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The genetic diversity and distribution of two endangered pit vipers (Bothriechis marchi and Cerrophidion wilsoni) within Cusuco National Park, Honduras

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The genetic diversity and distribution of two endangered pit vipers (Bothriechis marchi and Cerrophidion wilsoni) within Cusuco National Park, Honduras

Starbuck, Debbie

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THE GENETIC DIVERSITY AND DISTRIBUTION OF TWO ENDANGERED PIT VIPERS (*BOTHRIECHIS MARCHI* AND *CERROPHIDION WILSONI*) WITHIN CUSUCO NATIONAL PARK, HONDURAS

By

DEBBIE STARBUCK

A thesis submitted to the University of Plymouth in partial fulfilment for the degree of:

RESEARCH MASTERS

School of Biological and Marine Sciences

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Authors Declaration

At no time during the registration for the degree of Research Masters has the author been registered for any other University award without prior agreement of the College Quality Sub-Committee.

Work submitted for this research degree at the University of Plymouth has not formed part of any other degree either at the University of Plymouth or at another establishment.

A programme of advanced study was undertaken, which included taught modules.

Word count of main body of thesis: approx. 11,650

Signed; DStarbuck

Date; 23rd July 2021

THE GENETIC DIVERSITY AND DISTRIBUTION OF TWO ENDANGERED PIT VIPERS (*BOTHRIECHIS MARCHI* AND *CERROPHIDION WILSONI*) WITHIN CUSUCO NATIONAL PARK, HONDURAS – Debbie Starbuck

Abstract

The current extinction rate is being increasingly recognised as an escalating issue, with observations still indicating that humans are the leading cause of a sixth mass extinction. Cloud forests in particular are home to a variety of threatened species, however, many of these forests are highly fragmented resulting in many species and populations existing in isolation. These forests have become centres of endemism for many species, leading to a high representation within global biodiversity hotspots and "irreplaceable" protected areas.

This study primarily investigates the genetic diversity and distribution of two recently re-classified, sympatric pit vipers (*Bothriechis marchi* and *Cerrophidion wilsoni*) within Cusuco National Park, Honduras using samples acquired by Operation Wallacea Ltd. Twenty-nine *B. marchi* samples and forty-two *C. wilsoni* samples were successfully sequenced for the cytochrome oxidase subunit 1 (CO1), NADH dehydrogenase 4 (ND4), and two segments of the cytochrome b (CytB) gene to reveal eight haplotypes within the *B. marchi* population (haplotype diversity = 0.697044) compared to only two haplotypes within the *C. wilsoni* population (haplotype diversity = 0.493612). This low variation of mtDNA haplotype diversity within the *C. wilsoni* population may leave it vulnerable to future challenges.

The mapping of these haplotypes suggests a partial restriction of gene flow between the west and east of the park. This appears to be a larger issue for *B. marchi,* however ongoing research is required to confirm the significance and potential impact of this. The results also provide further support regarding the distribution of these species, indicating a higher abundance of *B. marchi* around the El Corticito camp on the west side of the park compared to a higher abundance of *C. wilsoni* around Cantiles camp in the centre of the park. At the El Danto camp

there is a good likelihood of encountering both species, however, the high levels of deforestation on this side of the park pose a significant threat.

The continuation of this deforestation could result in the loss of approximately 33-55% of all found mtDNA genetic diversity within the *B. marchi* population and cause significant change within the genetic structure of this population. In addition, 71% of all *B. marchi* encounters and 33% of *C. wilsoni* encounters between 2007 and 2018 were recorded on the west side of the park, highlighting a greater vulnerability of *B. marchi* to the current deforestation on this side of the park and indicating that a large percentage of both populations may be lost due to this deforestation should it continue.

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1. Introduction

While the modern extinction rate is being increasingly recognised as an escalating issue (Pereira *et al.*, 2010; Ceballos and Ehrlich, 2018), many species still remain listed as Data Deficient by the International Union for the Conservation of Nature (IUCN) resulting in an underestimation of the number of threatened species documented (Dirzo and Raven, 2003). To date, approximately 128,500 species have been assessed by the IUCN out of approximately 1.9 million named species, over 37,400 of which are threatened with extinction (International Union for the Conservation of Nature, 2021). A further 6.2-8.8 million eukaryotic species are predicted to exist globally and await description (Mora *et al.*, 2011), many of which will also be threatened or will have been made extinct before discovery.

Observations still indicate that humans are the leading cause of a sixth mass extinction known as the Holocene extinction (Ceballos and Ehrlich, 2018) or Anthropocene extinction which is due to climate change, habitat loss and fragmentation, the introduction of non-native species, and various other anthropogenic influences (Pimm et al., 1995; Wake and Vredenburg, 2008; Hoffmann et al., 2010). Habitat fragmentation in particular can lead to many long-term consequences such as inbreeding depression by reducing and isolating populations, which can then lead to loss of genetic diversity through inbreeding and genetic drift. This in turn can lead to numerous consequences such as chromosomal abnormalities, birth defects, and a low survival rate among juveniles that will reduce the fitness of the population (Ujvari *et al.*, 2002; Ferchaud *et al.*, 2011).

Through a variety of conservation tools, species can and have been rescued from the brink of extinction. These tools are many and varied including habitat management, captive breeding and reintroduction, and the preservation of the genetic health of a population (International Union for the Conservation of Nature, 2021).

1.1 Cloud forest conservation

One of the least studied ecosystems, yet one of the most affected by the current threat of climate change, are montane cloud forests (Foster, 2001). Defined as ‘tropical forests frequently covered in cloud or mist’ (Stadtmuller, 1987), they are spatially restricted and comprise between 2.5% (Cayuela *et al.*, 2006) and 14.2% (Mulligan, 2011) of forest ecosystems worldwide depending on whether the term is used to apply specifically to upper montane rain forest (Whitmore, 1998) or in a broader sense to include a range of broadleaved montane rain forests (Mulligan, 2011).

As ecologically unique sites that support a high diversity of flora and fauna, 86% of these cloud forest sites are found within the Global 200 Priority Forest Ecoregions as identified by the World Wide Fund for Nature (Olson and Dinerstein, 2002). While cloud forests are home to a variety of threatened species, many are highly fragmented and exist within sky islands; isolated mountains surrounded by radically different lowland environments (Fave *et al.*, 2015). The associated geographic barriers between these islands of suitable habitat causes allopatric speciation, with many species and populations existing in isolation (Le Soaut *et al.*, 2013). These populations then undergo genotypic or phenotypic divergence as they develop different mutations and independently undergo genetic drift (Fave *et al.*, 2015).

This isolation has led these forests to become centres of endemism for plants (Bubb *et al.*, 2004), invertebrates (Anderson and Ashe, 2000), herpetofauna (Wilson and McCranie, 2004a), and they also provide habitats for 10% of all range-restricted bird species (Stattersfield *et al.*, 1998). This leads to a high representation of Cloud Forests within global biodiversity hotspots (Myers *et al.*, 2000; Brooks *et al.*, 2006) and “irreplaceable” protected areas (Le Soaut *et al.*, 2013).

In addition to their well-recognised ecological importance (Rahbek *et al.*, 2019), cloud forests also provide a wide range of ecosystem services including nutrient cycling, carbon sequestration and storage, water catchment protection, and various biodiversity mediated services such as soil aeration, pest biocontrol, waste removal, and adjacent crop pollination (Bubb *et al.*, 2004).

The Mesoamerican biodiversity hotspot (Myers *et al.*, 2000) is particularly rich in cloud forests, however, they are considered critically threatened ecosystems that are experiencing high rates of habitat loss (Cayuela, Golicher and Rey-Benayas, 2006). In recent decades the mechanization of forestry, improved accessibility, and human population growth have resulted in an increase in habitat degradation and deforestation (Aldrich *et al.*, 1997; Hansen *et al.*, 2020) in addition to the threat of diseases (Scheele *et al.*, 2019) and climate change (Feeley *et al.*, 2011; Fadrique *et al.*, 2018).

Cloud forests are not only threatened by direct deforestation, but also by lowland deforestation. In Costa Rica, it has been found that clouds hang higher at approximately 1,100m above lowland covered with pastures compared to approximately 650m above lowland forest, suggesting that air over pastured land is warmer and drier, and therefore clouds form at higher altitudes (Zandonella, 2001). This indicates that protecting cloud forests without effort to protect forests at lower elevations may push the cloud banks further up the mountains, changing the ecology of the cloud forests at higher elevations.

1.2 Snake Conservation

Within ecosystems, snakes are important as they may be keystone species or ecosystem engineers, and as obligate predators, snakes may often reflect the populations of prey species (Beaupre and Douglas, 2009) and, therefore, can act as indicators of community and ecosystem health. A decline in the predatory pressure snakes exert on these prey populations may have serious consequences for the functioning of many ecosystems (Petranka and Murray, 2001; Franzreb, 2007; Reading *et al.*, 2010).

Both the Green Anaconda (*Eunectes murinus* (Linnaeus, 1758)) in the Brazilian Pantanal and the Reticulated Python (*Python reticulatus* (Schneider, 1801)) in Asia are considered key examples of snakes as keystone species, though no published accounts have utilised top predating snakes in keystone models. Various biologists have used snakes as indicator species (Stafford *et al.*, 1977; Matthews, Knapp and Pope, 2002; Lind *et al.*, 2005), however, the cryptic nature of snakes typically makes them more difficult to survey than the species in question which often prevents them from being good indicator species.

There are various possible reasons for the lack of published accounts utilising snakes in keystone models, such as that most conservation efforts for snakes typically occur within frameworks initially built around organisms considered more important for economic, recreational, or aesthetic reasons (Beaupre and Douglas, 2009). In addition, data collection for snakes can be difficult and resource-intensive. Despite this, snakes can be useful indicators of change in the local environment or ecosystem due to vulnerabilities to changes associated with habitat destruction, climate change, or other long-term trends (Mullin and Seigel, 2009). As the home ranges of many snakes typically remain consistent over the course of their lifetime, any changes can usually be linked to changes in the local environment (Bauerle, Spencer and Wheeler, 1975).

While it is difficult to evaluate the true status of snake populations due to their cryptic nature, there is evidence of global declines in reptiles as a whole (Gibbons *et al.*, 2000), alongside a general consensus among herpetologists that snake populations are also in decline (Mullin and Seigel, 2009).

The IUCN Red List considers 12% of global snake species to be Vulnerable (4.6%), Endangered (5%), or Critically Endangered (2.4%), with a further 20.1% listed as Data Deficient (International Union for the Conservation of Nature, 2021). This does not however take into account that over 25% of snake species listed by the Integrated Taxonomic Information System (ITIS, 2020) are missing from the IUCN Red List entirely, which would increase the number of unassessed or data deficient snake species to over 50%.

In 2010, sharp population declines in eight snake species were reported over a period of 10-20 years, with suggested causes including habitat quality deterioration, low prey availability, and a root cause of climate change (Reading *et al.*, 2010). A recent global review of reptiles on the IUCN Red List noted that species from families of ambush predators such as *Viperidae* and *Boidae* were at more risk of extinction compared to foraging species from families such as *Colubridae* (Bohm and others, 2013). This is in line with findings from an earlier, similar study on Australian elapid species that found most threatened taxa to be ambush predators; hypothesising that ambush predators may be more vulnerable as they rely on habitats with specific types of ground cover that is easily disrupted by anthropogenic activities (Reed and Shine, 2002).

1.2.1 Viper Conservation

The viper family (*Viperidae*) is defined by solenoglyphous maxillary dentition (Pough, Andrews and Cadle, 2011), by which it is meant that these snakes have long, hollow, articulated fangs that fold against the roof of the mouth when the jaws are closed. These fangs are the only teeth in the maxilla (upper jaw), and are capable of injecting venom deep into prey (Allaby, 2009).

The IUCN Red List considers 24.6% of *Viperidae* species to be Vulnerable (10.1%), Endangered (11%), or Critically Endangered (3.5%), with a further 8.8% listed as Data Deficient (International Union for the Conservation of Nature, 2021). However, the IUCN only lists 227 species within the *Viperidae* family while the Integrated Taxonomic Information System (ITIS) lists 341 species within the family (ITIS, 2020). This would indicate an additional 114 species that are not yet assessed, increasing the percentage of species that are unassessed or Data Deficient to above 39%.

The *Viperidae* family is further divided into the subfamilies of *Viperinae* (vipers) and *Crotalinae* (pitvipers). The latter is a diverse clade from Asia and the Western Hemisphere characterised by the heat sensitive pit (loreal) located on the head between the nostril and pre-ocular scales, and consisting of approximating 156 species throughout 18 genera (Pough, Andrews and Cadle, 2011).

New World pitvipers are estimated to have diverged from Old World pitvipers in the early Miocene, between 16 and 18 million years ago (Castoe *et al.*, 2009). From here, *Bothriechis* and *Cerrophidion,* two genera which are the focus of this study, originated between 12 to 16 million years ago and 7.5 to 11.5 million years ago, respectively (Castoe *et al.*, 2009).

1.2.2 *Bothriechis*

Bothriechis is a genus of venomous pit viper found in Central and South America and commonly referred to as the Palm pit-vipers. Species within this genus tend to possess a haemotoxic venom to aid with predation and digestion (Vitt and Caldwell, 2009).

This clade of 11 species has a complex geographic distribution with a disputed evolutionary history (Mason *et al.*, 2019). A mosaic of species distribution can be seen across Middle American montane habitats due to its general restriction of mid to highly elevated habitats (Campbell and Lamar, 2004). Biogeographic breaks and changing topologies separating montane populations are thought to have led to allopatric speciation (Doan *et al.*, 2016). For example: The Nicaraguan Depression has been suggested as a biogeographic break separating the Southern Middle American Isthmus (*B. lateralis, B. nigroviridis, B. nubestris,* and *B. supreciliaris*) from the nuclear Middle American species (*B. aurifer, B. bicolor, B. guifarroi, B. marchi, B. rowleyo,* and *B. thalassinus*) (Castoe *et al.*, 2009; Daza, Castoe and Parkinson, 2010). Alternatively, the Motagua-Polochic fault has been implicated as a break separating *B. guifarroi, B. marchi,* and *B. thalassinus* from *B.aurifer, B. bicolor,* and *B. rowleyi* (Castoe *et al.*, 2009; Daza, Castoe and Parkinson, 2010). The order of diversification is however questionable within these groups due to historic incongruences in inferred phylogenies (Mason *et al.*, 2019).

The earliest phylogeny based on allozyme and morphology data (Crother, Campbell and Hillis, 1992) indicated a biogeographic history based on a recovered clade containing southern and nuclear Middle American taxa (Figure 1a), however the later addition of mitochondrial data led to the recovery of an incongruent topology (Taggart, Crother and White, 2001) with a monophyletic Middle American clade (Figure 1b). The recent description of various new species has added to the biogeographic-phylogenetic conflict (Figure 1c) causing further ambiguity in regard to the evolutionary processes shaping *Bothriechis* (Solórzano *et al.*, 1998; Campbell and Smith, 2000; J. H. Townsend *et al.*, 2013; Doan *et al.*, 2016).

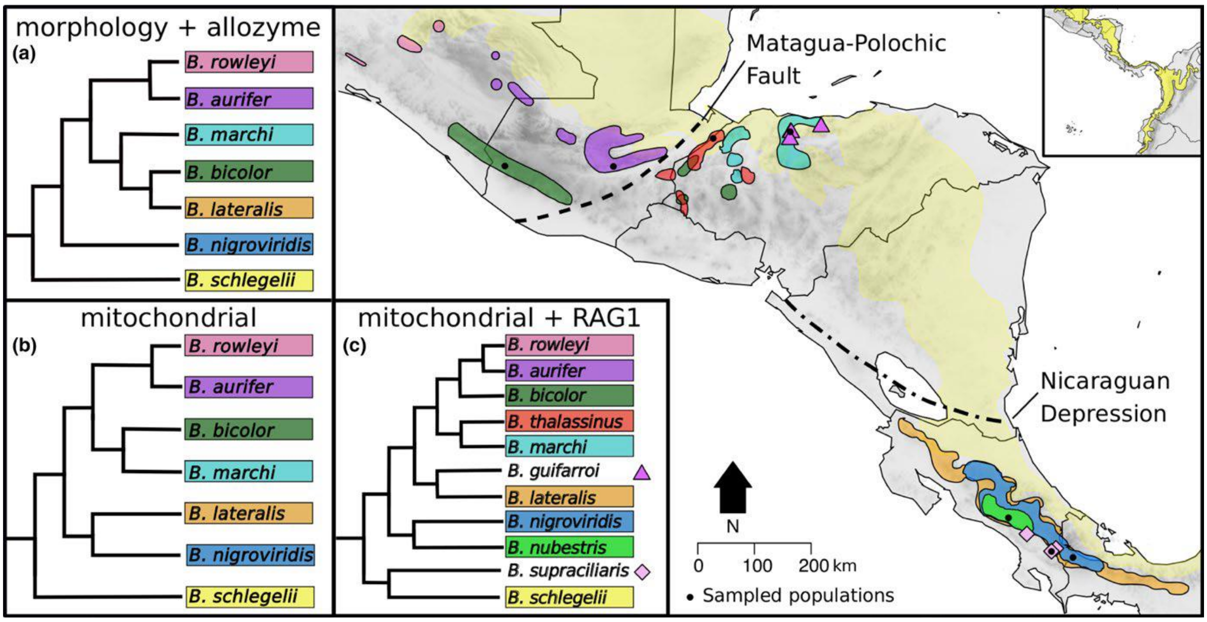

Figure 1; Phylogenetic hypotheses (a, b, c) for the evolution of Bothriechis in relation to their geographic distributions. (a) Phylogeny of Crother et al. (1992) based on morphological and allozyme characters. Notably, B. lateralis is nested with northern Middle American taxa suggesting a southward dispersion of this taxon to Costa Rica. (b) Phylogeny of Taggart et al. (2001) based on 12S sequences with a monophyletic northern Middle American clade suggesting a northward invasion. (c) Phylogeny of Doan et al. (2016) including all currently described species. Ranges are based on Campbell and Lamar (2004); Townsend et al. (2013); and Doan et al. (2016). Species with limited or poorly sample ranges shown as points and sampled populations are denoted with a black dot where known. Reproduced with permission (appendix 1) from (Mason *et al.*, 2019)

While Castoe *et al.* (2009) estimated the origin of *Bothriechis* at approximate 14 mya, a more recent study by Mason *et al.* (2019) suggests that the common ancestor of *Bothriechis* initially arose much earlier at approximately 18 mya, with many instances of diversification occurring in the late Miocene – early Pliocene (Figure 2). According to Mason's findings, the *B. nigroviridis* and *B. nubestris* lineage is estimated to have diverged from the other *Bothriechis* between 7.5 and 13 mya. The Mayan block (red) clade of *B. aurifer, B. bicolor,* and *B. rowleyi* as well as a largely Chortis (green) block consisting of *B. guiforroi, B. lateralis, B. marchi* and *B. thalassinus* are estimated to have diverged between 8.5 and 10 mya concordant with the West to East progression of the Chortis block along the Mayan block to its current position (Rogers, Mann and Emmet, 2007; Townsend, 2014). The *B. guifarroi/B. lateralis* and the *B.*

marchi/B. thalassinus lineages likely diverged shortly after whilst speciation of *B. aurifer, B. bicolor* and *B. rowleyi* likely occurred between 7 and 9 mya (Mason *et al.*, 2019).

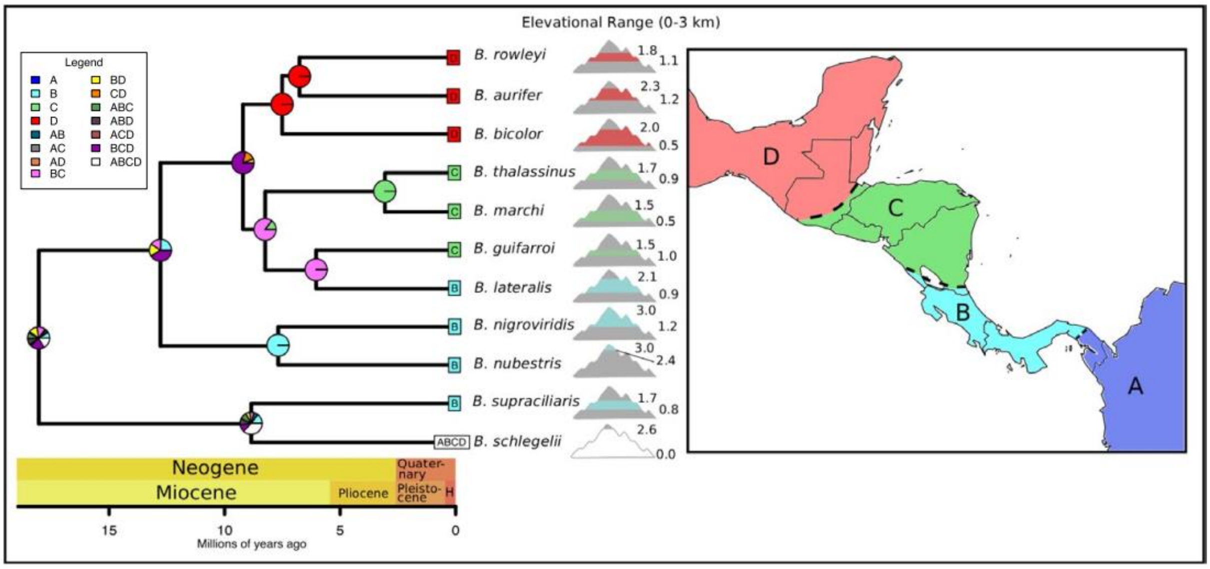

Figure 2; Ancestral area reconstruction from 'BioGeoBEARS' of Bothriechis. Piecharts on nodes indicate likelihood of originating in a given region. Biogeographic regions roughly correspond with South America (A; dark blue), the lower Middle American isthmus (B: light blue), the Chortís block region (C: green), and the Mayan block region (D: red). Mountain icons in the center of the figure indicate the elevational distributions of each species in km above sea level. Reproduced with permission (appendix 1) from (Mason *et al.*, 2019)

Of the 11 species listed within *Bothriechis* by ITIS, only 7 are listed by the IUCN meaning that 36.4% of *Bothriechis* species should be listed as either Not Assessed or Data Deficient by the IUCN. Those listed by the IUCN include two cloud forest endemics from Mexico; the Guatemalan palm viper (*Bothriechis aurifer*) and Rowley's palm viper (*Bothriechis rowleyi*). Both species are classified as Vulnerable due to severely fragmented ranges of less than 20,000 km caused by persecution from humans, collection for the pet trade, and ongoing habitat destruction primarily for agriculture (International Union for the Conservation of Nature, 2021).

Also listed and described in more detail further in this thesis is the Honduran palm pit viper (*Bothriechis marchi*), currently classified as Endangered and in decline due to a restricted geographic range and fractured habitat (Wilson and Townsend, 2014). Although it is not yet included by the IUCN, Guifarro's palm pit viper (*Bothriechis guifarroi*) has been recommended for classification as Critically Endangered due to its limited known area of occurrence in two cloud forest localities in Honduras, and the potential for anthropogenic damage to its habitat (Townsend *et al.*, 2013).

1.2.3 *Cerrophidion*

Although five species are currently recognised within this genus by ITIS, only three are featured by the IUCN Red List with one of these, the Petlalcala montane pit viper (*Cerrophidion petlalcalensis*), listed as Data Deficient. Both the Tzotzil montane pit piper (*Cerrophidion tzotzilorum*) and Godman's montane pit viper (*Cerrophidion godmani*) have been classified as of Least Concern (International Union for the Conservation of Nature, 2021), however, 60% of *Cerrophidion* species still lack classification. Neither the Costa Rica montane pit viper (*Cerrophidion sasai)* nor the Honduras montane pit viper (*Cerrophidion wilsoni*) are featured, however, neither were described until 2012 (Jadin *et al.*, 2012).

This genus typically ranges between approximately 1200 and 3500 metres above sea level throughout Central America (Campbell and Lamar, 2004; Kohler, Vesely and Greenbaum, 2005) and possess haemotoxic venom (Lomonte *et al.*, 2012). This venom has pro-coagulant effects that are particularly effective on mammals, aiding adult *Cerrophidion spp.* who are thought to primarily prey upon small mammals, lizards, and other snakes (Martins, Marques and Sazima, no date; Chaves *et al.*, 2014). Juveniles, however, typically prey upon invertebrates (Savage, 2005).

Castoe *et al.* (2009) found substantial genetic structure within *Cerrophidion godmani* consisting of four clades, however, Jadin *et al.* (2012) identified just three clades. These three clades were considered to be so divergent as to merit the recognition of two new species; *Cerrophidion wilsoni* and *Cerrophidion sasai.*

1.3 Conservation genetics

Conservation genetics refers to a subfield of population genetics that generally aims to understand the dynamics of genes within populations in order to support conservation and biodiversity (Frankham, 2005). At the core of conservation are three fundamental levels of biodiversity; genetic diversity, species diversity, and ecosystem diversity (Frankham, 2003). This diversity is important, as it provides a means for populations to adapt to changing environments, reducing extinction risk.

The extent and cause of population decline in snakes is often difficult to identify as snake population parameters are difficult to quantify (Altwegg et al., 2005; Waldron et al., 2006; Winne et al., 2007), however, it is clear that habitat fragmentation threatens biological diversity worldwide (Webb and Shine, 1998). Due to the typical low density of occurrence and limited dispersal ability, snake species are likely to be particularly sensitive to habitat fragmentation (Webb and Shine, 1998), although the extent of this can depend on various factors including body size, habitat type, and feeding preferences (Dodd and Barichivich, 2007). By studying the genetic variation and subsequent dispersal of these genes within a population, any fragmentation of habitats or prevention of gene flow can be identified and used to support conservation efforts to maintain the genetic health of a population and reduce vulnerability.

Key examples include the Hungarian meadow viper (*Vipera ursinii rakosiensis*) which was reduced to two fragmented populations in Hungary (Ujvari *et al.*, 2002) and one rediscovered population in Romania (Ghira, 2007). Chromosomal abnormalities, birth defects and a low survival rate among juveniles were reported as symptoms of inbreeding depression resulting from the fragmentation of the Hungarian populations, placing the species at risk of extinction (Ujvari et al., 2002). A study into the genetic variation between these two populations found a genetic differential, leading to the creation of a captive breeding program using genetically screened individuals to increase the genetic variability of the population (Pechy *et al.*, 2014).

A further example is the Orsini's viper (*Vipera ursinii*) which is considered one of the most threatened snake species in Europe due to its highly fragmented distribution (Ujvari et al., 2002; Ferchaud et al., 2011). Samples taken from 11 fragmented populations displayed 11 cytochrome b haplotypes with limited divergence, however, considerable genetic differentiation between the populations was observed (Ferchaud et al., 2011) leading to the possibility of captive breeding programs to increase the genetic variability within the populations.

In order to compare levels of genetic diversity among populations and species, comparisons with other studies are essential to determine whether results indicate a lower or higher level of diversity than what could be considered average. Table 1 details the results from five studies (Valenzuela, 2016; Nitschke *et al.*, 2018; Asadi *et al.*, 2019; Guo *et al.*, 2019; Kundu *et al.*, 2020) for comparison with an average haplotype diversity of 0.691649, however, it is difficult to make an exact comparison due to differences within each study such as species, sequence lengths, number of genes used, sample sizes, areas, and many other variables.

Table 1; Comparison of 5 studies detailing haplotype diversity of 17 snake species. n = sample size, k = total haplotypes, PS = polymorphic sites, H = haplotype diversity (Valenzuela, 2016; Nitschke *et al.*, 2018; Asadi *et al.*, 2019; Guo *et al.*, 2019; Kundu *et al.*, 2020)*. CytB = cytochrome B, ND4 =NADH dehydrogenase 4, C-mos = oocyte maturation factor Mos*

Reference	**Species**	**Sub-Group**	**n**	**k**	**PS**	**H**	**Genes**
(Kundu *et al.*, 2020)	*Ophiophagus hannah*	Overall	14	12	37	0.989	CytB
	Bungarus fasciatus	Overall	15	9	15	0.885	CytB
(Asadi *et al.*, 2019)	*Gloydius halys caucasicus*	Total	41	24	24	0.964	CytB, ND4, C-mos
		KD-EA	10	9	12	0.978	
		Cantiles	4	2	6	0.667	
		LarNP-CA	12	7	11	0.894	
		WA-Az	13	6	6	0.679	
(Guo *et al.*, 2019)	*P. mucrosquamatus*	CytB gene	174	57	153	0.838	CytB, ND4
		ND4 gene	179	34	76	0.711	
(Valenzuela, 2016)	*B. lojanus*	Overall	17	9	67	0.824	CytB, ND4
		Clade A	10	5	16	0.857	
		Clade B	7	4	10	0.533	
(Nitschke *et al.*, 2018)	*A. apraefrontalis*	WAC	12	6		0.879	CytB
	A. duboisii	WAC	11	4		0.491	
		Timor Sea	6	2		0.333	
	A. foliosquama	WAC	12	5		0.742	
		Timor Sea	8	4		0.821	
	A. laevis	WAC	44	12		0.817	
		Timor Sea	10	3		0.6	
		N&E AUS	7	2		0.286	
	A. tenuis/A. fuscus complex	WAC	6	3		0.733	
		Timor Sea	9	2		0.389	
	A. mosaicus	WAC	7	5		0.857	
		N&E AUS	6	2		0.533	
	E. annulatus	WAC	13	4		0.603	
		Timor Sea	8	3		0.714	
	H. elegans	WAC	19	6		0.819	
		N&E AUS	25	11		0.86	
	H. major	WAC	47	12		0.509	
		N&E AUS	9	2		0.222	
	H. ocellatus	WAC	26	5		0.351	
		N&E AUS	5	3		0.8	
	H. peronii	WAC	20	14		0.953	
		Timor Sea	3	2		0.667	
		N&E AUS	7	4		0.81	
	H. stokesii	WAC	16	5		0.65	
		N&E AUS	6	2		0.333	

1.4 Study Subjects

As the two most commonly seen species of pit viper within Cusuco National Park (CNP), this study focuses on the sympatric species; March’s palm pit viper (*Bothriechis marchi*) and the Honduras montane pit viper (*Cerrophidion wilsoni*).

1.4.1 Conservation Status

Although an IUCN conservation status of Critically Endangered has been claimed by Clegg using a preliminary study at CNP (Clegg, in prep), *B. marchi* is still currently classified as endangered by the IUCN Red List (Wilson and Townsend, 2014). Until this status was reached in 2014, however, the species was listed as Data Deficient. Due to the species’ small, restricted geographic range and the increasingly fractured habitat of lower montane and cloud forests at elevations of 500-1800m, *B. marchi* is considered in decline and a high priority species for conservation with a high Environmental Vulnerability Score (EVS) of 16 out of 19 (Wilson and McCranie, 2003; Wilson and Townsend, 2014), and is believed to have diverged from *Bothriechis’* common ancestor approximately 8.5-10mya (Mason *et al.*, 2019).

C. wilsoni, however, was initially classified under *Cerrophidion godmani* (Godman’s montane pit viper), currently listed as of Least Concern by the IUCN Red List (Chaves *et al.*, 2014). A phylogenetic and morphological study by Jadin *et al*. (2012) generated further support for three distinct species-level clades that are allopatrically distributed in the highlands of Middle America as suggested in previous studies (Castoe *et al.*, 2009; Daza, Castoe and Parkinson, 2010). Estimations of divergence times within these clades infer a shared common ancestor between 7.7 and 11.5mya, with the most recent divergence between *C. sasai* and *C. wilsoni* occurring between 3.1 and 6 mya (Castoe *et al.*, 2009; Daza, Castoe and Parkinson, 2010; Jadin *et al.*, 2012). While *C. wilsoni* has not yet been given a classification by the IUCN, it is scored as 12 on the Environmental Vulnerability Score (EVS) (Wilson and McCranie, 2004b) placing it as of less concern than *B. marchi*.

1.4.2 Ecology

B. marchi is known to be arboreal, however Lonsdale's (2021) findings suggest that juveniles tend to remain higher in the trees compared to larger individuals as is also noted in other *Bothriechis spp* in Guatemala. Most *Bothriechis spp* are also known to be nocturnal (Lomonte *et al.*, 2008), however, Lonsdale reported approximately a third of *B. marchi* individuals were found during the day. In contrast, *C. wilsoni* is a terrestrial, diurnal species (Chaves *et al.*, 2014), which can often be found in open and disturbed habitats, making this species an ideal comparison to measure the effects of habitat fragmentation within the park.

1.4.3 Habitat and distribution

B. marchi is primarily found in Northern Honduras, though may extend into eastern Guatemala (Campbell and Lamar, 2004). Although rare to be found below an elevation of 900 m, its reported range is between 500 m to above 1,500 m (Wilson and Townsend, 2014). This species generally prefers intact, closed-canopy mid elevation rainforest, and is often found near streams or rivers (Townsend and Wilson, 2008), while *C. wilsoni* inhabits lower montane wet forest such as cloud forest, pine-oak forest, lower montane dry forest, and high montane forest at higher elevations (Chaves *et al.*, 2014).

1.4.4 Feeding Habits

B. marchi was initially believed to feed almost exclusively on amphibians (McCranie and Castaneda, 2005; Townsend and Wilson, 2008; McCranie, 2011) which would leave *B. marchi* particularly affected by the reduction of amphibian populations locally due to chytridiomycosis (Kolby and Padgett-Flohr, 2009), which is causing enormous problems for amphibians as whole worldwide (Longcore, Pessier and Nichols, 1999; Bosch, Martinez-Solano and Garcia-Paris, 2001; Wilson and Townsend, 2014). Juveniles were observed consuming small mammals during a recent study however, suggesting that the species is more opportunistic than previously thought (Solis and Brown, 2015).

This movement between dietary niches may introduce interspecific competition with other species whose ecological niche also involves these small mammals as a food source, such as *C. wilsoni*. Competition between these two species to fill the same ecological niche may result in the least well-adapted species being outcompeted with disastrous consequences for that species (Pianka, 1981; Szyndlar and Rage, 1999). Alternatively, an evolutionary response may develop resulting in a form of niche partitioning to reduce competition, allowing for the successful coexistence of both species.

As the least understood of all *Cerrophidion* species (Schramer *et al.*, 2018), there is little information available in regard to the feeding habits of *C. wilsoni.* Historically, a single large rodent (unidentified) was reported as a prey item, and on the 24th July 2017 a *C. wilsoni* was observed preying on a giant whiptail lizard (*Aspidoscelis motaguae*) (Schramer *et al.*, 2018). As *C. wilsoni* was originally considered to be *C. godmani*, previous information detailing the feeding habits of *C. godmani* could be used to infer a greater understanding of the potential prey choices for *C. wilsoni.* The prey items listed in full in appendix 2 include arthropods, amphibians, birds, mammals, and reptiles including *Bothriechis aurifer* and another *C. godmani* (Schramer *et al.*, 2018). This indicates that *C. wilsoni* is a more generalist feeder allowing the species more adaptability with less reliance on streams in comparison with *B. marchi,* potentially reducing the risk of competition between the two species.

1.5 Deforestation and Genetic Fragmentation

The countries of Central America are the fastest growing of the American tropics, with the population of Honduras estimated in 2004 to double within 25 years (Wilson and McCranie, 2004a). This growth drives the need for additional resources, housing, transport infrastructure, agriculture, and many other forms of development that negatively affect the areas wildlife and surrounding habitat, causing the habitat degradation and destruction that are recognised as major threats to biodiversity today (Raven and Berg, 2001). Honduras has one of the worst deforestation rates globally in protected areas (Hansen *et al.*, 2013) as well as one of the highest general deforestation rates of any Central American country (Magrin *et al.*, 2014). By 1995, only 37% of the original forested area of the country remained and it has been predicted that only 0.5 million hectares will remain by 2085 (Wilson and Perlman, 2000). The trend in forest loss has been increasing since, with models predicting that the area of deforested land will double over the next 20 years (Hoskins, 2019).

Satellite imagery suggests that CNP lost more than 7% (approx. 1,759 ha) of forest between 2000 and 2017 with an average annual loss of 103 ± 99 ha (Hoskins, 2019; Martin *et al.*, 2021). Annual deforestation rates have been variable, but have been worse in recent years suggesting that this threat is accelerating (Martin *et al.*, 2021), which may further fragment populations alongside the natural fragmentation caused by the mountainous landscape. This could cause inbreeding depression, leading to a decrease in genetic variation which can then decrease the fitness of the populations. Within the small populations in CNP, the influence of genetic drift is increased which may lead to increased homozygosity and further loss of genetic variation.

Evidence of Genetic Fragmentation within CNP

A recent haplotype network analysis (appendix 3) of the mitochondrial structure of *B. marchi* within Cusuco National Park did not reveal a clear substructure in the population (Clegg, 2014), however, only one mtDNA gene was used in the study. Two of the 24 haplotypes found were restricted to a single sampling location (Base Camp) on the eastern side of CNP's core zone, which may indicate a lack of connectivity between the west and east sides of the park. However, investigation of additional mtDNA genes and, ideally, the inclusion of nuclear markers, would help further evaluate this hypothesis, especially given the maternal inheritance of mtDNA genes and the philopatric nature of most female snakes (Zhang and Hewitt, 2003).

Distribution and Resistance Models

Habitat suitability modelling using the software MaxEnt (Phillips, Dudik and Schapire, 2004; Phillips, Anderson and Schapire, 2006) has previously indicated a higher level of habitat fragmentation for *B. marchi* (Clegg, 2014; Master, 2017; Oliveira, 2021) compared to *C. wilsoni,* and predicted climate change scenarios also suggest that *B. marchi* may be more vulnerable to reduced future habitat suitability causing increased fragmentation (Master, 2017). Resistance models (appendix 4) indicate that the high elevations and fewer streams around Cantiles camp cause moderate resistance to movement for *B. marchi* that does not occur for *C. wilsoni* (Oliveira, 2021).

2. Aims and Objectives

This study aims to evaluate the levels of genetic diversity within the *B. marchi* and *C. wilsoni* populations in Cusuco National Park at a mtDNA level, and to assess the spatial distribution of haplotypes for evidence of genetic structuring. The study will seek to determine if either species is more genetically impoverished or has a greater genetic structure, and will determine whether limited gene flow is an issue for either species within Cusuco National Park. This aim will be supported by the analysis of previous spatial data, the creation of haplotype networks, and distribution mapping.

3. Methodology

Tissue samples, in the form of ventral scale clips, and data regarding the number of each species located per year and their locations were collected and provided by Stephen Green and others as part of an ongoing project within Cusuco National Park by Operation Wallacea Ltd (www.opwall.com).

Specialising in coral reef and tropical forest conservation and ecology, Operation Wallacea is an international conservation research organisation that has been monitoring cloud forest biodiversity in Cusuco National Park since 2006 (Green *et al.*, 2012). Research is conducted annually using seven research camps from mid-June to mid-August to investigate species abundance, diversity and distribution, as well as to monitor the effects of habitat degradation and anthropogenic disturbance on biodiversity over time (Green *et al.*, 2012).

3.1 Study Site

3.1.1 Sample Site; Cusuco National Park

The study site is a 234 km^2 protected area located within the Sierra de Omoa in Central America named Cusuco National Park, featuring approximately 23,440 ha of rare high altitude cloud forest within the Cordillera de Merendon in Northwest Honduras (Kolby, 2009; Opwall Trust, 2016). It is located to the east of Guatemala, beyond the Matagua-Polochic fault.

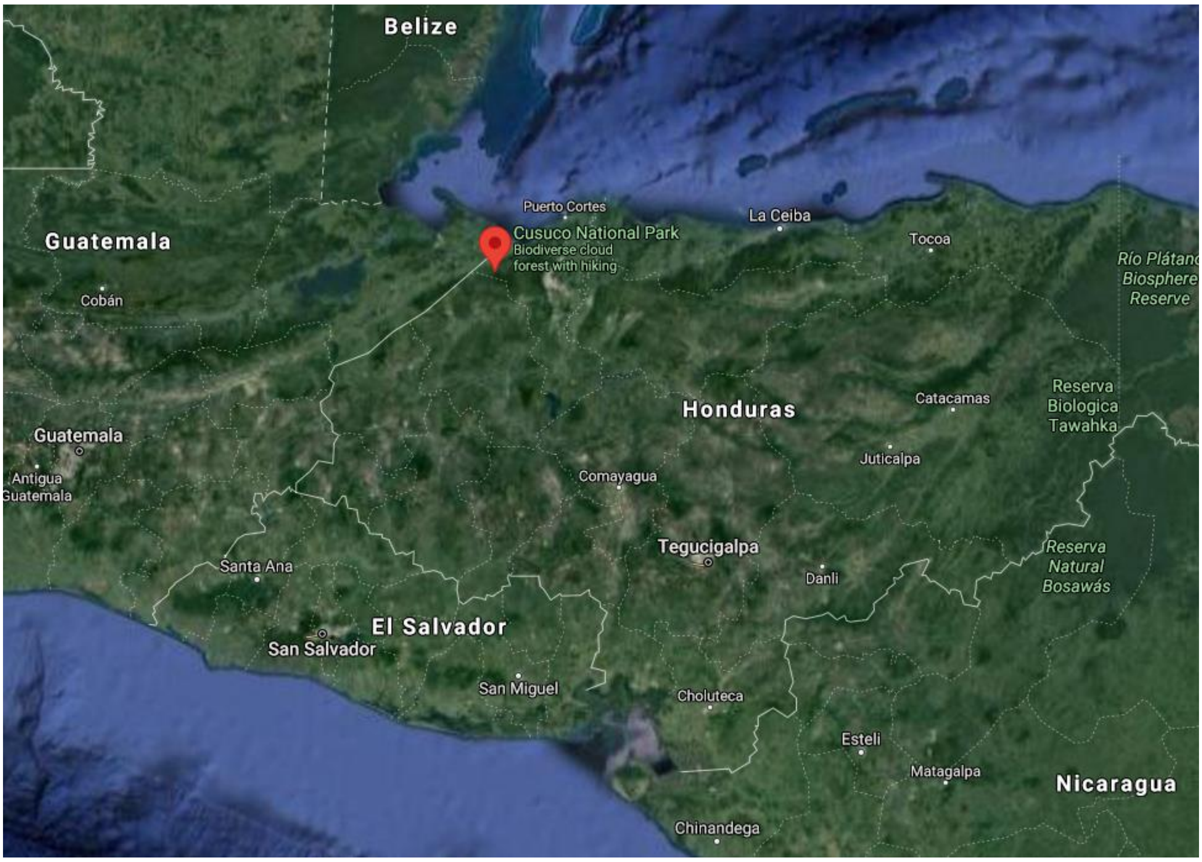

Figure 3; Satellite image of Cusuco National Park within Central America (Google Maps, 2021)

The park was established as part of the Sistema Nacional de Áreas Protegidas de Hondura (SINAPH) after the passing of the Cloud Forest Act (Act 87 – 1987) (Bonta, 2005; Martin and Blackburn, 2009), primarily to protect the watershed that supplies Sula Valley and the second-largest city in Honduras; San Pedro Sula (population ~700,000) (Townsend and Wilson, 2008). The Park is adjacent to three other protected areas, two of which are forest regions protected under Act 53 – 1959 (Zona Forestal No2) and Act 210-1985, as well as a Water Protection Zone under Act 46-1990.

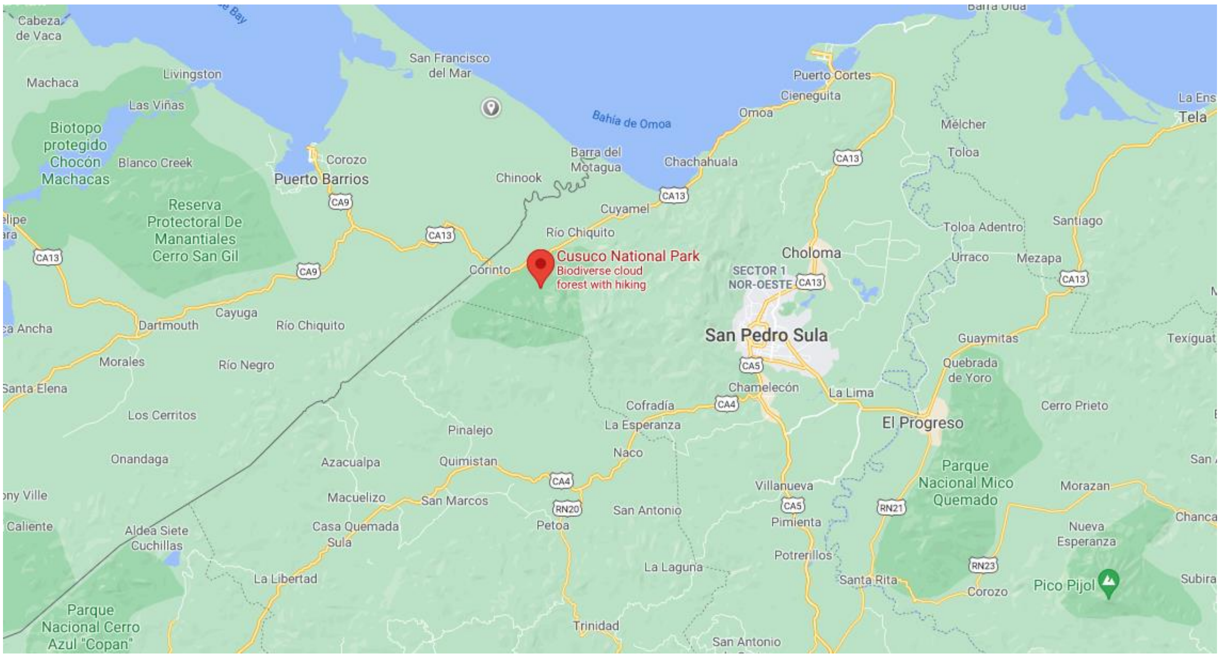

Figure 4; Map of Cusuco National Park within Honduras (Google Maps, 2021)

The park consists of a 76.9 km^2 core zone surrounded by a 157.5 m^2 buffer zone within which both primary and secondary forests are present (Kolby, 2009), including lower montane tropical rainforest with patches of both primary cloud forest and upper montane rainforest featuring a maximum elevation of 2245 m (Jocque *et al.*, 2013). Monthly average temperatures range from 12.9^{o}C to 23.1^{o}C with an annual rainfall of nearly 3000 mm (Wilson and McCranie, 2004b).

As well as being considered the 25th most irreplaceable site of over 173,000 protected areas for threatened amphibians, CNP is also listed within the top 100 irreplaceable sites for threatened birds and mammals making the park and its exceptional level of species richness a site of particular importance for biodiversity conservation (Le Soaut *et al.*, 2013). Despite this importance, CNP has suffered extensive deforestation primarily on the western side of the park (appendix 5). An apparently dramatic increase in deforestation was discovered in the Summer of 2013 during a routine annual biodiversity monitoring program funded by Operation Wallacea (Opwall Trust, 2016).

Survey data from this organisation along with the findings of other surveys completed within the park indicates that over 966 taxa have been detected to a species-level in CNP to date (Martin *et al.*, 2021). Of these species, 37.5% (n=338) are Mesoamerican endemics, with a further 67 threatened or near threatened species including nine Critically Endangered species. An additional three species are listed as data-deficient and 49 species are micro-endemics only found in the Merendon range.

CNP is acknowledged as an internationally important site particularly for threatened amphibians and reptiles (Saout *et al.*, 2013). Of the 102 species of herpetofauna that have been found within the park, 70.6% (n=72) are regional endemics and the remaining 30.4% (n=31) are threatened or near-threatened species, including 8 micro-endemics known only from CNP and all of which are IUCN listed. In addition, the park is a key stronghold to a further three Critically Endangered or Endangered species endemic to the Merendon Range.

Over half of the 45 snake species identified within CNP have been detected on less than 10 occasions between 2007 and 2017 with 8 of these species having been detected only once (Gilroy *et al.*, 2017). Of these 45 species, five are endemic to Honduras with three being endemic to CNP (Gilroy *et al.*, 2017). The two most frequently observed species within the park are the terrestrial *Cerrophidion wilsoni* and the arboreal *Bothriechis marchi*, both of which were considered endemic to Honduras (Gilroy *et al.*, 2017) however *C. wilsoni* has recently been discovered in Nicaragua near to the Honduras border (Fernandez *et al.*, 2017).

3.1.2 Sample Collection

As only qualified members of the Operation Wallacea team are permitted to process venomous snakes, tissue samples were only available from five of the eight sites located within the core area of Cusuco National Park in Honduras (Figure 5). Two of these sites, El Danto (1,550 m elevation) and El Cortecito (1,397 m elevation), were from the western side of CNP while the remaining three sites, Guanales (1,220 m elevation), Cantiles (1,828 m elevation) and Base Camp (1,546 m elevation), were located on the eastern side of CNP. Transects were discontinued from Santo Tomas camp due to excessive deforestation.

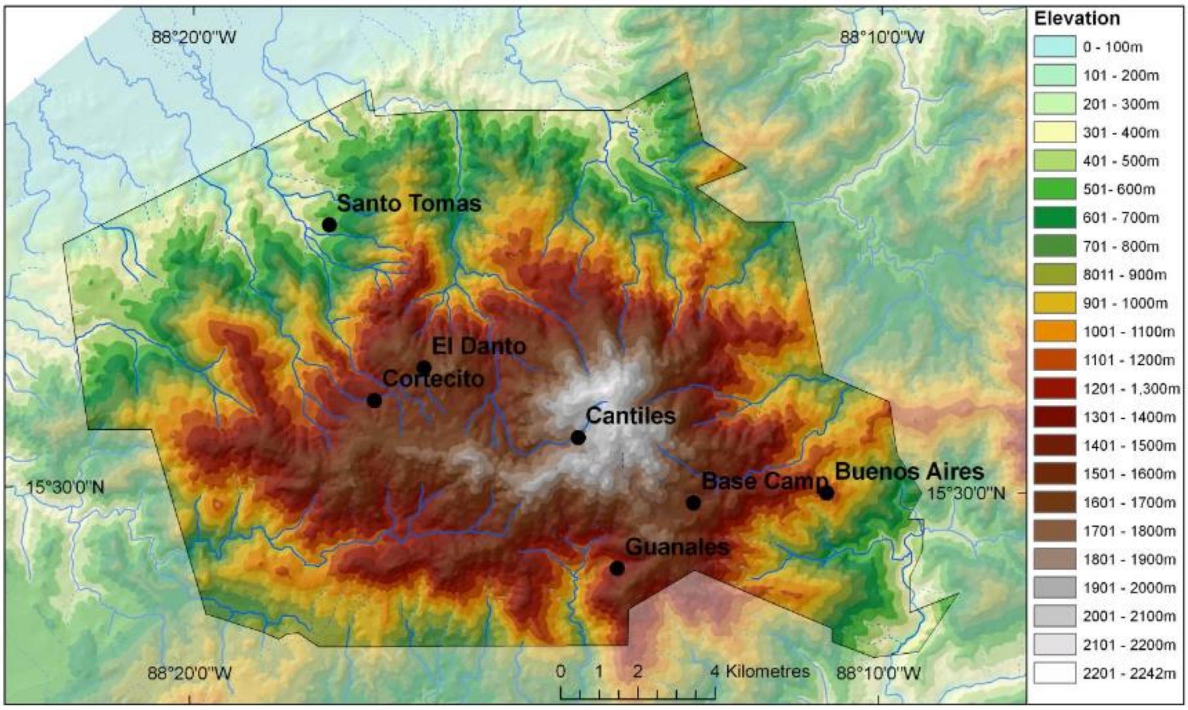

Figure 5; GIS map of the Cusuco National Park showing elevation and the locations of 7 Operation Wallacea research camps. An eighth camp, Capuca, to the north of base camp, was also surveyed for one season but does not form part of the core survey network and so is not displayed here (Long and Field, 2008)

Snakes were searched for during diurnal and nocturnal Visual Encounter Surveys (VES) both opportunistically and along designated transects leading out of the research sites and alongside nearby streams. Survey effort was variable between years but can be roughly approximated by the number of recorded transect surveys per year (Table 1). Any snakes encountered were captured and secured using appropriate techniques (snake hooks/tongs and clear plastic handling tubes), in order to be measured (SVL and tail), weighed, sexed and photographed. Up to three ventral scale clips were taken using a pair of fine scissors and stored in 1.5ml vials of 95% ethanol. These scales were exported to Cornwall College, Newquay, under the required export and import permits for use in this study (Green *et al.*, 2012).

Table 2; The total number of transects surveyed within five camps per year. Note that river surveys were only recorded as opportunistic surveys until standardised river transects were established in 2013, thus, explaining the noticeable increases in transect survey effort from 2013 to present. Data extracted from the 2017 Operation Wallacea Report (Gilroy et al., 2017) *and augmented with data from the 2018 field season.*

Camp	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	Total
Base Camp	3	9	18	24	20	54	55	69	55	48	21	**383**
Cantiles	10	12	0	27	14	25	27	44	29	31	21	**256**
El Corticito	4	4	5	20	11	24	21	22	23	15	22	**178**
El Danto	12	3	5	15	4	19	29	20	19	20	18	**188**
Guanales	4	11	4	38	28	47	33	49	41	42	26	**340**
Total	**33**	**39**	**32**	**124**	**77**	**169**	**165**	**204**	**167**	**156**	**108**	**1345**

3.2 Molecular Laboratory Methods

DNA was extracted from whole tissue samples using the DNeasy Blood & Tissue Kit (Qiagen, 2020a) at Cornwall College in Newquay.

3.2.1 Gene Primers & Protocols

The mitochondrial genomes of snakes are characterised by an accelerated evolutionary rate among vertebrates (Jiang *et al.*, 2007; Douglas and Gower, 2010) which is ideal for this study. Due to the lack of pre-existing microsatellite markers for either *B.marchi* or *C.wilsoni,* and the inability to develop suitable markers with the resources available, this study was limited to the markers available as part of the larger ongoing study by Operation Wallacea. Samples were sequenced for 5 mitochondrial genes; cytochrome oxidase subunit 1 (CO1), NADH dehydrogenase 4 (ND4), cytochrome b (CytB), and the 12S and 16S ribosomal RNA genes (12S & 16S). A set of primers were also tested for 3 nuclear genes; Recombination activating gene 1 (RAG1), recombination activating gene 2 (RAG2), and oocyte maturation factor Mos (C-MOS).

3.2.1.1 CO1

Forward primer CO1_F1 5’-acagtttaccgctttaatcagcca-3’ and reverse primer CO1_R1 5’-ataggtggcagttaaagtctgtctc-3’ were used for amplification, however only CO1_F1 was used during sequencing with an additional internal forward primer, CO1_F3_vip (Stümpel, 2012; Zinenko *et al.*, 2015). PCR was carried out using the TopTaq Mastermix Kit (Qiagen, 2020b). The amplification conditions began with an initial denaturation step at 95°C, followed by 35 cycles as follows; 45 seconds denature at 94°C, 45 seconds annealing at 57°C, and 120 seconds extension at 72°C. Amplification was concluded with a final extension of 5 minutes at 72°C.

Due to an abundance of primer dimer, various attempts using primer concentrations ranging from a 2.5ul volume to a 10ul volume were used in order to get the required sequences.

3.2.1.2 ND4

Forward primer NADH4 5’-cacctatgactaccaaaagctcatgtagaagc-3’ and reverse primer LeuR 5’-cattacttttacttggatttgcacca-3’ were used for amplification (Arevalo et al.,1994), however, the amplified PCR product was sequenced in the forward direction only due to the short PCR product of approximately 800bp.

PCR was carried out using the TopTaq Mastermix Kit (Qiagen, 2020b). The amplification conditions began with an initial denaturation step at 94°C for 3 minutes, followed by 35 cycles as follows; 30 seconds denature at 94°C, 30 seconds annealing at 60°C, and a 1 minute extension at 72°C. Amplification was concluded with a final extension of 10 minutes at 72^{C} (Arevalo, Davis, and Sites, 1994).

3.2.1.3 CytB

Although forward primer CytB_F1 and reverse primer CytB_RC were initially used for amplification (Stümpel, 2012; Zinenko *et al.*, 2015), it was unfortunately not possible to consistently sequence the entirety of the Cytochrome B gene for these samples, possibly due to DNA degradation. Therefore, shorter lengths from the beginning and end were successfully amplified using two separate primer pairs from Lawson, Slowinski, and Burbrink (2004).

Fragment one was amplified, and later sequenced, using primers L14910 5'-gacctgtgatmtgaaaaaccaycgttgt-3’ and H15149 5’-ccctcagaatgatatttgtcctca-3’ at a 5uM concentration (Lawson, Slowinski and Burbrink, 2004), while fragment two amplified and sequenced using primers L15584 5’-tcccattycacccatacca-3’ and H16064 5’-ctttggtttacaagaacaatgcttta-3’ also at a 5uM concentration (Lawson, Slowinski and Burbrink, 2004).

PCR was carried out using the TopTaq Mastermix Kit (Qiagen, 2020b). The amplification conditions began with an initial denaturation step at 95°C for 2 minutes, followed by 35 cycles as follows; 40 seconds denature at 94°C, 60 seconds annealing at 40°C, and 66 seconds extension at 72°C. Amplification was concluded with a final extension of 5 minutes at 72°C (Stümpel, 2012; Zinenko *et al.*, 2015).

3.2.1.4 12S & 16S

The 12S and 16S gene regions were amplified using primers from Pook e*t al.* (2009). The primers used for the 12S gene were L1091 5’-aaactgggattagataccccactat-3’ and H1557 5’-gtacacttaccttgttacgactt-3’, while the primers used for the 16S gene were L2510 5’-cgcctgtttatcaaaaacat-3’ and H3059 5’-ccggtctgaactcagatcacgt-3’ (Pook *et al.*, 2009).

PCR was carried out using the TopTaq Mastermix Kit (Qiagen, 2020b). The amplification conditions began with an initial denaturation step at 95°C for 3 minutes, followed by 35 cycles as follows; 30 seconds denature at 95°C, 45 seconds annealing at 43°C, and 60 seconds extension at 72°C. Amplification was concluded with a final extension of 5 minutes at 72°C.

3.2.1.5 Nuclear Genes

Three sets of nuclear gene markers were tested as follows; L2408 and H2928 for RAG1 (Fitness, Hitchmough and Morgan-Richards, 2012), L562 and H1306 for RAG2 (Hawlitschek, Nagy and Glaw, 2012), and G74 and G74 for C-mos (Saint *et al.*, 1998) under the PCR conditions outlined in Table 2.

Table 3; PCR conditions used for nuclear genes RAG1, RAG2, and C-mos

			RAG1	**RAG2**	**C-mos**
	Initial Denaturation	**Time**	1min	3min	3min
		Temp	94 °C	94 °C	94 °C
35 Cycles	**Denature**	**Time**	20sec	30sec	45sec
		Temp	94 °C	94 °C	94 °C
	Annealing	**Time**	15sec	30sec	45sec
		Temp	50 °C	47 °C	48 °C
	Extension	**Time**	90sec	60sec	60sec
		Temp	72 °C	72 °C	72 °C
	Final Extension	**Time**	5min	10min	6min
		Temp	72 °C	72 °C	72 °C

3.2.2 Sequencing & Purification

PCR products were purified using the Invitrogen Purelink PCR Purification Kit (ThermoFisher Scientific, 2021) and sequencing was completed by Macrogen-Europe using their EZsequence bag service (Macrogen, 2021)

3.3 Molecular Analysis

3.3.1 Consensus Sequences and Alignment

Sequence data was checked by eye, and consensus sequences created from forward and reverse sequence reads for the CO1 gene using the Bioedit software (Hall, 1999). Checked 'consensus' sequences were then aligned using the Molecular Evolutionary Genetics Analysis software (MEGA) (Kumar, Stecher and Tamura, 2015).

3.3.2 Haplotype Networks

Haplotype networks were created using Network 10.0 (Bandelt, Forster and Rohl, 1999), while the required Sequential Nexus file was generated using the online format conversion tool (Triad National Security, 2016). The median joining method was used for these networks, as it was designed for non-binary data, using the programs default parameters.

3.3.3 GIS Maps

Maps of snake encounter locations and resulting haplotype records were created on ArcMap 10.5 as part of ArcGIS (Esri, 2021), using map layers provided by Operation Wallacea (Burdgis, 2021). For snake encounter records with missing GPS coordinates (where these could not be traced back to a known location on the transect network), the record could only be linked back to the nearest research camp location. Therefore, to avoid records with identical spatial coordinates overlaying one another, the 'Disperse Markers' function within ArcMap was used. The system for this study is located under Projected Coordinate Systems>UTM>WGS 1984>Northern Hemisphere>**WGS 1984 UTM Zone 16N.**

4. Results

4.1 Abundance and Distribution

Between 2008 to 2018, a total of 192 *B. marchi* and 450 *C. wilsoni* encounters were recorded near to the 5 camps included in this study. This ranged from a single *B. marchi* encounter in 2010 to 34 encounters in 2015, while *C. wilsoni* encounters ranged from 8 encounters in 2010 to 78 encounters in 2014 (figure 6). Encounters of *C. wilsoni* have generally been higher than *B. marchi* encounters each year, with the exception of 2012 where they were roughly equal.

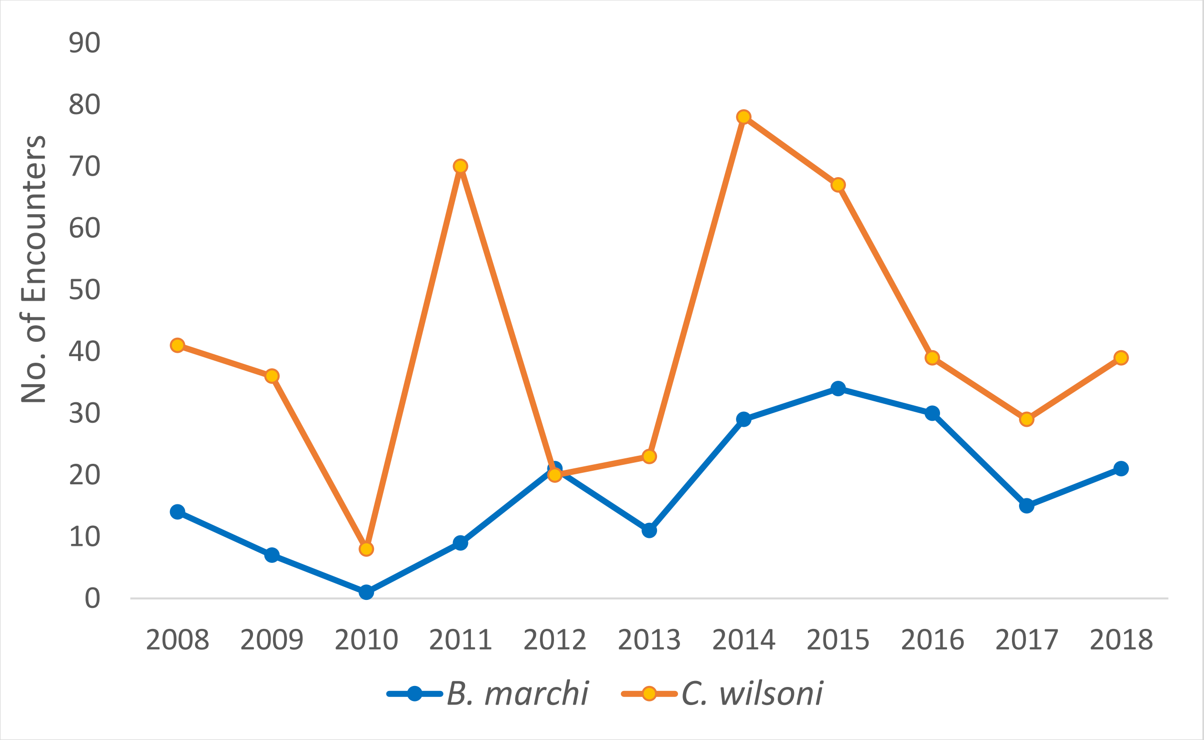

Figure 6; The total number of annual recorded encounters for B. marchi and C. wilsoni in Cusuco National Park, Honduras between 2008 and 2018

However, when corrected for survey effort (number of transect surveys completed in a given year) (figure 7), the average number of *B. marchi* encounters recorded per transect ranged between 0.42 in 2008 to 0.02 in 2018. The average number of *C. wilsoni* encounters per transect ranged from to 1.24 in 2008 to 0.06 in 2013 for *C. wilsoni*. Both species showed a decline in relative encounter rate during the study, with the highest encounter rates recorded for both species in 2008.

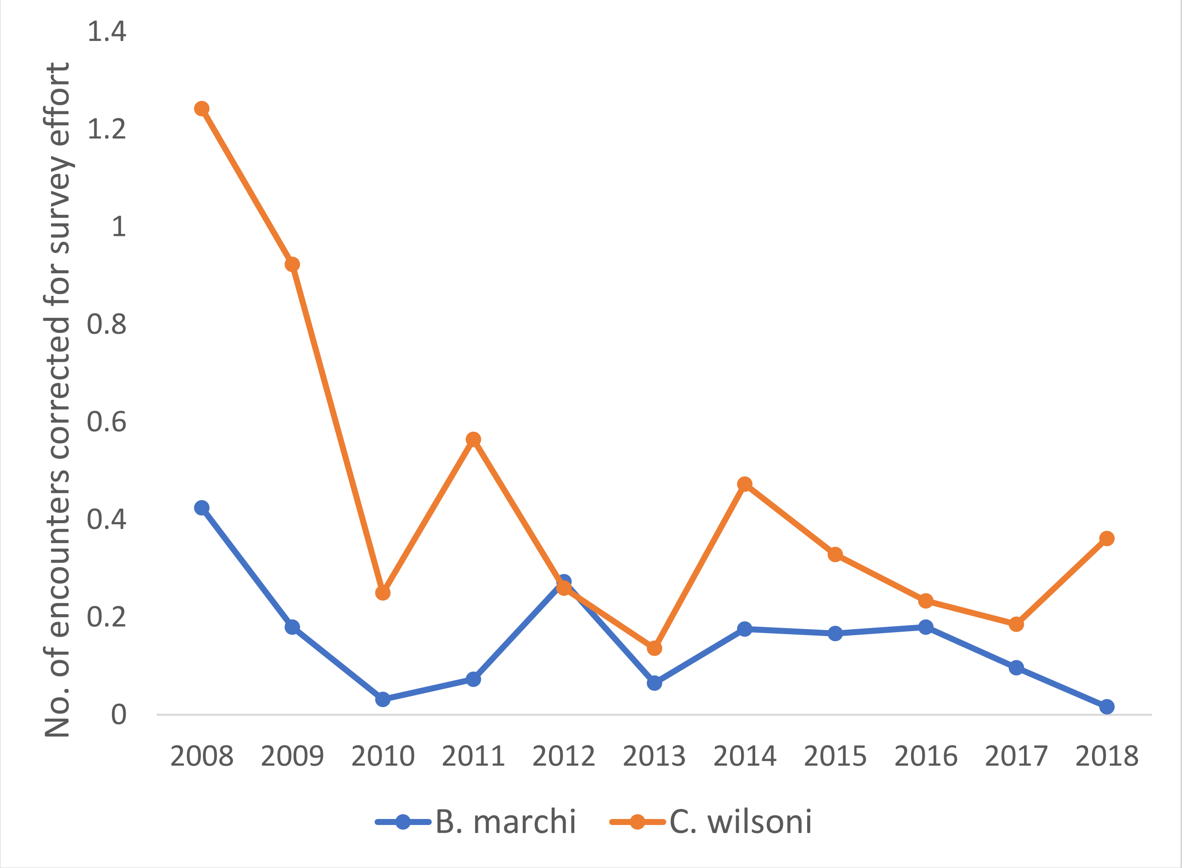

Figure 7; Total number of annual recorded encounters for B. marchi and C. wilsoni in Cusuco National Park, Honduras corrected for survey effort (table 1). The total includes opportunistic encounters.

The majority of *C. wilsoni* encounters were around Cantiles camp in the middle of the park (figure 8) with an average of 1 encounter per transect in this area, while Guanales appears to have the lowest abundance of both species with an average of below 0.1 sightings per transect and a low of just 0.02 *C. wilsoni* encounters per transect. The western camps of El Corticito and El Danto appear to have the highest abundance of *B. marchi* with approximate averages of 0.5 and 0.3 encounters per transect respectively.

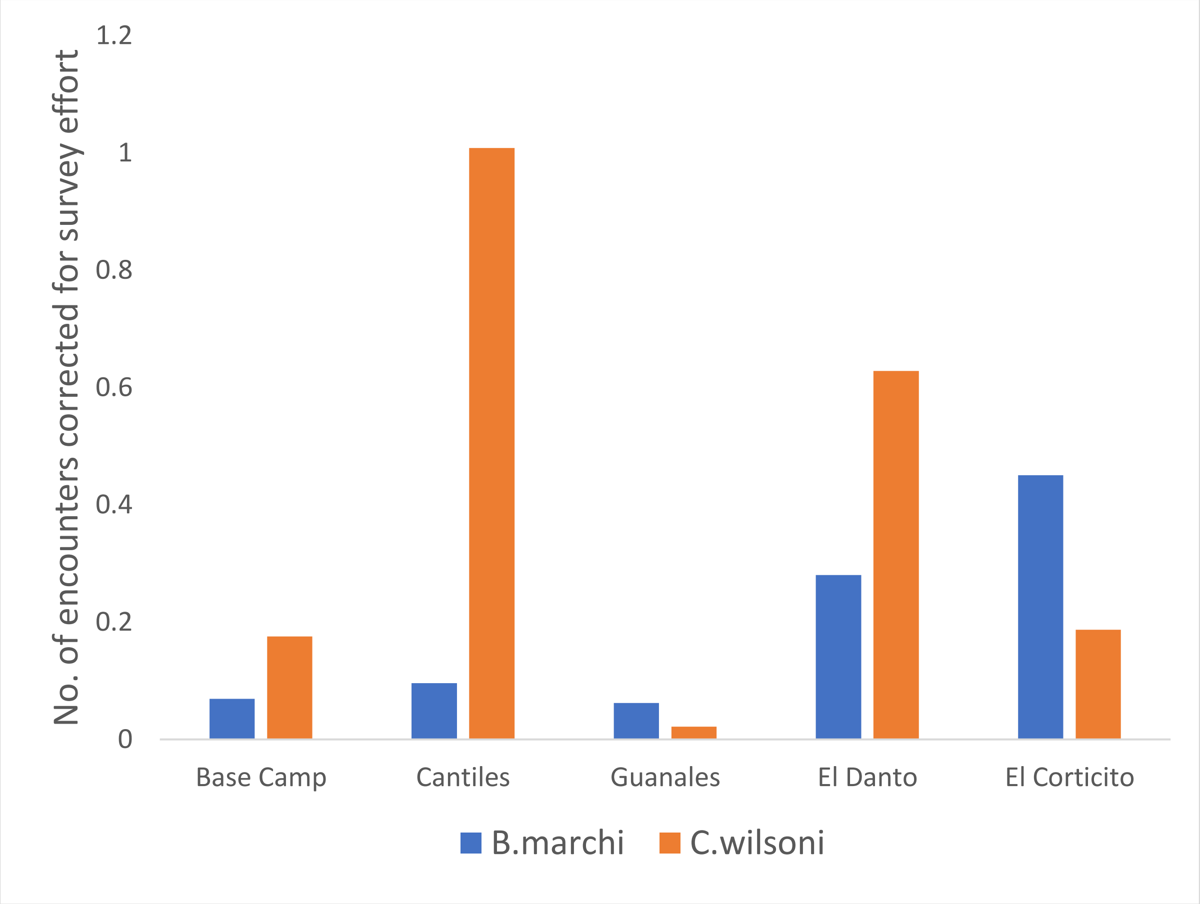

Figure 8; The number of annual recorded encounters of B. marchi and C. wilsoni in Cusuco National Park, Honduras corrected for survey effort for each research camp between 2008 and 2018, The total number of *B. marchi* encountered per camp varied annually as seen in figure 9. Guanales camp yielded the least encounters over ten years (n-20) ranging between 0 and 8 annually, while El Corticito displayed the most encounters (n=77) ranging between 0 and 20. El Danto camp also had a high number of encounters (n=46) ranging between 0 and 11.

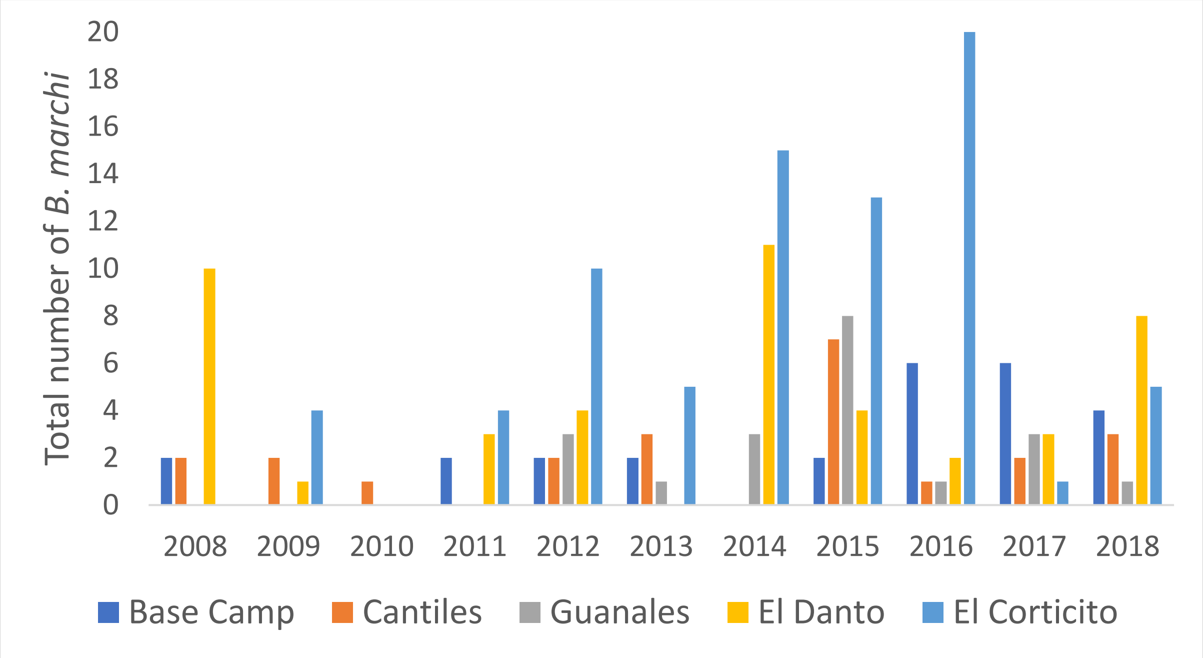

Figure 9; Total number of B. marchi recorded annually per research camp at Cusuco National Park, Honduras

Of 138 *B. marchi* encounter records featuring the location of capture between 2007-2018 (figure 10), 71% (n=98) of individuals were found on the North-Western side of the park with 27% (n=37) attributed to the El Danto camp and 43% (n=60) attributed to the El Corticito camp. An additional single individual was located outside the core zone at Santo Tomas camp. The remaining 29% found in the South-Eastern side of the park consisted of 12% (n=16) attributed to Cantiles camp, 10% (n=14) attributed to Base Camp, and 6% (n=9) attributed to Guanales camp. Again, a single individual was found outside the core zone at Buenos Aires

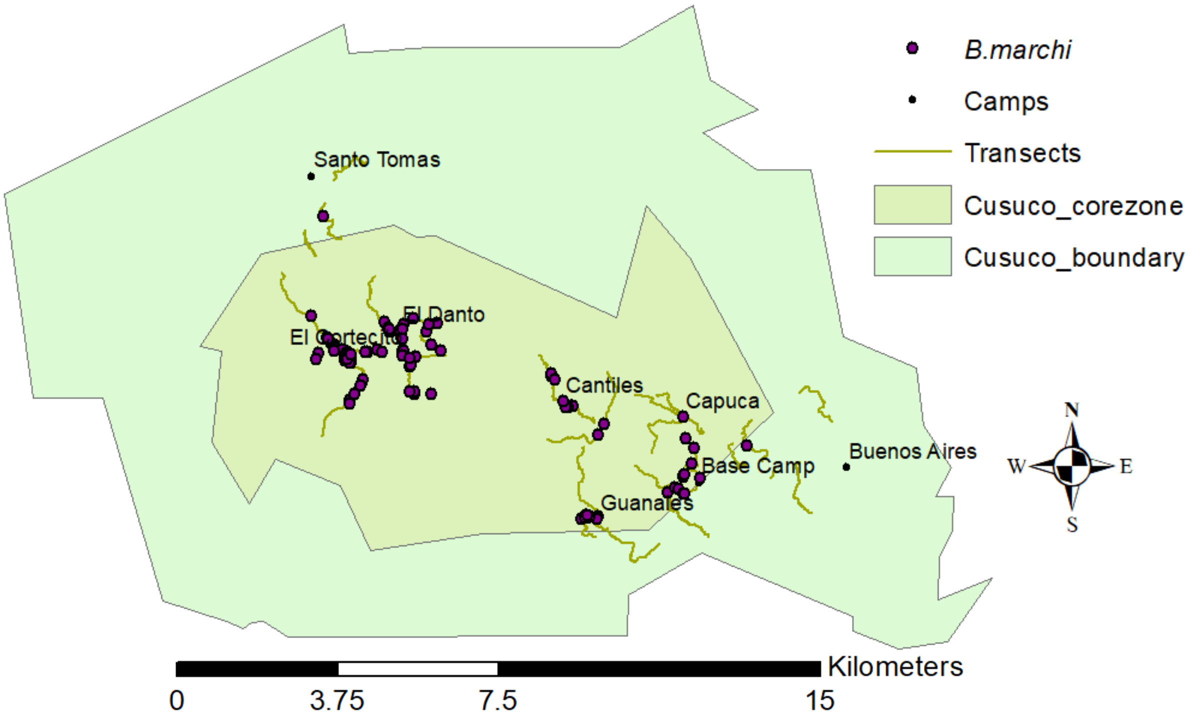

Figure 10; GIS map of recorded B. marchi encounters (n=138) within Cusuco National Park, Honduras between 2007-2018

The total number of *C. wilsoni* encountered per camp also varied annually as seen in figure 11 but were predominantly higher around Cantiles camp every year except for 2018. Guanales camp yielded the least encounters over ten years (n-7) ranging between 0 and 4 annually, while Cantiles recorded 54% of all encounters (n=242) ranging between 3 and 46. El Danto camp also had a high number of encounters (n=103) ranging between 1 and 17.

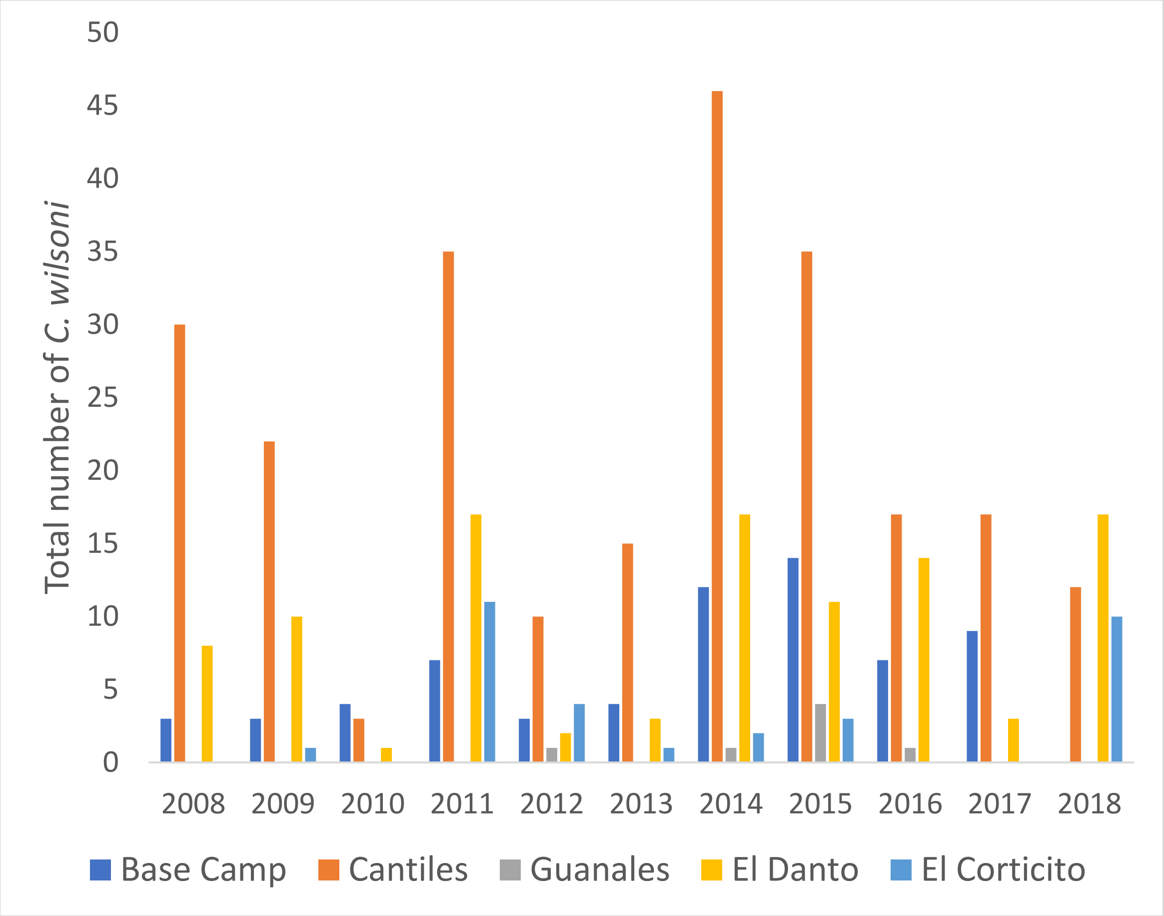

Figure 11; The total number of C. wilsoni recorded annually per research camp (and associated transect network) in Cusuco National Park, Honduras

Of the 450 *C. wilsoni* records featuring the location of capture between 2007-2018 (figure 12), 33% (n=147) of individuals were found on the northwest side of the park with 28% (n=124) attributed to the El Danto camp and 5% (n=23) attributed to the El Corticito camp. Two further individuals were located outside the core zone around Santo Tomas camp. The remaining 67% found on the southeast side of the park consisted of 49% (n=220) attributed to Cantiles camp, 12% (n=55) attributed to Base Camp, 2% (n=7) attributed to Guanales camp, and an additional 4% (n=19) attributed to the Capuca camp in 2015 as this was the only year in which Capuca was used.

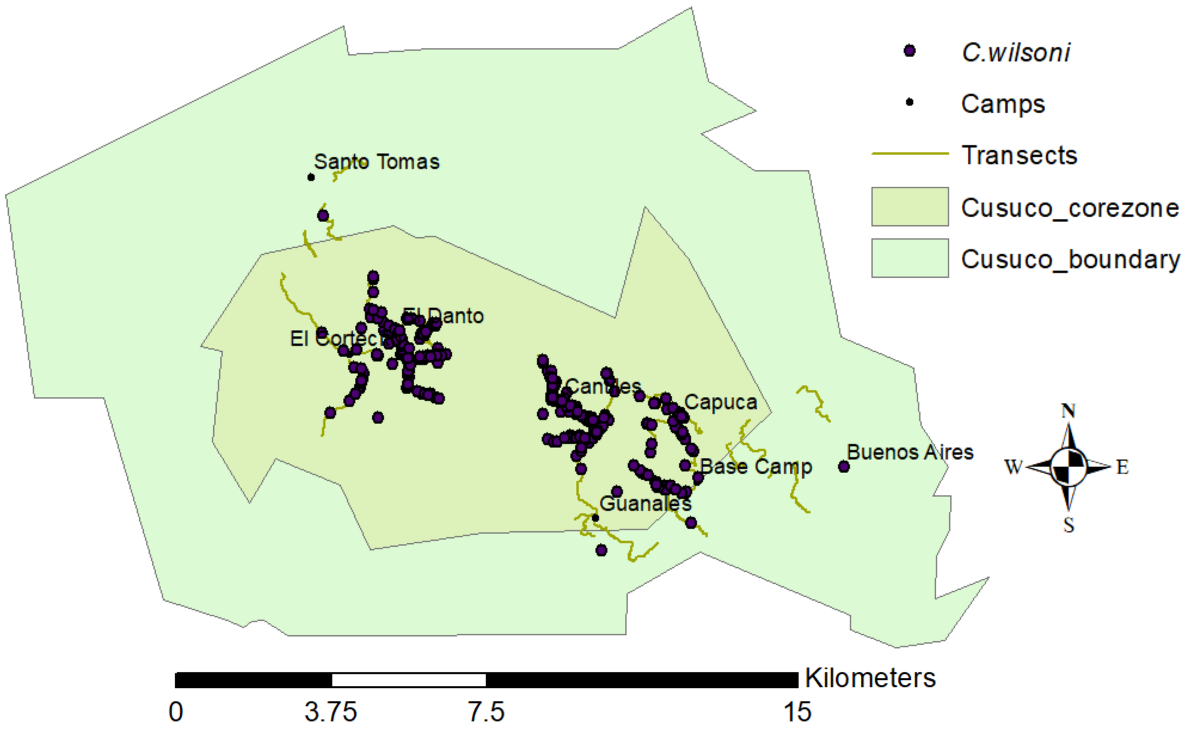

Figure 12; GIS map of recorded C. wilsoni encounters (n=450) in Cusuco National Park, Honduras between 2007-2018

4.2 Sequencing and haplotype networks

Due to the limited variability found across mtDNA sequences (as well as time constraints and a limited budget), nuclear genes, although amplified, were not sequenced. Table 3 displays the number of samples that were successfully sequenced for each mtDNA gene or gene segment. Concatenated sequences were created for those samples in which all gene fragments had been successfully sequenced, resulting in 29 concatenated sequences for *B. marchi* and 42 concatenated sequences for *C. wilsoni*. No variance was observed within the 12S and 16S sequences for either species, and so these were excluded from the final concatenated sequences.

Table 4; The number and length of B. marchi and C. wilsoni samples successfully sequenced for each gene or gene segment per species

	Bothriechis marchi		***Cerrophidion wilsoni***	
Gene	**Total**	**Length (bp)**	**Total**	**Length (bp)**
CO1 Consensus sequence	34	1254	52	1298
ND4 Forward (NADH4)	47	818	45	685
ND4 Reverse (LeuR)	25	838	0	0
CytB1 Forward (L14910/H15149)	29	357	45	382
CytB2 Forward (L15584/H16064)	38	468	45	474
12S Forward (L1091/H1557)	19	416	26	429
16S Forward (L2510/H3059	0	0	23	497

Within the *B. marchi* population, a total of eight haplotypes were found over 13 polymorphic sites resulting in a haplotype diversity of 0.697044. Within the *C. wilsoni* population, only two haplotypes were found with a haplotype diversity of 0.493612. Table 4 provides a breakdown of haplotype diversity between the camps.

Table 5; Haplotype diversity scores for B. marchi and C. wilsoni within Cusuco National Park, Honduras. n = sample size, k = number of haplotypes, PS = polymorphic sites, H = haplotype diversity

Species	Sampling location	n	k	PS	H
B. marchi	Overall	29	8	13	0.697044
	Base Camp	4	3	6	0.833333
	Cantiles	3	3	4	1
	El Corticito	17	4	6	0.551471
	El Danto	2	1	0	0
	Guanales	3	2	4	0.666667
C. wilsoni	Overall	42	2	1	0.493612
	Base Camp	13	2	1	0.512821
	Cantiles	17	2	1	0.514706
	El Corticito	1	1	0	0
	El Danto	10	2	1	0.355556
	Guanales	1	1	0	0

Thirteen single nucleotide polymorphisms (SNPs) were discovered throughout the combined *B. marchi* concatenated sequences as displayed in table 5 throughout the CO1 gene (base pairs 1-1219), the ND4 gene (bp 1220-2026), the initial CytB (CytB1) gene segment (bp 2027-2390) and the final CytB (CytB2) gene segment (bp 2391-2857). Of these thirteen SNPs; five were found within the CO1 gene, three within the ND4 gene, and five within the CytB gene of which a single variation was found within the first segment and the remaining four were in the final segment.

Table 6; Base point variations in B. marchi samples with accompanying haplotypes and genes within the concatenated sequence

	Nucleotide Position													
	CO1					**ND4**			**CytB1**	**CytB2**				
Sample	**110**	**239**	**440**	**539**	**575**	**1255**	**1387**	**1585**	**2304**	**2509**	**2560**	**2686**	**2828**	**Haplotype**
14	T	C	G	C	G	G	A	A	T	T	G	T	T	**A**
15	T	C	G	C	G	G	A	A	T	T	G	T	T	**A**
16	T	**T**	G	**T**	**A**	**A**	A	A	**A**	**C**	G	**G**	T	**D**
17	**C**	C	G	C	G	G	A	A	T	T	G	T	T	**G**
22	T	C	G	C	G	G	A	A	T	T	G	T	T	**A**
23	T	C	G	C	G	G	A	A	T	T	G	T	T	**A**
42	T	C	G	**T**	**A**	**A**	A	A	**A**	T	G	T	T	**B**
43	T	C	G	C	G	G	A	A	T	T	G	T	T	**A**
44	T	C	G	**T**	**A**	**A**	A	A	**A**	T	G	T	T	**B**
46	T	C	G	C	G	G	A	A	T	T	G	T	T	**A**
56	T	**T**	G	**T**	**A**	**A**	A	A	**A**	**C**	G	T	T	**C**
57	T	C	G	**T**	**A**	**A**	**G**	A	**A**	T	**A**	T	T	**F**
58	T	C	G	C	G	G	A	A	T	T	G	T	T	**A**
59	T	C	G	C	G	G	A	A	T	T	G	T	T	**A**
60	T	C	G	**T**	**A**	**A**	A	A	**A**	T	G	T	T	**B**
61	T	C	G	C	G	G	A	A	T	T	G	T	T	**A**
62	T	C	G	**T**	**A**	**A**	A	A	**A**	T	G	T	T	**B**
63	T	C	G	C	G	G	A	A	T	T	G	T	T	**A**
64	T	C	G	**T**	**A**	**A**	A	A	**A**	T	G	T	T	**B**
65	T	**T**	G	**T**	**A**	**A**	A	A	**A**	**C**	G	T	T	**C**
66	T	C	G	C	G	G	A	A	T	T	G	T	T	**A**
67	T	C	G	C	G	G	A	A	T	T	G	T	T	**A**
69	T	C	G	C	G	G	A	A	T	T	G	T	T	**A**
70	T	C	G	C	G	G	A	A	T	T	G	T	**C**	**H**
71	T	C	G	**T**	**A**	**A**	A	A	**A**	T	G	T	T	**B**
72	T	C	G	C	G	G	A	A	T	T	G	T	T	**A**
73	T	C	G	C	G	G	A	A	T	T	G	T	T	**A**
152	T	C	**A**	**T**	**A**	**A**	A	**T**	**A**	T	G	T	T	**E**
156	T	**T**	G	**T**	**A**	**A**	A	A	**A**	**C**	G	T	T	**C**

A haplotype network consisting of eight haplotypes was constructed (figure 13). Haplotype A (n=15) was the most prevalent, followed by haplotype B (n=6) which diverged from haplotype A by four SNPs in total. Haplotype C (n=3) diverged from haplotype B by a further two SNPs leading to the single individual in haplotype D which varied by a single additional SNP. Haplotypes E and F each diverged from haplotype B by two different SNPs each while the remaining haplotypes, G and H both diverted from haplotype A by a single SNP.

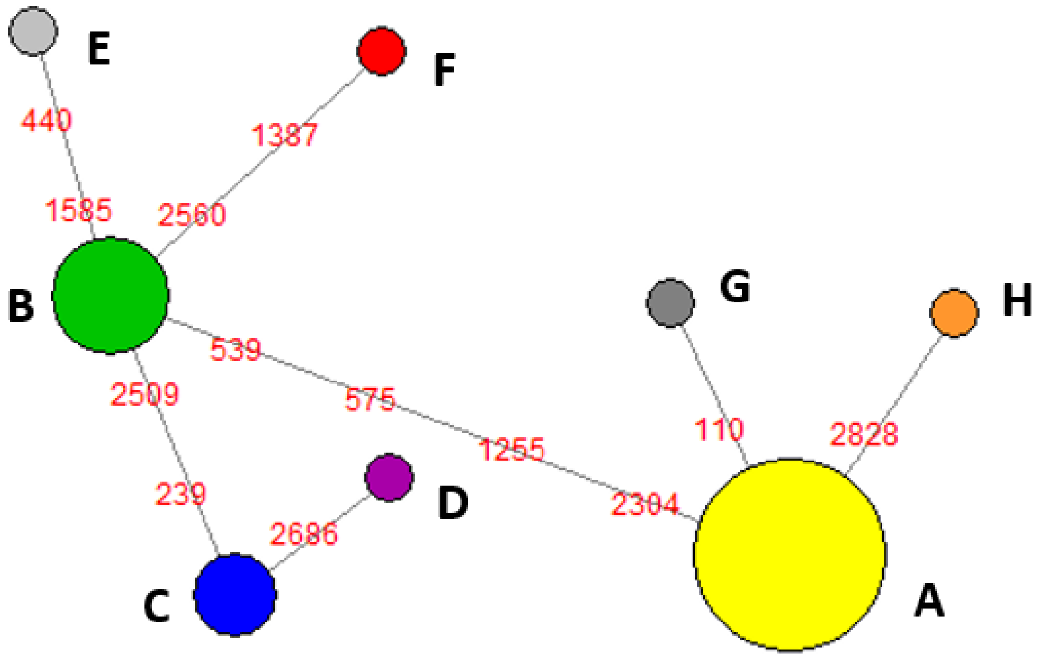

Figure 13; Haplotype network for B. marchi population in Cusuco National Park, Honduras. Sample sizes are; (A) n=15, (B) n=6), (C) n=3, (D) n=1, (E) n=1, (F) n=1, (G) n=1), and (H) n=1. Numbers indicate the position of single nucleotide substitutions within the concatenated mtDNA sequences

While 42 *C. wilsoni* individuals were successfully sequenced for the CO1, ND4 and both segments of the CytB genes, only one SNP was found within the ND4 gene. Within haplotype X (n=25), a guanine (G) base was found whereas within haplotype Y (n=17) this was replaced by an adenine (A) base (figure 14).

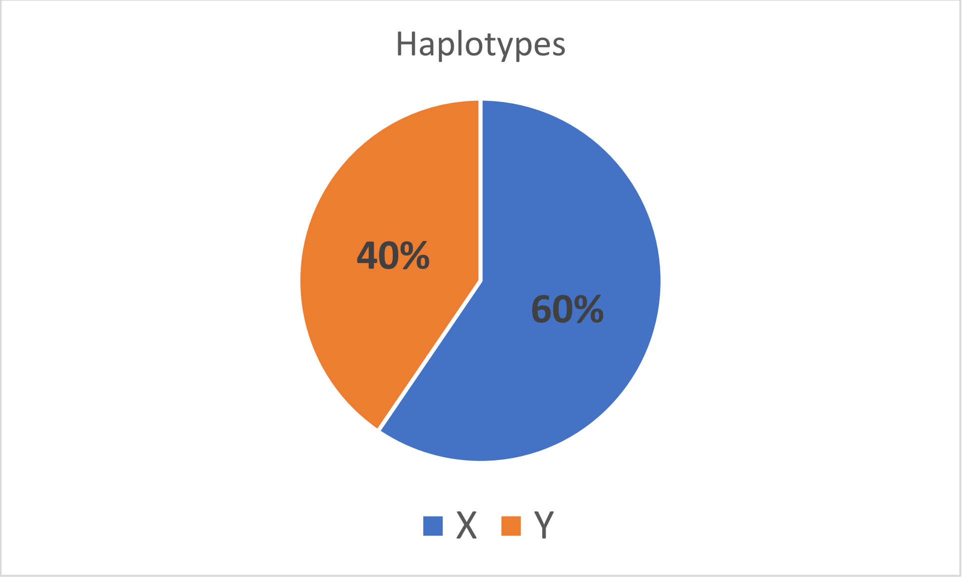

Figure 14; Pie chart displaying the proportion of two C. wilsoni haplotypes identified in Cusuco National Park, Honduras

4.3 Haplotype dispersal

Of the 15 *B. marchi* individuals attributed to Haplotype A, 87% (n-13) were discovered in the North-Western side of the park primarily around El Corticito (n=11) camp, however the only two sequenced individuals found near to El Danto camp were both also identified as Haplotype A. The remaining two individuals within Haplotype A were found on the southeast side of the park near to Guanales camp.

Within Haplotype B (n=6), 67% of individuals (n=4) were located in El Corticito camp in the Northwest side of the park, while one each of the remaining two were located in Cantiles and Guanales camps both in the Southeast of the park. All three of those identified as Haplotype C were found in the southeast, with two located in Base Camp and a single individual located in Cantiles.

The remaining haplotypes D-H were all represented by a single individual. Haplotypes D-F were all found in the Southeast side of the park with haplotype D found in Cantiles camp and haplotypes E and F both found in Base Camp. Haplotypes G and H were both found in the northwest side of the park at El Cortico camp.

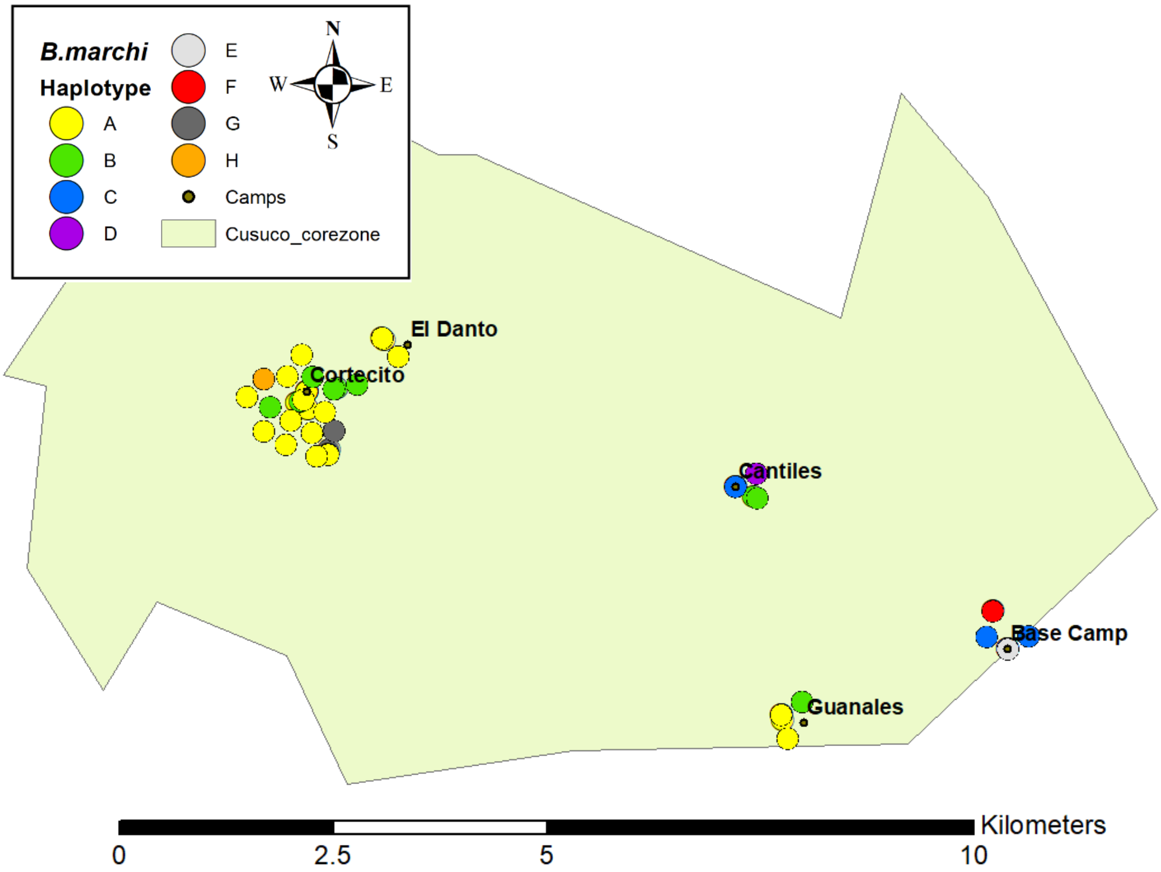

Figure 15; GIS map of B. marchi haplotypes within Cusuco National Park, Honduras. N=29, H=8. The data points within figure 15 have been dispersed to ensure visibility due to a number of these points existing in close proximity

Data points in figure 16 have also been dispersed to ensure visibility due to the proximity of a number of data points to each other. Of the *C. wilsoni* individuals successfully sequenced, 26% (n=11) were located in the northwest of the park, with all but one located around El Danto camp and the remaining individual was found at El Corticito camp. Of these individuals, including the individual found in El Corticito, 82% (n=9) were all attributed to haplotype X, while 18% (n=2) were attributed to haplotype Y. The remaining 74% of individuals found on the southeast side of the park were made up of 52% (n=16) haplotype X and 48% (n=15) haplotype Y.

Of the 17 individuals found near to Cantiles camp, 59% (n=10) were attributed to haplotype Y while the remaining 41% (n=7) were haplotype X. Within Base Camp, 13 individuals were located consisting of 61% (n=8) attributed to haplotype X and 39% (n=5) to haplotype Y. In addition, a single individual was located at Guanales camp attributed to haplotype X.

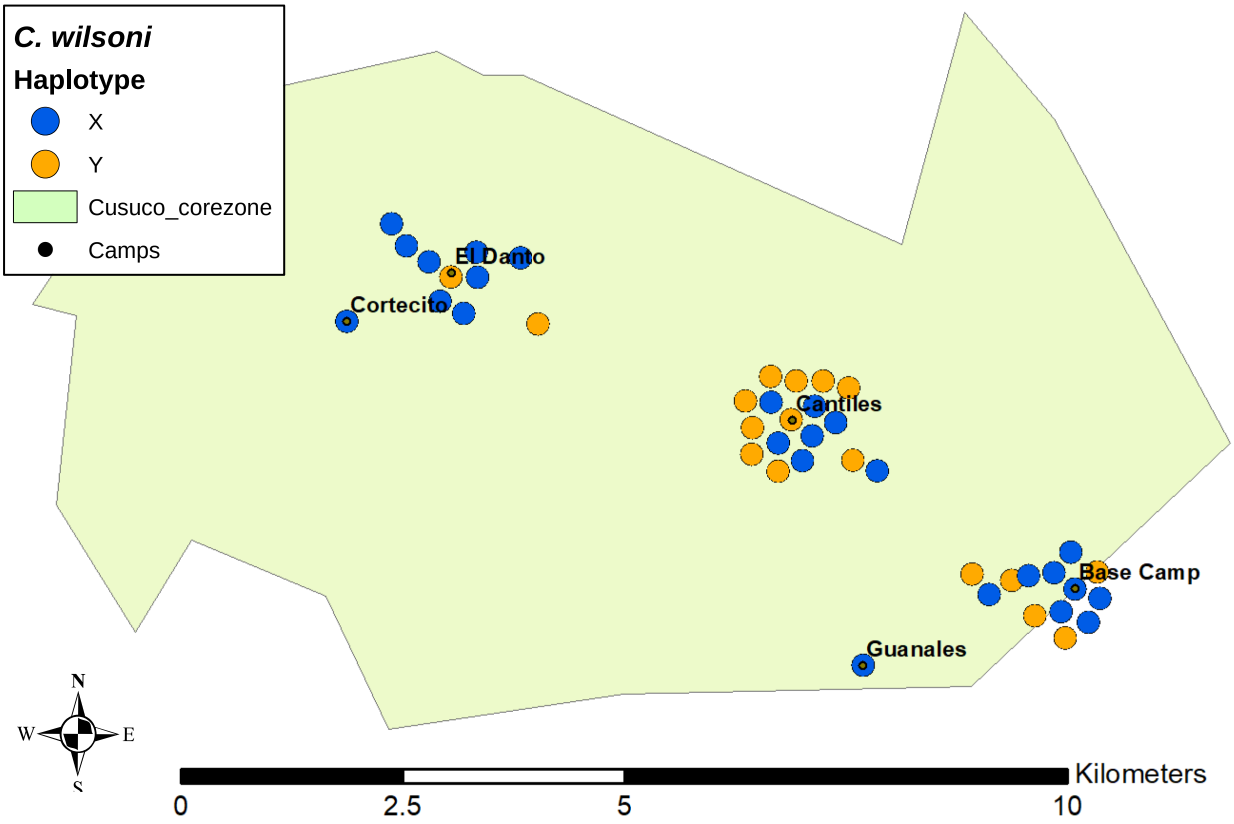

Figure 16; GIS map of C. wilsoni haplotypes in Cusuco National Park, Honduras. N=42, H=2

5. Discussion

The area around the western camps of El Danto and El Corticito are at risk of illegal deforestation (appendix 5), and these results indicate that continued deforestation may have severe consequences. Two of the *B. marchi* haplotypes found within the study, as well as a third suspected haplotype, have only been found in the western camps and so would be lost from the population, as would the majority of the individuals carrying the two most abundant haplotypes; A and B. This could result in the loss of approximately 33-55% of all found mitochondrial genetic diversity within the *B. marchi* population and significantly alter the genetic structure of this population. The high percentage of haplotypes represented by single samples despite such a low sample size indicates a high likelihood of further haplotypes within the population that have not yet been discovered. In addition, 71% of all *B. marchi* encounters between 2007 and 2018 were recorded on the west side of the park, indicating that a large percentage of the population may be lost due to this deforestation should it continue. A smaller but significant percentage of *C. wilsoni* could also be lost as 33% of encounters between 2007 and 2018 were also recorded at these western camps.

Despite appearing to be less abundant, it is clear that there is a much higher level of genetic variation amongst the *B. marchi* population compared to the *C. wilsoni* population within the mitochondrial genes successfully sequenced for this study. The arboreal nature of *B. marchi* may cause this species to be under-detected using current survey methods, however. It is also apparent that despite the generally increasing number of encounters recorded for both species each year (figure 6), the average number of encounters recorded per transect (figure 7) for both species has dropped significantly since 2008.

This could be a result of increasing survey efforts (table 2) causing increased disturbance to individuals so that they remain hidden, however it may indicate a large decrease in both populations between 2008 and 2010. The data used for these figures also included opportunistic sightings which may inflate the number of encounters, and changes in survey technique over the years will also have an effect. The total number of transects completed were much lower between 2008-2010 compared to later years. The locations of these survey efforts are also relevant as it is clear from figure 8 that *C. wilsoni* can be found in higher abundance around Cantiles camp due to the higher elevation, however, no transects occurred in this area in 2010 compared to 30% of all transects in 2008.

The higher number of *C. wilsoni* encounters in this area could be due to a large open area on one of the transects here, caused by a landslide pre-2008. It is undergoing succession but is still very open compared to other areas, which has made it a favoured hotspot for *C. wilsoni* to bask. This may mean that rather than being more abundant in this area, the *C. wilsoni* are simply easier to spot due to the lack of vegetation.

Comparatively, *B. marchi* is found in higher abundance around El Corticito camp as well as around El Danto camp, which is likely due to the number of rivers in this area that *B. marchi* relies upon for prey. Neither species are abundant around Guanales camp, however, with less than 0.1 encounters per transect for both species despite transects in this area accounting for 25% of survey effort between 2008 and 2018. Base camp accounted for 28.5% of survey effort in this time frame but yielded less than 0.2 encounters per transect for both species. As a good compromise with the second highest average rate of encounters per transect, El Danto would be the best camp to focus further survey efforts to maximise the number of encounters for both species.

The lack of genetic diversity found with the 12S and 16S genes is likely due to the slower rate of evolution within these genes compared to CytB and ND4 (Schuett *et al.*, 2002). This is also the key reason in which nuclear genes were not sequenced within this study, as mtDNA has a faster rate of base substitution (Gariboldi *et al.*, 2016) and so little variation was expected within the nuclear genes. As a result, however, this study only represents the female genetic contribution due to the maternal inheritance of mtDNA (Sato and Sato, 2012). This bias could be avoided in future studies with the use of microsatellites as they are highly polymorphic and biparentally inherited (Gariboldi *et al.*, 2016). It is important to note however that much of the variation seen within this study is likely from 'silent' mutations that are unlikely to have any adaptive significance in themselves.

Within the *B. marchi* samples, thirteen SNPs were found resulting in eight different haplotypes compared to only two *C. wilsoni* haplotypes. This could be the result of a variety of factors, beginning with recent estimations that the *Bothriechis* genus originated between 12 and 16.3 million years ago while the younger *Cerriphidion* genus originated between 7.6 and 11.5 million years ago (Castoe *et al.*, 2009) leaving *Bothriechis* in general with more time to accumulate mutations. The overall haplotype diversity within the *B. marchi* population of 0.697044 seems to be around average when compared to the studies in table 1 which provided a benchmark average of 0.691649, however, this would indicate that the haplotype diversity within the *C. wilsoni* population is below average at 0.493612. It is not possible to make a direct comparison however due to the variables within this study and those used within table 1.

In addition, Middle America is a relatively young land mass with a complex tectonic and geological history, lying at the active junction of four major tectonic plates and several tectonic blocks (Iturralde-Vinent, 2006; Marshall, 2007). This has shaped the diversity in this area with continual physiographical reshaping of the region since the Cretaceous period whilst simultaneously complicating attempts to decipher the events resulting in this high diversity (Iturralde-Vinent, 2006). These same events would have had an impact on when each species arrived in CNP, in which numbers, and how much variation already existed within the founding populations (founder effect). It is possible that *C. wilsoni* underwent a genetic bottleneck when diverging into the park due to low genetic diversity within the founding population. The answer to the higher diversity within the *B. marchi* population may also lie within the tectonic and geological history, as it is possible that historically the *B. marchi* population was much larger and less fragmented with greater gene flow between surrounding areas.

The presence of haplotypes A and B on both sides of the park indicates that either historically there was greater gene flow across the park, or that gene flow is not entirely restricted by the high elevation in the middle of the park (appendix 4), however a higher frequency of both haplotypes can be found around the western camps. This is also where the only representatives of haplotypes G (n=1) and H (n=1) can be found, which both branch from haplotype A, and are at risk of being lost due to the deforestation on this side of the park.

Haplotypes C-F however, which all stem from haplotype B, have only been found on the eastern side of the park. This is likely because these haplotypes are rarer than haplotypes A and B, and in terms of probability it is more likely that an individual moving from one area to another will be a representative of a more common haplotype. On the eastern side of the park, however, haplotype C (n=3) appears to be the most prevalent which may indicate an emergence of this haplotype as the dominant haplotype on this side of the park compared to haplotypes A and B on the western side.

Within the *C. wilsoni* population, haplotype X appears to be the most abundant haplotype representing 60% of the samples sequenced within this study. On the west side of the park, nine of the eleven samples represented haplotype X with only two found for haplotype Y. A more even split was found on the Eastern side of the park however, featuring 16 representatives of haplotype X alongside 15 representatives of haplotype Y. This could indicate a less-restricted gene flow across the park for *C. wilsoni* which could be due to the terrestrial nature of this species, however it is possible that the populations on either side of the park may have become more geographically isolated over time and that while both haplotypes can be found on both sides of the park, the western population may now be at risk of losing haplotype Y to genetic drift. With such a small sample size, this could only be confirmed with ongoing monitoring and a larger sequencing effort of samples from the western camps.

While it is difficult to make direct comparisons with other studies detailed in table 1, the number of haplotypes found for *B. marchi* and *C. wilsoni* appears to be comparatively low as many studies found more haplotypes within smaller populations using only one or two mtDNA genes. For example within the N&E AUS sub-group of *H. elegans* a total of 11 haplotypes were found within a population of 25 using the CytB gene only (Nitschke *et al.*, 2018), while only eight were found with the similar *B. marchi* size despite the addition of the CO1 and ND4 genes.

This study (Nitschke *et al.*, 2018) was, however, able to make use of the full CytB gene rather than segments from the beginning and end. Considering the variation within the CytB segments successfully sequenced within this study, it is likely that more variation will be found if the entirety of this gene can be sequenced for these samples in future. In addition, any population with less than two haplotypes in table 1 had a population of less than 10 (Nitschke *et al.*, 2018; Asadi *et al.*, 2019), compared to the sample size of 42 *C. wilsoni* used within this study.

The lower number of haplotypes found within the *B. marchi* and *C. wilsoni* populations compared to the other studies is likely due to the natural isolation of Cusuco National Park as a sky island. It is likely that despite the low sample sizes in the comparison studies, the overall populations are likely much higher than those within Cusuco National Park and so eight haplotypes with the *B. marchi* population could be considered reasonably high despite being lower when compared to other studies. The number of haplotypes with the *C. wilsoni* population, however, can be considered as below average.

6. Conclusions

There is some evidence of limited gene flow across the park that is likely due to an area of high resistance around Cantiles camp (appendix 4) that is a larger issue for *B. marchi*. Haplotype A appears to be the dominant haplotype on the west side of the park, however, it is possible that haplotype C may be emerging as the dominant haplotype on the east side. Should the deforestation on the west side of the park continue, approximately 33-55% of all found mitochondrial genetic diversity within the *B. marchi* population could be lost causing significant change within the genetic structure of this population.

The average number of encounters per transect has decreased significantly for both species since 2008, with an average of only 0.02 *B. marchi* encounters per transect in 2018. This could indicate a severe drop in population size, however, further research using more appropriate methods is required in order to reach a clearer picture of the overall population trends, as this was not the primary focus of this study.

It is clear that El Corticito and El Danto camps have a high abundance of *B. marchi* that will be at risk should the extensive deforestation in these areas continue. This is also a risk for a significant percentage of the *C. wilsoni* population which can be found in relatively high abundance around El Danto camp.

A total of eight haplotypes have been confirmed within the *B. marchi* population, however, when compared with Clegg's (2014) haplotype network it is likely that there is a ninth haplotype from a sample that was not used within this study. It is likely that *C. wilsoni* has undergone a genetic bottleneck when diverging into the park, and as a result only two haplotypes have been found within the *C. wilsoni* population. This may leave them less able to adapt to challenges that may arise such as changing environmental conditions, habitat loss/fragmentation, or new diseases.

When compared with other studies, *B. marchi* appears to have an average haplotype diversity of 0.697044, however *C. wilsoni* appears to have a below average haplotype diversity score of 0.493612. When the small, isolated nature of Cusuco National Park is taken into account, the number of haplotypes within the *B. marchi* population can be considered high while the number of haplotypes within the *C. wilsoni* population can be considered low.

Next Steps

Any further deforestation within CNP must be prevented wherever possible in order to protect the genetic diversity found within the park. Ongoing sequencing efforts, ideally using microsatellite markers or next generation sequencing, are required in order to provide a clearer picture of the genetic structure within the *B. marchi* population as it is suspected that further haplotypes await discovery, as well as to confirm any restrictions in gene flow across the park. A captive breeding project should be considered in order to increase the genetic variation within the *C. wilsoni* population, and so increase the resilience of the population to future challenges.

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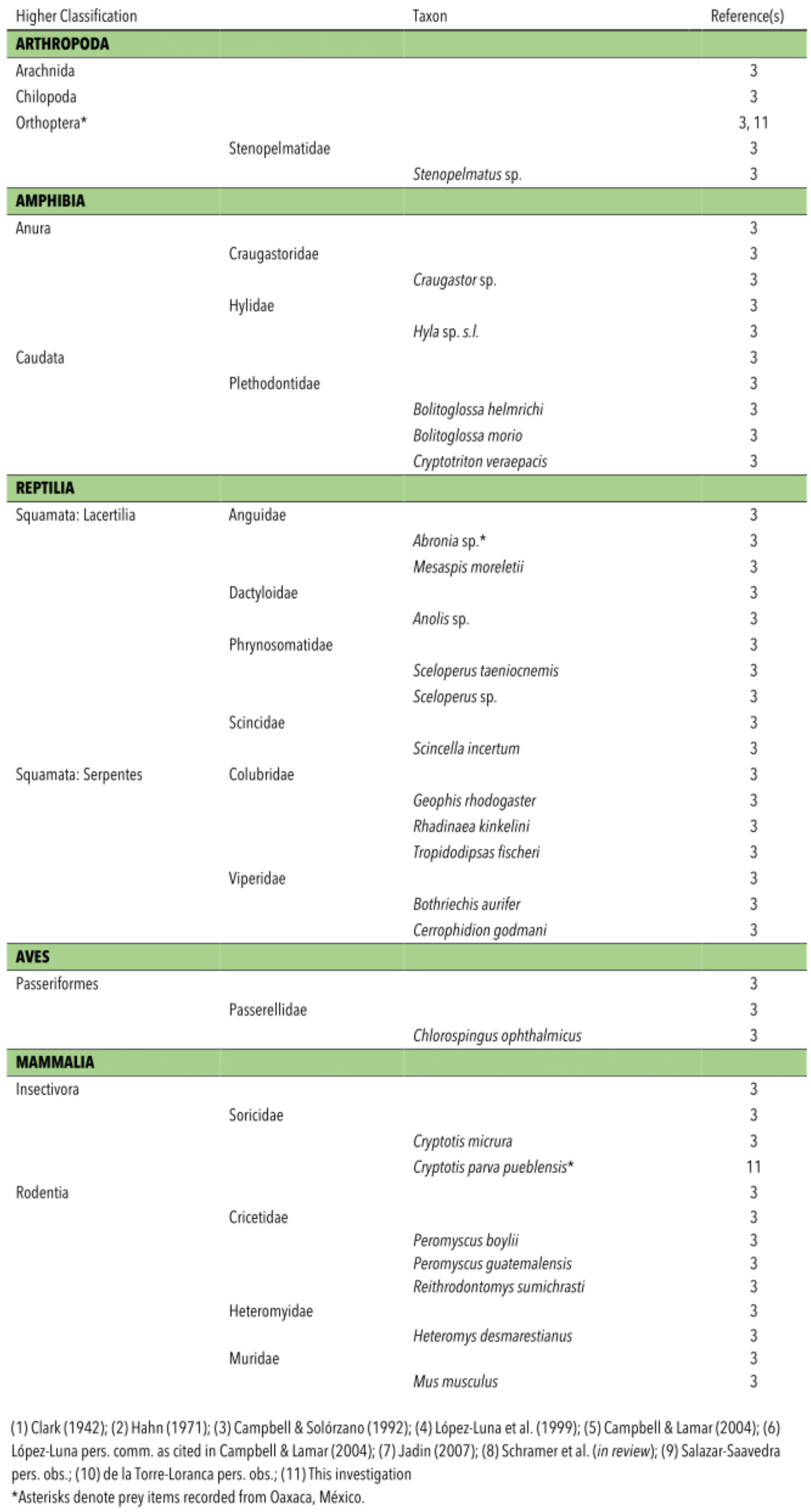
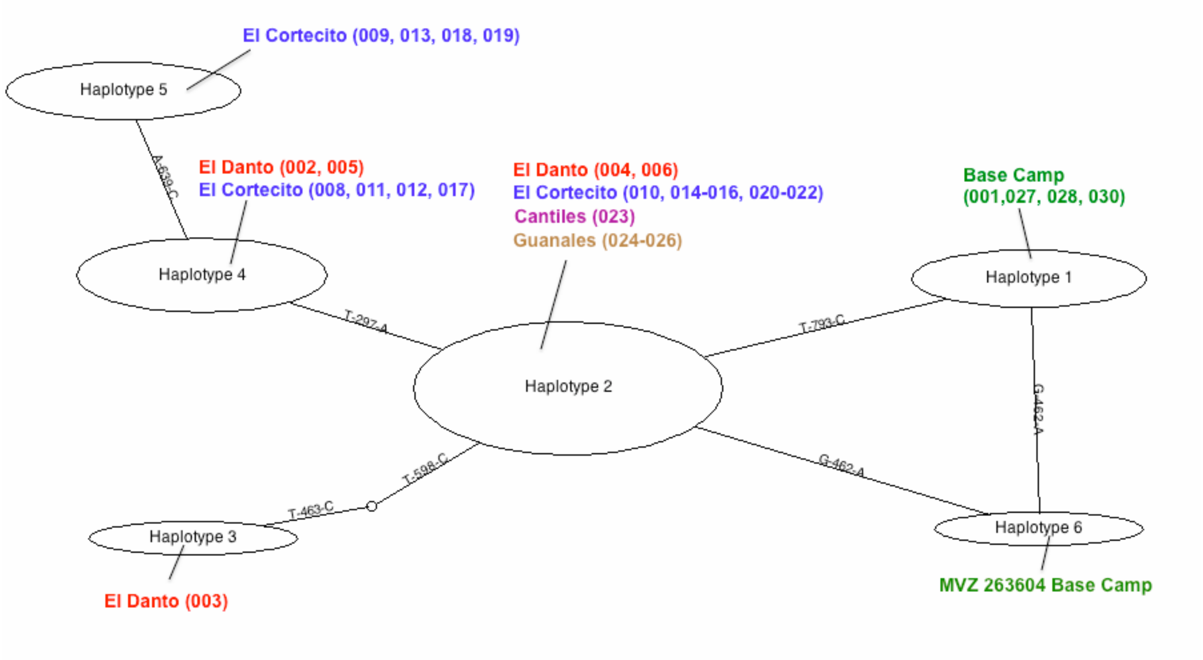


Higher Classification		Taxon	Reference(s)
ARTHROPODA			
Arachnida			3
Chilopoda			3
Orthoptera*			3, 11
	Stenopelmatidae		3
		Stenopelmatus sp.	3
AMPHIBIA			
Anura			3
	Craugastoridae		3
		Craugastor sp.	3
	Hylidae		3
		Hyla sp. *s.l.*	3
Caudata			3
	Plethodontidae		3
		Bolitoglossa helmrichi	3
		Bolitoglossa morio	3
		Cryptotriton veraepacis	3
REPTILIA			
Squamata: Lacertilia	Anguidae		3
		Abronia sp.*	3
		Mesaspis moreletii	3
	Dactyloidae		3
		Anolis sp.	3
	Phrynosomatidae		3
		Sceloperus taeniocnemis	3
		Sceloperus sp.	3
	Scincidae		3
		Scincella incertum	3
Squamata: Serpentes	Colubridae		3
		Geophis rhodogaster	3
		Rhadinaea kinkelini	3
		Tropidodipsas fischeri	3
	Viperidae		3
		Bothriechis aurifer	3
		Cerrophidion godmani	3
AVES			
Passeriformes			3
	Passerellidae		3
		Chlorospingus ophthalmicus	3
MAMMALIA			
Insectivora			3
	Soricidae		3
		Cryptotis micrura	3
		*Cryptotis parva pueblensis**	11
Rodentia			3
	Cricetidae		3
		Peromyscus boylii	3
		Peromyscus guatemalensis	3
		Reithrodontomys sumichrasti	3
	Heteromyidae		3
		Heteromys desmarestianus	3
	Muridae		3
		Mus musculus	3

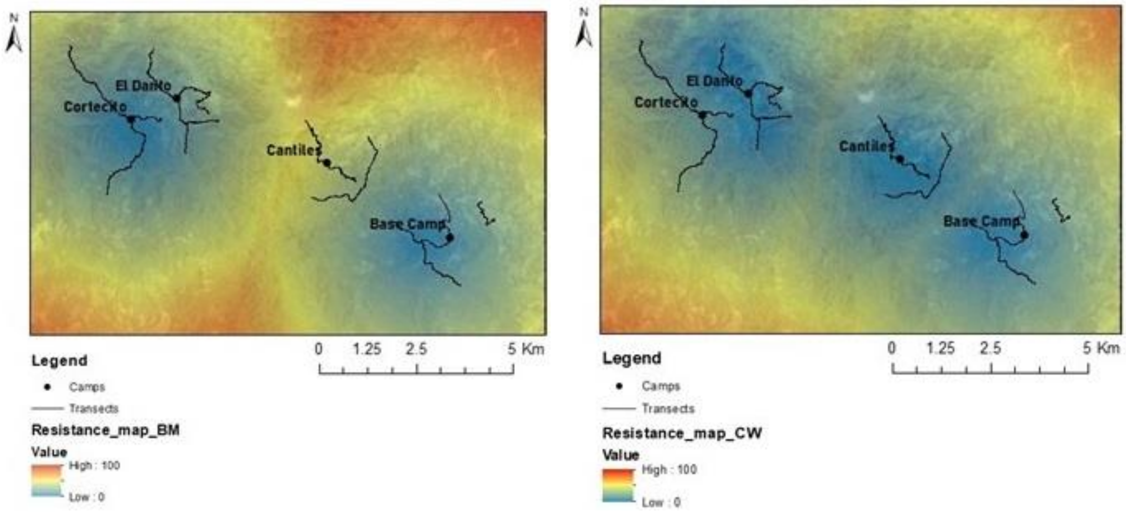
(1) Clark (1942); (2) Hahn (1971); (3) Campbell & Solórzano (1992); (4) López-Luna et al. (1999); (5) Campbell & Lamar (2004); (6) López-Luna pers. comm. as cited in Campbell & Lamar (2004); (7) Jadin (2007); (8) Schramer et al. (*in review*); (9) Salazar-Saavedra pers. obs.; (10) de la Torre-Loranca pers. obs.; (11) This investigation

*Asterisks denote prey items recorded from Oaxaca, México.

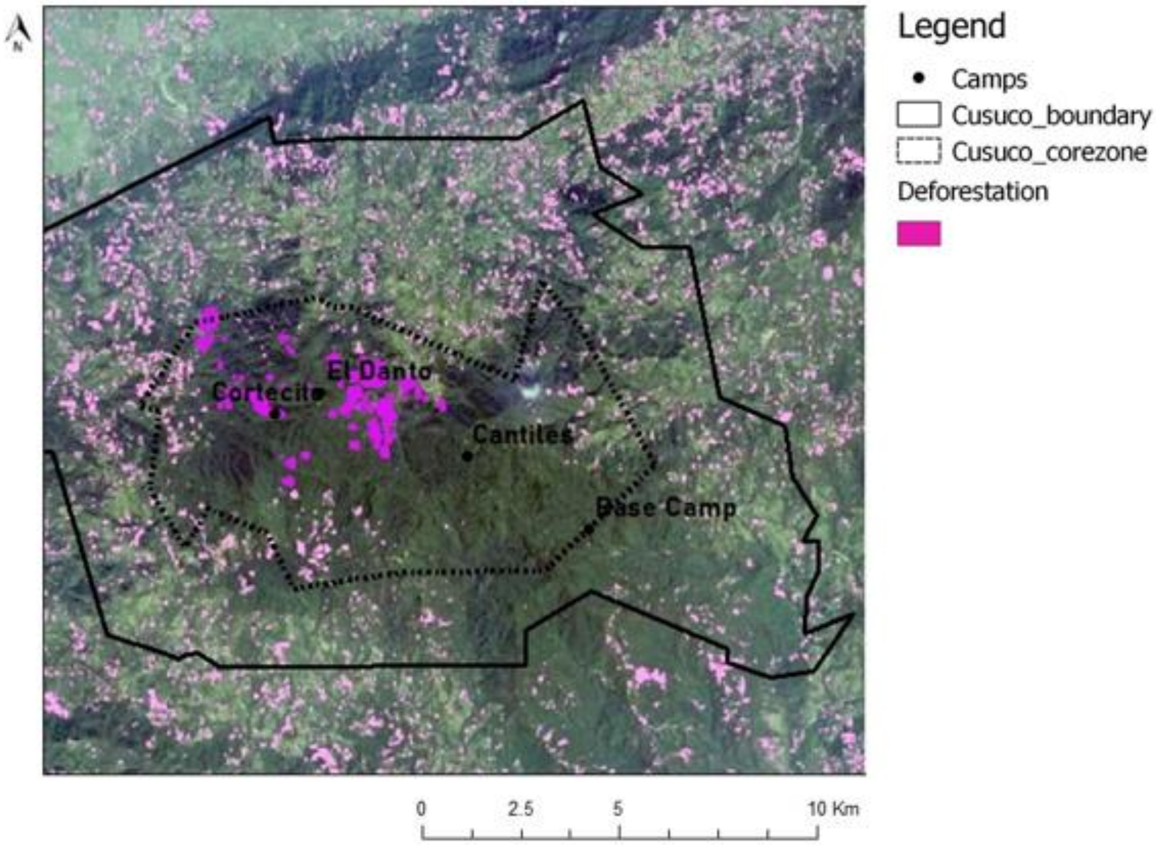
Appendix 2; All known prey items of Cerrophidion godmani (Schramer et al., 2018)



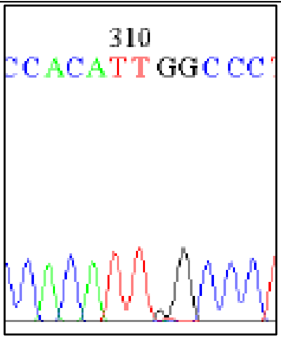
Appendix 3; Mitochondrial haplotype diversity of B. marchi population at Cusuco National Park. Numbers on branches indicate single nucleotide polymorphisms (Clegg, no date)



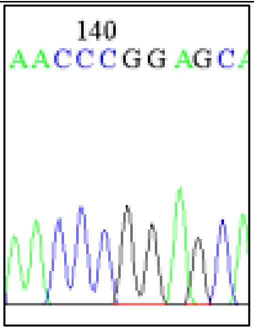
Appendix 4; Resistance surface for a) B. marchi b) C. wilsoni. Resistance surface calculated from linear negative transformation of the MaxEnt output and resistance values for deforestation and streams. Warm colours (red and orange) indicate higher resistance to movement, whereas cool colours (blue) indicate low resistance (Oliveira, 2021



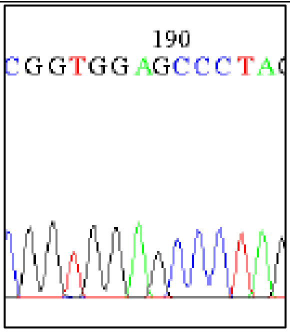
Appendix 5; Deforestation in Cusuco National Park (Oliveira, 2021)



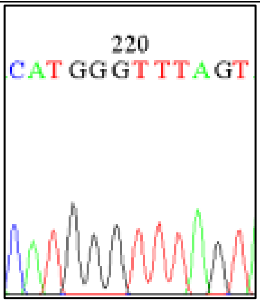
a) BM16 – expected TTTG, have TTGG



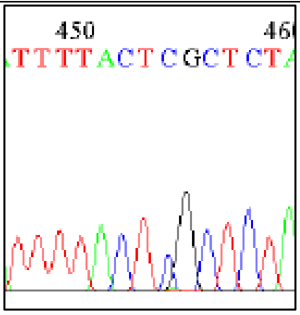
b) BM17 – Expected CCTG, have CCCG



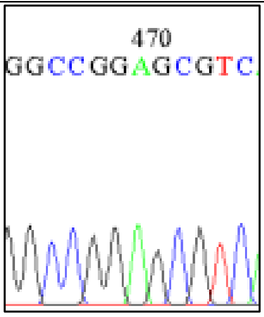
c) BM57 - expected GGG, have GAG



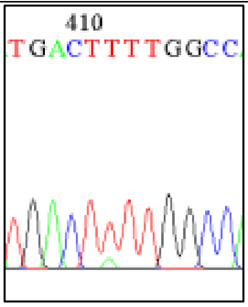
d) BM57 (second variation) – Expected TAGG, have TGGG



e) BM70 - Expected TTG, have TCG



f) BM152 – Expected GGGG, have GGAG



g) BM152 (second variation) – Expected TATT, have TTTT

Appendix 6; Evidence of single point variations