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Assessment of diversity of apicomplexan parasites in selected snake species

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‘How every biologist on Earth would envy her
if they could see what she was seeing!’

– Mary Malone in *His Dark Materials* by Philip Pullman

‘Very little in biology is not made more comprehensible
in the light of phylogenetic history’

– Morrison (2009)

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ABSTRACT

Parasites are a highly diverse group of organisms with essential roles in the ecosystems they inhabit. Cataloguing their biodiversity has not been an easy task, mostly due to the difficulties in simply detecting and identifying many of these organisms. The impairment is even greater for intracellular parasites, such as the members of the phylum Apicomplexa. These include some of the most notorious parasites of anthropogenic interest. Regardless, they remain one of the most poorly studied groups of organisms. Clearly, more sampling is needed, especially in wild hosts. Through molecular screening by PCR, parasites can be quickly and efficiently detected in host samples, and sequencing provides abundant characters for phylogenetic analysis. However, the limited taxa and character sampling represent a major setback to further advances in this field.

In this thesis, tissue samples from several wild snakes were analysed using hemogregarine-specific primers for the 18S rRNA gene. In snakes, the most common hemoparasites are species of *Hepatozoon*, but they also harbour a wide variety of other apicomplexans. For the first study of this thesis, tissue samples from four snake genera of the Mediterranean area with different dietary regimens were analysed. The saurophagous snakes were the ones who presented infections, and the retrieved *Hepatozoon* sequences were closely related to those previously found in lizards of the same region. This suggests that these parasites might exploit trophic associations for their transmission. In the second study, samples of snakes of the genus *Psammophis*, also saurophagous, were analysed. As these occupy a more desertic habitat, the inherent hypothesis was to test if ecological differences reflect in differences in infecting *Hepatozoon* lineages. However, these were similar to lineages previously found in Mediterranean species.

Other apicomplexan parasites were also found in these studies. A *Caryospora* infection was for the first time detected through molecular analysis of tissue samples. Also, *Sarcocystis* infections were identified and their phylogeny did not reflect host taxonomy, in contrast to what had been previously suggested in other studies. Nevertheless, more sampling is needed and primers for new molecular markers have to be developed. Moreover, all the hosts that these parasites use need to be identified for an accurate assessment of host specificity and for parasite description. Only then will we be able to obtain sufficient information to better understand the diversity and evolution of parasites. This knowledge would certainly help bring clarity into parasite taxonomy.

Keywords

Apicomplexa, parasite, Coccidia, *Sarcocystis*, *Caryospora*, hemogregarine, *Hepatozoon*, reptile, snake, *Hemorrhois*, *Malpolon*, *Natrix*, *Rhinechis*, *Psammophis*, diet, transmission, prevalence, detection, molecular screening, PCR, 18S rRNA gene, biodiversity, phylogeny, Africa, Middle East, Mediterranean, South Europe.

RESUMO

Os parasitas são um grupo de organismos altamente diversos com papéis essenciais nos ecossistemas que habitam. Catalogar a sua biodiversidade não tem sido tarefa fácil, sobretudo devido às dificuldades em simplesmente detectar e identificar muitos destes organismos. O impedimento é ainda maior para os parasitas intracelulares, como os membros do filo Apicomplexa. Estes incluem alguns dos mais notórios parasitas de interesse antropogénico. Independentemente disto, eles mantêm-se um dos grupos de organismos mais pobremente estudados. Claramente, mais amostragem é necessária, especialmente em hospedeiros selvagens. Através de rastreamento molecular por PCR, amostras de hospedeiros podem ser analisadas rápida e eficazmente, e a sequenciação fornece abundantes caractéres para análise filogenética. Contudo, a limitada amostragem de taxa e caractéres são um grande contratempo a mais avanços neste campo.

Nesta tese, amostras de tecido de várias cobras selvagens foram analisadas usando *primers* específicos para hemogregarinas do gene 18S rRNA. Em cobras, os hemoparasitas mais comuns são espécies de *Hepatozoon*, mas elas também hospedam uma larga variedade de outros apicomplexos. Para o primeiro estudo desta tese, foram analisadas amostras de tecido provenientes de quatro géneros de cobras da área mediterrânea com diferentes regimes alimentares. As cobras saurófagas foram as que apresentaram infecções, e as sequências de *Hepatozoon* adquiridas eram mais próximas daquelas que tinham sido anteriormente encontradas em lagartos da mesma região. Isto sugere que estes parasitas poderão aproveitar as associações tróficas para a sua transmissão. No segundo estudo desta tese, amostras de cobras do género *Psammophis*, também saurófagas, foram analisadas. Como estas ocupam um território mais desértico, a hipótese inerente era testar se diferenças ecológicas reflectem-se em diferenças nas linhagens infectantes de *Hepatozoon*. Contudo, estas eram semelhantes às linhagens anteriormente encontradas nas espécies mediterrâneas.

Outros parasitas apicomplexos foram também encontrados nestes estudos. Uma infecção por *Caryospora* foi pela primeira vez detectada através da análise molecular de amostras de tecido. Também, infecções de *Sarcocystis* foram identificadas e a sua filogenia não reflectiu a taxonomia dos hospedeiros, ao contrário do que tinha sido antes sugerido noutros estudos. Porém, mais amostragem é necessária e *primers* para novos marcadores moleculares têm de ser desenvolvidos. Além disto, todos os hospedeiros que estes parasitas usam têm de ser identificados para uma determinação exacta da sua especificidade e para a descrição de parasitas. Só então seremos capazes de obter informação suficiente para melhor compreender a diversidade e evolução dos parasitas. Este conhecimento certamente ajudaria a trazer clarificação à taxonomia de parasitas.

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INTRODUCTION

The primary material of this work is organized in five chapters. The “Introduction” includes the state of the art, as well as the importance and the general objectives. The “Materials and Methods” provides a detailed review of the techniques used. The proceeding two chapters consist of two works completed during this thesis, which comprise two separate papers that had been submitted for publication in the *Journal of Parasitology*, one of which has already been published. Lastly, in “Final Remarks”, the main conclusions drawn from this thesis and future perspectives are explored.

This current chapter is divided into three sections. Firstly, the “State of the Art” section gathers background knowledge about the themes covered in this thesis. Next, several aspects highlighting the significance of parasite studies, such as the ones that make part of this thesis, are compiled in “Importance”. Lastly, several issues established as starting points for the thesis are identified in the section “Objectives”.

State of the Art

Parasitism can be considered one of the most successful modes of life, judging by how often this strategy evolved independently (Poulin and Morand, 2000; Walker et al., 2011). The number of species classified as parasites changes according to the definition one adopts. The word parasite comes from the latinization of the Greek *παράσιτος* (*parásītus*), referring to someone who eats at the expense of another. In this sense, parasitism could be defined as the feeding by one species on another living organism without the death of the latter (contrasting with predation, wherein the prey dies). With such a broad definition, almost half of the known animals would be considered parasites (Windsor, 1998; Poulin and Morand, 2000). Stricter definitions are usually adopted, which require an intimate host-parasite relation and regard life history factors (such as the extent of the parasitic phases). Still, the diversity of parasites must be huge. It includes metazoans, fungi, amoebae, algae, bacteria, viruses and many other types of organisms.

Cataloguing the diversity of parasites has been a very arduous task due to several factors. For instance, many parasitic species, especially unicellular organisms, are hard to find (due to their small size) and sampling efforts have been inadequate, focusing primarily on species of agricultural, veterinary or medical interest (Poulin and Morand, 2000; Morrison, 2009). As such, the number of described parasitic species surely represents only a fraction of the total diversity of this group of organisms (Adl et al., 2007; Morrison, 2009). Not all free-living organisms have yet been quantified, and if one thinks that one single species may host several other parasitic ones, it might be expected that

the diversity of parasites equals or even excels that of their free-living counterparts, depending on the general scenario of host specificity (Poulin and Morand, 2000; Duszynski and Upton, 2009). The lack of a broad sampling effort and the limitation of studies to a restricted number of groups have acted as a drag on the description of the diversity and on the understanding of the phylogenetic relations of parasites (Poulin and Morand, 2000, Walker et al., 2011).

This section starts with a tour through the taxonomy of the eukaryotic domain, with a special focus on well-known parasites of animal hosts. The aim is to phylogenetically contextualize the parasitic groups studied in this thesis. Then, the narrative specifies toward those groups, with accounts on phylogeny, life cycle and epidemiology. To finalize, an overview on the main methods used to detect and study the diversity of parasites is given.

Taxonomic arrangement of the Eukaryota

Over the past few decades, the view of the phylogenetic relationships of the Eukaryota has changed dramatically, especially for unicellular organisms (Adl et al., 2005, 2007; Walker et al., 2011). Consequently, the phylogenetic positioning of protists has been the subject of great controversy. In many cases, names and estimated relationships have been changed so many times that the classification ceased to be clear and lost its facilitation purpose (Adl et al., 2007). Traditionally, the classification of protists has been based on morphological and life history characters, but these have proven to be insufficient to fully disentangle their phylogenetic relationships. The lack of power to distinguish between species of similar morphology and the nonexistent consensus on the relative weight of each character, led to a confusing view of protist evolution and their relationships to multicellular organisms, and also to an underestimation of their diversity (Sogin and Silberman, 1998; Adl et al., 2007).

The value of the ribosomal RNA (rRNA) genes for the inference of the evolution framework of eukaryotes was recognized even before they were put for such use. The critical role of rRNA in the cellular machinery resulted in an inevitable conservation of its genes, making them markers of choice for the reconstruction of phylogenetic relationships at a high level (Sogin and Silberman, 1998). The first molecular phylogenies based on sequences of the small subunit rRNA (SSU rRNA) gene showed a nearly coincidental separation of the ‘crown’ eukaryotes (animals, plants, fungi and some protists) atop a ladder-like sequential divergence of basal lineages (see Figure 1.1), which included prominent parasites such as *Giardia*, *Trichomonas*, *Trypanosoma* and the microsporidians (Sogin and Silberman, 1998; Sogin, 1989). This result corroborated the Archezoa hypothesis of Cavalier-Smith (1983, 1987), which stated that the ‘amitochondrial’ protists

represented basal lineages that diverged before the acquisition of mitochondrial endosymbiosis and other key eukaryotic innovations. This was the predominant view through the 1990s.

Although alluring and logic, the crowned perspective of the eukaryotic relationships has been rejected based on distinct lines of evidence (Walker et al., 2011). First, mitochondrially-derived organelles have been found in almost all of the supposedly 'amitochondriate' protists (Embley and Martin, 2006). Second, it was showed that several analysis errors and data artefacts, such as 'long branch attraction', had a misleading effect on the positioning of the organisms on the tree. The less conserved sequences clustered together regardless of whether the divergence was consequence of a rapid evolution (as the case of many parasites) or the accumulation of independent mutations during an extended length of time (as in the prokaryotic sequences used to root the eukaryotic sequences). Due to this artefact, highly divergent protists were mistaken for basal eukaryotes (Walker et al., 2011). When these findings were taken into account, the apparent primitive parasites and basal eukaryotes were either unequivocally placed elsewhere in the tree or were unresolved. Most notable is the example of the Microsporidia, which, initially thought as one of the most primitive eukaryotes, were found to actually be highly specialized members of the Fungi (Keeling and Fast, 2002).

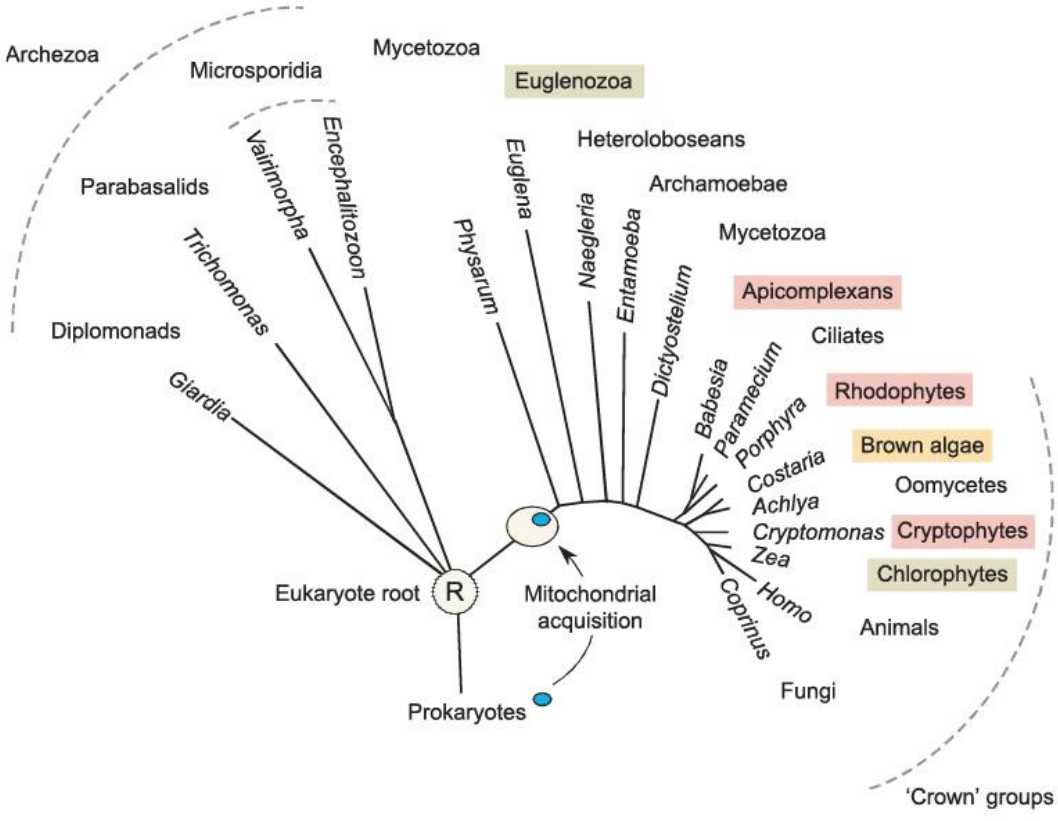

Figure 1.1 – Outline of the evolution of the Eukaryota based on the early studies with the SSU rRNA gene. Sequences from prokaryotes were used to root the tree. The image is presented as in the original publication: Embley and Martin (2006).

Nevertheless, SSU rRNA remains one of the most used phylogenetic markers, although strategies have changed and more care is taken into analysis and interpretation of molecular sequence data. Several genes were used to test specific hypothesis about smaller taxonomic groupings, and these resolved relationships would together constitute a consensus view of the Eukaryota phylogeny as a whole (Walker et al., 2011). Also, the resolved relations contributed to a renewed interpretation of the ultrastructural data that had been being produced using light and electron microscopy. All these reappreciations culminated in a proposal of reorganization of the classification scheme of the eukaryotes. In their publication, Adl et al. (2005) based on morphological, ultrastructural and molecular data divided the Eukaryota into six 'supergroups': Opisthokonta, Amoebozoa, Excavata, Rhizaria, Archaeplastida and Chromalveolata. Further research has still to be done and although some of these supergroups have been well supported, others are subject of debate and adjustments have been proposed. Moreover, the relations between supergroups are still dubious and a root remains to be found (Walker et al., 2011). This six-sided division has emphasized how diverse parasites are and how this lifestyle has evolved independently multiple times in Eukaryota evolution (see Figure 1.2. Note that only taxonomic groups with organisms that parasitize animals are indicated, in others groups there may exist eukaryote parasites of plants, fungi and even protists. Also, not all six supergroups correspond to those proposed by Adl et al. [2005]).

The most basal division in the Eukaryota seems to be between unikonts (composed by the Opisthokonta and the Amoebozoa) and the rest of the aforementioned supergroups (called bikonts in opposition) (Hampl et al., 2009). The supergroup Opisthokonta encompasses animals, fungi and their protist relatives. Numerous parasitic taxa exist in these groups. Notorious metazoan parasites include hematophagous arthropods (such as mosquitoes, ticks and fleas), tapeworms, hookworms, among many others. Within Fungi there are parasites of plants and animals, and parasitism is present in the several phyla of this kingdom, including the taxonomically illusive Microsporidia. The Amoebozoa is composed predominantly by amoeba and amoeboid flagellates. Some are known to be parasitic, including of vertebrates, like the intestinal parasites *Entamoeba*.

In the Bikonta, the first divergence of a main lineage is that between Excavata and the remaining supergroups (Hacket et al., 2007; Cavalier-Smith, 2009; Hampl et al., 2009). Various studies support the monophyly of the Excavates (Burki et al., 2008; Hampl et al., 2009). These contain some major human parasites like *Giardia lamblia*, *Trichomonas vaginalis*, *Leishmania* and *Trypanosoma* (*T. brucei* and *T. cruzi* are respectively the causative agents of African sleeping sickness and Chaga's disease). Results of different works put into question the organization of the three last supergroups

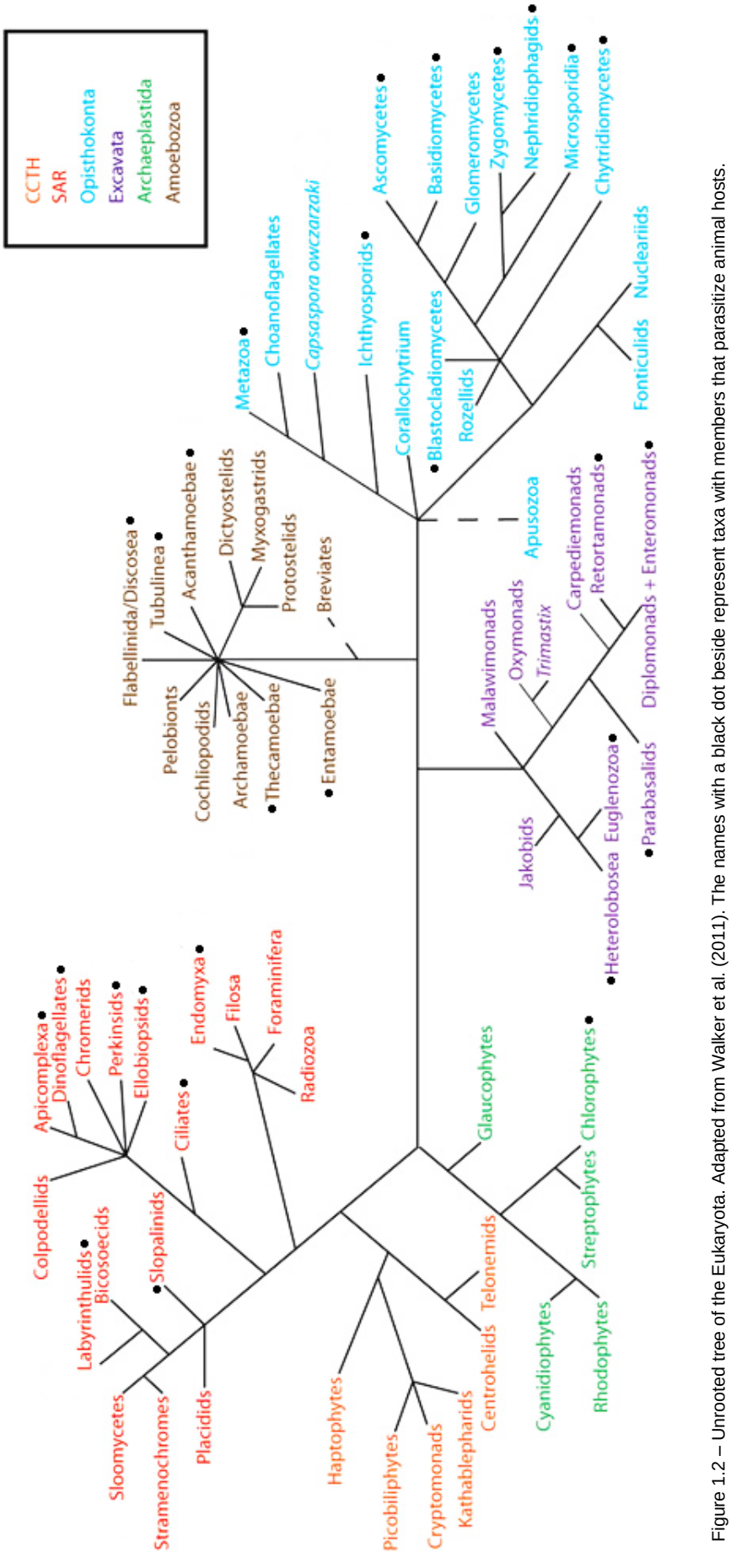

Figure 1.2 – Unrooted tree of the Eukaryota. Adapted from Walker et al. (2011). The names with a black dot beside represent taxa with members that parasitize animal hosts.

(Hacket et al., 2007; Burki et al., 2008; Hampl et al., 2009), with the possibility of polyphyly in the Chromalveolata and of paraphyly in the Archaeplastida. In Hampl et al. (2009), some chromalveolates cluster within the Archaeplastida, while in Burki et al. (2008), the same are sister-groups. These chromalveolates correspond to different Algae groups, such as Cryptophyta, Centrohelida, Telonemia and Haptophyta (referred collectively as CCTH or non-SAR Chromalveolates). Their grouping with the Archaeplastida links nearly all photosynthetic lineages and supports a possible unique origin of plastids (Burki et al., 2008). There are some Archaeplastid parasites of animals, like the non-photosynthetic algae of the genus *Prototheca*, which may infect dogs, cats, cattle and humans. By contrast, the CCTH clade presents no parasitic species (Walker et al., 2011).

On the branch opposed to the Archaeplastida and the CCTH, are the Rhizaria and the remaining Chromalveolata, namely the Stramenopiles and the Alveolata. These two groups with the Rhizaria form the SAR clade (Burki et al., 2007). Nuclear multigene phylogenies robustly support the monophyly of this clade, and place the Rhizaria basally to the Stramenopiles and the Alveolates (Hacket et al., 2007; Burki et al., 2008; Hampl et al., 2009). All these 3 groups have parasitic representatives (Walker et al., 2011). Rhizaria contains various parasites of molluscs, crustaceans and annelids, including *Haplosporidium nelson*, a pathogen of oysters with significant commercial importance. Several stramenopile taxa parasitize metazoans hosts, from flatworms (*Thraustochytrium caudivorum*) to humans (*Blastocystis hominis*). Alveolates include seven lineages that are separated into three well supported groups: the ciliates (which are basal to the other Alveolata); the dinoflagellates, perkinsids and ellopsids; and the apicomplexans, colpodellids and chromerids (Walker et al., 2011). Among these, the parasitic groups are: perkinsids, ellopsids and apicomplexans. The phylum Apicomplexa contains some of the most notable parasites, such as the genus *Plasmodium*, the causative agent of malaria. It also harbours the parasites of particular interest in this thesis.

It is worthy to mention that some eukaryotic taxa remain unplaced (Adl et al., 2005), because they have yet to be adequately described, do not consistently cluster with any of the other groups, or even have no close extant relatives (Walker et al., 2011).

Apicomplexa

The phylum Apicomplexa comprises a large, widely distributed and highly diverse group of obligate unicellular parasites. These parasitic protozoans are probably the most successful pathogens known to mankind. They have been found in all classes of vertebrates, from fish to mammals, as well as in a wide variety of both marine and terrestrial invertebrates (Kopečná et al., 2006; Frölich et al., 2012). It is believed that all animals harbour at least one unique species of apicomplexan parasites (Morrison, 2009).

This phylum includes many of the most prominent parasites of humans and domestic animals. It is estimated that half of the human population is at risk by *Plasmodium*, the causative agent of malaria. This is for sure the greatest of all human afflictions caused by an eukaryotic parasite, causing about one million deaths annually, mostly children (Rich et al., 2009; Frölich et al., 2012). This genus contains about 172 described species, with 89 occurring in reptiles, 32 in birds and 51 in mammals, of which 4 cause malaria in humans (Paul et al., 2003). *Toxoplasma gondii* is thought to be present as dormant cysts approximately in a third of humans (Su et al., 2010), varying by region. This apicomplexan parasite has none to mild symptoms in healthy individuals. However, in immunonaive pregnant women, it can seriously affect the development of her foetus, and in immunocompromised individuals, like AIDS patients and organ transplant recipients, acute illness caused by a new infection or reactivation of the cysts may occur (Aspinall et al., 2003; Frölich et al., 2012). Other apicomplexan genera which parasitize humans include species of *Cryptosporidium*, *Babesia*, *Cyclospora* and *Sarcocystis*. The Apicomplexa infecting livestock are also responsible for large economic costs in food production. For example, *Eimeria*, which causes coccidiosis in chickens, infects the intestinal mucosa leading to severe weight lose and even to the death of the host. Worldwide, it is responsible for losses over one billion dollars in the poultry industry each year (Sharman et al., 2010; Frölich et al., 2012). Apicomplexans are resistant to most known drugs and few vaccines exist (Frölich et al., 2012). Control efforts have concentrated in prevention of infection, but historically it has been arduous (Schmid-Hempel, 2011). Besides, these parasites present a wide variety of transmission modes: some are vector-borne, as the blood parasites *Babesia* and *Plasmodium*; others, e.g. *Cryptosporidium*, *Toxoplasma* and *Sarcocystis*, form highly resistant cysts and can reach the host through an array of contaminated materials, including earth, water and food.

With such characteristics and notorious members, one would expect that by now a great knowledge about the biology of the Apicomplexa had already been gathered. Unfortunately, that is not the case, perhaps with the exception of only a few highly anthropogenically important groups, such as *Plasmodium*. Unicellular endoparasites are hard to find and describe, with few easy to study characteristics (usually, life cycle patterns, cyst organization and ultrastructure, and host specificity), making them one of the most difficult organisms to work with (Morrison, 2009). The Apicomplexa is the largest group of such organisms and is accordingly the worst studied in terms of biodiversity. About 6000 species are described, but it is estimated that 1.2 to 10 million apicomplexan species exist (Adl et al., 2007). Thus, only about 0.1% of the total number of species of this phylum has been named, despite the usefulness this information may impart to understand these parasites.

General characteristics and life cycle

The invention of Electron Microscopy led to groundbreaking discoveries in the field of Parasitology, and the Apicomplexa were no exception. The description of unique intracellular structures resulted in the establishment of the group Apicomplexa (Levine, 1973) (Figure 1.3). The structure that characterizes and gives name to the Apicomplexa is the apical complex. It is found at the anterior end of certain life cycle stages, especially the infective stages, and is constituted by several components (Adl et al., 2005). The cytoskeletal components are the conoid, one or more polar rings, and subpellicular tubes. Associated to these are the secretory organelles rhoptries and micronemes. The apical complex is the machinery responsible for host cell recognition, adherence and penetration (Walker et al., 2011; Frölich et al., 2012). Other characteristic features are the dense granules on the posterior part, the acidocalcinomes and the apicoplast, a non-photosynthetic remnant chloroplast. The inner part of the membrane complex is composed by two parallel membranes that form the alveoli, which places the Apicomplexa within the Alveolates. The apicomplexans have another endosymbiotic derived compartment, the mitochondrion, and the universal eukaryotic organelles.

The Apicomplexa typically have a complicated life cycle, involving double or triple alternations of generations (Walker et al., 2011). They undergo a series of asexual and sexual reproductions involving one or more hosts. Inside the host, apicomplexans invade their cells to develop into new forms and divide, until the host cell is lysed and the parasites freed. Extracellularly, these parasites generally do not differentiate or divide (Morrissette and Sibley, 2002). For the cycle to continue they must find an adequate cell, which might be a similar cell, one of another type, or even of a new host species.

The apicomplexan life cycle is characterized by 3 distinct reproductive processes: Sporogony, Merogony and Gametogony (Leander et al., 2003). A diploid zygote is formed by the junction of the gametes. It then invades a cell and by meiotic division forms sporozoites, which are haploid infective stages that exit the host cell to infect others. This process of multiplication is called Sporogony. Within a new host cell, a sporozoite produces merozoites, which may be called many different names depending on the species. Unlike Sporogony, Merogony may involve multiple rounds of asexual division, during which the merozoites proliferate, invading new cells and giving origin to more merozoites. Eventually, merozoites can develop into gametocytes (also called gamonts), which produce the gametes, whose fusion originates the zygote. This last part of the cycle is the Gametogony and corresponds to the phase of sexual reproduction. In Figure 1.4, a scheme of the general life cycle is presented. The details of the life cycles may change between the apicomplexan groups: names and locations may be different; additional steps

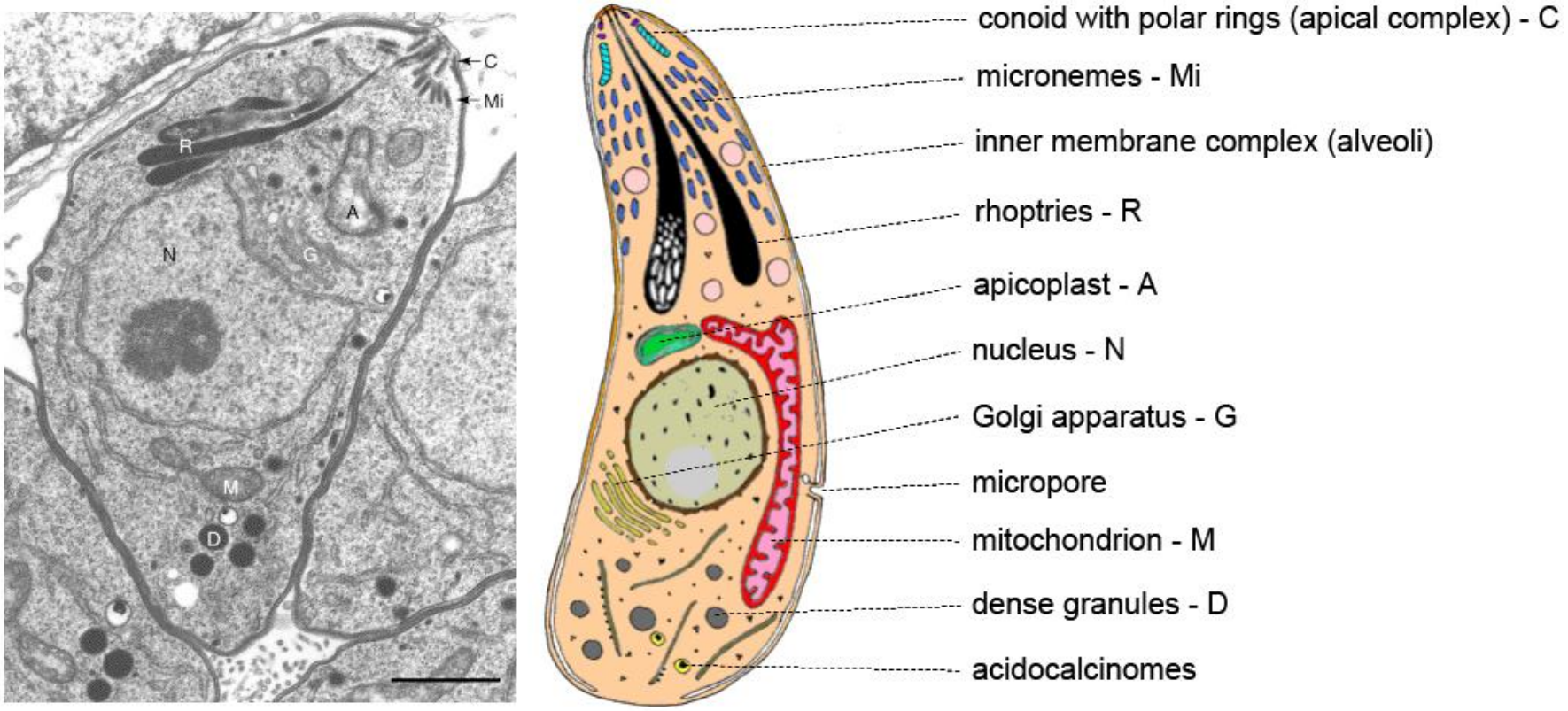

Figure 1.3 – Typical ultrastructural features of the Apicomplexa.
The image on the left corresponds to an electron microscopy photograph to a *Toxoplasma gondii* tachyzoite (type of merozoite). Letters are labelled on the right. The black bar equals 1 µm. Reference: Maréchal and Cesbron-Delauw (2001).
The middle portion of the figure is a schematic where the main cellular components of apicomplexans are represented. Reference: Šlapeta and Morin-Adeline (2011).

may be present; or some of the above described processes may be absent, for example, Merogony does not occur in some species (Adl et al., 2005).

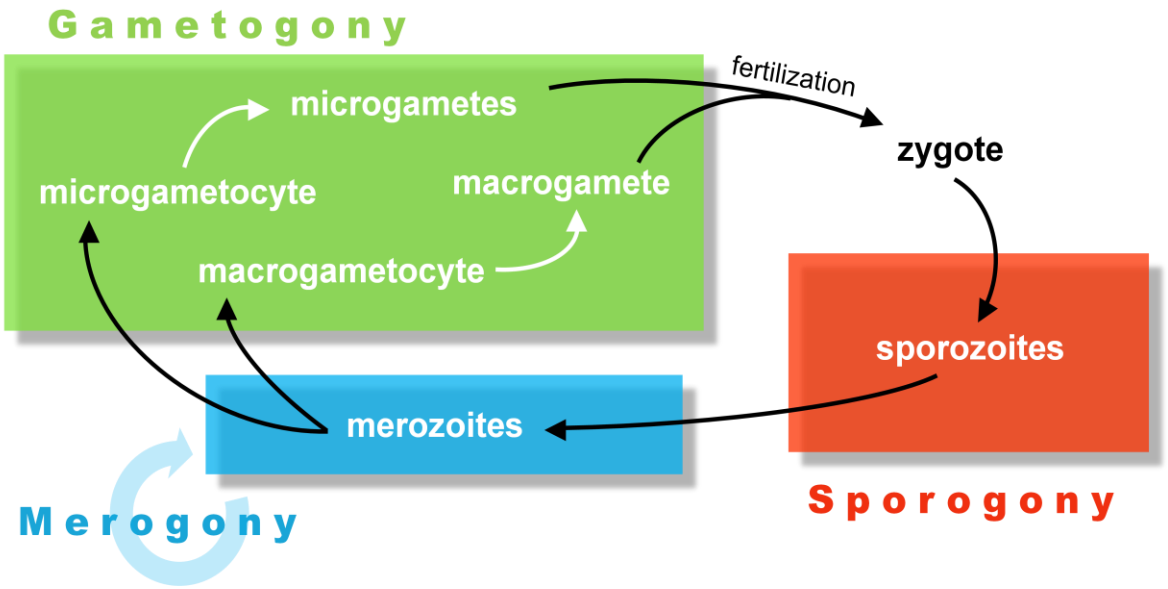

Figure 1.4 – General apicomplexan life cycle. Diagram by Beatriz Tomé.

Phylogenetic relationships

Between the two other main groups of the Alveolata, the Apicomplexa are more closely related to dinoflagellates than to the ciliates (Escalante and Ayala, 1995; Leander and Keeling, 2004). Some recently described alveolates were found to branch at the root of the Apicomplexa, giving some insight into the origins of this phylum. *Chromera velia*, a photosynthetic alveolate, seems to be more related to the Apicomplexa than to the Dinoflagellata, many of which are phototrophic (Moore et al., 2008). This scenario indicates a possible photosynthetic origin of the lineage leading to the apicomplexans; it also confirms the hypothesis that the apicoplast corresponds to the remnants of a red-algal derived chloroplast (Moore et al., 2008; Sato, 2011). This information might have great implications because, since plastids are not present in animals, the apicoplast can be a potential target for drug treatments (Fichera and Roos, 1997; Moore et al., 2008; Wiesner et al., 2008; Sato, 2011). According to phylogenetic analysis of the SSU rRNA gene, the chromerids along with another alveolates, the colpodellids, form a sister-grouping to a monophyletic Apicomplexa (Siddall et al., 2001; Moore et al., 2008). Species of *Colpodella* are free-living predators of protists. They have an apical feeding complex, which allows them to attach to the prey, penetrate through the cell membrane, and consume the cytoplasmatic content or ingest the whole cell (Siddall et al., 2001, Walker et al., 2011). Given the presence of this structure, Adl et al. (2005) placed the colpodellids

within the Apicomplexa. However, this phylum is generally regarded as parasitic and, combined with the remaining uncertainty on their placement, some authors consider the colpodellids as a separate group (Walker et al., 2011). Nevertheless, the relationship of apicomplexans with the colpodellids and the chromerids provides great insight into their evolutionary history: from the photosynthetic shared ancestry with the Dinoflagellata, to a predatory mode of life like the colpodellids, and finally reaching their current parasitic state (Leander and Keeling, 2004).

The historical main groups within the Apicomplexa are the gregarines, cryptosporidians, piroplasms, haemosporinids and coccidians (Barta et al., 2012). These designations have resisted, with modifications to their composition and taxonomic level, but only recently have their relative phylogenetic positions started to be understood (Leander et al., 2003). The lineage leading to the Gregarina is considered the first divergence within the Apicomplexa. Part of their life cycle occurs in extracellular spaces of insects and marine invertebrates, and they share some characteristics with the colpodellids, such as predation on host cells. These apparently ancestral features have hinted to the critical role of this group in the phylogenetic reconstruction of the apicomplexan relationships (Leander and Keeling, 2004). This suspicion was confirmed when gregarine sequences were added to the phylogenies, settling the position of another main apicomplexan group. The Cryptosporidia were originally included within the coccidians (Adl et al., 2005), but early phylogenetic analyses placed them as sister-group to all other apicomplexans (Escalante and Ayala, 1995; Morrison and Ellis, 1997; Zhu et al., 2000). When gregarine sequences started being included in phylogenetic studies, the two groups formed a clade (Leander et al., 2003; Leander and Keeling, 2004). This discovery had significant impact on how the biology of the Apicomplexa is understood. The gregarines are monoxenous (i.e. use only one host) parasites of insects and marine invertebrates, the cryptosporidians are also monoxenous but they parasitize vertebrates (this also applies to many “true” coccidians). Such implies a convergence of the exploitation of vertebrate host by monoxenous apicomplexans, both by cryptosporidians and coccidians. On the other hand, it also explains, for instance, why anticoccidial drugs have no effect on cryptosporidians (Leander and Keeling, 2004; Morrison, 2009).

Piroplasms, haemosporinids and coccidians form a clade of their own. The relative positions of these three groups change between studies and thus, the node of their divergence is sometimes presented as a tricotomy (Escalante and Ayala, 1995; Sogin and Silberman, 1997; Zhu et al., 2000; Barta et al., 2012). The Piroplasmida (e.g. *Plasmodium*) and the Haemosporida (e.g. *Babesia*, *Theileria*) both parasitize hematophagous invertebrates and the blood of vertebrates, forming together the class Hematozoa (synonym Aconoidasida) (Adl et al., 2005). The Coccidia are a very diverse

group that includes the tissue coccidia (Eimeriorina: Sarcocystidae), the enteric coccidia (Eimeriorina: Eimeriidae), the adeleorinid coccidia (Adeleorina: Adeleidae), and the hemogregarines (Adeleorina: various families) (Barta et al., 2012). They present many different life cycles: some have more than one host with a prey-predator type of transmission (Sarcocystidae), there are also monoxenous parasites of vertebrates (Eimeriidae) and of invertebrates (adeleorinids), and others are heteroxenous (i.e. have more than one host) with vertebrates and hematophagous invertebrates as hosts (hemogregarines), like the Hematozoa. The coccidian taxonomy is problematic, much due to the misuse of the phylogenetic tools and the incomplete taxon sampling (Kopečná et al., 2006; Morrison, 2009), with the majority of sequences belonging to species of anthropogenic importance, such as *Eimeria* and *Sarcocystis*.

In Figure 1.5, the hypothesised relations of the apicomplexan groups are illustrated. The taxonomy and phylogenetic relationships of the Apicomplexa have been subjected to controversy and numerous revisions. This has had important repercussions in how the evolution of the Apicomplexa is understood, being one example the order of emergence of the distinct types of life cycles. As these organisms leave no fossil record, the only way of solving this debate is through phylogeny reconstructions and dating by molecular clock. Escalante and Ayala (1995) addressed this issue and their results show that the Apicomplexa lineage is very ancient, with almost one billion years, placing its divergence from the ciliates and dinoflagellates in the Pre-Cambrian period. This coincides with the supposed time for the emergence of the multicellular kingdoms. Therefore, the monoxenous with invertebrate host life cycle must be the ancestral form. The radiation within the Apicomplexa that gave rise to the piroplasms, haemosporinids and coccidians is dated by the same authors to have happened about 824 million years ago. This precedes the origin of vertebrates and thus, the life cycles that involve the exploitation of vertebrate hosts must have arisen independently. Still, the evolutionary course on the main lineages remains obscure. The vector-transmitted apicomplexans retain their sexual reproduction in the invertebrate hosts, supporting that the heteroxenous parasites evolved from the monoxenous apicomplexans of invertebrate hosts (Kopečná et al., 2006). However, there are also monoxenous apicomplexans with vertebrate hosts. These may have evolved from the heteroxenous or in parallel with the host lineage that gave rise to the vertebrates (Escalante and Ayala, 1995). Only further sampling and phylogenetic analysis may answer these questions. For instance, in a recent study (Saffo et al., 2010), a century long mystery has been solved. *Nephromyces*, a beneficial symbiont of marine animals, was identified as a member of the apicomplexan Hematozoa. This opens an exception to the parasitic-only criterion of the Apicomplexa, and highlights how much we have yet to learn about the diversity of these organisms.

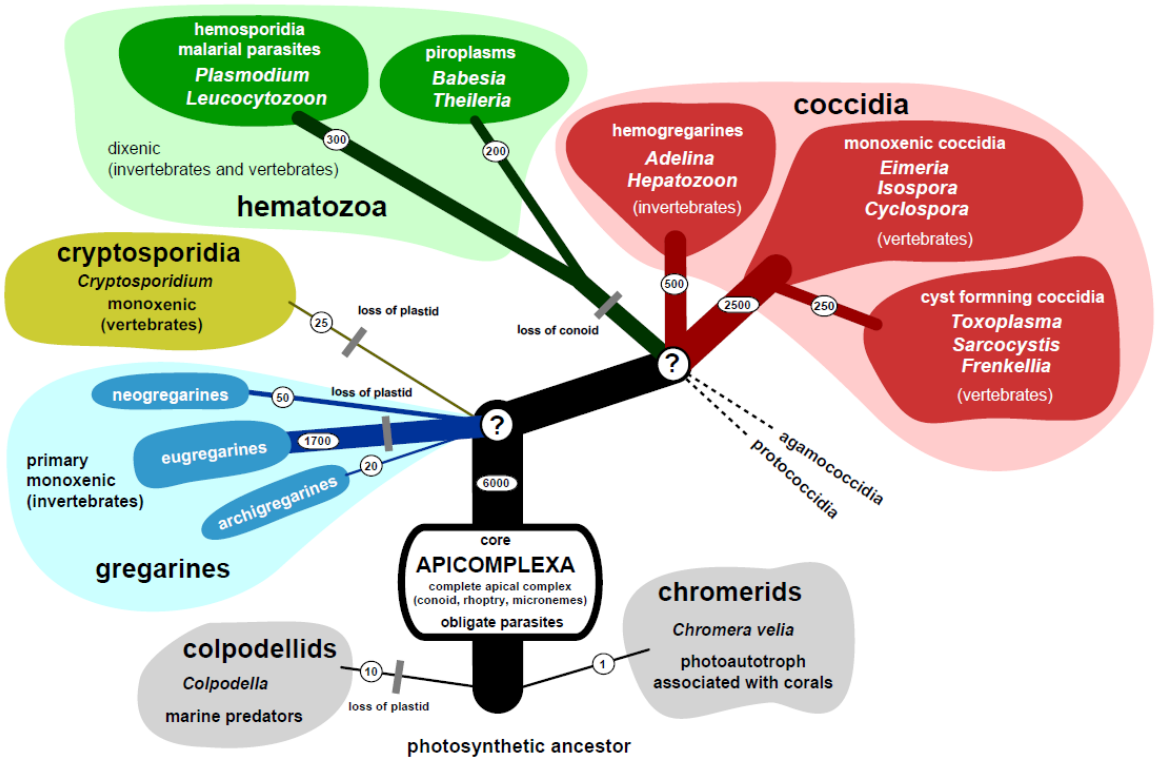

Figure 1.5 – Tree representing the main apicomplexan groups and their hypothetical relationships. The width and number on the branches refers to the named species and thus, the known diversity. Reference: Šlapeta and Morin-Adeline (2011).

In snake hosts

Infections with *Cryptosporidium* spp. have been reported in several species of snakes. These parasites of the digestive system cause lesions in the mucosa of the gastric tissue. Many of the reports on captive snakes refer to a severe clinical state, but cryptosporidiosis is believed to be rare and milder in wild individuals (Duszynski and Upton, 2009). Snakes are also host to the haemosporinid parasites *Plasmodium* and *Haemoproteus*. Six species of *Plasmodium* are found in ophidian hosts, while only *Haemoproteus mesnili* and *Haemoproteus balli* are attributed to snakes (Jacobson, 2007; Telford, 2009). Both genera are rarely associated with pathological conditions in reptiles.

The Coccidia are commonly found in reptiles. Both the families Eimeriidae (namely the genera *Caryospora*, *Cyclospora*, *Eimeria*, *Isospora*, *Tyzzeria* and *Wenyonella*) and Sarcocystidae (*Sarcocystis* and maybe *Toxoplasma*) have members that use snakes as hosts (Duszynski and Upton, 2009). Various species of *Eimeria* and *Isospora* are identified from snakes and lizards, with new species being described each year, few of which are associated with pathologic states (Jacobson, 2007). Fifty four species of *Caryospora* are reported from snakes (Duszynski and Upton, 2009). No lesions are stated in those descriptions. Sexual and asexual phases of *Caryospora* sp. can occur both in the intestinal epithelium of predatory reptiles (or birds) and in the dermal connective tissues of

rodents, a life cycle unique among coccidia (Upton et al., 1986). This parasite can thus be transmitted within the same host species and also in a facultative predator-prey dynamics (see Figure 1.6A).

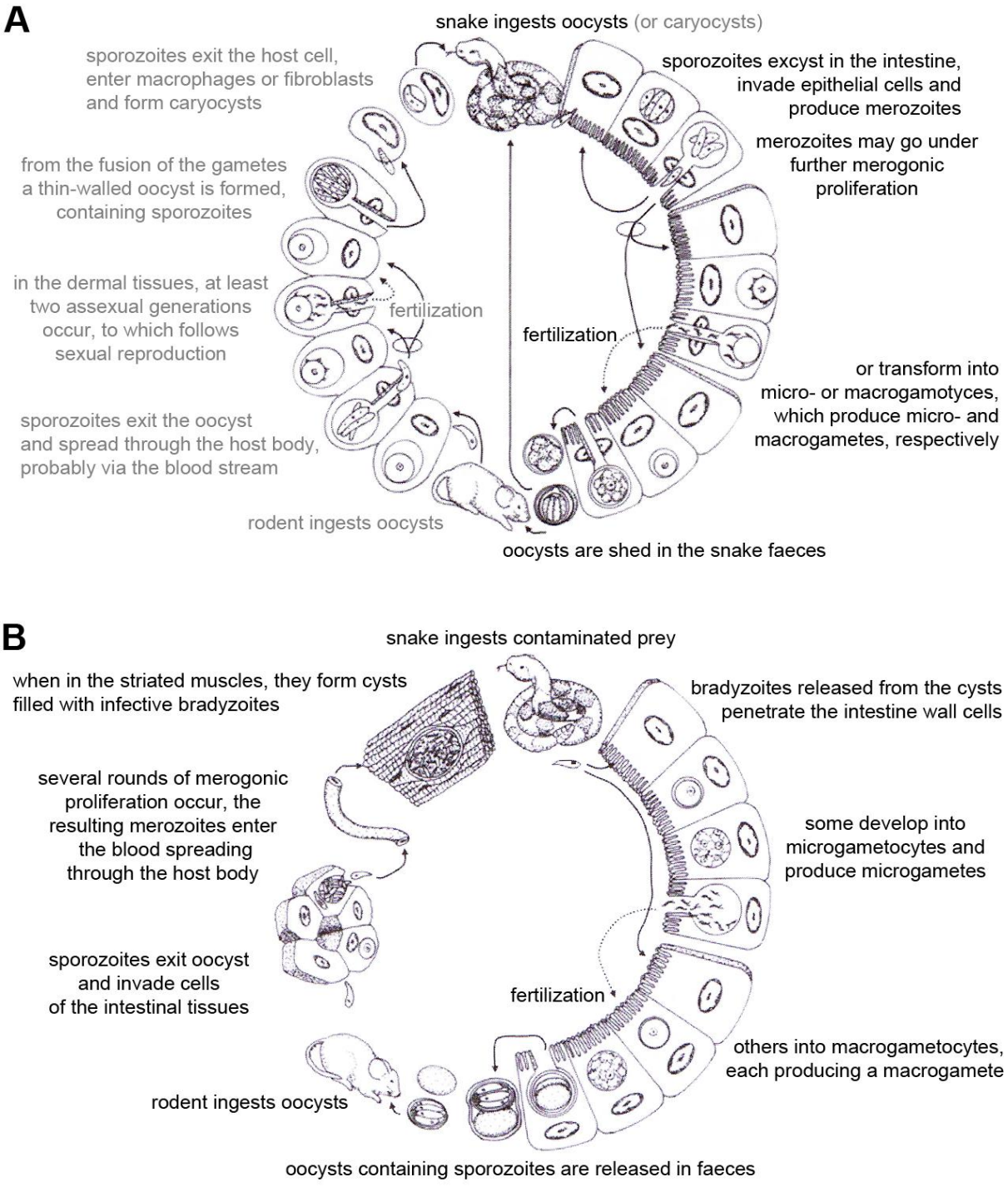

Figure 1.6 – Life cycle of two coccidian parasites of snakes. **A**: the typical life cycle of *Caryospora* spp., illustrating both the direct (black text) and the facultative heteroxenous (gray text) pathways. **B**: the characteristic setting of a heteroxenous *Sarcocystis* life cycle. Original source of both images: Duszynski and Upton (2009); some alterations, like incorporation of the text, were made.

Snakes commonly host species of *Sarcocystis* and several studies report a high prevalence of oocysts (encysted zygotes) in faeces (Šlapeta et al., 2003). Duszynski and Upton (2009) list twenty six species of *Sarcocystis* that parasitize snakes. Most members of this genus have a heteroxenous life cycle with a predator/prey or scavenger/prey relation (Figure 1.6B). It involves a definitive host (where sexual reproduction occurs), in which Gametogony and Sporogony take place in epithelial cells of the small intestine, and various muscles of an intermediate host (only involved in asexual phases of the cycle) (Duszynski and Upton, 2009). Studies have demonstrated that snake *Sarcocystis* are capable of infecting rodents (Jacobson, 2007) and the life cycle of thirteen named *Sarcocystis* spp. is described with a snake-rodent transmission (Šlapeta et al., 2003). Many *Toxoplasma*-like organisms have been reported from poikilotherms, with at least one named species from snake hosts, but probably these descriptions belong to other close genera (Duszynski and Upton, 2009). Moreover, mammalian *Toxoplasma* cysts may persist briefly in poikilotherms (although failing to further multiply), leading to erroneous attributions of host status to these vertebrates.

Snakes are also host to several blood-borne coccidia. Hemogregarines are the most common, widely distributed and speciose of reptilian hemoparasites, being present in all the orders of living reptiles. However, their definitive hosts are blood-feeding invertebrates and many species have been described based on the principle that the presence in a new host meant a new species, with most descriptions coming from vertebrate hosts (Telford, 2009). It is today acknowledged that description of development in both vector and reptile is essential for classification at the generic level (Jacobson, 2007; Barta et al., 2012). Members of the genus *Hepatozoon* are the hemoparasites most commonly seen in snakes (Jacobson, 2007; Telford, 2009) and, as so, are of main concern in the works of this thesis.

Hepatozoon

The genus *Hepatozoon* was established to accommodate blood parasites observed by Miller (1908) in leukocytes of laboratory rats. This author demonstrated that those parasites use both rats (as intermediate hosts) and mites (definitive hosts) in their rodent-mite life cycle (Smith and Desser, 1997). From his works, *Hepatozoon muris*, the type species of this genus, was described. Since then, species of *Hepatozoon* have been reported parasitizing all tetrapod groups and a variety of hematophagous invertebrates, such as ticks, mites, lice, fleas, reduviids and dipterans (Smith, 1996). Over 300 species are assigned to this genus, with about 120 described from more than 200 species of snakes (Smith, 1996; Smith et al., 1999; Sloboda et al., 2007). The taxonomy of these parasites traditionally requires information on morphological characters (ideally of the

developmental phases in both vertebrates and vectors), life cycle patterns and host specificity (Mathew et al., 2000; Perkins and Keller, 2001). However, the majority of the species descriptions lack complete accounts. Most include only measurements of the gametocytes in the peripheral blood of the vertebrate host, which might not be a reliable character (Moço et al., 2012), and ignore the phases in the invertebrate vector, which may also not be identified. Many species have been named just because they are found in a new host or a new locality (Smith et al., 1999). Due to these factors, the validity of many species is questionable and the systematics of the genus *Hepatozoon* is to some extent provisory (Mathew et al., 2000; Sloboda et al., 2007).

The significant differences in the morphology and location of life cycle stages, together with the wide range of vertebrate and invertebrate hosts, question the monophyly of the genus *Hepatozoon*. Based on morphological and developmental features, an analysis by Smith and Desser (1997) suggested that the genus is in fact paraphyletic. This has been supported by subsequent phylogenetic studies (Mathew et al. 2000; Barta et al., 2012) and it has been proposed that this genus should be divided at least into two, or even raised to the family level. Barta et al. (2012) identified four clades for *Hepatozoon* spp., which also included a sequence of *Hemolivia mariae* (the only representative of the genus in the analysis). The most basal clade comprised *Hepatozoon* species with mammals and ticks as hosts. Next, diverged a clade composed by the *Hemolivia* sequence and a single *Hepatozoon* sp. (both with tick and reptilian hosts). The two most derived clades were respectively composes by *Hepatozoon* spp. with tick and marsupial hosts, and by species with a variety of mammal, amphibian and reptilian hosts and several arthropods as vectors (ticks, fleas, mites and mosquitoes). This pattern suggests a coevolution association of these parasites with their definitive hosts. Furthermore, there could be a lesser association with the type of vertebrate host. At a higher scale, the analysis by these authors also proposes a sequence of events for the evolution of the type of life cycle of hemogregarines. The genus *Hepatozoon* is part of the suborder Adeleorina, which is composed by monoxenous parasites of invertebrates (adeleorinids) and heteroxenous parasites of vertebrates and hematophagous invertebrates (hemogregarines). The ancestral form probably was monoxenous, adeleorinids retained this trait and hemogregarines developed a variety of heteroxenous life cycles, probably when their hosts started feeding on the blood of vertebrates, but kept the sexual reproduction in the invertebrate hosts.

In another study, Allen et al. (2011) did a phylogenetic analysis of *Hepatozoon* spp. sequences retrieved from several vertebrate hosts and also reported a broad association with intermediate host taxonomy. Moreover, sequences from predators and prey seemed to be closely related. This pattern supports a prey-predator transmission dynamics, in

which an infected invertebrate is consumed by a first intermediate hosts, this becomes parasitized and when it is preyed upon, the predator also becomes infected. This kind of life cycle had already been documented in *Hepatozoon* spp. from snakes, but their findings suggest that this transmission pattern might be more widespread than it has been regarded. This would change how the life cycle of these parasites is perceived and how parasites control policies should be formulated. In fact, for *Hepatozoon americanus*, a tick-borne parasite of canids and the causative agent of American canine hepatozoonosis (an emerging disease often fatal for dogs), experiments have demonstrated that the prey-predator pathway is possible, and probably common (Johnson et al., 2008, 2009).

The available descriptions of life cycles of *Hepatozoon* spp. from ophidian hosts involve a three-host pattern with mosquitoes as definitive hosts, but it should be noted that this could not apply to all. In the hematophagous mosquito, the parasite suffers Sporogony in the haemocoel of the host (Smith, 1996). When a lizard or frog (called first intermediate hosts or paratenic hosts) ingests an infected mosquito, development of cysts occurs in the liver and lung tissues (Smith et al., 1999; Sloboda et al., 2007; Viana et al., 2012). The snake (second intermediate host) becomes infected when consuming parasitized paratenic hosts. Additionally, Sloboda et al. (2008) successfully infected mice after inoculating them with oocysts of *Hepatozoon ayorgbor* (which parasitizes the African ball python), and fed infected liver tissue of the mice to pythons, which also became infected. Thus, others vertebrates, besides lizards and amphibians, may serve as paratenic hosts. In the ophidian host, typically two, or more, rounds of Merogony take place in various visceral organs. Then, the merozoites enter the blood stream, where they invade erythrocytes and develop into gametocytes (Smith and Desser, 1997; Smith et al., 1999). This is the familiar form of identification of *Hepatozoon* spp. in the blood of snakes. Normally, one gametocyte is present per erythrocyte, but two or more can be found, and also free extracellular gametocytes have been observed (see Figure 1.7) (Smith et al., 1999; Sloboda et al. 2007). Finally, a mosquito takes a meal of blood contaminated with the gametocytes, macro- and micro-gametocytes leave the host cell and penetrate the

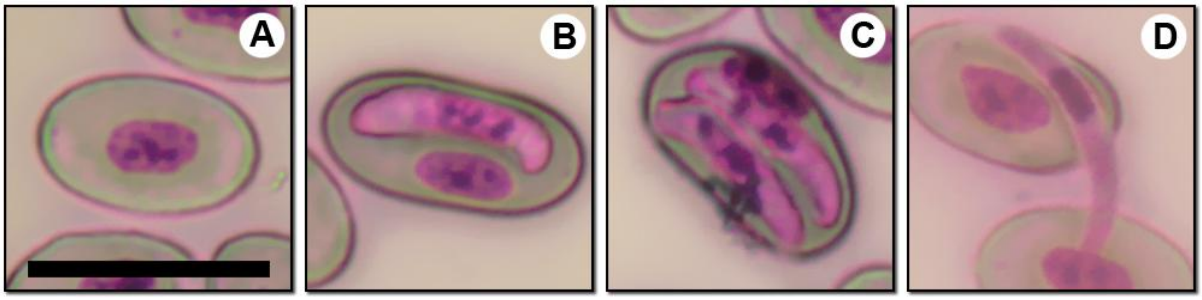

Figure 1.7 – *Hepatozoon* gametocytes in erythrocytes of snake hosts, at 400x magnification. **A**: uninfected erythrocyte. The black bar measures 20 µm. **B**: erythrocyte with one gametocyte. **C**: erythrocyte with two gametocytes. **D**: extraerythrocyte gametocyte. Photos taken by Beatriz Tomé.

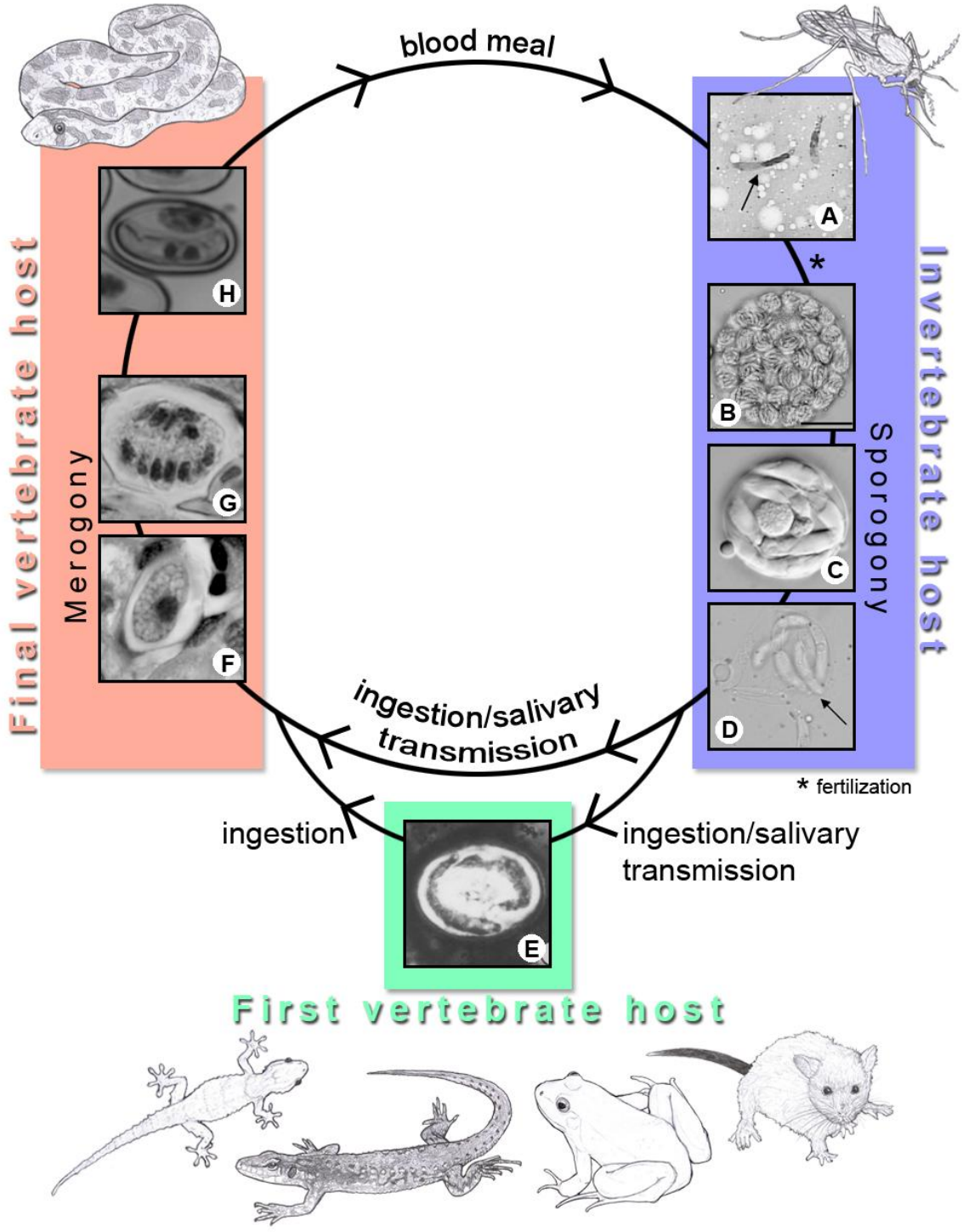

Figure 1.8 – Stages of the life cycle of *Hepatozoon* spp. with snake hosts.

A: free gametocytes in the digestive tract of a mosquito (O'Dwyer et al., 2011).

B: mature oocyst (with sporocysts) in the hemocoel of a mosquito (Sloboda et al., 2007).

C: sporocyst, after release from oocyst, in the hemocoel of a mosquito (Sloboda et al., 2007).

D: free sporocysts in the hemocoel of a mosquito (O'Dwyer et al., 2011).

E: cyst containing two cystozoites in a liver smear of an infected frog (Smith et al., 1994).

F: undivided meront in liver tissue of a snake (Sloboda et al., 2007).

G: divided meront in liver tissue of a snake (Sloboda et al., 2007).

H: gametocyte inside an erythrocyte in the blood of a snake (photo by Beatriz Tomé).

Illustrations and diagram by Beatriz Tomé.

intestinal wall of the invertebrate and enter the hemocoel (Telford, 2009). Here, gamete formation occurs via syzygy, which is the association of the gametocytes before the gametes are completely maturated. This is considered the main autapomorphy of the suborder Adeleorina (Kopečná et al., 2006). Lastly, fertilization occurs and a diploid zygote is formed, it undergoes Sporogony and develops into a sporulated oocyst. This contains several sporocysts filled with sporozoites, the infective unit for the vertebrate hosts. In Figure 1.8, this life cycle of ophidian *Hepatozoon* spp. is schematized.

It has been demonstrated that *Hepatozoon* spp. can be transmitted to snakes by three main pathways and, how exactly infection takes place in nature, remains an open question (Telford, 2009). Besides the prey-predator transmission, the two other modes are by direct ingestion of the invertebrate host (that seems rather unlikely to happen in natural conditions, hence the need of a first vertebrate host) (Telford et al., 2001, 2004; Sloboda et al., 2007) and by salivary transmission. This last has been supported by the presence of oocysts in the salivary glands and proboscides of mosquitoes (Telford et al., 2001, 2004, 2005, 2008) and experimental transmissions during which snakes were infected after being bitten by parasitized mosquitoes (Ebraheem et al., 2006; Rashdan and El-Sebaii, 2006).This way, obligate two-host, optional three-host and obligate three-host life cycles are possible for *Hepatozoon* spp. parasitizing snakes. Moreover, congenital transmission in ovoviviparous snakes has been reported (de Biasi et al., 1972; Lowichik and Yaeger, 1987). This flexibility in the transmission of *Hepatozoon* spp. points to a quite low specificity for the vertebrate hosts, at least for species detected in snakes. Genetically related lineages of *Hepatozoon* have been found in different snakes (Ujvari et al., 2004; Moço et al., 2012) and the reverse also happens. Smith (1996) refers that a *Hepatozoon* species can parasitize several snake species and that one same snake can be infected by more than one *Hepatozoon* species. Furthermore, host specificity is probably even lower for the first intermediate host, perhaps reflecting the trophic relationships of the final intermediate host, the snake (Sloboda et al., 2007). All this makes it hard to establish the host range for many parasite species and thus, species status should not be attributed to any new isolate from a given host until all possible features have been analysed (Smith et al., 1999). So, morphological and, additionally, phylogenetic data are essential for a correct taxonomical assignment.

Another aspect that is still not clear is the level of pathology of *Hepatozoon*, and other hemogregarines, in snakes. For example, Brown et al. (2006) describe the parasite-snake association as ‘surprisingly benign’ and ‘sometimes may have only trivial consequences for host fitness in natural populations’, adding that ‘coevolution weakens or eliminates fitness costs of parasitism’. On the other hand, Madsen et al. (2005) conclude that ‘only snakes harbouring lower levels of parasitemia were able to survive to old age’.

These discrepant results could be explained by differences in the snake species analysed and the type of parasite that they harboured. For instance, two *Hepatozoon* species are identified as responsible for canine hepatozoonosis: *H. canis* and *H. americanus*. While *H. canis* has mild effects on the dog, infection with *H. americanus* can lead to a fatal outcome. It appears that the former is well adapted to its canine host, whereas the last probably recently switched from a wild host, so that the coevolution period with the dog is very short (Baneth et al., 2003). The same might also be the case for species infecting snakes. For instance, Wozniak et al. (1994, 1996) report clinically significant inflammatory disease in captive unnatural reptilian hosts, while in natural hosts the effects seem less severe, including slight anaemia, varied degrees of erythrocyte hypertrophy, and some erythrocyte plasma membrane alterations. This is another area in which the current knowledge on *Hepatozoon* spp. is deficit. Studies of the diversity of these parasites in wild populations are therefore essential for identifying and comparing existing parasite lineages, and also to estimate their natural incidences.

Parasite presence and diversity detection techniques

The classical trademark tool of the parasitologist is microscopy. The Dutch Antoni van Leeuwenhoek is credited as the responsible for the conversion of the microscope from a novelty into a scientific tool. From his improvements and experiments, the microbiologic world was discovered, including the role of the protozoa as causative agents of diseases (de Waal, 2012). For a long time, microscopy was the only tool available for the detection and characterization of parasites, and today, is still widely used. It is the most unequivocal way of diagnosis of protozoan infection, by demonstration of the parasite presence in a variety of host samples (Ndao, 2009). The simplest method of microscopic examination is that of sample smears on a slide. In the case of protozoans circulating in the blood (such is the case of many Apicomplexa, especially Hematozoa and hemogregarines), smears of blood are used. However, sample preparation and examination is frequently time-consuming, labour intensive and proper identification, and diagnosis depends on skilled and experienced personnel (de Waal, 2012). Still, microscopy has been the primary source of parasite knowledge, though detection and morphological description. It offers advantages that few other methods have: quantification of infection intensity (in the case of blood samples, it is usually presented as the number of parasites per erythrocytes); differentiation between the distinct developmental stages of the life cycle; infection site discrimination (i.e. which tissue or cell is the parasite occupying); identification of mixed infections, that is, when more than one type of parasite is present in the host. Further, optical microscopy is usually inexpensive

and available in most laboratories, thus being easy to apply in routine parasite screening, such as in medical centres (Moody, 2002).

Parasite taxonomic assignments and phylogeny reconstruction were first based on knowledge drawn from microscopy, namely morphology, life cycle development, and host and geographic distribution. As microscopy techniques advanced, so did the understanding about the morphology, life history, diversity, evolution and epidemiology of the distinct parasite groups (e.g. the use of Electron Microscopy, and with it the discernment of many intracellular structures, indistinguishable until then). However, the application of these characters to systematic studies has many limitations. The loss of characters, the small size (sometimes extreme for certain life cycle phases, e.g. the ring stage of *Plasmodium* spp.), the high conservation of general structure within groups, and the lack of easily studied characters (in Apicomplexa, generally life cycle patterns, cyst organization and developmental phases morphology are used) make parasites, and unicellular parasites in particular, difficult to detect, identify and classify in a cladistic manner (Morrison, 2009; Perkins et al., 2011). Constructing a sizeable morphological data matrix is an enormous challenge and, from the analysis, character conflict and polytomies frequently occur (Perkins et al., 2011). These characteristic also make parasites prone to harbour cryptic diversity. Indeed, with molecular analysis, the report of cryptic diversity has become very common (de León and Nadler, 2010). For example, in a malaria parasite from Caribbean *Anolis* lizards, molecular data from the cytochrome *b* gene showed a clear separation between the erythrocyte-infecting and the leukocyte-infecting parasites (Perkins, 2000). This revealed the limited value of morphological similarity in defining species of these protozoans. So, it becomes necessary the application of other techniques, such as molecular tools, to fully understand the diversity of parasites and its distribution. Assessment of morphological variation is nevertheless extremely valuable in taxonomy, particularly for the description of holotypes (Adl et al., 2007).

Another method to detect parasites is through serology based-assays. When there is no sufficient sample or the parasites occur at very low densities (making them difficult to find through microscopy), serological tests have been employed to indirectly detect infections. These tools are similar in ease and time consumption to microscopy, but more sensitive and specific (Ndao, 2009), making them attractive to apply in clinical and field contexts. In a survey of *Hepatozoon canis*, Karagenc et al. (2006) did a comparison between the microscopic, serologic and molecular tools on their efficiency to detect infections. Of the three, serology was the most effective at parasite detection (36.8%), followed by PCR (25.8%) and then microscopy (10.6%).

Serologic tests can be divided into two main categories: antibody-detection assays (immunodiagnosis) and antigen-detection. Immunodiagnosis is limited by the persistence

of antibodies for a long time, even after the infection has been cleared, meaning that a positive result may not correspond to a current presence in the host. Moreover, cross reactions between closely related parasites are common, resulting also in false positives (de Waal, 2012). Alternatively, the parasite antigens may be used as target, instead of the host immunologic response, eliminating the time-lapse drawback. However, antigen assays are also prone to cross reactions and lack standardised reagents, leading to variation in results between laboratories (de Waal, 2012). Nevertheless, both types of assays have undergone improvements and are becoming increasingly available commercially.

During the last decade, molecular tools have become establish as an integral part of parasite detection in field surveys (Beck et al., 2009). Various methods have been applied to parasites (e.g. isoenzyme electrophoresis, Southern blot), but Polymerase Chain Reaction (PCR) is certainly the most commonly used (Ndao, 2009; de Waal, 2012).

PCR as a detection and phylogenetic tool in parasite studies

PCR has shown to be a simple, sensitive, reproductive and cost-effective technique, applicable to the detection of parasites in a variety of samples from animal hosts (Su et al., 2010). Using specific primers, a fragment of the parasite DNA can be amplified from a complex sample (e.g. host blood, which includes the host DNA, as well as a possible variety of microorganisms), and the success or absence of amplification informs if the sample is parasitized or not, respectively. As was mentioned previously, PCR seems to be more sensitive than microscopy to detect infections, and modifications to the original process, such as nested PCR, have helped to further increase sensitivity and specificity. With the same PCR protocol one can thus screen a large number of samples in an easy, accurate and timely manner. Still, traditional PCR has its own set of limitations, some of which can be overcome by variants of the technique.

One of the greatest flaws of traditional PCR is the impossibility of measurement of parasitemia levels (i.e. intensity of infection). However real-time PCR allows the quantification of a given DNA template concentration, thereby estimating the sample parasitemia values. Real-time PCR has been applied for infections of *Hepatozoon* spp. (Criado-Fornelio et al., 2007). Another limitation is the detection of mixed infections by PCR. For each pair of primers only a certain set of parasites (dependent on primer specificity) is detected per PCR reaction. To detect different parasite groups, several PCR reactions must be performed, increasing expenses and time consumed; while with microscopy, mixed infections can be identified directly at the same time. Nevertheless, multiplexed PCR protocols can be developed and multiple parasite detections made in the same reaction (Ndao, 2009). Despite all this, limitations still persist, especially when the

infecting parasites are very closely related or the specificity of the primers is low. When competing for a given pair of primers, the parasite DNA in higher concentration or greater similarity will anneal preferentially and, consequently, be more likely to be amplified. In this way, the other infecting parasite lineages will not be detected by PCR, and erroneous conclusions can be drawn regarding the true incidence and diversity of parasites in the host (Perkins et al., 2011). PCR is also prone to produce false positives, either by contamination or by unspecific or unexpected annealing. A crucial *a priori* step in PCR is the design of the primers. The desired specificity may vary in conformity with application objectives, but the design must always be based in both related and unrelated sequences, most notably of the host (especially for unicellular parasites, as they normally live inside host cells and their separation is nearly impossible). Comparatively, false positives are unlikely to happen with microscopy, save cases of misidentification. Also, there is the unavoidable risk of amplification of the DNA of a parasite that is present in the host but not truly parasitizing it (that is, the individual parasite ended up in a host that does not make part of its natural life cycle and cannot continue its development). Microscopy is many times argued to represent a lower expense in comparison with PCR, but that may not always be the case, namely when sophisticated microscopy techniques (e.g. electron microscopy) are necessary.

Through sequencing, one of the most advantageous features of PCR is made available. DNA sequences constitute an abundant source of characters for using in phylogenetic analyses (Perkins et al., 2011). The ribosomal RNA genes are the most used for phylogenetic reconstructions of protists, most notably the small subunit rRNA (also known as 18S rRNA) (Adl et al., 2007). The rRNA genes have certain features that make them particularly attractive for molecular studies: they have multiple copies in the genome; their transcripts are extremely abundant in the cell; and they possess simultaneously highly conserved regions that are helpful for primer design, due to their fundamental rule in translation, and more variable regions, which provide phylogenetic information (Perkins et al., 2011). The rRNA genes are typically organized in arrays of tandem repeats on several chromosomes, as shown in Figure 1.9. In Apicomplexa, 18S rRNA genes exist in a variable number of different single-locus copies (from a few to hundreds across species) that evolve in parallel and are differently expressed during the life cycle, thus being subjected to different selection pressures. Additionally, numerous insertion/deletions may lead to difficulties in alignments (Perkins and Keller, 2001), that may influence to some extent the phylogenetic output (Morrison and Ellis, 1997). Nevertheless, the 18S rRNA gene is considered to be a good marker for reconstructing the phylogenetic relationships among protists, and apicomplexans in particular (Morrison and Ellis, 1997; Šlapeta et al., 2003; Adl et al., 2007; Perkins et al., 2011).

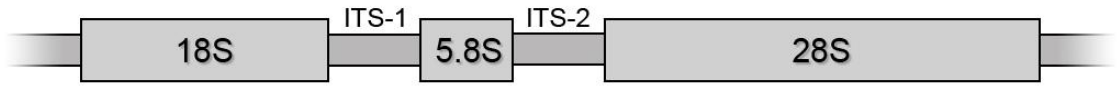

Figure 1.9 – Diagram depicting the physical organization of the nuclear genes of ribosomal subunits. Drawn by Beatriz Tomé, based on Le Blancq et al. (1997) and Ellis et al. (1998).

More molecular markers have been used in phylogenetic studies of parasites. For example, the internal transcribed spacer regions (ITS1 and ITS2) constitute other nuclear markers of choice for protozoans (Smith et al., 1999). As these genes are incorporated along with the ribosomal ones (see Figure 1.10), they share many of the advantages discussed above. Yet, as they are non-transcribed regions and hence under less selectional constrains, they normally have faster rates of evolution (Perkins et al., 2011). Mitochondrial genes are also a popular choice, as they offer many of the valuable features of these nuclear markers (i.e. high copy number, conserved sites for primer annealing) and some regions evolve at a higher rate than rRNA genes, making them useful for phylogenetic analysis at lower taxonomic levels. In addition, they are haploid and maternally only inherited, resulting in the lack of recombination, an attractive trait for phylogenetic markers. On the other hand, mitochondrial DNA structure differs between apicomplexan groups. Many species of Apicomplexa have very reduced mitochondrial genomes, generally containing only three protein-coding genes (cytochrome *c* oxidase subunit I, cytochrome *c* oxidase subunit III and cytochrome *b*) and can also present fragmented pieces of the mitochondrial large and small ribosomal genes (16S and 12S rRNA, respectively) (Wilson and Williamson, 1997; Hikosaka et al., 2010). Given the variable nature of the apicomplexan mitochondria, primers are hard to develop and, although largely used for malaria parasites, the use of mitochondrial genes for systematic purposes has not been extended to other genera (Perkins et al., 2011). Furthermore, introgression by hybridization, incomplete lineage sorting and presence of “numts” (duplication of mitochondrial loci translocated to the nuclear genome) may lead to incorrect phylogenetic tree reconstructions. The combined use of both mitochondrial and nuclear markers is therefore advised (Perkins et al., 2011). The development of more nuclear markers has not been an easy task, even with the increasing number of sequenced genomes. Complete and in-progress genome projects of parasite species are few, although they exist for several apicomplexans. Obviously, these are limited to species of high medical or veterinary importance, including *Plasmodium* spp., *Toxoplasma gondii*, *Babesia*, *Theileria* and *Cryptosporidium* (Walker et al., 2011). As sequencing costs decrease, a better taxonomic sampling of parasite genomes will be possible.

Some issues can be listed as the current major impediments to the construction of coherent and well-supported phylogenies in the field of Parasitology and particularly of Apicomplexa. For starters, data collection for parasites can mostly be described as fortuitous, rarely with phylogenetic analysis in mind. This applies both to taxon and character sampling (a problem shared by the morphological and molecular approaches of systematics). To find a given parasite, frequently extensive fieldwork is required. Hosts themselves may not be easy to collect (the case of snakes) and normally it is not possible to know if parasitized samples have been obtained until all are examined at the laboratory (Perkins et al., 2011). This small sampling capacity does not allow an adequate assessment of the variability within species and even in higher taxonomic levels, especially for rare parasite groups (Poulin and Morand, 2000). Not having a representative and balanced sampling of diversity may lead to a false determination of ancestral and derived characters, and other problems during phylogenetic analyses, resulting in biased tree estimates (Berney et al., 2004; Morrison, 2009). An example in the Apicomplexa already mentioned in this thesis is the placement of the cryptosporidians. Unsurprisingly, the apicomplexan taxa best sampled are those of medical and veterinary importance, comprehending the majority of the available sequences in databases such as GenBank. Unfortunately, wild host species lack parasite sampling, a scenario unlikely to change in the immediate future given the deficiency in funding, even with the potential of the attained data to help better understand the biology of human and domesticated animal parasites. Plus, for many of the already described parasites from wild animals, only type specimens are available and so molecular analysis is not possible, as it involves destructive processes (Perkins et al., 2011).

Most of the published sequences and phylogenies for the Apicomplexa are of the 18S rRNA gene. The choice for this gene is generally pragmatic: it has been shown to confer phylogenetic resolution often to the species level within the Apicomplexa; and, as it has the greater abundance of available sequences, is ideal for comparison of newly obtained sequences (Šlapeta et al., 2003; Barta et al., 2012). Although the studies with the 18S rRNA gene have provide most valuable information about the evolution of the Apicomplexa, a single-gene tree represents the evolutionary path of the specific gene, and not necessarily of the actual phylogenetic relationships of the taxa. Some multi-gene phylogenies are appearing for some genera, like *Cryptosporidium* and *Plasmodium*, but this is unlikely to extend to a reasonable number of other groups in the near future (Morrison, 2009).

All these sampling difficulties add to the issues known to happen during phylogenetic analysis and that can bias its output. For instance, long-branch attraction is known to occur when reconstructing Apicomplexa phylogenies. This happens due to the

heterogenic rates of evolution, consequence of the selective history of these organisms (exposed to the immunologic defences of a variety of hosts), with some highly fast-evolving groups, such as the haemosporidians (Berney et al., 2004; Morrison, 2009). Although the computational task increases with each added sequence, a wide sampling can help prevent some of these artefacts and enhance the analysis power (Morrison, 2009). Obtaining a robust phylogeny might be the key to a well-accepted classification and resolve the current chaotic state of parasite systematics (Adl et al., 2007). Molecular data can thus be incorporated as a taxonomic character, and can also help elucidate complex life cycles, such as by the screening of possible hosts (for many apicomplexan hemoparasites, the vector remains unknown) and the identification of stage forms (most descriptions of apicomplexan hemoparasites are based solely on certain stages present in the blood of vertebrate hosts).

Importance

The role of parasites in ecosystems has historically been considered of little significant, given their low representation in overall biomass. However, they influence ecological processes by modifying host fitness and, consequently, interspecific interactions, such as competition and trophic interactions (Prenter et al., 2004; Hudson et al., 2006). Thus, at a larger scale, parasites may shape energy flow and the distribution of biodiversity in an ecosystem. Despite all this, there is still much to be discovered about the biology of these organisms. The idea of a current man-mediated mass extinction has now long been acknowledged, but its effects on parasites remain poorly studied. The suggested four main drivers of this extinction phenomenon are habitat loss, species invasion, overkill and coextinction (i.e. one species goes extinct because of the disappearance of another species on which it depended). Given the obligate relationship of many parasites with their hosts, these specialist parasites must be at a high risk of coextinction (Dunn et al., 2009). Unsurprisingly, the idea of conservation of parasites lacks charisma and few studies have been done. Very few species of parasites are listed on the IUCN Red List of Threatened Species, and this still holds true even for metazoan parasites (Whiteman and Parker, 2005).

Although parasites should be conserved for their own biodiversity value, their essential ecological roles should have been sufficient to call the attention of biologists and conservation managers. Parasites evolve faster than their hosts and, as so, they are more diverse. Their extinction represents a great loss of evolutionary history and may also have great repercussions on their hosts. For instance, the loss of specialist parasites may leave hosts predisposed to infection by generalist parasite competitors and emergent

pathogens, which many times have greater health impacts (Dunn et al., 2009). Therefore, changes in the composition of parasite biotas may have grave effects on host diversity and evolution. However, this rarely is taken into consideration and, in captive hosts from zoos and conservation programs, parasite removal is routinely performed. Parasites also seem to have a role in the outcome of biological invasions in a number of distinct aspects (Prenter et al., 2004). The classic case is when native species are infected by a new parasite transported by the invading species. For example, avian malaria was introduced to Hawaii around 1830 with its mosquito vector and has been responsible by high rates of mortality in the naive native bird populations (Fonseca et al., 2000; Woodworth et al., 2005). Additionally, introduced bird species seem less susceptible to malaria than the native ones, leading to a lower competitive success of the latter and further contributing to their decline (van Riper et al., 1986). On the other hand, when in a new habitat, the selective pressures that act on the invader species change, for instance, they may be 'released' from their original parasites, resulting also in a competitive advantage over the native species. Therefore, the study of parasite ecology and evolution constitutes a significant tool for conservation management.

Parasites represent a significant portion of biodiversity, but only a small fraction of the estimated number of species has been named (Nadler and de León, 2011). This is particularly true for the phylum Apicomplexa, for which it is estimated that only 0.1% of the total diversity has been described (Adl et al., 2007; Morrison, 2009). The restricted taxon sampling is the greatest challenge in the reconstruction of the phylogenetic relationships of the apicomplexans (Kopečná et al., 2006; Morrison, 2009; Perkins et al., 2011). Additionally, the taxonomy of these organisms is in a problematic state due to the limitations of morphological approaches and the lack of molecular phylogenetic studies within individual groups of this phylum (Barta et al., 2012). Moreover, understanding their relative positions and their relation to other eukaryote organisms may elucidate how these parasites interact with their hosts (Walker et al., 2011). The information obtained from phylogenetic studies may aid parasitologists in many synergetic ways, such as: in the assessment of host and geographic distribution of parasites; in the identification of reservoir hosts of clinically important parasites; in the development of control and therapy programs; and in bringing clarification into parasite systematics (Littlewood, 2011).

Objectives

Clearly, more sampling is necessary to enhance our understanding on distribution, diversity and evolution of parasites, particularly in wild hosts that have never been assessed. Several studies in CIBIO focussed on the screening of apicomplexans,

specifically *Hepatozoon* spp., in reptile hosts and have successfully obtained DNA sequences using 18S rRNA specific primers, allowing the detection and the reconstruction of the phylogenetic relationships of these parasites (Harris et al., 2011, 2012; Maia et al., 2011). In this thesis, the aim is to focus screening in snake hosts, as these are ideal models for the study of parasites.

Snakes typically have a relatively long life expectancy, they are top predators and, as such, are expected to contain a wide variety of parasites. Particularly in the case of *Hepatozoon* species, snakes can be infected through different transmission modes, and according to which mode is the most predominant, there is an array of expected phylogenetic scenarios that can be hypothesized. For instance, if ophidian *Hepatozoon* species constitute distinct lineages and these are also closely related to those found in the respective prey of each snake species, this would suggest that prey-predator transmission is the most common. On the other hand, *Hepatozoon* spp. from snakes might form an individual clade, indicating a strong coevolution with these intermediate hosts. Comparisons with other host characteristics would also be of interest. For example, are these parasites host-specific, or does the same parasite infects different snake species? Can different *Hepatozoon* lineages be found in the same snake species, and can mixed infections be present in one same individual? Are some *Hepatozoon* lineages more common and intense than others, perhaps depending on the coevolutionary history and relation with these and other hosts? What is the range of the geographic distribution of the each ophidian *Hepatozoon* lineage?

Many other questions can be proposed, but they can only be answered when samples from different snake groups have been screened, parasites detected and their diversity assessed in a phylogenetic framework. Therefore, the main objectives of this thesis were the following:

1. Screen tissue samples from different snake groups with distinct dietary regimens using parasite-specific primers;

2. Compare the prevalence and distribution of the different parasites in the analysed snake hosts;

3. Reconstruct the phylogenetic relationships of the discovered parasite groups;

4. Investigate the significance of the retrieved phylogenies in parasite taxonomy, host specificity, coevolution and transmission patterns.

MATERIALS AND METHODS

In this chapter, the methodology used for this thesis is presented, from sampling procedures to sequence analysis. It should be noted that in the next two chapters the materials and methods are again mentioned, although briefly and concerning each study in particular. Here, a much more comprehensive description is provided.

Sample Collection

Snakes are elusive animals, as hard to find as to catch. Many of the samples used in this work were obtained from roadkills, that is, specimens that have been run over by vehicles. In these cases, a piece of muscle tissue was collected from the dead animal. When a live specimen was caught, the tip of the tail was preferred as sample material and the animal was released back to the capture location after sampling. The tissue samples were preserved in 96% ethanol. If natural bleeding from the cut of the tail tip occurred, blood was used to prepare blood smears for microscopic examination and also stored in Whatman filter paper (for more information on the preparation of blood smears and blood samples, see Appendix 1). The date of capture and the GPS coordinates of the sampling location were registered, and photographs of the animal taken. When possible, additional information (such as sex, age and size) was recorded and the animals were checked for the presence of ectoparasites (e.g., ticks and mites) and these also collected. The two types of samples collected, tissue and blood on filter paper, can successfully be used for extraction of both host and parasite DNA and, as such, are appropriate for screening for the presence of diverse hemoparasites.

The samples used in this thesis comprised a total of 202 specimens, which had been previously collected. They include 5 ophidian genera (see Table 2.1 and Figure 2.1) and locations are scattered through a large area, constituted by Southern Europe, the Middle East, Mediterranean Islands and the peri-Saharan region (Figure 2.2).

Table 2.1 – Discrimination of the number of samples per studied genus.

Genus	Number of samples
Hemorrhois	68
Malpolon	30
Rhinechis	24
Natrix	35
Psammophis	45
Total	202

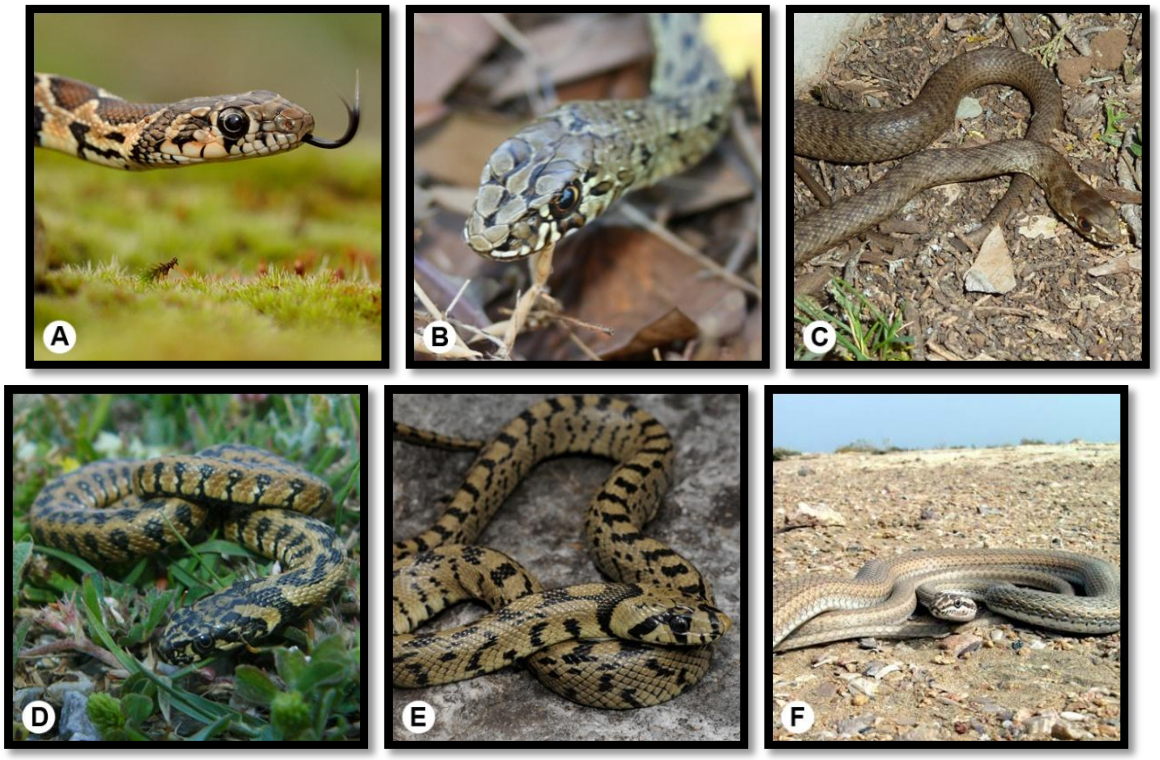

Figure 2.1 – Photographs of snakes of the different genera studied in this thesis.

A: *Hemorrhois hippocrepis*. Photographer: Filipe Caetano.

B: *Malpolon monspessulanus* from Penafiel, Portugal. Photographer: Beatriz Tomé.

C and **D**: *Malpolon monspessulanus* and *Natrix maura*, respectively. Both animals were caught during a fieldtrip to Morocco in May 2012, of which Beatriz Tomé took part. Photographer: Daniele Salvi.

E: *Rhinechis scalaris* from Portugal. Photographer: Benny Trapp.

F: *Psammophis schokari*. Photographer: Stefano Doglio.

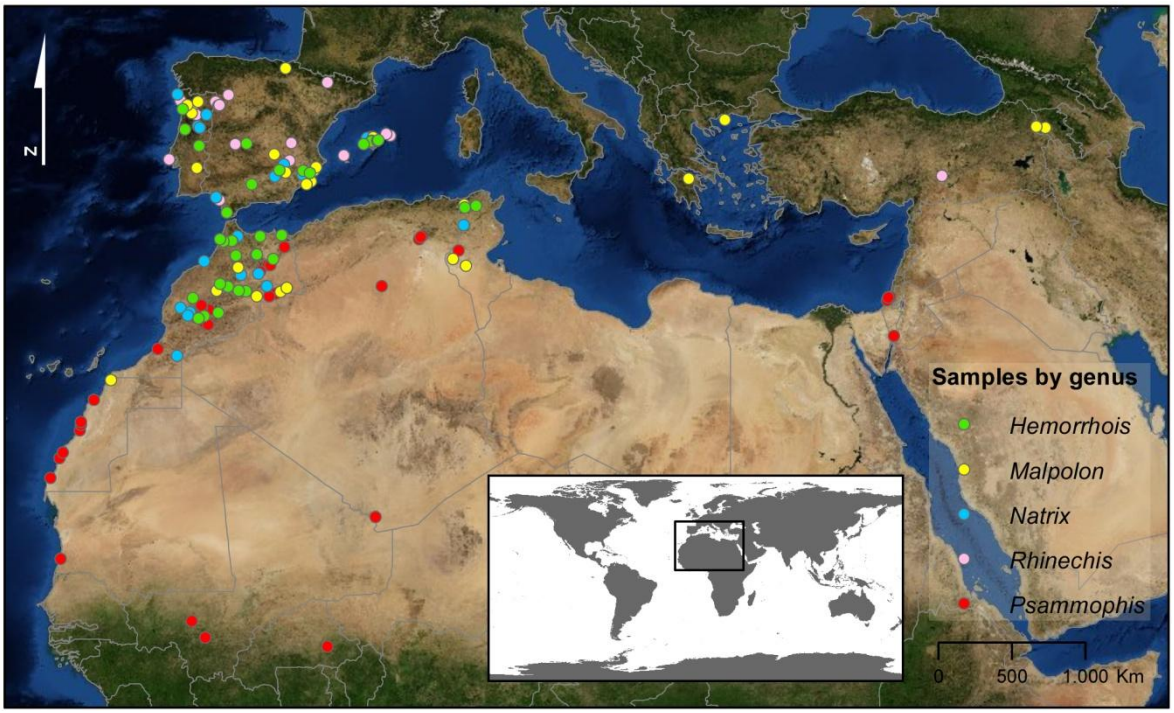

Figure 2.2 – Sampling geography distribution per genus.

DNA Extraction, Amplification and Electrophoresis

For the works included in this thesis, DNA was extracted from tissue samples preserved in 96% ethanol, employing the high salt method (Sambrook et al., 1989). This technique will next be briefly described, but a more detailed account of the procedure can be found in Appendix 2. First, a small amount of tissue is cut in tinier pieces, which are gathered into an eppendorf tube. Lysis buffer and proteinase K are added, so the tissue is digested and the cellular contents freed. Ammonium acetate is used to precipitate the proteins and, after centrifuging, the supernatant is collected to a new eppendorf tube. Then, cold isopropanol is used to precipitate the DNA into a pellet during centrifugation. The pellet DNA is washed with cold 70% ethanol and left at room temperature, for the ethanol to evaporate. Lastly, ultrapure water is added to hydrate the DNA.

Various markers had previously been surveyed for the detection of various parasites from host tissue samples. Primers designed to target the 18S rRNA gene region have yielded satisfactory results at CIBIO, retrieving sequences from reptile samples matching several apicomplexan parasites (Harris et al., 2011; 2012; Maia et al., 2011). Namely, the primers used in this thesis for the screening of host samples were the pairs HepF300/HepR900 and HEMO1/HEMO2 (in Table 2.2 information regarding the sequences and the publication references can be found). The two pairs of hemogregarines-specific primers amplify partially overlapping fragments of the 18S rRNA gene. The Hep primers, whose product of amplification is about 600 bp long, were found to be less specific than the HEMO primers, which amplify a fragment with 900 bp (Figure 2.3). During this thesis, a third pair of primers, CR-1 and CR-2 (see Table 2.2 for further details), designed to amplify a fragment with around 900 bp of the 28S rRNA gene of Coccidia, was also used.

Table 2.2 – Detailed information on the primers used in the works of this thesis.

Name	Gene	Sequence (5’ → 3’)	Reference
HepF300	18S rRNA	GTTTCTGACCTATCAGCTTTCGACG	Ujvari et al., 2004
HepR900		CAAATCTAAGAATTTCACCTCTGAC	
HEMO1	18S rRNA	TATTGGTTTTAAGAACTAATTTTATGATTG	Perkins and Keller, 2001
HEMO2		CTTCTCCTTCCTTTAAGTGATAAGGTTCAC	
CR-1	28S rRNA	CTGAAATTGCTGAAAAGGAA	Ellis et al., 1998
CR-2		CCAGCTACTAGATGGTTCGA	

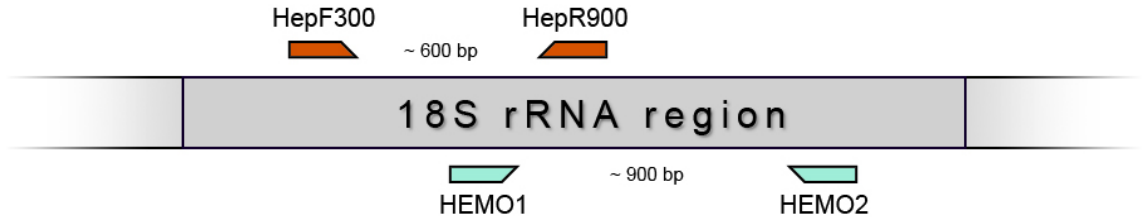

Figure 2.3 – Schematics of the relative positions of the primers used to amplify the 18S rRNA region. Diagram by Beatriz Tomé.

PCR protocols and conditions are described in Table 2.3 and Table 2.4 for each pair of primers. PCRs were run on a Biometra TProfessional Standard gradient Thermocycler. With every PCR run, two types of controls were added: a positive control (a sample previously known to be infected) to ensure that the PCR reaction had been successful; and a negative control, composed of all reagents except DNA, to check for possible contaminations. All PCR reagents were first prepared in a premix and equally distributed by the reaction wells. DNA was the last to be added, to each corresponded well separately.

The PCR products were run in 2% agarose, containing 1 µl of GelRed Nucleic Acid Stain (10,000x in water, BIOTIUM) per 50 µl of agarose solution. The gel was put into a mold and, once solidified, a mixture of 2 µl of PCR product and 2 µl of methylene blue solution was loaded into each gel well. Also, a reference ladder was loaded, in order to determine the size of the amplicons. The gel was placed in the electrophoresis apparatus and run at 200 volts. After the electrophoresis run, the PCR results were visualized using an ultraviolet transilluminator and each gel was photographed.

Table 2.3 – List of reagents and respective concentrations for the Hep, HEMO and CR primers.

Reagent	Concentration		
	Hep	HEMO	CR
Water	To 20 µl	To 20 µl	To 20 µl
Buffer	1 X	1 X	1 X
$MgCl_2$	1.5 mM	3.75 mM	3.75 mM
dNTPs	0.125 mM each	0.2 mM each	0.2 mM each
BSA	0.4 mg/µl	0.4 mg/µl	0.4 mg/µl
Primer forward	0.6 µM	0.5 µM	0.8 µM
Primer reverse	0.6 µM	0.5 µM	0.8 µM
Taq	1 U	0.5 U	0.5 U
DNA	2 µl	2 µl	2 µl

Table 2.4 – PCR protocols for the Hep, HEMO and CR primers.

Step	Function	Hep			HEMO			CR		
		ºC	time	#cycles	ºC	time	#cycles	ºC	time	#cycles
Initial denaturation	Denaturation	94	3’	1	94	3’	1	94	7’	1
Thermal cycling	Denaturation	94	30’’		94	30’’		94	1’	
	Annealing	60	30’’	35	48	30’’	37	60	30’’	35
	Extension	72	1’		72	1’20’’		72	1’	
Final Extension	Extension	72	10’	1	72	10’	1	72	10’	1
Hold	Hold	12	∞	1	12	∞	1	12	∞	1

Sequencing and Phylogenetic Analysis

Positive PCR products were sent to be purified and sequenced by a commercial sequencing facility (Macrogen Inc.). The received sequences were blasted to the NCBI database on GenBank to identify the most similar available sequences. Most matched *Hepatozoon* sequences, but a few samples were found to be infected with other apicomplexan parasites (this is further discussed in the “Results” sections of the third and fourth chapters). The parasite sequences were corrected manually and aligned, using the ClustalW software (Thompson et al., 1994) implemented in the program BioEdit (Hall, 1999), against alignments of previous studies and new sequences from GenBank.

The best-fitting models of nucleotide substitution for each alignment were chosen according to the Akaike Information Criterion, using Modeltest 3.06 (Posada and Crandall, 1998) and jModelTest 0.1 (Posada, 2008). For the phylogenetic analysis, two methods were chosen: Maximum Likelihood (ML) and Bayesian Inference (BI). The ML analysis was performed using PAUP* 4.10 (Swofford, 2002) and PhyML 3.0 (Guindon and Gascuel, 2003). While, the BI analysis was carried out in Mr. Bayes v.3.2 (Huelsenbeck and Ronquist, 2001). The specific analysis procedures for each studied dataset are presented in the “Materials and Methods” sections of the two following chapters.

HEPATOZOON INFECTION PREVALENCE IN FOUR SNAKE GENERA: INFLUENCE OF DIET, PREY PARASITEMIA LEVELS, OR PARASITE TYPE?

This chapter corresponds to one of the works developed during this thesis, which was published in the *Journal of Parasitology* of the American Society of Parasitologists in October 2012.

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Abstract

Hepatozoon spp. (Apicomplexa: Haemogregarinidae) are the most commonly reported hemoparasites from snakes. Of over 300 *Hepatozoon* species identified, more than 120 were described from snakes. However, recent genetic assessments have found *Hepatozoon* lineages recovered from both prey and predators, indicating that diet may play an important role in the infection of final vertebrate hosts. Here 4 different snake genera with different diets were assessed. *Hepatozoon* spp. prevalence varied greatly between the genera, but only lineages already identified from potential prey, i.e., gecko and lacertid lizards, were recovered from the snakes. Interestingly, the *Hepatozoon* spp. lineage known from geckos was the most common in the snakes, but this does not reflect their diet. Higher parasitemia levels, reported for some geckos relative to lacertid lizards, may play a role. Alternatively, this lineage may be more effective at parasitizing snakes or may occur, despite being unrecorded, in other vertebrate groups consumed by snakes.

Introduction

The phylum Apicomplexa is a large protist group composed by a diverse array of obligatory parasitic organisms. However, regardless of its high medical and veterinary importance, it is estimated that only 0.1% of the diversity of this phylum has been described. Even within this percentage there is a bias in studies toward a few genera, although more than 300 are recognized (Morrison, 2009). Species of *Hepatozoon* are among the most abundant and widely distributed hemoparasites. This genus was first

described in rats by Miller (1908), but it has been observed in all tetrapod groups, as well as in numerous hematophagous invertebrates, such as mosquitoes, mites, fleas, and ticks, which are their definitive hosts (Smith, 1996; Smith and Desser, 1997; Telford, 2009). More than 300 *Hepatozoon* species have been identified, with less than 50 in mammals (Allen et al., 2011), while more than 120 are described just from snakes (Smith et al., 1999).

Species of *Hepatozoon* are the hemoparasites most commonly found in snakes (Jacobson, 2007; Telford, 2009). *Hepatozoon* species have a complex heteroxenous life cycle, which generally involves a definitive invertebrate host and an intermediate vertebrate host. It is characterized in snakes by the location of gamonts within red blood cells, normally 1 per cell, though, more rarely, 2 or more can be found (Smith et al., 1999; Jacobson, 2007). At least in some *Hepatozoon* spp. infecting snakes, gametogenesis, fertilization, and sporogony take place in an invertebrate vector, after a blood meal containing infected erythrocytes, and then cystic development occurs in a first vertebrate host (a lizard or a frog, for example) that has ingested an infected invertebrate. Then merogonic development occurs in the visceral organs of a second and final vertebrate host, the snake, which has fed on the first vertebrate, culminating in the formation of the merozoites and their release into the bloodstream, where they enter the erythrocytes and develop into gamonts (Smith, 1996; Smith et al., 1999).

Traditionally, *Hepatozoon* species descriptions were accomplished using morphological analysis of the different life stages in the vertebrate intermediate and invertebrate definitive hosts and/or host specificity and life cycle patterns (Mathew et al., 2000; Telford et al., 2004). Host specificity seems to be quite low for snakes, and the same is true for the invertebrate host (see Sloboda et al., 2007). However, this has been used as a taxonomic criterion to describe new species. Moreover, morphological and life cycle data are often incomplete or incongruent, resulting many times in taxonomic assignments that do not reflect the real phylogenetic relationships (Telford et al., 2004; Sloboda et al., 2007; Moço et al., 2012). Consequently, molecular data may constitute an indispensable additional tool for taxonomists. In the present study, the main goal was to assess the prevalence and diversity of *Hepatozoon* species in different snake species across North Africa, Iberia, the Mediterranean region, and the Caucasus, using specific primers that amplify a part of the 18S rRNA region. *Hepatozoon* spp. infections have been reported in several snake species across the globe using microscopy and molecular techniques (Smith et al., 1999; Telford et al., 2004; Sloboda et al., 2007; Telford et al., 2008; Vilcins et al., 2009; Allen et al., 2011; Harris et al., 2011; Moço et al., 2012). In the aforementioned study area, *Hepatozoon* spp. were detected in several lizards (Amo et al., 2004, Amo, López, and Martín, 2005; Amo, Fargallo, et al., 2005; Maia et al., 2011), with

varying prevalence values. However, available information regarding apicomplexan hemoparasites in snakes from the study area is still limited.

Laboratory studies have shown that various *Hepatozoon* species are capable of directly infecting snakes from the invertebrate host, either by salivary transmission via a bite or by ingestion of the invertebrate host. However, this is unlikely in nature; hence, the necessity of the first vertebrate host (Sloboda et al., 2007; Telford, 2009). The role of this first vertebrate host indicates the possible importance of diet for the understanding of the life cycle of ophidian *Hepatozoon* species. It is postulated that the infection pattern by *Hepatozoon* spp. in a given snake species may be correlated with its diet, as has been suggested in some studies of other predators (Allen et al., 2011; Viana et al., 2012). Four snake genera with known and distinct dietary regimens were chosen to be included in this study to test the prey-predator transmission hypothesis. If there is in fact influence of diet on parasite infection cycles, it is expected that this will be identified from the phylogeny obtained from *Hepatozoon* spp. infecting the different snakes. Alternatively, another pattern may be found, perhaps reflecting the final host phylogeny.

Materials and Methods

Sample collection

A total of 157 samples making up 4 different genera of snakes from the Iberian Peninsula, the Maghreb and Mediterranean region, and the Caucasus was analyzed for the presence of *Hepatozoon* parasites (see Table 3.1 and Figure 3.1 for more details). For each specimen, the capture location was registered with a GPS device, digital photographs were taken, and tissue samples for molecular analysis (in the form of tail tips) were collected and preserved in 96% ethanol. Many specimens were road kills, but all live animals were then released back at the capture site.

DNA extraction, amplification, and sequencing

DNA extraction was performed using standard high-salt methods (Sambrook et al., 1989). Molecular detection of *Hepatozoon* spp. parasites was carried out through PCR reactions using the HepF300 and HepR900 primers, which were designed to amplify a part of the 18S rRNA of these parasites (Ujvari et al., 2004). PCR cycling consisted of 94 C for 30 sec, 60 C for 30 sec, and 72 C for 1 min (35 cycles) (see Harris et al., 2011, for more details). Positive and negative controls were run with each reaction. The positive PCR products were purified and sequenced by a commercial sequencing facility (Macrogen Inc., Seoul, Korea). New sequences were deposited in GenBank under the accession numbers JX244266 to JX244269.

Table 3.1 - Total number of samples analyzed and of positive *Hepatozoon* sequences obtained for each of the studied host genera. The observed prevalence is calculated from the total number of *Hepatozoon* sequences and the total number of analyzed host samples.

Genus	Total analyzed	Total *Hepatozoon* sequences	Observed prevalence (%)
Hemorrhois	67	12	18
Malpolon	30	1	3
Natrix	35	0	0
Rhinechis	24	0	0
	157	13	8

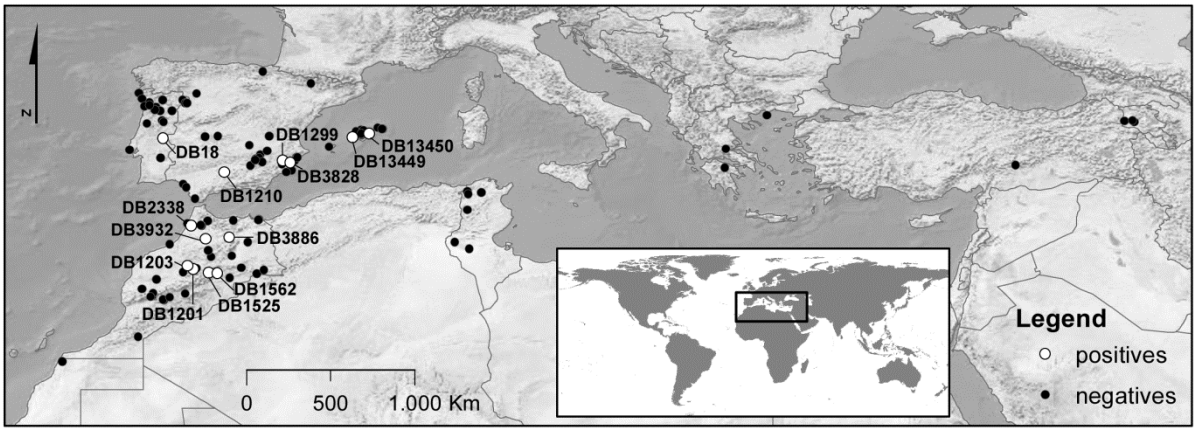

Figure 3.1 - Map of the study area containing the locations of the analyzed samples. The black dots indicate samples negative for *Hepatozoon* parasites, and the white circles indicate positive ones, which are also identified with the assigned codes.

Phylogenetic analysis

The retrieved sequences were blasted in GenBank and 13 matched *Hepatozoon* spp. However, 2 parasite sequences from *Hemorrhois* sp. were excluded from the analysis given their poor quality. Of the remaining 11, 4 unique haplotypes were aligned against previously published *Hepatozoon* spp. sequences, using the dataset from Harris et al. (2012). The alignment of the sequences was performed using ClustalW software implemented in the program BioEdit (Hall, 1999). Phylogenetic relationships were estimated using maximum likelihood (ML) and Bayesian inference. ML was performed in PAUP*4.10 (Swofford, 2002), with node support estimated using bootstrapping with 100 replicates, and the model of evolution chosen using Modeltest 3.06 (Posada and Crandall, 1998). Bayesian analysis was implemented using Mr. Bayes v.3.2 (Huelsenbeck and Ronquist, 2001) with parameters estimated as part of the analysis. The analysis was run for 1 x 10^7 generations, saving 1 tree every 1,000 generations. The log-likelihood values of the sample point were plotted against the generation time, and all the trees prior to reaching stationary were discarded as burn-in samples (25%). Remaining trees were

combined in a 50% majority consensus tree. Following Harris et al. (2012), *Adelina bambarooniae* was designated as an outgroup (see Figure 3.2).

Results

From the 157 analyzed host samples, 13 were positive for *Hepatozoon* spp. parasites, resulting in a global prevalence of 8%. However, the prevalence values differed significantly between genera. For species of *Natrix* and *Rhinechis*, no infections were found, while *Hemorrhois* and *Malpolon* spp. presented, respectively, prevalences of 18% and 3% (see Table 3.1).

The phylogenetic analysis yielded 4 *Hepatozoon* haplotypes infecting the snakes in the study area, 2 similar to those previously found in lacertids and skinks (Lineage D; Figure 3.2) and the other 2 to those of geckos (Lineage B; Figure 3.2). Within lineage D, *Hepatozoon* parasites were found in 2 *Hemorrhois* samples from Morocco (DB1203 and DB1562), which are more closely related with those previously detected in the lizards *Eumeces algeriensis* and *Chalcides polylepis*, also from Morocco. Within lineage B, the only positive *Malpolon* sample (DB1201) and the remaining 8 positives from *Hemorrhois* hosts clustered in a clade shared with *Hepatozoon* spp. from rodents, snakes, and geckos, closer to the sequences reported in the gecko hosts *Tarentola*, *Ptyodactylus*, and *Quedenfeldtia*. These 9 sequences are either identical to the already published sequences from geckos (represented in Figure 3.2 by DB1201) or differ by just 1 mutation (DB1210).

The number of positive PCR products obtained with the HEP primers was considerably superior to the actual number of *Hepatozoon* spp. positives, confirmed after sequencing. Most of the PCR positives were apparently fungi, considering the BLAST search results. Since this region of 18S rRNA is not variable enough to ascertain with certainty the type of fungi and since the fungi could be from the samples or contamination during handling, these were not considered further. Nevertheless, this highlights the difficulty of relying solely on positive PCRs and the need of verification by sequencing when reporting parasite infections. Additionally, 2 analyzed individuals (1 *Hemorrhois* and 1 *Malpolon*) were infected with different *Sarcocystis* spp., whose closest match on GenBank was a *Sarcocystis lacertae*. Detection of *Sarcocystis* spp. with these primers in reptiles has been previously reported (Harris et al., 2012).

Discussion

For the first time, the prevalence and phylogenetic relationships of *Hepatozoon* spp. from several snake species of the Mediterranean area was assessed. Instead of

constituting a separated phylogenetic unit, the ophidian *Hepatozoon* spp. clustered with those of other lizard and gecko hosts, from the same approximate geographic location. This objectively shows that the phylogeny of these parasites does not reflect vertebrate host phylogeny. Barta et al. (2012) also indicate vertebrate host phylogeny is not reflected by the relationships of adeleorinid parasites, but rather highlight the importance of the invertebrate host, particularly at a deeper taxonomic level, where there is a clear separation between parasites using leeches as their hosts and those using arthropods. It seems that diet is one of the driving factors of *Hepatozoon* spp. transmission in snakes,

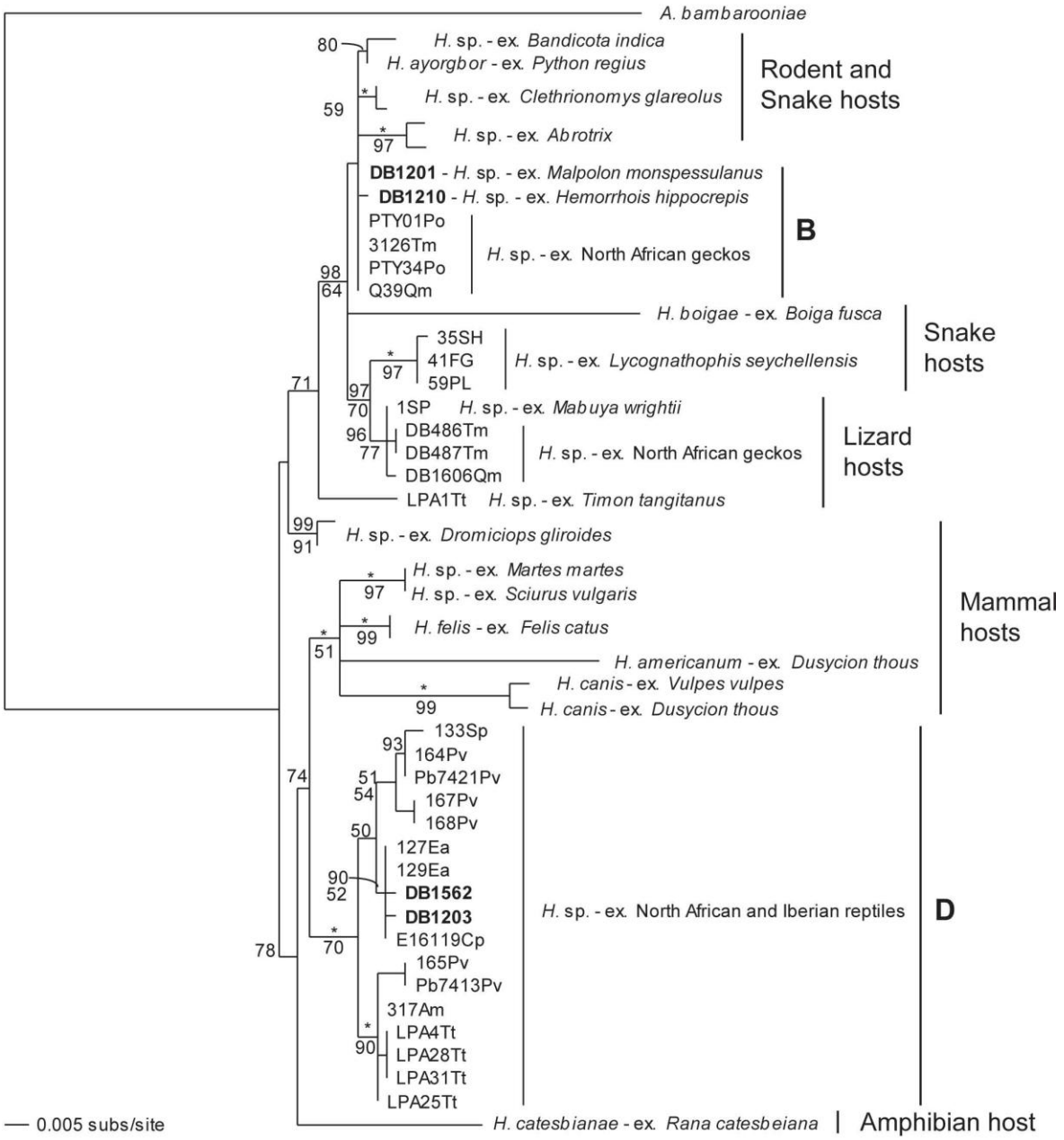

Figure 3.2 - Estimate of relationships based on an ML analysis. Bootstrap values for ML are given below relevant nodes, while Bayesian posterior probabilities are given above them. When values were 100%, this is indicated with an asterisk. The 4 haplotypes indicated in bold are those from the snakes identified in this study.

as shown by the presence of genetic lineages of *Hepatozoon* spp. previously found in lizards in species of snakes whose diet is partially constituted by these kinds of organisms. Unfortunately, the 2 genera with a much lower consumption of reptiles (species of *Natrix* and *Rhinechis*) presented no infections.

A study of *Hemorrhois hippocreppis* describes the diet of this species as 45% mammals, 28% reptiles, 17% birds, and 10% amphibians (Pleguezuelos and Fahd, 2004). Among the reptiles consumed, the authors report a higher frequency of lacertids compared to geckos. In our study, we found a higher prevalence of *Hepatozoon* spp. related to those found in geckos rather than to those infecting lacertids in *Hemorrhois* spp. hosts. This raises several questions, and several hypotheses can be presented that might explain this result.

First, the *Hepatozoon* spp. predominantly found in these snakes may also be found in other groups of organisms besides geckos, but there have simply not been enough studies to detect them. This is supported by the clustering of *Hepatozoon* spp. from this lineage next to those from mammals, including rodents (Lineage B; Figure 3.2). However, this seems unlikely, given no *Hepatozoon* spp. lineage previously identified in mammals was retrieved from our samples, including from species whose diet is almost exclusively mammals, e.g., *Rhinechis* sp., which may indicate that these snakes are not commonly infected by this kind of *Hepatozoon* spp. Extensive sampling of small mammals from the Iberian Peninsula and North Africa would be needed to test this hypothesis. A second hypothesis suggests that the *Hepatozoon* spp. from geckos are transmitted more easily or multiply more, or infection is more persistent in snake hosts than the *Hepatozoon* spp. from lacertids, and that these are more readily detected. This could be tested through experimental infection of snakes with different lineages of *Hepatozoon* spp. from lacertids and geckos. Also, snakes of different genera may differ in immune responsiveness and their ability to clear infection. Finally, if geckos and lacertids have very different infection loads, this would represent differential probabilities of infection for both types of parasite for each consumed individual. For example, a study reported that 41 out of 43 individuals had infection intensities of 10, or less, infected cells per 2,000 erythrocytes (Amo, López, and Martín, 2005), and another study reported a maximum of 81 hemogregarines per 10,000 erythrocytes (Sacchi et al., 2011), both for the wall lizard *Podarcis murallis*. In contrast, in the gecko *Ptyodactylus hasselquistii*, Hussein (2006) reported up to 410 per 10,000 erythrocytes. If these figures reflect the actual differences between *Hepatozoon* spp. loads in both groups, it might explain our findings, since snakes would in reality be consuming more *Hepatozoon* spp. from geckos, even if the actual number of geckos consumed were fewer. A first step to address this would be to assess infection load in other geckos from the region, particularly the common *Tarentola mauritanica*. Additionally,

the detected infections (either in snakes or lizards) may represent ‘‘dead ends’’ and not be part of the actual life cycle of these *Hepatozoon* spp. Further studies on the potential invertebrate hosts are essential to assess this possibility.

Hepatozoon spp. are commonly found in all tetrapod groups, and recent genetic assessments have reported them in both prey and predators. In fact, experimental studies have shown that infections can occur in multiple vertebrate hosts, which can transmit infection when consumed as prey, e.g., Viana et al. (2012). Hence, diet may play an important role in this parasite’s life cycle (Allen et al., 2011). Here, we report the occurrence of *Hepatozoon* spp. in 2 snake genera that are known to regularly feed on lizards, with the phylogenetic analysis showing that the isolates found in these prey and predators to be closely related. Although many factors may play a role in parasite transmission, including higher parasitemia levels in geckos than in other lizards, it is possible that infections from geckos can be more effectively transmitted to the final vertebrate hosts. This warrants further investigation.

Acknowledgments

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Literature Cited

The bibliography used in this article is provided along with the general references of this thesis.

MOLECULAR ASSESSMENT OF APICOMPLEXAN PARASITES IN THE SNAKE *PSAMMOPHIS* FROM NORTH AFRICA: DO MULTIPLE PARASITE LINEAGES REFLECT THE FINAL VERTEBRATE HOST DIET?

This chapter corresponds to the second study of this thesis. It is here presented as it was submitted on 27 September 2012 for publication in the *Journal of Parasitology* of the American Society of Parasitologists.

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Abstract

The Apicomplexa are intracellular pathogens of animals, with the Coccidia being the largest group. Among these are the hemogregarines, which include some of the most common hemoparasites found in reptiles. Several studies have reported a possible pattern of prey-predator transmission for some of these parasites. Snakes from the Mediterranean region have been found to be parasitized with *Hepatozoon* spp. similar to those in lacertids and geckos, supporting the prey-predator transmission hypothesis. Here we analyzed specimens of the saurophagous genus *Psammophis* from North Africa, an ecologically different region. Through molecular analysis of tissue samples we detected three different apicomplexan parasites: *Caryospora*, *Sarcocystis* and *Hepatozoon*. *Caryospora* was detected in a *P. schokari* individual from Algeria, constituting the first time these parasites have been detected from a tissue sample through molecular screening. The obtained *Sarcocystis* phylogeny does not reflect the relationships of their final hosts, with the parasites identified from snakes forming at least 3 unrelated groups, indicating that it is still premature to predict definitive host based on the phylogeny of these parasites. Three unrelated lineages of *Hepatozoon* parasites were identified in *Psammophis*, each closely related to lineages previously identified from different lizard groups, on which these snakes feed on. This once again indicates that diet might be a key element in transmission, at least for *Hepatozoon* species of saurophagous snakes.

Introduction

The Apicomplexa are a group of highly successful and widespread intracellular pathogens of animals that pose a serious threat to human health and activities. Despite this, it remains one of the less studied phyla in terms of biodiversity, with the majority of studies focused on a limited number of genera, such as *Plasmodium*, *Toxoplasma*, *Babesia*, and others of direct anthropogenic interest (Morrison, 2009). This may be historically explained by their obligatory endoparasitic lifestyle, which makes them one of the hardest group of organisms to work with, given they are difficult to identify morphologically, with few diagnostic characteristics (Morrison, 2009; Perkins et al., 2011). In this situation, molecular techniques are particularly useful tools for both diagnostic and phylogenetic purposes and, as such, they have become firmly established in recent years (Beck et al., 2008; de Waal, 2012).

The Coccidia form the largest group of apicomplexan organisms, presenting a diverse array of forms and life cycles. Coccidians can be divided into two suborders Eimeriorina and Adeleorina, whose monophyly seems to be supported by phylogenetic studies (e.g. Perkins and Keller, 2001; Barta et al., 2012). The former is the largest and includes important parasites of reptiles, such as *Eimeria*, *Isospora*, *Sarcocystis* and *Caryospora*. Adeleorina encompasses monoxenous parasites of invertebrates and the heteroxenous hemogregarines (Barta et al., 2012). These are commonly found in reptiles, generally parasitizing red blood cells. In snakes, the genus *Hepatozoon* is the most often identified hemogregarine, although it has been described in all tetrapod groups (Smith et al., 1999; Sloboda et al., 2007). Several studies report a possible connection between the lineages of *Hepatozoon* parasites found in predators and those found in the respective prey (Allen et al., 2011; Viana et al., 2012). This might be the reflection of the transmission patterns between hosts, suggesting that prey-predator transmission for these parasites may be more widespread than previously thought.

In a recent assessment of snakes from the Mediterranean region (Tomé et al., 2012), two *Hepatozoon* types were identified from two of the studied genera (*Hemorrhois* and *Malpolon*), similar to lineages previously found in lacertids and geckos. Coincidently, those two genera are known to have a diet that includes such lizards. These findings support the hypothesis that, at least for some *Hepatozoon* species, vertebrate host diet might influence the transmission and phylogenetic relationships of these parasites (Viana et al., 2012). However, other possible explanations were presented, such as differences in prey parasitemia levels and in effectiveness at parasitizing snakes between *Hepatozoon* types, and the possibility that these infections represent “dead end” infections. Previously,

Sloboda et al. (2007) proposed that, for *Hepatozoon* parasites, vertebrate host ecology might be important in determining host specificity, rather than vertebrate host phylogeny.

To distinguish between these competing hypotheses, it is necessary to assess snakes from an ecologically different but comparative region. Furthermore, these should be saurophagous, like the infected genera *Malpolon* and *Hemorrhois* from the Mediterranean region, so that the influence of diet is also assessed. Consequently, we analyzed specimens of the snake genus *Psammophis*, using it as a model of comparison with the Mediterranean snakes, due to its more desertic ecological preferences. In North Africa, *Psammophis schokari* is known to have a saurophagous diet (Cottone and Bauer, 2009), and has been studied phylogeographically (Rato et al., 2007), so the geographic distribution of genetic variability within the vertebrate host is known. If the hypothesis of Sloboda et al. (2007) is correct, we expect to find distinct lineages in the *Psammophis* snakes compared with those obtained in Tomé et al. (2012), since they have different ecological niches. If the link with diet is primarily determining the parasites detected, we expect to find *Hepatozoon* lineages also found in animals *Psammophis* preys on, that is, lineages already found in lizards from this region (Maia et al., 2011).

Materials and Methods

Sample collection

Our dataset comprised a total of 45 tissue samples from different specimens of several *Psammophis* species from 9 countries in North Africa and the Middle East (see Figure 4.1 for the distribution points). For each specimen, the GPS coordinates of the capture point were recorded, digital photographs taken, identification made by experienced herpetologists, and tails tips stored in 96% ethanol for molecular. Many specimens were roadkills, but live snakes were released at the capture point after processing.

DNA extraction, amplification and sequencing

DNA extraction was performed using standard high salt methods (Sambrook et al., 1989). To test for the presence of *Hepatozoon* parasites, two pairs of hemogregarine-specific primers targeting different but overlapping parts of the 18S rRNA region were used: HepF300/HepR900 (Ujvari et al., 2004) and HEMO1/HEMO2 (Perkins and Keller, 2001). PCR cycling for the Hep primers consisted of 94ºC for 30 sec, 60ºC for 30 sec and 72ºC for 1 min (35 cycles). For the HEMO primers, the protocol was the same except for the annealing temperature, which was 48ºC (see Harris et al. 2011 for more details). Later, a third pair of primers designed to amplify a part of the 28S rRNA gene of Coccidia,

CR-1 and CR-2 (Ellis et al., 1998), and that are known to amplify some Apicomplexa groups (e.g., Ellis et al., 1998; Šlapeta et al., 2003; Zhu et al., 2008), were also tested to see if they amplified any of the parasites identified with the more widely used Hep primers. Briefly, amplification temperatures and times were 94ºC for 1 min, 55ºC for 30 sec and 72ºC for 1 min (35 cycles). Positive and negative controls were run with each reaction. The positive PCR products were sent to be purified and sequenced by a commercial sequencing facility (Macrogen Inc., Seoul, Korea). New sequences were deposited in GenBank under the accession numbers *** to *** (to be added after final acceptance).

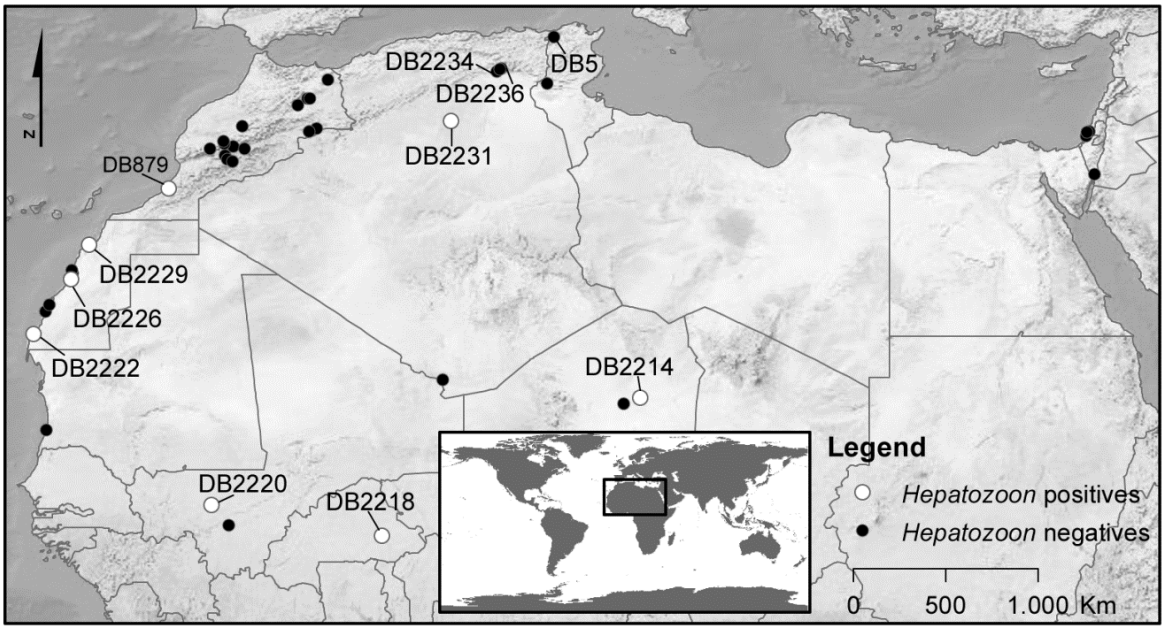

Figure 4.1 - Map of the study area containing the locations of the analyzed samples. Black dots indicate samples negative for *Hepatozoon* parasites, while the white circles indicate positive ones, which are identified with the assigned codes. Also identified are the samples positive for *Sarcocystis* (DB2234 and DB5) and *Caryospora* (DB2236) infections.

Phylogenetic analysis

Due to the genetic differences between the parasites detected, separate phylogenetic analyses were performed for each of the parasite groups (*Hepatozoon*, *Sarcocystis* and *Caryospora*). For each dataset, sequences generated in this study were aligned with related sequences from GenBank. These were aligned using ClustalW software implemented in the program BioEdit (Hall, 1999). The final three datasets contained 53, 24 and 15 sequences with 1396, 616 and 580 bp in length, respectively.

Two different phylogenetic analyses (Maximum Likelihood and Bayesian Inference) were conducted. Maximum Likelihood (ML) analysis with random sequence addition (100 replicate heuristic searches) was used to assess evolutionary relationships, using the software PhyML 3.0 (Guindon et al., 2010). Support for nodes was estimated using the

bootstrap technique (Felsenstein, 1985) with 1000 replicates. The AIC criterion conducted in jModeltest 0.1 (Posada, 2008) was used to choose the model of evolution and the parameters employed. Bayesian analysis was implemented using Mr. Bayes v.3.1 (Huelsenbeck and Ronquist, 2001) with parameters estimated as part of the analysis. The analysis was run for 10×10^6 generations, saving one tree each 1000 generations. The log-likelihood values of the sample point were plotted against the generation time and all the trees prior to reaching stationary were discarded, ensuring that burn-in samples were not retained. Remaining trees were combined in a 50% majority consensus tree, in which frequency of any particular clade represents the posterior probability (Huelsenbeck and Ronquist, 2001). Following Barta et al. (2012), *Haemogregarina balli* and *Dactylosoma ranarum* were used as outgroups for rooting the phylogenetic tree for the *Hepatozoon* sequences. Following Šlapeta et al. (2003) and Harris et al. (2012), *Besnoitia* and *Hylokossia* were used to root the estimate of relationships of the *Sarcocystis*, while following Jirku et al. (2009), *Choleoeimera* sp. and *Eimeria tropidura* were used to root the estimate of relationships of the *Caryospora* dataset.

Results

From the 45 analyzed samples, 8 were positive for *Hepatozoon* infections, 1 for *Sarcocystis* and another 1 for *Caryospora*, using the Hep primers. These primers have previously been reported to detect other Apicomplexa besides hemogregarines, such as *Eimeiria* and *Sarcocystis* (Harris et al., 2012; Tomé et al., 2012), although this constitutes the first example of amplification of a *Caryospora* sequence from a tissue sample. The HEMO primers yielded only positives for *Hepatozoon*, matching the Hep pair, resulting in an overall prevalence of 18% for these parasites. As found in Tomé et al. (2012), we again identified fungal sequences obtained with the Hep primers, so some amplifications with these primers did not correspond to apicomplexan infections.

With the Hep primers, it was clear that sample DB2222 was infected with two different *Hepatozoon* lineages. The electropherogram presented twin peaks at positions that differed between known *Hepatozoon* haplotypes until a deletion in one sequence led to the two being out of phase, and thus unreadable. The same situation occurred with the sequence from the reverse direction. This is, to our knowledge, the first example of a mixed infection of *Hepatozoon* identified through molecular screening of tissue samples. On the other hand, the HEMO primers only amplified one of the lineages, which may indicate preferential amplifications of some lineages over others. Sequence from one sample (DB2226) blasted with *Hepatozoon*, although it had poor quality. Therefore, this sample and DB2222 were not included in the following phylogenetic analyses. With a third

pair of primers, CR-1 and CR-2, just one of the analyzed samples was positive and a 28S rRNA sequence of a *Sarcocystis* sp. was obtained. This sample, DB2234, was the same for which the Hep primers detected a *Sarcocystis* infection.

To assess relationships of the different parasites, three estimates of phylogeny were produced. Regarding *Caryospora*, the newly sequenced sample is most closely related to *C. bigenetica* (Figure 4.2). Like *Cyclospora*, *Caryospora* appears as a lineage within a paraphyletic clade of *Eimeria* sequences. Otherwise, relationships within this group are similar to those previously estimated using the same marker (Harris et al. 2012). Regarding *Sarcocystis*, isolates from multiple unrelated snake hosts available in GenBank

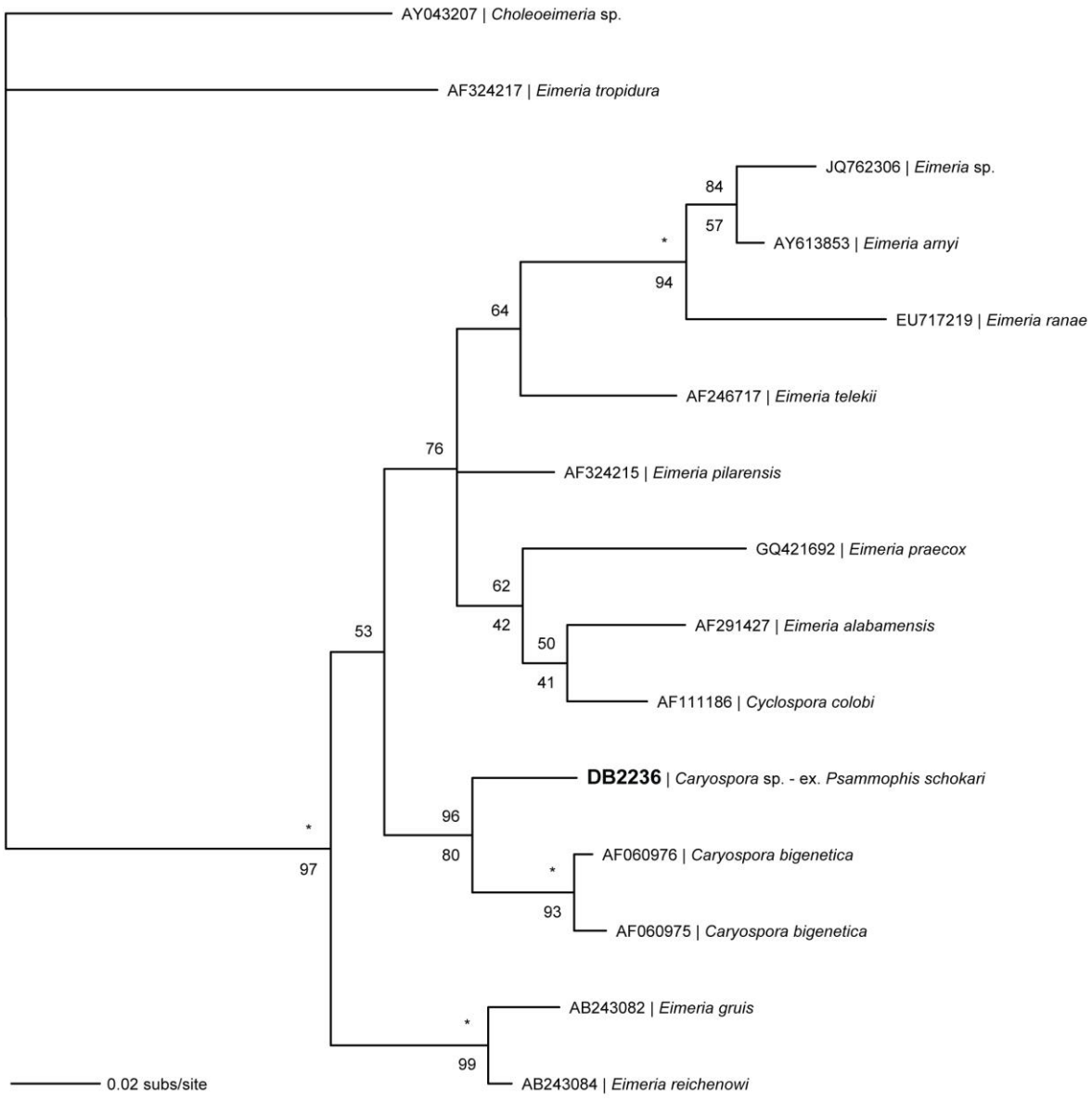

Figure 4.2 **-** Estimate of relationships for *Caryospora* based on a Bayesian analysis using the GTR+G model. Bootstrap values for ML are given below relevant nodes, while Bayesian Posterior Probabilities are given above them. When values were 100%, this is indicated with a *. The new sequence is indicated in bold.

were included in the analysis of this group of parasites (Figure 4.3), but these did not form a monophyletic group, as previously suggested in a study with more limited sampling (Tian et al., 2012). Apart from this, relationships were again similar to those previously proposed (Harris et al., 2012). Finally, the estimate of relationships of *Hepatozoon* sequences based on the combined data from the Hep and HEMO primers was similar to that of Maia et al. (2011) and Barta et al. (2012), with the newly sequenced haplotypes being similar, or nearly so, to known haplotypes from other reptiles (Figure 4.4).

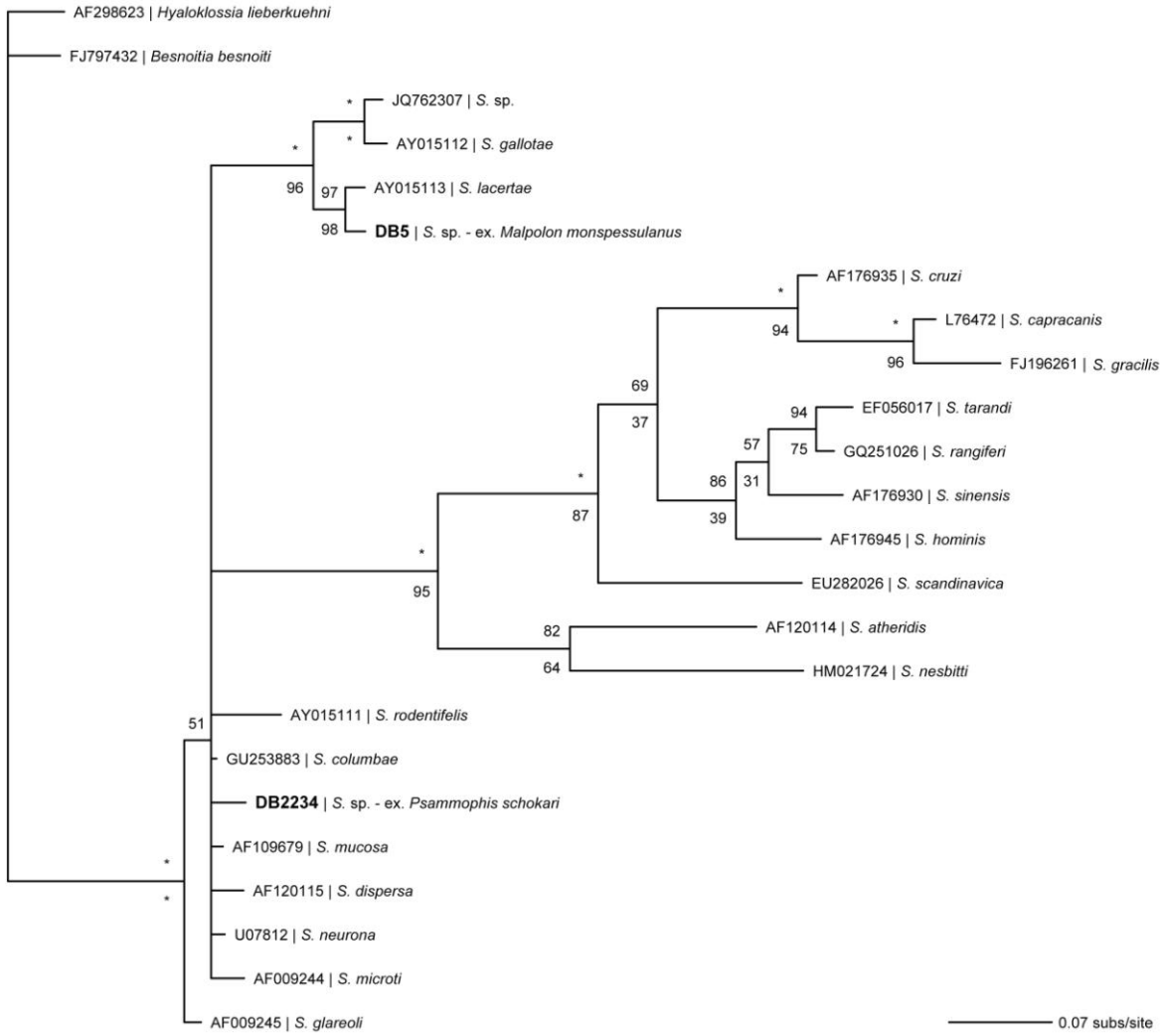

Figure 4.3 **-** Estimate of relationships for *Sarcocystis* based on a Bayesian analysis using the HKY+I+G model. Bootstrap values for ML are given below relevant nodes, while Bayesian Posterior Probabilities are given above them. When values were 100%, this is indicated with a *. The new sequences are indicated in bold.

Discussion

Just as in the Mediterranean snakes (Tomé et al., 2012), in this assessment we found the *Psammophis* individuals to be infected with *Hepatozoon* lineages similar to

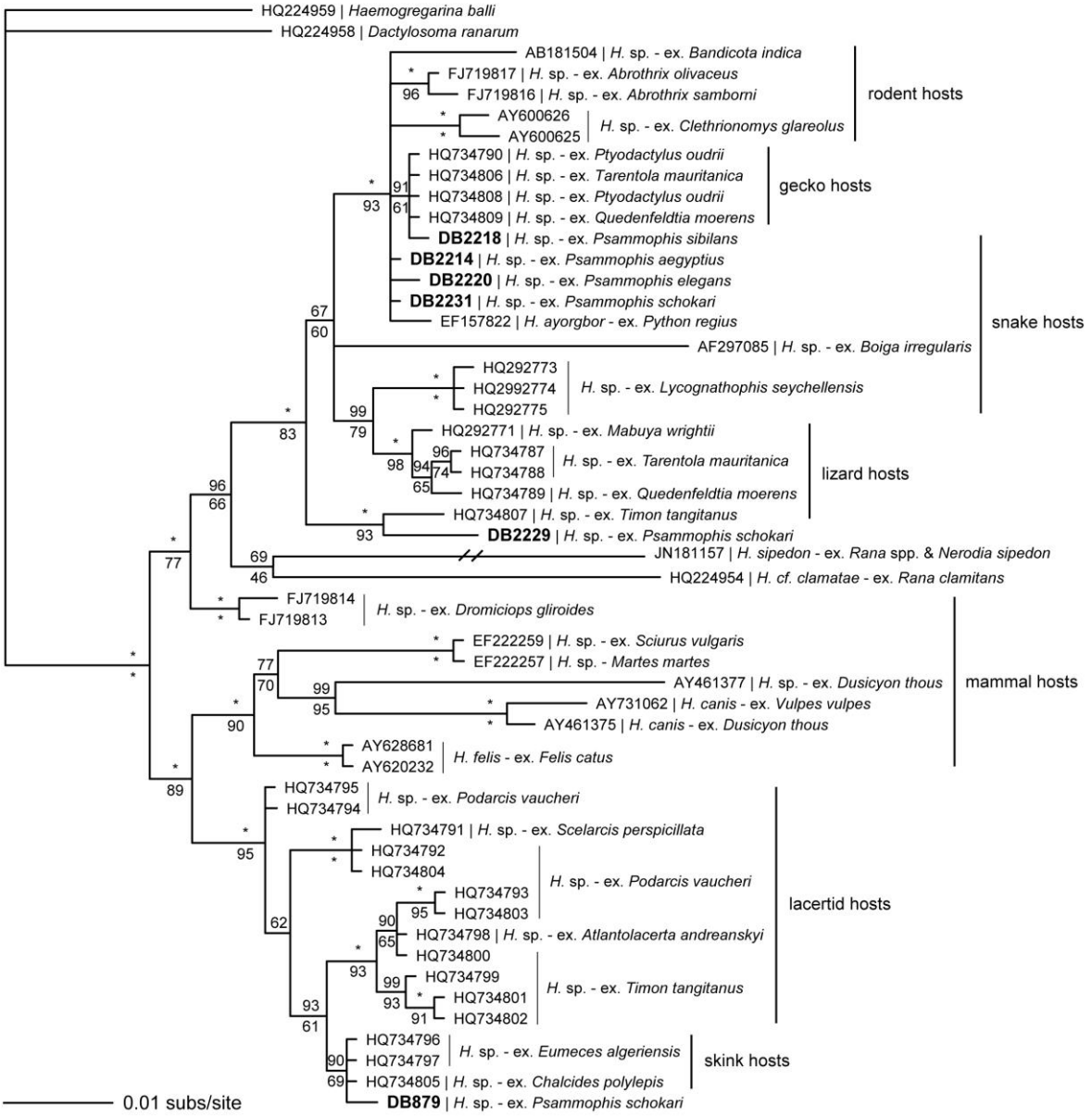

Figure 4.4 **-** Estimate of relationships for *Hepatozoon* based on a Bayesian analysis using the TVM+G model. Bootstrap values for ML are given below relevant nodes, while Bayesian Posterior Probabilities are given above them. When values were 100%, this is indicated with a *. The new sequences are indicated in bold. The branch length leading to *H. sipedon* was shortened to half purely for presentation purposes.

those that have been previously reported from several lizard groups, supporting the idea that saurophagous snakes are primarily infected by the same parasites that infect the lizards they consume. The six new *Hepatozoon* sequences used in the phylogenetic analyses clustered together with parasites from different lizard types (geckos, skinks and lacertids), probably reflecting the mixed diet of these snakes. In fact, all these groups of lizards have been reported as prey items for *P. schokari* (Cottone and Bauer, 2009). Despite occupying a very different ecological niche, these retrieved lineages were very similar to those found in the Mediterranean snakes, thus not supporting the hypothesis

that the ecological niche of the vertebrate host is a determinant in host specificity. On the other hand, diet seems again to be a key element in this matter, at least for saurophagous snakes.

Sporocysts of *Sarcocystis* spp. have been identified in fecal samples from different *Psammophis* snakes: *P. schokari* from Israel (Paperna and Fikelman, 1998) and *P. orientalis* from Eastern Kenya (Šlapeta et al., 2003). From our *Psammophis* tissue samples, we identified one individual infected with *Sarcocystis*. Phylogenetically, it is unrelated either to the isolate from a *Malpolon* snake sample (first reported in Tomé et al., 2012), or to the other published sequences from snakes (e.g., *S. atheridis*). It has been previously suggested that *Sarcocystis* phylogeny reflects the relationships of their final hosts (Doležel et al., 1999). Our findings show that, at least for snakes, this is not the case, with *Sarcocystis* identified from snakes forming at least 3 unrelated groups. In Tian et al. (2012), the authors attempted to use phylogenetic analysis to predict the probable definitive host of *Sarcocystis nesbitti*. They suggest that it should be a snake based on the clustering of their sequence with other *Sarcocystis* with snake hosts, based on the assumption that definitive host associations reflect the phylogenetic relationships of the parasites. However, our results contradict these conclusions, indicating that it is still premature to predict definitive host based on the phylogeny of *Sarcocystis* given that, at least in snakes, it does not correspond to final host phylogenetic relationships. Clearly, more sampling is needed to fully understand these parasites phylogenetic relationships and life cycles.

Several species of *Caryospora* have been described from *Psammophis* specimens from Sub-Saharan Africa (Hoare, 1933; Bray, 1960; Šlapeta et al., 2003) and from the Arabian Peninsula (Modrý et al., 1999; Alyousif et al., 2004). We were able to amplify an 18S rRNA sequence using the Hep primers that seems to be from a *Caryospora* specimen. The host, DB2236, is a *P. schokari* from northern Algeria, a region where there are no descriptions of *Caryospora* from *Psammophis* snakes. Only two species of *Caryospora* are described from *P. schokari*, *Caryospora maxima* (Modrý et al., 1999) and *Caryospora schokari* (Alyousif et al., 2004), from Jordan and Saudi Arabia, respectively. Unfortunately, since they were not characterized genetically, it is not possible to say which, if either, corresponds to the *Caryospora* sequence from our study.

To conclude, we report the presence of several apicomplexan parasites in *Psammophis* snakes from North Africa. For *Hepatozoon* parasites, 3 main types were found, each closely related to lineages previously identified from different lizard groups. *Psammophis* are known to feed on these lizards, supporting transmission by predation as a common infection mode of these parasites for wild snakes. Still, invertebrate hosts need to be identified to completely understand the life cycle, phylogeny and distribution of these

Hepatozoon spp. The Hep primers were designed to amplify a part of the 18S rRNA gene of *Hepatozoon* parasites, however they have been reported to detect other apicomplexan parasites (Harris et al., 2011, 2012), as well as other organisms such as stramenopiles (Maia et al., 2012a) and fungi (Tomé et al., 2012). Therefore, the use of sequencing is advised to assure the identity of positive PCR products. In this study, we were able to detect infections of two other apicomplexan groups and gather additional information of the diversity of parasites found in snakes. We assessed the utility of primers for the 28S rRNA gene, but these only worked for the *Sarcocystis* infection. However, at least for studies of *Sarcocystis* they could be a useful marker. Still, for the genus *Hepatozoon*, primers for additional genes remain to be found, so future studies should focus on the development and optimization of primers for this, and also for other apicomplexan groups. Nevertheless, our results constitute further evidence that many different apicomplexans can be identified from tissue samples using molecular tools, and again highlight the value of this technique to gain new insight into Apicomplexa diversity and distribution.

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Literature Cited

The bibliography used in this article is provided along with the general references of this thesis.

FINAL REMARKS

Through the works of this thesis, new insights into the biology of the Apicomplexa have been provided, in particular of parasite species that use snakes as hosts. The studies in this thesis constitute the first large-scale molecular survey study on the diversity of parasites of snakes from the Mediterranean region and North Africa. The most commonly found parasites in the assessed snakes were species of the genus *Hepatozoon*. However, other apicomplexans were detected, suggesting that these vertebrates are host to a wide variety of parasites.

In the first study (Tomé et al. 2012), snakes of four distinct genera of the Mediterranean region were screened for the presence of *Hepatozoon* parasites using hemogregarine-specific primers. Infections were detected only in individuals known to have a dietary regimen largely composed by lizard prey. The retrieved *Hepatozoon* sequences clustered in separate clades, close to those from lizard hosts of the same proximate geographic area. This shows that *Hepatozoon* spp. from snakes do not form a single group and, thus, the phylogenetic relationships of these parasites do not reflect final vertebrate host phylogeny. Instead, the diet of snakes seems to be a key factor in the transmission of *Hepatozoon* parasites in these hosts. Although, the discrepancy between the prevalence of the distinct *Hepatozoon* lineages and the relative portion of the different lizards consumed by *Hemorrhois* snakes raised some questions, it also provided several exploratory hypotheses for the future.

Screening of mammals from the Mediterranean area is needed to find if these host any *Hepatozoon* spp. and confirm the true phylogenetic position of some of the lineages found in this study. However, no *Hepatozoon* infections were detected in the genus with the higher uptake of mammals (i.e. *Rhinechis*), suggesting that, if *Hepatozoon* spp. parasitize mammals in this region, they seem to not infect snakes, at least of the studied genera. In fact, species of *Hepatozoon* have been detected in rodents, cats and canids of Spain and France (Criado-Fornelio et al., 2006, 2009). No infections were also detected in the samples from *Natrix* spp., although, for example, *Hepatozoon sipedon* has been shown to use as vertebrate hosts frogs and natricine snakes from Canada (Smith et al., 1994). From these results, this kind of cycle seems to not happen in the Mediterranean region, or at least it was not detected in this study. Only more sampling of these various hosts can answer this question. On the other hand, if the ‘gecko type’ *Hepatozoon* lineages are in fact more related to the ones found in geckos, this would indicate a differential detection of the different parasite lineages, which can be explain in several ways. These include differences in infectability and prey parasite load. Another interesting

hypothesis is that the used primers preferentially amplify certain lineages over others. Different amplification preferences by primers would explain why detection results many times vary across studies. Also, assessing the complete life cycle of these *Hepatozoon* spp., including the identification of the invertebrate hosts, is essential to fully understand their diversity and transmission patterns, and confirm whether the detected infections in the studied snakes correspond to 'dead ends'.

For the second study of this thesis, samples from snakes of the genus *Psammophis* were also screened with parasite-specific primers. As expected from the previous results, these saurophagous snakes were parasitized by *Hepatozoon* spp. that are similar to lineages found in several lizard species. This again indicates a correlation between diet and *Hepatozoon* infection in snakes. Once more, the 'gecko type' was the most frequent. Additionally, it was the first time a mixed infection by different *Hepatozoon* lineages was identified though molecular screening in a single individual (see Figure 5.1).

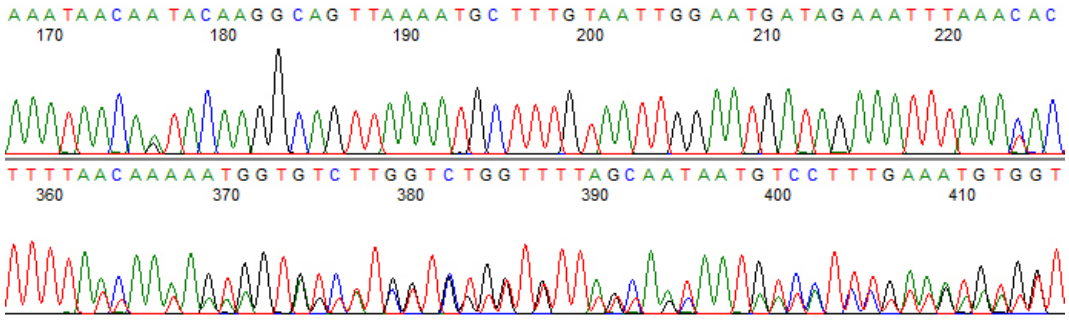

Figure 5.1 – Parts of the chromatogram of the mixed *Hepatozoon* infection sequence from the DB2222 *Psammophis* individual of the second study of this thesis. Note the double peaks.

The *Psammophis* snakes occupy a different niche from the Mediterranean snakes of the previous study, with more desertic characteristics, as it can be seen in Figures 2.1 and 2.2. Despite this distinction, the retrieved sequences were similar to the Mediterranean species, so, for these parasite species, ecological niche of the host seems to not be a determinant in host specificity. This would also suggest a highly motile and widely distributed invertebrate definitive host, such as mosquitoes, especially for the 'gecko type' *Hepatozoon* spp. So again, the diversity of these parasites in their definitive hosts needs to be assessed to completely understand the observed phylogenetic and distribution patterns.

With the same pair of primers (HepF300 and HepR900), infections by several apicomplexan parasites were detected in an array of reptile hosts in the works of these thesis and also in other publications from CIBIO (Harris et al., 2011, 2012; Maia et al., 2011, 2012b). For instance, in the second work of this thesis, it was reported for the first time the detection of a *Caryospora* sp. through molecular screening of a tissue sample.

These primers have also been demonstrated to detect other organisms, such as stramenopiles (Maia et al., 2012a) and several fungi (Tomé et al., 2012). Given this lack of specificity, PCR results alone are not trustworthy as proof of infection by a particular parasite. Validation by sequencing, comparison with published sequences (e.g. BLAST search) and phylogenetic analysis should be performed to avoid false positives and subsequent biased conclusions. If the limitations of a given method are recognized and taken into consideration, all its advantages can be fruitfully employed. Molecular screening through PCR is a helpful technique, with its own set of flaws, many of which can be overcome. It undoubtedly has provided new knowledge about the biodiversity of the Apicomplexa, and other organisms, that inhabit the assessed vertebrate hosts, contributing to increase lineage sampling, the main limitation in parasite phylogenetic studies.

Summing up, from the obtained results it can be concluded that ophidian *Hepatozoon* species do not cluster in a monophyletic group, and the same can be said for ophidian *Sarcocystis* spp. Instead, *Hepatozoon* spp. from saurophagous snakes form different groups, which are closely related to lineages from various lizards, supporting prey-predator transmission as a main infection mode for these parasites. An obvious result is that close *Hepatozoon* lineages can be found in different snake species and across a wide geographic area, so their specificity seems to be very low for these intermediate hosts. In contrast, the same group of snakes, and even a single individual, can be infected with very distinct lineages of these parasites.

Coevolutionary patterns are still hard to discern. From the results of this thesis, a connection between the lineages retrieved from snakes and their supposed lizard prey can be observed. Other similar studies have suggested the same pattern (e.g. Allen et al., 2011). Additionally, morphological and experimental studies also pinpoint prey-predator transmission as a common infection mode for the *Hepatozoon* spp. parasitizing top predators, like dogs (Johnson et al., 2009), snakes (Smith and Desser, 1998), and caimans (Viana et al., 2012). Based on the available information, one can conjecture that *Hepatozoon* spp. have evolved in close relation with their invertebrate host, as suggested in Barta et al. (2012). Then, in a lower taxonomic level, they have specialized in groups of vertebrate hosts, in a more or less specific manner, depending on the individual strategy of each species of parasite.

It is also important to look to the life cycle of these parasites and try to understand how its structure influences host specificity in each phase. In the vertebrate hosts, *Hepatozoon* spp. reproduce asexually. This is a phase of pure proliferation, in which the final objective is to get to the host blood in a suitable concentration, so gametocytes have a good probability of passing to the hematophagous invertebrate when it feeds. In turn,

the invertebrate is the definitive host, that is, where sexual reproduction occurs. Given its importance, it can be expected that host specificity in this phase is higher. Using vertebrates for proliferation is actually a smart strategy for parasites of hematophagous organisms. It increases the transmission probability, helps maintaining parasite intraspecific diversity (as different parasite individuals can be acquired by the same vertebrate host, or they can meet in the invertebrate as it feeds on various vertebrates, providing an efficient means for sexual recombination), and also vertebrates serve as reservoirs for the parasites, particularly useful given that the invertebrates hosts are much shorter-lived relative to vertebrates.

Moreover, taking into consideration the host specificity of the invertebrate hosts, which may also be parasites, can help comprehend the vertebrate host distribution of their parasites. *Hepatozoon* spp. use a large variety of invertebrate hosts, with very distinct characteristics. If an invertebrate host is very host specific, it is expected that the parasite vertebrate host preferences will match those of the invertebrate, as further development in an unsuitable vertebrate would be pointless (the so called 'dead ends'). On the other hand, strategies like the exploitation of the trophic relationships would help the parasite get to its final vertebrate host. But this would only be sensible if the invertebrate parasitize both prey and predator, or if it is naturally consumed by the prey vertebrate and feeds on the predator. This is also applicable to low-specific invertebrates, but in this case transmission strategies can evolve in other directions. The existence of a wide variety of *Hepatozoon* life cycles (as referred to in the 'State of the Art' section) is evidence for this possibility. Only when parasite species descriptions start to fully include information about the invertebrate host, and host specificity in general, can these hypotheses be assessed.

Based on the results of this thesis, some speculations can be made regarding the identity of the invertebrate hosts of the *Hepatozoon* species detected in the screened reptiles, although with reservations. For example, see Figure 4.4, starting in the first divergence of *Hepatozoon* spp.; the clade below is composed by parasites that use ticks and mammals as hosts, and also several lizards and snakes. Snakes, lacertids and skinks often harbour ticks between their scales or in parts where the skin is more exposed, such as in the eardrums. Also, these lineages seem to present higher genetic structuring, consistent of a more limited dispersion of host species, and such is the case of ticks. So ticks are a probable candidate for definitive host of the *Hepatozoon* spp. of this clade.

The individuals in the clade above include *Hepatozoon* spp. that use many possible different invertebrates as hosts, such as fleas, mosquitoes, mites and also ticks. Very close to the 'gecko type' lineages in this clade is *Hepatozoon ayorgbor*, which uses *Culex* mosquitoes as definitive hosts (Sloboda et al., 2007). Mosquitoes are much more motile, and probably more generalists, than the other hematophagous invertebrates, which would

fit with the wide distribution of these lineages (as seen in the snake hosts) and with their lower phylogenetic resolution. In contrast, the *Hepatozoon* spp. from the rodent hosts are thought to use mites or fleas. Mites also constitute reasonable potential definitive hosts for these lineages, as mites frequently parasitize geckos and other reptiles.

This is still very speculative, especially because sampling and phylogenetic resolution are still in a limited state. Although it is the most used marker, 18S rRNA is a slow-evolving gene and becomes of little use when studying diversity in a specific and population level. So, faster evolving markers would be necessary to increase resolution and help untangle the phylogenetic relations of these and other parasites. As the capacity of phylogenetic analysis is improved, it would also be interesting to integrate ecological and geographic data. This would give valuable information regarding how the several types of life cycles have evolved and potentially aid in parasite control management. For instance, it would help elucidate how widespread is the three host life cycle, if it evolved from a common ancestor or if convergence happened, and how flexible is host specificity. Right now, the perspectives appear limitless.

Ongoing research and future perspectives

At CIBIO, several people have been studying the diversity of parasites of reptiles and amphibians, producing a variety of publications using microscopy and molecular tools with an integrative viewpoint (e.g. Harris et al., 2011, 2012; Jorge et al., 2011; Perera et al., 2012 Maia et al., 2012b). New MSc students are also starting their projects on parasites of various herpetofauna, further enhancing the research capacity. There is a huge amount of available tissue samples from reptiles and amphibians, over 13000, most still to be screened for parasites. In addition, there is also a collection of thousands of blood slides from several locations and seasonal periods, ideal to test hypothesis about the development and ecology of hemoparasites. Also, new procedures are being developed, like real-time PCR protocols. Besides providing estimates of the parasitemia level, this technique also allows a more thorough evaluation and comparison between the methodologies used, like the type of sample and the DNA extraction techniques, factor that may lead to differences across studies.

In the first work of this thesis, one of the proposed future studies is to assess *Hepatozoon* parasitemia loads in geckos, in particular of the genus *Tarentola*. There are over 300 blood slides of *Tarentola* spp. in CIBIO. These include specimens from several locations of Iberia, Morocco, and islands of the Cape Verde and Sardinia. Besides, allowing testing the parasitemia load hypothesis, other studies can be done like comparison between continent and islands. This can help understand how parasite

strategies evolve in different conditions and how it affects the vertebrate hosts. For instance, will prevalence and intensity of infections be different? Is it low, like in the case of reptiles from the Seychelles (Harris et al., 2011), or higher? Are the parasite lineages the same, similar or completely separated? Are there several lineages, or a single colonization event for these parasites? How is the ecology of the *Hepatozoon* parasites affected by the different conditions in the islands? For example, *Sarcocystis gallotiae*, which infects the endemic *Gallotia* lizards of the Canary Islands, developed a dihomoxenous life cycle (Šlapeta et al., 2001; Harris et al., 2012). Whilst the parasite would normally, for example, alternate between lizards and snakes, as there were no predators in the islands to fill the role of final host, the same lizard species serves both as definitive and intermediate host in the dihomoxenous life cycle, with the *Sarcocystis* parasite being transmitted via cannibalistic behaviour by predation of tails.

To obtain a complete understanding of the life cycle and phylogeny of the *Hepatozoon* and other apicomplexan hemoparasites, the hematophagous invertebrate host needs to be identified. In CIBIO, ectoparasites of reptiles have been collected for almost 10 years, including ticks and mites (known to transmit *Hepatozoon* spp. to reptiles), among others, like leeches. Additional field work would be needed to collect more possible invertebrate hosts, especially as the lineages found in the reptiles studied are so diverse. Nevertheless, the available collection is already extensive.

As it has been referred numerous times in this thesis the main obstacle for the reconstruction of the phylogeny of the Apicomplexa is the lack of taxa and character sampling. Molecular screening of host samples is a time and cost-effective technique that provides a vast number of characters for phylogenetic analysis of the detected parasites. However, the scarcity of reliable alternative markers to the 18S rRNA gene and the difficulty in developing efficient and specific primers hold back the applicability of this technique. So for the future, more sampling and the design of new primers are mandatory for further advance in this field of Parasitology.

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APPENDIX 1

Preparation, fixation and staining of blood smears

When preparing blood smears in slides, some particular aspects should be taken into account:

- The information should be written in pencil because ink from pens can be washed off by methanol during the fixing steps.
- Each slide will have two purposes: an edge is used to spread the blood, while one of the larger sides contains the actual smear (as displayed in the diagram below).
- More than one smear can be made per slide, including from different individuals although with explicit identification.

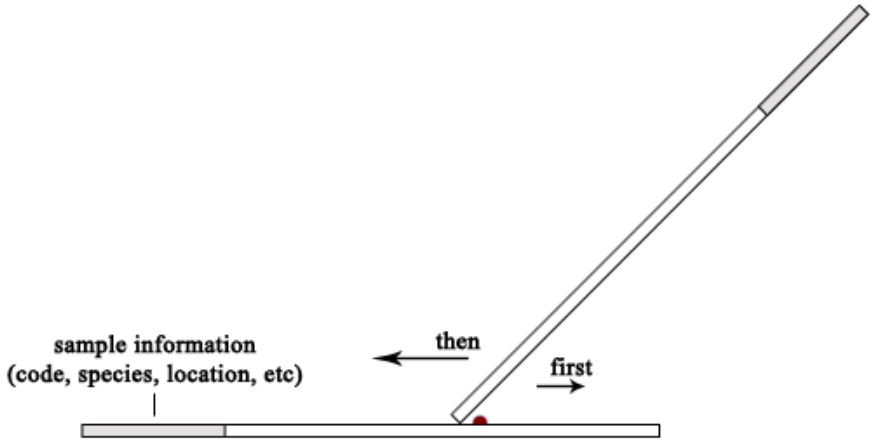

Diagram by Beatriz Tomé.

Making a smear

1. Place a small drop of blood near the end of the slide.

2. Using another slide (the 'spreader') at an angle of approximately 45º, pull against the drop, let the blood spread along the edge by capillarity, and then push the 'spreader' across the other slide (see image above). This should be done as quickly as possible given the blood might clot very rapidly, especially in hot conditions and in some host groups, such as geckos.

3. Let air dry.

4. A slide should not be used as spreader more than once, because the leftover blood in the edge will quicken the coagulation of the sampled blood. And the slide used as 'spreader' should always be changed between sampled individuals to avoid

contaminations. Also, the ‘spreader’ can be turned over to be used as the receiver of the next smear.

Fixing

5. Smears should be fixed with 100% methanol as soon as possible, preferably in the same day. A container with individual slots, so the slides do not touch each other, should be used both for fixing and staining.
6. Immerse the slides with the blood smears into absolute methanol for 1-2 minutes.

Staining

7. First, the staining solution has to be prepared. It is composed of a mixture of 1:9 of respectively Giemsa and distilled water, and the pH should be around 7.0.
8. Dip the fixed blood smears in the staining solution for 50-55 minutes.
9. Wash the blood smears in a bath of distilled water for 1-2 minutes twice, changing the water before the second time.
10. Leave the stained blood smears to dry at room temperature.
11. The same staining solution is reusable for a few stains on the day of preparation.

Preparation of blood samples in filter paper

Blood can be stored in Whatman filter paper, as exemplified in the image below and following steps, and later be successfully used for DNA amplification.

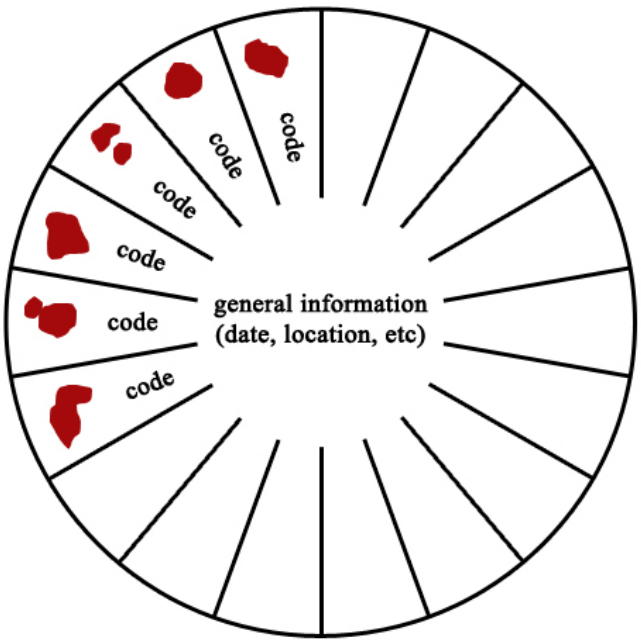

Illustration by Beatriz Tomé.

1. With a pencil identify the paper disk, preferentially including details such as location, date and collector.
2. Write the code for the sample and place a few drops of blood on the paper in the respective division.
3. Several samples can be stored in the same sheet of paper, as exemplified in the diagram in the previous page.
4. Leave the blood dots to air dry.
5. Each filter paper should be kept in separate zip-lock plastic bags with some silica gel to avoid damage by humidity.
6. Alternatively, more than one disk can be placed in the same bag if each two are separated by a blank paper disk. Blood dots must already have dry!
7. The disks can be kept at room temperature until returning to the laboratory, but then they should be placed in a freezer at around -20ºC.

(Based on Telford [2009] and on resources from the site of the Schall Lab at the University of Vermont: http://www.uvm.edu/~jschall/techniques.html)

APPENDIX 2

High-salt method of saline extraction

Needed reagents:

- Lysis buffer (0,5M tris; 0,1M EDTA; 2% SDS; pH 8,0; autoclavated)
- Ammonium acetate (7M; pH 8,0; autoclavated)
- Proteinase K (25 mg/ml)
- Ice-cold isopropanol
- Ice-cold ethanol (70%)
- Ultra-pure water (or other hydration solution)

1. Clean the working bench with bleach and ethanol. In a glass plate, also cleaned with bleach and ethanol, draw separate divisions to avoid sample mixture.

2. Label eppendorf tubes with the codes for the respective sample and pipette 600 µl of lysis buffer to each tube.

3. Using a sterilized scalpel, separate a small amount of tissue sample and cut it in fine pieces. Transfer them into the correspondent eppendorf tube.

4. Add 5-20 µl of proteinase K (depending on the amount of tissue), vortex and incubate at 56ºC overnight.

5. After the tissue is digested, put the tubes in the freezer for 30 minutes.

6. Add 300 µl of ammonium acetate, shake the tubes manually (vortex is not advised as it can damage the DNA), and centrifuge for 15 minutes at 14000 rpm at -4ºC. If precipitated proteins remain in the supernatant, add more 100 µl of ammonium acetate.

7. Label new eppendorf tubes and transfer the supernatant into these, add 600 µl of ice-cold isopropanol and mix, inverting the tubes several times. Put them in the freezer for 3 hours to overnight.

8. Centrifuge for 10-30 minutes at 14000 at -4ºC and discard the supernatant. It is advised to direct the opening of the tubes to the centre of the centrifuge, since the pellet of DNA will be formed on the opposite side during centrifugation.

9. Add 1000 µl of ice-cold 70% ethanol and mix by tapping the bottom of the tube with the finger until the DNA pellet is released. This will wash the DNA from impurities.

10. Centrifuge for 15 minutes at 14000 at -4ºC and discard the supernatant.

11. Let the ethanol evaporate at room temperature or in an incubator for a few hours.

12. When the ethanol has completely evaporated, add 50-200 µl of ultra-pure water (or other DNA hydration solution) and leave to hydrate at room temperature in an agitator for a couple of hours to overnight.