Characterization of the ticks infecting tortoises in the Mediterranean Basin and their role in parasite transmission

Diogo José Mendes Silveira
MSc in Biodiversity, Genetics and Evolution
Faculty of Sciences of the University of Porto
2016

Supervisor
Dr. Ana Perera Leg, Researcher, CIBIO

Co-supervisors
Dr. David James Harris, Researcher, CIBIO
Dr. Eva Martínez-Graciá, Researcher, Universidad Miguel Hernández

Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, _____/_____/_____

Acknowledgements

The period during the development of this thesis has ended up being probably the most peculiar time of my life, and I cannot find the words to express all I want to say about it nor to everyone who has helped me accomplish this dream of making a contribution to the scientific community. Nonetheless, I shall try. First, I want to address my supervisors to whom I cannot thank enough for all they have done, and put up with.

To my supervisor Ana Perera, who is one of the best, nicest people I have ever met! For all the times you guided and helped me, for always trying, pushing me, and motivating me to do more and better. For all your dedication and friendship and for believing and not giving up on me even after I had given up on myself, I cannot thank you enough. And I can only feel so lucky to have met you and have such a wonderful person as my supervisor.

To my co-supervisor James Harris, thank you for allowing me to be part of the AP group and to accepting me as your student even though the only ideas I had about a thesis project when we first talked was that I wanted to work with evolutionary genetics and maybe get some fieldwork experience. For all that you taught and showed me, and for taking me with you to do field work in Morocco, which is one the best experiences I had in my life both as a person and as a biologist, I am really grateful.

To everyone who was part of the Ecolab over these last two years: João Maia, Beatriz, Isabel, Daniela, Henrique, Amanda, Victoria, Sandra, Ana, Joana and Joana, Coelho, Kevin, Walter, Adri, Alex. Thank you for making it so easy and fun to work with you, and for always lending me a hand whenever I needed. Days in the lab are never dull with you around. You are amazing.

To my friends Nuno, Joana, Ana, Sónia, Diana, Rafaela, Zé, Renata, Pedro, Rui and to my cousin Pedro: I would trade all the peach ice tea in the world just to continue having you by my side, and having the support you have always showed to me. And you know very well how I feel about my peach ice tea, so this is serious. You are the best friends anyone can ask for and I love you all!

To all my brothers and sisters-in-arms who have been a part of GEMAC and Emcadeamentos. When I felt I was “getting stuck between a sword and wall”, you were the ones who literally put a sword in my hand and showed me how to fight back. I may not be “Brecha”, but I’m soon becoming a master. Woot woot woot woot woot woot.

And saving the best for last: to my sister Sara and brother Miguel, and to my grandparents, for always being proud of me and keep cheering me on. And to my parents, for all their patience, sacrifices, their love, support, help, advice and everything have you done for me. You have made me who I am and if I am able to finish this MSc, it is because of you.

To everyone listed above, this thesis is dedicated to you. I could not have done it without you. Thank you all from the bottom of my heart.

Resumo

Devido à forte relação com os seus hospedeiros, os parasitas têm sido considerados dos melhores modelos para o estudo de padrões coevolutivos, especialmente quando têm hospedeiros específicos. Entre todos os organismos parasitas, as carraças são reconhecidas como sendo dos parasitas mais importantes e influentes na sociedade porque geralmente conseguem alimentar-se em vários hospedeiros incluindo humanos, animais de estimação e gado, e podem servir de vetores para muitos patógenos perigosos. No entanto, as carraças também podem ser sensíveis às suas necessidades ecológicas, e a sua distribuição pode muitas vezes ser correlacionada com fatores ambientais, em vez de especificidade com o hospedeiro. Isto tem resultado em chamadas de atenção para a necessidade de incorporar diversas fontes de informação, incluindo genética e ecológica, aquando da avaliação da distribuição das carraças, e de como elas se podem reestruturar sob diferentes modelos ambientais.

Nesta tese, usamos os genes 12S e CO1 de ADN mitocondrial para estudar os padrões de distribuição genética da carraça *Hyalomma aegyptium*, que é dependente de hospedeiros do género *Testudo*, na região Norte-Africana do Magreb, e algumas populações Turcas, e compará-la com estudos previamente publicados sobre os padrões de distribuição genética do hospedeiro *Testudo graeca* nas mesmas regiões, com o intuito de procurar possíveis padrões de coevolução. Carraças amostradas também foram testadas para a transmissão de patógenos da família de bactérias Anaplasmatacea, e do protozoário dependente do género *Testudo, Hemolivia mauritanica.*

Os resultados preliminares identificam duas linhagens distintas de *H. aegyptium* no norte de África, que não seguem estritamente os mesmos padrões de distribuição genética do hospedeiro *Testudo graeca*. Ao invés, os nossos resultados parecem indicar que diferentes fatores ecológicos e ambientais, em vez de eventos de especiação do o hospedeiro, podem ter tido uma influencia maior na modelação da paisagem genética de *H. aegyptium*, o que reforça a necessidade de incorporar informação ecológica neste tipo de análises.

Entre os patógenos encontrados neste estudo, há a realçar a inclusão dos primeiros registos de uma estirpe de *Wolbachia* sp. e do endossimbionte de carraças *Midichloria mitochondrii* em *H. aegyptium*, assim como o primeiro registo de *Ehrlichia* sp. identificada em carraças na Argélia.

Palavras-Chave

Hyalomma aegyptium, *Testudo graeca*, Norte de África, Turquia, co-evolução entre hospedeiros e parasitas, 12S rRNA, CO1, parasitas, patógenos, triagem molécular, carraças, diversidade genética.

Abstract

Due to the strong relationship with their hosts, parasites have long been considered one of the best models to study coevolutionary patterns, especially in parasites with specific hosts. Among the many parasitic organisms, ticks are known to be some of the most important and influential parasites on modern society because they can generally feed on many host species including humans, pets and cattle, and can be vectors for a multitude of dangerous pathogens. However, ticks can also be very sensitive to their ecological needs, and tick distribution is often more correlated to environmental factors rather than host specificity. This has led to recent calls for the need to incorporate several data sources, including genetics and ecology, when assessing tick distributions, and how they may reshape under climate change models.

In this thesis, we use the mitochondrial DNA genes 12S rRNA and CO1 to assess the genetic distribution patterns of the *Testudo* host dependent tick *Hyalomma aegyptium* in the North African Maghreb region and some Turkish populations, and compare them to previously published data on the genetic distribution patterns of the host *Testudo graeca* in the same regions to look for possible coevolutionary patterns. Sampled ticks were also screened for several possible pathogens from the Anaplasmatacea family of bacteria, and for the *Testudo* dependent Apicomplexan protozoan parasite *Hemolivia mauritanica*.

Preliminary results identify two distinct lineages in North African *H. aegyptium* which do not follow strictly the genetic distribution patterns of the host *Testudo graeca*. Instead, these results may indicate that different ecological and environmental factors, rather than host speciation events, could have had a greater influence on modeling the genetic landscape of *H. aegyptium*, which reinforces the need to incorporate ecological data in these types of analysis.

Among the pathogens found in screened samples, this study includes the first records of a strain of *Wolbachia* sp. and the tick endosymbiont *Midichloria mitochondrii*, on *H. aegyptium*, as well as the first record of *Ehrlichia* sp. identified from ticks in Algeria.

Keywords

Hyalomma aegyptium, *Testudo graeca*, North Africa, Turkey, Host-parasite coevolution, 12S rRNA, CO1, parasite, pathogen, molecular screening, ticks, genetic diversity.

Table of contents

List of tables

List of Figures

List of Abbreviations

km: Kilometer

mya: millions of years ago

DNA: Deoxyribonucleic acid

mtDNA: Mitochondrial Deoxyribonucleic acid

rRNA: Ribossomal Ribonucleic acid

12S rRNA: 12S ribossomal RNA

CO1: Cytochrome Oxidase C subunit 1

Bp: base pair

PCR: Polymerase Chain Reaction

dntp: Deoxynucleotide triphosphate

Mgcl2: Magnesium chloride

BSA: Bovine Serum Albumin

AMOVA: Analysis of Molecular Variance

Introduction

Parasites

Since the beginning of life, organisms have been constantly under selective pressures that force them to change in order to adapt to their environment. It is thanks to this process called evolution that currently there exists so many different life forms and survival strategies, one of the most successful ones being parasitism. It is estimated that nearly half of the current global biodiversity are parasitic organisms (Dobson et al., 2008; Morand, 2015). Parasites are described as being dependent on a host to fulfill one or more necessities, such as nutrients or an energy source, motility or a habitat (Combes, 2001). While this relationship is beneficial for the parasite, it is detrimental for the host (Combes, 2001). The effect of parasites on the host is very variable, causing damage to the host in a multitude of ways, being in some cases responsible for the hosts' death which has led them to being perceived in a most negative way.

As parasites evolved to better exploit their hosts, hosts have also developed mechanisms such as a complex immune system to defend themselves against these parasites, which in turn forces parasites to readapt to the host. This dynamic process is known as the "Red Queen hypothesis" (Van Valen, 1973), as both parasites and hosts continuously create selective pressures on each other, modeling the direction of their own evolutionary paths and generating the incredibly wide variety of parasite-host interactions that currently exist (Poulin and Morand, 2000).

In fact, parasites have become so diverse and widespread that almost all types of life forms, including parasites themselves, are subjected to parasitism to a certain extent (Poulin and Morand, 2014). Due to the strong relation with their hosts, parasites have thus become a preferred model for studying co-evolutionary processes (Page, 2003; Criscione & Blouin, 2004). These interactions are so strong that they can be responsible for parasites and hosts to not only mutually influence each other's fitness, but also genetic diversity and demographic dynamics (Burdon, 1992; Barret et al., 2008). However, despite their importance and consequences for their hosts, parasites are still much understudied, due partly to the difficulties associated to their detection and isolation, among others. Fortunately the development of molecular techniques has changed this, and in the last years, there has been a considerable increment in the number of parasitological studies (Gomez and Nichols, 2013; Poulin, 2014).

Ticks

One of the most successful and widespread groups of parasites are ticks. As part of the Acari subclass of Arachnids, ticks along with mites make up the Order Parasitiform. All ticks are obligate blood-feeding ectoparasites of wild and domestic terrestrial or semi-aquatic vertebrates. Despite being mostly associated to endotherm animals such as mammals and birds, some ticks can also parasitize amphibians and reptiles. Known for being potential vectors for the transmission of a wide range of human and animal pathogens, ticks are of huge economic importance given their consequences in domestic species, as they can be responsible for spreading diseases between hosts if not discovered in time (Pastiu et al., 2012), which can lead to more serious situations. For this reason, most research regarding ticks is focused on their role as vectors, which pathogens they carry and how they are transmitted, since it brings more benefits to disease prevention, pathogen detection and identification, and other health related issues, while studies on ticks themselves are more neglected (Araya-Anchetta et al, 2015).

Ticks (Suborder Ixodida) are divided into three distinct families: The Nuttalliellidae, a small monotypic family living in South Africa, the Argasidae (soft ticks), and the Ixodidae (hard ticks) (Mans et al., 2012, Fig. 1) which can easily be distinguished by morphological traits (Black et al 1994, Guglielmone et al 2014). Hard ticks have their capitulum or gnathosoma (head) exposed and projected forward from the body in the nymph and adult stages, which is also a key trait in genera identification (Fig. 2a and 2b), and they present a hard scutum (shield), that is absent in both Argasidae and Nuttaliellidae. This scutum, which can cover up the full dorsal region in male ticks or just a small portion of the back starting from the base of the capitulum in females, allows to distinguish easily males and females along with the clear difference in body size with females being much larger than males (Fig. 2c and 2d). It is estimated that approximately 700 described species and subspecies of ticks are part of the Ixodidae family which is divided into 14 genera. However, more than 600 of those species are distributed amongst the following six genera: *Ixodes* (245), *Amblyomma* (102), *Haemaphysalis* (155), *Dermacentor* (30), *Rhipicephalus* (70), and *Hyalomma* (Koch, 1844) (30) (Camicas et al 1998; Guglielmone et al., 2010, 2014).

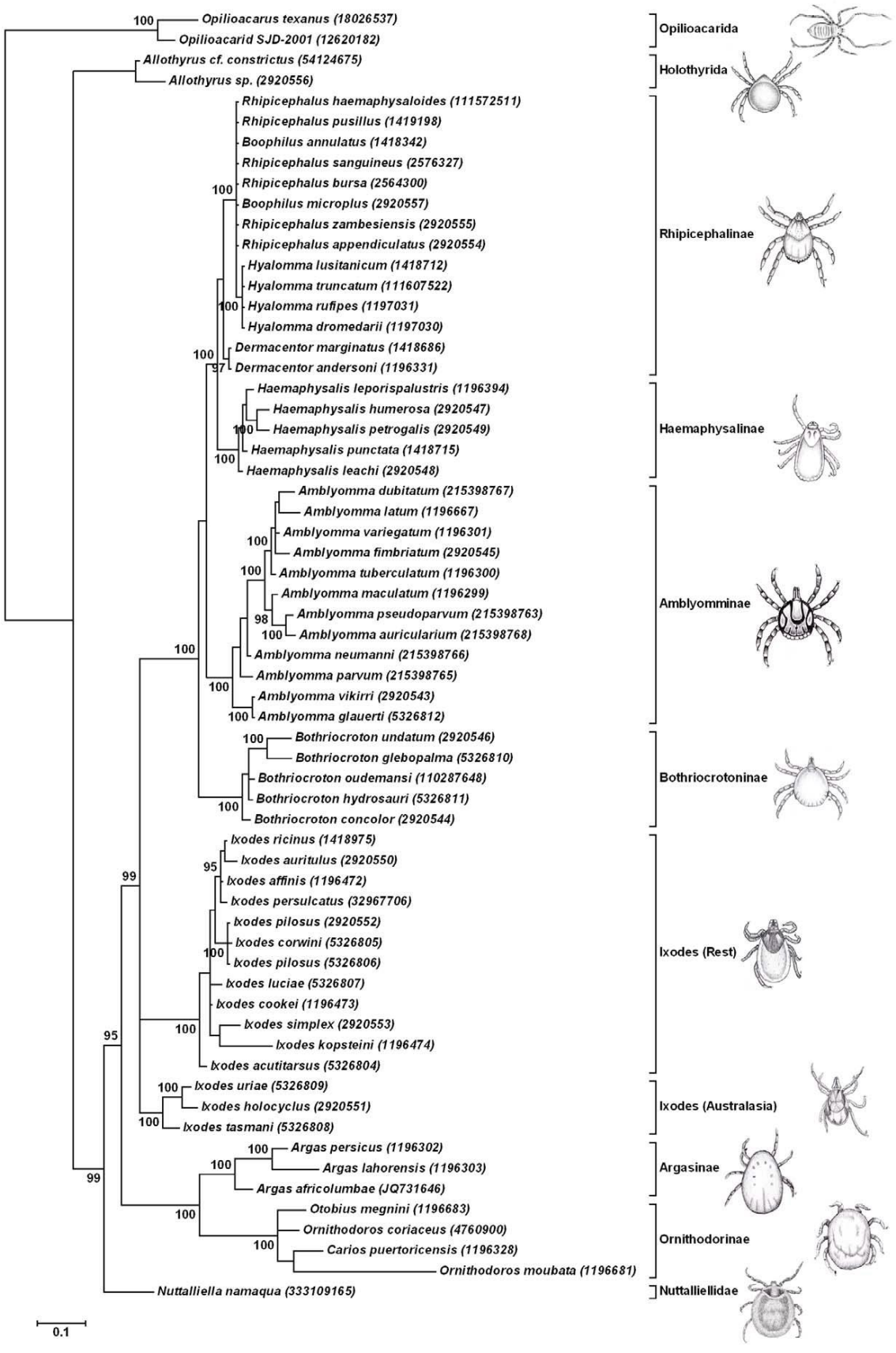
Opilioacarus texanus (18026537)
Opilioacarid SJD-2001 (12620182)
Allothyrus cf. constrictus (54124675)
Allothyrus sp. (2920556)
Rhipicephalus haemaphysaloides (111572511)
Rhipicephalus pusillus (1419198)
Boophilus annulatus (1418342)
Rhipicephalus sanguineus (2576327)
Rhipicephalus bursa (2564300)
Boophilus microplus (2920557)
Rhipicephalus zambesiensis (2920555)
Rhipicephalus appendiculatus (2920554)
Hyalomma lusitanicum (1418712)
Hyalomma truncatum (111607522)
Hyalomma rufipes (1197031)
Hyalomma dromedarii (1197030)
Dermacentor marginatus (1418686)
Dermacentor andersoni (1196331)
Haemaphysalis leporispalustris (1196394)
Haemaphysalis humerosa (2920547)
Haemaphysalis petrogalis (2920549)
Haemaphysalis punctata (1418715)
Haemaphysalis leachi (2920548)
Amblyomma dubitatum (215398767)
Amblyomma latum (1196667)
Amblyomma variegatum (1196301)
Amblyomma fimbriatum (2920545)
Amblyomma tuberculatum (1196300)
Amblyomma maculatum (1196299)
Amblyomma pseudoparvum (215398763)
Amblyomma auricularium (215398768)
Amblyomma neumanni (215398766)
Amblyomma parvum (215398765)
Amblyomma vikirri (2920543)
Amblyomma glauerti (5326812)
Bothriocroton undatum (2920546)
Bothriocroton glebopalma (5326810)
Bothriocroton oudemansi (110287648)
Bothriocroton hydrosauri (5326811)
Bothriocroton concolor (2920544)
Ixodes ricinus (1418975)
Ixodes auritulus (2920550)
Ixodes affinis (1196472)
Ixodes persulcatus (32967706)
Ixodes pilosus (2920552)
Ixodes corwini (5326805)
Ixodes pilosus (5326806)
Ixodes luciae (5326807)
Ixodes cookei (1196473)
Ixodes simplex (2920553)
Ixodes kopsteini (1196474)
Ixodes acutitarsus (5326804)
Ixodes uriae (5326809)
Ixodes holocyclus (2920551)
Ixodes tasmani (5326808)
Argas persicus (1196302)
Argas lahorensis (1196303)
Argas africolumbae (JQ731646)
Otobius megnini (1196683)
Ornithodoros coriaceus (4760900)
Carios puertoricensis (1196328)
Ornithodoros moubata (1196681)
Nuttalliella namaqua (333109165)
0.1
Opilioacarida
Holothyrida
Rhipicephalinae
Haemaphysalinae
Amblyomminae
Bothriocrotoninae
Ixodes (Rest)
Ixodes (Australasia)
Argasinae
Ornithodorinae
Nuttalliellidae

Figure *1*- Phylogenetic tree of the major Subfamilies of the Suborder Ixodida based on Bayesian Analysis of 18S rRNA, as presented by Mans et al., 2012: "The Mitochondrial Genomes of *Nuttalliellidae Namaqua* (Ixodoidea: Nuttalliellidae) and *Argas africolumbae* (Ixodoidae: Argasidae): Estimation of Divergence Dates for the Major Tick Lineages and Reconstruction of Ancestral Blood-Feeding Characters".

According to Dobson and Barker (1999), ticks may have originated in Australia (the Gondwana part that gave origin to Australia) between 408-362 million of years ago (mya). Murrell et al. (2000 and 2001) further stated that some hard tick members (Rhipicephalinae subfamily, which includes the *Rhipicephalus* and *Hyalomma* genera) might have originated much later, in the Afrotropical region around 50 mya and the *Hyalomma* genus to have separated and evolved in the Oriental region in the early Miocene (~19 mya). From here it expanded into Eurasia and crossed again back to Africa through a land bridge 14 mya (Cox and Moore, 1993). This hypothesis is consistent with the earlier scenario proposed by Balashov (1994), that suggested an Asian origin for the *Hyalomma* genus, and it explains the current distribution of the genus across the Afrotropical, Oriental and Palearctic regions. Fossil records, for the most part, are in accordance with the dating from mentioned studies, however some inconsistencies exist that suggest an earlier origin on the *Hyalomma* genus (De La Fuente, 2003). More recently, Burger et al. (2012), based on mitochondrial genomes, highlighted the need for a taxonomic revision on Ixodidae tick phylogeny. The same year, Mans et al. (2012) proposed an alternative hypothesis to the one proposed by Dobson and Barker (1999), suggesting a Southern African origin around 319 mya. However, Dobson and Barker's hypothesis has yet to be disproved (Barker et al., 2014). It is also still unclear if the Rhipicephalinae biogeography will need revision as studies have remained consistent, but been largely untouched in recent years (Murrell et al., 2005; Barker and Murrell, 2003, 2004; Apanaskevich and Horak, 2008; Burger et al., 2014; Barker et al., 2014).

TAXONOMIC KEY: THE IXODID GENERA OF THE MEDITERRANEAN REGION

	IXODES	AMBLYOMMA	HYALOMMA	DERMACENTOR	RHIPICEPHALUS	BOOPHILUS	HAEMAPHYSALIS
PALPS	Long & Slender (♀)	Long	Long	Medium & Broad	Medium	Short	Short, 2nd segment laterally extended
COLOUR PATTERN	Absent	Present	Absent	Present	Absent	Absent	Absent
EYES	Absent	Flat - Convex	Convex	Present	Flat - Convex	Present (indistinct)	Absent
FESTOONS (11)	Absent	Present & ventral scutes	Present (Some species reduced in number)	Present	Present	Absent	Present (Some species reduced in numbers)
ANAL GROOVE							
ADANAL PLATES ♂	Several flat ventral plates	Platelets	Anals, & Accessory & Sub-anals	Absent	Anals & Accessory +/-	Anals & Accessory	Absent
LEGS	Slender & Uniform colour	Annulated	Annulated	Uniform colour	Uniform colour	Slender, Yellow, Slightly beaded	Slender & Uniform colour
OTHER				Enlarged coxa 4	Caudal process + /-	Caudal process absent	

Table 1- Main traits used for identification of the main genera of the Ixodidae family within the Mediterranean Region. Original table obtained from: https://www.researchgate.net/post/How_to_describe_a_tick_morphologically_And_what_molecular_markers_can_be_used_for_identification

The genus *Hyalomma*

Of the many ticks spread across Africa, the genus *Hyalomma* includes some of the most common tick parasites of livestock in Africa and Saudi Arabia, including sheep, cows, horses, goats and camels (Diab et al., 1987; Walker, 2003, revised 2014) However, the main issue with tick infestation is not their presence, but the possible pathogens that they might carry and transmit. *Hyalomma* ticks have been found to be the vectors of several important agents including *Borrelia sp*. which causes the dangerous Lyme disease. Despite this, there are still relatively few studies regarding all of the genus.

Hyalomma ticks can be easily identified from other genera through their unique combination of some morphological traits (Walker, 2003, revised 2014) (Table 1). They have long palps and mouthpieces that come out of the capitulum, along with a pair of convex eyes close to the border of the scutum near the basis of the capitulum. The legs usually present rings on their junctions, which can be a factor to identify some species, and the anal groove is completely posterior with a longitudinal fissure. Male *Hyalomma* ticks are also the only ones that present a full set of adanal, sub-anal and

accessory plates surrounding the anal groove. These ticks have also developed different behaviors from other Ixodid ticks when it comes to feeding habits. Most of the tick genera usually exhibit an ambush-like behavior where they wait motionless atop grass leaves until they receive stimuli from passing prey, after which they raise their first pair of legs in the direction of the prey in a behavior called “questing” (Mironov, 1939; Wilkinson, 1953). Next the tick uses its legs to grab onto the prey as it passes by.

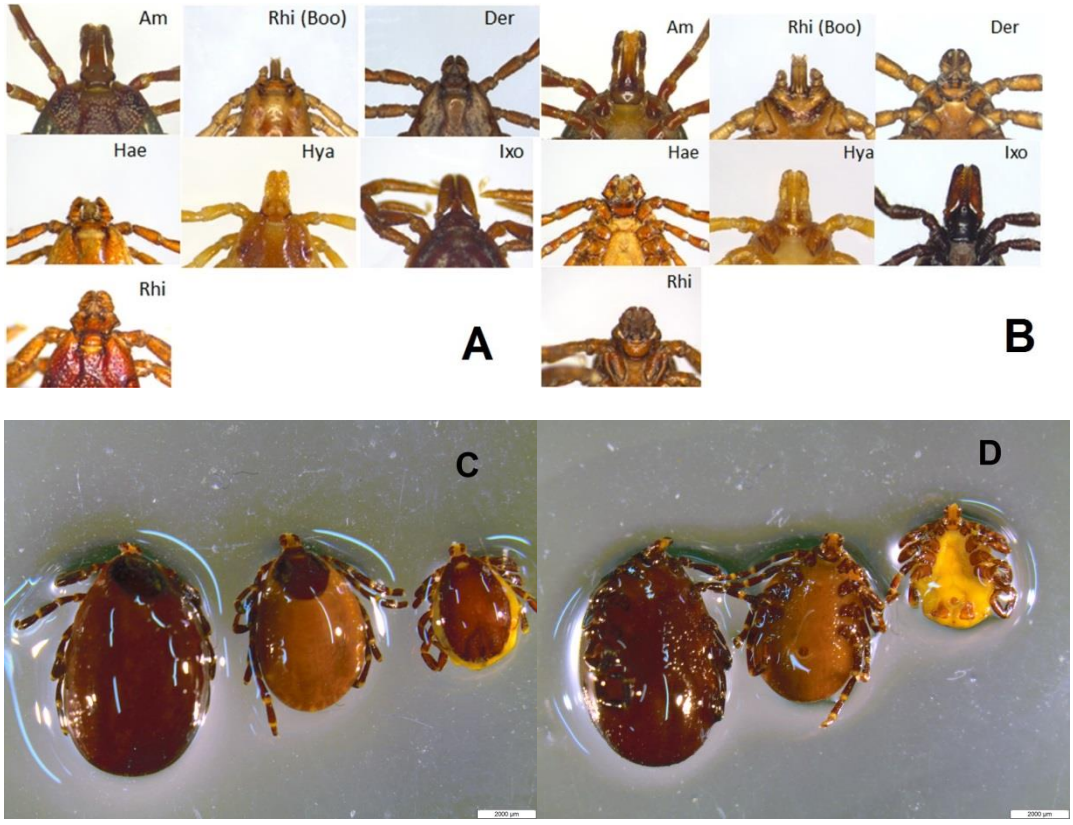

Figure 2. Morphological differences of the gnathosoma of different Ixodidae genera: *Amblyomma* (Am), *Rhipicephalus (Boophilus)* (Rhi (Boo)), *Dermacentor* (Der), *Haemaphysalis* (Hae), *Hyalomma* (Hya), *Ixodes* (Ixo), *Rhipicephalus* (Rhi). 2A - Dorsal view of the gnathosoma of several tick genera. 2B – Ventral view of the gnathosoma Original images from (http://bristoltickid.blogs.ilrt.org/). 2C – Body size differences between a fully engorged female, semi-engorged female and a male of *Hyalomma aegyptium* tick and dorsal view of their scutum. 2D – ventral view of *H. aegyptium*.

However, *Hyalomma* in general have also evolved to becoming active hunters and run after their hosts instead, but can still resort to questing in certain environments or climatic conditions (Balashov, 1960; Apanaskevich and Oliver, 2014).

Like most Ixodidae, *Hyalomma* life cycle usually involves three hosts. After a feeding event, the ticks needs to detach themselves from the host before moulting, and thus

need to find a new host after they have moulted into the next stage of their life cycle. So each life stage (larvae, nymph, and adult) will occur in a different host. Fertilized females tend to ovulate near nests of suitable hosts so it is easier for the larvae to find a host after hatching (Apanaskevich and Oliver, 2014). Larvae and nymph feeding and moulting times may differ substantially between species (Troughton and Levin, 2007; Široký et al., 2011). However, studies conducted in laboratories may not reflect actual feeding and moulting periods in natural conditions. After reaching the last stage of their life cycles, adults search for their host which in some cases may be specific.

Hyalomma aegyptium

Among the *Hyalomma* ticks, *H. aegyptium* (Linnaeus, 1758) is commonly known as the tortoise tick due to its high specificity with *Testudo* tortoise hosts at the adult stage of their life cycles (Hoogstraal & Kaiser, 1960; Široký *et al.*, 2006; 2011). This specificity is sometimes so high that the presence of *H. aegyptium* has been considered as an indirect indicator of the presence of the host (Široký et al., 2009; Tiar et al., 2016). It is also the most common tick species infecting tortoises in North Africa and the Mediterranean basin, and it is rare to find them on other hosts especially during the adult stage of development (Hoogstraal and Kaiser, 1960; Apanaskevich, 2003; Široký et at., 2007, Tiar et al., 2016). *Hyalomma aegyptium* is widely distributed across the Paleartic region, ranging from the Mediterranean, Atlantic Moroccan and North African coastline, Balkan region and Middle East, where it can be found in different habitats including forests, woodlands and even deserts, and has recently been reported in some countries of central Asia (Kolonin, 1983; Apanaskevich, 2003; Vatansever et al., 2008; Gazyağci et al., 2010; Razmjo et al., 2013; Gharbi et al., 2015; Koc et al., 2015).

Hyalomma aegyptium can be easily identified by the presence of adanal plates on males with semi-rectangular like-shape of equal length and width (Fig. 3) when comparing to other *Hyalomma* ticks on North Africa, and by the presence in females of a more rounded scutum (Fig. 2C). Moreover, both males and females have a wider and divergent spur on the coxa of the most posterior pair of legs (Fig. 3). These characters has lead them to be considered a distinct subgenus *Hyalomma* (previously *Hyalommasta*), different from the other species of the genus that would belong to the *Hyalommina* and *Euhyalomma* subgenera (Apanaskevich, 2003).

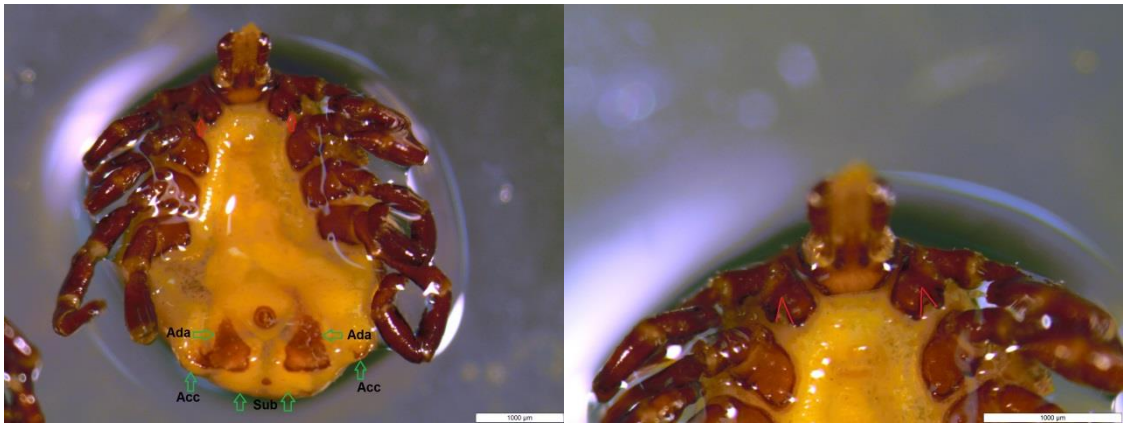

Figure 3 – Ventral view of a male *Hyalomma aegyptium* tick with highlights in key traits for identification. Red arrows highlight the divergence of the spur of the coxa I. Green arrows point to ventral plates only found in male ticks. Acc – Accessory shields, Sub – Sub-anal plates, Ada – Adanal plates which are smaller and broader in *H. aegyptium* than other *Hyalomma* species.

One of the main barriers for the distribution of *H. aegyptium* is believed to be its high preference, at the adult stage, to tortoise hosts of the genus *Testudo,* especially to *T: graeca*. This host specificity may suggest a possible co-evolutionary pattern between *H. aegyptium* and *T. graeca*. Although larvae and nymphs of *H. aegyptium* tend to also attach themselves to tortoises, they are less specific and have been reported to feed on other animals such as lizards, birds, small mammals and even humans (Apanaskevich, 2003; Kolonin, 2013; Vatansever et al. 2008; Pastiu et al., 2012; Bursali et al., 2010; Kar et al., 2013; Guglielmone et al., 2014).

Pathogens associated to *H. aegyptium*

Unlike many other ticks in North Africa*, H. aegyptium* does not pose such an immediate threat to humans, nor human related animals or activities, such as cattle breeding and agriculture. Despite this, pathogens carried by *H. aegyptium* should not be under looked as *H. aegyptium* is still known to be the vector of several important and dangerous pathogens. A resume of some of the most important is presented in table 2.

Pathogenic agent	Associated condition	Typically Affected hosts	Reference
Anaplasma phagocytophilum	Anaplasmosis	Sheep, Cattle and Humans	Pastiu et al., 2012
Borrelia burgdorferi s.l.	Lyme disease	Humans, other Mammals and Birds	Kar et al., 2011
Borrelia turcica	Borreliosis - Relapsing fever	Humans	Güner et al., 2004
Crimean-Congo hemorrhagic fever Virus (CCHFV)	Crimean-Congo hemorrhagic fever	Humans	Široký et al., 2014
Coxiella burnetii	Q-fever	Humans	Široký et al., 2010
Ehrlichia canis	Ehrlichiosis	Canines	Pastiu et al., 2012
Francisella-like Endosymbionte (FLE)			Ivanov et al., 2011
Hemolivia mauritanica		*Testudo graeca*	Sergent and Sergent, 1904
Hepatozoon kisrae		*Agama stellio*	Paperna et al., 2002
Rickettsia aeschlimannii	Maculopapular skin rash	Humans	Bitam et al., 2009
Rickettsia spp	Rickettsiosis - Spotted fever and Typhus fever	humans	Kar et al., 2011
Theileria annulata	Tropical theileriosis	Cattle	Ray, 1950

Table 1 – Known associated organisms carried by *Hyalomma aegyptium* and respective associated medical conditions with notes on the most commonly associated hosts, and first records of transmission by *H. aegyptium*.

Other factors influencing tick distribution

Much like with other parasites, connectivity patches between tick populations in tick distribution has been proven to be one of the most important factors in tick abundance and persistence and population structuring (Estrada-Peña et al., 2002, 2003; McKoy et al., 2003; Li et al., 2014). In more generalist species this connectivity is maintained by the wide range of suitable hosts and environments, which enables a higher degree of gene flow among more distant populations, which also promotes a certain lack of clear structuring (Mixson et al., 2006; Cangi et al., 2013; Porreta et al., 2013; Ogrzewalska et

al., 2016). However, in tick species with more specific hosts, even those with lesser dispersion capability, it has been suggested that connectivity could be sustained through the more juvenile life stages which tend to have a more generalist behavior than their adult stages (McKoy et al., 2003; Barret et al., 2008), or are more tolerant to adverse climatic environments (Cangi et al., 2013). This could result in different genetic structures for ticks when compared to their hosts. In the case *of H. aegyptium*, it has long been observed that juvenile stages have the ability to survive long periods of time without feeding and can survive outside the host's natural range (Hoogstraal and Kaiser, 1960). They can also infect hosts other than *Testudo* tortoises (Hoogstraal and Kaiser, 1960; Barnard and Durden, 2000; Burridge and Simmons, 2003; Pastiu et al., 2012) including birds (Hoogstraal et al., 1963). As such, it is possible that the juvenile stages of *H. aegyptium* may play an important role in defining a genetic structure through the use of secondary hosts with wider dispersion ranges, as it has also been suggested for other tick species (Ogden et al., 2008; Cangi et al., 2013).

Environmental factors (both biotic and abiotic) have also been described as taking an important role on tick distribution. Tick development, behavior and survivability are greatly influenced by temperature (Semtner et al., 1971; Gilbert et al., 2014) which is a key factor in regulating tick metabolism by maintaining the humidity equilibrium between the tick and its environment (Needham and Teal, 1991). In order to keep their metabolism at an optimal equilibrium, ticks spend most their questing periods under the protection of existing vegetation and the microclimate it creates (Pfäffle et al., 2013). Studies have determined that the type of vegetation and the microclimate that it creates to be one of the main factors responsible to limiting or enabling the increase of tick abundance in a given area (Lancaster, 1957; Semtner et al., 1971; Semtner and Hair 1973a, 1973b; Cumming, 2002). And variations to vegetation or this microclimate have great implications on off-host tick survivability and development, as it compromises the tick's metabolic rate, through change in their humidity equilibrium (Semtner et al., 1971; Semtner and Hair 1973a, 1973b; Norval, 1977; Fujimoto, 1989; Needham and Teal, 1991; Dantas-Torres and Otranto, 2011; Estrada-Peña et al., 2011).

Testudo graeca

Testudo graeca, commonly known as the spur-thighed tortoise, is a small testudine species that is distributed across North Africa and the Mediterranean Basin, the Middle East and southwest Asia (Fritz et al., 2009) (Fig. 4). Individuals from this species can weigh up to 2 Kilogram and have a convex shell that can reach 35 cm in size. They exhibit a clear sexual dimorphism; females tend to be larger than males. *Testudo graeca* can easily be identified by the presence of conical shaped spurs on each thigh (although some may appear a bit flattened due to attrition), from which it receives its common name.

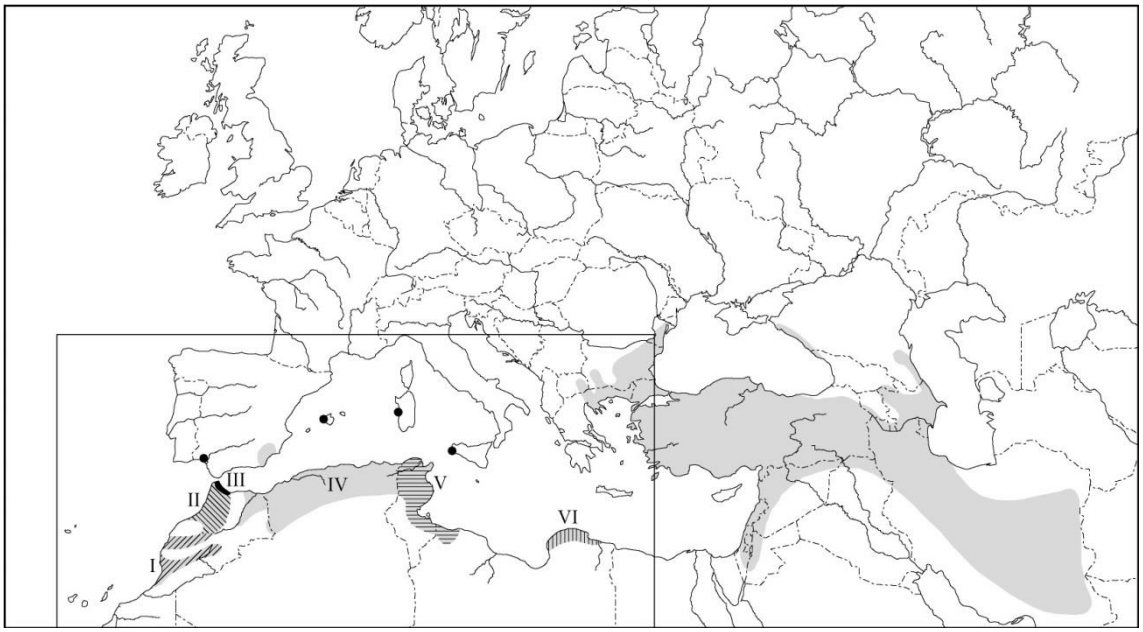

Figure 4 - Current known distribution of *Testudo graeca*. Highlighted area corresponds to the distribution of the North African Clade (Fritz et al., 2007), with annotations on the distribution of the main lineages and subspecies of this clade. I – *T. graeca soussenssis*; II, III – *T. graeca marokkensis*; IV – *T. graeca graeca*; V – *T. graeca nabeulensis*; VI – *T. graeca cyrenaica*. Figure adapted from Fritz et al. (2009) "Mitochondrial phylogeography of *Testudo graeca* in the Western Mediterranean: Old complex divergence in North Africa and recent arrival in Europe".

Historically, *T. graeca* most probably originated from either *Testudo burtschaki* (8.0-10.0 mya) or *Testudo eldarica* (6.0-9.0 mya) (Danilov, 2005; see Harzhauser and Piller, 2007 for dating) in the Caucasian region (Fritz et al., 2007). Currently, *T. graeca* has different morphotypes which were in the past considered as up to 15 different subspecies (Guyot-Jackson, 2004). However, in recent years, studies including genetic tools have shown that many of these subspecies are not completely differentiated from each other (Harris et al., 2003; van der Kuyl et al., 2005; Fritz, et al., 2007, 2009). The

high morphological plasticity of the species to different environmental pressure could explain the wide variety of phenotypes present in *T. graeca* (Carretero et al., 2005; Fritz et al., 2007) and their current distribution in such diverse habitats (Anadón et al., 2012).

Currently the consensus on this species is that it is divided in six mtDNA clades (A-F – table 3), each one represented by a different subspecies, with exception of the North African clade which is divided in 5 different lineages, each of one considered as a different subspecies (Fritz et al., 2007, 2009). Although the high morphological plasticity of this species, does not allow subspecies to be identified using a single trait or morphotype (Fritz et al., 2007), they present clear non-overlapping geographical distributions (Fritz et al., 2007, 2009) (table 3). Despite having a well-defined phylogeny, the biogeography of *T. graeca* is still debatable as the current distribution can only be explained by a series of complex events that repeatedly isolated and reconnected the ancient *Testudo graeca* populations. According to fossil records, *T. graeca* is expected to have reached North Africa from the East Caucasus region around 2.0-2.5 mya (de Lapparent de Broin, 2000) through temporary land bridges that would have connected the actual Maghreb and Caucasus regions in the late Miocene (Fritz et al., 2007). After this, North African *Testudo* lineages are estimated to start diverging into its current subspecies between 1.5-0.5 mya (Fritz et al., 2009; Anadón et al., 2015).

Like most land tortoises, *T. graeca* populations present a slow growth rate (Shine and Iverson, 1995; Díaz-Paniagua *et al.,* 2001; Sanz-Aguilar *et al.*, 2011). On one side hatchlings and juveniles are easy targets for predators and as such present a very low survival rate (García et al., 2003). On the other, adults have a high survival rate since they are harder prey for predators and are more resistant to harsh habitats. This species usually lives in semi-arid regions and its abundance and dispersion is heavily influenced by lithology and topology and by climatic factors such as annual rainfall and annual temperatures ranges (Anadón et al., 2006a, 2006b, 2007, 2009, 2010, 2012, 2015), along with more biotic factors that include the presence of very specific flora that are the basis of its diet (El Mouden et al., 2006). However, *T. graeca* natural habitats are being destroyed due to human activities such as deforestation, agriculture and overgrazing (Bayley and Highfield, 1996; Slimani et al., 2001). These actions in addition with the growing collection of specimens in North Africa and Southeast Spain for illegal pet trade have been the major causes for the rapidly decrease in population sizes for these tortoises, which already lead to the disappearance of some population across Morocco and Spain (Znari et al., 2005; Pérez et al., 2004, 2012) and the

inclusion of *T. graeca* on the IUCN Red List under the "Vulnerable" tag (van Djik et al., 2004).

mtDNA Clade	**Subspecies based on mtDNA clades**	**Subspecies distribution range**
A	*Testudo graeca armeniaca* (Chkhikvadze and Bakradze, 1991)	Central west coast of Caspian Sea; eastern and parts of central Caucasus Region including Araks River Valley (Armenia, Turkey)
B (North Africa)	*Testudo graeca soussensis* (Pieh, 2001)	Southern Morocco (north of the High Atlas Mts. And in the Souss Valley)
	Testudo graeca marokkensis (Pieh and Perälä, 2004)	Morocco (northeast and southwest of the Rif Mountains)
	Testudo graeca graeca (Linnaeus, 1758)	Algeria, eastern Morocco, south-eastern Spain and Mallorca
	Testudo graeca nabeulensis (Highfield, 1990)	Eastern Algeria, Tunisia, Sardinia and Sicily
	Testudo graeca cyrenaica (Pieh and Perälä, 2002)	Cyrenaica Peninsula
C	*Testudo graeca ibera* (Pallas, 1814)	Southeast Europe, western Asia Minor, Russian and Georgian Black Sea coast,
D	*Testudo graeca terrestris* (Forsskål, 1775)	Southern and eastern Asia Minor, Levant
E	*Testudo graeca buxtoni* (Boulenger, 1921)	Northwestern and central Iran, in the eastern Caucasus Region probably also adjacent parts of other countries
F	*Testudo graeca zarudnyi* (Nikolsky, 1896)	East Iran

Table 3 – *Testudo graeca* subspecies based on mtDNA clades and lineages as described by Fritz et al., (2007, 2009), with notes on their respective distribution ranges

Aside from the destruction of natural populations, illegal pet trade also has indirect consequences, such as the introduction of exotic and invasive species. This is particularly dramatic, given that in many cases, these introduced species carry their parasites into their new environments. Such is the case of the illegal trade of *T. graeca*, where it has already been reported as being the gateway for the arrival of the tick *H. aegyptium* in countries such as Italy, Romania, Poland and the United States of America (Burridge and Simmons, 2003; Brianti et al., 2010; Nowak, 2010; Richter et al., 2013; Mihalka, 2015). Naturally this becomes a more serious issue when considering the possible diseases and parasites that both *T. graeca* and *H. aegyptium* may be carrying with them.

The Atlas Mountains

As part of the landscape surrounding the Mediterranean basin, a natural hotspot of biodiversity, the Maghreb region and North African mountains (Fig. 5) are home to an incredible diversity of both animal and plants. Reptiles are especially abundant all across this region and can be found in a multitude of habitats that can go from the more fertile lands closer to the Mediterranean Sea, to the more arid areas of the Saharan desert (Schleich et al., 1996).

Maghreb Mountains were formed during many tectonic events over millions of years, many of which are not yet fully understood (de Lamotte et al., 2000). Currently, three major events are invoked to explain the three main mountain systems present in North Africa: The Anti-Atlas, The Atlas Mountains, and the Er-Rif and Tell Atlas. It is believed that the Anti-Atlas mountains were the first to rise after the collision of the North American plate and the conjoined African and European plates in the Paleozoic (~300 mya) (Helg et al., 2004), and this is the reason of their disposition along the Atlantic line, perpendicular to the remaining mountains. The Anti-Atlas extends over 600 km, and its peaks extend up to between 2500-2700 m of altitude. Its southern stretch serves as a western border for the Sahara desert, while the Northern section is flanked by a section of the High Atlas.

Millions of years later a second major event during the Cenozoic period involving the collision of the African and European plates caused the beginning of the uplift of the actual Atlas Mountains (de Lamotte et al, 2009). Due to previous tectonic events that led to the weakening of the north-western part of the African tectonic plate (Missenard *et al.*, 2006) and several thermal anomalies in the post Miocene (Babault et al., 2008), the Atlas Mountains uplift was irregular and asymmetrical resulting in several distinct formations: The High Atlas and Middle Atlas in Morocco, the Saharan Atlas in western Algeria, and the Aurès Mountains in eastern Algeria and Tunisia. Together these mountain ranges form a continuous mountainous landscape with more than 2500 km that extends from the Moroccan Atlantic coast to the east Tunisian Mediterranean coast. The high Atlas begins at the Atlantic coast in central Morocco and stretches over 700 km and entering into Algeria, and with a maximum altitude of over 4000m. The Middle Atlas, similar to the Anti-atlas, is positioned along the Atlantic coast, instead of the Mediterranean coast, despite being created at the same time as the High and Saharan Atlas. This is attributed to different tectonic movements occurring in different direction and the already previously weakened tectonic plate around that area (de Lamotte et al, 2009). These mountains range about 350 km and with a maximum

altitude of over 4000 km. The Saharan Atlas, as its name implies, serves as the northernmost border of the Saharan desert along with the Aurès Mountains. Unlike the High or Middle Atlas, this part of the Atlas had a much slower rise which resulted in lower altitudes (de Lamotte et al., 2009), with its peak just over 2200m in altitude in the Saharan Atlas, and over 2300m in the Aurès Mountains.

The last set of elevations, Tell-Riff system was more recently formed and involved a series of out-of-sequence thrusts between the African and European plates (de Lamotte et al., 2000) with the major uplift happening phase occurring between 30 and 20 Ma (Oligocene - Miocene) (Beauchamp et al., 1999). The Tell-Rif system extends for more than 2000 km and peaks at over 2300 in the Rif mountains and 2400 in the Tell-Atlas. Although the orogeny periods of the Atlas Mountains ranges is still debatable, more recent studies do tend to place it in a post-Miocene scenario (Ayarza et al., 2005; Babault et al., 2008).

These mountains not only serve as geological and geographical borders, but also as environmental borders. It is possible to witness a drastic change in landscapes whenever a mountain range is crossed. The lands north of the Tell-Riff system tend to be much more rich and fertile, providing a much greener landscape due to their Mediterranean climate influence, providing a suitable environment for the growth of taller trees such as oaks, olives and pistachio trees (Schleich et al., 1996).

In the High plateau, which is surrounded by the Aurès Mountains, Middle, Saharan and Tell-Atlas, lands tend to be more arid than the coastal regions and as such, they have a greater desert influence. As latitude decreases, getting closer to the equatorial line, temperatures in these regions tend to be higher and can reach over 45ºC during the dry summers. Humidity also tends to be lower as the Middle and Tell-Atlas and Aurès Mountains serve as barriers for humidity coming from both the Atlantic and Mediterranean. Oaks and Olives are scarcely found while tall grass becomes predominant (Schleich et al., 1996).

Finally, south of the Saharan-Atlas, the Saharan desert becomes the least sustainable environment to life, where only the most adapted species can survive. The completely arid climate can be recognized by the lack of permanent natural surface watercourses, and very rare precipitation events which lead to typically very shallow and widely dispersed vegetation. Moreover, extreme temperatures fluctuations (from 60ºC during the day down to below 0ºC during the night), make the Sahara desert one of the harshest environments on Earth (Schleich et al., 1996).

With so many diverse climates and environments, it is not surprising that the Atlas Mountains can hold so much species and genetic diversity, and this has been already shown in several organisms, including sand flies *Phlebotomus papatasi* (Prudhomme

et al., 2012), the scorpions *Buthus elmoutaouakili* (Husemann et al., 2012) and *Androctonus mauritanicus* (Coelho *et al.*, 2014), the reptiles *Psammophis schokari* (Rato et al., 2007) and *Agama impalearis* (Gonçalves *et al.*, 2012), and the freshwater turtle *Mauremys leprosa* (Fritz *et al.*, 2005 and Fritz *et al.*, 2006). Aside from the Atlas Mountains, North Africa also presents other natural barriers such as the Moulouya river or the Souss river that serve as barriers for the distribution of animals such as North African dipodils (Rodentia: *Muridae*) (Nicolas et al., 2014) and the Spur-thighed tortoise *Testudo graeca* (Álvarez et al., 2000; Pieh, 200*1)*. All these animals typically follow a West-East gradient of diversity, promoted by consecutive events of contraction into climate refuggia and dispersion during the glacial phases of the Pleistocene (Husemann et al., 2013), however other animals such as the land snail *Cornu aspersum* (Guiller and Madec, 2010) and the frog *Hyla meridionalis* (Recureo et al., 2007) appear to follow different distribution patterns, suggesting that the events that are responsible for the wide diversity of life in North Africa and the Mediterranean basin, may not be so simple as too always promote the same common patterns of diversity.

Figure 5 – Main mountain ranges (in red) and countries (in blue) of the North African Maghreb region.

Molecular Tools

For as long as we can recall, humans have had a tendency to group and classify their surroundings, specially living things. One of the first people to have tried to classify and describe living things was the Greek philosopher Aristotle who started dividing between plants and animals, and started grouping them according to arbitrary traits. For centuries this approach was used with different traits used for grouping. During the XVIII century, Linnaeus started to use morphological traits to arrange living organisms into groups according to a hierarchical order, and he is now recognized as the father of modern taxonomy. However, Linnaeus' system was limited as using only morphological characters did not account for events such as convergent evolution, species with high morphological plasticity or cryptic species (Perkins et al., 2011). This becomes even more evident in microscopic species and simpler life-forms whose evolutionary pressures may have led them to adapt in similar ways, or in animals with more complex life-cycles that may imply a change in morphology through metamorphosis.

The introduction of molecular tools such as DNA amplification through Polymerase Chain Reaction (PCR) and sequencing over the last century has thus become the current solution to these issues. By directly analyzing DNA sequences, biologists have achieved a greater insight on the evolution of life organisms, and currently can describe and characterize different clades from a genetic perspective. Just as evolutionary events presented species and broader taxa defining morphological traits which can be used for their classifications and identification, the same goes for DNA. Since DNA is the template for which all characteristics on an individual are expressed, it is possible to find common sequences or variations for all individuals under the same species or clade and thus this enables the use of DNA as a tool for species identification (Herbert et al., 2004a, 2004b; Lv et al., 2014).

DNA also allows us to study the evolutionary relationships between species and clades. Methods like the construction of phylogenetic trees can be performed through many approaches such as UPGMA (Sneath and Sokal, 1973), Neighbor-joining (NJ) (Saitou and Nei, 1987), Maximum parsimony (MP) (Fitch, 1971), Maximum likelihood (ML) (Felsenstein, 1981) and Bayesian inference (Huelsenbeck and Ronquist, 2001). Further, plotting methods such as hierarchical trees and haplotype networks allow us to graphically display at different levels the evolutionary relationships between the studied organisms. And by using faster evolving genes, and more sensitive analysis, it is possible to study patterns at a single species and population level.

One of the most critical issues in these types of studies is what genes should be used when inferring genetic relations. Although at first the use of any single gene may seem enough to construct a complete and accurate tree or network, we should take into account that it may not truthfully represent the actual evolutionary pattern for the whole genome. Because different genes are under different evolutionary pressures, they may also present more or less diversity. As such, different genes may translate in quite different evolutionary patterns (Sunnucks, 2000). One other issue is to choose between nuclear or mitochondrial DNA, but they both have their advantages and disadvantages. While mtDNA does follow a more linear transmission and is entirely haploid, since it does not suffer recombination, it only describes maternal lineages and we have to consider possible introgression cases from hybridization between sister-species which will greatly influence results. On the other hand, nuclear DNA is more diverse and is not limited to only the maternal lineage. However, events such as recombination and diploidism may prove to be an obstacle. Ideally, phylogenetic studies should consider a multiloci approach including both nuclear and mitochondrial DNA (Ballard & Whitlock 2004). Furthermore, if possible the use of morphological traits should be included; however the use of all three elements is often impossible, although it provides the basis for an integrative taxonomical analysis which many scientists support (Padial et al., 2010; Schlick-Steiner et al., 2010). Mitochondrial DNA in animals is usually a circular molecule made up by around 17000 base pairs which translate 37 genes. A key trait for mtDNA is that it does not present introns between coding regions, thus leaving the mtDNA molecule with very few non-coding regions. Additionally, mtDNA presents a much higher single nucleotide substitution rate than nuclear DNA (Brown et al., 1979), which also leads to a higher accumulation of single nucleotide mutations throughout its entire genome which proves more useful when studying more recent evolutionary events. This study will focus on the mitochondrial genes 12S rDNA and cytochrome oxidase subunit 1.

Just like other mitochondrial genes, 12S and CO1 have the advantage of being found in a high number of copies throughout the genome, making them easier to amplify, and also provide conservative primer sites. If we consider that these genes also tend to evolve at a higher rate, this makes them a valuable choice when performing studies with closely related species, or single-species studies (Perkins et al., 2011). 12S rDNA has been one of the most used genes to uncover tick phylogeny in the past (Norris et al., 1999), and is still used today in some studies as it is a gene with a high resolution for species identification (Lv et al., 2014) although it may not be ideal for studies at an intra-species level (Cruickshank, 2002). Regarding CO1, it has been the prime choice

for DNA barcoding for many taxa (Hajibabaei et al., 2006; Herbert et al., 2004a, 2004b), including Ixodidae ticks (Lv et al., 2014) and may be the most precise marker for species identification (Xia et al., 2012; Lv et al., 2014). Also, due to CO1 being a much more variable marker, it is better suited to use at lower taxonomic levels (Cruickshank, 2002). Additionally, records of both markers for *H. aegyptium* and other *Hyalomma* species already exist in the NCBI Genbank database, which will allow for an easier confirmation of this species identification and more reliable support when identifying the species through sequence analysis. For all these reasons, the use of both CO1 and 12S rDNA may prove successful for this study when considering that *H. aegyptium* genetics across North Africa is still much understudied and that this combination of markers has already been useful to resolve relationships among the rhipicephaline Subfamily in Ixodid ticks (Murrell et al., 1999, 2000).

Parasite screening and identification

Before the development of molecular based tools, parasite detection, and identification was performed using visual methods and relied entirely on the visual confirmation of the parasite and its identification using exclusively morphological traits and other additional characteristics, such as infecting tissue or host. With the introduction of optic microscopy techniques in the XVII century, researchers were able to actually see microscopic organisms which were hidden from the naked eye, and new morphological traits were described which greatly refined parasite identification. Direct observation of animal tissues or blood smears under microscopic augmentation became the norm for screening and identification for microscopic parasites, and such methods are still used and are still fundamental in parasitology studies which involve measuring infection levels. Currently more advanced observation tools such as electronic microscopy have enabled a more refined look into microscopic organisms; however both optic and electronic microscopy methods are limited to the resolution and power of used magnification methods, and proper parasite screening for a single host is usually a lengthy and tiresome process.

With the development of molecular based tools, scientists now have the means to overcome many of the obstacles imposed by traditional methods. Techniques such as Polymerase Chain Reaction (PCR), which allows for isolation and amplification of target DNA fragments, have now become the basis for the detection of many parasites. This new molecular approach has become a most valuable tool for the detection and identification of simpler and smaller organisms which do not present observable

defining traits, or are too small or exist in so few numbers that are almost impossible to discover, which include many parasitic organisms (Weiss, 1995). Regarding ticks, these techniques are now the easiest form of screening for pathogens carried by the ticks, and has led to the discovery of several parasites (Sparagano et al., 1999). And by using specific primers, it is possible to create and optimizes protocols to target sequences for a desired species, where a positive amplification is indicative of presence.

Objectives

From an evolutionary perspective, parasite-host systems are prime models for coevolutionary studies. By comparing phylogeographic patterns and biogeographic history of both host and parasite, it is possible to infer if coevolution is a plausible event between them, and what events have led to their current distribution and relationship. As such, the *Testudo graeca – Hyalomma aegyptium* system does seem in theory like a great model to study coevolution, as *H. aegyptium* ticks have been described as highly dependent on their *Testudo* hosts, and are the main tick species infecting their *Testudo* host (Syroki et al., 2006, 2007; Tiar et al., 2016). By going further and adding a third layer involving parasitic pathogens transmitted by vector parasites, it is even possible to assess not only possible coevolution between host-vector-parasites, but also to start assessing infectionary history of said parasites and their evolutionary role in this three-way relationship.

Additionally, despite being one of the main vectors for many pathogenic parasites around the world, information on ticks in general is still lacking when it comes to gathering new information on species dispersion and genetics or answering any other question rather than: what pathogens can they be carrying? Or, in what hosts species were they found? Furthermore, we tend to greatly overlook tick influence in hosts and ecosystems which do not present an immediate connection to human activity, ignoring the fact that such neglected ticks and other vectors along with their wildlife hosts may also serve as natural reservoirs for many pathogenic agents (Daszak, 2000; Cutler 2010). Such is the case of *Hyalomma aegyptium* where studies and reports on this species have mostly focused on identifying possible transmitting parasites, or simply notes on occurrences on their hosts (Tiar et al., 2016)

Taking these factors into consideration, this study presents itself as an attempt to assess the follow issues: i) providing a first insight on the mtDNA genetic distribution patterns of *Hyalomma aegyptium* in the Maghreb region; ii) assessing possible evidence for cophylogenetic patterns between the *H. aegyptium* ticks and their *T. graeca* hosts in the Maghreb region; iii) perform a screening for more organisms infecting *Hyalomma aegyptium*.

Methods and materials

Sampling and storing

Ticks that were actively feeding were collected from *T. graeca* hosts across several sites in Morocco, Algeria, Tunisia and Turkey and immediately stored in 96% ethanol in 1.5ml Eppendorf tubes labeled with the corresponding code. Ticks were transported to the lab for species identification and further analysis. Sample sites with respective coordinates are presented in Table 4.

Country	Location	Code	Latitude	Longitude	Number of samples
Algeria	Ain Chorfa	AAC	35,230	0,140	13
Algeria	Guertoufa	AGU	35,200	1,160	8
Algeria	Zemmora	AKH	35,710	0,750	5
Algeria	Moudjbara	AMB	34,570	3,410	13
Algeria	Saf Saf	ASS	35,490	0,230	14
Algeria	The´niet el Had	ATH	35,890	2,010	7
Algeria	Zemmouri	AZE	36,460	3,410	9
Morocco	Balcon d'Ito	MBA	33,54	-5,32	1
Morocco	Had Kourt	MHK	34,651	-5,66	1
Morocco	Kaytun	MKA	34,687	-5,396	10
Morocco	Kariat Bamohammed	MKB	34,478	-5,311	7
Morocco	El Ksiba	MKS	32,575	-6,035	1
Morocco	Larache	MLA	35,166	-6,121	4
Morocco	Oulad Yakoub	MOY	34,025	-6,717	4
Morocco	Raba Harcha	MRH	34,053	-5,468	6
Morocco	Sefrou	MSE	33,854	-4,858	6
Morocco	Zoumi	MZO	34,77	-5,52	2
Morocco		MAR1	35,525	-5,713	2
Morocco		MAR2	31,530	-7,563	1
Morocco		MAR3	31,461	-7,407	1
Morocco		MAR4	34,098	-6,685	1
Morocco		MAR5	33,61048	5,15957	1
Tunisia	Rass el Melah	TRA	36,909	11,105	3
Turkey		TUR1	37,863	36,358	4
Turkey		TUR2	37,859	36,365	2
Turkey		TUR3	38,332	31,012	2
Turkey		TUR4	38,047	36,636	3

Table 4 – Sample sites arranged by country and population code with respective GPS coordinates, and number of samples used for each location. Sites with no location refer to non-specific locations such as roadside GPS points.

Tick identification

Collected ticks were identified morphologically using the dichotomous keys based on Földvári (2005), and Walker et al., (2003) and resources available at http://bristoltickid.blogs.ilrt.org/.

Pooling, DNA Extraction, isolation and quantification

For each sampling location, five individual ticks from different individual hosts were amplified. In sites where the number of hosts sampled was less than five, when possible, more than one tick was picked for DNA extraction in order to obtain five sequences for the geographical distribution analysis.

After morphological identification under the microscope, DNA was extracted from individual ticks using saline methods (with ammonium acetate) (Sambrook et.al, 1989). Small incisions were performed in the abdomen cavity and under the scutum of the ticks in order to improve the efficiency of the lysis buffer and proteinase K on the tick internal tissues, facilitating digestion of possible host blood still existing in the tick, allowing to indirectly screen not only the ticks but also tortoise blood. Extracted DNA was hydrated in 50µl of deionized water.

PCR reactions and sequencing

A partial fragment of the mitochondrial 12S rRNA gene was amplified using the SR-J-14199 forward and SR-N-14594 (Kambhampati & Smith,1995) reverse primers, and the CO1 gene was amplified using the TY-J-1449 (Black and Piesman, 1994) forward and CI-N-2312 (Murrel et al., 2000) reverse primers (Table 5). PCR conditions for amplification of both markers were adapted from Murrell et al. (1999, 2000). One µl of extracted DNA was added to 24µl PCR reaction mix containing 1.5 units of GoTaq polymerase (Promega) and with a final concentration of 1xGoTaq Buffer, 200µM of each dNTP, 0.2µM of each respective primer and 2.5mM $MgCl_2$. The initial denaturation step was performed at 94ºC for 3min followed by 35 cycles consisting of a denaturation step at 94ºC for 30s, annealing at 52ºC for 30s and elongation at 72ºC for 1min, and a final elongation at 72ºC for 10 min for the 12S gene. PCR reaction for the CO1 gene differed from the 12S PCR by having 40 cycles after the initial denaturation, and annealing temperature being 46ºC. 3µl of PCR product were subjected to

electrophoresis in a 2% agarose gel pre-stained with gel-red and run at 300V for 20min. Positive PCR reactions for each gene were sequenced through Sanger Method, using the respective forward primers. Sequencing was performed by an external company (Cogenics, Netherlands).

Primer name	Target region	Sequence 5'--->3'	Notes
SR-J-14199	12S rRNA	TACTATGTTACGACTTAT	Kambhampati & Smith (1995)
SR-N-14594	12S rRNA	AACTAGGATTAGATACCC	Kambhampati & Smith (1995)
TY-J-1449	Cytochrome oxidase subunit 1	AATTTACAGTTTATCGCCT	Black and Piesman (1994)
CI-N-2312	Cytochrome oxidase subunit 1	CATACAATAAAGCCTAATA	Murrel et al. (2000)

Table 5 – Primers used for amplification of *H. aegyptium* mtDNA.

Sequence analysis

All obtained sequences were subjected to NCBI BLAST tool for genetic confirmation of species and checked for possible sequencing errors and aligned against each other in Geneious 4.8.5 (Biomaters, 2009) for both 12S rRNA and CO1 genes. Additionally, every *H. aegyptium* confirmed sequence obtained will be submitted to the NCBI GenBank database. TCS software (Clement et al., 2000) was used to create haplotype networks using a statistical parsimony algorithm (Templeton et al., 1992). This type of approach allows inferring relative age of haplotypes, where more central haplotypes with more connections are considered older, while more peripheral haplotypes with fewer connections have appeared more recently (Posada and Crandall, 2001).

AMOVA analysis

Analysis of molecular variance (AMOVA) (Excoffier et al, 1992) was performed using ARLEQUIN. This analysis allows for assessing the genetic structure and differentiation of populations by testing hypothesis related to gene flow or isolation within a species through comparison of predetermined groups of sample sites. AMOVA also relies on F-statistics to estimate the percentage of genetic variability and level of structure found among populations (FST), among populations within groups (FSC) and among groups

(FCT) and respective fixation indexes (Weir and Cockerham, 1984). Fixation indexes were only considered significant at P value < 0.05.

An AMOVA was run with populations grouped according to the geographical distribution of *Testudo* subspecies in order to search for a possible differentiation between population groups which could be an indicator of hosts and parasites sharing the same genetic distribution patterns. This hypothesis considers that genetic distribution of North African *Hyalomma aegyptium* would be similar to that of their *Testudo graeca* host, so the first AMOVA analysis grouped sampled populations in accordance to the distribution of *Testudo graeca* subspecies. Samples from North of the High and Middle Atlas were considered as a single group. Individuals from East of the Middle Atlas and the Aurès Mountains was considered another group, and populations East of the Aurès mountains made up a third group. Because populations within the three groups are coincidently confined within country borders, these three groups will hence be referred to as the Moroccan, Algerian and Tunisian group respectively. All populations from Turkey will also be grouped together to form the Turkish group.

A second AMOVA was run with taking more into account the geographical distribution of tick haplotypes, and trying to define group based on that information, rather than being biased on host geographical patterns. Four groups were also defined in likelihood of the first hypothesis, although these will be defined by other natural borders (Fig. 6). The first group is defined by all populations West and North of the Middle Atlas, the Er-Rif mountains, and populations North of the Tell Atlas up to the Aurès mountains, and includes populations AAC, AKH, ASS, ATH, AZE, MHK, MKA, MKB, MLA, MOY, MRH, MZO, MAR1, and MAR4. Contrasting with the first group, the second group consists of all population around and south of the Middle Atlas and South of the Tell Atlas, which include AGU, AMB, MBA, MKS, MSE, MAR2, MAR3 and MAR5. The first group's range seems to follow a more coastal or littoral distribution when compared to the second group which is more from the interior of the region, will thus be referred to as the littoral and interior groups respectively. The third and fourth groups corresponding to Tunisian and Turkish sites will be kept as they were.

Haplotype diversity (*H*d), nucleotide diversity (π) and the average number of nucleotide differences (*Pi*) was calculated for all the defined groups using DNAsp v5 (Librado and rosas, 2009) to better assess which set of groups better explains genetic distribution of *H. aegyptium*.

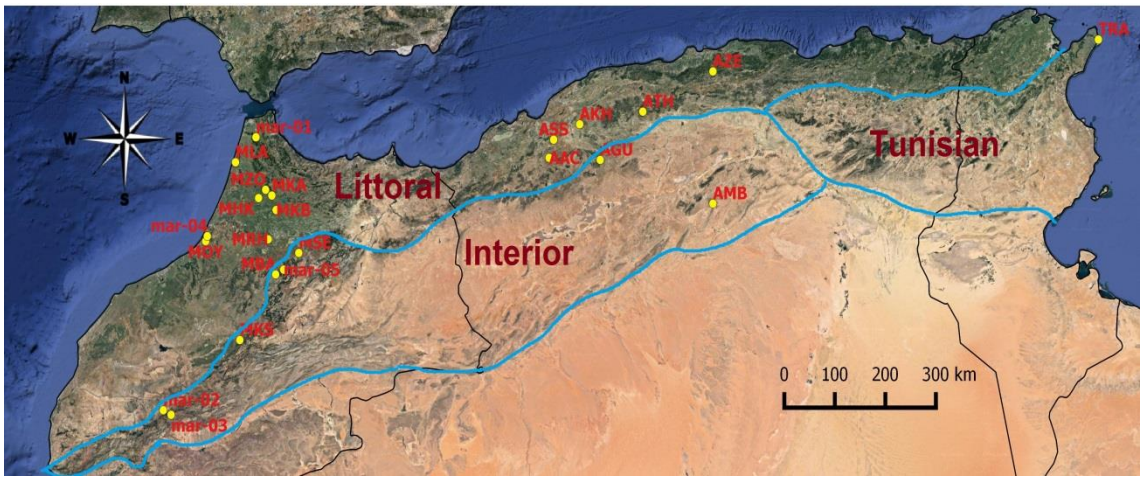

Figure 6 – Different AMOVA grouping distribution of North African sites. First set of groups is defined by *Testudo* subspecies distribution range, which in this case overlaps with country borders (depicted in black lines). Second set of groups is depicted by mountain ranges defined by blue lines and is devided in the "littoral", "interior", "Tunisian" and "Turkish" (not observed in map) groups.

Pathogens detection

All the ticks' samples that were extracted previously were screened for several pathogens. Two sets of wide spectrum primers were used to broaden the search for possible parasites existing within feeding *H. aegyptium* ticks. Primers EHR16SD/EHR16SR (Hornok et al., 2008) target a specific 345bp fragment of the 16S rRNA gene from the Analasmataceae family of bacteria which include *Anaplasma*, *Ehrilichia*, *Neorickettsia* and *Wolbachia* (Parola et al., 2000, 2003) (Table 6). And primers HepF300/HepR900 which target a fragment of *Hepatozoo*n 18S rRNA (Ujvari et al., 2004), but can also be used to detect *Hemolivia mauritanica* (Harris et al., 2011) (Table 6). Template DNA used was the same as used for 12S and CO1 amplification for each sample as DNA had been extracted in a way that would also retain DNA of possible existing pathogens.

For EHR16S PCR, primers 1µl of template DNA was used in a 15µl PCR mix consisting of 1x final buffer concentration, 300µM of each dNTP, 0.3µM of each respective primer, 0.4 mg/µl of BSA enhancement solution, and 2.5mM $MgCl_2$. with 0.75 units of PlatinumTaq (Invitrogen). Initial denaturation lasted for 5min. at 95ºC followed by -1ºC touchdown cycles between 65ºC and 55ºC annealing temperature with a 30s denaturation step at 95ºC, 30s annealing step and 1min. elongation step at 72ºC to increase primer specificity to non-tick DNA. Last 35 cycles were performed

with 55ºC annealing temperature with equal temperature and duration for remaining steps and a final elongation step at 72ºC lasting 5min. For PCR reactions using Hep primers, the protocol described in Harris et al. (2011) was followed, but adjusted to a final volume of 15µl.

All pathogens screening PCR products were subjected to electrophoresis in a 2% agarose gel pre-stained with a gel-red and run at 300V for 20min. Positive PCR reactions for each gene were sequenced through Sanger Method, using the respective forward primers. Protocol was the same as described for ticks. Sequences obtained were compared to published sequences in the NCBI database through BLAST in order to identify the pathogen.

Primer name	Target region	Sequence 5'--->3'	Notes	Target pathogens
EHR16SD	16S rRNA	GGTACCYACAGAAGAAGTCC	Hornok et al. (2008)	Bacteria from the Anaplasmataceae Family
EHR16SR	16S rRNA	TAGCACTCATCGTTTACAGC	Hornok et al. (2008)	
HepF300	18S rRNA	GTTTCTGACCTATCAGCTTTCGACG	Ujvari et al. (2004)	*Hepatozoon* and *Hemolivia mauritanica*
HepR900	18S rRNA	CAAATCTAAGAATTTCACCTCTGAC	Ujvari et al. (2004)	

Table 6 - Primers used for pathogen screening and main target groups

Results

Species identification

All the tick samples analyzed were identified morphologically as *Hyalomma aegyptium*, and BLAST analysis of all obtained sequences for both 12S and CO1 had respectively between a 96-99% and 97-99% identification match against previously published sequences for this species. As such, all sampled ticks were considered to be of *H. aegyptium* and further subjected to molecular analysis.

DNA sequence analysis

The alignment of 12S rRNA sequences resulted in a total data set of 123 individual sequences with 346bp length. For this gene, a total of 12 different haplotypes with 16 variable sites were found, and are described in Table 7. The CO1 data set comprises of 119 individual sequences with 680bp of length with a total of 46 variable sites, which can be used to describe 26 different haplotypes as seen in Table 8. Geographical distribution of all haplotypes of both markers is further explored below (see FIG. 7 for 12S and FIG 8 for CO1). For parsimony networks the same color scheme was used to represent same lineages and possible groups based on haplotypes. The full list of haplotype distribution across all sampled sites can be observed in Table 9.

12S rRNA

At a 95% connection limit, the parsimony network for 12S rRNA is entirely connected, with two main haplogroups, A and B (2 and 10 different haplotypes respectively) with 1.0% divergence between them, and separated by only three steps (Fig. 7). Single event mutations are represented by black lines, and missing links are represented by hollow dots. One mutation event that was considered a single duplication of a small 5bp fragment is marked with a cross on its respective line.

Regarding haplogroup A, it is distributed across the north of Morocco, Algeria and Tunisia. The two haplotypes within this group (Ac1 and Az1) are separated by a single event mutation. The two samples with Az1 haplotype were found exclusively on a

single locality from central Morocco (MZO) where no other haplotype was found (Table 9). Haplotype Ac1, on the contrary, was found in several localities from northern Algeria (AAC, AKH, ASS, ATH, AZE), northern Morocco (MAR4, MHK, MKA, MKB, MKS, MLA, MRH, MSE) and Tunisia (TRA).

	Nucleotide Position															
	10	67	68	69	70	71	72	76	86	146	149	154	172	245	247	327
Consensus	G	-	-	-	-	-	-	T	A	A	-	A	T	T	C	C
Ac1																
Az1								C								
Bc1	A										A		A			T
Bc2	A									T	A		A			T
Bm1	A						T				A		A			T
Bm2	A						T				A	G	A			T
As1	A								G		A		G			T
As2	A								G		A		A			T
As3	A	T	T	C	A	A	T		G		A		G			T
Bt1.1	A										A		A	C		T
Bt1.2	A						T				A		A	C		T
Bt2	A										A		A		T	T

Table 7 - Alignment of variable positions found in 12 unique haplotypes of 12S rRNA sequences, compared to the alignment consensus sequence (123 individuals, 346bp)

Haplogroup B has higher diversity (10 haplotypes) and includes samples from Morocco, Algeria and Turkey. It has a central haplotype Bc.1 connected to other less frequent haplotypes: Bm, Bs and Bt1 and Bt2, based on geographical distribution. All samples tested from AGU, AMB, both from Algeria, MBA, MOY, MAR1, MAR2, MAR5 from Morocco and TUR from Turkey were exclusive to the haplogroup B, although in several localities, multiple haplotypes were observed. The central haplotype Bc1 is the most widespread haplotype from the B haplogroup, appearing in every country except Tunisia. In Algeria it appears twice in AAC and ATH, three times in AGU and once AMB; in Morocco, it appears once in MBA, MKB, MAR2 and MAR5, twice in MOY and MAR1, and five times in MSE; and in Turkey once in TUR1 and TUR4, and twice in TUR3. Haplotype Bm.1 which is found mainly on the southernmost Algerian population (AMB) is also found less frequently in other northern Algerian localities (once in AAC and ATH and twice in AGU), and is closely related to Bm.2 which is composed by two individuals from AGU. Haplotype Bc.2 was attributed to a single sample also from AGU. Haplotypes from Bs were found on only three populations: ASS, MOY and MRH; Bs.1 is predominant in ASS with one sample taken from both MOY and MRH; Bs.2 was

attributed to a single sample from ASS; Bs.3 strangely consists of a single sample from MRH. Remaining haplotypes from haplogroup B were discovered only on Turkish populations. Bt1.1 was found only on populations TUR1 and TUR4, while Bt1.2 was found only on TUR2. Lastly, Bt2 is attributed to a single sample from TUR4.

While both haplogroups are found in population of both Morocco and Algeria, all tested samples collected from Tunisia belong entirely to haplogroup A and all tested samples from Turkey belong to haplogroup B.

Cytochrome Oxidase 1

Haplotype network for Cytochrome oxidase 1 follows a similar pattern to 12S rRNA, which is to be expected as both are of mitochondrial origin, so the same nomenclature for identical haplotype groups will be used. However, CO1 does allow for a more detailed network as it presents more haplotypes for each clade which also allows inferring more refined genetic patterns across the sampled populations. Much like with 12s rRNA, at 95% connection limit, the CO1 parsimony network is also constituted by two main haplogroups: A and B. However, for this marker, these groups are not connected revealing a bigger divergence between them (3.3% divergence). 12 different haplotypes and three missing nodes are represented in haplogroup A, while haplogroup B has 14 different haplotypes and five missing nodes (Fig. 8). For this marker, both haplogroups still follow the same general distribution like 12S, with haplogroup A being distributed across Morocco, Algeria and Tunisia, while Haplogroup B similarly being found in Morocco, Algeria and Turkey. However,

Haplotype Ac1 was observed both in Morocco (MKA, MKB, MLA, MRH, MSE and MZO), and Algeria (AAC, AKH, ASS, ATH and AZE), but was not found in Tunisia. Instead, haplotypes At1 and At2, which are connected to Ac1 by two branching mutation events, were exclusive to Tunisia (TRA) where no other haplotypes were found. On the contrary, haplotypes in this network found in MZO all belong to Ac1, and not to separate haplotypes. Haplotypes Ac2 and Ac3 were found exclusively on the Algerian location ASS, and haplotype Ac6 was also exclusive to a single sample found in AKH. In Morocco, haplotypes Ac4 and Ac5 were found once in AAC, haplotype Ac7 was found once in MKA and Ac8 was found once in MAR4. These haplotypes were exclusive to their sites. Haplotypes Am1 and Am2 were connected to Ac1 by more than one mutation event, and were found once in MKA and MHK respectively.

Haplogroup B for CO1, similar to 12S, consists of a central haplotype Bc1 connected to other less frequent haplotypes Bo, Bs and Bt2 also based on geographical distribution. All Algerian samples from AGU and AMB, Moroccan samples from, MOY and MAR3 and all Turkish samples (TUR) belong to haplogroup B. Haplotype Bc1 is the most distributed haplotype from this group and can be found in Algeria (AAC, AGU, AMB, ATH), and Morocco (MKB, MOY and MSE), and haplotype Bc2 is found on a single sample exclusive to MSE. Unlike with 12S, Bc1 does not appear in Turkey. Haplotype Bs1 is mostly found in Algerian site ASS, but is also found once in ATH and MRH. Haplotype Bs2 was found only once in two separate Moroccan sites MOY and MAR3, haplotype Bs3 was found exclusively on one sample from MRH, and haplotypes Bs4 and Bs5 were found exclusively on single samples from Algerian sites AMB and AAC respectively. Haplotype Bo1 belongs to a single sample obtained from the Moroccan location MOY, and is not found anywhere else. Turkish samples (TUR) were found divided in two groups: Bt1 which is connected to Bs1, and Bt2 which is connected to Ac1. Bt1.1 is found twice in TUR1 and once in TUR4, while Bt1.2 is found only on both samples from TUR2. Haplotype Bt2.1 is found once in TUR1 and twice in TUR4, haplotype Bt2.2 was found once and exclusively in TUR1, and haplotypes Bt2.3 and Bt2.4 were exclusive to both samples from TUR3.

The samples which belonged to haplotypes Bm in the 12S network did not appear as distinct haplotypes from Ac1 in the CO1 network. As such, the Bm haplotypes were not recognized for this network. However, two exception were found which are here renamed has haplotypes Bs4 and Bs5.

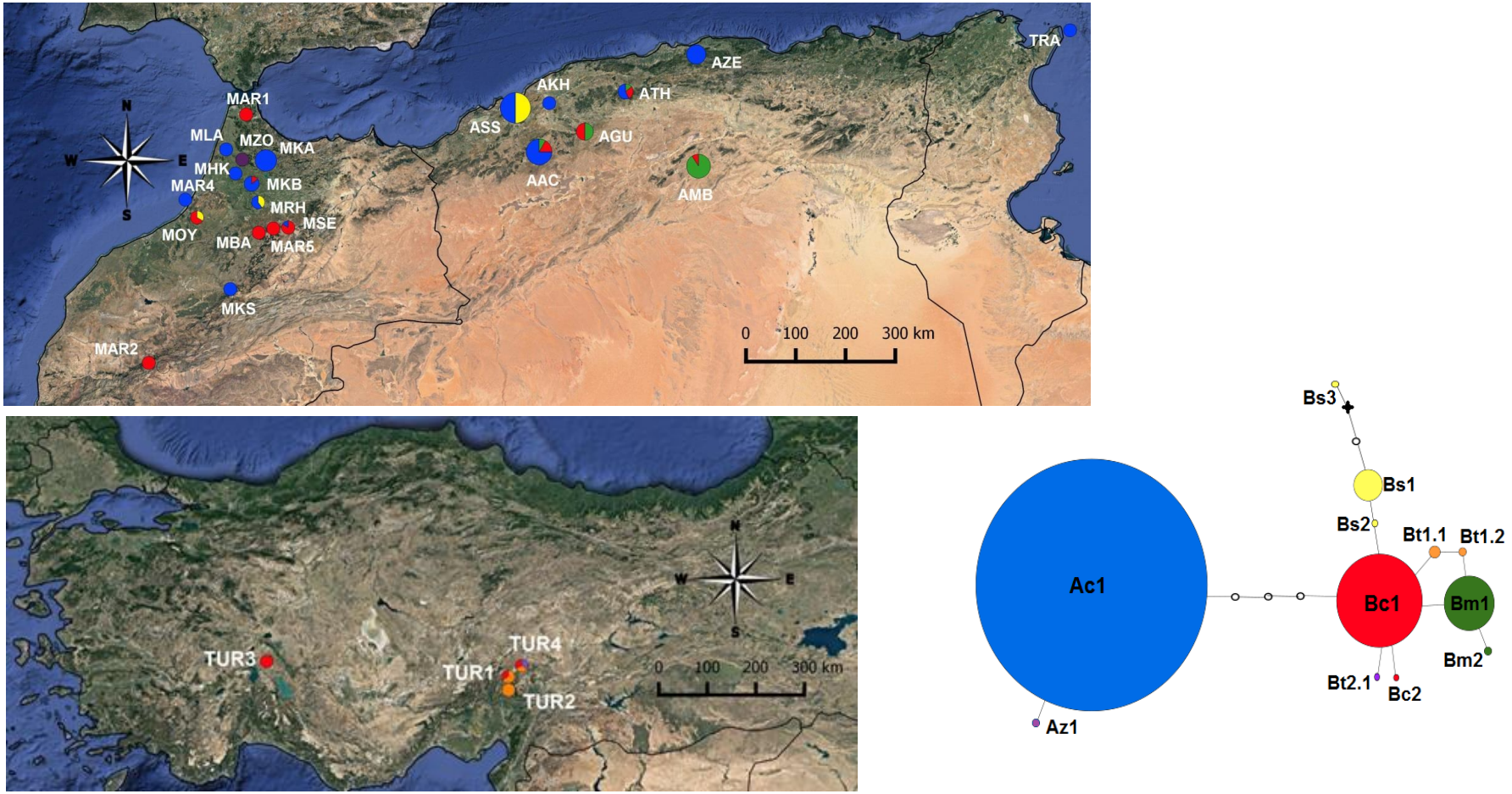

Figure 7 - Haplotype distribution maps of 12S rRNA of *Hyalomma aegyptium* in North Africa (above) and Turkey (bellow) and respective haplotype network (right). Diagram size on maps is proportional to the number of samples sequenced for that site, but sites with less than 3 samples were upscaled. Different colours in network represent different haplotype groups, with lines representing sinle nucleotide polymorphisms and a cross representing a single event duplication. Observed haplotypes are labeled according to table 7 and size is proportional to the number of sequences. Hollow dots represent missing aleles. Colors on the map diagrams represent respective haplotype groups with the same color on the haplotype network.

	Nucleotide Position																																													
	13	14	27	29	35	38	44	53	62	74	83	101	127	140	169	203	224	236	281	293	299	302	314	317	320	338	344	380	382	388	392	446	485	494	497	509	578	587	607	617	637	640	644	655	665	667
Consensus	A	G	A	T	T	A	T	G	G	T	C	A	G	C	G	G	G	G	A	C	A	A	A	G	T	A	A	T	A	G	T	C	T	A	A	G	C	G	G	G	C	A	G	A	A	C
Ac1		A																																												
Ac2		A																																				A								
Ac3		A																					G																							
Ac4		A										G																																		
Ac5		A													A																															
Ac6		A																																							T					
Ac7		A								A																																				
Ac8		A																								G																				
Am1		A																																								T		T		
Am2		A																																					T					T		G
At1																												C																		
At2																																					T									

Bc1				A	C				A		T		A	T		A	A	A	G	T				A		T	G				A	T		G		A	T								T	
Bc2				A	C				A		T		A	T		A	A	A	G	T				A		T	G				A	T		G		A	T						A		T	
Bo1	G			A	C				A		T		A	T		A	A	A	G	T				A		T	G			A	A	T		G		A	T								T	
Bs1				A	C			A	A		T		A	T		A	A	A	G	T				A		T	G				A	T		G		A	T								T	
Bs2				A	C			A	A		T		A	T		A	A	A	G	A				A		T	G				A	T		G		A	T								T	
Bs3			T	A	C			A	A		T		A	T		A	A	A	G	T				A		T	G				A	T		G		A	T								T	
Bs4				A	C			A	A		T		A	T		A	A	A	G	T				A	C	T	G				A	T		G		A	T								C	
Bs5				A	C			A	A		T		A	T		A	A	A	G	T	G			A	C	T	G				A	T		G		A	T			T					C	
Bt1.1				A	C			A	A		T		A	T		A	A	A	G	T		G		A		T	G				A	T		G		A	T								T	
Bt1.2				A	C	G		A	A		T		A	T		A	A	A	G	T		G		A		T	G		G		A	T		G		A	T								T	
Bt2.1				A	C				A		T		A	T		A	A	A	G	T			G	A		T	G				A	T		G	G	A	T								T	
Bt2.2				A	C				A		T		A	T		A	A	G	G	T			G	A		T	G				A	T		G	G	A	T								T	
Bt2.3				A	C				A		T		A	T		A	A	A	G	T			G	A		T	G				A	T	C	G	G	A	T								T	
Bt2.4				A	C		A		A		T		A	T		A	A	A	G	T			G	A		T	G				A	T		G	G	A	T								T	

Table 8 - Alignment of variable positions found in 46 unique haplotypes of CO1 sequences, compared to the alignment consensus sequence (119 individuals, 680 bp)

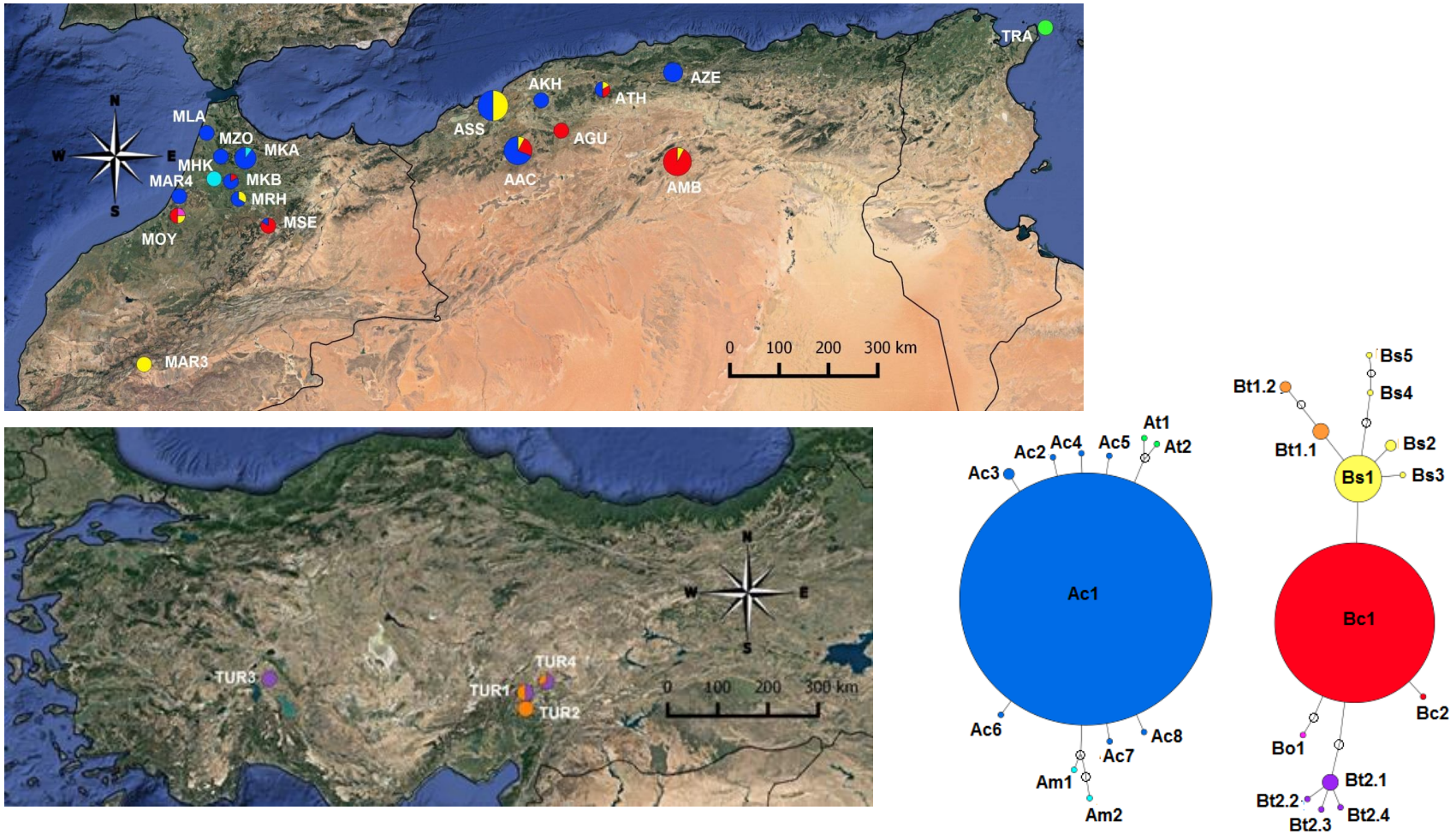

Figure 8 - Haplotype distribution maps of CO1 of *Hyalomma aegyptium* in North Africa (above) and Turkey (bellow) and respective haplotype network (right). Diagram size on maps is proportional to the number of samples sequenced for that site, but sites with less than 3 samples were upscaled. Different colours in network represent different haplotype groups, with lines representing sinle nucleotide. Observed haplotypes are labeled according to table 8 and size is proportional to obtained sequences. Hollow dots represent missing aleles. Colors on the map diagrams represent respective haplotype groups with the same color on the haplotype network.

Population	Number of hosts sampled	Number of ticks sampled	Haplotypes found	
			12s rRNA	CO1
AAC	10	13	Ac1 (9), Bc1 (2), Bm1 (1)	Ac1 (7), Ac4 (1), Ac5 (1), Bc1 (3) Bs5 (1)
AGU	8	8	Bc1 (3), Bc2 (1), Bm1 (2), Bm2 (2)	Bc1 (7),
AKH	2	5	Ac1 (5)	Ac1 (4), Ac6 (1),
AMB	13	13	Bc (1), Bm1 (10)	Bc1 (12), Bs4 (1)
ASS	9	14	Ac1 (7), Bs1 (6), Bs2 (1)	Ac1 (4), Ac2 (1), Ac3 (2), Bs1 (7)
ATH	7	7	Ac1 (4), Bc1 (2), Bm1 (1)	Ac1 (3), Bc1 (2), Bs1 (1)
AZE	9	9	Ac1 (9)	Ac1 (9)
MBA	1	1	Bc1 (1)	
MHK	1	1	Ac1 (1)	Am2 (1)
MKA	10	10	Ac1 (10)	Ac1 (8), Ac7 (1), Am1 (1)
MKB	2	7	Ac1 (6), Bc1 (1)	Ac1 (5), Bc1 (1)
MKS	1	1	Ac1 (1)	
MLA	2	4	Ac1 (4)	Ac1 (3)
MOY	1	4	Bc1 (2), Bs1 (1)	Bc1 (2), Bo1 (1), Bs2 (1)
MRH	1	6	Ac1 (3), Bs1 (1), Bs3 (1)	Ac1 (4), Bs1 (1), Bs3 (1)
MSE	2	6	Ac1 (1), Bc1 (5)	Ac1 (1), Bc1 (4), Bc2 (1)
MZO	2	2	Az1 (2)	Ac1 (1)
MAR1	1	2	Bc1 (2)	
MAR2	1	1	Bc1(1)	
MAR3	1	1		Bs2 (1)
MAR4	1	1	Ac1 (1)	Ac8 (1)
MAR5	1	1	Bc1 (1)	
TRA	1	3	Ac1 (3)	At1 (1), At2 (1)
TUR1	1	4	Bc1 (1), Bt1.1(2),	Bt1.1 (2), Bt2.1 (1), Bt2.2 (1)
TUR2	1	2	Bt1.2 (2)	Bt1.2 (2)
TUR3	1	2	Bc1 (2)	Bt2.3 (1), Bt2.4 (1)
TUR4	1	3	Bc1 (1), Bt1.1 (1), Bt2 (1)	BT1.1 (1), Bt2.1 (2)

Table 9 – *Hyalomma aegyptium* haplotypes discovered in each sampling location and number of individuals (inside parenthesis) carrying each haplotype in that location for both 12S rRNA and CO1 genes. Missing values indicate that it was not possible to obtain any sequence for that location and respective gene.

AMOVA Analysis

AMOVA analysis for the first set of groups related to *Testudo* subspecies distribution did not suggest a clear phylogeographic structure for those groups (Table 10) for both 12S rRNA and Cytochrome Oxidase 1. Results suggest that for both genes the highest degree of variation was among populations within the same groups (46.97%/44.99% for 12s and COI respectively) followed by the variation within populations (12s: 43.48%, COI: 42.46%), while variation among groups (12s: 9.56%, COI: 12.55%) was much lower than the other sources of variation. Lack of structure between groups is further suggested by the low fixation indexes between groups (FCT) (Fct = 0.096, P = 0.148 / Fct = 0.126, P = 0.119 for 12s and COI respectively), although Fct values for this set of populations groups are not statistically significant. However, a high degree of structure was observed in populations within the same group (FSC) (Fsc = 0.519, P < 0.001 / Fsc = 0.514, P < 0.001 for 12s and COI respectively), suggesting that populations are still structured in at least one group.

Marker	Source of variation	d.f.	Sum of squares	Percentage of Variation	F-Statistics	P
12S rRNA	Among groups	3	21.243	9.56%	Fct = 0.096	0.148
	Among populations Within groups	22	83.799	46.97%	Fsc = 0.519	0
	within populations	97	61.739	43.48%	Fst = 0.565	0
Cytochrome Oxidase 1	Among groups	3	100.057	12.55%	Fct = 0.126	0.119
	Among populations Within groups	18	324.736	44.99%	Fsc = 0.514	0
	within populations	97	260.585	42.46%	Fst = 0.575	0

Table 10 - Analysis of molecular variance for *Hyalomma aegyptium* for 12S rRNA and CO1 genes, between the four defined groups based on the phylogeography of *Testudo graeca* host subspecies: Morocco, Algeria, Tunisia and Turkey.

For the second set of groups taking more into account the genetic distribution of *H. aegyptium*, AMOVA analysis revealed a certain degree of structure for both 12S rRNA

and CO1 (Table 11). Unlike the first set of groups, in this analysis the highest degree of variation was actually variation among groups (51.19% / 55.50%), while variation among populations within groups had the lowest percentage of variation (14. 36% / 11.33%) and percentage of variations within all sampled populations registered at 34.45% and 33.17%,respectively. Fixation indexes indicate a clear population structure among set groups (Fct = 0.512 , P < 0.001 / Fct = 0.555 , P < 0.001), however the chosen parameters do not seem to be completely responsible for all of the populations structure as the fixation index for populations within groups may also reveal an underlying structure within at least one of the defined groups (Fsc = 0.294 , P < 0.001 / Fsc = 0.255 , P < 0.001).

Marker	Source of variation	d.f.	Sum of squares	Percentage of Variation	F-Statistics	P
12S rRNA	Among groups	3	64.251	51.19%	Fct = 0.512	0
	Among populations Within groups	22	40.791	14.36%	Fsc = 0.294	0
	within populations	97	61.739	34.45%	Fst = 0.655	0
Cytochrome Oxidase 1	Among groups	3	288.718	55.50%	Fct = 0.555	0
	Among populations Within groups	18	136.075	11.33%	Fsc = 0.255	0
	within populations	97	260.585	33.17%	Fst = 0.668	0

Table 11 - Analysis of molecular variance for *Hyalomma aegyptium* for 12S rRNA and CO1 genes, between the four defined groups based on haplotype geographical distribution among all sampled sites: Littoral, Interior, Tunisia and Turkey.

Haplotype Diversity

Because the haplotypic diversity analysis in DNAsp excludes sites with alignment gaps, this analysis was performed solely on CO1 since many important haplotypes occurring in the 12S rRNA differentiate between each other through nucleotide insertions which would not be taken into consideration (Table 7). This would result in the analysis considering the existence of fewer haplotypes which would lead to an inaccurate estimate to calculate haplotype diversity within this gene. CO1 overall haplotype

diversity (Hd) for all sites was calculated at Hd = 0,760 with a nucleotide diversity (π) of π = 0,017. Considering the two different AMOVA grouping hypothesis, the Moroccan defined group presented 11 different haplotypes with a diversity of Hd = 0,659 and π = 0,0152, while Algeria presented 10 different haplotypes but with higher Hd = 0,703 and π = 0,0165. On the other hand, when analyzing diversity considering the littoral/interior grouping, diversity within groups tends to be lower with Hd = 0,613 and π = 0,0133 in the more littoral group with a total of 16 different haplotypes, and Hd = 0,279 and π = 0,0029 in the more interior group with only 5 haplotypes. The Turkish group which is common for both hypotheses presented 6 different haplotypes with Hd = 0,873 and π = 0,0050. Since in Tunisia only two sampled were obtained with a different haplotype each, this analysis was not performed in this group.

Pathogen Identification

From all PCRs performed for parasite detections, a total of 59 were positive and revealed the presence of at least four different parasites existing in sampled populations. All samples were sequenced with forward primer, but sequence quality did not allow for an accurate identification of different haplotypes, so this study limits itself to species identification. HEP primers only revealed the presence of *Hemolivia mauritanica* which was found in Morocco, Algeria and Turkey, and was not found in Tunisia. Interestingly, only one sample from Morocco (MSE) was positive for *H. mauritanica* in all of tested Moroccan samples. In contrast, in Algeria 5 (AAC, AGU, AKH, ASS and ATH) of the seven localities sampled were positive for *H. mauritanica*, and , while in Turkey all sites (4 localities) revealed positively infected ticks.

The broad spectrum EHR primers on the other hand allowed detection for two more possible parasites: *Ehrlichia sp*. and *Wolbachia sp.* and the tick mitochondrial symbiont M*idichloria mitochondrii.* Despite Blast analysis allowing for a reliable identification for *C. midichloria mitochondrii* (96-99% identity), *Ehrlichia sp.* (99% identity) *and Wolbachia sp*. (99% identity), it is not sensitive enough to provide an accurate species match for *Ehrlichia* or *Wolbachia* genus. Both M*idichloria mitochondrii* and *Ehrlichia sp*. were also found in Morocco and Algeria, while only *M. mitochondrii* was found in one site in Turkey. *Midichloria mitochondrii* was found in Moroccan sites MBA, MKA, MKB, MLA, MSE and MZO, while *Ehrlichia* was only found in MKS and not in other Moroccan sites. The same Algerian sites AAC, AGU, AMB and ATH were positive for infestation for both *Midichloria mitochondrii*. and *Ehrlichia sp*. And a single sample from the

Moroccan site MSE revealed positive for *Wolbachia sp.* A more details can be found in Table 13, and a parasite location map in Figure 9.

Co-infection was also observed in four distinct sites. In ACC, AGU, ATH and Tur2 co-parasitism events were observed where ticks tested positive for both M*idichloria mitochondrii.* and *Hemolivia mauritanica.* However, only in AGU a single event of co-parasitism between *Ehrlichia sp.* and *H. mauritanica* was observed.

Population	**Number of hosts sampled**	**Number of ticks sampled**	**Positive infections**	***Midichloria mitochondrii***	***Ehrlichia sp.***	***Wolbachia sp.***	***Hemolivia mauritancia***	**Co-Infested Ticks**
AAC	10	10	6	3	1		4	+(2)
AGU	8	8	6	1	4		3	+(1) x(1)
AKH	2	5	2				2	
AMB	13	13	4	1	3			
ASS	9	9	5				5	
ATH	7	7	4	2	2		2	+(1) x(1)
AZE	9	9	0					
MBA	1	1	1	1				
MHK	1	1	0					
MKA	10	10	1	1				
MKB	2	6	1	1				
MKS	1	1	1		1			
MLA	2	2	1	1				
MOY	1	4	0					
MRH	1	4	0					
MSE	2	6	3	2		1	1	
MZO	2	2	2	2				
MAR1	1	1	0					
MAR2	1	1	0					
MAR3	1	1	0					
MAR4	1	1	0					
MAR5	1	1	0					
TRA	1	3	0					
TUR1	1	4	2				2	
TUR2	1	2	1	1			1	+(1)
TUR3	1	2	1				1	
TUR4	1	3	2				2	

Table 12 – Table describing: the number of discovered *Testudo graeca* tortoises infected with *Hyalomma aegyptium* ticks and number of ticks screened for parasites in each sampled site; number of positively infected ticks for each site is presented in the third column with following notes on which and how many parasites were tested positive for each infected site; and number of co-infected ticks discovered. Co-infection between *Hemolivia mauritanica* and *Midichloria mitochondrii* is marked by "+" and co-infection between *H. mauritanica* and *Ehrlichia sp*. is marked by "x" with number of ticks carrying both organisms inside parenthesis.

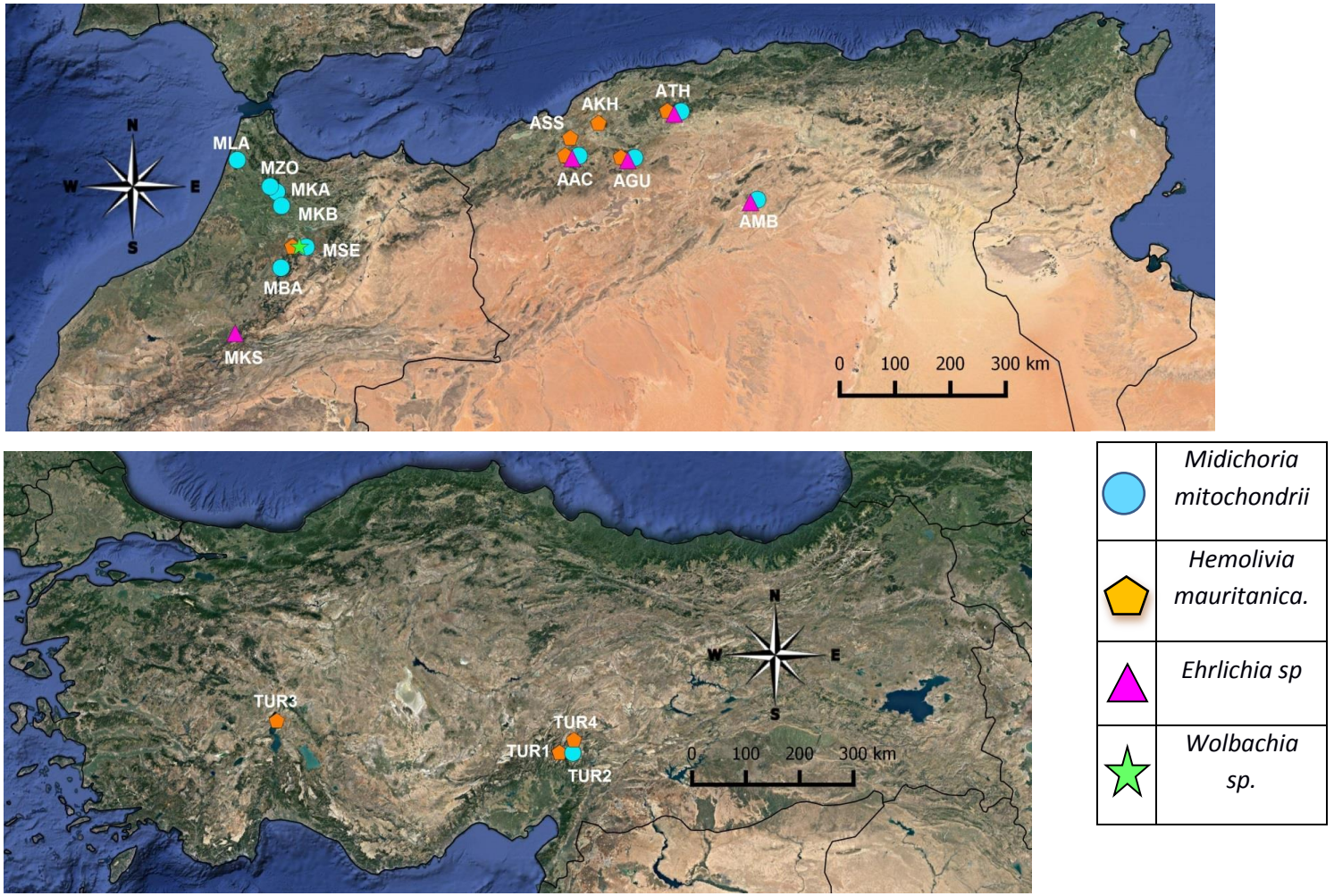

Figure 9 - Map of North Africa (above) and Turkey (below) representing different parasites found in *H. aegyptium* and their respective sites. A legend representing each of the parasites is included (right).

Discussion

The genetic distribution of *H. aegyptium*

Despite a great effort in developing new protocols that can be used to easily detect tick transmitted parasites, not much is known about the genetic diversity of ticks themselves (Araya-Anchetta et al., 2015). This study represents the first attempt to characterize the genetic landscape for the North African spur-tortoise tick, *Hyalomma aegyptium,* in the North African Maghreb region and its relationship with Turkish populations through analysis of mitochondrial DNA, which has enabled the presentation of an early assessment of the genetic variation within this species. The sampled regions showed high overall diversity. However, when compared to other ixodids, *H. aegyptium* presented lower values than other species for the same CO1 locus (Rees et al., 2003; Cangi et al., 2013; Lv et al., 2014; Kanduma et al., 2016). This might be due to the small number of samples obtained in several sites, which causes biases in the detection of the more common haplotypes. In addition, our database, although it covers most of the range of *T. graeca* host in the Maghreb Region, it misses samples from important areas between the Algerian and Moroccan sites, and between the Tell-Atlas and Aurès Mountains. Because of this situation, the obtained results in this study may not reveal the full genetic complexity of the sampled regions.

The parsimony haplotype network analysis for both 12S and CO1 reveals the presence of two distinct haplogroups representing two different evolutionary lineages of *H. aegyptium*. Both haplogroups were found in Morocco and Algeria, but whereas Haplogroup A was mostly predominant in Eastern Morocco and North of Tell-Atlas in Algeria, Haplogroup B was mostly distributed around the Moroccan Middle and High Atlas and South of the Tell-Atlas mountains. Tunisian sites only revealed haplotypes originating from Haplogroup A. And haplotypes found in Turkey all belonged to Haplogroup B, however according to the CO1 network they appear to have two distinct origins. Despite the great genetic deviation between the two haplogroups (1% divergence for 12S rRNA and 3% divergence in CO1), it is apparent that its distribution is not completely isolated from each other. Along the Tell-Atlas (AAC, ASS and ATH) and west of the Middle Atlas (MKB, MRH and MSE), haplotypes from both haplogroups were discovered meaning these areas probably serve as contact zones between the two lineages of *H. aegyptium*. Although these areas are usually recognized as barriers for many species (Schleich et al., 1996), such does not seem to

be the case for *T. graeca*, as the subspecies *T. graeca marokkensis* has been recorded as occupying the entire region North-east of the High-Atlas, and surrounding the Middle Atlas up to the Riff-Regions; and *T. graeca graeca* is recorded to occupy the entire region both North and South of the Tell-Atlas mountains (Fritz et al., 2009; Anadón et al., 2015). This distribution of *T. graeca*'s subspecies could explain the geographic position of the *H. aegyptium* contact zones. Sites MAR1, MAR4, MKS and MOY also bring up questions on the distribution patterns of *H. aegyptium*. MAR1 in the Riff Region appears as a single sample from haplogroup B surrounded by other sites presenting only samples belonging to haplogroup A, and MKS which is represented only by Haplogroup A is found in the High Atlas where no other sample from this group was found. On the other hand, MOY and MAR4, near the coast west of the Middle Atlas, are two sites very close to each other that are only 10.5 km from each other and have apparent physical or geographical barrier between them. However, while samples from MAR4 belong entirely to Haplogroup A, all samples from MOY belong to Haplogroup B. It is possible that other factors, rather than just host distribution (Fritz et al., 2009), might be influencing this pattern in ticks but it is just as likely that man could have played a role in these cases during the recent heavy collection and commercialization of *T. graeca* in Morocco and Algeria (Groombridge, 1982; Lambert, 1983) which might also have enabled the translocation and introduction of these new haplotypes creating these unexpected patterns, difficult to explain by natural expansion processes.

Evaluation of possible co-evolutionary patterns between *H. aegyptium* and *T. graeca*

The presence of two distinct haplogroups in the Maghreb region could be explained by one of two likely *a priori* scenarios: i) *Hyalomma aegyptium* populations have been evolving together with *T. graeca* populations since its arrival in North Africa; under this scenario, *H. aegyptium* would be under the same pressures and events which caused *T. graeca* to diverge into its current North African subspecies. Or ii) *Hyalomma aegyptium* in North Africa has been more influenced by different selective pressures or events which would translate into a different evolutionary pattern compared to the host (Semtner and Hair 1973a; Needham and Teal, 1991; Cumming, 2002; Estrada-Peña et al., 2013; Pfäffle et al., 2013;). AMOVA analysis revealed high Fst values for both 12S and CO1 (in both cases $Fst > 0.5$, $P < 0.01$) which indicates a high level of structure amongst the overall data, and that there is not a gene flow among all sampling sites

(Frankham et al., 2002; Frankham, 2010). Usually this either implies great levels of genetic drift among populations, either promoted by distance or other barriers for species dispersion such as mountains, rivers or environmental factors. However, due to insufficient prior information on the origins of *H. aegyptium*, it is currently impossible to estimate how colonization and diversification in North Africa occurred.

If we consider the first scenario, then the landscape of the two obtained haplogroups would follow a similar pattern as the one observed in *T. graeca*. This would imply that in theory, *H. aegyptium* would follow an east-west gradient of genetic variation which is commonly observed in *T. graeca* (Fritz et al., 2009; Anadón et al., 2015), and other species in the Maghreb Region (Husemann et al., 2014). However, upon observing the haplotype distribution across the sampled region, the expected pattern for this hypothesis was not clear. Instead, a more North-South diversity gradient was observed. AMOVA analysis based on this hypothesis, where groups were defined based on current North African and Turkish *T. graeca* subspecies distribution, indicates low structure amongst groups (12S Fct = 0.0956, CO1 Fct = 0.126; P > 0.5), where only 9.56% and 12.55% of variation, respectively, could be explained with these groups, meaning that at least two or more groups cannot be genetically differentiated by this test. Tunisian and Turkish samples are represented by a single haplogroup each and CO1 parsimony network reveals they are well separated from the Moroccan and Algerian haplotypes. This means that the Moroccan and Algerian groups are responsible for this lack of structure, and cannot be differentiated from each other. This goes highly against the obtained phylogeny of the North African *T. graeca* proposed by Fritz et al. (2009) and Anadón et al. (2015) where North-Moroccan, Algerian and Tunisian tortoises are from three distinct lineages with very little overlap.

For the second scenario, AMOVA analysis resulted in higher Fct values for both markers than in the previous scenario (12S Fst = 0.512, CO1 Fst = 0.555; P < 0.01), accounting for 51.19% and 55.50% of variation. This means that our defined barriers for this hypothesis on the distribution of *H. aegyptium* are capable of defining a greater portion of the overall *H. aegyptium* population structure, and that all defined groups for this hypothesis are sufficiently considered distinct from each other. Haplotype diversity within groups in this scenario is also lower than in the first scenario, providing more evidence on the homogeny of each individual group when considering the Littoral/Interior genetic landscape. This North-South distribution of haplotypes is very atypical in the North African regions, especially considering the strong dependence that *H. aegyptium* has with its host for its survival, suggesting that other factors may be influencing the North African *H. aegyptium* landscape.

Despite our second defined group for the AMOVA analysis being more prominently based on the Atlas Mountains serving as geographical barriers, it also largely overlaps with the variation in vegetation landscapes, and other environmental factors such as temperature, humidity and precipitation seen in the Maghreb region (Schleich et al., 1996). The high Fsc values still suggest that within groups there is still some structure that could not be explained by our simple model, so it is most likely that the observed genetic distribution of *H. aegyptium* could have also been influenced by other different factors. Possibly, different environments of the *H. aegyptium* distribution areas could be responsible for some of the observed structuring, while the connectivity between the Algerian and Moroccan populations could be maintained through secondary hosts or the *H. aegyptium* juvenile forms. Nonetheless, our findings on the genetic distribution, and haplotype diversity of *H. aegyptium*, are not consistent with a latitudinal co-evolutionary trend with *T. graeca* in North Africa, and should be explained by other events. We propose two possible occurrences that may have led to the observed patterns: an allopatric differentiation to the different climate areas of North Africa; or *H. aegyptium* arrived in the Maghreb Region in two different occasions.

Possible scenarios for the genetic distribution of *H. aegyptium*

The allopatric differentiation scenario consists of *H. aegyptium* arriving together with *T. graeca* In North Africa. And during the glaciation events of the Pleistocene which have caused *T. graeca* to contract into different refugia (Graciá et al., 2013; Anadón et al., 2015) and begin its own differentiation, *H. aegyptium* also contracted and adapted in two different environments. Upon the more recent expansion events of *T. graeca* (Anadón et al., 2015), *H. aegyptium* also expanded but became conditioned by the developed different environmental adaptations.

The different colonization events scenario implies the arrival of *H. aegyptium* from two different origins and in two distinct periods (possibly promoted by the same events proposed by Ahmadzade et al. (2013)), with each colonization event occupying and adapting to a distinct distribution and environment sometime during the Pleistocene. Both hypotheses rely heavily on the sensitivity of adult ticks to their environment and the need of sustainable intermediaries to promote connectivity patches between populations, but can explain the close genetic origin of the Tunisian samples from the more northern and littoral sites.

Dlugosh and Parker (2008) have presented two cases with plants which are consistent and comparable with our proposed hypothesis. In the first case, a single invasion of *Hypericum canariense* in the Tenerife islands resulted in high levels of morphological and genetic adaptations to the different environments even after strong bottleneck events. In the second case, *Verbascum thapsus* in California was the result of several introductions from Europe, and each introduction event ended up adapting to differently to its surroundings and occupying different distribution ranges. However, neither one of our proposed scenarios can provide an accurate explanation for the two apparent separate clades observed in Turkey. And the distribution of haplotypes in North Africa would probably still not be fully explained by such simple models. Similar difficulties in obtaining an accurate history in ticks have been recently predicted by several authors who have also addressed the need to incorporate both genetic and ecological factors with geographical history and host biogeography in tick studies in order to fully understand tick distribution patterns (Estrada-Peña and De La Fuente, 2014a, 2014b; Poulin, 2014; Miller et al., 2016). We think that *H. aegyptium* genetic distribution may well only be entirely explained with the incorporation of such sources of information, and with wider sampling ranges from not only North Africa, but ideally covering the entire global distribution range for this species.

Detection of pathogens and other co-existing organisms

Hemolivia mauritanica

H. aegyptium is a known vector for *Hemolivia mauritanica* for more than a hundred years, although only recently molecular tools have been developed to facilitate its detections (Harris et al., 2013). This pathogen had previously been screened in *H. aegyptium* collected from Morocco, Algeria and Tunisia by Harris et al. (2013), although infection was only observed in Algeria in sites AAC, AGU, AKH, ASS and ATH. Our study confirms previous results, regarding the presence of *Hemolivia mauritanica* in Algeria, and was not found in Tunisia. Moreover, one single sample taken from the Moroccan site MSE was positive for *H. mauritanica*. Despite this most recent positive sample, *H. mauritanica* has been previously reported infecting *T. graeca* and *H. aegyptium* in both Morocco and Algeria (Široký et al., 2009). All Turkish sites presented positive results for the presence of *H. mauritanica*, which is also consistent with the high prevalence reported for this pathogen observed by Široký et al.,(2005).

Ehrlichia sp.

Ehrlichia sp. Is a genus of rickettsial bacteria, which is transmitted to vertebrates by ticks, and usually attacks the white blood cells of its hosts.. It has been previously described in *H. aegyptium* ticks from Romania (Pastiu et al., 2012) and as being transmitted through non *Hyalomma* ticks in Morocco, and through several tick species including *Hyalomma* in Tunisia (Sarih et al., 2006), and Turkey (Aktas et al., 2009) and in dogs from Algeria (Dahmani et al., 2015). From our samples, *Ehrlichia* sp. was more common in Algeria where it was found on four sites: AAC, AGU, AMB and ATH, while the only other site where *Ehrilichia* was found was MKS in Morocco. *Ehrlichia* was not found in either Tunisia, or in Turkey, despite records showing its transmission through other tick species in these countries. It is possible that *Ehrlichia* might not simply be transmitted through *H. aegyptium* in these areas, but our lack of positive samples for these countries is most likely due to the low sampling available. Our found records are as best of our knowledge, the first report that *Ehrlichia* sp. is being carried by *H. aegyptium* ticks in North Africa and *H. aegyptium* as the first tick found infected in Algeria.

Wolbachia sp.

A single rare event of infection by *Wolbachia* sp. was discovered infecting one tick sampled from MSE. Although *Wolbachia* is mostly considered an endosymbiont in ticks (Noda et al., 1997), its presence is still uncommon. Few studies have focused on the detection of this group in ticks, and detection has mostly been a rare occasion, and in our studied area, *Wolbachia* in ticks has also only been previously found in Morocco (Sarih et al., 2006; Seng et al., 2009). This is as best to our knowledge the first record of *Wolbachia* sp. present in *H. aegyptium*.

Midichloria mitochondrii

Midichloria mitochondrii is a common tick endosymbiont of the mitochondria (Sassera et al., 2006) which has been described as infecting ticks from several genera including *Hyalomma* (Epis et al., 2008; Cafiso et al., 2016), and it has also been described in ticks from Morocco (Seng et al., 2009). Although, the functional relationship between M*idichloria mitochondrii* and ticks is still unknown, the 100% prevalence of this endosymbiont in females of *Ixodes ricinus* (the most common and studied European

tick) suggests some sort of underlying mutualistic relationship (Cafiso et al., 2016). To the best of our knowledge, this is the first reported case of M*idichloria mitochondrii* present in *H. aegyptium*, and was found in populations from Morocco, Algeria and Turkey making it the most common organism we found in *H. aegyptium*.

Conclusions and Future prospects

The field of genetics in ticks is still much under studied, and is in need of a new approach to provide more accurate data sets. As some ticks can be heavily dependent on both their environments and hosts, a more integrative approach combining and correlating genetics with ecological factors and host and geographical history would be the ideal way to ensure a complete evolutionary history for these parasites.

In the case of *Hyalomma aegyptium* in North Africa, we observed that simply trying to compare host and parasite genetic distribution ranges is not enough to explain the current genetic distribution of the parasite. As instead, our preliminary analysis points to a possible higher correlation between the genetic distributions of *H. aegyptium* with different environments, which has probably been further influenced by host movement during the Pleistocene period. As such, it in order to assess the full history of *H. aegyptium* it would be needed to take into account not only more nuclear genetic data and the environmental factors, but also the hosts' history, and the needed to increase the sampling to include representatives from the distribution range of the *Testudo* hosts.

Such extensive studies would also be highly valuable to pathogen detection, since such sources of information would be fundamental to create predicative models for pathogen presence and dispersion.

Although this study only used two primer sets for pathogen detection, it has already provided new insight into what pathogens *H. aegyptium* could be carrying. We believe that screening for a broader range of parasites would result in even more pathogens being discovered in *H. aegyptium*. This is highly important because although *H. aegyptium* is not typically associated with human related activities, it can still be serving as a reservoir and be carrying highly dangerous pathogens.

As a final statement we recommend that the scientific community should not overlook the importance of studies relating to pathogen-transmitting vectors just because they do not pose such immediate threats to us, as they can quite literally come back to bite you.

References

Ahmadzadeh, F., Flecks, M., Roedder, D., Boehme, W., Ilgaz, Ç., Harris, D. J., Engler, J. O., Üzüm, N., & Carretero, M. A. (2013). Multiple dispersal out of Anatolia: biogeography and evolution of oriental green lizards. Biological Journal of the Linnean Society, 110(2), 398-408.

Aktas, M., Altay, K., Dumanli, N., & Kalkan, A. (2009). Molecular detection and identification of *Ehrlichia* and *Anaplasma* species in ixodid ticks. Parasitology Research, 104(5), 1243-1248.

Álvarez, Y., Mateo, J.A., Andreu, A.C., Díaz-Paniagua, C., Diez, A., Bautista, J.M. (2000). Mitochondrial DNA haplotyping of *Testudo graeca* on both continental sides of the Straits of Gibraltar. Journal of Heredity, 91, 39-41.

Anadón, J. D., Giménez, A., Perez, I., Martinez, M., & Esteve, M. A. (2006). Habitat selection by the spur-thighed tortoise *Testudo graeca* in a multisuccessional landscape: implications for habitat management. Biodiversity & Conservation, 15(7), 2287-2299.

Anadón, J. D., Giménez, A., Martínez, M., Martínez, J., Pérez, I., & Esteve, M. A. (2006). Factors determining the distribution of the spur-thighed tortoise *Testudo graeca* in south-east spain: a hierarchical approach. Ecography, 29(3), 339-346.

Anadón, J. D., Giménez, A., Martínez, M., Palazón, J. A., & Esteve, M. A. (2007). Assessing changes in habitat quality due to land use changes in the spur-thighed tortoise *Testudo graeca* using hierarchical predictive habitat models. Diversity and Distributions, 13(3), 324-331.

Anadón, J. D., Giménez, A., Ballestar, R., & Pérez, I. (2009). Evaluation of local ecological knowledge as a method for collecting extensive data on animal abundance. Conservation Biology, 23(3), 617-625.

Anadón, J. D., Giménez, A., & Ballestar, R. (2010). Linking local ecological knowledge and habitat modelling to predict absolute species abundance on large scales. Biodiversity and Conservation, 19(5), 1443-1454.

Anadón, J. D., Giménez, A., Graciá, E., Pérez, I., Ferrández, M., Fahd, S., El Mouden, H., Kalboussi, M., Jdeidi, T., Larbes, S., Rouag, R., Slimani, T., Znari, M., & Fritz, U. (2012). Distribution of *Testudo graeca* in the western Mediterranean according to climatic factors. Amphibia-Reptilia, 33(2), 285-296.

Anadón, J. D., Graciá, E., Botella, F., Giménez, A., Fahd, S., & Fritz, U. (2015). Individualistic response to past climate changes: niche differentiation promotes diverging Quaternary range dynamics in the subspecies of *Testudo graeca*. Ecography, 38(9), 956-966.

Apanaskevich, D. A. (2003). [Host-parasite relationships of the genus *Hyalomma* Koch, 1844 (Acari, Ixodidae) and their connection with microevolutionary process]. Parazitologiia, 38(6), 515-523.

Apanaskevich, D. A., & Horak, I. G. (2008). The genus *Hyalomma* Koch, 1844: V. Re-evaluation of the taxonomic rank of taxa comprising the *H.(Euhyalomma) marginatum* Koch complex of species (Acari: Ixodidae) with redescription of all parasitic stages and notes on biology. International Journal of Acarology, 34(1), 13-42.

Apanaskevich, D. A., & Oliver Jr, J. H. (2014). Life cycles and natural history of ticks. Biology of Ticks, 1, 59-73.

Araya-Anchetta, A., Busch, J. D., Scoles, G. A., & Wagner, D. M. (2015). Thirty years of tick population genetics: A comprehensive review. Infection, Genetics and Evolution, 29, 164-179.

Ayarza, P., Alvarez-Lobato, F., Teixell, A., Arboleya, M. L., Teson, E., Julivert, M., & Charroud, M. (2005). Crustal structure under the central High Atlas Mountains (Morocco) from geological and gravity data.Tectonophysics, 400(1), 67-84.

Babault, J., Teixell, A., Arboleya, M. L., & Charroud, M. (2008). A Late Cenozoic age for long-wavelength surface uplift of the Atlas Mountains of Morocco. Terra Nova, 20(2), 102-107.

Balashov, Y. S. (1960). Water balance and the behavior of *Hyalomma asiaticum* in desert areas. Medical Parasitology (Moscow) 3, 313-320.

Balashov, Y. S. (1994). Importance of continental drift in the distribution and evolution of ixodid ticks. Entomological Review, 73(8), 42-50.

Ballard, J. W. O., & Whitlock, M. C. (2004). The incomplete natural history of mitochondria. Molecular Ecology, 13(4), 729-744.

Barker, S. C., & Murrell, A. (2003). Phylogeny, evolution and historical zoogeography of ticks: a review of recent progress. In Ticks and Tick-Borne Pathogens (pp. 55-68). Springer Netherlands.

Barker, S. C., & Murrell, A. (2004). Systematics and evolution of ticks with a list of valid genus and species names. Parasitology, 129(1), 15-36.

Barker, S. C., Walker, A. R., & Campelo, D. (2014). A list of the 70 species of Australian ticks; diagnostic guides to and species accounts of *Ixodes holocyclus* (paralysis tick), *Ixodes cornuatus* (southern paralysis tick) and *Rhipicephalus australis* (Australian cattle tick); and consideration of the place of Australia in the evolution of ticks with comments on four controversial ideas. International Journal of Parasitology, 44(12), 941-953.

Barnard S. M., Durden L. A. (2000). A Veterinary Guide to the Parasites of Reptiles. Vol. 2, Arhropods (Excluding Mites). Krieger Publishing Company, Malabar, Florida.

Barrett, L. G., Thrall, P. H., Burdon, J. J., & Linde, C. C. (2008). Life history determines genetic structure and evolutionary potential of host–parasite interactions. Trends in Ecology & Evolution, 23(12), 678-685.

Beauchamp, W., Allmendinger, R. W., Barazangi, M., Demnati, A., El Alji, M., & Dahmani, M. (1999). Inversion tectonics and the evolution of the High Atlas Mountains, Morocco, based on a geological-geophysical transect. Tectonics, 18(2), 163-184.

Biollaz, F., Bruyndonckx, N., Beuneux, G., Mucedda, M., Goudet, J., & Christe, P. (2010). Genetic isolation of insular populations of the Maghrebian bat, Myotis punicus, in the Mediterranean Basin. Journal of Biogeography, *37*(8), 1557-1569.

Bitam, I., Kernif, T., Harrat, Z., Parola, P., & Raoult, D. (2009). First detection of *Rickettsia aeschlimannii* in *Hyalomma aegyptium* from Algeria. Clinical Microbiology and Infection, 15, 253-254.

Black, W. C., & Piesman, J. (1994). Phylogeny of hard-and soft-tick taxa (Acari: Ixodida) based on mitochondrial 16S rDNA sequences. Proceedings of the National Academy of Sciences, 91(21), 10034-10038.

Brianti, E., Dantas-Torres, F., Giannetto, S., Risitano, A., Brucato, G., Gaglio, G., & Otranto, D. (2010). Risk for the introduction of exotic ticks and pathogens into Italy through the illegal importation of tortoises, *Testudo graeca*. Medical and Veterinary Entomology, 24(3), 336-339.

Brown, W. M., George, M., & Wilson, A. C. (1979). Rapid evolution of animal mitochondrial DNA. Proceedings of the National Academy of Sciences, 76(4), 1967-1971.

Burdon, J. J. (1992). Host population subdivision and the genetic structure of natural pathogen populations. Advances in Plant Pathology, 8, 81-94.

Burger, T. D., Shao, R., Beati, L., Miller, H., & Barker, S. C. (2012). Phylogenetic analysis of ticks (Acari: Ixodida) using mitochondrial genomes and nuclear rRNA genes indicates that the genus *Amblyomma* is polyphyletic. Molecular Phylogenetics and Evolution, 64(1), 45-55.

Burger, T. D., Shao, R., & Barker, S. C. (2014). Phylogenetic analysis of mitochondrial genome sequences indicates that the cattle tick, *Rhipicephalus (Boophilus) microplus*, contains a cryptic species. Molecular Phylogenetics and Evolution, 76, 241-253.

Bursali, A., Tekin, S., Orhan, M., Keskin, A., & Ozkan, M. (2010). Ixodid ticks (Acari: Ixodidae) infesting humans in Tokat Province of Turkey: species diversity and seasonal activity. Journal of Vector Ecology, 35(1), 180-186.

Burridge, M. J., & Simmons, L. A. (2003). Exotic ticks introduced into the United States on imported reptiles from 1962 to 2001 and their potential roles in international dissemination of diseases. Veterinary Parasitology, 113(3), 289-320.

Cafiso, A., Bazzocchi, C., De Marco, L., Opara, M. N., Sassera, D., & Plantard, O. (2016). Molecular screening for *Midichloria* in hard and soft ticks reveals variable prevalence levels and bacterial loads in different tick species. Ticks and Tick-borne Diseases.

Camicas, J. L., Hervy, J. P., Adam, F., & Morel, P. C. (1998). The ticks of the world (Acarida, Ixodida). Nomenclature, described stages, hosts, distribution. Orstom éditions, Paris, France.

Cangi, N., Horak, I. G., Apanaskevich, D. A., Matthee, S., Das Neves, L. C., Estrada-Peña, A., & Matthee, C. A. (2013). The influence of interspecific competition and host

preference on the phylogeography of two African Ixodid tick species. PloS one, 8(10), e76930.

Carretero, M. A., Znari, M., Harris, D. J., & Mace, J. C. (2005). Morphological divergence among populations of *Testudo graeca* from west-central Morocco. Animal Biology, 55(3), 259-279.

Clement, M., Posada, D. C. K. A., & Crandall, K. A. (2000). TCS: a computer program to estimate gene genealogies. Molecular Ecology, 9(10), 1657-1659.

Coelho, P., Sousa, P., Harris, D. J., & van der Meijden, A. (2014). Deep intraspecific divergences in the medically relevant fat-tailed scorpions (*Androctonus, Scorpiones*). Acta Tropica, 134, 43-51.

Combes, C. (2001). Parasitism: the ecology and evolution of intimate interactions. University of Chicago Press.

Cox, C. B., and Moore, P. D. (1993). Biogeography: An Ecological and Evolutionary Approach, Blackwell Sci., Oxford.

Criscione, C. D., & Blouin, M. S. (2004). Life cycles shape parasite evolution: comparative population genetics of salmon trematodes. Evolution, 58(1), 198-202.

Cruickshank, R. H. (2002). Molecular markers for the phylogenetics of mites and ticks. Systematic and Applied Acarology, 7(1), 3-14.

Cumming, G. S. (2002). Comparing climate and vegetation as limiting factors for species ranges of African ticks. Ecology, 83(1), 255-268.

Cutler, S. J., Fooks, A. R., & Van Der Poel, W. H. (2010). Public health threat of new, reemerging, and neglected zoonoses in the industrialized world. Emerging Infectious Diseases, 16(1), 1-7.

Dahmani, M., Loudahi, A., Mediannikov, O., Fenollar, F., Raoult, D., & Davoust, B. (2015). Molecular detection of *Anaplasma platys* and *Ehrlichia canis* in dogs from Kabylie, Algeria. Ticks and Tick-Borne Diseases, 6(2), 198-203.

Danilov, I. G. (2005). Die fossilen Schildkröten Europas. In Handbuch der Reptilien und Amphibien Europas. Bd. 3, 3B, Schildkröten (Testudines) II (Cheloniidae, Dermochelyidae, Fossile Schildkröten Europas) (p. 330).

Dantas-Torres, F., & Otranto, D. (2013). Species diversity and abundance of ticks in three habitats in southern Italy. Ticks and Tick-borne Diseases, 4(3), 251-255.

Daszak, P., Cunningham, A. A., & Hyatt, A. D. (2000). Emerging infectious diseases of wildlife--threats to biodiversity and human health. Science, 287(5452), 443-449.

De La Fuente, J. (2003). The fossil record and the origin of ticks (Acari: Parasitiformes: Ixodida). Experimental & Applied Acarology, 29(3-4), 331-344

de Lapparent de Broin, F. (2000). African chelonians from the Jurassic to the present: phases of development and preliminary catalogue of the fossil record.

de Lamotte, D. F., Saint Bezar, B., Bracène, R., & Mercier, E. (2000). The two main steps of the Atlas building and geodynamics of the western Mediterranean. Tectonics, 19(4), 740-761.

de Lamotte, D. F., Leturmy, P., Missenard, Y., Khomsi, S., Ruiz, G., Saddiqi, O., Guillocheau, F., & Michard, A. (2009). Mesozoic and Cenozoic vertical movements in the Atlas system (Algeria, Morocco, Tunisia): an overview. Tectonophysics, 475(1), 9-28.

Diab, F. M., AI-Khalifa, M. S., Hussein, H. S.and AI-Asgah, N. A (1987). Ticks (Acari: Ixodidae) Parasitizing Indigenous Livestock in Northern and Eastern Saudi Arabia. Arab Gulf Journal of Scientific Research, B - Agricultural. & Biological Sciences, 5, 301-319.

Díaz-Paniagua, C., Keller, C., & Andreu, A. C. (2001). Long-term demographic fluctuations of the spur-thighed tortoise *Testudo graeca* in SW Spain. Ecography, 24(6), 707-721.

Dlugosch, K. M., & Parker, I. M. (2008). Founding events in species invasions: genetic variation, adaptive evolution, and the role of multiple introductions. Molecular Ecology, 17(1), 431-449.

Dobson, S. J., & Barker, S. C. (1999). Phylogeny of the Hard Ticks (Ixodidae) Inferred from 18S rRNA Indicates That the Genus *Aponomma* is Paraphyletic. Molecular Phylogenetics and Evolution, 11(2), 288

Dobson, A., Lafferty, K. D., Kuris, A. M., Hechinger, R. F., & Jetz, W. (2008). Homage to Linnaeus: How many parasites? How many hosts?. Proceedings of the National Academy of Sciences, 105(Supplement 1), 11482-11489.

El Mouden, E. H., Slimani, T., Kaddour, K. B., Lagarde, F., Ouhammou, A., & Bonnet, X. (2006). *Testudo graeca graeca* feeding ecology in an arid and overgrazed zone in Morocco. Journal of Arid Environments, 64(3), 422-435.

Epis, S., Sassera, D., Beninati, T., Lo, N., Beati, L., Piesman, J., Rinaldi, L., McCoy, K. D., Torina, A., Sacchi, L., & Clementi, E. (2008). *Midichloria mitochondrii* is widespread in hard ticks (Ixodidae) and resides in the mitochondria of phylogenetically diverse species. Parasitology, 135(04), 485-494.

Estrada-Peña, A. (2002). Understanding the relationships between landscape connectivity and abundance of *Ixodes ricinus* ticks. Experimental & Applied Acarology, 28(1-4), 239-248.

Estrada-Peña, A. (2003). The relationships between habitat topology, critical scales of connectivity and tick abundance *Ixodes ricinus* in a heterogeneous landscape in northern Spain. Ecography, 26(5), 661-671.

Estrada-Peña, A., Martínez Avilés, M., & Muñoz Reoyo, M. J. (2011). A population model to describe the distribution and seasonal dynamics of the tick *Hyalomma marginatum* in the Mediterranean Basin. Transboundary and Emerging Diseases, 58(3), 213-223.

Estrada-Peña, A., Guglielmone, A. A., & Mangold, A. J. (2013). The distribution and ecological'preferences' of the tick Amblyomma cajennense (Acari: Ixodidae), an ectoparasite of humans and other mammals in the Americas. Annals of Tropical Medicine and Parasitology, 98(3), 283-292.

Estrada-Peña, A., & de la Fuente, J. (2014). The ecology of ticks and epidemiology of tick-borne viral diseases. Antiviral Research, 108, 104-128.

Estrada-Peña, A., & de la Fuente, J. (2014). Toward a multidisciplinary approach to the study of tick-borne diseases. Frontiers in Cellular and Infection Microbiology, 4, 118.

Excoffier, L., Smouse, P. E., & Quattro, J. M. (1992). Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics, 131(2), 479-491.

Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. Journal of Molecular Evolution, 17(6), 368-376.

Fitch, W. M. (1971). Toward defining the course of evolution: minimum change for a specific tree topology. Systematic Biology, 20(4), 406-416.

Földvári, G., (2005). Studies of ticks (Acari: Ixodidae) and tick-borne pathogens of dogs in Hungary. PhD dissertation, Szent István University, Postgraduate School of Veterinary Science.

Frankham, R., Briscoe, D. A., & Ballou, J. D. (2002). Introduction to conservation genetics. Cambridge University Press.

Frankham, R. (2010). Challenges and opportunities of genetic approaches to biological conservation. Biological Conservation, 143(9), 1919-1927.

Fritz, U., Fritzsch, G., Lehr, E., Ducotterd, J. M., & Müller, A. (2005). The Atlas Mountains, not the Strait of Gibraltar, as a biogeographic barrier for *Mauremys leprosa* (Reptilia: Testudines). Salamandra, 41(3), 97-106.

Fritz, U., Barata, M., Busack, S. D., Fritzsch, G., & Castilho, R. (2006). Impact of mountain chains, sea straits and peripheral populations on genetic and taxonomic structure of a freshwater turtle, *Mauremys leprosa* (Reptilia, Testudines, Geoemydidae). Zoologica Scripta, 35(1), 97-108.

Fritz, U., Hundsdörfer, A. K., Široký, P., Auer, M., Kami, H., Lehmann, J., Mazanaeva, L. F., Türkozan, O. & Wink, M. (2007). Phenotypic plasticity leads to incongruence between morphology-based taxonomy and genetic differentiation in western Palaearctic tortoises (*Testudo graeca* complex; Testudines, Testudinidae). Amphibia-Reptilia, 28(1), 97-121.

Fritz, U., Harris, D. J., Fahd, S., Rouag, R., Martínez, E. G., Casalduero, A. G., Široký, P., Kalboussi, M., Jdeidi, T.B. & Hundsdörfer, A. K. (2009). Mitochondrial phylogeography of *Testudo graeca* in the Western Mediterranean: old complex divergence in North Africa and recent arrival in Europe. Amphibia-Reptilia, 30(1), 63-80.

Fujimoto, K. (1989). Ecological studies on ixodid ticks. 6. The effects of temperature on the oviposition, development and survival of *Ixodes ovatus* Neumann(Acarina: Ixodidae). Japanese Journal of Sanitary Zoology, 40(3), 187-193.

García, C. ,Gorostiza, A., Ballestar, R., Yelo, N. , Anadón, J. D., Pérez, I., Sánchez-Zapata, J. A., Botella, F., Giménez, A. (2003). Predation of the spur-thighed tortoise *Testudo graeca* by carnivorous fauna in Southeastern Spain. 2nd International Congress on Chelonian Conservation. Senegal.

Gazyağci, S., Aşan, N., & DEMİRBAŞ, Y. (2010). A common tortoise tick, *Hyalomma aegyptium* Linne 1758 (Acari: Ixodidae), identified on eastern hedgehog (*Erinaceus concolor* Martin 1838) in Central Anatolia. Turkish Journal of Veterinary and Animal Sciences, 34(2), 211-213.

Gharbi, M., Rjeibi, M. R., Rouatbi, M., Mabrouk, M., Mhadhbi, M., Amairia, S., Gharbi, M., Rjeibi, M.R., Rouatbi, M., Mabrouk, M., Mhadhbi, M., Amairia, S., Amdouni, Y & Boussaadoun, M. A. (2015). Infestation of the spur-thighed tortoise (*Testudo graeca*) by *Hyalomma aegyptium* in Tunisia. Ticks and Tick-borne Diseases, 6(3), 352-355.

Gilbert, L., Aungier, J., & Tomkins, J. L. (2014). Climate of origin affects tick (*Ixodes ricinus*) host-seeking behavior in response to temperature: implications for resilience to climate change?. Ecology and Evolution, 4(7), 1186-1198

Gómez, A., & Nichols, E. (2013). Neglected wild life: parasitic biodiversity as a conservation target. International Journal for Parasitology: Parasites and Wildlife, 2, 222-227.

Gonçalves, D. V., Brito, J. C., Crochet, P. A., Geniez, P., Padial, J. M., & Harris, D. J. (2012). Phylogeny of North African *Agama* lizards (Reptilia: Agamidae) and the role of the Sahara desert in vertebrate speciation. Molecular Phylogenetics and Evolution, 64(3), 582-591.

Graciá, E., Giménez, A., Anadón, J. D., Harris, D. J., Fritz, U., & Botella, F. (2013). The uncertainty of Late Pleistocene range expansions in the western Mediterranean: a case study of the colonization of south-eastern Spain by the spur-thighed tortoise, *Testudo graeca*. Journal of Biogeography, 40(2), 323-334.

Groombridge, B., & Wright, L. (1982). The IUCN Amphibia-Reptilia Red Data Book (Vol. 1). IUCN.

Guglielmone, A. A., Robbins, R. G., Apanaskevich, D. A., Petney, T. N., Estrada-Peña, A., Horak, I. G., & Barker, S. C. (2010). The Argasidae, Ixodidae and Nuttalliellidae (Acari: Ixodida) of the world: a list of valid species names. Zootaxa, 2528, 1–28.

Guglielmone, A. A., Robbins, R. G., Apanaskevich, D. A., Petney, T. N., Estrada-Peña, A., & Horak, I. (2014). The hard ticks of the world. Springer, Dordrecht.

Guiller, A., & Madec, L. (2010). Historical biogeography of the land snail *Cornu aspersum*: a new scenario inferred from haplotype distribution in the Western Mediterranean basin. BMC Evolutionary Biology, 10(18), 1.

Güner, E. S., Watanabe, M., Hashimoto, N., Kadosaka, T., Kawamura, Y., Ezaki, T., Güner, E.S., Watanabe, M., Hashimoto, N., Kadosaka, T., Kawamura, Y., Ezaki, T., Kawabata, H., Imai, Y., Kaneda, K. & Masuzawa, T. (2004). *Borrelia turcica sp. nov.,* isolated from the hard tick *Hyalomma aegyptium* in Turkey. International Journal of Systematic and Evolutionary Microbiology, 54(5), 1649-1652.

Guyot-Jackson, G. (2004). Numéro spécial *Testudo*. Manouria, 7(22), 1-52.

Hajibabaei, M., Janzen, D. H., Burns, J. M., Hallwachs, W., & Hebert, P. D. (2006). DNA barcodes distinguish species of tropical *Lepidoptera*. Proceedings of the National Academy of Sciences of the United States of America, 103(4), 968-971.

Harzhauser, M., & Piller, W. E. (2007). Benchmark data of a changing sea—palaeogeography, palaeobiogeography and events in the Central Paratethys during the Miocene. Palaeogeography, Palaeoclimatology, Palaeoecology, 253(1), 8-31.

Harris, D. J., Znari, M., Mace, J. C., & Carretero, M. A. (2003). Genetic variation in *Testudo graeca* from Morocco estimated using 12S rRNA sequencing. Revista Española de Herpetologia, 17, 5-9.

Harris, D. J., Maia, J. P., & Perera, A. (2011). Molecular characterization of *Hepatozoon* species in reptiles from the Seychelles. The Journal of Parasitology, 97(1), 106-110.

Harris, D. J., Graciá, E., Jorge, F., Maia, J. P., Perera, A., Carretero, M. A., & Giménez, A. (2013). Molecular detection of *Hemolivia* (Apicomplexa: Haemogregarinidae) from ticks of North African *Testudo graeca* (Testudines: Testudinidae) and an estimation of their phylogenetic relationships using 18S rRNA sequences. Comparative Parasitology, 80(2), 292-296.

Hebert, P. D., Penton, E. H., Burns, J. M., Janzen, D. H., & Hallwachs, W. (2004). Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. Proceedings of the National Academy of Sciences of the United States of America, 101(41), 14812-14817.

Hebert, P. D., Stoeckle, M. Y., Zemlak, T. S., & Francis, C. M. (2004). Identification of birds through DNA barcodes. PLoS Biol, 2(10), e312.

Helg, U., Burkhard, M., Caritg, S., & Robert-Charrue, C. (2004). Folding and inversion tectonics in the anti-Atlas of Morocco. Tectonics, 23(4).

Highfield, A. C., & Bayley, J. R. (1996). The trade in tortoise-derived souvenir products in Morocco. Traffic Bulletin-Wildlife Trade Monitoring Unit, 16, 33-35

Hoogstraal, H., & Kaiser, M. N. (1960). Some host relationships of the tortoise tick, *Hyalomma (Hyalommasta) aegyptium* (L.)(Ixodoidea, Ixodidae) in Turkey. Annals of the Entomological Society of America, 53(4), 457-458.

Hoogstraal, H., Kaiser, M. N., Traylor, M. A., Guindy, E., & Gaber, S. (1963). Ticks (Ixodidae) on birds migrating from Europe and Asia to Africa, 1959-61. Bulletin of the World Health Organization, 28(2), 235.

Hornok, S., Földvári, G., Elek, V., Naranjo, V., Farkas, R., & de la Fuente, J. (2008). Molecular identification of *Anaplasma marginale* and rickettsial endosymbionts in blood-sucking flies (Diptera: Tabanidae, Muscidae) and hard ticks (Acari: Ixodidae). Veterinary Parasitology, 154(3), 354-359.

Huelsenbeck, J. P., & Ronquist, F. (2001). MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics, 17(8), 754-755.

Husemann, M., Schmitt, T., Stathi, I., & Habel, J. C. (2012). Evolution and radiation in the scorpion *Buthus elmoutaouakili* Lourenço and Qi 2006 (Scorpiones: Buthidae) at the foothills of the Atlas Mountains (North Africa). Journal of Heredity, 103(2), 221-229.

Husemann, M., Schmitt, T., Zachos, F. E., Ulrich, W., & Habel, J. C. (2014). Palaearctic biogeography revisited: evidence for the existence of a North African refugium for Western Palaearctic biota. Journal of Biogeography,41(1), 81-94.

Ivanov, I. N., Mitkova, N., Reye, A. L., Hübschen, J. M., Vatcheva-Dobrevska, R. S., Dobreva, E. G., Ivanov, I.N., Mitkova, N., Reye, A.L., Hübschen, J.M., Vatcheva-Dobrevska, R.S., Dobreva, E.G., Kantardjiev, T.V. & Muller, C. P. (2011). Detection of new *Francisella*-like tick endosymbionts in *Hyalomma spp.* and *Rhipicephalus spp.* (Acari: Ixodidae) from Bulgaria. Applied and Environmental Microbiology, 77(15), 5562-5565.

Kambhampati, S., & Smith, P. T. (1995). PCR primers for the amplification of four insect mitochondrial gene fragments. Insect Molecular Biology, 4(4), 233-236.

Kanduma, E. G., Mwacharo, J. M., Githaka, N. W., Kinyanjui, P. W., Njuguna, J. N., Kamau, L. M., Kariuki, E., Mwaura, S., Skilton, R.A. & Bishop, R. P. (2016). Analyses of mitochondrial genes reveal two sympatric but genetically divergent lineages of Rhipicephalus appendiculatus in Kenya. Parasites & Vectors, 9(1), 353.

Kar, S., Yilmazer, N., Midilli, K., Ergin, S., Alp, H., & Gargili, A. (2011). Presence of the Zoonotic *Borrelia burgdorferi sl*. and *Rickettsia spp*. in the Ticks from Wild Tortoises and Hedgehogs. Marmara Üniversitesi Saglik Bilimleri Enstitüsü Dergisi, 1(3), 166.

Kar, S., Dervis, E., Akın, A., Ergonul, O., & Gargili, A. (2013). Preferences of different tick species for human hosts in Turkey. Experimental and Applied Acarology, 61(3), 349-355.

Koc, S., Aydın, L., & Cetin, H. (2015). Tick species (Acari: Ixodida) in Antalya City, Turkey: species diversity and seasonal activity. Parasitology Research, 114(7), 2581-2586.

Kolonin, G. V. (1983). World distribution of ixodid ticks. Genera *Hyalomma, Aponomma, Amblyomma*. Izd Nauka, Moscow.

Kolonin, G. V. (2013). Reptiles as hosts of ticks. Russian Journal of Herpetology, 11(3), 177-180.

Lambert, M. R. K. (1983). Some factors influencing the Moroccan distribution of the western Mediterranean spur-thighed tortoise, *Testudo graeca graeca* L., and those precluding its survival in NW Europe. Zoological Journal of the Linnean Society, 79(2), 149-178.

Lancaster, J. L. (1957). Control of the lone star tick. University of Arkansas, College of Agriculture and Home Economics, Agricultural Experiment Station.

Latif, A. A., and Walker, A. R. (2004). An introduction to the biology and control of ticks in Africa. International Consortium on Ticks and Tick Borne Diseases (ICTTD-2) Project.

Li, S., Vanwambeke, S. O., Licoppe, A. M., & Speybroeck, N. (2014). Impacts of deer management practices on the spatial dynamics of the tick *Ixodes ricinus*: a scenario analysis. Ecological Modelling, 276, 1-13.

Librado, P. & Rozas, J. (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics, 25, 1451–1452.

Lv, J., Wu, S., Zhang, Y., Chen, Y., Feng, C., Yuan, X., Jia, G., Deng, J., Wang, C., Wang, Q. & Mei, L. (2014). Assessment of four DNA fragments (COI, 16S rDNA, ITS2, 12S rDNA) for species identification of the Ixodida (Acari: Ixodida). Parasites & Vectors,7(1), 1.

Mans, B. J., de Klerk, D., Pienaar, R., de Castro, M. H., & Latif, A. A. (2012). The mitochondrial genomes of *Nuttalliella namaqua* (Ixodoidea: Nuttalliellidae) and *Argas africolumbae* (Ixodoidae: Argasidae): estimation of divergence dates for the major tick lineages and reconstruction of ancestral blood-feeding characters. PLoS One, 7(11), e49461.

McCoy, K. D., Boulinier, T., Tirard, C., & Michalakis, Y. (2003). Host-dependent genetic structure of parasite populations: differential dispersal of seabird tick host races. Evolution, 57(2), 288-296.

Mihalca, A. D. (2015). Ticks imported to Europe with exotic reptiles. Veterinary Parasitology, 213(1), 67-71.

Miller, M. J., Esser, H. J., Loaiza, J. R., Herre, E. A., Aguilar, C., Quintero, D., Alvarez, E. & Bermingham, E. (2016). Molecular Ecological Insights into Neotropical Bird–Tick Interactions. PloS one, 11(5), e0155989.

Mironov, V. S. (1939). On the Behaviour of the Siberian Forest Tick *Ixodes persulcatus schulze*. Medical Parasitology 8(1), 123-136.

Missenard, Y., Zeyen, H., Frizon de Lamotte, D., Leturmy, P., Petit, C., Sébrier, M., & Saddiqi, O. (2006). Crustal versus asthenospheric origin of relief of the Atlas Mountains of Morocco. Journal of Geophysical Research: Solid Earth, 111(B3).

Mixson, T. R., Lydy, S. L., Dasch, G. A., & Real, L. A. (2006). Inferring the population structure and demographic history of the tick, *Amblyomma americanum* Linnaeus. Journal of Vector Ecology, 31(1), 181-192.

Morand, S. (2015). (macro-) Evolutionary ecology of parasite diversity: From determinants of parasite species richness to host diversification. International Journal for Parasitology: Parasites and Wildlife, 4(1), 80-87.

Murrell, A., Campbell, N. J., & Barker, S. C. (1999). Letter to the editor. Molecular Phylogenetics and Evolution, 12(1), 83-86.

Murrell, A., Campbell, N. J., & Barker, S. C. (2000). Phylogenetic analyses of the rhipicephaline ticks indicate that the genus *Rhipicephalus* is paraphyletic. Molecular Phylogenetics and Evolution, 16(1), 1-7.

Murrell, A., Campbell, N. J., & Barker, S. C. (2001). A total-evidence phylogeny of ticks provides insights into the evolution of life cycles and biogeography. Molecular Phylogenetics and Evolution, 21(2), 244-258.

Murrell, A., Dobson, S. J., Walter, D. E., Campbell, N. J., Shao, R., & Barker, S. C. (2005). Relationships among the three major lineages of the Acari (Arthropoda: Arachnida) inferred from small subunit rRNA: paraphyly of the Parasitiformes with respect to the Opilioacariformes and relative rates of nucleotide substitution. Invertebrate Systematics, 19(5), 383-389.

Needham, G. R., & Teel, P. D. (1991). Off-host physiological ecology of ixodid ticks. Annual Review of Entomology, 36(1), 659-681.

Nicolas, V., Ndiaye, A., Benazzou, T., Souttou, K., Delapre, A., & Denys, C. (2014). Phylogeography of the North African dipodil (Rodentia: Muridae) based on cytochrome-b sequences. Journal of Mammalogy, 95(2), 241-253.

Noda, H., Munderloh, U. G., & Kurtti, T. J. (1997). Endosymbionts of ticks and their relationship to *Wolbachia spp*. and tick-borne pathogens of humans and animals. Applied and Environmental Microbiology, 63(10), 3926-3932

Norris, D. E., Klompen, J. S. H., & Black, W. C. (1999). Comparison of the mitochondrial 12S and 16S ribosomal DNA genes in resolving phylogenetic relationships among hard ticks (Acari: Ixodidae). Annals of the Entomological Society of America, 92(1), 117-129.

Norval, R. A. I. (1977). Studies on the ecology of the tick *Amblyomma hebraeum* Koch in the Eastern Cape Province of South Africa. II. Survival and development. The Journal of Parasitology, 740-747.

Nowak, M. (2010). The international trade in reptiles (Reptilia)—The cause of the transfer of exotic ticks (Acari: Ixodida) to Poland. Veterinary Parasitology, 169(3), 373-381.

Ogden, N. H., Lindsay, L. R., Hanincova, K., Barker, I. K., Bigras-Poulin, M., Charron, D. F. Heagy, A., Francis, C.M., O'callaghan, C.J., Schwartz, I. & Thompson, R. A. (2008). Role of migratory birds in introduction and range expansion of *Ixodes scapularis* ticks and of *Borrelia burgdorferi* and *Anaplasma phagocytophilum* in Canada. Applied and Environmental Microbiology, 74(6), 1780-1790

Ogrzewalska, M., Schwarcz, K., Bajay, M. M., Bajay, S. K., Pinheiro, J. B., Zucchi, M. I., Pinter, A. & Labruna, M. B. (2016). Characterization of Genetic Variability and Population Structure of the Tick *Amblyomma aureolatum* (Acari: Ixodidae). Journal of Medical Entomology, tjw049.

Padial, J. M., Miralles, A., De la Riva, I., & Vences, M. (2010). The integrative future of taxonomy. Frontiers in Zoology, 7(1), 1.

Page, R. D. (2003). Tangled trees: phylogeny, cospeciation, and coevolution. University of Chicago Press.

Paperna, I., Kremer-Mecabell, T., & Finkelman, S. (2002). *Hepatozoon kisrae n. sp.* infecting the lizard *Agama stellio* is transmitted by the tick *Hyalomma cf. aegyptium*. Parasite, 9(1), 17-27.

Parola, P., Roux, V., Camicas, J. L., Baradji, I., Brouqui, P., & Raoult, D. (2000). Detection of ehrlichiae in African ticks by polymerase chain reaction. Transactions of the Royal Society of Tropical Medicine and Hygiene, 94(6), 707-708.

Parola, P., Cornet, J. P., Sanogo, Y. O., Miller, R. S., Van Thien, H., Gonzalez, J. P., Raoult, D., Telford III, S.R. & Wongsrichanalai, C. (2003). Detection of *Ehrlichia spp., Anaplasma spp., Rickettsia spp*., and other eubacteria in ticks from the Thai-Myanmar border and Vietnam. Journal of Clinical Microbiology, 41(4), 1600-1608.

Paștiu, A. I., Matei, I. A., Mihalca, A. D., D'Amico, G., Dumitrache, M. O., Kalmár, Z., Sándor, A.D., Lefkaditis, M., Gherman, C.M. & Cozma, V. (2012). Zoonotic pathogens associated with *Hyalomma aegyptium* in endangered tortoises: evidence for host-switching behaviour in ticks?. Parasites & Vectors, 5(1), 1.

Pérez, I., Giménez, A., Sánchez-Zapata, J. A., Anadón, J. D., Martínez, M., & Esteve, M. Á. (2004). Non-commercial collection of spur-thighed tortoises (*Testudo graeca graeca*): a cultural problem in southeast Spain. Biological Conservation, 118(2), 175-181.

Pérez, I., Tenza, A., Anadón, J. D., Martínez-Fernández, J., Pedreño, A., & Giménez, A. (2012). Exurban sprawl increases the extinction probability of a threatened tortoise due to pet collections. Ecological Modelling, 245, 19-30.

Perkins, S. L., Martinsen, E. S., & Falk, B. G. (2011). Do molecules matter more than morphology? Promises and pitfalls in parasites. Parasitology,138(13), 1664-1674.

Pfäffle, M., Littwin, N., Muders, S. V., & Petney, T. N. (2013). The ecology of tick-borne diseases. International Journal for Parasitology, 43(12), 1059-1077

Pieh, A. (2001). *Testudo graeca soussensis*, eine neue Unterart der Maurischen Landschildkrote aus dem Sousstal (Sudwest-Marokko). Salamandra-Bonn-, 36(4), 209-222.

Porretta, D., Mastrantonio, V., Amendolia, S., Gaiarsa, S., Epis, S., Genchi, C., Otranto D. & Urbanelli, S. (2013). Effects of global changes on the climatic niche of the tick *Ixodes ricinus* inferred by species distribution modelling. Parasites & Vectors, 6(1), 1.

Posada, D., & Crandall, K. A. (2001). Intraspecific gene genealogies: trees grafting into networks. Trends in Ecology & Evolution, 16(1), 37-45.

Poulin, R. (2014). Parasite biodiversity revisited: frontiers and constraints. International Journal for Parasitology, 44(9), 581-589.

Poulin, R., & Morand, S. (2000). The diversity of parasites. Quarterly Review of Biology, 277-293.

Poulin, R., & Morand, S. (2014). Parasite biodiversity. Smithsonian Institution.

Prudhomme, J., Gunay, F., Rahola, N., Ouanaimi, F., Guernaoui, S., Boumezzough, A., Bañuls, A.L., Sereno, D. & Alten, B. (2012). Wing size and shape variation of *Phlebotomus papatasi* (Diptera: Psychodidae) populations from the south and north slopes of the Atlas Mountains in Morocco. Journal of Vector Ecology, 37(1), 137-147.

Rato, C., Brito, J. C., Carretero, M. A., Larbes, S., Shacham, B., & Harris, D. J. (2007). Phylogeography and genetic diversity of *Psammophis schokari* (Serpentes) in North Africa based on mitochondrial DNA sequences. African Zoology, 42(1), 112-117.

Ray, H. N. (1950). Hereditary transmission of *Theileria annulata* infection in the tick, *Hyalomma aegyptium* Neum. Transactions of the Royal Society of Tropical Medicine and Hygiene, 44(1), 93-104.

Razmjo, M., Bahrami, A., Hosseini, E., & Bahrami, A. M. (2013). The first report of lizard and turtle ticks from Ilam, Western Province of Iran. Advances in Bioresearch, 4(3).

Recuero, E., Iraola, A., Rubio, X., Machordom, A., & García-París, M. (2007). Mitochondrial differentiation and biogeography of *Hyla meridionalis* (Anura: Hylidae): an unusual phylogeographical pattern. Journal of Biogeography, 34(7), 1207-1219.

Rees, D. J., Dioli, M., & Kirkendall, L. R. (2003). Molecules and morphology: evidence for cryptic hybridization in African *Hyalomma* (Acari: Ixodidae). Molecular phylogenetics and evolution, 27(1), 131-142.

Richter, S. H., Eydal, M., Skírnisson, K., & Ólafsson, E. (2013). Tick species (Ixodia) identified in Iceland. Icelandic Agricultural Sciences 26, 3-10

Saitou, N., & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution, 4(4), 406-425.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). Molecular cloning (Vol. 2, pp. 14-9). New York: Cold Spring Harbor Laboratory Press.

Sanz-Aguilar, A., Anadón, J. D., Giménez, A., Ballestar, R., Graciá, E., & Oro, D. (2011). Coexisting with fire: the case of the terrestrial tortoise *Testudo graeca* in Mediterranean shrublands. Biological Conservation, 144(3), 1040-1049.

Sarih, M. H., M'Ghirbi, Y., Bouattour, A., Gern, L., Baranton, G., & Postic, D. (2005). Detection and identification of *Ehrlichia spp*. in ticks collected in Tunisia and Morocco. Journal of Clinical Microbiology, 43(3), 1127-1132.

Semtner, P. J., Barker, R. W., & Hair, J. A. (1971). The ecology and behavior of the lone star tick (Acarina: Ixodidae) II. Activity and survival in different ecological habitats. Journal of Medical Entomology, 8(6), 719-725

Semtner, P. J., & Hair, J. A. (1973). The ecology and behavior of the lone star tick (Acarina: Ixodidae). IV. The daily and seasonal activity patterns of adults in different habitat types. Journal of Medical Entomology, 10(4), 337-344.

Semtner, P. J., & Hair, J. A. (1973). The ecology and behavior of the lone star tick (Acarina: Ixodidae) V. Abundance and seasonal distribution in different habitat types. Journal of Medical Entomology, 10(6), 618-628.

Seng, P., Sarih, M., Socolovschi, C., Boudebouch, N., Hassar, M., Parola, P., Raoult, D. & Brouqui, P. (2009). Detection of Anaplasmataceae in ticks collected in Morocco. Clinical Microbiology and Infection, 15, 86-87.

Sergent, E., & Sergent, E. T. (1904). Sur une hémogrégarine, parasite de *Testudo mauritanica*. Comptes Rendus Des Seances de la Societe de Biologie et ses Filiales, 56, 130-131.

Schleich, H. H., Kästle, W., & Kabisch, K. (1996). Amphibians and reptiles of North Africa. Koeltz, Koenigstein, 627.

Schlick-Steiner, B. C., Steiner, F. M., Seifert, B., Stauffer, C., Christian, E., & Crozier, R. H. (2010). Integrative taxonomy: a multisource approach to exploring biodiversity. Annual Review of Entomology, 55, 421-438.

Shine, R., & Iverson, J. B. (1995). Patterns of survival, growth and maturation in turtles. Oikos, 343-348.

Siroký, P., Kamler, M., & Modrý, D. (2005). Prevalence of *Hemolivia mauritanica* (Apicomplexa: Adeleina: Haemogregarinidae) in natural populations of tortoises of the genus *Testudo* in the east Mediterranean region. Folia Parasitologica, 52(4), 359.

Široký, P., Petrželková, K. J., Kamler, M., Mihalca, A. D., & Modrý, D. (2006). *Hyalomma aegyptium* as dominant tick in tortoises of the genus *Testudo* in Balkan countries, with notes on its host preferences.Experimental & Applied Acarology, 40(3-4), 279-290.

Široký, P., Mikulíček, P., Jandzík, D., Kami, H., Mihalca, A. D., Rouag, R., Kamler, M., Schneider, C., Záruba, M. & Modrý, D. (2009). Co-distribution pattern of a haemogregarine *Hemolivia mauritanica* (Apicomplexa: Haemogregarinidae) and its vector *Hyalomma aegyptium* (Metastigmata: Ixodidae). Journal of Parasitology, 95(3), 728-733.

Široký, P., Kubelová, M., Modrý, D., Erhart, J., Literák, I., Špitalská, E., & Kocianová, E. (2010). Tortoise tick *Hyalomma aegyptium* as long term carrier of Q fever agent Coxiella burnetii—evidence from experimental infection. Parasitology Research, 107(6), 1515-1520.

Široký, P., Erhart, J., Petrželková, K. J., & Kamler, M. (2011). Life cycle of tortoise tick *Hyalomma aegyptium* under laboratory conditions. Experimental and Applied Acarology, 54(3), 277-284.

Široký, P., Bělohlávek, T., Papoušek, I., Jandzik, D., Mikulíček, P., Kubelová, M., & Zdražilová-Dubská, L. (2014). Hidden threat of tortoise ticks: high prevalence of Crimean-Congo haemorrhagic fever virus in ticks *Hyalomma aegyptium* in the Middle East. Parasites & Vectors, 7(1), 1.

Sneath, P. H., & Sokal, R. R. (1973). Numerical taxonomy. The principles and practice of numerical classification. W.H. Freeman and Company, San Francisco.

Sparagano, O. A. E., Allsopp, M. T. E. P., Mank, R. A., Rijpkema, S. G. T., Figueroa, J. V., & Jongejan, F. (1999). Molecular detection of pathogen DNA in ticks (Acari: Ixodidae): a review. Experimental & Applied Acarology,23(12), 929-960.

Slimani, T., El Mouden, E. H., & Ben Kaddour, K. (2001). Structure et dynamique d'une population de *Testudo graeca graeca* L. 1758 dans les Jbilets Centrales, Maroc, Chelonii 3. In Proceeding International Congress on *Testudo* Genus, March (pp. 7-10).

Sunnucks, P. (2000). Efficient genetic markers for population biology. Trends in Ecology & Evolution, 15(5), 199-203.

Tavassoli, E., Rahimi-Asiabi, N., & Tavassoli, M. (2007). *Hyalomma aegyptium* on spur-thighed tortoise (*Testudo graeca*) in Urmia Region West Azerbaijan, Iran. Iranian Journal of Parasitology, 2(2), 40-47.

Templeton, A. R., Crandall, K. A., & Sing, C. F. (1992). A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. Genetics, 132(2), 619-633.

Tiar, G., TIAR-SAADI, M., Benyacoub, S., Rouag, R., & Široký, P. (2016). The dependence of *Hyalomma aegyptium* on its tortoise host *Testudo graeca* in Algeria. Medical and Veterinary Entomology.

Troughton, D. R., & Levin, M. L. (2007). Life cycles of seven ixodid tick species (Acari: Ixodidae) under standardized laboratory conditions. Journal of Medical Entomology, 44(5), 732-740.

Ujvari, B., Madsen, T., & Olsson, M. (2004). High prevalence of *Hepatozoon spp.*(Apicomplexa, Hepatozoidae) infection in water pythons (*Liasis fuscus*) from tropical Australia. Journal of Parasitology, 90(3), 670-672.

van der Kuyl, A. C., Ballasina, D. L., & Zorgdrager, F. (2005). Mitochondrial haplotype diversity in the tortoise species *Testudo graeca* from North Africa and the Middle East. BMC Evolutionary Biology, 5(1), 1.

van Dijk, P.P., Corti, C., Mellado, V.P. & Cheylan, M. (2004). *Testudo graeca.* The IUCN Red List of Threatened Species 2004

Van Valen, L. (1973). A new evolutionary law. Evolutionary theory, 1, 1-30.

Vatansever, Z., Gargili, A., Aysul, N. S., Sengoz, G., & Estrada-Peña, A. (2008). Ticks biting humans in the urban area of Istanbul. Parasitology Research, 102(3), 551-553.

Walker, A. R. (2003). Ticks of domestic animals in Africa: a guide to identification of species. Edinburgh: Bioscience Reports.

Weir, B. S., & Cockerham, C. C. (1984). Estimating F-statistics for the analysis of population structure. Evolution, 1358-1370.

Weiss, J. B. (1995). DNA probes and PCR for diagnosis of parasitic infections. Clinical Microbiology Reviews, 8(1), 113-130.

Wilkinson, P. R. (1953). Observations on the sensory Physiology and Behaviour of larvae of the cattle tick, *Boophilus Microplus* (Can.)(Ixodidae). Australian Journal of Zoology, 1(3), 345-356.

Xia, Y. U. N., GU, H. F., Peng, R. U. I., Chen, Q. I. N., ZHENG, Y. C., Murphy, R. W., & ZENG, X. M. (2012). COI is better than 16S rRNA for DNA barcoding Asiatic salamanders (Amphibia: Caudata: Hynobiidae). Molecular Ecology Resources, 12(1), 48-56.

Znari, M., Germano, D. J., & Macé, J. C. (2005). Growth and population structure of the Moorish Tortoise (*Testudo graeca graeca*) in Westcentral Morocco: possible effects of over-collecting for the tourist trade. Journal of Arid Environments, 62(1), 55-74.

Appendix

Sample code	Country	Population Code	Region	Latitude	Longitude	12S haplotype	CO1 Haplotype	EHR detection	HEP detection
AAC 1.1	Algeria	AAC	Ain Chorfa	35,230	0,140	Ac1	Ac1	*Midichloria mitochondrii*	*Hemolivia mauritanica*
AAC 2.1	Algeria	AAC	Ain Chorfa	35,230	0,140	Ac1	Ac1		
AAC 4.1	Algeria	AAC	Ain Chorfa	35,230	0,140	Ac1	Ac1		
AAC 4.2	Algeria	AAC	Ain Chorfa	35,230	0,140	Bc1	Bc1	*Midichloria mitochondrii*	*Hemolivia mauritanica*
AAC 5.1	Algeria	AAC	Ain Chorfa	35,230	0,140	Ac1	Ac1		*Hemolivia mauritanica*
AAC 6.1	Algeria	AAC	Ain Chorfa	35,230	0,140	Bc1	Bc1		
AAC 6.2	Algeria	AAC	Ain Chorfa	35,230	0,140	Ac1	Ac4	*Midichloria mitochondrii*	
AAC 7.1	Algeria	AAC	Ain Chorfa	35,230	0,140	Ac1	Ac1		*Hemolivia mauritanica*
AAC 8.1	Algeria	AAC	Ain Chorfa	35,230	0,140	Ac1	Ac5	*Ehrlichia sp.*	
AAC 8.2	Algeria	AAC	Ain Chorfa	35,230	0,140	Bm1	Bs5		
AAC 9.1	Algeria	AAC	Ain Chorfa	35,230	0,140	Ac1	Ac1		
AAC 10.1	Algeria	AAC	Ain Chorfa	35,230	0,140	Ac1	Ac1		
AAC 12.1	Algeria	AAC	Ain Chorfa	35,230	0,140		Bc1		
AGU 1.1	Algeria	AGU	Guertoufa	35,200	1,160	Bc1	Bc1	*Midichloria mitochondrii*	*Hemolivia mauritanica*
AGU 2.1	Algeria	AGU	Guertoufa	35,200	1,160	Bm1	Bc1	*Ehrlichia sp.*	*Hemolivia mauritanica*
AGU 3.1	Algeria	AGU	Guertoufa	35,200	1,160	Bc2	Bc1		
AGU 04	Algeria	AGU	Guertoufa	35,200	1,160	Bm1			*Hemolivia mauritanica*
AGU 6.1	Algeria	AGU	Guertoufa	35,200	1,160	Bc1	Bc1	*Ehrlichia sp.*	
AGU 7.1	Algeria	AGU	Guertoufa	35,200	1,160	Bm1	Bc1		
AGU 10.1	Algeria	AGU	Guertoufa	35,200	1,160	Bm1	Bc1	*Ehrlichia sp.*	
AGU 11.1	Algeria	AGU	Guertoufa	35,200	1,160	Bc1	Bc1	*Ehrlichia sp.*	
AKH 1.1	Algeria	AKH	Zemmora	35,710	0,750	Ac1	Ac1		*Hemolivia mauritanica*
AKH 3.1	Algeria	AKH	Zemmora	35,710	0,750	Ac1	Ac1		*Hemolivia mauritanica*
AKH 3.2	Algeria	AKH	Zemmora	35,710	0,750	Ac1	Ac6		
AKH 3.3	Algeria	AKH	Zemmora	35,710	0,750	Ac1	Ac1		
AKH 3.4	Algeria	AKH	Zemmora	35,710	0,750	Ac1	Ac1		
AMB?	Algeria	AMB	Moudjbara	34,570	3,410	Bm1	Bc1		
AMB ? 2.1	Algeria	AMB	Moudjbara	34,570	3,410	Bm1	Bc1		
AMB 1100.1	Algeria	AMB	Moudjbara	34,570	3,410	Bm1	Bc1		
AMB 02.1	Algeria	AMB	Moudjbara	34,570	3,410		Bc1		

AMB 04.1	Algeria	AMB	Moudjbara	34,570	3,410	Bc1	Bc1		
AMB 5.1	Algeria	AMB	Moudjbara	34,570	3,410	Bm1	Bc1		
AMB 6.1	Algeria	AMB	Moudjbara	34,570	3,410	Bm1	Bc1		
AMB 8.1	Algeria	AMB	Moudjbara	34,570	3,410	Bm1	Bs4	*Ehrlichia sp.*	
AMB 9.1	Algeria	AMB	Moudjbara	34,570	3,410	Bm1	Bc1	*Ehrlichia sp.*	
AMB 10.1	Algeria	AMB	Moudjbara	34,570	3,410	Bm1	Bc1	*Ehrlichia sp.*	
AMB 12.1	Algeria	AMB	Moudjbara	34,570	3,410	Bm1	Bc1		
AMB 13.1	Algeria	AMB	Moudjbara	34,570	3,410	Bm1	Bc1	*Midichloria mitochondrii*	
AMB 16.1	Algeria	AMB	Moudjbara	34,570	3,410		Bc1		
ASS 1.1	Algeria	ASS	Saf Saf	35,490	0,230	Bs1	Bs1		
ASS 2.1	Algeria	ASS	Saf Saf	35,490	0,230	Bs2	Bs1		
ASS 3.1	Algeria	ASS	Saf Saf	35,490	0,230	Bs1	Bs1		*Hemolivia mauritanica*
ASS 05	Algeria	ASS	Saf Saf	35,490	0,230	Bs1	Bs1		*Hemolivia mauritanica*
ASS 5.1	Algeria	ASS	Saf Saf	35,490	0,230	Ac1	Ac1		
ASS 5.2	Algeria	ASS	Saf Saf	35,490	0,230	Bs1			
ASS 5.3	Algeria	ASS	Saf Saf	35,490	0,230		Bs1		
ASS 7.1	Algeria	ASS	Saf Saf	35,490	0,230	Ac1	Ac1		
ASS 7.2	Algeria	ASS	Saf Saf	35,490	0,230	Ac1	Ac3		
ASS 8.1	Algeria	ASS	Saf Saf	35,490	0,230	Ac1	Ac1		
ASS 8.2	Algeria	ASS	Saf Saf	35,490	0,230	Bs1	Bs1		*Hemolivia mauritanica*
ASS 9.1	Algeria	ASS	Saf Saf	35,490	0,230	Ac1	Ac3		
ASS 11.1	Algeria	ASS	Saf Saf	35,490	0,230	Bs1	Bs1		*Hemolivia mauritanica*
ASS 11.2	Algeria	ASS	Saf Saf	35,490	0,230	Ac1	Ac2		
ASS 12.1	Algeria	ASS	Saf Saf	35,490	0,230	Ac1	Ac1		*Hemolivia mauritanica*
ATH 1.1	Algeria	ATH	The´niet el Had	35,890	2,010	Ac1	Ac1		
ATH 2.1	Algeria	ATH	The´niet el Had	35,890	2,010	Ac1		*Ehrlichia sp.*	
ATH 3.1	Algeria	ATH	The´niet el Had	35,890	2,010	Ac1	Ac1	*Midichloria mitochondrii*	
ATH 4.1	Algeria	ATH	The´niet el Had	35,890	2,010	Bc1	Bs1	*Midichloria mitochondrii*	*Hemolivia mauritanica*
ATH 5.1	Algeria	ATH	The´niet el Had	35,890	2,010	Bc1	Bc1		
ATH 6.1	Algeria	ATH	The´niet el Had	35,890	2,010	Ac1	Ac1		
ATH 07	Algeria	ATH	The´niet el Had	35,890	2,010	Bm1	Bc1	*Ehrlichia sp.*	*Hemolivia mauritanica*
AZE 01	Algeria	AZE	Zemmouri	36,460	3,410	Ac1	Ac1		
AZE 02	Algeria	AZE	Zemmouri	36,460	3,410	Ac1	Ac1		
AZE 04	Algeria	AZE	Zemmouri	36,460	3,410	Ac1	Ac1		
AZE 05	Algeria	AZE	Zemmouri	36,460	3,410	Ac1	Ac1		
AZE 06	Algeria	AZE	Zemmouri	36,460	3,410	Ac1	Ac1		

AZE 07	Algeria	AZE	Zemmouri	36,460	3,410	Ac1	Ac1		
AZE 08	Algeria	AZE	Zemmouri	36,460	3,410	Ac1	Ac1		
AZE 09	Algeria	AZE	Zemmouri	36,460	3,410	Ac1	Ac1		
AZE 10	Algeria	AZE	Zemmouri	36,460	3,410	Ac1	Ac1		
MBA 1	Morocco	MBA	Balcon d'Ito	33,540	-5,320	Bc1		*Midichloria mitochondrii*	
MHK 2.0	Morocco	MHK	Had Kourt	34,651	-5,660	Ac1	Am2		
MKA02.1	Morocco	MKA	Kaytun	34,687	-5,396	Ac1	Am1	*Midichloria mitochondrii*	
MKA03.1	Morocco	MKA	Kaytun	34,687	-5,396	Ac1	Ac7		
MKA04.1	Morocco	MKA	Kaytun	34,687	-5,396	Ac1	Ac1		
MKA05.1	Morocco	MKA	Kaytun	34,687	-5,396	Ac1	Ac1		
MKA06.1	Morocco	MKA	Kaytun	34,687	-5,396	Ac1	Ac1		
MKA07.1	Morocco	MKA	Kaytun	34,687	-5,396	Ac1	Ac1		
MKA09.1	Morocco	MKA	Kaytun	34,687	-5,396	Ac1	Ac1		
MKA10.1	Morocco	MKA	Kaytun	34,687	-5,396	Ac1	Ac1		
MKA11.1	Morocco	MKA	Kaytun	34,687	-5,396	Ac1	Ac1		
MKA12.1	Morocco	MKA	Kaytun	34,687	-5,396	Ac1	Ac1		
MKB1.1	Morocco	MKB	Kariat Bamohammed	34,478	-5,311	Ac1	Ac1		
MKB1.2	Morocco	MKB	Kariat Bamohammed	34,478	-5,311	Ac1			
MKB1.3	Morocco	MKB	Kariat Bamohammed	34,478	-5,311	Ac1	Ac1		
MKB2.1	Morocco	MKB	Kariat Bamohammed	34,478	-5,311	Ac1	Ac1		
MKB2.2	Morocco	MKB	Kariat Bamohammed	34,478	-5,311	Bc1	Bc1		
MKB2.3	Morocco	MKB	Kariat Bamohammed	34,478	-5,311	Ac1	Ac1	*Midichloria mitochondrii*	
MKB2.4	Morocco	MKB	Kariat Bamohammed	34,478	-5,311	Ac1			
MKS 1	Morocco	MKS	El Ksiba	32,575	-6,035	Ac1		*Ehrlichia sp.*	
MLA 1.1	Morocco	MLA	LarAche	35,166	-6,121	Ac1			
MLA 1.2	Morocco	MLA	LarAche	35,166	-6,121	Ac1	Ac1		
MLA 2.2	Morocco	MLA	LarAche	35,166	-6,121	Ac1	Ac1		
MLA 2.3	Morocco	MLA	LarAche	35,166	-6,121	Ac1	Ac1	*Midichloria mitochondrii*	
MOY 1.1	Morocco	MOY	Oulad Yakoub	34,025	-6,717		Bc1		
MOY 1.2	Morocco	MOY	Oulad Yakoub	34,025	-6,717	Bs2	Bs2		
MOY 1.3	Morocco	MOY	Oulad Yakoub	34,025	-6,717	Bc1	Bc1		
MOY 1.4	Morocco	MOY	Oulad Yakoub	34,025	-6,717	Bc1	Bo1		
MRH1.2	Morocco	MRH	Raba Harcha	34,053	-5,468	Ac1	Ac1		
MRH1.3	Morocco	MRH	Raba Harcha	34,053	-5,468		Ac1		
MRH1.4	Morocco	MRH	Raba Harcha	34,053	-5,468	Bs1	Bs1		
MRH1.5	Morocco	MRH	Raba Harcha	34,053	-5,468	Bs3	Bs3		

MRH1.6	Morocco	MRH	Raba Harcha	34,053	-5,468	Ac1	Ac1		
MRH1.7	Morocco	MRH	Raba Harcha	34,053	-5,468	Ac1	Ac1		
MSE 1.1	Morocco	MSE	Sefrou	33,854	-4,858	Bc1	Bc1	*Midichloria mitochondrii*	
MSE 1.2	Morocco	MSE	Sefrou	33,854	-4,858	Bc1	Bc1		
MSE2.1	Morocco	MSE	Sefrou	33,854	-4,858	Bc1	Bc1	*WolbAchia sp.*	
MSE2.2	Morocco	MSE	Sefrou	33,854	-4,858	Bc1	Bc1		
MSE2.3	Morocco	MSE	Sefrou	33,854	-4,858	Ac1	Ac1		*Hemolivia mauritanica*
MSE2.4	Morocco	MSE	Sefrou	33,854	-4,858	Bc1	Bc2	*Midichloria mitochondrii*	
MZO 1	Morocco	MZO	Zoumi	34,770	-5,520	Az1		*Midichloria mitochondrii*	
MZO.m13	Morocco	MZO	Zoumi	34,770	-5,520	Az1	Ac1	*Midichloria mitochondrii*	
23997.1	Morocco	MAR1		35,525	-5,713	Bc1			
23997.2	Morocco	MAR1		35,525	-5,713	Bc1			
23995.1	Morocco	MAR2		31,530	-7,563	Bc1			
24010.1	Morocco	MAR3		31,461	-7,407		Bs2		
23998.1	Morocco	MAR4		34,098	-6,685	Ac1	Ac8		
38412	Morocco	MAR5		33,610	-5,160	Bc1			
TRA 1	Tunisia	TRA	Rass el Melah	36,909	11,105	Ac1	At1		
TRA 1.2	Tunisia	TRA	Rass el Melah	36,909	11,105	Ac1	At2		
12068.1	Turkey	TUR1		37,863	36,358	Bt1.1	b1.1		*Hemolivia mauritanica*
12068.2	Turkey	TUR1		37,863	36,358	Bt1.1	b1.1		*Hemolivia mauritanica*
12100.1	Turkey	TUR1		37,863	36,358		Bt2.2		
12100.2	Turkey	TUR1		37,863	36,358	Bc1	Bt2.1		
12110.1	Turkey	TUR2		37,859	36,365	Bt1.2	Bt1.2		
12110.2	Turkey	TUR2		37,859	36,365	Bt1.2	Bt1.2	*Midichloria mitochondrii*	*Hemolivia mauritanica*
12131.1	Turkey	TUR3		38,332	31,012	Bc1	Bt2.3		
12131.2	Turkey	TUR3		38,332	31,012	Bc1	Bt2.4		*Hemolivia mauritanica*
12069.2	Turkey	TUR4		38,047	36,636	Bt2	Bt2.1		
12069.3	Turkey	TUR4		38,047	36,636	Bc1	Bt2.1		*Hemolivia mauritanica*
12069.4	Turkey	TUR4		38,047	36,636	Bt1.1	Bt1.1		*Hemolivia mauritanica*