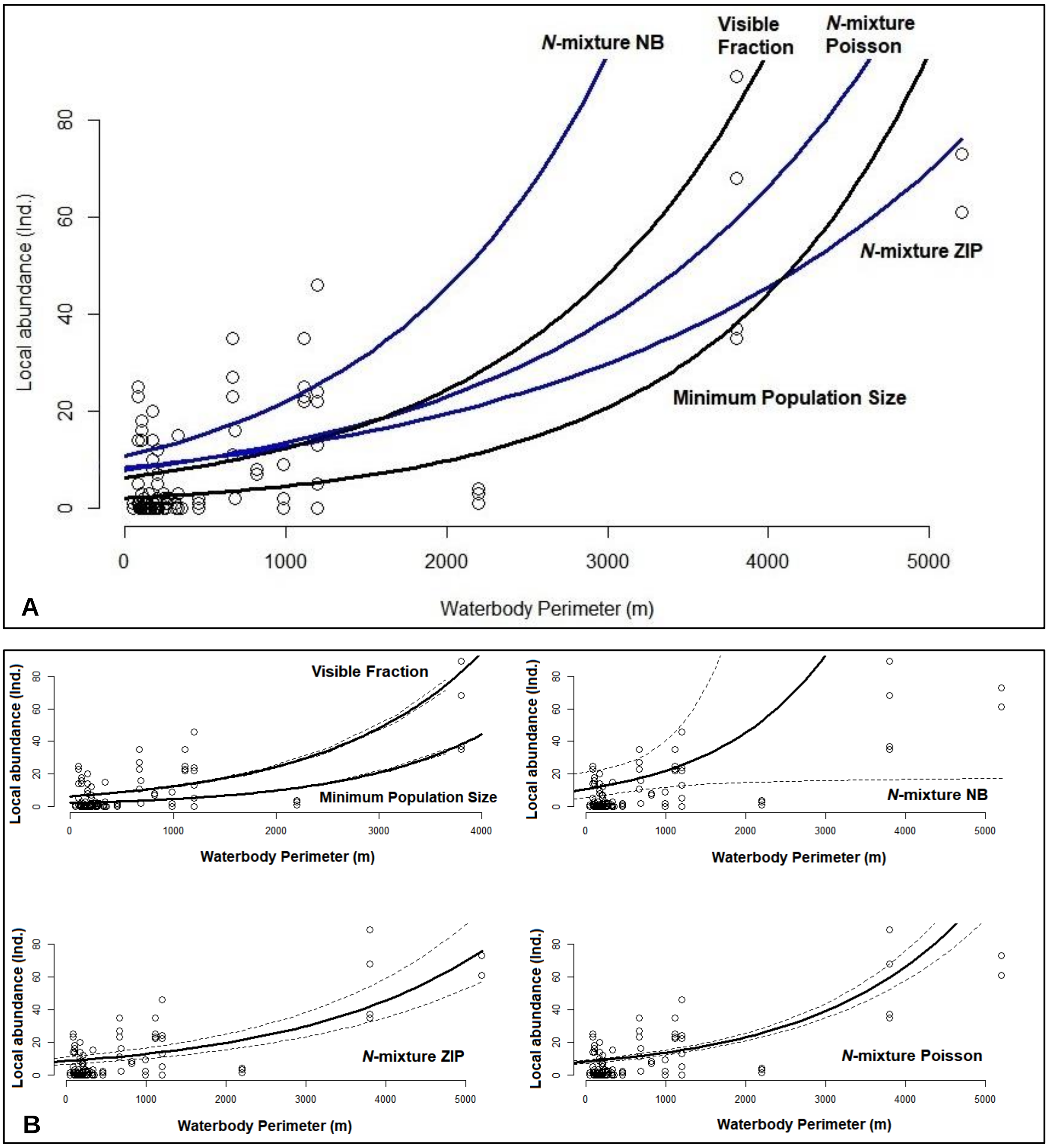


Figure 22. Generalized linear models between local *Crocodylus moreletii* abundance and waterbody perimeter in the region of Calakmul. Open circles represent all baseline crocodile count values (i.e. number crocodiles observed during the survey). In the uppermost figure (A), black lines represent fitted values from estimations through King's visible fraction method and Minimum population size using only baseline count data, and dark blue lines represent fitted values from binomial *N*-mixture modelling using a Poisson, Negative Binomial and a Zero Inflated Poisson approach. In the bottom figures (B), dashed lines in graphs represent the upper and lower confidence intervals.

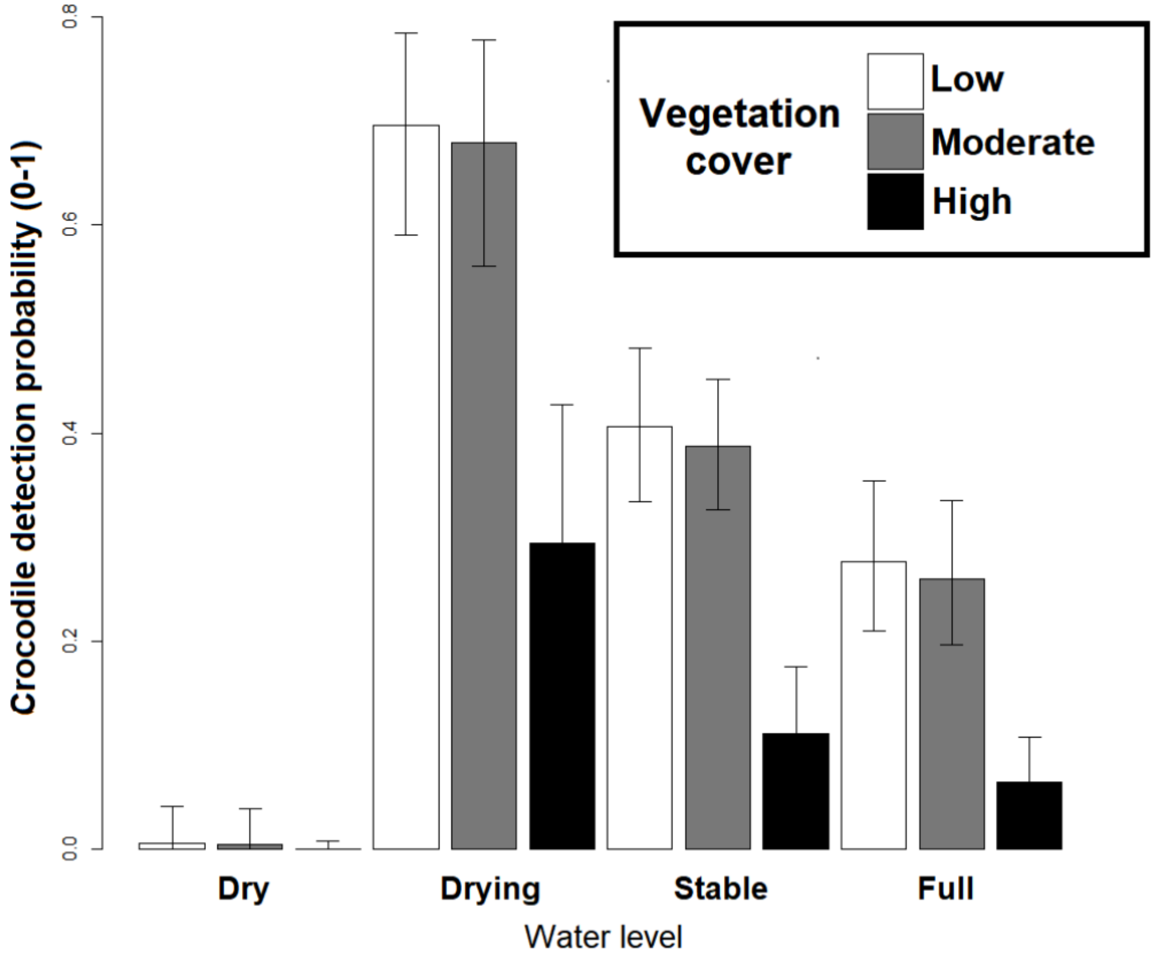


Figure 23. *Crocodylus moreletii* detection probability estimations in function of water level and vegetation cover inside the waterbody calculated through Zero Inflated Poisson *N*-mixture modelling. Error bars represent 95% confidence intervals.

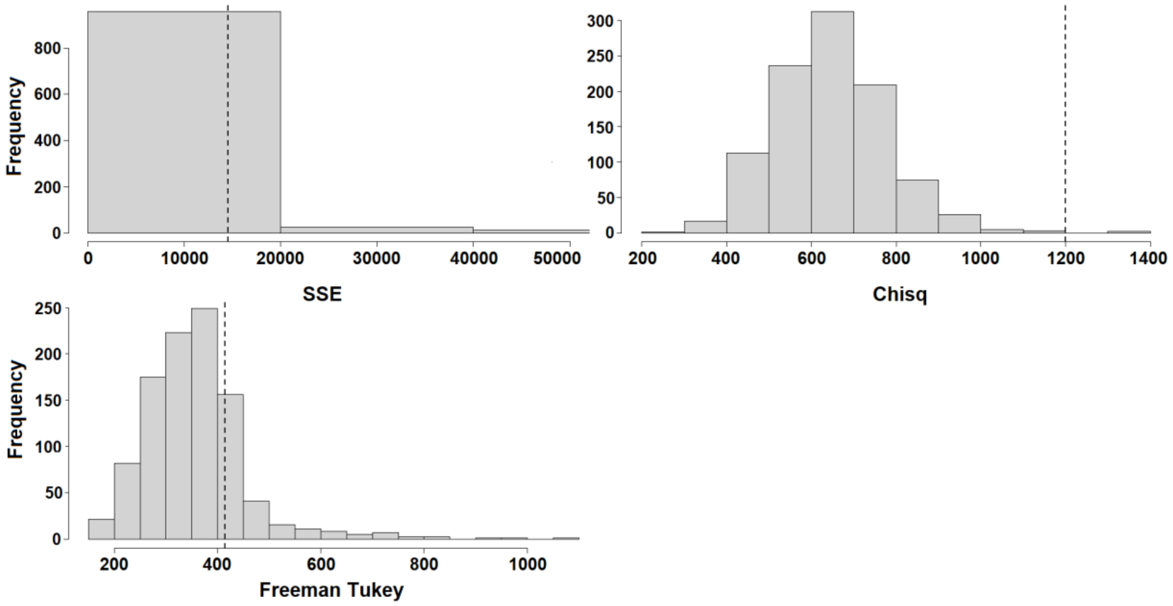


Figure 24. Graphical assessment of model fit by parametric bootstrapping of the *N*-mixture modelling approaches. The dashed line is the observed statistic. The histogram approximates the expected sampling distribution.

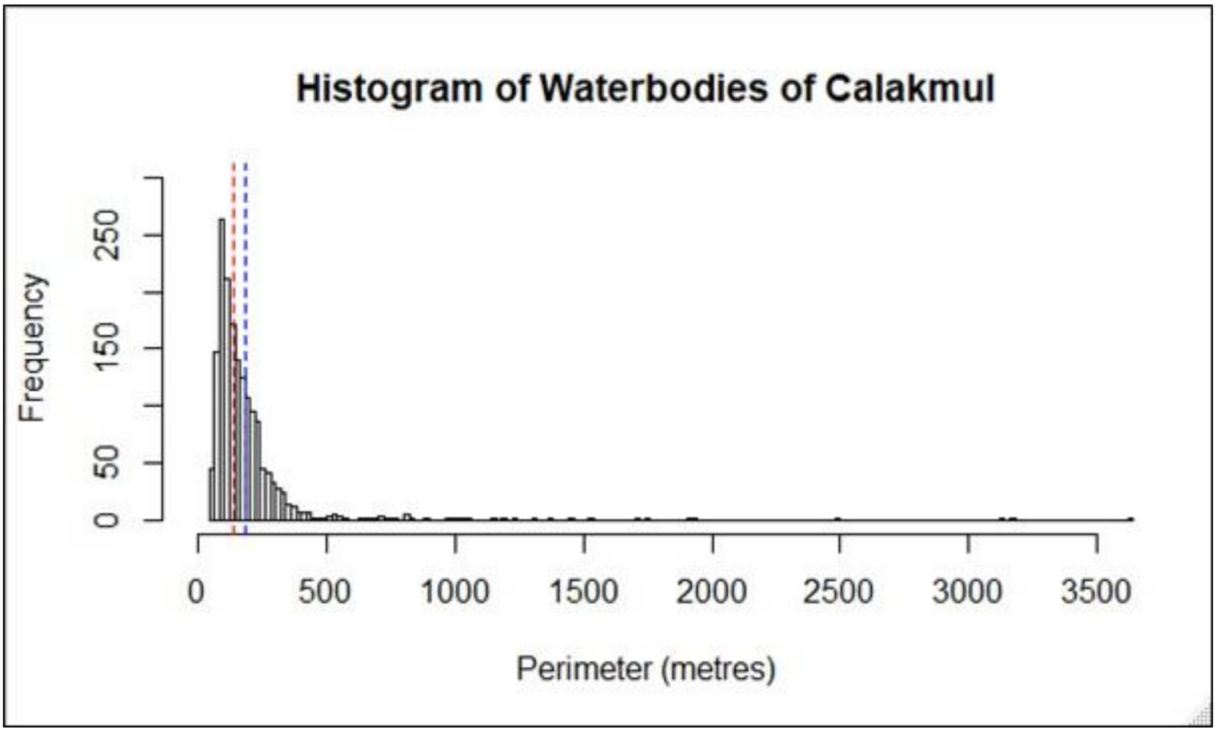


Figure 25. Perimeter distribution of the 1663 semi-temporary and permanent waterbodies across the region of Calakmul (Campeche, Mexico). Data extracted from a GIS hydrological dataset published for this region (García-Gil, 2000). Dashed lines represent the median (red) and mean (blue) waterbody perimeter values.

Table 4. Estimates of total population size of *Crocodylus moreletii* occurring in the region of Calakmul based on five different estimation approaches. MPS – crocodile count data alone (without considering detection probability); VFM – King's visible fraction method; MPS^{GLM} – Generalized linear model considering the relation between MPS and waterbody perimeter; VFM^{GLM} – Generalized linear model considering the relation between VFM and waterbody perimeter; ZIP – Zero Inflated Poisson *N*-mixture approach. Range inside parentheses represent the lower and upper prediction range within a 95% confidence for total abundance of crocodiles in Calakmul.

Approach	**Total population size**
MPS	9,941 (6,651 – 13,230)
VFM	15,184 (14,014 – 16,355)
MPS^{GLM}	4,273 (4,112 – 4,440)
VFM^{GLM}	12,177 (11,543 – 12,846)
N-Mixture ZIP	15,138 (11,681 – 19,653)

3.4 Discussion

Allowing for the separate estimation of abundance and detection probabilities from replicated counts of unmarked individuals (Zipkin et al., 2014; e.g. Kéry & Royle, 2016), *N*-mixture models have in recent years become applied to taxa ranging from mosquitoes to megafaunal mammals (Kidwai et al., 2019; Manica et al., 2019). In the present study, we applied a set of such models to multi-year and multi-site data for the Morelet's crocodile *C. moreletii* in southern Yucatan, where it inhabits particularly dynamic waterbodies and serves as an important flagship species for a large expanse of protected forest. We reveal that Calakmul represents a global *C. moreletii* stronghold and show that *N*-mixture models offer a flexible approach for abundance estimates when ecological conditions cause wide variations in detectability.

Count data are often used as a proxy for true abundance, but require the implicit assumption that the relationship between observed counts and actual population sizes remains constant (e.g. Kéry et al., 2005). For crocodylians, count data may indeed serve as abundance surrogates to capture population temporal and spatial trends related with for example habitat structure or human induced changes to the environment, but only when conditions during multiple surveys allow for the recording of similar proportions of individuals (Bayliss, 1987; Platt & Thorbjarnarson, 2000). As this was likely not the case in our study, we assumed that local crocodile abundances remained overall constant and did not allow λ to vary with respect to survey year, water level and vegetation cover, due to the more evident effect on these variables on the observational process rather than on local abundance (Figure 23). In Calakmul, decreasing water availability caused by disruptions in the timing and intensity of precipitation resulted in marked shifts in water level and vegetation cover of aguadas across the study period (Barão-Nóbrega, 2019; Márdero et al., 2019; Reyna-Hurtado et al., 2019). Under such conditions, the observer's ability to detect individuals will widely vary both temporally as well as spatially, requiring the effects of environmental conditions to be taken into account for obtaining accurate estimates (see also Fujisaki et al., 2011).

Given that the majority of *C. moreletii* habitat across its range is represented by rivers and lakes where spotlight searches are conducted over larger areas than in the case of our more confined aguadas (Cedeño-Vázquez et al., 2006b; Sánchez-Herrera et al., 2011; Tellez et al., 2017), we argue that our surveys provide particularly accurate information. The non-linear rise in abundances with increasing size of aguadas for the MPS, VFM and *N*-mixture ZIP curves is likely linked to larger waterbodies representing more hydrologically stable environments, therefore supporting higher relative numbers of reproductive individuals. Small to medium sized aguadas are generally more prone to desiccation (Barão-Nóbrega, 2019), and are often inhabited by only 1-2 adult *C. moreletii* which might not locally reproduce. It also needs to be borne in mind that the relationship between waterbody surface and volume with perimeter, while depending on the overall shape, is non-linear in general. Given that detection rates are not accounted for, the low abundance values for the MPS approach are not surprising. The VFM and the *N*-mixture ZIP model, on the other hand, consider detectability and reveal remarkably converging abundances for waterbodies below 1200 metres in perimeter, a size class which accounts for 98% of aguadas in the study area (García-Gil et al., 2002; Barão-Nóbrega, 2019). The lower abundance estimates as predicted by the *N*-mixture ZIP model for larger sites, where relative variation around mean counts for repeated surveys are low, suggests that this model generally assumes higher detectabilities under such scenarios; that surface area is related to encounter rates for crocodylians has been previously documented (e.g. Da Silveira et al., 2008; Fujisaki et al., 2011).

N-mixture models yield unbiased estimates of abundance and detectability in simulated datasets of closed populations (Royle, 2004; Kéry & Royle, 2016), but benchmarks to assess the performance of *N*-mixture models from field data are difficult to obtain (Kéry et al., 2005). A particular feature of our dataset is a wide range of count values across sites, with zero detections being a common occurrence (no *C. moreletii* were recorded for about 40% of aguadas, and approximately 60% of all surveys yielded in no counts). This likely led to the limited fit of our data to assumptions of specific distributions, which is a common problem in count data (Lee & Nelder, 2000; Ver Hoef & Boveng, 2007; Kéry & Royle, 2016). Estimates of local abundances in Drying aguadas were also possibly slightly biased downward, as such situations can lead to temporary emigration or hiding behaviour in dens and burrows within or in the vicinity of waterbodies (Platt, 2000; Barão-Nóbrega et al., 2016b). That the geographic specifier significantly improved the performance of our models however also suggests a high degree of philopatry, supporting the assumption of closed populations; low levels of dispersal are also evidenced by genetic data on the relatedness structure within and between aguadas (Barão-Nóbrega et al., unpublished). A particular strength of the *N*-mixture models was their ability to directly relate detectability with ecological parameters. While our findings confirm existing studies on the general nature of such relationships (Montague, 1983; Wood et al., 1985; Bayliss et al., 1986; Cherkiss et al., 2006; Da Silveira et al., 2008; Fujisaki et al., 2011), they enabled an accurate quantification for the estimation and interpretation of *C. moreletii* abundances specifically for our study setting.

The comparison between the three binomial *N*-mixture models suggested that the ZIP distribution provided best confidence overall. The NB model overall exhibited lower AIC values, but performed poorly during the residual diagnostic analysis and revealed excessively large confidence intervals which are likely linked to model unidentifiability (the “good fit/bad prediction dilemma”; see Joseph et al., 2009; Dennis et al., 2015; Kéry & Royle, 2016; Kéry, 2018 for detailed discussion on this topic). It also resulted in abundance estimates which appeared unrealistically high, linked to low projected detection probabilities related to water level and vegetation cover (see also Mazzotti et al., 2019 for a similar finding on the closely related *Crocodylus acutus*). Hierarchical modelling of abundance from unmarked individuals using *N*-mixture models will remain a rich ground for both theoretical and applied investigations also in the future (Kéry & Royle, 2016; Kéry, 2018; Bötsch et al., 2019).

Extrapolating our abundance data across Calakmul requires that the surveyed aguadas are unbiased representatives for the entire area. While a randomization process for site selection was not possible due to logistic constraints (landowner permission and site accessibility), we did not take previous information on the presence or absence of *C. moreletii* into account, and based our inferences on a large sample size of sites. Comparing our overall population sizes derived for Calakmul with country-wide estimates for *C. moreletii* numbers (largely based on the MPS approach, the total population size in Mexico has been estimated at 78,157 – 104,815 individuals; Álvarez, 2005; Rivera-Téllez et al., 2017), reveals that our study area represents a significant stronghold for the study species (Figure 26). The importance of Calakmul for *C. moreletii* is further reinforced by the finding that it still harbours genetically pure individuals, whereas genetic pollution through increased hybridization with the American crocodile *C. acutus* is common across other parts of its range (Chapter 4; Pacheco-Sierra et al., 2018).

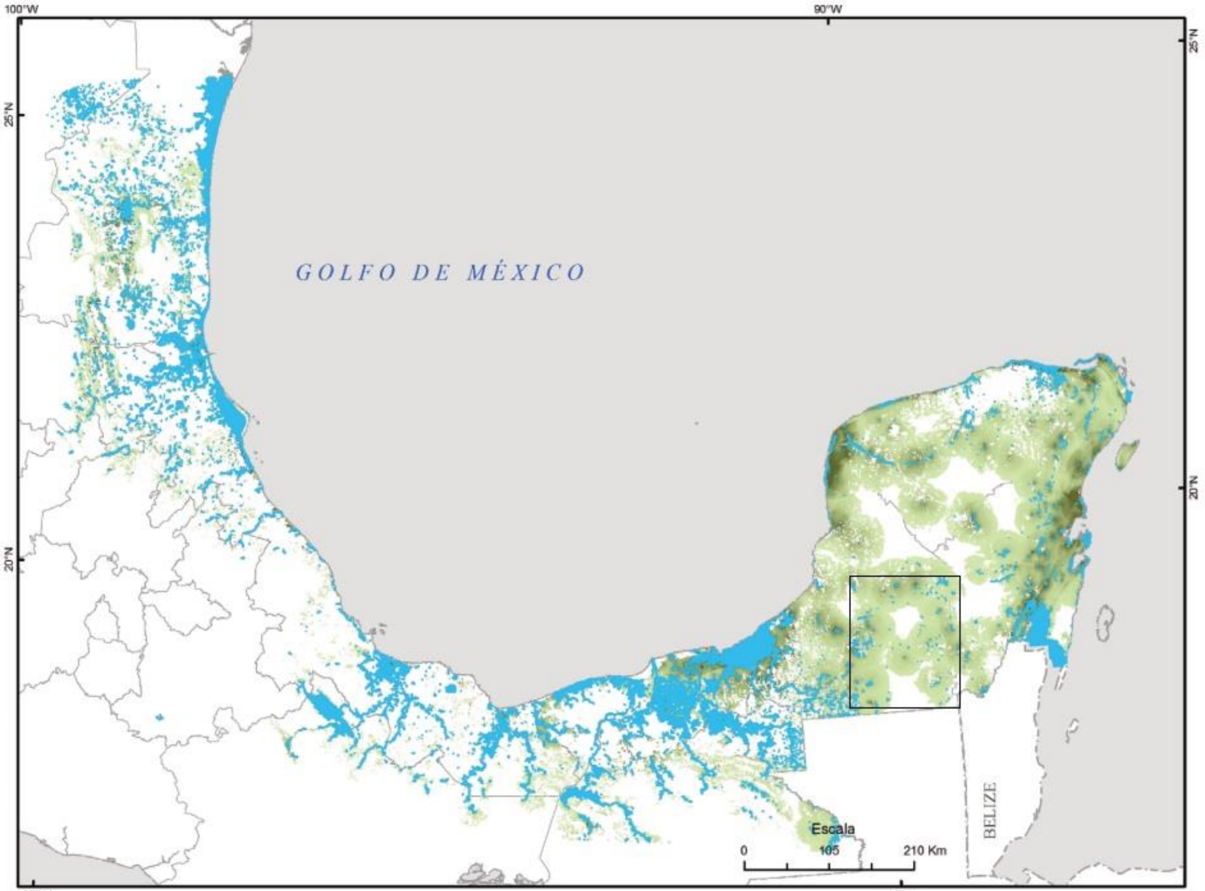


Figure 26. Map of potential *C*. *moreletii* distribution in Mexico generated by CONABIO (*Comisión Nacional para el Conocimiento y Uso de la Biodiversidad*). Blue layers represent the major riverine and lacustrine hydrological systems of Mexico, and therefore the potential habitat for the species. Shades of green represent the modelled probability of occurrence (0 to 1; where 1=10000). Adapted from Rivera-Téllez et al. (2017).

3.5 Final Considerations

Long-term monitoring data using landscape-level systematic surveys provide useful information to describe spatial and temporal patterns of relative density in crocodylians (Fujisaki et al., 2011; Waddle et al., 2015). This study constitutes the first population size estimates for the south-central region of the Yucatan Peninsula, and reveals a healthy population of *C. moreletii*, likely involving multiple active reproduction areas across the region. Because covariates enable insights into population responses to local environmental conditions, we confirm that *N*-mixture models applied to spotlight count data result in particularly insightful estimates of crocodylian detection and abundance. Future studies could expand the existing field surveys and *N*-mixture models to investigate whether further factors such as annual precipitation, water quality, surrounding forest structure, human activity and reproductive activities account for local presence and abundance. On a larger scale, we also recommend the use of *N*-mixture approaches to analyse existing and future *C. moreletii* spotlight count data collected across its range (Álvarez, 2005; Sánchez-Herrera et al., 2011; Rivera-Téllez et al., 2017), to provide more accurate baseline information for future conservation management plans at species level.

Chapter 4 – Screening for evidence of hybridization and assessing genetic structure of Morelet’s crocodile (*Crocodylus moreletii*) across the region of Calakmul (Campeche, Mexico) using Next Generation Sequencing (dd-RADseq)

Unpublished data and information

4.1 Introduction

The foundation for species’ evolutionary flexibility and adaptation capabilities when exposed to environmental change relies on genetic variation. When compared with large populations inhabiting zones of high genetic connectivity, small and isolated populations are especially susceptible to genetic drift, and have a tendency to reduce genetic variability over time through erosive processes (Slatkin, 1987, 2017). Furthermore, they are susceptible to deleterious effects caused by inbreeding, which result in a decrease of individual heterozygosity (Amos & Balmford, 2001). Naturally fragmented populations of sexually reproducing species may possess adaptations to avoid inbreeding, such as some level of kin recognition, delayed maturation and sex-biased dispersal (Pusey & Wolf, 1996). Gene flow among fragmented populations often depends on geographical distance and resistance of a given landscape to dispersal (Ricketts, 2001; Revilla et al., 2004). Connectivity amongst occupied sites in metapopulations requires occasional dispersal between sub-populations (Hanski, 1998; Nelson-Tunley et al., 2016). Analysis of genetic structure within a region can reveal the degree of population isolation (Hedrick, 2005) and the rate of successful migrations between locations (Slatkin, 1987).

Over the last two decades, advances in Next Generation Sequencing (NGS) techniques have enabled parallel sequencing of millions of DNA molecules in single studies, allowing the use of thousands of Single Nucleotide Polymorphisms (SNPs) as powerful molecular markers for ecological and evolutionary studies (Metzker, 2010; Elshire et al., 2011). SNPs are single base pair positions along a sequence at which the nucleotide present can vary between individuals in a population, with the least common variant existing in at least 1% of individuals as a common threshold (Brookes, 1999). Restriction site-associated DNA sequencing (RADseq) combines NGS technology with the use of restriction enzymes and has become a molecular tool to characterise thousands of genetic markers across large numbers of individuals in non-model organisms at reasonable costs (Davey & Blaxter, 2011; Andrews et al., 2016).

Our knowledge about population genetic processes in crocodylian species has increased significantly over the last decades (e.g. Densmore III & Glenn, 2008; Meredith et al., 2011; Grigg & Kirshner, 2015), allowing a better understanding of their natural history, phylogeography, population structures, gene flow patterns, as well as introgression and hybridization with other species. The use of microsatellite markers in crocodylian conservation studies gradually increased from the late 1990s to the early and mid-2000s (Glenn et al., 1996; Vashistha et al., 2020), and has often been used together with mitochondrial DNA markers to reveal the genetic structure of populations (e.g. Fitzsimmons et al., 2002; Villela et al., 2008; Velo-Antón et al., 2014; Pacheco-Sierra et al., 2016; Roberto et al., 2020). The power of RADseq approaches is increasingly being applied to generate SNP datasets to investigate questions relevant for crocodylian conservation programs, such as the identification of evolutionary lineages, hybridization between species, population structure and genetic diversity (Muniz et al., 2018; Nguyen et al., 2018; Pacheco-Sierra et al., 2018; Cao et al., 2020).

Although hybridization is considered a potential threat to populations of endangered crocodylians (e.g. Milian-Garcia et al., 2016), information on its incidence, geographical extent and driving factors among wild crocodylians is still limited. Hybridization between the American crocodile (*Crocodylus acutus*) and the Morelet's crocodile (*C. moreletii*) has been reported in coastal regions of sympatry between both species in Mexico (e.g. Cedeño-Vázquez et al., 2008; González-Trujillo et al., 2012; Serrano-Gomez et al., 2016), and at inland sites outside the distribution range of *C. acutus* in Belize (Ray et al., 2004; Hekkala et al., 2015) and Mexico (Pacheco-Sierra et al., 2016), including remote locations up to 450 km from the coastline. Populations routinely identified as *C. moreletii* are very likely to be admixed (i.e. of hybrid origin), and the IUCN conservation status and CITES classification of *C. moreletii* may therefore need to be revised (Pacheco-Sierra et al., 2018). Admixture in the North of the Yucatan Peninsula can be traced back to 2-3 million years ago, and pure *C. moreletii* populations might currently only occur in "islands" in Northern Mexico, sheltered from hybridization due to their remoteness and reduced connectivity (Pacheco-Sierra et al., 2016; Pacheco-Sierra et al., 2018).

Relatedness patterns in wild populations constitute valuable information to many areas of research in conservation, population genetics, behavioural ecology and evolution (Allendorf & Luikart, 2007; Wang, 2011; Pew et al., 2015). Understanding mating systems and identifying patterns of relatedness based on fieldwork alone is often difficult, and molecular methods are regularly used to investigate relationships amongst individuals within a population (Blouin et al., 1996; Archie et al., 2006).For example, interpretations of behavioural patterns and social structure often become clearer when information on relatedness and the framework of inclusive fitness are considered (Emlen, 1995; Bourke, 2011). More generally, genetic marker-based relatedness has been increasingly used in wildlife studies to, amongst others, estimate heritability (e.g. Ritland, 2000; Visscher et al., 2006), investigate spatial structure and isolation by distance (e.g. Hardy & Vekemans, 2002; Vekemans & Hardy, 2004), examine social structures and kin selection (Girman et al., 1997; Peters et al., 1999), infer sex-based migration (e.g. Piertney et al., 1998; Knight et al., 1999), and estimate population sizes (e.g. Nomura, 2008).

Mating systems can generally be classified as monogamous, polygamous by polygyny (one male mating with more than one females) or polyandry (one female mating with more than one males), and promiscuous (when males and females mate with multiple individuals; Emlen & Oring, 1977; Nunney, 1993). Crocodylians have traditionally been linked to a polygynous mating system (Grigg & Kirshner, 2015), although molecular studies further revealed multiple paternity in egg clutches of many alligatorid (Davis et al., 2001; Amavet et al., 2008; Hu & Wu, 2010; Muniz et al., 2011; Oliveira et al., 2014) and crocodile species (McVay et al., 2008; Lewis et al., 2013; Budd et al., 2015; Milian-Garcia et al., 2016). Multiple paternity is considered ubiquitous in reptiles (Uller & Olsson, 2008), with multiple males contributing to a single clutch of offspring potentially increasing effective population sizes (N_e) and therefore maintaining genetic diversity within a population (Sugg & Chesser, 1994).

The Yucatan Peninsula is one of the most recognizable parts of Mexico, and known as one of the areas that supported the Mayan civilization (Sharer, 1998; González-Sánchez et al., 2017). In the region of Calakmul (southern Campeche, Mexico), due to the geological characteristics that cause rapid filtration of the rain (García-Gil et al., 2002), aquatic habitat occurs in the form of semi-temporary natural ponds (aguadas) sustained by rainfall during the majority of the year. The shape and size of these waterbodies vary seasonally in response to fluctuations in annual precipitation. Aguadas represent the only local source of standing surface water during most of the year (Reyna-Hurtado et al., 2010; Barão-Nóbrega, 2019), occurring at relatively low densities across the region (on average less than one non-dry waterbody per 535 hectares, García-Gil, 2000; Delgado-Martínez & Mendoza, 2020).

Calakmul is at higher elevation than the surrounding parts of the Yucatan Peninsula (Figure 30), and contains a largely unconnected above-ground hydrological network suitable for *C. moreletii* (García-Gil et al., 2002; Gunn et al., 2002). Limiting dispersal and gene flow could likely lead to pronounced genetic structure in *C. moreletii* across the region. Moreover, although *C. moreletii* populations genotyped along more coastal regions in the Yucatan Peninsula are mainly composed of admixed individuals (Pacheco-Sierra et al., 2018), natural geographical restrictions to crocodile movement within and into Calakmul suggest that it may be the only region in the Mexican Yucatan Peninsula to still harbour genetically pure *C. moreletii* (without admixture with *C. acutus*), as non-admixed populations have only been reported to occur in isolated locations (Pacheco-Sierra et al., 2016; Pacheco-Sierra et al., 2018).

Although non-admixed individuals of *C*. *moreletii* and *C*. *acutus* have morphological features that differentiate them (e.g. head morphology, caudal scalation; Figures 27, 28), hybrid individuals are not always morphologically discernible in the field as they exhibit a complex mosaic of characters, particularly in sympatric zones (Cedeño-Vázquez et al., 2008; Pacheco-Sierra et al., 2016). Although illustrative guides for morphological identification of *C. moreletii* and possible hybrids have been attempted (e.g. see Sánchez-Herrera et al., 2011), field identification can be unreliable when dealing with admixed individuals because nearly all possible combinations of morphological traits can be obtained. As the phenotype of hybrid individuals likely depends on the level of introgression, cryptic hybrids (resembling one of the parental species phenotypically but not genotypically) can be mistaken for non-admixed individuals (Pacheco-Sierra et al., 2016, and references therein). Individuals from Calakmul do not seem to exhibit any morphological characteristics usually associated with *C*. *acutus* (Barão-Nóbrega, unpublished data). However, admixture is not always apparent from morphological observations alone, particularly after several generations of backcrossing (Rhymer & Simberloff, 1996; Medina et al., 2013; Pacheco-Sierra et al., 2016).

This study uses SNPs generated through a double-digest RADseq approach (dd-RADseq) to investigate the population structure of *C. moreletii* within the region of Calakmul. The hypotheses explored in this chapter are that (1) Calakmul harbours largely non-admixed *C. moreletii* individuals; (2) reduced gene flow has led to distinct spatial genetic differentiation of *C. moreletii* across Calakmul; and (3) population structure is linked to extended family networks (i.e. each locality will be inhabited by closely related individuals).

4.2 Material and Methods

4.2.1 Sample collection

Fieldwork was conducted between 2017 and 2018 in 18 waterbodies across six regions where they are within 20 km from each other (Figure 29). In total, 85 crocodiles (Table 6) were captured at night with a pole with a break-away noose and physically restrained using a pole-snare (Ketch-All Animal Restraining Pole), ropes and tapes (following Sánchez-Herrera et al., 2011). Captured crocodiles were marked by removal of an individual combination of up to three vertical tail scutes (Sánchez-Herrera et al., 2011; Barão-Nóbrega et al., 2016b). All procedures were performed on site, and individuals were released within a maximum period of 30 minutes after capture. Tail scutes were stored in Eppendorf tubes with 96% ethanol until processed. All field activities were performed in compliance with the guidelines for use of live amphibians and reptiles in field and laboratory research (Beaupre et al., 2004).

Additional blood and tissue samples from previously described non-admixed parental populations (Pacheco-Sierra et al., 2016; Pacheco-Sierra et al., 2018) of *C. acutus* (Banco Chinchorro and Cozumel islands, Quintana Roo – four tissue samples) and *C. moreletii* (Cienega de Cabezas, San Luís Potosi – four blood samples; Laguna Las Ilusiones – three tissue samples) were acquired to be sequenced in conjunction with the samples from Calakmul (Figure 29). Despite multiple extraction attempts no genetic material was successfully obtained from one *C. acutus* and all seven *C. moreletii* samples (probably a result of DNA degradation). Therefore, samples from *C. moreletii* crocodiles captured in the region of Altamira (Tamaulipas, Mexico) approximately 140 km from the reported non-admixed population were used as replacement reference samples, despite the likelihood of admixture in this location (Pacheco-Sierra et al., 2016).

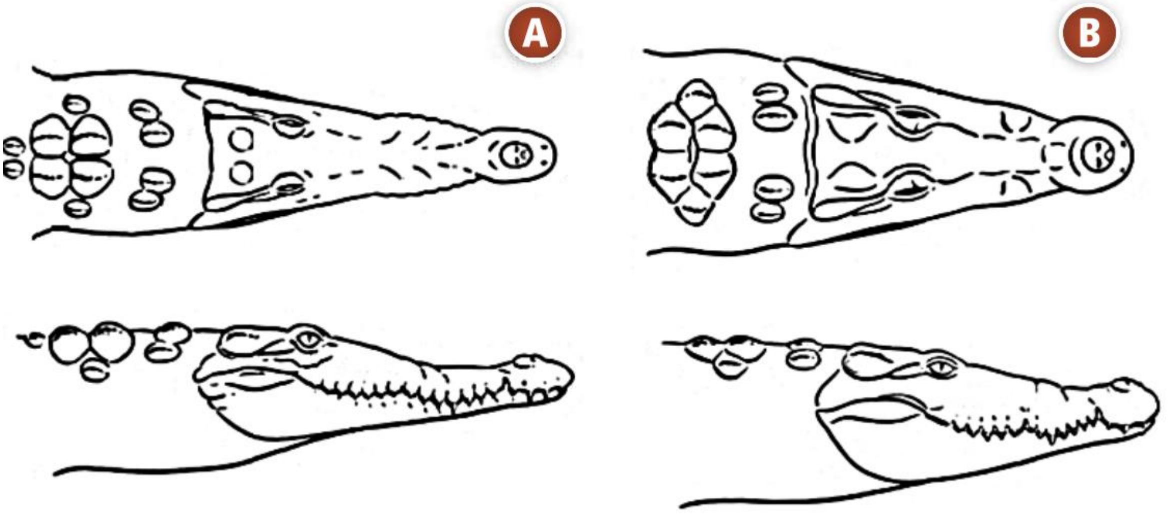


Figure 27. Lateral and dorsal views of the head in *Crocodylus acutus* (A) and *Crocodylus moreletii* (B), highlighting the proportion between cranial length and width, the pre-orbital protuberance (more evident in *C. acutus*) and also the different pattern and number of nuchal osteoderms. Adapted from Sánchez-Herrera et al. (2011).

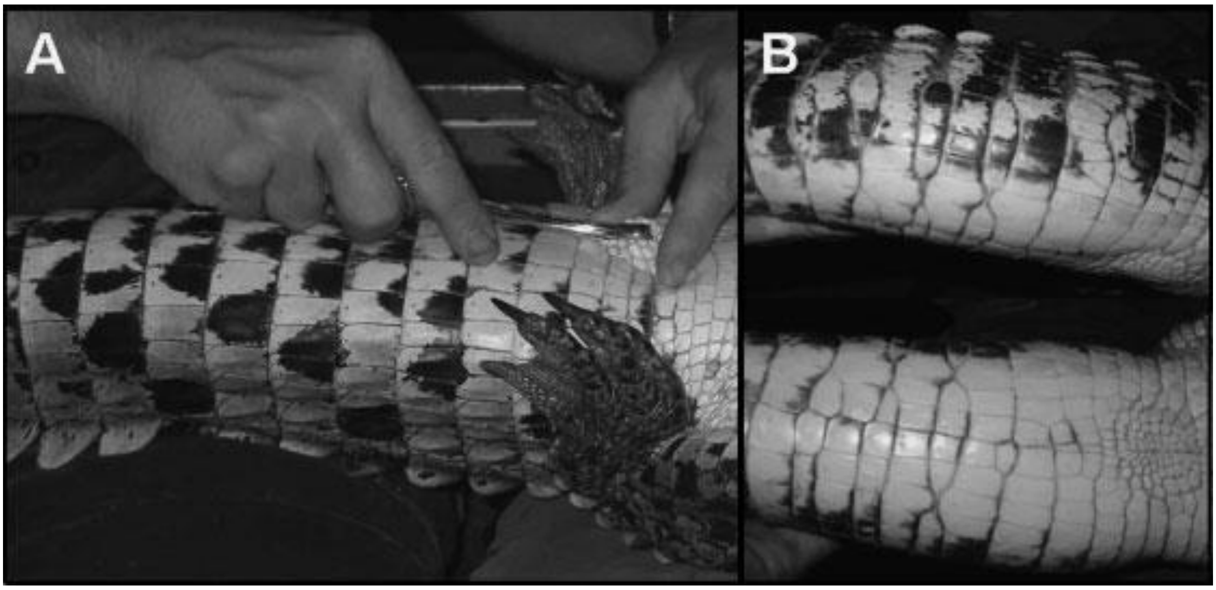


Figure 28. Side and ventral views of caudal scalation in the proximal end of tail of *Crocodylus acutus* (A) and *Crocodylus moreletii* (B). All around symmetrical appearance in rows of scales can be observed in *C. acutus*, but not in *C. moreletii*, where irregularly shaped scale group intrusions are present. Adapted from Cedeño-Vázquez et al. (2008).

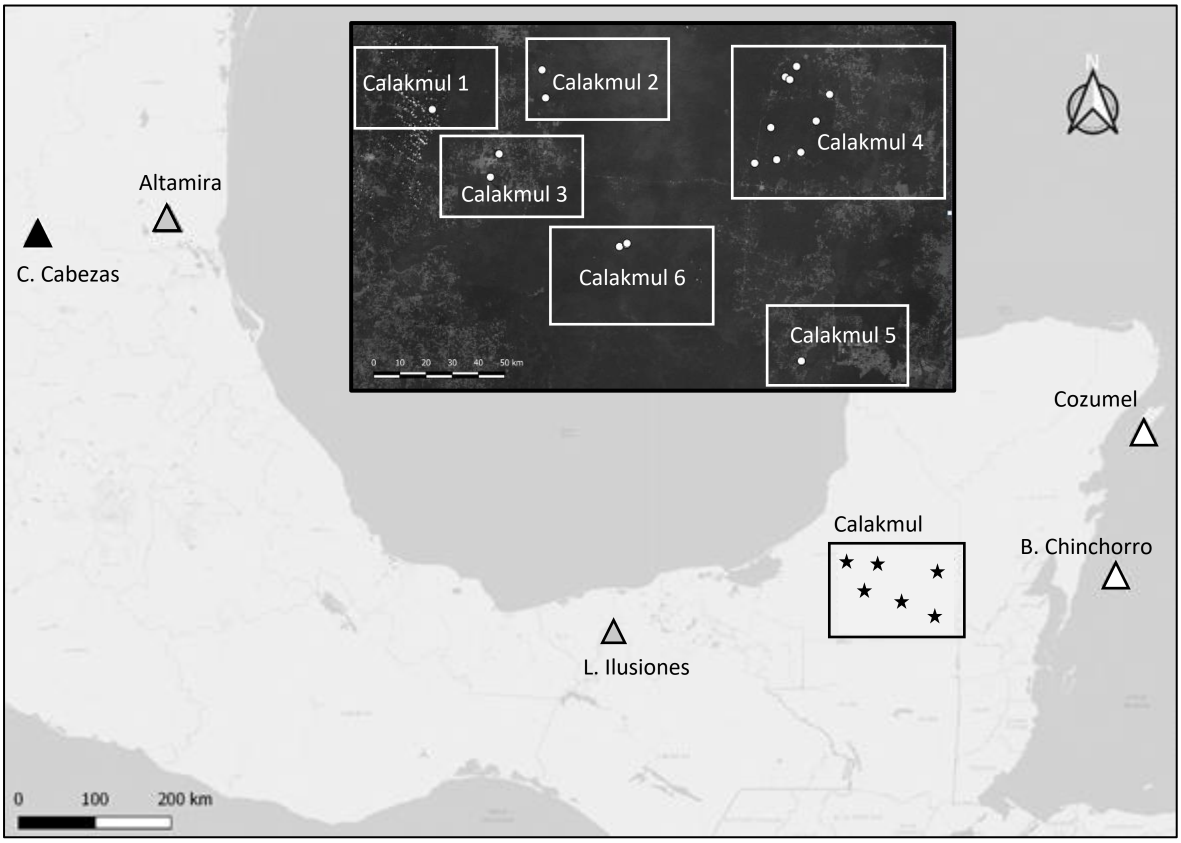


Figure 29. Six main collection regions within Calakmul (black stars), and origin of reference samples obtained from non-admixed populations of *Crocodylus acutus* (white triangles) and *Crocodylus moreletii* (black triangle) within Mexico (Pacheco-Sierra et al., 2018). The grey triangles represent back-up samples obtained from areas (Altamira, Tamaulipas; Laguna Ilusiones, Tabasco) verified to have significant incidence of admixture amongst individuals (Pacheco-Sierra et al., 2018). White circles in the highlighted section indicate waterbodies within each region of Calakmul where *C. moreletii* samples were obtained.

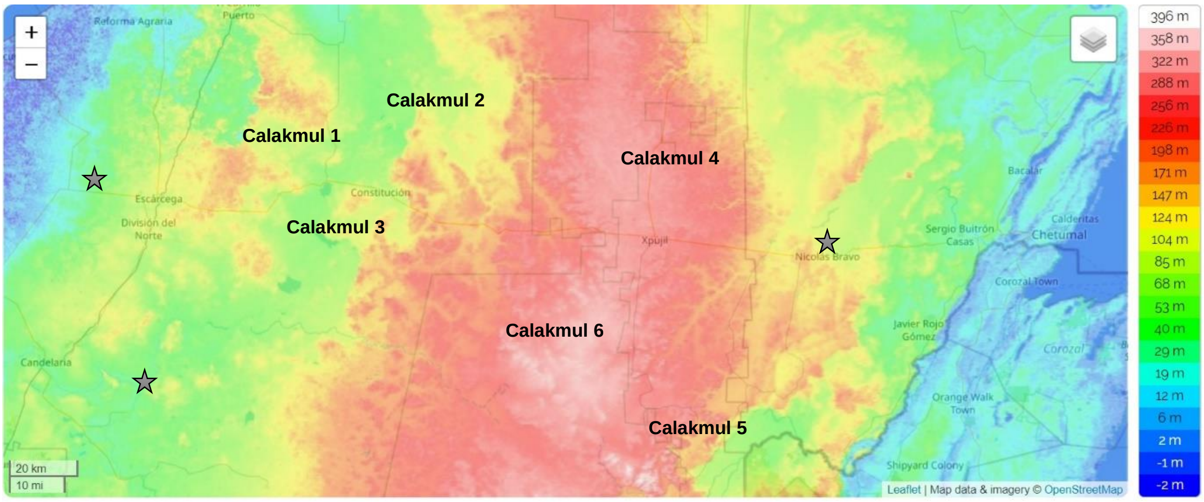


Figure 30. Elevation profile of the southern portion of the Mexican Yucatan Peninsula and location of the six regions in Calakmul where *Crocodylus moreletii* samples were obtained. Gray stars indicate closest localities where admixed individuals have been reported (Pacheco-Sierra et al., 2016; Pacheco-Sierra et al., 2018). Map adapted from http://topographic-map.com/maps/tfba/Calakmul/.

4.2.2 Double digest RAD Sequencing (dd-RADseq)

Laboratory work was conducted in September 2018 at the Aquatic Ecology and Systematics Department of El Colegio de la Frontera Sur (ECOSUR) in Chetumal, Mexico. Genomic DNA was isolated and purified from approximately 20 mg of tissue from tail scutes with the use of DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. DNA concentration was assessed using a microvolume spectrophotometer (Eppendorf™) and normalised to approximately 20 ng/uL. To verify standardised concentration, and to ensure that the extracted DNA was of high molecular weight and free of contaminants and degradation, every sample was visualised on a 1.5% agarose gel (Figure 31). High quality DNA of 95 samples was sent to Floragenex (OR, USA) for genotyping by sequencing library construction (GBS, Elshire et al., 2011) and sequencing, following the protocol of Truong et al. (2012). The complexity of genomic DNA was reduced by double-digesting with a combination of rare and frequent cutting endonucleases (*PstI* and *MseI*, respectively), followed by ligation with Floragenex adaptors with individual indexes. 1x100bp single end sequencing was performed on the resulting PCR-generated library using an Illumina HiSeq 4000 (CA, USA).

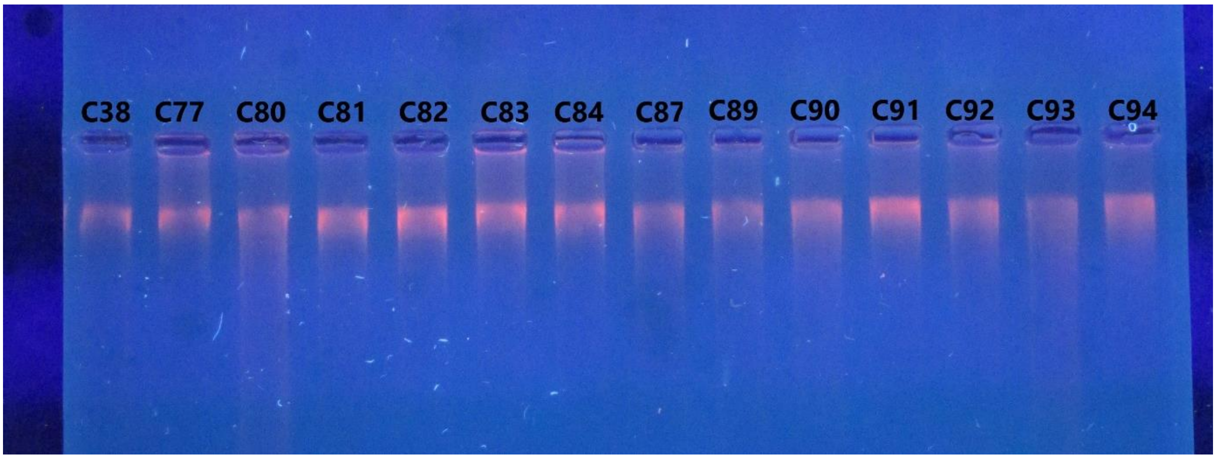


Figure 31. Example of a 1.5% agarose gel electrophoresis run on crocodile DNA extracted from caudal vertical scutes.

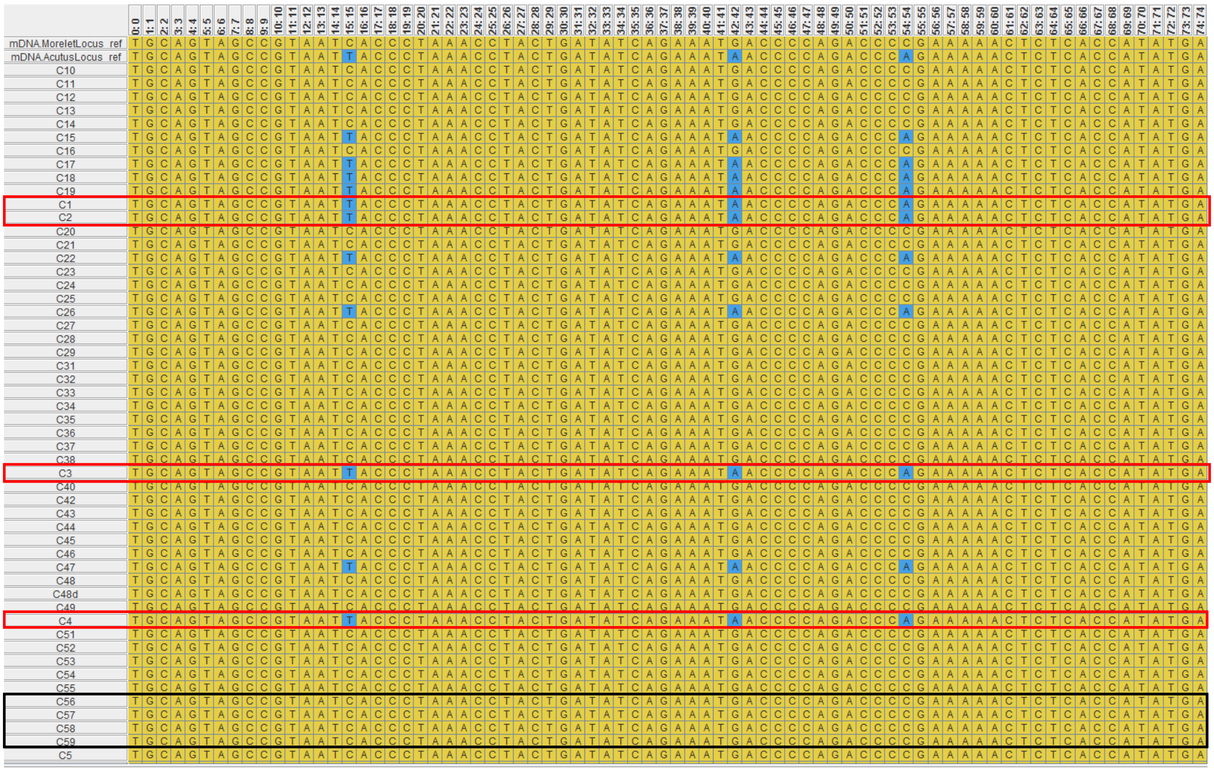
4.2.3 SNP discovery and genotyping using STACKS

Returned raw sequences were demultiplexed and processed using the software STACKS 2.0 (Catchen et al., 2013), following the protocol described by Rochette and Catchen (2017). Sequences were checked for correct restriction sites and adaptor sequences using the *process_radtags* pipeline. Reads with an uncalled base were discarded, as were reads containing a 15 bp window in which the average quality dropped below a Phred score of 10 (i.e. a 90% probability of being correct). Barcodes and RAD-tags containing 1 mismatch to an expected sequence were retained.

In the absence of reference genomes for *C. moreletii* or *C. acutus*, the whole genome sequence (WGS) of *Crocodylous porosus* (Green et al., 2014) was obtained from Ensembl (https://www.ensembl.org/Crocodylus_porosus/Info/Index; see also Pacheco-Sierra et al., 2018) for single nucleotide polymorphism (SNP) discovery and genotyping in the software STACKS 2.0 (Rochette et al., 2019). Two reference-aligned assemblies were produced by mapping the clean demultiplexed sequences against the *C. porosus* genome using BWA-MEM (Li & Durbin, 2009) and GSNAP (Wu et al., 2016). The best reference-aligned assembly (GSNAP) was then analysed using the *ref_map* pipeline in STACKS 2.0 (Rochette et al., 2019). The procedures developed by Paris et al. (2017) and further elaborated by Rochette and Catchen (2017) were followed to identify the optimal set of parameters. Firstly, to enable efficient testing of parameter values, a subset of samples representing different geographical regions was selected. This consisted of three to six representatives of each sampling area (40 individuals in total). The *ref_map* pipeline was then run with varying parameter values while measuring the number of polymorphic loci found across at least 80% of all samples (the r80 loci, Paris et al., 2017) to identify a stable set of values. Prior to exporting the genotype calls from STACKS for downstream analyses, the dataset was further filtered using the *populations* pipeline. In addition to only retaining the r80 loci, loci with a minor allele frequency below 0.05, an observed heterozygosity above 0.70 and absent in at least one of the eight crocodile sample groups (*C. acutus* and *C*. *moreletii* reference samples plus all six *C*. *moreletii* populations, i.e. geographical regions, in Calakmul) were removed. Called variants were exported into variant call format (VCF), and STRUCTURE and haplotype format through the *populations* pipeline to generate suitable datasets for different population genetic analysis.

4.2.4 Identifying mitochondrial reads

As the reference-aligned assembly does not include the mitochondrial genome, mitochondrial reads were identified by separately mapping the clean demultiplexed reads against both the mitochondrial genomes of *C. acutus* (NCBI Reference Sequence: NC_015647.1; Man et al., 2011) and *C. moreletii* (NCBI Reference Sequence: NC_015235.1; Meganathan et al., 2011). Mapped reads were sorted and indexed using SAMTOOLS v.1.4 (Li et al., 2009). The software IGV (Robinson et al., 2011) was used to visually assess where the reads map against the *C*. *moreletii* and *C*. *acutus* mitochondrial genomes, and four regions where all crocodile samples had mapped reads were identified in both cases (following Barth et al., 2020). As these four identified regions were detected on the mitochondrial genomes of both species, the decision was made to continue with the use of reads mapped against *C*. *acutus*. Sequences from all samples mapped to these four regions were extracted and converted to FASTA format using SAMTOOLS, BCFTOOLS v.1.6 (Li, 2011), and SEQTK v.1.0 (https://github.com/lh3/seqtk). Sequences corresponding to the regions 2278-2872, 5836-5930, 8392-8485 and 9528-9622 of the *C*. *acutus* mitochondrial genome were aligned with default settings in MAFFT v.7.397 (Katoh & Standley, 2013), and the software TASSEL v.5.0 (Bradbury et al., 2007) was used to visually assess the mapped sequences across all samples (Figure 32). All four mapped regions were further aligned against other mitochondrial isolates and partially sequenced genomes of *C*. *acutus* (GenBank References: JF502241.1; JF315769.1, JF315757.1, JF315747.1, JF315714.1, HM636894.1, JF315729.1; Meredith et al., 2011; Oaks, 2011) and C*. moreletii* (GenBank References: JF315768.1, JF315752.1; HQ585889.1; Meganathan et al., 2011; Oaks, 2011) using the NCBI database BLAST tool (Zhang et al., 2000; Morgulis et al., 2008). Regions 2278-2872 and 9528-9622 were kept and the two resulting alignments concatenated. Regions 5836-5930 and 8392-8485 were discarded due to being undiagnostic at the level of species.



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                       0:0 1:1 2:2 3:3 4:4 5:5 6:6 7:7 8:8 9:9 10:10 11:11 12:12 13:13 14:14 15:15 16:16 17:17 18:18 19:19 20:20 21:21 22:22 23:23 24:24 25:25 26:26 27:27 28:28 29:29 30:30 31:31 32:32 33:33 34:34 35:35 36:36 37:37 38:38 39:39 40:40 41:41 42:42 43:43 44:44 45:45 46:46 47:47 48:48 49:49 50:50 51:51 52:52 53:53 54:54 55:55 56:56 57:57 58:58 59:59 60:60 61:61 62:62 63:63 64:64 65:65 66:66 67:67 68:68 69:69 70:70 71:71 72:72 73:73 74:74
mDNA.MoreletLocus ref  T G C A G T A G C C G T A A T C A C C C T A A A C C T A C T G A T A T C A G A A A T G A C C C C A G A C C C C G A A A A A C T C T C A C C A T A T G A
mDNA.AcutusLocus ref   T G C A G T A G C C G T A A T T A C C C T A A A C C T A C T G A T A T C A G A A A T A A C C C C A G A C C C A G A A A A A C T C T C A C C A T A T G A
C10                    T G C A G T A G C C G T A A T C A C C C T A A A C C T A C T G A T A T C A G A A A T G A C C C C A G A C C C C G A A A A A C T C T C A C C A T A T G A
C11                    T G C A G T A G C C G T A A T C A C C C T A A A C C T A C T G A T A T C A G A A A T G A C C C C A G A C C C C G A A A A A C T C T C A C C A T A T G A
C12                    T G C A G T A G C C G T A A T C A C C C T A A A C C T A C T G A T A T C A G A A A T G A C C C C A G A C C C C G A A A A A C T C T C A C C A T A T G A
C13                    T G C A G T A G C C G T A A T C A C C C T A A A C C T A C T G A T A T C A G A A A T G A C C C C A G A C C C C G A A A A A C T C T C A C C A T A T G A
C14                    T G C A G T A G C C G T A A T C A C C C T A A A C C T A C T G A T A T C A G A A A T G A C C C C A G A C C C C G A A A A A C T C T C A C C A T A T G A
C15                    T G C A G T A G C C G T A A T T A C C C T A A A C C T A C T G A T A T C A G A A A T A A C C C C A G A C C C A G A A A A A C T C T C A C C A T A T G A
C16                    T G C A G T A G C C G T A A T C A C C C T A A A C C T A C T G A T A T C A G A A A T G A C C C C A G A C C C C G A A A A A C T C T C A C C A T A T G A
C17                    T G C A G T A G C C G T A A T T A C C C T A A A C C T A C T G A T A T C A G A A A T A A C C C C A G A C C C A G A A A A A C T C T C A C C A T A T G A
C18                    T G C A G T A G C C G T A A T T A C C C T A A A C C T A C T G A T A T C A G A A A T A A C C C C A G A C C C A G A A A A A C T C T C A C C A T A T G A
C19                    T G C A G T A G C C G T A A T T A C C C T A A A C C T A C T G A T A T C A G A A A T A A C C C C A G A C C C A G A A A A A C T C T C A C C A T A T G A
C1                     T G C A G T A G C C G T A A T T A C C C T A A A C C T A C T G A T A T C A G A A A T A A C C C C A G A C C C A G A A A A A C T C T C A C C A T A T G A
C2                     T G C A G T A G C C G T A A T T A C C C T A A A C C T A C T G A T A T C A G A A A T A A C C C C A G A C C C A G A A A A A C T C T C A C C A T A T G A
C20                    T G C A G T A G C C G T A A T C A C C C T A A A C C T A C T G A T A T C A G A A A T G A C C C C A G A C C C C G A A A A A C T C T C A C C A T A T G A
C21                    T G C A G T A G C C G T A A T C A C C C T A A A C C T A C T G A T A T C A G A A A T G A C C C C A G A C C C C G A A A A A C T C T C A C C A T A T G A
C22                    T G C A G T A G C C G T A A T T A C C C T A A A C C T A C T G A T A T C A G A A A T A A C C C C A G A C C C A G A A A A A C T C T C A C C A T A T G A
C23                    T G C A G T A G C C G T A A T C A C C C T A A A C C T A C T G A T A T C A G A A A T G A C C C C A G A C C C C G A A A A A C T C T C A C C A T A T G A
C24                    T G C A G T A G C C G T A A T C A C C C T A A A C C T A C T G A T A T C A G A A A T G A C C C C A G A C C C C G A A A A A C T C T C A C C A T A T G A
C25                    T G C A G T A G C C G T A A T C A C C C T A A A C C T A C T G A T A T C A G A A A T G A C C C C A G A C C C C G A A A A A C T C T C A C C A T A T G A
C26                    T G C A G T A G C C G T A A T T A C C C T A A A C C T A C T G A T A T C A G A A A T A A C C C C A G A C C C A G A A A A A C T C T C A C C A T A T G A
C27                    T G C A G T A G C C G T A A T C A C C C T A A A C C T A C T G A T A T C A G A A A T G A C C C C A G A C C C C G A A A A A C T C T C A C C A T A T G A
C28                    T G C A G T A G C C G T A A T C A C C C T A A A C C T A C T G A T A T C A G A A A T G A C C C C A G A C C C C G A A A A A C T C T C A C C A T A T G A
C29                    T G C A G T A G C C G T A A T C A C C C T A A A C C T A C T G A T A T C A G A A A T G A C C C C A G A C C C C G A A A A A C T C T C A C C A T A T G A
C31                    T G C A G T A G C C G T A A T C A C C C T A A A C C T A C T G A T A T C A G A A A T G A C C C C A G A C C C C G A A A A A C T C T C A C C A T A T G A
C32                    T G C A G T A G C C G T A A T C A C C C T A A A C C T A C T G A T A T C A G A A A T G A C C C C A G A C C C C G A A A A A C T C T C A C C A T A T G A
C33                    T G C A G T A G C C G T A A T C A C C C T A A A C C T A C T G A T A T C A G A A A T G A C C C C A G A C C C C G A A A A A C T C T C A C C A T A T G A
C34                    T G C A G T A G C C G T A A T C A C C C T A A A C C T A C T G A T A T C A G A A A T G A C C C C A G A C C C C G A A A A A C T C T C A C C A T A T G A
C35                    T G C A G T A G C C G T A A T C A C C C T A A A C C T A C T G A T A T C A G A A A T G A C C C C A G A C C C C G A A A A A C T C T C A C C A T A T G A
C36                    T G C A G T A G C C G T A A T C A C C C T A A A C C T A C T G A T A T C A G A A A T G A C C C C A G A C C C C G A A A A A C T C T C A C C A T A T G A
C37                    T G C A G T A G C C G T A A T C A C C C T A A A C C T A C T G A T A T C A G A A A T G A C C C C A G A C C C C G A A A A A C T C T C A C C A T A T G A
C38                    T G C A G T A G C C G T A A T C A C C C T A A A C C T A C T G A T A T C A G A A A T G A C C C C A G A C C C C G A A A A A C T C T C A C C A T A T G A
C3                     T G C A G T A G C C G T A A T T A C C C T A A A C C T A C T G A T A T C A G A A A T A A C C C C A G A C C C A G A A A A A C T C T C A C C A T A T G A
C40                    T G C A G T A G C C G T A A T C A C C C T A A A C C T A C T G A T A T C A G A A A T G A C C C C A G A C C C C G A A A A A C T C T C A C C A T A T G A
C42                    T G C A G T A G C C G T A A T C A C C C T A A A C C T A C T G A T A T C A G A A A T G A C C C C A G A C C C C G A A A A A C T C T C A C C A T A T G A
C43                    T G C A G T A G C C G T A A T C A C C C T A A A C C T A C T G A T A T C A G A A A T G A C C C C A G A C C C C G A A A A A C T C T C A C C A T A T G A
C44                    T G C A G T A G C C G T A A T C A C C C T A A A C C T A C T G A T A T C A G A A A T G A C C C C A G A C C C C G A A A A A C T C T C A C C A T A T G A
C45                    T G C A G T A G C C G T A A T C A C C C T A A A C C T A C T G A T A T C A G A A A T G A C C C C A G A C C C C G A A A A A C T C T C A C C A T A T G A
C46                    T G C A G T A G C C G T A A T C A C C C T A A A C C T A C T G A T A T C A G A A A T G A C C C C A G A C C C C G A A A A A C T C T C A C C A T A T G A
C47                    T G C A G T A G C C G T A A T T A C C C T A A A C C T A C T G A T A T C A G A A A T A A C C C C A G A C C C A G A A A A A C T C T C A C C A T A T G A
C48                    T G C A G T A G C C G T A A T C A C C C T A A A C C T A C T G A T A T C A G A A A T G A C C C C A G A C C C C G A A A A A C T C T C A C C A T A T G A
C48d                   T G C A G T A G C C G T A A T C A C C C T A A A C C T A C T G A T A T C A G A A A T G A C C C C A G A C C C C G A A A A A C T C T C A C C A T A T G A
C49                    T G C A G T A G C C G T A A T C A C C C T A A A C C T A C T G A T A T C A G A A A T G A C C C C A G A C C C C G A A A A A C T C T C A C C A T A T G A
C4                     T G C A G T A G C C G T A A T T A C C C T A A A C C T A C T G A T A T C A G A A A T A A C C C C A G A C C C A G A A A A A C T C T C A C C A T A T G A
C51                    T G C A G T A G C C G T A A T C A C C C T A A A C C T A C T G A T A T C A G A A A T G A C C C C A G A C C C C G A A A A A C T C T C A C C A T A T G A
C52                    T G C A G T A G C C G T A A T C A C C C T A A A C C T A C T G A T A T C A G A A A T G A C C C C A G A C C C C G A A A A A C T C T C A C C A T A T G A
C53                    T G C A G T A G C C G T A A T C A C C C T A A A C C T A C T G A T A T C A G A A A T G A C C C C A G A C C C C G A A A A A C T C T C A C C A T A T G A
C54                    T G C A G T A G C C G T A A T C A C C C T A A A C C T A C T G A T A T C A G A A A T G A C C C C A G A C C C C G A A A A A C T C T C A C C A T A T G A
C55                    T G C A G T A G C C G T A A T C A C C C T A A A C C T A C T G A T A T C A G A A A T G A C C C C A G A C C C C G A A A A A C T C T C A C C A T A T G A
C56                    T G C A G T A G C C G T A A T C A C C C T A A A C C T A C T G A T A T C A G A A A T G A C C C C A G A C C C C G A A A A A C T C T C A C C A T A T G A
C57                    T G C A G T A G C C G T A A T C A C C C T A A A C C T A C T G A T A T C A G A A A T G A C C C C A G A C C C C G A A A A A C T C T C A C C A T A T G A
C58                    T G C A G T A G C C G T A A T C A C C C T A A A C C T A C T G A T A T C A G A A A T G A C C C C A G A C C C C G A A A A A C T C T C A C C A T A T G A
C59                    T G C A G T A G C C G T A A T C A C C C T A A A C C T A C T G A T A T C A G A A A T G A C C C C A G A C C C C G A A A A A C T C T C A C C A T A T G A
C5                     T G C A G T A G C C G T A A T C A C C C T A A A C C T A C T G A T A T C A G A A A T G A C C C C A G A C C C C G A A A A A C T C T C A C C A T A T G A
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Figure 32. Visual assessment across crocodile samples of the mapped sequences corresponding to mitochondrial genome region 9528-9622 of *Crocodylus moreletii* (first row) and *Crocodylus acutus* (second row) in TASSEL v.5.0 (Bradbury et al., 2007). Individuals highlighted within the red and black rectangles indicate, respectively, external reference samples of *C*. *acutus* and *C*. *moreletii*.

4.2.5 Genetic structure and introgression

Genetic structure analyses were firstly conducted just on samples from *C*. *moreletii* in Calakmul using the ADEGENET package (Jombart, 2008; Jombart & Ahmed, 2011) for *R* statistical software (*R* Development Core Team, 2019) in *R*Studio version 1.1.456 (*R*Studio Team, 2016). A Principal Component Analysis (PCA) was first performed to visualise structure in the patterns of genetic variability between individuals. After transforming the data using PCA, a Discriminant Analysis of Principal Components (DAPC; Jombart et al., 2010) was used to identify clusters of genetically related individuals. While PCA characterises the overall variability between individuals, including both between-group and within-group variability, DAPC seeks to maximise between-group variation whilst minimising within-group variation. Individuals were assigned into genetic clusters using *k*-means, a clustering algorithm that identifies a given number (*k*) of groups. This was performed without providing prior information on the populations of origin, and was run sequentially using increasing values of *k*. These runs were compared using the Bayesian Information Criterion (BIC), with the preferred value of *k* defined by the elbow of the curve of BIC values when graphed against increasing values of *k*.

The presence of introgressed *C*. *acutus* DNA amongst *C*. *moreletii* samples was then examined using an array of analytical procedures. Firstly, a spatially independent analysis based on Bayesian inference of admixture proportions, that is the proportion of each individual's genome derived from each source population *i* (q_i), was performed using fastSTRUCTURE (Raj et al., 2014). A maximum likelihood structure approach implemented in the software NGSadmix (Skotte et al., 2013) was also used. Both methods identify admixture proportions, but fastSTRUCTURE uses SNP calls whilst NGSadmix uses the genotype likelihood estimates produced through the software ANGSD (Korneliussen et al., 2014) without calling genotypes. In both cases, the probability of individual assignment into population clusters (*K*) was estimated without prior information on the origin of individuals, conducting several tests with a maximum number of populations from $K = 2$ to $K = 10$, with default convergence criterion and priors, and five replicates for each value. The optimal *K* value was estimated for fastSTRUCTURE results with the Python-based tool CHOOSEK (included in the package) and for NGSadmix results using CLUMPAK (Kopelman et al., 2015). Admixture proportions at $K = 2$ were used to detect genetic admixture between *C*.

moreletii and *C*. *acutus*, and only individuals exhibiting point estimates of $q_i > 0.95$ with the lower bound of the 95% credible intervals (CI) for $q_i > 0.8$ were assumed as non-admixed (following Rodriguez et al., 2008; Pacheco-Sierra et al., 2016).

The software fineRADstructure v.0.3.1 (Malinsky et al., 2018) was also used to infer genomic variation among individuals by clustering them according to similarity of their RAD haplotypes. This software exploits information drawn from stacks containing several SNPs, whereas different stacks are assumed to be unlinked, to derive a co-ancestry matrix based on the most recent coalescent events (i.e. the sharing of identical or nearest-neighbour haplotypes among individuals). Crocodile haplotypes exported using the *populations* tool in STACKS were converted to fineRADstructure input format using the script *Stacks2fineRAD.py* (Malinsky et al., 2018). The co-ancestry matrix was inferred using RADpainter, and the MCMC clustering algorithm in fineSTRUCTURE v.4 (Lawson et al., 2012) was used to infer clusters of shared ancestry, setting the number of burn-in iterations to 100,000, the sample iterations to 100,000, and the thinning interval to 1,000. Finally, to reflect the relationships within the co-ancestry matrix, the inferred clusters were arranged according to a tree inferred with fineSTRUCTURE, using 100,000 hill-climbing iterations, and allowing for all possible tree comparisons (following Barth et al., 2020).

4.2.6 Crocodile relatedness in the region of Calakmul

For analysis of crocodile relatedness, a new dataset containing only genotype calls for *C*. *moreletii* samples in Calakmul was created in STACKS using the same methods as described above. Only the 400 loci with the highest number of heterozygosity across individuals were retained, and exported into VCF and GENEPOP formats. Pairwise relatedness between individuals was calculated using RELATED (Pew et al., 2015), a software package for *R* based on the source code of COANCESTRY (Wang, 2011). Using the simulation function, four relatedness estimation methods (Queller & Goodnight, 1989; Li et al., 1993; Lynch & Ritland, 1999; Wang, 2002) were compared by using allele frequencies within the dataset to generate 100 simulated pairs in each of the following categories: parent-offspring, full-siblings, half-siblings, and unrelated individuals. Wang's relatedness estimator (Wang, 2002) overall provided the highest correlation coefficient between calculated and expected relatedness estimates and thus was used as a measure of relatedness across the region.

Further relatedness analysis and parentage assignment was conducted using COLONY 2.0 (Wang, 2009; Jones & Wang, 2010), which is a likelihood-based method that implements groupwise approach for sibship reconstruction to infer genealogies. The *C*. *moreletii* SNP dataset containing the 400 loci with the highest number of heterozygosity was converted to COLONY format using the script *Genepop2Colony*, available in the software package GENEPOPEDIT (Stanley et al., 2017), a *R* software package designed for manipulating multilocus data. A full likelihood model with medium precision and without setting a sibship prior was used in COLONY, allowing for polygamous mating in both sexes. Samples of adult crocodiles in Calakmul regions 4 and 6 were treated as potential paternal and maternal candidates, whereas all other samples were treated as potential offspring. Network graphics displaying the inferred full-sibship and half-sibship dyads and “Best (ML) Configuration” assignments of parent-offspring were constructed using CYTOSCAPE 3.8.0 (Shannon et al., 2003), following Ursprung et al. (2011).

4.3 Results

4.3.1 SNP discovery and genotyping using stacks

The number of reads retained after demultiplexing, verification of RAD-tags, and quality filtering ranged between 2.31 and 5.42 * 10^6 per individual, with a mean of 3.07 * 10^6 reads. Two samples from Calakmul were excluded from the data set due to a low number of reads (~ 5,200 and 70,000). The remaining 93 samples were processed and filtered through the *ref_map* pipeline and *populations* tool in STACKS, resulting in a final selection of 4,980 polymorphic loci shared by both species of crocodile and identified in at least 80% of all individuals.

Seven samples (five from Calakmul 2, and two from Calakmul 4) had RAD sequences mapping to the 2278-2872 and 9528-9622 regions of the *C*. *acutus* mitochondrial genome (Figures 37,39, 40).

Table 5. Geographic distance and mean F_{st} values between the six regions in Calakmul where *Crocodylus moreletii* samples were obtained. Distance values in each region pair represent Euclidian distance between their two closest sample locations. F_{st} values were calculated using the *populations* pipeline in STACKS (Rochette et al., 2019).

	Calakmul 1	Calakmul 2	Calakmul 3	Calakmul 4	Calakmul 5	Calakmul 6
Calakmul 1	---	0.079	0.176	0.075	0.197	0.095
Calakmul 2	40 km	---	0.100	0.065	0.111	0.088
Calakmul 3	30 km	20 km	---	0.057	0.203	0.077
Calakmul 4	115 km	80 km	90 km	---	0.026	0.078
Calakmul 5	155 km	130 km	130 km	75 km	---	0.063
Calakmul 6	80 km	60 km	50 km	60 km	80 km	---

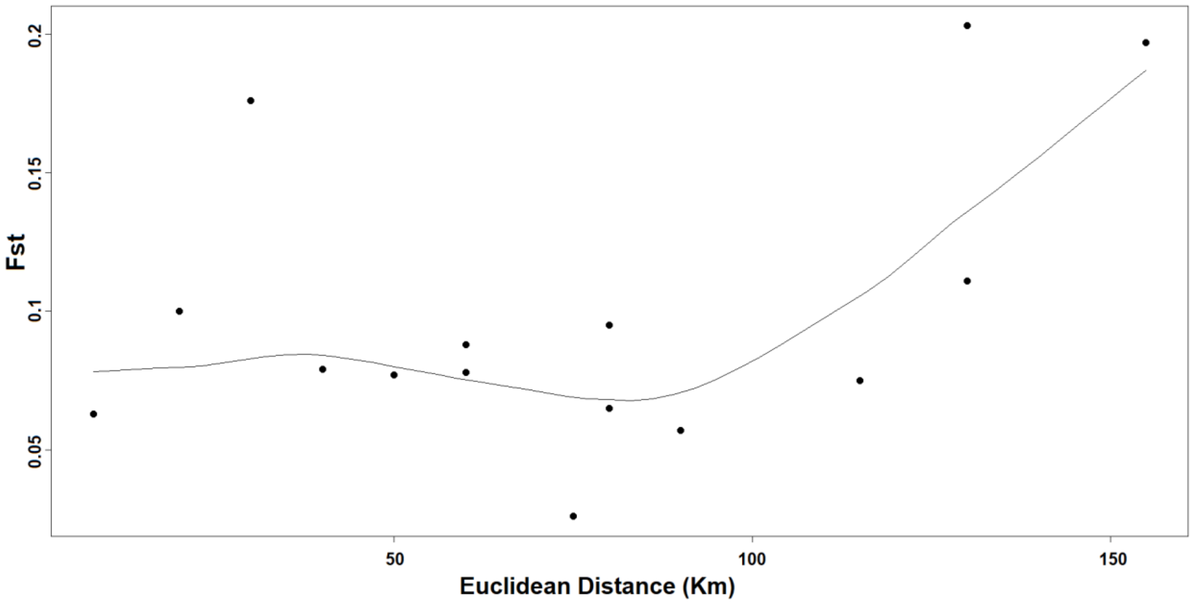

Figure 33. Relationship between genetic and geographical distances in *C. moreletii* across Calakmul. The line represents a smooth curve fitted by locally estimated scatterplot smoothing (Loess; Cleveland, 1979)

4.3.2 Genetic structure across Calakmul

The PCA revealed a probable occurrence of mislabelling, as one sample from the Calakmul region 6 was found to cluster with region 4 (Figure 34). This sample was removed from the dataset. The PCA highlighted differences between the six *C. moreletii* populations along the first two PC axes (Figure 34A), but no apparent structure was observed along the third axis (Figure 34B). The first PC axis separated populations into two main groups (Calakmul regions 1-3 and 4-6), whilst the second axis separated Calakmul region 6 from regions 4 and 5.

The *k*-means algorithm revealed that the most likely number of clusters (*n* = 3, based on the elbow of the BIC curve; Figure 35) is less than the number of sampling regions. However, the assignment of individuals into genetic clusters based on SNPs did not universally correspond with these regions (Figures 36, 37). Individuals from Regions 1, 2 and 3 were assigned into the same cluster. Calakmul Region 4 to a large extent represents its own genetic cluster containing 95% (33/35) of individuals. The remaining 5% (n = 2) were assigned into the cluster formed by Calakmul regions 1, 2 and 3. All three individuals from Region 5 were grouped with one of the clusters formed by Region 4. Region 6 was also to a large extent its own genetic cluster, with 90% of crocodiles being assigned to one cluster and the remaining 10% to the Cluster formed by Region 4 and 5.

The DACP further demonstrated the results observed through PCA, illustrating its increased power to discriminate between three major groups (Figure 38).

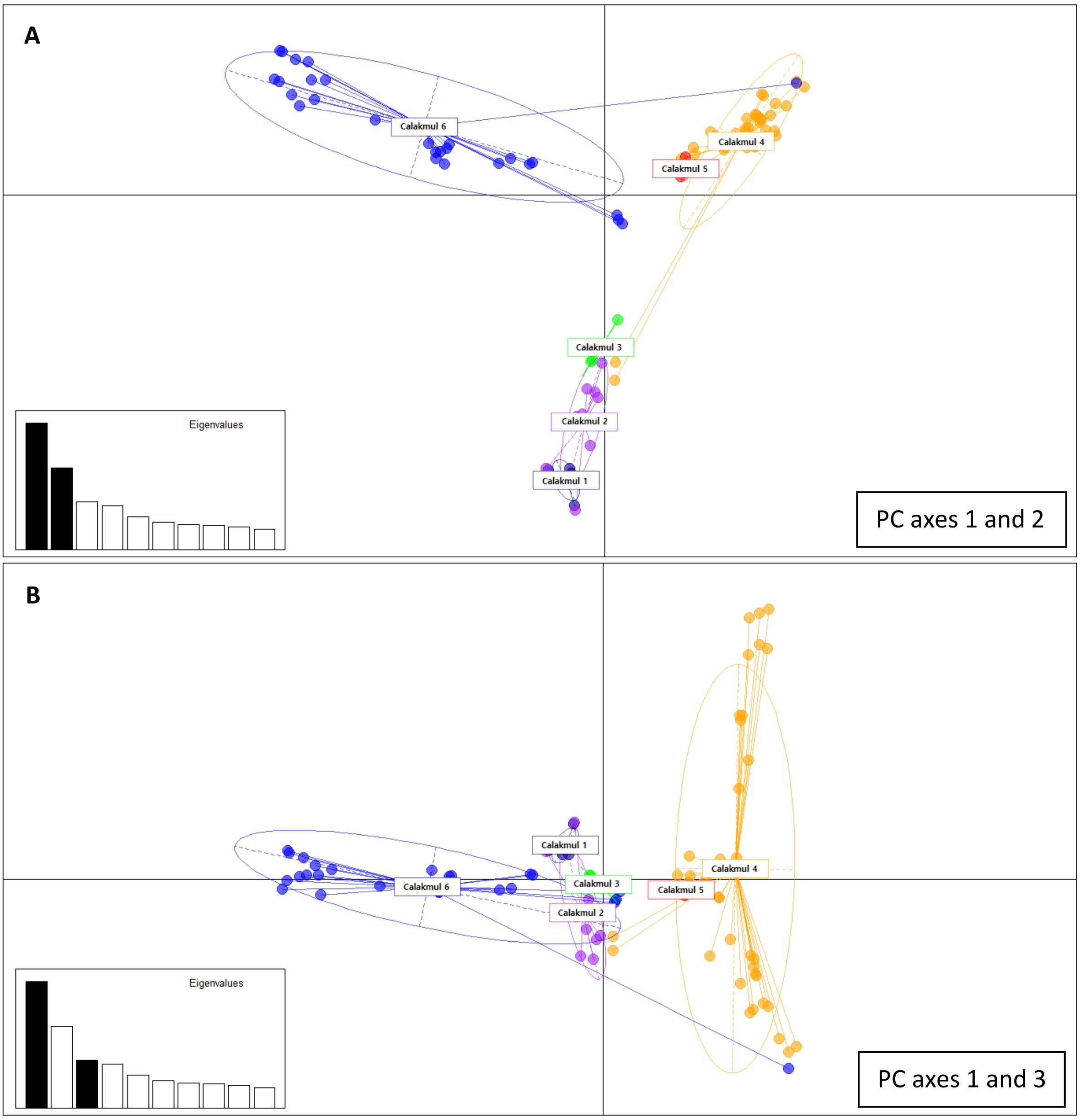

Figure 34. PCA plots generated from the SNP dataset of *C. moreletii* from Calakmul. The first two PC axes indicate differentiation across sample regions (A), whilst no structure is apparent along the third axis (B). Calakmul labels discriminate the six major areas within the region where crocodile samples were collected (see figure 37).

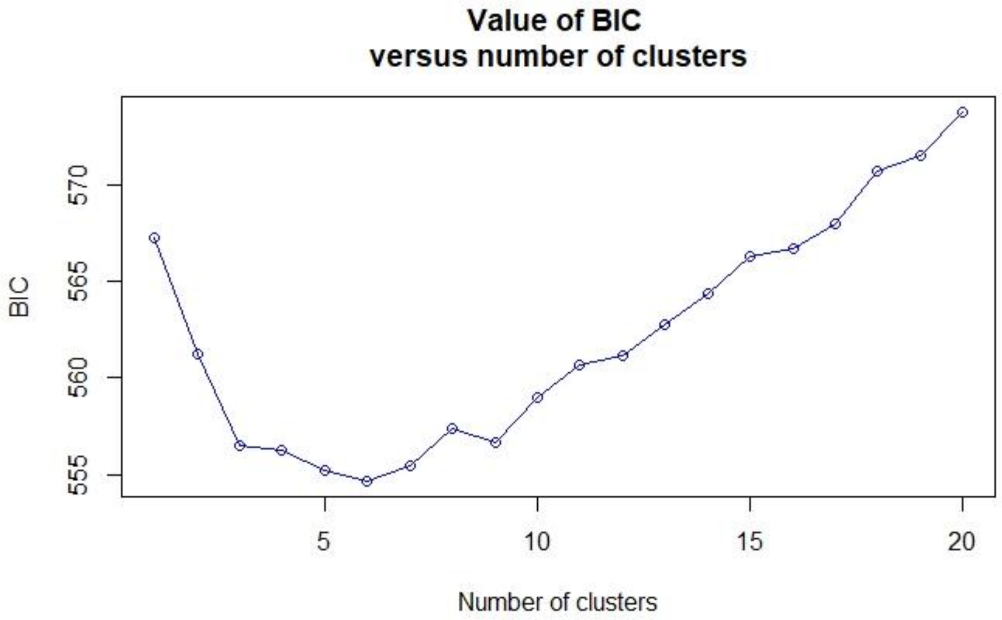

Figure 35. Inference of number of clusters identified within the SNP dataset of *C. moreletii* from Calakmul. Number of clusters selection (*k* = 6) was based on the elbow of the curve of BIC values.

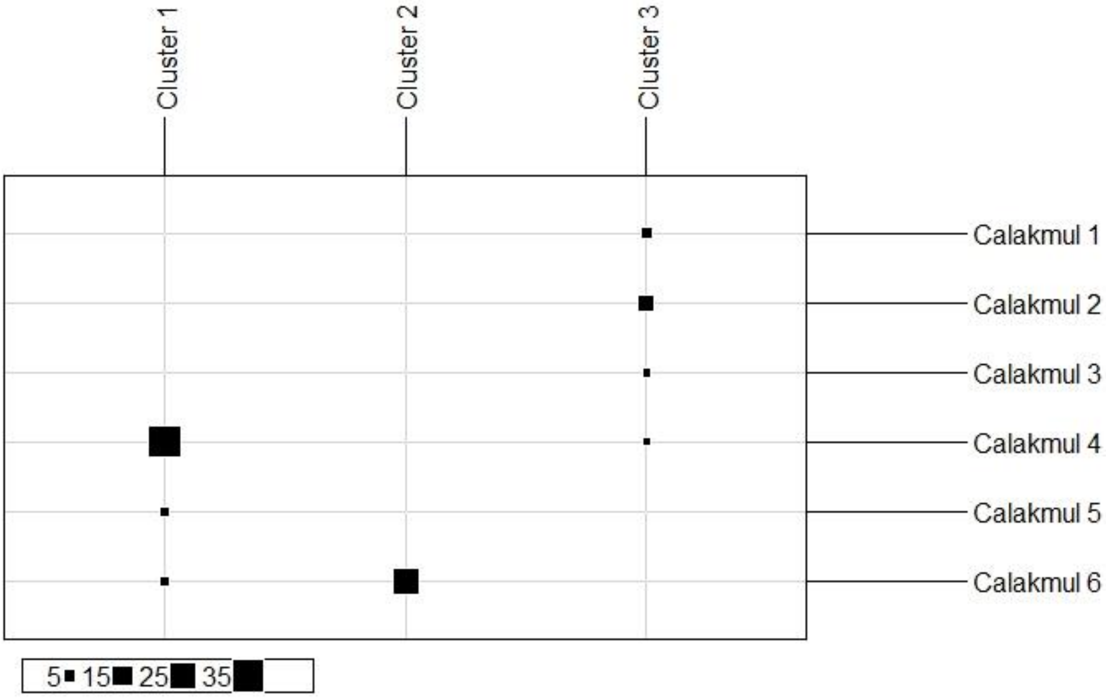

Figure 36. Assignment of *Crocodylus moreletii* samples into the six genetic clusters inferred by the *k* means algorithm in relation to where samples originate from. Calakmul labels discriminate the six major areas within the region where crocodile samples were collected (see figure 37).

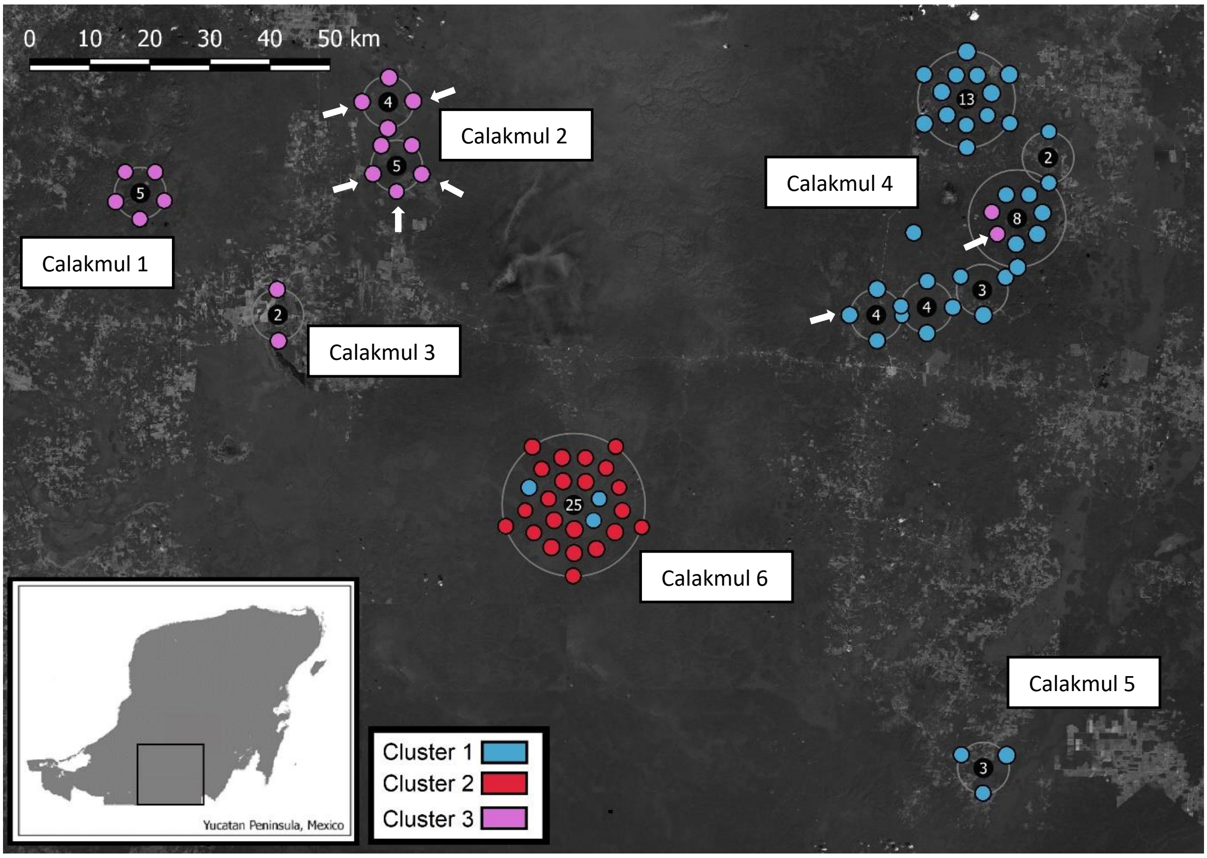

Figure 37. Spatial distribution of *Crocodylus moreletii* from the three genetic clusters inferred by the *k* means algorithm. Calakmul labels discriminate the six major areas within the region where crocodile samples were collected. White arrows indicate samples with reads matching regions 2278-2872 and 9528-9622 of the *Crocodylus acutus* mitochondrial genome.

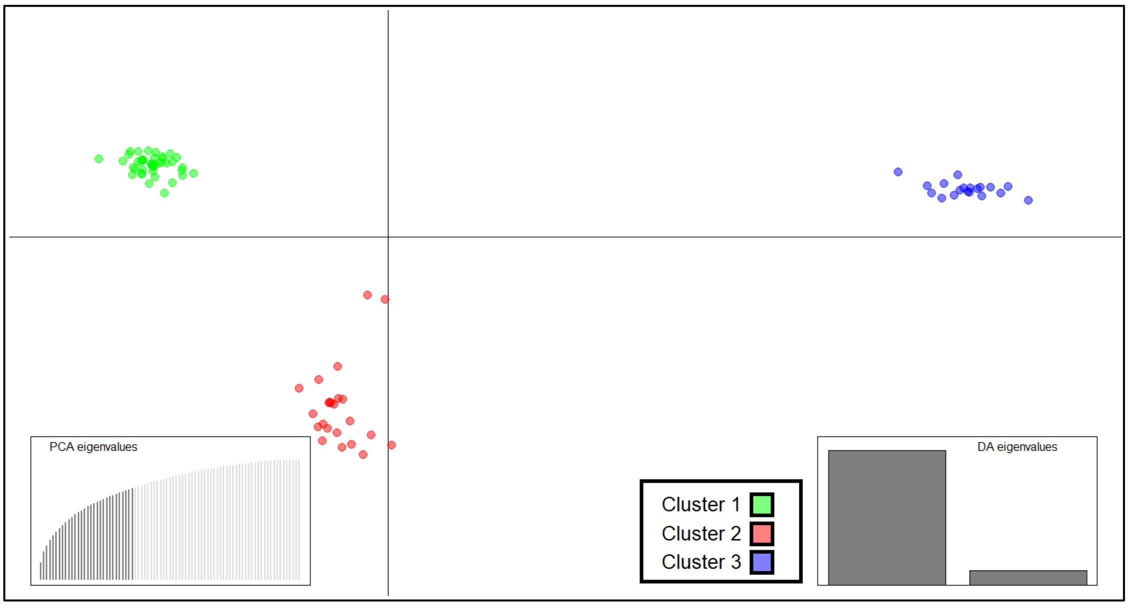

Figure 38. DAPC plot generated from the SNP dataset of *C. moreletii* from Calakmul. The first two PC axes indicate differentiation across sample regions.

4.3.3 Admixture proportions and co-ancestry

Bayesian admixture proportions (q_i) for $K = 2$ estimated in fastSTRUCTURE identified 100% (84/84) and 86% (72/84) of crocodile samples from the region of Calakmul as non-admixed *C*. *moreletii* at q_i thresholds of 0.95 and 0.99 (Figure 39), respectively. Calakmul regions three and six had no samples with signs of introgression (Figure 39). However, significant *C*. *acutus* admixture proportions were observed in all external reference samples of *C*. *moreletii* (0.17 ± 0.01; 0,1 – 0.18), confirming existing expectations (Pacheco-Sierra et al., 2016). Bayesian assignments based on the CHOOSEK method suggested that the number of clusters that best explain the structure was $K = 6$, demonstrating genetic sub-structuring also within Calakmul (Figure 39). At this value of K, no samples retained an admixture proportion matching *C*. *acutus*.

Admixture proportions (Q-scores) estimated from genotype likelihoods for $K = 2$ in NGSadmix identified 96% (81/84) and 74% (62/84) of crocodile samples from the region of Calakmul as non-admixed *C*. *moreletii* at a threshold of 0.95 and 0.99 (Figure 40), respectively. Similar to what was observed in fastSTRUCTURE, significant *C. acutus* admixture proportions (0.18 ± 0.04; 0,11 – 0.23) were apparent in all external reference samples of *C*. *moreletii*. Assignment results based on the CLUMPAK method indicated that the number of clusters that best explain the structure within the dataset was $K = 4$. At this value of K, no crocodile samples from Calakmul exhibited a *C*. *acutus* Q-score above 0.05, and only seven exhibited values above 0.01.

The clustered co-ancestry matrix and the cladogram resulting from the fineRADstructure haplotype similarity analysis suggested that the reference samples of *C*. *moreletii* had higher estimated levels of coancestry with *C. acutus* when compared with samples from Calakmul, a further indicative of their admixed origin (Figure 41). Running the same analysis using only samples from Calakmul revealed further sub-structuring into three main clusters (Figure 42).

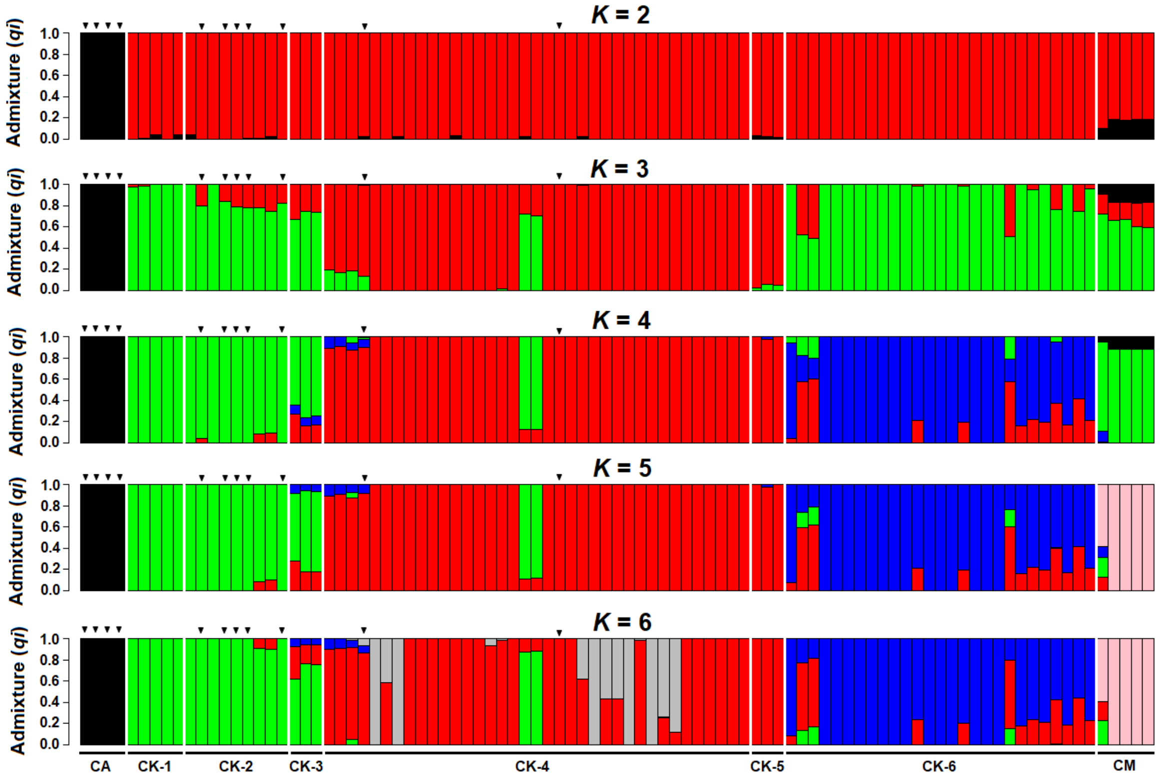

Figure 39. Genetic structure and Bayesian admixture proportions (*q_i*) from fastSTRUCTURE (Raj et al., 2014) with *K* = 2 to 6, where solid lines are 95% credible intervals and different colours represent each admixture proportion. Each vertical bar corresponds to one crocodile sample. Inverted black triangles represent samples with reads matching regions 2278-2872 and 9528-9622 of *Crocodylus acutus* mitochondrial genome. CK represents crocodile samples from the six areas within the study region. CA and CM represent, respectively, the external reference samples of *Crocodylus acutus* and *C. moreletii*. Bayesian assignment results based on the CHOOSEK method indicated that the number of clusters that best explain the structure was *K* = 6.

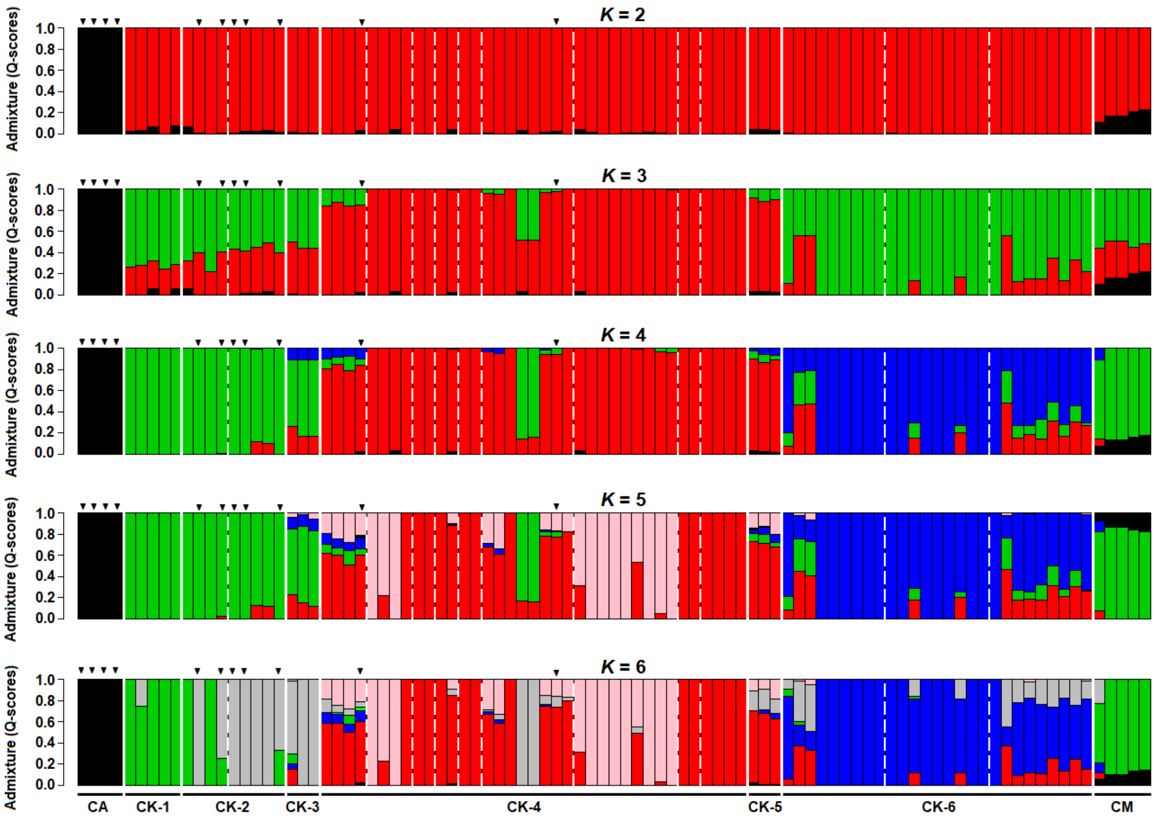

Figure 40. Genetic structure and ancestry proportions (Q-scores) from NGSadmix (Skotte et al., 2013) with *K* = 2 to 6, where solid lines are 95% credible intervals and different colours represent each admixture proportion. Each vertical coloured bar corresponds to one crocodile sample. Dashed white lines delimit different sample locations within each region of Calakmul (CK). Inverted black triangles represent samples with reads matching regions 2278-2872 and 9528-9622 of *Crocodylus acutus* mitochondrial genome. CA and CM represent, respectively, the external reference samples of the *C. acutus* and *C. moreletii*. Assignment results based on the CLUMPAK method indicated that the number of clusters that best explain the structure was *K* = 4.

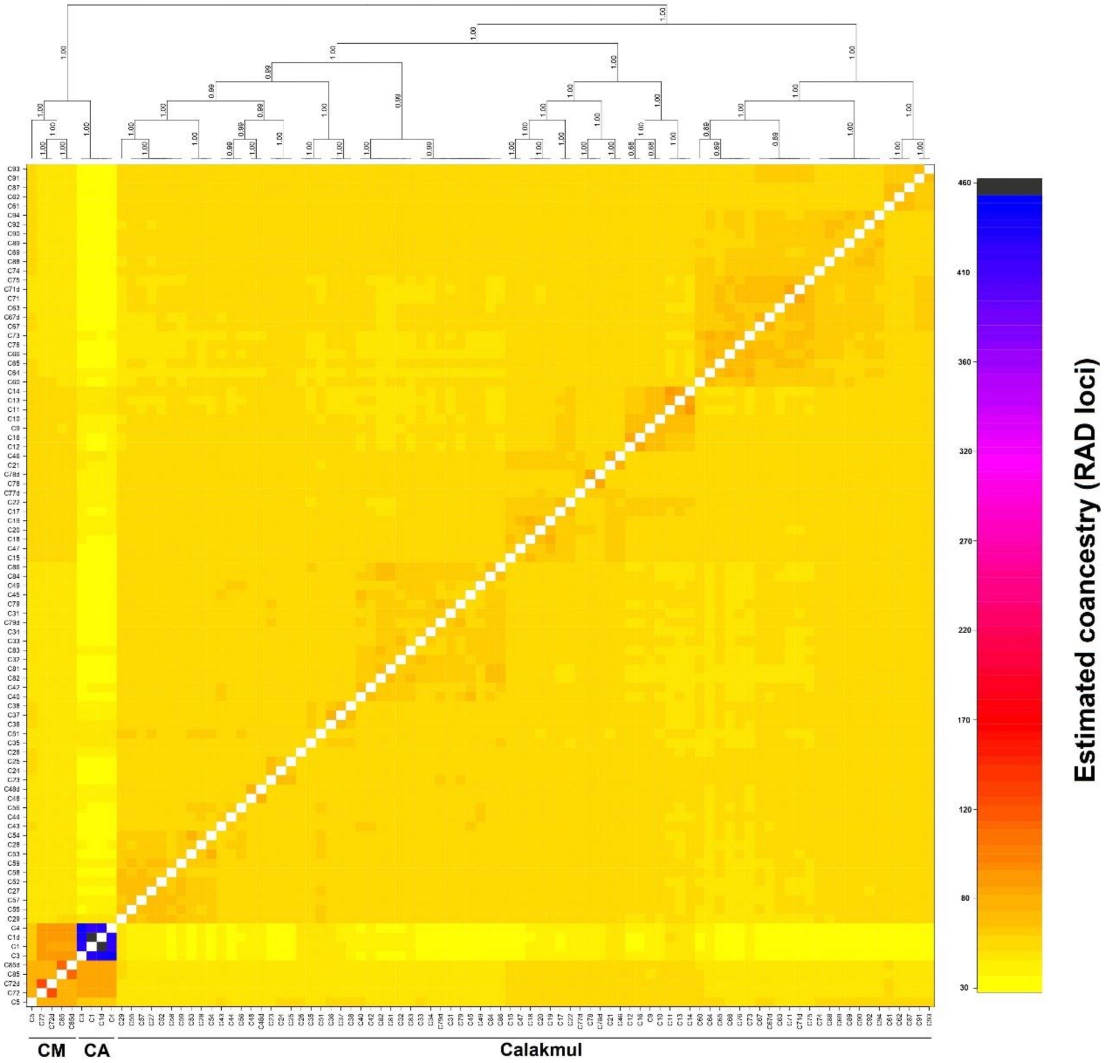

Figure 41. Individual coancestry based on haplotype similarity across all *C*. *moreletii and C*. *acutus* samples. Coancestry was investigated based on RAD loci with fineRADstructure (Malinsky et al., 2018). Heatmap colours indicate numbers of RAD loci with estimated shared coancestry. Individuals are listed on both axes in the same order, clustered according to the tree shown on top of the heatmap (Lawson et al., 2012). Individuals from Calakmul cluster together with further substructure visible (further detail in Figure 42). External *C*. *moreletii* individuals (CM) clustered together with *C. acutus* (CA) further confirming admixture within these crocodile samples.

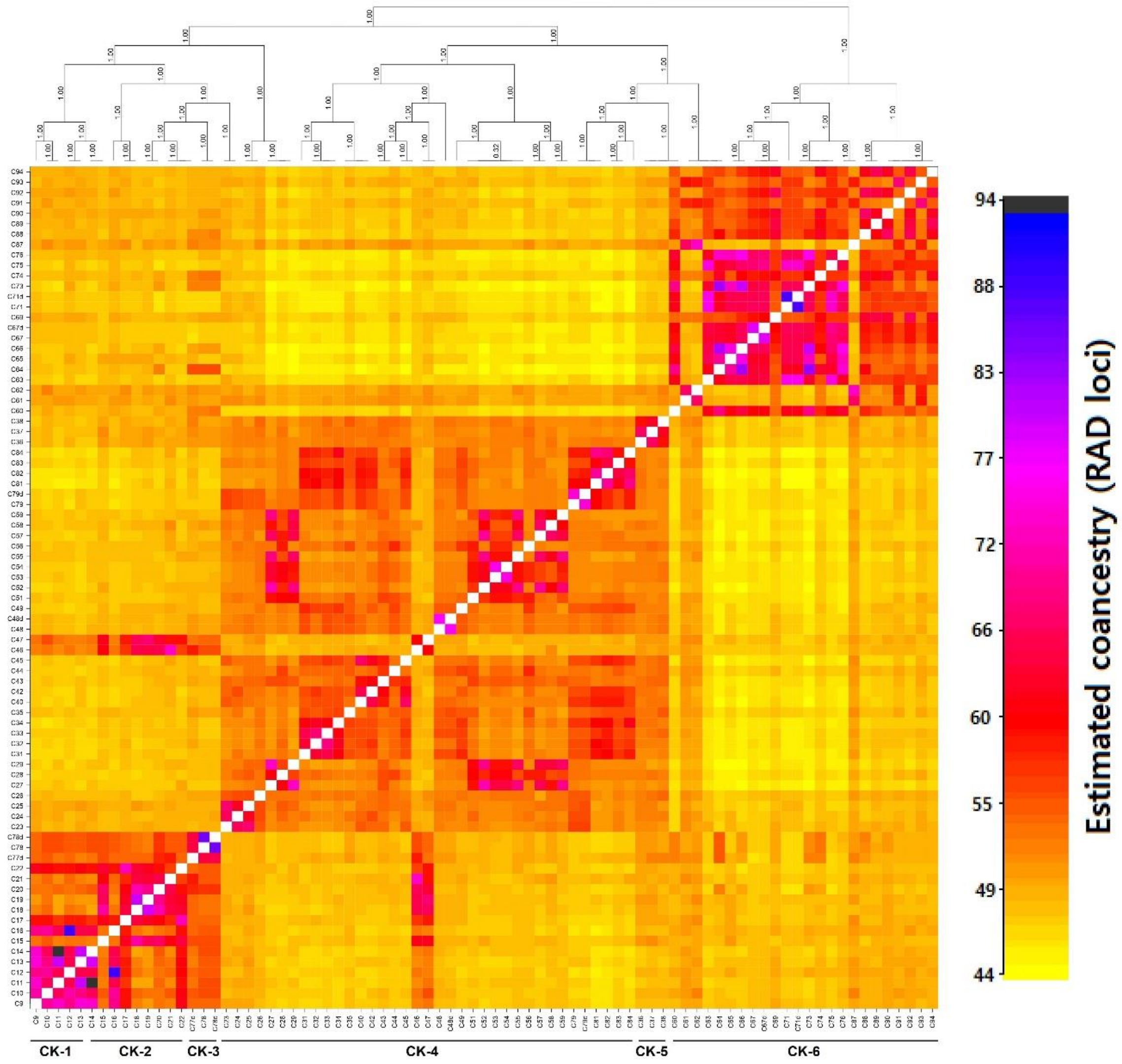

Figure 42. Individual coancestry based on haplotype similarity for *C. moreletii* samples from the region of Calakmul, indicating the presence of three main clusters with further substructure visible within each group. Coancestry was investigated based on RAD loci with fineRADstructure (Malinsky et al., 2018). Heatmap colours indicate numbers of RAD loci with estimated shared coancestry. Individuals are listed on both axes in the same order, clustered according to the tree shown on top of the heatmap (Lawson et al., 2012).

4.3.4 Individual relatedness

Relative intervals of individual relatedness (R^W) were delineated based on 100 simulated pairs of known relatedness (Figure 43) and identified as Full-sibling ($R^W \geq 0.4$; share both parents), Half-sibling (R^W ranging from 0.15 to 0.39; share one parent) or Unrelated ($R^W < 0.15$). For pairwise relatedness within the same region of Calakmul between juvenile and adult crocodiles, Parent-Offspring relationships were identified for R^W values greater than 0.4 and Other for R^W values ranging from 0.15 to 0.39.

From the 2850 pairwise relatedness values estimated for the samples from Calakmul, 89% (n = 2550) yielded a R^W value less than 0.15 and were classified as unrelated. From the remaining 300 R^W values, 81% (n = 241) were assigned as Half-sibling, 12% (n = 37) as Full-sibling, 6% (n = 18) as Other, and 1% (n = 4) as Parent-Offspring.

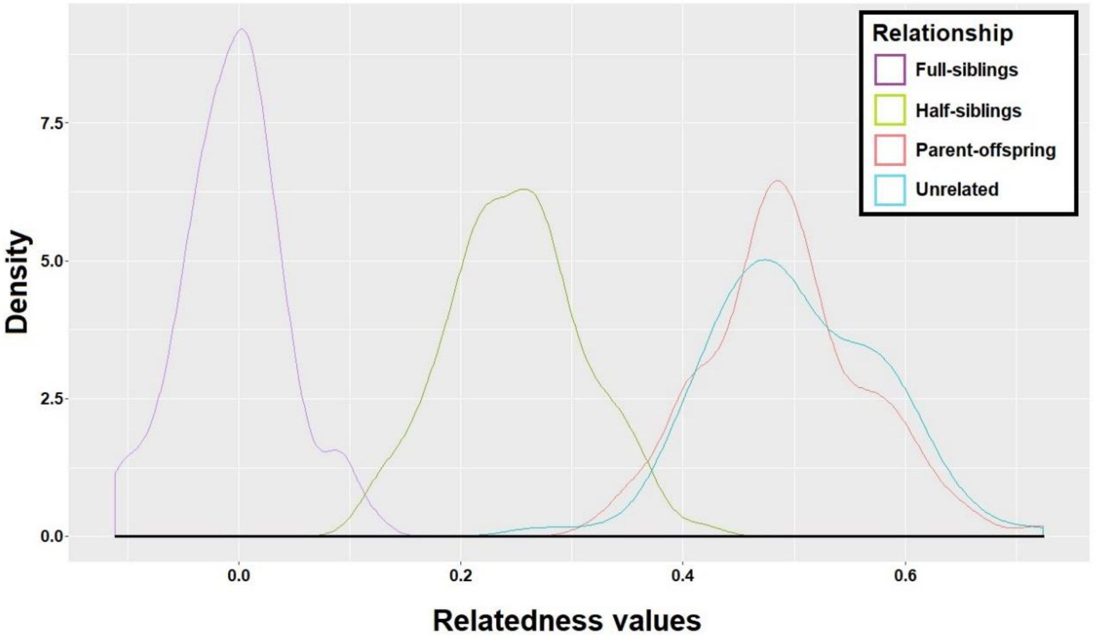

Figure 43. Relatedness values (R^W) for 100 simulated pairs with known relatedness estimated through Wang's relatedness estimator (Wang, 2002) in RELATED (Pew et al., 2015; Wickham, 2016), based on allele frequencies from the 400 loci with highest number of heterozygosity across *C*. *moreletii* individuals in Calakmul. Relationship curves are well differentiated, demonstrating the elevated statistical power of the 400 SNP loci in estimating pairwise relatedness amongst crocodile samples.

The resulting relatedness network indicated that, apart from two crocodiles from Region 2 being grouped with individuals from Group 1, no relatedness between crocodiles of different regions was identified (Figure 44). Calakmul regions 1, 2, and 5 were composed of a single cluster of connected individuals each. For Calakmul region 4, two large clusters were identified, in addition to a smaller unit of three individuals, and four individuals unrelated to any other crocodile. For Region 6, two large clusters and a smaller unit of five individuals were identified.

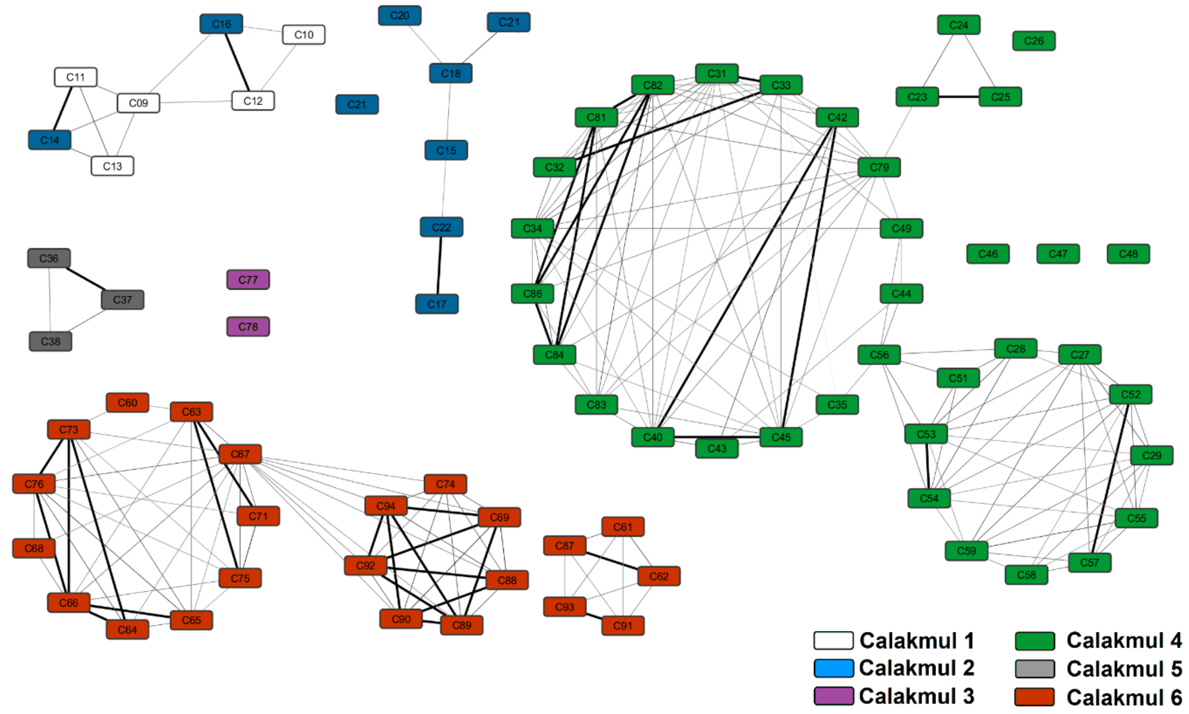

Figure 44. Pairwise relatedness (R^W) network generated in for *C. moreletii* estimated through Wang's relatedness estimator (Wang, 2002) in RELATED (Pew et al., 2015), based on allele frequencies. Each coloured node represents a crocodile, with different colours indicating one of the six sampled regions in Calakmul. Distances and locations of nodes do not contain any spatial information. Black lines represent R^W values higher than 0.4 (Parent-offspring or Full-siblings) and grey lines indicate an R^W between 0.15 and 0.4 (Half-siblings or Other). This network was constructed using CYTOSCAPE 3.8.0 (Shannon et al., 2003).

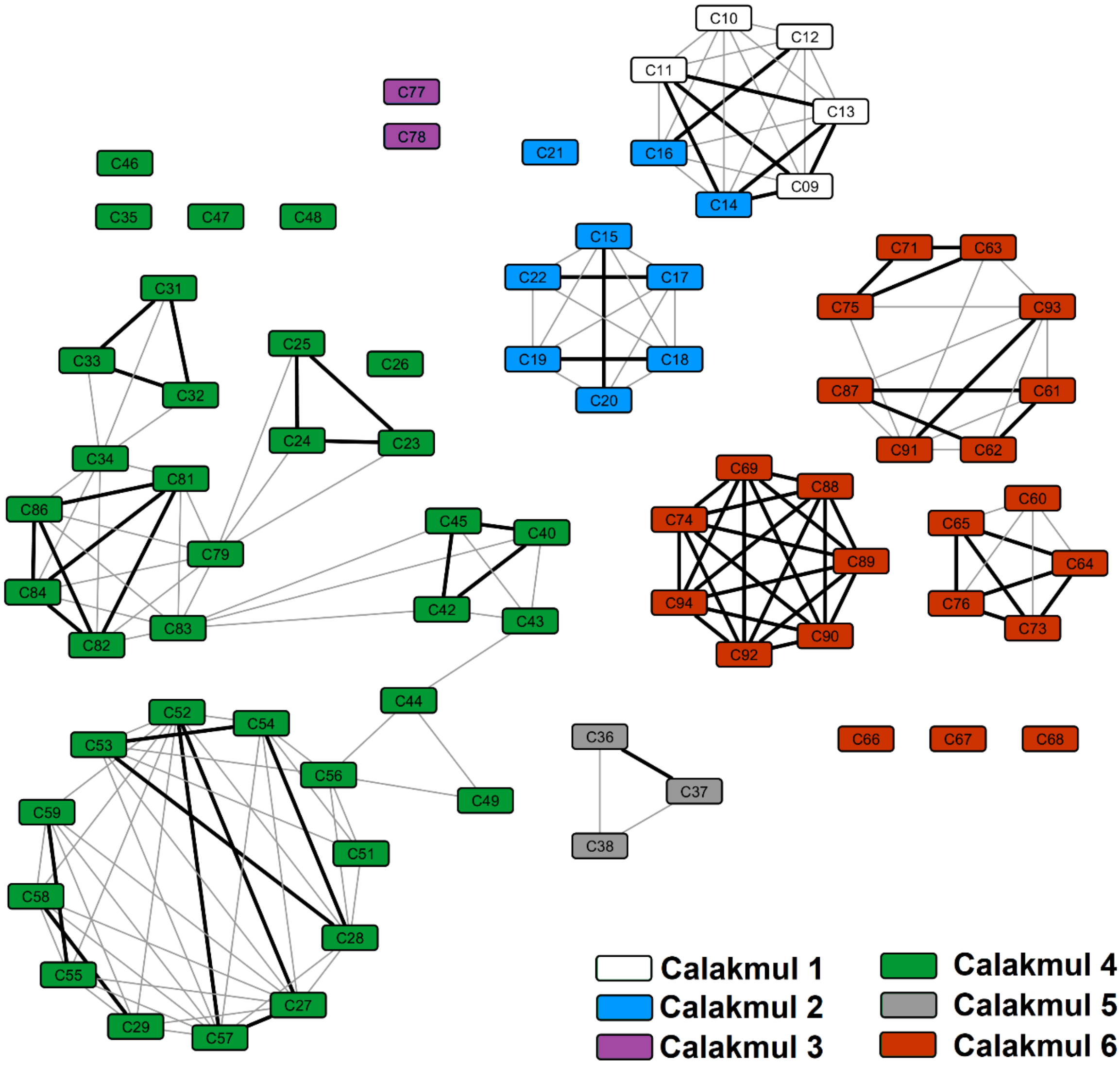

Figure 45. Pairwise relatedness network for *C. moreletii* based on the full-sibship (black lines) and half-sibship (grey lines) dyads inferred through maximum likelihood in COLONY (Wang, 2009). Each coloured node represents a crocodile, with different colours indicating one of the six sampled regions in Calakmul. Distances and locations of nodes do not contain any spatial information. Unconnected coloured nodes in Calakmul 2 (C21), Calakmul 3 (C77), Calakmul 4 (C47, C48) and Calakmul 6 (C66, C67, C68) represent individuals that were passed as potential parental genotypes in the analysis, due to being significantly larger (adults; total length ≥ 2 metres) that the rest of the crocodiles of those regions. This network was constructed using CYTOSCAPE 3.8.0 (Shannon et al., 2003).

Full-sibship and half-sibship dyads for *C. moreletii* inferred through maximum likelihood in COLONY revealed 170 links amongst sampled individuals, where 68 represent Full-siblings and 102 Half-siblings (Figure 45). A similar structure to the network based on R^W was observed for Calakmul regions 1, 2, 3, 5 and 6. However, Region 4 was sub-divided into 5 clusters and four individuals unrelated to any other crocodile.

For parental assignment, COLONY only assigned four individuals to a known female genotype within the dataset, and all remaining individuals were assigned to an unknown female (n = 19) and male (n = 21). The inferred parental network for crocodile samples (Figure 46) revealed a polygamous system with most females and males sharing offspring with more than one partner.

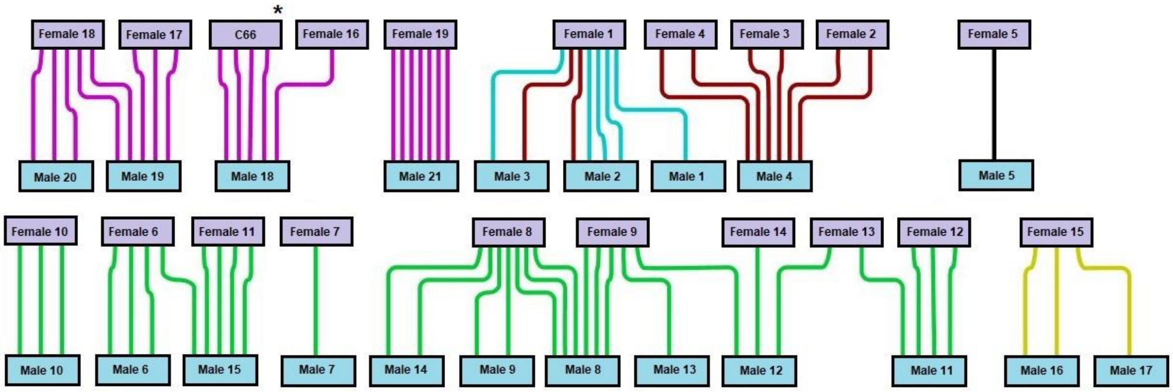

Figure 46. Parental assignment network in *C. moreletii* inferred through maximum likelihood in COLONY (Wang, 2009). Each coloured line represents a crocodile sample, with different coloured lines indicating one of the six sampled regions in Calakmul (Calakmul 1 – blue lines; Calakmul 2 – brown lines; Calakmul 3 – black line; Calakmul 4 – green lines; Calakmul 5 – yellow lines; Calakmul 6 – magenta lines). Light blue and light purple coloured rectangular nodes represent, respectively, the inferred paternal and maternal genotypes for each crocodile sample. Distances and locations of nodes do not contain any spatial information. Asterisk indicates parental assignment to a known female genotype. This network was constructed using CYTOSCAPE 3.8.0 (Shannon et al., 2003).

4.4 Discussion

This study investigates the presence of *Crocodylus moreletii* – *Crocodylus acutus* hybrids and provides evidence that the region of Calakmul, unlike what has been reported in other parts of the Yucatan Peninsula (Pacheco-Sierra et al., 2016; Pacheco-Sierra et al., 2018), still holds a healthy population of non-admixed *C. moreletii* individuals. Furthermore, population structure assessment amongst samples from Calakmul revealed that the six study regions were grouped into three genetic clusters. Coupled with high relatedness values and significant number of pairwise Half-sibling and Full-sibling relationships, spatial population structure is therefore linked to extended family networks within each region.

4.4.1 Screening for presence of hybrid individuals in Calakmul

Hybridization has long been a topic of interest to many fields in biology, and an increasing matter of concern to conservation biologists (Rhymer & Simberloff, 1996; Allendorf et al., 2001; Grabenstein & Taylor, 2018; McFarlane et al., 2020). Regions of secondary contact, where individuals from genetically different populations reproduce and form admixed offspring, have been recognized as fertile grounds for evolutionary studies on speciation, selection, recombination and maintenance of species boundaries (Anderson & Thompson, 2002; Payseur & Rieseberg, 2016; Ottenburghs et al., 2017). Although natural hybridization regularly occurs and is an important part of the evolutionary history of many species (Mallet, 2005; Abbott et al., 2013; Payseur & Rieseberg, 2016), interpreting the evolutionary significance of hybridization and determining the role of hybrid populations in developing conservation plans can be more difficult than is usually appreciated (Allendorf et al., 2001; Genovart, 2008). Conservation of hybrid individuals, especially those originating from natural hybridization, has been an ongoing topic of controversy and debate (Jackiw et al., 2015; Pacheco-Sierra et al., 2016), in great part due to hybrids often having higher fitness which allow for adaptation to novel environments (Allendorf et al., 2001; Edmands, 2007). On the other hand, hybridization and introgression have contributed to the extinction of species and is especially concerning when rare species come into contact with more abundant ones, whether owing to natural or anthropogenic causes (Allendorf et al., 2001), as it can result in the formation of localized hybrid swarms and eventual genetic swamping of the rarer species (Rhymer & Simberloff, 1996; Genovart, 2008).

Although hybridization between crocodiles has long been documented, its importance for conservation concerns has been raised only recently, and there is still some prevalent controversy regarding the management strategies and conservation policies when dealing with hybrids (Hekkala et al., 2015; Pacheco-Sierra et al., 2018). Hybridization between *C. moreletii* and *C. acutus* has long been suggested based on morphological assessments (e.g. Ross & Ross, 1974; Ross & Mayer, 1983) with individuals exhibiting a gradient of morphological characteristics from both species (e.g. Hekkala, 2004; Cedeño-Vazquez et al., 2011). One of the first studies making use of molecular tools to report hybridization between two crocodile species (*Crocodylus siamensis* and *Crocodylus rhombifer*) was conducted on individuals living in captivity (Fitzsimmons et al., 2002) and provided evidence that hybridization could also occur in the wild amongst other species within the genus. Since then, a significant number of studies have employed mitochondrial DNA and microsatellite information to investigate hybridization between *C*. *moreletii* and *C*. *acutus* in Mexico (e.g. Cedeño-Vázquez et al., 2008; Rodriguez et al., 2008; González-Trujillo et al., 2012; Pacheco-Sierra et al., 2016) and Belize (e.g. Ray et al., 2004; Hekkala et al., 2015), highlighting the occurrence of bidirectional gene flow between the two species and identifying a large number of hybrid populations especially in coastal areas (Ray et al., 2004; Cedeño-Vázquez et al., 2008; Rodriguez et al., 2008; Pacheco-Sierra et al., 2016).

It was nevertheless not until recently that hybridization between *C*. *moreletii* and *C*. *acutus* was examined on a large-scale, encompassing samples from both species across their historic distribution ranges in Mexico (Pacheco-Sierra et al., 2016; Pacheco-Sierra et al., 2018), with the latter study investigating hybridization between these two species through 12,800 SNP loci produced through RADseq. These two studies revealed that, contrary to previous expectations, hybridization between the two species is geographically extensive, extending well beyond their historical region of sympatry, with admixed individuals found throughout the Gulf of Mexico and the Caribbean. Furthermore, and contrary to what was previously believed, the studies demonstrated that non-admixed populations of both *C*. *moreletii* and *C*. *acutus* could only be found in confined locations within the species' Mexican distribution range. For

C. *moreletii*, such locations consist of upstream continental lagoons in Northern Mexico that represent “islands” with little connection with other water bodies, decreasing opportunities for gene flow with admixed individuals as well as *C. acutus*. Despite previous assumptions, hybridization between the two species does not appear to be a result of anthropogenic influences, as admixture is due to historic processes and involves an extended mosaic hybridization pattern (Pacheco-Sierra et al., 2016; Pacheco-Sierra et al., 2018). Two distinct hybrid *C. moreletii* lineages have been identified. The first lineage is represented by individuals inhabiting the Gulf of Mexico and is genetically closer to the parental lineage from Northern Mexico with little introgression from *C. acutus*, whilst the second lineage is characterised by higher levels of admixture and mostly inhabits coastal areas along the Yucatan Peninsula (Pacheco-Sierra et al., 2016; Pacheco-Sierra et al., 2018).

Although Calakmul represents only a small geographical region within the southern central portion of the Yucatan Peninsula, the results presented herein suggest that Calakmul represents a stronghold for non-admixed populations of *C*. *moreletii* surrounded by largely admixed populations along more coastal regions (Cedeño-Vázquez et al., 2008; Rodriguez et al., 2008; Pacheco-Sierra et al., 2016). A substantial proportion of individuals (~74%) were entirely free from *C*. *acutus* introgression, and admixture proportions as quantified by both Bayesian (fastSTRUCTURE) and maximum-likelihood (NGSadmix) approaches were below 0.05 for all but three individuals from Calakmul, values which are generally interpreted as indicative of non-admixture (Rodriguez et al., 2008; Hekkala et al., 2015; Pacheco-Sierra et al., 2016). These findings are of great conservation importance, as they provide first-hand evidence that Calakmul is the last refuge within the Mexican Yucatan Peninsula still holding a non-admixed lineage of *C*. *moreletii*. It is important to highlight that the only other two non-admixed *C. moreletii* populations inhabit isolated locations in the very opposite end of Mexico. Although *C*. *moreletii* is a species protected by the Mexican law as conservation dependant (NOM-059-ECOL-2001) and internationally listed by IUCN (Least Concern) and CITES (Appendix II), conservation guidelines and protection for the *C*. *moreletii* – *C*. *acutus* complex are in urgent need of review due to the vast majority of individuals and populations routinely identified as *C. moreletii* being in fact admixed (Pacheco-Sierra et al., 2016). Naturally occurring *C. moreletii* – *C. acutus* hybrid populations need to be considered for their conservation value (Jackiw et al., 2015; Pacheco-Sierra et al., 2018), but, more importantly, the three non-admixed *C. moreletii* populations require different categorization than their hybrid counterparts due to their more endangered nature (Pacheco-Sierra et al., 2016).

Isolated pure C. *moreletii* populations within the historic species distribution range coupled with hybrids increasingly becoming reproductively isolated from their parental lineages could in the long-term lead to a process of incipient speciation by hybridisation (Nolte & Tautz, 2010; Pacheco-Sierra et al., 2018). Alternatively, a process of despeciation might occur if hybrid lineages eventually are able to genetically absorb the last refuges of non-admixed populations via admixture (Grant & Grant, 2014; MacLeod et al., 2015). The two parental populations of Northern Mexico exhibit low population numbers and low, homogenised levels of genetic diversity (average heterozygosity, H_o = 0.13) making them vulnerable to an extinction vortex (Pacheco-Sierra et al., 2018). Crocodiles in Calakmul exhibit higher levels of genetic diversity (H_o = 0.19), and field observations indicate the presence of a healthy population of approximately 12,000 individuals, with multiple active reproduction areas across the region (Barão-Nóbrega et al. unpublished data; Chapter 3). Although hybrid individuals are widely distributed in the Yucatan Peninsula, crocodiles disperse mainly through hydrological networks (coastline, mangroves, lagoons, cenotes, flood zones, rivers; Lee, 2000; Sigler & Gallegos, 2017), and as such the non-admixed nature of the populations in Calakmul is likely a direct result of ecological isolation through its general elevated topographic profile and poor overall hydrological connectivity to surrounding areas (García-Gil et al., 2002; Gunn et al., 2002). Unless there is a significant increase in human-mediated hybridization in the future resultant from translocations or dispersal facilitated by future development projects (e.g. Mayan train; Pskowski, 2019; Godoy, 2020; Palacios, 2020; Villa, 2020), the region of Calakmul is very likely to remain an isolated stronghold harbouring non-admixed *C. moreletii*. This is particularly the case for the regions within the Biosphere Reserve (Calakmul 4 and 6), which are the most geographically isolated (Figure 30; Gunn et al., 2002).

When aiming to identify admixed and non-admixed individuals in studies evaluating hybridization, samples from both parental species are usually required for adequate statistical power and to avoid biased estimations of hybrid indexes or admixture proportions (Anderson & Thompson, 2002; Gompert & Buerkle, 2010; Pacheco-Sierra et al., 2016). The present study lacks comparable sequence information from known parental populations of *C. moreletii*, but the high statistical power offered by the large number of molecular SNP markers (n ~ 5000) used in the analyses (Morin et al., 2009; Willing et al., 2012) nevertheless allowed for unambiguous conclusions. Although the analyses suggest crocodile populations in Calakmul to be largely composed of non-admixed individuals, they also reveal traces of introgressed *C. acutus* nuclear DNA ($0.05 \geq q_i \geq 0.01$) in 19 samples (24%), corroborated by two *C. acutus* mitochondrial DNA sequences (i.e. SNP loci) detected in seven individuals (Figures 32, 34, 35). Admixture proportions inferred at different levels of K also clearly confirm the admixed nature of the external reference samples of *C. moreletii* from Northern Mexico. Although these external *C. moreletii* samples exhibited substantial nuclear introgression (Figures 34, 35), their two mapped mitochondrial SNP loci matched the mitochondrial reference genome of *C. moreletii* (Figure 32). It is worthwhile mentioning however, that the identified mtDNA sequences stemmed from single reference individuals, and so their diagnostic value would require further confirmation.

One of the most striking patterns of introgression is represented by nuclear-mitochondrial discordance (observed difference between mitochondrial and nuclear markers, often in the form of substantial mitochondrial introgression in combination with no or little nuclear introgression; Toews & Brelsford, 2012), which in the past two decades, has been increasingly identified across a wide range of vertebrate and invertebrate species (see Bonnet et al., 2017, and references therein). Multiple mechanisms such as incomplete lineage sorting, genetic drift, spatial expansion, positive selection for introgressing mitochondrial variants and negative selection against introgression in the nuclear genome have been proposed and discussed to explain nuclear-mitochondrial discordance (see Toews & Brelsford, 2012; Bonnet et al., 2017 for detailed discussion). Many taxa displaying patterns of biogeographic nuclear-mitochondrial discordance represent groups that were isolated for long periods of time, and are either currently in secondary contact or have experienced contact at some point in their past (Toews & Brelsford, 2012), which is the case of *C. moreletii* populations in the Yucatan Peninsula (Ray et al., 2004; Cedeño-Vázquez et al., 2008; Rodriguez et al., 2008; Pacheco-Sierra et al., 2016). In Calakmul, introgression of *C. acutus* mitochondrial DNA was observed in only a small fraction of the overall population (mainly in Calakmul 2) and was likely generated by past contact with backcrossed individuals with the discordant nuclear-mitochondrial combination (Bonnet et al., 2017), as extensive mitochondrial DNA introgression at population-level would also require elevated levels of gene-flow at nuclear genes (Toews & Brelsford, 2012; Bonnet et al., 2017). Crocodylians have long generation times and are prone to exhibit low levels of mitochondrial DNA genetic structure (Glenn et al., 2002; Ray et al., 2004; Rodriguez et al., 2008; González-Trujillo et al., 2012), which can be partially explained by their low metabolic rates (Martin & Palumbi, 1993), low mutation rates during DNA replication (Bromham, 2002), and high longevity (Galtier et al., 2009). The increasing number of studies across several taxa reporting nuclear-mitochondrial discordance highlight its prevalence and importance in shaping genetic variation in natural populations (Wiens et al., 2010; Toews & Brelsford, 2012; Bonnet et al., 2017). As such, whenever possible, molecular studies employing RADseq, particularly the ones addressing hybridization, should attempt to map both nuclear and mitochondrial markers for a better understanding of the genetic nature of the studied taxa.

Recent data have suggested that hybridization between *C*. *moreletii* – *C*. *acutus* is an ancient process that initiated around 2-3 million years ago in the North of the Yucatan Peninsula (Pacheco-Sierra et al., 2018). As such, and taking into consideration the geohydrologic nature of the study region, the detection of SNP alleles diagnostic for *C*. *acutus* within Calakmul samples suggests a more likely result of historical introgression events rather than contemporary hybridization, even for the highest q_i estimates above the 0.05 threshold (~ 0.07) observed in three samples in Calakmul 1. Although these three samples were just above the more conservative threshold used in this study ($0.95 < q_i < 0.05$; following Rodriguez et al., 2008; Hekkala et al., 2015; Pacheco-Sierra et al., 2016), they still fall within the ancestry coefficient cut-off range often used in genetic assignments of wild populations ($0.90 < q_i < 0.10$; Burgarella et al., 2009; Chattopadhyay et al., 2016; Chattopadhyay et al., 2019).

One of the advantages of the use of thousands of SNPs as genomic markers, in relation to microsatellite markers for example, is its higher resolution for identifying admixture, particularly in systems where backcrossing has occurred for multiple generations (Pacheco-Sierra et al., 2018; McFarlane et al., 2020). The molecular approaches used in this study are based on approximately 5,000 SNPs shared by at least 80% of individuals and present in all eight sample origin groups (both external *C*. *moreletii* and *C*. *acutus* plus all six Calakmul regions), which enables a high degree of confidence to support the initial hypothesis that Calakmul is the only location within the Yucatan Peninsula to still hold a pure parental linage of *C*. *moreletii*. Although further exploration of this dataset can be pursued using other software packages also used to investigate genetic structure and hybridization (BAPS – Corander et al., 2008; INTROGRESS – Gompert & Buerkle, 2010; BGC – Gompert & Buerkle, 2012; HYBRIDDETECTIVE – Wringe et al., 2017), the results herein provide a solid basis to demonstrate the largely non-admixed nature (in relation to the *C*. *acutus* parental samples) of the surveyed locations in Calakmul. Considering sequence data from additional locations within the Peninsula and a higher number of samples from known parental populations of both species could shed further light into the matter. Attempts to screen the VCF file containing the dataset used by Pacheco-Sierra et al. (2018) in the hope of finding comparable called SNP variants proved unsuccessful (although both studies used *C*. *porosus* as reference genome to call SNPs, the methods for library preparation and SNP calling were different).

4.4.2 Population structure across Calakmul

A way to understand the evolutionary history of a given species is through its temporal and spatial distribution of intraspecific genetic variability (Beebee & Rowe, 2008), which provides important information regarding the evolutionary processes and space-time dynamics within and between populations (Smith et al., 2001; Slatkin, 2017). Population genetic studies have provided insights into gene flow, population structure and genetic diversity of crocodylian species (e.g. Muniz et al., 2018; Cao et al., 2020; Roberto et al., 2020; Rossi et al., 2020), including *C. moreletii* (e.g. Dever & Densmore III, 2001; Ray et al., 2004; Pacheco-Sierra, 2010; González-Trujillo et al., 2012; Versfeld, 2016). Demographic and genetic structure in water-dependent species, such as crocodiles, particularly in locations where restrictions to gene flow are influenced by topographic changes and seasonal isolation, are highly influenced by aquatic habitat availability and water-level fluctuations (Monsen & Blouin, 2003; Velo-Antón et al., 2014), thereby impacting upon genetic drift between isolated populations (Allendorf & Luikart, 2007; Versfeld, 2016). For the majority of the year, aquatic habitat in Calakmul largely consists of small to medium size semi-temporary ponds (< 1 ha; García-Gil, 2000; García-Gil et al., 2002; Barão-Nóbrega, 2019) that occur at relatively low densities across the region (on average less than one non-dry waterbody per 535 hectares, García-Gil, 2000; Delgado-Martínez & Mendoza, 2020). Spatial aggregations of crocodiles in these waterbodies, which generally only support one or two reproductively active pairs, coupled with field observations on overall demographic structure and philopatric behaviour (Barão-Nóbrega, unpublished data), already suggested that individuals inhabiting the same area represent a genetic unit (i.e. local population).

Topography and geographical distance appear to govern the genetic population structure of *C. moreletii* in the region of Calakmul, as different analytical approaches independently highlight a pattern of pronounced differentiation between geographically distinct locations. Marked genetic distances ($F_{st} > 0.05$; see also Dever et al., 2002; Ray et al., 2004; Versfeld, 2016) were observed in all pairwise comparisons amongst Calakmul regions (Table 5; Figure 33), with the exception of Calakmul 4 and Calakmul 5 ($F_{\mathrm{st}} \sim 0.02$). Differentiation was observed between the Northwest (regions 1 and 2), Northeast (regions 4) and Southcentral (regions 6) quadrants of Calakmul (Figures 37, 38, 41). The proximal causes of lack of the genetic differentiation from Calakmul regions 3 and 5, which were assigned into genetic clusters from Calakmul regions 2 and 4, respectively, are currently unknown (Figure 41). It might be in part due to the relatively low number of samples from these regions (three samples from each location), although such numbers can be adequate to resolve population structure when the number of SNP markers is large (> 1,000; Willing et al., 2012). Low connectivity, in great part due to the elevation profile (Figure 30), geographical distance (Table 5; Figure 33) and hydrological network of the landscape between sampled sites in Calakmul 3 and 5 and the genetic clusters they were assigned to suggest that if a more representative number of samples from additional waterbodies within those regions were available, regional clusters for those regions might likely emerge. Future studies addressing landscape genetics across Calakmul will be able to shed further light into the influence of topography on *C. moreletii* genetic structure. Significant differentiation between geographically distant and hydrologically isolated populations of the same species has also been reported in other crocodylian species (e.g. *Caiman crocodilus*, *Melanosuchus niger* – Farias et al., 2004; *Crocodylus johnstoni* – Cao et al., 2020; *C. acutus* – Rossi et al., 2020).

Calakmul is prone to highly dynamic changes to structural characteristics of waterbodies over time due to shifts in precipitation patterns and consequently in water availability across the region (Reyna-Hurtado et al., 2010; Barão-Nóbrega, 2019;

Márdero et al., 2019; Delgado-Martínez & Mendoza, 2020), which influence the spatial-temporal occurrence and abundance of the local aquatic fauna (Slater et al., 2020). Although *C. moreletii* movement over terrestrial habitat have been previously reported (e.g. Barão-Nóbrega et al., 2016b), and long-distance movement in response to seasonal changes to aquatic environment (e.g. droughts or floods) or in response to seasonal reproduction is common in crocodylian species (Lang, 1989), data from this study suggests a high degree of philopatry in Calakmul, possibly due to the combinatory effect of the rather uneven nature of the terrain and the absence of permanent hydrological corridors between aguada habitats across the region. The carrying capacity of waterbodies is also restricted due to their often-ephemeral nature and overall relative small dimensions (min – max perimeter = 40 – 3639 metres; Q1 = 98 m; Q3 = 207 m; with 98% of locations exhibiting a perimeter of less than 1000 metres; Chapter 3 – Figure 25). Crocodile populations inhabiting habitats with such dynamic features might be more prone to increased demographic and environmental vulnerability when compared to more stable environments throughout the Yucatan Peninsula, which in turn could negatively impact their genetic diversity and increase risk to their adaptive capacity due to stochastic changes (Saccheri et al., 1998; Madsen et al., 1999; Velo-Antón et al., 2014). On the other hand, *C. moreletii* seems to be well adapted to live in such dynamic environments as average heterozygosity of populations in Calakmul (H_o = 0.19) was similar to the reference C. *moreletii* samples (H_o = 0.18), and also to what has been reported based on SNP data, on average, for hybrid crocodile populations in other parts of the Peninsula (H_o = 0.185; Pacheco-Sierra et al., 2018). Isolated groups of the West African crocodile (*Crocodylus suchus*) inhabiting mountain rock pools (gueltas) with seasonal hydrographic connectivity in Mauritania have been reported to exhibit high genetic differentiation and viable genetic diversity even when local population sizes were as small as five individuals (Velo-Antón et al., 2014). Although no permanent aquatic dispersal corridors for *C. moreletii* exist in Calakmul, during the peak of the rainy season (October – December), temporary dispersal corridors between aguada sites within a region are occasionally formed by flooding in lowland forests (Reyna-Hurtado et al., 2010; Reyna-Hurtado et al., 2019). These periodical and short-timed events allow temporal seasonal connectivity to otherwise isolated aguada habitats, which likely resulted in the extended family network pattern within each region observed in the dataset.

Analysing spatial genetic diversity at a fine geographic scale amongst and within regions highlighted that *C. moreletii* were genetically more similar within than between Northwest, Northeast and Southcentral areas of Calakmul. This result strongly suggests that long-distance dispersal events are rare, and that these areas in Calakmul harbour demographically uncoupled populations. It should be noted, however, that crocodiles are long-lived vertebrates (Galtier et al., 2009; Grigg & Kirshner, 2015). Despite being largely undisturbed by human settlements and activities until the 1960s (Vester et al., 2007), verbal accounts of translocations by local communities and authorities within the last 40 years (González-Jauregui, personal communication; Barão-Nóbrega, unpublished information), could partly explain high ancestry coefficients for individuals across regions. Nonetheless, altogether the results of this study do not indicate that these individuals represent first generation migrants (Fukuda et al., 2019). Detectable genetic connectivity between populations, despite large distance separation and isolation, has been previously reported for *C. moreletii* and *C. acutus* in other parts of the Yucatan Peninsula (Dever et al., 2002; Ray et al., 2004).

4.4.3 Relatedness and mating system

An important contribution to the conservation of a species is knowledge of its genetic mating system, which determines the transmission of gene genealogies across generations and thus directly influences the genetic structure of populations at a fine scale (Rowe & Hutchings, 2003; Allendorf & Luikart, 2007; Oliveira et al., 2014). For example, in mating systems in which only one male, or a limited number of males are responsible for the majority of progeny, the effective population size of this population is expected to be smaller than that found in populations which have a mating system where several males contribute to the next generation (Trontti et al., 2006). However, direct observation and monitoring of reproductive interactions between individuals in their natural habitat is often difficult (Sullivan et al., 1995; Wink & Dyrcz, 1999; Uller & Olsson, 2008). Many interpretations of social structure and behavioural patterns rely on relatedness information (Emlen, 1995; Bourke, 2011). Therefore, molecular markers are routinely used as an indirect approach to document genealogies and provide insight into mating systems of vertebrate groups including fish (DeWoody & Avise, 2001), amphibians (e.g. Gopurenko et al., 2006), snakes (e.g. McCracken et al., 1999), turtles (e.g. Valenzuela, 2000), birds (e.g. Foerster et al., 2006) and crocodylians (e.g. Davis et al., 2001; McVay et al., 2008; Oliveira et al., 2014; Ojeda et al., 2016).

In recent years, SNP loci have increasingly been used for determining genetic relationships, largely due to the increased level of statistical power offered by these markers than a moderate number of microsatellite loci at assigning paternity and estimating relatedness among individuals (Thrasher et al., 2018; Dodds et al., 2019). For example, variance in estimates of pairwise relatedness (*r*) for known parent–offspring pairs is dramatically reduced when using SNP loci in comparison to microsatellites (Thrasher et al., 2018). This power of SNP loci might be particularly important for systems with low availability of demographic data to determine family relationships between individuals in conjunction with estimated *r*-values (Dodds et al., 2019). Although SNP markers are routinely used to study parentage and relatedness in fish (e.g. García-Fernández et al., 2018), reptiles (e.g. Çilingir et al., 2019) and birds (e.g. Thrasher et al., 2018), the present study to my knowledge is the first to utilize ddRAD-seq data for relatedness inferences in a wild crocodylian population.

In this study, 400 SNP loci contained sufficient information to accurately estimate relatedness and assign paternity amongst *C*. *moreletii* individuals. However, number of SNP loci required to perform robust parentage and relatedness analyses in other populations of *C*. *moreletii* or other crocodylian species will inherently be linked to specific characteristics of the studied population (Saunders et al., 2007; Tortereau et al., 2017; Thrasher et al., 2018). Parentage and relatedness analyses were performed using the pedigree reconstruction programs COLONY and RELATED, and several other programs are also readily available (e.g. see Weinman et al., 2015 for comparisons amongst programs). The *R* package SEQUOIA (Huisman, 2017), for example, although not used here it has been suggested to be specifically tailored to handle SNP data, and is able to reconstruct multigenerational pedigrees with as few as 100 SNPs which can be useful for studies with limited social information or incomplete population sampling (Thrasher et al., 2018).

Pairwise relatedness values of *C. moreletii* individuals in Calakmul demonstrated that spatial population structure is tightly linked to extended family networks. Such intertwined family structure often promotes inbreeding (Falconer & Mackay, 1996; Mauger et al., 2017), which in Calakmul might be counteracted by occasional long-distance migrants (e.g. crocodiles C24 and C46 in Calakmul 4; Figure 44) which likely represent juvenile and sub-adult individuals (Hutton, 1982; Messel & Vorlicek, 1982; Lang, 1989). Occasional migrants amongst different aguadas within and between regions (e.g. Calakmul 1 and 2; Figure 44) highlight the species' adaptative capabilities to an hydrological dynamic environment, which likely helps to maintain local populations by diminishing the negative effects associated with stochastic events and genetic drift (Velo-Antón et al., 2014). Dispersal is likely to take place during the peak of the rainy season when short-timed corridors between otherwise unconnected aguada habitats are formed due to flooding of surrounding lowland forest (Barão-Nóbrega, 2019; Slater et al., 2020). During these events, rather large males also attempt to find reproductively active females for mating, and are rarely found in small-to-medium sized aguadas inhabited by females and their offspring (Barão-Nóbrega, personal observation).

The reconstruction of parental and maternal genotypes assigned most individuals to unknown parents, which was expected due to the low number of samples from large reproductive adults in all regions. In Calakmul 6, one out of three candidate mothers (large females, samples C66, C67, C68) sired four out of 20 locally residing juvenile individuals (Figure 46). The expectation was that more individuals would be matched against one of the three large females, as 2/3 of these potential offspring samples originated from the two aguadas where the females were captured. It is however noteworthy that the majority of these juveniles are more distantly related to at least one of these three females, which also share ancestry among themselves (Figure 44). Furthermore, all three sampled aguadas in Calakmul 6 are located within a large section of continuous lowland floodable forest, encompassing 3,500 hectares and surrounded by hills on all sides. When this forest is flooded, movement of crocodiles between sites is enabled through water flow resulting from temporary whirlpools in sinkholes draining water underground (Barão-Nóbrega, personal observation; Gunn et al., 2002).

Mate fidelity has been identified in several crocodylian species, along with polyandric behaviour and multiple paternities (Grigg & Kirshner, 2015; Vashistha et al., 2020; and references therein). Parental assignment analysis of *C. moreletii* in Calakmul highlighted some degree of mate fidelity (e.g. Female 19 - Male 21 in Calakmul 6;

Figure 46), but also polyandry and multiple paternity as reproductive strategies (Figure 46). An interesting note regarding observed multiple paternities arises by cross referencing parental assignment results with field-observations and morphometric data of captured individuals. In most cases, multiple paternity within clutches was discarded due to either evidence against it or by not having enough data to support it. On the other hand, for example the three crocodile samples in Calakmul 5 (C36, C37 and C38) are likely part of the same clutch, as they originated from individuals of the same length and age (1-year old hatchlings) captured during the same night in close proximity to one another (less than 15 metres), but one of them was identified as Half-sibling of the other two (Figures 44, 45), which strongly suggests multiple paternity within clutches also occurs in Calakmul.

Polyandry has been already reported based on evidence from microsatellite markers in many crocodylian species (Davis et al., 2001; Hu & Wu, 2010; Amavet et al., 2012; Oliveira et al., 2014; Ojeda et al., 2016) including *C. moreletii* in Belize (McVay et al., 2008), and it is believed to increase the genetic variability of offspring (Yasui, 1998). Although differential reproductive success (i.e. high reproductive skew) associated with multiple paternity has been reported in polyandrous mating systems (Portnoy et al., 2007; e.g. Lance et al., 2009), it also often increases effective population sizes and contributes to the maintenance of genetic diversity in isolated populations (Chesser & Baker, 1996; Muniz et al., 2011; Rafajlović et al., 2013).

In crocodylians, polyandry is the result two main forms reproductive behaviour. The first consists of multiple copulations without storage of semen in the female reproductive tract between one reproductive period and the next; and the second form involves single or multiple copulations with sperm storage between reproductive periods (Gist et al., 2008). These two forms of polygamy have been confirmed in *Alligator mississipiensis* (Davis et al., 2001), but might not be applicable to all other crocodylian species. For *C. moreletii*, although copulations with multiple males without sperm storage is the most likely scenario in larger and more stable hydrological environments, the particular landscape and dynamic hydrological characteristics of Calakmul might further determine the local mating system. Female fidelity has been shown to play a significant role in multiple paternity analyses as cases of sperm storage have been reported, and even after mating with multiple males the offspring of a given female may only have a single sperm donor (Lance et al., 2009). Male fidelity however, could be a reflectance of small number of competing males and low mating encounters owing to habitat fragmentation and population structure (Grigg & Kirshner, 2015; Vashistha et al., 2020).

4.5 Final Considerations

Assessing genetic structure and gene flow among populations, as well as identifying landscape features that have an impact on gene flow, are key to identify spatial targets for conservation efforts. Revealing the genetic patterns of non-admixed *C. moreletii* populations helps to understand population processes in given environments, and contribute to the prioritization of conservation actions (Crooks & Sanjayan, 2006; McRae et al., 2012; Velo-Antón et al., 2014).

The present study for the first time describes the population structure and mating system of *C. moreletii* in the southcentral portion of the Mexican Yucatan Peninsula, information which can become embedded into local management programs and conservation plans. In summary, the study shows that: 1) unique in the Yucatan Peninsula, Calakmul is largely composed by non-admixed individuals; 2) different large regions within Calakmul are inhabited by demographically rather decoupled *C. moreletii* populations; 3) the mating system is polygamous, with polyandrous females and clutches exhibiting significant levels of multiple paternity.

Table 6. Details on capture location, size class, gender and assigned crocodile group of the 95 samples used for genotyping-by-sequencing (ddRAD-Seq). Location of reference samples of populations of both *Crocodylus moreletii* and *Crocodylus acutus* species were based on current literature (Pacheco-Sierra et al., 2016; Pacheco-Sierra et al., 2018). DLN – Dos Lagunas Norte; AO – Alvaro Obrégon; NB – Nuevo Becal; ZNS – Zona Nucleo Sur

Species	**Sample**	**Area**	**Location**	**Size class**	**Sex**	**Crocodile Group**
C. acutus	CA1	Cozumel	Faro Celarain	Adult	Male	Reference Acutus
	CA2	Cozumel	L. Xtacum	Adult	Female	Reference Acutus
	CA3	Banco Chinchorro	L. Chandez	Adult	Male	Reference Acutus
	CA4	Banco Chinchorro	L. Chandez	Adult	Male	Reference Acutus
	CA5	Yum-Balam	Yum-Balam	Adult	Male	Reference Acutus
C. moreletii	CMT1	Tamaulipas	L. Altamira	Adult	?	Reference Moreletii 1
	CMT2	Tamaulipas	L. Altamira	Adult	?	Reference Moreletii 1
	CMT3	Tamaulipas	L. Altamira	Adult	?	Reference Moreletii 1
	CMP4	San Luís Potosi	Cienega cabezas	Yearling	?	Reference Moreletii 2
	CMP5	San Luís Potosi	Cienega cabezas	Yearling	?	Reference Moreletii 2
	CMP6	San Luís Potosi	Cienega cabezas	Yearling	?	Reference Moreletii 2
	CMT7	Tabasco	L. Ilusiones	Adult	?	Reference Moreletii 3
	CMT8	Tabasco	L. Ilusiones	Adult	?	Reference Moreletii 3
	CMT9	Tabasco	L. Ilusiones	Adult	?	Reference Moreletii 3
	C9	Miguel Colorado	L. Mocú	Juvenile	?	Calakmul 1
	C10	Miguel Colorado	L. Mocú	Sub-adult	?	Calakmul 1
	C11	Miguel Colorado	L. Mocú	Sub-adult	?	Calakmul 1
	C12	Miguel Colorado	L. Mocú	Adult	?	Calakmul 1
	C13	Miguel Colorado	L. Mocú	Adult	?	Calakmul 1
	C18	Balam-Kú Reserve	Pueblo Viejo	Adult	Male	Calakmul 2
	C19	Balam-Kú Reserve	Pueblo Viejo	Adult	Male	Calakmul 2
	C20	Balam-Kú Reserve	Pueblo Viejo	Adult	Male	Calakmul 2
	C21	Balam-Kú Reserve	Pueblo Viejo	Yearling	?	Calakmul 2
	C22	Balam-Kú Reserve	Pueblo Viejo	Adult	Male	Calakmul 2
	C14	Balam-Kú Reserve	X'Toc	Adult	Male	Calakmul 2
	C15	Balam-Kú Reserve	X'Toc	Adult	Male	Calakmul 2
	C16	Balam-Kú Reserve	X'Toc	Adult	Male	Calakmul 2
	C17	Balam-Kú Reserve	X'Toc	Adult	Male	Calakmul 2
	C78	Balam-Kú Reserve	Silvituc	Hatchling	?	Calakmul 3
	C77	Balam-Kú Reserve	Maravilhas	Adult	Male	Calakmul 3
	C23	Calakmul Reserve	Zoh Laguna	Yearling	?	Calakmul 4
	C24	Calakmul Reserve	Zoh Laguna	Juvenile	Male	Calakmul 4
	C25	Calakmul Reserve	Zoh Laguna	Yearling	?	Calakmul 4
	C26	Calakmul Reserve	Zoh Laguna	Juvenile	Female	Calakmul 4
	C27	Calakmul Reserve	Chux (DLN)	Juvenile	Male	Calakmul 4
	C28	Calakmul Reserve	Chux (DLN)	Juvenile	Male	Calakmul 4
	C29	Calakmul Reserve	Chux (DLN)	Juvenile	Female	Calakmul 4
	C30	Calakmul Reserve	Chux (DLN)	Juvenile	Male	Calakmul 4
	C31	Calakmul Reserve	Braso fuerte (NB)	Yearling	?	Calakmul 4
	C32	Calakmul Reserve	Braso fuerte (NB)	Yearling	?	Calakmul 4
	C33	Calakmul Reserve	Braso fuerte (NB)	Yearling	?	Calakmul 4
	C40	Calakmul Reserve	Chumaquil (NB)	Adult	Male	Calakmul 4

C41	Calakmul Reserve	Chumaquil (NB)	Adult	Female	Calakmul 4
C42	Calakmul Reserve	Chumaquil (NB)	Juvenile	Female	Calakmul 4
C43	Calakmul Reserve	Chumaquil (NB)	Juvenile	Female	Calakmul 4
C44	Calakmul Reserve	Chumaquil (NB)	Juvenile	Female	Calakmul 4
C45	Calakmul Reserve	Chumaquil (NB)	Yearling	?	Calakmul 4
C46	Calakmul Reserve	Chumaquil (NB)	Sub-adult	Female	Calakmul 4
C47	Calakmul Reserve	Chumaquil (NB)	Juvenile	Female	Calakmul 4
C48	Calakmul Reserve	Chumaquil (NB)	Sub-adult	Male	Calakmul 4
C79	Calakmul Reserve	Papagayo (AO)	Sub-adult	Female	Calakmul 4
C80	Calakmul Reserve	Porto Rico (AO)	Juvenile	Male	Calakmul 4
C81	Calakmul Reserve	Porto Rico (AO)	Hatchling	?	Calakmul 4
C82	Calakmul Reserve	Porto Rico (AO)	Hatchling	?	Calakmul 4
C83	Calakmul Reserve	Porto Rico (AO)	Juvenile	Male	Calakmul 4
C84	Calakmul Reserve	Porto Rico (AO)	Juvenile	Male	Calakmul 4
C49	Calakmul Reserve	Valeriana (Bel-Ha)	Sub-adult	Female	Calakmul 4
C51	Calakmul Reserve	Valeriana (Bel-Ha)	Adult	Female	Calakmul 4
C52	Calakmul Reserve	Valeriana (Bel-Ha)	Juvenile	Male	Calakmul 4
C53	Calakmul Reserve	Valeriana (Bel-Ha)	Juvenile	Male	Calakmul 4
C54	Calakmul Reserve	Valeriana (Bel-Ha)	Juvenile	Male	Calakmul 4
C55	Calakmul Reserve	Valeriana (Bel-Ha)	Adult	Female	Calakmul 4
C56	Calakmul Reserve	Valeriana (Bel-Ha)	Sub-adult	Male	Calakmul 4
C57	Calakmul Reserve	Valeriana (Bel-Ha)	Juvenile	Male	Calakmul 4
C58	Calakmul Reserve	Valeriana (Bel-Ha)	Juvenile	?	Calakmul 4
C59	Calakmul Reserve	Valeriana (Bel-Ha)	Sub-adult	Male	Calakmul 4
C34	Calakmul Reserve	Flores Magón	Sub-adult	Female	Calakmul 4
C35	Calakmul Reserve	Flores Magón	Adult	Male	Calakmul 4
C36	Calakmul Reserve	Manuel Rejón	Yearling	?	Calakmul 5
C37	Calakmul Reserve	Manuel Rejón	Yearling	?	Calakmul 5
C38	Calakmul Reserve	Manuel Rejón	Yearling	?	Calakmul 5
C60	Calakmul Reserve	Km26 (ZNS)	Sub-adult	Male	Calakmul 6
C61	Calakmul Reserve	Km26 (ZNS)	Sub-adult	Female	Calakmul 6
C62	Calakmul Reserve	Km26 (ZNS)	Sub-adult	Female	Calakmul 6
C63	Calakmul Reserve	Km26 (ZNS)	Sub-adult	Female	Calakmul 6
C64	Calakmul Reserve	Km26 (ZNS)	Juvenile	Male	Calakmul 6
C65	Calakmul Reserve	Km26 (ZNS)	Yearling	Male	Calakmul 6
C66	Calakmul Reserve	Km26 (ZNS)	Adult	Female	Calakmul 6
C67	Calakmul Reserve	Km26 (ZNS)	Adult	Female	Calakmul 6
C68	Calakmul Reserve	Ramonal I (ZNS)	Adult	Female	Calakmul 6
C69	Calakmul Reserve	Ramonal I (ZNS)	Juvenile	Male	Calakmul 6
C71	Calakmul Reserve	Ramonal I (ZNS)	Juvenile	Male	Calakmul 6
C73	Calakmul Reserve	Ramonal I (ZNS)	Juvenile	Female	Calakmul 6
C74	Calakmul Reserve	Ramonal I (ZNS)	Yearling	Male	Calakmul 6
C75	Calakmul Reserve	Ramonal I (ZNS)	Juvenile	Female	Calakmul 6
C76	Calakmul Reserve	Ramonal I (ZNS)	Juvenile	Male	Calakmul 6
C86	Calakmul Reserve	Ramonal II (ZNS)	Juvenile	Male	Calakmul 6
C87	Calakmul Reserve	Ramonal II (ZNS)	Sub-adult	Female	Calakmul 6
C88	Calakmul Reserve	Ramonal II (ZNS)	Juvenile	Female	Calakmul 6
C89	Calakmul Reserve	Ramonal II (ZNS)	Juvenile	Male	Calakmul 6
C90	Calakmul Reserve	Ramonal II (ZNS)	Juvenile	Female	Calakmul 6
C91	Calakmul Reserve	Ramonal II (ZNS)	Hatchling	Female	Calakmul 6
C92	Calakmul Reserve	Ramonal II (ZNS)	Hatchling	Female	Calakmul 6
C93	Calakmul Reserve	Ramonal II (ZNS)	Juvenile	Male	Calakmul 6
C94	Calakmul Reserve	Ramonal II (ZNS)	Juvenile	Male	Calakmul 6

Chapter 5 – General discussion and concluding remarks

5.1 Key Findings

This PhD project had three major aims: 1) Update current knowledge on aquatic habitat in CBR, by providing descriptive information characterizing waterbodies in the region (aguadas) through details on their general structure and water quality; 2) Provide baseline data on *C. moreletii* populations in CBR by estimating local abundances in aguadas and total population sizes across the region; 3) Describe the population structure of *C. moreletii* in Calakmul (CBR and surrounding areas) by using a genotyping by sequencing (ddRAD-Seq) approach to characterize the species genetic profile across the region. In investigating these questions, the following key findings were made:

1) The shape and size of aguadas varied seasonally in response to fluctuations in annual precipitation, and prolonged drought periods which increasingly affect the region severely limit water availability for wildlife (Chapter 2).

2) Aguada contain generally nutrient-rich water, with most sites exceeding the maximum limits defined by ecological criteria for freshwater environments in Mexico (CE-CCA-001/89 1989). This suggests that, due to the specific characteristics and dynamics of the region, existing guidelines may not be suitable for assessing water quality in waterbodies such as the aguadas of Calakmul (Chapter 2).

3) A structured citizen science project for aguada monitoring in Calakmul, involving local communities and organized surveying campaigns twice a year was initiated as an effective method for large-scale aquatic habitat surveillance in the long-term (Chapter 2).

4) *N*-mixture models applied to spotlight count data have provided insightful estimates of crocodylian detection rates and local abundances in a dynamic environment, enabling insights into population responses to local environmental conditions by facilitating pooling of information of both small aguadas and large lakes and allowing comparisons between highly heterogeneous survey sites (Chapter 3).

5) Extrapolating abundance estimates across Calakmul yielded in an estimated ~12,000 *C. moreletii* present, highlighting the region is an important global stronghold for this species (Chapter 3).

6) Calakmul still harbours a demographically healthy population of non-admixed *Crocodylus moreletii*, unlike what has been reported in other parts of the Yucatan Peninsula where genetic pollution through increased hybridization with the American crocodile (*Crocodylus acutus*) is common (Chapter 4).

7) *Crocodylus moreletii* shows a distinct genetic structure across Calakmul, with the six main geographical regions where crocodiles were sampled being grouped into three different genetic clusters (Chapter 4).

8) The spatial population structure within each region is determined by, and reflected in extended family networks as revealed by parentage inferences (Chapter 4).

5.2 General Discussion

Calakmul Biosphere Reserve (CBR) in southern Mexico is an UNESCO World Heritage Site of Culture and Nature, due to a forest containing outstanding biodiversity and surrounding multiple ancient Maya ruins sites, including the city of Calakmul with a population of up to 150,000 people during the height of its power between 250BC – 900AD (Folan, 1988; Gunn et al., 2002; Faust & Folan, 2015). This area is characterised by tropical deciduous forest, which differs from regular tropical rainforests by lower amounts of yearly rainfall than what is typically expected within a tropical rainforest environment (Murphy & Lugo, 1986; Bullock & Solis-Magallanes, 1990). Although many species that occur in humid rainforests are present in Calakmul (e.g. Duellman & Klaas, 1964), their temporal abundance and distribution patterns within the reserve are directly influenced by fluctuations in water availability (Hernández-Ordóñez et al., 2015).

One important feature of the region of Calakmul is the absence of rivers and permanent water sources (García-Gil, 2000; García-Gil et al., 2002). The forest structure is heavily affected by distance from semi-temporary lakes, locally known as aguadas, which are formed in the wet season and act as a crucial resource for a wide variety of species (Hernández-Huerta et al., 2000; Martínez-Kú et al., 2008; Reyna-Hurtado et al., 2019; Sánchez-Pinzón et al., 2020). With reduced water availability compared to other types of forest, these aguadas act as a hub for fauna, offering drinking and bathing spots for large mammals such as tapirs (Reyna-Hurtado et al., 2016) and providing semi-permanent homes for fish (Valencia Díaz, 2001; Vega-Cendejas et al., 2013), amphibians and reptiles (Cedeño-Vázquez et al., 2006a; Colston et al., 2015). Aguadas in Calakmul appear to have been systematically drying up (Ericson et al., 2001; Morales & Pantí, 2015; Slater et al., 2020), which may have potential long-term ramifications for species that depend on these freshwater habitats (Zacharias & Zamparas, 2010; Reyna-Hurtado et al., 2019; Sánchez-Pinzón et al., 2020; Slater et al., 2020).

Chapter 2 provided evidence that the prolonged drought affecting the region during the 2016 – 2019 monitoring period (Márdero et al., 2019; Reyna-Hurtado et al., 2019) severely and detrimentally limited water availability in aguada habitats, which inherently impacted the surrounding forested habitat, human communities and species that rely on these water sources. Although drought severely reduced the amount of water held by aguadas in Calakmul, its impacts varied by geographical zone, with the Southern Core and Buffer zones experiencing similarly severe declines in water availability during the study period. Local populations of felid and ungulates species in these most affected areas, for example, migrated further south towards more humid environments (Slater et al., 2020). Informal interviews during the study period also showed that, in areas severely affected by drought, locals that lived from agriculture or beekeeping traded these activities for other type of employment to sustain their families (Barão-Nóbrega, unpublished information).

After several years of drought, the soil in many aguadas had completely dried up by 2019, losing the layer of putrefying litter that prevents water seepage. In these conditions, despite the precipitation events in the rainy season, the aguadas did not always fill due to water being absorbed by herbaceous and arbustive vegetation that colonized desiccated sites or infiltration through the fine soil and limestone karst substrate. A possible action plan to counteract this process involves human intervention by scything of the opportunistic vegetation, adding water to the dry soil to create mud and trample a significant amount of leaf-litter into it so for decomposition and to serve as building blocks for the re-impermeabilization of the sediment. This method of aguada rehabilitation was trialled by Operation Wallacea and CBR's reserve management immediately before the peak of rains in 2019 in sites that have been desiccated for more than 4 years, and has shown promising results: manipulated aguadas were able to accumulate rainfall for the first time in many years (Slater et al., 2020).

An emergent threat to wildlife and aquatic environments in the region of Calakmul is the Mayan Train megaproject (https://www.trenmaya.gob.mx/) currently being promoted by the Mexican government as an engine for social development and energise the regional economy across the Yucatan Peninsula (Pskowski, 2019). It has unleashed controversy between those who back the administration's propaganda and those who question the railway because of its potential environmental, social and cultural impacts, as well as the risk of fuelling illegal activities (Godoy, 2020). A major worry is the inherent negative environmental impacts on the environmental services provided by the affected ecosystems, particularly in areas critical to the recharge of groundwater aquifers in the Yucatan Peninsula: 1) the geo-hydrogeological network encompassing the ring of cenotes (sinkholes) around the Chicxulub crater in northwest Yucatan (https://whc.unesco.org/en/tentativelists/5784/); and 2) the forests of the Calakmul region, where harvested waters are transported and collected, eventually flowing into the main coastal systems of the Yucatan Peninsula and feeding six of its nine sub-basins (Palacios, 2020). Attempts to mitigate the trainline impacts in the region are currently being explored (i.e. identifying the less impactful route to both the environment and wildlife), and Operation Wallacea has been invited to take active part in the environmental impact assessment discussion, particularly related to the protection of aguada habitats in the region.

It is not yet clear how herpetofauna assemblages in Calakmul change in relation to size, hydroperiod or the type of vegetation within and surrounding aguadas (Slater et al., 2020). Moreover, little is known about the response mechanisms and local migration patterns employed by herpetofauna (particularly turtles and crocodiles) triggered by water availability. Prolonged droughts limit water availability in aguadas across the CBR, and so it is important to understand how these habitat changes affect species such as *C. moreletii,* that depend on them. This was the main incentive for the quantification of the environment-dependent abundances and habitat preferences of *C. moreletii* in CBR, as accurate estimates of relative abundance and total population sizes provide baseline information for population ecological investigations (Sutherland, 2006; Bötsch et al., 2019; Costa et al., 2020).

In **Chapter 3**, spotlight count data were analysed to estimate local abundances and total population size of *C. moreletii* in the region of Calakmul through methodologies which have been historically used for studies involving crocodylian species (minimum population size and visible fraction), in comparison to a binomial *N*-mixture approach. The findings highlight that *N*-mixture modelling can provide reliable estimates of *C. moreletii* abundance and population size from spotlight count data. This study constitutes the first population size estimates for the south-central region of the Yucatan Peninsula, and reveals a healthy population composed of multiple active reproduction areas across the region. The *N*-mixture models applied to *C*. *moreletii* spotlight count data resulted in particularly insightful estimates of crocodile detection and abundance in Calakmul, by allowing comparisons between highly heterogeneous survey sites and facilitating information of both small aguadas and large lakes to be pooled together. *N*-mixture models may then provide an overall more comprehensive framework for the monitoring and management of crocodylian populations in general, as abundance and detection probability can be accurately estimated also in situations involving a high degree of environmental stochasticity. *N*-mixture approaches can provide a rich ground for investigations aiming to analyse information relevant to future conservation management plans and should be more commonly integrated in survey methodologies and monitoring schemes of not only crocodylians, but also other groups due to the versatility of these models to analyse count data and provide insight to population responses to environmental conditions (e.g. see Ward et al., 2017; Duarte et al., 2018; Kidwai et al., 2019; Costa et al., 2020). As for *C. moreletii*, the national monitoring program in Mexico (Sánchez-Herrera et al., 2011) should consider integrating a *N*-mixture modelling framework to analyse their survey data to better understand *C. moreletii* population dynamics at both local and national scales. Furthermore, this monitoring program would also be a suitable testing ground for the creation of a potential user-friendly platform for *N*-mixture modelling analyses of abundance (similar to what has been done for example for distance sampling; http://distancesampling.org/), allowing a wider range of local managers to analyse their data in a easy to follow standardized system. One of the current deterrents for the use of *N*-mixture approaches is the amount of time and knowledge needed to carry out the analyses when using packages in *R* (Fiske & Chandler, 2011; *R* Development Core Team, 2019).

Chapter 4 demonstrated the power of these RADseq technologies to discern genetic structure amongst wild crocodile populations across Calakmul, and to investigate the presence of *Crocodylus moreletii – Crocodylus acutus* hybrids. The knowledge that can be acquired from the genome of a study species depends on the type of molecular marker used (Amos & Balmford, 2001; Vega et al., 2017; Vashistha et al., 2020). Microsatellite markers were firstly employed in 1994 in non-model organisms to discern patterns of genetic variation, not previously possible using mitochondrial DNA or other methods of DNA fingerprinting, in a study involving a remnant population of the northern-hairy nosed wombat *Lasiorhinus krefftii* (Taylor et al., 1994). Since then, microsatellites have revolutionised population genetics, providing insights into the structure and demographic history of populations and species, the relationships and origins of individuals, dispersal patterns of offspring, and the impact of landscape features on migration and population structure (Selkoe & Toonen, 2006; Sarre & Georges, 2009). Over the past decade, a revolution of similar magnitude is underway (Andrews et al., 2016), as novel technologies such as RADseq offer the potential to genotype individuals at thousands of loci in both coding and non-coding regions (Brookes, 1999; Davey & Blaxter, 2011). The power of RADseq approaches is also increasingly applied to generate SNP datasets to investigate questions relevant for crocodylian conservation programs, such as the identification of evolutionary lineages, hybridization between species, population structure and genetic diversity (Muniz et al., 2018; Nguyen et al., 2018; Pacheco-Sierra et al., 2018; Cao et al., 2020), and over the coming years, studies using these techniques are sure to multiply.

The results from Chapter 4 show that the region of Calakmul, unlike what has been reported in other parts of the Yucatan Peninsula (Pacheco-Sierra et al., 2016; Pacheco-Sierra et al., 2018), still holds a healthy population of non-admixed *C. moreletii* individuals. This finding is of high conservation significance as the only other two non-admixed *C. moreletii* populations inhabit isolated locations at the very opposite ends of Mexico, exhibit low population numbers and low, homogenised levels of genetic diversity, making them vulnerable to an extinction vortex (Pacheco-Sierra et al., 2018). Although *C. moreletii* is a species protected by the Mexican law as conservation dependant (NOM-059-ECOL-2001) and internationally listed by IUCN (Least Concern) and CITES (Appendix II), conservation guidelines and protection for the *C. moreletii – C. acutus* complex are in urgent need of review, as the vast majority of individuals and populations routinely identified as *C. moreletii* are in fact admixed (Pacheco-Sierra et al., 2016). Naturally occurring *C. moreletii* – *C. acutus* hybrid populations need to be considered for their conservation value (Jackiw et al., 2015; Pacheco-Sierra et al., 2018), but, more importantly, the three non-admixed *C. moreletii* populations identified require different categorization than their hybrid counterparts due to their more endangered nature (Pacheco-Sierra et al., 2016).

Conservation recommendations and actions based on nominal species alone (e.g. *C. moreletii*) can lead to a false sense of protection, while in reality certain lineages of that species can be threatened and may be in a need to lineage-specific conservation measures (Muniz et al., 2018). It is therefore important to identify intraspecific units below the species level for conservation purposes so that the often-limited resources can be utilized optimally (Ryder, 1986). Evolutionary Significant Units (ESUs) and Management Units (MUs) have long been recognized as appropriate units of conservation (e.g. see Ryder, 1986; Moritz et al., 1995; de Guia & Saitoh, 2006; Casacci et al., 2013; Zhao et al., 2020). ESUs often represent lineages adapted to local environmental conditions that once extinct may not be replaced by emigrants (Funk et al., 2012), and are the logical units for species monitoring since preserving the genetic diversity of each unit will also conserve genetic diversity and evolutionary potential of the species as a whole (Moritz et al., 1995). The three non-admixed lineages of *C. moreletii* are natural candidates for differentially optimized management strategies and should be recognized as ESUs as they are highly vulnerable to becoming genetically absorbed by the surrounding hybrid lineages (see Pacheco-Sierra et al., 2016; Pacheco-Sierra et al., 2018). This is particularly relevant if there is significant increase in human-mediated hybridization resultant from translocations or facilitated dispersal. Ignoring independent evolutionary lineages could lead to incorrect inferences about a species' resilience and adaptive potential in a changing environment by implicitly or explicitly assuming the functional and evolutionary equivalence of these lineages (Frankham et al., 2004; Muniz et al., 2018).

Demographic and genetic structure in aquatic species, such as crocodiles, particularly in locations where restrictions to gene flow are influenced by topographic changes and seasonal isolation, are highly influenced by aquatic habitat availability and water-level fluctuations (Monsen & Blouin, 2003; Velo-Antón et al., 2014). In Calakmul, topography and geographical distance appear to govern the genetic population structure of *C. moreletii* in the region of Calakmul, as different analytical approaches independently highlight a pattern of pronounced differentiation between geographically distinct locations. At a local scale, spatial aggregations of crocodiles in aguada habitats, which generally only support one or two reproductively active pairs, coupled with field observations on overall demographic structure and philopatric behaviour indicate that individuals inhabiting the same area represent a genetic unit (i.e. local population). Although no permanent aquatic dispersal corridors for *C. moreletii* exist in Calakmul, during the peak of the rainy season (October – December), temporary dispersal corridors between aguada sites within a region are occasionally formed by flooding in lowland forests (Reyna-Hurtado et al., 2010; Reyna-Hurtado et al., 2019). These periodical and short-timed events allow temporal seasonal connectivity to otherwise isolated aguada habitats, which likely resulted in the extended family network pattern within each region observed in the relatedness analyses. Future studies should attempt to combine information on crocodile movements and relatedness using techniques such as radio-telemetry. Attempts to carry out such an experiment were conducted during this PhD project using TGB transmitters (VHF & GPS + Wireless communication), but unfortunately proved unsuccessful as units for data transfer could not become relocated. If ever recovered, the units will still provide insight into crocodile dispersion patterns in the region, GPS movement data are still stored after battery depletion. Although unsuccessful, this telemetry experiment laid the stepping stones for the development of a project aimed at upgrading drones with low-cost autonomous devices to track multiple signals from VHF transmitters at the same time, which will significantly boost signal tracking capabilities to ground teams in densely forested environments such as the CBR (Desrochers et al., 2018; Muller et al., 2019).

The environmental and ecological features shaping the hydrological dynamics of aquatic habitats and the population structure of the Morelet's crocodile across Calakmul are more complex than what has been explored in this study, and there are still many questions to be answered: 1) With cyclical droughts in the CBR and following the recent "2020 reset" due abnormally high rainfall, should a similar pattern of spatial and temporal desiccation across the region be expected in the next five years? 2) Does crocodile adaptation to desiccation in aguadas vary across locations, sex, and size classes? Also, are crocodiles more likely to migrate over land in the forest to reach a location that still has water or to aestivate in crocodile dens within the aguada or in the surrounding forest?; 3) Does population structure in aguada habitats in the adjacent El-Mirador National Park (Guatemala) mirror what has been registered in Calakmul? If so, does it also harbour non-admixed populations of *C*. *moreletii*? 4) Should crocodiles inhabiting the eco-region encompassing Calakmul – El-Mirador be considered as one or multiple ESUs?

The results herein represent valuable baseline information of the habitat characteristics available to aquatic fauna such as crocodiles, and can serve as a baseline and also provide the foundation for more comprehensive future studies involving aquatic environment not only in CBR, but also across the entire Selva Maya. Taken together, the findings of this PhD project constitute valuable information for management actions towards the conservation of *C. moreletii* populations in aguada habitats, where it generally serves as an umbrella species for the biodiversity that occurs in them. In Calakmul Biosphere Reserve, the information produced during this PhD project has already become embedded into management programs and conservation plans by local environmental authorities and supported the reserve's application for aguadas to become wetlands of international importance under the Ramsar Convention (https://www.ramsar.org/), which further highlights the importance of aquatic habitats in Calakmul.

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7. Appendix I – Ethics Approval Letter (University of Salford)

**Research, Innovation and Academic
Engagement Ethical Approval Panel**

Research Centres Support Team
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30 March 2017

Jose Nobrega

Dear Jose,

RE: ETHICS APPLICATION ST1617-56 - Population structure, genetics and ecology of Morelet's crocodile (Crocodylus moreletii) in Calakmul Biosphere Reserve, Campeche, México

Based on the information you provided, I am pleased to inform you that your application ST1617-56 has been approved.

If there are any changes to the project and/ or its methodology, please inform the Panel as soon as possible by contacting S&T-ResearchEthics@salford.ac.uk

Yours sincerely,

Anthony Higham

8. Appendix II – Aguadas of Calakmul Database

Overview

Location and general structure information on 101 waterbodies (aguadas) in the region of Calakmul (Calakmul Biosphere Reserve and Balam-Ku State Reserve) in Campeche, Mexico. This work is part of the PhD project of José António L. Barão-Nóbrega at University of Salford in collaboration with Operation Wallacea. The Aguadas of Calakmul Database was launched in the form of a website, using the Google sites platform, and made available to the general public. On this website, the user can find general information on how the database works, instructions on how to visualise the spatial distribution and summary details of monitored aguadas in Google Earth (Map Aguadas Calakmul Google Earth; Figure 47) and access the Aguadas of Calakmul Attribute table which contains general structure data (e.g. water levels, dominant vegetation, etc..) collected during my visits to surveyed locations between 2017 and 2019. Furthermore, the user can also find additional links for geographical information on other waterbodies within the region (Garcia 2000), general information on freshwater plant communities (Moreno-Casasola et al. 2012), soil and vegetation characteristics (Monsivais-Huertero et al. 2016) and other aspects related to aguadas (UNESCO's Man and the Biosphere Programme status report) in Calakmul Biosphere Reserve.

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Author's notes

This open access database serves as a working tool for anyone who might want to develop a project related with waterbodies in the region of Calakmul. It aims to provide a solid starting point on location and general structure of what we consider the most relevant / accessible aguadas in the region, which was something I did not have when I started my PhD project. Furthermore, it also aims to serve as the baseline database structure for a citizen science waterbody monitoring project in the region.

Aguada Km26 - Pond

Permanent waterbody. Dominant vegetation - swamp. Vegetation Cover - Low. Hydric State: July 2017 → Stable; March 2018 → Full; July 2018 → Stable. For additional information on this location please see the attribute table spreadsheet.

Additional photos

José António Lemos Barão-Nóbrega ©2019 Operation Wallacea & University of Salford

Figure 47. Aguada data visualization tool available online (Barão-Nóbrega, 2019) using Google Earth (Gorelick et al., 2017). Each blue waypoint represents an aguada. Further information by following this link: https://sites.google.com/view/baraonobrega-aguadas-calakmul/home.

9. Appendix III – Herpetological diversity of Calakmul (Campeche, Mexico): an updated species list with new distribution notes

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Abstract

The herpetofauna occurring in the Mayan jungle is one of the richest assemblages in the Americas, primarily because of the considerable variation in habitat within these forests. We herein provide a list of amphibian and reptile species for the region of Calakmul, located in the southern part of the Yucatan Peninsula (Campeche, Mexico). This species list was compiled considering the results of herpetological surveys we conducted between 2015-2019 in Calakmul Biosphere Reserve, species information gathered from previous literature and sighting records reported on the website iNaturalist. All this information pooled together resulted in the identification of 109 herpetofauna species (23 amphibians and 86 reptiles) in the region of Calakmul, belonging to 34 different families. Furthermore, we hereby present new distribution records for seven snake, two lizard and two frog species not previously observed in this region. Approximately 38% (n = 41) of the listed herpetofauna species reported to occur in Calakmul are listed under a threat category by the environmental Mexican legislation. The list presented in this work increases to 109 the number of herpetofauna species known to occur in the region of Calakmul, and to 135 the number of amphibians and reptiles in the state of Campeche, which therefore becomes the Mexican state of the Yucatan Peninsula with the highest herpetofauna diversity, followed by Quintana Roo with 133 species.

Key words: Amphibians; Calakmul; Herpetofauna; Mexico; Reptiles; Species List; Yucatan Peninsula

Introduction

Mexico is one of the world's most biologically rich countries (García-Frapolli et al. 2009; Mittermeier and Goettsch-Mittermeier 1997), largely due to the high biodiversity found in the southern regions of the Yucatan Peninsula, home to the largest expanse of mature seasonal tropical forests remaining in Mesoamerica (Carr III and Stoll 1999; Vester et al. 2007). Calakmul Biosphere Reserve (CBR) is located in southern Mexico and together with state reserves Balam-Ku and Balam-Kin encompass more than 1.2 million hectares of protected forest, respectively under jurisdiction and management of federal and Campeche state authorities. CBR is an UNESCO World Heritage Site of Culture and Nature due to the multiple ancient Mayan ruin sites, including the major city of Calakmul and the remnants of the ancient Mayan agroforestry that has given place to a forest of outstanding biodiversity (UNESCO 2016). This reserve is composed of tropical semi-deciduous forest. In most of the reserve, canopy height ranges between 15 to 40 meters and 20% of trees lose their leaves during the dry season. Canopy in the northern parts of the reserve ranges from 8 to 20 meters in height and 40% of trees are deciduous (Chowdhury 2006). As with other areas of the Selva Maya (Ross and Rangel 2011), forest adjacent to ruin sites in Calakmul has notably larger trees, a different tree species composition and higher fruit production than other forested areas (Slater 2019). Surface water in CBR occurs only in some areas where the terrain allows the accumulation of water, which create semi-temporary natural ponds, locally known as aguadas (Barão-Nóbrega 2019). These aguadas are sustained by retaining water provided by the annual precipitation gradient and constitute the only source of water to both the fauna and the human communities of the region (Reyna-Hurtado et al. 2010). These waterbodies are of particular importance to herpetofauna species that require aquatic habitat such as frogs, freshwater turtles and crocodiles (Cedeño-Vázquez et al. 2006; Colston et al. 2015)

Operation Wallacea is an UK-based non-governmental organization specialized in biodiversity assessments and monitoring of protected areas. Utilizing the expertise of university academics and students, this organization has been performing annual biodiversity surveys in CBR since 2012. In 2015, an indexed species list of amphibians and reptiles of CBR was compiled and published, reporting the presence of 20 amphibian and 69 reptile species from 24 total families (Colston et al. 2015). Since then, taxonomic changes affected the nomenclature of many species, with some even subject to taxonomic division (e.g *Pseudelaphe flavirufa* Cope 1867 and *Holcosus undulatus* Wiegmann 1834; González-Sánchez et al. 2017; Meza-Lázaro and Nieto-Montes de Oca 2015). Operation Wallacea has continued performing annual biodiversity surveys in CBR and found nine new herpetofauna records not previously reported for the area, with some even representing new records for the state of Campeche and the Mexican Yucatan Peninsula. We herein update and summarize current knowledge on the species of amphibians and reptiles in the region of Calakmul (CBR and surrounding areas) and discuss their conservation implications for the region. We also discuss other herpetofauna species that we believe may also occur in the region but have not been detected yet.

Figure 48. Location of Calakmul Biosphere Reserve within the southern Yucatan Peninsula in Mexico. Operation Wallacea's annual survey sites are numerated from one to six. All other localities represent opportunistic sightings or survey locations mentioned in previous literature (Calderón-Mandujano et al*.* 2003; Calderón-Mandujano et al*.* 2010; Colston et al*.* 2015). Locations 7, 16 and 17 are located within Balam-Ku State Reserve.

Material and Methods

Calakmul Biosphere Reserve (18.60583 N, 89.94444 W; WGS84; Figure 48) is an expanse of tropical forest that covers an area of 723,000 hectares, and is part of the Selva Maya that encompasses Mexico, Guatemala and Belize, spanning over 10.6 million hectares and making it the largest continuous section of tropical forest in Mesoamerica (Vester et al. 2007). The southern Yucatan is characterized by a warm, sub-humid climate with a mean annual temperature of 24.6 ºC. A precipitation ecocline goes from the northwest (ca. 900mm) to the southeast (ca. 1400 mm) of the reserve (Vester et al. 2007), over the 120 km from the north of the reserve to the Guatemalan border (Lawrence and Foster 2002), significantly influencing forest structure and tree species composition (Chowdhury 2006; Martínez and Galindo-Leal 2002).

Herpetofauna surveys in CBR were carried out each year between June and August from 2015 to 2019 through transects, timed searches around waterbodies and opportunistic funnel trapping in six different sampling localities (Dos Naciones, KM20, KM27, KM40, Mancolona and Hormiguero; Figure 48), as part of Operation Wallacea's annual biodiversity monitoring project in the region. For further detailed information on surveyed locations and methods, please see Colston et al. (2015). All data were collected by teams of students led by university academics and local indigenous experts. Due to permit restrictions within CBR, no specimens were collected but digital photographs for all new records were taken and represent digital vouchers (Colston et al. 2015), curated by Operation Wallacea (José António L. Barão-Nóbrega and Pedro E. Nahuat-Cervera) and University of Texas (Eric Smith and Gregory Pandelis). Fieldwork was performed in compliance with the protocols described by Beaupre et al. (2004). Ethics approval was yearly granted to Operation Wallacea's long-term monitoring project, in collaboration with Pronatura Peninsula Yucatan, by Mexico's Secretariat of Environment and Natural Resources (SEMARNAT; SGPA/DGVS/005403/18).

We compiled an updated species list for the region of Calakmul by combining our herpetological survey results between 2015-2019 in CBR with *ad libitum* herpetofauna sightings from JALBN's field-work activities in the region, opportunistic amphibian and reptile data records from the iNaturalist community (research grade records only; iNaturalist 2019) and herpetofauna species information from literature review on previous studies conducted within and around the reserve (Calderón-Mandujano et al. 2003; Calderón-Mandujano et al. 2008; Calderón-Mandujano et al. 2010; Colston et al. 2015; Neri-Castro et al. 2017). We decided to base our taxonomic classification on the works of González-Sánchez et al. (2017), Lee (2000), Meza-Lázaro and Nieto-Montes de Oca (2015), Wilson et al. (2013a,b) and updated the scientific names of species until September 2019, based on Frost (2019) for amphibians and Uetz and Hošek (2019) for reptiles.

Results

Operation Wallacea's herpetofauna survey records, pooled together with data from pre-existing literature and opportunistic sightings, indicate the occurrence of 109 species of amphibians and reptiles within the region of Calakmul (Table 7). From this total, 23 are amphibian species belonging to nine different families, while the remaining 86 species are reptiles distributed amongst 25 families. For amphibians, the richest family in terms of species is Hylidae (35% of the total amphibian species richness detected), while for reptiles the richest families are Colubridae (23%), Dipsadidae (20%) and Dactyloidae (8%). Approximately 38% (n = 41) of the herpetofauna species reported to occur in Calakmul (Table 7) are listed under a threat category by the environmental Mexican legislation (NOM-059-SEMARNAT-2010). From these 41 species, 71% (n = 29) are indicated as in need of Special Protection (*Protección especial*), 24% (n = 10) as Threatened (*Amenazada*), and 5% (n = 2; *Cachryx defensor* Cope 1866 and *Claudius angustatus* Cope 1865) as in Danger of Extinction (*Peligro de Extinción*).

During this study, we encountered a total of eleven herpetofauna species (nine in CBR and 2 in the surrounding areas) that had never been previously reported for the region. The frog *Engystomops pustulosus* (Cope 1864), the lizards *Ctenosaura similis* (Gray 1831) and *Holcosus gaigeae* (Smith and Laufe 1946) and snakes *Coniophanes bipunctatus* (Günther 1858), *Pseudelaphe phaescens* (Dowling 1952) and *Stenorrhina freminvillei* (Duméril, Bibron and Duméril 1854) represent new records for the region of Calakmul (Figures 49A, 49C, 49D, 50A, 50B, 50C). The snakes *Tantilla cuniculator* (Smith 1939), *Tantilla schistosa* (Bocourt 1883) and *Scaphiodontophis annulatus* (Duméril, Bibron and Duméril 1854) represent new records for the state of Campeche (Figures 51A, 51B, 51C). The frog *Eleutherodactylus leprus* (Cope 1879) and the snake *Sibon dimidiatus* (Günther 1872) represent new records for the Mexican Yucatan Peninsula (Figures 49B, 51D). All new records mentioned above correspond to adult individuals. Number of observed individuals goes as following: one individual for *E*. *pustulosus*, *C. similis*, *C. bipunctatus*, *S. dimidiatus, S. freminvillei* and *T*. *cuniculator*; two individuals for *S. annulatus, T*. *schistosa* and *P. phaescens;* more than five individuals for *E*. *leprus* and *H. gaigeae*.

Figure 49. New records encountered in Calakmul: (A) *Engystomops pustulosus* (UTADC 9453), (B) *Eleutherodactylus leprus* (UTADC 9452), (C) *Ctenosaura similis* (UTADC 9451) and (D) *Holcosus gaigeae* (UTADC 9454). Photos by PENC.

Figure 50. New records encountered in Calakmul: (A) *Coniophanes bipunctatus* (UTADC 9450), (B) *Pseudelaphe phaescens* (UTADC 9455), (C) *Tantilla cuniculator* (UTADC 9459). Photos (A) and (B) by PENC and (C) by A Ross.

Figure 51. New records encountered in Calakmul: (A) *Stenorrhina freminvillei* (UTADC 9458), (B) *Tantilla schistosa* (UTADC 9460), (C) *Scaphiodontophis annulatus* (UTADC 9456), (D) *Sibon dimidiatus* (UTADC 9457). Photos (A) by PENC, (B) by AT and (C) and (D) by JO.

Table 7. List of amphibians and reptiles encountered in the region of Calakmul. Numbers indicate source of species records (1 – Colston et al. 2015; 2 – This study; 3 – Neri-Castro et al. 2017; 4 – Calderón et al. 2003; 5 – Calderón-Mandujano et al. 2008; 6 – Calderón-Mandujano et al. 2010; 7 – iNaturalist.org 2019). AO – Álvaro Obregón; BKC – Balam-Ku Conejo; CR – Calakmul Ruins; DN – Dos Naciones; HM – Hormiguero; MC – Mancolona; NDZ - Nadzca'an; NM – Narciso Mendoza; NB – Nuevo Becal; PA – Plan de Ayala. * represents new records for the region of Calakmul, ** represents new records for the state of Campeche, *** represents new records for the Mexican Yucatan Peninsula. NNTV indicates species not native to the Yucatan Peninsula. Information inside brackets in localities column represents either the digital voucher number or iNaturalist registry ID.

Family	Species	Localities
Bufonidae	*Incilius valliceps* (Wiegmann 1833)	Km20[1,2], Km40[1,2], DN[1,2], HM[1,2], MC[1,2], NDZ[1], BKC[2]
Bufonidae	*Rhinella horribilis* (Wiegmann 1833)	Km20[1,2], Km40[1,2], DN[1,2], HM[1,2], MC[1,2], NDZ[1], NB[2]
Eleutherodactylidae	*Eleutherodactylus leprus* (Cope 1879) ***	DN[2] (UTADC 9452)
Hylidae	*Dendropsophus ebraccatus* (Cope 1874)	Km20[1,2], DN[1,2], NM[4], PA[4]
Hylidae	*Dendropsophus microcephalus* (Cope 1886)	Km20[1,2], DN[1,2], HM[1,2], MC[1,2]
Hylidae	*Scinax staufferi* (Cope 1865)	Km20[1,2], DN[1,2], HM[1,2], MC[1,2]
Hylidae	*Smilisca baudinii* (Duméril & Bibron 1841)	Km20[1,2], DN[1,2], HM[1,2], MC[1,2], NDZ[1], BKC[2]
Hylidae	*Tlalocohyla loquax* (Gaige & Stuart 1934)	Km20[1,2], Km40[1,2], DN[1,2], HM[1,2], MC[1,2]
Hylidae	*Tlalocohyla picta* (Günther 1901)	Km20[1]
Hylidae	*Trachycephalus typhonius* (Linnaeus 1758)	Km20[1,2], HM[1,2], MC[1,2], Bel-Ha[2]
Hylidae	*Triprion petasatus* (Cope 1865)	Km20[1,2], DN[1,2], HM[1,2], MC[1,2]
Leptodactylidae	*Engystomops pustulosus* (Cope 1864) *	BKC[2] (UTADC 9453)
Leptodactylidae	*Leptodactylus fragilis* (Brocchi 1877)	Km20[1,2], Km40[1,2], MC[1,2], BKC[2]
Leptodactylidae	*Leptodactylus melanonotus* (Hallowell 1861)	Km20[1,2], DN[1,2], MC[2], BKC[2]
Microhylidae	*Gastrophryne elegans* (Boulenger 1882)	Km20[1,2], DN[1,2], HM[2], MC[2]
Microhylidae	*Hypopachus variolosus* (Cope 1866)	Km20[1,2], Km40[1,2], DN[1,2], HM[1,2], MC[1,2], NDZ[1], BKC[2]

Phyllomedusidae	*Agalychnis callidryas* (Cope 1862)	Km20[1,2], DN[1,2], HM[1,2], MC[1,2]
Ranidae	*Lithobates brownorum* (Sanders 1973)	Km20[1,2], Km27[1,2], Km40[1,2], DN[1,2], HM[1,2], MC[1,2], BKC[2]
Ranidae	*Lithobates vaillanti* (Brocchi 1877)	Km20[1,2], HM[1,2], MC[2]
Rhinophrynidae	*Rhinophrynus dorsalis* (Duméril & Bibron 1841)	Km20[1,2], DN[1,2], HM[1,2], BKC[2], Bel-Ha[2]
Plethodontidae	*Bolitoglossa mexicana* (Duméril, Bibron & Duméril 1854)	Km20[1], DN[1,2]
Plethodontidae	*Bolitoglossa rufescens* (Cope 1869)	Km20[1]
Plethodontidae	*Bolitoglossa yucatana* (Peters 1882)	Km20[1], DN[1], AO[4]

Family	**Species**	**Locality**
Crocodylidae	*Crocodylus moreletii* (Duméril & Bibron 1851)	Km27[1,2], HM[1,2], MC[1,2], BKC[2], CR[2], PA[2], AO[2], NB[2], NM[2]
Corytophanidae	*Basiliscus vittatus* (Wiegmann 1828)	Km20[1,2], Km27[1,2], Km40[1,2], DN[1,2], HM[1,2], MC[1,2], NB[5], BKC[2]
Corytophanidae	*Corytophanes cristatus* (Merrem 1820)	Km20[1,2], Km27[1,2], Km40[1,2], DN[1,2], HM[1,2], MC[1,2]
Corytophanidae	*Corytophanes hernandesii* (Wiegmann 1831)	Km40[2], DN[1,2], HM[1,2]
Corytophanidae	*Laemanctus serratus* (Cope 1864)	Km20[1,2], Km48[5], HM[1,2], NDZ[1]
Corytophanidae	*Laemanctus longipes* (Wiegmann 1834)	CR[6]
Dactyloidae	*Norops beckeri* (Boulenger 1881)	Km20[1,2], AO[4], CR[4], Bel-Ha[4]
Dactyloidae	*Norops biporcatus* (Wiegmann 1834)	DN[1,2], HM[1,2]
Dactyloidae	*Norops lemurinus* (Cope 1861)	Km20[1,2], Km27[1,2], Km40[1,2], DN[1,2], HM[1,2], MC[1,2], NDZ[1], BKC[2]
Dactyloidae	*Norops rodriguezii* (Bocourt 1873)	Km20[1,2], Km27[1,2], Km40[1,2], DN[1,2], HM[1,2], MC[1,2], NDZ[1], BKC[2]
Dactyloidae	*Norops sagrei* (Duméril & Bibron 1837) [NNTV]	Km20[1,2], HM[2], DN[1,2], MC[1,2]
Dactyloidae	*Norops tropidonotus* (Peters 1863)	Km20[1,2], Km27[1,2], Km40[1,2], DN[1,2], HM[1,2], MC[1,2], NB[5], BKC[2]
Dactyloidae	*Norops ustus* (Cope 1864)	Km20[1,2], Km27[1,2], Km40[1,2], DN[1,2], HM[1,2], MC[1,2]
Eublepharidae	*Coleonyx elegans* (Gray 1845)	Km20[1,2], Km27[1,2], Km40[1,2], DN[1,2], HM[1,2], MC[1,2], NDZ[1], NB[5]
Gekkonidae	*Hemidactylus frenatus* (Duméril & Bibron 1836) [NNTV]	Km20[1,2], DN[1,2], HM[1,2], MC[1,2], BKC[2]
Iguanidae	*Cachryx alfredschmidti* (Köhler 1995)	Km10[4], Km48[5], CR[4]

Iguanidae	*Cachryx defensor* (Cope 1866)	HM[1,2], NDZ[1]
Iguanidae	*Ctenosaura similis* (Gray 1831) *	Silvituc[2] (UTADC 9451)
Mabuyidae	*Marisora brachypoda* (Taylor 1956)	HM[2], AO[7], MC[5]
Phrynosomatidae	*Sceloporus chrysostictus* (Cope 1866)	Km20[1,2], Km27[2], Km50[4], DN[1,2], HM[1,2], MC[1,2], NDZ[1], NB[5], BKC[2]
Phrynosomatidae	*Sceloporus lundelli* (Smith 1939)	Km20[2], MC[1,2], HM[2], CR[4]
Phyllodactylidae	*Thecadactylus rapicauda* (Houttuyn 1782)	Km20[1,2], Km48[5], DN[1,2], HM[1,2], MC[1,2], CR[5]
Scincidae	*Mesoscincus schwartzei* (Fischer 1884)	Km20[1,2], MC[1,2], HM[2], DN[2]
Scincidae	*Plestiodon sumichrasti* (Cope 1867)	Km20[1,2], DN[2], MC[2], CR[4]
Sphaerodactylidae	*Sphaerodactylus glaucus* (Cope 1866)	Km20[1,2], DN[1,2], HM[1,2], MC[1,2]
Sphenomorphidae	*Scincella cherriei* (Cope 1893)	Km20[1,2], HM[2] Km50[4], MC[4], CR[5]
Teiidae	*Aspidoscelis angusticeps* (Cope 1878)	Km20[1,2], Km27[1,2], Km40[1,2], HM[1,2], MC[1,2], BKC[2]
Teiidae	*Aspidoscelis deppii* (Wiegmann 1834)	NM[4]
Teiidae	*Holcosus gaigeae* (Smith & Laufe 1946) *	Km20[2], DN[2] (UTADC 9454)
Teiidae	*Holcosus hartwegi* (Smith 1940)	Km20[1,2], Km27[1,2], Km40[1,2], DN[1,2], HM[1,2], MC[1,2], NDZ[1], NB[5]
Boidae	*Boa imperator* (Daudin 1803)	Km20[1,2], Km27[2], NDZ[1]
Colubridae	*Drymarchon melanurus* (Duméril, Bibron & Duméril 1854)	Km20[2], HM[1,2], DN[2], MC[1,2,] NB[2]
Colubridae	*Drymobius margaritiferus* (Schlegel 1837)	Km20[1,2], Km27[1,2], Km40[1,2], HM[1,2], MC[1,2], NDZ[1], NB[5]
Colubridae	*Ficimia publia* (Cope 1866)	Km20[1,2], HM[1,2], DN[2], AO[5], CR[5]
Colubridae	*Lampropeltis abnorma* (Bocourt 1886)	Km20[1,2], HM[1,2], MC[1,2], Bel-Ha[4]
Colubridae	*Leptophis ahaetulla* (Linnaeus 1758)	Km20[1,2], Km40[1,2], HM[2], DN[1,2]
Colubridae	*Leptophis mexicanus* (Duméril, Bibron & Duméril 1854)	Km20[1,2], Km27[1,2], Km40[1,2], DN[1,2], HM[1,2], MC[1,2], NB[5], BKC[2]
Colubridae	*Mastigodryas melanolomus* (Cope 1868)	Km20[1,2], Km40[1,2], HM[2] DN[1,2], MC[1,2], NDZ[1], NM[4], NB[2]
Colubridae	*Oxybelis aeneus* (Wagler 1824)	Km20[1,2], NDZ[1], CR[4], PA[4], AO[4]
Colubridae	*Oxybelis fulgidus* (Daudin 1803)	MC[1,2], NB[4], PA[4]
Colubridae	*Phrynonax poecilonotus* (Günther 1858)	Km20[1,2], MC[2], DN[2]
Colubridae	*Pseudelaphe flavirufa* (Cope 1867)	Km20[1,2], Km27[1,2], Km40[1,2], DN[1,2], HM[1,2], BKC[2]

Colubridae	*Pseudelaphe phaescens* (Dowling 1952) *	HM^{2} (UTADC 9455)
Colubridae	*Senticolis triaspis* (Cope 1866)	MC^{1}
Colubridae	*Spilotes pullatus* (Linnaeus 1758)	Km27^{1,2}, Km40^{1,2}, HM^{1,2}, MC^{1,2}
Colubridae	*Stenorrhina freminvillei* (Duméril, Bibron & Duméril 1854) *	MC^{2} (UTADC 9458)
Colubridae	*Symphimus mayae* (Gaige 1936)	Km20^{1,2}, Km50^{4}, DN^{1,2}, MC^{4}
Colubridae	*Tantilla cuniculator* (Smith 1939) **	HM^{2} (UTADC 9459)
Colubridae	*Tantilla moesta* (Günther 1863)	DN^{2}, Km20^{2,3}
Colubridae	*Tantilla schistosa* (Bocourt 1883) **	HM^{2}, MC^{2} (UTADC 9460)
Colubridae	*Tantillita lintoni* (Smith 1940)	DN^{2}, MC^{4}
Dipsadidae	*Coniophanes bipunctatus* (Günther 1858) *	Bel-Ha^{2} (UTADC 9450)
Dipsadidae	*Coniophanes imperialis* (Baird & Girard 1859)	Km20^{1,2}, Km27^{1,2}, Km40^{1,2}, DN^{1,2}, MC^{1,2}, HM^{2} NDZ^{1}, NB^{5}, BKC^{2}
Dipsadidae	*Coniophanes schmidti* (Bailey 1937)	Km20^{1,2}, Km27^{1,2}, Km40^{1,2}, DN^{1,2}, HM^{1,2}, MC^{1,2}, NDZ^{1}, BKC^{2}
Dipsadidae	*Dipsas brevifacies* (Cope 1866)	Km20^{1,2}, Km27^{1,2}, Km40^{1,2}, HM^{1,2}, MC^{1,2}, DN^{2}
Dipsadidae	*Imantodes cenchoa* (Linnaeus 1758)	Km20^{1,2}, Km48^{5}, DN^{1,2}, HM^{1,2}, MC^{1,2}
Dipsadidae	*Imantodes gemmistratus* (Cope 1861)	Km20^{1,2}, HM^{1,2}, MC^{2}, Bel-Ha^{4}
Dipsadidae	*Imantodes tenuissimus* (Cope 1867)	Km20^{1,2}, Km27^{1,2}, Km40^{1,2}, MC^{2}, HM^{2}
Dipsadidae	*Leptodeira frenata* (Cope 1886)	Km20^{1,2}, HM^{1,2}, MC^{1,2}, NDZ^{1}, CR^{5}, BKC^{2}
Dipsadidae	*Leptodeira polysticta* (Günther 1895)	DN^{1,2}, HM^{2}, NB^{5}, CR^{5}
Dipsadidae	*Ninia diademata* (Baird & Girard 1853)	Km20^{1,2}, Km40^{1,2}
Dipsadidae	*Ninia sebae* (Duméril, Bibron & Duméril 1854)	Km20^{1,2}, HM^{1,2}, DN2, MC4, CR^{4}
Dipsadidae	*Pliocercus elapoides* (Cope 1860)	CR^{5}
Dipsadidae	*Sibon dimidiatus* (Günther 1872) ***	DN^{2} (UTADC 9457)
Dipsadidae	*Sibon nebulatus* (Linnaeus 1758)	Km20^{1,2}, Km48^{5}, DN^{1,2}, CR^{4}, AO^{4}, NM^{4}
Dipsadidae	*Sibon sanniolus* (Cope 1866)	Km20^{1,2}, DN^{2}, CR^{4}, NM^{4}
Dipsadidae	*Tropidodipsas fasciata* (Günther 1858)	Km20^{1,2}, Km27^{1,2}, HM^{1,2}, MC^{2}, AO^{5}
Dipsadidae	*Tropidodipsas sartorii* (Cope 1863)	Km20^{1,2}, Km27^{1,2}, Km40^{1,2}, DN^{1,2}, HM^{1,2}, MC^{1,2}, NDZ^{1}

Dipsadidae	*Xenodon rabdocephalus* (Wied-Neuwied 1824)	Km20[1,2], Km48[5], MC[1,2], CR[4], NM[4]
Elapidae	*Micrurus diastema* (Duméril, Bibron & Duméril 1854)	Km20[1,2], Km27[1,2], Km40[1,2], DN[1,2], MC[1,2], NDZ[1], NB[5], CR[5]
Natricidae	*Thamnophis marcianus* (Baird & Girard 1853)	AO[4], MC[4]
Natricidae	*Thamnophis proximus* (Say 1823)	Becan[7] (iN 1729318)
Sibynophiidae	*Scaphiodontophis annulatus* (Duméril, Bibron & Duméril 1854) **	DN[2] (UTADC 9456)
Typhlopidae	*Amerotyphlops microstomus* (Cope 1866)	AO[6]
Viperidae	*Agkistrodon russeolus* (Gloyd 1972)	Km20[1,2], NDZ[1], CR[4]
Viperidae	*Bothrops asper* (Garman 1883)	Km20[1,2], Km27[1,2], Km40[1,2], DN[1,2], HM[2], NDZ[1], MC[5], BKC[2]
Viperidae	*Crotalus tzabcan* (Klauber 1952)	Km20[1,2], DN[1,2], HM[1,2], NDZ[1], CR[4], AO[4], NB[4]
Emydidae	*Terrapene yucatana* (Boulenger 1895)	Km20[2], HM[1,2], AO[2], CR[4]
Emydidae	*Trachemys venusta* (Gray 1855)	Km20[1,2], Km27[1,2], HM[1,2], Bel-Ha[2]
Geoemydidae	*Rhinoclemmys areolata* (Duméril, Bibron & Duméril 1851)	Km20[1,2], Km48[5], MC[2], HM[1,2], DN[2], AO[5]
Kinosternidae	*Kinosternon acutum* (Gray 1831)	HM[1,2], DN[2]
Kinosternidae	*Kinosternon creaseri* (Hartweg 1934)	Km20[1,2], NDZ[1], HM[2], DN[2]
Kinosternidae	*Kinosternon leucostomum* (Duméril, Bibron & Duméril 1851)	Km20[1,2], DN[1,2], MC[2], HM[1,2], NDZ[1]
Kinosternidae	*Kinosternon scorpioides* (Linnaeus 1766)	Km20[1,2], Km27[1,2], HM[1,2], MC[1,2], NDZ[1], NB[5]
Staurotypidae	*Claudius angustatus* (Cope 1865)	Km20[1,2], Km27[2], DN[2], HM[1,2], NDZ[1]
Staurotypidae	*Staurotypus triporcatus* (Wiegmann 1828)	NB[4], Arroyo Negro[6,7]

Discussion

The 109 species of amphibians and reptiles found in CBR and surrounding areas represent, respectively, approximately 84% and 75% of total species richness registered for the state of Campeche and Yucatan Peninsula (González-Sánchez et al. 2017) and approximately 60% of the observed herpetofauna richness in the entire Mayan jungle (188 species; Lee 2000). From the total number of species found in Calakmul, nearly 21% are endemic to the Yucatan Peninsula (e.g. *Bolitoglossa yucatana* Peters 1882; *Cachryx defensor*; *Tantilla cuniculator*; *Terrapene yucatana* Boulenger 1895). Field observations and data from Operation Wallacea's monitoring project between 2014 and 2018 in CBR indicate a decline in overall herpetofauna abundance and diversity paired with a significant reduction in water availability across the region due to the ongoing prolonged drought the reserve is experiencing (Slater 2019). As site conservation may be one of the most effective means of reducing biodiversity loss (Eken et al. 2004), our data highlights the importance of increasing our knowledge of the assemblage and distribution of herpetofauna species within the Yucatan Peninsula when taking measures to preserve the fauna of this region.

Our results from 2015 to 2019 in CBR point out that only about 65% of the species we found were recorded in three or more areas. This result is not very different from the percentage previously reported for the reserve (50%; Colston et al. 2015) and further suggests significant differences in species richness and diversity within Calakmul. Topography and latitude-correlated rainfall differences across this region could be accountable for differences in local microhabitat differences (Lyons and Willig 2002; Stevens 1989), which may result in species variation among areas (Garda et al. 2013; Vitt et al. 2007).

The significant percentage (38%) of herpetofauna species occurring in Calakmul listed in the risk categories proposed by the Mexican environmental legislation (NOM-059-SEMARNAT-2010) highlights the importance of considering amphibian and reptiles when planning future development projects that would involve habitat loss or alteration in the region (e.g. Mayan train; Pskowski 2019), as degradation and loss of habitat has been indicated as one of the major threats to amphibian and reptile conservation in Mexico (Flores-Villela and García-Vázquez 2014; Parra-Olea et al. 2014). It is worthwhile mentioning that unlike CBR, which has federal jurisdiction and management, other areas of the Calakmul region (e.g. Balam Ku Balam Kin state reserves) are not under the same legal protection framework which restricts the conservation efforts of environmental authorities operating in these areas.

Although the frog *E*. *leprus* and the snake *S*. *dimidiatus* represent new records for the Mexican part of the Yucatan Peninsula, it was speculated that the distribution of these species could encompass the region of Calakmul as they have both been reported in Uaxactún (northern Guatemala), which is relatively close (70km; Lee 2000) to the southern portion of CBR (González-Sánchez et al. 2017). This may also be the case for other amphibians and reptiles, hence we believe that species typically reported in neighbouring regions (e.g., Tabasco) or countries (i.e., Belize and Guatemala), such as the lizards *Celestus rozellae* (Smith 1942), *Norops capito* (Peters 1863)*, Norops uniformis* (Cope 1885), *Sceloporus serrifer* (Cope 1866)*, Sphaerodactylus millepunctatus* (Hallowell 1861), the snakes *Adelphicos quadrivirgatum* (Jan 1862)*, Conophis lineatus* (Duméril, Bibron and Duméril 1854)*, Masticophis mentovarius* (Duméril, Bibron and Duméril 1854)*, Oxyrhopus petolarius* (Linnaeus 1758)*, Tretanorhinus nigroluteus* (Cope 1861)*, Bothriechis schlegelii* (Berthold 1846) *Porthidium nasutum* (Bocourt 1868) and the turtle *Dermatemys mawii* (Gray 1847) might also occur within the region of Calakmul. Some of these species require very specific habitat characteristics to thrive (Lee 2000), which would likely limit their distribution to very confined areas / habitats within Calakmul. For example, species like *D*. *mawii* and *T*. *nigroluteus* inhabit permanent and semi-permanent waterbodies with a relative slow water flow (Chaves et al. 2016; Lee 2000; Vogt et al. 2006). Information gathered during author JALBN’s PhD activities in the region indicates the presence of areas across Calakmul, away from our regular survey sites, that seem to offer such conditions (Barão-Nóbrega 2019).

Conclusion

The list presented in this work increases to 109 the number of herpetofauna species known to occur in the region of Calakmul, and to 135 the number of amphibians and reptiles in the state of Campeche, which therefore becomes the Mexican state of the Yucatan Peninsula with the highest herpetofauna diversity, followed by Quintana Roo with 133 species. Furthermore, our study increases to 148 the total number of amphibian and reptile species recorded in the Yucatan Peninsula (González-Sánchez et al. 2017; Ortiz-Medina et al. 2019). Future studies should be performed during different seasons and in other areas within Calakmul, focusing sampling efforts on fossorial amphibians and reptiles (e.g. genus *Tantilla* and *Tantillita*), which are hard to detect and therefore deeply understudied.

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We would like to thank the communities of Bel-Ha, Conhuas, Dos Naciones, Hormiguero, Mancolona, Nuevo Becal and Silvituc (Campeche, Mexico) for granting us the opportunity to work in their ejidal land and for providing guide services and logistical support. We especially thank O Platas-Vargas, V Isoart, V Corradi and C Acton, as field activities for this study would have not been possible without their assistance. We also thank JR Cedeño-Vázquez for providing detailed information on the localities of reptile records from Calderón-Mandujano *et al.* (2010); E Smith and G. Pandelis for providing assistance in obtaining digital vouchers; A Lopez-Cen and D Sima-Pantí for their assistance with obtaining yearly research permits; JM Hutton, T Johnson, R Bhattacharyya-Dickson, N Weigner, M Viteri, A Padilla, A Romero, AP Couto, T Pinto, R Smith, R Piraccini, J Phangurha, O. Sawościanik, P Taylor, E Cambranis, J Daw and the rest of the Operation Wallacea staff and student volunteers for logistical assistance and data collection. We thank SEMARNAT and CONANP for providing permits over the period of study (SGPA/DGVS/005403/18), as part of Operation Wallacea’s long-term biodiversity monitoring project in collaboration with Pronatura Peninsula de Yucatan.

10. Appendix IV – Distribution and concentrations of Persistent organic pollutant (POP) residues in sediments of waterbodies in the region of Calakmul (Campeche, Mexico)

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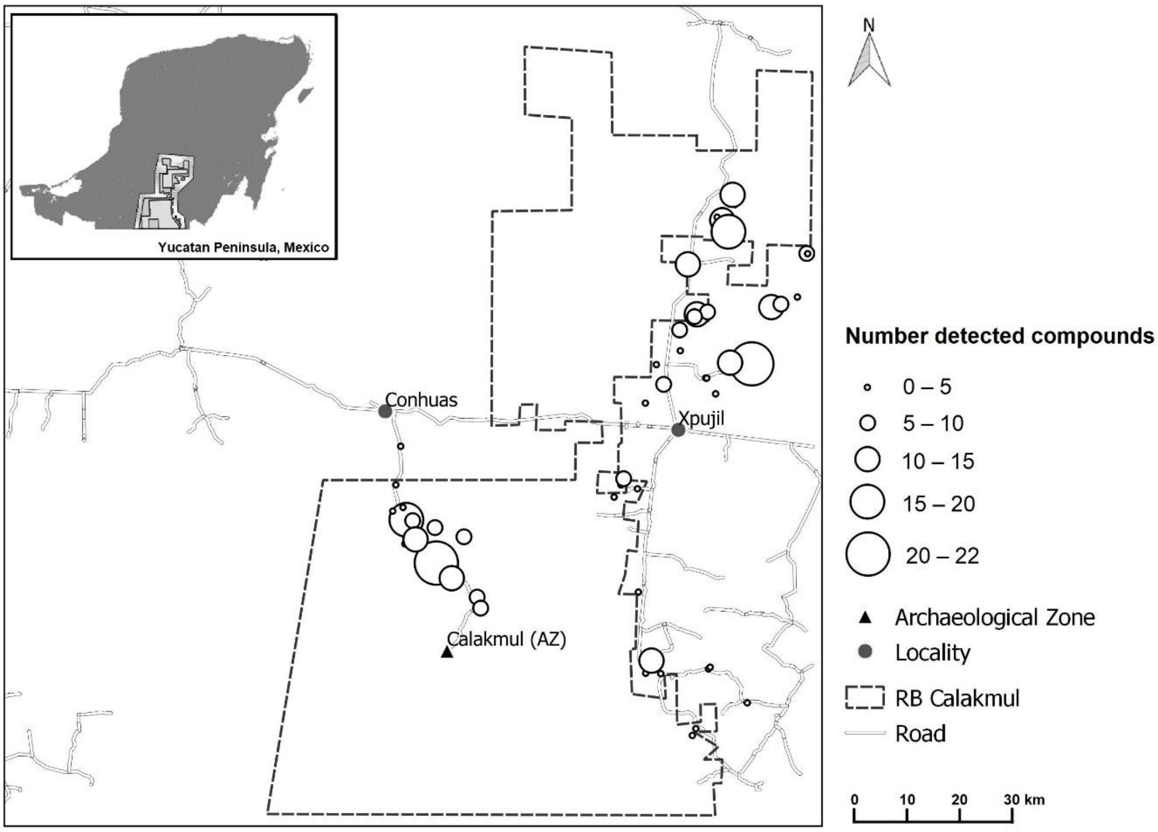

Figure 52. Number of detected Persistent Organic Pollutants (PAHs and OCs) in sediment samples from aguadas in the region of Calakmul (Biosphere Reserve and surrounding areas) in southern Campeche, Mexico.

Table 8. Number of detections and mean concentrations of Polycyclic aromatic hydrocarbons (PAHs) and in sediment samples from 66 waterbodies across the region of Calakmul (Calakmul Biosphere Reserve and surrounding areas) in southern Campeche, Mexico. All concentrations are displayed in ug/g dry weight. Mean concentration and standard deviation were only calculated for compounds detected in more than 10% of sediment samples. TEL represents the threshold effect level values listed on NOAA Screening Quick Reference Tables for organics in sediments (Burton Jr, 2002; Buchman, 2008).

Compound (Nº rings)	**Detections N (%)**	**Concentrations mean ± sd (min - max)**	**Toxicity guideline (TEL)**
Naphthalene (2)	3 (5%)	(0.002 - 0.077)	0.035
Acenaphthene (3)	8 (12%)	0.003 ± 0.017 (0.002 - 0.050)	0.007
Acenaphthylene (3)	22 (33%)	0.020 ± 0.212 (0.001 - 0.371)	0.006
Anthracene (3)	28 (42%)	0.005 ± 0.009 (0.001 - 0.058)	0.047
Fluoranthene (3)	20 (30%)	0.004 ± 0.0023 (0.001 - 0.017)	0.111
Phenanthrene (3)	9 (14%)	0.005 ± 0.012 (0.002 - 0.089)	0.042
LMW PAHs			
Fluorene (4)	7 (10%)	0.003 ± 0.003 (0.001 - 0.022)	0.021
Pyrene (4)	19 (29%)	0.004± 0.004 (0.002 - 0.037)	0.053
Benz(a)anthracene (5)	34 (52%)	0.015 ± 0.07 (0.002 - 0.199)	0.032
Benzo(b)fluoranthene (5)	7 (11%)	0.004 ± 0.0323 (0.002 - 0.259)	0.07
Benzo(k)fluoranthene (5)	15 (23%)	0.005 ± 0.011 (0.002 - 0.168)	0.06
Benzo(a)pyrene (5)	9 (14%)	0.009 ± 0.4201 (0.002 - 0.220)	0.032
Dibenzo(a,h)anthracene (5)	12 (18%)	0.014 ± 0.366 (0.005 - 0.099)	0.006
Chrysene (5)	19 (29%)	0.006 ± 0.015 (0.002 - 0.204)	0.057
Indeno(1,2,3-cd)pyrene (6)	10 (15%)	0.002 ± 0.003 (0.002 - 0.020)	NA
Benzo(g,h,i)perylene (6)	18 (27%)	0.006 ± 0.007 (0.002 - 0.167)	NA
HMW PAHs			
Σ by number of rings			
ΣPAHs (2)	3 (5%)	(0.002 - 0.077)	---
ΣPAHs (3)	32 (49%)	0.045 ± 0.615 (0.406)	---
ΣPAHs (4)	29 (44%)	0.011 ± 0.032 (0.214)	---
ΣPAHs (5)	40 (60%)	0.050 ± 0.490 (0.605)	---
ΣPAHs (6)	18 (27%)	0.007 ± 0.031 (0.167)	---

Table 9. Number of detections and mean concentrations of organochlorinated compounds in sediment samples from 66 waterbodies across the region of Calakmul (Calakmul Biosphere Reserve and surrounding areas) in southern Campeche, Mexico. All concentrations are displayed in ug/g dry weight. Mean concentration and standard deviation were only calculated for compounds detected in more than 10% of sediment samples. TEL represents the threshold effect level values listed on NOAA Screening Quick Reference Tables for organics in sediments (Buchman, 2008).

Compound	**Detections N (%)**	**Concentrations mean ± sd (min - max)**	**Toxicity guideline (TEL)**
Aldrin	7 (11%)	0.002 ± 0.001 (0.001 - 0.003)	NA
Chlordane (cis)	25 (38%)	0.002 ± 0.001 (0.001 - 0.009)	NA
Chlordane (trans)	9 (13%)	0.001 ± 0.001 (0.001 - 0.004)	NA
Dieldrin	12 (18%)	0.001 ± 0.001 (0.001 - 0.004)	0.003
Endosulfan (I)	11 (17%)	0.001 ± 0.001 (0.001 - 0.005)	NA
Endosulfan (II)	14 (21%)	0.002 ± 0.003 (0.001 - 0.070)	NA
Endosulfan (Sulfate)	18 (27%)	0.002 ± 0.002 (0.001 - 0.009)	NA
Endrin	10 (15%)	0.003 ± 0.005 (0.001 - 0.099)	0.003
Endrin (Aldehyde)	8 (12%)	0.002 ± 0.001 (0.001 - 0.010)	NA
Endrin (Ketone)	8 (12%)	0.002 ± 0.0034 (0.001 - 0.024)	NA
DDD (pp)	11 (17%)	0.004 ± 0.004 (0.001 - 0.025)	0.004
DDE (pp)	24 (36%)	0.002 ± 0.004 (0.001 - 0.097)	0.001
DDT (pp)	12 (18%)	0.003 ± 0.002 (0.001 - 0.01)	NA
HCH (α)	2 (3%)	(< 0.001 - 0.003)	NA
HCH (β)	14 (21%)	0.002 ± 0.001 (0.001 - 0.014)	NA
HCH (δ)	12 (18%)	0.001 ± 0.000 (0.001 - 0.002)	NA
HCH (γ)	3 (5%)	(< 0.001 - 0.004)	0.001
Heptachlor	6 (9%)	(< 0.001 - 0.001)	NA
Heptachlor Epoxide	6 (9%)	(< 0.001 - 0.004)	< 0.001
Metoxichlor	20 (30%)	0.009 ± 0.018 (0.001 - 0.147)	NA
Σ by group			
Σ Deines	25 (38%)	0.004 ± 0.007 (0.101)	NA
Σ DDTs	31 (47%)	0.003 ± 0.006 (0.104)	0.007
Σ Endosulfans	29 (44%)	0.003 ± 0.005 (0.070)	NA
Σ HCHs	19 (29%)	0.001 ± 0.002 (0.014)	NA
Σ Heptachlors	9 (14%)	0.001 ± 0.001 (0.004)	NA
Σ other PCBs	32 (49%)	0.006 ± 0.017 (0.153)	NA

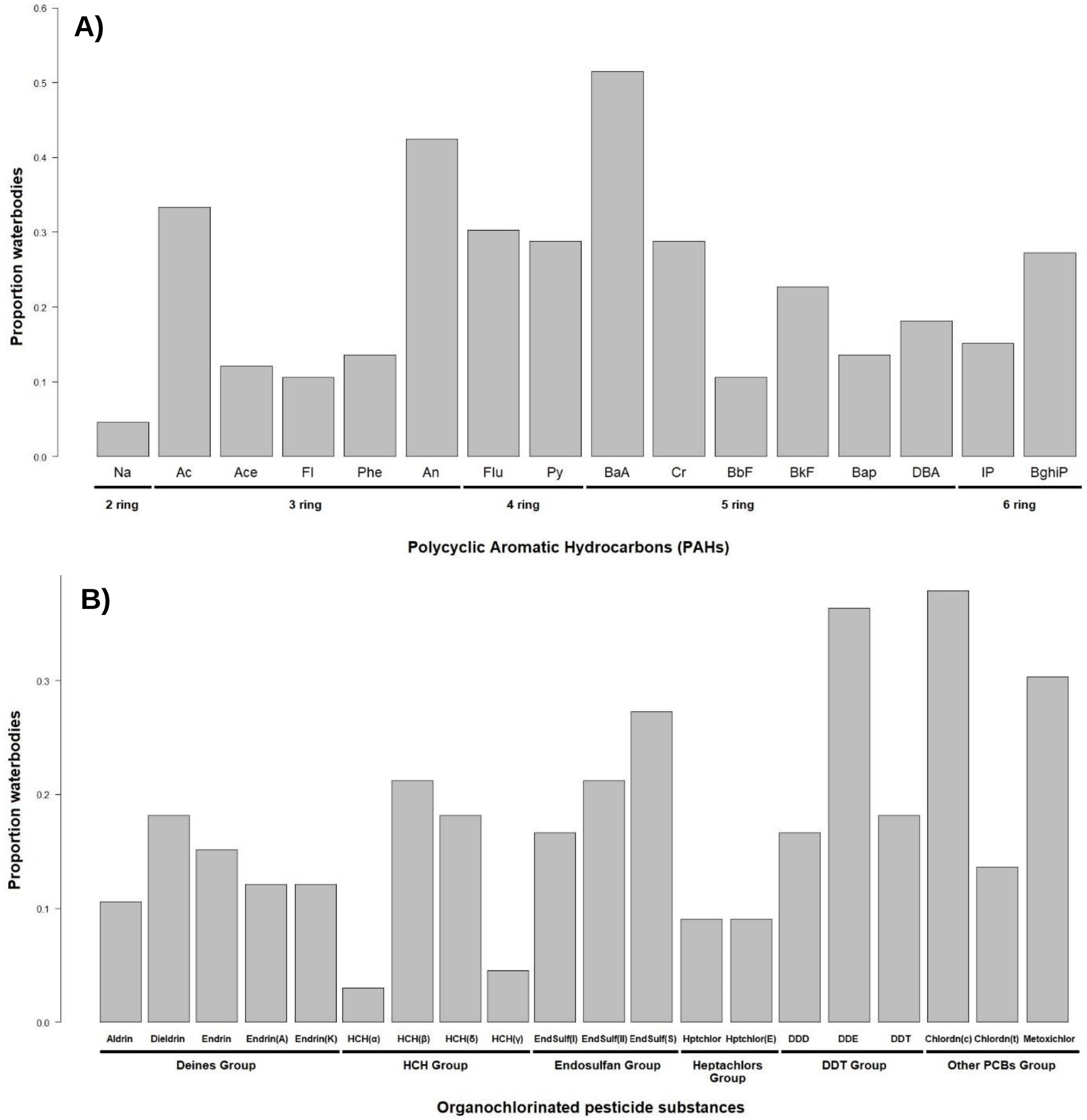

Figure 53. Polycyclic Aromatic Hydrocarbons (PAHs) of low (2 – 3 ring) and high (4 – 6 rings) molecular weights (A) and Organochlorinated pesticide (OCs) substances (B) detected in surface sediments of 66 waterbodies in the region of Calakmul (Biosphere Reserve and surrounding areas) in southern Campeche, Mexico. Bars indicate proportion of sampled waterbodies where compounds were detected. (Calakmul Biosphere Reserve and surrounding areas) in southern Campeche, Mexico. Full names of PAHs and OCs compounds are listed in Tables 8 and 9, respectively.

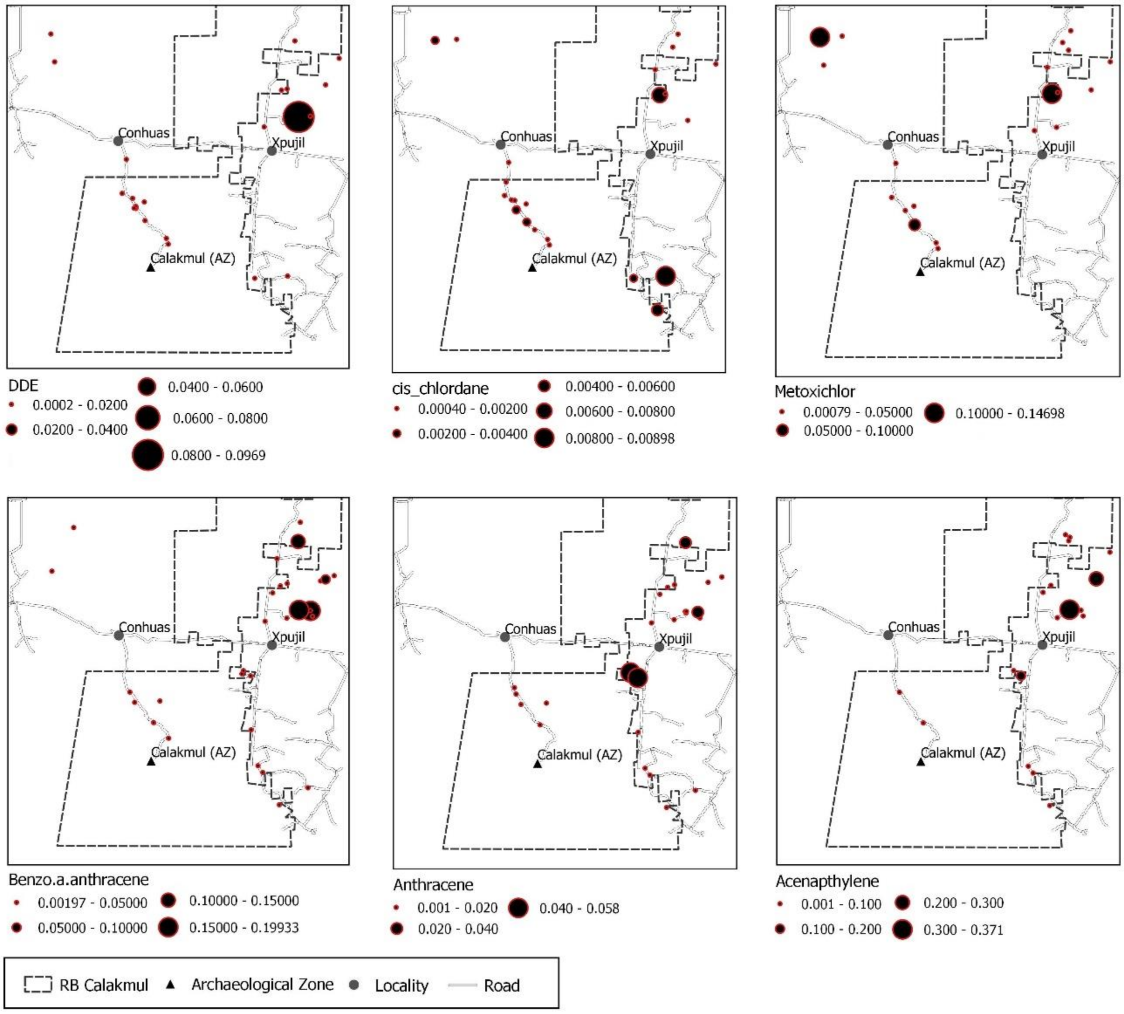

Figure 55. Concentrations in µg/g (dry weight) of the three most detected Organochlorinated pesticide (DDE, cis Chlordane and Metoxichlor) and Polycyclic Aromatic Hydrocarbon (Benzo.a.anthracene, Anthracene and Acenapthylene) substances quantified in sediment samples from aguadas in the region of Calakmul (Biosphere Reserve and surrounding areas) in southern Campeche, Mexico.