Evolution and community structure of parasites in Galápagos giant tortoises

Leandro Dario Patino Patino

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The candidate confirms that the work submitted is his/her own, except where work which has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

The blood samples and ticks for the years 2005 and 2006, used for Chapters 2 and 3, were collected during my work in the Galápagos Genetics, Epidemiology & Pathology (GGEPL) and in partnership with the DMV Marilyn Cruz and the DMV Pamela Martinez, also other members of staff, volunteers and students working in the laboratory at that time. The microscope analysis of ~ 1000 blood films prepared for the years before 2013, were also conducted in collaboration with them. I was involved in microscope analysis of ~30% of samples including Isabela and Santiago. Dr Cruz and Dr Martinez, analysed the remaining samples collected across all the tortoise populations included in this study. The GGEPL operated in Galápagos from 2003 to 2010 as a collaboration among the Galápagos National Park Directorate, the University of Leeds, Zoological Society of London, the University of Guayaquil and the NGO Concepto Azul. During this time the Laboratory was supported by grants from the UK government's Darwin Initiative scheme (reference numbers: 162-12-17 & EIDPO15), awarded to Dr Simon Goodman, Dr Virna Cedeño, and Prof Andrew Cunningham. The DNA extraction and obtention of sequence data of ticks and nematode larvae presented in chapters 3 and 4, respectively, were done in collaboration with undergraduate and master students which were under my technical supervision, and the academic supervision of my main supervisor, Dr. Simon Goodman. Jane Hosegood and Rita Velez (MSc students) were involved in the analysis of nematode larvae, while, Rachel Muris (undergraduate student), Leigha Little and Alvaro Garcia (both MSc students) were involved in the analysis of ticks. Morphological analysis of ticks was done by Leigha Little. Students performed DNA extractions and generated a subset of PCR products sent for sequencing. The sequence data was incorporated with my own data, and analysed independently. Dr Gabriel Gentile (University Tor Vergata, Rome) and Dr Gisella Caccone (Universty of Yale US) contributed 10 ticks from Wolf collected in 2015. Dr. Gabriele Gentile also share his data of land iguana haemogregarines. Final morphological identification of nematode larvae was done by Dr. Lynda M. Gibbons.

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Abstract

A central theme in ecology is to understand the distribution and abundance of organisms and the factors influencing these patterns. This thesis investigated the taxonomic identity and biogeography of blood parasites, *Amblyomma* ticks and gastro-intestinal helminths of Galápagos tortoise, *Chelonoidis* spp. Blood parasite and ticks were assessed for co-phylogeographic patterns with their tortoise host. The patterns of helminths diversity was examined and whether factor such as host colonization history and local ecology determine their distribution and community composition. Microscope and phylogenetic analysis of 18S rDNA identified the blood parasite as a haemogregarine of the genus *Hepatozoon*. It was represented by just two haplotypes restricted to the northern volcanoes of Isabela. Thirty-seven tortoise blood samples yielded the same haemogregarine haplotype for Alcedo and Wolf volcanoes, unique to *Chelonoidis* spp. The only tortoise that was haemogregarine positive from Darwin yielded a different haplotype, related to haemogregarines reported from Galápagos land iguanas. Molecular analysis of the COI gene of *Amblyomma* ticks revealed 3 different species, one infesting tortoises of Alcedo and Wolf volcanoes, one in tortoises of Santiago and one of tortoises from Pinzón. Galápagos tortoise ticks from Alcedo and Wolf has been described before as *A. unsingeri,* while tortoise ticks from Santiago and Pinzón have been described as belonging to *A. pilosum.* The restricted distribution of tortoise haemogregarines impeded testing them for co-phylogeographic patterns. Ticks showed no agreement with the phylogeography of their tortoise host. Coprological and metabarcoding methods revealed the presence of Platyhelminths, Acanthocephala, and Nematoda. Metabarcoding however, exceeded the traditional method in sensitivity for parasite detection and identification. At least seven families of Nematoda were identified with most taxa widespread across the Galápagos archipelago suggesting little effect of host colonisation for the common taxa in their distribution. At least three genera were found only on one or two islands suggesting their potential local acquisition or exclusion. These results are relevant for understanding the diversity and ecology of *Chelonoidis* spp. parasites, the management andconservation of this reptile and as a model for other wild species.

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AIC	Akaike Information Criterion ()
BLAST	Basic Local Alignment Search Tool
BCa	Bootstrap confidence interval ()
GNPD	Galápagos National Park Directorate ()
GGEPL	Galápagos Genetic Pathology and Epidemiology Laboratory "Fabricio Valverde"
Mya	million years ago
PCR	Polymerase chain reaction
NGS	Next Generation Sequencing
CR	Control region
COI	Cytochrome Oxidase
DEFRA	Department for Environment Food & Rural Affairs ()
MEGA	Molecular Evolutionary Genetic Analysis
MUSCLE	Multiple Sequence Comparison by Log Expectation
NCBI	National Center for Biotechnology Information ()
AMOVA	Analysis of Molecular Variance
BEAST	Bayesian Evolutionary Analysis Sampling Trees
ESS	Effective sample size
MCMC	Markov chain Monte Carlo
GLM	Generalized Linear Models
ZIM	Zero Inflated Model
SENESCYT	Secretariat of Education Science and Innovation (by its Spanish acronym).
USEARCH	Ultra-fast sequence analysis

Chapter 1. General Introduction

Parasites are ubiquitous and an integral component of ecosystems often overlooked in conservation, biodiversity and ecological research (Gomez and Nichols 2013). The can have wide ranging effects on hosts and ecosystem, including influencing food webs, regulating community composition, and host genetic diversity (Dobson and Hudson 1986; Jørgensen 2015; Lafferty *et al.* 2006), as well as causing disease (Daszak *et al.* 2001). In addition parasite and host stablish unique relationships as result of millions of years of coexistence and coadaptation. The importance of this association was highlighted as early as 1891 with parasites proposed as zoogeographical markers for their hosts (von Ihering 1891). This view has been strengthened by the finding that some parasites-host systems shows congruent molecular phylogenies providing, in those cases, an important model for better understanding their reciprocal evolution (Stefka *et al.* 2011). Parasites also can be sensitive to ecological changes (Lafferty and Kuris 1999; Marcogliese 2005) which in turn make them susceptible to extinction or may exacerbate their pathogenic characteristics.

In this thesis I study the neglected parasites of the Galápagos giant tortoise (*Chelonoidis* spp.) aiming to understand their evolution and ecological relevance. In previous work with these reptiles, I and co-workers identified a blood parasite, ticks and several new putative species of nematodes infesting some tortoise populations (Fournie *et al.* 2015). Based on samples collected previously, and in the framework of this thesis, I use a set of conventional parasitological and molecular techniques to determine the taxonomic identity and biogeography of these parasitic taxa. I look for signals of co-evolution in blood parasites, ticks and their tortoise hosts and try to explain the evolutionary history of these parasites in the Galápagos Islands. Based on geological and ecological data available for the Galápagos, I also look for factors influencing the spatial distribution of parasites in the context of individual parasitic infection (blood parasite and ticks) or multiple helminthic infections. Together this can provide insights into the evolutionary and ecological processes which may have shaped the structure of parasite communities in *Chelonoidis* spp., and which may also be relevant to the development of multiparasite communities more generally.

1.1. Galápagos giant tortoises as a system for parasite evolutionary studies

The Galápagos giant tortoise is renowned both for its uniqueness and for its contribution to the development of Darwin's theory of natural selection (Ciofi *et al.* 2006). The number of tortoise species is controversial. However, at least five taxa are extinct from different islands, comprising Floreana (*C. elephantopus*), Santa Fe (*C.* sp.), Fernandina (C. *phantastica*), Rábida (*C* sp.) and Pinta (*C. abingdoni*) (Poulakakis *et al.* 2012). At least nine species still exist now, four occurring on separate islands (*C. darwini* in Santiago; *C. ephippium* in Pinzón; *C. chatamensis* in San Cristobal, and *C. hoodensis* in Española), two occurring in Santa Cruz (*C. nigra* in the east and *C. donfaustoi* in the west) and three inhabit the largest island, Isabela (*C. becki* in Wolf volcano, *C. microphyes* in Darwin volcano, and *C. vicina* in Alcedo and the southern volcanoes of the island), see Fig. 1.1. The designated species differ in a number of morphological characters, such as carapace shape (domed vs. saddlebacked), maximum adult size, and length of the neck and limbs, which are related to habitat and diet in the range of each population (Burns *et al.* 2003). Tortoises with domed-shaped shell are found in larger, wetter and more elevated islands with diversity of vegetation zones; tortoises with saddlebacked shell are found in smaller and drier islands (Figure 1.2a-b).

These animals present a striking example of evolution in a large vertebrate following a single colonisation event, radiating across islands, and subsequent divergence under restricted gene flow (Caccone *et al.* 2002). DNA-based phylogenetics, has allowed the reconstruction of their evolutionary history and identified both the origins of the lineage and the relationships among the extant species. Galápagos giant tortoises originated from a mainland common ancestor and differentiated among and within islands following a single colonisation (Burns *et al.* 2003; Caccone *et al.* 2002; Caccone *et al.* 1999; Ciofi *et al.* 2006) (Fig. 1.3). The divergence from the closest extant mainland relative, *Chelonoidis chilensis,* probably occurred 6–12 million years ago (mya), whereas the deepest split in the Galápagos lineage occurred 1.5–2.0 mya. The estimated time of colonisation of the youngest island, Isabela, at about 0.2–0.3 mya, is consistent with the oldest lava flow on this island, which is dated to no more than 0.5 mya (Caccone *et al.* 2002).

The Galápagos tortoises inhabit the Galápagos archipelago which lies approximately 1,000 km west of the South American mainland and has never been attached to any continental land mass. This isolation means most endemic fauna derive from either single or a small number of colonisation events, rather than a regular influx of migrants. Such a system provides the opportunity to study the radiation of species from a limited founding stock without the confounding signals of recurrent colonisation (Parent *et al.* 2008). The archipelago consists of ten large islands (greater than 10 km^2), six smaller islands and over forty islets spread over 45000 km^2 of sea (Jackson 2009). As the islands differ in size and degree of isolation, there is also the chance to examine the interplay between evolutionary diversification and demographic processes following initial colonisation. Furthermore, their temporal geological origin is well known, supplying a framework to reconstruct the biogeographic history of various species. The Galápagos Islands arose 3-4 mya from a tectonic hotspot that lies beneath the Nazca plate, which is travelling in an eastward direction. As a result, island ages decrease from east to west. Their biological colonisation usually follows this progression, as shown for the case of the Galápagos tortoises in Fig. 1.3 (Caccone *et al.* 2002; Parent *et al.* 2008).

As with other oceanic islands, the Galápagos archipelago has provided a convenient model for phylogenetic studies on several individual species (Parent *et al.* 2008). It has also been considered a perfect ecosystem for studies of host-parasite interaction (Stefka *et al.* 2011; Whiteman *et al.* 2007). Parasites in particular are being studied either, as a means to infer the evolutionary history of their host or to understand the processes underlying their diversification. For example, Štefka *et al.* (2011) and Whiteman *et al.* (2007) studying the Galápagos mockingbirds (*Mimus* spp.) and the Galápagos hawk (*Buteo galapagoensis*), respectively, found strong correlations in phylogeographic patterns between each host species and their respective ectoparasites.

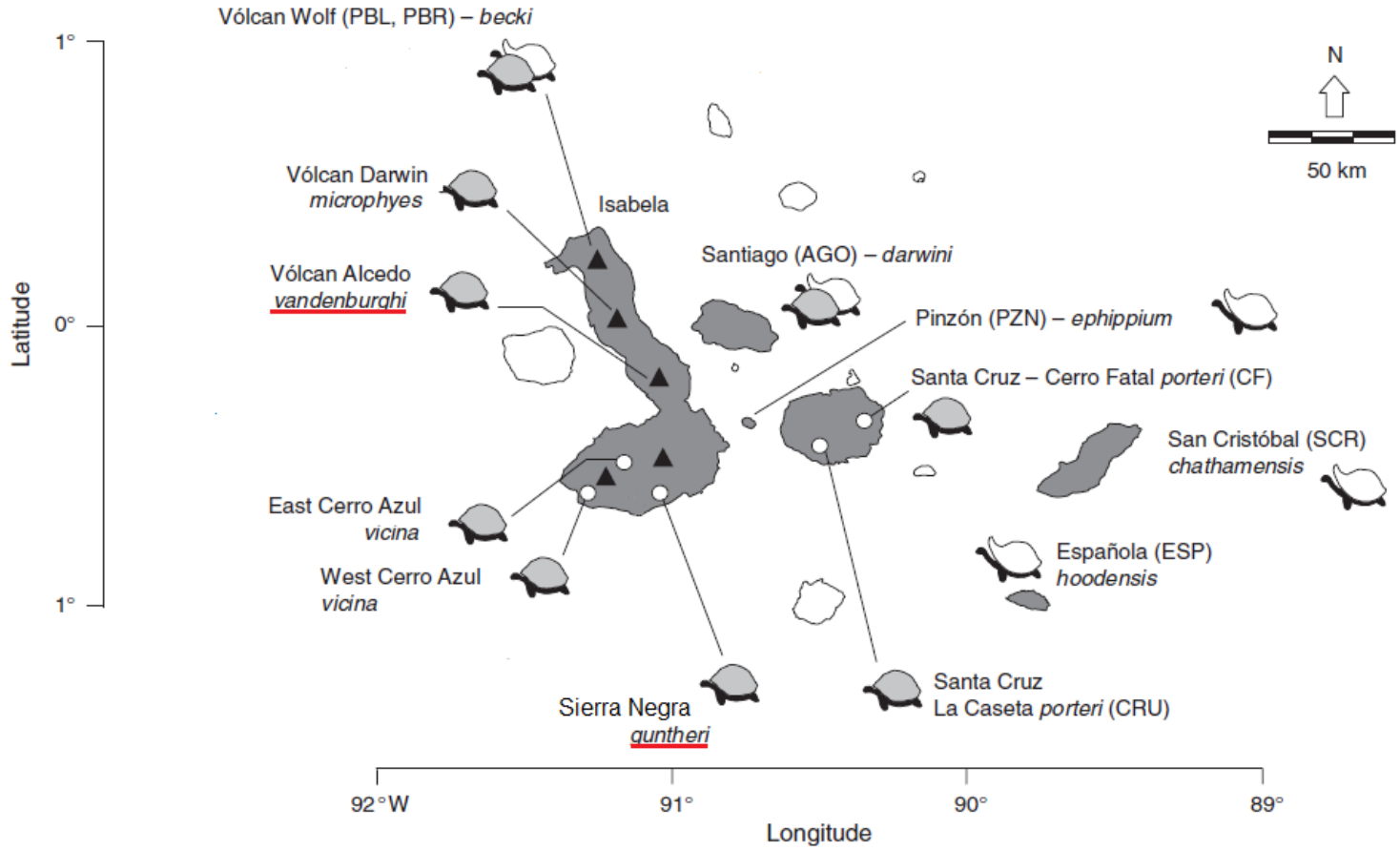

Figure 1.1. Distribution of giant tortoises in the Galápagos archipelago. Italicised names indicate current taxonomic designations, ▲: volcanoes on Isabela, underlined taxa in Isabela represent the former taxon name now unified as vicina. Cartoons represent the shell morphology observed in each population. From Poulakakis *et al.* (2008).

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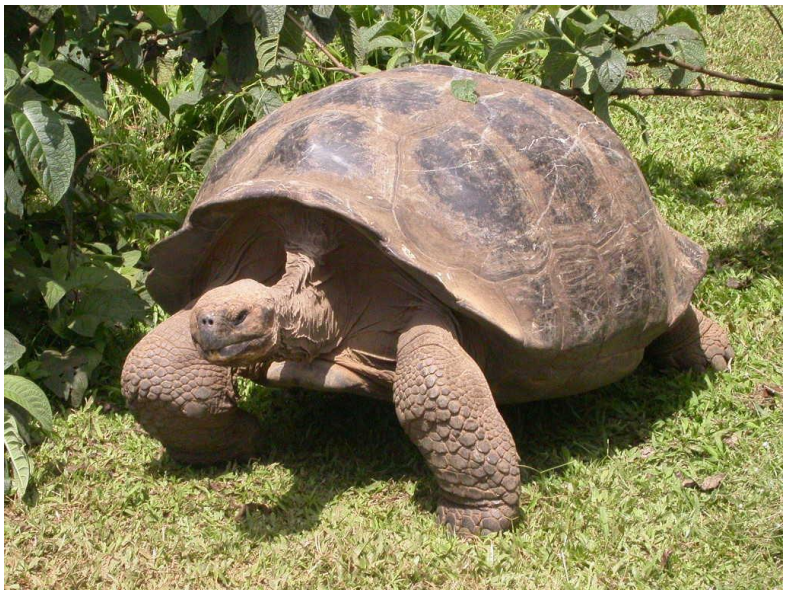


a)

b)

Figure 1.2 Galápagos tortoises with different shell morphology; a) domed-shaped shell; b) saddlebacked shell.

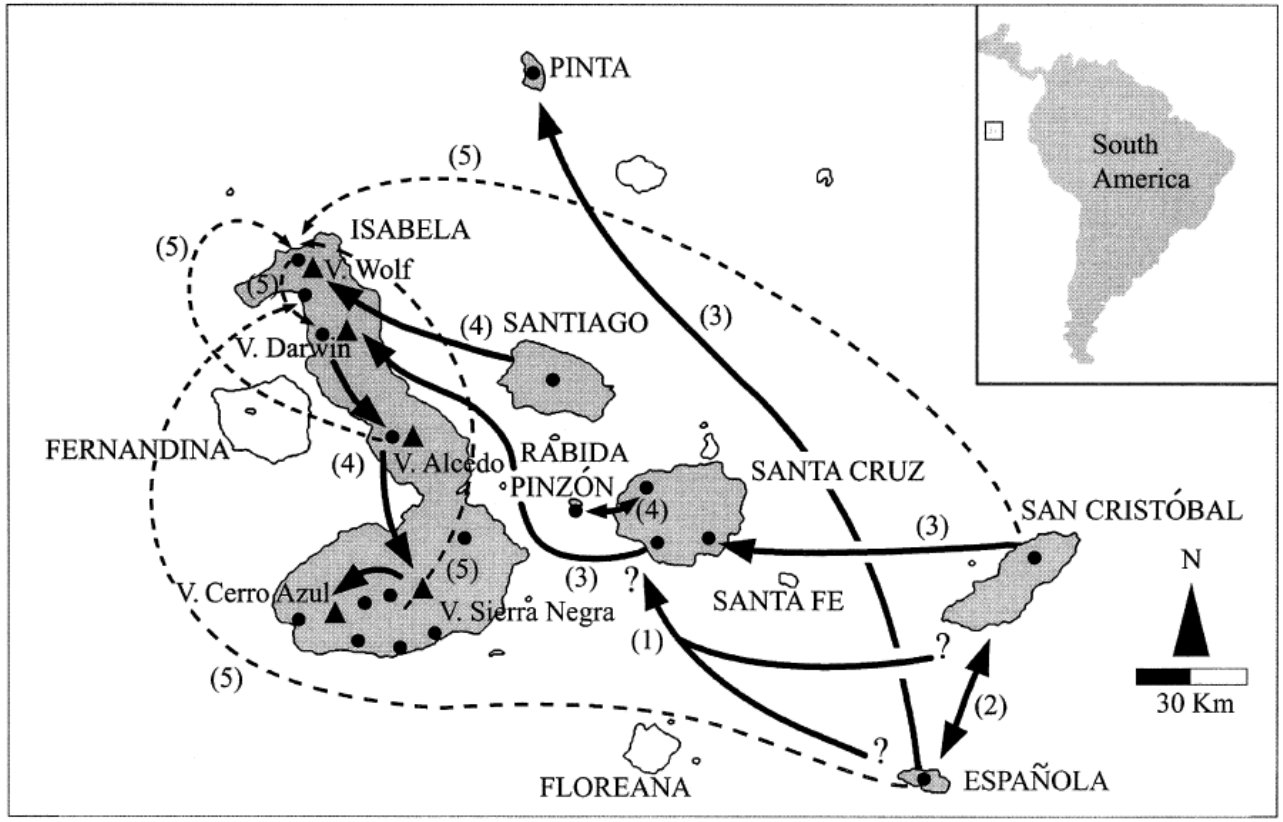


Figure 1.3. Schematic of the proposed phylogeographic history of Galápagos tortoises. The older islands of San Cristóbal and Española are the likely first islands colonized from mainland progenitors, but the genetic data cannot identify which. The arrows represent colonisation events within Galápagos with the numbers indicating very approximate temporal order. Solid arrows represent hypothetical natural colonisation events and the dashed arrows possibly human-induced translocations. From Caccone *et al.* (2002)..

1.2. Conservation and management of the Galápagos tortoise

The Galápagos tortoise remains vulnerable throughout its range (IUCN 2016), limited to populations on six islands within this remote, oceanic archipelago. In the last census in 2004, the total number of tortoises was estimated at around 20,000 individuals (Márquez *et al.* 2004), compared with 100s of thousands prior to human impacts. The decline of the populations began in the 17th century when buccaneers and whalers collected tortoises as a source of fresh meat. It is estimated that 200,000 animals were killed for food then. Additionally, at least 650 animals were removed to other continents by scientific expeditions. As a result, populations were extirpated on some islands and others were dramatically reduced in number and distribution. Furthermore, only three of the remaining species appear to have the potential for natural self-replacement (Beheregaray *et al.* 2003; Caccone *et al.* 1999). All populations face major contemporary threats including introduced species, such as goats, black rats, donkeys, pigs, cats and dogs. Some of these species offer strong competition for food or predate intensively eggs and hatchlings. In addition some populations still suffer from illegal hunting (IUCN 2016).

The critical status of most tortoise populations led the Galápagos National Park Directorate to establish captive breeding programmes in 1965. This management action has increased the population size of the endangered tortoise populations by reintroducing offspring obtained either from captive breeders, or from eggs or hatchlings collected in the wild and reared through vulnerable ages in captivity. The first breeding center, Fausto Llerena (named after park rangers devoted to the conservation of Galápagos), was stablished in Santa Cruz to help the recovery of tortoises of this island, Española, Pinzón and Santiago. Another breeding center, Arnaldo Tupiza, was built in Isabela in 1994 to repopulate threatened tortoise populations of that island. The last breeding center, Jacinto Gordillo, was implemented in San Cristóbal in 2004 to protect and help to recover the tortoise population of this island. The reintroduction of Galápagos tortoises started in 1970 and to date many young tortoises have been reintroduced to their islands of origin including Española (~ 2000), Pinzón (~ 837), Santiago (~ 1033), and two populations from the South of Isabela (~1000) (Galapagos Conservancy 2016; Macfarland *et al.* 1974).

Since 2015 a giant tortoise restoration initiative has been taking place (Galapagos Conservancy 2016). Among other activities the programme aims to reintroduce tortoises to all islands where they once existed before human impacts, to help restore island ecosystems to close to their original state. Where species are extinct, genetically similar species to those lost from the original habitat are selected. One of these islands is Santa Fe which is now home to 201 tortoises born and reared in the breeding center of Santa Cruz, which originated from Española progenitors. The programme has identified and brought into captivity 32 hybrid tortoise specimens found inhabiting Wolf volcano on Isabela Island, which are thought to have resulted from tortoises translocated from other islands by humans in the recent past (most probably pirates or whales dumping unwanted tortoises before sailing away from the archipelago). Some of these hybrid tortoises appear to be F1 or F2 backcrosses of the now extinct tortoises from Pinta and Floreana (Garrick 2012). It is hoped to carry out a highly targeted breeding programme with the long-term goal of restoring the genetic background of Pinta and Floreana tortoises for reintroduction to the respective islands (Galapagos Conservancy 2016).

1.3. Parasites of Galápagos tortoises

The role of parasites as a potential limiting factor of wild tortoise populations is not well understood. Most studies of helminth infections in tortoises have been carried out in captive populations kept in zoological parks (Chavarri *et al.* 2012). With few exceptions, nematodes are the only helminths infecting terrestrial chelonians, and most of them belong to the orders Oxyurida and Ascaridida, which are considered to be transmitted by the faecal-oral route (Chavarri *et al.* 2012). There are isolated reports of mortality associated with large ascarid infestations (Rideout *et al.* 1987); in contrast, oxyurids can be very prevalent and are considered to have an almost commensal relationship with their host.

With regard to parasites of wild Galápagos tortoise populations, very little is known, with just two previous studies reporting the presence of one nematode species (Bursey and Flanagan 2002) and coccidian species (Couch *et al.* 1996). In the last 12 years, in the framework of a project to establish tortoise' health parameters I and co-workers attempted to assess the diversity of parasitic nematode communities and the spatial variability of their distributions within four wild tortoise populations comprising three

species across three Galápagos Islands. We identified five different nematode egg types: oxyuroid, ascarid, trichurid and two types of strongyle. In wild tortoises, nematode egg complements varied according to tortoise species and island (Fig. 1.4) (Fournie *et al.* 2015).

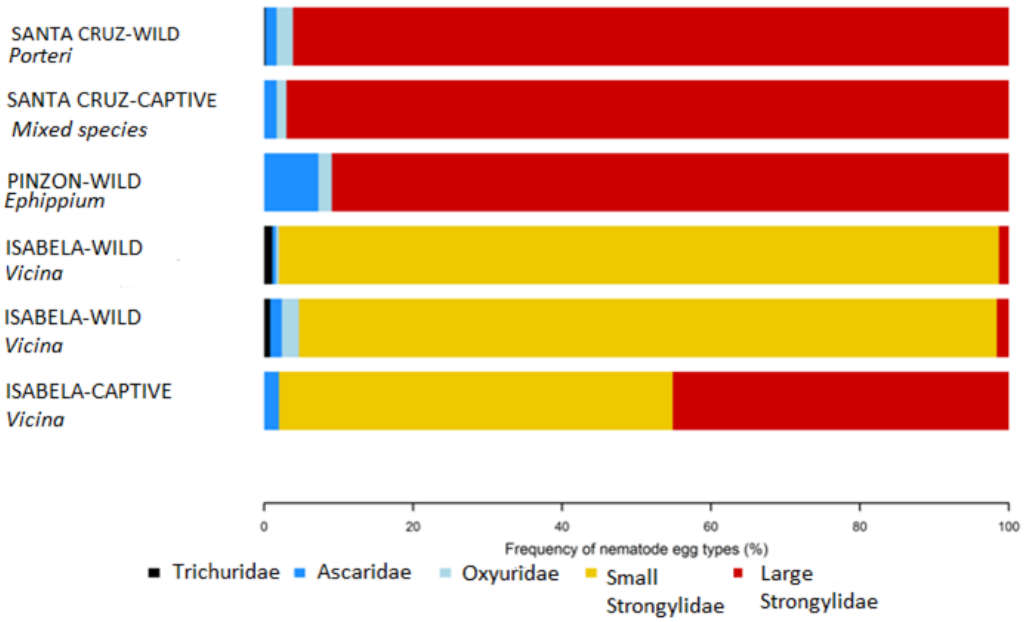


Figure 1.4. Relative frequency of nematode egg types according to location (relative frequencies are expressed as a percentage). After the name of the Island, the status of the sampled population is described. Analysis of captive tortoises are also shown in the figure. From Fournie *et al.* (2015).

Haemogregarines were also observed in blood smears from two tortoise populations of Isabela Island, those from Alcedo and Wolf Volcanoes. Subsequently, the parasite was found by PCR in three tortoise populations from Isabela and in tortoises from Pinzón Island. No haemogregarines were found by either method in tortoise population from other islands. Ticks were also observed and collected from tortoises of Alcedo, Wolf, Pinzón and Santiago but they were not observed in the remaining tortoise populations sampled throughout the Galápagos archipelago. The ticks infesting Galápagos tortoises have previously been reported to comprise three *Amblyomma* (Ixodidae) species and one of the genus *Argas* syn *Microargas* (Argasidae). *Amblyomma* ticks are represented by *A. usingeri*, found in tortoises from North Isabela; *A. macfarlandi* reported for tortoises on Cerro Azul in southern Isabela and on Santa Cruz Island; and *A. pilosum* found on Pinzón and Santiago but without reference to the host (Keirans *et al.* 1973a). Microargas ticks are represented by *Argas*

transversas and have been found just in Isabela (Tagus cove, located at west of Darwin Volcano) and on a tortoise of Santa Cruz (Hoogstraal *et al.* 1973).

Information about parasites and their distribution is relevant for the conservation management of the *Chelonoidis* spp., especially as they have been subject to an intensive captive breeding programme during the past 3 decades. Reintroduction programmes are confronted with several problems, e.g. releasing of immunologically naive animals into an area where parasites are endemic (Cunningham 1996), mixing and contamination with parasites during captive breeding, and introduction of new parasites by releasing animals in the wild population (Dybdahl and Storfer 2003; Mathews *et al.* 2006). In Galápagos, health-screening of juvenile giant tortoises has not been achieved comprehensively before their translocation, although this is changing in the last 12 years. Released juveniles carrying new nematode species to the wild populations could potentially modify the structure and the composition of the original nematode community.

Though the nematode species have potentially co-evolved with their hosts, they could infect allopatric hosts, and could have a different effect than on the original population (Dybdahl and Storfer 2003). Moreover, small and inbred host populations with reduced genetic variability could have a high susceptibility to new parasites (Whiteman *et al.* 2007). Translocations, breeding and re-introduction programs aim to prevent the extinction of threatened populations; nevertheless, the role that parasites play can be decisive in the success of such programs (Chavarri *et al.* 2012). On the other hand, captive bred tortoises should be managed to try and maintain the natural parasite communities of their source populations. As in other species these are potentially an evolved component of the Galápagos ecosystem (Whiteman *et al.* 2007).

1.4. Parasites as inferential tool for evolutionary history of their host

For some species the evolutionary histories of parasites run in parallel with host lineages. As hosts speciate, their parasites may also become reproductively isolated, potentially leading to co-speciation. Therefore, that the evolutionary history of parasites may reflect the evolutionary history of their hosts (Stefka *et al.* 2011). A

classic example of co-speciation is represented by the pocket gophers species (*Rodentia, Geomyoidea*), distributed in North America, and their ectoparasitic chewing lice (Mallophaga, Phthiraptera). In this model the extremely asocial behaviour between host species provided few opportunities to the parasite to colonize new species leading it to co-evolve with their specific host species (Page 1993). The evolutionary rate of parasite DNA is faster relative to that within the homologous loci of their hosts (Page 1993). Thus, this property of parasites can make them a powerful tool, providing additional information for inferring host evolution (Whiteman *et al.* 2007). Co-speciation however, is not universal for all parasite-host assemblages, especially for generalist parasites. This means that co-phylogenetic analyses are always required before making the assumption of co-speciation. Phylogenetic incongruences in turn might help to elucidate the factors influencing the independent evolution of some parasitic species.

1.5. Macroevolution of parasites and speciation

The origin of any parasite species in a parasite fauna has three general explanations. First, a parasite species may have been inherited by the host species from its ancestor. Second, a parasite species may have colonized the host species, jumping ship from another, sympatric host species. Third, a parasite species may be the outcome of an intra-host speciation event, i.e. an ancestral parasite species giving rise to one or more daughter species all within the same host species, without host speciation. Concerning extinction parasites, could be lost during host speciation events or later as a result of changes in the ecological characteristics of the host species, competition with other parasites or genetic drift (Vickery and Poulin 1998). In the colonization of new areas parasites could also be missed by chance in the sample of hosts from the original population (missing the boat) (MacLeod *et al.* 2010; Paterson and Gray 1997) .

Speciation mechanism in parasites can involve both allopatry and sympatry (Huyse *et al.* 2005), Fig. 1.5. For Allopatry, Huyse *et al.* (2005) pointed out two mechanisms; first an ancestral parasite species can be subdivided geographically together with its ancestral host species (vicariance); second, it may involve host-switching which can be followed by speciation through a peripheral-isolates mode or the new host will be added to the species range of the parasite. Sympatric speciation occurs when species arise in the absence of a physical barrier. He also added that host-switching can be

defined as allopatric depending on the parasites involved, this might also be a form of sympatric speciation when infective, free-living stages of both host-adapted populations are in syntopy.

Huyse *et al.* (2005) concluded that the genetic structure of parasite populations correlates with host mobility, mode of reproduction of the parasite, complexity of the parasite life cycle, parasite infra-population size and host specificity. Also, that the importance of these factors varies from one parasite species to the next. For this reason he emphasizes, that a phylogenetic comparative approach is crucial to disentangle the various processes that drive parasite diversification.

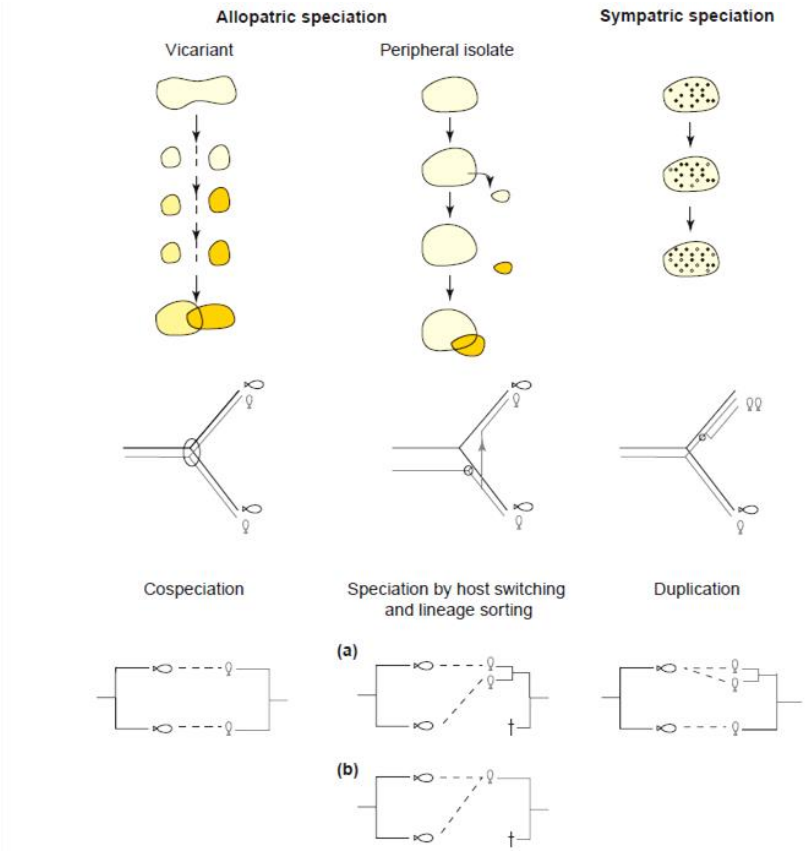


Figure 1.5. Possible ways of parasite speciation. Allopatric speciation could happen in two forms vicariance or peripheral isolation. The latter in turn could involve a) host switching or b) lineage sorting. Modified from Huyse *et al.* (2005).

1.6. Macroevolution and determinants of diversity of parasite communities

In nature, host species are exposed to a variety of parasites. As a result, it is common that a variety of parasites may infect them simultaneously. These multiple infections are however part of a more broad pattern of parasite assemblages. The basic assemblage comprises all parasites species infecting one individual host and is known as infra-community. The next, includes all species found in a host population and is known as component community. The highest organizational level of parasites is the sum of the component community in the host species and represents the meta-community or parasite fauna (Morand 2011).

The foundation for predictive hypotheses regarding the role of ecological factors in determining parasite communities come from theoretical ecology (with determinants such as latitudinal gradients, host geographical range, host size) and epidemiological theory (with determinants such as host population size, host population density, host population longevity). Other determinants have been associated with host behavioural ecology (sociality, grooming and preening behaviour). Furthermore, parasite species richness seems to be an attribute of host species like any other host life history trait (Krasnov *et al.* 2008; Morand 2011).

Parasite-parasite interactions also seem to influence directly or indirectly the composition of parasite communities (Petney and Andrews 1998) through interspecific competition (e.g. mixed species helminth infection) and/or intraspecific competition (e.g. genetically diverse strains of microparasites). Fundamentally, competition between parasites may be direct or indirect, through competition for resources (e.g. blood) or immune system (i.e. immunosuppression or cross-immunity) (Cox 2011; Fenton *et al.* 2010; Morand 2011). During multiple infections with two or more parasite species, the infection intensity of one (or more) parasite(s) might be enhanced by the other parasite(s) (synergic interactions) or, on the contrary, be suppressed (antagonist interactions) (Fenton *et al.* 2010; Morand 2011) .

1.7. Thesis aims and outline:

This thesis investigates the taxonomic identity and biogeography of blood parasites, *Amblyomma* ticks and gastro-intestinal helminths of *Chelonoidis* spp. For blood parasite and ticks I assessed whether they have co-phylogeography patterns with their tortoise host. For helminths, I examined whether host colonization history and local ecology determine their distribution and community composition.

Chapter 2 aims to characterise the haemoparasite species infecting Galápagos giant tortoises, their biogeography and diversity across the different tortoise species. I discuss possible vectors, routes of transmission, the likely origin of the parasites, and their relationship with haemoparasites already described elsewhere.

Chapter 3 aims to determine the current distribution of Galápagos tortoise ticks, to evaluate genetic distinctiveness in relation to current morphological classification, to evaluate the genetic structure, and to test the pattern and timing of their evolutionary diversification correlates with that of their tortoise host. Using ticks collected from marine and land iguana I also assess the possible origin of the ticks infecting Galápagos tortoises and the ecological and geological factors influencing their evolution in Galápagos.

Chapter 4 aims to survey the prevalence and abundance of nematode taxa in wild Galápagos tortoise populations using traditional coprological methods. I examine temporal variation in a population (*C. nigra*; Santa Cruz west) surveyed in a former study performed by Fournie *at al.* (2015). I also investigate the helminth parasites of reintroduced captive bred tortoises on Española Island and evaluate possible associations between prevalence and infection intensity with host sex, origin and location.

Chapter 5 aims to use high throughput parallel amplicon sequencing of 18S rDNA to characterize the nematode community structure of the Galápagos tortoises analysed by coprological methods in chapter 4; I evaluate the ability of NGS based methods to resolve nematode OTUs to genus or species scale; and compare the relative abundances of taxa detected with results from conventional microscopical techniques. I use the data to identify helminth community variation.

Chapter 6 aims to discuss the results in the context of the biogeographic and conservation history of Galápagos tortoises, and the relevance of tortoise parasites to biosecurity and management in the tortoise captive breeding programme. It also provides ideas for further research directions.

Chapter 2. Characterisation and biogeography of blood parasites infecting Galápagos giant tortoises (*Chelonoidis* spp.).

2.1. Introduction

Reptilia represents an ancient vertebrate class that arose around 320 million years ago. The first reptiles probably co-evolved with their own parasites so the factors influencing the distribution of one might help to understand the factors influencing the biogeography of the others (Lainson and Naiff 1998). Blood parasites, also termed as haemoparasites, are among the common infectious agents of this vertebrate group and are globally widespread. They comprise taxonomically diverse organisms, including haemogregarines and haemosporidia from the Phylum Apicomplexa, trypanosomatid flagellates and *Leishmania* from the Phylum Euglonozoa, and filariid worms from the Phylum Nematoda (Telford 1984). Of these, the haemogregarines are the most common, with ~ 400 species described so far. Four accepted genera of haemogregarines infect reptiles: *Haemogregarina*, *Hepatozoon, Karyolysus* and *Hemolivia* (Kvicerova *et al.* 2014; Telford 2009; Wozniak *et al.* 1994b). Species in the genus *Haemogregarina* are the most common haemoparasites found in semi-aquatic chelonians (e.g. families Chelydridae, Emydidae, Chelydae, Geomydidae), while species in the genera *Haemolivia* and *Hepatozoon* are the most common haemoparasites found in terrestrial chelonians (Cook *et al.* 2014; Karadjian *et al.* 2015).

A feature shared by all reptile haemoparasites is their heteroxenous life cycle requiring both a vertebrate and a haematophagous invertebrate (e.g. mosquito, simuliid fly, tick, leech) host. The life cycle, however, differs amongst the different taxonomic groups. In haemogregarines, asexual reproduction (merogony and gamogony) takes place in the vertebrate intermediate host, while sexual reproduction (sporogony) occurs in the invertebrate definitive host (Criado-Fornelio *et al.* 2006; Smith 1996). Most haemogregarine parasites are transmitted via the ingestion of infected invertebrates (Kim *et al.* 1998; Telford *et al.* 2012; Wozniak *et al.* 1994a), with exceptions reported only for species of the genus *Haemogregarina* which are transmitted via the bite of leeches, their vector and definitive host (Paperna 2006). Reptiles feeding on other vertebrates might acquire haemogregarines after ingesting

the infected prey (e.g. lizards, mice), which act as paratenic hosts (Tome *et al.* 2014). Congenital infection has been described in some snake species, including *Nerodia fasciata, Crotalus durissus* and *Boa constrictor* (Siroky *et al.* 2007; Siroky *et al.* 2004; Telford *et al.* 2005; Wozniak *et al.* 1994a).

The diversity and ecology of reptile haemoparasites are poorly known. Most studies are focused on morphology and/or prevalence, while epizootiological data are rarely provided (Telford 1984; Telford 2009). The little attention paid to them is probably related to their low pathogenicity. Reptile haemoparasites appears to have low virulence for their natural hosts and do not generally cause deleterious effects (Telford 2009). Nevertheless, infections of immunologically naive animals can lead to inflammatory disease (Brygoo 1963). Thus, surveillance for haemoparasites has been recommended for reptiles held in captivity and for animals involved in conservation programmes (Wozniak *et al.* 1994b).

Reptile haemoparasites can be useful for developing a better understanding of the ecology and evolution of their host species (Holmes 1993; Windsor 1998). As some parasite species co-evolved with their hosts, they can provide useful markers for host phylogeny, ecology and biogeography (Marcogliese 2004). In addition, the study of the parasites themselves is relevant for the characterisation of biodiversity. For example, information about their biogeography might be useful for understanding factors influencing parasite distribution, transmission and evolution.

The study of reptile haemoparasites is a challenging task. The traditional diagnostic method involves microscopic examination of stained blood smears, in which parasite stages can be seen within or outside of the blood cells. This method is advantageous for obtaining haemoparasite morphometric data, but is of limited use for taxonomy (Telford 2009). In the case of haemogregarines in particular, a confident generic assignment based on morphology requires knowledge of the sporocyst development pattern and the parasite fertilization mechanism (sysygy or syngamy). Accomplishing this goal demands the collection and processing of definitive hosts, which is not always feasible even if these are known. Moreover, the generic identification of haemogregarines based on morphological features alone is confounded by homoplasy

(Cook *et al.* 2014; Cook *et al.* 2015). There is, therefore, a recent major shift to using molecular methods for supplementing their identification.

Sequencing of 18S ribosomal DNA gene (18S rDNA) has advanced the characterisation and clarification of haemogregarine taxonomy and phylogeny (Barta *et al.* 2012; Karadjian *et al.* 2015; Kvicerova *et al.* 2014). The use of this method has led to the reassignment of some species (Cook *et al.* 2014; Cook *et al.* 2015) and a suggestion that the genus *Hepatozoon* be split into *Hepatozoon* and *Bartazoon*. The division of *Hepatozoon* into two genera is based on the split of their members into two different clades and the current knowledge on the life cycle of the parasites in this group. According to this arrangement, *Hepatozoon* would retain haemoparasites infecting vertebrates of the order Carnivora, while *Bartazoon* will contain a complex of haemoparasites infecting reptiles, amphibians, marsupials, birds and rodents (Karadjian *et al.* 2015).

Little is known about haemoparasites infecting Galápagos giant tortoises *Chelonoidis* spp. Although there is an unpublished report of haemogregarines infecting tortoises in Galápagos (Landazuri 2000), there is no information about their taxonomy, prevalence or distribution. In Galápagos, haemogregarines have also been reported parasitizing endemic lava lizards (*Microlophus albemarlensis*), marine iguanas (*Amblyrhynchus cristatus*) on Santa Cruz Island (Ayala and Hutchings 1974), and the three Galápagos land iguana species: *Conolophus subcristatus*, *C. pallidus* and *C. marthae* (Fulvo 2010). The lava lizard and marine iguana parasites have only been described morphologically, and references to their taxonomy or epizootiology are lacking. Recent molecular studies detected different haemogregarine 18S haplotypes in the native Galápagos mosquito *Aedes taeniorhynchus*, but without knowledge of the intermediate host or parasite morphology (Bataille *et al.* 2012). The only detailed assessment of haemoparasites in reptiles from the Galápagos archipelago to date has been the work of Fulvo (2010) for Galápagos land iguana species. Surveys of these species across the Galápagos archipelago have yielded a surprising diversity of 18 haemogregarine haplotypes, with 12 of them present in one population on the north of Isabela (Wolf Volcano). The presence in Galápagos of haematophagous invertebrates, including ticks of the genus *Amblyomma*, provides suitable conditions for the circulation of haemoparasites.

Knowledge of the haemoparasites of *Chelonoidis* spp. is important for several reasons. Such knowledge would allow the identification of any host-taxon-specific parasite lineages and inform biosecurity measures for the conservation management of Galápagos biodiversity. This is particularly important as it is likely that infected tortoises are translocated across islands as part of current tortoise captive breeding and repatriation programme. The phylogeography of the tortoise haemoparasites might shed additional light on the biogeography of the host species. In addition, as the tortoises inhabit different islands and habitat types, identification of haemoparasite biogeography could enable exploration of environmental factors influencing haemoparasite distribution. In this chapter, I characterise the haemoparasite species infecting Galápagos giant tortoises, their biogeography and diversity across the different tortoise species. I discuss possible vectors, routes of transmission, the likely origin of the parasites, and their relationship with haemoparasites already described elsewhere.

2.2. Material and Methods

2.2.1. Sample collection

The samples used in this study comprised whole blood in EDTA and blood films collected and prepared during the years 2005, 2006, and 2014. The sampling in 2005-2006 was done from wild tortoises from across the Galápagos archipelago and from captive individuals in the three breeding centres established on the islands (see Fig. 1.1). The sampling in 2014 was from wild tortoises on Santiago Island and from the Cerro Azul population on Isabela. Sampling in 2005-2006 was performed within the framework of a programme to establish the haematological parameters of *Chelonoidis* spp. and was led for the Galápagos National Park Directorate (GNPD) by the Galápagos Genetic Pathology and Epidemiology Laboratory (GGEPL) "Fabricio Valverde". The sampling of 2014 was conducted only for the purpose of surveying haemoparasites. By this year the GGEPL activities had finished, therefore a specific sampling permit (coded PC-9-13) was granted by the GNPD.

In all cases, sampling involved drawing of 3 to 10 ml of peripheral blood from the brachial vein of tortoises using sterile disposable syringes and 21G needles. A drop of fresh blood was used for preparing a blood film in the field and the remainder was aliquoted into tubes containing EDTA. A code was recorded for each sample, as well

as the tortoise's sex (male, female or juvenile if sexual characteristics were not evident) and, if present, the identification number (implanted microchip or iron brand on shell) marked previously by the GNPD. Each bled tortoise was marked using temporary paint (which lasted for approximately one week) to prevent repeated collection. Each tortoise was examined for ticks and any found were collected and fixed in ethanol. In the field, the blood samples were kept cold on wet ice. On some islands they were transported to the laboratory on the day of collection, but on others they were kept on ice in the field for up to five days. Once in the laboratory, the samples in EDTA were stored at -80°C for future molecular analysis while the blood smears were processed for examination (see below). For a description of the tortoise samples collected, including study sites, dates of sampling and the number of each tortoise species sampled, see Tables 2.1 and 2.2.

2.2.2. Blood film analysis

Blood films were fixed in ethanol either in the field or in the laboratory. They were stained with a standard Wright-Giemsa procedure between four and six hours after their preparation (Houwen 2002). A total of 1032 blood smears (752 from wild giant tortoises and 280 from captive individuals) were prepared during 2005 and 2006 (Table 2.1 and 2.2). During these years they were analysed by light microscopy for presence/absence of haemoparasites. Screening was performed using a ×400 magnification on a Zeiss axioscope at the GGEPL. Positive blood films identified in 2005-2006 were reviewed in 2014 using light microscopy. The degree of parasitaemia (the demonstrable presence of parasites in the blood) was calculated as the number of infected red blood cells per 100 red blood cells, with ~10^4 erythrocytes examined per blood film using the method described by (Siroky *et al.* 2004). Blood film examination also included a survey of the white cells present among the ~10^4 erythrocytes examined, the number of these cells were relative lower on comparison with erythrocytes. A total of 97 blood films were prepared and screened by light microscopy in 2014 (Table 2.1). The reassessment of old blood films and the analysis of new ones were performed on Galápagos in a new laboratory facility belonging to the Agencia de Control y Regulación de la Bioseguridad. Photographs and measurements of the length and width of observed haemoparasites were done in 2014 using a Leica DM 2700 light microscope with a camera attachment. Each observed

parasite was measured three times and the median value was used to report the morphometric data.

The power to detect infections relative to sample size was estimated in two ways. Firstly I estimated the sample size required for detecting at least one tortoise infected with haemogregarines at an infection prevalence of 5%, using the equation "n=log (1-C)/log (1-P)" where n is number of sampled individuals, C is the desired probability of finding at least one infected animal in such a sample and P is the prevalence of infection in a defined population of animals (Digiacomo and Koepsell 1986). The second followed the method of Smith (2015), implemented as an Excel spreadsheet macro, available online at the web page of the "Risk Project" by the Mississippi State University (Smith 2015). This calculation considers the population size and sensitivity of the test. For this approach the test sensitivity was set at 80 percent. In both cases the confidence interval was set at 95 percent.

Table 2.1. Details of samples collected/blood films prepared from wild *Chelonoidis* spp. M=male, F=Female, J=juvenile.

Sampling site (tortoise spp).	Wild tortoises (M/F/J)	Date of sampling
Isabela, Wolf (*C. becki*)	30 (15/15/0)	20-21/04/2005
Isabela, Darwin (*C. microphyes)*	13 (9/1/3)	28/04/2005
Isabela, Alcedo (*C. vandenburghi syn vicina*)	100 (67/12/21)	22-26/04/2005
Isabela, Sierra Negra (*C. guntheri syn vicina*)	70 (23/27/20)	18-22/04/2006; 5/07/2006
Isabela, Cerro Azul (*C. vicina)*	11 (8/3/0)	25/04/2006
Isabela, Cerro Azul (*C. vicina)*	57 (40/17/0)	10-12/02/2014
Pinzón (*C. ephippium*)	44 (19/20/5)	15-17/03/2006
Santiago (*C. Darwini*)	35 (32/3/0)	19-21/05/2006
Santiago (*C. Darwini*)	40 (39/1/0)	17-21/01/2014
Santa Cruz west (*C. Porteri*)	289 (210/64/15)	Throughout 2005 and 2006
Santa Cruz east (*C. donfaustoi*)	55 (17/23/15)	Throughout 2005 and 2006
San Cristóbal (*C. chatamensis*)	105 (44/16/45)	9-11/7/2005

Table 2.2. Details of samples collected/blood films prepared from captive *Chelonoidis* spp.

Breeding center (tortoise spp).	Wild tortoises (M/F/J)-	Date of sampling
Santa Cruz (*C. hoodensis*)	36 (4/32/0)	Throughout 2005 and 2006
Santa Cruz (mixed C. spp)	91 (63/28/0)	Throughout 2005 and 2006
Isabela, Sierra (*C. guntheri*)	52 (0/0/52)	05/03/2005; 27-28-04/2006
Isabela, Cerro Azul (*C. vicina)*	53 (26/25/2)	6/03/2005; 25/05/2006; 27/07/2006
San Cristóbal (*C. chatamensis*)	48 (31/5/12)	4-08/2005; 26/01/2006; 30/03/2006

2.2.3. Detection of blood parasites by PCR

Molecular analysis was conducted on a subset of tortoise samples analysed by light microscopy. Samples were screened by PCR for all individuals in populations where ticks were present, and in ~30% of samples collected in islands where neither haemogregarines nor ticks were detected. The final screening comprised 453 samples collected in 2005-2006 with 213 from Isabela (100 Alcedo, 30 Wolf, 13 Darwin, 70 Sierra Negra and 11 Cerro Azul); 35 from Santiago, 44 from Pinzon; 75 from Santa Cruz west, 25 from Santa Cruz east, and 50 from San Cristóbal. It also included all the 97 blood samples collected in 2014 (40 from Santiago and 57 from Isabela-Cerro Azul). Captive tortoises were excluded based on the results of microscopic analysis and absence of ticks. Ticks collected from 2005 to 2014 were also screened for haemoparasites using PCR. Aliquots of each blood sample and the fixed ticks were transported to the United Kingdom under export permits: DPNG 84-2013, 064-2014, and CITES 0981315, 0981325 and importation permits: DEFRA TARP/2013/213 and CITES 516095/01 and 522826/01.

The DNA isolation and PCR analyses were conducted at the University of Leeds. Genomic DNA was isolated using the DNeasy Blood & Tissue kit (Qiagen) following the manufacturer's instructions. In the case of ticks, DNA was extracted from the head and abdomen of engorged individuals. Each tissue was treated individually; before DNA isolation samples were frozen on dry ice and crushed using sterilized pestles. A total of 57 ticks were analysed, comprising individuals collected from tortoises on Isabela-Wolf (n=13), Isabela-Alcedo (n=8), Santiago (n=25), and Pinzón (n=11).

Based on the results of light microscopy, PCR for haemoparasites was targeted at haemogregarines. Haemogregarine 18S rDNA was screened using two set of primers used frequently for characterising these groups of haemoparasites (Table 2.3). One set included the forward primer HepF300 and the reverse Hep900, designed based on *Hepatozoon* sequences (Ujvari *et al.* 2004). The second set included the forward primer HEMO1 and the reverse HEMO2 designed to target haemogregarine DNA from different genera (Perkins and Keller 2001). These primer sets yield non overlapping PCR products of ~ 600 base pairs (bp) and ~ 900 bp respectively. The PCR reactions were performed as described in the references above.

Nested PCR analyses were also conducted for the detection of haemosporidia (*Haemoproteus* and *Plasmodium*) in blood samples positive for haemoparasites by light microscopy but negative for haemogregarines by PCR. A first PCR reaction was done using primers HAEMF and HAEMR2. This was followed by a second reaction using primers HAEMNF and HAEMNR2. The primers sequences are given in the Table 2.3. The reactions were performed as described by (Waldenstrom *et al.* 2004).

2.2.4. DNA sequence analysis for taxon identification and taxon prevalence within Galápagos samples

Amplicons of the expected size were purified and sent for sequencing in both directions by the commercial company Beckman Coulter Genomic, UK. Sequences were trimmed and quality checked in BioEdit version 7.00 (Hall 1999). Taxonomic identity of sequences was evaluated using BLASTN (Altschul *et al.* 1990) and compared to those available in GenBank. Next sequence alignments were generated by combining sequences obtained in the current study with sequences previously obtained from marine iguana, land iguana and Galápagos mosquitoes. Details of these sequences are given in Table 2.4. The haemogregarine sequences of Galápagos land iguanas were kindly provided by Dr. Gabriel Gentile, University of Tor Vergata, Italy, and were obtained by Fulvo (2010). The haemogregarine sequences from mosquitoes are accessible in GenBank (*Hepatozoon* sp. MG1 ID: JQ080302, *H.* sp. MG2 ID: JQ080303, *H.* sp. MG3 ID: JQ080304) (Bataille *et al*. 2012). Sequence alignments were performed in BioEdit 7.00; using Clustal W.

2.2.5. Generation of sequences for phylogenetic analysis

Generation of sequence data for the phylogenetic analysis of Galápagos tortoise haemogregarines was carried out in two stages. The first stage was done with sequences of 18S rDNA obtained with primers Hep300-Hep900. The second stage was performed using a ~1050 bp fragment of the same gene obtained with primer Hep900 and a new primer named HEMO3 (Table 2.3) designed for this study in order to obtain a larger fragment of the gene. Primer HEMO3 was designed using the on-line software Primer-Blast (Ye *et al.* 2012) and using as input a sequence already acquired using primers HEMO1 and HEMO2. The PCR reactions with HEMO3-Hep900 were done in a 50 µl volume containing 0.5 uM of each primer, 0.2 mM of each dNTP, 0.5 unit of Taq DNA polymerase (Invitrogen), and 2.5 mM of MgCl2 and 1 ul of DNA. The PCR program used

was 94° C for 5 min, followed by 35 cycles of 94° C for 30 s, 52° C for 45 s and 72°C for 60 s, and finally 72° C for 7 min. PCR products were sequenced and analysed as described above.

2.2.6. Phylogenetic data analysis

The data set for phylogenetic analysis involved representatives of all the haplotypes of reptile haemogregarines available in GenBank to date (Table 2.4). The sequences were retrieved using MEGA version 7.0 (Tamura *et al.* 2013) and aligned using MUSCLE (Edgar 2004).

Phylogenetic relationships between haplotypes were inferred using MrBayes v.3.1.1 (Huelsenbeck and Ronquist 2001). The analysis was run either under the conditions of the Tamura 3-parameter + Gamma Distributed model (T92+G), or the Tamura 3-parameter + Gamma Distributed with Invariant Sites (T92+G+I) identified as the best fit model for the data using MEGA 7.0. Multiple simulations were run for 10 million generations with the first 500,000 trees discarded as burn-in period after confirming the convergence of chains. Trees were sampled every 1,000 generations and a 50% consensus tree was constructed from the results. Each analysis was repeated three times. The resulting consensus tree was visualised and edited using the online software iTol (Letunic and Bork 2016).

Table 2.3. Primers used for screening of haemogregarines and haemosporidia in Galápagos giant tortoises.

Primer name	Sequence	Reference
HepF300	GTTTCTGACCTATCAGCTTTCGACG	Ujvari *et al.* 2004
Hep900	CAAATCTAAGAATTTCACCTCTGAC	
HEMO1	ATTGGTTTTAAGAACTAATTTTATGATTG	Perkins and Keller
HEMO2	CTTCTCCTTCCTCCTTTAAGTGATAAGGTT	2001
HAEMF	ATGGTGCTTTCGATATATGCATG	Waldenstrom *et al.*
HAEMNR2	AGAGGTGTAGCATATCTATCTAC	2004
HEMO3	CTTGCGTTAGACACGCAAAG	This thesis

Table 2.4. Haemogregarine haplotypes used for phylogenetic analysis of Galápagos tortoise haemogregarines. The GenBank accession number is given for each haplotype, also the host family, host species and geographic region. At the time of submitting this thesis the haemogregarine haplotypes from *Conolophus* spp. had not been submitted to GenBank, they were provided by Dr. Gabriel Gentile from the University of Tor Vergata, Italy. Table continue in the following four pages.

Accession number	Isolate code	Host Family	Host species	Geographic Region
JQ080302	Hepatozoon sp. MIG1	Culicidae	*Aedes taeniorhynchus*	Galápagos
JQ080303	Hepatozoon sp. MIG2	Culicidae	*Aedes taeniorhynchus*	Galápagos
JQ080304	Hepatozoon sp. MIG3	Culicidae	*Algyroides marchi*	Galápagos
JX531938	Hepatozoon sp. JPMCM-2012	Lacertidae	*Amblyomma fimbriatum*	Spain
EU430235	Hepatozoon sp. 777a	Ixodidae	*Amblyomma fimbriatum*	Australia
EU430231	Hepatozoon sp. 770a	Ixodidae	*Amblyomma moreliae*	Australia
EU430232	Hepatozoon sp. 782	Ixodidae	*Aponomma varanensis*	Australia
EU430233	Hepatozoon sp. 797	Ixodidae	*Aponomma varanensis*	Australia
JQ670909	Hepatozoon sp. CS-2012	Ixodidae	*Atlantolacerta andreanskyi*	Thailand
JQ670910	Hepatozoon sp. CS-2012	Ixodidae	*Caiman crocodilus yacare*	Thailand
HQ734798	Hepatozoon sp. 317am	Lacertidae	*Caiman crocodilus yacare*	Morocco
KJ413113	Hepatozoon sp. MRA-2014b	Alligatoridae	*Caiman crocodilus yacare*	Brazil
KJ413132	Hepatozoon sp. MRA-2014b	Alligatoridae	*Cerastes cerastes*	Brazil
KJ413133	Hepatozoon sp. MRA-2014b	Alligatoridae	*Chersina angulata*	Brazil
KJ408510	Hepatozoon sp. BT-2014	Viperidae	*Crotalus durissus terrificus*	Morocco
KJ702453	Haemogregarina fitzsimonsi	Testudinidae	*Crotalus durissus terrificus*	South Africa
KC342528	Hepatozoon sp. ex rattlesnake BR-2012	Viperidae	*Crotalus durissus terrificus*	Brazil
KC342527	Hepatozoon sp. ex rattlesnake BR-2012	Viperidae	*Crotalus durissus terrificus*	Brazil
KC342524	Hepatozoon sp. ex rattlesnake BR-2012	Viperidae	*Crotalus durissus terrificus*	Brazil
KC342525	Hepatozoon sp. ex rattlesnake BR-2012	Viperidae	*Crotaphopeltis hotamboeia*	Brazil
KC342526	Hepatozoon sp. ex rattlesnake BR-2012	Viperidae	*Dolichophis caspius*	Brazil
KJ408512	Hepatozoon sp. BT-2014	Colubridae	*Elaphe carinata*	Niger
KJ408513	Hepatozoon sp. BT-2014	Colubridae	*Elaphe carinata*	Turkey
KF939626	Hepatozoon sp. YLW-2014	Colubridae	*Elaphe carinata*	Shanghai China

Accession number	Isolate code	Host Family	Host species	Geographic Region
KF939621	Hepatozoon sp. YLW-2014	Colubridae	*Eumeces algeriensis*	Shanghai China
KF939622	Hepatozoon sp. YLW-2014	Colubridae	*Furcifer* sp.	Shanghai China
HQ734796	Hepatozoon sp. 127ea	Scincidae	*Hemidactylus mabouia*	Morocco
KM234649	Hepatozoon domerguei	Chamaeleonidae	*Hemidactylus mabouia*	Madagascar
KM234618	Hepatozoon sp. DJH-2014c	Gekkonidae	*Hemidactylus mabouia*	Brazil
KM234615	Hepatozoon sp. DJH-2014c	Gekkonidae	*Hemorrhois hippocrepis*	Brazil
KM234616	Hepatozoon sp. DJH-2014c	Gekkonidae	*Hemorrhois nummifer*	Brazil
JX244268	Hepatozoon sp. DB1562	Colubridae	*Hierophis viridiflavus*	Morocco
KJ408514	Hepatozoon sp. BT-2014	Colubridae	*Hierophis viridiflavus*	Turkey
KJ408515	Hepatozoon sp. BT-2014	Colubridae	*Kinixys zombensis*	Italy
KJ408516	Hepatozoon sp. BT-2014	Colubridae	*Kinixys zombensis*	Italy
KR069083	Hemolivia parvula NMBZAF P 371	Testudinidae	*Liasis fuscus*	South Africa
KR069082	Hemolivia parvula RC140409A1	Testudinidae	*Liasis fuscus*	South Africa
AY252104	Hepatozoon sp. ex Liasis fuscus	Pythonydae	*Lycognathophis seychellensis*	Australia
AY252103	Hepatozoon sp. Boiga	Pythonydae	*Lycognathophis seychellensis*	Australia
HQ292773	Hepatozoon sp. 35SH	Colubridae	*Mabuya wrightii*	Seychelles
HQ292774	Hepatozoon sp. 41FG	Colubridae	*Mauremys leprosa*	Seychelles
HQ292771	Hepatozoon sp. 1SP	Scincidae	*Natrix tessellata*	Seychelles
KF257929	Haemogregarina stepanowi 5013	Geoemydidae	*Oplurus* sp.	Algeria
KJ408526	Hepatozoon sp. BT-2014	Colubridae	*Phyllopezus pollicaris*	Turkey
KM234650	Hepatozoon sp. JPM-2014c	Opluridae	*Podarcis bocagei*	Madagascar
KM234613	Hepatozoon sp. DJH-2014c	Phyllodactylidae	*Podarcis bocagei*	Brazil
KJ189393	Hepatozoon sp.	Lacertidae	*Podarcis hispanica*	Portugal
JX531955	Hepatozoon sp. JPMCM-2012	Lacertidae	*Podarcis vaucheri*	Portugal
KJ189426	Hepatozoon sp.	Lacertidae	*Podarcis vaucheri*	Portugal
HQ734793	Hepatozoon sp. 165pv	Lacertidae	*Podarcis vaucheri*	Morocco
KJ659859	Hepatozoon sp. DJH-2014b	Lacertidae	*Podarcis vaucheri*	Morocco
HQ734792	Hepatozoon sp. 164pv	Lacertidae	*Psammophis elegans*	Morocco
HQ734794	Hepatozoon sp. 167pv	Lacertidae	*Psammophis schokari*	Morocco

Accession number	Isolate code	Host Family	Host species	Geographic Region
KC696568	Hepatozoon sp. DB2220	Lamprophiidae	*Python regius*	Algeria
KC696564	Hepatozoon sp. DB2229	Colubridae	*Quedenfeldtia moerens*	Algeria
EF157822	Hepatozoon ayorgbor	Pythonydae	*Sacalia quadriocellata*	Ghana
HQ734789	Hepatozoon sp. db1606qm	Gekkonidae	*Salvator komaini*	Morocco
KM887507	Haemogregarina sacaliae VN_34_13	Geoemydidae	*Spalerosophis dolichospilus*	Vietnam
HM585204	Hepatozoon sp. CCS-2010	Varanidae	*Tarentola mauritanica*	Thailand
KJ408528	Hepatozoon sp. BT-2014	Colubridae	*Testudo graeca*	Morocco
HQ734787	Hepatozoon sp. db486tm	Gekkonidae	*Testudo graeca*	Algeria
KF992709	Hemolivia mauritanica SY 72 10	Testudinidae	*Testudo graeca*	Syria
KF992712	Hemolivia mariae 4955	Scincidae	*Egernia stokesii*	Australia
KP881349	Hemolivia stellata	Ixodidae	*Amblyomma rotundatum*	Brazil
KF992713	Hemolivia sp	Geoemidydae	*Rhinoclemmys pulcherrima*	Nicaragua
KF992710	Hemolivia mauritanica Vendelin	Testudinidae	*Testudo marginata*	Mediterranean region
KC512766	Hemolivia sp 1DJH 2013	Ixodidae	*Hyalomma aegyptium*	North Africa
HQ734807	Hepatozoon sp. lpa1tt	Lacertidae	*Varanus salvator komaini*	Morocco
HM585205	Hepatozoon sp. CCS-2010	Varanidae	*Varanus salvator salvator*	Thailand
HQ317910	Hepatozoon sp. V46Hep_Th	Varanidae	*Varanus scalaris*	Thailand
HM585210	Hepatozoon sp. CCS-2010	Varanidae	*Varanus scalaris*	Thailand
AY252108	Hepatozoon sp. ex Varanus scalaris	Varanidae	*Varanus scalaris*	Australia
AY252109	Hepatozoon sp. ex Varanus scalaris	Varanidae	*Varanus scalaris*	Australia
KJ461941	Karyolysus sp IR289BLVHU	Ixodidae	*Ixodes ricinus*	Hungary
KJ461942	Karyolysus sp LT33BRO	Lacertidae	*Lacerta trilineata*	Romania
KX011040	Karyolysus paradoxa	Varanidae	*Varanus albigularis*	South Africa
KJ461944	Karyolysus sp IR289BLVHU	Ixodidae	*Ixodes ricinus*	Hungary
KJ461945	Karyolysus sp OPZVPL	Macronyssidae	*Ophionyssus sp*	Poland
KJ461943	Karyolysus sp LV268BHU	Lacertidae	*Lacerta viridis*	Hungary
KJ461940	Karyolysus sp LA780BPL	Lacertidae	*Lacerta agilis*	Poland
KJ461946	Karyolysus sp ZV752BPL	Lacertidae	*Zootoca vivipara*	Poland
KM234612	Hepatozoo sp DJH 2014c	Phyllodactylidae	*Phyllopezus pollicaris*	Brazil

Accession number	Isolate code	Host Family	Host species	Geographic Region
HQ224959	Haemogregarina balli	Chelydridae	*Chelydra serpentina serpentina*	North America
ND	BL24_1	Iguanidae	*Conolophus subcristatus*	Galápagos Baltra
ND	CUM30_1	Iguanidae	*Conolophus subcristatus*	Galápagos Fernandina
ND	CUM66_1	Iguanidae	*Conolophus subcristatus*	Galápagos Fernandina
ND	B4_1	Iguanidae	*Conolophus subcristatus*	Galápagos
ND	W97_2	Iguanidae	*Conolophus subcristatus*	Galápagos Isabela
ND	W179_1	Iguanidae	*Conolophus subcristatus*	Galápagos Isabela
ND	W87_1	Iguanidae	*Conolophus subcristatus*	Galápagos Isabela
ND	W50_1	Iguanidae	*Conolophus subcristatus*	Galápagos Isabela
ND	W254_1	Iguanidae	*Conolophus subcristatus*	Galápagos Isabela
ND	W201_2	Iguanidae	*Conolophus subcristatus*	Galápagos Isabela
ND	W31_1	Iguanidae	*Conolophus subcristatus*	Galápagos Isabela
ND	W132	Iguanidae	*Conolophus subcristatus*	Galápagos Isabela
ND	W176_2	Iguanidae	*Conolophus subcristatus*	Galápagos Isabela
ND	W29_2	Iguanidae	*Conolophus subcristatus*	Galápagos Isabela
ND	W176_1	Iguanidae	*Conolophus subcristatus*	Galápagos Isabela
ND	CD36_1	Iguanidae	*Conolophus subcristatus*	Galápagos Santa_Cruz
ND	CD51_1s	Iguanidae	*Conolophus subcristatus*	Galápagos Santa_Cruz
ND	SF14_1	Iguanidae	*Conolophus pallidus*	Galápagos Santa Fe

2.3. Results

2.3.1. Blood film analysis

Light microscopy revealed haemoparasites in 37 out of 1032 blood films analysed in 2005-2006. No parasites were detected in any of the 97 blood films collected and analysed in 2014. Haemoparasites were observed in just two wild tortoise populations: those inhabiting Wolf (*C. becki*) and Alcedo (*C. vandenburghi*) volcanoes on Isabela Island. All the infections were intraerythrocytic, no parasite was observed in the screened lymphocytes. The point prevalence was 27% (8/30) for tortoises sampled from Wolf and 29% (29/100) for tortoises sampled from Alcedo; while the mean value of parasitaemia was 0.11% and 0.04%, respectively. For each of the other islands the power analysis indicated that the number of samples tested was sufficient to detect at least one infected individual at the lower prevalence found in Isabela (27%; Table 2.5). In most cases the sample sizes would allow detection at prevalences as low as 5%-10% with 95% probability. The only exception in this last case was the tortoise population from Darwin Volcano where the sample size was too small to detect infection at a prevalence lower than 27%.

At least three haemoparasite morphologies were observed. Based on measurements of 60 haemoparasites they were classified as follows: Type I — 'crescent shaped', 7.62 ± 0.42 x 2.03 µm ± 0.35 (n=26) with a range of length: 6.81-8.47 by width: 1.55-2.55 µm; Type II — 'slender and elongated', 6.44 ± 0.5 by 1.58 ± 0.14 µm (n=32) with a range of length: 4.95-7.41 by width: 1.33-1.83 µm; and Type III — 'pear shaped', curved with rounded or bluntly pointed ends (n=2). All three morphologies resemble haemogregarine development stages reported in other tortoise species and are generally considered as trophozoites, juvenile and mature gamonts, (Types I, II, III respectively; Fig. 2.1). For comparison, the haemoparasites observed in Galápagos lava lizards by Ayala and Hutchings (1974) were piriform, bullet-shaped or rounded. Their measurements were 7.9 ± 1.2 by 4.1 ± 1.2, µm and a range of 6.2-10 by 2.5-8 µm. The haemoparasites of Galápagos tortoise were morphologically more similar to sausage-shaped haemoparasites observed in marine iguana. However, marine iguana parasites were typically larger with measurements 13.1 ± 1.4 x 5.3 ± 1.2 µm and a range of 9-16.6 x 3-7.61 µm.

Table 2.5. Estimation of the sample size required for detecting at least one tortoise infected with haemogregarines at a prevalence of infection of 5%, 10% and 27%. Two methods were used: DiGiacomo *et al*., 1986 before the slash and Smith, 2015 after the slash. *The tortoise population size in Santa Cruz east is 20 but they have been sampled multiple times.

Island, population and tortoise species	Estimated population	Prevalence and minimum sample size			Analysed by microscope
		5%	10%	27%	
Isabela, Wolf (*C. becki*)	1000-2000	59/71-72	29/35-36	10/12	30
Isabela, Darwin (*C. microphyes)*	500-1000	59/68-71	29/35	10/12	13
Isabela, Alcedo (*C. vandenburghi syn vivina*)	3000-5000	59/73	29/36	10/12	100
Isabela, Sierra Negra (*C. guntheri*)	100-300	59/52-65	29/30	10/12	70
Isabela, Cerro Azul (*C. vicina)*	400-600	59/67-69	29/35	10/12	11
Isabela, Cerro Azul (*C. vicina)*	400-600	59/67-70	29/35	10/12	57
Pinzón (*C. ephippium*)	150-200	59/58-62	29/32	10/12	44
Santiago (*C. Darwini*)	500-700	59/68-70	29/35	10/12	35
Santiago (*C. Darwini*)	500-700	59/68-70	29/35	10/12	40
Santa Cruz, west (*C. Porteri*)	2000-3000	59/72-73	29/36	12	289
Santa Cruz, east (*C. donfaustoi*)	20*	59/20	29/17	10/9	55
San Cristóbal (*C. chatamensis*)	500-700	59/68-70	29/35	12	105

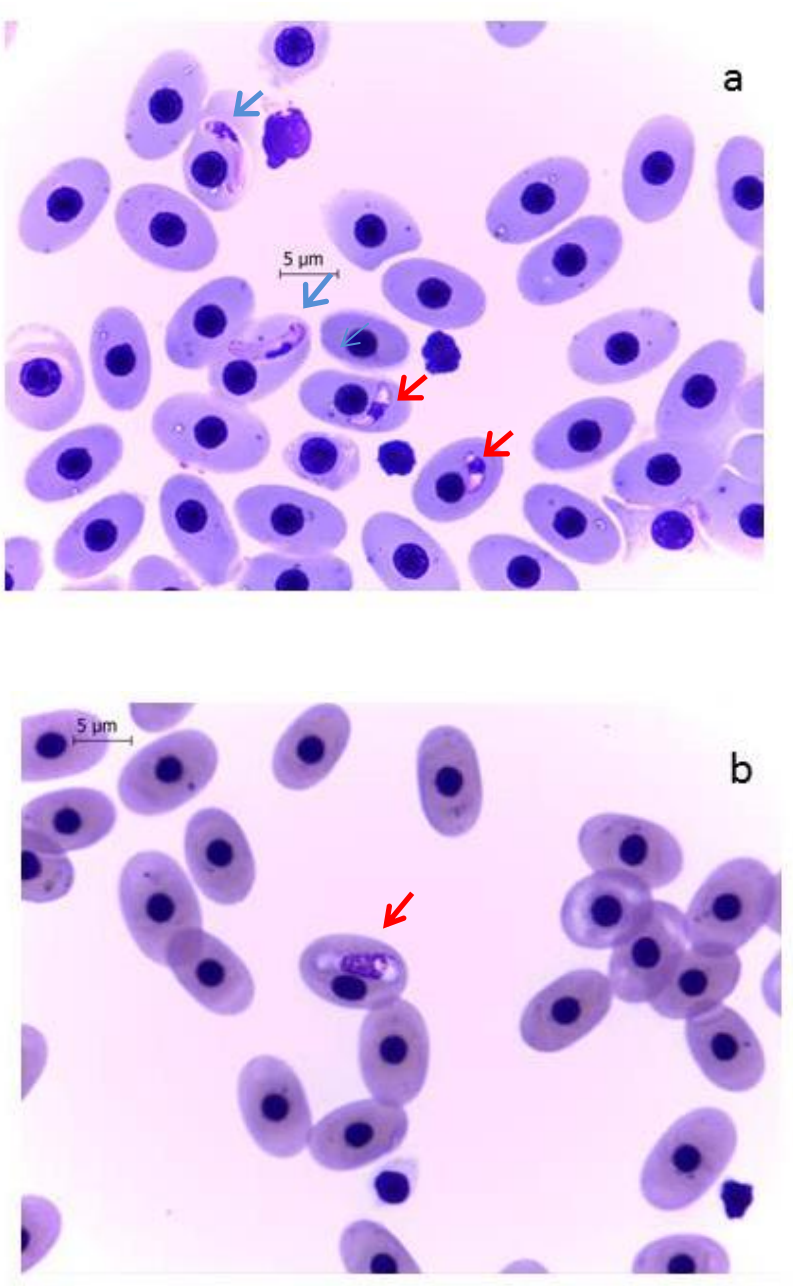


Figure 2.1. Possible developmental stages of haemogregarine parasites observed in erythrocytes of *Chelonoidis* spp. Erythrocytes of reptiles are nucleated and parasites are observed into the cytoplasm of these cells. Figure a) contains parasite morphologies type I and II (trophozoites —red arrows— and juvenile gamonts —blue arrows—); Figure b) contains parasite morphology type III (mature gamont—red arrow).

2.3.2. Screening of blood parasites by PCR

A subset of 453 wild tortoise blood samples collected in 2005-2006 and 97 collected in 2014 were analysed for the presence of haemogregarine DNA using PCR. As an initial trial to assess the effectiveness of PCR-based haemogregarine detection in Galápagos giant tortoises, analysis was performed on all 130 samples taken from the tortoise populations of Wolf and Alcedo on Isabela Island where haemogregarine-like parasites were observed.

A comparison of the results obtained by microscope and PCR surveys is shown in Fig. 2.2. Primers Hep300-Hep900 yielded haemogregarine 18S rDNA sequence in 27 % (8/30) of samples from Wolf and 22 % (22/100) of samples from Alcedo. For each population, haemogregarines were found in 100 % (8/8) and 92 % (22/29) of samples positive for parasites via microscopy. PCR using primers HEMO1-HEMO2 gave haemogregarine sequences in 27% (8/30) of Samples from Wolf Volcano and in 17 % (17/100) from Wolf. For this primer set haemogregarines were found in 100% (8/8) of microscope positive samples from Alcedo and (17/29) of microscope positive samples from Wolf.

The samples amplified with primers HEMO1 and HEMO2 were also amplified with primers Hep300-Hep900. Seven samples positive for haemogregarines on microscopy were not confirmed by PCR. Of these, four were negative with both set of primers and the remaining three yielded ambiguous sequences. These negative samples were subsequently analysed using a nested PCR for haemosporidia, with which they gave negative results. Re-analysis of the ambiguous sequences using the same or fresh DNA gave the same ambiguous output.

Primer set Hep300-Hep900 was chosen for the analysis of the remaining tortoise blood samples and ticks. The tortoise samples comprised 323 collected in 2005-2006 (13 from Darwin-Isabela, 70 from Sierra Negra-Isabela, 11 from Cerro Azul-Isabela, 44 from Pinzón, 35 from Santiago, 75 from El Chato-Santa Cruz, 25 from El Fatal-Santa Cruz and 50 from San Cristóbal), and 97 collected in 2014 (40 from Santiago and 57 from Cerro Azul-Isabela). Haemogregarine DNA was detected in just one tortoise. This animal, of

the species *C. microphyes*, had been sampled from Darwin Volcano on Isabela Island in 2005-2006.

Ticks were found infesting two tortoise populations of Isabela (Alcedo and Wolf), and tortoises on Santiago and Pinzon. Ticks were present on all tortoises sampled in Alcedo and Wolf, but at prevalence lower than 19% in the other two islands. The head and abdomen of 57 ticks were analysed using PCR: Isabela-Wolf (n=13), Isabela-Alcedo (n=8), Santiago (n=25), and Pinzón (n=11). Haemogregarine DNA was amplified from two ticks, both of which came from Wolf. The taxonomic identity of thes ticks will be examined in Chapter 3.

2.3.3. Analysis of DNA sequences for taxonomic identity and taxon prevalence

BLAST analysis of haemoparasite sequences from Galápagos tortoises returned 99% sequence identity with a variety of published sequences for *Hepatozoon* spp. The top matches for sequences obtained from Alcedo and Wolf tortoises included *Hepatozoon* sequences from the marsupial mammal *Dromiciops gliroides* from Chile (*H.* sp. DG1; accession number FJ719813), and haplotypes from diverse reptiles from different geographic locations including Madagascar (*H.* sp. JPM-2014c from the colubrid *Madagascarophis colubrinus*; accession number KM234647), China (*H.* sp. YLW-2014 from the colubrid *Elaphe Carinata*; accession number KF939627), North Africa (*H.* sp. pty01po from the lizard *Ptyodactylus oudrii*; accession number HQ734790), Seychelles i.e. *H.* sp. 1SP from *Mabuya wrightii* (accession number HQ292771), and the Mediterranean basin i.e *H.* sp. BT-2016 from the gecko *Tarentola deserti* (accession number KU680460). The sequence obtained from Darwin tortoises showed 99% sequence identity with the *H. fitzsimonsi* voucher RC140411C1 (accession number KR069084) isolated from the tortoise *Kinixys zombensis* in South Africa.

Sequence alignments of 400 nucleotide of 18S rDNA from Galápagos haemogregarines is shown in Fig. 2.3. The comparison among Galápagos tortoise haemogregarines, revealed the presence of two haplotypes. One was found in all sequences obtained from tortoises sampled in Alcedo and from tortoises and ticks sampled in Wolf. Hereafter it will be referred as haemogregarine haplotype Alcedo-Wolf. The second haplotype was found in the only positive tortoise from Darwin, hereafter referred as haemogregarine

haplotype Darwin. The nucleotide identity between these two sequences was 98.5%. There were six nucleotide differences including 5 transitions (2 from C to T, 1 from T to C, 1 from A to G and 1 from G-A), and one insertion (Fig. 2.3). The nucleotide identity among the haemogregarine haplotype Alcedo-Wolf and other haemogregarine haplotypes previously identified from Galápagos land iguana and Galápagos mosquitos ranged from 96.0% to 98.0%. The nucleotide identity among the haemogregarine haplotype Darwin and the other haemogregarine haplotypes identified in Galápagos ranged from 96.0% to 99.0%. Haemogregarine haplotype Darwin showed higher identity (99.0%) with a sequence detected in Galápagos mosquito (*H.* sp. MIG1 ID: JQ080302) than with haemogregarine haplotype Alcedo-Wolf. A matrix showing these values is showed in Table 2.6.

2.3.4. Phylogenetic analysis

Two phylogenetic trees were inferred using Bayesian methods. One was built using a sequence of ~400 nucleotides obtained using primers Hep300-Hep900, the other with a sequence of ~1050 nucleotides obtained using primers Hep900-Hemo3. The output of these analyses is shown in Fig. 2.4 and 2.5, respectively. Neither of the two Galápagos tortoise haemogregarine haplotypes has been previously reported. Both clustered within the major *Hepatozoon* clade (now split between *Hepatozoon* and *Bartazoon*), however they separated into different clusters. The haemogregarine haplotype Alcedo-Wolf formed a polytomy within the *Hepatozoon* clade, clearly separated from the other tortoise haemogregarine genera (*Haemogregarina* and *Hemolivia*). The haemogregarine haplotype Darwin clustered with some of the haemogregarines identified from Galápagos land iguana, one of three identified from the Galápagos mosquitoes (*A. taenorinchus*) and with *H. fitzsimonsi* isolated from the African tortoise *C. angulata.*

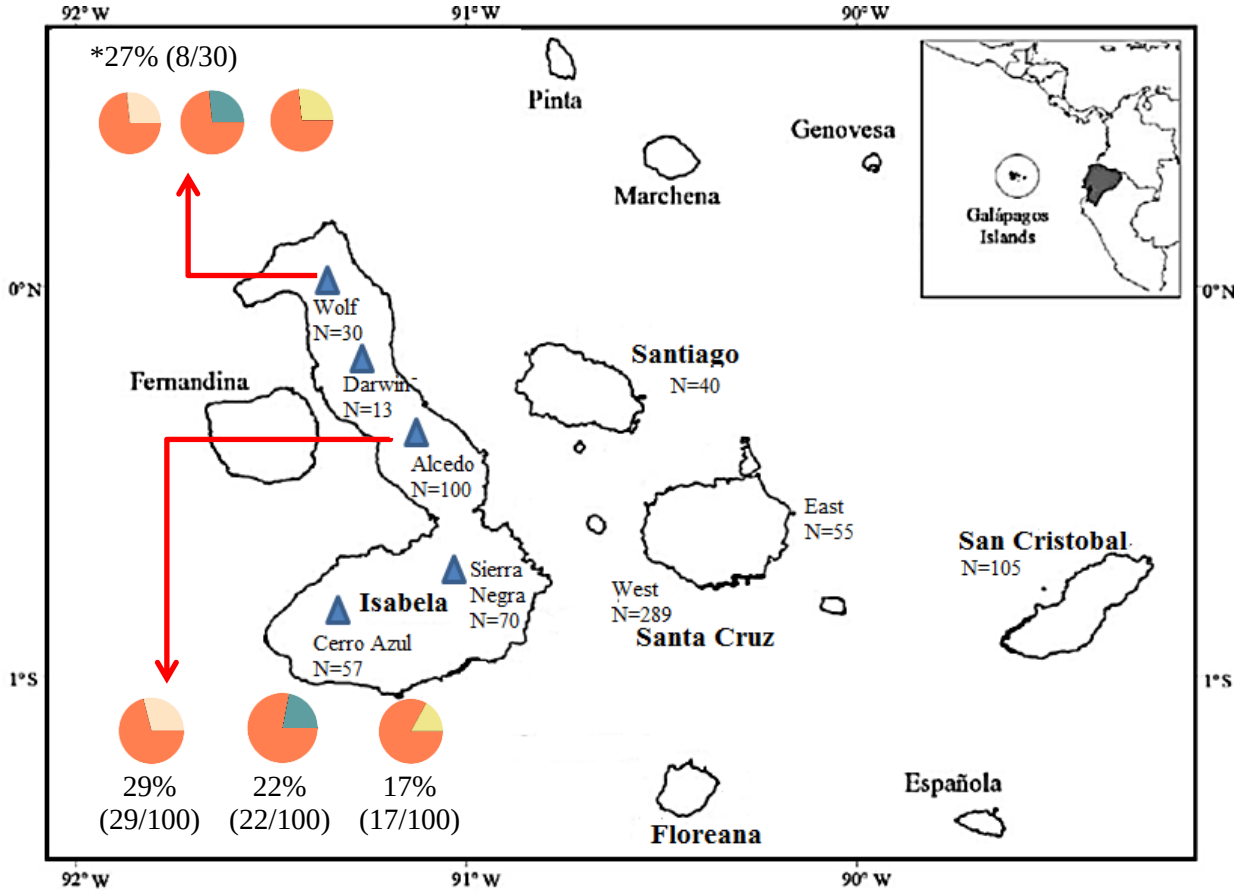


Figure 2.2. Comparison of microscope and PCR survey of haemogregarines in Galápagos tortoises. Only samples for Alcedo and Wolf volcanoes were positives both by microscope and PCR. Pie charts with pink slices represent microscope results, blue slices primer Hep300-Hep900, yellow slices primers Hemo1-Hemo2. N=number of samples collected by tortoise populations. All samples were screened for blood parasites by microscope. All samples of Isabela and a 30% of samples from the other tortoise populations were analysed by PCR. The haplotype found in Darwin is not showed.

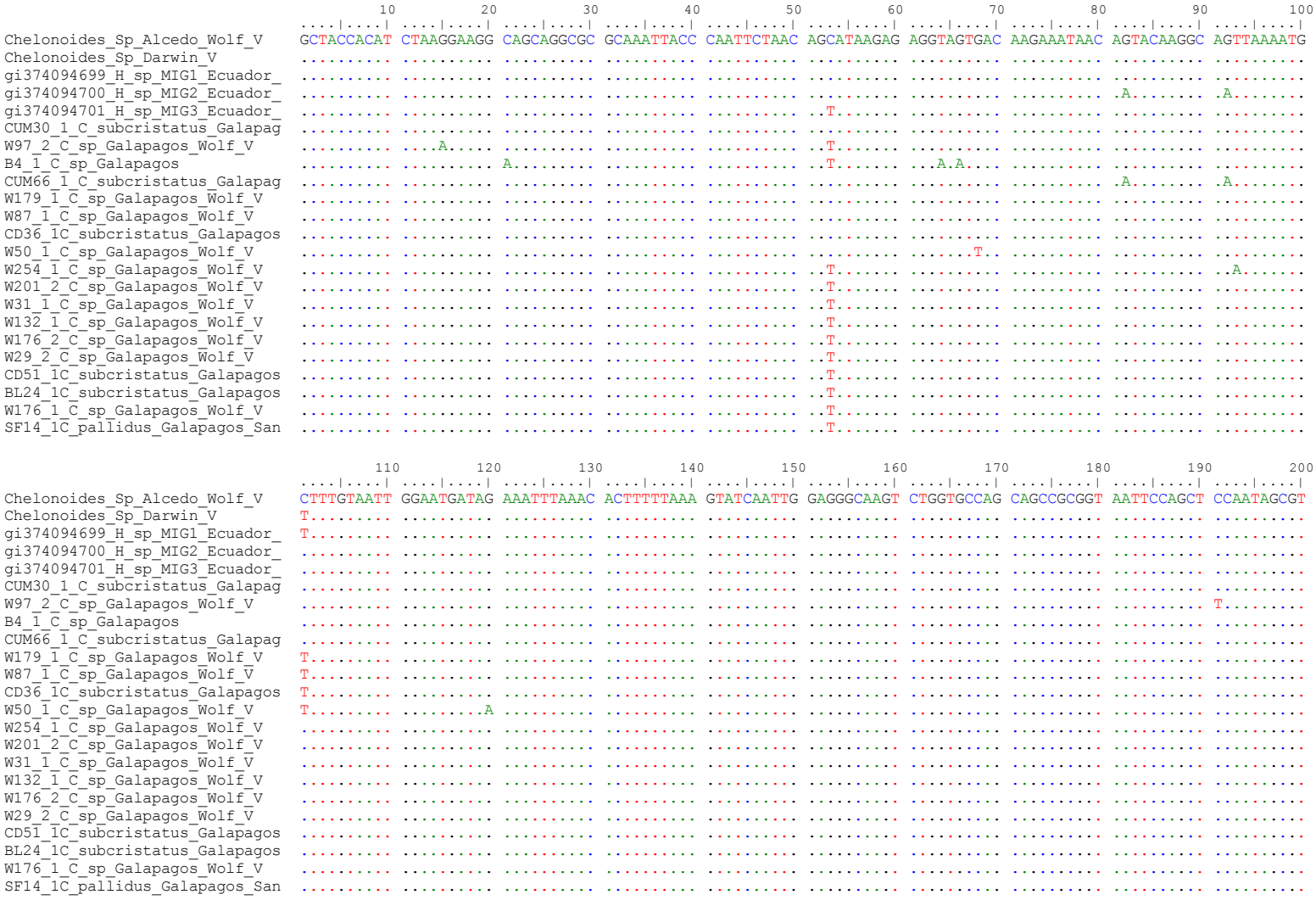


Figure 2.3a. Alignment of 400 bp of18S rDNA of haemogregarines haplotypes found in the Galápagos Islands. The DNA of haemogregarines infecting Galápagos tortoises was amplified with primers Hep300-Hep900. Continued on the next page.

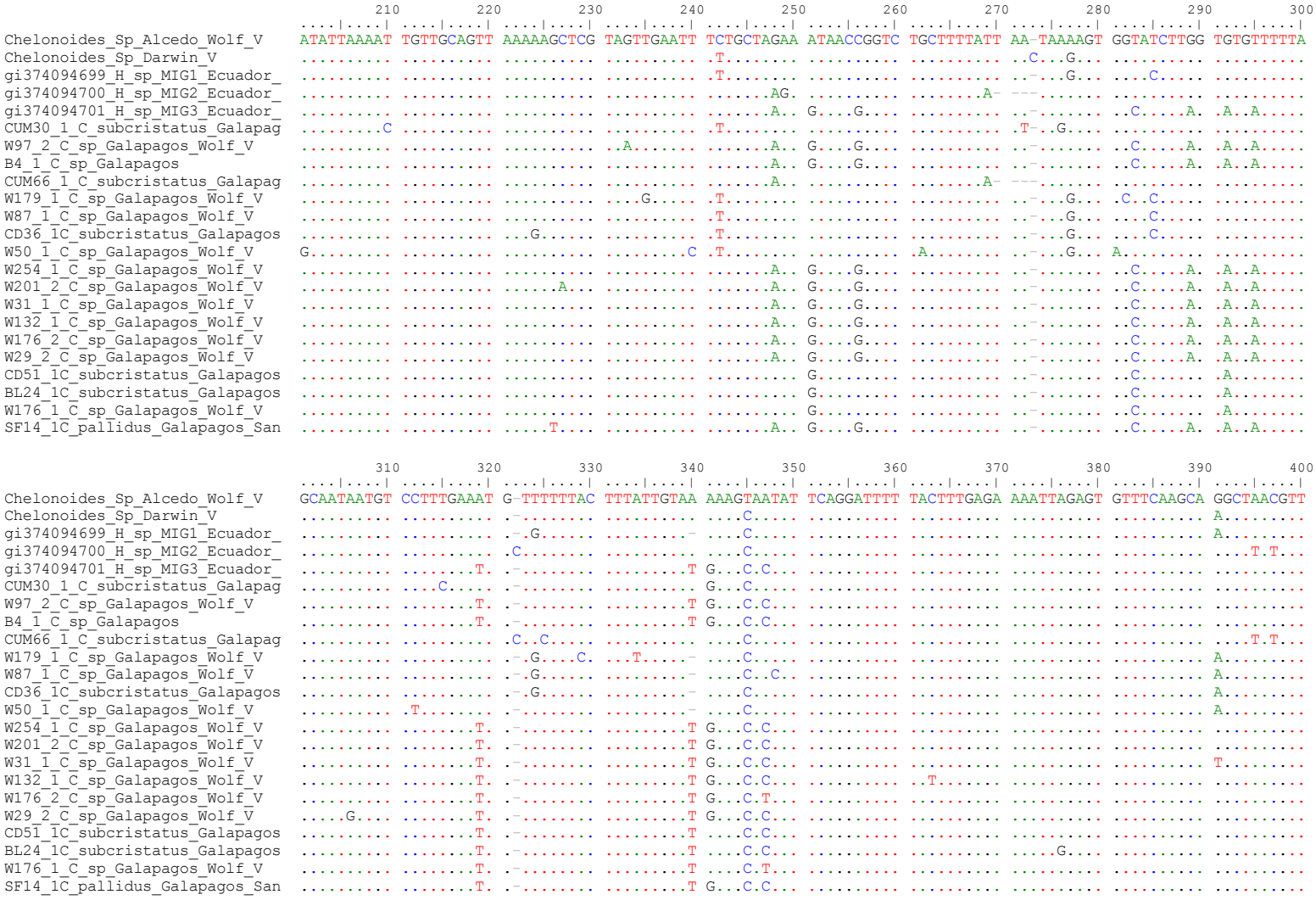


Figure 2.3b. Alignment of haemogregarines haplotypes found in Galápagos.

Table 2.6. Percentage of nucleotide identity among haemogregarine haplotypes found in Galápagos. C_Sp AW (haemogregarine tortoise haplotype from Alcedo and Wolf; C_Sp_D (haemogregarine tortoise haplotype from Darwin); MIG1, MIG2 and MIG3 (haemogregarine haplotypes obtained from Galápagos mosquitoes); the remaining codes correspond to haemogregarine haplotypes obtained from land iguanas.

	C_Sp AW	C_Sp_D	MIG1	MIG2	MIG3	CUM30	W97	B4	CUM66	W179			W50	W254	W201	W31	W132	W176	W29	CD51	BL24	W176	SF14
C_Sp AW																							
C_Sp_D	0.985																						
MIG1	0.980	0.990																					
MIG2	0.970	0.960	0.96																				
MIG3	0.967	0.957	0.96	0.95																			
CUM30	0.982	0.977	0.97	0.96	0.96																		
W97	0.960	0.95	0.95	0.94	0.99	0.95																	
B4	0.960	0.95	0.95	0.94	0.99	0.95	0.99																
CUM66	0.970	0.96	0.96	0.99	0.95	0.96	0.94	0.94															
W179	0.970	0.98	0.99	0.95	0.95	0.96	0.94	0.94	0.95														
W87	0.977	0.99	1.00	0.95	0.95	0.97	0.95	0.95	0.95	0.99													
CD36	0.977	0.99	1.00	0.95	0.95	0.97	0.95	0.95	0.95	0.99	0.99												
W50	0.967	0.98	0.98	0.94	0.94	0.96	0.94	0.94	0.94	0.97	0.97	0.97											
W254	0.965	0.96	0.95	0.95	1.00	0.96	0.99	0.99	0.95	0.94	0.95	0.95	0.94										
W201	0.965	0.96	0.95	0.95	1.00	0.96	0.99	0.99	0.95	0.94	0.95	0.95	0.94	1.00									
W31	0.965	0.96	0.96	0.95	1.00	0.96	0.99	0.99	0.95	0.95	0.95	0.95	0.94	1.00	1.00								
W132	0.965	0.96	0.95	0.95	1.00	0.96	0.99	0.99	0.95	0.94	0.95	0.95	0.94	1.00	1.00	1.00							
W176	0.967	0.96	0.96	0.95	1.00	0.96	0.99	0.99	0.95	0.95	0.95	0.95	0.94	1.00	1.00	1.00	1.00						
W29	0.965	0.96	0.95	0.95	1.00	0.96	0.99	0.99	0.95	0.94	0.95	0.95	0.94	1.00	1.00	1.00	1.00	1.00					
CD51	0.980	0.97	0.97	0.96	0.99	0.97	0.98	0.98	0.96	0.96	0.97	0.97	0.96	0.99	0.99	0.99	0.99	0.99	0.99				
BL24	0.977	0.97	0.97	0.95	0.99	0.97	0.98	0.98	0.95	0.96	0.96	0.96	0.95	0.98	0.98	0.98	0.98	0.98	0.98	1.00			
W176	0.980	0.97	0.97	0.96	0.99	0.97	0.98	0.98	0.96	0.96	0.97	0.97	0.96	0.98	0.98	0.98	0.98	0.99	0.98	1.00	1.00		
SF14	0.965	0.96	0.95	0.95	1.00	0.96	0.99	0.99	0.95	0.94	0.95	0.95	0.94	1.00	1.00	1.00	1.00	1.00	1.00	0.99	0.98	0.98	0.98

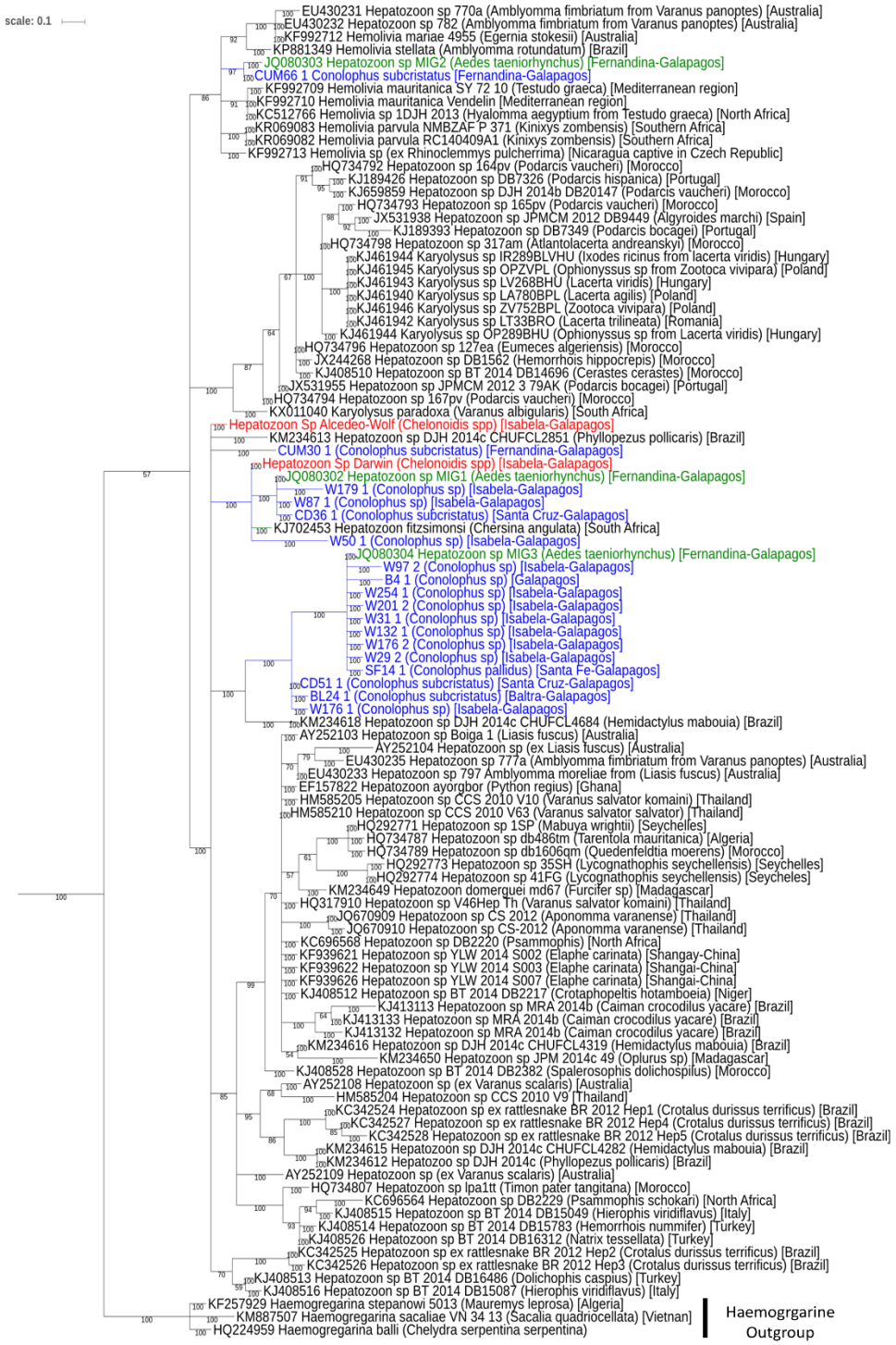


Figure 2.4. Phylogeny of haemogregarines of reptiles based on partial sequence of 450 nucleotides of 18S rDNA, inferred using Bayesian analysis under Tamura 3-parameter + Gamma model (T92+G). Labels contain GenBank accession numbers, names of haemogregarine isolated, host especies and sampling location both given in brackets. Galápagos tortoise haemogregarines are labelled in red, haemogregarines of Galápagos iguana and Galápagos mosquitoes are shown in blue and green. Posterior probabilities are indicated for each node.

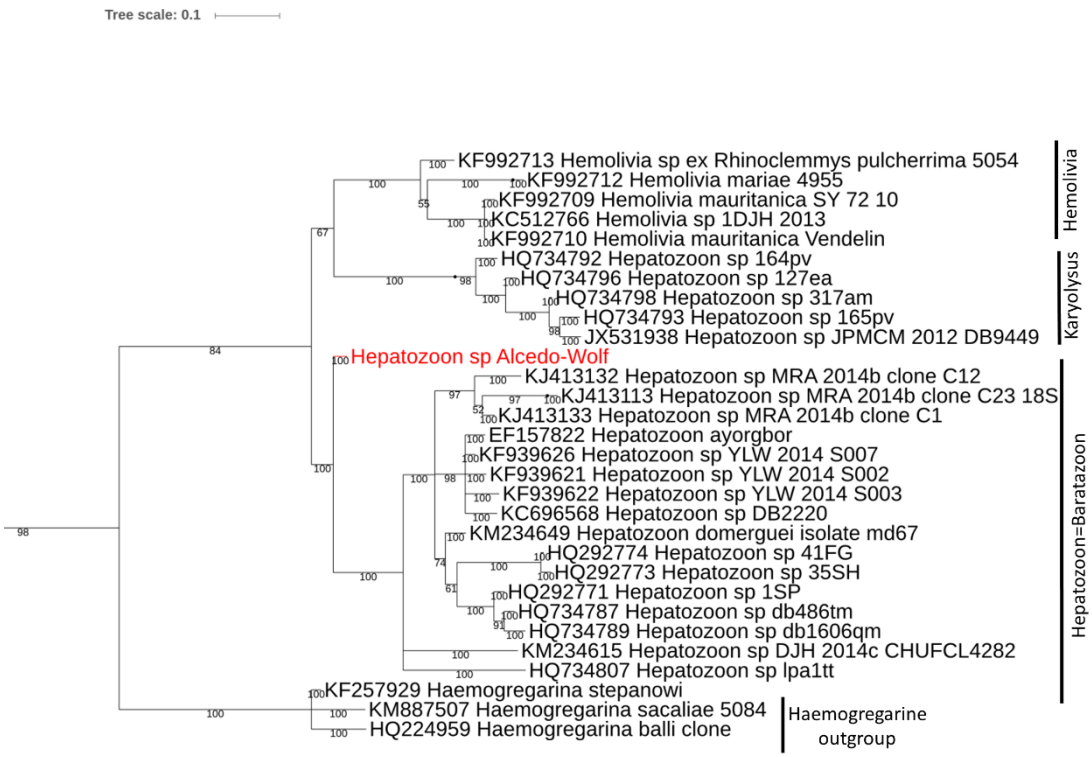


Figure 2.5. Phylogenetic analysis of the haemogregarines of reptiles based on partial sequence of 1050 nucleotides of 18S rDNA inferred using Bayesian analysis under Tamura 3-parameter + Gamma model (T92+G). Labels contain GenBank accession numbers and names of haemogregarine isolated. The Galápagos tortoise haemogregarine haplotype is labelled in red. Posterior probabilities are indicated for each node.

2.4. Discussion

Blood parasites have been reported previously infecting the Galápagos lava lizard, Galápagos marine and land iguana and Galápagos giant tortoises. Light microscopy and molecular analyses of blood samples from *Chelonoidis* spp. allowed identification of their blood parasite as haemogregarines and suggest that this is the only, or at least the most common haemoparasite present. However, they were detected only in tortoises inhabiting the northern volcanoes of Isabela: Alcedo (*C. vandenburghi*), Wolf (*C. becki*) and Darwin (*C. microphyes*). Microscopic examinations of blood smears revealed infection in tortoises from Alcedo and Wolf with a prevalence of 27% and 29%, respectively. DNA sequencing confirmed these findings and also yielded a haemogregarine sequence from one out of thirteen tortoises sampled in Darwin. *Amblyomma* ticks were found infesting tortoises from Alcedo and Wolf volcanoes on Isabela Island, and also on the islands of Santiago and Pinzón. Two engorged ticks collected from tortoises on Wolf volcano were positive for haemogregarine DNA, suggesting a role for ticks as the vector of the haemoparasite.

The phylogenetic analysis is consitent with the results of Kvicerova *et al.* (2014) and Haklova-Kocíková *et al.* (2014) which found *Haemolivia*, *Karyulysus* and *Hepatozoon* syn *Bartazoon* (Karadjian *et al*., 2015) splitting into different clades. Analysis of 450bp of the tortoise haemogregarine 18S rDNA yield two haplotypes, one in tortoises from Alcedo and in tortoises and ticks from Wolf (haplotype 'Alcedo-Wolf'), and the other in the single haemogregarine positive tortoise from Darwin (haplotype 'Darwin'). The phylogenetic reconstruction clustered the Galápagos haemogregarines within the genus *Hepatozoon* syn *Bartazoon*. Given that *Bartazoon* is a new proposal for classifying this blood parasite the haemoparasites of this study will be referred to with their general name of haemogregarines. The haemogregarine haplotype Alcedo-Wolf seems unrelated to the other reptile haemogregarines reported from Galápagos or elsewhere. Conversely, the Darwin haplotype clustered with haplotypes previously identified from Galápagos land iguanas, one of three of Galápagos mosquitoes and *Hepatozoon fitzsimonsi*, a haemogregarine identified from South African tortoises.
The distribution and diversity of Galápagos tortoise haemogregarines contrasts with the haemogregarines of Galápagos land iguanas. This last haemogregarines were detected in all *Conolophus* populations sampled across the Galápagos archipelago, and comprised 18 haplotypes one of them clustering within *Hemolivia*. It also differs

from the diversity found in Galápagos mosquitoes represented by three haplotypes, one of them also related to *Hemolivia*.

The tortoises inhabiting Alcedo and Wolf, which are separated by distance and lava flows which act as barrier to dispersal, are considered separate species which came from different colonizing sources (Poulakakis *et al.* 2012). It is believed that Wolf volcano was colonized by tortoises from Santiago Island and the other volcanoes by tortoises from Santa Cruz (Caccone *et al.* 1999; Poulakakis *et al.* 2012). Hence, the finding of a common haemogregarine haplotype in Alcedo and Wolf was unexpected. It suggests either that this is an ancient haplotype once common for all *Chelonoidis* spp. or that the parasite has been acquired more recently transported from one population to the other.

Galápagos tortoise haemogregarines could have been transported between volcanoes thougth migration of infected tortoises. Previous research on the biogeography of *Chelonoidis* spp. has shown that Wolf Volcano includes some tortoises with maternal lineages from Alcedo what indicates that movement between volcanoes has taken place, either naturally or through anthropogenic means (Caccone *et al.* 2002). Alternativelly, the movement of haemogregarines among these distant volcanoes might have been also mediated via introduced ungulates such as goats or donkeys. These animals could have transported infected final hosts (e.g. ticks) for the Galápagos tortoise haemogregarine. There is at least one case where *Amblyomma* ticks have been reported infecting vertebrates of different orders. It is the case of *Amblyomma marmoreum* which at an inmature stage is propense to feed in reptile and mammals (Allan *et al.* 1998). Thus, the transportation of ‘haemogregarine infected’ tortoise ticks to different volcanoes by introduced ungulates is a reasonable possibility.

The circulation of a unique tortoise haemogregarine haplotype in both Alcedo and Wolf volcanoes suggest that this parasite could be specific for *Chelonoidis* spp. This is supported by the phylogeny and by the absence of this haplotype from the extensive sampling of *Conolophus* spp. Furthermore, the Galápagos tortoise haemogregarine seen in blood smears from Alcedo and Wolf differs morphologically from the lava lizard and marine iguana parasite (Ayala and Hutchings, 1974). The possibility that

the Alcedo-Wolf haemogregarine circulates in the other reptile species cannot be excluded, but their ecological niches do not overlap with those of tortoises, making spill over unlikely. Exceptions occur with land iguanas, which are syntopic with tortoises on Wolf and Darwin volcanoes. Some species of haemogregarine show low host specificity to both their definitive and intermediate hosts (Smith, 1996). Thus, the similarity of the haemogregarine haplotype found in a tortoise from Darwin with that from *Conolophus* spp. could indicate a transfer of that parasite between those hosts. The PCR-positive tortoise blood from Darwin, however, was negative for parasites on microscopical examination of the blood smear. This could indicate a low level of infection or a lack of active infection (e.g. only parasite DNA present), which would be consistent with infection of an aberrant host. Given this particularity the circulation of more haemogregarine haplotypes infecting *Chelonoidis* spp. in Wolf volcano cannot be discarded. For this population the sample size and the number of haemogregarine-positive tortoises were low.

The detection of the Alcedo-Wolf haemogregarine haplotype in ticks from Isabela suggests that these arthropods are a vector for this parasite. It is known that ticks within the genera *Hyalomma* and *Amblyomma* are competent at transmitting either *Haemolivia* or *Hepatozoon*. For example *Hyalomma aegypticum* is the vector and definitive host for *Haemolivia mauritinaca* infecting *T. graeca* across North Africa and the Middle East (Harris *et al.* 2013; Paperna *et al.* 2002). The ticks parasitizing Galápagos tortoises have been described as *Amblyomma usingeri* (north volcanoes of Isabela: Alcedo, Darwin and Wolf), *A. macfarlandi* (south volcano of Isabela Cerro Azul, and Santa Cruz Island), and *A. pilosum* (Santiago and Pinzon islands) (Keirans *et al.* 1973b). The behaviour of some tick species within this genus (climbing vegetation to locate a new host) (Yonow 1995) could favour their inadvertent ingestion by tortoises and so would allow the transmission of the tortoise haemogregarine. The presence of *A. usingeri* on Wolf and Alcedo is consistent with the distribution of the Galápagos tortoise haemogregarine. In the case of Darwin volcano, the tortoises were tick-free at the time of sampling, but they have been reported previously as being infested with *A. usingeri* (Keirans *et al.* 1973b). The tortoise habitat on this volcano is very arid and could influence the circulation of the ticks. This archnida have a questing behaviour that ussualy involve climbing vegetation and wait for its potential host (Godfrey *et al.* 2011).

The absence of haemogregarines in any other tortoise population (assuming sampling power for a detection threshold of infection ≤ 5%) might be explained by several factors. One is the absence of competent vectors, which would suggest that the tick *A. pilosum* infecting tortoises in Santiago and Pinzón is not competent for tortoise haemogregarines. Ticks and haemogregarines were both absent from the tortoise populations of San Cristóbal, Santa Cruz, Cerro Azul and Sierra Negra volcanoes on Isabela. Seasonal circulation of these parasites in these islands is unlikely as samples were collected multiple times, especially in San Cristóbal and Santa Cruz. To my knowledge, *Amblyomma* ticks have never been reported from San Cristóbal, and none have been observed on Santa Cruz or the Southern volcanoes of Isabela since 1971 (Keirans *et al.* 1973b).

The lack of *Amblyomma* ticks in ancient Galápagos tortoise populations such as San Cristóbal and Santa Cruz suggest either that they have never occurred there or that they have gone extinct. Since *Amblyomma* ticks have been reported historically in tortoises from Santa Cruz and tortoises (and presumably all their parasites) are thought to have colonised Galápagos via San Cristobal o Española (the older islands and the closest to the southamerica mainland), the second scenario is more likely. It is worth noting that ticks of the genus *Argas (Microargas)* were collected in 1964 from Galápagos tortoises on Santa Cruz Island (Hoogstraal *et al*. 1973). There is at least one case where ticks of the genus *Argas* (*Argas brumpti*) contained sporogonic stages of these parasites, in the case of *Hemolivia argantis* whose vertebrate host has not been identified (Telford 2009). However, since in Galápagos, these ticks were recorded on an island where haemogregarines appear to be absent, they would not be implicated as a current vector of the Galápagos tortoise haemogregarines.

Similar to ticks, a second alternative for the absence of haemogregarines in some Galápagos tortoise populations is also extinction, either alone or in conjunction with its intermediate and/or its final host. Parasites are susceptible to the same threats that affect free-living species, but could face higher risk of extinction due to several factors, such as dependence on a host population threshold, host connectivity, life cycle involving more than one host, and changes in host ecology (Gomez and Nichols 2013). Haemogregarines in Galápagos have all these constraints. Tortoises in these islands have been subject to hunting and pressures of introduced species since the

18th-19th centuries. Both factors led to the extinction of at least six tortoise species while many others (including those from Isabela, Santiago and Pinzón) were drastically reduced (Macfarland *et al.* 1974). This tortoise population decline may have also depleted their parasite population, and parasite connectance as well. The presence of goats since the 18th century also altered the vegetation of most islands (Rivera-Parra *et al.* 2012) which in turn could have affected the life cycle of parasites such as ticks. Some islands such as Pinzon have long periods of dryness where tortoises usually feed on cactus instead of grass (De Vries 1984), such behaviour might reduce the probability of ingesting ticks disrupting haemogregarine transmission. Failure of haemogregarines to co-colonise new islands with their hosts or to persist if they did reach them (i.e. stochastic parasite founder effects and drift), is an additional factor to take into account (Torchin *et al.* 2003).

Other haematophagous invertebrates have been implicated in the transmission of haemogregarines from the genus *Hepatozoon*. These include the mites *Ophyonyssus* sp., and *Hirstella* sp., the tsetse fly *Glossina palpalis,* the phlebotomine *Lutzomyia vexator oxidentalis* and the reduviid bugs *Triatoma arthurneivae* and *T. rubroria*. Mosquitoes have been also found as amenable vectors but only in laboratory assays. For Galápagos the haematophagous invertebrates are represented by one species of horse-fly (*Tabanus vittiger*) (Marchena and Santa Cruz), 11 species of biting midge (*Forcipomyia* spp. and *Dasyhelea* spp.) and three species of mosquito (*Culex quinquefasciatus*, *Aedes aegypti* and *A. taeniorhynchus*) (Bataille *et al.* 2012; Borkent 1991). None of these insects has been reported from the volcanoes of northern Isabela, although this is probably due to an absence of sampling effort. At least 202 species of mites have been described for Galápagos but none of them have been associated to Galápagos tortoises so far (Schatz 1998). *C. quinquefasciatus, A. aegypti* and at least seven biting midge species are recent introductions (Bataille *et al.* 2012) but are restricted to human populated islands were the haemoparasite has not been detected.

Aedes taeniorhynchus, however, appears to be ubiquitous in highland areas of Isabela, as well as on Santa Cruz and Santiago islands. This mosquito is a strong flier and would be expected to be present on Alcedo, Wolf and Darwin volcanoes. It normally feeds on birds and mammals but on Galápagos it is known to also feed on reptiles, including tortoises (Bataille *et al.* 2012). Three haemogregarine haplotypes have been

reported from this species of mosquito from specimens caught in coastal areas of Fernandina Island and Isabela northwest. None of which were related to the Alcedo-Wolf haplotype infecting Galápagos tortoises, but one was related to the Darwin haplotype. While the transmission of haemogregarines via biting insects has been suggested, it has not been proven; instead the ingestion of an infected insect is thought to be the typical transmission route for *Hepatozoon* spp. (Telford 2009). It seems unlikely that the ingestion of mosquitoes, or other biting flies, by Galápagos tortoises occurs often enough to allow haemogregarine persistence.

Unfortunately the lack of haemoparasites in most tortoise population limits the use of this parasite as a tool for studying the biogeography of its tortoise host. Nevertheless these results provide a baseline for future studies of Galápagos giant tortoise haemoparasites and have helped to determine the biogeography of tortoise haemogregarines. My work suggests that the tick, *A. usingeri*, present on Isabela Island, is the most likely definitive host and vector of Galápagos giant tortoise haemogregarines. A combination of anthropogenic activities, ecological differences among island and founding events might have influenced the distribution of these parasites. Based on the current acepted classification the haemogregarines of land iguana and mosquitos are represented by the genera *Hepatozoon- Bartazoon* and *Hemolvia*, while the haemogregarines of Galápagos tortoises would be represented by *Hepatozoon-Bartazoon*. Future studies are required to identify the origin of the Galápagos tortoise haemogregarine haplotypes, and to study their life cycle and ecology.

Chapter 3. Phylogeography and evolution of Galápagos tortoise ticks

3.1. Introduction

Phylogeography is a discipline concerned with the phylogenetic analysis of populations distributed across a landscape with the aim of understanding the historical processes governing their geographical distribution, and the mechanisms driving speciation (Avise *et al.* 1987; Nieberding *et al.* 2004a). Phylogeography is applied specially to intraespecific lineages. Comparative phylogeography studies sympatric species with the aim of provide insight into the role of historical factors in their observed distribution. Conconcordant phylogeography would indicate that the species differentiated in response to similar, possibly the same, geological or environmental events (Nieberding *et al.* 2004a). Incongruence indicates that taxa reacted differently, highlighting independent colonisation events, differences in dispersal characteristics or species-specific ecological requirements (Crandall *et al.* 2008). The phylogeographies of species linked by a close biotic interaction such as host-parasites show a degree of congruence that tends to increase with the obligate character of the parasite (Hafner and Nadler 1990). The concordance often decreases if the parasite is either not specific or heteroxenous (Barbosa *et al.* 2012).

Comparative phylogeography between hosts and their parasites could provide insights about parasite evolution and its population history (Criscione and Blouin 2007). Those studies can be particularly informative when placed within the context of host phylogeography (Koehler *et al.* 2009). For example, they could provide insight into episodes of host-switching due to newly arrived hosts or ‘cryptic isolating events’, where geographic isolation of hosts creates divergence in parasite populations and serves as a driver for diversification (Koehler *et al.* 2009). In addition, since parasites can have a higher molecular evolution rate, the phylogeography of a specific parasite may also provide valuable information on the phylogeography of its host. It has been sugestted that parasite phylogeny can potentially elucidate host population history or demography and can serve as biological tags for identifying host-source populations (Criscione and Blouin 2007).

One example of phylogenetic congruence was found between the nematode *Heligmosomoides polygyrus* and its rodent host *Apodemus sylvaticus* which showed spatial and temporal congruences in the differentiation of both species lineages inhabiting Western Europe, Italy and Sicily. The rate of molecular evolution of the cytochrome b gene was estimated to be 1.5-fold in the parasite than in its host suggesting that the pasite could be useful for studying undetected historical events of its host (Nieberding *et al.* 2004b). Similar patterns of phylogenetic congruences can be also obtained from some ectoparasitic species. For example, a study of an ischnoceran louse (Insecta: Phthiraptera) and a hippoboscid fly (Insecta: Diptera) asociated to the Galápagos hawk (*Buteo galapagoensis*) revealed phylogenetic congruence between the population structures of the ischnoceran louse and its hawk host inhabiting different islands in the Galápagos (Whiteman *et al.* 2007).

Mitochondrial DNA (mtDNA) sequence data has proven useful in molecular phylogenetics. Some genes of the mtDNA are characterized by faster mutation rates than nuclear genes, recombine rarely and in most species is inherited maternally (Callejon *et al.* 2012; Walker and Avise 1998). The gene encoding the mitochondrial cytochrome c oxidase 1 (COI) has been used to study evolutionary relationships among recently diverged rapidly evolving taxa and also to resolve deep branch phylogenies in which multiple substitutions are a critical problem (Callejon *et al.* 2012). A non-coding mitochondrial sequence named the Control Region (CR) is another useful sequence. CR is under fewer functional and structural constraints, leading to a high average substitution rate evolving faster than mitochondrial coding genes. Thus, it is typically used for high resolution analysis of recent population structure (Avise 2012).

The study of the phylogeography of parasites and their use as markers for host biogeography is acquiring importance. However, many ectoparasite species such as ticks (Arachnida: Acari: Parasatiforme: Ixodida) have received little attention (Callejon *et al.* 2012; Gómez and Nichols 2013; Mihalca *et al.* 2011). Ticks comprise hematophagus, obligate ectoparasites of vertebrates and are distributed worldwide. There are ~ 896 described species; almost all contained into two major families the Ixodidae (hard ticks) and the Argasidae (soft ticks). A third family, Nuttalliellidae, contains only a single species (Black and Piesman 1994). The Ixodidae family

contains 702 of the valid tick species and is further divided into two lineages: the Prostriata, containing only one subfamily (Ixodinae) and the single genus *Ixodes*, and the Metastriata, containing three subfamilies: Amblyomminae (genera *Amblyomma* syn *Aponomma* and *Bothriocroton*), Rhipicephalinae syn Hyalomminae (genus, *Dermacentor*, *Rhipicephalus* syn *Boophilus*, *Rhipicentor*, *Hyalomma*, *Cosmiomma*, *Nosomma*, *Margaropus*, and *Anomalohimalaya*) and Haemaphysalinae (genus *Haemaphysalis*) (Burger *et al.* 2013; Klompen *et al.* 2002). Phylogeographic studies are available for several ticks species but most of them motivated by their economic importance as pest of livestock, and its association with a variety of animal and human pathogens (Araya-Anchetta *et al.* 2015). Ticks are known to transmit disease-causing protozoa, viruses, and bacteria including, blood parasites, rickettsias, arbovirus, among others (Araya-Anchetta *et al.* 2015).

Ticks are present in the Galápagos Islands. At least eleven species have been reported infecting endemic vertebrates and six of them have been associated with reptiles (Schatz 1991). Very little is known of these ticks beyond basic morphological descriptions. For Galápagos tortoises in particular, three species of tick have been described from at least four islands of the archipelago. These are *Amblyomma usingeri* collected from tortoises of the north of Isabela in Wolf, Darwin and Alcedo Volcano; *A. macfarlandi* collected in tortoises from the south of Isabela in Cerro Azul volcano and in Santa Cruz island; and *A. pilosum* collected from the islands Pinzón and Santiago but without reference to the host (Keirans *et al.* 1973b). According to Keirans *et al.* (1973b) *A. usingeri* has also been also collected from Galápagos land iguanas (*Conolophus*, Iguanidae, Squamata). *A. darwini* and *A. williamsii* have been described as ticks of iguana marine species (*Amblyrinchus*, Iguanidae, Squamata) (Bequaert 1932) and *A. boulingeri* have been described infesting Galápagos lava lizard (*Microlophus*, Tropiduridae, Squamata) and land iguana (Hirts and Hirts 1910, cited in (Schatz 1991).

Despite the prominence of Galápagos reptiles in the evolutionary literature e.g. (Benavides *et al.* 2009; Gentile and Snell 2009; Poulakakis *et al.* 2012), there is no information concerning the biogeography of their *Amblyomma* ticks. This data however would be important from both the evolutionary perspective and disease ecology/conservation, if any pathogenic parasites is associated to these ticks. To my

knowledge no challenge of their taxonomic status has been done since their description in the 20th century, no genetic data and ecological data is available. In this chapter I aim to evaluate genetic distinctiveness in relation to current morphological classification, to evaluate the genetic structure, and to test the pattern and timing of their evolutionary diversification correlates with that of their tortoise host. Using ticks collected from marine and land iguana I will also assess the possible origin of the ticks infecting Galápagos tortoises and the ecological and geological factors influencing their evolution in Galápagos. Giving the oblígate parasitic charasteristic of ticks, the colonization history of tortoises and the geological age of Galápagos islands it would be expected both species display congruence in their phylogeography and in their diversification history.

3.2. Materials and Methods

3.2.1. Sample collection

This study involved Galápagos tortoise ticks collected across the Galápagos Island during the years 2005, 2006, and 2013 to 2014 (Table 3.1). The tortoise populations surveyed during these years are described in materials and methods of chapter 2, section 2.21, Fig. 2.1 and Table 2.1. In 2005 and 2006 all tortoises included in a haematological study for the Galápagos National Park Directorate (GNPD) were examined for ticks, and representative specimens collected when present. Similarly, in 2013 and 2014 tortoises from Santa Cruz west, Santa Cruz east, Santiago, Isabela-Cerro Azul, and San Cristóbal, were screened for ticks during collection of blood for the haemoparasite survey described in Chapter 2. As decribed in Chapter 2, ticks circulated only in four tortoises populations, those inhabiting Alcedo (*C. vandenburghi*) and Wolf (*C. becki)* volcanoes in Isabela and these living in Pinzón (*C. ephippium)* and Santiago (*C. darwini).* The ticks of Pinzón used in this study were collected from tortoises of that island brought into captivity to the breeding Centre of Santa Cruz in 2010. Pinzón ticks were not collected during 2005-2006. Sampling was done under GNPD permit coded PC-9-13. Ticks collected from Galápagos marine and land iguanas were also included here for genetic comparison with tortoise' ticks. These specimens were also collected under research led by the GNPD from 2004 to 2010. Immediately after collection all samples were placed in ethanol 70% and stored at room temperature in a laboratory facility on the Galápagos Island, they were

transported to the United Kingdom in 2014 (exportation permit: DPNG 064-2014, and importation permit DEFRA TARP/2013/213).

3.2.2. PCR analysis and DNA sequencing

Prior to DNA isolation the tick specimens used for this study were photographed in order to perform gross morphological analysis. DNA extraction was done from individual ticks using the GeneJET Genomic DNA Purification Kit (Thermo scientific). This kit have has been shown to be most effective in comparison with other methods commonly used for DNA isolation from tissues samples (Ammazzalorso *et al.* 2015). For non-engorged adult tick DNA was extracted from the legs in order to conserve the rest of the tick body for future morphological identification. For engorged ticks the hypostome (mouth parts) and legs was used in order to avoid contamination with gut content. For nymph stages the entire specimen was processed. Prior to DNA extraction tissues were frozen in dry ice and crushed with a sterilised pestle in a microfuge tube. Some samples were also treated in combination with a bead-beating approach. According to Halos et al. 2004 (Halos *et al.* 2004) combining these methods would yield a greater quantity of tick DNA. At the final step, the isolated DNA was eluted in 50 µl of buffer, rather than the recommended 100 µl, in order to obtain a higher final concentration due to the small amount of starting tissue.

Table 3.1. Number of ticks samples included in this study. The Galápagos reptile host, sampling location and dates of collection is indicated. The tick collected in 2013 and 2014 corresponded to different tortoise indiviuals, the tick collected in 2005 and 2006 were placed in the same vial and it was not possible to partition them by individual host.

Host	Number of samples	sampling location	dates of collection
Giant tortoise	22	Isabela, Wolf V. (*C. becki*)	20-21/04/2005-2014
	18	Isabela, Alcedo V. (*C. vandenburghi syn vicina*)	22-26/04/2005
	37	Santiago (*C. Darwini*)	17-21/01/2014
	31	Breeding Center Santa Cruz (Pinzón tortoises) (*C. ephippium*)	2015
Land Iguana	4	Isabela, Wolf V. *Conolophus* spp.	2015
Marine Iguana	2	San Cristóbal and Santa Fé *Amblyrynchus* spp.	2004

Partial sequences of COI gene and from the mitochondrial CR were chosen as markers for population analysis of Galápagos tortoise ticks. Amplification of COI was done with the forward primer LCO1490 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') and the reverse primer HCO2198 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') (Folmer *et al.* 1994). These primers target a sequence of ~710 bp, they were originally designed for the amplification of this region in a wide range of invertebrates, and have been used to this end in ticks (Cruickshank 2002). PCR for COI was conducted in a volume of 25µl containing 1.5mM Magnesium Chloride, 0.2mM each dNTP, 1.0µM of each primer, 1.0 unit of Taq polymerase (Promega) and 1µl of the DNA. The thermal cycling program comprised: 94˚C for 5 minutes; followed by 35 cycles with 94˚C for 30 s, 50˚C for 45 s and 72˚C for 60 S; and finally 72˚C for 7 minutes.

The CR marker was amplified with the forward primer DLIx3 (5'-TAA CCG TCK GCK GCT GGC ACA A-3') in combination with the reverse primer DLIx4 (5'-AGA TAA YCC TTT AYT XAC AG -3'). These primers were used previously to amplify a ~340bp of this DNA region in *Amblyomma variegatum* (Beati *et al.* 2012). The PCR conditions were the same as above for COI. The PCR program included an initial cycle of 94˚C for 5 minutes followed by 4 cycles of 94˚C for 30 s, a "touchdown" annealing temperature from 57°C to 52°C for 45 s and at 72˚C for 45 s, then 36 cycles more but using an annealing temperature of 52 °C for 45 s. A final extension was done a 72° C for 7 min.

PCR products were purified and sent for sequencing in both directions by the commercial company Beckman Coulter Genomic, United Kingdom. Electropherograms were assessed for sequence quality and processed to remove ambiguous data at the 5' and 3' ends in BioEdit version 7.00 (Hall 1999). The forward and reverse reads were used to generate complementary sequences for each sample. For downstream analysis, sequences were aligned in MEGA version 7.0 (K *et al.* 2013; Tamura *et al.* 2013) and aligned using MUSCLE (Edgar, 2004).

3.2.3. Genetic diversity of Galápagos tortoise ticks

The genetic diversity of ticks was analysed independently for COI and CR markers. Analysis included the number of haplotypes (*H*, which describe the number and

frequencies of mitochondrial haplotypes) and haplotype diversity (*h*, which describe the probability that two randomly chosen haplotypes are different in the sample), Haplotype diversity estimate is analogous to the heterozygosity measure calculated for diploid loci; h values closer to 0 indicate low diversity, while *h* values closer to 1 indicate high diversity. For fast evolving loci *h* will often approach 1 in a population containing a high proportion of unique haplotypes. So, it is also informative to estimate DNA nucleotide diversity (π) (Nei 1987) which is an estimate that quantifies the mean divergence between sequences. Another important measure is the number of segregating sites (*S*) which represent the polymorphisms between related genes in the alignment. The estimates for these parameters were obtained using DnaSP 5 (Librado and Rozas 2009). Uncorrected pairwise genetic distance (hereafter referred as p-distance) was also calculated within and between populations. The p-distance method represents the proportion of nucleotides sites at which two haplotypes differ. It is the product of the number of nucleotide differences by the total number of nucleotides compared. This analysis was done in MEGA version 7.0 (Tamura *et al.* 2013), with gaps treated with partial deletion and standard deviation estimated by 500 bootstrap pseudoreplicates.

3.2.4. Population Subdivision

Genetic analysis of population subdivision was performed using COI and CR independently. One of the main methods for analysing population genetic structure for allelic data is through F-statistics (F_{ST} also known as the fixation index). F_{ST} estimates genetic differentiation between populations by measuring the degree of inbreeding within a subpopulation relative to the total population (all the subpopulation combined), it ranges from 0 (no differentiation) to 1 (complete differentiation). An analogue of F_{ST} for sequence data is phi-st (Φst) and was applied in this study. A second method for analysing population structure is via an analysis of molecular variance (AMOVA), which allows the hierarchical partitioning of genetic variation within and among populations and the estimation of *F*-statistics and/or their analogues. The statistical significance of these genetic differentiation statistics is tested by by permutating (randomising) samples among populations and recalculating the statistics, the *p-value* is the proportion of permuted data sets which give an F_{ST} or Φst value equal to or greater than the observed value. Phi-st and

AMOVA estimates were obtained using the software Arlequin 3.5 (Excoffier and Lischer 2010). Statistical significance was tested with 20,000 permutations.

3.2.5. Haplotype networks

DNA sequence variation is commonly analysed using phylogenetic trees and ⁄ or haplotype networks (Mardulyn 2012). An advantage of network analysis over trees is that it displays multifurcating lineages, the co-existence of ancestor and descendants and the reticulated evolution that accompanies hybridisation and recombination (Freeland 2011). An haplotype network displays the frequency and distribution of each haplotype, which allows making inferences about demographic history of populations. The presence of internal nodes (median vectors) is interpreted as unsampled or extinct ancestral haplotypes. The software package PopART (Leigh 2016) was used to reconstruct phylogenetic relationships among haplotypes of COI, and CR markers. It was done using a median-joining network method with an epsilon parameter of 0, in order to avoid over-complex networks, which tends to occur with higher values.

3.2.6. Demographic history

Compared to a neutral case of a randomly mating, constant sized population, with no selection processes such as population structure, changes in population size, or selective sweep can influence the pattern of variation in population samples of DNA sequences. Population expansions or selective sweeps in particular tend to mean that most extant haplotypes in a population will be derived from a recent common ancestor rather than deeper in the genealogy. Tests for demographic expansion compute the haplotype frequency deviation from evolutionary neutrality and are based on comparison of observed genetic variability (e.g. haplotype or nucleotide diversity) to what would be expected under mutation-drift equilibrium or population growth. Raggedness statistic (r), Fu and Li's D*, Fu and Li's F*, Fu's Fs and Strobeck's S are some of the statistical estimating this deviation. Fu and Li's D* and F* are based on the difference between the number of singletons and the total number the mutations (D*) or the average number of pairwise nucleotide difference (F*), while Fu's Fs is based on the probability of the observed number of haplotypes or greater occurring under conditions of neutrality. Significant values for these statistics may indicate recent population growth, background selection or positive selective sweeps. Similarly a mismatch distribution a plot of the frequency distribution for the number

of nucleotide site differences between pairs of sequences. Populations that have experienced a sudden or exponential growth or decline produce a smooth, unimodal wave in the distribution of pairwise sequence differences (the mismatch distribution) corresponding to that event, whereby stable populations produce more steadily sloped (non-wave-like) distributions and population structure or diversifying selection can give multimodal distributions All these analyses where applied in this study using the software DNAsp. They were performed using COI and CR independently.

3.2.7. Phylogenetic analysis

Phylogenetic analysis was carried out with ticks COI and and the combined COI/CR data set. In the case of COI it also included the haplotypes of Galápagos marine iguana and Galápagos land iguana detected in this study. Galápagos ticks were analysed together with Amblyomminae species from outside the Galápagos and with Ixodinae ticks used as an outgroup (see Table 3.2). The non Galápagos ticks sequences were retrieved using MEGA version 7.0 (Tamura *et al.* 2013), and all the dataset were aligned using MUSCLE (Edgar 2004). Phylogenetic relationships between haplotypes were inferred using MrBayes v.3.1.1 (Huelsenbeck and Ronquist 2001). COI analysis was run under the conditions of the Hasegawa-Kishino-Yano model with Gamma Distributed rates and invariant sites (HKY+G+I). For the COI/CR, the best fit model was the Tamura 3 parameters model with Gamma Distributed rates (T92+G). Due to difficulties in obtaining reliable alignment of the CR among distantly related tick species, the analysis of combined COI/CR data set included only the haplotypes identified in this study, and three Amblyomma species from outside Galápagos as an outgroup. Separate partitions were set for each locus, using the respective models. The models for each partition were identified as the best fit model for the data by the implementation of ModelTest in MEGA 7.0. Multiple simulations were run for 10 million generations with the first 500,000 trees discarded as burn-in period after confirming the convergence of chains. Trees were sampled every 1,000 generations and a 50% consensus tree was constructed from the results. The resulting consensus tree was visualised and edited using the online software iTol (Letunic and Bork 2016).

3.2.8. Divergence time estimates

Divergence times among Galápagos ticks haplotypes and key references from Amblyomminae, Hyalomminae and Ixodinae (Table 3.2), were estimated for COI sequences. Divergence time estimation was restricted to COI because this marker has

been used previously for divergence estimation of ticks. CR sequences were not obtained successfully for Galápagos iguana ticks, and reliable alignments for CR sequences in other tick species could not be generated due to excessive insertions and deletions. The estimate of divergence times is based on the hypothesis that DNA sequences evolve at roughly constant rates so they provide a "molecular clock" of evolution. The calibration of molecular clocks is based on the approximate date when two genetic lineages diverged. This date should ideally be obtained from fossil record or known geological events. The next step is to calculate the amount of sequence divergence that has occurred since that time between the lineages of interest.

A Bayesian relaxed-clock analysis was conducted in the software BEAST (Drummond *et al.* 2012), using a calibration of 35-50 my for Hyalominae and 35-40 my for Ixodinae. These values have been obtained from fossil records of hyalomids and ixodis ticks, respectively (Gou *et al.* 2013). Setting of the analysis included an uncorrelated lognormal distribution, and a Yule speciation process. Simulations were run for 10 million generations, with sampling every 1000 generations. Adequate sampling and convergence of the chain to stationary distribution were confirmed by inspection of MCMC samples using Tracer 1.5 (Drummond *et al.* 2012). The effective sample size (ESS) values of all parameters were greater than 200, which were considered a sufficient level of sampling. The sampled posterior trees were summarized using TreeAnnotator 1.7.1 (Drummond *et al.* 2012). A 95% higher posterior density distribution (HPD) was used as confidence interval for parameter estimates. The tree topology was visualized and edited with FigTree 1.4.3 (Drummond *et al.* 2012).

Table 3.2. Amblyomminae and Ixodinae ticks species used for phylogenetic analysis of Galápagos tortoise and Galápagos iguana ticks The host class and the geographical distribution is given.

GenBank Accesion number	Tick subfamily	Tick specie	Host	Georgaphic distribution.
AB113317	Amblyomminae	*Amblyomma triguttatum*	Mammalia	Australia
FJ584425	Amblyomminae	*Bothriocroton hydrosauri*	Reptilia	Australia
FJ584429	Amblyomminae	*Amblyomma limbatum*	Reptilia	Australia
GU062743	Amblyomminae	*Amblyomma variegatum*	Aves, mammalia	Africa
HM193875	Amblyomminae	*Amblyomma pattoni*	Reptilia	Asia
HM193892	Amblyomminae	*Amblyomma testudinarium*	Mammalia, aves	Asia
JN863728	Amblyomminae	*Bothriocroton undatum*	Mammalia, Reptilia	Europe, Australia, Asia
JN863729	Amblyomminae	*Amblyomma elaphense*	Reptilia	America
JN863730	Amblyomminae	*Amblyomma fimbriatum*	Reptilia	Australia, Asia, America
JN863731	Amblyomminae	*Amblyomma sphenodonti*	Reptilia	Oceania
KF200085	Amblyomminae	*Amblyomma calcaratum*	Mammalia	America (neotropical region)
KF200093	Amblyomminae	*Amblyomma sabanerae*	Reptilia	America
KF200103	Amblyomminae	*Amblyomma longirostre*	Aves, mammalia	America (neotropical region)
KF200109	Amblyomminae	*Amblyomma oblongoguttatum*		America
KF200124	Amblyomminae	*Amblyomma cajennense*	Mammalia, aves	America
KF200128	Amblyomminae	*Amblyomma pecarium*		
KF200137	Amblyomminae	*Amblyomma auricularium*	Mammalia	America
KF200138	Amblyomminae	*Amblyomma nodosum*	Mammalia	America
KF200139	Amblyomminae	Amblyomma varium	Mammalia	America
KF200142	Amblyomminae	*Amblyomma sp MJM-2013*		

GenBank Accesion number	Tick subfamily	Tick specie	Host	Georgaphic distribution.
KF200145	Amblyomminae	*Amblyomma calcaratum*		
KF200158	Amblyomminae	*Amblyomma ovale*	Mammalia, aves	America
KF200159	Amblyomminae	*Amblyomma geayi*	Mammalia, aves	America
KF200160	Amblyomminae	*Amblyomma cajennense*		
KF200167	Amblyomminae	*Amblyomma naponense*	Mammalia	
KF200170	Amblyomminae	*Amblyomma dissimile*	Reptilia	America
KF200171	Amblyomminae	*Amblyomma tapirellum*	Mammalia	America
KM821513	Amblyomminae	*Bothriocroton concolor*	Mammalia	Austalia, Asia, America
KM839245	Amblyomminae	*Amblyomma maculatum*	Mammalia	America
KP862672	Amblyomminae	*Amblyomma eburneum*	Mammalia	Africa
KP987771	Amblyomminae	*Amblyomma rotundatum*	Reptilia, amphibia	America
KT307491	Amblyomminae	*Amblyomma eburneum*		
KT307492	Amblyomminae	*Amblyomma lepidum*	Mammalia	Africa
KT307493	Amblyomminae	*Amblyomma tholloni*	Mammalia	Africa
KT382870	Amblyomminae	*Amblyomma geoemydae*		
KU892221	Amblyomminae	*Amblyomma hebraeum*	Mammalia, reptilian, aves	Africa
KP941755	Amblyomminae	*Amblyomma americanum*	Mammalia, aves	America
AB075954	Haemaphysalinae	*Haemaphysalis flava n*	Mammalia	Asia
JX573135	Haemaphysalinae	*Haemaphysalis formosensis*		
JX573136	Haemaphysalinae	Haemaphysalis parva	Mammalia	Asia
JX573137	Haemaphysalinae	*Haemaphysalis hystricis*		Asia
AJ437089	Rhipicephalinae syn Hyalomminae	*Hyalomma truncatum*	Mammalia, aves	Africa

GenBank Accesion number	Tick subfamily	Tick specie	Host	Georgaphic distribution.
AJ437098	Rhipicephalinae syn Hyalomminae	*Hyalomma marginatum*	Mammalia	Europe
EU827736	Rhipicephalinae syn Hyalomminae	*Hyalomma lusitanicum*	Mammalia	Europe
JQ737074	Rhipicephalinae syn Hyalomminae	*Hyalomma rufipes*	Aves, Mammalia	Africa, Europe, Asia
KR075985	Rhipicephalinae syn Hyalomminae	*Hyalomma asiaticum*	Mammalia	Asia
KT989616	Rhipicephalinae syn Hyalomminae	*Hyalomma aegyptium*	Mammalia, Reptilia	Europe
KU364325	Rhipicephalinae syn Hyalomminae	*Hyalomma asiaticuma*		
KU556745	Rhipicephalinae syn Hyalomminae	*Rhipicephalus sanguineus*	Mammalia	America
HM193891	Rhipicephalinae syn Hyalomminae	*Dermacentor marginatus*	Mammalia	Europe
KM831304	Rhipicephalinae syn Hyalomminae	*Dermacentor variabilis*	Mammalia	America
AB231669	Ixodinae	*Ixodes pavlovskyi*		
AY945440	Ixodinae	*Ixodes ricinus*	Mammalia	Europe, Africa
GU437873	Ixodinae	*Ixodes bakeri*	Mammalia	Africa
KM821524	Ixodinae	*Ixodes hirsti*	Aves	Australia
KM821527	Ixodinae	*Ixodes cornuatus*	Mammalia	Australia
KU935457	Ixodinae	*Ixodes persulcatus*	Mammalia	Asia, Europe

3.3. Results

3.3.1. Morphological variation

Photographs of Galápagos tortoise ticks collected from Isabela, Santiago and Pinzón tortoises are shown in Figs. 3.1a-3.1i. The gross morphology of each tick specimen was comparatively similar. On Isabela, (Alcedo and Wolf volcanoes), the female scutum is ornate, with patches of green and orange colours, and presence of setae. The males have a pseudoscutum, with patches of dark and light colouration across dorsal area. Setae are present, but very few and short. Ticks from Isabela are quite distinctive from the ticks collected from Santiago and Pinzón tortoises. The ticks from Santiago and Pinzón tortoises do not show much morphological differentiation from each other. Female ticks of Pinzón and Santiago have a greyish-brown coloured scutum, with long white setae found across the dorsal and ventral regions. Male ticks have a pseudoscutum that is grooved and further body grooves. Setae grow across the body. Females were differentiated from males by the genital aperture. In males, the aperture is convex, where as females have concave apertures. The features of the tortoise ticks of Isabela are in agreement with the features described for *A. usingeri* while the features of the tortoise ticks of Santiago and Pinzón are in agreement with those described for *A. pillosum*. With information of Little L. (2016).

3.3.2. PCR analysis and DNA sequencing

Of the tortoise ticks included in molecular analysis 90% (86/96) had positive PCR results for COI, 78% (75/96) for CR, and 75% (72/96) for both genes. The number of successful amplifications by tortoise population is shown in Table 3.3. For COI amplification success ranged from 44% in ticks from Isabela-Alcedo to 95% in Santiago. For CR it ranged from 27% in Isabela-Wolf to 100% in Santiago. Negative samples remained the same after being analysed with fresh isolated DNA or modification of PCR stringency. A preliminary analysis using BLAST confirmed the identity of all the Galápagos ticks analysed here as belonging to the Genus *Amblyomma*.

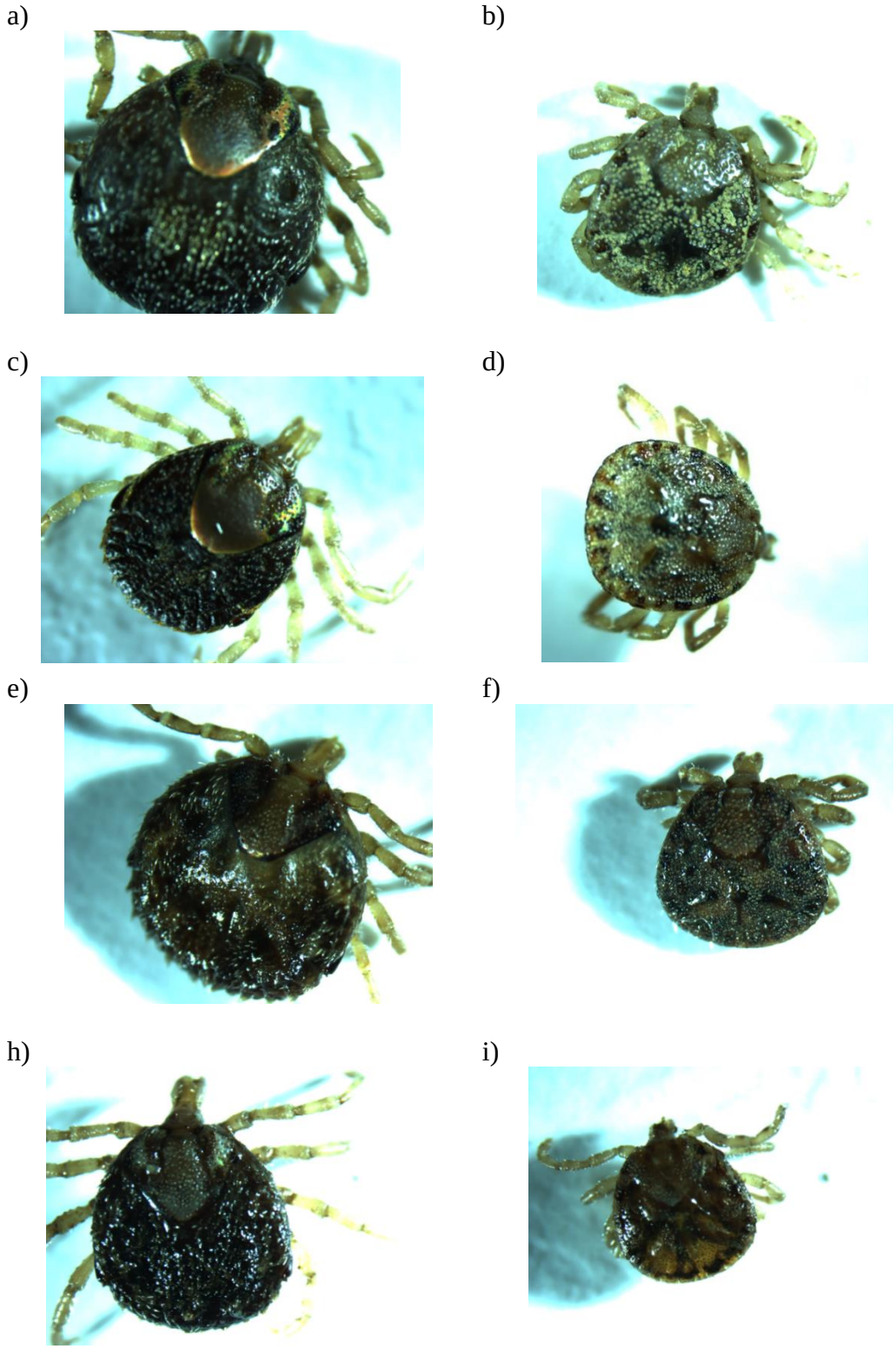


Figure 3.1. Photographs of Galápagos tortoise ticks collected from Isabela, Santiago and Pinzón, a-b) female and male of Alcedo, c-d) female and male of Wolf, e-f) female and male from Pinzón, h-i) female and male of Santiago. In pictures 'a' and 'c' (female ticks from Isabela Island) note the patches of green and orange colours on the tick's scutum. Of the photographed ticks their length (from apices of scutum to posterior body margin) ranged from 3-3.50 mm and width from 2.7-3.4 mm. Pictures were made with a Leica M165 FC, 2 x objective.

3.3.3. Genetic diversity of Galápagos tortoise ticks

Alignments of 658 bp (obtained after trimming) of COI sequences of Galápagos tortoise ticks revealed the presence of 18 haplotypes across the archipelago. The number of haplotypes found by population (*H*), haplotype and nucleotide diversity (*h* and π, respectively), number of segregating sites (*S*), and p-distance within populations are shown in Table 3.3. Each of the populations surveyed have unique haplotypes, but one haplotype was shared between Galápagos tortoise ticks of Alcedo and Wolf. The lowest variability was found in Santiago and the highest in Alcedo, with haplotype numbers ranging from 2 to 7, haplotype diversity from 0.060 to 0.95, and nucleotide diversity from 0.0003 to 0.0105, respectively. The p-distance within populations was lower for tortoise ticks from Santiago and Pinzón in comparison with Isabela. Across tick populations COI nucleotide diversity was 0.0040, the number of polymorphic sites was 73. The four COI sequences from land iguana ticks yielded 3 haplotypes, and the two COI sequences from marine iguana comprised two haplotypes.

The analysis of 446 bp CR sequences from Galápagos tortoise ticks yields 19 haplotypes. As for COI, each tick population had unique haplotypes, with three shared between Alcedo and Wolf. The statistics for this sequence are also shown in Table 3.3. The lowest number of haplotypes was found in Pinzón (4) and the highest in Santiago (8). The lowest haplotype diversity was for Pinzón (0.71) and the highest for Alcedo (0.95), the lower nucleotide diversity was also found in Pinzón (0.004048) and the higherst for Wolf (0.008108). The p-distance was again lowest for Pinzón (0.041) and highest for Wolf (0.0063). Across all tick populations the nucleotide diversity was 0.032 and the number of polymorphic sites was 43.

Graphical information of the COI and CR haplotypes obtained from Isabela, Santiago and Pinzón, and their frequencies is shown in Fig. 3.2.

Table 3.3. Description of PCR and haplotype analysis results. In PCR results N=samples analysed, n=tick sequences obtained. The haplotype analysis results shows the number of haplotypes found by tick population (*H*), haplotype diversity (h), nucleotide diversity (π), number of segregating sites (S), and p-distance within populations and standard deviation.

DNA Marker, reptil host, and location of sampling	PCR results		Haplotype analysis results				
	N	*n*	*H*	*h*	π	*S*	p-distance
COI tortoises							
Alcedo	18	8	7 (COI 1, COI 2, COI 3, COI 4, COI 5, COI 6, COI 7)	0.94	0.0106	16	0.0098 ±0.0024
Wolf	22	13	5 (COI 4, COI 8, COI 9, COI 10 COI 11)	0.58	0.0031	10	0.0037 ±0.0012
Pinzon	31	26	5 (COI 12, COI 13, COI 14, COI 15, COI 16)	0.72	0.0016	4	0.0015 ±0.0009
Santiago	37	35	2 (COI 17, COI 18)	0.06	0.0003	10	0.0002 ±0.0001
COI iguanas							
Land iguana	4	4	3 (COI 12, COI 13, COI 19)				0.0420 ±0.0054
Marine iguana	2	2	2 (COI 20, COI 21)				NA
CR tortoises							
Alcedo	18	6	5 (CR 1, CR 2, CR 3, CR 4, CR 5)	1.00	0.0059	6	0.0050 ±0.0020
Wolf	22	6	5 (CR 2, CR 3, CR 4, CR 6, CR 7)	1.00	0.0081	8	0.0063 ±0.0023
Pinzon	31	26	4 (CR 8, CR 9, CR10, CR 11)	0.71	0.0041	4	0.0041 ±0.0021
Santiago	37	37	8 (CR 12, CR 13, CR 14, CR 15, CR 16, CR 17 CR 18, CR 19)	0.84	0.0045	6	0.0045 ±0.0021

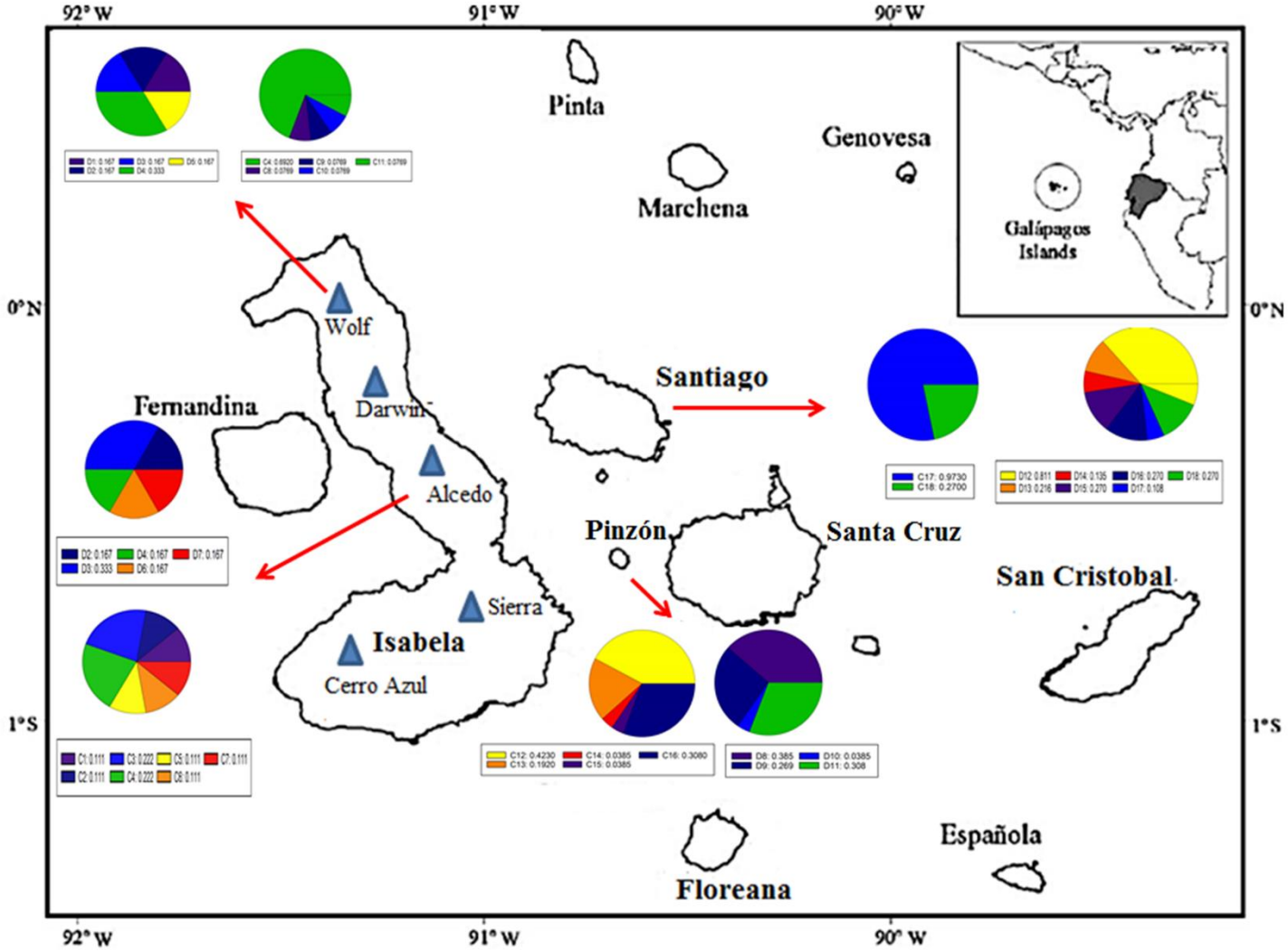


Figure 3.2 Distribution and frequency of COI and CR haplotypes on Isabela, Santiago and Pinzón.

Genetic distance between populations, in terms of p-distance, is shown in Table 3.4. For COI, Alcedo and Wolf have a value of 0.007, while the other comparisons ranged from 0.042 to 0.081. In pairwise comparisons with ticks from land iguanas, tortoise ticks from Pinzon have the lower genetic distance (0.022), while the values for the other populations ranged from 0.052 to 0.063. Between populations, genetic distance for CR was lowest between Alcedo and Wolf (0.005), and ranged from 0.042 to 0.066 in the remaining pairwise analyses.

3.3.4. Population Subdivision

The results of a pairwise analysis are shown Table 3.5 Santiago and Pinzon populations were significantly different from each other and from the two Isabela populations, as shown by the Φst value of >0.95 (*p value*<0.001) for all the pairwise relationships. The Φst value between Alcedo and Wolf was non-significant at 0.048 (*p-value* 0.13). Similar results were found for CR in all pairwise comparison, Santiago and Pinzón have Φst value of > 0.89 (*p-value* <0.001) and the Φst value between Alcedo and Wolf was non-significant at -0.10345 (*p-value* 0.88). The lack of significant differentiation in Isabela indicates that there is no, or very limited, differentiation between the tortoise ticks of these volcanoes. AMOVA analysis of COI showed that 95.18% of the variation occurred among populations and 4.82% within populations (F_{ST} 0.9650, *p-value* <0.001). For CR, 90.525% of the variation occurred among populations and 9.48% within populations (F_{ST} 0.90519, *p-value* <0.001).

3.3.5. Haplotype networks

Network analysis of COI and CR exhibited three clusters of tortoise tick haplotypes, each belonging to a different island (Fig. 3.3 and 3.4, respectively). The clusters for the Pinzon and Santiago haplotypes are separated by 26 mutations, while Santiago and Isabela haplotype clusters are differentiated by 45 mutations. The haplotypess of Pinzón and Isabela are separated by 71 nucleotide differences. Within Isabela (Alcedo and Wolf volcanoes) distance between COI haplotypes varied from one to nine substitutions. The most frequent haplotype on wolf (9 copies), is the joint most frequent haplotype on Alcedo (2 copies). The COI network includes the three haplotypes obtained from ticks on land iguanas collected on Wolf volcano (coloured magenta). One haplotype clustered with tortoise tick haplotypes from Wolf, but the

two others were identical to two different haplotypes of Pinzon tortoises, the most frequent, with ten and eight copies respectively. Within Pinzon COI haplotypes varied from one to two mutations. Santiago has two unique haplotypes which varied by 3 mutations.

The CR haplotypes in Isabela differed by one to six nucleotides. Within Santiago there were 8 haplotypes which varied by 1 to 5 nucleotides, while Pinzon had 4 haplotoypes differing by 1 to 4 nucleotides. Among tick populations the CR variation was smaller than with COI. Pinzon and Santiago haplotypes were separated by 15 mutations; Santiago and the closest Isabela haplotype have 20 nucleotide differences. For both networks, medians were present between each cluster, representing possible extinct or unsampled haplotypes. The number of median vectors, or hypothesised links between the haplotype nodes was higher for CR, which supports the higher levels of mutation found in a non-coding sequence. The positions of Pinzon and Santiago in relation to Isabela varied according the marker used in the analyses. However, the number of mutations between clusters for CR is more similar compared with COI, making it harder to resolve the relative ordering of populations in the network

3.3.6. Demographic Analyses

The results of demographic analysis comprising Raggedness statistic, Fu and Li's D*, Fu's Fs, and Strobeck's S tests are shown in Table 3.6. There were no significant values indicating deviation from neutrality. Mismatch analysis is shown in Figs. 3.5a -3.5h. Mismatch distributions need to be interpreted with caution because there are relatively few haplotypes within populations, mostly differing by a few nucelotides. In general it was expect to get similar signals from both sequences since the mtDNA genome should act as a single locus. However, in Alcedo the COI showed a multimodal distribution while the CR data set was unimodal. Pinzon and Santiago showed unimodal distributions with both datasets, while for Santiago the observed distribution values were similar to the expected model of population expansion.

Table 3.4. Genetic distance between tick populations, in terms of p-distance. The p-distance for COI are showed over the diagonal. The p-distance for CR are below the diagonal. Ticks from land iguanas were included. Standard deviations are given for each value.

Tick population	Tick population				
	Alcedo	Pinzon	Santiago	Wolf	Land_Iguana
Alcedo		0.081 ±0.010	0.076 ±0.010	0.007 ±0.002	0.063 ±0.008
Pinzon	0.055 ±0.010		0.042 ±0.008	0.080 ±0.010	0.022 ±0.003
Santiago	0.065 ±0.011	0.042 ±0.008		0.075 ±0.010	0.051 ±0.008
Wolf	0.005 ±0.002	0.056 ±0.010	0.066 ±0.011		0.062 ±0.008

Table 3.5. Pairwise analysis for population subdivision among tortoise ticks. F_{ST} =Φst values for COI are showed over the diagonal, Φst for CR are below the diagonal; * $P > 0.05$, all other values, $P<0.001$, permutation test.

	Wolf	Alcedo	Pinzon	Santiago	Land Iguana
Wolf		0.04817*	0.97214	0.985	0.7902
Alcedo	-0.10345*		0.95589	0.97309	0.68118
Pinzon	0.92109	0.92543		0.9817	0.44161
Santiago	0.91662	0.91977	0.89574		0.90911

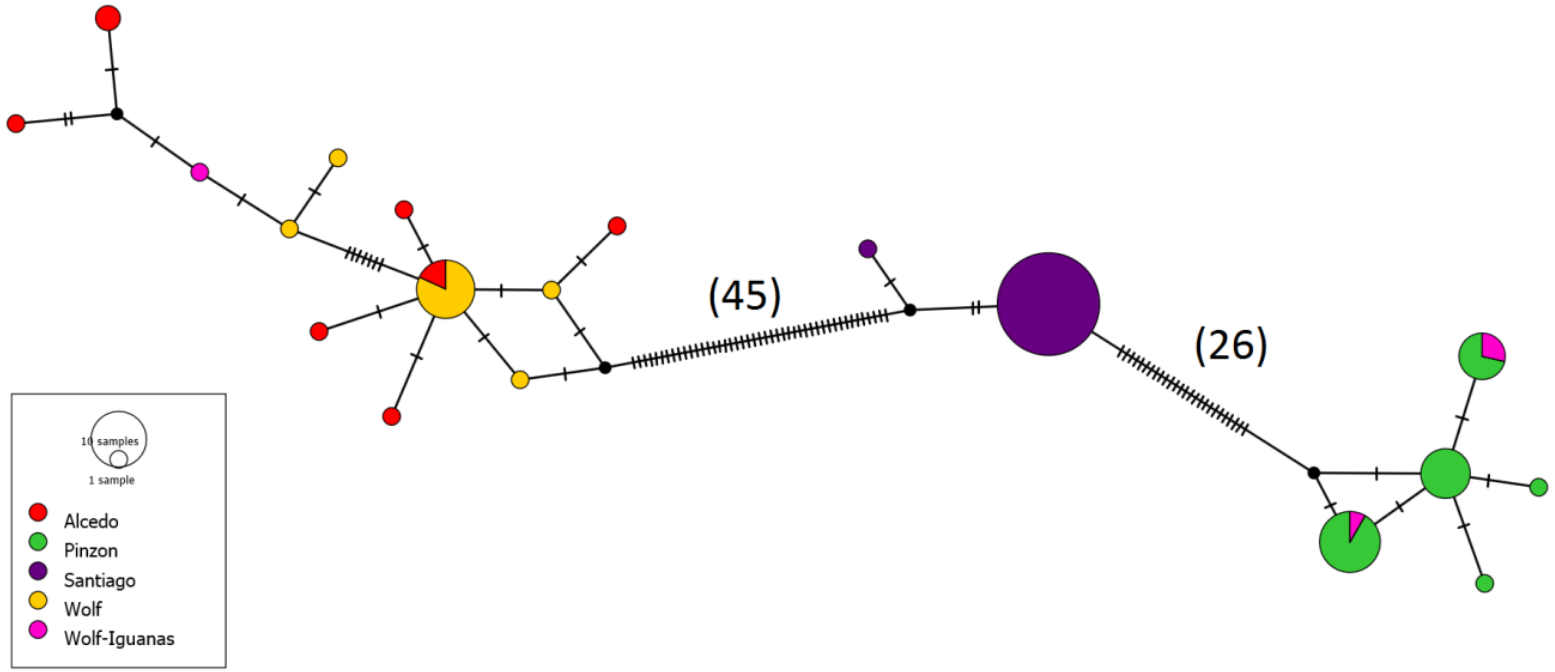


Figure 3.3. Median Joining haplotype network for the COI gene. Populations are represented in red (Alcedo), green (Pinzon), purple (Santiago), yellow (Wolf) and pink (Wolf Iguanas). Each mutation between haplotypes is indicated by a stroke across the connection that joins it to another, haplotype, or a black node, longer links have the number of mutations in brackets.

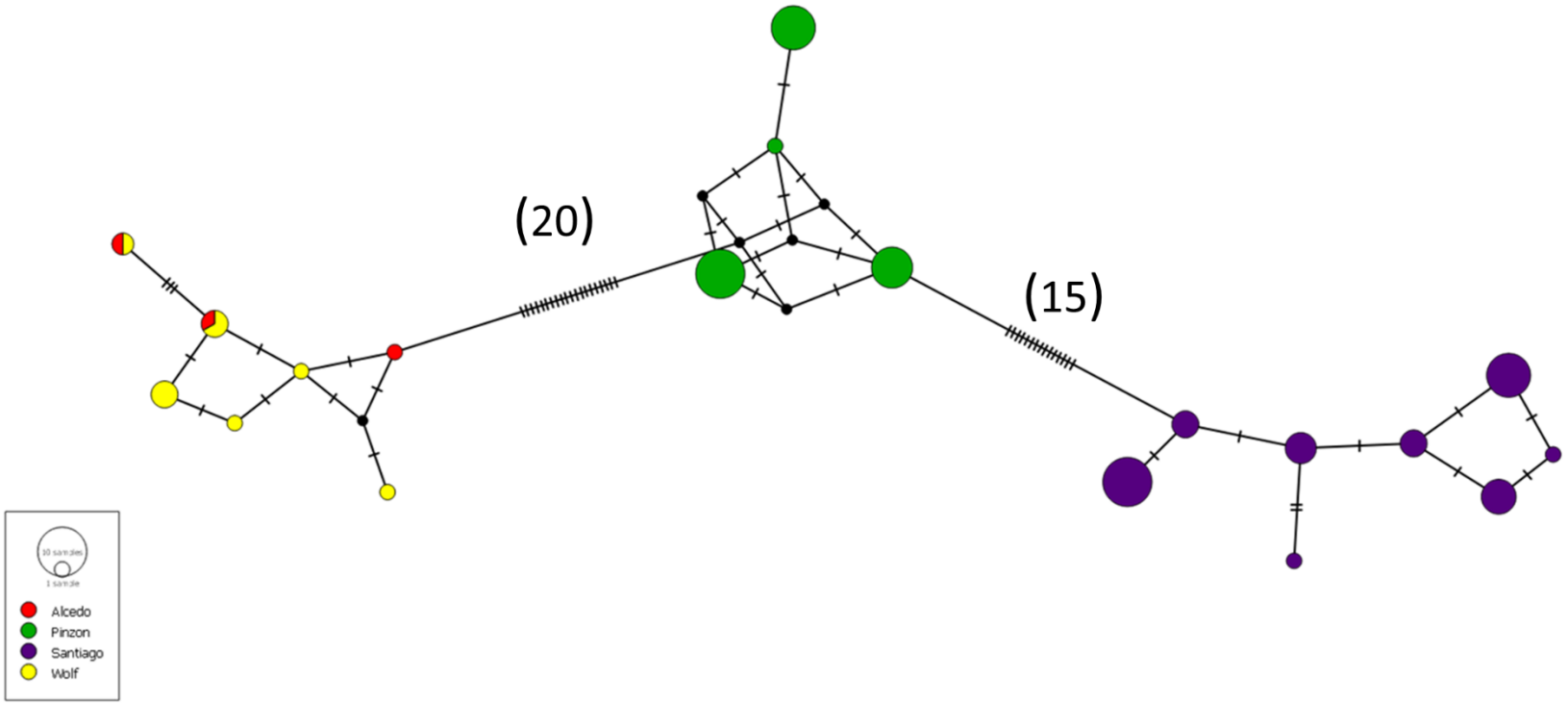


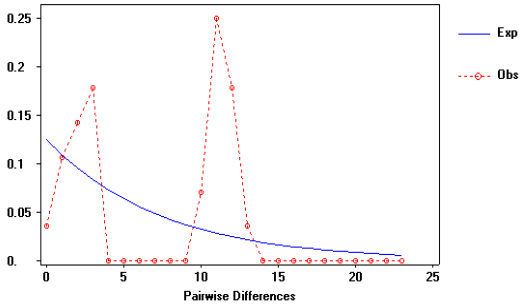
Figure 3.4. Median Joining haplotype network for CR haplotypes. Populations are represented in red (Alcedo), green (Pinzon), purple (Santiago) and yellow (Wolf). Each mutation between haplotypes is indicated by a stroke across the connection that joins it to another haplotype, or a black node, longer links have the number of mutations in brackets.

Table 3.6. Tests for polymorphism used to assess demographic history in Galápagos tortoise ticks.

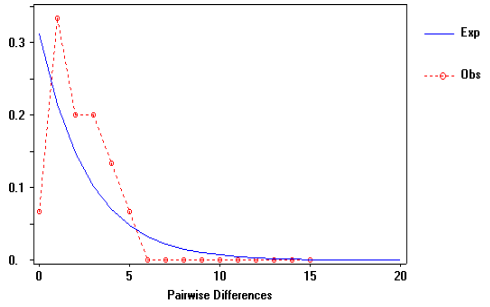
Population	Raggedness statistic	Fu and Li's D*	Fu and Li's F*	Fu's Fs	Strobeck's S	Mismatch distribution curve
COI						
Alcedo	0.077	0.00810	0.04676	0.653	0.886	Multimodal
Wolf	0.1289	0.20065	-0.12398	0.421	0.651	Multimodal
Pinzon	0.1276	-0.89691	-0.79457	-0.832	0.869	Unimodal
Santiago	0.9007	0.64908	0.33482	16.953	0.000	Similar to model
CR						
Alcedo	0.1022	1.07467	1.53704	1.896	0.306	Unimodal
Wolf	0.0667	-0.41639	-0.43037	-1.672	0.978	Multimodal
Pinzon	0.3269	1.07467	1.53704	1.896	0.306	Unimodal
Santiago	0.0564	-0.42396	0.03449	-0.911	0.849	Unimodal

The significance of the deviation from neutrality is calculated with coalescent simulation (**<0.01)

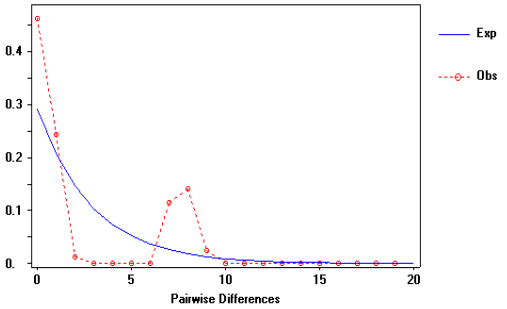
a)Alcedo COI



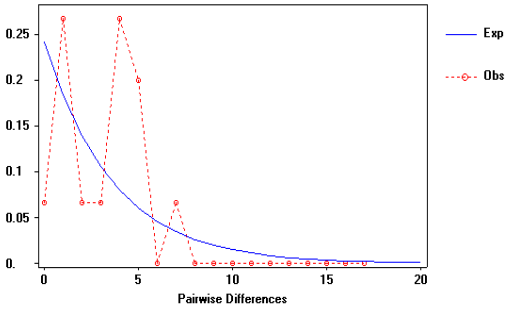
e)Alcedo CR



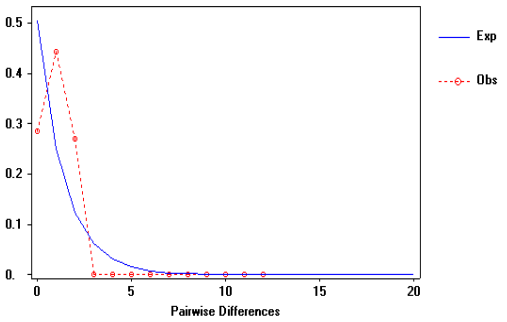
b) Wolf COI



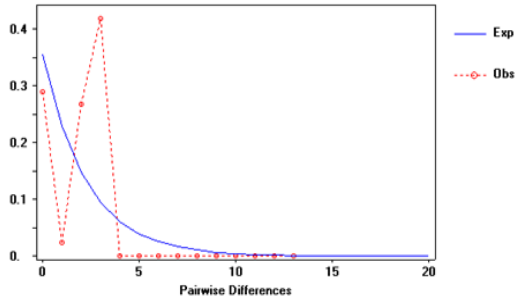
f) Wolf CR



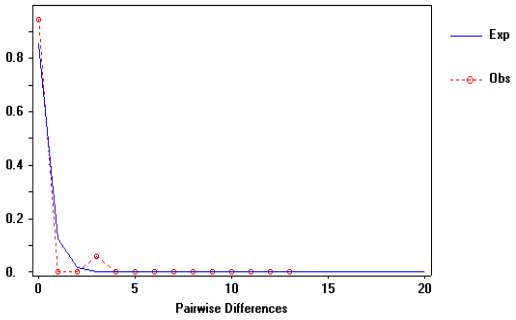
c) Pinzon COI



g) Pinzón CR



d) Santiago COI



h)Santiago CR

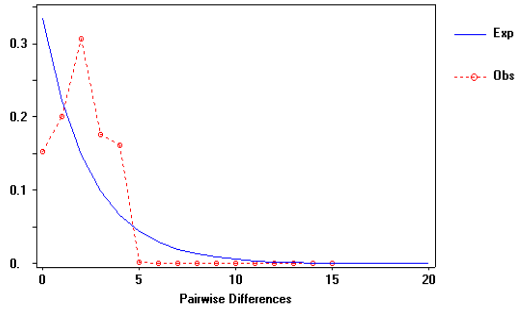


Figure 3.5. Mismatch analysis of Galápagos tortoise ticks. Results for each marker is showed in colums. COI is in the right column, CR in the left. The population in marker name is given for each column.

3.3.7. Phylogenetic analysis

The Bayesian phylogenetic analyses of COI and combined COI/CR data are shown in Figs. 3.6 and 3.7. In both cases Galápagos tortoise tick sequences form a monophyletic clade with respect to the other taxa. However, the Galápagos topology differs slightly between the 2 datasets. For COI data there is a sequential split of 2 Isabela haplotype clusters, while for the combined data Isabela sequences form a monophyletic group. In both cases support for the Isabela cluster topologies is as low as 0.55, and there is no partitioning of Alcedo and Wolf haplotypes. For both datasets Santiago and Pinzón haplotypes form well supported monophyletic clusters as a sister lineage to the Isabela groups. Two of the three haplotypes from land iguana ticks sampled on Wolf, also group in the Pinzon cluster as in the haplotype networks. Marine iguana ticks form an independent lineage, distant from the ticks of Galápagos tortoises and Galápagos land iguanas, with their closest sequence being *A. sabanerae*, a tick present in America along the pacific coast, recorded as infesting *Rhinoclemmys* (*Geomidae* rodent) species (Garces-Restrepo *et al*. 2013). For COI, support values at nodes of intermediate depth are low and resolution of the reference taxa is poor.

3.3.8. Divergence estimation

In the BEAST derived tree (Fig 3.8), the topology of Galápagos tortoise tick haplotypes is concordant the combined COI-CR dataset, but again support for intermediate depth nodes involving reference taxa is poor. The divergence analysis estimated a rate of evolution among lineages of hard ticks of 0.012 substitution per site per million years (HPD 95% 8.22×10^{-3}-0.015). This implies divergence times among the major tick lineages as follows: Hyalominae formed 22.28 million years ago (mya) (HPD 95% 15.22-30.30), Ixodinae formed 36.67 mya (HPD 95% 28.37-44.66) and Amblyominae formed 38.68 mya (HPD 95% 29.29–48.75). The divergence of Galápagos tortoise ticks and their closest relative occurred ~29 mya (HPD 95% 20-38). The split within Galápagos, between the Isabela sequences and the Santiago-Pinzon group is 8.2 mya (HPD 95% 13-4.5); the split between Santiago and Pinzón dates to 3.2-3.6 mya (HPD 95% 1.5-5.5), and coalence time of haplotypes

collected in Alcedo and Wolf occurred ~1.5 mya (HPD 95% 0.5-3). The split of marina iguana their closest relative occurred 23 mya (HPD 95% 14-31.5).

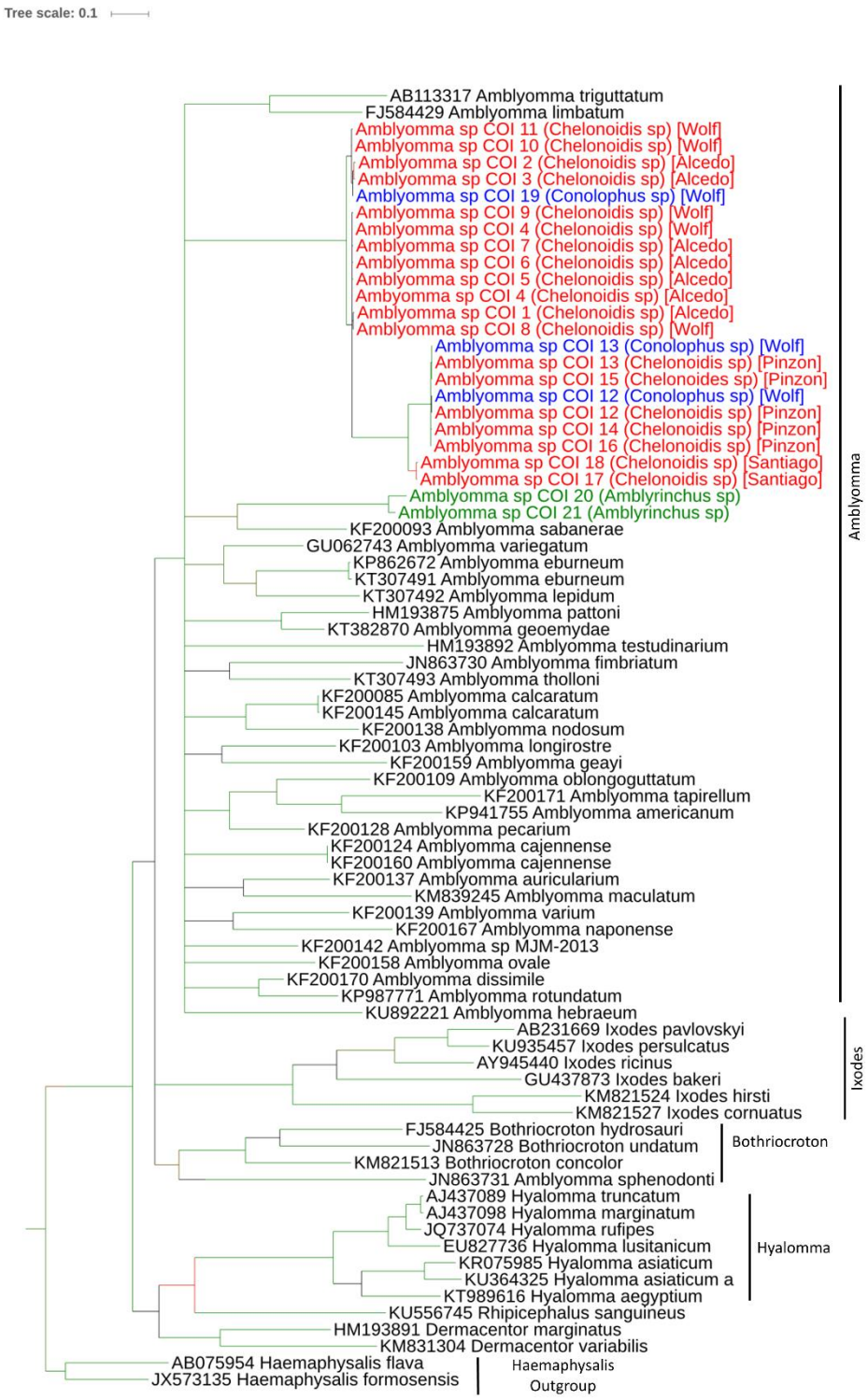


Figure 3.6. Phylogeny of Galápagos tortoise ticks based on partial sequence of COI, inferred using Bayesian analysis under Hasegawa-Kishino-Yano model with Gamma Distributed rates and invariant sites (HKY+G+I). The labels show the GenBank accession number, tick species name, host and sampling locations for Galápagos ticks. Galápagos tortoise ticks are shown in red, ticks of Galápagos land iguana and Galápagos marine iguana are shown in blue and green. Posterior probabilities of 100% are showed in green, lower values (max 75%) are showed in red.

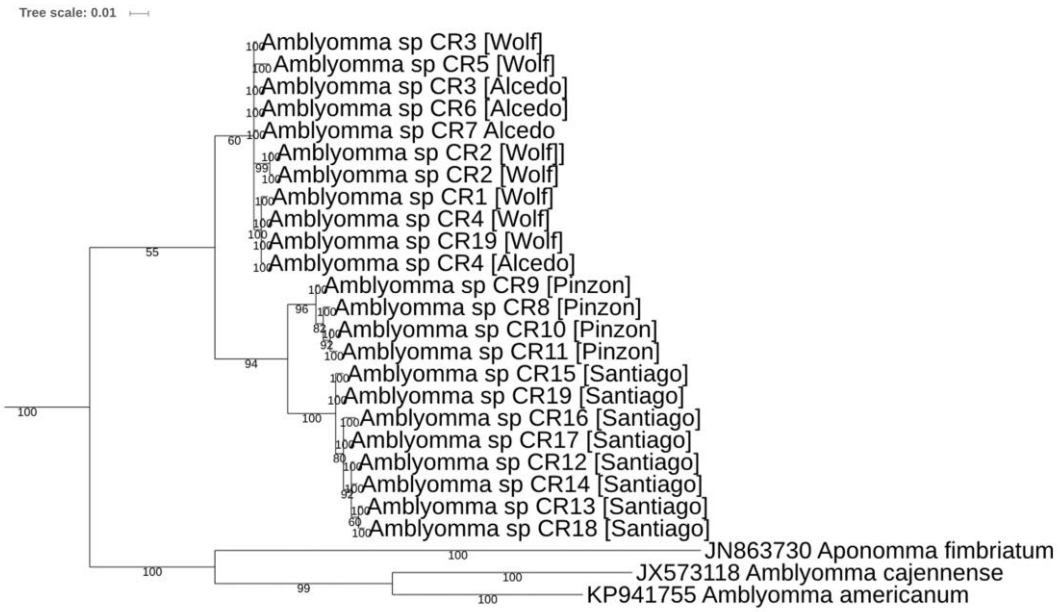


Figure 3.7. Phylogeny of Galápagos tortoise ticks based on concatenated sequences of COI and CR. The phylogeny was inferred using Bayesian analysis under Tamura 3 parameters model with Gamma Distributed rates (T92+G). The labels show the GenBank accession number and tick species. *A. cajennense*, *A americanum* and *A fimbriatum* were used as outgroup. Posterior probabilities are indicated for each node.

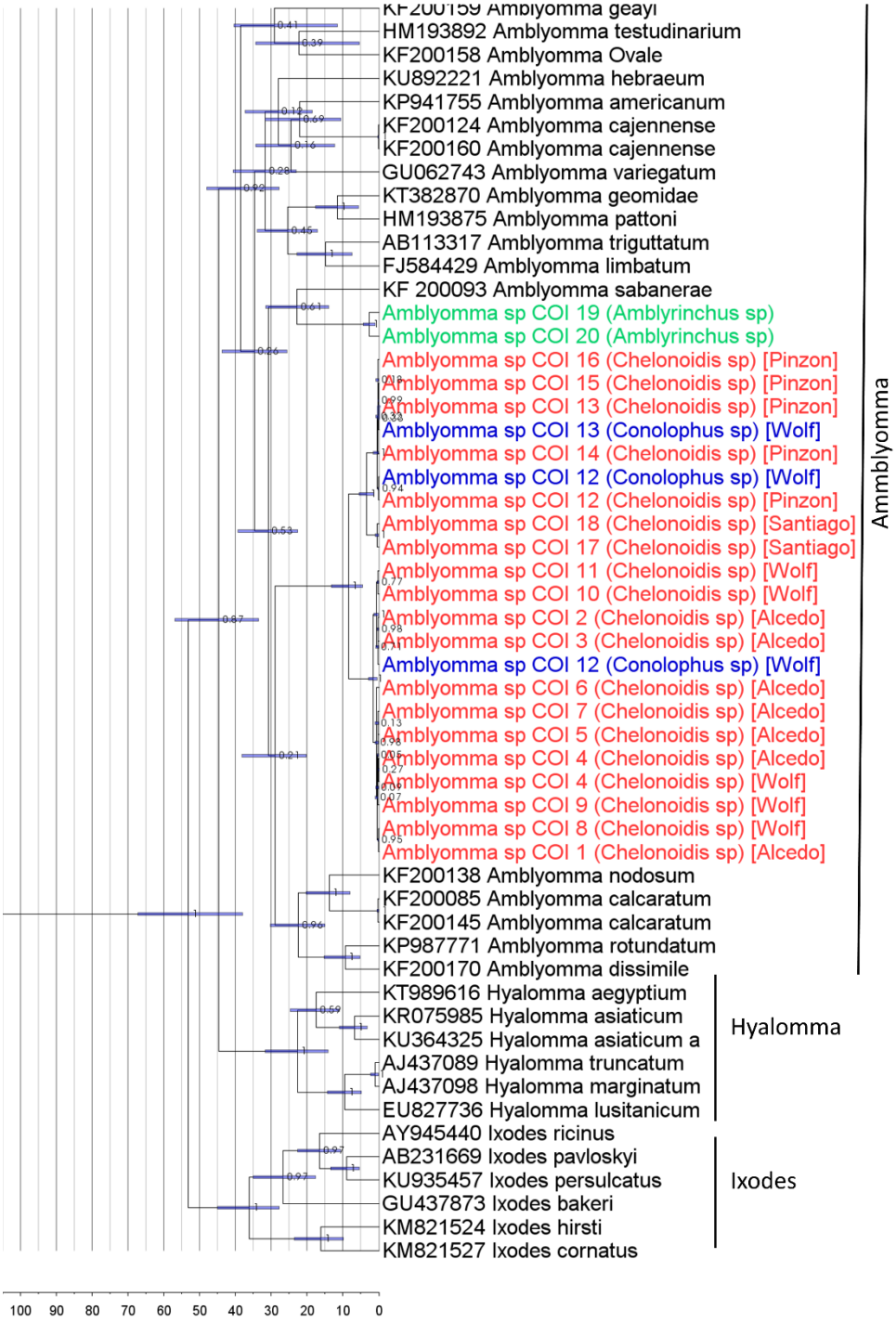


Figure 3.8. Temporal framework of Galápagos tortoise tick evolution on the Galápagos Islands. The labels show the GenBank accession number and tick species. Sampling location is shown for the Galápagos specimens, tortoise ticks are labelled in red. Ticks of Galápagos marine iguana were also included and are labelled in green. The tree is based on COI sequences. It was calibrated using fossil records of Hyalomminae and Ixodinae ticks. Numbers at nodes indicate support. Time estimates and 95% credibility intervals from a timetree analysis are shown in blue bars.

3.4. Discussion

Keiran *et al*. (1973) described two species of *Amblyomma* ticks collected from Galápagos tortoises: *A. usingeri* (in north volcanoes of Isabela: Alcedo, Darwin and Wolf), and *A. macfarlandi* (in the south volcano of Isabela "Cerro Azul", and in the south of Santa Cruz Island). They also described a third species, *A. pilosum*, for Santiago and Pinzon islands. These last specimens were collected without reference to the hosts although tortoises in those islands have been recorded as being infested with ticks long ago (Heller 1903). Data collected in 2005-2006 and 2013-2014 across the Galápagos archipelago, and included in this thesis, allowed confirmation of *Amblyomma* infestation in tortoise populations of Alcedo and Wolf volcanoes and in tortoises of Santiago and Pinzón. Gross morphology of tortoise ticks collected in Isabela agrees with the description provided by Keirans *et al*. (1973) for *A. usingeri*; and gross morphology of ticks sampled in Santiago and Pinzón agrees with the descriptions given for *A. pilosum*. Network and phylogenetic analysis of mitochondrial DNA grouped the specimens according to the island where they circulated which agrees with their geographical separation. No genetic structure was found among tortoise within islands, but there was strong differentiation among islands, including between the ticks collected in Santiago and Pinzon which have to date been classed as the same species based on morphology. The extent of the differentiation between Pinzon and Santiago, including an estimated divergence time of 4.8 (HPD 95% 13-4.5) mya implies the presence of a cryptic species. Haplotypes of two ticks sampled from land iguanas on Wolf volcano were identical to 2 different haplotypes of ticks on tortoises from Pinzón held in the Santa Cruz breeding centre. This result suggests a potential transfer of Pinzón ticks to Isabela in the past.

The genetic similarity among ticks collected in Alcedo and Wolf volcanoes confirms they belong to the same species, *A. usingeri*. The sharing of haplotypes between these distant volcanoes, separated by Darwin volcano and extensive lava field suggests a movement of ticks among them. It is also consistent with the finding of the same tortoise haemogregarine haplotype as reported in chapter 2. As indicated there, each of these volcanoes are populated predominantly by different tortoise species (*C. guntheri* in Alcedo and *C. becki* in Wolf) resulting from different colonization sources (Santiago Island, and Santa Cruz islands, respectively) (Poulakakis *et al.* 2012).

However, mitochondrial lineages of Alcedo tortoises also circulate in Wolf suggesting natural or human induced movement of tortoises between these locations (Caccone *et al.* 1999). Movement of tick infested tortoises between volcanoes could have caused the introduction to Wolf. However there is no evidence of migration of tortoises to Alcedo. Alternativelly, the movement of *A. usingeri* between volcanoes might have been also mediated by introduced ungulates such as donkey and goats which roamed Alcedo and Wolf until 2006, when they were eradicated (Carrion *et al.* 2007; Rivera-Parra *et al.* 2012). It has been shown that immature stages of *Amblyomma* species of reptiles are prone to infest mammals; one example is the tick *Amblyomma marmoreum* introduced to United States and is found on three species of exotic captive tortoises and in dogs (Allan *et al.* 1998). Thus, the translocation of *A. usingeri* by the introduced animals in the Galápagos is a reasonable possibility.

The percentage of genetic differentiation between tick species on Santiago and Pinzon (4.82% in the COI marker and 9.48 for CR) (classified morphologically by Keiran *et al*. as belonging to a single species, *A. pilosum,* is high in comparison with other studies of reptile ticks. For example, within species COI differentiation of 0.6-1.9% is reported for *A. sphenodonti* (an ectoparasite of the tuatara *Sphenodon punctatus*), for populations distributed across different New Zealand islands (Miller *et al.* 2007). In *Rhipicephalus sanguineus* (an ectoparasite of mammals) populations from southern Italy, central Spain and eastern Greece differentiation ranged from 0.4-3.5% (Dantas-Torres *et al.* 2013). Hebert *et al.* (2003) found that a difference of >3% in COI was sufficient for diagnosis of species of lepidopterans. Given this percentage of especies diagnosis and the geographical isolation of the ticks from Santiago and Pinzon they classification as separate species needs to be further investigated.

One of the central aims of this research was to investigate if the evolution of tortoise ticks follows the evolution of their host in phylogenetic terms. Tortoises from Santiago Island are thought to have colonized volcano Wolf, while tortoises from other islands, presumably from Santa Cruz, are thought to have colonized Alcedo and the other southern volcanoes of Isabela (Caccone *et al.* 2002; Poulakakis *et al.* 2012). The phylogenetic and network analyses presented here suggest independent evolutionary histories for ticks and tortoises. For Wolf volcano in particular, there is no evidence of a distinct haplotype clade originating from Santiago. This conclusion

should be robust inspite of the ambiguity of Isabela haplotype topologies in the Bayesian phylogenetic trees for COI and combined COI/CR datasets.

With the current data the phylogeograpic history of the Galápagos tortoise ticks is a conundrum. The lack of congruence among the ticks and tortoise colonization history suggest two scenarios: 1) *A. usingeri* descended from an extinct Galápagos tortoise tick haplotype of Santiago or other older Galápagos island or 2) that they have been transferred from another reptile host. The extinction of ticks infesting tortoises in Galápagos is reasonable as some tortoise species have already become extinct, and ticks reported before in some tortoise populations had not been observed again. For example, Keirans *et al*. (1973) analysed *Amblyomma* ticks labelled by Craig McFarland as collected of tortoises from Cerro Azul volcano and Santa Cruz. *Amblyomma* ticks were recorded in tortoise populations from Cerro Azul in 1905-1906 during the expedition of the California Academy of Sciences lead by John Van Denburgh where he wrote: "at Tagus and Iguana coves I noticed that the tortoises were covered with ticks all over the skin and along the cracks between the plates of the plastron" (Van Denburgh 1914). Iguana Coves corresponds to Cerro Azul Volcano where the ectoparasite was not found in two sampling expedition (2005 and 2014) reported in this thesis. Likewise no ticks were observed in Santa Cruz or in tortoise populations other than from Isabela's Wolf and Alcedo Volcanoes, Santiago and Pinzón. Except from Keiran *et al*'s. notes, there is no former or new evidence of the circulation of tortoise ticks in Santa Cruz. Thus, unless convincing data is found the presence of ticks in this location should be considered questionable. Nevertheless, and given the historical records, *Amblyomma* ticks of tortoises might have gone extinct at least in one tortoise population, this from Cerro Azul. The specimes analysed by Keirans *et al.* are deposited in the Rocky Mountain Laboratory Hamilton, Montana U.S. Genetic analysis of this samples might help to further undertand the relationship of Galápagos tortoise ticks.

The second scenario mentioned above related to the transfer of ticks to Galápagos tortoises from another Galápagos reptile host would be also possible. This scenario finds support in the current data, especially with the clustering of 3 tick haplotypes collected from Galápagos land iguana with tick haplotypes collected from Galápagos tortoises. This result suggest that ticks found in Galápagos tortoises are not host

specific and host switches from tortoises to land iguana or vice versa would be common. Host switching would be likely in Isabela, especially in Wolf Volcano, where these hosts are syntopic. This finding is further supported by the data of Keiran *et al*. (1973) who identified ticks collected from both reptile hosts as *A. usingeri*. In Galápagos, iguanas are represented by three species of land iguana (genus *Conolophus*) and a species of marine iguana (genus *Amblyrhynchus*) (Rassmann 1997). One species on land iguana, the pink iguana (*Conolophus marthae*), inhabit only in Wolf Volcano, the restriction of this specie to Isabela coincides with the restriction of tortoise ticks in this island.

Historically, land iguanas also coexisted with Galápagos tortoises in Santiago Island, making possible the transfer of ticks between these hosts if they shared habitats. Land iguanas were observed in this island by Charles Darwin in 1835. However, no live iguanas were found in Santiago during the expedition of the California Academy of Science in 1905-1906 (Snell *et al.* 1984). The cause of extinction of land iguanas in this island is unknown but it is attributed to predation by feral dogs (Snell *et al.* 1984). The circulation of ticks in tortoises from Pinzón is more difficult to explain as current or ancient occurrence of land iguanas has not been reported for this island (Steadman *et al.* 1991). An unexpected result was found however, for this location where the tortoise tick haplotypes clustered with two tick haplotypes collected from land iguana from Wolf volcano. A new microscope analysis of these two ticks of Wolf confirmed their consistent morphology with *A. pillosum*. This finding highlights two facts; first, that the transmission of ticks among these reptiles ―even without being syntopic― is possible, and second, that given the geographical isolation among these ticks/tortoise populations it could represent a human mediated translocation from Pinzón to Wolf. It is known that in the 19th century buccaneers and whalers used tortoise as source of meat and translocated individuals between islands (Townsend 1925). Evidence of translocation was reported by Caccone *et al.* (2002) through the finding on Wolf Volcano of tortoise haplotypes from distant islands such as Española and San Cristobal and Floreana. Introduced ungulates have been also transported among islands (Carrion *et al.* 2007) and would have been another source for translocation of ticks if they are able to infect them.

The implications of other Galápagos reptiles as host of the ticks found in Galápagos tortoises cannot be disregarded. However, an analysis of two ticks from marine iguana (*Amblyrhynchus subcristatus*) shows they were unrelated to the haplotypes found in Galápagos tortoises and land iguana and belong to a different cluster of *Amblyomma* ticks (*A. sabanerae*). *Amblyrhynchus* is a monospecific ancient lineage forming an archipelago-endemic clade with three species of Galápagos land iguanas (Rassmann *et al.* 2004). It would be expected marine and land iguana share phylogenetically related ticks but the current data reject this expectation. Until now two *Amblyomma* species have been described infesting marine iguana named *A. darwini* and *A. williamsii (Bequaert 1932).* The arrival of Galápagos iguanas to the Galápagos Islands with more than one species of ticks, as implied by the current data, would be possible, and needs to be further studied. The sharing of tortoise ticks with the Galápagos lava lizard (*M. tropiduridae*) would be also possible especially in Pinzón where both reptiles share habitat. Lava lizards also carry *Amblyomma* ticks and the species *A. boulengeri* has been described from specimens of Santa Cruz Island (Keirans *et al.* 1973b). Dias (1958), synonymised *A. boulengeri* with *A. pilosum* but it was later given its own valid taxon (Keirans *et al.* 1973b). This suggests that the two species are morphologically similar and would be adapted to infest both hosts.

The divergence time estimated here for the Galápagos tortoise tick is inconsistent with the subaerial age of some of the Galápagos islands. According to the COI analysis the first diversification involved the tick lineages of Isabela and those inhabiting Pinzon and Santiago. It occurred ~ 8.2-8,6 mya while the oldest extant islands of San Cristobal and Española are thought to have emerged between 3-4 mya (Geist *et al.* 2014). The next split was between the tortoise ticks of Santiago and Pinzón occurring ~ 3.2-3.6 mya, it also predates the formation of the current observed islands and the diversification of tortoises into the archipelago estimated to have occurred 1.26 mya (Caccone *et al.* 1999). The most recent divergence was between ticks of Alcedo and Wolf; it occurred 1.5 mya before the formation of Isabela (0.35-0.53 Mya) and their colonization by any reptile host (Poulakakis *et al.* 2012). The difference between the divergence times of Galápagos tortoises and their ticks in younger island of the Galápagos support the claim that ticks evolved independently from this host. The split of ticks agrees with the split of tortoise and also with an initial estimated of the split

of iguanas from their continental ancestors, calculated at 6-12 Mya and 10-20 Mya, respectively (Caccone *et al.* 1999; Tzika *et al.* 2008).

It has been suggested that the diversification of both Galápagos tortoises and Galápagos iguanas from their closest continental ancestors might have occurred on the mainland and prior to their colonization of the Galápagos. Alternatively it might have occurred in now submerged island of the archipelago located at the east of San Cristóbal and Española. The estimated age of this now drowned island is 14 million years and is thought it was available for colonization 9 mya (Caccone *et al.* 1999; Tzika *et al.* 2008). Either of these evolution histories could be also true for the ticks analysed here. However, the initial estimated of the split of Galápagos iguanas from the mainland ancestor has been challenged by markers of nuclear DNA (ncDNA) which yield a deepest diversification estimated of 4.5 Mya (MacLeod *et al.* 2015). This new estimated is in agreement with the origin of the Galápagos Islands and the estimates of divergence of other Galápagos taxa including tortoises, lava lizard and Darwin Finches. Nevertheless, other Galápagos species including Galápagos leaf-toed geckos are also thought to be older than the islands in the archipelago having diverged early as 13.2 mya (Torres-Carvajal *et al.* 2016).

Given the discrepancies found in Galápagos iguana between studies using mtDNA and nDNA it is worth to note that inferences obtained from a single kind of marker should be interpreted with caution. A primary drawback of mtDNA is that the analysis corresponds to the study of a single locus (Godinho *et al.* 2008). A phylogenetic tree obtained from mtDNA may differ from the population or species tree as this markers can be affected by natural selection, introgression from one species into another or the stochastic variance that characterizes a sample of gene trees collected from a set of populations or species (Ballard and Whitlock 2004). In some species complex processes of population structure, can only be properly addressed through the use of several and complementary types of molecular markers as the combined use of mitochondrial and nuclear markers. Despite the limitations of mtDNA analysis, in the timesacale of interest this bias may not have a big impact.

In this study Galápagos tortoise ticks of Santiago Island displayed a low nucleotide and haplotype diversity. This result suggests that the population has suffered a

bottleneck and might be now expanding. This claim is further supported by the mismatch distribution analysis, where the observed values are similar to the expect values for an expanding populations. A bottleneck of this tick population might have been caused by individual or a combination of several factors. One of these might be reduction of the host population. By 1959 only 500 tortoises were living in this island as result of its human exploitation as source of food or oil, and the destruction of nest and predation of hatchings by introduced species (Van Denburg 1907). A reduction of tortoises might have also caused the loss of ticks and the availability of host to infect. Another important factor might have been the clearing of vegetation by introduced ungulates which could have altered the reproductive cycle of ticks (Rivera-Parra *et al*. 2012). Many islands have substantial changes to native vegetation, particularly San Cristobal, it would provide e a similar potential for extinction of ticks due decrease in tortoise population and changes to vegetation.

In this study the COI and/or CR markers fail to be amplified on some ticks. The higher percentage of unsuccessful amplification was found in tortoise ticks from Isabela Island. This could have result from poor preservation. These ticks were collected in 2005 and preserved in ethanol. The GGEPL laboratory that conducted this sampling closed in 2010 and since them the samples have been kept in the Galápagos but in suboptimal conditions. In addition tick DNA extraction is often problematic as it needs the accurate lysis of the hard chitinous exoskeleton and avoidance of potential co-extraction of whole blood DNA from hosts (Hill and Gutierrez 2003; Hubbard *et al.* 1995). Despite the improvement of the current methods of DNA extraction, the DNA extracted from these organisms appears to be highly susceptible to degradation (Ammazzalorso *et al.* 2015; Halos *et al.* 2004). This last factor might have caused the failed amplification of freshly collected tick (Santiago and Pizón). Failing of amplification of the CR marker in ticks collected from marine iguana might be also explained by a lack of primers specificity due to the high divergence of this mitochondrial region (Avise 2012).

In conclusion the analysis of the *Amblyomma* ticks found on the giant Galápagos tortoises has shown three distinctive populations on three islands; Isabela, Santiago and Pinzon. The Santiago and Pinzon populations were thought to be part of the same species but with a variation of 4.3% they could represent two separate species. There

is limited genetic differentiation between the tortoise ticks collected from Alcedo and Wolf implying that there is evidence of gene flow. Several posibilities including transfer for introduced animal may be responsible. The earliest predicted divergence time of the Galápagos ticks from their closest relative was before the Galapagos Islands were formed, suggesting the lineage split from closest ancestor occurred on the mainland. There is not phylogeographic concordance among ticks in their tortoise's hosts which suggest they might be arrived to Galápagos with other reptile species. The sharing of ticks between tortoise and land iguana revealed the ticks are nonspecific and are able to switch hosts. This initial study has raised interesting questions which can be persued further with more extensive sampling of ticks and adding more sequence data for nuclear and mitocondrial loci.

Chapter 4. Characterisation and biogeography of helminth communities of Galápagos tortoises *Chelonoidis* spp.

4.1. Introduction

The study of helminth communities is a subject of growing interest. A key aim with regard to their ecology has been to develop a conceptual framework about their hierarchical organization (Poulin 2004) and to formulate hypotheses about the processes that regulate their community composition and structure (Esch 1990). Studies in birds, fish and mammals suggest that migration, seasonal changes, diet, habitat preferences and host age, among others, are important factors influencing the composition of their helminth parasite communities. This information is lacking for most reptile species, for many of which, their helminthic fauna is unknown (Aho 1990; Bush 1990). Parasitism is a fundamental factor driving the dynamics of wild animal populations and might influence the structure and the diversity of ecological communities and ecosystems (Dobson and Hudson 1986; Scott 1988). Moreover, parasites can present serious threats to wildlife conservation, particularly when acting in conjunction with anthropogenic factors (Daszak *et al.* 2001). The impact of helminth infection in chelonians is not well understood (Chavarri *et al.* 2012). While numerous species have been described, relatively few are considered pathogens (Jacobson 2007; Rideout *et al.* 1987).

Most hosts harbour mixed infections of a variety of helminth species which are acquired over the course of the lifetime of the infected organisms (Petney and Andrews 1998). Co-infecting parasites interact directly or indirectly among each other and with their host (Cox 2011). These interactions, in turn, might influence the invasion of new parasite species, infection intensity and host susceptibility to new infections (Ezenwa *et al.* 2010; Knowles 2011; Telfer *et al.* 2010). Assessing parasite identity and collecting quantitative data on their prevalence (percentage of hosts infected in a population) and intensity of infection (mean number of parasites per infected host) is relevant for determining their distribution and community structure (Aho 1990). Understanding factors that influence the formation of parasite assemblages might provide information not just for host conservation but also for the conservation of potentially uniquely evolved “parasite-host” systems. One major

impediment for achieving this task is that quantitatively censusing parasite populations often requires the sacrifice of the host. Lethal sampling raises ethical and logistical considerations, especially in the case of threatened and rare species or species with low population sizes (Jorge *et al.* 2013). In addition, the aggregation of parasites in host populations requires the sampling of a large number of hosts (Mes 2003; Scott 1988).

Non-invasive analysis of immature helminths (eggs and larvae) in faecal samples is an alternative approach for performing these studies. Coprological analysis has been performed in wild reptile species (Jorge *et al.* 2013), including tortoises (Chavarri *et al.* 2012; Traversa *et al.* 2005), as in other charismatic vertebrates (Bertelsen *et al.* 2010; Lynsdale *et al.* 2015). A disadvantage, however, is that the analysis of parasites in faeces may not represent the true parasite intensity. The amount of eggs observed in these samples is affected by several factors, including diurnal fluctuation in parasites egg-laying, uneven distribution in the faecal pat and the halting of the worms' ovulation by the immunological system of the host. Another constraint is that the eggs of many species of nematodes are not distinguishable; morphologically similar eggs may belong to a mixture of species. Nonetheless, coprological analysis can provide useful data on the parasite taxa infecting a host species (MAFF 1986).

Helminth parasites of Galápagos tortoises are poorly characterised. A parasite baseline is relevant for conservation management and to explore the impact of parasitism on these hosts (Fournie *et al.* 2015). Furthermore, since the colonisation history of the Galápagos archipelago by tortoises is well understood (Poulakakis *et al.* 2012), Galápagos tortoises could form an interesting model system for understanding the formation of parasite communities. Although nematodes have been implicated as a contributory cause of mortality events in *C. porteri* on Santa Cruz Island (Butler 1996), at the time of writing, there are only three published studies concerning the helminths of *Chelonoidis* spp. These studies comprise i) the identification of five species of oxyurids from adult specimens isolated from a Galápagos tortoise which was held and died in the United States (Walton 1942); ii) the morphological identification of a larval nematode (*Atractis marquensi*) found in a necropsied tortoise in Santa Cruz Island (Bursey and Flanagan 2002); and iii) the coprological analysis of nematode eggs in three wild populations and from captive individuals of the three

breeding centres on the Islands. This last study included the molecular identification of two common nematode larvae (*Atractis* sp and *Labiduris* sp) found in Santa Cruz (Fournie *et al.* 2015).

In the coprological analysis performed by Fournie *et al*. (2015) in Galápagos tortoises is reported the presence of five nematode superfamilies: Strongyloidea, Trichinelloidea, Oxyuroidea, Ascaridoidea, and Cosmocercoidea. The prevalence and spatial distribution of the first four taxa varied among islands, suggesting the influence of potential evolutionary and ecological factors affecting their biogeography. In this chapter I build on the work of Fournie *et al*. (2015) by surveying the prevalence and abundance of nematode taxa in additional tortoise species/populations comprising *C. donfaustoi* (Santa Cruz east), *C. darwini* (Santiago), and *C. vicina* (Isabela-west Cerro Azul), and I examine the temporal variation in a population (*C. nigra*; Santa Cruz west) surveyed by both studies. I also investigate the helminth parasites of reintroduced captive bred tortoises on Española Island. I used McMaster and Baerman techniques (MAFF 1986) to quantify the prevalence and intensity infection of nematode eggs and larvae found in these tortoise species. In addition, I evaluate possible associations of prevalence and infection intensity with host sex and location.

4.2. Materials and methods

4.2.1. Sampling

Fresh tortoise faecal samples were collected between January and May of 2013 and 2014 under Galápagos National Park Directorate (GNPD) permit number PC-09-13. Samples were obtained from four wild populations (Santa Cruz west "El Chato", Santa Cruz east "Cerro Fatal", Santiago, and Isabela south west "Cerro Azul Volcano"), from repatriated individuals on Española (born and raised in captivity and released to the wild at an age of 2-3 years), and from captive specimens from the breeding centre on San Cristóbal (which was populated with wild-caught tortoises in 2003) (see Figure 1.1 for sampling locations). Sampling on San Cristóbal was only successful for captive tortoises in the breeding centre, since wild tortoises did not void faeces, possibly due to dryness of the habitat and scarcity of food at the time of the collecting expeditions.

Sampling involved placing the tortoises into dorsal recumbancy and stimulating their cloaca with an index finger while wearing disposable latex gloves. When faeces were voided, the glove was inverted to contain them (MAFF 1986). A code was recorded for each sample collected in this way, as well as the tortoise's age class (adult, juvenile), sex (male, female – for adult animals) and, if present, the identification number (microchip or iron brand on shell) previously given by the GNPD. Each sampled tortoise was marked using temporary paint (lasting one week) to prevent repeated collection. Fresh stools were also collected when found on the ground and an individual code was assigned to them. A summary of the samples collected is shown in Table 4.1.

In Santa Cruz Island, the samples were taken directly to the laboratory of the Agency for the Control and Regulation of the Galápagos Biosecurity. In the other islands, they were kept chilled (on wet ice in an insulated container) for three to five days before being taken to the laboratory. On the same day of sampling, 15 g of faeces were used for larvae isolation (see below). The remaining sample was placed at ~4°C until being evaluated for nematode eggs. In the laboratory the samples were stored in a fridge. Nematode egg counts were conducted within five days of sample collection.

4.2.2. Isolation and characterisation of helminth larvae and eggs

Faecal samples were examined for nematode larvae using a modification of the Baermann technique (MAFF 1986). This involved wrapping 15 g of faeces in cotton gauze and placing it into a funnel with a sealed stem. The funnel was then filled with distilled water at 37° C and allowed to stand for 4 hours. Then, the faecal matter and half of the volume of water was discarded; the stem of the funnel was opened and the remaining water collected in a Petri dish. Presence of nematode larvae in the Petri dish was examined by eye in the field or under a dissecting microscope in the laboratory. All the nematode larvae found were washed with distilled water, counted and preserved in 70% ethanol.

Faeces were examined for helminth eggs using a modified McMaster procedure using saturated NaCl solution, specific gravity 1.23. Briefly, 4.5 g of faeces obtained from the core of the faecal sample were diluted in 40.5 ml of saline solution, shaken until all the faecal matter was disassociated, sieved through mesh with an aperture of 0.15

mm, collected in a bowl and stirred. Three ml were withdrawn with a Pasteur pipette, and used to fill a McMaster counting chamber. The filled McMaster chamber was left to stand for 5 minutes, then it was analysed under the microscope at 100x magnification.

Eggs with similar morphologies were considered as belonging to the same taxon and were counted and multiplied by 10 in order to give the number of eggs per gram of faeces (MAFF 1986). Eggs were identified to superfamily level according to the reptile helminth eggs described in (Jacobson (2007)). Estimation of the sample size required to detect at least one tortoise infected with nematodes at a prevalence of infection of 5%, 10% and 20% was done according to (Digiacomo and Koepsell 1986) and (Smith 2015) as described in section 2.2.2.

Eggs were then processed for later metabarcoding analysis (Chapter 5). Nematode larvae and eggs were transported to the United Kingdom (export permits: DPNG 84-2013, 064-2014, and CITES 0981315, 0981325 and import permits: DEFRA TARP/2013/213 and CITES 516095/01 and 522826/01).

Eggs were classified to the superfamily level based on morphology. For each group the mean of length and width, minimum and maximum values, and ratios were reported. Fournie *et al*. (2015) previously documented the presence of egg morphologies classed as ‘Large’ and ‘Small’ strongyle. In order to assess quantitatively whether egg mixtures contained eggs drawn from different size distributions, the length and width (in µm) of a subsample of strongyle eggs were measured using the Axioscope at 400x magnification as described above. Each measure was taken three times using the software Open Lab 4.0.2 and photomicrographs were taken for most of the eggs measured.

A subset of 50 individual larvae isolated from different tortoises and from different populations (10 Santa Cruz west, 10 Santa Cruz east, 10 Isabela, 10 Santiago and 10 Española) was chosen for morphological analysis. Each larva was observed at 200x magnification using an Axioscope microscope (Carl Zeiss EL-Elnsatz 451889) and photographed using a Coolpix camera attached to it, complemented with the software Open Lab 4.0.2 (Improvision 2001). Then, the larvae were grouped according to

similar morphological characteristics. A representative of each group (n=5) was cleared using lactoglycerol (equal parts of glycerol and lactic acid) in order to observe their internal structures (Hooper 2005). They, along with the photomicrographs of the larvae, were sent to an expert in parasitic worm identification, Dr Lynda Gibbons at the Royal Veterinary College, London, for morphological identification. Only a subset of larvae was sent for morphological analysis to allow the remainder to be used for genetic analysis.

Table 4.1. Faecal samples collected from Galápagos tortoises. M=male, F=female, J=juvenile.

Island, Species	Faecal samples collected directly from tortoises (M/F/J)	Faecal samples collected from the ground	Total number of faecal samples collected	Date of sampling
Santa Cruz (W), *C. porteri*	49 (24/16/9)	11	60	29 Mar-10 Apr 2013
Santa Cruz (E), *C. donfaustoi*	16 (12/3/1)	5	21	13-15 May 2013
Santiago, C. *darwini*	42 (41/1/0)	3	45	17-21 Jan 2014
Isabela south west *C. vicina*	49 (30 /15/4)	0	49	10-12 Feb 2014
Española *C. hoodensis*	21 (12/3/6)	15	36	2-28 May 2013
S. Cristóbal breeding centre *C. chatamensis*	23 (21/2/0)	2	25	10-14 Mar 2014

4.2.3. Statistical analyses

Statistical analyses of prevalence and abundance were performed independently for nematode larvae and nematode eggs across *Chelonoidis* spp. populations for each nematode family. All estimations were done using R either in R studio or in the software Quantitative Parasitology (QP 0.1) available on-line (R Development Core Team 2016; Rozsa *et al.* 2000). Descriptive statistics included the prevalence (proportion of tortoise faecal samples containing helminth larvae or helminth eggs), median intensity (median number of larvae or helminth eggs found in the faecal samples-the zeros of uninfected host are excluded), mean intensity (mean number of larvae or helminth eggs found in the faecal samples-the zeros of uninfected host are excluded), and variance (Rozsa *et al.* 2000). The 95% confidence interval (CI) for each prevalence was calculated using the exact Sterne method (Reiczigel 2003), whereas the CIs for the remaining measures were estimated by bootstrap confidence interval (BCa) (Bradley Efron 1994). A measure of aggregation (aggregation index) for each type of helminth larvae and helminth eggs was obtained using the ratio of the variance to the mean number of parasites per host (Shaw *et al.* 1998). Egg count distributions were visualised using frequency distribution histograms (transformed to proportions).

Logistic regression was used for the comparison of parasite prevalences (Ziadinov *et al.* 2010). Generalized Linear Models (GLM) (Wilson and Grenfell 1997) and zero inflated (ZI) models (Chipeta *et al.* 2015) were used for the comparison of parasite abundances (mean intensity) and for testing island and host sex as factors influencing this parameter. They were performed under the assumption of negative binomial distribution. Santa Cruz west was selected as the reference population. Differences between this reference population and the others were assessed using the Wald test implemented in each of the models. The null hypothesis of no significant differences between populations was accepted at *p-values*>0.05. Selection between GLM and ZI as the best model was done in reference to the model yielding the lower residual deviance and Akaike Information Criterion (AIC) values (Crawley 2013) and according to the Vuong closeness test. The statistic tests the null hypothesis that the two models are equally close to the true data generating process, against the alternative that one model is closer (Vuong 1989).

The proportion of faecal samples containing nematode eggs found in this study was compared with the proportion found by Fournie *et al*. (2015). A direct comparison was only possible for Santa Cruz west as this tortoise population was the only one sampled in both studies. An indirect comparison was done for southern Isabela populations - Cerro Azul in this thesis and Roca Union and San Pedro in Fournie *et al*. (2015), which are all located on the southern coast of Isabela (see Figure 1.1), and all are comprised of the same species (*C. vicina*).

Fournie *et al*. (2015) documented the presence of egg morphologies classed as 'Large' and 'Small' strongyle on Isabela Island, but did so without the ability to measure eggs accurately. Measurements of eggs were taken in order to explore the presence of different modal classes. Length and width of eggs assigned to different morphological types were explored through a boxplot for identification of outliers. If outliers were found, their picture was re-analysed and eliminated if they were not identifiable as nematode eggs. Measured eggs were compared through their frequency distribution. Length, width and length:width ratio were tested for conformity to a normal distribution using a Shapiro-Wilk test. An analysis of variance was done using the Kruskal-Wallis test and a Spearman correlation analysis was performed between the length and width of eggs.

4.2.4. Molecular identification and phylogeny of nematode larvae

Individual ethanol-fixed larvae were washed three times with saline solution and then crushed with a sterilised pestle in a separate microfuge tube. DNA was isolated using the InvitrogenTM Charge Switch® gDNA Mini Tissue Kit, following the manufacturer's instructions. Larval identification at the molecular level was attempted using primers targeting three different genes: 18S ribosomal DNA (18S rDNA), the internal transcript spacer (ITS1) and mitochondrial Cytochrome Oxidase I (COI) (Table 4.2). The PCR reactions were performed as described in references describing the use of each primer set (references listed in Table 4.2). PCR products were purified using the ultra-clean Gel spin kit (Mobio cat No 12400) and submitted to a commercial company (Beckman Coulter Genomic, UK) for sequencing in both directions. Sequence data were assessed for quality, trimmed to remove poor quality 5' and 3' sequence and, if required, any ambiguous base calling was corrected manually using

BioEdit version 7.00 (Hall 1999). Edited sequences were compared to those available in GenBank using BLASTn (Altschul *et al*., 1997).

Phylogenetic analysis was carried out with the datasets shown in Table 4.3. It involved the the two most similar matches found in GenBank and 72 nematode species representing different orders from the class Chromadorea. This class includes the majority of gastrointestinal nematodes that parasitise vertebrate species (Holterman *et al.* 2006; Nadler 2016). Six sequences from non-parasitic nematodes were used as an outgroup (Meldal *et al.* 2007). All sequences were retrieved using MEGA version 7.0 (Tamura *et al.* 2013) and aligned using MUSCLE (Edgar 2004). The best fit sequence evolution model was evaluated using ModelTest (Posada and Buckley 2004) implemented in MEGA 7.0. On the basis of AIC values, the Tamura 3-parameter + Gamma Distributed model (T92+G), and the Tamura 3-parameter + Gamma Distributed with Invariant Sites (T92+G+I) were identified as the best fits. For both datasets, phylogenetic relationships between haplotypes were inferred using MrBayes v.3.1.1 (Huelsenbeck and Ronquist 2001). Multiple simulations were run for 10 million generations with the first 500,000 trees discarded as burn-in period after confirming the convergence of chains. Trees were sampled every 1,000 generations and a 50% consensus tree was constructed from the results and. Each analysis was repeated three times. The resulting consensus tree was visualised and edited using the online software iTol (Letunic and Bork 2016).

Table 4.2. List of primers used for amplification of nematode DNA.

TARGET GENE	PRIMER NAME	SEQUENCE 5'-3'	REFERENCE
18S rDNA	NEM18_S_F	CGC GAA TRG CTC ATT ACA ACA GC	(Floyd *et al.* 2005)
	NEM18_S_R	GGG CGG TAT CTG ATC GCC	
	NEMF1	CGC AAA TTA CCC ACT CTC	(Waite *et al.* 2003)
	S3	AGT CAA ATT AAG CCG CAG	
	SSUF04	GCT TGT CTC AAA GAT TAA GCC	(Blaxter *et al.* 1998)
	SSUR26	CAT TCT TGG CAA ATG CTT TCG	
	SSUF3M	GCT TGT CTC AAA GAT TAA GCC ATG C	(Blaxter 2004)
	SSUR9	AGC TGG AAT TAC CGC GGC TG	
	SSUF3M	GCT TGT CTC AAA GAT TAA GCC ATGC	
	SSUR26	CAT TCT TGG CAA ATG CTT TCG	
	SSUF02	GGA AGG GCA CCA CCA GGA GTG G	
	SSUR82	TGA TCC TTC TGC AGG TTC ACC TAC	
	SSUF4	GCT TGT CTC AAA GAT TAA GCC	
	SSUR 22	GCC TGC TGC CTT CC TTG GA	
ITS1	RDNA2	TTG ATT ACGTCC CTGCCCTTT	(Powers *et al.* 1997)
	RDNA2 1.58	ACG AGC CGA GTG ATC CAC CG	
	RDNA2	TTG ATT ACG TCC CTG CCC TTT	
	RDNA 1.44	GTA GGT GAA CCT GCA GAT GGA	
COI	JB2S3	ATG TTTT GAT TTT ACC WGS WTT TGG	(Derycke *et al.* 2010)
	JB5R	AGC ACC TAA ACT TAA AAC ATA RTG RAA RTG	
	COI JB3	TTT TTT GGG CAT CCT GAG GTT TAT	
	COIJB4	TAA AGA AAG AAC ATA ATG AAA ATG	
	JB3	ATG TTTT GAT TTT ACC WGS WTT TGG	
	JB5	AGC ACC TAA ACT TAA AAC ATA ATG AAA ATG	

Table 4.3. Sequences of 18S rDNA used for the phylogenetic reconstruction of the phylum Nematoda and of the order Ascaridida. Species without a stated reference were obtained through a direct search into the National Center for Biotechnology Information (NCBI) database. .

Nematode species	Accesion number	Order
Closest macht in GenBank		
Atractis sp.	KT364749	Ascaridida
Labiduris sp.	KT364750	Ascaridida
Reference species		
Anisakis pegreffii	EF180082	Ascaridida
Anisakis sp.	U94365	Ascaridida
Anisakis sp.	U81575	Ascaridida
Ascaridia galli	EF180058	Ascaridida
Ascaris lumbricoides	U94366	Ascaridida
Ascaris suum	U94367	Ascaridida
Ascarophis arctica	DQ094172	Spirurida
Aspidodera sp.	EF180070	Ascaridida
Baylisascaris procyonis	U94368	Ascaridida
Baylisascaris transfuga	U94369	Ascaridida
Brumptaemilius justini	AF036589	Rhigonematida
Camallanus cotti	EF180071	Spirurida
Camallanus lacustris	DQ442663	Spirurida
Camallanus oxycephalus	DQ503463	Spirurida
Camallanus sp.	DQ442664	Spirurida
Contracaecum eudyptulae	EF180072	Ascaridida
Contracaecum microcephalum	AY702702	Ascaridida
Contracaecum multipapillatum	U94370	Ascaridida
Cruzia americana	U94371	Ascaridida
Dentostomella sp.	AF036590	Oxyurida
Dujardinascaris waltoni	EF180081	Ascaridida
Goezia pelagia	U94372	Ascaridida
Heterakis gallinarum	DQ503462	Ascaridida
Heterakis sp.	AF083003	Ascaridida
Heterocheilus tunicatus	U94373	Ascaridida
Hysterothylacium fortalezae	U94374	Ascaridida
Hysterothylacium pelagicum	U94375	Ascaridida
Hysterothylacium reliquens	U94376	Ascaridida
Iheringascaris inquies	U94377	Ascaridida
Leidynema portentosae	EF180073	Oxyurida
Nematodirus battus	U01230	Strongylida
Nemhelix bakeri	DQ118537	Ascaridida
Oxyuris equi	EF180062	Oxyurida
Parascaris equorum	U94378	Ascaridida
Paraspidodera sp.	AF083005	Ascaridida
Passalurus sp.	EF180061	Oxyurida
Physaloptera alata	AY702703	Spirurida
Physaloptera sp.	EF180065	Spirurida
Porrocaecum depressum	U94379	Ascaridida

Table 4.3. (cont.)

Nematode species	Accesion number	Order
Porrocaecum streperae	EF180074	Ascaridida
Procamallanus pacificus	DQ442665	Spirurida
Procamallanus pintoi	DQ442666	Spirurida
Procamallanus rebecae	DQ442667	Spirurida
Protozoophaga obesa	EF180075	Oxyurida
Pseudoterranova decipiens	U94380	Ascaridida
Raillietnema sp.	DQ503461	Ascaridida
Raphidascaris acus	DQ503460	Ascaridida
Rhabdochona denudata	DQ442659	Spirurida
Rhigonema thysanophora	EF180067	Rhigonematida
Rondonia rondoni	DQ442679	Ascaridida
Skrjabinema sp.	EF180060	Oxyurida
Spinitectus carolini	DQ503464	Spirurida
Spirocama llanusistiblenni	EF180076	Spirurida
Sulcascaris sulcata	EF180080	Ascaridida
Terranova caballeroi	U94381	Ascaridida
Terranova scoliodontis	DQ442661	Ascaridida
Thelastoma krausi	EF180068	Oxyurida
Toxascaris leonina	U94383	Ascaridida
Toxocara canis	U94382	Ascaridida
Toxocara cati	EF180059	Ascaridida
Toxocara vitulorum	EF180078	Ascaridida
Turgida torresi	EF180069	Spirurida
Turgida turgida	DQ503459	Spirurida
Wellcomia siamensis	EF180079	Oxyurida
Wellcomia sp.	EF180066	Oxyurida
Outgroup species		
Aduncospiculum halicti	U61759	Diplogasterida
Caenorhabditis elegans	X03680	Rhabditida
Plectus aquatilis	AF036602	Araeolaimida
Pristionchus pacificus	AF083010	Diplogasterida
Rhabditis myriophila	U13936	Rhabditida
Tylocephalus auriculatus	AF202155	Araeolaimida

4.3. Results

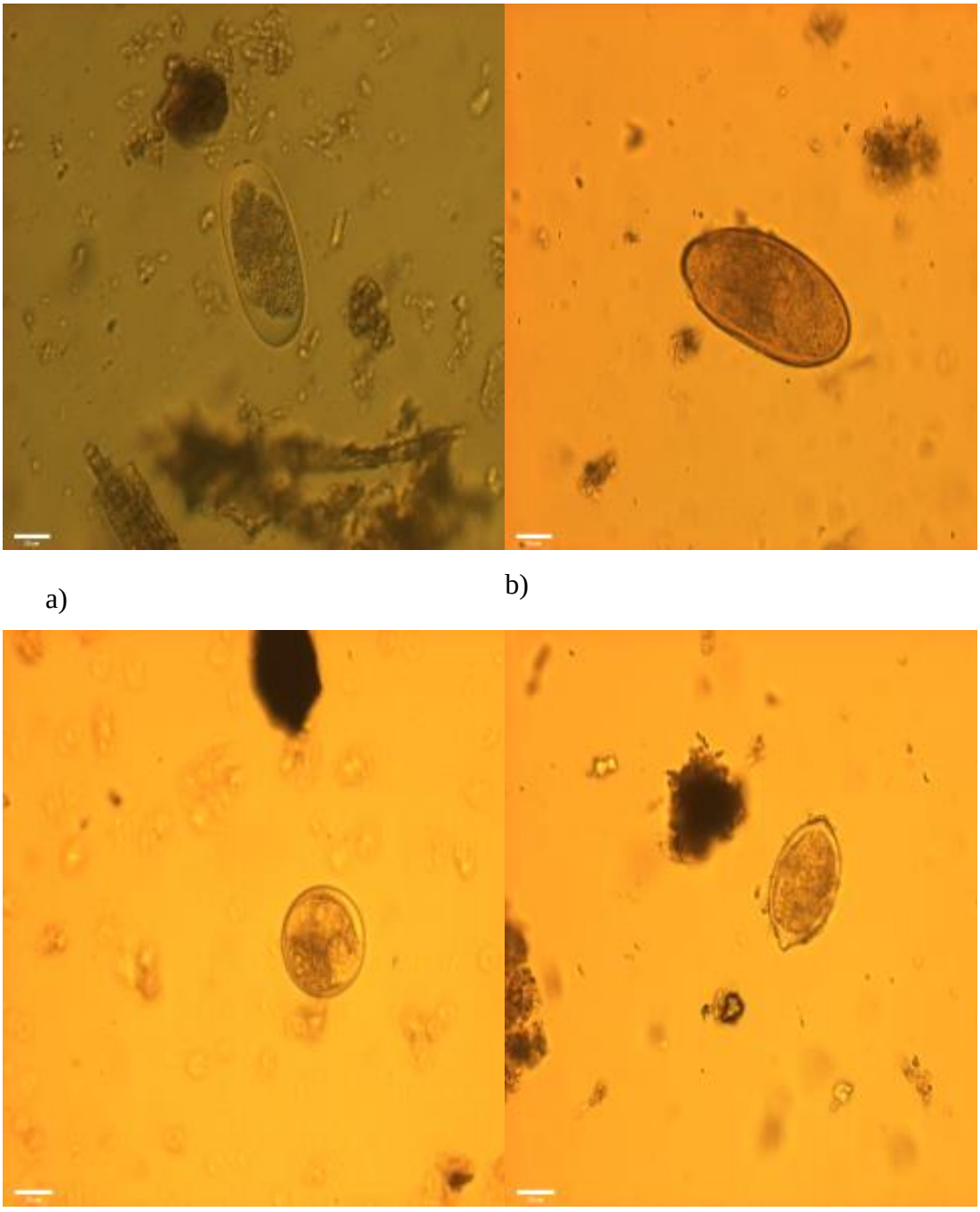
4.3.1. Characterisation and distribution of helminth eggs

A combination of McMaster and direct smear methods detected nematode eggs in all wild tortoise populations and in repatriated individuals on Española. No nematode eggs were observed in the captive tortoises on San Cristóbal. Four types of nematode egg, assigned to the superfamilies Strongyloidea, Oxyuroidea, Ascaridoidea and Trichinelloidea, were found at the metapopulation level (Fig. 4.1a - 4.1d). Discrimination between different nematode taxa and even between different Phyla from egg data is challenging. Oxyurids eggs are difficult to differentiate from ciliate protozoan cysts. So the current assignment should be interpreted with caution. The difficulty of identification apply also for Strongyloidea as the eggs of different superfamilies into the Order Strongylida are very similar. Following Fournie at (2015) however, I will consider putative oxyurid eggs as oxyurids and strongylids as Strongyloidea. On this basis, strongyle eggs were found in all the populations examined, while eggs from each of the other families were absent from at least one population. Specifically, trichurid eggs were absent from Santa Cruz east; oxyurid and ascarid eggs were absent from Isabela-Cerro Azul; and ascarid eggs were absent from Santa Cruz east and Española. The complement of nematode superfamilies observed here at the metapopulation level was the same as that reported by Fournie *et al*. (2015).

The prevalence as proportion of tortoise faecal samples containing each of the different egg types is shown in Table 4.4. Strongyle were the most commonly observed. The proportion of faecal samples containing strongyle eggs ranged from 0.25 (CI: 0.13-0.42) in Española to 0.77 (0.63-0.86) in Santa Cruz west. The proportion for oxyurid eggs ranged from 0.22 (CI: 0.09-0.50) in Santa Cruz east to 0.45 (CI: 0.28-0.65) in Santiago. Estimations of the sample sizes required to detect at least one faecal sample containing a particular type of nematode egg at proportion of infection of 0.05, 0.10% and 0.20% with a confidence interval of 95% are shown in Table 4.5. The number of samples analysed from each tortoise population was enough to enable the detection of an infection percentage of 10% with a 95% confidence interval. Comparisons of the proportion of faecal samples containing each type of nematode egg for each tortoise

population sampled in this study and those reported by Fournie *et al*. (2015) are shown in Figs. 4.2 and 4.3. Across both studies, similar prevalence values were found for strongyle eggs, ascarid eggs and trichurid eggs. Oxyurid eggs, however, were observed to be at a higher prevalence in this study. In both cases strongyle eggs were the most prevalent and trichurid eggs were the least common.

Of the different egg types observed within the current study, only strongyle eggs were observed frequently enough to draw statistical conclusions (Table 4.6). Logistic regression showed that "island", but not "sex", has an effect in the prevalence of strongyle eggs (Wald test: deviance 29.06, df 4, *p-value* <0.01). Prevalence on Santiago and Española was significant lower in comparison with Santa Cruz west (Wald test, *p-values* 0.01 – ≤0.001), see Table 4.7. Strongyle egg counts showed an over-dispersed distribution skewed to the right (Fig. 4.4a - 4.4e). The ZI model, which had a better fit to the data than the GLM (Vuong test, *p-value* <0.001), showed an effect of "island" on strongyle egg abundance (Wald test: deviance 32.28, df 4, *p-value* <0.001); no effect was found for "sex" (see Table 4.8). The abundance of strongyle eggs was significantly lower on Santiago and Española than on Santa Cruz west (ZI model, Wald test: *p-values* 0.01- <0.001).



a) b)

c) d)

Figure 4.1. Photomicrograph of nematode eggs observed in faecal samples of *Chelonoidis* spp. at 400x a) Strongyle, b) Oxyurid, c) Ascarid, d) Trichurid. Bar = 20 µm.

Table 4.4. Proportions of tortoise faecal samples that were infected with different families of nematode eggs.

Nematode eggs	Island and tortoise species				
	Santa Cruz (W), *C. porteri*	Santa Cruz (E), *C. donfaustoi*	Santiago, *C. darwini*	Isabela, *C. vicina*	Española, *C. hoodensis*
Oxyurids (positive/N)	0.28 (17/60)	0.22 (5/21)	0.45 (20/45)	0.00 (0/49)	0.30 (11/36)
CI 95% (Sterne)	0.18-0.43	0.09- 0.50	0.28- 0.65	0.00-0.01	0.17-0.50
Trichurids (positive/N)	0.17 (10/60)	0.00 (0/21)	0.17 (8/45)	0.12 (6/49)	0.05 (2/36)
CI 95% (Sterne)	0.00-0.10	0.00- 0.21	0.01- 0.23	0.04-0.27	0.00- 0.10
Ascarids (positive/N)	0.11 (7/60)	0.00 (0/21)	0.14 (6/45)	0.00 (0/49)	0.00 (0/36)
CI 95% (Sterne)	0.05-0.23	0.00- 0.21	0.05-0.32	0.00-0.09	0.01- 0.20
Strongyles (positive/N)	0.77 (46/60)	0.56 (12/21)	0.49 (22/45)	0.70 (33/49)	0.25 (9/36)
CI 95% (Sterne)	0.63-0.86	0.33- 0.76	0.35-0.65	0.55-0.81	0.13-0.42

Table 4.5. Estimation of the sample size required for detecting at least one tortoise infected with nematodes at proportion of infection of 0.05, 0.10 and 0.20, at a confidence interval of 95%. Two methods were used: Di Giacomo *et al*., 1986/Smith, 2015.

Island, population and tortoise specie	Estimated population	Minimum sample size for detection at specified prevalence			Analysed by microscope
		0.05	0.010	0.020	
Santa Cruz (W) (*C. porteri*)	2000-3000	59/72-73	29/36	14/17	60
Santa Cruz (E) (*C. donfaustoi*)	20	59/20	29/17	14/12	21
Santiago (*C. Darwini*)	500-700	59/68-70	29/35	14/17	45
Isabela (*C. vicina)*	400-600	59/67-69	29/35	14/17	49
Española, *(C. hoodensis)*	1000-1500	59/71-72	29/36	14/17	36
San Cristóbal (*C. chatamensis*)	500-700	59/68-70	29/35	14/17	20

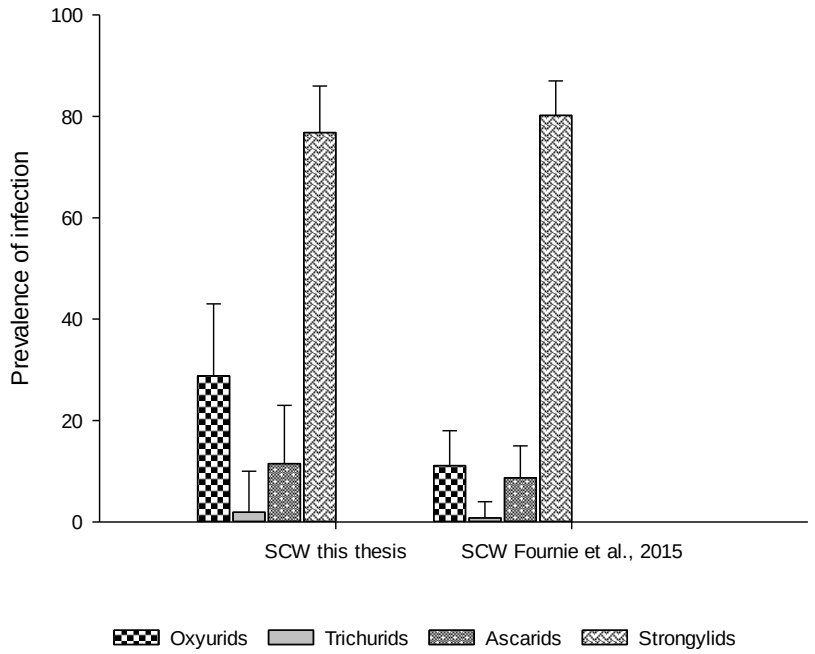


Figure 4.2. Comparison of the proportion of infection of nematode eggs found in this thesis and the study of Fournie *et al*. (2015) in Santa Cruz Island. SCW=Santa Cruz west. Error bars are showed for each parasite taxa.

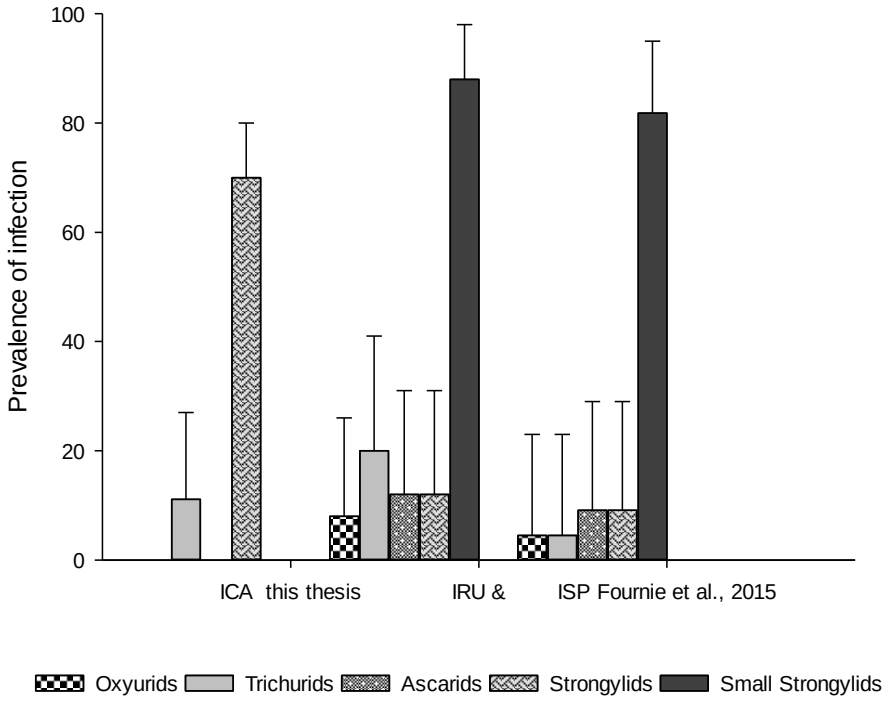


Figure 4.3. Comparison of the proportion of infection of nematode eggs found in this thesis and the study of Fournie *et al.* (2015) in Isabela Island. Different tortoise population from Isabela South were analysed in each study. ICA=Isabela "Cerro Azul, IRU=Isabela "Roca Unión, ISP=Isabela "San Pedro". Error bars are showed for each parasite taxa.

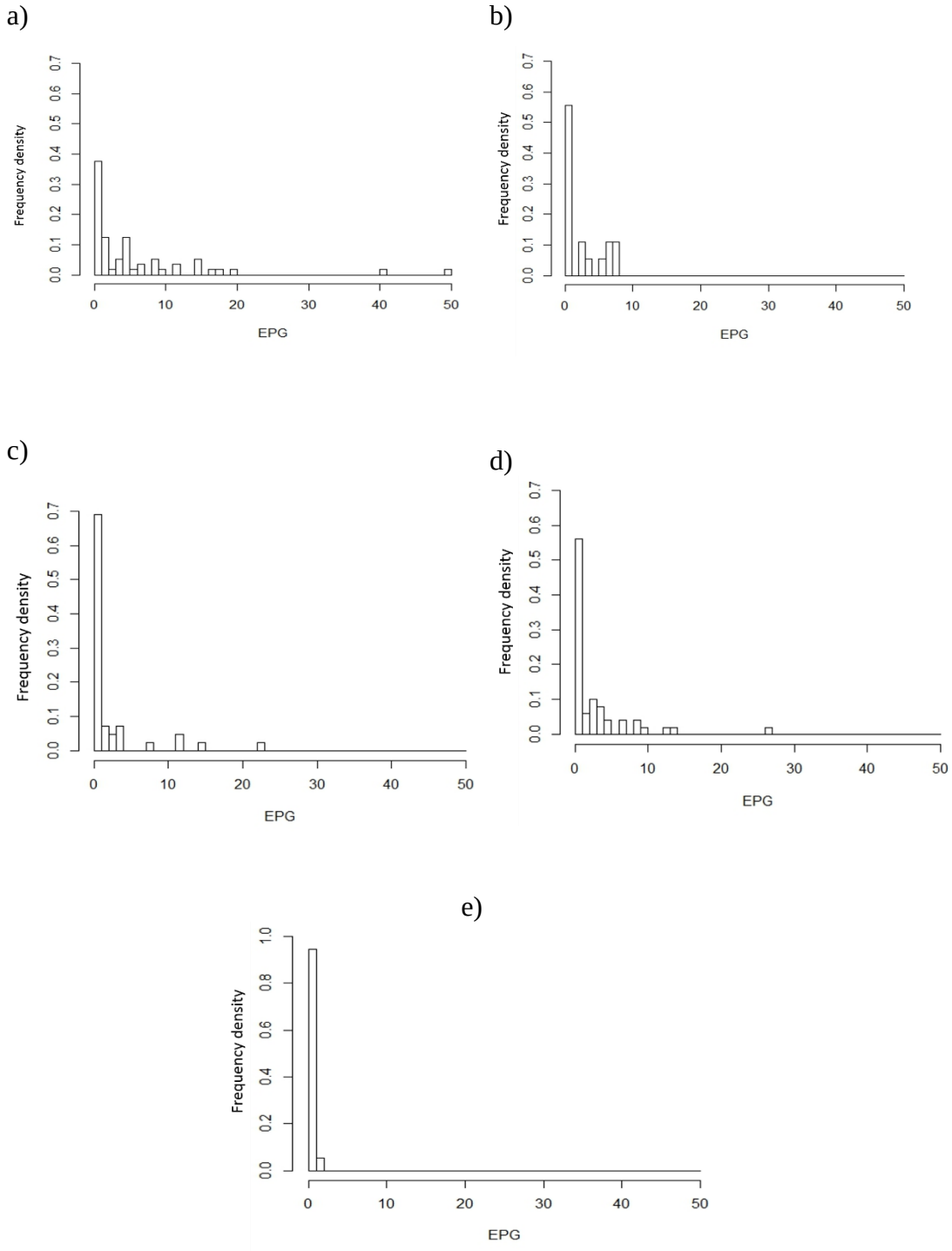


Figure 4.4. Frequency distribution (in proportions) of strongyle eggs from faecal samples of *Chelonoidis* spp. a) Santa Cruz west - *C. porteri*, b) Santa Cruz east - *C. donfaustoi,* c) Santiago - *C. darwini*, d) Isabela south west-Cerro Azul - *C. vicina*, e) Española- *C. hoodensis.* Frequency density represents the frequency divided by class interval (class interval was set at 1). EPG=Egg per gram of faeces. Note that the scale of the Y axis for Española differs from the others to accommodate the major percentage of tortoises where strongyle eggs were not detected.

Table 4.6. Statistics of Strongyle eggs quantified from different Galápagos tortoise populations, median and mean intensity, variance mean ratio and aggregation parameter, k. E=east, W=west.

Nematode eggs statistics	Island and tortoise species				
	Santa Cruz (W) *C. porteri*	Santa Cruz (E), *C. donfaustoi*	Santiago, *C. darwini*	Isabela *C. vicina*	Española, *C. hoodensis*
Median intensity	50	50	20	30	10
CI 95% Bca 2000 replications	30.0-70.0	10 - 80	10 - 40	10 - 40	10 - 20
Mean intensity	80	48.0	48.6	44.6	12.2
CI 95% Bca 2000 replications	56.7-122	29.6- 62	30-82.4	31.1-69.3	10 -14.4
Variance/mean	141.74	37.5	94.84	75.31	10.88
k (ML estimate):	0.36	NA	0.1543	NA	NA

Table 4.7. Comparison of prevalence of larvae infection among different tortoise populations using logistic linear regression. The asterisk indicates *p-value*s ≤ 0.05.

Islands	Coefficient Estimate	Std.Error	z value	Pr(>|z|)
(Intercept)	1.20	0.32	3.78	0.00 *
Santa Cruz East	-0.97	0.57	- 1.71	0.088
Santiago	-1.20	0.44	- 2.71	0.01 *
Isabela Cerro Azul	-0.35	0.44	-0.79	0.43
Española	-2.29	0.50	-4.61	4.12e-06 *

Table 4.8. Comparison of infection intensity among different tortoise populations using zero inflated model under the assumption of negative binomial distribution.

Islands	Coefficient Estimate	Std.Error	z value	Pr(>|z|)
Count model				
(Intercept)	4.37	0.15	30.13	< 2e-16 *
Santa Cruz east	-0.52	0.34	-1.54	0.12
Santiago	-0.51	0.25	-1.99	0.05 *
Isabela Cerro Azul	-0.59	0.22	-2.73	0.01*
Española	-1.94	0.36	-5.36	8.19e-08 *
Zero inflation model				
(Intercept)	-1.23	0.33	-3.78	0.00*
Santa Cruz east	0.98	0.58	1.67	0.09
Santiago	1.20	0.45	2.66	0.01*
Isabela Cerro Azul	0.33	0.46	0.72	0.47
Española	2.24	0.51	4.37	1.26e-05 *

4.3.2. Egg size morphology

Measurements of 103 strongyle eggs and 245 oxyurid eggs from the different tortoise populations were compared. Trichurid and ascarid eggs were not analysed in this way due to the low sample sizes. The length and width statistics (mean, min, max, standard deviation, and ratio of length to width) are summarized in Tables 4.9 and 4.10. For stronglyle eggs, the mean length ranged from 73.36 µm for Santiago (± SD 3.32) to 78.77 µm for Santa Cruz west (± SD 6.47), while the width mean ranged from 37.54 µm in Santiago (± SD 1.63) to 39.61 µm in Santa Cruz west (± SD 2.84). Almost all strongyle egg measurements fit a normal distribution, except for the width of eggs collected in Santa Cruz west (Shapiro-Wilk test, *p-value* < 0.01). For oxyurid eggs, their mean length ranged from 76.93 µm in Española (± SD 7.17) to 84.71 µm for Santiago (± SD 7.62), the width mean ranged 42.50 µm for Española (± SD 3.68) to 48.05 µm for Santa Cruz east (± SD 6.20). When looking at length:width ratios, the strongyle egg measurements fit to a normal distribution, but oxyurid eggs collected from Santa Cruz east did not fit to it (Shapiro-Wilk test, *p-value*s = < 0.01).

Frequency distributions of the length and width of strongyle and oxyurid eggs are shown in Figs. 4.5 and 4.6; outliers are shown in the supplementary material (S1). Strongyle egg length showed a potentially bimodal distribution for the tortoise population of Isabela (Fig. 4.5e), but a unimodal distribution for each of the other tortoise populations. Across all tortoise populations oxyurid eggs showed no detectable bimodal distribution neither for length or width. An analysis of variance showed no difference in the length of width of eggs measured from different islands. The individual measurements of length and width of both egg types are plotted in Figs. 4.7 and 4.8. The correlation between length and width of strongyle eggs from each of the tortoise populations analysed were low to moderate (r=0.20-0.44), the correlation values for oxyurid eggs were moderate to high (r=0.4-07).

Table 4.9. Morphometric data and analysis of Strongyle eggs observed in Galápagos tortoise faeces. Length and width are given in µm

Nematode egg measurements	Island and tortoise species		
	Santa Cruz (W), *C. porteri*	Santiago,*C. darwini*	Isabela, *C. vicina*
strongyle eggs (N)	66	11	23
Min length	63.00	68.00	69.00
Max length	95.00	79.00	85.00
mean	78.77	73.36	73.48
SD	6.47	3.32	3.86
p-value Shapiro-Wilk test	0.99	0.82	0.08
Min width	36.00	35.00	33.00
Max width	46.00	41.00	45.00
mean	39.61	37.54	38.30
SD	2.84	1.63	2.97
p-value Shapiro-Wilk test	< 0.01	0.29	0.75
Correlation	0.44	0.52	0.20
Min Ratio	1.432	1.65	1.63
Max Ratio	2.351	2.24	2.30
mean Ratio	1.99	1.92	1.94
SD ratio	0.16	0.18	0.17
p-value Shapiro-Wilk test	0.19	0.31	0.86

Table 4.10. Morphometric data and analysis of Oxyurid eggs observed in Galápagos tortoise faeces. Length and width are given in µm.

Nematode egg measurements	Island and tortoise species			
	Santa Cruz (W), *C. porteri*	Santa Cruz (E), *C. donfaustoi*	Santiago,. *C. darwini*	Española, *C. hoodensis*
Oxyurids eggs (N)	75	25	38	107
Min length	63.00	67.00	71.00	63.00
Max length	103.00	96.00	100.00	95.00
Mean	81.44	79.65	84.71	76.93
SD	8.71	7.31	7.62	7.17
p-value Shapiro-Wilk test	0.32	0.27	0.57	0.18
Min width	37.00	38.00	38.00	34.00
Max width	56.00	58.00	56.00	51.00
Mean	46.05	48.91	46.32	42.5
SD	4.67	5.03	4.15	3.68
p-value Shapiro-Wilk test	0.38	0.71	0.57	0.35
Correlation	0.63	0.41	0.54	0.72
Min Ratio	1.52	1.31	1.73	1.39
Max Ratio	2.78	2.52	2.68	2.45
mean Ratio	2.07	1.64	2.14	1.95
SD ratio	0.27	0.24	0.24	0.23
p-value Shapiro-Wilk test	0.59	<0.01	0.09	0.66

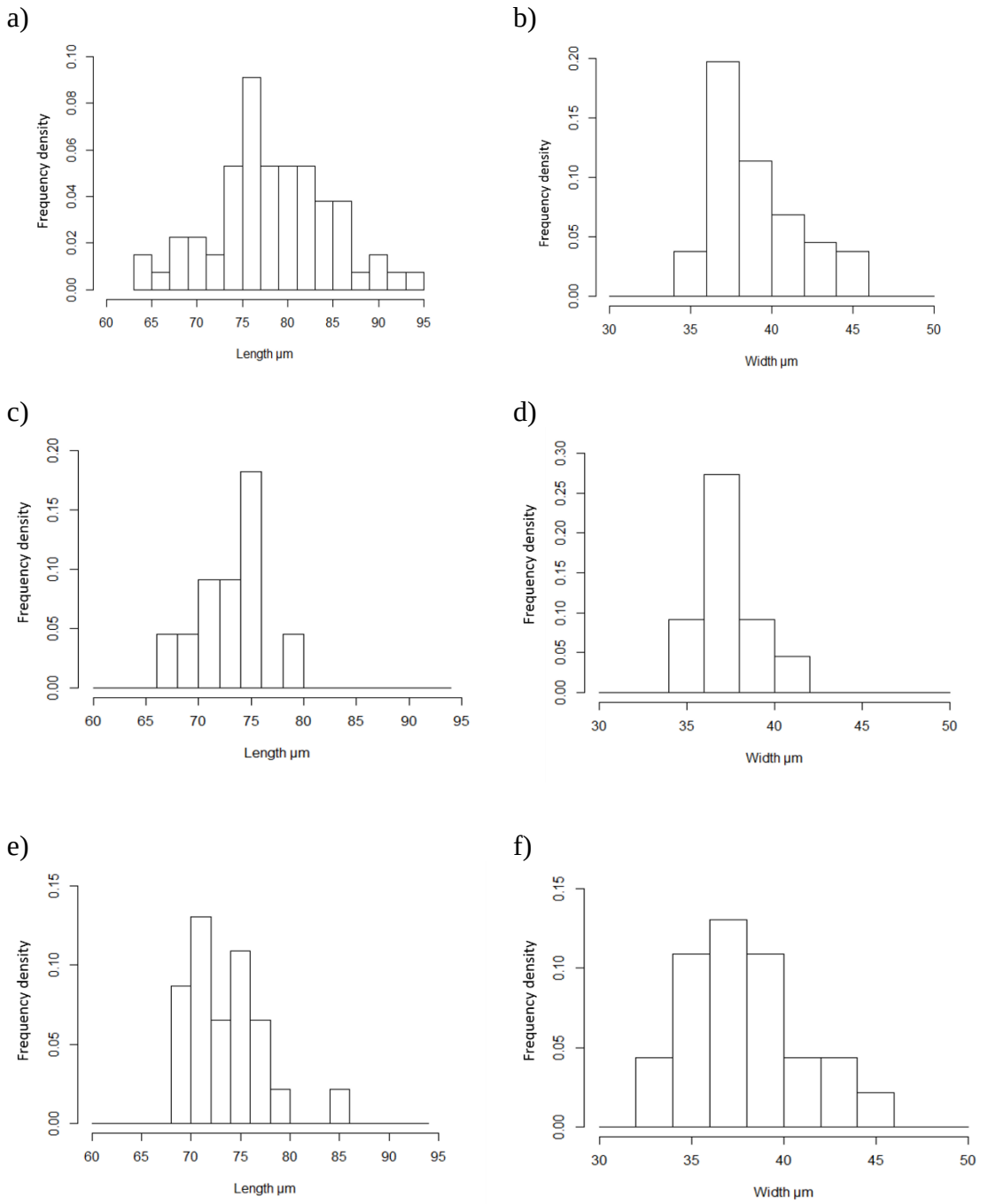


Figure 4.5. Frequency distributions (in propotions) of length and width of strongyle eggs: a-b) Santa Cruz west, c-d) Santiago, e-f) Isabela. Santa Cruz east is not included due the low number of strongyle eggs measured from that island. Frequency density represents the frequency divided by class interval. The class interval was set at 2 µm.

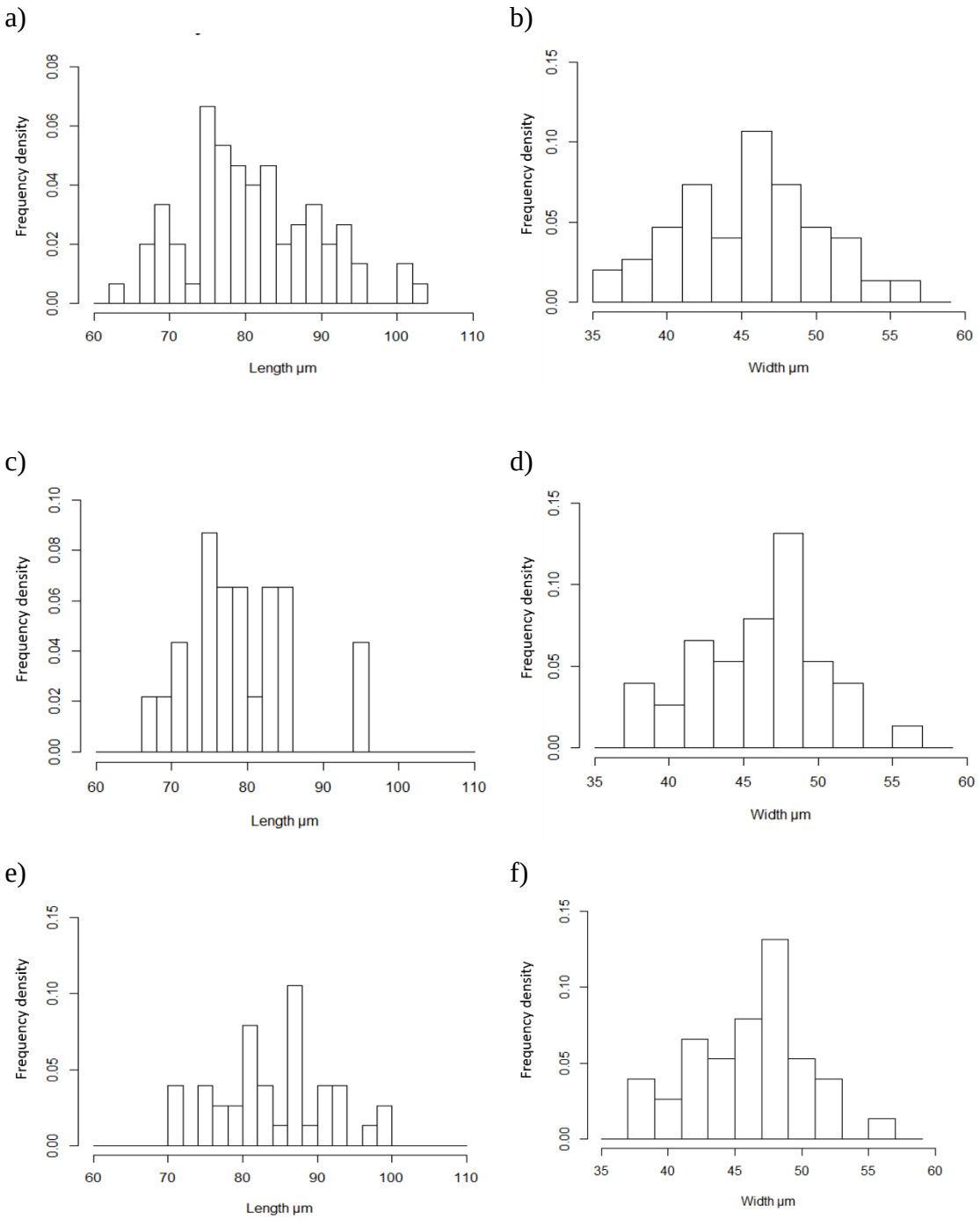


Figure 4.6. Frequency distributions (in propotions) of length and width of oxyurid eggs: a-b) Santa Cruz west, c-d) Santa Cruz east, e-f) , Santiago. Isabela is not included due that no oxiurids was detected in this study. Frequency density represents the frequency divided by class interval. The class interval was set at 2 µm.

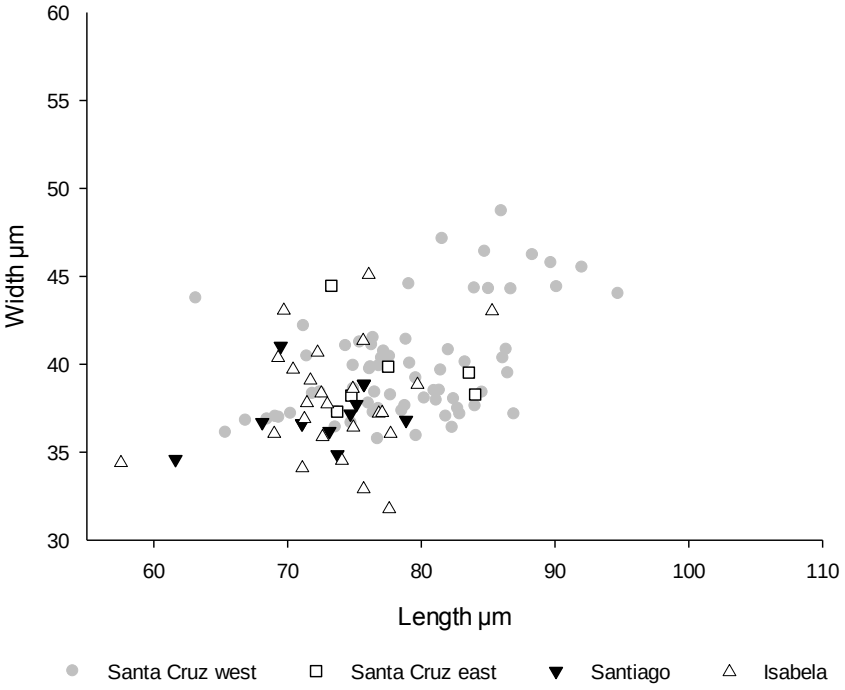


Figure 4.7. Measurements of length and width of strongyle eggs from different tortoise populations.

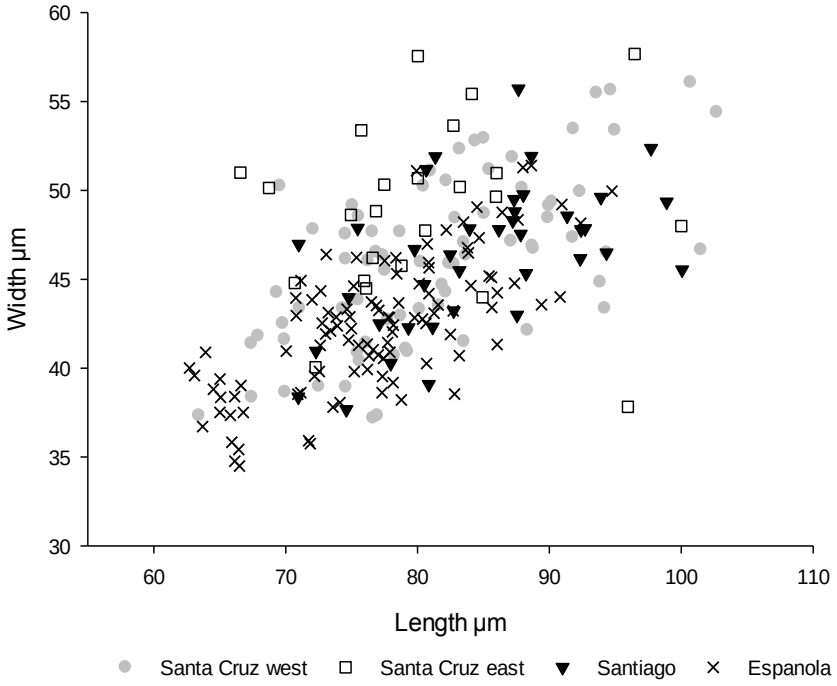


Figure 4.8. Measurements of length and width of oxyurid eggs from different tortoise populations.

4.3.3. Characterisation and distribution of helminth larvae

Using the Baermann technique, I isolated 919 nematode larvae from wild tortoise faecal samples (182 from Santa Cruz east, 141 from Santa Cruz west, 132 from Santiago and 464 from Isabela), 323 from faeces collected from repatriated tortoises on Española and none from the captive tortoises on San Cristóbal. A subset of 50 larvae chosen from individual tortoises on different islands all appeared identical when observed using light microscopy and on subsequent expert analysis of photomicrographs. The five specimens which were clarified for more detailed analysis were all in the 1st developmental stage and all were identified as belonging to the Family Atractidae, superfamily Cosmocercoidea (Fig. 4.9). It was not possible to characterise the larvae beyond the family level using morphology alone.

Descriptive statistics were calculated assuming that all the larvae belonged to the family Atractidae (Table 4.11). Tortoises from Española had the highest proportion of infected faecal samples (0.72, CI 0.56 - 0.85); while samples from Santa Cruz west had the highest median intensity (12.00, CI 5.00 - 20.00). Tortoise faeces from Isabela had the highest mean intensity (13.80, 8.98 - 20.20) of nematode larvae. Faecal samples from Santiago had the highest aggregation index (variance/mean 29.91), but the lowest proportion of parasitised samples (0.33 CI 0.19 - 0.49). The frequency distribution of nematode larvae on each island was over-dispersed and positively skewed (Fig. 4.10a - 4.10e).

Using a logistic regression model, "island" was found to have an effect on the prevalence of nematode larvae (Wald test test: deviance 15.5, df 4, *p-value* =0.004), Española had a higher prevalence compared with Santa Cruz west (Wald test, *p-value* <0.05), see Table 4.12. The ZI negative binomial model provided a better fit than the standard negative binomial GLM (Vuong test, *p-value* 0.037), and showed no significant effect of either "island" or "sex". The ZI model values are showed in Table 4.13.

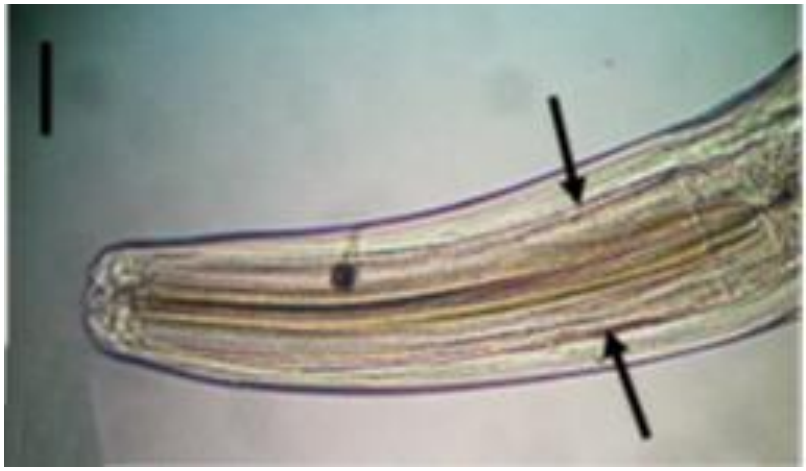


Figure 4.9. Photomicrograph of an *Atractis* sp. nematode detected in a Galápagos giant tortoise faecal sample. Anterior end of body showing sclerotised anterior region of the oesophagus [or pharynx]. Scale bars = 50 μm. Picture reproduced from Fournie *et al*., 2015.

Table 4.11. Descriptive statistics for nematode larvae isolated from tortoise faecal samples collected from the wild from different islands in the Galápagos archipelago, showing prevalence, median and mean intensity, variance mean ratio and aggregation parameter, k. CI= confidence interval, BCa= bootstrap confidence interval, based on 2000 replications. E=east, W=west.

Descriptive Statistics	Tortoise population sampled				
	Santa Cruz (W), *C. porteri*	Santa Cruz (E), *C. donfaustoi*	Santiago, *C. darwini*	Isabela, *C. vicina*	Española, *C. hoodensis*
Prevalence (positive/N)	0.49 (16/33)	0.72 (15/21)	0.33 (15/45)	0.58 (28/49)	0.72 (26/36)
CI 95% (Sterne)	0.32 - 0.65	0.47 - 0.88	0.19 - 0.49	0.44 - 0.71	0.56 - 0.85
Median intensity CI 95 0%	12.00 5.00 - 20.00	4.00 1.00 - 13.00	3.50 2 - 10	6.00 3.00 - 15.00	5.50 3.00 - 9.00
Mean intensity CI 95% s	13.20 8.94 - 19.10	10.90 5.46 - 25.80	9.57 4.36 - 22.8	13.80 8.98 -20.20	9.62 5.81 - 18.40
Variance/mean	14.81	26.95	29.91	23.02	24.08
k (ML estimate):	0.22	0.39	0.12	0.24	0.44

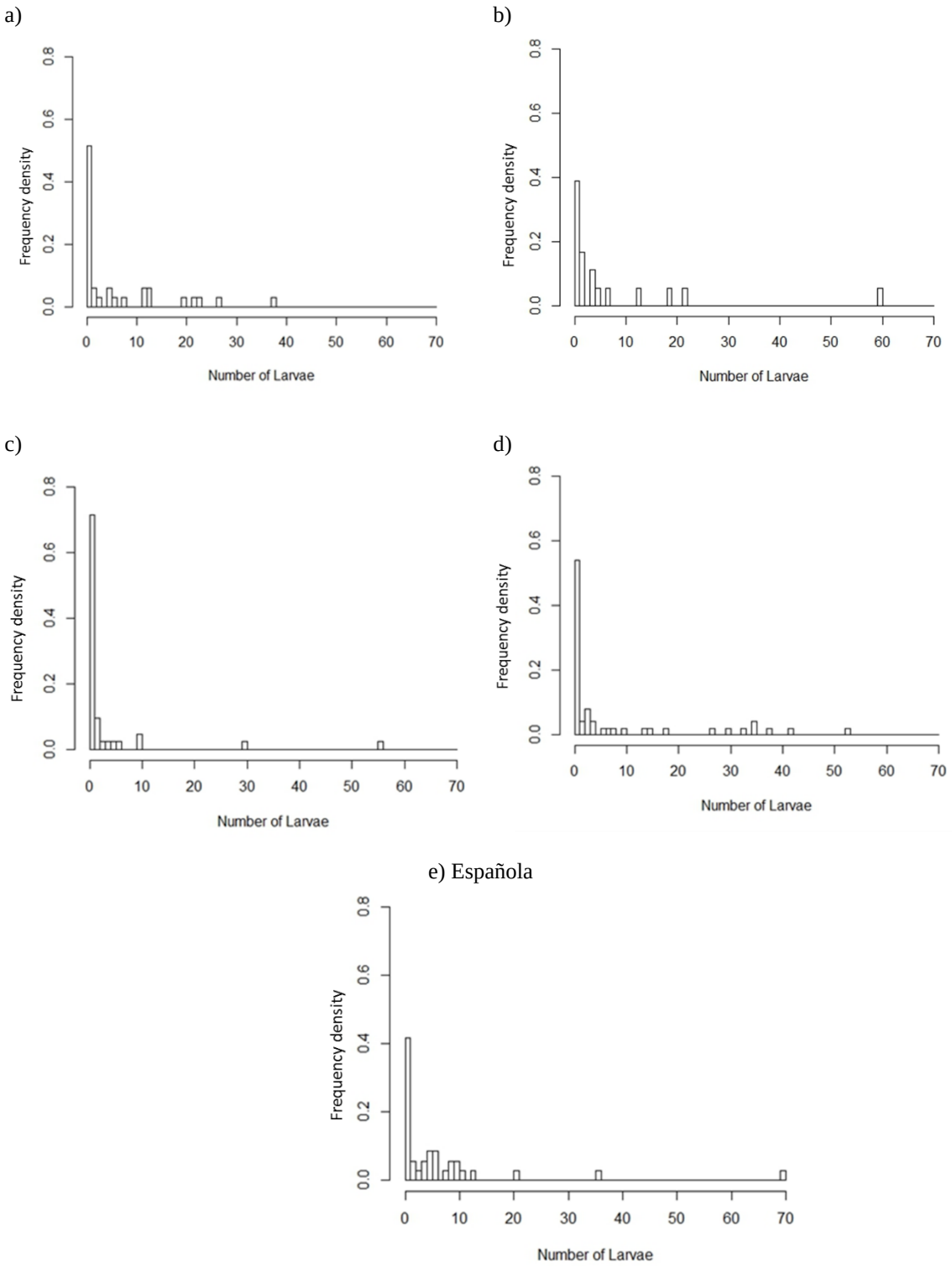


Figure 4.10. Frequency distribution of nematode larvae from *Chelonoidis* spp: a) Santa Cruz west - *C. porteri*, b) Santa Cruz east - *C. donfaustoi*, c) Santiago - *C. darwini*, d) Isabela Cerro Azul - *C. vicina*, e) Española - *C. hoodensis*. Frequency density represents the frequency divided by class interval. The class interval was set at 1.

Table 4.12. Comparison of prevalence of nematode larvae infection among different tortoise populations using logistic regression. The asterisk indicates *p-value*s ≤ 0.05.

Islands	Coefficient Estimate	Std.Error	z value	Pr(>|z|)
(Intercept)	-0.06	0.35	-0.17	0.86
Santa Cruz East	1.02	0.63	1.61	0.11
Santiago	-0.63	0.48	-1.32	0.19
Isabela	0.38	0.45	0.85	0.40
Española	1.02	0.51	1.99	0.05 *

Table 4.13. Comparison of infection intensity among different tortoise populations using a zero inflated model under the assumption of a negative binomial distribution.

Islands	Coefficient Estimate	Std.Error	z value	Pr(>|z|)
Count model				
(Intercept)	2.28	0.37	6.10	1.05e-09 ***
Santa Cruz east	-0.21	0.53	-0.40	0.69
Santiago	-0.39	0.53	-0.71	0.48
Isabela Cerro Azul	0.06	0.46	0.12	0.90
Española	-0.34	0.46	-0.74	0.46
Zero inflation model				
(Intercept)	-0.65	0.59	-1.11	0.27
Santa Cruz east	-8.46	138.88	-0.06	0.95
Santiago	0.73	0.70	1.04	0.30
Isabela Cerro Azul	-0.62	0.80	-0.77	0.44
Española	-10.05	128.98	-0.08	0.94

4.3.4. Molecular identification and phylogeny of nematode larvae

Three sets of primers for the 18S rDNA gene (Nem18SF-SSUR9, SSUF3M-SSUR9 and NemF-Nem18R) were effective at amplifying nematode larval DNA, while primers targeting ITS1 and COI genes failed to successfully amplify DNA from any nematode sample. Sanger sequencing yielded 42 18S rDNA nematode sequences (17 for Santa Cruz, 10 for Isabela, 10 for Santiago and 5 for Española Island). Following BLAST analysis they matched with previous nematode sequences identified from Galápagos tortoises by Fournié *et al*. (2015), which had been named as *Atractis* sp. and *Labiduris* sp. (Accession numbers KT364749 and KT364750 respectively). Forty sequences in this study matched the *Atractis* sp. (99% nucleotide identity), and were found across all the islands where larvae were recovered, while two sequences matched with *Labiduris* sp. (99% nucleotide identity) and were identified only from Santa Cruz west. From here on, I refer to the sequences obtained in the current study as "Atractis LP" and "Labiduris LP". As expected from the highly conserved nature of 18S rDNA, no polymorphism was found within the sequences corresponding to either species. The sequence homology between the two sequences was 94.6% (Fig. 4.11).

A phylogenetic tree built to position the sequences within the Phylum Nematoda is presented in Fig. 4.12. Sequences from *Atractis* sp. and *Labiduris* sp. formed different well supported clades (98%-100% posterior probability). The *Atractis* sp. sequences formed a sister group with *Rondonia rondoni* which belongs to the family Atractidae (supefamily Cosmocercoidea) and parasitise freshwater fish. The phylogenetic position of *Labiduris* sp. was unresolved. It formed a sister clade of a cluster represented by *Falcaustra catesbeianae* which belong to the family Kathlaniidae (Cosmocercoidea) and *Paraquiremia Africana* of the family Quimperiidae (superfamily Seuratoidea).

Figure 4.11. Comparison of the two 18SrDNA sequences obtained from Galápagos tortoise nematode larvae.

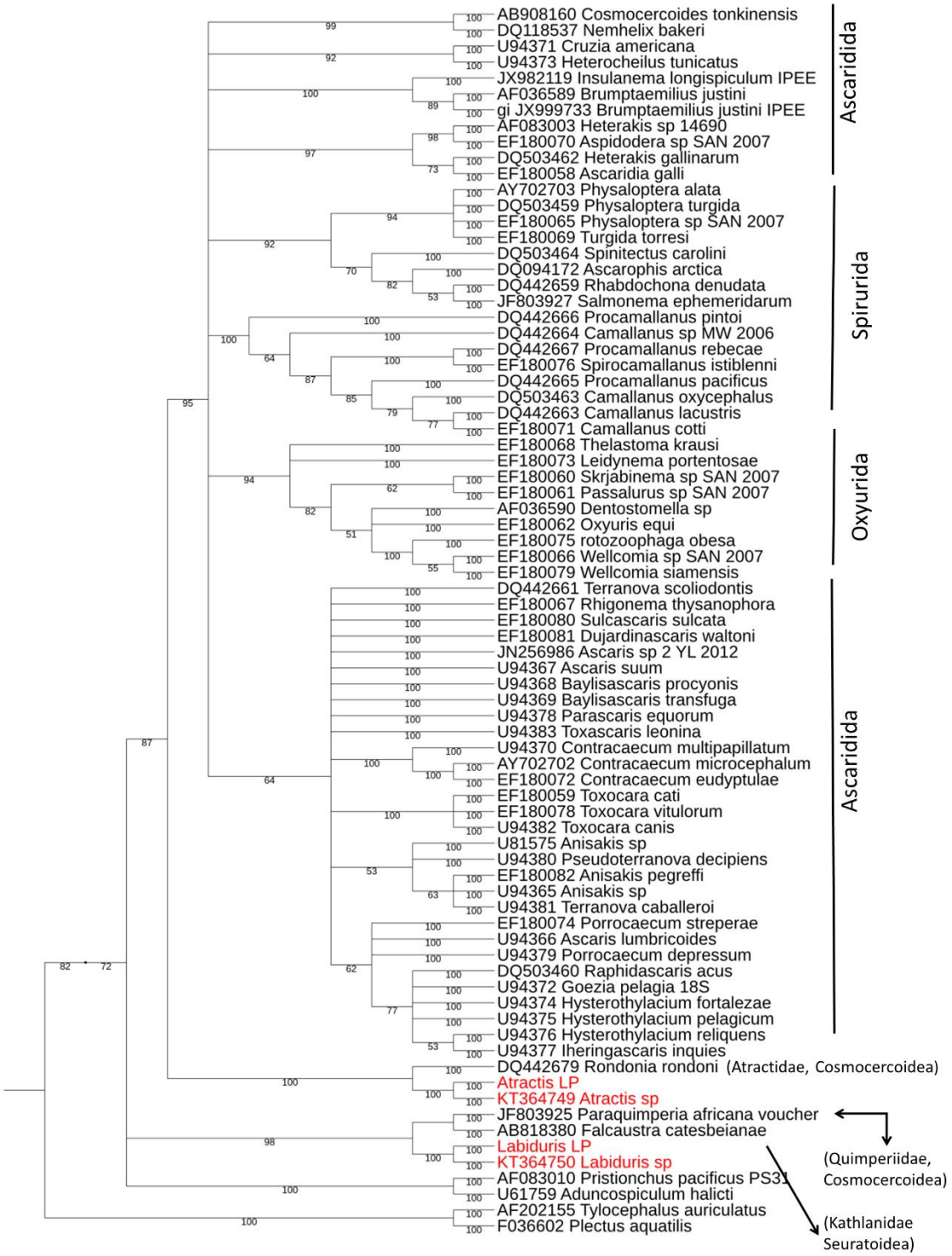


Figure 4.12. Bayesian tree of the phylum Nematoda, showing the placement of *Labiduris* LP and *Atractis* LP (labelled in red). The labels show the GenBank accession number and nematode species. Nematode families and superfamilies are given in brackets. Posterior probabilities are indicated for each node. Model (T92+G).

4.4. Discussion

Coprological analysis is used widely to study the parasites of wild animals. This non-invasive method has proved useful for parasite identification and for studying the patterns of parasite prevalence and spatial distribution particularly for species of conservation interest, where lethal sampling is not an option (MAFF 1986). Here I used faecal examination to investigate the helminths of the flagship species of the Galápagos Islands, the Galápagos giant tortoises. Based on analysis of nematode eggs and larvae I explored the nematode community infecting *Chelonoidis* spp., their prevalence, infection intensity, temporal variation, distribution and the factors that might influence this patterns. These results however should be interpreted with caution as the assumption of eggs/larvae counts representing true parasite species complement or abundance has not been proven, since absence or low number of eggs/larve may result from low worm intensity, low fecundity of parasites or intermittence in their shedding (Traversa *et al.* 2005). Any such limitation is exacerbated by the collection of only one faecal sample per individual host and low sample sizes associated with high parasite aggregation patterns (Pacala and Dobson 1988).

Acknowledging the above limitations, the coprological survey presented here revealed nematodes as common helminths of *Chelonoidis* spp. Nematode larvae and eggs were found in tortoise populations that have been always wild — Santa Cruz west (*C. porteri*), Santa Cruz east (*C. donfaustoi*), Santiago (*C. darwiini*) and Isabela south west-Cerro Azul (*C. vicina*) — and in tortoises raised in captivity but then repatriated to the wild — Española (*C. hoodensis*). Gastrointestinal helminths appear to be absent in captive individuals on San Cristóbal (*C. chatamensis*) which were captured from the wild on that island in 2003 as part of a conservation breeding programme.

Across the Galápagos Islands, the nematode community of *Chelonoidis* spp. was composed of five superfamilies: Strongyloidea, Oxyuroidea, Ascaridoidea and Trichinelloidea, identified on the basis of morphology of nematode eggs; and Cosmocercoidea identified using morphology and DNA analysis of larvae. Based on DNA sequencing Cosmocercoidea were represented by the genus *Atractis* sp, family Atractidae. The larvae identified as *Labiduris* was expected to cluster with Atractidae but the DNA analysis did not conform to it. Further analysis are required to clarify the

assignement and phylogenetic position of this nematode larvae. The identification of some nematodes species is problematic as similar but independently ecological challenges have given rise to similar phenotypes (Bik *et al.*, 2010; Choudhury. and Nadler; Nadler *et al.*, 2016). Molecular assignment is also confronted with sparse taxon sampling as many genera still remain unsampled. To date the taxonomy of the phylum Nematoda is regarded as 'work in progress', and the use of taxon sampling and multi loci approach has been suggested for a more streamlined systematic (Bik *et al.*, 2010). Althougth the asignement of one of the nematode larvae of *Chelonoidis* spp. to *Labiduris* is still uncertain I will keept this name throught the thesis.

The DNA sequence of either *Atractis* sp. and *Labiduris* sp. had 99% similarity with sequences obtained previously by Fournie *et al.* (2015) suggesting they could be the same two species described there. The only adult nematode species described from wild Galápagos tortoises in Santa Cruz west is *Atractis marquezi* (Bursey and Flanagan 2002), so it is possible that the *Atractis* sp. larvae found are of this species. In this study Strongyloidea eggs and Atractidae larvae were found in all the parasitised tortoise species, including those repatriated to Española. *Labiduris* larvae were detected only in Santa Cruz. Oxyuroidea eggs were absent from tortoises on Isabela and Trichinelloidea eggs were absent from tortoises in Santa Cruz east. Ascaridoidea eggs were absent from both of these populations and also from the tortoises on Española.

My results are in agreement with those of Fournie *et al.* (2015) who used the same coprological methods for studying the biogeography of the Galápagos tortoise helminths. Identical nematode taxa were identified across Galápagos in the two studies and a similar pattern of prevalence was found for Santa Cruz west, the only wild tortoise population included in both studies. Also, as in Fournie *et al*. (2015), strongyle eggs were found in all the tortoise populations examined and helminths were not detected in captive tortoises from San Cristóbal. A contrasting result was found however on Isabela where Fournie *et al*. (2015) reported oxyurid and ascarid eggs, but neither of these were detected on Isabela in the current study. This might be result of sampling effort: for the Fournie *et al*. (2015) survey, both oxyurid and ascarid eggs were observed at ≤12%, but the sample size used for my thesis would allow the detection of infection at a prevalence of ≥10% at a confidence interval of 95%. Therefore, parasite eggs present at low prevalence might not have been detected in the current study. The distribution of nematode larvae of

Chelonoidis spp. has not been described before so, this information is reported for first time.

Fournie *et al.* (2015) documented the presence of egg morphologies classed as 'Large' and 'Small' strongyle on Isabela Island, but did so without the ability to measure eggs accurately. While I found a range in strongyle egg size, I found weak evidence of a bimodal distribution on Isabela but little evidence elsewhere. The measurements presented in this thesis however, were performed after the eggs had been frozen; and freezing can cause nematode eggs to shrink (van Wyk and van Wyk 2002). The mean ratio of length: width of strongyle eggs fitted a normal distribution, but that for oxyurid eggs collected from Isabela did not. These results are similar with the study of Stear et al. (2005) in Scottish sheep where he found some types of nematode egg fitted a normal distribution, while others did not. They attributed this finding to the contribution of different nematode species which are indistinguishable from egg counts (Stear *et al.* 1995).

It is worth noting that the different studies sampled different locations on Isabela Island: Roca Union and San Pedro by Fournie *et al.* (2015) (near Sierra Negra Volcano) and Cerro Azul for the current study (see figure 1.1). Based on geographic isolation and morphological differences, the tortoises of Roca Union and San Pedro were formerly considered as belonging to the subspecies *C. ghuntheri*, with the Cerro Azul tortoises belonging to *C. vicina.* Recently, however, based on mitochondrial DNA sequence data, all of the tortoises in these populations have been grouped as one species, *C. vicina* (Ciofi *et al.* 2006). Nevertheless, according to mitochondrial DNA data, the tortoises from western Cerro Azul diverged from those elsewhere in southern Isabela around 294,000 years ago (Ciofi *et al.* 2006). The two coprological parasitology studies compared here were performed ten years apart. Despite some differences in the findings between the two studies for tortoises on Isabela, the general finding of the same parasite taxa across the archipelago indicates that the community structure of gastrointestinal helminths in *Chelonoidis* spp. might be relatively stable over time.

Studies in vertebrates suggest that colonisation of new areas is an important factor influencing their parasite communities (MacLeod *et al.* 2010). Insular species such as the Galápagos tortoises are an amenable model for testing the generality of these findings. In

Galápagos, tortoise colonisation started in the islands closest to the South American mainland (San Cristóbal or Española), then progressed more or less sequentially westwards (Poulakakis *et al.* 2012). Given this colonisation pattern, it could be hypothesised that the nematodes seen today arrived with the original tortoise colonists. If this was the case, tortoises living on the first island to be colonised might be expected to carry a higher diversity of parasites, while tortoises living on islands colonised subsequently might have lost some parasites due to stochastic processes such as founder effects and drift. Based on the nematode taxa observed in wild tortoise populations, stochastic effects during colonisation may have had little effect in determining their parasite community into the archipelago. The same nematode species complement found in Santa Cruz west were found by Fournie et al. (2015) in two populations of Isabela, one of the last island to be colonised. In relation to Fournie results and those from this thesis tortoise colonisation will have little effect at least for the distribution of Strongyloidea and Atractidae nematodes.

In this study Santa Cruz west is considered as the oldest remaining continuously intact population of tortoises on Galápagos (Macfarland *et al.* 1974). Although there are tortoises on islands colonised earlier than Santa Cruz (i.e. Española and San Cristóbal), these populations were decimated by human activities and the extant tortoises are either a remnant population (San Cristóbal) or captive-reared, reintroduced animals (Española) (Macfarland *et al.* 1974). Moreover, in some islands as San Cristóbal there are substantial changes in their native vegetation. Tortoise parasite diversity on these islands, therefore, is unlikely to represent the community structure prior to the discovery of the islands by people.

Strikingly, no evidence of helminth infection was detected in the tortoises held in the breeding centre on San Cristóbal. Loss of parasites in the captive tortoises of this island might be a consequence of small founding population for this breeding programme. However, Fournie (2015) detected nematode infections on coprological examination of captive tortoises in the other two captive breeding centres on Galápagos so the loss of parasites during the establishment of these breeding facilities would be rare. Alternatively, a pre-existing nematode community of wild tortoises on San Cristóbal might have become extinct, as a result of human impacts.

Further work is required to investigate if nematode infections of the wild tortoises on San Cristóbal are present. Unfortunately, despite two attempts to do so, no faecal samples were obtained from wild tortoises of this island. On each sampling expedition, the habitat was found to be very dry, no fresh faeces were found and the tortoises did not void them after being stimulated. On San Cristóbal, the remnant wild tortoise population lives in a xeric habitat, receiving a mean annual precipitation of as little as 97.5 mm, while in others areas of San Cristóbal and on the central islands (e.g. Santa Cruz, Isabela) mean annual rainfall varies from 214.1 to 1694.2 mm (Hamman 1997). It is known that environmental factors influence both development and survival of nematodes (Stromberg 1997). Thus sampling during or shortly after a period of precipitation would be required to maximise the chances of detecting evidence of nematodes via coprological examination.

Within any nematode species, variation among populations in standard infection parameters (prevalence, intensity and abundance) is common (Poulin, 1998; Krasnov et al., 2006). In a meta-analysis peformed by Poulin et al. (2006), infection intensity and abundance per host appears to vary less than prevalence and thus were considered parasite species-specific attributes. Prevalence on the other hand had a higher variation and was considered to be more dependent on local extrinsic factors (Arneberg et al., 1997; Krasnov et al., 2006; Poulin, 2006). Whether these patterns can be generalized to all parasites will depend on additional observations over a range of parasite and host species (Santiago-Alarcon *et al.* 2008). In concordance with Poulin et al. (2006), in the current study I found "island" to have an effect on the prevalence of nematode larvae but also on both the prevalence and abundance of strongyle eggs. Despite this statistical finding the values of prevalence and parasite intensity of these nematode taxa were respectively similar for almost all tortoise populations. The noticeable differences in these parameters were only found for strongyles of Santiago and for strongyles and *Atractis* larvae of the reintroduced tortoises of Española both in comparison with Santa Cruz west.

The relative similarity in the prevalence and intensity of strongyles and Atractidae across the Galápagos suggests the successful colonisation and adaptation of these different nematode groups to different islands. Their colonization success would be possibly due to the presence of similar biotic and abiotic conditions. *Chelonoidis* spp. populations that have been always wild, and included in this study, inhabited islands that differs in area and altitude (Santa Cruz : 986 km^2 , 864 m, Santiago 585 km^2, 907 m, and Isabela 4588

Km^2, 1707 m) (Jackson 2009). All occupy habitats over 100 m which are expected to have similar patterns of precipitation, vegetation and solar radiation (Macfarland, Villa and Toro 1974).

In the presence of stable extrinsic factors, the variation of parasite intensity and prevalence might be explained by nematode life histories (Dobson *et al.* 1992). In *Chelonoidis* spp. that have been always wild, Cosmocercoidea larvae and Strongyloidea eggs were found in higher prevalence and intensity than the other nematode taxa identified here. The life cycles of the gastrointestinal nematode parasites of Galápagos tortoises is unknown. However, parasite species of the family Atractidae—Cosmocercoidea (represented here by *Atractis*), are ovoviviparous. The larvae develop in utero until the 3rd (infective) stage and are auto-infective. Transmission to new hosts occur mainly via the faeco-oral route and, in some cases/species, during host copulation (Anderson 1992). During sampling, Atractidae larvae were observed and collected from the external surface of some tortoise's cloaca, a position that could facilitate their transportation through the male sexual organ to the female cloaca. Whether it is a way of *Atractidae* transmission in tortoises needs to be investigated. Cosmocercoidae belong to the order Ascaridida whose eggs were also present in some of the faecal samples surveyed. Those eggs have been considered as belonging to the superfamily Ascaridoidea but they could also represent a mixture of superfamilies and families of Ascaridida.

With regard to the superfamily Strongylidae, eggs are expelled to the environment where they embryonate rapidly (1-2 days) under suitable conditions of moisture and temperature. Hatched larvae grow and moult to second-, and third stage (5–6 days); this last stage is the infective stage and third stage larvae can survive in the environment for weeks or months, depending on the species and the environmental conditions (Anderson 1992). The long survival time of the larvae and their ability to migrate amongst vegetation enhances their ability to be ingested by a new, suitable host.

Parasite life history might also explain the low prevalence and intensity of Oxyuroidea, Trichinelloidea and Ascaridoidea (provided these eggs are different from Cosmocercoidae). The transmission of parasites within these superfamilies involves the ingestion by a suitable host of embryonated eggs voided in faeces. The eggs hatch in the host's gastrointestinal tract where the larvae either develop directly into adults or do so

after migrating to the lungs and being coughed up and swallowed. Once the worms develop into adults in the gut, they mate and produce eggs which are passed in faeces again. The eggs remain viable in the environment for at least 2-3 weeks (often months), where they can persist until being ingested by a suitable host (Anderson 1992). Vegetation contaminated with faeces is probably not an attractive food for tortoises, so the transmission of these parasites will be less frequent than those transmitted through free living larvae. In contrast to larvae which migrate out from the faecal pat, embryonated eggs in faeces might also be more vulnerable to desiccation (Stromberg 1997).

Given the probable life history of the nematodes infecting Galápagos tortoises the differences in the prevalence and infection intensity of Strongyloidea and Atractidae in Santiago and Española might be explained by the high human impact on these islands. In the case of Santiago there was a marked reduction in the tortoise population between the 18th and 19th century. This was one of the main islands where tortoises were hunted for food and oil (Van Denburg 1907). Such an event may have depleted both hosts and parasite abundance. Another factor might be the high density of introduced ungulates, especially goats that were introduced to this island in the 18th century and were eradicated only in 2006 (Rivera-Parra et al. 2012). These invasive species cleared most of the vegetation on the island and this might have reduced the transmission ability of parasites depending on it for infecting new hosts. The tortoises of Española also were heavily reduced in the past; just 14 wild individuals lived there by the 1960s. Those last individuals were taken to captivity in Santa Cruz to implement the first *Chelonoidis* spp. conservation breeding programme. The first captive-bred hatchlings were obtained between 1970 and 1971, and these were reintroduced to Española in 1975 (Macfarland and Reeder 1975). Since then >1500 young tortoises have been re-introduced to this island (Gibbs *et al.* 2008).

The prevalence patterns for nematode eggs and *Atractis* larvae also differ for the Española tortoises compared with those elsewhere. Oxyurids were the most prevalent eggs, contrasting with the tortoise populations on other islands that have been always wild, where strongyle eggs predominate. The older tortoises in Española should be ≤ 40 years old the age distribution would differ compared to the other islands sampled in this study. Thus the variation in oxyurid prevalence could be associated to differences in feeding habits, with younger tortoises being more coprophagic than older, which are essentially

vegetarian (Gagno 2005). An additional factor might be that young reintroduced tortoises are released together in the same areas which could intensify the probability of infection (Cruz Márquez 1991). Oxyurids are common parasites in other tortoise species and there is evidence of an elevated prevalence in young age-classes (Gagno 2005).

As Española was tortoise free for several years, the parasites found there nowadays might have originated from a recent co-introduction with their tortoise hosts. Altered prevalences in strongyle eggs and *Atractis* larvae on Española might be also have occurred if a low percentage of the reintroduced tortoises were infected prior to repatriation, resulting in a low level of co-introduction of these parasite taxa. Fournie et al. (2015) found nematode eggs in adult and juvenile captive tortoises in the Santa Cruz breeding centre, where multiple species from multiple locations are held. It is possible that the parasites originated from the wild-caught adult tortoises from Española or that, accidental cross contamination from other tortoise species occurred during the long period of captive management of *C. hoodensis*. Infection of the reintroduced tortoises on Española with parasites from other reptile taxa on the island is an alternative possibility but so far, there have been no reports of nematodes transferring from Squamata to chelonians. The other reptiles inhabiting Española comprise one species of snake (*Philodryas biserialis*), one species of leaf-toed gecko (*Phyllodactillus bauri*) and one species of lava lizard (*Tropidurus grayi*) (Jackson 2009). Unfortunately no Española tortoise parasite data exist prior to the extinction of *C. hoodensis* in the wild, or from the founding animals used in the captive breeding programme.

Altogether this study shows the successful colonization of different Galápagos islands for at least two nematode superfamilies, and differences in the parasitic nematode species complement for at least one tortoise populations/islands. Confirmation of the absence of some nematodes would require increasing the sample size or using more sensitive methods for nematode eggs detection. McMaster technique with flotation solutions other than NaCl (Cringoli *et al.* 2004), such as salt-sugar flotation (Mes *et al.* 2007), and FLOTAC (Cringoli *et al.* 2010) are interesting alternatives. Different flotation solutions provide different densities that might lead to greater buoyancy of nematode eggs. Another alternative is the use of high-throughput parallel sequencing that would allow the simultaneous sequencing of all the nematode taxa present in a sample (Porazinska *et al.*

2009). The results of a metabarcoding approach for studying the nematode communities in faecal samples of Galápagos tortoises will be presented in chapter 5.

In conclusion this study reports the presence of four superfamilies of nematode eggs and two nematode larvae species within a fifth superfamily, Cosmocercoidae, infecting Galápagos giant tortoises in the wild. All of these nematode taxa were present on Santa Cruz Island, one of the first islands to be colonised by the tortoises, but also on Isabela, one of the last tortoise populations to be established. Despite the relative stability of parasites parameters "Island" was a significant factor in driving parasite species complement; reduced species complements were found in islands with the greatest human impacts on tortoise populations; represented here by Santiago and Española. Founding effects might have impacted the nematodes of captive population of San Cristóbal, where no helminth was detected there in two different surveys. Further research is required to investigate whether human activities have affected the parasites of wild tortoises of that island now restricted to a xeric habitat. Tortoises from Española which are subject to an intensive captive bred programme carried at least 4 of the six nematode taxa identified in Santa Cruz. It suggests either they retained some of their natural parasite fauna while in captivity or there was potential cross contamination from other species (in captivity) and parasite co-introduction during repatriation to the island. Together, these results are relevant for understanding the factors influencing the parasite communities of Galápagos tortoises as well as informing current day biosecurity.

Chapter 5. Metabarcoding of nematode communities using high-throughput sequencing technology

5.1. Introduction

In nature most wild species are concurrently or sequentially infected with multiple parasites (Petney and Andrews 1998). Characterizing and quantifying the abundance of these parasites is relevant for determining their biogeography, ecology, and effects on the host health. For gastrointestinal (GI) helminths doing these tasks comprehensively usually require the sacrifice of the host (Budischak *et al.* 2015). Lethal sampling however, involves ethical and logistical considerations, especially for threatened and rare species (Jorge *et al.* 2013). It also restricts the host species that can be studied, obtaining adequate sample sizes, the geographic scope of sampling, and the research questions which can be addressed (Budischak *et al.* 2015). Non-invasive coprological methods have become an alternative for studying GI helminths of wildlife (Chavarri *et al.* 2012; Wang *et al.* 2013). However, the accuracy of quantifying parasites from faecal samples depends on intrinsic factor of the method chosen for the analysis. These methods rely on the separation of parasitic forms from faecal debris using sedimentation procedures that might be biased to particular taxa (Cringoli *et al.* 2004). In addition, it relies on the counting of immature stages of parasites (eggs and larvae), making it difficult to identify them to fine scale taxonomic levels such as genera or species (MAFF 1986).

Non-invasive coprological methods in combination with DNA technology are a promising tool to better approach the studies of GI helminth communities (Gasser *et al.* 2008). Mitochondrial and ribosomal DNA sequences have been used as molecular markers for identifying nematodes. Voucher helminth specimens have allowed the development of species or genus specific PCR primers facilitating single parasite characterization, for review see Gasser (1999). Multiplex PCR and Real-time PCR assays each combining several nematode species specific primers has been also developed for simultaneous identification of more than one parasite group simplifying substantially the analyses of mixed parasite populations (Zarlenga *et al.* 2001). DNA

technology however, has been optimized for helminth parasites of veterinary concern, and mainly for livestocks. Thus, the analysis of helminths of wildlife, especially mixed infections, is still hampered by the lack of knowledge of the helminths species infecting wildlife hosts.

The difficulty of studying multiple helminth infections of wildlife using molecular methods can now be tackled with high-throughput sequencing technology also known as Next Generation Sequencing (NGS). Amplicon-based approaches, through PCR-targeted sequencing (also known as metabarcoding or metagenetic) of selected genomic markers offers an unprecedented opportunity to comprehensively examine helminth parasites (Bass *et al.* 2015; Escobar-Zepeda *et al.* 2015; Tanaka *et al.* 2014). Such methods have been widely used due to its convenience to perform taxonomic and phylogenetic classification in large and complex samples of microorganism, such as human gut microbiota. The methods are well established for prokaryotes, but now applications in eukaryotes are increasing (Andújar *et al.* 2015; Sapkota and Nicolaisen 2015). In all cases, the method can be performed using consensus primers allowing simultaneous sequencing of millions of DNA molecules representing multiple species of taxa of interest (Gruber *et al.* 2002; Hudson 2008). This is a remarkable advantage over Sanger sequencing which can retrieve only up to 96 individual sequences per run and is not suitable for sequencing of mixed DNA templates (Escobar-Zepeda *et al.* 2015).

In metazoa one of the preferred markers for metabarcoding is the 18S rDNA. For example Porazinska *et al.* (2009) applied this methodology for nematodes. They first validated the method using defined experimental nematode communities, and then analysed nematode assemblages of tropical soils. They used consensus nematode primers targeting 200 bp of the SSU and LLU of 18S rDNA that also allowed sequencing of this locus from other eukaryotes. A similar approach was used for a survey of soil nematodes in unmanaged flowerbed and agriculture soils of Japan (Morise *et al.* 2012) and to characterize intestinal nematodes in wild rats (Tanaka *et al.* 2014) and the GI "nemabiome" of cattle from mid-west USA Avramenko *et al.* (2015). All these studies have demonstrated the suitability of metabarcoding for identification of nematodes and have revealed an unexpected diversity, abundance and patterns of communities structure.

However, metabarcoding is still to be widely adopted for ecological research of eukaryotic parasites of wildlife, and to my knowledge, it has not yet been combined with non-invasive sampling of wild vertebrates. In this chapter I aim to use high throughput parallel amplicon sequencing of 18S rDNA to characterize the nematode community structure of the Galápagos tortoises previously analysed by coprological methods; evaluate the ability of NGS based methods to resolve nematode OTUs to genus or species scale; and compare the relative abundances of taxa detected with results from conventional microscopical techniques. I use the data to identify helminth community variation among tortoise populations.

5.2. Materials and Methods

5.2.1. Faecal sampling, isolation of nematode eggs mixtures and DNA extraction.

This study complements the previous analysis of nematode eggs using conventional coprological methods (McMaster and Baerman techniques) presented in Chapter 4. Fresh tortoise faecal samples were collected between January and May of 2013 and 2014 from different islands of the Galápagos as described on material and methods of chapter four. The collection include samples obtained from the tortoises of Santa Cruz west, Santa Cruz east, Santiago, Isabela and Española described in Table 4.1. It also included 32 samples of the breeding centre of Santa Cruz, not analysed by coprological methods.

Nematode eggs mixtures from individual tortoises were obtained from the solution prepared for the McMaster analysis, which included the dilution and homogenization of 4.5 g of faeces in 40.5 ml of saturated NaCl solution (see Chapter 4). In order to get a good representation of the nematodes eggs present in each sample they were then concentrated by centrifugation following a method similar to that described by Bott *et al.* (2009). Whereas Bott *et al.* (2009) used saturated sodium nitrate as the flotation solution (in order to detect Strongylid eggs), I used sodium chloride to allow flotation of additional nematode taxa (Ballweber *et al.* 2014; MAFF 1986). Concentration of eggs was done as follows: a 5 ml volume of the same NaCl solution prepared for McMaster analysis was transferred to a tube of 50 ml, diluted in 45 ml of sterile filtered

distilled H_2O and centrifuged. Then the supernatant was removed and the pellet was washed in the same way to remove exces of ClNa. The pellet was resupended in 500 ul of ultrapure H_2O and transferred to microtubes of 1.5 ml and storage at -20 °C.

DNA extraction of each nematode egg mixture was done using Powersoil kits (Cat No. 1288-S) from Mo Bio Laboratories Inc. This kit has been validated in several studies using faecal samples for the genetic characterization of gut microbiota of mammals including humans (Kennedy *et al.* 2014). DNA extraction was carried following the manufacturer's protocol for wet soil samples. The protocol requires a bead beating step which was done using the TisueLyser LT (QIAGEN, Cat No. 69980) at 50 Hz for five minutes (3000 oscillations/minute).

5.2.2. Design of primers for PCR libraries and testing of amplicon size

Amplicon sequencing (sequencing of PCR libraries) was performed using an Illumina Miseq platform. This platform allows the sequencing of paired-end reads of PCR products with sizes ranging from 2 x 150 bp to 2 x 300 bp (Caporaso *et al.* 2011). The first steps of any metabarcoding projects involve the selection of primers which amplify the target DNA of all the taxa of interest and generate amplicons of suitable size for the sequencing platform. For this study I chose two set of consensus primers (Table 5.1) targeting the 18S rDNA of the phylum Nematoda, which would generate amplicons of ~400 bp originally published by the Blaxter Lab, University of Edinburgh (http://xyala2.bio.ed.ac.uk/research/barcoding/sourhope/nemoprimers.shtml). The taxonomic breadth of the primers was tested using the DNA of nematodes from different Orders and Superfamilies. Those comprised *Ascaris lumbricoides* (Ascaridida—Ascaridoidea), 2 nematode genera identified so far in Galápagos tortoise faeces *Atractis* sp. (Ascaridida—Cosmocercidae) and *Labiduris* sp., and samples of *Caenorhabditis elegans* (Rhabditida—Rhabditidae), and *Trichuris trichiura* (Trichocephalida—Trichinelloidea). DNA of the last three species were kindly provided by Dr. Ian Hope and Dr. Rupert Quinnel (University of Leeds). A final set of 2 primers which amplified the 18S rDNA of all the nematode species listed above and yielded PCR products of ~400 bp was chosen for assembling a initial pair of barcode primers as required for Illumina sequencing libraries. Barcode sequencing library primers contain long nucleotide sequences (~ 75 nucleotides) which in orientation 5' to 3' comprise i) an adaptor or sequence complementary to the platform sequencing

primers; ii) a barcode sequence used to "tag" each PCR product — present just in the forward or reverse primer; iii) a linker sequence to avoid dimerization; iv) the sequence targeting the DNA of interest; see Fig. 5.1. The Primers were assembled according to the Earth and Microbioma Project guidelines (http://www.earthmicrobiome.org), replacing the consensus sequence used in that project for bacteria identification for the sequences identified here as appropriate for nematode 18S rDNA. The barcode was placed in the forward primer as recommended elsewhere as it would allow the designing and use of more reverse primer combinations. The barcode sequence library primers were then retested on the DNA of *Atractis* sp., *Labiduris* sp., *C. elegans, A. lumbricoides* and *T. trichiura*. After verifying successful amplification of the target species, one hundred barcoded primers were designed in order to obtain 3 x 100 libraries of nematode eggs mixtures of individual Galápagos tortoises.

Table 5.1. Consensus primers for the 18S rDNA gene if the phylum Nematoda tested for metabarcoding of Galápagos tortoise nematodes. Originally published by the Blaxter Lab, University of Edinburgh, http://xyala2.bio.ed.ac.uk/research/barcoding/sourhope/nemoprimers.shtml).

Primer name	Sequence	Position in *C. elegans* sequence
SSU_F_04	GCTTGTCTCAAAGATTAAGCC	30-49
Nem_18S_F	CGCGAATRGCTCATTACAACAGC	111-123
SSU_R_22	GCCTGCTGCCTTCCTTGGA	429-411111-123

Figure 5.1. Representation of barcoded sequencing library primers. Green lines represent the doble strand of DNA to be amplified the mulrticolor lines represent primers. In the primers the blue region represent an adaptor or sequence complementary to the platform sequencing primers; the orange region represent a barcode sequence used to "tag" each PCR product — present just in the forward or reverse primer; the red region a linker sequence to avoid dimerization; the reaminingh region represent the sequence targeting the DNA of interest.

5.2.3. Libraries and Next Generation sequencing

Libraries were prepared following Costello *et al.* (2009). The PCR mix was prepared within a PCR hood, all surfaces and pipettes were previously decontaminated using DNA AWAY (Molecular BioProducts) and UV irradiation of 30 minutes.

Each sample was amplified in triplicate using 96 well plates. Individual PCR reactions in a 25 µl volume contained 0.6 µM forward and reverse primers, 3 µl template DNA, 1x HotMasterMix (5 PRIME), and certified DNA-free PCR water (MO BIO). Thermal cycling was done in an Eppendorf Mastercycler gradient (22331, Hamburg). The cycling programme consisted of an initial denaturation at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 90 seconds, with a final extension of 10 minutes at 72°C. After the PCR each triplicate sample was mixed in one well and 5 µl was subjected to electrophoresis on a 1% agarose gel prepared in 0.5 TBE, and containing GelRed nucleic Acid gel stain (Biotium, BT41003) at 3x concentration. The PCR products were visualized in a gel documentation device (Ingenious, Syngene Bio Imaging). Positive samples were cleaned using the UltraClean-Up kit (MO BIO) according to the manufacturer's instructions and then were quantified using the Quant-iT dsDNA Assay Kit (Invitrogen, Q33130**)**.

Three pools of tagged amplicons were prepared. An initial pool contained 100 samples and was used for a first sequencing run in order to test the technology. The remaining samples were allocated into two additional pools analysed in a second sequencing run. Pools were intended to contain equal number of samples of each island. To achieve that, the codes numbers of all samples were randomized among sets of 100 samples using the statistical software *R* 3.3.2 (R Development Core Team 2016). The pools were prepared according to the randomisation results, using equimolar ratios.

To achieve equimolar ratios of all samples, each of them were diluted to 10 nM, and then equal volumes were added to a pool. Pools were quantified again using the Quant-iT dsDNA Assay method. Then, they were quantified in an Agilent 2200 TapeStation to detect the presence of spurious DNA not in the expected size range. When spurious bands were observed, the pools were purified again using AMPure XP beads at a ratio

of 0.8:1. According to the manufacturer, this ratio is commonly used for eliminating DNA up to ~ 100 bp. The concentration and quality of pooled amplicons were analysed again until no spurious DNA band was detectable. The sample, along with aliquots of sequencing primers at a concentration of 100 µM was submitted to Edinburgh Genomics (University of Edinburgh, Scotland). Sequencing was performed on an Illumina 2500 MiSeq instrument.

5.2.4 Bioinformatic Sequence Analysis

All analysis were under the Linux operating system using a virtual or local computer set up either in Amazon computing cloud or using a personal computer, respectively. Raw data was downloaded from the server of the sequencing centre to the Linux computer. Samples were unzipped and then separated by islands in different files. Reads were processed using the USEARCH algorithm version 9.0.1001_i86linux32, and using default parameters (Edgar 2013). The processing included merge or assemble of forward and reverse paired, read quality filtering of the merged sequences with up to 1% of errors, dereplication (condensing of identical sequences into one group), chimeric and sequence filtering, and OTUs clustering. For this last step I used a cuttof of 25 sequences, meaning that OTUs will be represented by sequences repeated >25. The pipeline for this analysis is given in supplementary material (S2).

5.2.5 Sequence annotation and descriptive analysis

Taxonomy annotation of sequences was done in two steps. Firstly sequences were annotated using the SILVA database (SILVA_128_SSURef_Nr99) (Quast *et al.* 2013). It was downloaded locally and curated to discard Prokariote sequences. Anotation was done using the 'sintax' comand of USEARCH (see supplementary material, S2) using the OTUs as query sequences and the curated database as target sequences. This step was done to describe OTUs at the level of phylum and class. In a second step the OTUs of Nematodes and Platyhelminths were extracted using a linux code and then annotated using BLAST. This annotation step was done in a local instance at Saint James Hospital using a local Blast database version 2.2.31, downloaded in January 2016. It queries a NCBI's nucleotide database which contains sequences from GenBank, EMBL, DDBJ, PDB and Refseq. BLAST analysis was used to describe only sequences of helminths and at the level of orders, families and genera.

BLAST anottation was performed because it contains more updated sequences than SILVA.

The statistical software *R* 3.3.2 (R Development Core Team 2016) was used for generating graphical information.

5.3. Results

5.3.1. Testing of primers and amplicon size

The primer set 18F-SSUR22 amplified the DNA of *Atractis* sp., *Labiduris* sp., *C. elegans*, *A. lumbricoides* and *T. trichiura*. Thus they were chosen for designing a first primer set for use on the the Illumina sequencing platform. Testing of this new primer set with the DNA of the nematode species mentioned above lead to recovery of the expected DNA sequences. Subsequently this 18F-SSUR22 were incorporated into 99 more tagged primer sets for amplicon sequencing of the intestinal nematodes of Galápagos tortoises.

5.3.2. Libraries and Next Generation sequencing

Of 300 samples included in this study 297 yielded products in the expected ~400 bp size range. The result of the amplicon sequencing expressed in raw reads, merged and dereplicated reads, singletons and OTUs are showed in Table 5.2. The greatest number of raw reads was obtained in in Santa Cruz west (5.8 million) the least was obtained in the in the breeding center (1.6 million) of the same island. The percentage of reads that merged successfully from raw sequences ranged from 90.23 in Santa Cruz east to 72.41 for the Breeding Centre. The dereplication of merged reads led to condensed unique sequences ranging from 706,531 in Santa Cruz west to 287,131 in the Breeding Centre. An important percentage of sequences were singletons (a read with a sequence that is present exactly once), they ranged from 76.2% in tortoises from Santa Cruz east to 81.2% in the Breeding Centre. Singletons were discarded before clustering OTUs. The numbers of OTUs ranged from 400 in the breeding Centre of Santa Cruz to 1128 in Isabela Island.

Table 5.2. Result of the amplicon sequencing expressed in raw reads, merged and dereplicated reads, singletons and OTUs.

Tortoise population	N	Merged/Raw reads (%)	Filtered/Merged reads (%)	Dereplicated reads from filtered	Singleton/Dereplicated reads (%)	OTUs
Santa Cruz west	60	5,821,530/7,180,627 (81.07)	5,515,385/5,821,530 (94.7)	706,531	538,671/706,531 (76.2)	1054
Santa Cruz east	21	2,367,349/2,623,576 (90.23)	276,183/236,749 (96.12)	311,094	237,697/311,094 (76.40)	466
Santiago	45	2,781,584/3,747,225 (74.23)	2,613,044/2,781,584 (93.9)	344,969	272,270/344,969 (78.9)	583
Isabela	49	3,509,700/4,479,938 (78.34)	3,232,738/3,509,700 (92.1)	672,676	544,162/672,676 (80.9)	1128
Española	36	3,445,898/4,166,157 (82.71)	3,191,315/3,445,898 (92.6)	408,578	324,219/408,578 (79.4)	538
Breeding Centre Santa Cruz	32	1,635,628/2,258,808 (72.41)	1,485,142/1,635,628 (90.8)	287,131	233,058/287,131 (81.2)	400

5.3.3. Sequences annotation

The sequences annotation was done in two steps. Firstly the OTUs were annotated against an 18S rDNA SILVA database. This analysis yielded an unexpected diversity of eukaryotic kingdoms, including at least 25 phyla representing from unicellular organism (e.g. Apicomplexa), to fungi (e.g. Ascomycota, Basidiomycota), and metazoa animals (e.g Mollusca, Nematoda, Platyhelminthes, Arthropoda).

The phyla and the corresponding classes sequenced from tortoises sampled across the Galápagos Island is shown in Table 5.3. With regard to helminths, two classes belonged to the phylum Nematoda (Chromadorea and Enoplea) and four classes belonged to Platyhelminths (Rhabditophora, Turbelaria, Cestoda and Trematoda). Both Nematodes classes contains parasite species of vertebrates and were distributed across different islands. The Platyhelminths classes Cestoda, and Trematoda also contain parasites of vertebrates but were found only in the tortoise population of Santa Cruz east. The parasitic phylum Acanthocephala, was also detected but only in Isabela Island.

Since Nematoda are the main focus of this chapter and given their wider distribution in Galápagos in relation to Platyhelminths and Acanthocephala, only nematode sequences will be considered further for the purposes of this thesis, and emphasis will be given to putative taxa related to known parasitic species of vertebrates. A second mapping of sequences involved the selection of all the OTUs annotated as Nematoda with the Silva database, which were passed to BLAST for more detailed annotation. The orders found across the Galápagos archipelago, the families and genus detected in each order, and whether they contain parasitic species is summarised in Table 5.4.

Nematodes were represented by 12 orders and 31 families. The taxa identified as being parasites of vertebrate comprised at least five orders, nine superfamilies and the same number of families. They comprised Ascaridida (Ascaridoidea—Ascaridae, Cosmocercoidea—Atractidae, Seuratoidea—Cucullanidae,), Strongylida (Strongyloidea—Strongylidae, Ancylostomatoidea—Ancylostomatidae, Trichostrongyloidea—Mackerrastrongylidae), Trichocephalida (Trichineloidea—

Trichuridae), Oxyurida (Thelastomatoidea—Pharyngodonidae) and Rhabditida (Rhabditoidea—Strongyloididae). Of the remaining taxa at least five orders and eight families contained parasites of invertebrates, one order and three families contained parasites of plants, and four orders with ten families contained free living organisms.

Table 5.3. Phyla and corresponding classes of organisms sequenced form tortoise faecal sampled collected across the Galápagos Island.

Phylum	Class
Acantocephala	Palaecantocephala
Apicomplexa	Conoidasida
Archamoebae	Entamoebea
Archamoebae	Entamoebida
Arthropoda	Arachnida
Arthropoda	Ellipura
Arthropoda	Insecta
Arthropoda	Malacostraca
Arthropoda	Ostracoda
Arthropoda	Rhabditophora
Ascomycota	Dothideomycetes
Ascomycota	Eurotiomycetes
Ascomycota	Lecanoromycetes
Ascomycota	Pezizomycetes
Ascomycota	Saccharomycetes
Ascomycota	Sordariomycetes
Basidiomycota	Agaricomycetes
Basidiomycota	Exobasidiomycetes
Basidiomycota	Tremellomycetes
Basidiomycota	Wallemiomycetes
Breviatea	Breviata
Cercozoa	Imbricatea
Cercozoa	Thecofilosea
Chlorophyceae	Chlamydomonadales
Chlorophyceae	Sphaeropleales
Choanoflagellida	Craspedida
Chytridiomycota	Chytridiomycetes
Ciliophora	Intramacronucleata
Dinoflagellata	Dinophyceae
Discosea	Flabellinia
Labyrinthulomycetes	Labyrinthulaceae
Mollusca	Gastropoda
Nematoda	Chromadorea*
Nematoda	Enoplea*
Neocallimastigomycota	Neocallimastigomycetes
Ochrophyta	Chrysophyceae
Ochrophyta	Diatomea
Peronosporomycetes	Phytophthora
Phragmoplastophyta	Embryophyta
Platyhelminths	Rhabditophora
Platyhelminths	Turbelaria
Platyhelminths	Cestoda
Platyhelminths	Trematoda
Protalveolata	Colpodellida
Rotifera	Bdelloidea
Tardigrada	Eutardigrada
Trebouxiophyceae	Chlorellales
Trebouxiophyceae	Trebouxiales
Tubulinea	Arcellinida
Vertebrata	Lepidosauria

Table 5.4. Classes, orders and families of Nematoda found in faecal samples of Galápagos tortoises sampled across the Galápagos archipelago. Whether they contain parasitic species is indicated and an example of annotated species is given.

Phylum Nematoda					
Class	Order	Family	Example of annotated Species	Family contains parasitic species	Habitat/host
Chromadorea	Araeolaimida	Plectidae	*Plectus andrassyi*	No	Soil, fresh water
Chromadorea	Araeolaimida	Rhabdolaimidae	*Rhabdolaimus aquaticus*	No	Soil, fresh water
Chromadorea	Ascaridida	Ascarididae	*Ascaris* sp.	Yes	Vertebrates
Chromadorea	Ascaridida	Atractidae	*Atractis* sp.	Yes	Vertebrates
Chromadorea	Ascaridida	Cucullanidae	*Truttaedacnitis truttae*	Yes	Vertebrates
Chromadorea	Desmodorida	Microlaimidae	*Prodesmodora circulata*	No	Soil, fresh water
Chromadorea	Dorylaimida	Dorylaimoidea	*Mesodorylaimus japonicus*	No	Soil, fresh water
Enoplea	Mermithida	Mermithidae	*Mermis nigrescens*	Yes	Invertebrates
Chromadorea	Monhysterida	Monhysteridae	*Tridentulus* sp.	Yes	Invertebrates
Chromadorea	Oxyurida	Thelastomatidae	*Leidynema portentosae*	Yes	Invertebrates
Chromadorea	Oxyurida	Pharyngodonidae	*Parapharyngodon echinatus*	Yes	Vertebrates
Chromadorea	Rhabditida	Bunonematidae	*Bunonema franzi*	No	Soil, fresh water
Chromadorea	Rhabditida	Cephalobidae	*Eucephalobus oxyuroides*	No	Soil, fresh water
Chromadorea	Rhabditida	Panagrolaimidae	*Nematoda* sp.	No	Soil, fresh water
Chromadorea	Rhabditida	Rhabditidae	*Poikilolaimus oxycercus*	Yes	Invertebrates
Chromadorea	Rhabditida	Strongyloididae	*Strongyloides cebus*	Yes	Vertebrates
Chromadorea	Rhabditida	Teratocephalidae	*Teratocephalus lirellus*	No	Soil, fresh water
Chromadorea	Strongylida	Strongylidae	*Cylicocyclus insignis*	Yes	Vertebrates
Chromadorea	Strongylida	Ancylostomatidae	*Necator americanus*	Yes	Vertebrates
Chromadorea	Strongylida	Mackerrastrongylidae	*Tetrabothriostrongylus*	Yes	Vertebrates
Enoplea	Trichocephalida	Trichuridae	*Trichuris trichiura*	Yes	Vertebrates
Enoplea	Triplonchida	Prismatolaimidae	*Prismatolaimidae env. Sample**	No	Soil, fresh water
Chromadorea	Tylenchida	Aphelenchoididae	*Bursaphelenchus arthuroides*	Yes	Plants
Chromadorea	Tylenchida	Sphaerulariidae	*Deladenus siricidicola*	Yes	Invertebrates

Phylum Nematoda					
Class	Order	Family	Example of annotated Species	Family contains parasitic species	Habitat/host
Chromadorea	Tylenchida	Anguinidae	*Ditylenchus dipsaci*	Yes	Plants
Chromadorea	Tylenchida	Neotylenchidae	*Fergusobia* sp.	Yes	Invertebrates
Chromadorea	Tylenchida	Tylenchidae	*Filenchus discrepans*	Yes	Invertebrates
Chromadorea	Tylenchida	Allantonematidae	*Howardula phyllotretae*	Yes	Invertebrates
Chromadorea	Tylenchida	Meloidogynidae	*Meloidogyne ethiopica*	Yes	Plants

The number of reads for the 12 Nematoda orders sequenced from tortoise faecal samples collected across the Galápagos Islands is shown in Table 5.5 and Fig. 5.2. Taking into account all the tortoise populations the highest number of reads was for Ascaridida (4,864,273) and the lowest for Triplonchida (31). Of the five orders containing parasitic nematode of vertebrates the highest number of reads was also for Ascaridida and the lowest for Oxyuridida (1,660).

A graphical representation of the proportion of reads of the nematodes orders found by tortoise population and the nematode orders distribution is shown in Figs. 5.3 and 5.4. Of the five orders containing parasitic nematodes of vertebrates Ascaridida, Trichocephalyda Strongylida and Rhabditida were present in all tortotoise populations. Of these orders the first three have obligate parasitic species of vertebrates while the latter also contain free living species. Thus, analysis at the family level would provide a better resolution for identifying the distribution of Rhabditida nematodes parasitizing vertebrates.

Of the orders containing obligate parasitic species of vertebrates Ascaridida was the most abundant in terms of number of reads. Among islands it ranged from 2,092, 239 in Isabela to 93,473 in Santa Cruz west, for Thichocepahlyda it ranged from 38,669 in the Breeding Centre of Santa Cruz to 1,084 in Santa Cruz east, and for Strongylida the range was from 31,702 in the Breeding Centre of Santa Cruz to 226 in Santa Cruz east. Of the nematode Orders without parasitic species of vertebrates only one, Tylenchida, was found in all islands. The number of reads for this order ranged from 56,615 in the Breeding Centre of Santa Cruz to 28 in Española.

Table 5.5. Number of reads for Nematoda orders sequenced of tortoise faecal samples collected across the Galápagos Islands , *=orders containing parasitic nematodes, **orders containing parasitic and non parasitic nematodes/

	Breeding Centre	Española	Isabela	Santa Cruz east	Santa Cruz west	Santiago	Total
Araeolaimida	0	16	19,120	46	27	13,406	32,615
Ascaridida*	771,025	75,2832	2,092,239	93,473	551,714	602,990	4,864,273
Desmodorida	15	0	410	0	0	18,100	18,525
Dorylaimida	0	0	3273	0	0	0	3,273
Mermithida	991	0	0	0	1,349	2,191	4,531
Monhysterida	127	0	391	195	14,543	26,635	41,891
Oxyurida*	0	1,040	264	16	225	115	1,660
Rhabditida**	72,886	2,944	16,922	1,294	2,144	7,741	103,931
Strongylida*	149	54	178	226	880	180	1,667
Trichocephalida*	38,669	4,836	29,655	1,084	15,762	6,544	96,550
Trichocephalida	0	0	0	0	0	31	31
Tylenchida	56,615	28	9,848	36,566	43,180	3,107	149,344

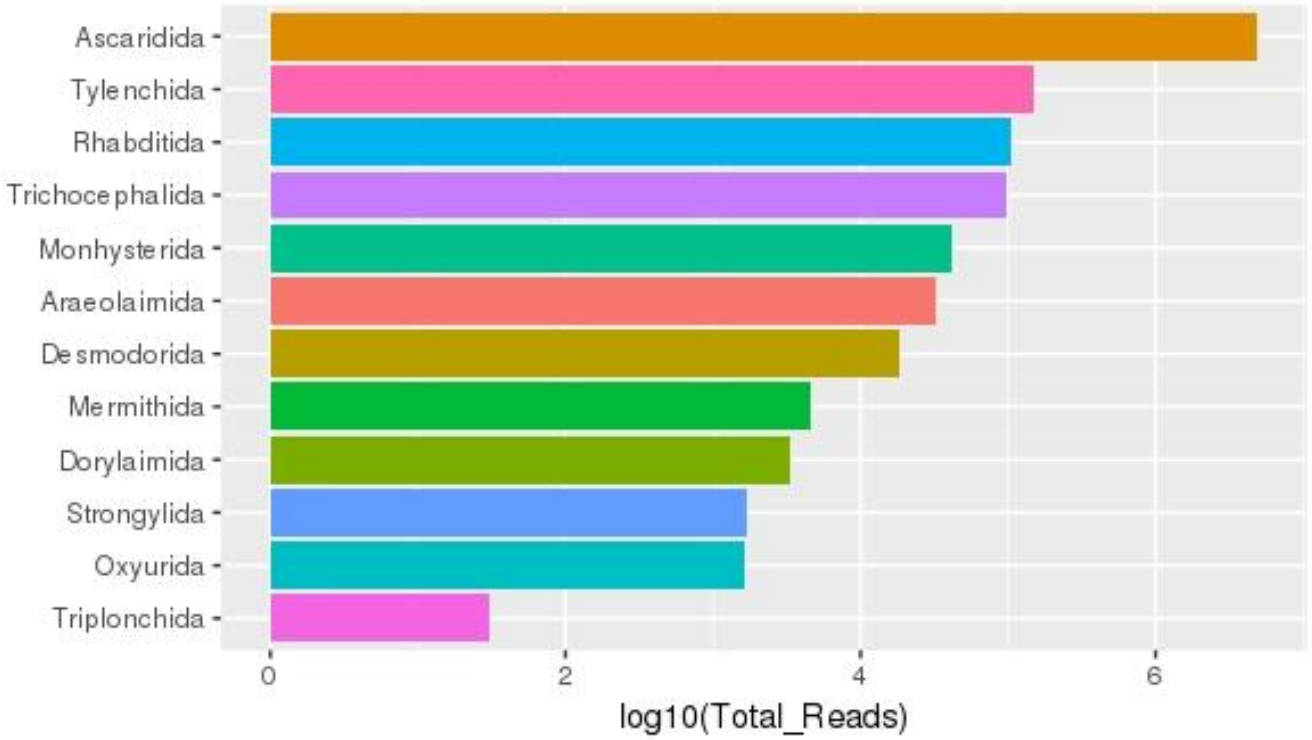


Figure 5.2. Abundance of sequences reads for nematode orders sequenced from tortoise faecal samples collected across the Galápagos Islands.

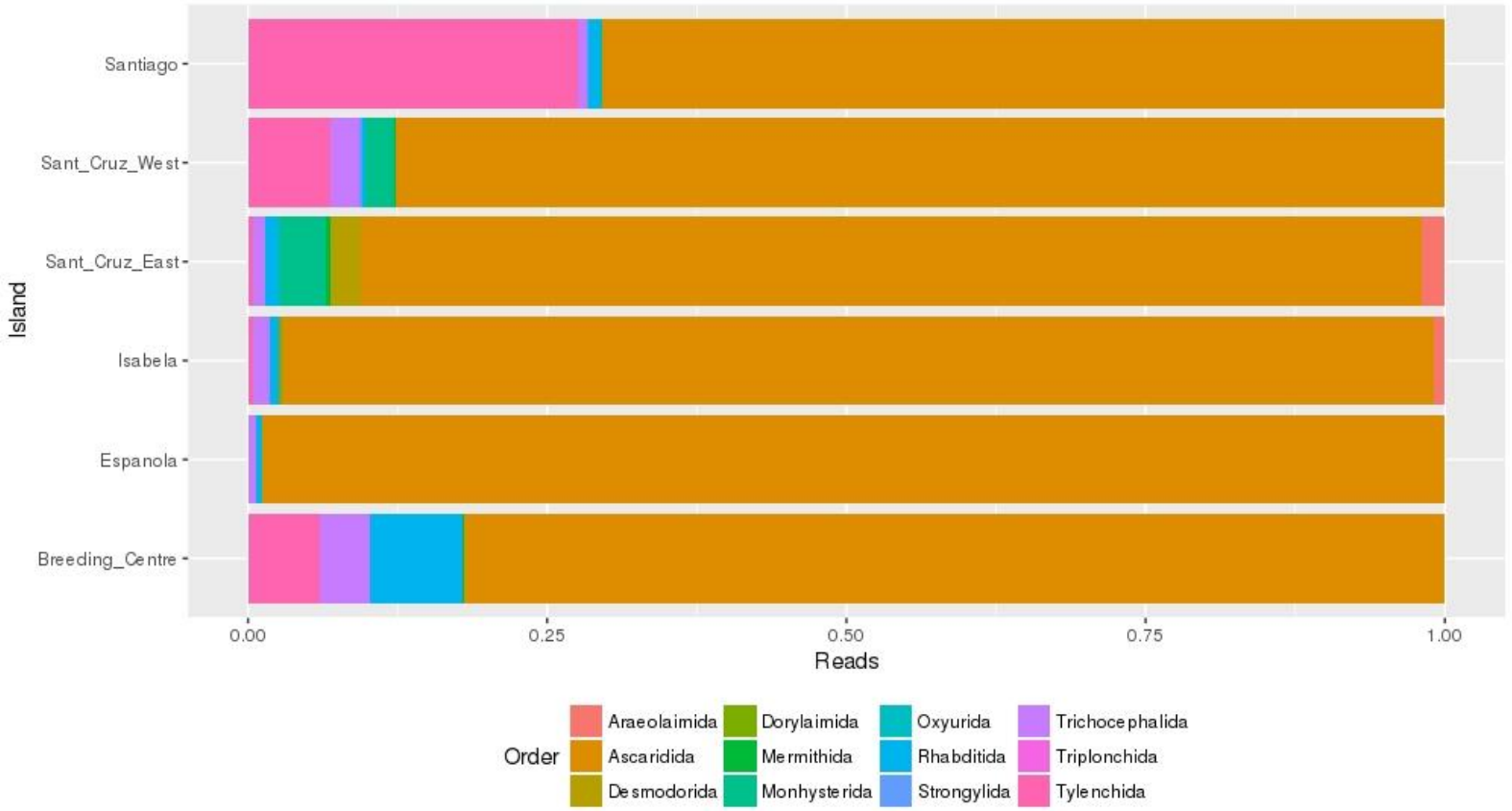


Figure 5.3. Proportion of sequence reads for nematode orders sequenced from tortoise faecal samples collected across the Galápagos Islands.

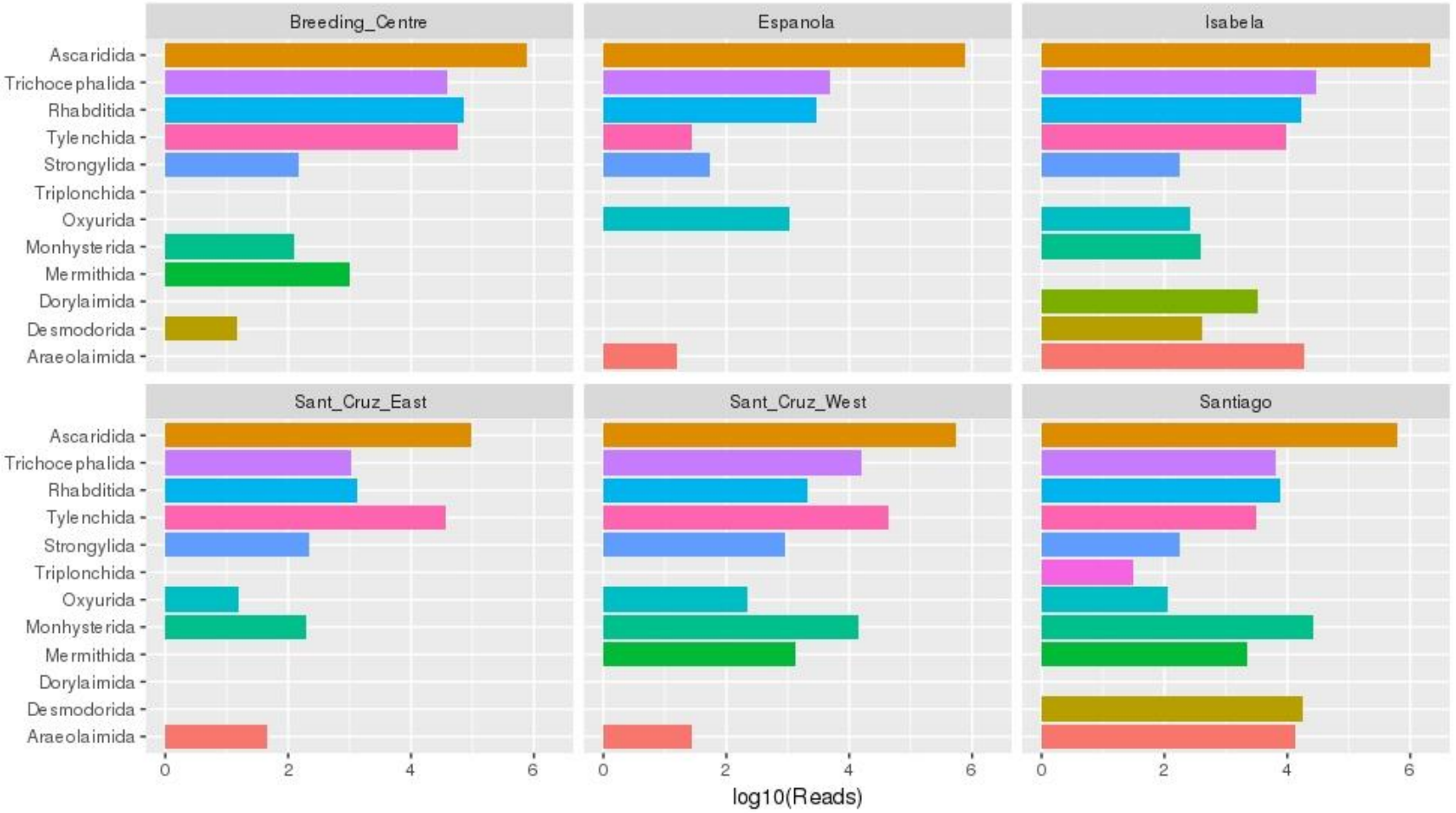


Figure 5.4. Distribution of nematode orders sequenced from tortoise faecal samples collected across the Galápagos Islands.

The number of reads of the 31 families identified across Galápagos tortoise populations and its graphical representation is showed in Table 5.6 and Fig. 5.5. Among all the families detected across tortoise populations Atractidae has the highest number of reads (4,161,755) and Cucullanidae had the lowest (11), both families belong to the order Ascaridida. Atractidae and Cucullanidae also had the highest and lowest number of reads respectively, when comparing the nine families containing parasitic species of vertebrates.

The proportion of reads of the nematodes families found by tortoise population and their distribution is given in Figures 5.6 and 5.7. Three out of nine families containing parasitic nematodes of vertebrates were detected in all the tortoise populations included in this study. Those families comprised Ascaridae and Atractidae (Ascaridida), Ancylostomatidae and Mackerrastrongylidae (Strongylida) and Trichuridae (Trichocephalida). The remaining nematode families containing parasite of vertebrates were detected at lower frequency: Cucullanidae (Ascaridida) was only detected in Española, Strongylidae (Strongylida) in Isabela and Santa Cruz east, and Strongyloididae (Rhabditida) in Isabela.

Of the vertebrate parasite families present in all islands, Atractidae was the most abundant in terms of number of reads. It ranged from 1,848,867 in Isabela to 82,325 in Santa Cruz east. The lowest abundance was for Mackerrastrongylidae ranging from 185 in Santa Cruz east to 11 in Isabela. For the parasitic families not present in all islands, Strongylidae had 19 reads in Isabela and 11 reads in Santa Cruz east, and Strongyloididae had 557 reads on Isabela.

Table 5.6. Number of reads of nematode families sequenced for tortoise faecal samples collected across the Galápagos Islands. The parasitic families are labelled with an asterisk.

Family	Breeding Centre	Española	Isabela	Santa Cruz east	Sant Cruz West	Santiago	Total
Allantonematidae	18	13	271	36,413	42,861	0	79,576
Ancylostomatidae*	28	43	0	30	740	0	841
Anguinidae	114	0	909	0	31	0	1,054
Aphelenchoididae	0	0	3,406	77	186	61	3,730
Ascarididae*	179,344	82,547	243,372	11,148	106,741	79,355	702,507
Atractidae*	591,681	670,285	1,848,867	82,325	444,962	523,635	4,161,755
Bunonematidae	0	0	861	0	28	0	889
Cephalobidae	0	372	5995	0	0	0	6,367
Cucullanidae*	0	0	0	0	11	0	11
Diplogasteridae	4,805	0	0	1,085	0	767	6,657
Dorylaimoidea	0	0	3,273	0	0	0	3,273
Mackerrastrongylidae*	121	11	159	185	140	180	796
Meloidogynidae	29	15	2,085	0	0	2,843	4,972
Mermithidae	991	0	0	0	1,349	2,191	4,531
Microlaimidae	15	0	410	0	0	18,100	18,525
Monhysteridae	127	0	391	195	14,543	26,635	41,891
Neodiplogasteridae	0	0	0	162	1,128	48	1,338
Neotylenchidae	56,454	0	0	76	78	0	56,608
Panagrolaimidae	41,063	2,241	176	0	142	5,175	48,797

Family	Breeding Centre	Española	Isabela	Santa Cruz east	Sant Cruz West	Santiago	Total
Pharyngodonidae*	0	983	0	0	0	0	983
Plectidae	0	16	18,838	46	27	13,406	32,333
Prismatolaimidae	0	0	0	0	0	31	31
Rhabditidae	27,018	331	431	24	107	1,751	29,662
Rhabdolaimidae	0	0	282	0	0	0	282
Sphaerulariidae	0	0	0	0	0	83	83
Strongylidae*	0	0	19	11	0	0	30
Strongyloididae*	0	0	557	0	0	0	557
Teratocephalidae	0	0	8,902	23	739	0	9,664
Thelastomatidae	0	57	264	16	225	115	677
Trichuridae*	38,669	4,836	29,655	1,084	15,762	6,544	96,550
Tylenchidae	0	0	3177	0	24	120	3,321

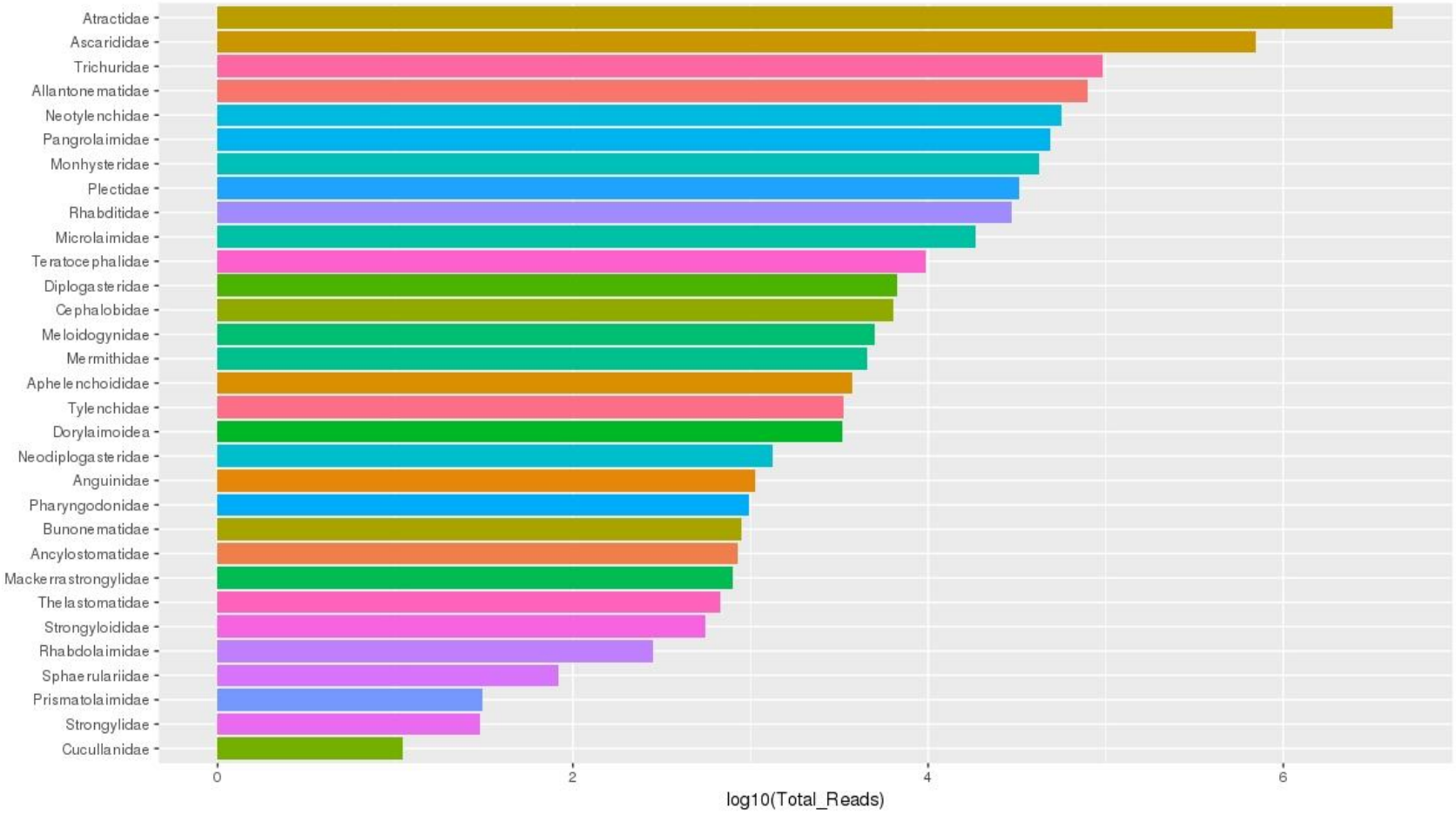


Figure 5.5. Number of reads for Nematoda families sequenced for tortoise faecal samples collected across the Galápagos Islands.

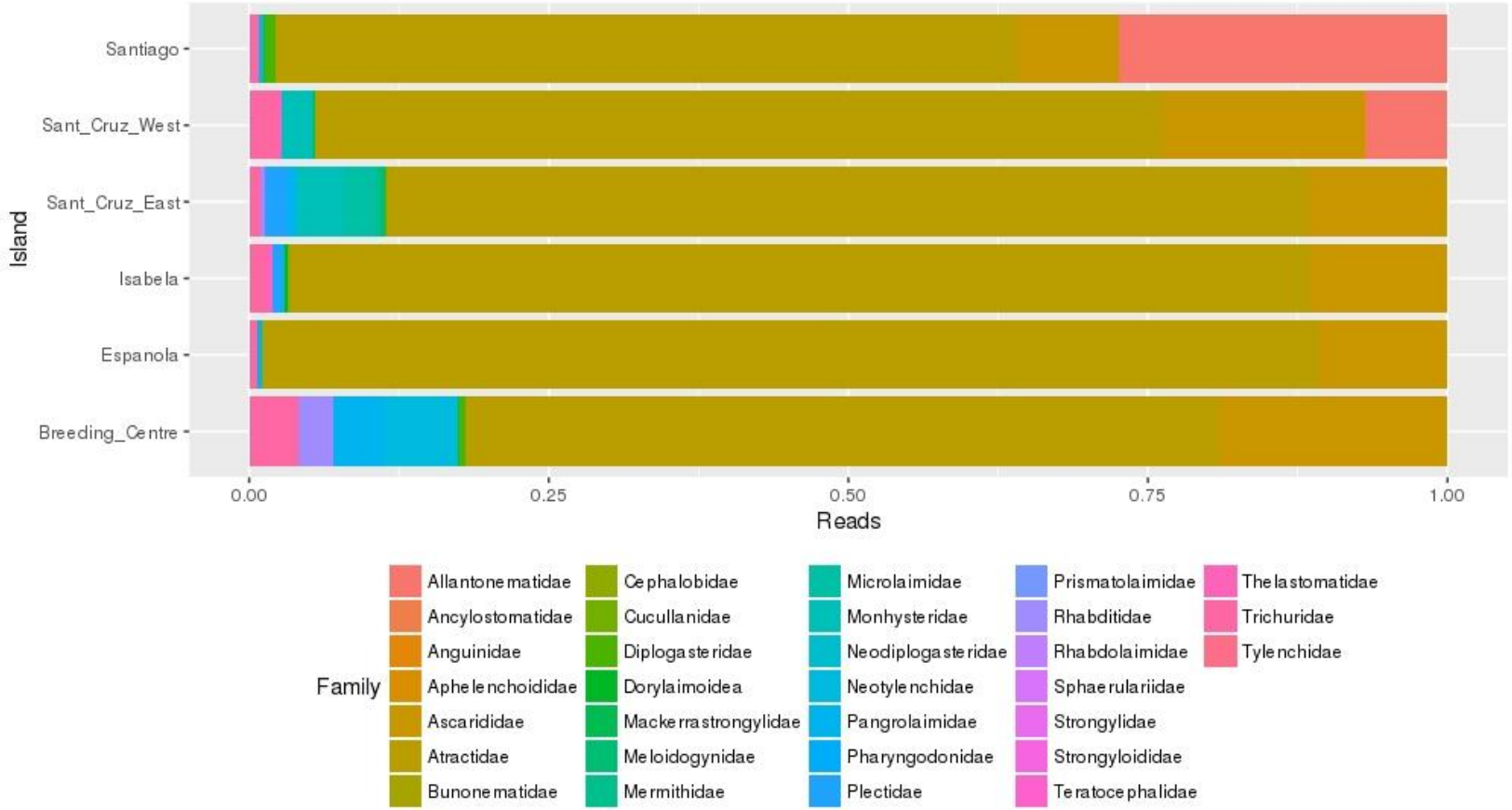


Figure 5.6. Proportion of sequence reads for nematode families sequenced for tortoise faecal samples collected across the Galápagos Islands.

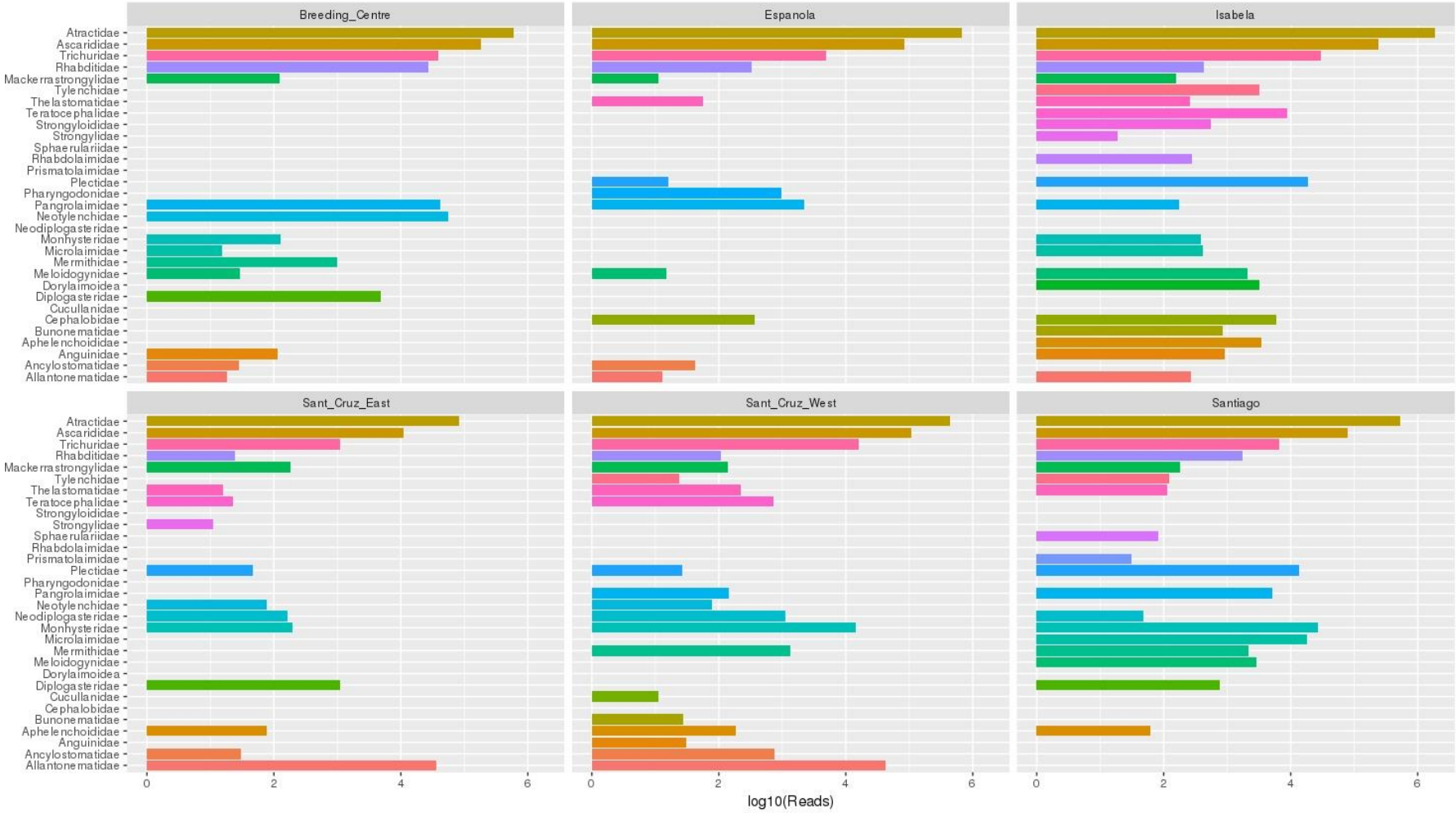


Figure 5.7. Distribution of nematode families sequenced for tortoise faecal samples collected across the Galápagos Islands.

The analysis at the genus level at the cutoff of 25 reads excluded two Nematoda families Strongylidae and Cucullanidae. Based on the remaining 29 families 48 genera were present across the Galápagos archipelago (Table 5.7). Seven taxa contain parasites of vertebrates and were represented by Ascaris and Porrocaecum (Ascarididae—Ascaridida), *Atractis*, *Labiduris* (*sensu latu*) and *Rondonia* (Atractidae—Ascaridida), *Necator* (Ancylostomatidae—Strongylida), *Tetrabothriostrongylus* (Mackerrastrongylidae—Strongylida), *Trichuris* (Trichuridae—Trichocephalida), *Parapharyngodon* (Pharyngodonidae—Oxyurida) and *Strongyloides* (Strongyloididae—Rhabditida).

The number of reads and a comparison of their abundance across the tortoise populations included in this study are also showed in Table 5.8 and Figure 5.8. Across the Galápagos the highest number of reads was found for Atractis (4,066,367) and the lowest for Zygotylenchus (27). With regard to the genera containing parasites of vertebrates the highest number of reads was also found for Atractis but the lowest for Parapharyngodon (983).

The proportion of reads for the nematode families found by tortoise population and their distribution is showed in Figures 5.9 and 5.10a and b. Of the genera containing parasites of vertebrates *Atractis*, *Labiduris* and *Trichuris* were present in all the tortoise populations sampled (Figure 5.9). Among populations the number of reads for *Atractis* ranged from 665,695 in Española to 78,839 in Santa Cruz east. The number of reads for *Labiduris* ranged from 53,175 in Isabela to 3,486 in Santa Cruz east. In the case of *Trichuris* the number of reads varied from 38,669 in the breeding centre to 1,084 in Santa Cruz east.

Table 5.7. Number of reads of nematode genera sequenced for tortoise faecal samples collected across the Galápagos Islands. The parasitic genera are labelled with an asterisk.

	Breeding Centre	Española	Isabela	Santa Cruz east	Santa_Cruz_ west	Santiago	Total
Acrobeles	0	0	5,995	0	0	0	5,995
Acrostichus	278	0	0	71	0	0	349
Aphelenchoides	0	0	944	26	43	61	1,074
Aporcelaimellus	0	0	2,462	0	0	0	2,462
Ascaris*	0	82,547	243,372	11,148	106,741	79,355	523,163
Atractis*	581,416	665,695	179,5615	78,839	426,762	518,040	4,066,367
Bitylenchus	44	0	124	0	0	11,212	11,380
Bunonema	0	0	861	0	28	0	889
Bursaphelenchus	0	0	0	0	143	0	143
Caenorhabditis	6,821	237	381	0	107	1,653	9,199
Ceratoplectus	0	0	153	0	27	0	180
Deladenus	0	0	0	0	0	83	83
Diplogaster	4,527	0	0	0	0	0	4,527
Ditylenchus	114	0	2,530	0	31	0	2,675
Eumonhystera	127	0	227	195	14,543	4,191	19,283
Fergusobia	56,454	0	0	76	78	0	56,608
Fictor	0	0	0	77	0	48	125
Filenchus	0	0	3,177	0	99	120	3,396
Geomonhystera	0	0	143	0	0	0	143

	Breeding Centre	Española	Isabela	Santa Cruz east	Santa_Cruz_ west	Santiago	Total
Howardula	0	0	271	36,413	42,861	0	79,545
Labiduris*	10,229	4,590	53,175	3,486	17,974	5,595	95,049
Leidynema	0	0	264	0	225	115	604
Meloidogyne	29	0	2085	0	0	2,843	4,957
Mermis	991	0	0	0	1,349	2,191	4,531
Mesodorylaimus	0	0	3,273	0	0	0	3,273
Mononchoides	0	0	0	85	1,128	0	1,213
Necator*	28	43	0	30	740	0	841
Neodiplogaster	0	0	0	340	0	0	340
Neotobrilus	0	0	0	674	0	767	1,441
Panagrolaimus	41,063	2,290	243	0	132	5,160	48,888
Parapharyngodon*	0	983	0	0	0	0	983
Plectus	0	0	1,8685	0	0	13,394	32,079
Poikilolaimus	0	0	0	0	0	59	59
Porrocaecum*	179,344	0	0	0	0	0	179,344
Prismatolaimidae	0	0	0	0	0	31	31
Prodesmodora	0	0	392	0	0	18,100	18,492
Rhabditis	20,197	69	50	0	0	39	20,355
Rhabdolaimus	0	0	282	0	0	0	282
Rondonia*	0	0	77	0	164	0	241

	Breeding Centre	Española	Isabela	Santa Cruz east	Santa_Cruz_ west	Santiago	Total
Strongyloides*	0	0	557	0	0	0	557
Teratocephalus	0	0	8,902	0	739	0	9,641
Tetrabothriostrongylus*	121	0	159	185	140	180	785
Thelastoma	0	57	0	0	0	0	57
Trichuris*	38,669	4,836	29,655	1,084	15,762	6,544	96,550
Tridentulus	0	0	0	0	0	22,444	22,444
Tylopharynx	0	0	0	0	1,669	0	1,669
Zeldia	0	372	0	0	0	0	372
Zygotylenchus	0	0	0	27	0	0	27

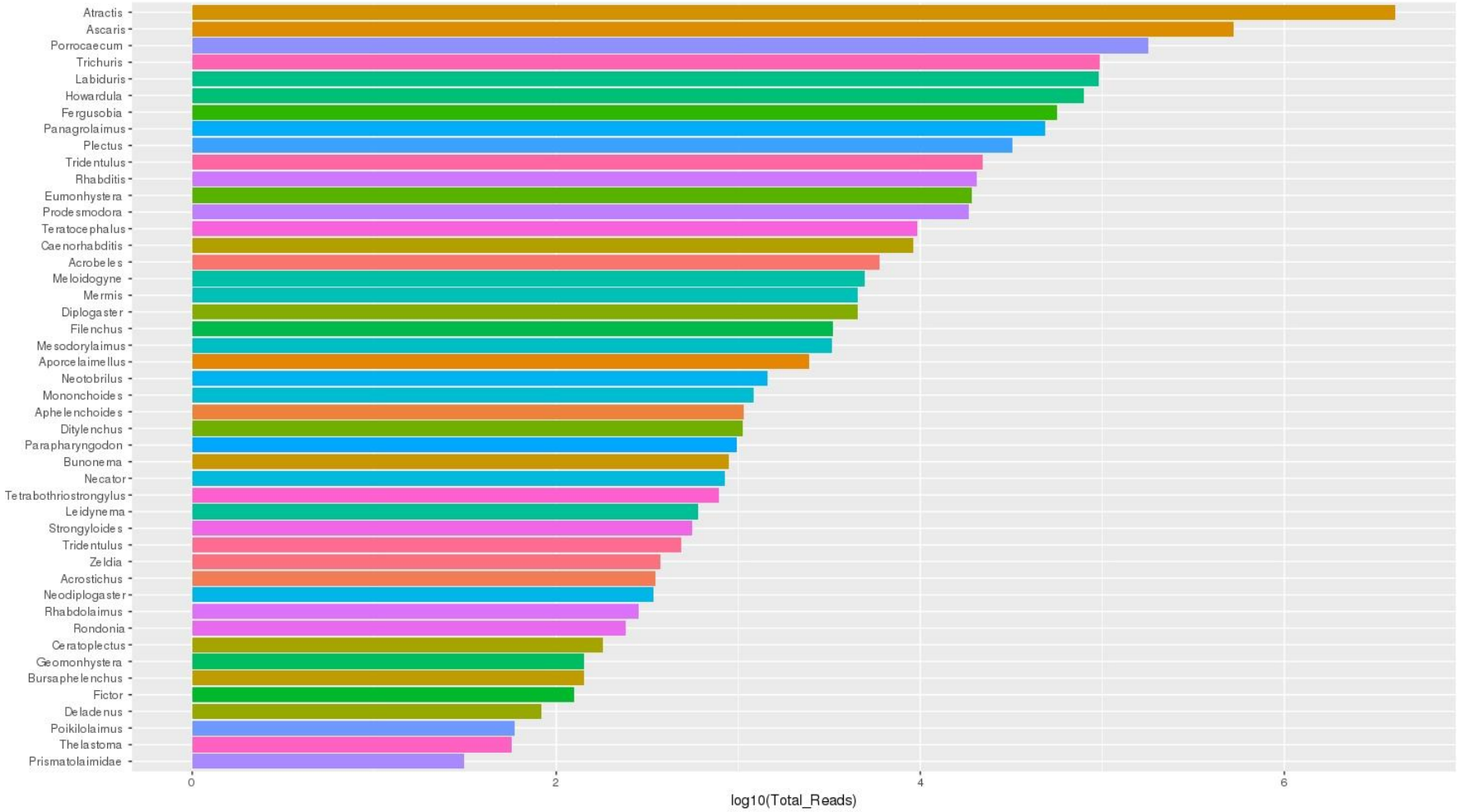


Figure 5.8. Number of sequences reads for nematode genera sequenced of tortoise faecal samples collected across the Galápagos Islands.

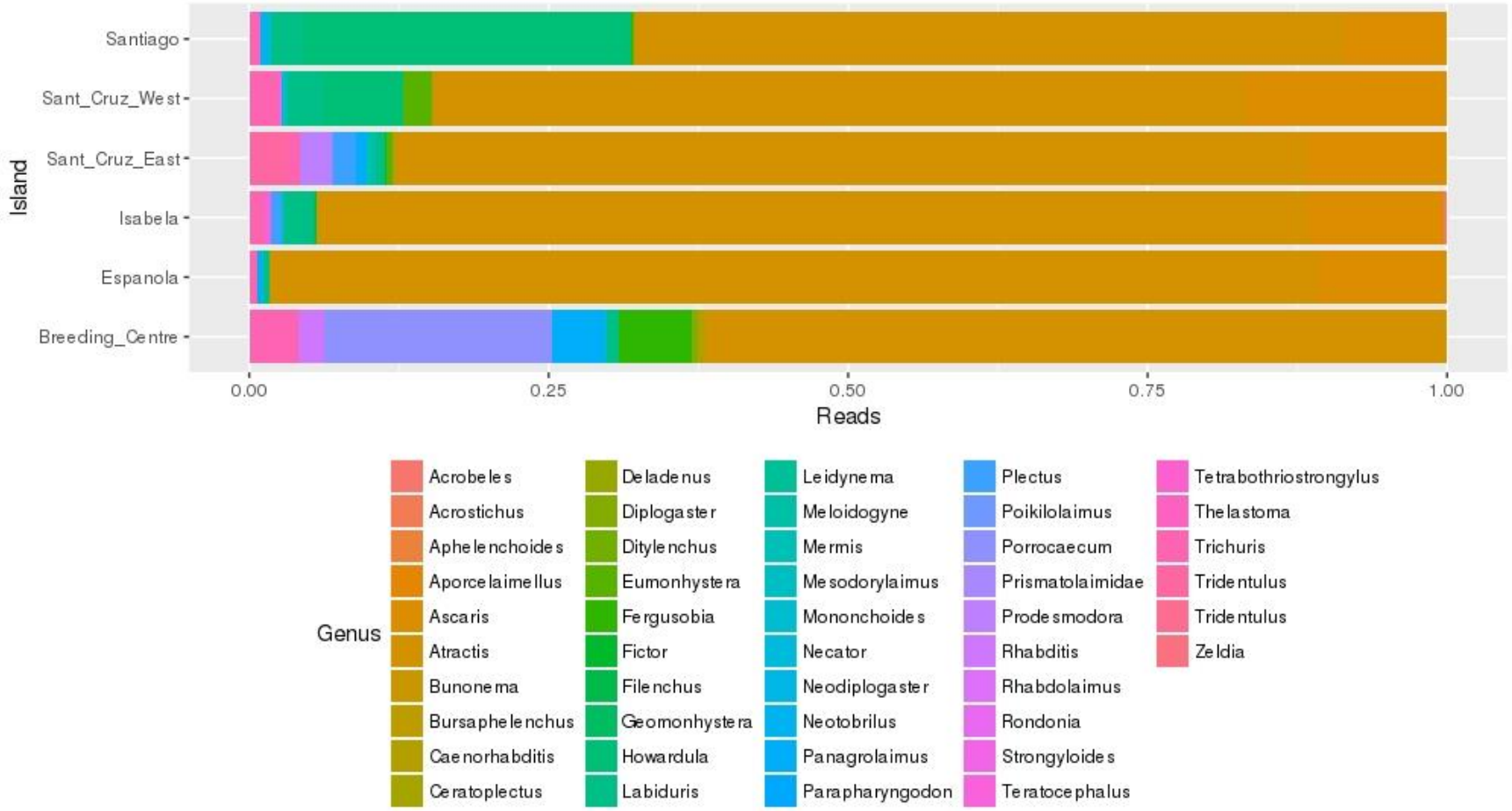


Figure 5.9. Proportion of sequence reads for nematode genera sequenced of tortoise faecal samples collected across the Galápagos Islands.

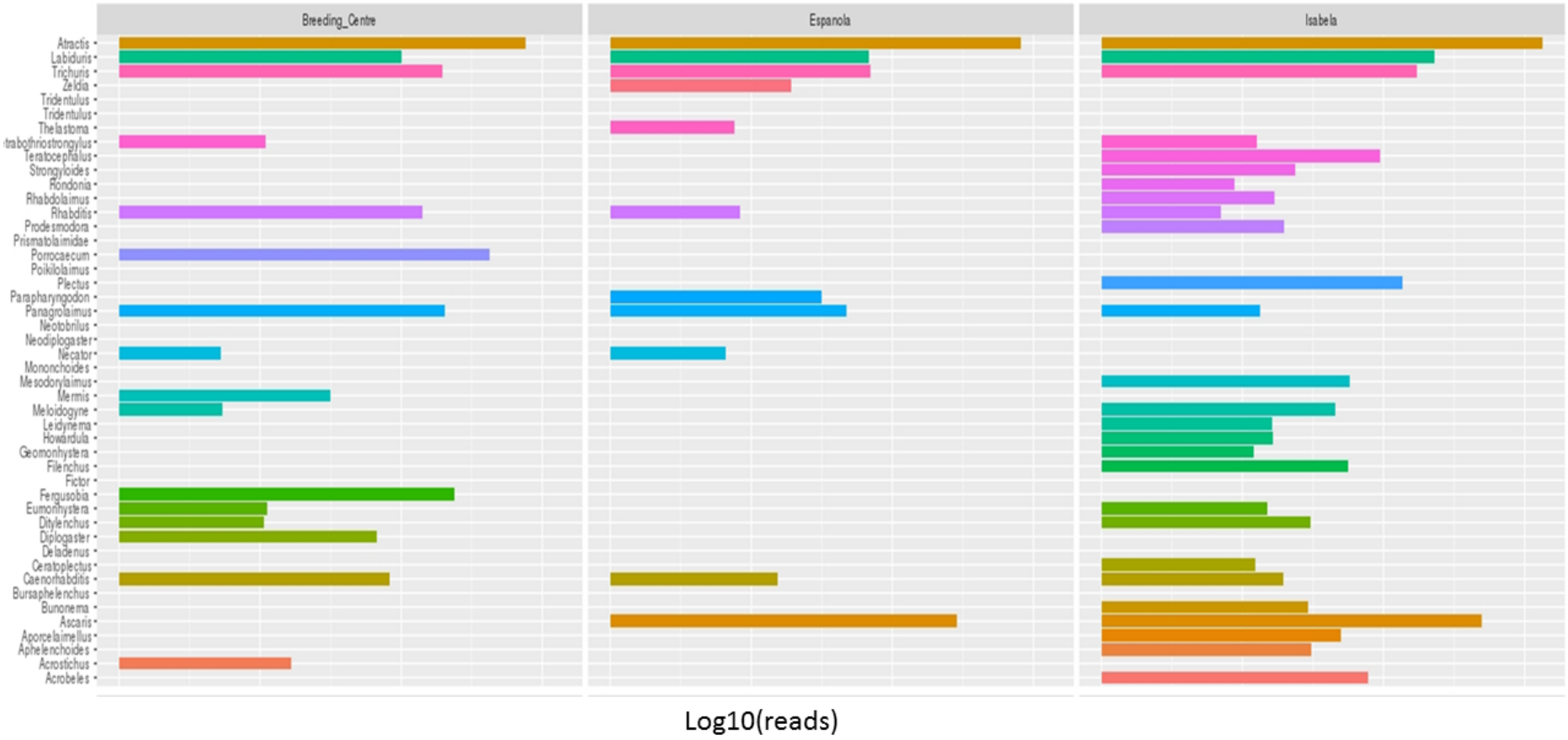


Figure 5.10a. Distribution of nematode genera sequenced for tortoise faecal samples collected across the Galápagos Islands.

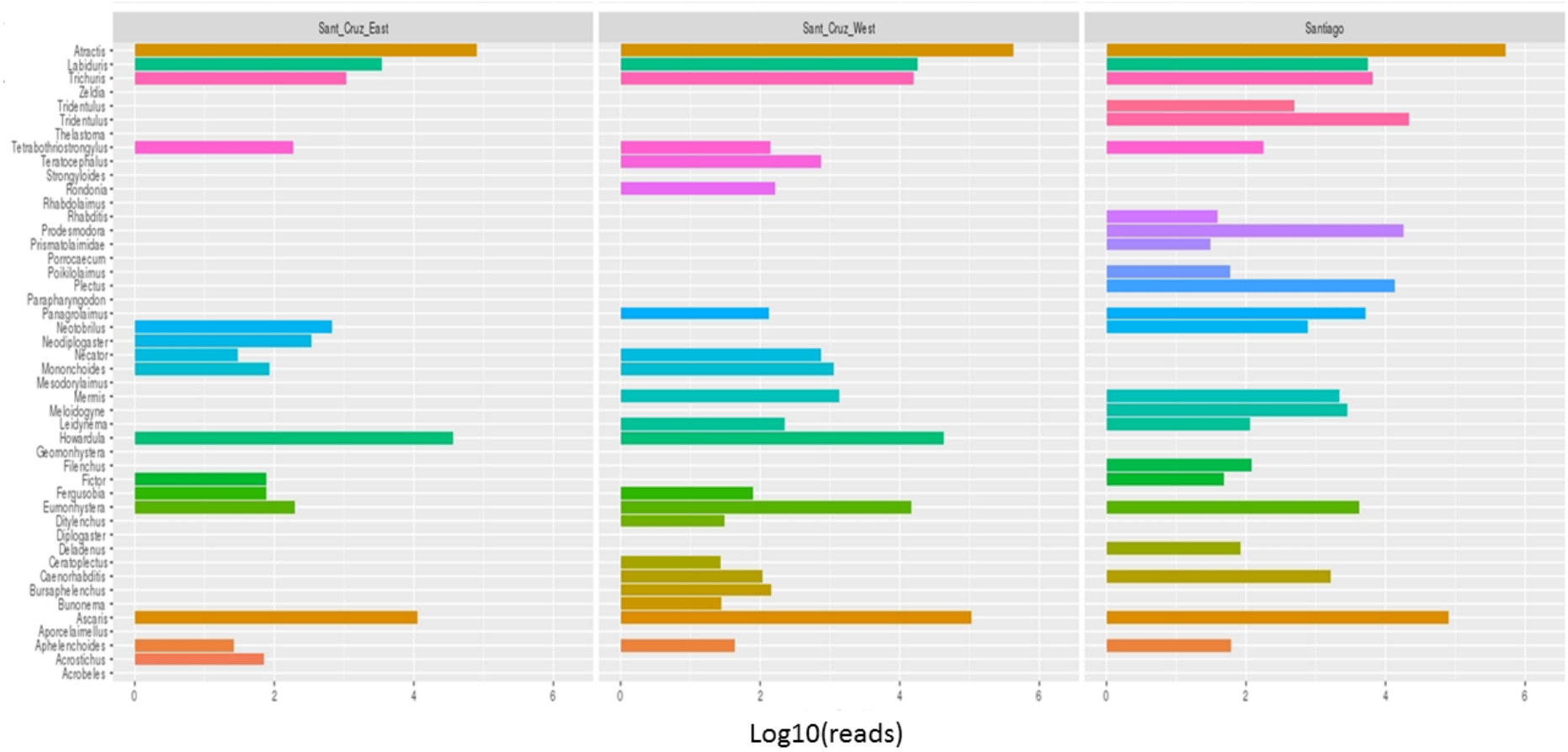


Figure 5.10b. Distribution of nematode families sequenced for tortoise faecal samples collected across the Galápagos Islands.

5.4. Discussion

Metabarcoding of faecal samples of *Chelonoidis* spp. allowed the taxonomic and biogeographical characterization of the helminth parasites infecting this species. This molecular technique far exceeded the resolution of the microscope analysis in characterizing the helminth community. It allowed detection of helminths that were missed during the microscope survey and provided a finer scale view of taxonomic diversity, potentially to the level of genus. Moreover, it also yielded an unexpected diversity of organisms representing not only helminths but at least 25 eukaryote phyla belonging to different kingdoms. Given that the focus of this thesis was on parasitic helminths and particularly on nematodes, the non-helminth sequences will not be discussed further here, and their detailed analysis will be left for future work.

In total the PCR sequencing of the 18S rDNA target yielded more than 24 million sequence reads. A maximum of ~28% of raw sequences was lost after merging forward and reverse reads and a further ~8% were discarded after filtering for chimeric sequences. The number of OTUs for all the eukaryotes taxa across all islands was 4169. The figures obtained before and after the processing of crude reads to obtain OTUs are concordant with previous studies of metazoan organisms including nematodes (Morise *et al.* 2012; Porazinska *et al.* 2010). The high percentage of sequences discarded during the merging process is attributed to no compatible overlaps of forward and reverse reads, or ambiguous sequence characters ("N"s). Despite the discard rate, the millions of remaining sequences obtained by NGS technology allowed characterisation of the target organisms in sufficient depth.

The parasitic helminth community of *Chelonoidis* spp. comprised the phylum Nematoda (classes Chromadorea and Enoplea); the phylum Platyhelminths (classes Cestoda and Trematoda) and the phylum Acanthochepala (class Palaecanthocephala). Nematode species were distributed across the tortoise population included in this study, while platyhelminths and acatochephalans were restricted to just one population. Cestoda (Cyclophyllidea—Anoplocephalidae—*Monieza*) and trematoda (Echinostomida—Philophthalmidae—*Philophthalmus*) were found only in Santa Cruz east and Palaecanthocephala (Echinorhynchidae—*Acanthocephalus*) was found solely

in Isabela. Finding Philophthalmidae sequences in faecal samples was unexpected. This trematode is commonly found infecting the eyes of avian and mammal species, it has and heteroxenous life cycle requiring a gastropod for its development and is unlikely to be passed through the faeces of their hosts (Church *et al.* 2013). In this thesis some faecal samples were collected from soil, so the finding of this parasite family might represent a contamination of faeces instead of tortoise intestinal infection.

The cestoda Cyclophyllidea—Anoplocephalidae and the Acanthocephalans have been reported parasitizing the intestines of reptiles. Steelman (1939) found a Cyclophyllidea parasite in the three toe box turtle (*Terrapene triunguis*) in Oklahoma. Anoplocephalidae is also common in other reptiles, in a study of pet reptiles imported to Slovenia, Rataj *et al.* (2011) found Anoplocephalidae parasites in Green Iguanas (Iguana iguana) and in the Sudan Spiny-tailed Lizards (*Uromastyx* spp.). Acanthocephala have been reported infecting at least a chelonian species, yellow-bellied terrapins *(Trachemys scripta)* from South Carolina (Esch *et al.* 1979). It has been also found in other reptiles including lizards and salamanders (Hughes and Moore 1943; McAllister and Bursey 2007). A definitive explanation for the restricted distribution of these parasites in Galápagos is elusive. The presence of the parasite in just one population might be due to accidental or incidental infections. Alternatively, its absence in other islands might be due to a failing to detect them as a result of a complex life cycle, location into their host and low egg shedding in faecal samples. Genera in both Anoplocephalidae and Acanthocephala have hetorexenous life cycles requiring a gastropod as an intermediate host – absence of suitable intermediate hosts could restrict their distribution. For both groups of parasites reptiles might act as a paratenic host (Jacobson 2007).

In relation to nematodes, the finer resolution of the metabarcoding method calls for a reassignment of the taxa observed originally by microscope. In comparison to Fournie *et al.* (2015) sequence analyses confirmed the morphological assignment of nematode eggs to three superfamiles (Ascaridoidea, Cosmocercoidea and Trichinelloidea), but not of other two Strongyloidea and Oxyuridea. According to sequence data the eggs assigned in Fournie *et al.* (2015) to “Strongyloidea” might be represented instead by three superfamilies, two into the order Strongylida (Ancylostomatoidea, and Trichostrongyloidea) and one into the order Rhabditida (Rhabditoidea). Oxyuridea

sequences were not detected in all tortoise populations as reported in chapter 4 and in Fournie *et al.* (2015) suggesting the eggs assigned to this taxa might have been misidentified. A review of pictures in papers presenting nematode eggs of reptiles including Rataj *et al.* (2011), Traversa *et al.* (2005) and Wolf (2015) suggest that the egg forms identified as Oxyuridea by Fournie *et al.* may instead be ciliate cysts. The metabarcoding analysis implemented in this thesis yield sequences of parasitic Oxyurida only on Española, and these sequences corresponded to the superfamily Thelasthomatoidea. Cilliate sequences were abundant in all islands.

Following the annotation step of the nematode sequences obtained by metabarcoding seven parasitic superfamilies of vertebrates, represented by seven families, and ten genera were identified. Altogether they comprised Ascaridoidea—Ascarididae (genera *Ascaris* and *Porrocaecum*); Cosmocercoidea—Atractidae (genera *Atractis*, *Labiduris* and *Rondonia*); Trichinelloidea—Trichuridae (*genus Trichuris*); Ancylostomatoidea—Ancylostomatidae (genus *Necator*); Trichostrongyloidea—Mackerrastrongylidae (*genus Tetrabothriostrongylus*); Rhabditoidea—Strongyloididae (*genus Strongyloides*); and Thelasthomatoidea—Pharyngodonidae (*genus Parapharyngodon*). Most superfamilies and families have been reported from chelonians (Jacobson 2007), except for Ancylostomatoidea which is commonly reported as a parasite of mammals (Chilton *et al.* 2006). Given that the annotation to OTUs relies on the sequences available in the GenBank the assignment to unexpected targets have to be interpreted with caution.

Given the reassignment of the nematode taxa identified by metabarcoding their distribution across the Galápagos was also reanalysed at a finer scale. Taking into account potential ambiguities in the classification of tortoise nematodes at the genus scale I will refer to this level only for *Atractis* and *Labiduris* (*sensu latu*) which have been confirmed to circulate in Galápagos tortoises on the basis of presence larval nematodes. In the other cases I will refer to families and where more than one genus was identified, I will refer to them as families/genus. Seven out of ten nematode parasitic taxa have widespread distributions in the tortoise populations included in this study. They comprised *Atractis*, *Labiduris* and Trichuridae found in all tortoise populations, and Ascarididae-*Ascaris*, Mackerrastrongylidae and Ancylostomatidae, found in a least four populations. The remaining nematode taxa were identified just in

one tortoise population. Pharyngodonidae was found only on Española, Ascarididae-*Porrocaecum* was found only in the Santa Cruz breeding centre, Atractidae-*Rondonia* was found in Isabela and Santa Cruz west and *Strongyloides* were detected only in Isabela.

In comparison with microscope data there was a clear concordance only in the distribution of *Atractis*. Diverse families comprising Mackerrastrongylidae (Trichostrongyloidea—Strongylida), Ancylostomatidae (Ancylostomatoidea—Strongylida) and Strongyloididae (Rhabditoidea—Rhabditida) might represent the eggs previously assigned to the Superfamily Strongyloidea (Strongylida) in Fournie *at al.* (2015). In that study Strongyloidea eggs were found across all the populations examined, which is concordant with the distribution of OTUs assigned here to Mackerrastrongylidae. Metabarcoding revealed that Strongyloididae was present only on Isabela. This superfamily might corresponded to the egg of different size observed in this island by Fournie *et al.* (2015).

Although the eggs of Strongyloididae (Rhabditida) and those from Strongylida overlap in size, the eggs of Strongyloididae (Rhabditida) can be differentiated by the presence of larvae. The pictures displayed by Fournie *et al.* (2015) show that the egg labelled as "small strongyle" contain a developing nematode larvae inside it. In relation to the inconsistency of finding Ancylostomatidae in tortoises I revisited the BLAST output for these OTUs. Beside to having 100% of nucleotide identity with Ancylostomatidae, they also have the same percentage of identity with *Chabertia ovina*, another member of the Strongylida parasitizing mammals. Therefore OTU assignment from metabarcoding data is not trouble free and accurate annotation may require individual review of multiple top sequence matches. One disadvantage is the limited resolution of the 18S rRNA gene among closely related species, the short length of the sequence obatained with the current NGS technology and the conserved nature of certain region of the gen (Wu *et al.* 2015). Additional problems may arise due to PCR or sequencing errors. Although denoising and chimera checking can reduce the number of potentially spurious sequences arising from both PCR and sequencing errors, such processing cannot eliminate all biases (Poretsky *et al.* 2014). Given this known problems with metabarcoding, whether the OTUs related to Ancylostomatidae/*Chabertia* is a real sequence needs to be further investigated.

A striking difference between the metabarcoding method and the coprological studies of Fournie *et al.* and from Chapter 4, is the relative abundance of Ascaridida and "Strongylida" in the Galápagos tortoise nematode community. By microscope, Strongylida were the most commonly egg found, by metabarcoding Ascarids sequences were several order of magnitude more abundant. It is still unclear whether the number of sequences found in metabarcoding methods is proportional to the number of individuals present in one sample, especially when dealing with sequences of rDNA characterised by their high abundance (Porazinska *et al.* 2009), other factor to consider is a potential variation in the copy number of this gene in different taxa (Torres-Machorro *et al.* 2010). Nevertheless, it worth to mention some technical factors that might contribute to this disagreement. Firstly, there may be underestimation of Ascarid eggs with the coprological technique used in this thesis. The observation of some parasites by coprological methods depends on the amount of the material examined, use of sample dilutions, whether or not samples are concentrated via centrifugation, the length of time allowed for flotation, and the type and specific gravity of the flotation solution used (Ballweber 2014). Another factor to consider is that the faecal sample used for metabarcoding were frozen and it could have caused the rupture and loss of some Strongylida eggs, which could be more frail with respect to freezing conditions, compared with Ascarid eggs (van Wyk and van Wyk 2002).

Giving the wide distribution of Ascarididae finding an alternate ascarid ('*Porrocaecum*') in the Santa Cruz breeding centre, is noteworthy. The distribution pattern of these nematode sequences might have result from and local acquisition or could represent and ecological exclusion of these nematode genera. Alternatively, it might have result from a sequencing error. One way to identify sequencing errors from real sequences is through the number of reads obtained for the putative taxa. For classification of OTUs at the genus level I used a cuttof > 25 sequences, which mean that only sequences present in that amount in individual faecal tortoises, will be considered in the analysis. The number of sequences retrieved for *Porrocaecum* was 179,344; thus, it could be considered a real sequence. This genus have been reported parasitizing reptiles, in Arkansas-North America, including anurans, lizards, and snakes (McAllister 2015).

With regard to wild Galápagos tortoise species, the wider distribution of most parasitic nematode taxa support the idea that for this reptile species the colonization of new areas has little influence in the distribution of its parasites. The finding of unique parasite sequences with restricted distribution suggests that local acquisition of parasites would be possible. However, it need to be looked on a case by case basis. In comparison with the results of coprological methods used in chapter 4 and in Fournie *et al.* the metabarcoding analysis performed here confirms that the reintroduced tortoises of Española carry a higher diversity of nematodes than initially detected by coprological method.

The primers used for metabarcoding of nematodes of Galápagos tortoises have been used previously for phylogenetic studies of these worms. In this thesis and given the complex mixtures of DNA co-extracted from faecal samples, these amplified DNA from many other eukaryotes, yielding sequences of aditional metazoa phyla. On the negative side this might have reduced the sequencing deep of nematode taxa, while in the positive they provide additional information about other eukaryotic microbiota and might enrich the understanding of the greater animal community present in the Galápagos tortoise gut. Since no faecal samples could be collected from wild tortoises in San Cristobal, and because no nematode eggs were detected in samples from the San Cristobal breeding centre, the San Cristobal faecal samples were not included in the metabarcoding. This was to allow for greater sample sizes for other populations. Given the high diversity of other organisms found in tortoise faeces, and the sensitivity of metabarcoding, this was an unfortunate decision in hindsight.

Metagenomic analyses aimed specifically at prokaryotic communities have already expanded the dimensions of known microbial diversity by several orders of magnitude. Similarly, metabarcoding aimed at nematodes has the potential to greatly expand our understanding of another diverse component of the microbiota. The combination of coprological and metabarcoding analysis outlined here provides a new tool for characterizing nematode communities infecting Galápagos tortoise, but is potentially useful for other wild hosts. It has various advantages over lethal sampling methods including the ability to sample large numbers of hosts. Given that helminth co-infection is pervasive in wildlife (Petney & Andrews 1998), this approach can help extend our basic understanding of fundamental aspects of host–nematode interactions in a range

of wildlife systems. Overall, this combined parasitological and molecular strategy is a viable tool for non-invasive studies of species-specific nematode abundance and community composition in wild hosts. Some drawbacks might still be present and might include the failing to detect parasites a lower prevalence and abundance, however the lack of parasite detection could be overcome is large number of samples are collected.

Chapter 6. General Discussion

A central theme in ecology is to understand the distribution and abundance of organisms and the factors influencing this patterns. Human and livestock parasites have received much attention in this context but less attention has been paid to parasites infecting wildlife species, despite their potential significance for conservation biology (Esch 1990; Poulin 2007). Parasites play an important role in shaping the population dynamics of their hosts and are important evolutionary drivers of biodiversity (Anderson & May, 1978; Dobson & Hudson, 1986). At the same time parasites and their hosts represent evolved communities which need to be maintained (Koh *et al.,* 2004). These evolved relationships are frequently overlooked in conservation programmes involving the movement of animals between populations or the repatriation of captive individuals, leading to 'parasite pollution' and disruption of natural parasite communities (Cunningham *et al.,* 2003; Gompper & Williams, 1998; Koh *et al.,* 2004).

This thesis investigated neglected parasites of the Galápagos giant tortoise (*Chelonoidis* spp.). I assessed the taxonomic identity and biogeography of blood parasites, *Amblyomma* ticks and gastro-intestinal helminths infecting this reptile. For blood parasite and ticks I assessed whether they have concordant phylogeographic patterns with their tortoise host. In the case of helminths I examined whether host migration and local ecology determine their distribution and whether these factors are important determinants for the formation of helminth communities. I compared the use of traditional parasitological methods with metabarcoding of faecal samples to provide information on parasite communities. Ultimatelly I discussed the findings in the context of the ecology of the Galápagos Island and the evolution and conservation management of the charismatic Galápagos tortoises.

6.1. Taxonomic and biogeographical characterisation of Galápagos tortoise parasites

Based on microscope and phylogenetic analysis of 18S rDNA the blood parasite infecting *Chelonoidis* spp. was identified as a haemogregarine of the genus *Hepatozoon* syn *Bartazoon* (Karadjian *et al.,* 2015). This haemogregarine differs morphologically from haemogregarines reported from Galápagos lava lizards (Microlophus spp.) and Galápagos marine iguanas (*Amblyrhynchus* spp.) and

genetically from haemogregarines reported from Galápagos land iguanas (*Conolophus* spp.) (Ayala and Hutchings 1974; Fulvo 2010). Tortoise haemogregarines have an impoverished diversity compared to those of land iguanas, being represented by just two haplotypes restricted to the northern volcanoes of Isabela (Alcedo, Darwin and Wolf). In contrast the land iguanas have 18 haplotypes, and haemogregarines are found in all populations of land iguanas across the Galápagos archipelago.

Morphological and molecular analysis of the COI gene of ticks confirmed their previous classification as belonging to the genus *Amblyomma* (Keirans *et al.* 1973). Keirans described three *Amblyomma* species infesting *Chelonoidis* spp: *A. macfarlandi* found in Wolf, Darwin and Alcedo volcanoes of Isabela Island, *A. usingeri* found in volcano Cerro Azul of Isabela and in Santa Cruz Island, and *A. pillosum* detected in Pinzon and Santiago. In this study ticks infecting Galápagos tortoises were found only on four populations, two of Isabela Island (Wolf and Alcedo), and the tortoise populations of Santiago and Pinzón. Analysis of COI and D-loop sequences of tick specimens from these tortoise populations revealed a substantial genetic differentiation of 4.3% between ticks of Pinzon ad Santiago which would warrant the assignment of distinct species status to the ticks of each of these islands.

The combination of traditional parasitological methods with metabarcoding of faecal samples of Galápagos tortoises allowed the taxonomic and biogeographical characterization of the helminth parasites infecting this species. Microscope surveys of tortoise faecal samples revealed the presence of at least five nematode superfamilies, while metabarcoding analysis yielded an unexpected diversity of eukaryotic organisms from at least 25 phyla, inhabiting the tortoise gut or associated with their faeces. The data should ultimately allow most of these taxa to be distinguished to at least the genus level.

Of known parasitic nematodes, microscope and metabarcoding analysis suggest the presence of at least ten genera representing seven families and seven superfamilies. Altogether they comprised *Ascaris* and *Porrocaecum* (Ascarididae—Ascaridoidea—Ascaridida), *Atractis*, *Labiduris* and *Rondonia* (Atractidae—Cosmocercoidea—

Ascaridida), *Necator* (Ancylostomatidae—Ancylostomatoidea—Strongylida), *Tetrabothriostrongylus* (Mackerrastrongylidae— Trichostrongyloidea—Strongylida), *Trichuris* (Trichuridae—Trichineloidea—Trichocephalida), *Parapharyngodon* (Pharyngodonidae—Thelasthomatoidea—Oxyurida) and *Strongyloides* (Strongyloididae—Rhabditoidea—Rhabditida).

According to metagenomic analysis three genera *Atractis*, *Labiduris* and *Trichuris* were found in all of the tortoise populations sampled for this study. Three were present in a least four islands and comprised *Ascaris*, absent from the breeding centre of Santa Cruz, *Tethrabosthriostrongylus*, absent from Española and *Necator*, absent from Isabela and Santiago. Three genera were less common and comprised *Parapharyngodon* found only on Española, *Porrocaecum* found only in the breeding centre, *Rondonia* and *Strongyloides* detected only in Santa Cruz west. The metagenomic analysis also revealed the presence of other parasitic helminths. Those comprised Platyhelminths of the classes Cestoda and Trematoda found in the tortoise population of Santa Cruz west and a parasite of the phylum Acanthocephala, detected in Isabela Island.

6.2. Analysis of co-phylogeography between haemogregarines, ticks and their hosts

Using the novel taxonomic and biogeographical characterization of *Chelonoidis* spp. parasites, I assessed to what extent the evolutionary history of haemogregarines, ticks and their tortoise hosts are concordant. In the case of haemogregarines the co-evolution question could not be answered because of its narrow distribution in the archipelago. Nevertheless, the results obtained for this parasite allow drawing hypothesis about their colonization history. Tortoises carried a haemogregarine haplotype genetically distinct to those found in the extensive analysis of land iguanas suggesting the haemogregarines found in tortoises could be exclusive for this reptile. Haemogregarines, however, have a heteroxenous life cycle requiring also a haematophagous invertebrate (e.g. mosquito, simuliid fly, tick, leech) for completing their life cycle and acting as a vector (Telford 1984). The complex life cycle of haemogregarines, and if they are exclusive parasites of *Chelonoidis* spp., implies a concerted colonization of the Galápagos involving tortoises, haemogregarines and its final host.

In this thesis *Amblyomma* ticks are proposed as the vector of tortoise haemogregarines. Ticks of the genera *Amblyomma* and *Hyalomma* have previously been identified as final hosts for *Hepatozoon* (Kim *et al.* 1998; Telford *et al.* 2012; Wozniak *et al.* 1994a). DNA sequences of Galápagos tortoise haemogregarine were found in two ticks collected from tortoises of Wolf Volcano which suggests that *A. macfarlandi* could be a vector of the haemogregarine parasite. Despite this result it was striking to find only two infected ticks in Isabela and a lack of haemogregarines in Santiago and Pinzon where *A. pilosum* (*sensu latu*) circulate.

The finding of only two *A. usingeri* specimens infected with haemogregarines might be due to a lack of sampling effort and poor preservation of samples. During 2005-2006 there was no systematic collection to identify ticks by host and to identify which came from infected tortoises and during that time samples were kept in suboptimal conditions. This was not the case for ticks collected during 2012-2015 in Pinzón and Santiago. On those islands hemogregarines were not detected even in blood films. An explanation for the lack of haemogregarines in tortoises of Pinzón and Santiago is elusive. It might involve the lack of competence of *A. pilosum* for transmitting the parasites, or the extinction of the parasite due to human impacts. Santiago and Pinzon represent two of the more highly disturbed islands by human activities. In both islands tortoises have been reduced to very low numbers, for Santiago one species of iguana has gone extinct.

There was no agreement between the phylogeography of ticks and their tortoise hosts. Previous genetic studies of tortoises suggest that tortoises from Santiago Island colonized volcano Wolf on Isabela Island, while tortoises from other islands, presumably from Santa Cruz, colonized Alcedo and the other southern volcanoes of Isabela (Caccone *et al*. 2002; Poulakakis *et al*. 2012). In contrast to their tortoise hosts, the results of this thesis show no evidence that tortoise ticks from Wolf (*A. usingeri*) originated from Santiago. Moreover, the data suggest that *A. usingeri* colonized Isabela in a single event and then dispersed to different volcanoes (Wolf and Alcedo). This differs from the tortoise colonization model on this island which suggests it occurred in two independent occasions (Poulakakis *et al.* 2012; Ciofi *et al.* 2006).

In host-parasite assemblages the degree of phylogenetic congruence tends to increase with the obligate character of the parasite (Hafner and Nadler 1990). This concordance is known to decrease if the parasite is either not specific or heteroxenous (Nieberding *et al.* 2004a).(Barbosa *et al.* 2012; Crandall *et al.* 2008). The question of whether ticks are host specialists or host generalists has been subject to much debate over the last half-century. However, recent studies suggest that there are a range of specialist and generalist species. If the latter holds for these ticks, the lack of congruence between ticks and their tortoise hosts suggest that ticks of tortoises could have been acquired from another Galápagos species such as land iguanas. This would be favoured in places where the taxa are syntopic such as Wolf volcano on Isabela Island.

Tick haplotypes collected from Galápagos land iguanas on volcano Wolf clustered with tick haplotypes of Galápagos tortoises sampled there. This result suggests the sharing of ticks among these taxonomic different hosts. This is further supported by Keiran *et al*. (1973), who described *A. usingeri* from tick samples collected from both reptile hosts. No land iguanas live nowadays in Pinzón or Santiago but they were reported inhabiting Santiago in 1853 (Snell *et al.* 1984). Strikingly, 2 tortoise tick haplotypes sampled in Wolf also clustered with haplotypes of Pinzón. This could represent a human mediated translocation from Pinzón to Wolf through the movement of tortoises or introduced ungulates (Carrion *et al.* 2007; Townsend 1925). Sharing of Galápagos tortoise ticks with other Galápagos reptiles would be also possible. Nevertheless different tick species have been described for them. *A. darwini* and *A. williamsii* have been described infesting marine iguana (Bequaert 1932), *and A. boulengeri* for lava lizard. A sequence of a marine iguana tick was obtained in this study. As expected from former descriptions, it clustered in a different clade of that of Galápagos tortoise ticks.

6.3. Comparison of traditional parasitological methods with metabarcoding

Next generation sequencing was superior in comparison with microscope analysis for characterizing the helminths of Galápagos tortoise present in faecal samples. The first method allowed detecting helminths that were missed during the microscope survey, moreover, allowed for taxonomic resolution at the level of genus and led to the reassignment of some parasites misidentified with the microscope method. NGS

confirmed the presence of three superfamiles or orders identified by microscope methods (Trichuroidea, Ascaridoidea, Cosmocercoidea and Strongylida but not Oxyuroidea.

According to sequence data the strongyle eggs observed by microscope seem to represent three superfamilies, two in the order Strongylida (Ancylostomatoidea—Ancylostomatidae, Trichostrongyloidea—Mackerrastrongylidae) and one in the order Rhabditida (Rhabditoidea—Strongyloididae). Oxyurid sequences were not detected with the frequency reported by microscope analysis which indicates the eggs assigned to this taxa were misidentified. In this thesis Oxyurids were found only on Española and assigned to the family Pharyngodonidae (Thelasthomatoidea—Oxyurida). It differs from the assignment in Fournie *et al*. which was given to Oxyuridea.

The primers used for NGS have been used previously for phylogenetic studies of nematodes. In this thesis and given the complex mixtures of DNA co-extracted from faecal samples, the primers amplified many other eukaryotes yielding sequences from at least 25 phyla. On the negative side this might have reduced the number of nematode sequenced, while on the positive side this provides information about other eukaryotic microbiota and might enrich the understanding of the greater community present in the Galápagos tortoise gut. Because of the lack of faeces from wild tortoises on San Cristobal, and the absence of nematode eggs in the faecal samples from the San Cristobal breeding centre, the San Cristobal breeding centre samples were not included in the NGS analyses to allow increased sample sizes for other populations. Given the high diversity of parasites found, this was an unfortunate decision in hindsight.

The combination of coprological and metabarcoding analysis outlined here provides a new tool for characterizing nematode communities infecting wild hosts. It has various advantages over lethal sampling methods including the ability to sample large numbers of hosts. Given that helminth co-infection is pervasive in wildlife (Petney & Andrews 1998), this approach can help extend our basic understanding of fundamental aspects of host–nematode interactions in a range of wildlife systems. Overall, this combined parasitological and molecular strategy is a viable tool for non-invasive studies of species-specific nematode abundance and community composition

in wild hosts. Some drawbacks might still be present and might include the failure to detect parasites at lower prevalence and abundance, however the lack of parasite detection could be overcome if a large number of samples are collected.

6.4. Factors influencing parasitic helminth distribution

After analysing co-phylogeographic signals of haemogregarines and ticks with their tortoise hosts I examined whether tortoise colonization history and local ecology determine the distribution of their parasitic helminths. I also investigated whether these factors are important determinants for the formation of helminth communities. Colonisation history of tortoises appears to have little influence at least for six parasitic nematode genera, three of which were present in all the tortoise populations sampled (*Atractis, Labiduris* and *Trichuris)* and three of which were found in at least four populations (*Ascaris, Tethrabosthriostrongylus, Necator*). Four genera were present only in one tortoise population. Three of them were probably acquired locally (*Porrocaecum, Parapharyngodon, Strongyloides*) and one (*R. rondoni*) might represent a non-biological sequence. The presence of Platyhelminths of the classes Cestoda and Trematoda found in the tortoise population of Santa Cruz west and a parasite of the phylum Acanthocephala detected in Isabela Island might be also associated to local acquisition of parasites.

6.5. Implications for the conservation management of Galápagos tortoises

Galápagos giant tortoises are the subject of an intensive captive breeding and reintroduction program. Since the 1960s, more than 5000 tortoises have been repatriated to their ancestral island of origin, with >1500 tortoises repatriated to Española and >1000 tortoises reintroduced to Pinzón. This management program has successfully repopulated endangered tortoise populations, but to date it has lacked a comprehensive protocol of health surveillance and a baseline of the parasites potentially co-introduced or acquired in the new habitat.

Health assessment of Galápagos tortoises was initiated in 2005 with the establishment of the Galápagoas Genetic Epidemiology and pathology Laboratory (GGEPL). From 2010 such monitoring has been intermittent due to a lack of continuity of the laboratory facility. This thesis has demonstrated the presence of haemogregarine, tick

and helminthic parasites infecting Galápagos tortoises and forms a baseline to resume and reinforce a health surveillance protocol for the management programme already in place.

This study shows the presence of haemogregarines and ticks only in some Galápagos tortoise populations. Although haemogregarines and their hosts are usually co-adapted, steps should be taken to keep these parasites among their original tortoise hosts populations and avoid spillover to uninfected populations. At least one case haemogregarines have caused pathological effects (Brygoo 1963). This information is relevant as in December 2015 thirty-two hybrid tortoises from Wolf volcano on Isabela Island were brought to a breeding centre in Santa Cruz Island with the goal of recovering the extinct tortoise species of Floreana and Pinta islands. Surveillance of these tortoise parasites is desirable either for a baseline of parasites and for putting in place the required biosecurity measures to avoid any transfer of parasites to other captive tortoises.

With regard to ticks no co-phylogenetic signal was detected with their hosts. However ticks showed very strong genetic structure among the three different islands where they were collected. Tortoises from Pinzón islands resident in the breeding centre in Santa Cruz carry *Amblyomma* ticks. Steps should be taken to avoid transfer to other tortoises or other reptile species of the breeding centre. At the same time captive Pinzon tortoises should be managed to conserve its potentially unique tick species.

In relation to nematodes, they were found in the reintroduced tortoise population of Española which implied a potential co-introduction from the breeding centre of Santa Cruz. In this study at least four genera are widely distributed across the archipelago. However, it is likely that they have diversified genetically among different islands, leading to unique evolutionary and ecological parasite-host-location associations. This potential unique association should be considered for the conservation management of *Chelonoidis* spp., and steps should be taken to avoid mixing of parasites between distinct tortoise populations. Porocaecum which is unique to the breeding centre – if that is it a true tortoise parasite we need to know where it came from, and there have to be steps to prevent its introduction into non-native ranges

The lack of biosecurity measures to prevent the mixing of parasites from different tortoise populations might disrupt millennia of host parasite co-evolution. On the other hand although nematodes have likely co-evolved with their hosts, ecological changes and novel host-parasite or parasite-parasite interactions might alter parasite dynamics and so the impact of parasites on their hosts. Those impacts could be amplified in small and inbred host populations that typically have reduced genetic variability an increased susceptibility to new parasites. Recommendations to minimise the impact of conservation programs on host-parasite interactions are available elsewhere (Mathews *et al.* 2006; Woodford and Rossiter 1993).

6.6. Further research directions

The results of this study need to be followed-up with the analysis of haemogregarines and ticks in other Galápagos reptiles. Ticks and other haematophagous invertebrates, circulating particularly in Alcedo and Wolf volcanoes (Isabela Island) should be analysed to determine their role as vector of the different haemogregarines detected so far. This thesis has detected a variety of nematode genera providing a baseline that could help improve the intestinal helminth surveillance before tortoises are released to the wild. One way would be improving the microscope analysis of faecal samples by testing more sensitive methods of helminth eggs detection. Another way would be designing genus specific primers that allow helminth detection via PCR. A protocol of surveillance using both methods would be ideal. Another further step is to expand the metagenomic analysis of helminths of other Galápagos reptiles across the entire archipelago. It would provide a vast amount of data for understanding helminth specificity and the local evolution of parasite communities.

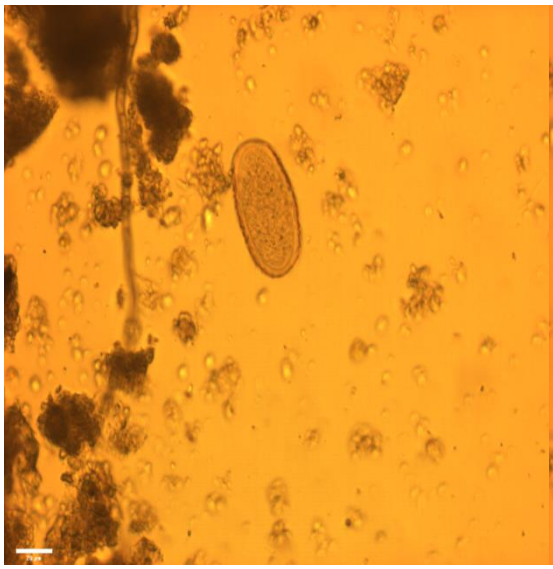
Another desirable step would be the morphological identification of the parasites identified here by molecular analysis. For Strongylida eggs this would require the culture of nematode eggs to larvae stage 3. The post mortem investigation of all tortoises that die in the captive breeding centres is also recommended as this could allow collection of adult nematodes required for more accurate morphological analysis. Searching for other macroparasites, as well as microparasites, in dead and live tortoises would allow a broader assessment of the potential pathogens that could threaten wild tortoise populations. Extending the molecular analysis of Galápagos giant tortoise nematodes will allow exploration of the potential co-evolution between

these parasites and their host(s). It may also give deeper insights into the biogeography and evolution of the tortoises, since the faster life-history of nematodes may record events not captured by the phylogenetic signals present in the genomes of the tortoises themselves.

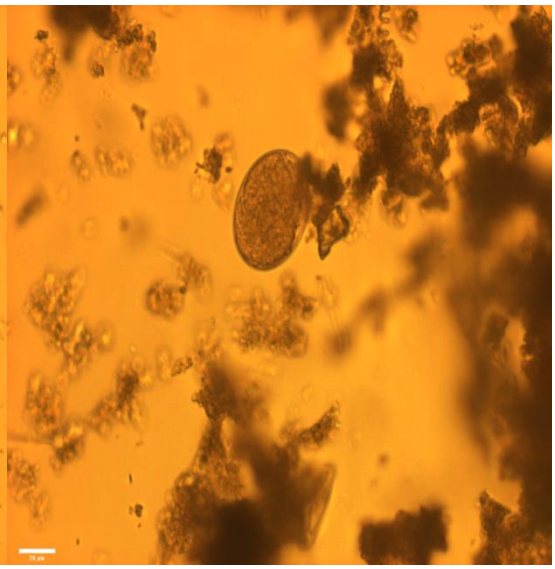
The conservation of endangered species should not just be focused on the protection of specific species populations, but should also target the protection of their ecological communities, including their parasites every effort should be made to minimise such ecological and evolutionary loss in the World Heritage Site and Biosphere Reserve that is the Galápagos Islands.

Supplementary information

S1 Parasitic forms observed during the coprological analysis of *Chelonoidis* spp. whose measures resulted in outliers in relation to the most common counted eggs



LP94_ST1



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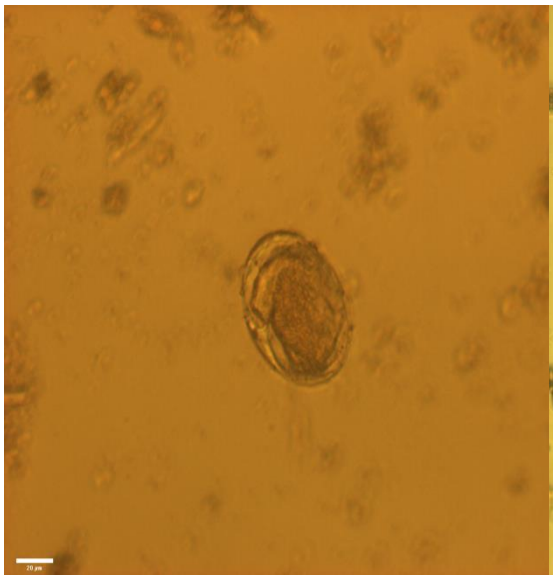
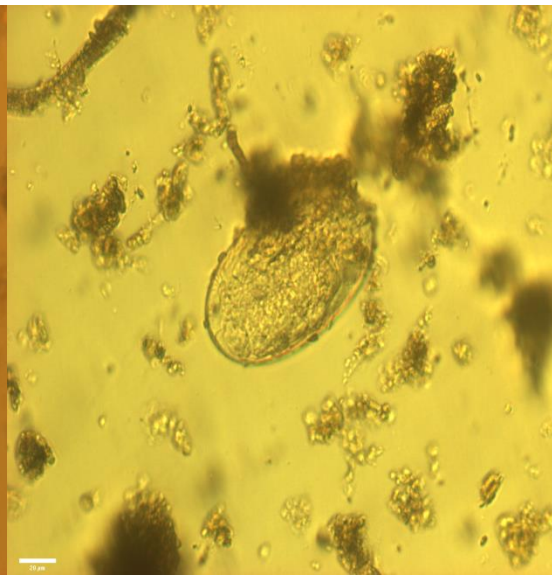
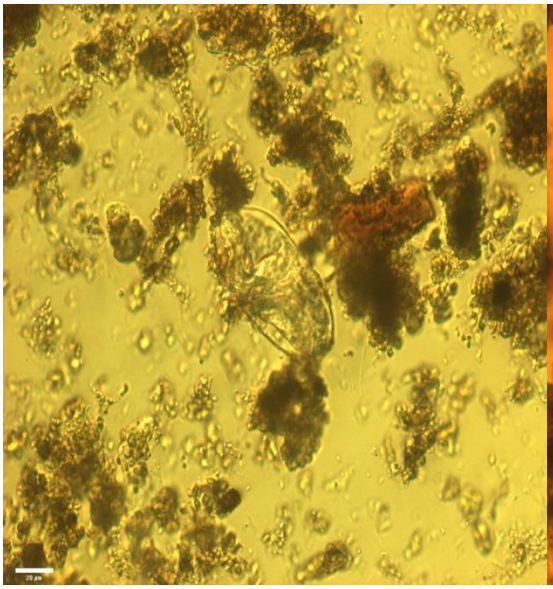


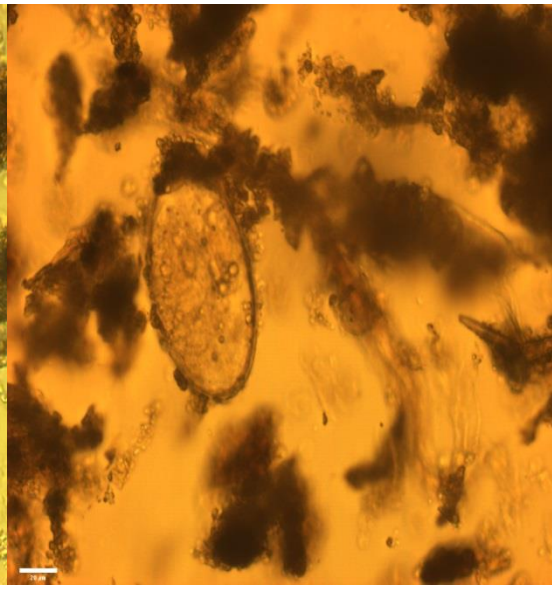
LPS19_ST4 (87x49 µm)



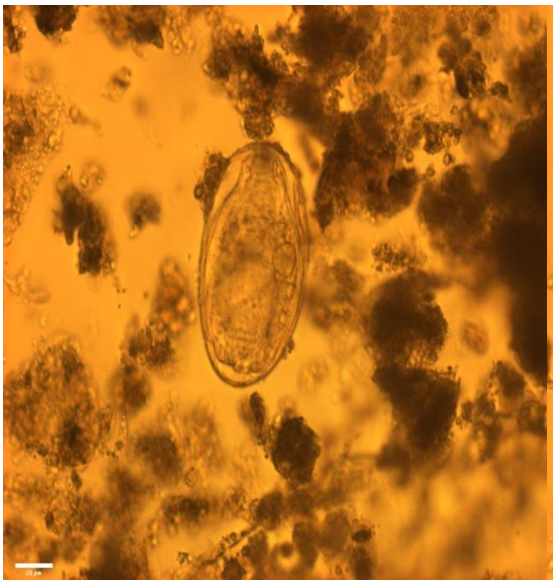
LPI34_ST1 (101x57 µm)



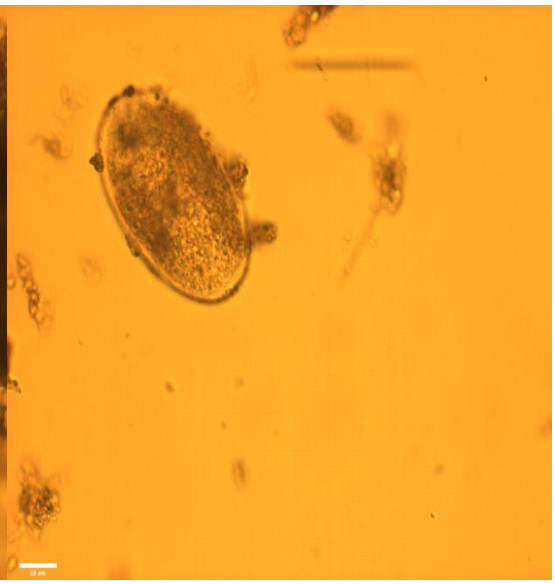
LPI34_ST4 (97x55 µm)



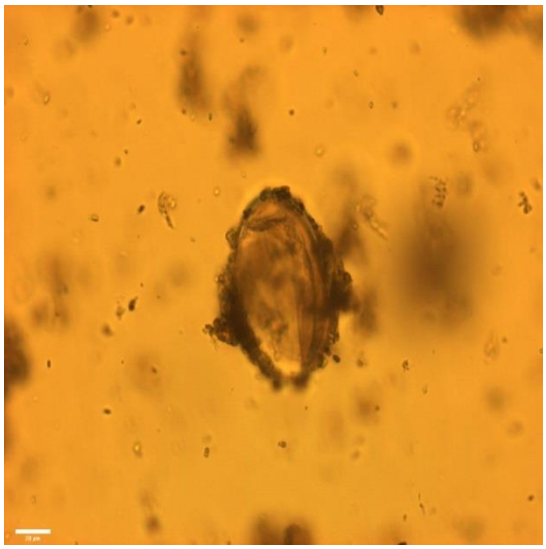
LPI6_ST1 (96x53) µm



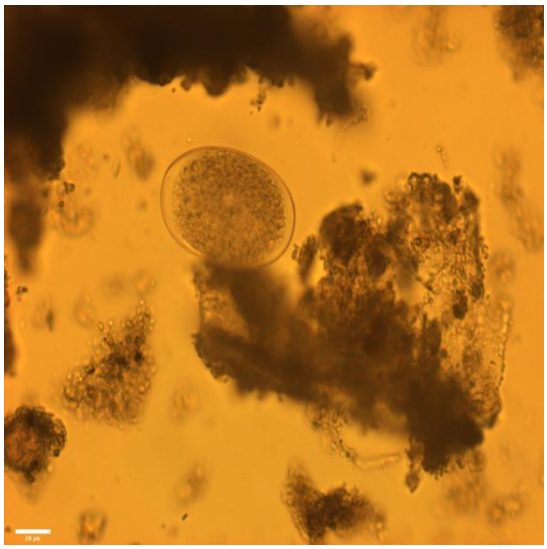
LPI40_ST1 (99x58 µm)



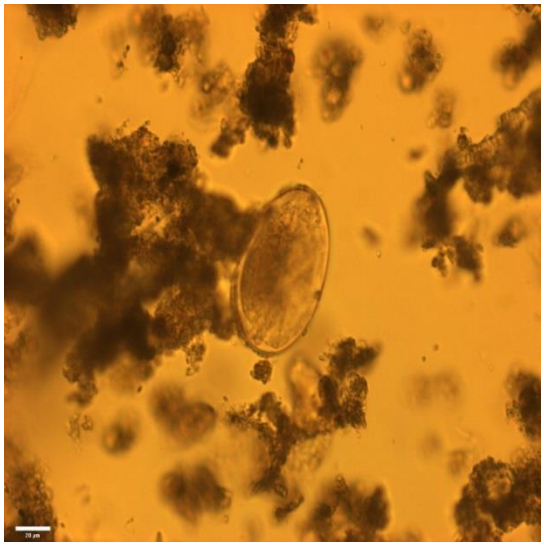
LPI61_ST1 (100x58 µm)



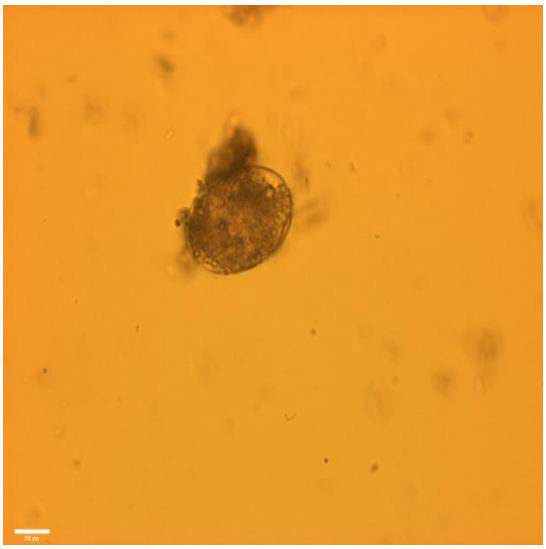
LPI38_ST2 (81x50 µm)



LPI42_ST1 (81x50 µm)



LPI52_ST (86x48 µm)



LPI52_ST (86x48 µm)

S2. Pipeline of the bioinformatics analysis used to annotate DNA sequences resulting of the metabarcoding analysis in Chapter 5

Action	Software	Script	Criteria
See Fasta Q reports (optional)	**MultiQC**	multiqc .	Next-generation reads specify a Phred score for each base, also known as a Quality or Q score. The Q score is an integer, typically in the range 2 to 40. Q indicates the probability that the base call is incorrect (P_e). For example, Q=2 means that the error probability is 63%, so the machine is reporting that the base is more likely to be wrong than right, while Q=20 corresponds to an error probability of 1%.
Merging Paired reads-Merging Forward and reverse files.	**USEARCH**	usearch -fastq _mergepairs N_Replicatesfaeces/*1.sanfastq -reverse N_Replicatesfaeces/*2.sanfastq -fastqout $out rfmerged.fq -relabel @ -log $out rfmerge.log -fastq_maxdiffs 10 -fastq_maxdiffpct 10	-fastq_maxdiffs Maximum number of mismatches in the alignment. Default 5. Consider increasing if you have long overlaps. -fastq_maxdiffpct Maximum number of mismatches as an integer percentage. Default 5. Consider increasing if you have long overlaps
Read quality filtering	**USEARCH**	usearch -fastq_filter merged.fq -fastq_maxee 1.0 -relabel Filt -fastaout filtered.fa	Filter reads by quality score.The maximum number of expected errors is specified by the -fastq_merge_maxee option. The number of expected error is a floating-point number. Using 1.0 specifies that the merged read should have zero as the most probable number of errors according to its Q scores.

Action	Software	Script	Criteria
Dereplication	**USEARCH**	usearch -fastx_uniques $out rffiltered.fa -sizeout -relabel Uniq -fastaout $out rfuniques.fa -log $out rfuniques.log	Identify the set of unique read sequences and record the number of occurrences (abundance) for each sequence.
Discarding singletons	**USEARCH**	usearch -unoise $out rfuniques.fa -fastaout $out rfdenoised.fa -relabel Den -log $out rfunoise.log -mina mpsize 4	In typical data sets, a large majority of unique read sequences are singletons, most of which are expected to have at least one error. Most such singletons can be discarded without loss of sensitivity, as the correct sequence will also be present. A small fraction typically has >3% errors, and these can induce a large number of spurious OTUs
OTU clustering	**USEARCH**	usearch -cluster_otus $out rfuniques.fa -minsize 2 -otus $out rfotus.fa -relabel Otu -log $out rfcluster_otus.log	OUT clustering is performed using UPARSE-OUT. It is a greedy clustering method that uses a single representative sequence to define each cluster (OTU), using the following algorithm. A database of OTU sequences is initially empty. Unique read sequences are considered in order of decreasing abundance, motivated by the expectation that more abundant reads are more likely to be correct amplicon sequences. If the read matches an existing OTU within the identify threshold (default 97%), the OTU abundance is updated but the database is otherwise unchanged. Otherwise, a model of the read is constructed by the UPARSE-REF algorithm (below) using the current OTU database as a reference. If the model is chimeric, the read is discarded; otherwise, the read is added to the database and thus becomes the representative sequence for a new OTU.

Action	Software	Script	Criteria
Taxonomy annotation	**USEARCH**	usearch -sintax $out rfotus.fa -db SILVA_128_SSURef_Nr99_tax_silva_trunc_euk_proc.fasta -strand plus -tabbedout $out rfotus_sintax.txt -log $out rfsintax.log	Taxonomy annotations can be added to OTU sequence labels by using the sintax command and a database of interest (in my case I used SILVA).
Making OUT tables for Downstream analysis	**USEARCH**	usearch -usearch_global $out rfmerged.fq -db $out rfotus.fa -strand plus -id 0.97 -log $out rfmake_otutab.log \-otutabout $out rfotutab.txt -biomout $out rfotutab.json -mothur_shared_out $out rfotutab.mothur	This commands allows to prepare OUT tables for downstream analysis.

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